NGS:
technologies and challenges

Olga Vinnere Pettersson, PhD
Project Coordinator
NGI/SciLifeLab, Uppsala Node

@OlgaVPettersson

Version 8.4

Outline

- INTRO: Sequencing service at NGI-SciLifeLab
- NGS general knowledge: current technologies
- NGS Challenges:
  - Sequencing artefacts
  - NGS sample quality requirements
- Next global challenge: the Earth Biogenome Project

NATIONAL GENOMICS INFRASTRUCTURE

NATIONAL GENOMICS INFRASTRUCTURE

SciLifeLab

We are non-profit

We have technology and knowledge

We want to help you to do GREAT research

We do not want co-authorship

Let us help YOU

NGI Support

Pre-sequencing

- **Project design** via discussions with users
- **Advise** in sample collection and preparation
- Case-to-case **DNA extraction service**

Post-sequencing:

- Control over produced data: making sure data meet our **high standards** in terms of quality and yield.
- Primary **analysis of human genomes** is enabled
- **Genome assembly** of PacBio data is offered as a service
- Data is delivered to **UPPMAX** (Uppsala Multidisciplinary Center for Advanced Computational Science)

Collaborative projects for technology and method development

Education

Current Technologies

illumina®

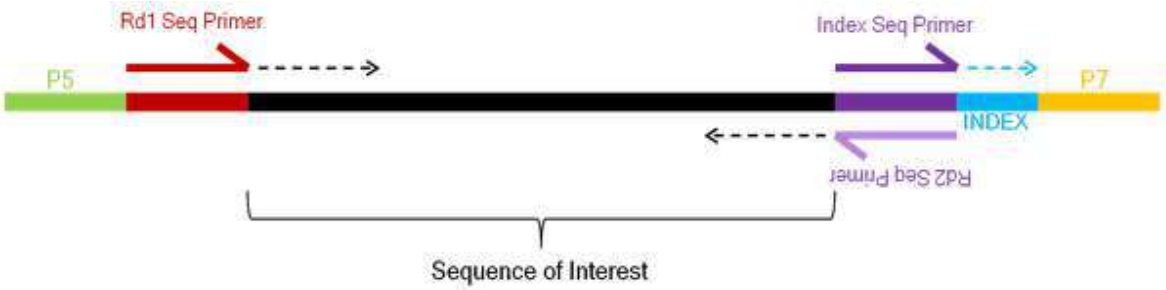
Current leader on the NGS market

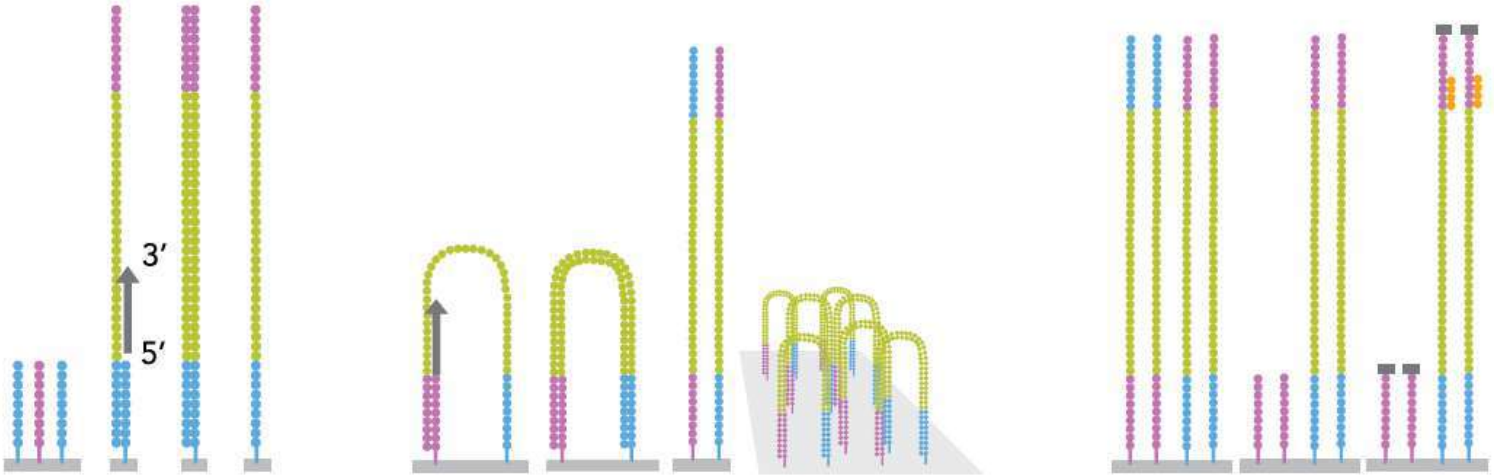
Instrument	Run time	Max output	Max reads/run	Max read length
iSeq	9.5 – 19 hrs	1.2 Gb	4 mln	PE 150
MiniSeq	4-24 hrs	7.5 Gb	25 mln	PE 150
MiSeq	4-55 hours	15 Gb	25 mln	PE 300
NextSeq series	12-48 hours	120-300 Gb	0.4 – 1 bln	PE 150
NovaSeq 6000	13-44 hours	6 Tb	20 bln	PE 250

RIP: HiSeq 2500 & HiSeq X

Used for everything

Illumina: bridge amplification





ThermoFisher SCIENTIFIC

Ion S5 XL

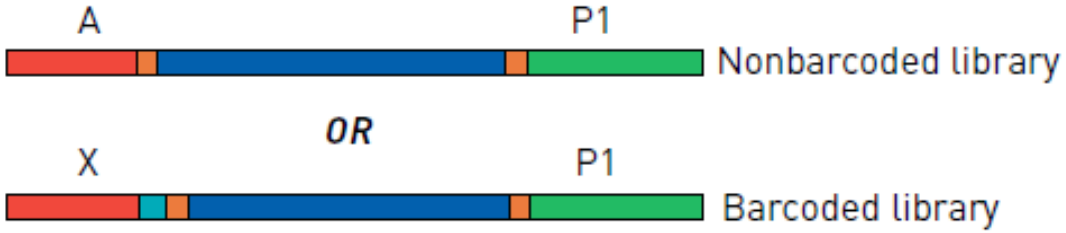
Chip:	Run time	Output	Max reads/ run	Max read length
510	2.5-4 hrs	0.3 - 0.5 Gb	2-3 mln	SE 400 bp
520	2.5-4 hrs	0.6-2 Gb	3-6 mln	SE 600 bp
530	2.5-4 hrs	3-8 Gb	15-20 mln	SE 600 bp
540	2.5-4 hrs	10-15 Gb	60-80 mln	SE 400 bp
550	2.5-4 hrs	18-20 Gb	100-130 mln	SE 200 bp

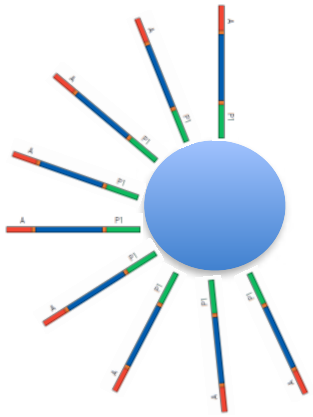
RIP: IonTorrent PGM, IonProton

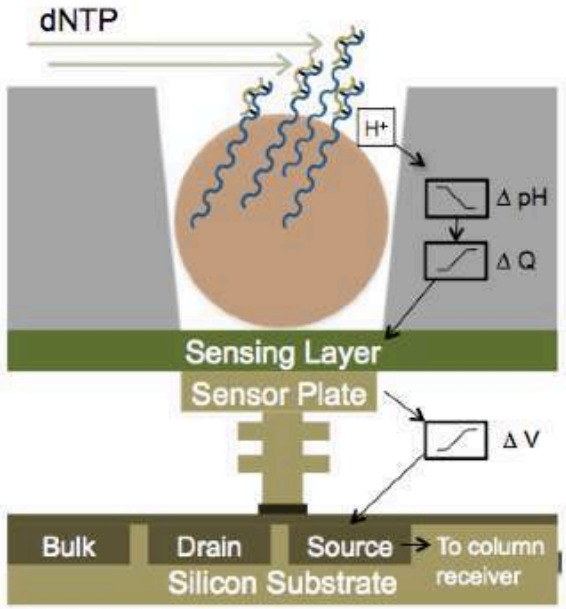
Clinical applications mainly
Standard analysis directly on the instrument

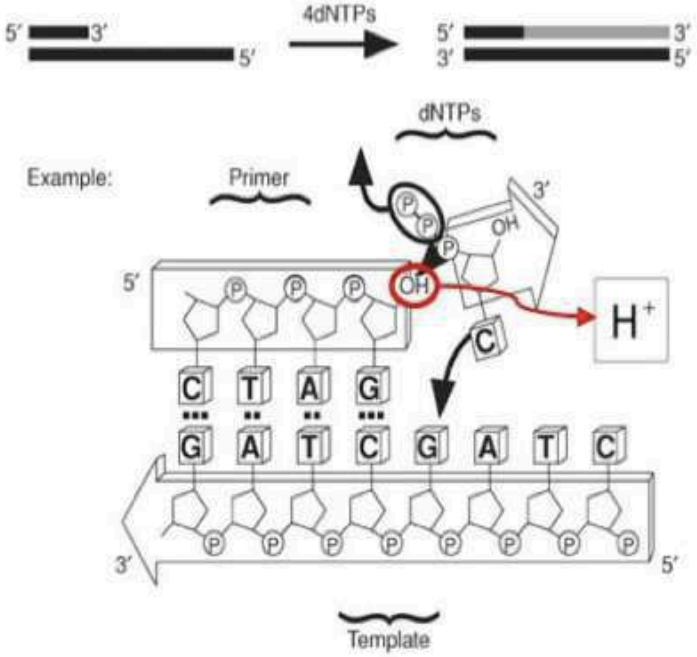
Multiplex-PCR panels

Ion Torrent: H^+ ion-sensitive field effect transistors

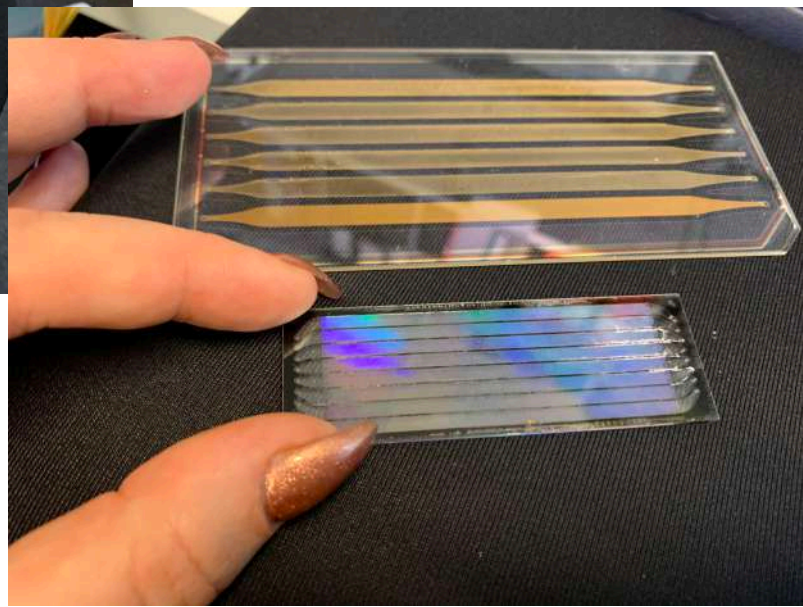




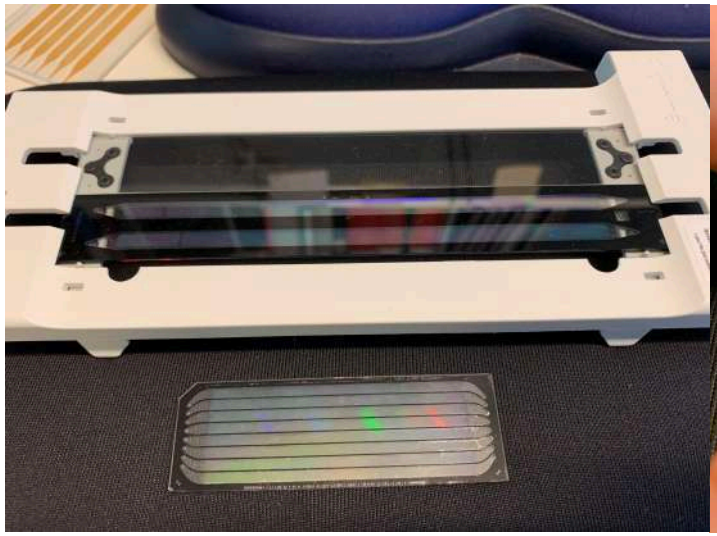


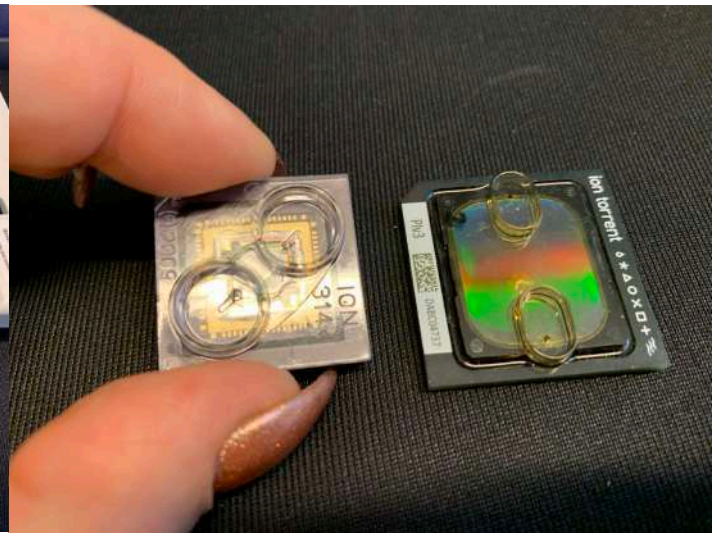


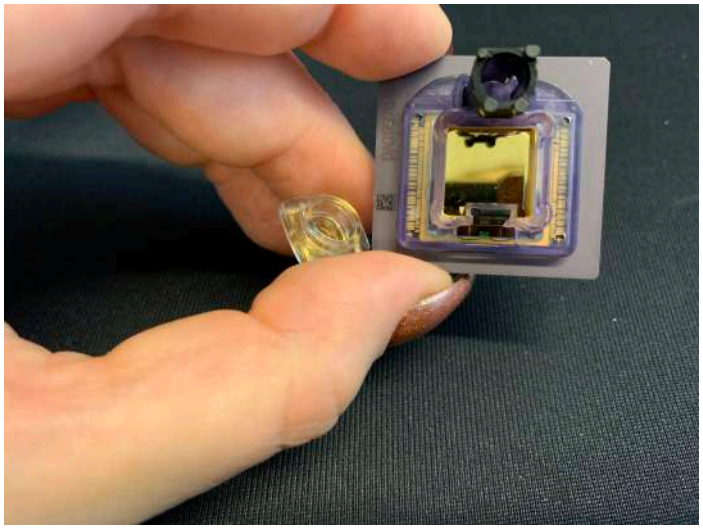
How it looked yesterday

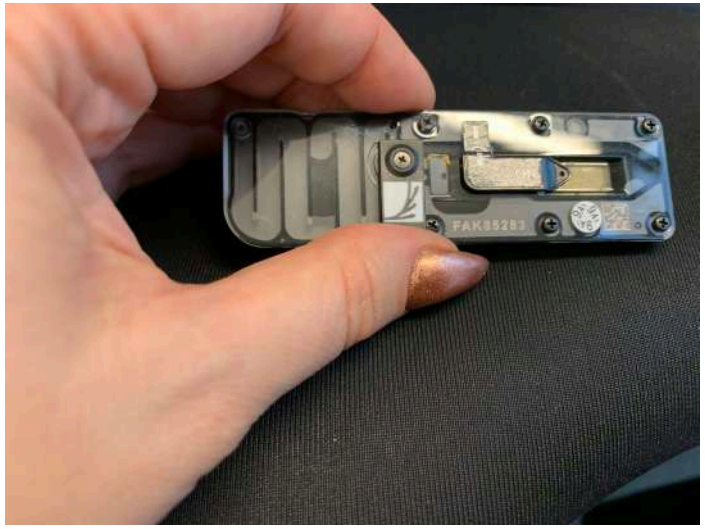


How it looks like now









PACBIO®

Instrument	Run time /SMRT	Output /SMRT	Max reads / SMRT	Max read length*
RSII	30 min – 6 hrs	500 Mb – 2 Gb	50 000	40 kb
Sequel	30 min – 20 hrs	2 – 35 Gb	200 000	60 kb
Sequel II				
HiFi	30 hrs	*320 Gb*	*4 mln*	20 kb
CLR	15 hrs	*300 Gb*	*3 mln*	120 kb

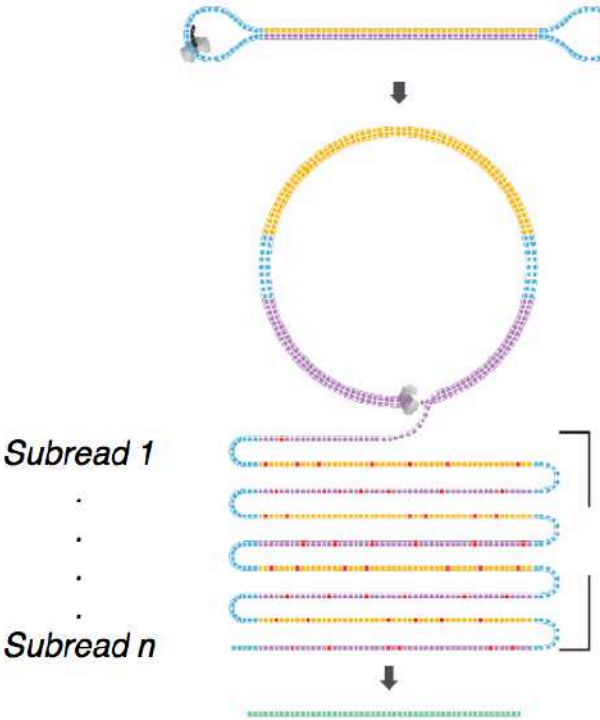
Single Molecule Real Time sequencing: SMRT

PacBio

TWO MODES OF SMRT SEQUENCING

Circular Consensus Sequencing (CCS) Mode

Inserts 10-20 kb



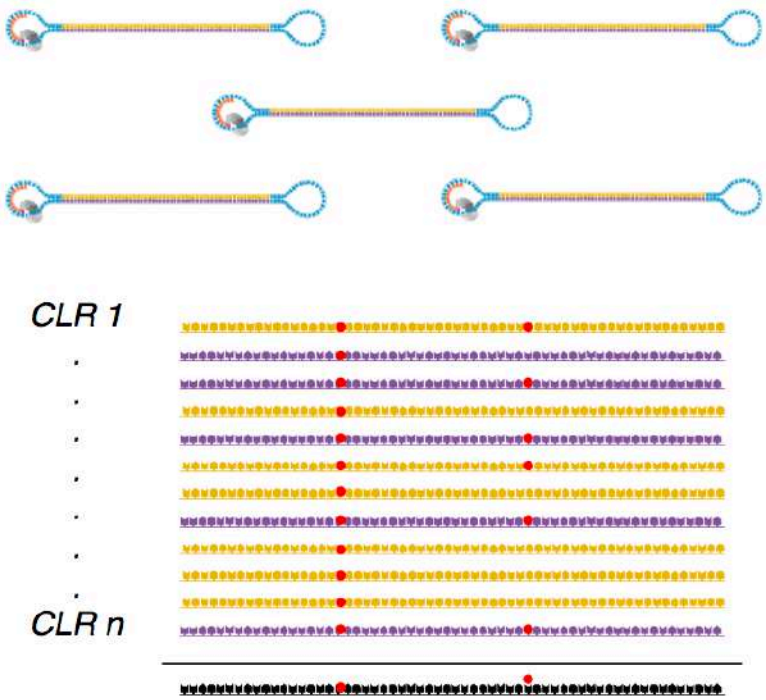
Qv 20+

HiFi READS

Single-molecule consensus sequence

Continuous Long Read (CLR) Sequencing Mode

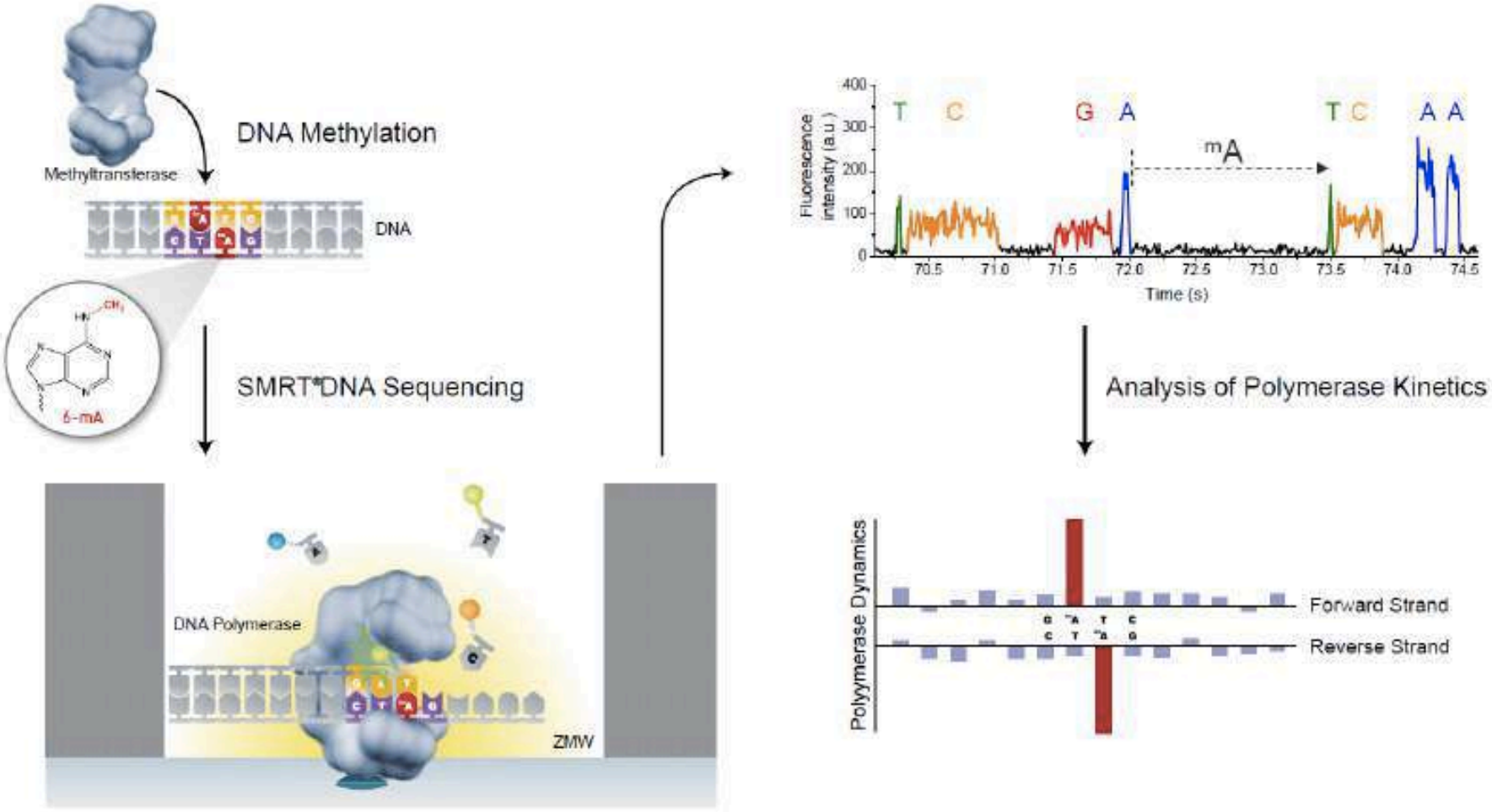
Inserts >25 kb, up to 175 kb



LONG READS

Multi-molecule consensus sequence

Base Modification: Discover the Epigenome



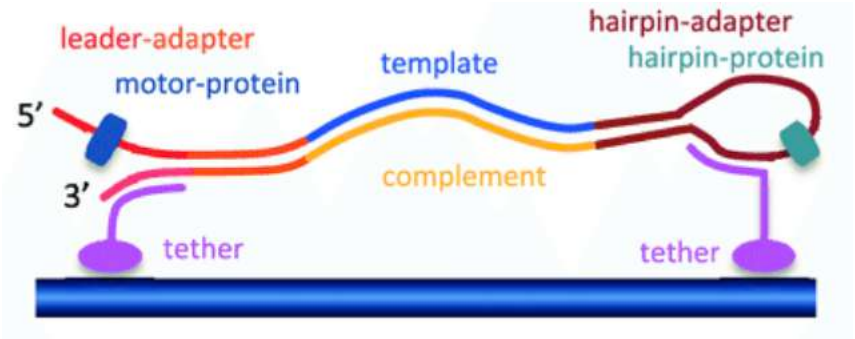
Detect base modifications using the kinetics of the polymerization reaction during normal sequencing

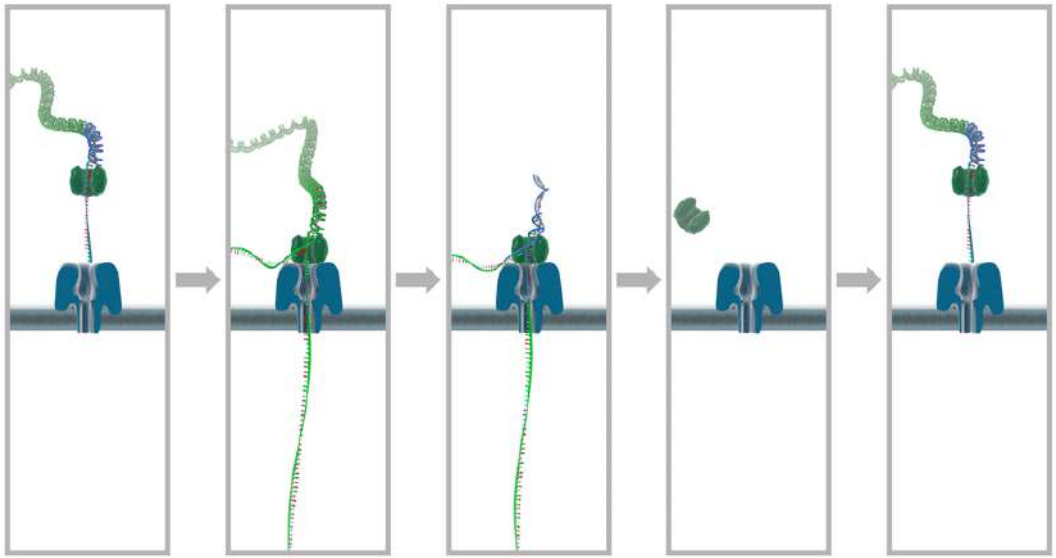
Oxford NANOPORE™ Technologies

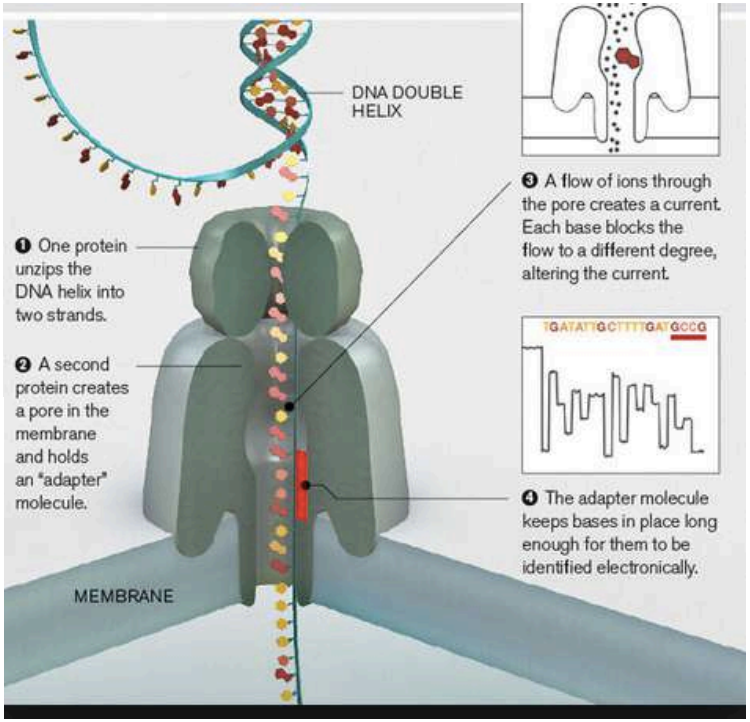
Instrument	Run time /FC	Output / FC	Nr of pores	Max read length
Flongle	16 hrs	1 Gb	126	1 Mb
MinION	24 hrs	2-15 Gb	512	1 Mb
GridION	24 hrs	2-15 Gb	512	1 Mb
PromethION	72 hrs	10 – 150 Gb	3 000	2 Mb

Q&A: *”It depends”…*

ONT: DNA + Motor + Pore







Main advantages of ONT: SPEED and PORTABILITY

Rapid Confirmation of the Zaire Ebola Virus in the Outbreak of the Equateur Province in the Democratic Republic of Congo: Implications for Public Health Interventions

Placide Mbala-Kingebeni, Christian-Julian Villabona-Arenas, Nicole Vidal, Jacques Likofata, Justus Nsio-Mbeta, Sheila Makiala-Mandanda, Daniel Mukadi, Patrick Mukadi, Charles Kumakamba, Bathe Djokolo ... Show more

Clinical Infectious Diseases, Volume 68, Issue 2, 15 January 2019, Pages 330–333, https://doi.org/10.1093/cid/ciy527

Published: 29 June 2018 **Article history** ▾

ORIGINAL ARTICLE BRIEF REPORT

A Novel Coronavirus from Patients with Pneumonia in China, 2019

Na Zhu, Ph.D., Dingyu Zhang, M.D., Wenling Wang, Ph.D., Xinwang Li, M.D., Bo Yang, M.S., Jingdong Song, Ph.D., Xiang Zhao, Ph.D., Baoying Huang, Ph.D., Weifeng Shi, Ph.D., Roujian Lu, M.D., Peihua Niu, Ph.D., Faxian Zhan, Ph.D., et al., for the China Novel Coronavirus Investigating and Research Team

RESEARCH ARTICLE | Full Access

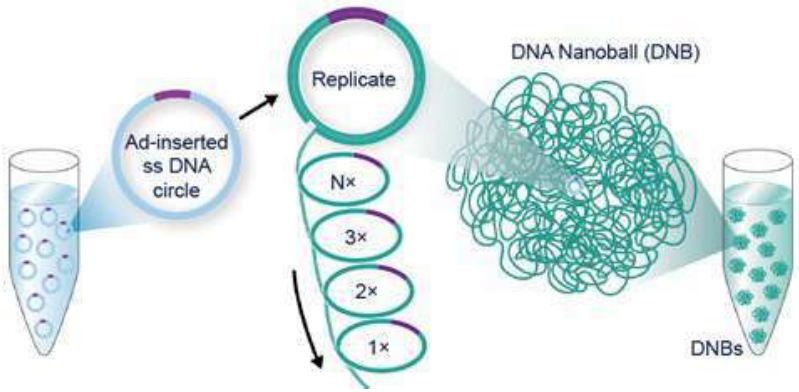
Semi-quantitative characterisation of mixed pollen samples using MinION sequencing and Reverse Metagenomics (RevMet)

Ned Peel, Lynn V. Dicks, Matthew D. Clark, Darren Heavens, Lawrence Percival-Alwyn, Chris Cooper, Richard G. Davies, Richard M. Leggett, Douglas W. Yu

First published: 15 July 2019 | https://doi.org/10.1111/2041-210X.13265

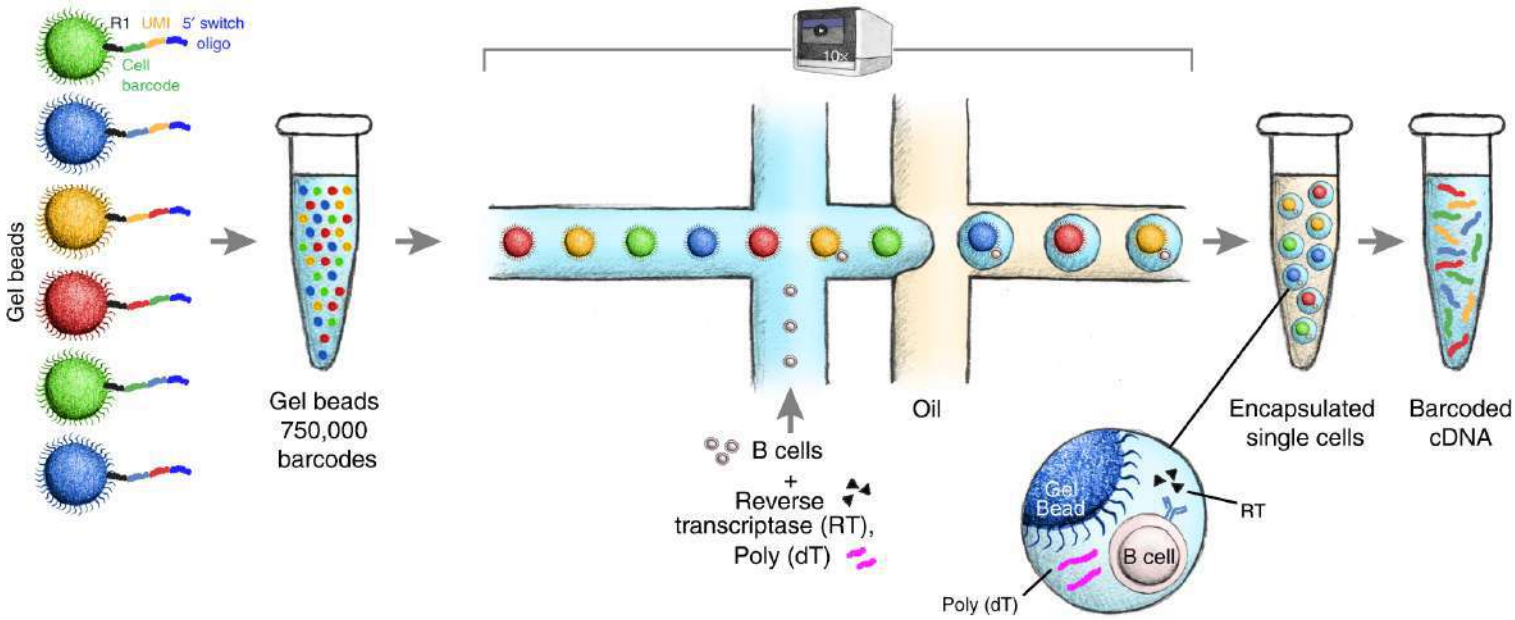
Other technologies & methods

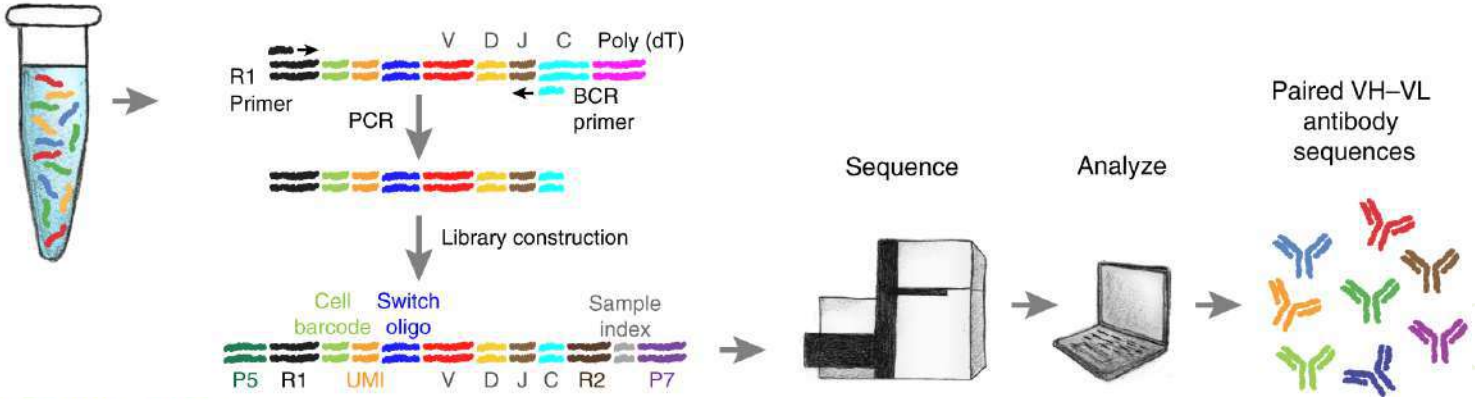
New Sequencing technology coming to NGI for *evaluation*:



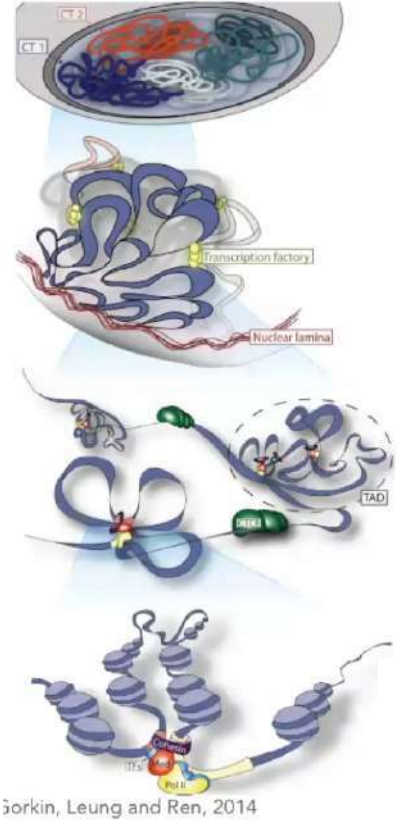
Output similar to Illumina

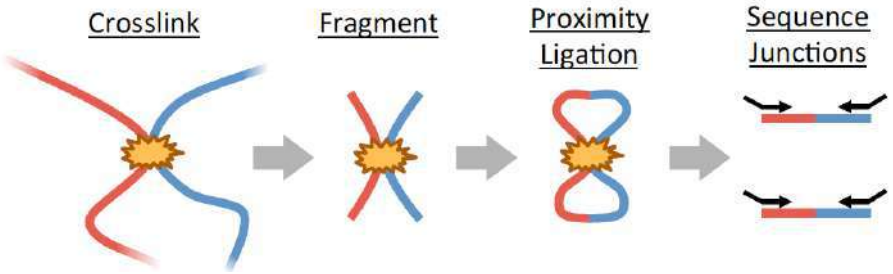
10x Genomics (Chromium)

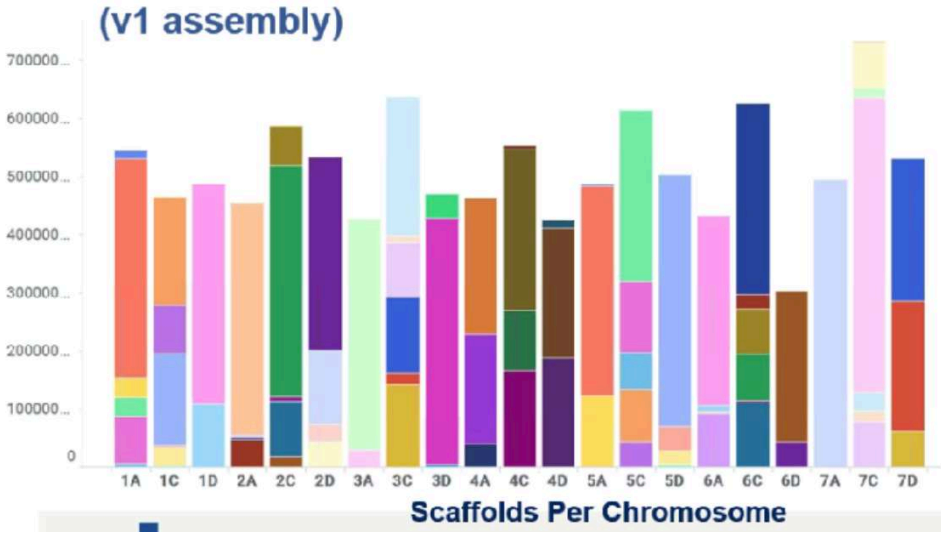


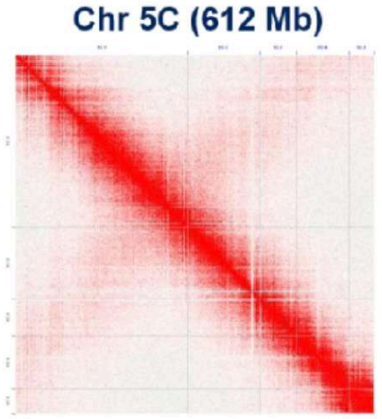


Hi-C / OmniC: linking reads to chromosomes









Start with a tissue!

Capture DNA bound to the same nucleosome

Make a library and sequence on Illumina NovaSeq

Technologies and Applications at NGI

NGS technologies

Short read NGS

Whole genome re-sequencing
RNA-seq
Exome
Targeted re-seq
Panels
Amplicons up to 600 bp

Long-read NGS

De novo genome sequencing
Whole-transcript sequencing
Structural variant resolving
Allele phasing
Targeted re-seq
Amplicons up to 13 kb

Research and development

NGS Technologies: SUMMARY

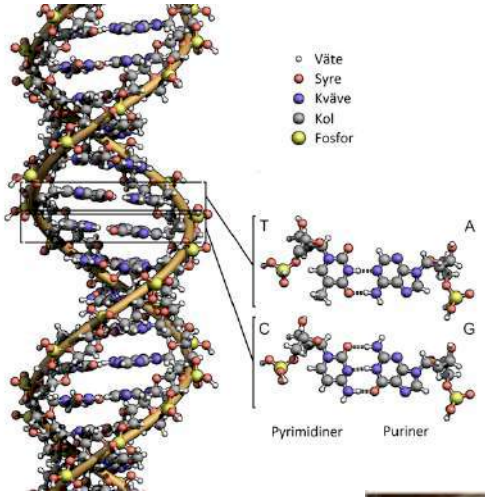
- Development goes VERY FAST
- All technologies have their PROs and CONs
- One technology does not suit all the applications
- In some projects, several technologies should be combined

Making sense of genomics data:

Understanding sequencing bias

Sequencing artefacts: what are they?

Sequencing a representative, completely randomized subsample:
it starts with input material



What textbook tells you

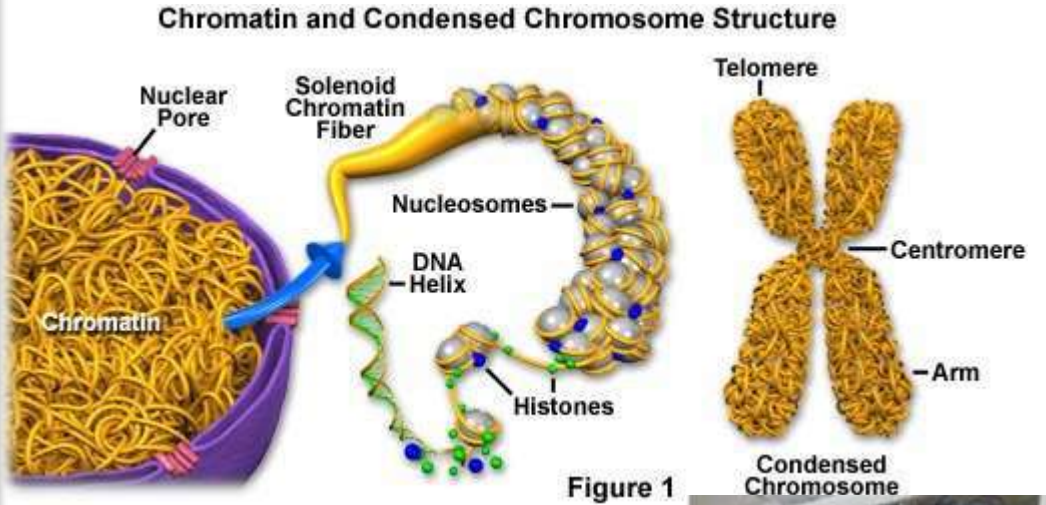


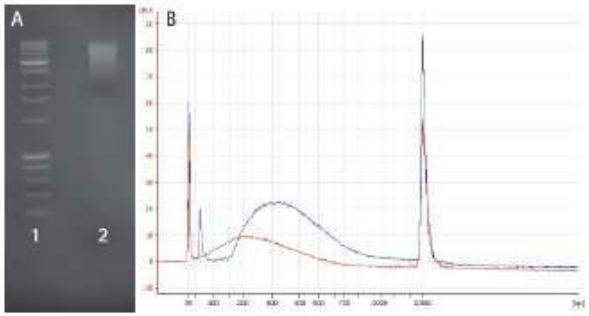
Figure 1

Brutal reality

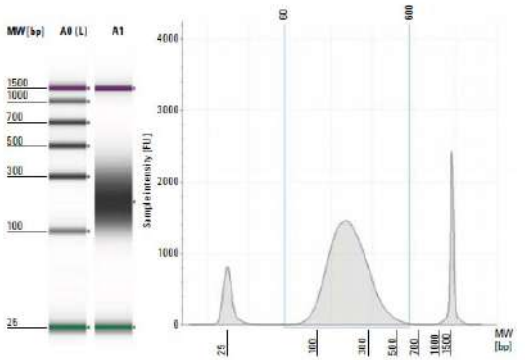
Do not forget:
DNA in solution behaves differently

Sequencing artefacts: what are they?

Sequencing a representative, completely randomized subsample:
continues with library preparation

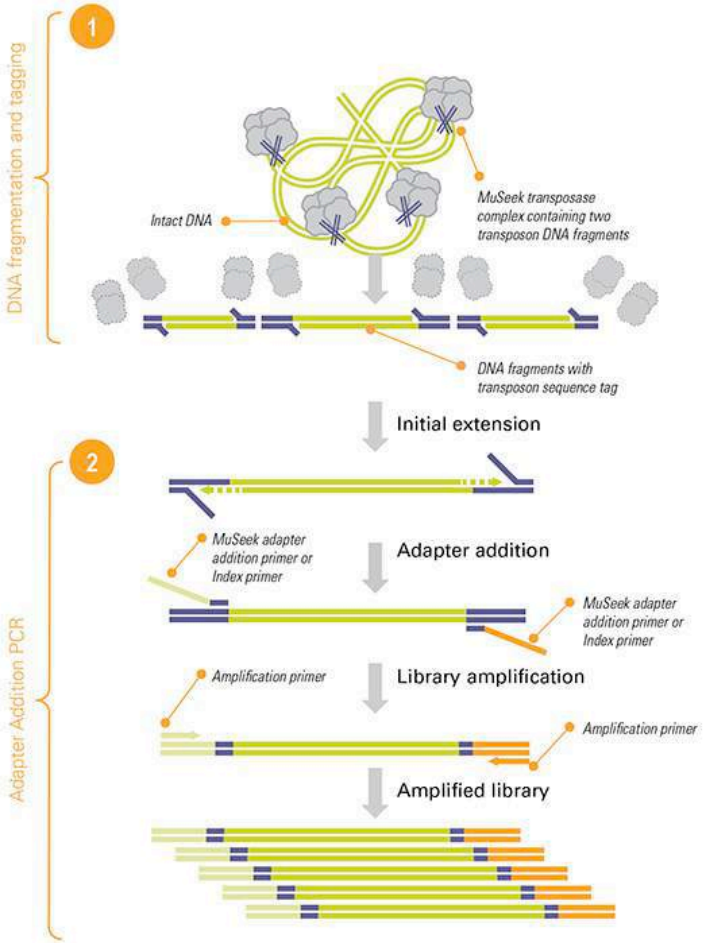


Input sample



Shearing and size-selection

Loosing molecules all the way



Less material -> more amplification cycles

Sequencing artefacts: what are they?

PCR bias – important source of sequencing artefacts

PCR steps involved in any NGS but PacBio and Oxford Nanopore:

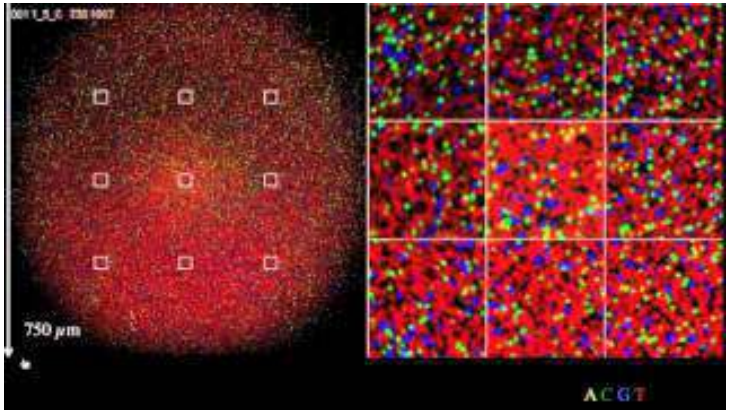
1. Library amplification
2. Amplification during templating (Illumina – on glass; Ion – emPCR)

Main PCR bias:

1. Size: shorter fragments amplify faster -> higher sequencing signal and coverage
2. Polymerase errors
   - slippage in low complexity regions
   - incorporation of erroneous bases & indels
3. GC-bias (fragments with high GC diminish to 1/10th from initial amount)

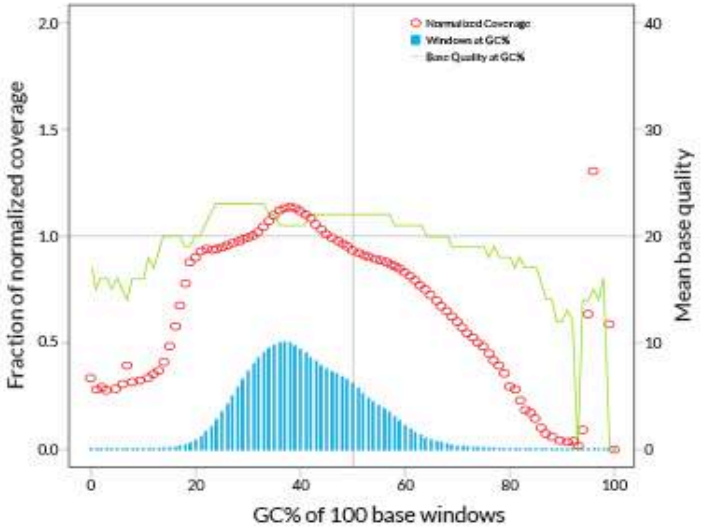
Sequencing artefacts: what are they?

PCR bias – important source of sequencing artefacts

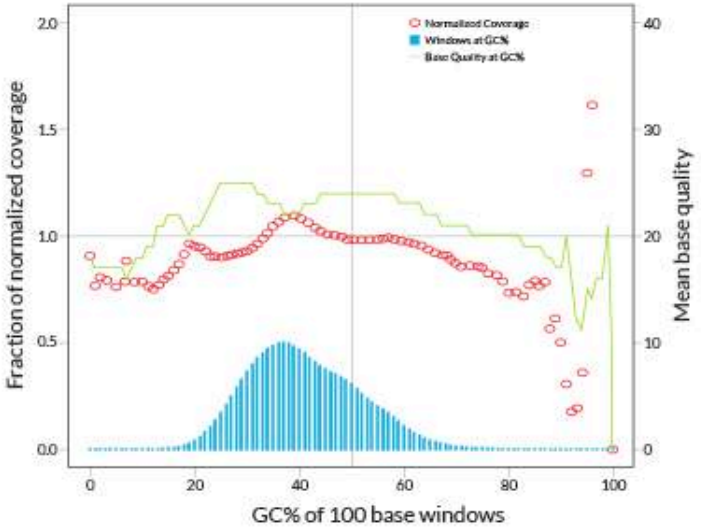


Clusters with shorter fragments grow faster -> quality signal from smaller clusters worsens

GC bias & genome coverage



Heavily amplified library

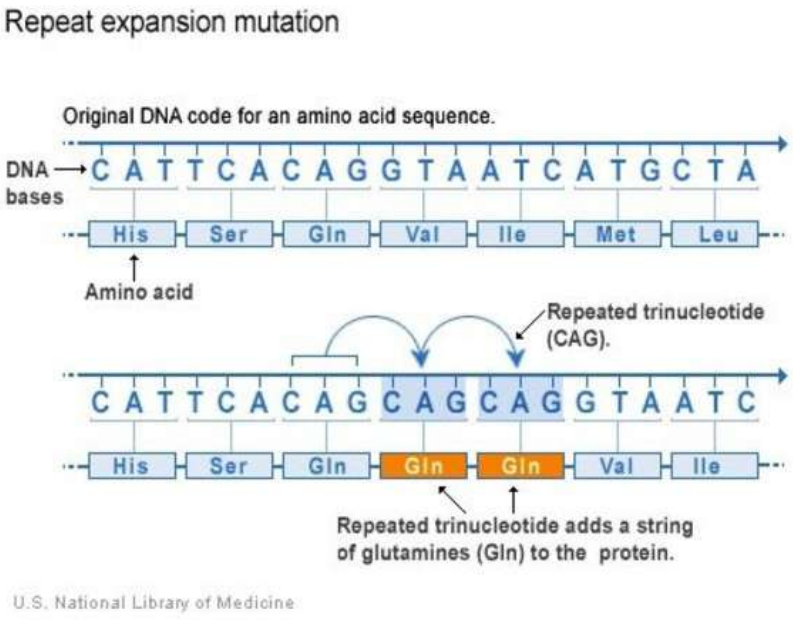


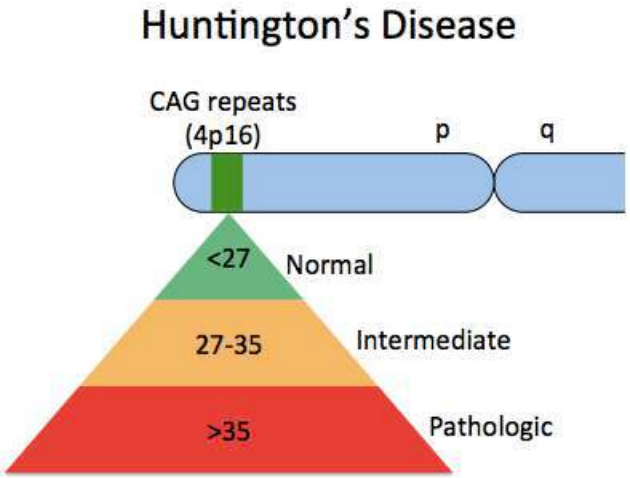
PCR-free library

Sequencing artefacts: what are they?

PCR bias – important source of sequencing artefacts

Polymerase slippage – low complexity regions





Huntington's disease:

- Inherited disorder resulting in brain cell death
- Decline of motoric and cognitive functions
- Common onset: 30-50 years of age
- No cure
- Causative genetic variant: CAG-repeat expansion in *HTT* gene

Sequencing bias: SUMMARY

- Keep in mind that they are there
- Coverage varies across the genome
- One technology does not suit all the applications

BREAK

SAMPLE QUALITY REQUIREMENTS

Garbage in – garbage out:

Sequencing success always depends on the **sample quality**.

NGS-quality DNA and
PCR-quality DNA
are two completely different things.

THE NUCLEIC ACIDS

Chemistry and Biology

Edited by

ERWIN CHARGAFF
Department of Biochemistry
Columbia University
New York, N. Y.

J. N. DAVIDSON
Department of Biochemistry
University of Glasgow
Glasgow, Scotland

Volume I

a. Extraction with Strong Salt Solution. Deproteinization with Chloroform

(1) *Sodium Deoxyribonucleate of Calf Thymus.*[98] Fresh frozen calf thymus glands (54.5 kg.) were minced and suspended in 0.9% sodium chloride (54 l.) and milled to produce a fine suspension. This suspension was centrifuged (6300 r.p.m.) and the solid material resuspended in 0.9% sodium chloride (45.5 l.) and milled and centrifuged as before. The tissues, which were now free of material containing pentose, were suspended in 10% sodium chloride (214 l.) with vigorous mechanical stirring at 0°. At this stage the viscosity of the solution increased considerably. After extraction at 0° for 48 hours, the insoluble material was removed by centrifuging (6300 r.p.m.) and the deoxypentose nucleoprotein precipitated from the resultant solution (pH 6.5) by the addition of an equal volume of industrial methanol. The precipitated solid was washed with 70%, then 100% industrial methanol and dried in a vacuum at room temperature. Yield, 1.69 kg. of a very slightly yellow fibrous solid.

THE PREPARATION OF DEOXYRIBONUCLEIC ACIDS BY THE *p*-AMINOSALICYLATE–PHENOL METHOD

K. S. KIRBY
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London (Great Britain)

(Received February 17th, 1959)

A general method for isolation of high molecular weight DNA from eukaryotes

Nikolaus Blin and Darrel W. Stafford

Department of Zoology, University of North Carolina, Chapel Hill, NC 27514, USA

Received 24 June 1976

ABSTRACT

A new method for isolation of high molecular weight DNA from eukaryotes is presented. This procedure allows preparation of DNA from a variety of tissues such as calf thymus or human placenta and from cells which were more difficult to lyse until now (e.g. Crypthecodinium cuhnii, a dinoflagellate). The DNA obtained in such a way has an average molecular weight of about 200 x 10^6 d and contains very few, if any, single strand breaks.

INTRODUCTION

Isolation of large quantities of nick-free, high molecular weight DNA from eukaryotic organisms has heretofore presented considerable technical difficulties. DNA prepared by conventional techniques has been a heterogeneous population of molecules ranging in molecular weight from 10 x 10^6 to 20 x 10^6 d (1, 2). The single strand molecular weight was often around

1983: P C R

ELSEVIER

Journal of Microbiological Methods
Volume 19, Issue 3, March 1994, Pages 167-172

A general method for the extraction of DNA from bacteria

Michael W Lema, Arnold Brown, Jo H Calkins

Show more

https://doi.org/10.1016/0167-7012(94)90066-3

Protocol | Published: November 1990

A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue

Thomas H. Tai & Steven D. Tanksley

Plant Molecular Biology Reporter **8**, 297–303(1990) | Cite this article

A simple, rapid, inexpensive and widely applicable technique for purifying plant DNA

S Gilmore, PH Weston and JA Thomson

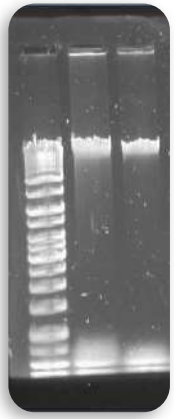
Australian Systematic Botany 6(2) 139 - 148
Published: 1993

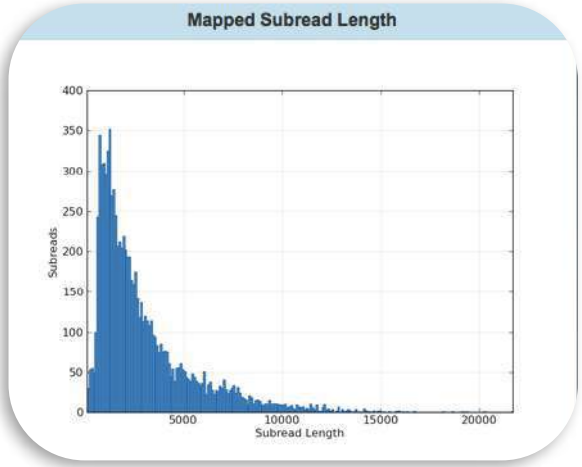
Simple, Efficient, and Nondestructive DNA Extraction Protocol for Arthropods

Aloysius J. Phillips, Chris Simon

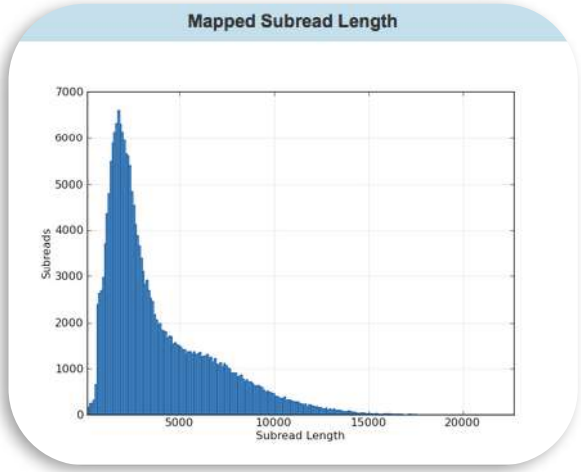
Annals of the Entomological Society of America, Volume 88, Issue 3, 1 May 1995, Pages 281–283, https://doi.org/10.1093/aesa/88.3.281
Published: 01 May 1995 **Article history** ▾

2013: a wake-up call





Polished Contigs	223	Max Contig Length	36,298
N50 Contig Length	2,932	Sum of Contig Lengths	480,087



Polished Contigs	9	Max Contig Length	1,508,929
N50 Contig Length	1,353,702	Sum of Contig Lengths	7,813,244

For Long Reads one needs to have *long and pure* DNA

SciLifeLab

DNA quality and inhibition of sequencing

Short-read technologies: PCR inhibition
Long-read technologies are PCR-free, but one sequences native DNA “as is”.

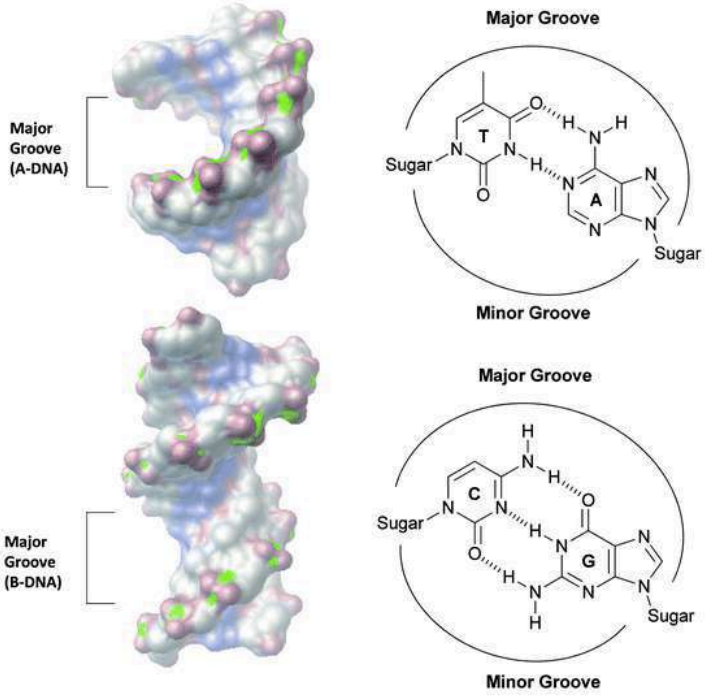
DNA-binders:

- Proteins
- Polyphenols
- Secondary metabolites (e.g. toxins)
- Pigments
- Polysaccharides

Polymerase inhibitors:

- Salts
- Phenol
- Alcohols

Physical inhibiting factors – debris



*Hamilton & Arya, Nat. Prod. Rep., 2012, **29**, 134-143*

What do absorption ratios tell us?

Pure DNA 260/280: 1.8 – 2.0

< 1.8:

Too little DNA compared to other components of the solution; presence of organic contaminants: proteins and phenol; glycogen - **absorb at 280 nm**.

> 2.0:

High share of RNA.

Pure DNA 260/230: 2.0 – 2.2

<2.0:

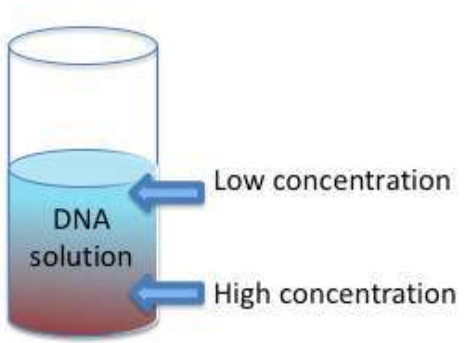
Salt contamination, humic acids, peptides, aromatic compounds, polyphenols, urea, guanidine, thiocyanates (latter three are common kit components) – **absorb at 230 nm**.

>2.2:

High share of RNA, very high share of phenol, **high turbidity,** dirty instrument, wrong blank.

Photometrically active contaminants:
phenol, polyphenols, EDTA, thiocyanate, protein,
RNA, nucleotides (fragments below 5 bp)

How to make a correct DNA measurement



LMW-DNA

HMW-DNA

- Thaw DNA completely
- Mix gently (**never vortex!**)
- Put the sample on a thermoblock: 37°C, 15-30 min
- Mix gently
- **Dilute 1:100** (if HMW)
- Mix gently
- Make a measurement with an appropriate blank

- **NANODROP is Bad**. Point.
- Use Qubit, or PicoGreen.

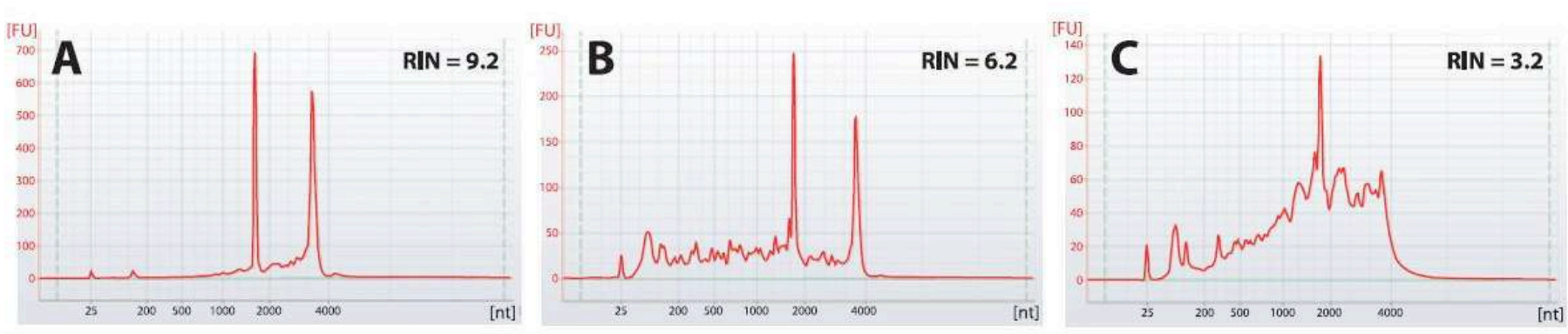
Sample prep: RNA

mRNA degrades FAST

Freeze sample or place it in RNA-later within 30 sec *(if possible)*

Chose a correct kit for your particular application!
Always treat samples with DNase

Differential expression, miRNA – **RIN value over 8.0**
Aim for 4 biological replicates

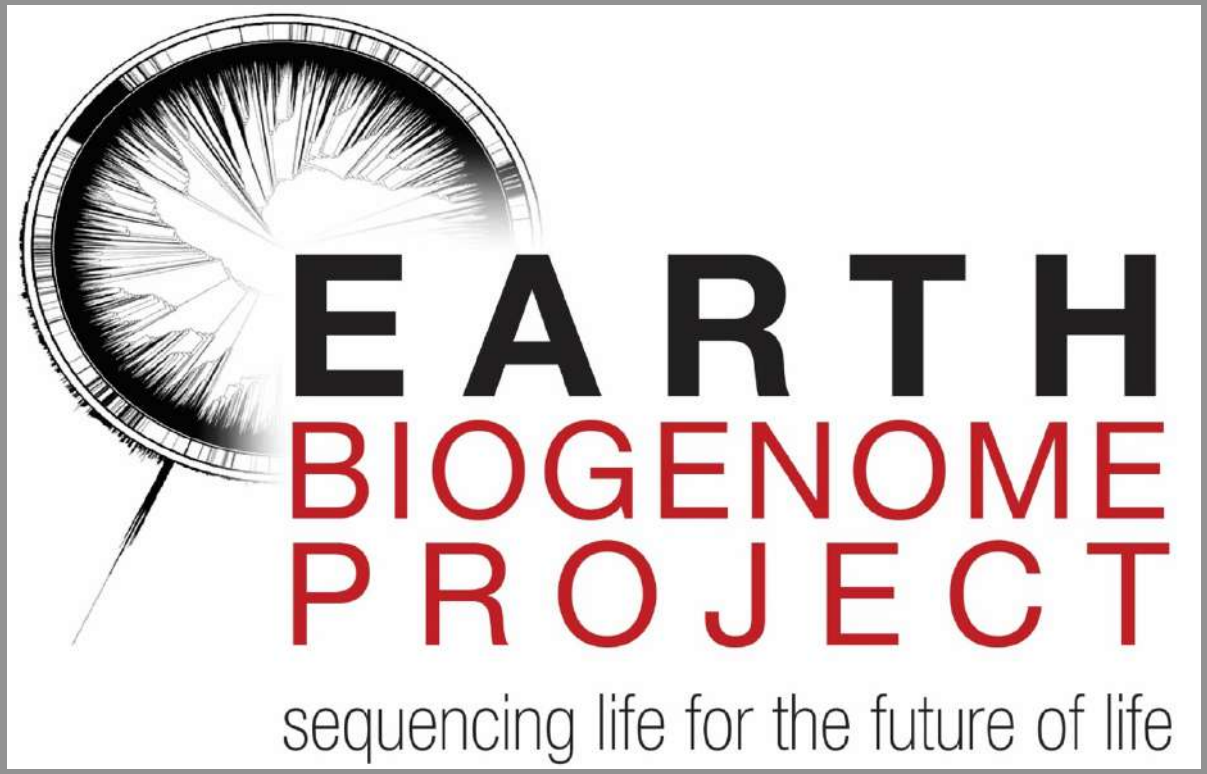


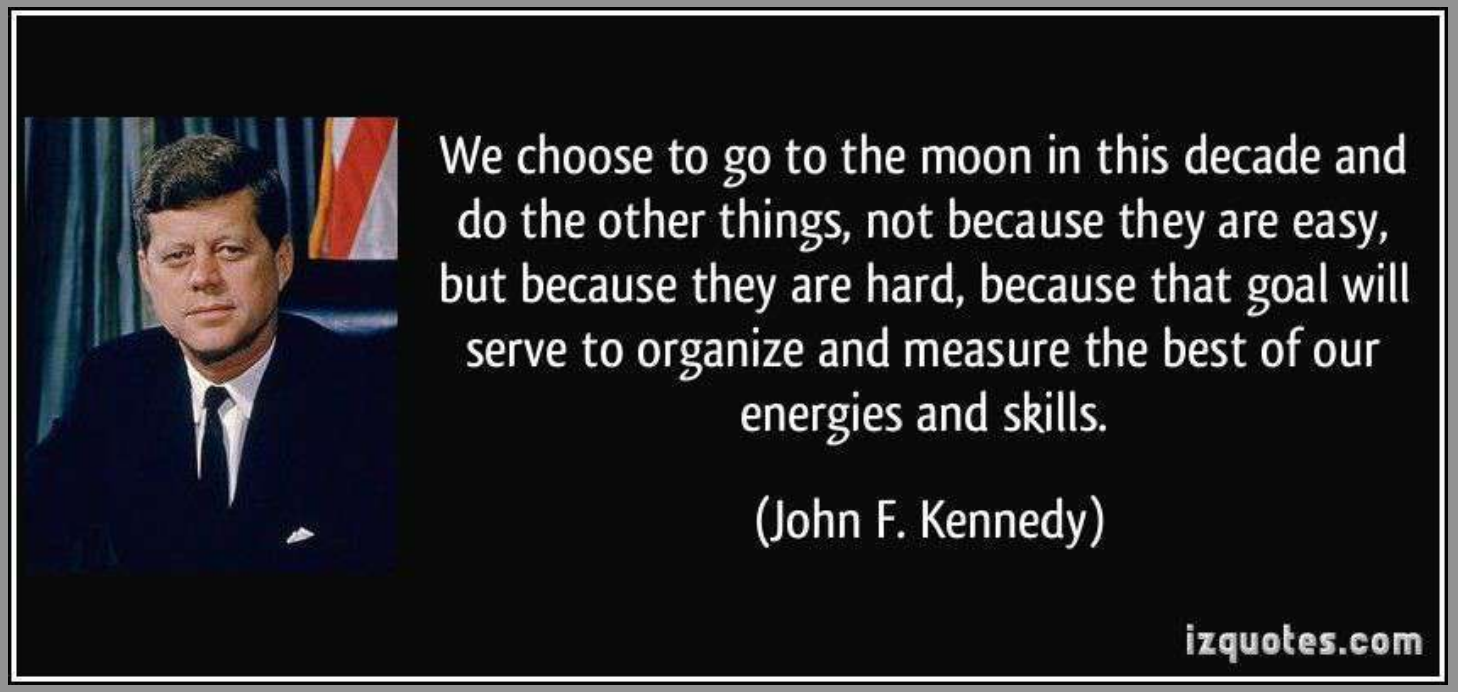
Sample prep: SUMMARY

- Sequencing success depends on the sample quality, DNA or RNA
- DNA quality is **essential** for PacBio and ONT sequencing
  … as well as PCR-free Illumina libraries & linked reads!

NGS and its challenges: SUMMARY

- Technologies develop VERY FAST.
- Beware of sequencing bias.
- Sequencing result depends on sample quality.
- Consult experts when it comes to experimental design and technology choice.


EARTH
BIOGENOME
PROJECT
sequencing life for the future of life

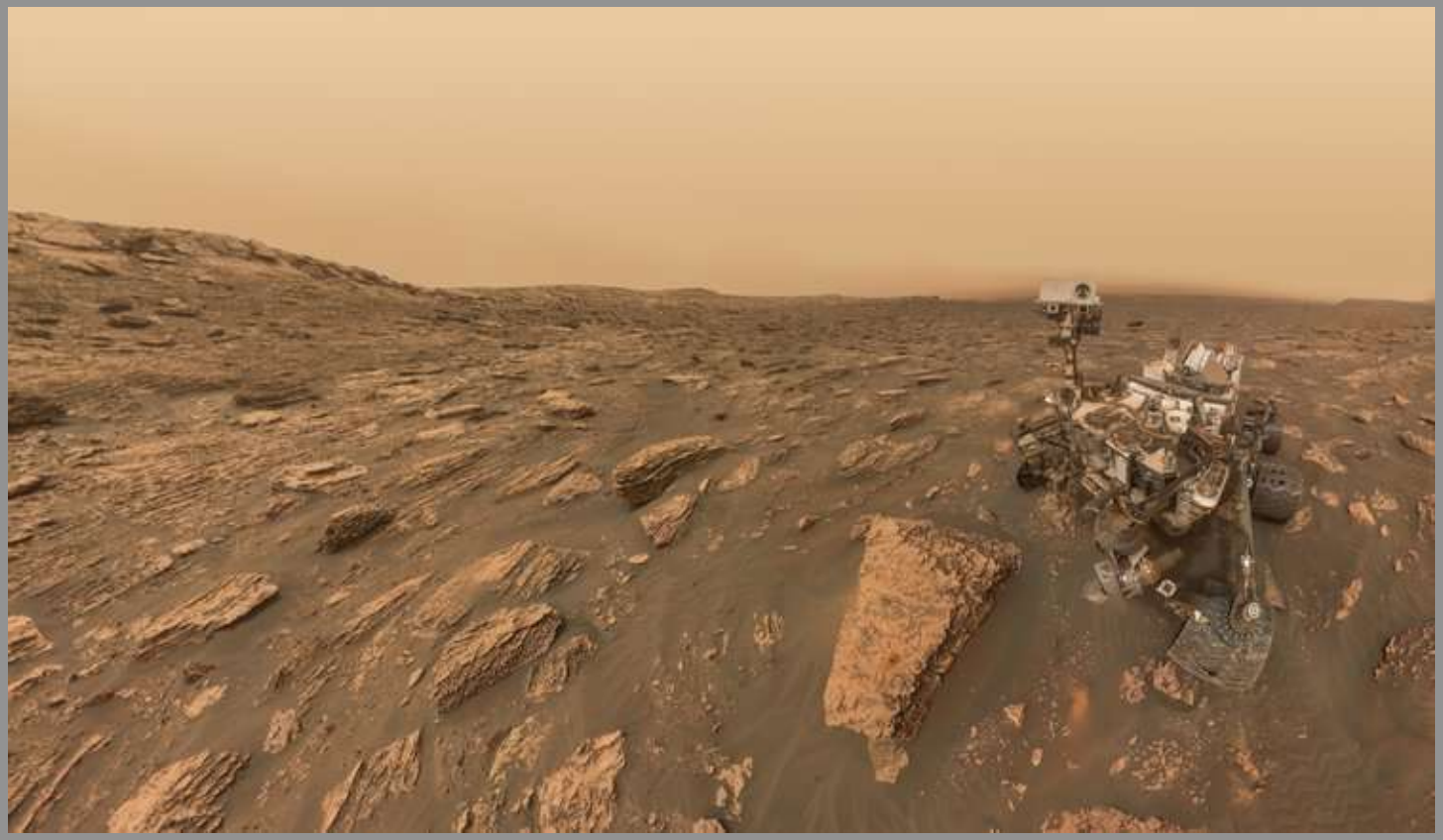


USA Moon program cost, 1962-1967:

28 bln USD (283 bln)

Hubble space telescope cost 1986-2010:

10 bln USD



Manned mission to Mars, 2024-2030:

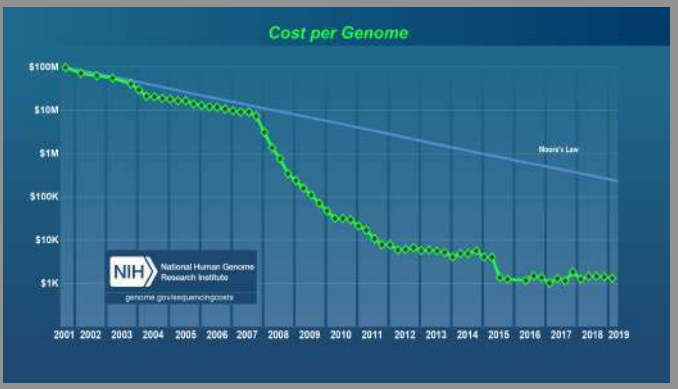
6 bln USD start-up + 4 bln USD per launch

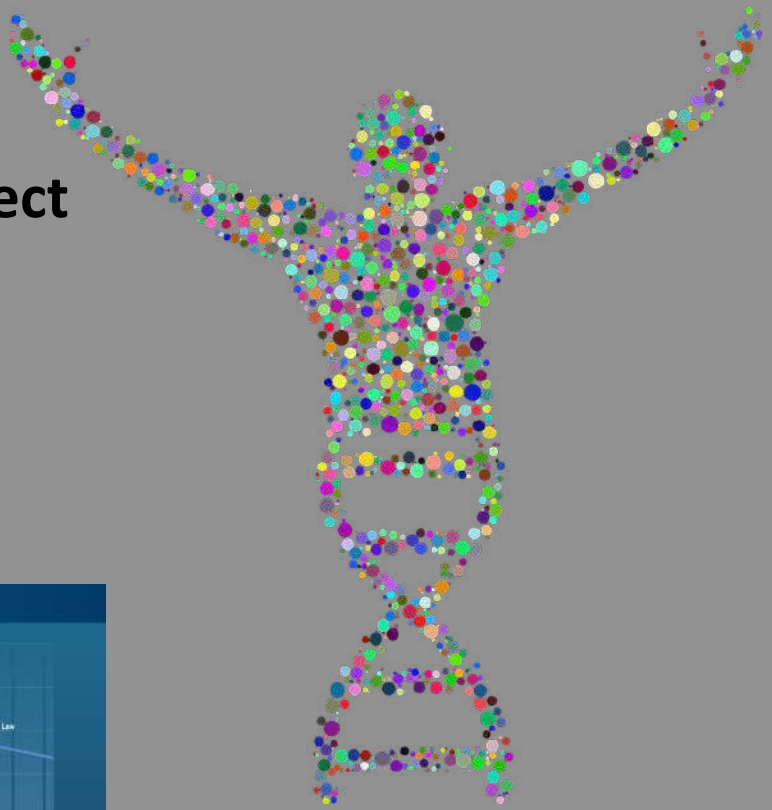
Meanwhile on Earth: 6th Mass Extinction Event

WWF estimate: 1 species is lost every 5 minutes

Human Genome Project
1990-2003

Spent: USD 14.5 bln
Output: USD 965 bln





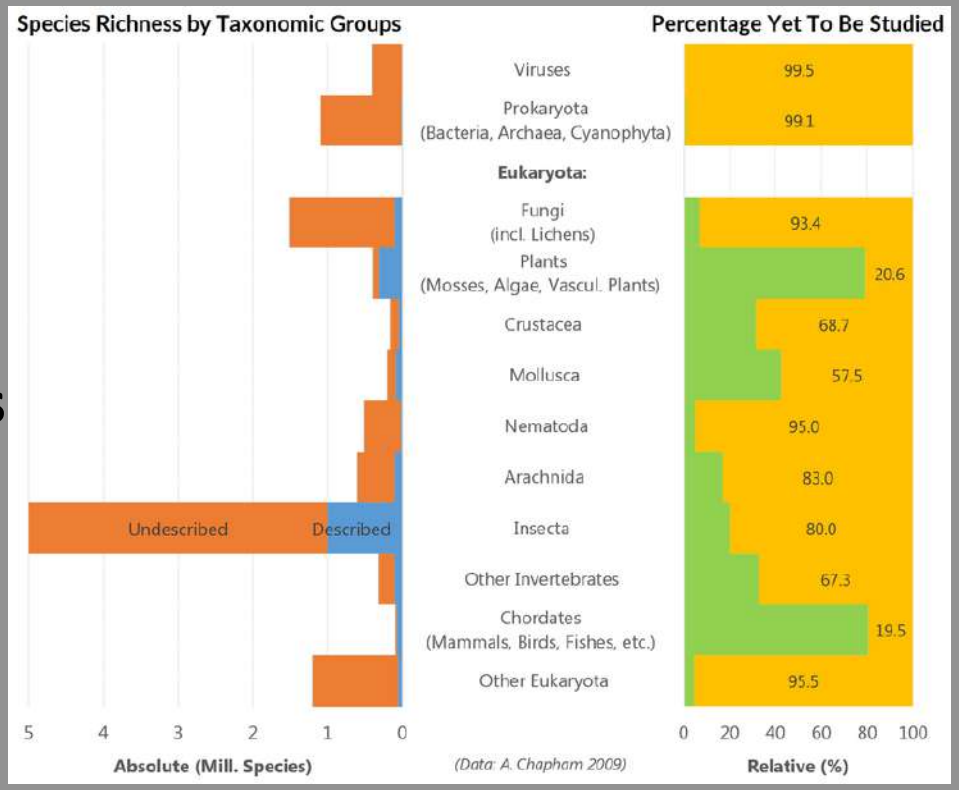
UK 10K
SweGen
Genomics england
TCGA
FINNGEN
SAN ANTONIO Cancer Genome Project
deCODE genetics
Go NL GENOMEoftheNETHERLANDS
WHOLE GENOMES SEQUENCED AT BROAD INSTITUTE
华大科技 BGI tech
PGP China Personal Genome Project 中国个人基因组项目
GENOME PROJECT 2.0
wellcome sanger institute
UK Biobank
Whole Genome Sequencing Project

Homo sapiens

N species = 1

Global Biodivers

N species = 1 trillion



EARTH BIOGENOME PROJECT

HARRIS LEWIN
UNIVERSITY OF CALIFORNIA, DAVIS

CHAIR

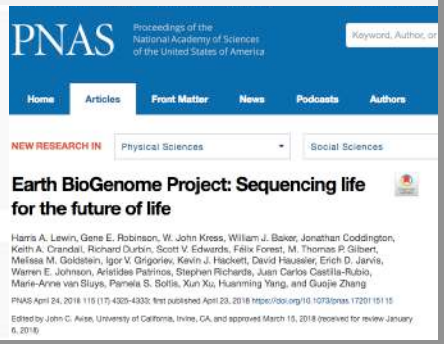
JOHN KRESS
SMITHSONIAN INSTITUTION

CO-CHAIR

GENE ROBINSON
UNIVERSITY OF ILLINOIS, URBANA-CHAMPAIGN

CO-CHAIR

> Our task now is to resynthesize biology; put the organism back into its environment; connect it again to its evolutionary past; and let us feel that complex flow that is organism, evolution, and environment united.
>
> Carl R. Woese, *New Biology for a New Century*



PNAS Proceedings of the National Academy of Sciences of the United States of America

Keyword, Author, or

Home | Articles | Front Matter | News | Podcasts | Authors

NEW RESEARCH IN Physical Sciences | Social Sciences

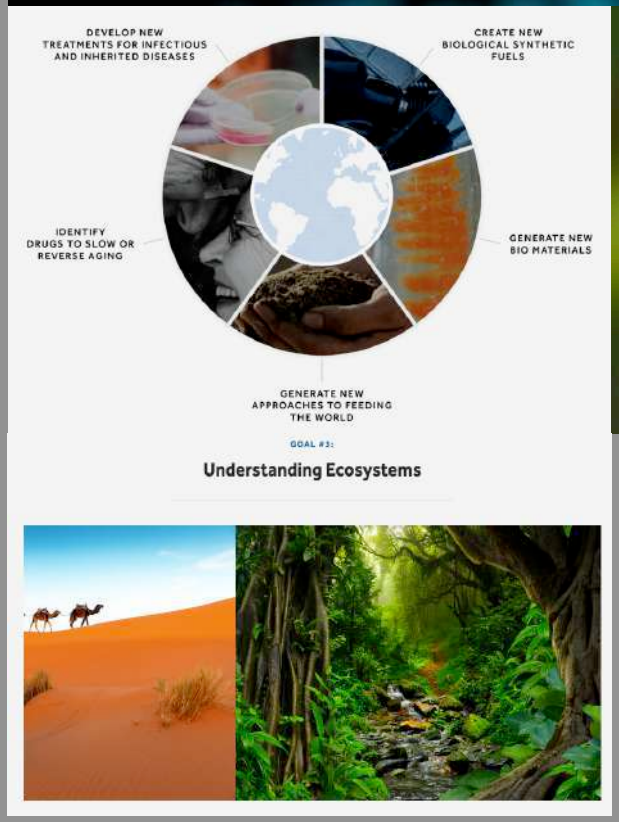
Earth BioGenome Project: Sequencing life for the future of life

Harris A. Lewin, Gene E. Robinson, W. John Kress, William J. Baker, Jonathan Coddington, Keith A. Crandall, Richard Durbin, Scott V. Edwards, Félix Forest, M. Thomas P. Gilbert, Melissa M. Goldstein, Igor V. Grigoriev, Kevin J. Hackett, David Haussler, Erich D. Jarvis, Warren E. Johnson, Aristides Patrinos, Stephen Richards, Juan Carlos Castilla-Rubio, Marie-Anne van Sluys, Pamela S. Soltis, Xun Xu, Huanming Yang, and Guojie Zhang

PNAS April 24, 2018 115 (17) 4325-4333; first published April 23, 2018 https://doi.org/10.1073/pnas.1720115115

Edited by John C. Avise, University of California, Irvine, CA, and approved March 15, 2018 (received for review January 6, 2018)

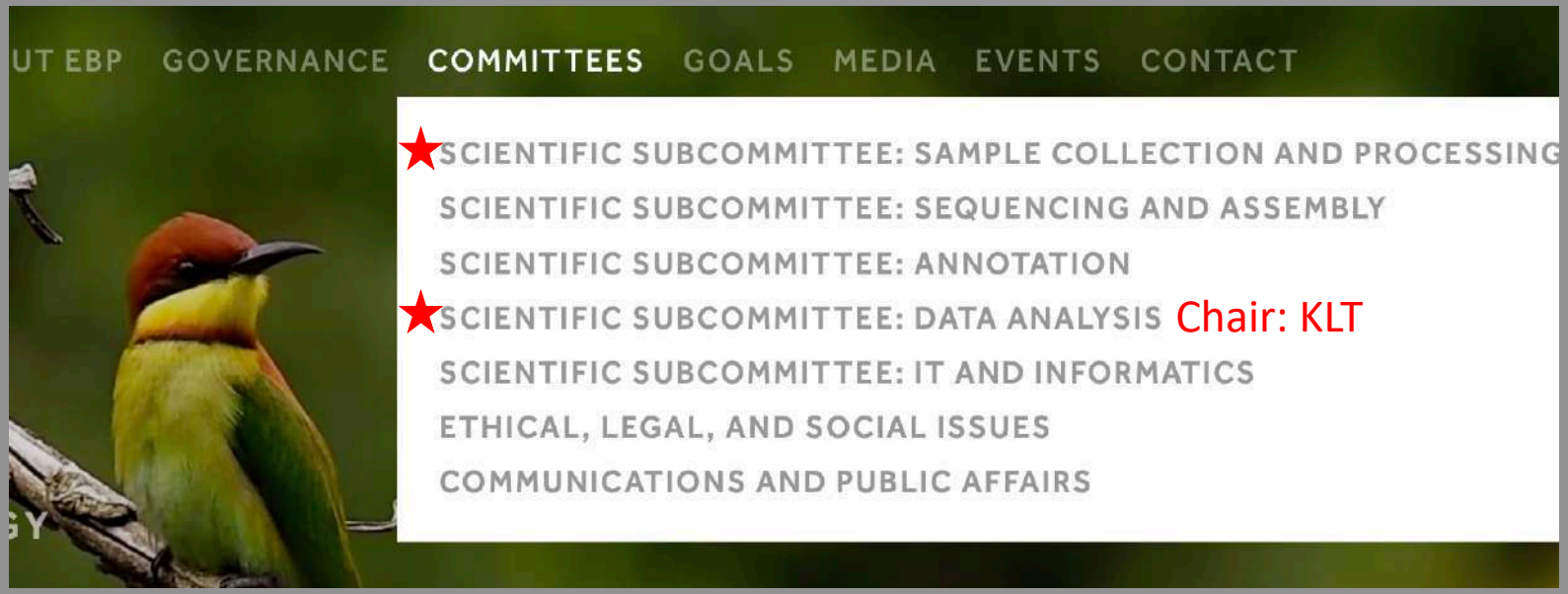
EARTH BIOGENOME PROJECT



EBP committees:

- Sample prep
- Informatics
- Genome sequencing
- Phylogenetics
- Genome assembly
- Legislation
- Genome annotation
-

EBP working groups



OVP

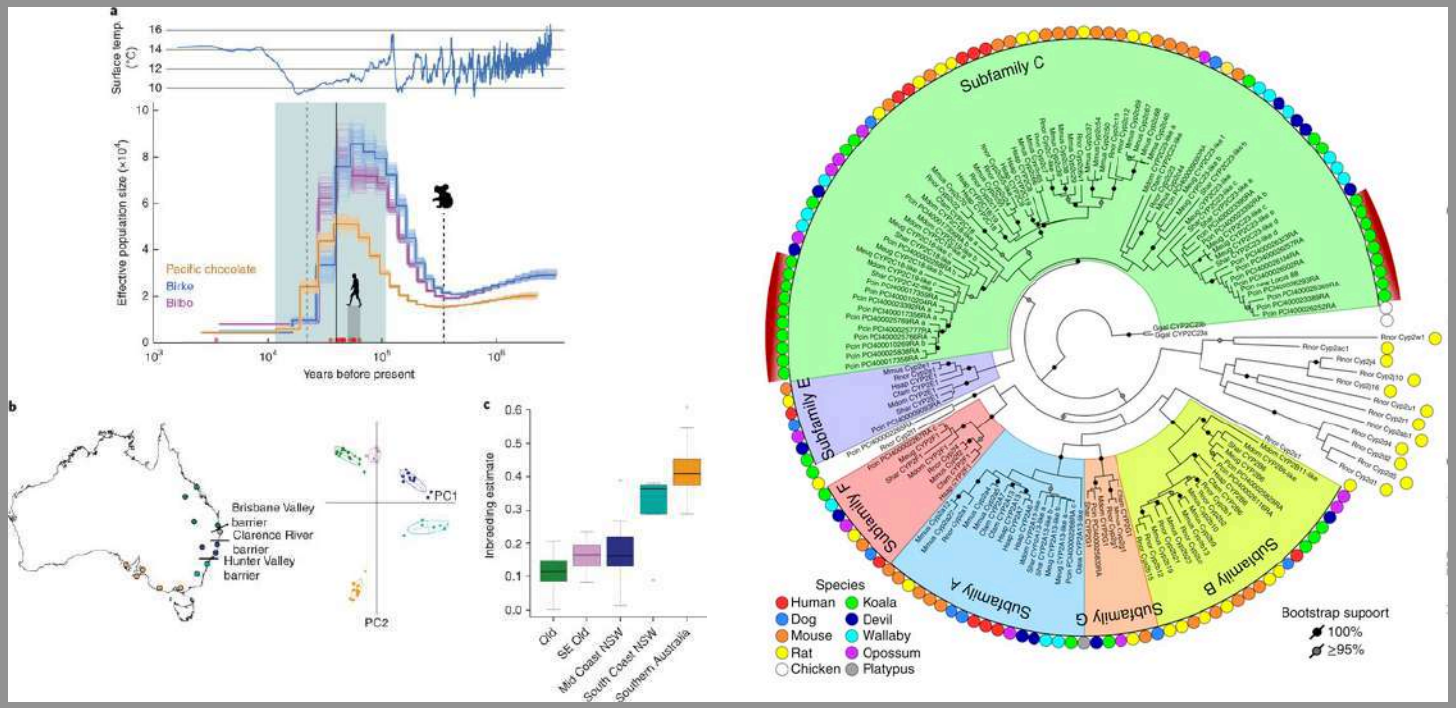
Chair: KLT

nature genetics

Article | Open Access | Published: 02 July 2018

Adaptation and conservation insights from the koala genome

Rebecca N. Johnson ✉, Denis O'Meally, [...] Katherine Belov



Swe-EBP

- September 29 - first Swedish meeting
- Oct 5 - Steering board selected

- Next meeting: November 30

- TO BE CONTINUED!

THANK YOU!

@OlgaVPettersson