Molecular Genetics Applications:
I. TECHNIQUES
(Ch.'s 15, 17, 18)

TODAY: Students should be able to…...

1. Diagram and describe the methods of **PCR, DNA sequencing, Restriction Enzymology, Hybridization techniques, and CRISPR** ……
   a) How were these methods mostly derived from ***knowledge of DNA structure and function***?
   b) What are some ***applications of each in research and medicine***?

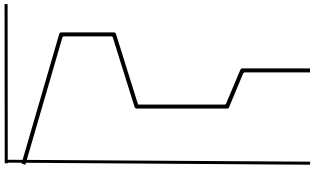
2. Describe some of the applications of ***DNA Hybridization*** in molecular cloning. What is a "*probe*"?

Cool Visuals!: http://www.hhmi.org/biointeractive/dna/animations.html

1

13.7) Practical Applications of DNA Replication:
A. Polymerase Chain Reaction

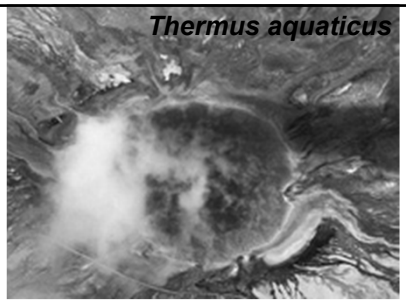
- The Polymerase Chain Reaction technique uses DNA polymerases to repeatedly replicate DNA in the test tube (*in vitro*).
- ***Specific DNA Primers flank target sequence.***
  - Usually, must know something about target sequence.
  - Primers tell the polymerase what to replicate, and where to stop!!
- **Reaction:**
  1. ***Thermostable (Taq) DNA Polymerase***,
  2. DNA Template,
  3. ***Primers***,
  4. Buffer Salts (neutral pH, Mg++),
  5. dNTPs



http://www.dnai.org/b/index.html → manipulations → tech. → ampl.
http://www.dnalc.org/ddnalc/resources/pcr.html

2

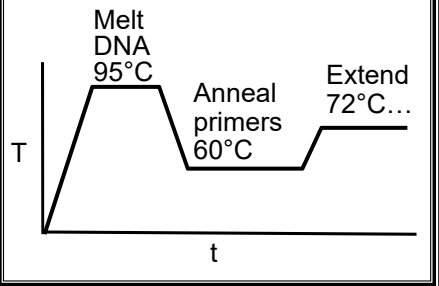
PCR Cycles



- **Many cycles of temperature-shifts:**

1. **Denature** Template (just 1 molecule needed!) = 95°C
2. **Anneal** Primers (large molar excess) = ~50-60°C
3. **Elongate** new strands = 72°C …..
4. Repeat 25-35 times!!
5. Produces AMPLIFIED, specific DNA fragment – for cloning, analysis, etc.

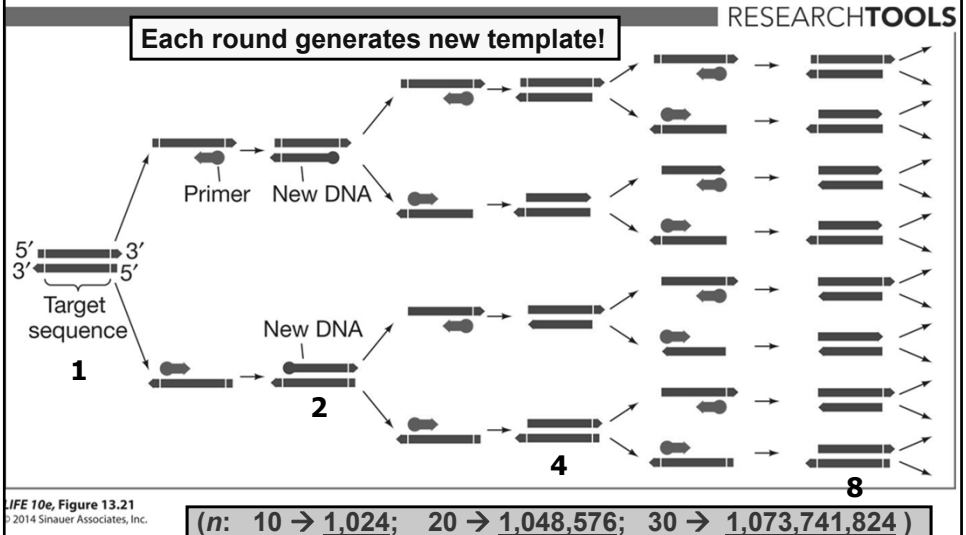
1 PCR CYCLE:



http://www.sumanasinc.com/webcontent/anisamples/molecularbiology/pcr.html

3

The Polymerase Chain Reaction (PCR)



LIFE 10e, Figure 13.21
© 2014 Sinauer Associates, Inc.

(*n*: 10 → 1,024; 20 → 1,048,576; 30 → 1,073,741,824)

***2^n* = the number of times the DNA is amplified!!**

- ***n*** = number of PCR cycles *(x number of starting DNA molecules)*
- ***This is MOLECULAR "CLONING" of one DNA sequence at a time!!***

4

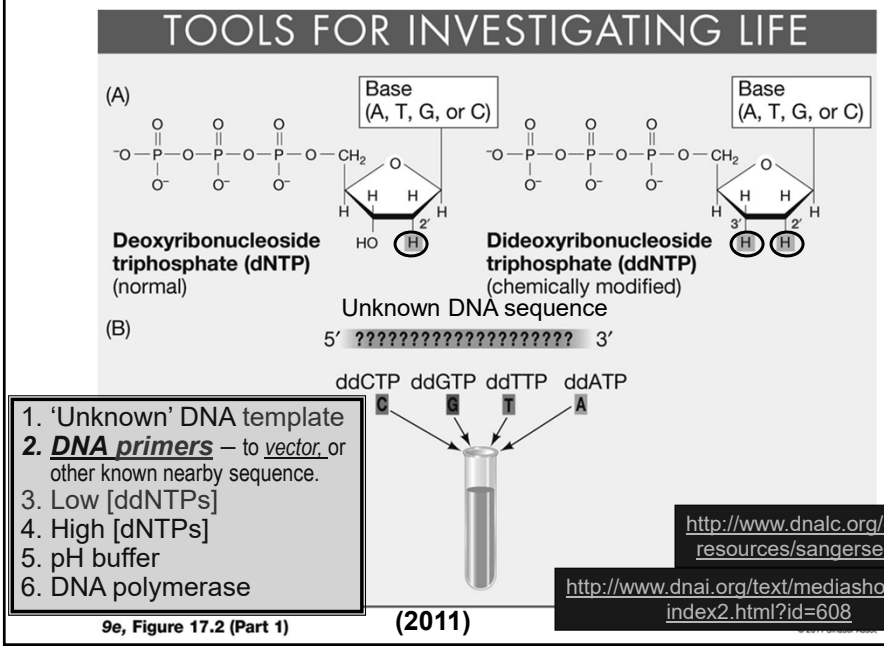
B. DNA Sequencing (Ch. 17)

- *The principles of DNA replication can be used to determine the nucleotide sequence of DNA.*
- ***ddNTPs* = *dideoxy "chain-terminators"*;** no free 3'OH, so synthesis stops when incorporated.
  - ddNTP put in at nucleotide early in sequence, = shorter frag
  - ddNTP incorporated later = longer frag (come off gel later)!
- Reaction = DNA Polymerase, ***DNA primers***, buffer salts, high [dNTPs], **very low [ddNTPs]** – each separately labeled with a different fluorescent dye.

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

5

DNA Sequencing (Ch. 17)



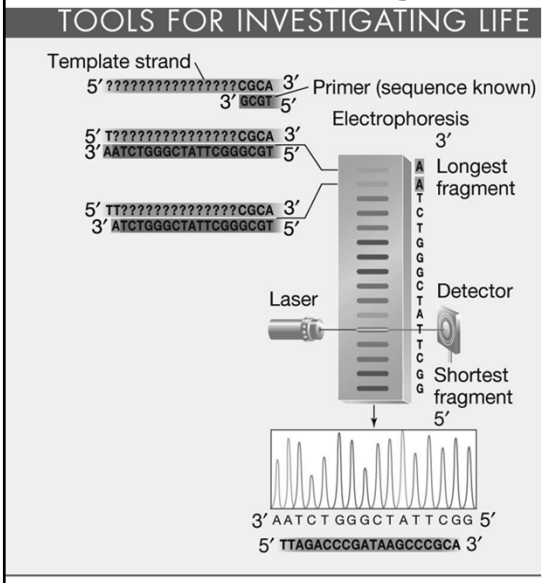
1. 'Unknown' DNA template
2. ***DNA primers*** – to *vector,* or other known nearby sequence.
3. Low [ddNTPs]
4. High [dNTPs]
5. pH buffer
6. DNA polymerase

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

http://www.dnai.org/text/mediashowcase/index2.html?id=608

6

DNA Sequencing Gel



17.2 (Part 2)

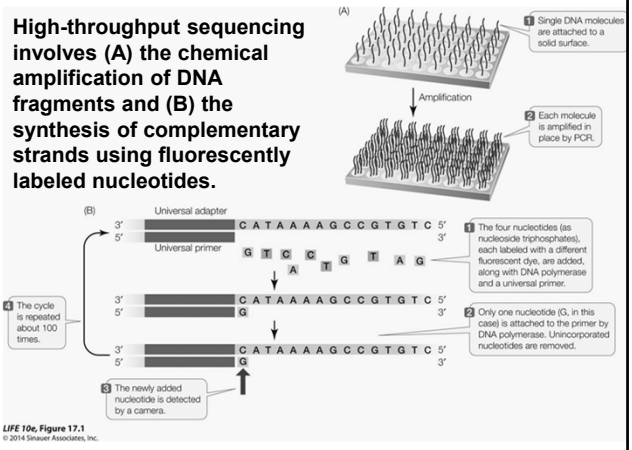
- Read Gel, or fluorescence signal detected by computer.
- Shortest/First fragments = nearest 5' end of newly synthesized strand.
  - (3' end, complementary to template sequence).
  - Each frag. "tagged" with terminal ddNTP.
    - Fluorescent label.
- Longer Frags. = near 3' end of sequence.
  - More sequence was polymerized → approached end of molecule.

http://highered.mcgraw-hill.com/sites/0072556781/student_view0/chapter15/animation_quiz_1.html
http://bioweb.uwlax.edu/GenWeb/Molecular/Theory/DNA_sequencing/dna_sequencing.htm

7

Figure 17.1 High-Throughput DNA Sequencing

1. DNA is cut into small fragments physically or using enzymes.
2. The fragments are denatured using heat, separating the strands.
3. Short, synthetic oligonucleotides are attached to each end of each fragment, and these are attached to a solid support (plate, beads).

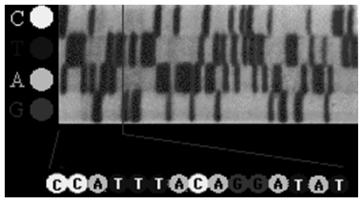


4. Fragments are amplified by PCR. **Sequencing:**
   a) Universal primers, DNA polymerase, and the 4 nucleotides (dNTPs, tagged with fluorescent dyes) are added.
   b) One nucleotide is added to the new DNA strand in each cycle, and the unincorporated dNTPs are removed by washing the support plate/beads.
   c) Fluorescent color of new nucleotide at each location is detected with a camera.
   d) Fluorescent tag is removed and the cycle repeats.

8

Sequencing the Human Genome

- The completed identification and sequencing of the **~18,000 human genes** *(MUCH smaller than predicted!)* …..
  - Genomics, Proteomics…



- already is leading to new methods of "molecular medicine".
  - Gene Therapy, and
  - Pharmacogenomics

http://www.pbs.org/wgbh/nova/genome/sequencer.html#

http://smcg.cifn.unam.mx/enp-unam/03-EstructuraDelGenoma/animaciones/secuencia.swf
http://www.wiley.com/college/pratt/0471393878/student/animations/dna_sequencing/index.html
http://www.biostudio.com/case_freeman_dna_sequencing.html

9

Other DNA Applications, based on Strx.:

15.1 Cleaving & Rejoining DNA

- **Genomic DNA is Huge!!**
  - Need to break it into relatively small fragments (**300bp to 6,000bp** {= 6 Kbp}) to analyze
    - (= Manageable sizes!!)

- ***Restriction enzymes***
  - made by microbes as a **defense mechanism against viruses**
  - bind to DNA at **specific sequences** and **cut** it.

10

Natural Function of Bacterial *Restriction Endonucleases*

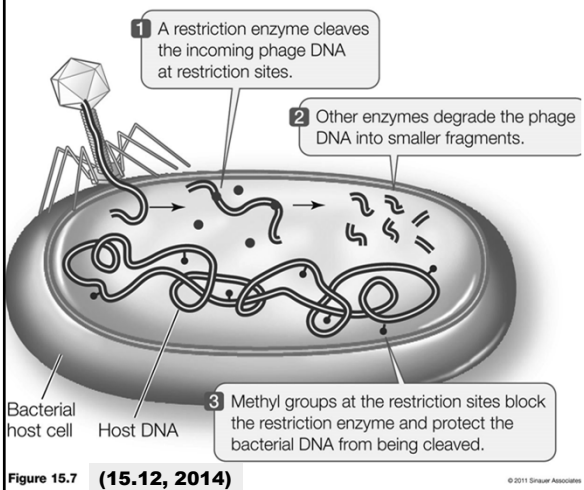


Figure 15.7 (15.12, 2014)

- Chop-up "Foreign" DNA!!
  - Bacterial defense mechanism!
- (unmethylated at endonuclease target sites)
- Reminder:
  - ***Endonuclease*** = cuts phosphodiester bonds within a DNA molecule;
  - ***Exonuclease*** = chews off single NT's 1 at a time, from the ends inward

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

11

Cleaving and Rejoining DNA

- DNA fragments generated from cleavage by restriction enzymes can be separated by size using ***Gel Electrophoresis***.
  - Cleave each DNA molecule at specific places **("sticky ends")**
  - Recognize specific DNA target sequences
  - ***"Restriction sites" (= Palindromic sequences):***
    - Eg: **Eco-RI = 5'_____G/AATTC_____**
      _____CTTAA/G_____

      Madam I'm Adam

      racecar

    - **Hin-dIII = 5'_____A/AGCTT_____**
      _____TTCGA/A_____

      Yo, Banana Boy!

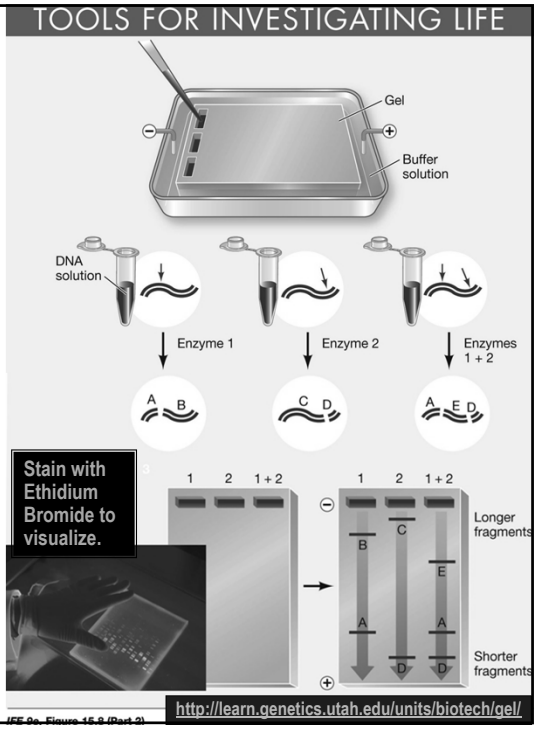
- The fragments' sequences can be further identified by ***Hybridization*** with a ***Probe***.

http://highered.mcgraw-hill.com/olc/dl/120078/bio37.swf

12

15.2 Restriction Digest & DNA Agarose Gel Electrophoresis

- ***DNA fragments (or RNA) can be separated by size***
- ***Through an aqueous gel matrix***
  - ***(Agarose, from seaweed)***
- Polysaccharide H-bonding forms porous gel
  - slows large molecules more than small molecules (=faster)
  - Electrical current drives macromolecule migration (to + electrode)



13

15.3 DNA Hybridization

- ***Annealing*** – complementary base-pairing between two DNA strands
  - If DNA strands = from different sources = "***Hybridization***"
- If one strand is labelled with a visible signal (fluorescent or radioiactive), it is called a "*PROBE*"
  - Used to detect it's complementary sequence on a gel or in tissues!!

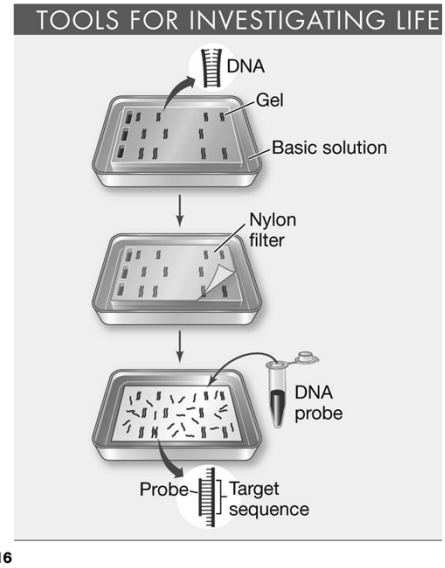


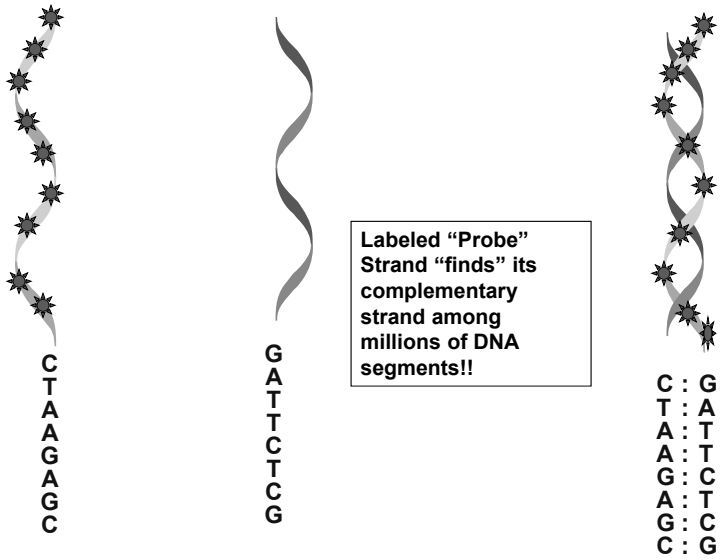
Figure 15.16

http://highered.mcgraw-hill.com/sites/0072556781/student_view0/chapter14/animation_quiz_4.html

14

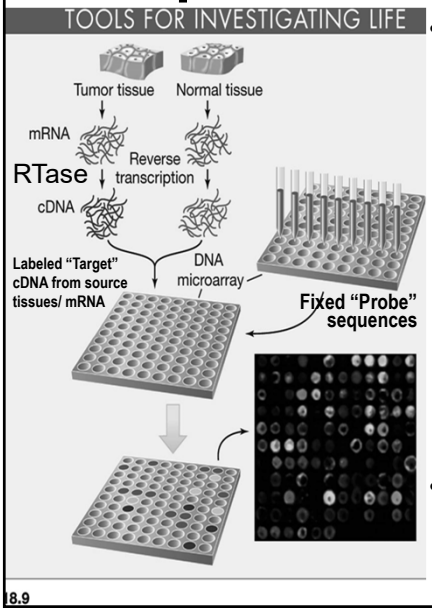
Hybridization **of matching DNA or RNA sequences:**

A "Probe" strand can find its match among millions!!!



15

Ch.18) Additional Tools for DNA Manipulation: DNA MicroArrays

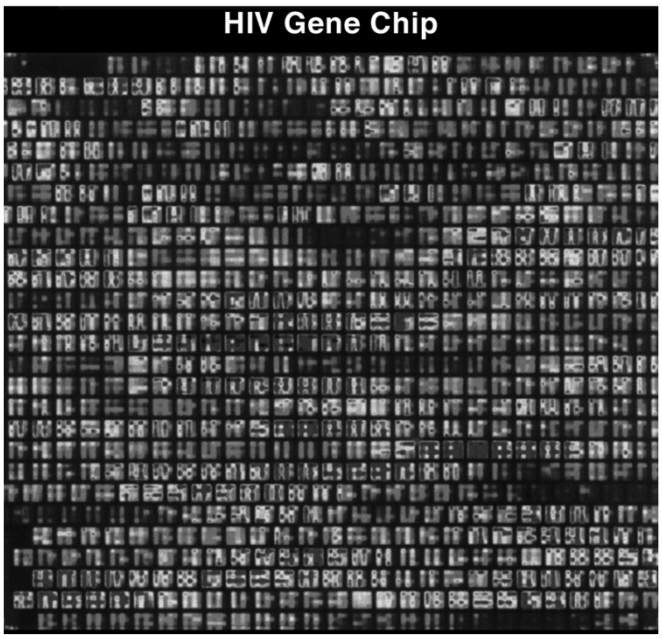


- ***DNA chip technology*** permits the screening of thousands of sequences at the same time.
  - ~40,000 Dots of oligo-DNA on 1 slide!! (1"x 3")
  - Each represents a different gene from organism
  - Computers analyze each "array" and identify which genes hybridize with each of your probes
  - EG: Cancerous Liver tissue vs. Normal, Healthy liver tissue
    - **Green** = genes on in NORMAL
    - **Red** = Genes on in CANCER
    - **Yellow** = Genes on in both (overlapping signals)
- ***Genes of interest = those ON or OFF only in cancer – may have role in regulating cancerous growth!!!***

16

HIV Gene Chip

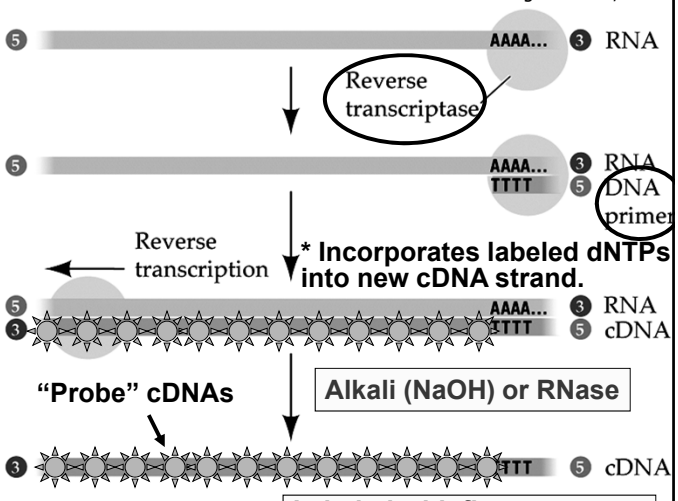
- **1000s of genes analyzed at once on one 1" x 2" slide!!!**
- Eg: Rapid identification of HIV strain causing an infection
- **→More effective treatment!**



17

Making "Probe" cDNA

figure 16.12, 2001



***cDNA** (complementary DNA) = complementary to an mRNA*

- **mRNA** = an expressed Gene!!
- So, **mRNAs** isolated from a tissue or group of cells under certain conditions = ***"SnapShot" of genes turned on under those conditions***

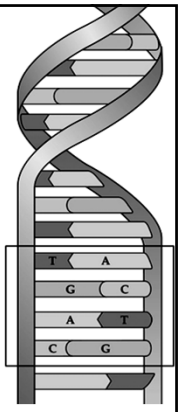
Labeled with fluorescent molecules (RED or GREEN)

- Can also use labeled **ssDNA** or **mRNA**
- Use labeled cDNA's to detect ***which genes are turned on or off under the tested conditions,*** or during a specific stage of development

18

DNA Hybridization

- **Target DNA** will "find" and bind to its specific complementary sequences on the **array Probes**
  - ***Hybridization*** = Watson-Crick specific base-pairing of complementary nucleic acid chains (A-T, G-C).
- The **labeled "spots" on the array indicate genes that are turned ON** in the source tissue/cells (bound by "Target" cDNA).
  - Very little messenger RNA (cDNA) needed – little biological material (even single cells!)!



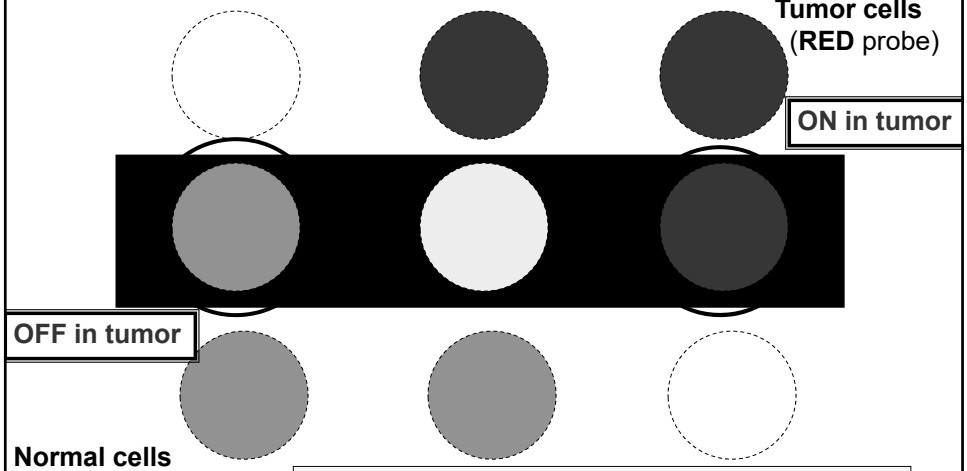
Spot #: 1285 0365 5927

[randomly-selected from thousands of spots]

19

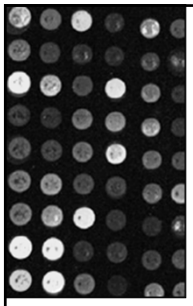
Computer overlays data from two samples for comparison

(3 randomly-selected spots from a microarray)

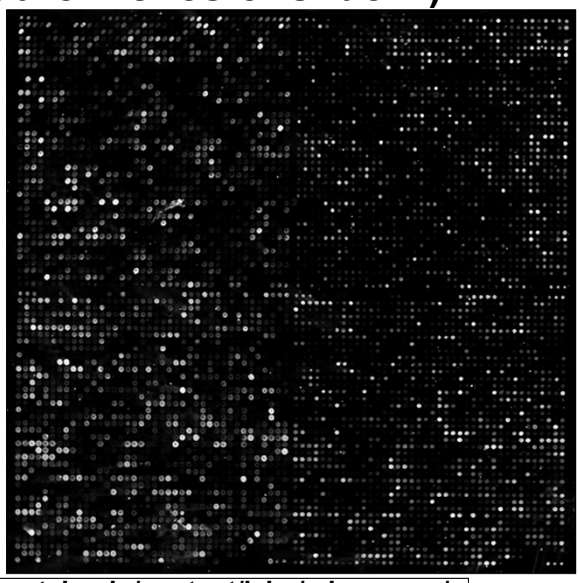


- **Identify genes that normally restrict**
- **Or abnormally promote tumor growth**

20



6,000 Yeast Genes
(on 3 square inches of slide!!!)



Interesting genes are those that are turned ON or OFF under the conditions tested!!

- e.g.: Lack of O_2 (anaerobic) vs. abundant O_2 (aerobic)

http://learn.genetics.utah.edu/content/labs/microarray/

21

22

II. CLONING & APPLICATIONS

TODAY's Objectives:

Students should be able to:

Ch. 18: Molecular Cloning

1. Describe and diagram how vectors and various enzymes are used to clone foreign genes into bacteria.

2. What is the difference between a cDNA library and a genomic DNA library?

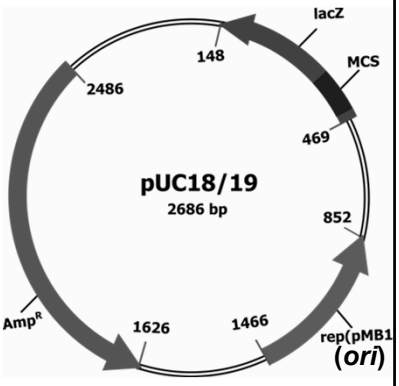
http://www.blackwellpublishing.com/trun/artwork/Animations/cloningexp/cloningexp.html

23

Ch. 18: Cloning Genes

(Lab Time)

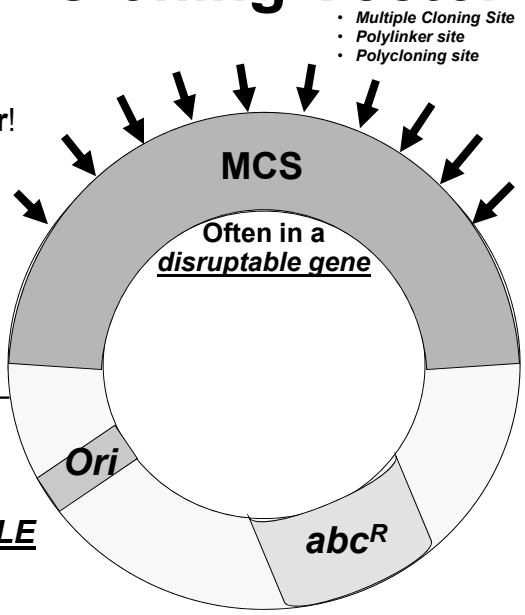
- Common hosts for recombinant DNA experiments:
  - **Bacteria, yeasts, and cultured plant cells**
- Newly introduced DNA must be part of a ***replicon*** (replicating unit) if it is to be propagated in host cells.
  - One way to assure this is to introduce it as part of a carrier DNA, or vector, that has a replicon (ie: with an ***Ori***).
- Specialized ***VECTORS*** transfect bacteria, yeasts, and plant cells.
  - These must contain:
    - a **replicon (self-replicating signals: *Ori*, centromere),**
    - **Restriction sites**, and
    - **genetic markers** to identify their presence in host cells.



24

18.1) "Generic" Cloning Vector

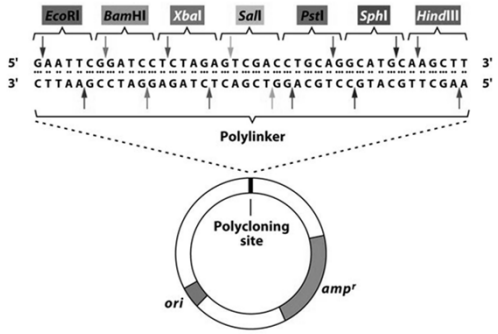
1. ***Replicon*** – an ***Ori***, and a centromere & telomere if for a eukaryote; prok = *Ori* & **circular**!
2. ***Selectable marker*** –
   - eg: antibiotic resistance ***REPORTER gene***
   - or a complementary nutritional gene for an auxotrophic host
3. ***Multiple Cloning Site*** – with recognition sites for multiple ***Restriction Endonucleases***
4. **MCS** often in a ***DISRUPTABLE Reporter GENE***
   - (eg: ***lacZ*** → Blue, artificial artificial product if intact)

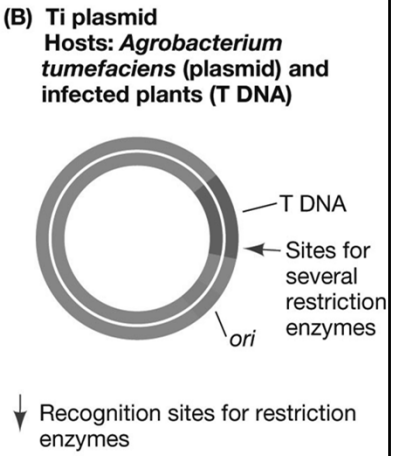


25

Vectors for Cloning

A Polycloning Site is a Cluster of Unique Restriction Sites





http://www.sumanasinc.com/webcontent/anisamples/molecularbiology/plasmidcloning_fla.html

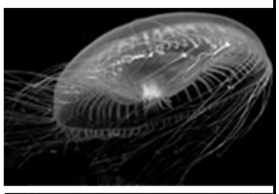
https://www.hhmi.org/biointeractive/genetic-engineering
http://www.hhmi.org/biointeractive/dna/animations.html

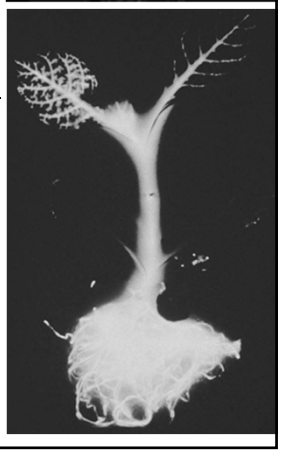
LIFE 9e, Figure 18.3

26

Cloning Genes

- Naked DNA may be introduced into a host cell by chemical or **mechanical means ("transformation!!")**.
  - In this case, the ***DNA must integrate into the host DNA by itself (if linear).***
  - https://www.dnalc.org/resources/animations/transformation2.html





- When vectors carrying recombinant DNA are incubated with host cells,
  - **nutritional, antibiotic resistance, or fluorescent markers** can identify which cells contain the vector.

27

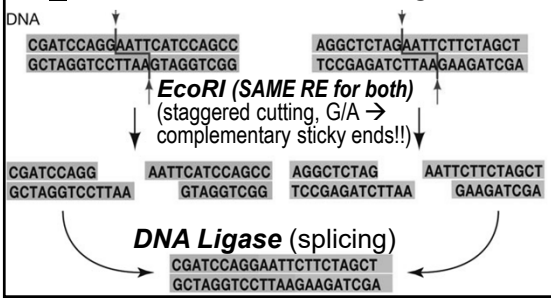
18.2) Sources of Genes for Cloning

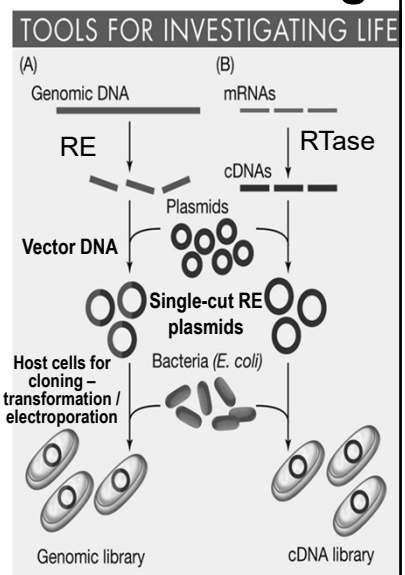
❖ The cutting of DNA by a restriction enzyme produces many fragments.
  - that can be individually and randomly combined with a vector (by ***DNA Ligase***!!)
  - and inserted into a host to create a ***GENE LIBRARY***.

1. Genomic DNA Libraries

2. cDNA Libraries

Figs 18.2, 18.6





18.6 **Each Colony in the Library has a fragment of gDNA or cDNA – a "volume" in the library!!**

28

cDNA

1) The mRNA's produced in a certain tissue at a certain time can be extracted and used to create ***complementary DNA (cDNA)*** by ***Reverse Transcription***.

2) This cDNA is then used to make a ***library***.
   a) Ligated into a vector
   b) Each cDNA (or genomic DNA restriction fragment) is taken up into bacterial cells in a vector
   c) Each bacterial "clone" of recombinant vector is a "Volume" in the DNA Library!!!
   d) Screen colonies with a labeled Probe for your sequence of intrest = Colony Hybridization

https://cooper7e.sinauer.com/animation0412.html

http://www.learnerstv.com/animation/animation.php?ani=167&cat=biology

29

cDNA – via *Reverse Transcriptase*

cDNA (complementary DNA) = complementary to an mRNA

- mRNA = an expressed Gene!!
- So, mRNAs isolated from a tissue or group of cells under certain conditions **= *"SnapShot" of genes turned on under those conditions***

- ***Best for finding many genes specifically associated with a certain process***
  - **Eg: Stress responses,**
  - **drought in plants,**
  - **protein deficiency on muscle responses,**
  - **yeast cell response to anaerobic conditions or heat stress, etc……**

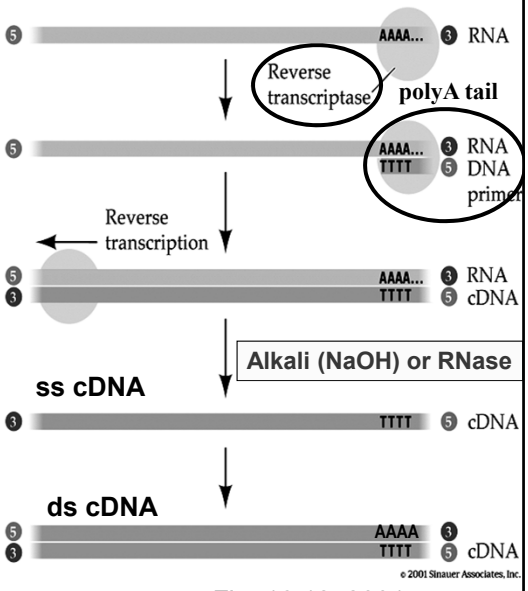
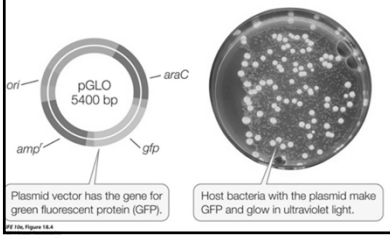


Fig. 16.12, 2001

30

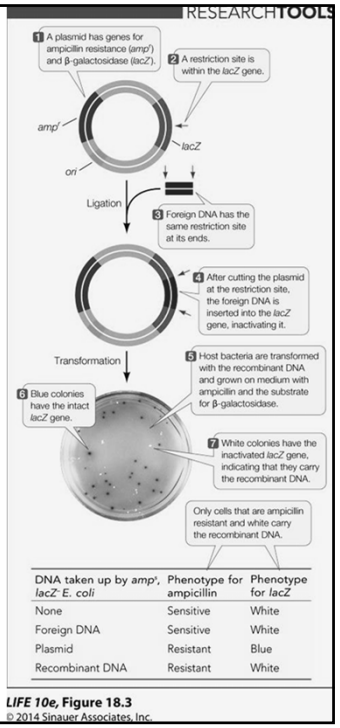
18.3) Selection for Recombinant DNA

- Disruptable Reporter Gene → **LacZ/ X-gal** or **pGLO** systems common now.
  - 2 STEPS IN CLONING:
  1) Select for transformed bacteria ("**transformants**", antibiotic-resistant)
  2) Screen for RECOMBINANT transformants (*lacZ*-, lack β-galactosidase, so **white**, NOT blue colonies on X-gal substrate)
  3) I.D. gene (specific colony clone) by ***Colony Hybridization*** with probe.



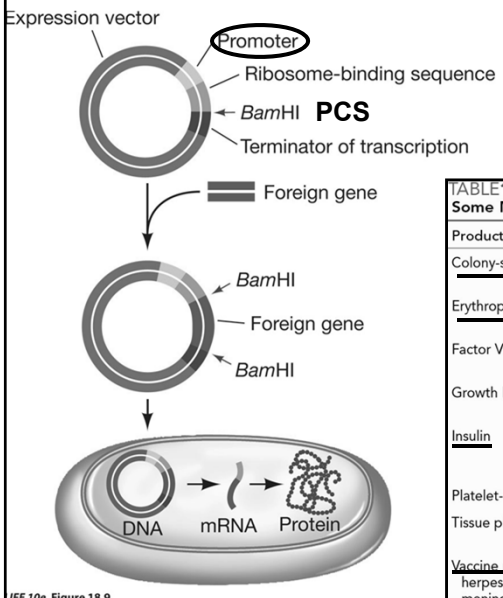
https://cooper7e.sinauer.com/animation0412.html

https://youtu.be/0DT093M4Ukc



31

18.4) Expression Vectors



How to find gene of interest in colony library:
1) Colony Hybridization with homologous probe
2) Antibody detection of protein product
3) Test for activity of the expressed protein

TABLE 18.1
Some Medically Useful Products of Biotechnology

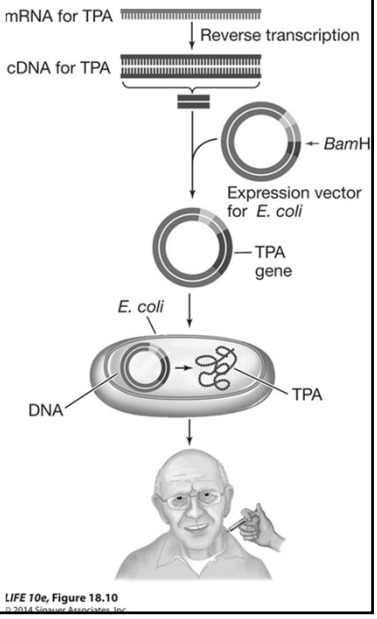
Product	Use
Colony-stimulating factor	Stimulates production of white blood cells in patients with cancer and AIDS
Erythropoietin	Prevents anemia in patients undergoing kidney dialysis and cancer therapy
Factor VIII	Replaces clotting factor missing in patients with hemophilia A
Growth hormone	Replaces missing hormone in people of short stature
Insulin	Stimulates glucose uptake from blood in people with insulin-dependent (Type I) diabetes
Platelet-derived growth factor	Stimulates wound healing
Tissue plasminogen activator	Dissolves blood clots after heart attacks and strokes
Vaccine proteins: Hepatitis B, herpes, influenza, Lyme disease, meningitis, pertussis, etc.	Prevent and treat infectious diseases

LIFE 10e, Table 18.1
© 2014 Sinauer Associates, Inc.

32

A. Biotechnology Is Changing Medicine, Agriculture, and the Environment:

1. ***Many medically useful products are being made using biotechnology:***
2. Example: The manufacture of ***tissue plasminogen activator*** **(TPA).**
   a) After wounds heal, blood clots are dissolved by ***plasmin***. Plasmin is stored as an inactive form called ***plasminogen***.
   b) Conversion of plasminogen is activated by TPA.
   c) TPA can be used to treat strokes and heart attacks, but large quantities are needed.
      • Can be made using recombinant DNA technology.



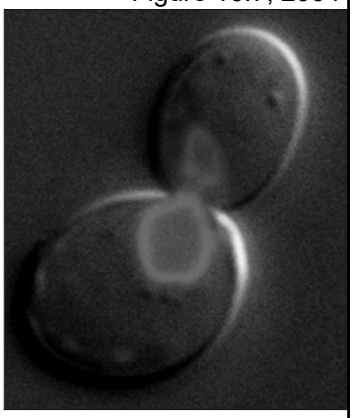
LIFE 10e, Figure 18.10

33

Biotechnology: Applications of DNA Manipulation

- The ability to clone genes has made possible many new applications of biotechnology,
  – such as the large-scale production of eukaryotic gene products.
- For a vector carrying a gene of interest to be ***expressed*** in a host cell,
  – the gene must be adjacent to appropriate sequences for its transcription and translation in the host cell.
  – ***= Tscn start (promoter), RBS (ribosomal binding sequence), PCS, [start and stop codons in recombinant gene], Tscn stop.***

Figure 16.7, 2004



Jellyfish Green Fluorescent Protein "*Reporter" gene* in dividing yeast cells

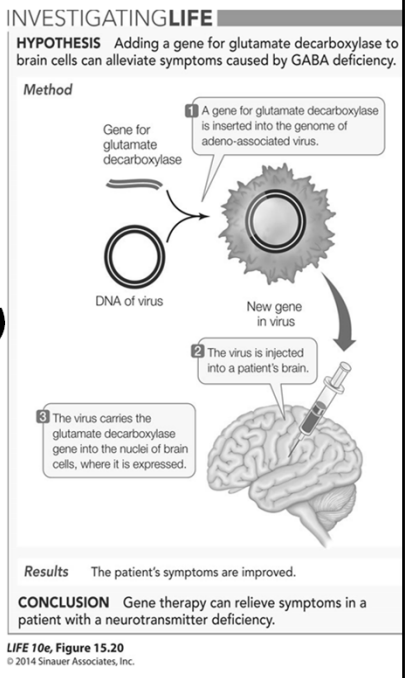
34

B. "Gene Therapy"

- just a form of "***transformation***" with DNA.
  - **Andrew Feigin** and his colleagues showed that a virus can be used to insert a therapeutic gene into the brains of patients with **Parkinson's Disease**.
  - Used the **glutamate decarboxylase** gene – encodes the enzyme that catalyzes the synthesis of the neurotransmitter, GABA.
- Use **Viral *"vector" (carrier)***
  - to transport the functional ("good") form of a gene into a patient's cells
  - to restore function lost in diseased gene.

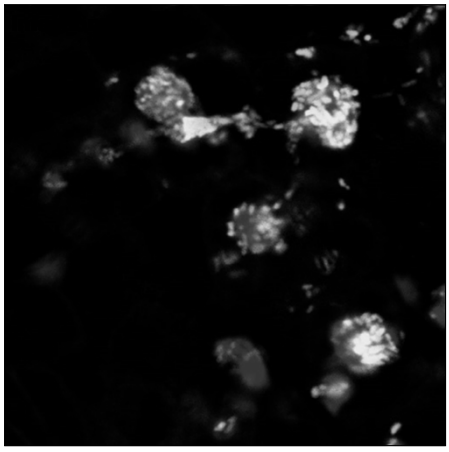
  - *& "Ex vivo" methods*

http://learn.genetics.utah.edu/content/genetherapy/



35

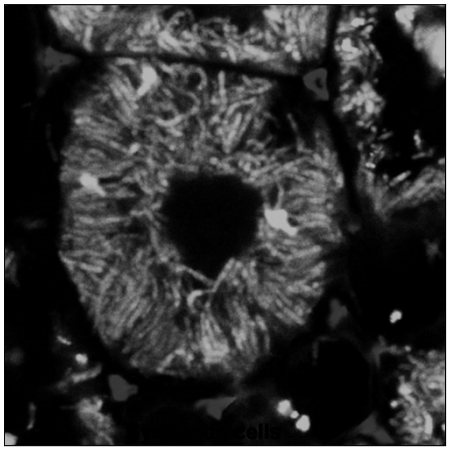
C. Expression Vectors for Studying Agriculture & the Environment



[a lot like our pGLO in the lab!!! But with a *constitutive* promoter]

Transgenic, GFP-expressing bacteria invading plant root tissues

- Symbiotic plant cell wall proteins labeled Red
- Allows visualization of LIVE biological processes!!



36

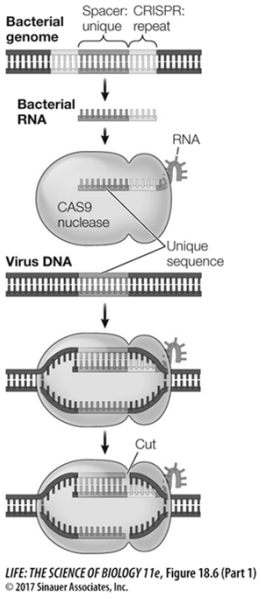
18.5) GENE EDITING: Several Tools Are Used to Modify DNA and Study Its Function

- Another way to study a gene is to inactivate it, or change the sequence so that an altered gene product is made. https://youtu.be/ZImVkI8QTW8 *** short
  http://time.com/4377130/crispr-genome-editing/
- **CRISPR/Cas9 technology** can add, disrupt, or change the sequence of specific genes.
  - It emulates a mechanism found in some bacteria and archaea for viral defense.

https://youtu.be/2pp17E4E-O8 = MIT

https://youtu.be/MnYppmstxIs = Bozeman

https://youtu.be/TdBAHexVYzc = J. Doudna



LIFE: THE SCIENCE OF BIOLOGY 11e, Figure 18.6 (Part 1)
© 2017 Sinauer Associates, Inc.

37

CRISPR/Cas9 Technology:

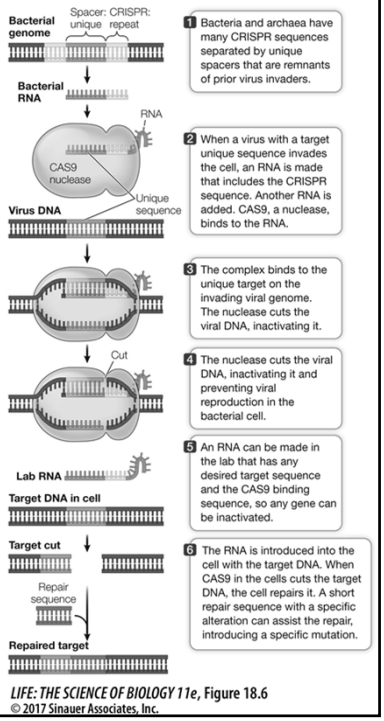
Tools Used to Modify DNA and Study Its Function

- **CRISPR** (***c***lustered ***r***egularly ***i***nterspaced ***s***hort ***p***alindromic ***r***epeat **s**equences):
  - **Short palindromic DNA** sequences (form hairpin/stem-loop structures in RNA!!) with **unique 24-bp spacer DNA in between**.
    - **Spacers are fragments of DNA from viruses** that previously infected the cell. They are hereditary!
      - A series of "WANTED" posters (or a vaccination card!) of previous viral parasites that the cell can now recognize and Destroy!!
  - When a virus with a sequence similar to a spacer invades the cell, the **CRISPR-spacer unit is transcribed to RNA**.
  - The spacer attaches to the viral genome by base pairing; **Cas9 protein** joins the complex and cuts the viral DNA.

38

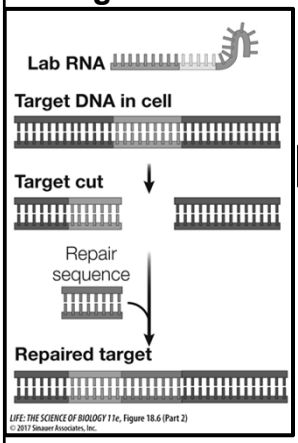
Several Tools Are Used to Modify DNA and Study Its Function

- **CRISPR** has been adapted to **edit any gene**. All that is needed is the RNA complementary to the target (a ***"guide" RNA***) and the **CAS9 enzyme**.
  - **CAS** = **C**RISPR-**AS**sociated genes (nucleases, helicases)
  - In the lab, genes can be inactivated, specific mutations can be induced, or mutations can be repaired.
  - **https://vimeo.com/112757040** ***
  - https://youtu.be/2pp17E4E-O8



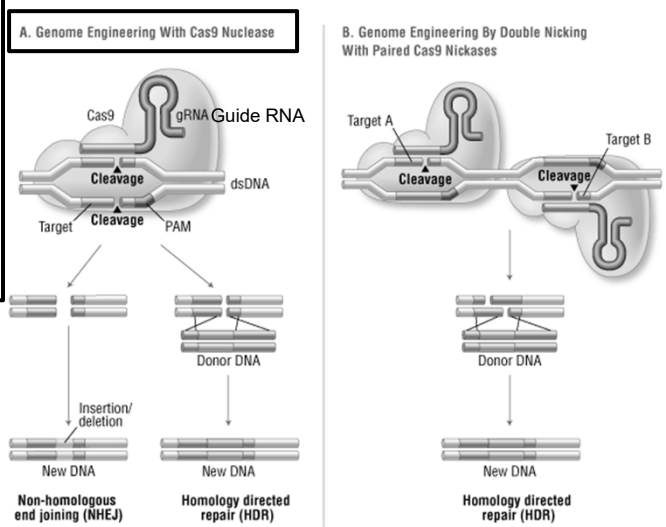
39

Figure 18.6 Inactivating or Mutating a Gene by CRISPR



https://youtu.be/qO6rusT24xo (3D)

https://www.neb.com/tools-and-resources/feature-articles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology



- ***PAM (protospacer adjacent motif)* for *S. pyogenes* Cas9 binding.**

40