Moderní analytická instrumentace pro genetický výzkum, lékařskou diagnostiku a molekulární identifikaci organizmů

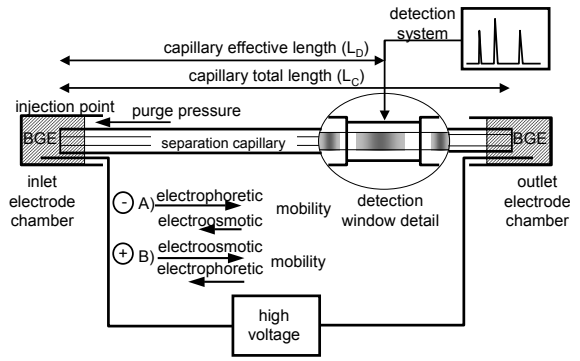
Karel Klepárník

Oddělení bioanalytické instrumentace
Ústav analytické chemie
Akademie věd České republiky
Brno

Capillary electrophoresis

CE

Capillary electrophoresis scheme



Stellan Hjertén

Uppsala

Sweden

James W. Jorgenson

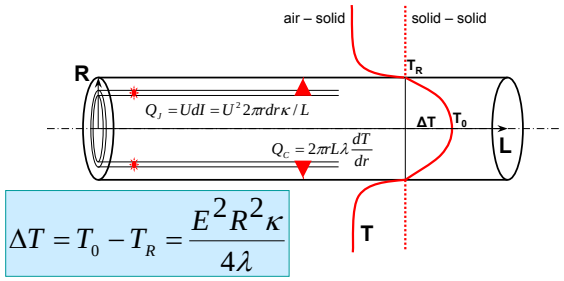
Department of Chemistry
Venable and Kenan Laboratories
The University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-3290 USA

Jorgenson, J. W.; Lukacs, K. D. *Anal. Chem* **1981**, *53*, 1298-1302.

Jorgenson, J. W.; Lukacs, K. D. *Science* **1983**, *222*, 266-272.

Prof. Milos Novotny
Indiana University, Bloomington
Department of Chemistry

Why capillary electrophoresis?



$$\Delta T = T_0 - T_R = \frac{E^2 R^2 \kappa}{4\lambda}$$

Miniature capillary:

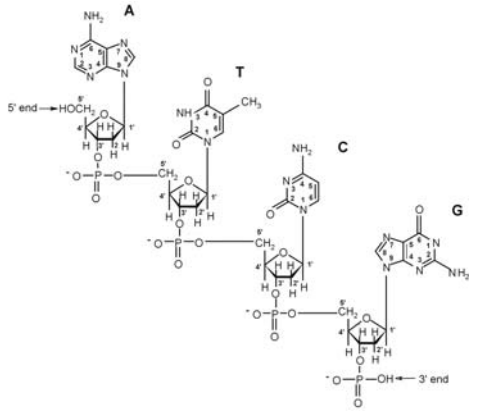
1) high resistivity ➥ low current at high voltage ➥ low heat production

2) efficient heat transport ➥ low temperature difference inside the capillary

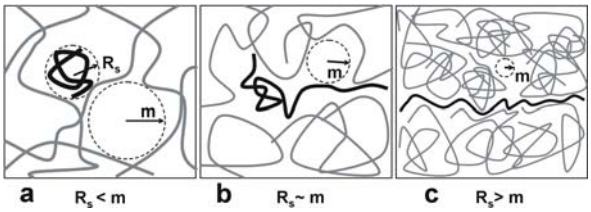
DNA electromigration

K. Klepárník, P. Boček, DNA diagnostics by Capillary Electrophoresis
Chemical Reviews 107, 5279 – 5317, 2007.

DNA primary structure



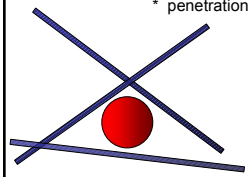
DNA electromigration regimes



Ogston regime

Ogston (1958): * distribution of spaces in a random network of rigid rods available to a spherical molecule

* penetration probability



$$P_{D\geq r} = \exp-(2\pi\nu Lr^2 + \frac{4\pi\nu r^3}{3})$$

ν average density of number of fibers
$2L$ fiber length
D „pore" radius
r particle radius

Rodbard Chrambach (1970): $P_{D\geq r} = \frac{\mu}{\mu_0} = \frac{V_a}{V_0} = \exp-(K_r c)$

μ el. mobility
μ_0 free electrolyte el. mobility
V_a accessible volume
V_0 void volume
K_r retardation coef.
c gel concentration
d gel fibre radius

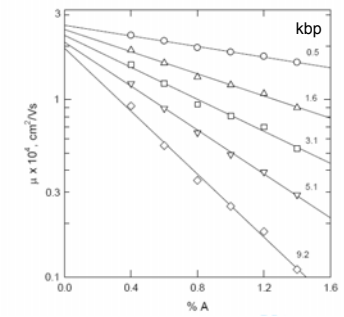
$$K_r \approx (r+d)^{n=1-3}$$

Ferguson plot (1964): $\log\mu = \log\mu_0 - K_r c$

Fergusson plot

$$\log\mu = \log\mu_0 - K_r c$$

$$\mu_0 = \frac{q}{6\pi r\eta}$$



Ferguson plots of DNA molecules in agarose gels. The logarithm of the mobility, extrapolated to zero electric field strength at each gel concentration, is plotted as a function of agarose concentration, %A.

Biased Reptation Model

$$\mu \propto \frac{1}{M}$$

$$\mu = \frac{q}{\xi} \times \frac{\langle h_x^2 \rangle}{L^2}$$

L tube legth
ξ friction inside
x field direction

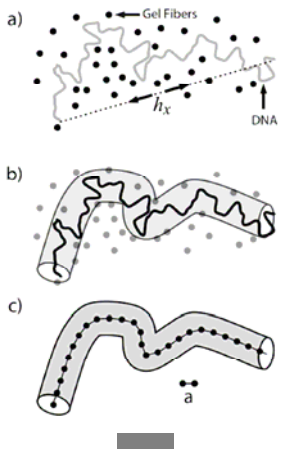
$$\mu = \frac{q}{3\xi} \times \left[\frac{1}{N} + \frac{\varepsilon^2}{3} \right] \quad \varepsilon << 1$$

$$\varepsilon = \frac{qEa}{2k_B T}$$

N reptation segments
ε scaled el. field
q segment charge
a segment length

$\mu/\mu_0 \sim$ 1/(3N) $N << \varepsilon^{-2}$

$\varepsilon^2/9$ $N >> \varepsilon^{-2}$



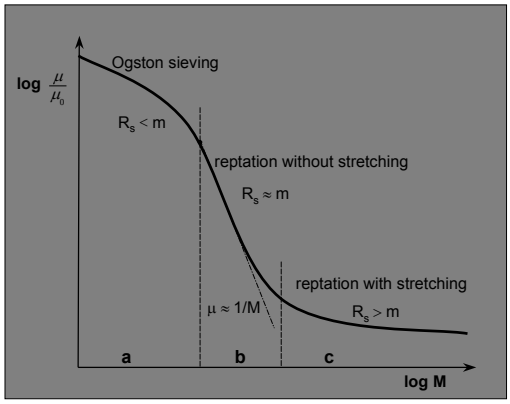
Pierre-Gilles de Gennes

1932 – 2007

Ecole de Physique et Chimie (Paris)

Nobel Prize in Physics in 1991

Dependence of DNA electrophoretic mobility on molecular mass



Jean-Louis Viovy
Curie Institute
Paris, France

Garry W. Slater

University of Ottawa

Poymerase chain reaction

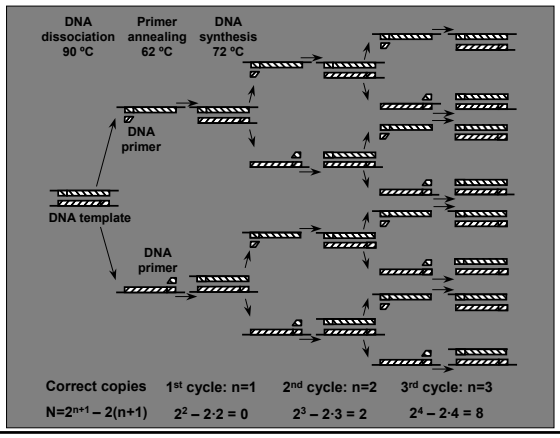
PCR amplification

Kary B. Mullis
born 1944
La Jolla, CA, USA
University of British Columbia

The Nobel Prize in Chemistry 1993

For his invention of the polymerase chain reaction (PCR) method

PCR amplification scheme



Human Genome Project

J. CRAIG VENTER, Ph.D., PRESIDENT, CELERA GENOMICS
REMARKS AT THE HUMAN GENOME ANNOUNCEMENT
THE WHITE HOUSE
MONDAY, JUNE 26, 2000

Mr. President, Honorable members of the Cabinet, Honorable members of Congress, distinguished guests. Today, June 26, 2000 marks an historic point in the 100,000-year record of humanity. We are announcing today that for the first time our species can read the chemical letters of its genetic code. At 12:30 p.m. today, in a joint press conference with the public genome effort, Celera Genomics will describe the first assembly of the human genetic code from the whole genome shotgun sequencing method. Starting only nine months ago on September 8, 1999, eighteen miles from the White House, a small team of scientists headed by myself, Hamilton O. Smith, Mark Adams, Gene Myers and Granger Sutton began sequencing the DNA of the human genome using a novel method pioneered by essentially the same team five years earlier at The Institute for Genomic Research in Rockville, Maryland. The method used by Celera has determined the genetic code of five individuals....

…There would be no announcement today if it were not for the more than **$1 billion that PE Biosystems invested in Celera and in the development of the automated DNA sequencer** that both Celera and the public effort used to sequence the genome…

J. Craig Venter

The Institute for Genomic Research (**TIGR**)

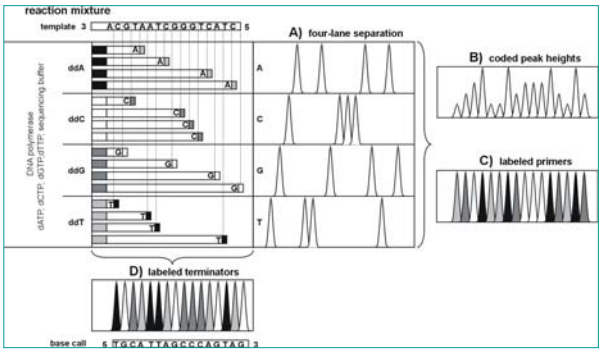
The first president of **Celera Genomics**

The completed sequence of the human genome was published in February 2001 in Science.

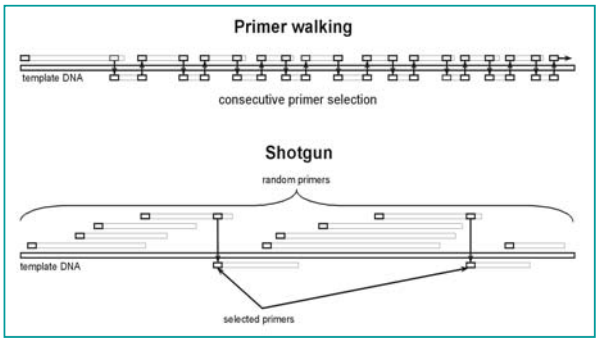
Venter, C. J. et al. *Science* **2001**, *291*, 1304-1351.

DNA sequencing

Analysis of Sanger sequencing fragments

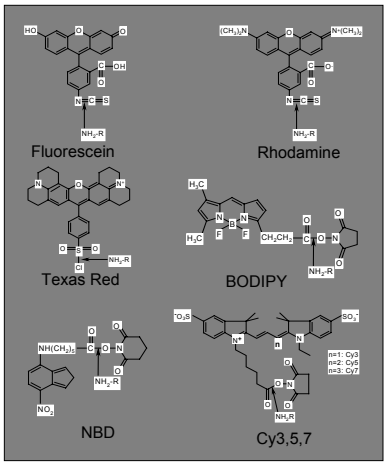


DNA sequencing strategy

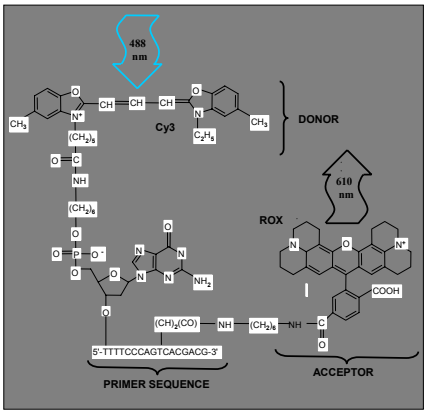


Fluorescence chemistry

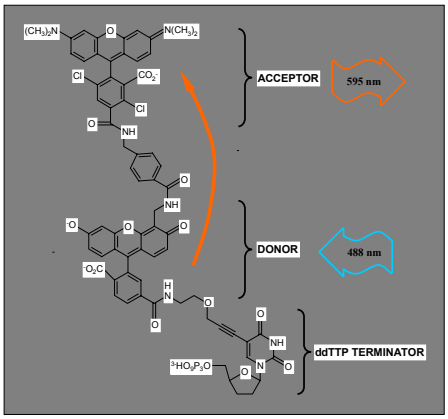
Fluorescent lebels



Sequencing primer attached to Fluorescence Resonance Energy Transfer

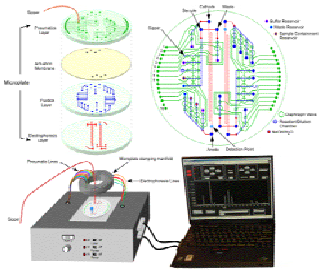


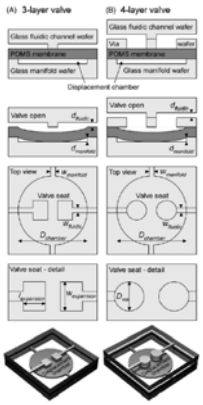
Dideoxy terminator attached to Fluorescence Resonance Energy Transfer



Prof. Richard A. Mathies

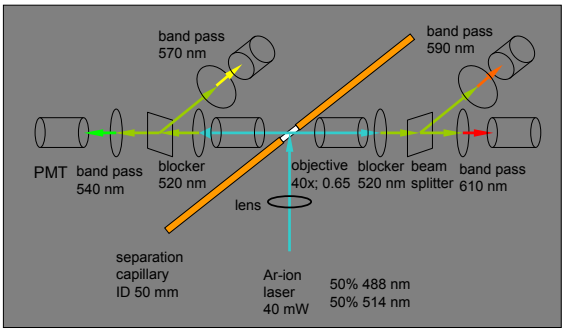
University of California at Berkeley
Department of Chemistry
Berkeley, CA



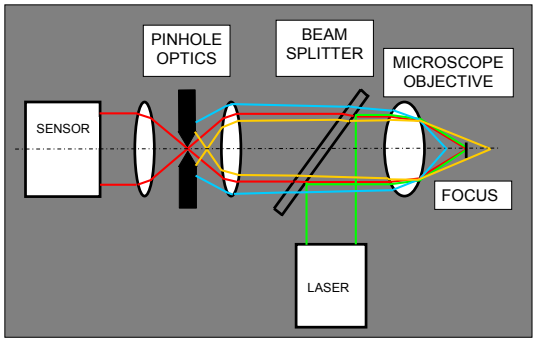


LIF detection

Four channel LIF detection arrangement

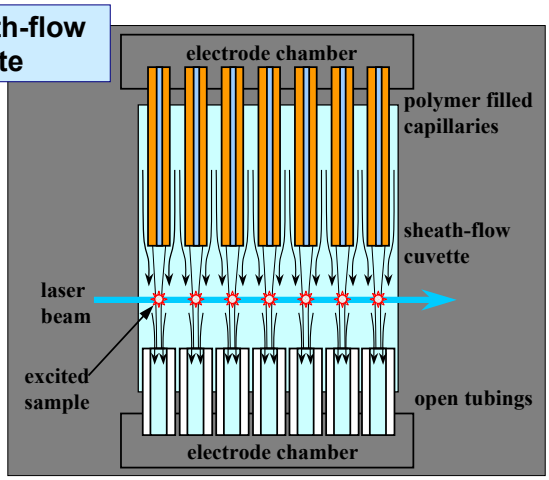


SCHEME OF CONFOCAL DETECTOR

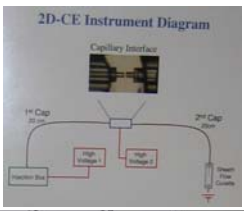


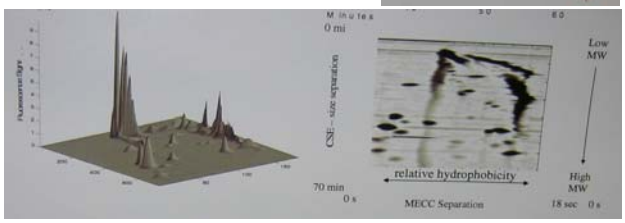
Prof. Edward S. Yeung
Ames Laboratory
U.S. Department of Energy
Iowa State University.

Sheath-flow cuvette



Prof. Norman Dovichi
University of Washington
Seattle, WA, USA

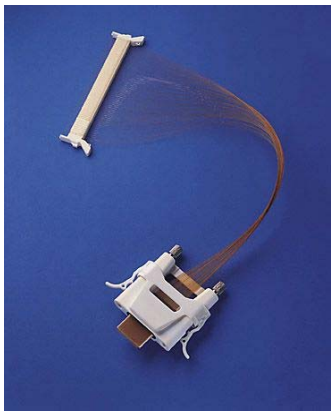




Prof. Hideki Kambara
senior chief scientist
Hitachi Central Research Laboratory
Tokyo, Japan

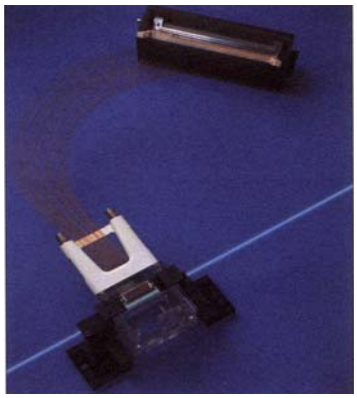
ABI PRISM® 3700 DNA Analyzer

96 active
eight reserve capillaries

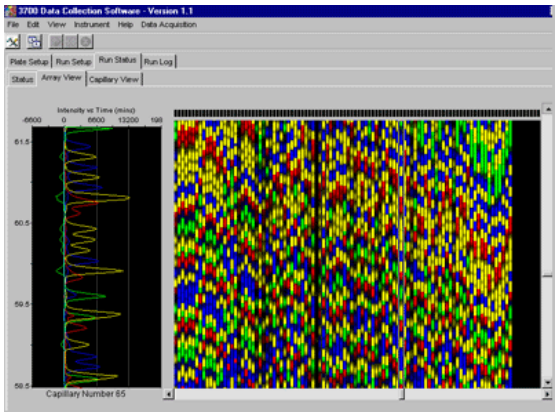


ABI PRISM® 3700 DNA Analyzer

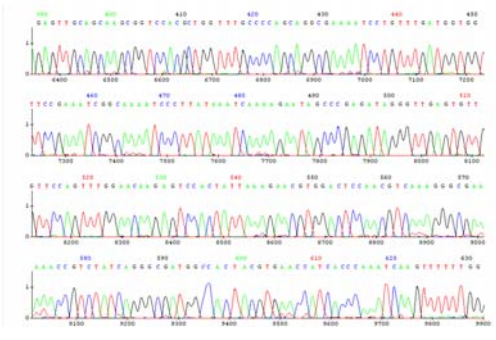
Sheath flow cuvette



ABI PRISM® 3700 DNA Analyzer



DNA sequencing record



PE Applied Biosystems

ABI PRISM 3700

accuracy > 98.5% to 550 base
96 samples per run in 3 hours
laser Ar-ion 488 and 514.5 nm
detection in sheath flow
concave spectrograph and cooled CCD

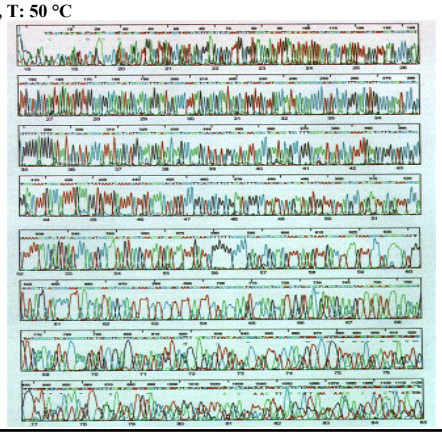
Molecular Dynamics

MEGABACE 1000

accuracy > 98.5% to 550 base
96 samples per run in 2 hours
laser Ar-ion 488 nm
energy transfer dyes
confocal scanning with 4 filters and 2 PMTs

DNA sequencing over 1000 bases in 1.5 hour

Separation matrix: LPA 2.0% (w/v) 5.5 MDa
E: 150 V/cm, T: 50 °C

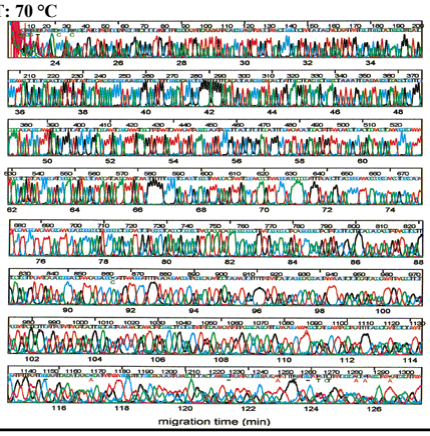


Barry L. Karger
Director, James L. Waters
Professor of Analytical Chemistry

The Barnett Institute
Northeastern University
Boston MA

DNA sequencing up to 1300 bases in 2 hours

Separation matrix: LPA 2.0% (w/w) 17 MDa, 0.5% (w/w) 270 kDa
E: 125 V/cm, T: 70 °C



DNA mutation analysis

Restriction (amplification) fragment legth polymorphism

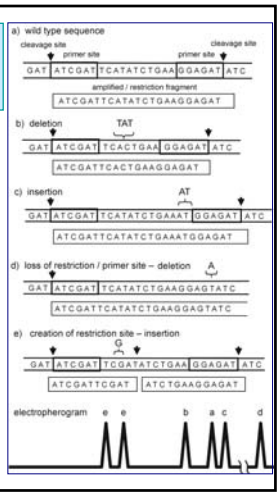
RFLP (AFLP)

Size based separation of ds or ss DNA fragments

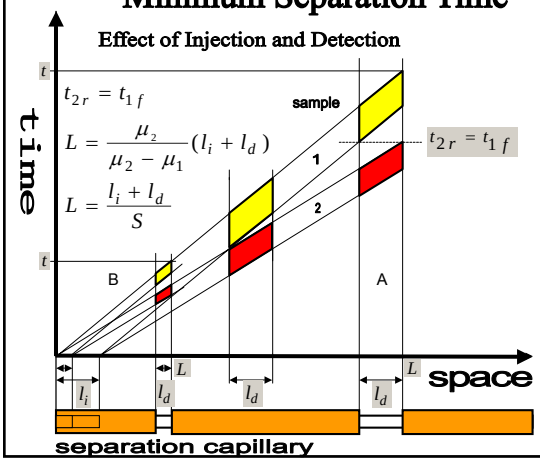
Resolution:

ss > 1000

ds > 400



Minimum Separation Time

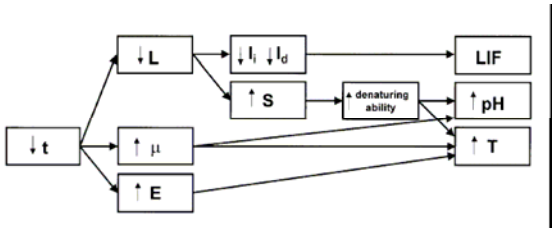


$t_{2r} = t_{1f}$

$L = \frac{\mu_2}{\mu_2 - \mu_1}(l_i + l_d)$

$L = \frac{l_i + l_d}{S}$

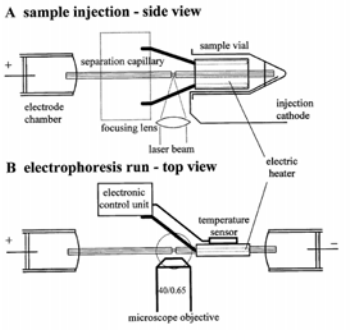
Optimization strategy in RFLP (AFLP) method



Miniaturized CE system with capillary heater and LIF detection

Capillary effective length

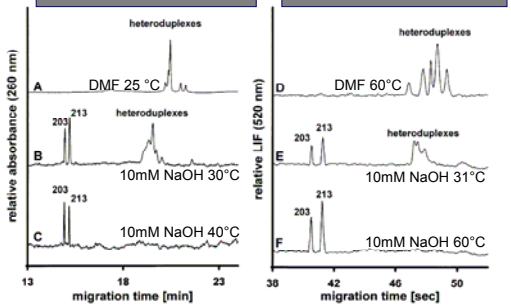
2.5 cm



Effect of detection, denaturing and temperature

AD
Capillary 30 cm
2% agarose
0.1 M Tris/TAPS + 7 M urea
173 V/cm

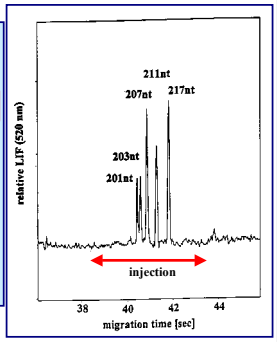
LIF
Capillary 2.5 cm
4% agarose,
0.1 M Tris/TAPS + 7 M urea
600 V/cm



Effective transient ITP stacking

Polymorphism of CT, CA, GC repeats in endothelin 1 gene

Multiplex PCR amplification:
heterozygote: 207, 217 and 201, 211
homozygote: 203
Denaturing: sample dissolved in 0.05 M NaOH
Separation medium: 4% agarose BRE (FMC)
in 0.1 M Tris -TAPS and 7 M urea
Fluorescent dye: SYBR Green II (492/513 nm)
Capillary: 2.5 (5) cm, 50 µm ID
Temperature: 60 °C
Electric field strength: 600 V/cm
Injection: 5 sec, 600 V/cm

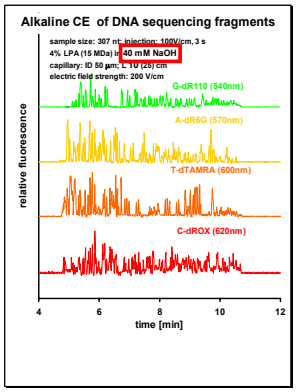


DNA denaturing

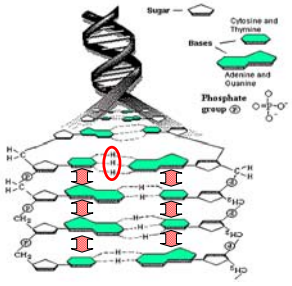
DNA separations under alkaline conditions

- effective denaturing - DNA sequencing
- fast separations - high effective charge of polyanions
- effective stacking - maximum mobility of OH^- ions
- compatibility - fluorescent labels and sieving media

Effective denaturing



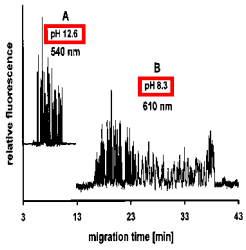
- Hydrogen bonding contributes little to the stability of the double helix.
- Hydrophobic forces largely stabilize DNA secondary structure.

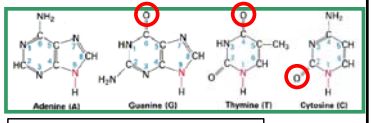


High effective charge of DNA fragments

Migration of DNA sequencing fragments at pH 12.6 and 8.3

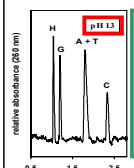
Sample: mixture of all fragments labeled with dideoxy terminators G-dR110 (545 nm), A-dR6G (570), T-dTAMRA (595), C-dROX (625)
Capillary: L 19.5 (25) cm, ID 50 µm, A) uncoated, B) PVA coating
4% LPA (15 MDa) in A) 0.04M NaOH, B) 0.1M Tris-Taps + 7M urea
30°C; 200 V/cm





Dissociation of DNA bases

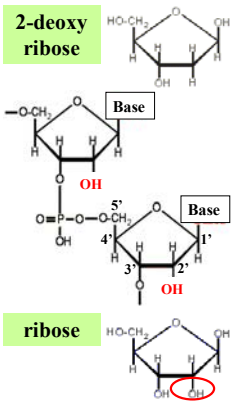
Sample: mixture of 10^{-4} M H, G, A, T, C in 0.1M NaOH
Capillary: ID 50 mm; L 4.6 (34.6) cm; bare fused-silica
Electrolyte: 0.1 M NaOH with 2% agarose (BRE-FMC)
Temperature: 40 °C; Electric field strength: 145 V/cm



		pKa'	
H	Hypoxanthine	8.94	12.10
G	Guanine	9.20	12.30
A	Adenine	9.80	
T	Thymine	9.90	13.00
C	Cytosine		12.50

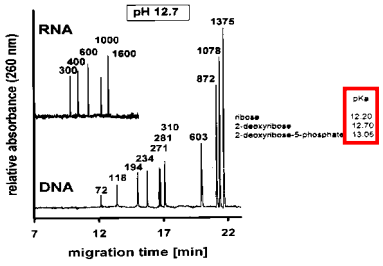
High effective charge of RNA fragments

2-deoxy ribose



Migration of RNA and DNA fragments under alkaline conditions

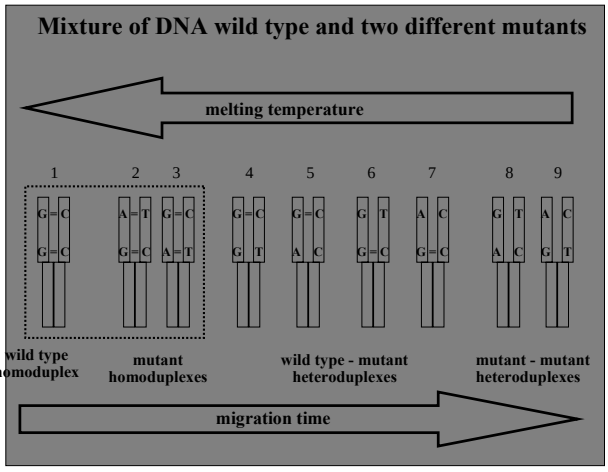
Sample: size standards RNA and DNA
Uncoated capillary: ID 50 µm; L 30.0 (34.6) cm
Electrolyte: 2% agarose in 0.05M NaOH
T: 40°C; E: 145 V/cm



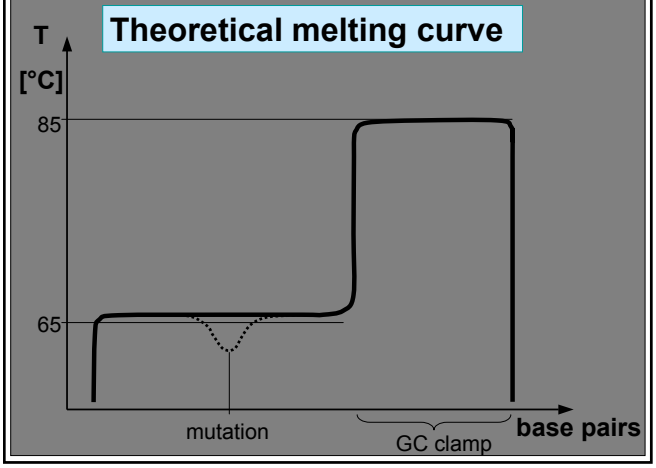
Constant Denaturant Capillary Electrophoresis

CDCE

Mixture of DNA wild type and two different mutants

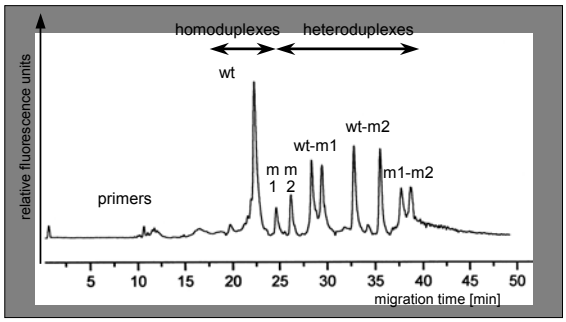


Theoretical melting curve



Mutation detection by CDCE

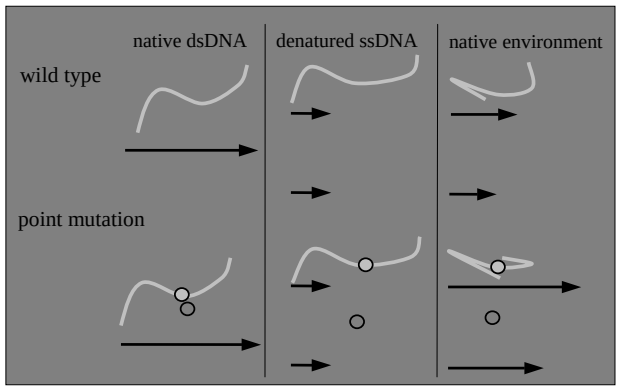
Fragments amplified on p53 gene
mutation: 2 substitutions A>G at different positions.
Separation conditions: 14% solution of LPA (180 kDa) in 50 mM Tris – TAPS buffer at 79°C; L_C – 55 cm; L_D – 40 cm; E – 360 V/cm.



Single Strand Conformation Polymorphism

SSCP

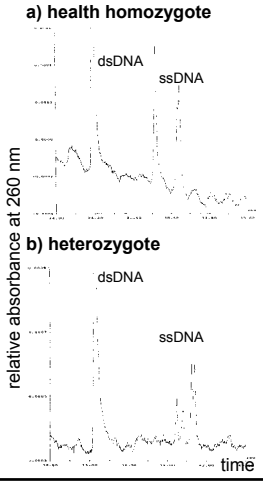
Principle of SSCP technique



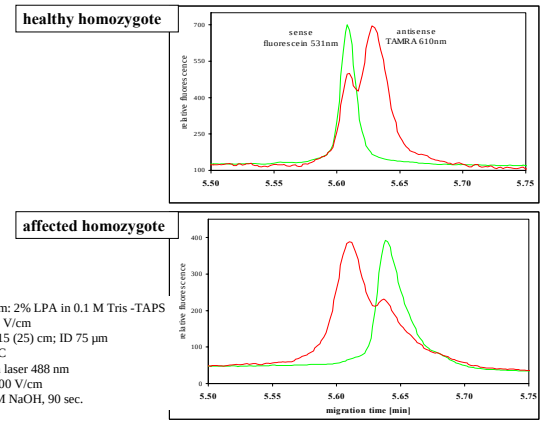
Phenylketonuria

SSCP analysis
Detection of point mutation C > T in phenylalanine hydroxylase gene on chromosome 12

Separation conditions:
2% solution of agarose SeaPrep in 1xTBE with 10% formamide
T - 30°C
LC - 55 cm
LD - 50 cm
E – a) 183 V/cm, b) 135 V/cm.



SSCP analysis: in-capillary alkaline denaturing



Separation medium: 2% LPA in 0.1 M Tris -TAPS
Electric field: 280 V/cm
Capillary: length 15 (25) cm; ID 75 µm
Temperature: 27°C
Excitation: Ar-ion laser 488 nm
Injection: 5 sec, 200 V/cm
Denaturing: 0.1 M NaOH, 90 sec.

Single Strand Conformation Polymorphism (SSCP)

Cystinuria type I

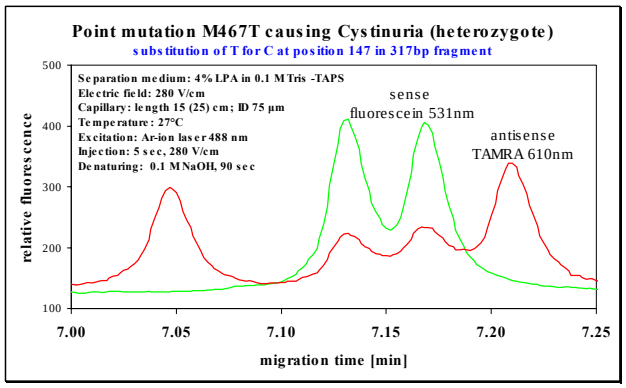
A disorder of amino acid transport characterized by a renal cystine transport defect resulting in urinary tract calculus disease.

Genome: The most frequent is point mutation M467T, a substitution of Thymine for Cytosine in position 1400 in SLC3A1 gene on short arm 2p of human chromosome 21.

Proteome: The mutation causes the substitution of Methionin for Threonin in position 467 of transport protein rBAT, a 90 kDa glycoprotein.

Sample: PCR fragment of 317 bp. The mutation is in position 146. Sense primer is labeled by fluorescein (520 nm) on 5´-end. Antisense primer is labeled by TAMRA (610 nm) on 5´-end.

SSCP analysis: in-capillary alkaline denaturing



Comparison of migration order with theoretical structures

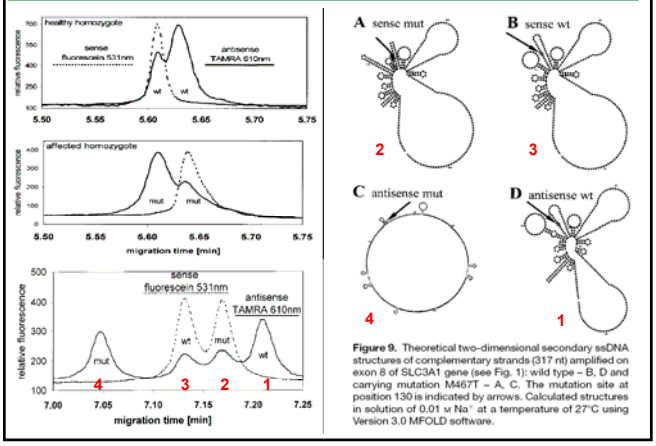
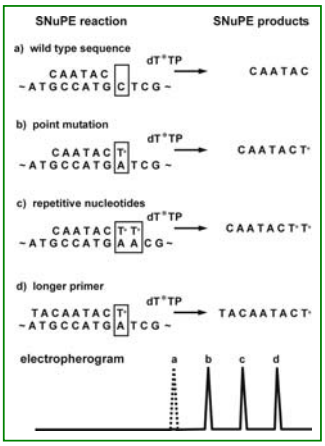


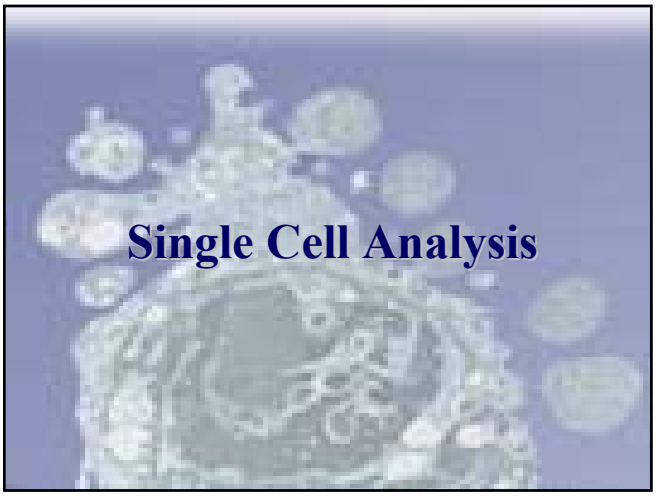
Figure 9. Theoretical two-dimensional secondary ssDNA structures of complementary strands (317 nt) amplified on exon 8 of SLC3A1 gene (see Fig. 1): wild type – B, D and carrying mutation M467T – A, C. The mutation site at position 130 is indicated by arrows. Calculated structures in solution of 0.01 M Na^+ at a temperature of 27°C using Version 3.0 MFOLD software.

Single nucleotide primer extension

Minisequencing

SNuPE

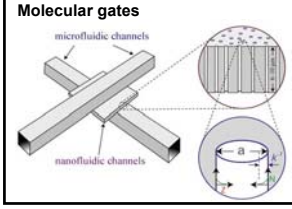


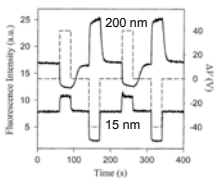


Jonathan V. Sweedler
Department of Chemistry
University of Illinois
Urbana, IL

Director of the Biotechnology Center
Associated with the Beckman Institute, Biotechnology Center, Neuroscience Program and Bioengineering Program

Molecular gates





Single cell analysis

Typical eucaryotic somatic cell

diameter: 5 - 10 µm

volume: 500 fl

total mass: 500 pg

DNA

nucleus: 20 % of cell mass

DNA mass: 5 pg (MCF 7 cells)

Proteins

10% of cell mass = 2 fmol

10 000 proteins expressed

conc. of a protein: 200 zmol

(1 zmol = 600 copies)

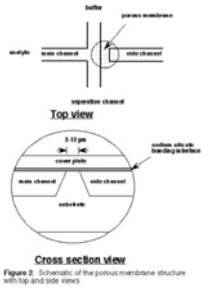
Microtechnologies

"lab-on-chip"

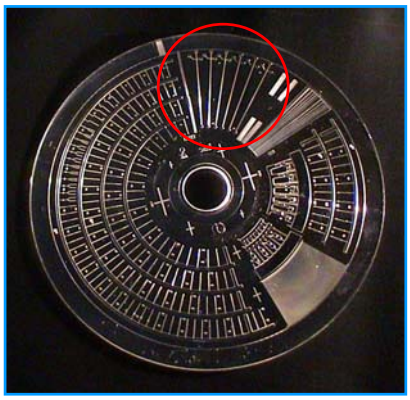
Prof. Dr. Andreas Manz
Head of the ISAS
Dortmund

J. Michael Ramsey
Minnie N. Goldby Distinguished Professor of Chemistry

Department of Chemistry
The University of North Carolina at Chapel Hill
Chapel Hill, NC USA



CD microfluidic device - Gyros AB



Confocal microscope detection system

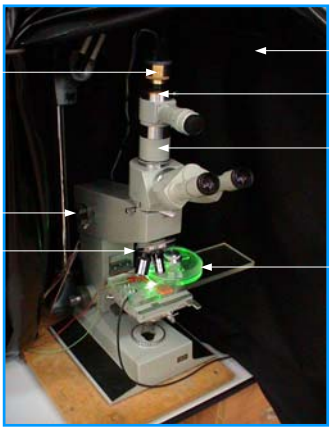
PMT

CCD camera

laser 532 nm

objective 25 x

0.65



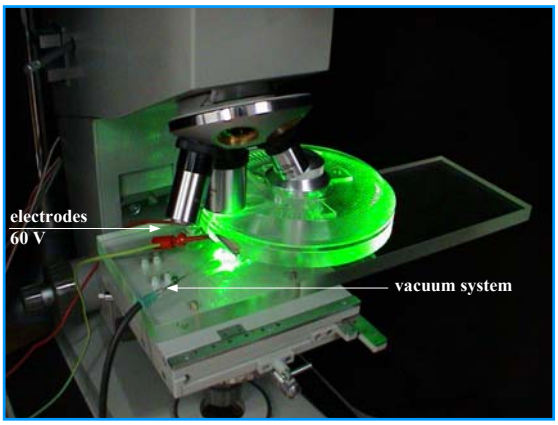
lightproof curtain

610 nm BP

550 nm LP

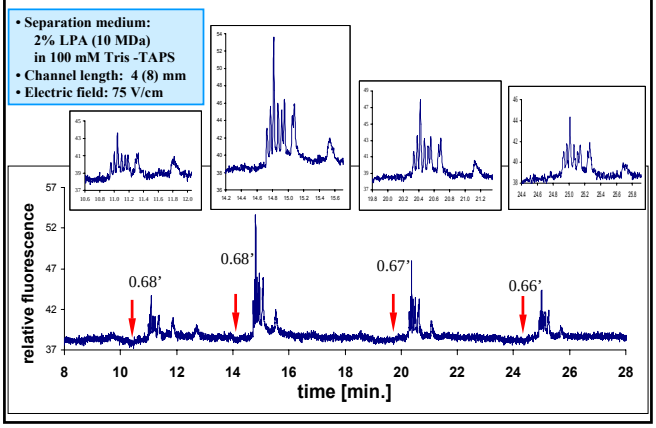
CD assembly

CD device assembly and detection system

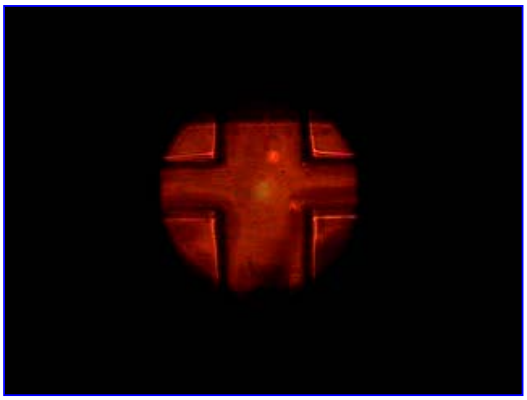


Separation of DNA restriction fragments

- Separation medium: 2% LPA (10 MDa) in 100 mM Tris -TAPS
- Channel length: 4 (8) mm
- Electric field: 75 V/cm



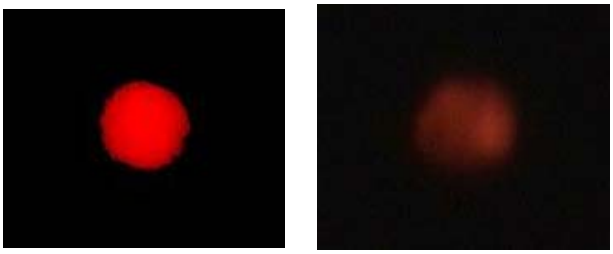
Microfluidics - cell handling



Alkaline cell lysis

Intact cell nucleus

Cell lysis
in 0.1 M NaOH after 2 min



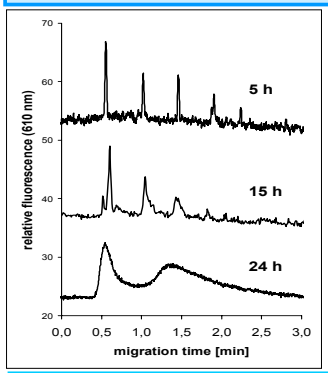
Cardiomyocyte cell nucleus stained by ethidium bromide
LIF microscopy: excitation - 532 nm; emission - 610 nm

Alkaline cell lysis

Cl^- are beeing replaced by OH^-

Electric field strength

DNA fragmentation in apoptotic cardiomyocytes treated by doxorubicin (2 µg/ml)



Cell lysis: 3 min

Electrophoresis:
2 % LPA (10 MDa) + 0.1 M NaOH
electric field strength 60.2 V/cm
temperature 25 °C

Separation channel:
10 mm x 50 µm x 20 µm
effective migration distance 6 mm

LIF detection:
excitation 532 nm
emission 610 nm
DNA stained by EtBr

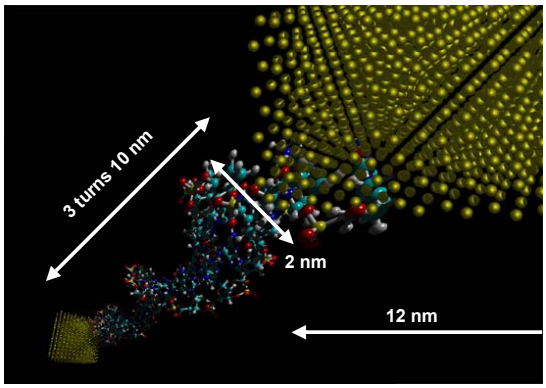
K. Klepárník, M. Horký, Electrophoresis 2003, 24, 3778 - 3783.

Nanotechnologies

"cell as a test tube"

Single molecule detection

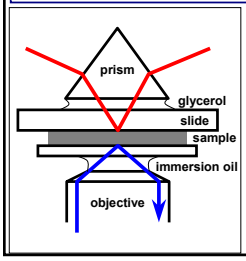
Gold nanoparticles linked by DNA molecule



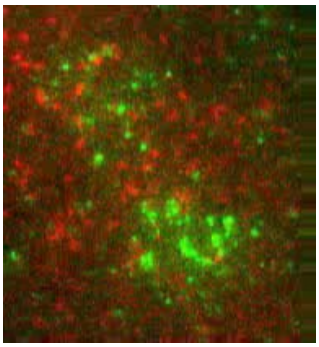
Single molecule imaging

Membrane proteins
CD58-Cy3 (*green*)
ICAM-1-Cy5 (*red*)
in a glass-bound planar phospholipid bilayer
under two PMA/ionomycin-treated Jurkat cells.

Total Internal Reflection Fluorescence Microscopy



Disadvantages:
- Photo bleaching
- Molecules at the cell surface
- Two lasers: 514, 633 nm



Parallel single molecule sequencing by synthesis

Helicos

The HeliScope™ Sequencer

2 . 10⁹ b/day

10⁹ reads/run
25 – 55 bp read lengths

454 LIFE SCIENCES

Genome Sequencer FLX System

3 . 10⁸ b/day

100 Mb/7.5 hour run
400 000 reads/7.5 hour
200 – 300 bp read lengths

Solexa

Illumina Genome Analyzer

6 . 10⁸ b / day

3 . 10⁹ b / 5 days run
50 . 10⁶ oligo clusters
36 – 50 bp read lengths

The HeliScope™ Sequencer

Photocleavable dideoxy nucleotides

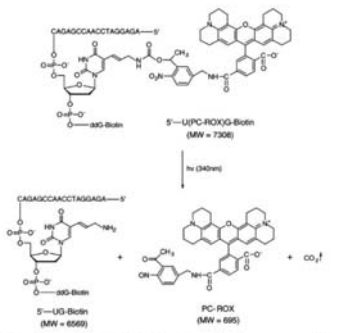


Fig. 2. DNA product 5'-U(PC-ROX)G-biotin, formed by incorporating a dUTP-PC-ROX into a primer in a polymerase reaction and its photocleavage, producing DNA fragment 5'-UG-biotin and PC-ROX. MW, molecular weight.