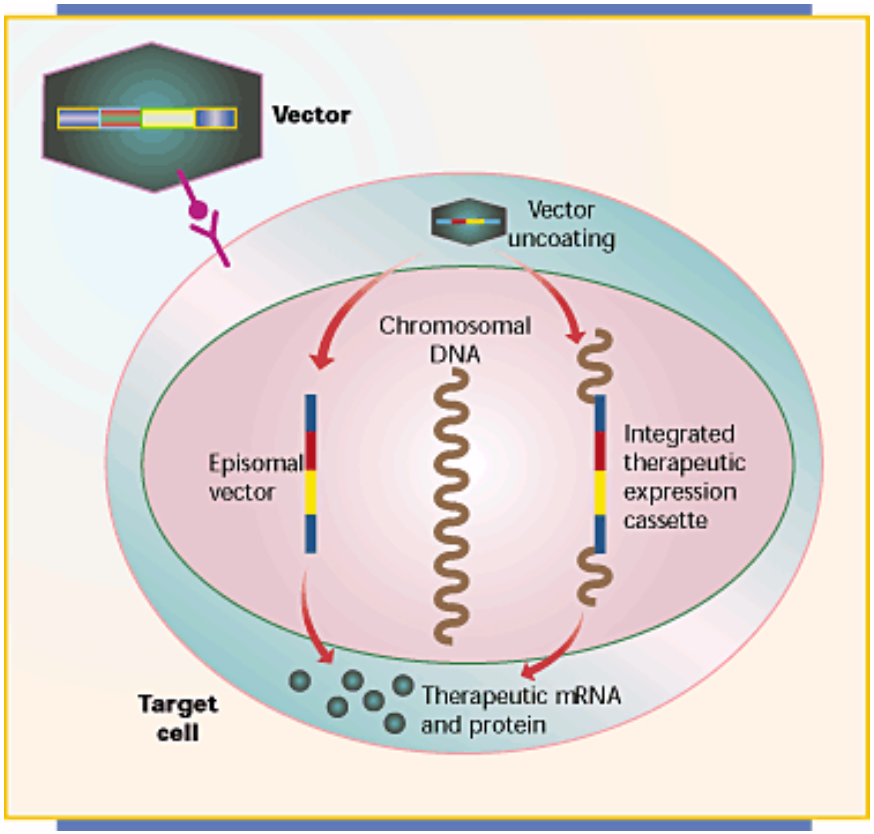
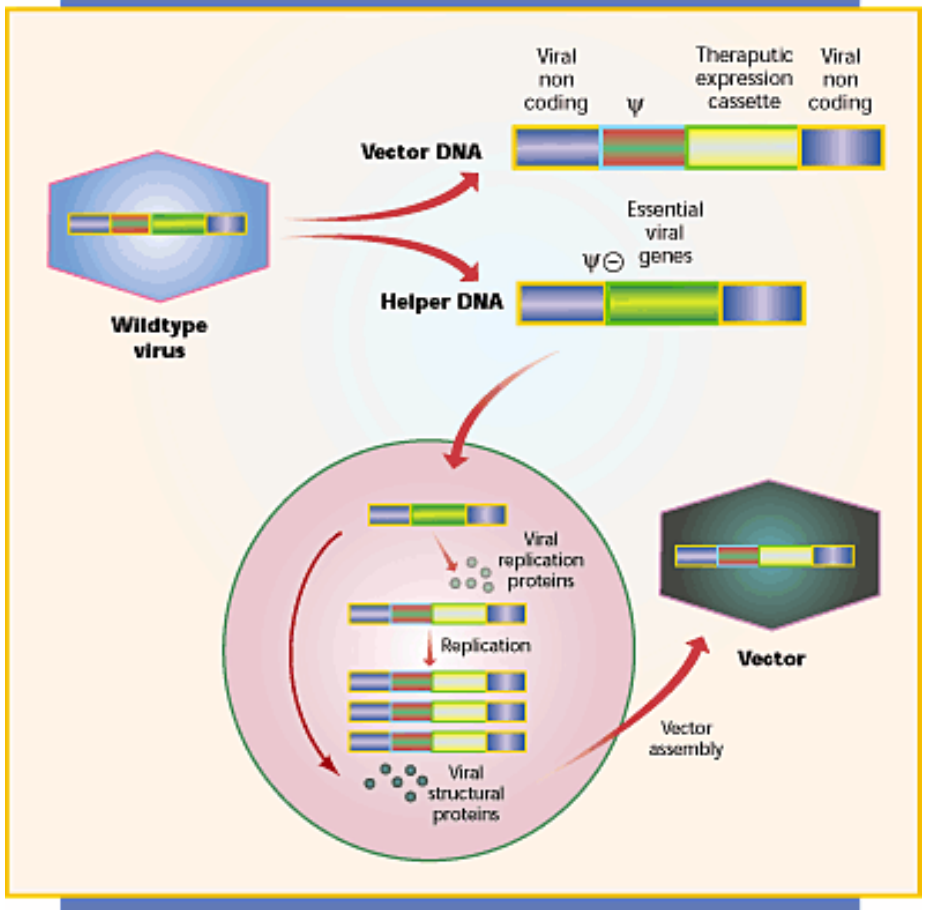
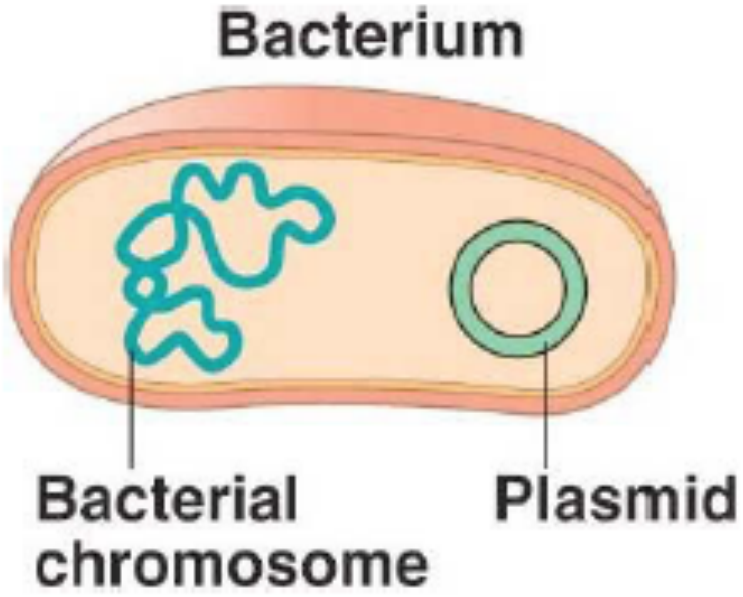
I vettori virali:

come si costruiscono e come funzionano





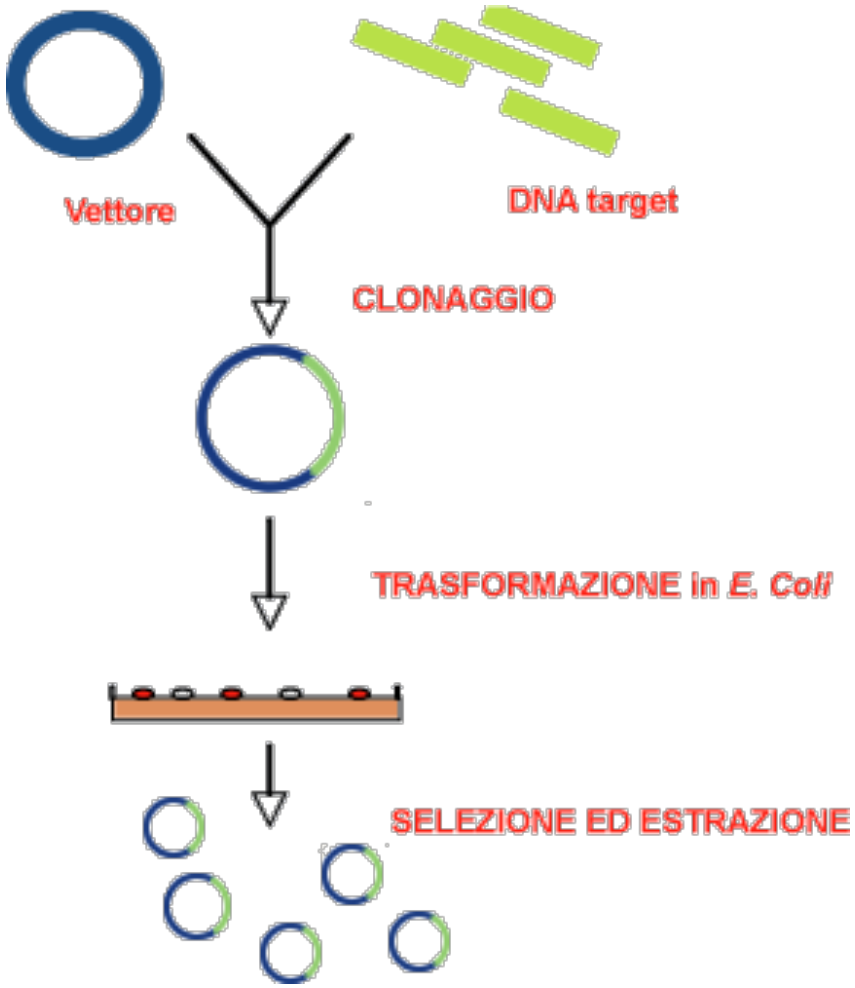
Recombinant DNA technology
1. Plasmids



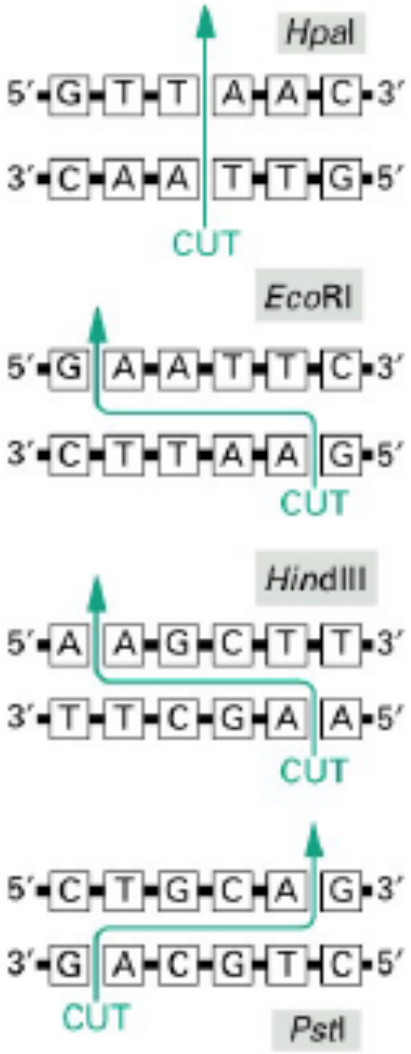
In 1952, Joshua Lederberg coined the term plasmid, in reference to any extrachromosomal heritable determinant. Plasmids are fragments of double-stranded DNA that can replicate independently of chromosomal DNA, and usually carry genes. Although they can be found in Bacteria, Archaea and Eukaryotes, they play the most significant biological role in bacteria where they can be passed from one bacterium to another by horizontal gene transfer, usually providing a context-dependent selective advantage, such as antibiotic resistance.

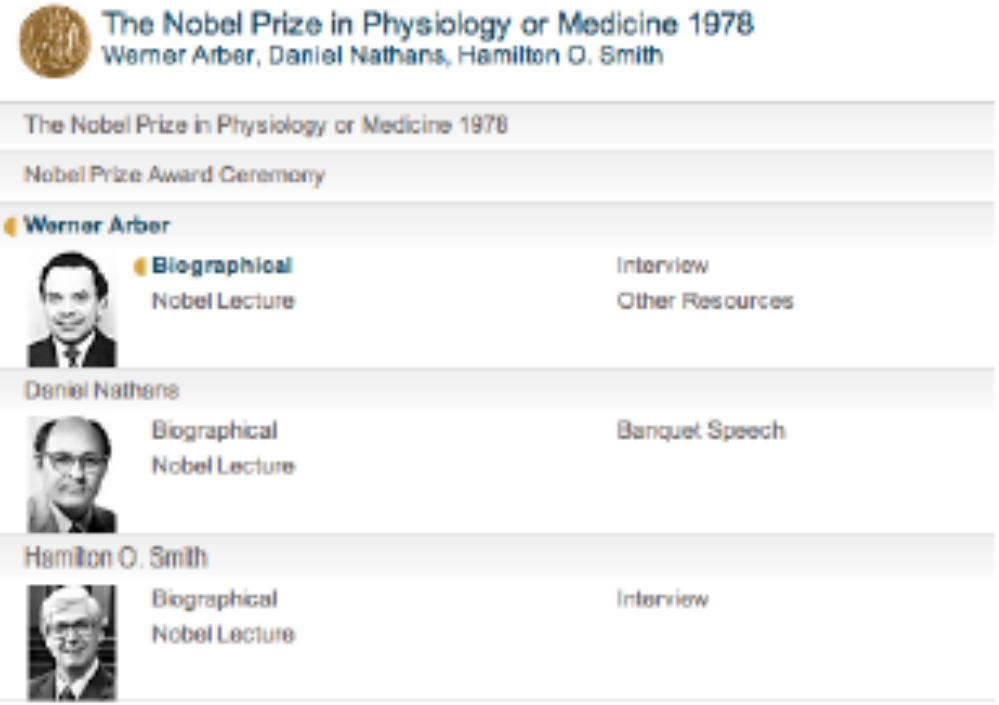
CLONAGGIO

1. Il clonaggio di DNA è una tecnica che permette di **duplicare frammenti di DNA**
2. E' necessario avere un **"vettore"** per portare il frammento di DNA di interesse dentro una cellula.
3. Con il clonaggio si può **selezionare un frammento di DNA e produrne copie illimitate**
4. E' alla base dell'ingegneria genetica:
   - Produzione di libreria di DNA
   - PCR
   - Sequenziamento del DNA
5. Studiare un frammento permette di capire un meccanismo, modificarlo, studiare la proteina prodotta…

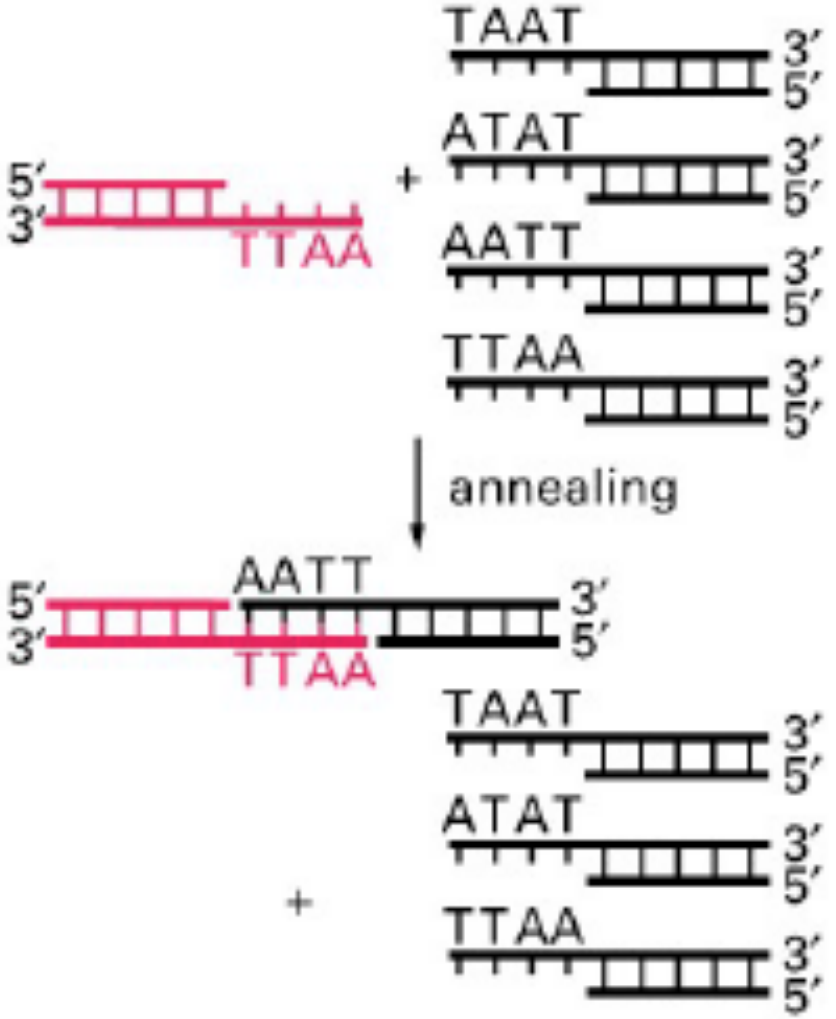


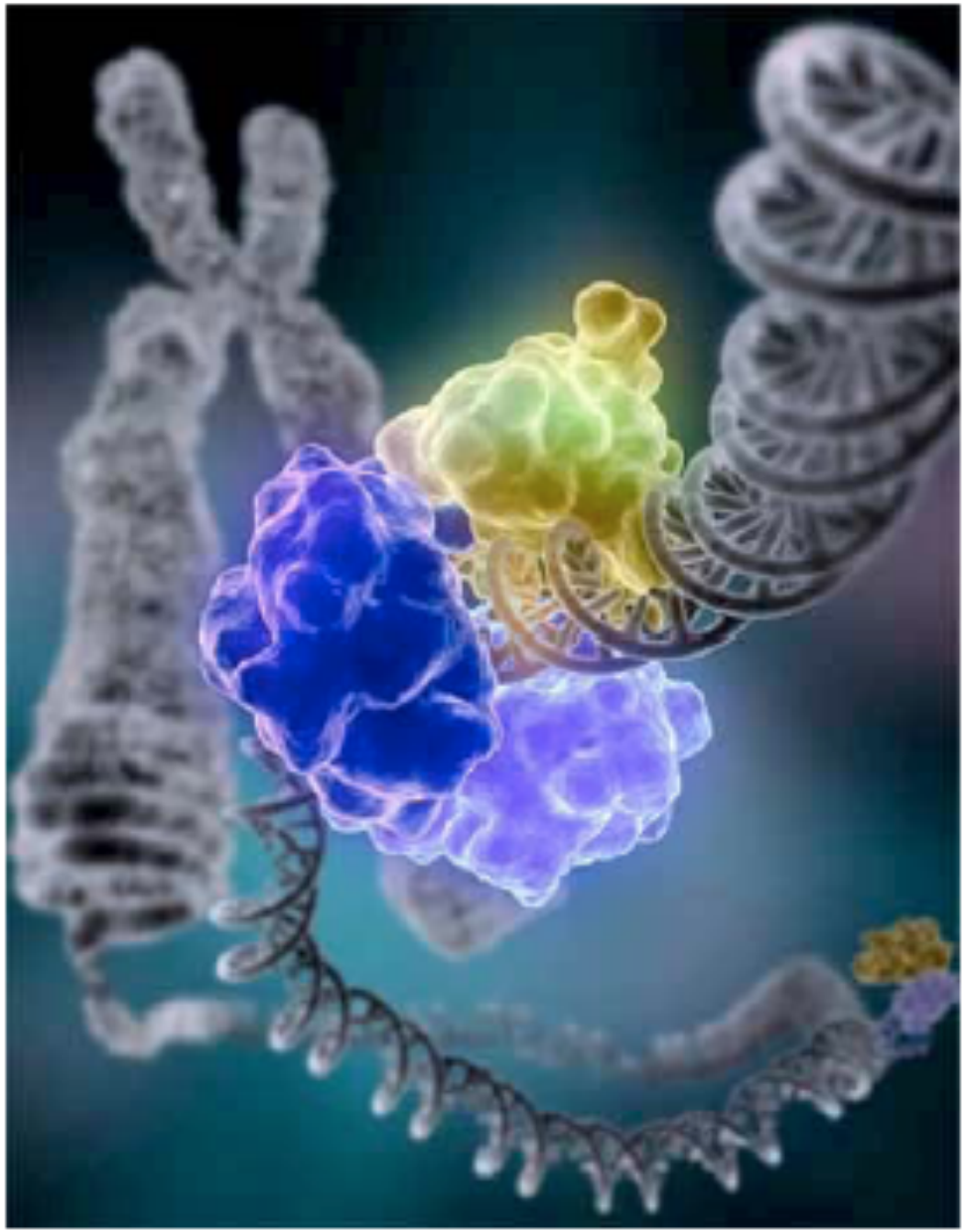
Recombinant DNA technology
2. Restriction enzymes & other DNA enzymes





Recombinant DNA molecules

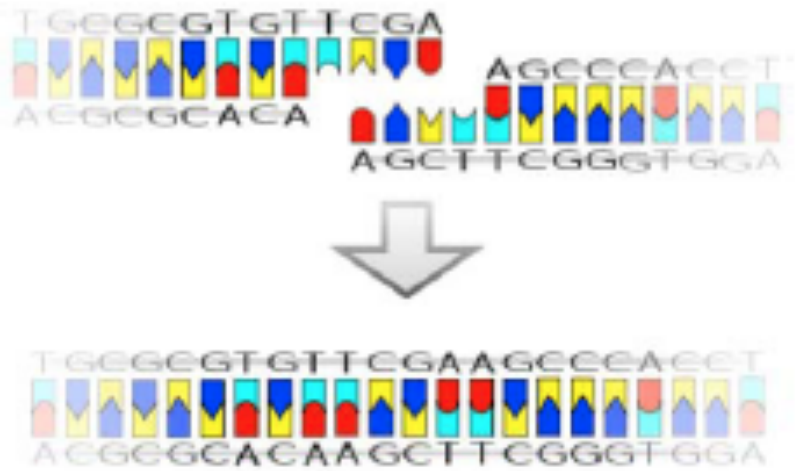




DNA ligase

Ligase mechanism

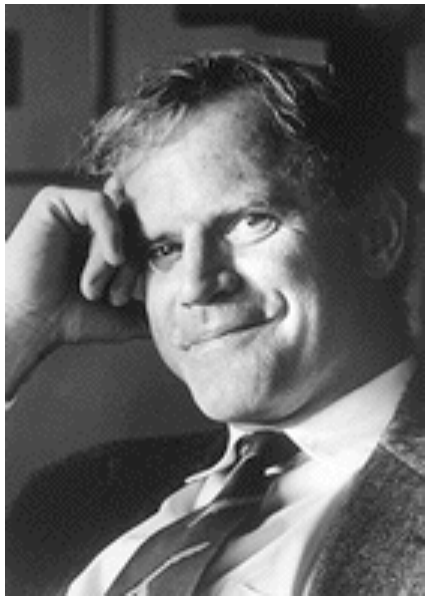
The mechanism of DNA ligase is to form two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. ATP is required for the ligase reaction.
A pictorial example of how a ligase works (with sticky ends):



Ligase will also work with blunt ends, although higher enzyme concentrations and different reaction conditions are required.

Sources of Biological Evidence

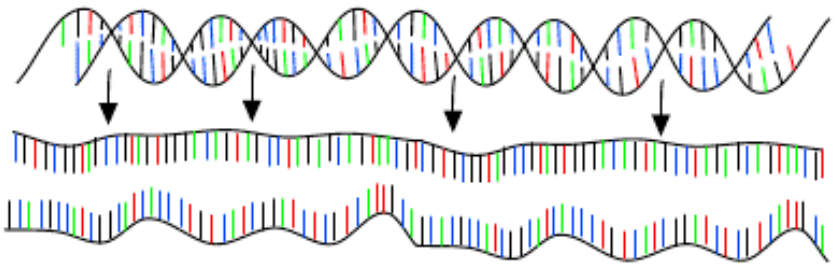
- Blood
- Semen
- Saliva
- Urine
- Hair
- Teeth
- Bone
- Tissue



Kary B. Mullis

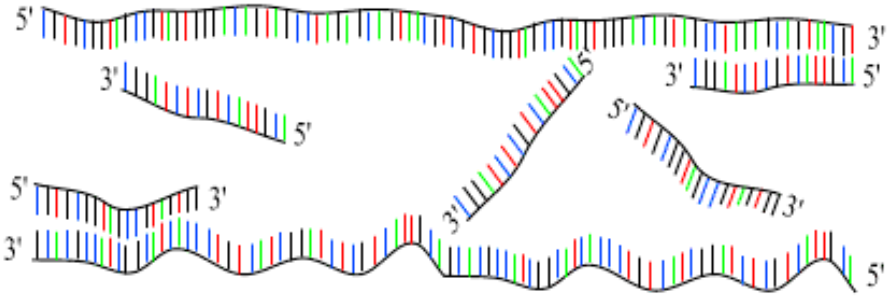
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

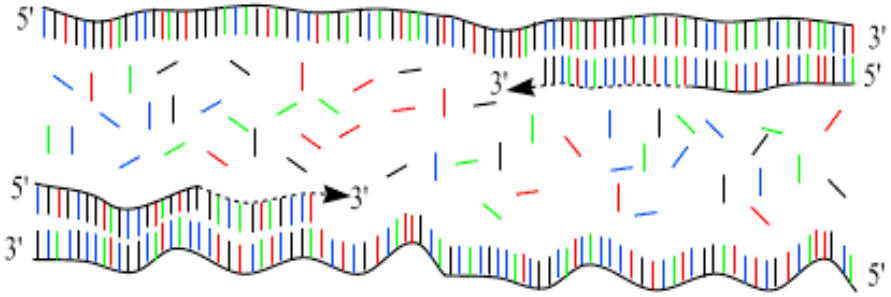
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

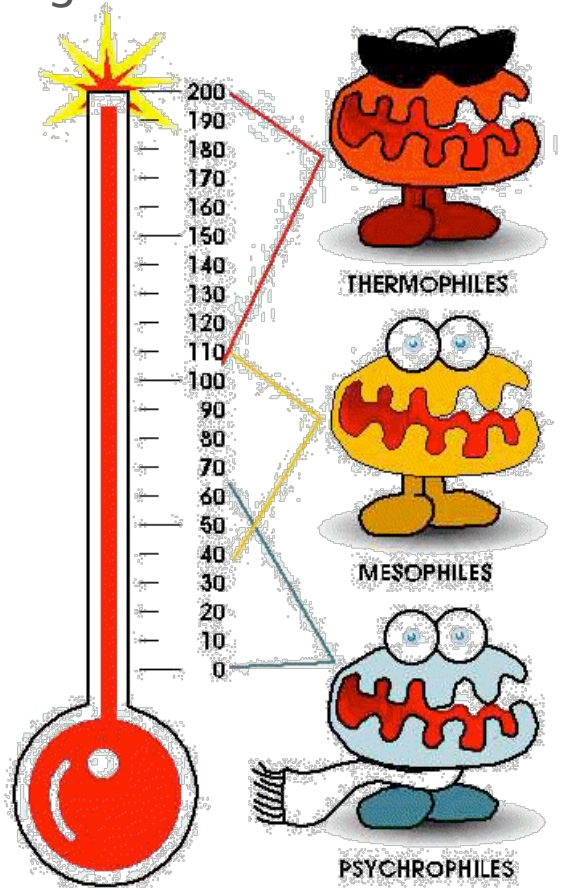
2 minutes 72 °C

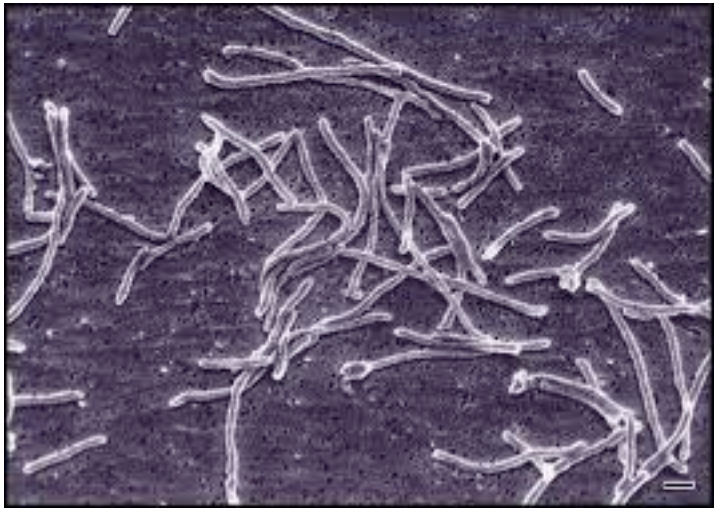
only dNTP's

(Andy Vierstraete 1999)

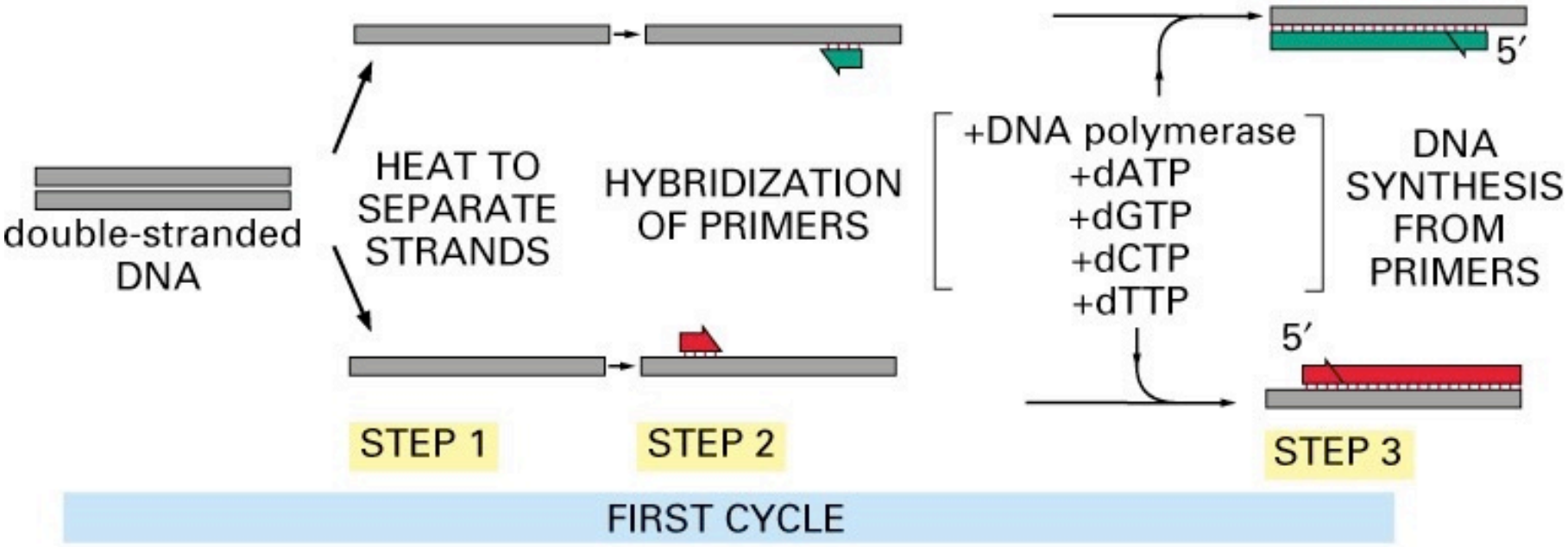
Taq polymerase is a DNA polymerase derived from Thermus Aquaticus

Thermus Aquaticus is a Gram Positive bacterium that is classified under a group called thermophiles. Thermophiles are defined as organisms that thrive and reproduce at temperatures that are above 45 Degrees Celsius. Specifically, Thermus Aquaticus optimally thrives and reproduces at 70 degrees celsius.

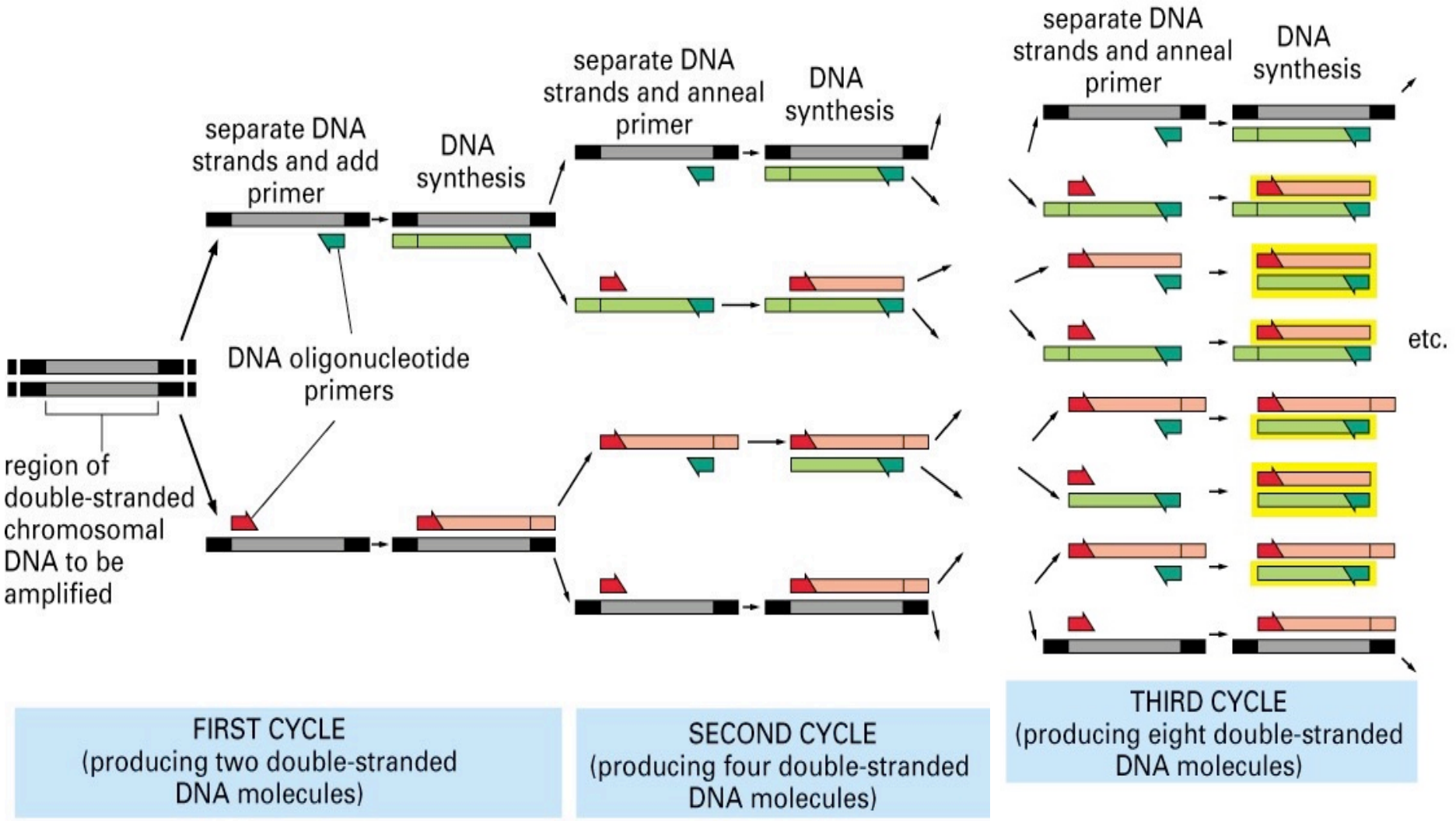




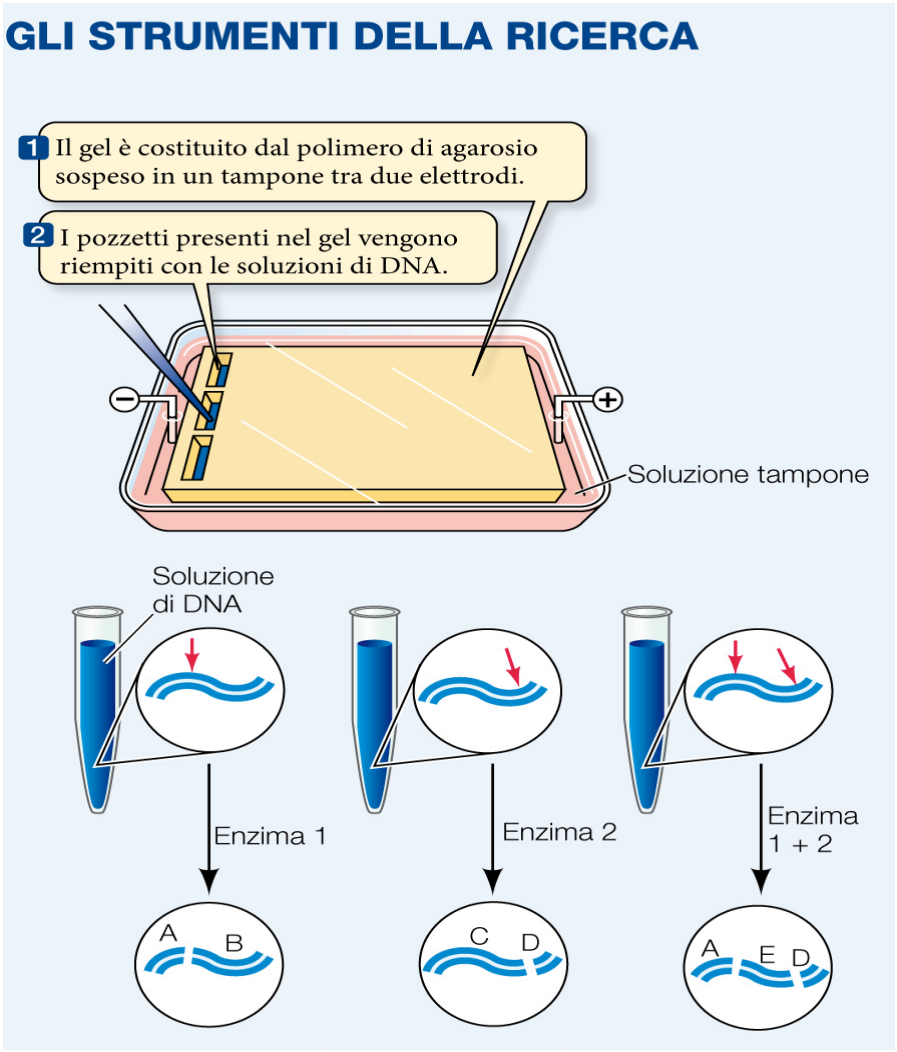
PCR amplification

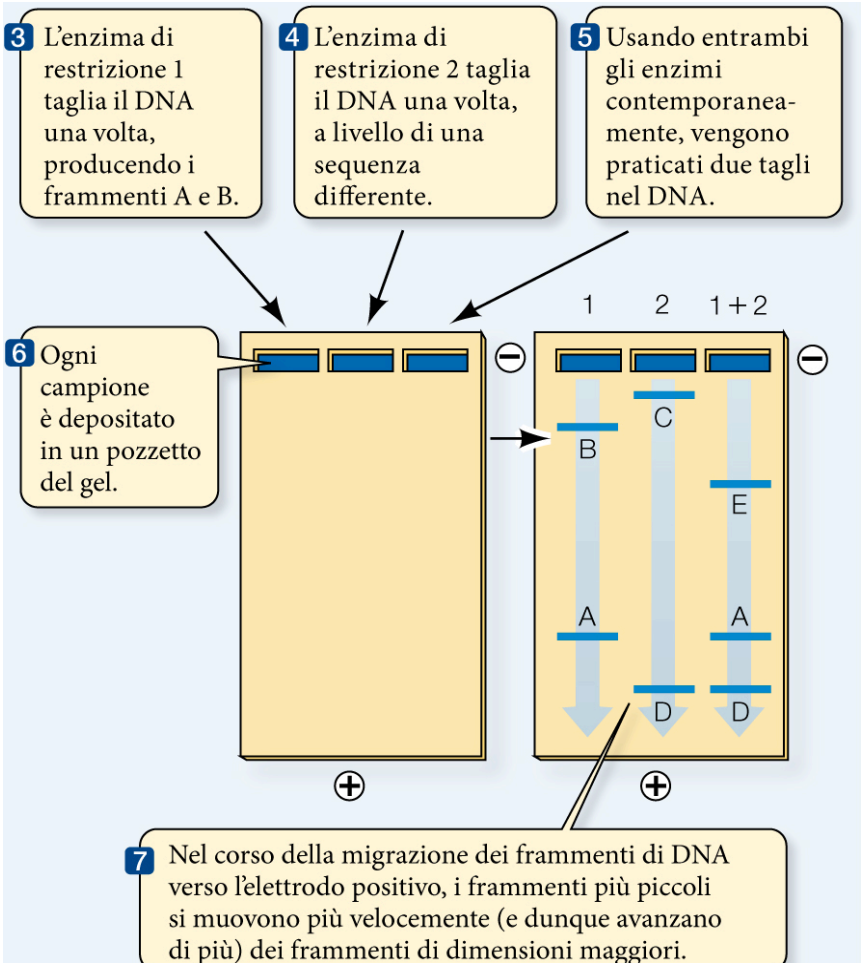


PCR cycles

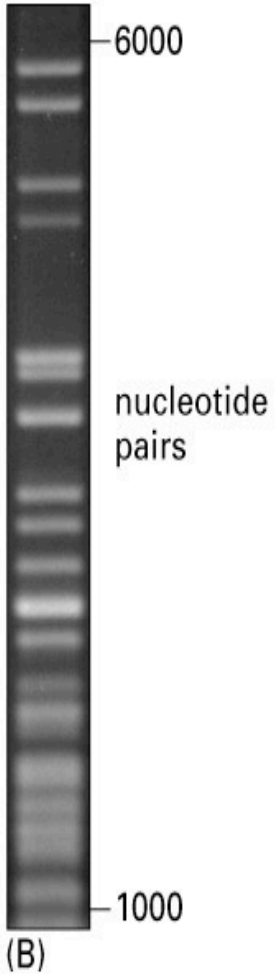

separate DNA strands and add primer
DNA synthesis
separate DNA strands and anneal primer
DNA synthesis
separate DNA strands and anneal primer
DNA synthesis
DNA oligonucleotide primers
region of double-stranded chromosomal DNA to be amplified
etc.
FIRST CYCLE
(producing two double-stranded DNA molecules)
SECOND CYCLE
(producing four double-stranded DNA molecules)
THIRD CYCLE
(producing eight double-stranded DNA molecules)

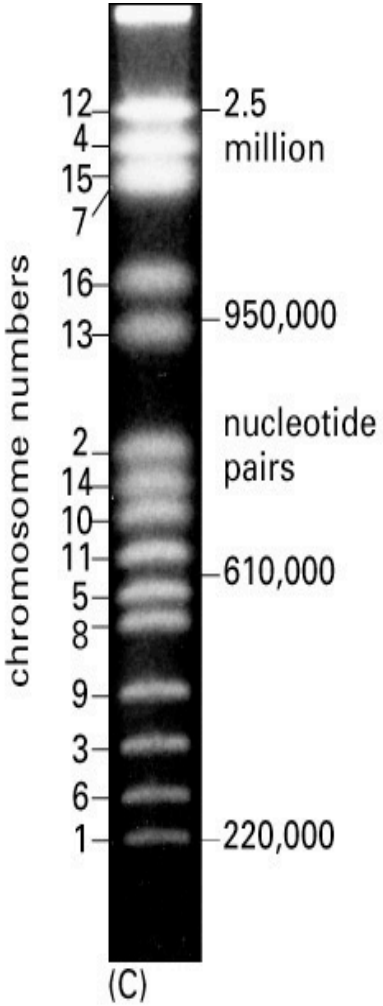
L’elettroforesi su gel

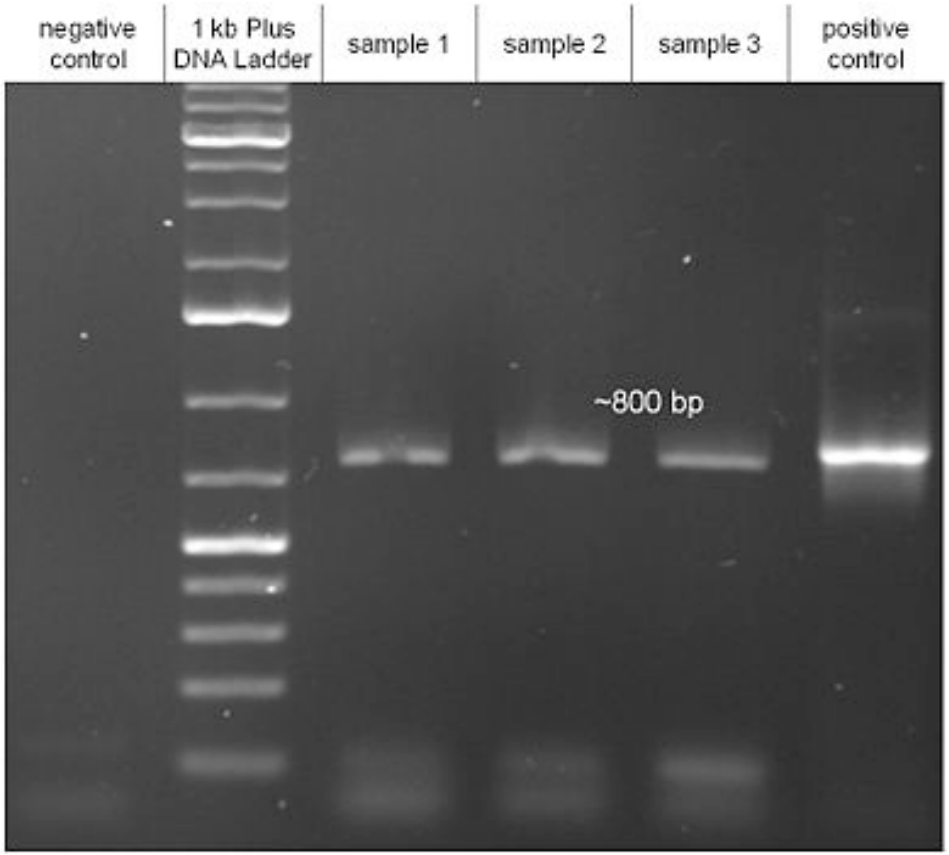




Gel Electrophoresis Separates DNA Molecules of Different Sizes





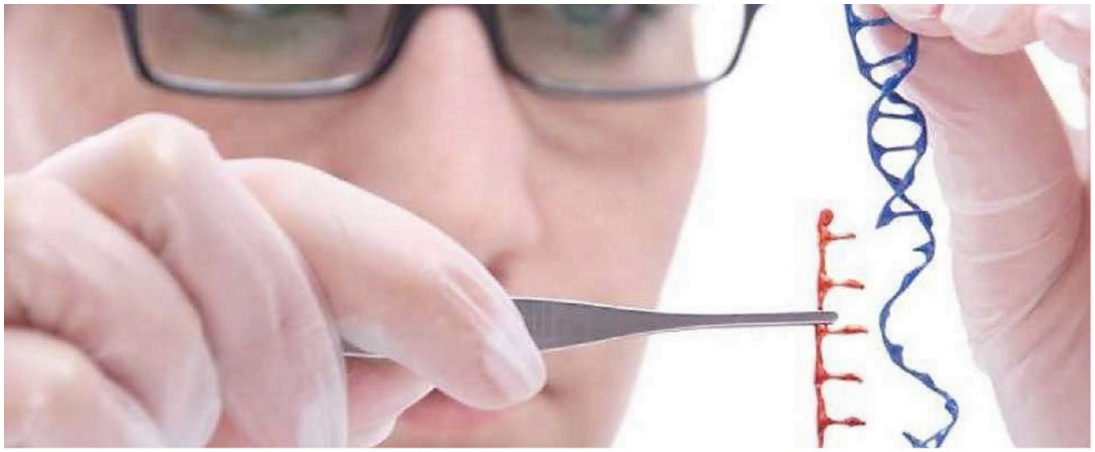


UTILIZZI DELLA PCR:

Diagnostica e prognostica

1. Ricerca di MUTAZIONI: Diagnosi di malattie genetiche monogeniche (alkaptonuria, fibrosi cistica…)
2. Ricerca di POLIMORFISMI: Prevedere la gravità di una malattia in corso o la predisposizione di una persona a incorrere in una malattia
3. Diagnosi di infezioni virali (HIV, HCV)
4. Test genetici (paternita')
5. Medicina forense (ricerca persone scomparse, identificazione in caso di distrastri e tragedie, database di persone con precedenti penali)

Le Scienze
EDIZIONE ITALIANA DI SCIENTIFIC AMERICAN



07 aprile 2015

Nuove forbici molecolari per l'editing genetico

Specifici geni possono essere modificati *in vivo* grazie a una versione più piccola dell'enzima Cas9, una forbice molecolare già utilizzata per ingegnerizzare il genoma. In uno studio sui topi, i ricercatori sono riusciti a modificare l'attività del gene che controlla il colesterolo, diminuendone i livelli nel sangue

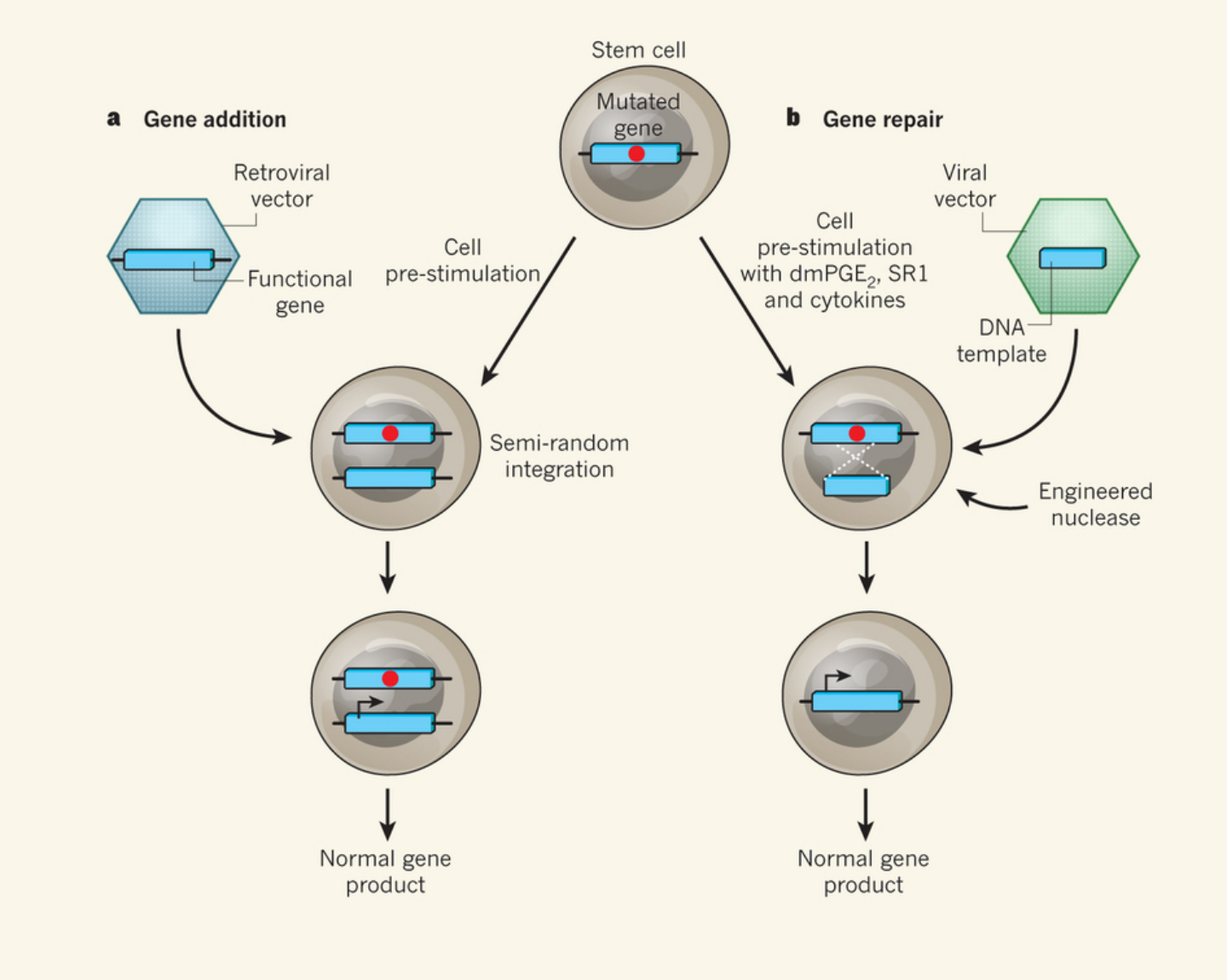
03 dicembre 2015

Interventi di editing genetico sempre più mirati e sicuri

Cambiando solo tre amminoacidi dei 1400 circa che formano la proteina Cas9 - le "nanoforbici" usate nella nuova tecnica di modificazione del genoma chiamata CRISPR/Cas9 - è possibile assicurarsi che l'enzima non sbagli bersaglio e vada a colpire una parte indesiderata del DNA *(red)*

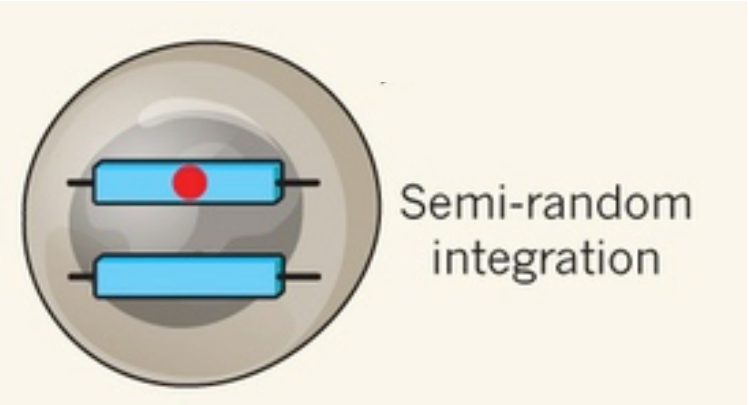
Clustered Regularly Interspaced Short Palindromic Repeats

Two strategies for genetic therapy: gene addition and genome editing



Fischer. Nature (2014) 510:226.

Two strategies for genetic therapy: addition and editing

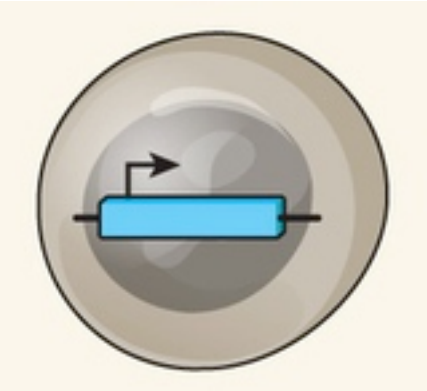


- Gene addition:
  - Feasible with existing technology; clinical trials ongoing.
  - Early trial results appear exciting.
  - Challenges:

    1. Will enough of the added gene be made in the cells with the integration? Will enough of the blood stem cells have the added gene?
    2. Is the benefit durable? Will the added gene continue to function over days, weeks, months, years, decades?
    3. Is the added gene safe? Will its semi-random integration into the genome change the function of other genes in the genome?

Fischer. Nature (2014) 510:226.

Two strategies for genetic therapy: addition and editing



- Gene editing:
  - Promise of permanent repair of the underlying disease-causing mutation.
  - Promise of specific beneficial change at the intended genomic site (e.g. β-globin gene) without impacting remainder of genome.
  - Challenges:

    1. Technology is in a relatively early stage and needs to be further developed.
    2. Can enough cells be edited to have therapeutic impact?
    3. Will the editing be exquisitely specific, or will other regions of the genome aside from the target be affected?

Fischer. Nature (2014) 510:226.

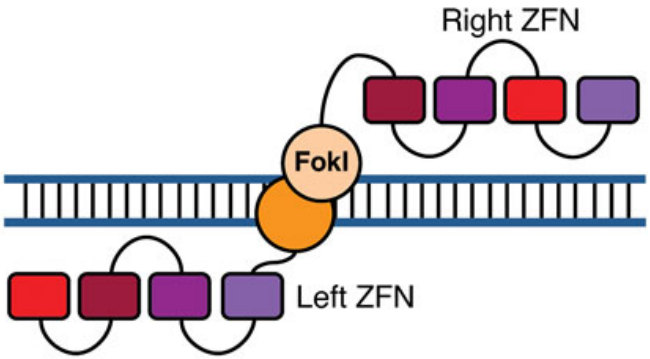
Genome editing tools are sequence-specific nucleases

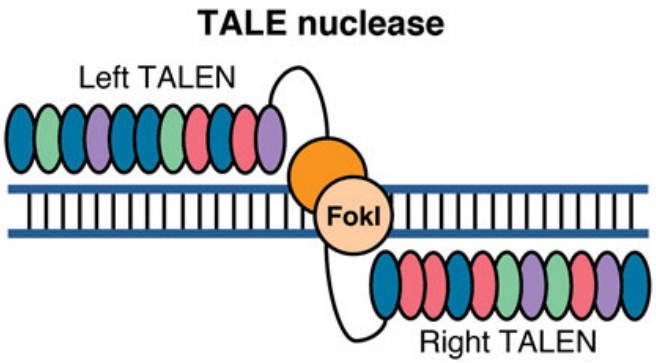
Genome editing tools have two features:

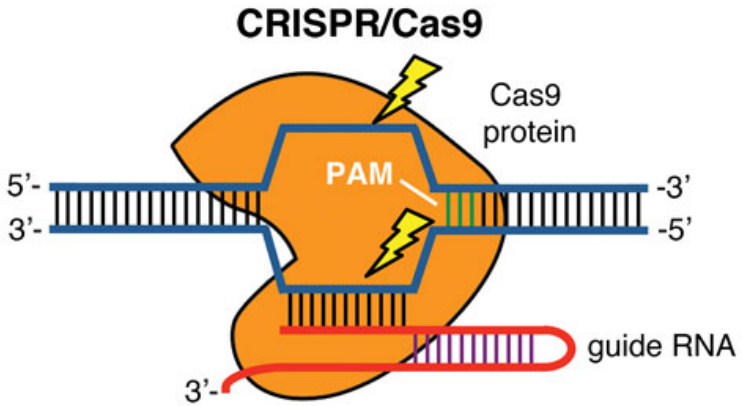
1) Recognize specific DNA sequences (i.e. specific genes or non-coding elements)

2) Cut DNA ("nuclease"), then a scar is left behind

van der Oost. Science (2013) 339:768.



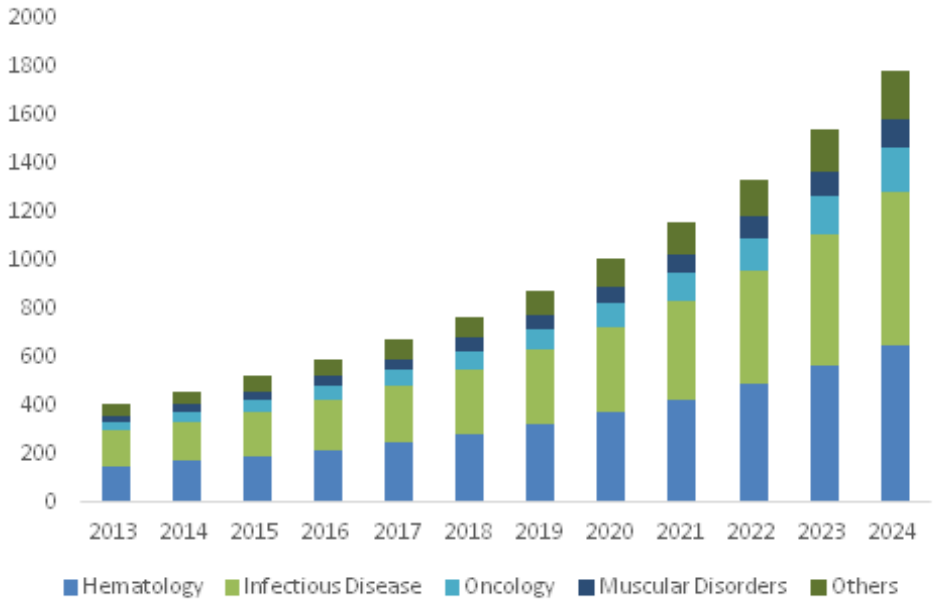




Ci sono varie tecnologie per fare "gene editing"

- **Zinc finger**
- **TALEN**
- **Crispr-Cas9**

Gene Editing Market Size

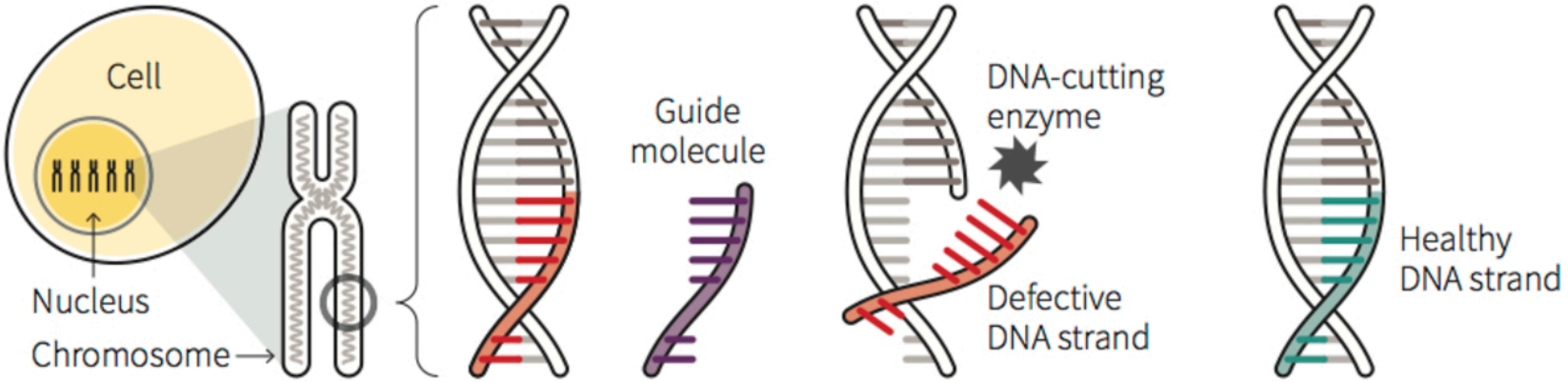


Clustered Regularly Interspaced Short Palindromic Repeats

DNA editing

A DNA editing technique, called CRISPR/Cas9, works like a biological version of a word-processing programme's "find and replace" function.

HOW THE TECHNIQUE WORKS



A cell is transfected with an enzyme complex containing:

- Guide molecule
- Healthy DNA copy
- DNA-cutting enzyme

A specially designed synthetic guide molecule finds the target DNA strand.

An enzyme cuts off the target DNA strand.

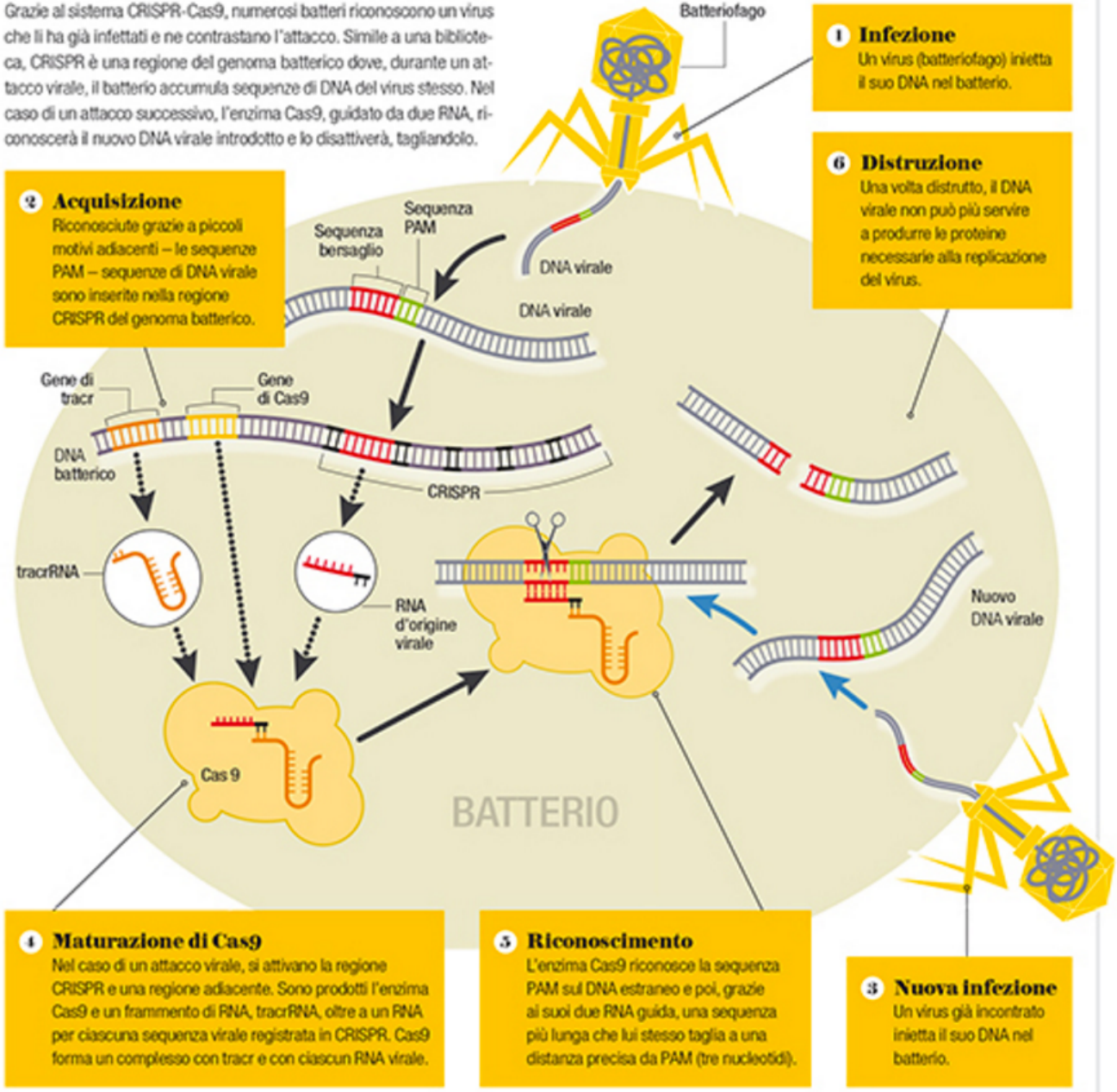
The defective DNA strand is replaced with a healthy copy.

Sources: Reuters; Nature; Massachusetts Institute of Technology

UN'ARMA CONTRO I VIRUS

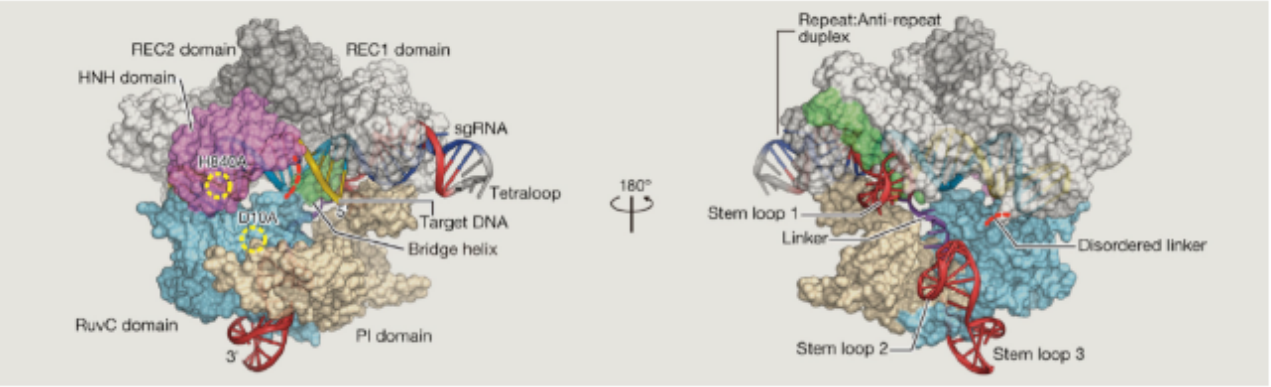
Come il sistema CRISPR-Cas9 protegge il batterio

Grazie al sistema CRISPR-Cas9, numerosi batteri riconoscono un virus che li ha già infettati e ne contrastano l'attacco. Simile a una biblioteca, CRISPR è una regione del genoma batterico dove, durante un attacco virale, il batterio accumula sequenze di DNA del virus stesso. Nel caso di un attacco successivo, l'enzima Cas9, guidato da due RNA, riconoscerà il nuovo DNA virale introdotto e lo disattiverà, tagliandolo.



Which are the crucial components of the CRISPR system?

(1) **Cas9 endonuclease**



Cas9 is an RNA driven endonuclease able to induce DSBs in a desired locus

It is characterized by two catalytic domain that cleavage the DNA:

- HNH domain

- RuvC domain

Which are the crucial components of the CRISPR system?

(2) crRNA

The spacers are composed by 32 nt

The CRISPR array is first transcribed as a single crRNA, which directs the nucleolytic activity

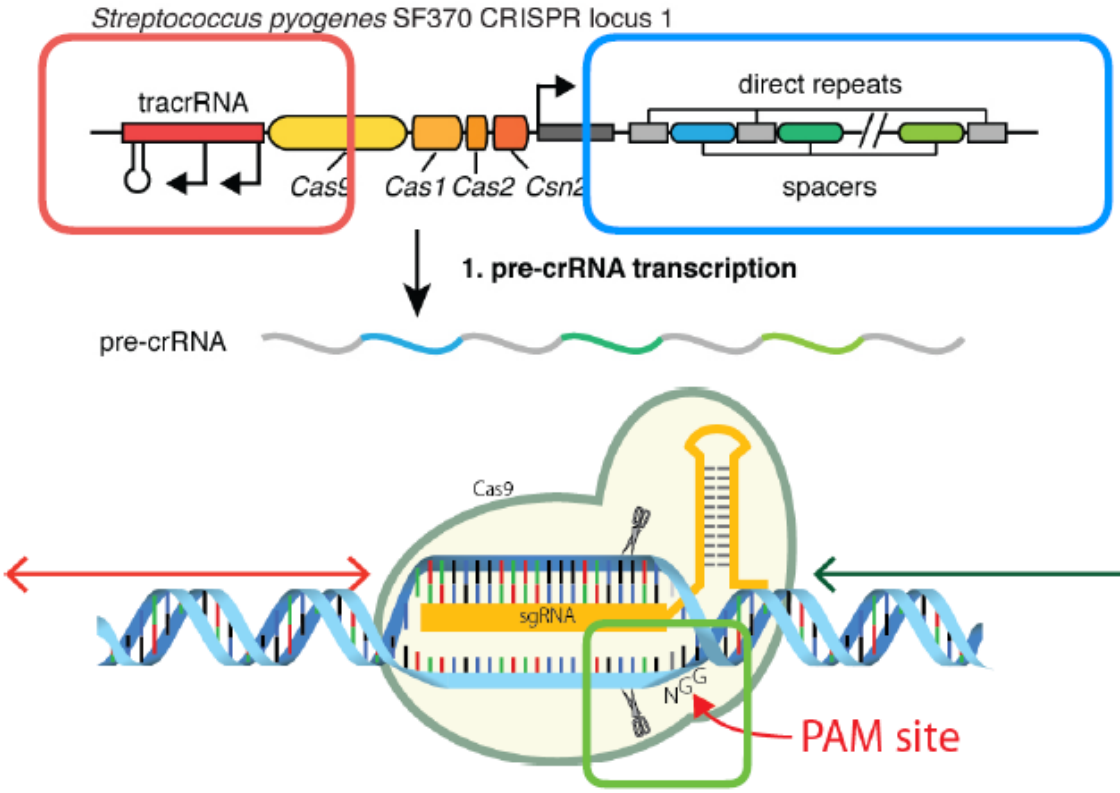
crRNAs recognize sequences of DNA with a Watson-Crick interaction

(3) tracrRNA

The trans-activating crRNA hybridizes with crRNA to facilitate RNA-guided targeting

(4) PAM

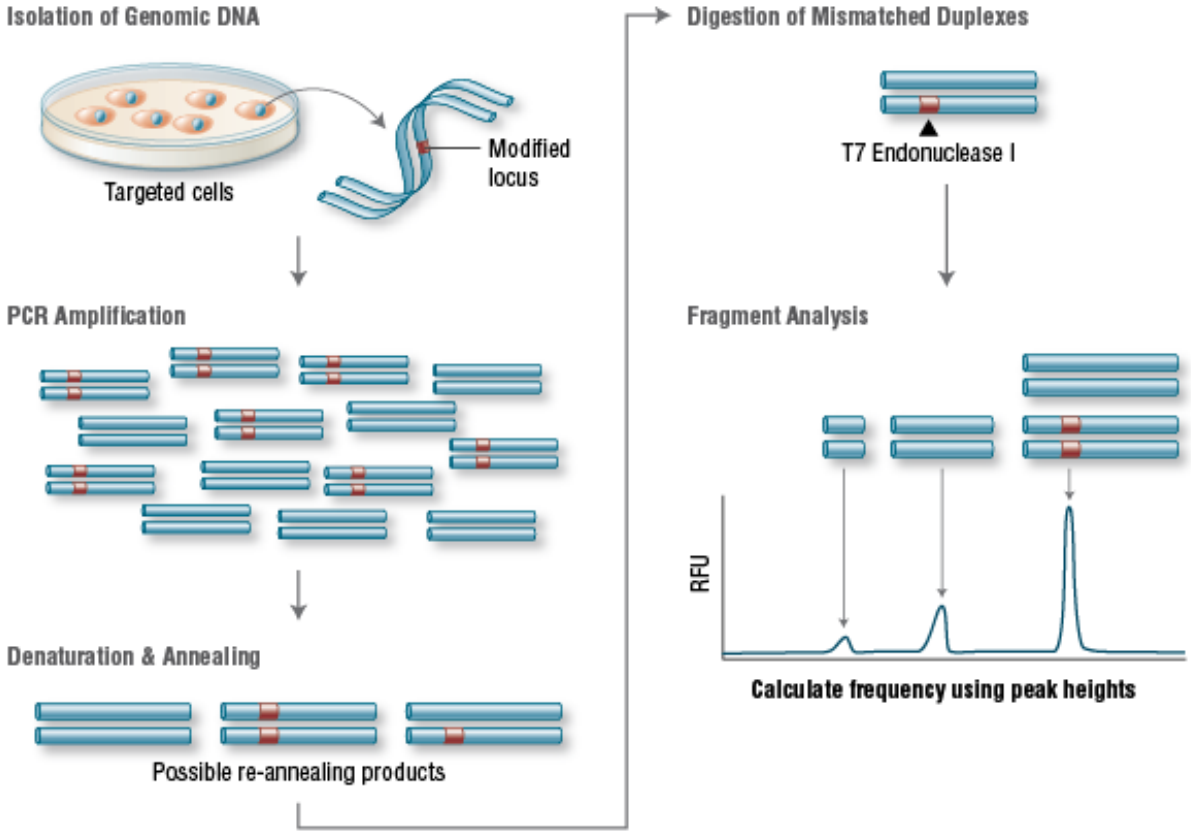
A short sequence of 3 nt called protospacer adjacent motif (PAM) (5'-NGG, in the CRISPR type II system) is required at the 5' of the crRNA



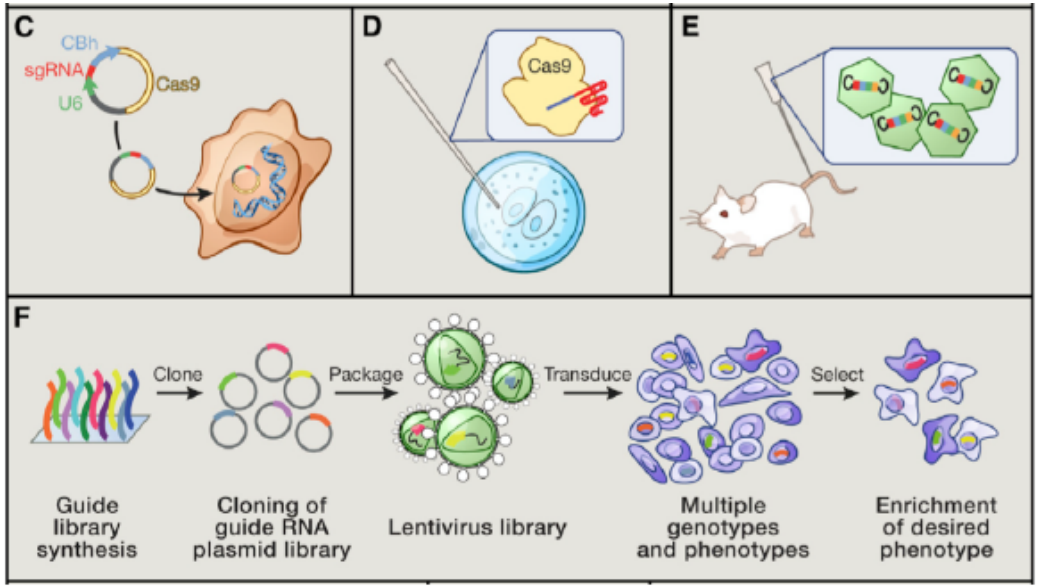
These two RNAs can be modified in vitro in order to create a single gRNA sequence (**sgRNA**), able to direct very efficiently the Cas9 activity

IN PRATICA????
Come possiamo creare le guide e vedere se funzionano??

- Si disegnano le guide (prediction bioinformatica)
- Si ordinano gli oligos
- Si clonano in un plasmide con il promotore adeguato e sequenze spacer
- Si trasfettano/trasducono insieme a Cas9 nelle cellule bersaglio
- Si guarda se la proteina di interesse è rimossa/ riparata in caso di templato substrato



CRISPR/Cas9 system possible application



C) Cells can be transfected with plasmids coding for the Cas9 sequence, for the guideRNA and for the DNA template to perform gene correction

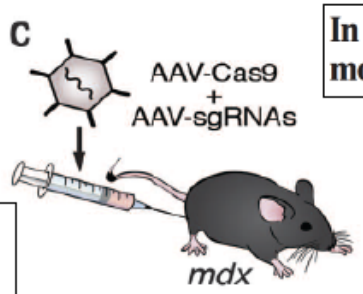
D) The purified Cas9 protein can be microinjected into fertilized zygotes to create animal models

E) To detect a somatic effect, viral vectors encoding for CRISPR sequence can be injected inside animal models

F) Functional high-throughput screening can be perform by infecting cells with viral vectors encoding for library of gRNAs and select for the desired phenotype

Systemic delivery of CRISPR-Cas9 restores dystrophin expression in mouse

Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy



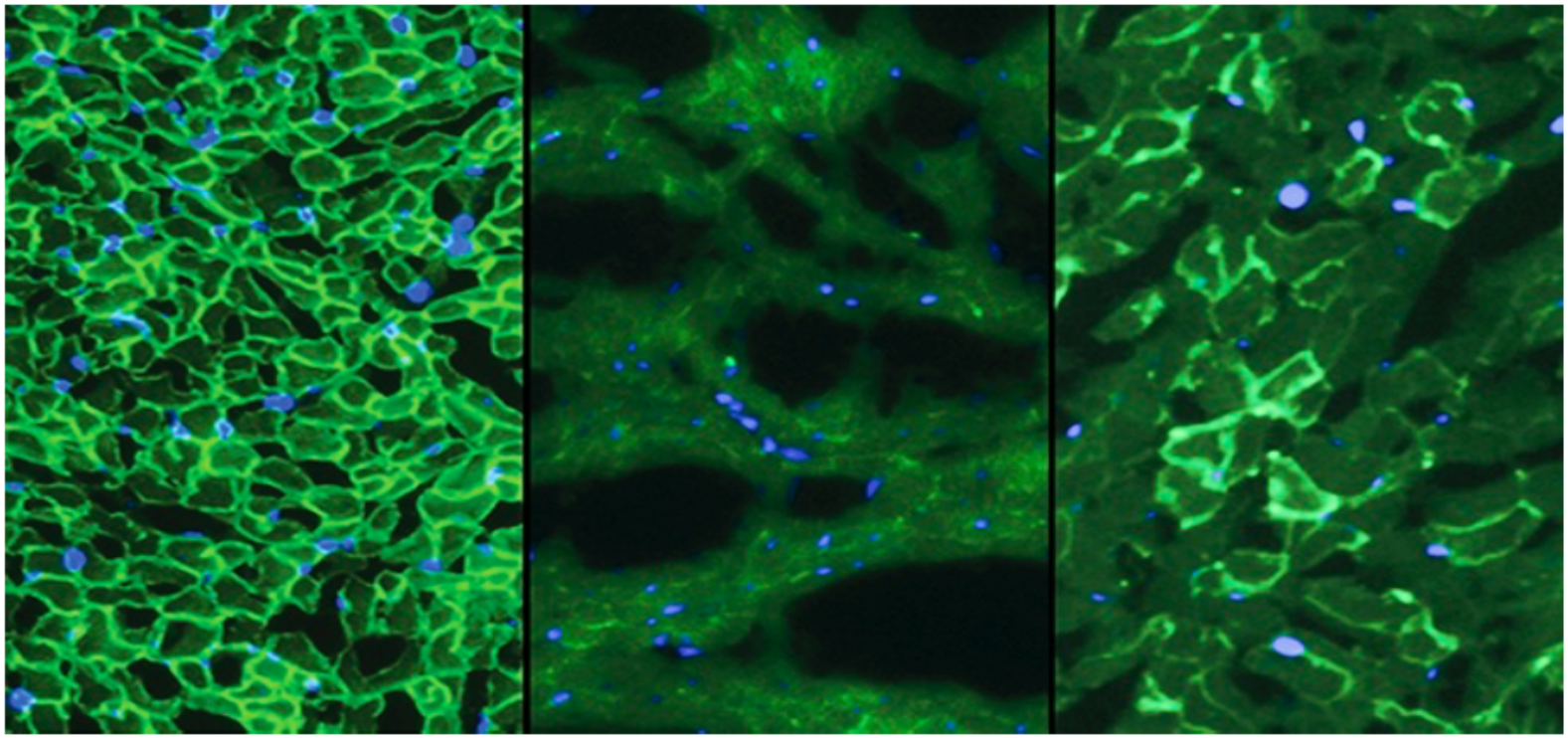
In vivo gene editing in dystrophic mouse muscle and muscle stem cells

Science

AAAS

Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA

In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy



WT

Muscular Dystrophy

Muscular Dystrophy + CRISPR/Cas 9

CRISPR/Cas9 system possible application

Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA

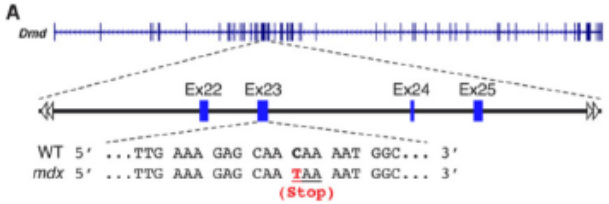
Chengzu Long,[1*] John R. McAnally,[1*] John M. Shelton,[2] Alex A. Mireault,[1] Rhonda Bassel-Duby,[1] Eric N. Olson[1†]

Duchenne Muscular Dystrophy (DMD)

It's a **X-linked pathology**, caused by a point mutation in the gene of Dystrophin (Dmd). This gene codifies for a large cytoskeletal structural protein, crucial for muscle cells and membrane integrity.

Animal model

mdx mice carrying a single point mutation (nonsense mutation) in the exon 23 of Dmd gene.



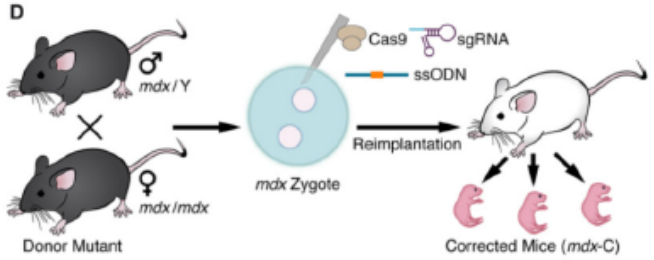
CRISPR/Cas9 system to correct the mutation

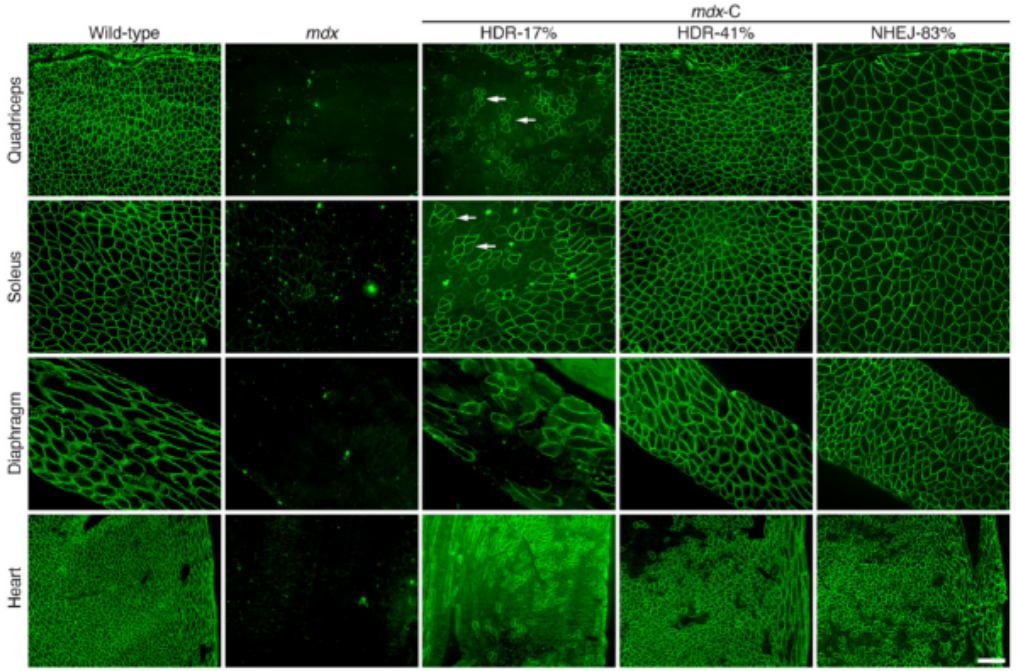
Cas9 mRNA, the sgRNA against Dmd exon 23 and ss oligonucleotide (ssODN) as donor DNA for gene correction were microinjected inside mdx Zigote. This was then reimplantated in pseudopregnant female mice.

CRISPR/Cas9 system possible application

Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA

Chengzu Long,[1*] John R. McAnally,[1*] John M. Shelton,[2] Alex A. Mireault,[1] Rhonda Bassel-Duby,[1] Eric N. Olson[1†]





Dystrophin immunofluorescence (green) in wilt-type mice is present in all muscles and is completely absence in mdx mice. In the HDR-41% and in the NHEJ-83% mdx-C mice muscles are composed by dystrophin-positive myofibers only.

Long *et al.* apply genome editing to "correct" the disease-causing mutation in mice genetically destined to develop the disease. This germline editing strategy kept muscles from degenerating, even in mice harboring only a small percentage of corrected cells. Although not feasible for humans, this proof of concept sets the stage for applying genome editing to specific cell types involved in the disease.

Curare l'emofilia?

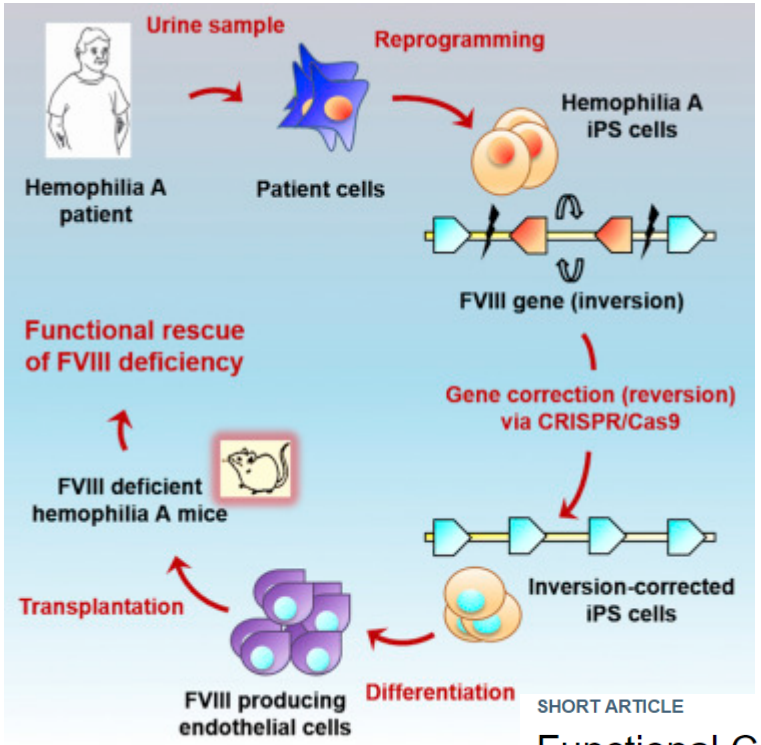
Research Article

Transparent Process Source Data Open Access

CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse

Yuting Guan, Yanlin Ma, Qi Li, Zhenliang Sun, Lie Ma, Lijuan Wu, Liren Wang, Li Zeng, Yanjiao Shao, Yuting Chen, Ning Ma, Wenqing Lu, Kewen Hu, Honghui Han, Yanhong Yu, Yuanhua Huang, Mingyao Liu, Dali Li

Author Affiliations



The X-linked genetic bleeding disorder caused by deficiency of coagulator factor IX, hemophilia B, is a disease ideally suited for gene therapy with genome editing technology.

These studies suggest that CRISPR/Cas-mediated *in situ* genome editing could be a feasible therapeutic strategy for human hereditary diseases, although an efficient and clinically relevant delivery system is required for further clinical studies.

Cell 2015

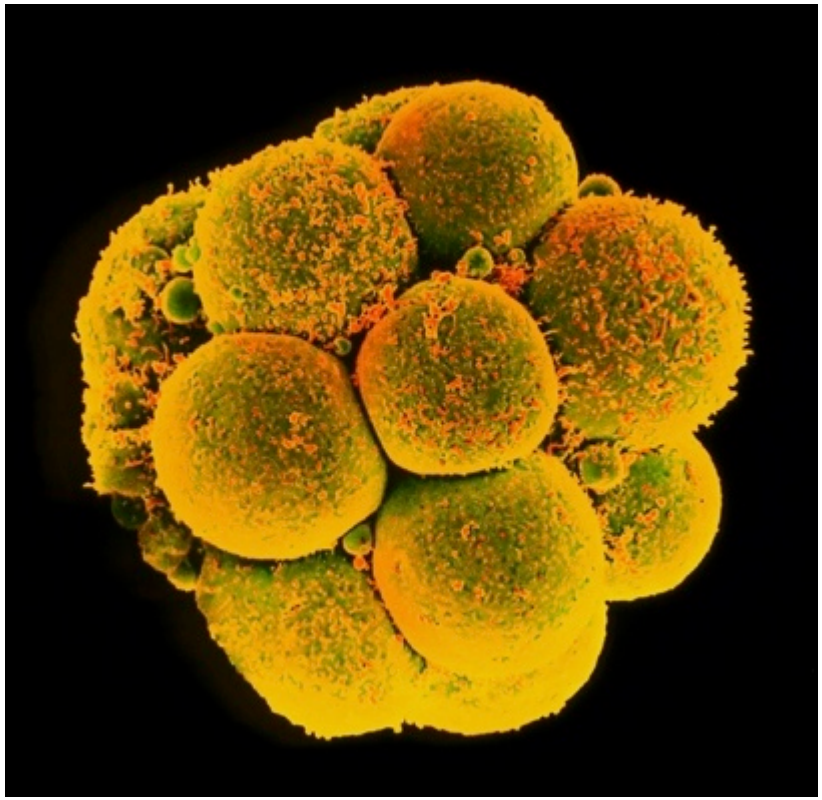
SHORT ARTICLE

Functional Correction of Large Factor VIII Gene Chromosomal Inversions in Hemophilia A Patient-Derived iPSCs Using CRISPR-Cas9

Chul-Yong Park[6], Duk Hyoung Kim[6], Jeong Sang Son[6], Jin Jea Sung, Jaehun Lee, Sangsu Bae, Jong-Hoon Kim[7], Dong-Wook Kim[7], Jin-Soo Kim[7]

CRISPR debate fueled by publication of second human embryo–editing paper

By **Jocelyn Kaiser** | Apr. 8, 2016 , 3:45 PM



NATURE | NEWS

Second Chinese team reports gene editing in human embryos

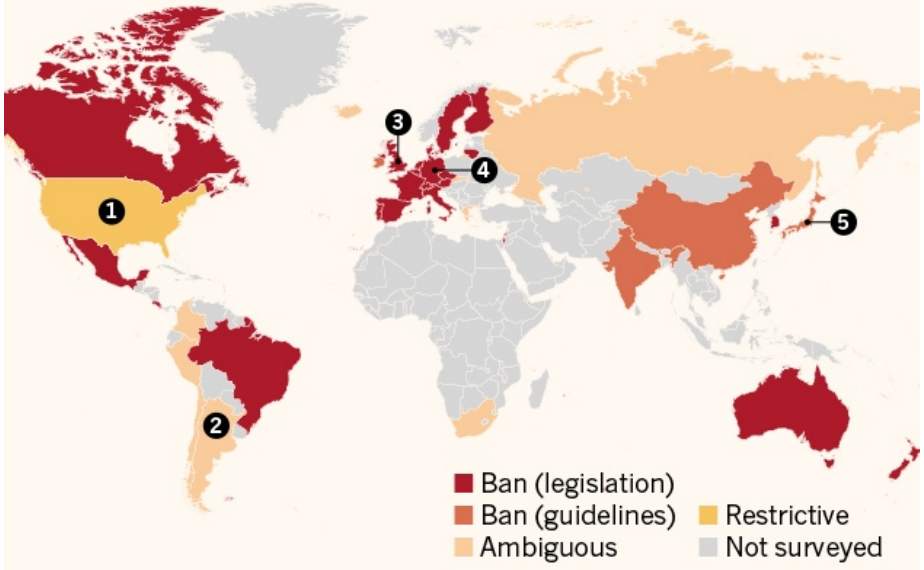
Study used CRISPR technology to introduce HIV-resistance mutation into embryos.

Ewen Callaway

A far scattare il cortocircuito tra ricerca scientifica ed etica è stato il passo compiuto da un gruppo di ricercatori cinesi che nell'aprile del 2015 ha annunciato di aver preso un'ottantina di embrioni umani (ancorché difettosi e destinati comunque a distruggersi) e di averli sottoposti al taglia e incolla per correggere il gene responsabile della talassemia.
L'esperimento, ripetuto un anno dopo per rendere gli embrioni resistenti all'Hiv, ha violato la moratoria che qualche mese prima gli stessi ricercatori pionieri della tecnica si erano autoimposti.

CRISPR EMBRYOS AND THE LAW

Regulations governing genetic modification in human embryos vary. Some countries ban the practice through legislation that carries criminal penalties; others have unenforceable guidelines.



1. THE UNITED STATES does not allow the use of federal funds to modify human embryos, but there are no outright genome-editing bans. Clinical development may require approval.

2. ARGENTINA bans reproductive cloning, but research applications of human-genome editing are not clearly regulated.

3. THE UNITED KINGDOM's independent Human Fertilisation and Embryology Authority may permit human-genome editing for research, but the practice is banned in the clinic.

4. GERMANY has strict laws on the use of embryos in assisted reproduction. It also limits research on human embryos, and violations could result in criminal charges.

5. JAPAN, like China, India and Ireland, has unenforceable guidelines that restrict the editing of a human embryo's genome.

Where in the world could the first CRISPR baby be born?

A look at the legal landscape suggests where human genome editing might be used in research or reproduction