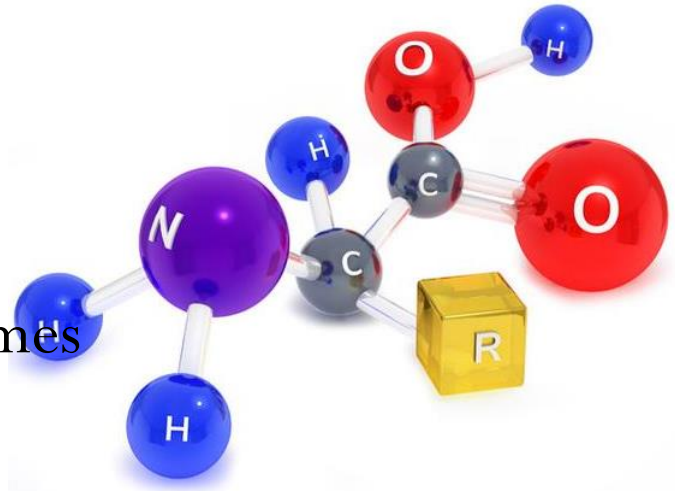
BIOTECHNOLOGY IN PHARMACEUTICAL PRODUCTION

Lecture

The Production of Commercial Products by Recombinants Microorganisms

Molecular biotechnology can be used to enhance the production of many commercially important compounds e.g.

- Vitamins
- Amino acids
- Antibiotics
- Restriction enzymes
- Ascorbic acid
- Microbial synthesis of the dye indigo
- Production of xanthan gum

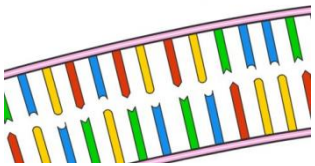


Xanthan gum

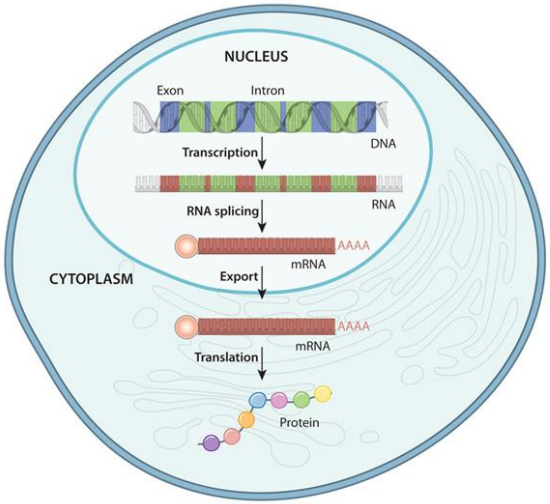
Therapeutic Agents

- Before the advent of molecular biotechnology most human proteins were available in only small (limited) quantities.
- Today hundreds of genes for human proteins have been…

…cloned



… sequenced



… expressed in the host cells

… tested as therapeutic agents (drugs) in humans

Types of biomolecules produced through recombinant DNA technology

Recombinant Hormones:

- Insulin (and its analogs), growth hormone, follicle stimulating hormone, salmon calcitonin.

Blood products:

- Albumin, thrombolytics, fibrinolytics, clotting factors (Factor VII, Factor IX, tissue plasminogen activator, recombinant hirudin)

Cytokines and growth factors

- Interferons, interleukins and colony stimulating factors (Interferon, α, β and γ, erythropoietin, interlukin-2, GM-CSF, GCSF)

Monoclonal antibodies and related products:

- Mouse, chimeric or humanized; whole molecule or fragment; single chain or bispecific; and conjugated (rituximab, trastuzmab, infliximab, bevacizumab).

Types of biomolecules produced through recombinant DNA technology

Recombinant Vaccines:

- Recombinant protein or peptides, DNA plasmid and anti-idiotype (HBsAg vaccine, HPV vaccine)

Recombinant Enzymes:

- Dornase– α (Pulomozyme), Acid glucosidase (Myozyme), α –L- iduronidase (Aldurazyme) and Urate Oxidase

Miscellaneous products:

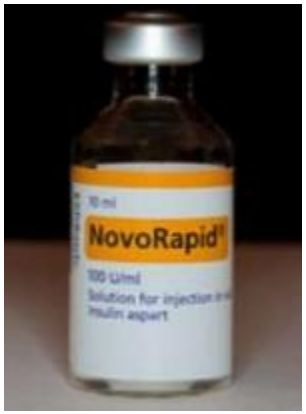
- Bone morphogenic protein, conjugate antibody, pegylated recombinant proteins.

Economical profits in market

- The market for recombinant therapeutics has considerably improved with generation of new molecules and new expression systems.
- Even though the overall world market has been around $30-40 billion it is expected to reach around $75 billion by end of this decade.

Human insulin

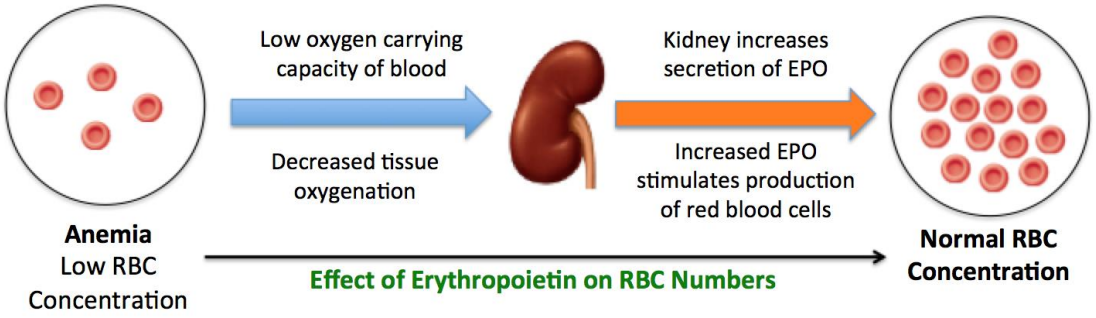
Bacteral cells



GM-insulin

GM-technology of production insulin

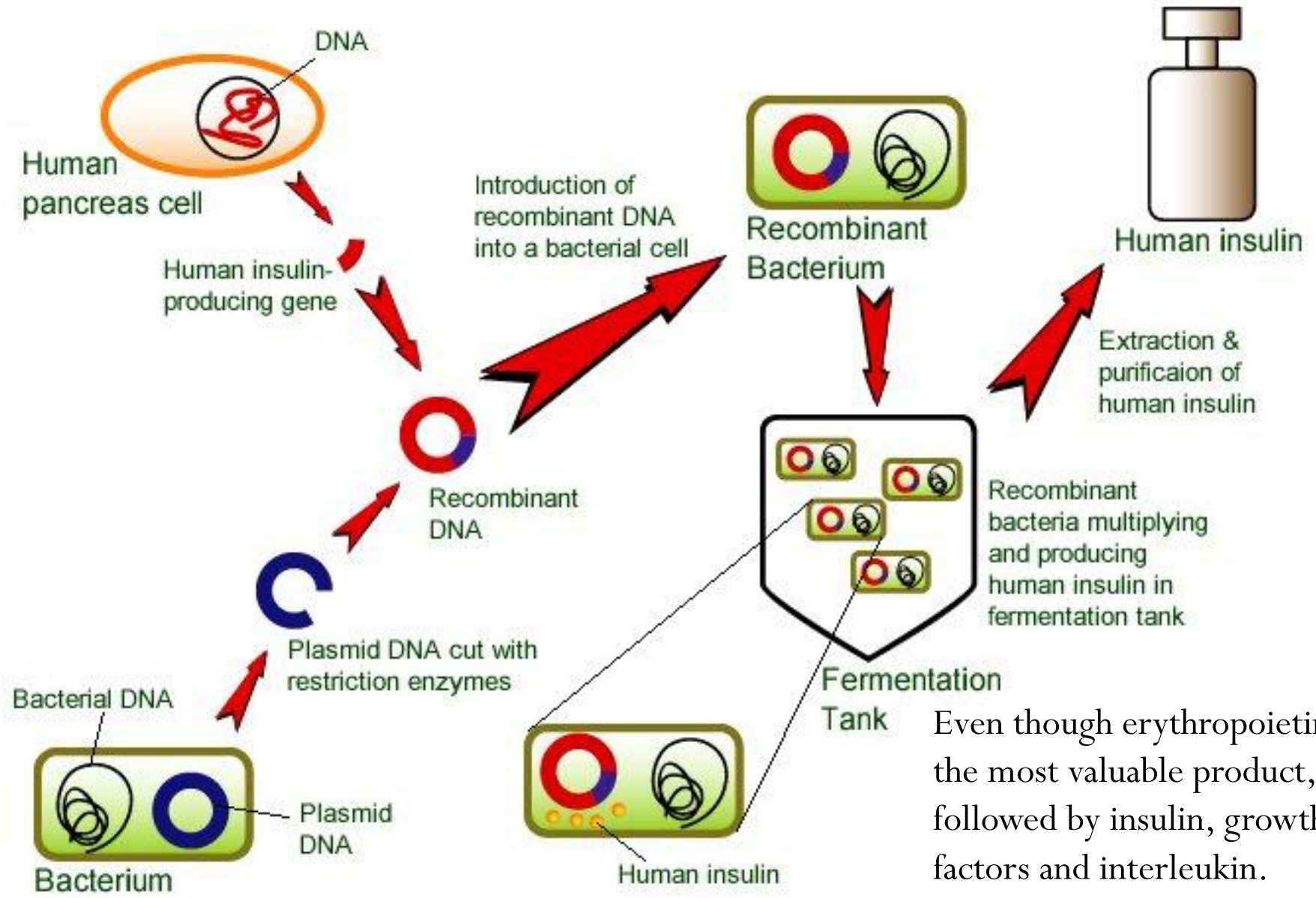
In fact the major money producer has been few biomolecules such as insulin, erythropoietin, interferon and hormones. These few molecules take a major share of biopharmaceutical market.



GM-technology of Erithropoietin production

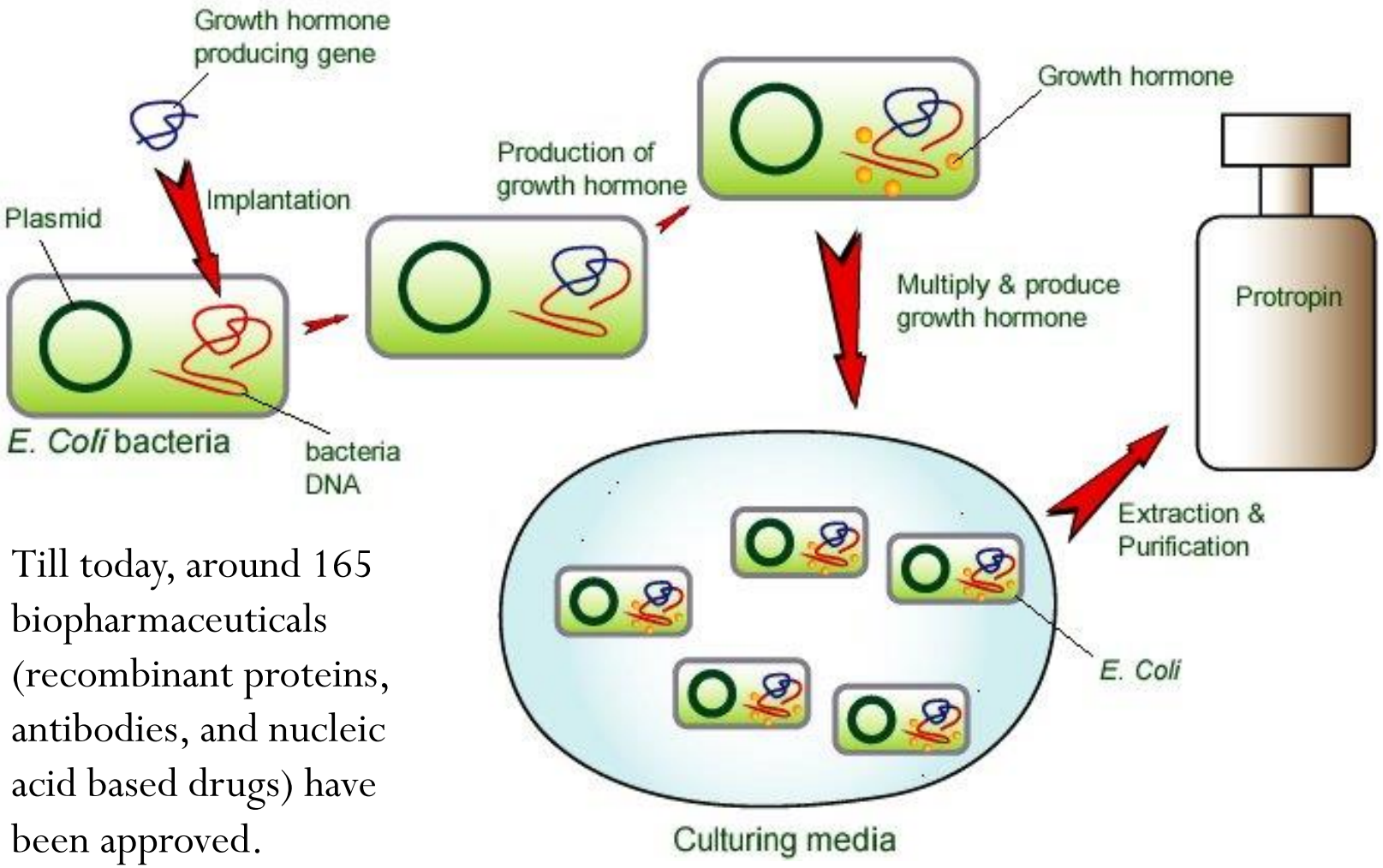
It is projected that erythropoietin market will be around $10 billion by next five years.

Human Insulin Production



Even though erythropoietin is the most valuable product, it followed by insulin, growth factors and interleukin.

Protropin Production



Till today, around 165 biopharmaceuticals (recombinant proteins, antibodies, and nucleic acid based drugs) have been approved.

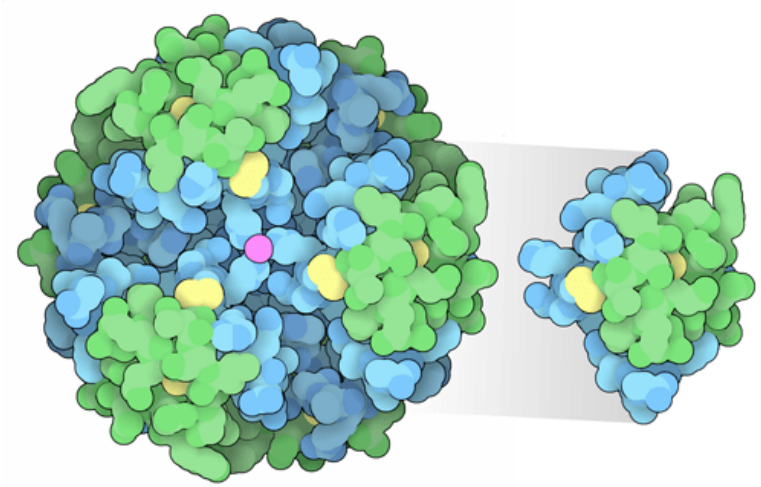
Human therapeutics from recombinant DNA technology

- One of the greatest benefit of the recombinant DNA technology has been the production of human therapeutics such as hormones, growth factors and antibodies which are not only scarcely available but also are very costly for human use.

Ever since the recombinant insulin was produced by Eli Lilly in 1982, considerable efforts has been made world wide to clone and express many therapeutically important proteins, which are otherwise difficult to produce either by extraction from the natural sources or by chemical synthesis.

Colonel Eli Lilly, American soldier, pharmacist, chemist, and businessman who founded the Eli Lilly and Company pharmaceutical corporation.

Profits of Human Therapeutic Proteins from Recombinant DNA Technology



Therapeutic proteins are preferred over conventional drugs because of their…

- Higher specificity
- Less toxic
- Neither teratogenic
- Absence of side effects
- Neither carcinogenic

- Further, once the biologically active form of a protein is identified for medical application, its further development into a medicinal product involves fewer risks than chemical drugs.

Notable diseases include diabetes, hemophilia, hepatitis, myocardial infarction and various cancers

Help the body to fight infection or to carry out specific functions such as blood factors, hormones, growth factors, interferones and interleukins.

Improvement of pharmacokinetic parameters

Lower cost

Availability of large amount of pure molecules has helped in development of its different modified form to have improved pharmacokinetic parameters.

Pegylated proteins

Pegylated proteins and controlled release formulation of biomolecules have become reality with improved characteristics. It has led to development of new molecules having improved performances.

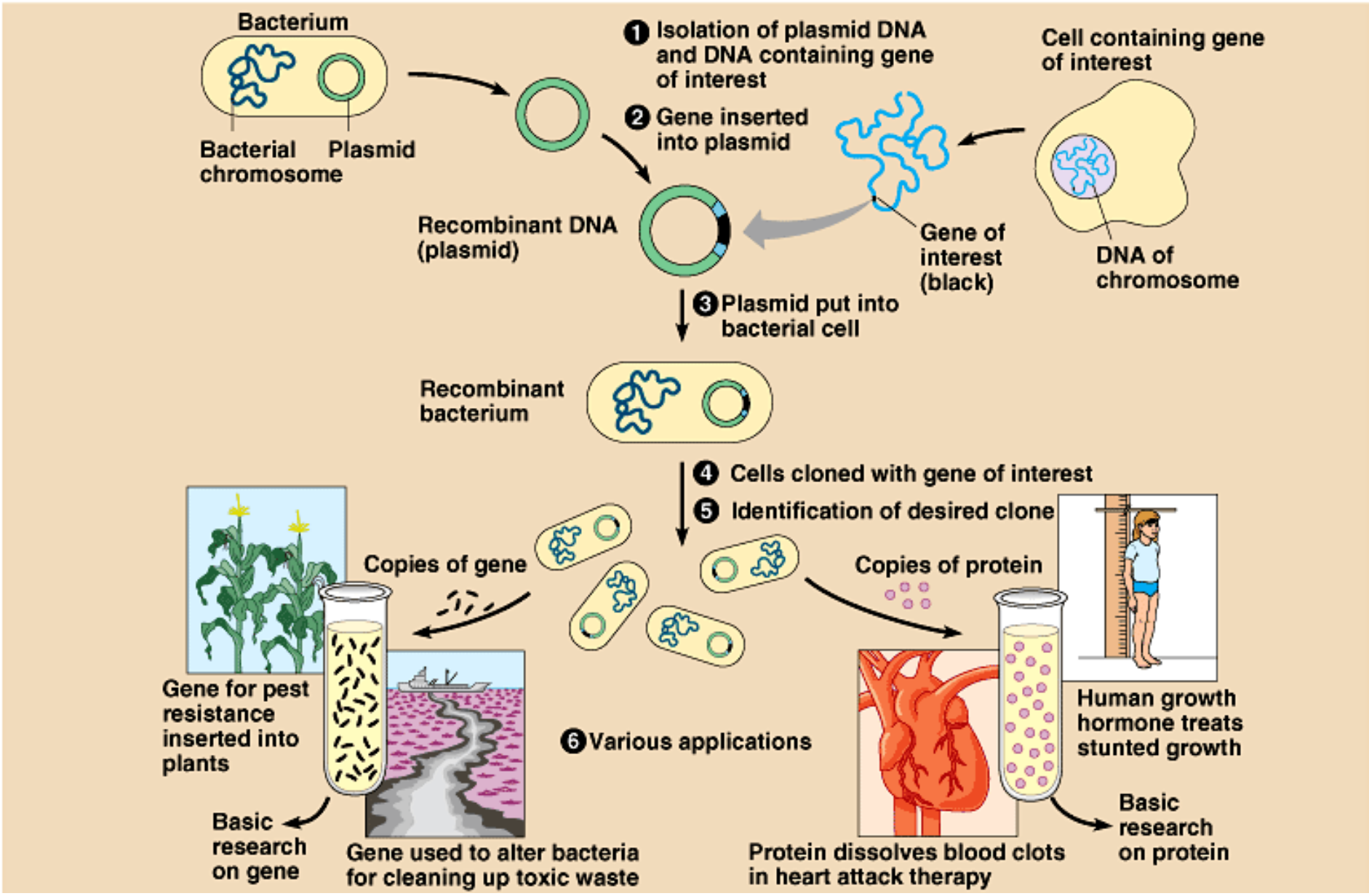
Peg-insulin | Peg-interferon | Peg-antibodies | Peg-growth factors

It has led to long acting, slow release, acid stable etc.

The most notable applications of the recombinant technology

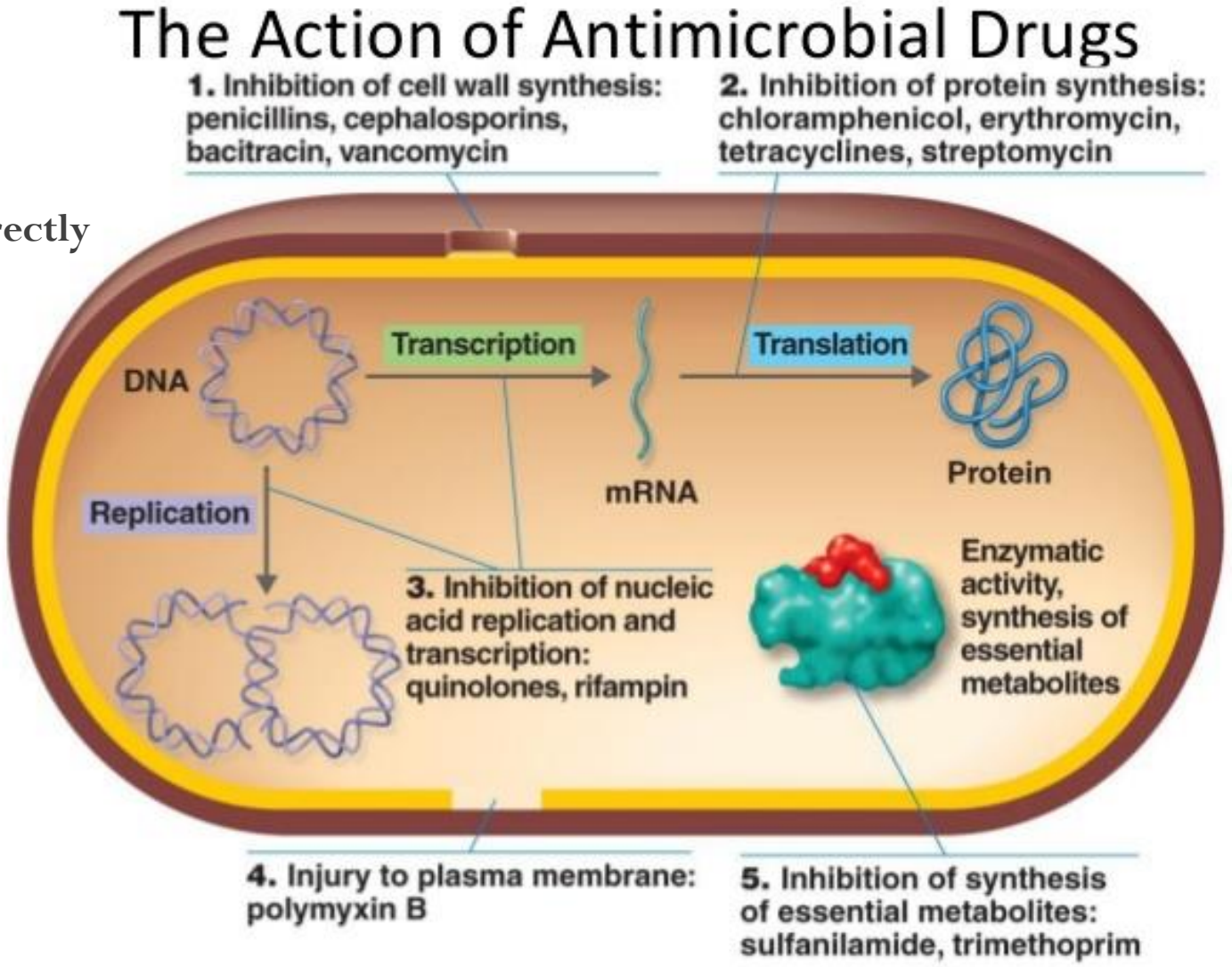
- Large scale production of therapeutic protein such as insulin, hormones, vaccine and interleukins using recombinant microorganisms.
- Antibiotics large scale production.
- Production of humanized monoclonal antibodies for therapeutic application.
- Production of insect resistant cotton plant by incorporation of insecticidal toxin of Bacillus thuringiensis (Bt cotton plant).
- Production of golden rice (rice having vitamin A) by incorporating three genes required for its synthesis in rice plant.
- Bioremediation by the use of recombinant organisms.
- Use of genetic engineering techniques in forensic medicine.

Applications of Gene Modified Technology



Using Microbes Against Other Microbes

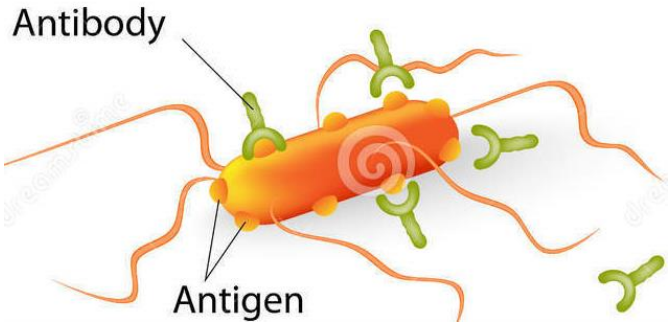
6. Kill directly



Vaccines

In every modern vaccine the main or sole active ingredient consists of killed microorganisms, nonvirulent microorganisms, microbial products (e.g., toxins), or microbial components that have been purified.

All these active ingredients are antigens: substances that can stimulate the immune system to produce specific antibodies.

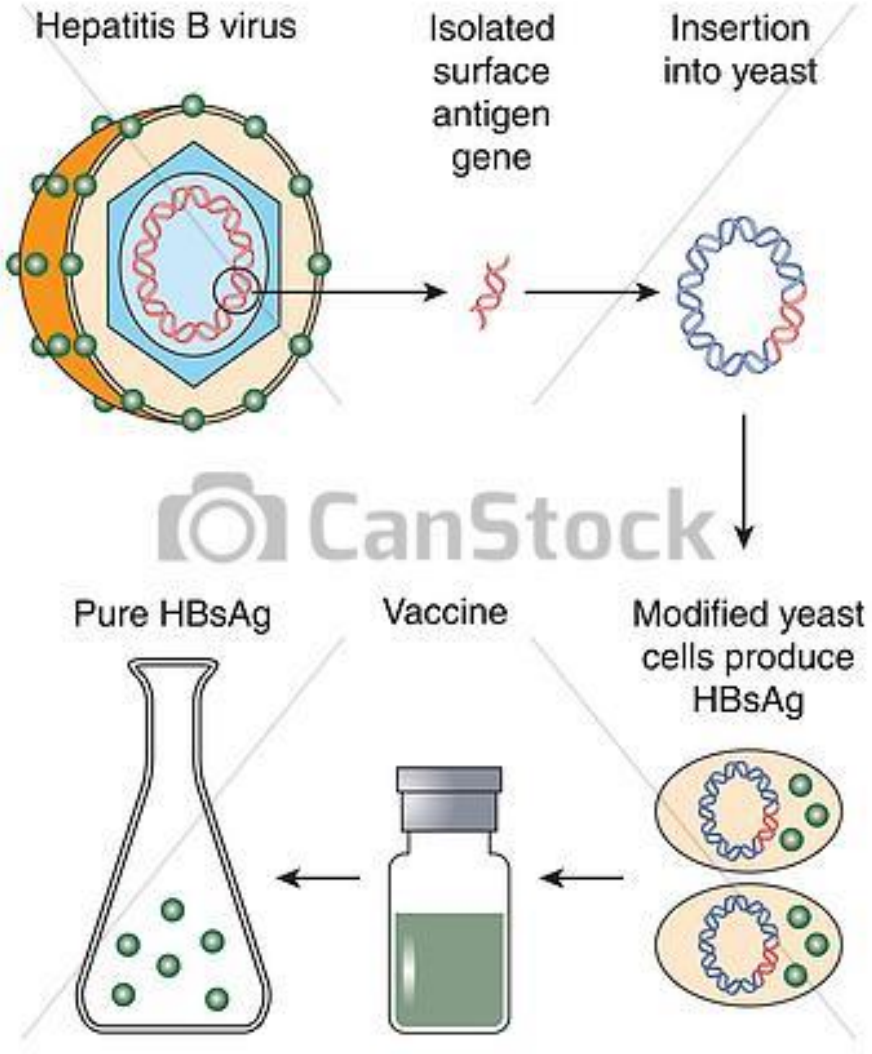


The immune system destroys bacteria and viruses whose antigens correspond to the antibodies.

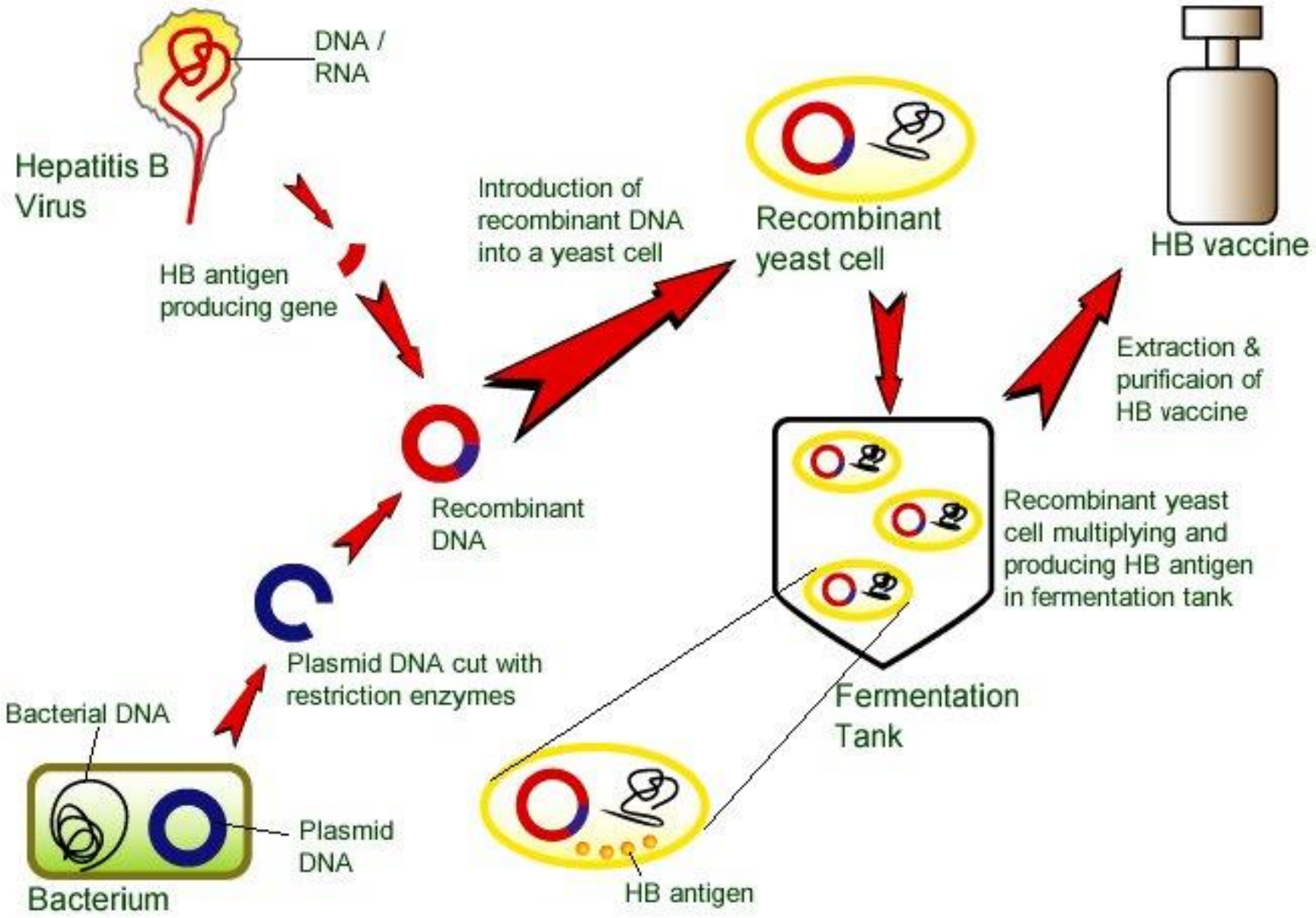
Although conventionally produced vaccines are generally harmless, some of them may, rarely, contain infectious contaminants.

Genetic Modified Vaccines

- Vaccines whose active ingredients are recombinant antigens do not carry this slight risk.
- More than 350 million persons worldwide are infected with the virus that causes hepatitis B, a major cause of chronic inflammation of the liver, cirrhosis of the liver, and liver cancer.
- Hepatitis B kills a million people each year worldwide.

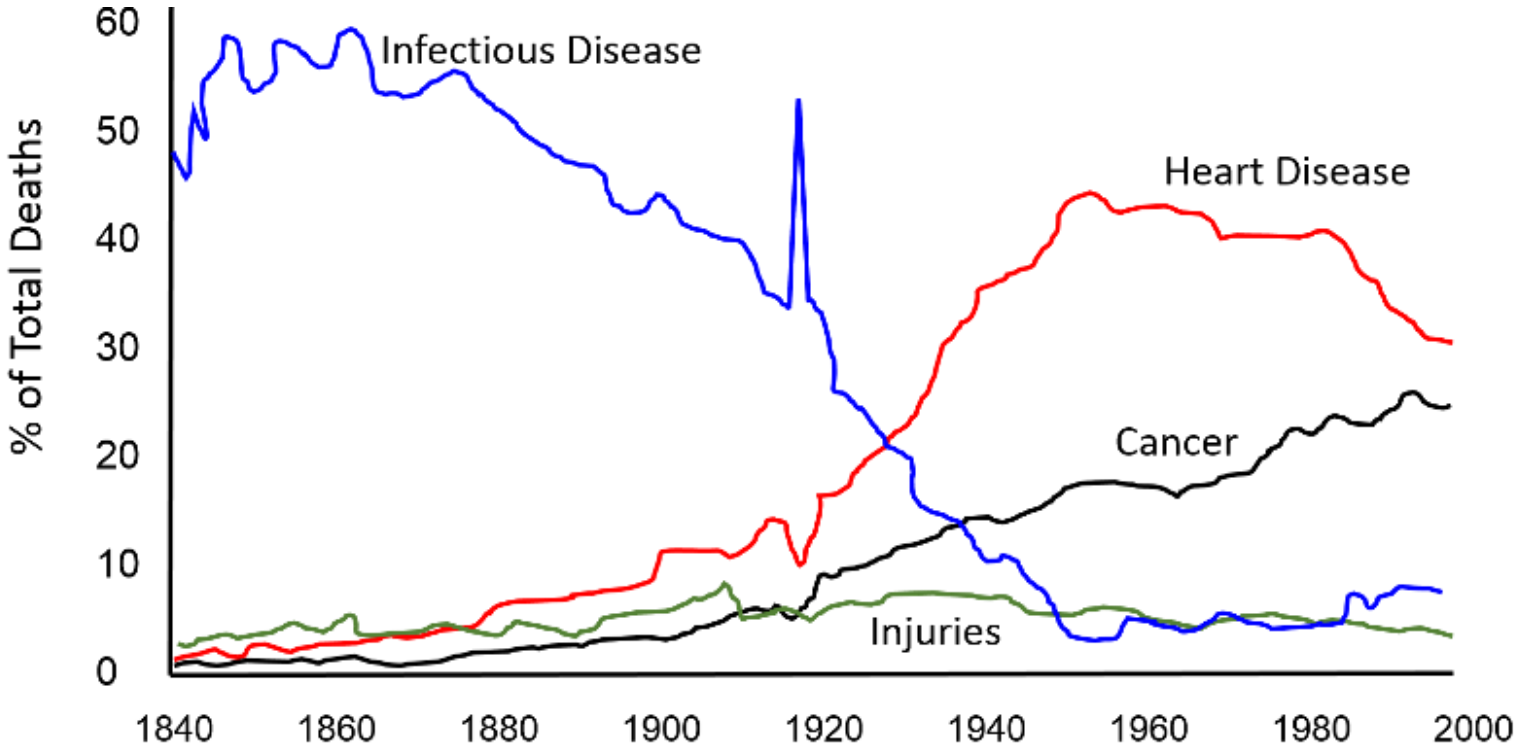


Production of Recombinant HB Vaccine



Vaccines

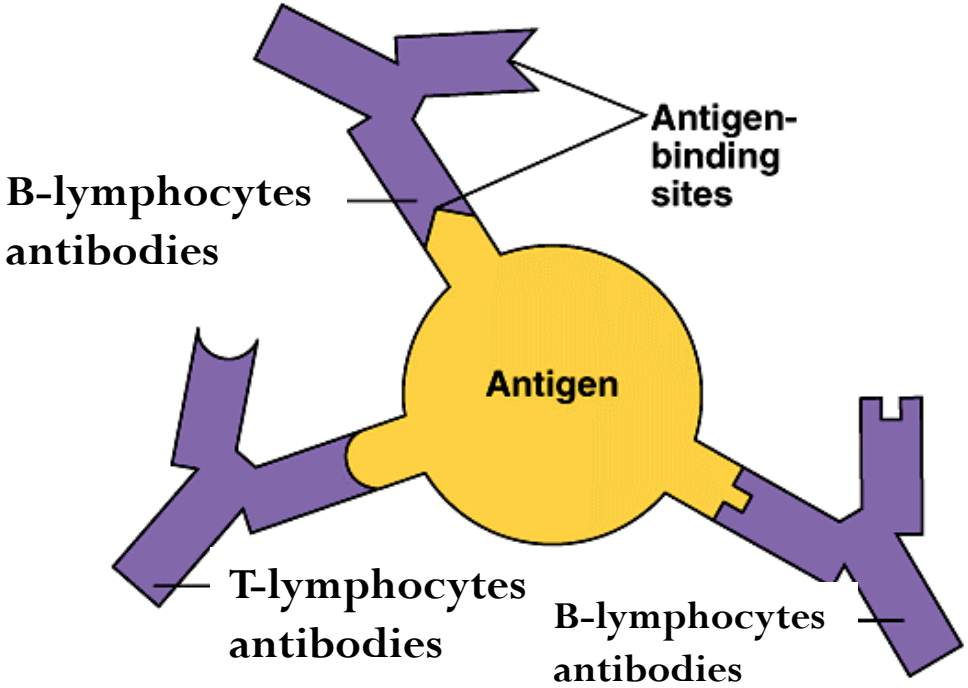
- First was a vaccine against Smallpox (cowpox provides immunity)
- DPT – diphtheria, pertussis, and tetanus
- MMR – measles, mumps, and rubella
- OPV – oral polio vaccine.

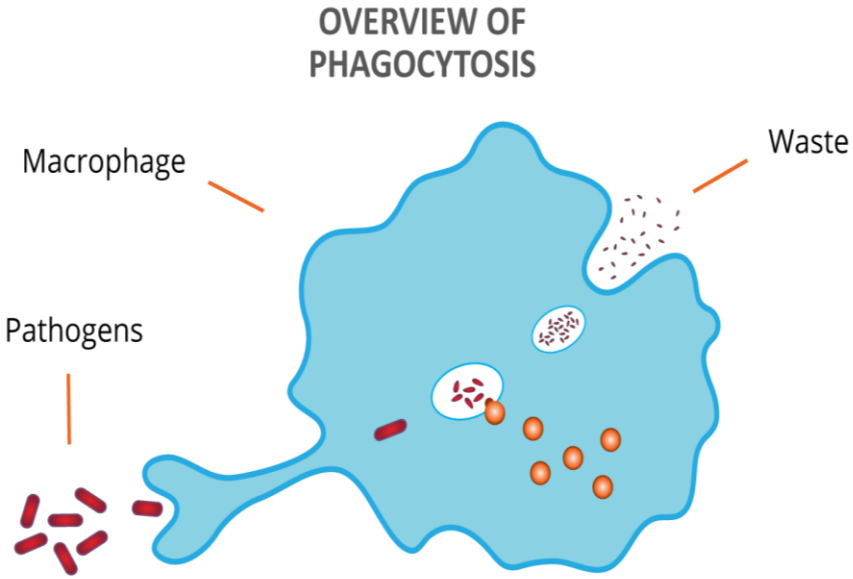


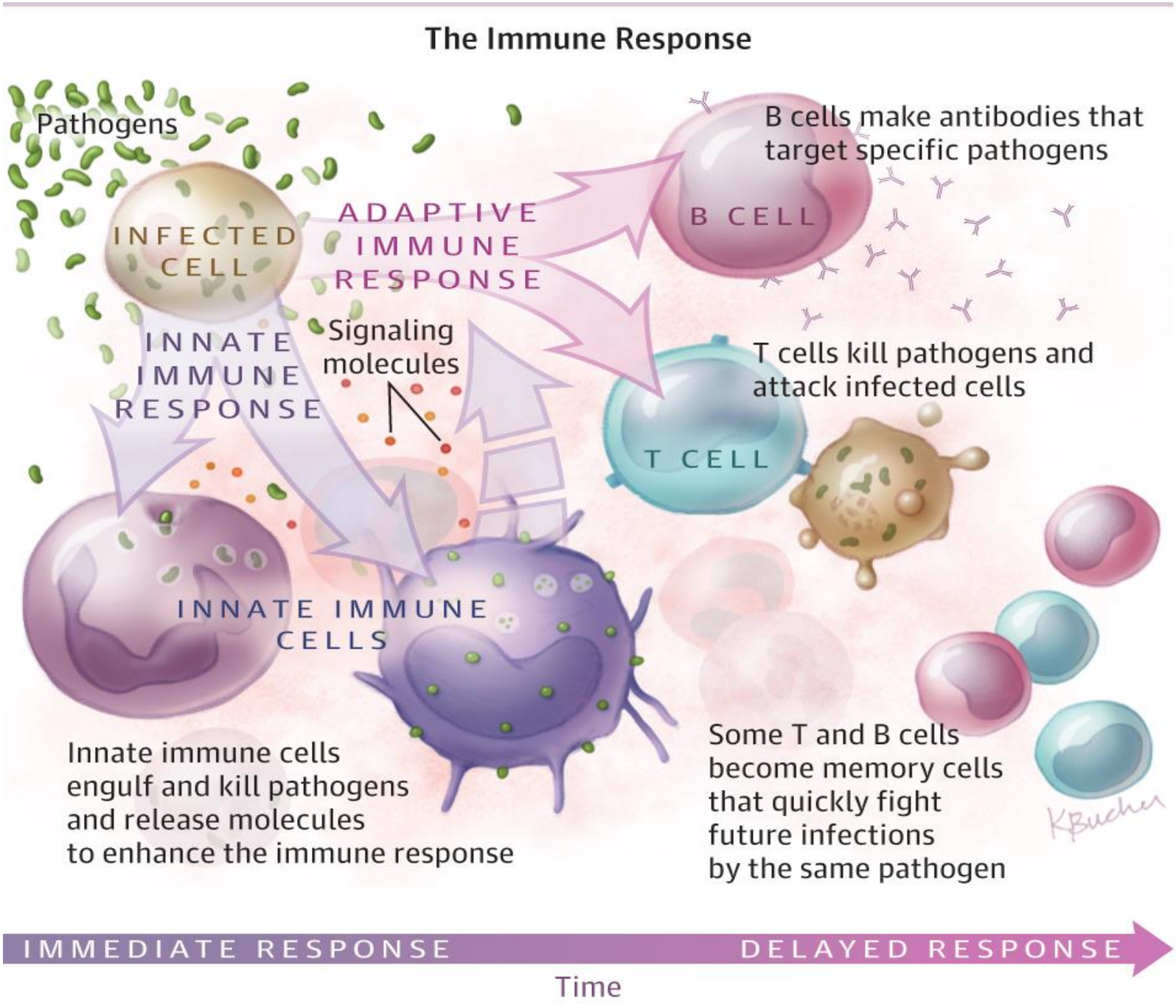
A Primer on Antibodies

- Antigen – foreign substances that stimulate an immune response.

  There some types of leukocytes or white blood cells.






The Immune Response
Pathogens
INFECTED CELL
ADAPTIVE IMMUNE RESPONSE
B CELL
B cells make antibodies that target specific pathogens
INNATE IMMUNE RESPONSE
Signaling molecules
T cells kill pathogens and attack infected cells
T CELL
INNATE IMMUNE CELLS
Innate immune cells engulf and kill pathogens and release molecules to enhance the immune response
Some T and B cells become memory cells that quickly fight future infections by the same pathogen
IMMEDIATE RESPONSE
DELAYED RESPONSE
Time

How are vaccines made?

- They can be part of a pathogen (e.g. a toxin) or whole organism that is dead or alive but attenuated (doesn't cause disease)
- Subunit (toxin) or another part of the pathogen
- Attenuated (doesn't cause disease)
- Inactivated (killed).

How are vaccines made?

- They can be part of a pathogen (e.g. a toxin) or whole organism that is dead or alive but attenuated (doesn't cause disease).

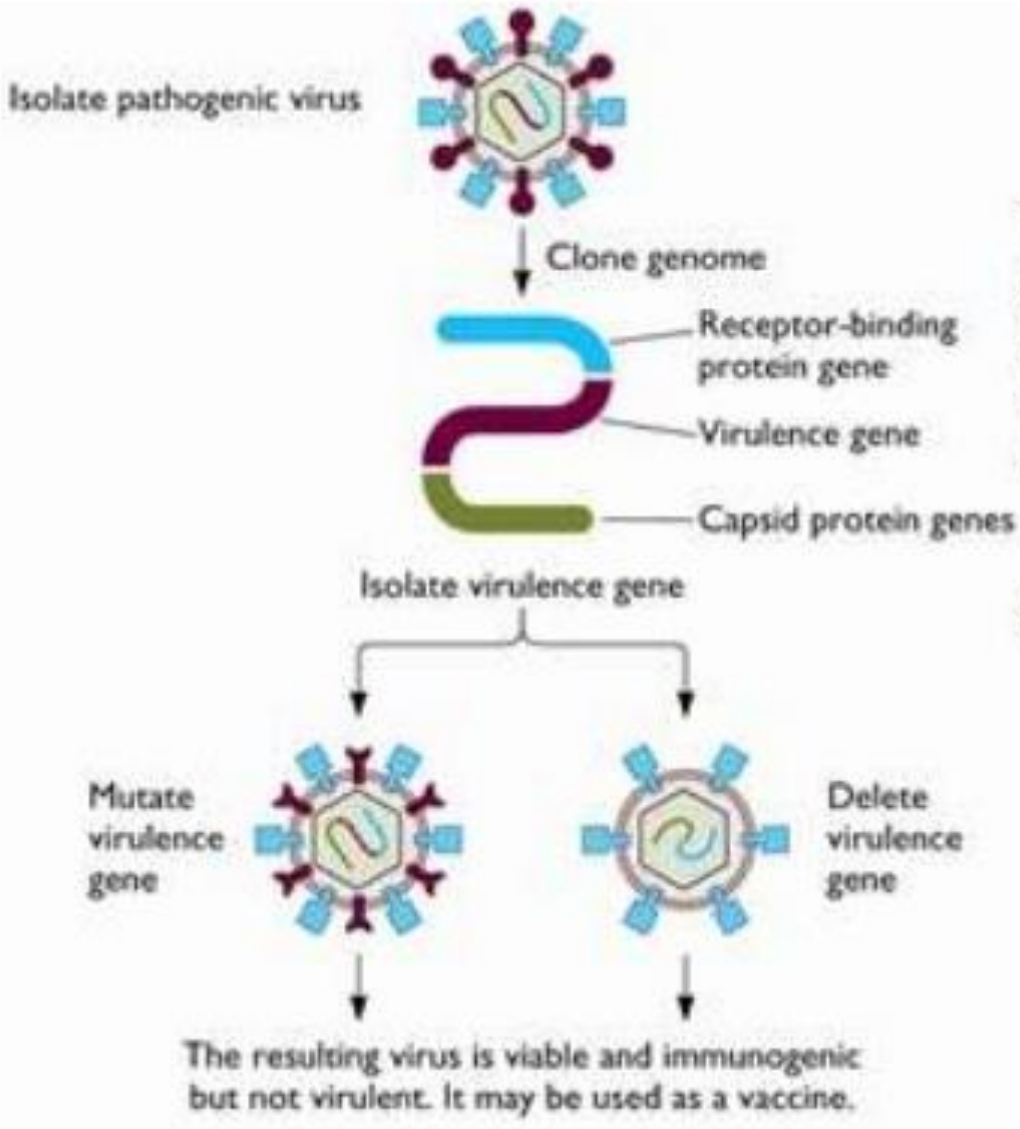
Subunit (toxin) or another part of the pathogen

Inactivated (killed)

Doesn't cause disease

Antibodies usually bind to surface proteins of the pathogen or proteins generated after the disruption of the pathogen. Binding of antibodies to these proteins will stimulate an immune response.

Construction of recombinant attenuated virus

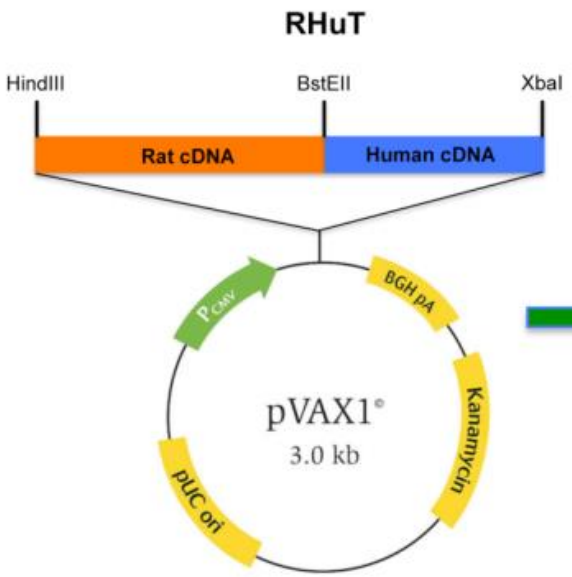


1. Isolate virus
2. Clone genome
3. Isolate virulence gene
4. Mutate or delete virulence gene
5. Resulting virus is
   - Viable
   - Immunogenic
   - Not virulent
   - Can be used as a vaccine

Recombinant DNA technology to produce a new generation of vaccines

- Virulence genes are deleted and organism is still able to stimulate an immune response.
- Live nonpathogenic strains can carry antigenic determinants from pathogenic strains.
- If the agent cannot be maintained in culture, genes of proteins for antigenic determinants can be cloned and expressed in an alternative host e.g. E. coli.

DNA Vaccine



E.coli

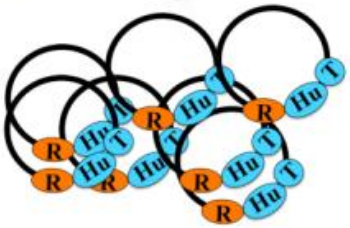
E.coli antibiotic resistent clone

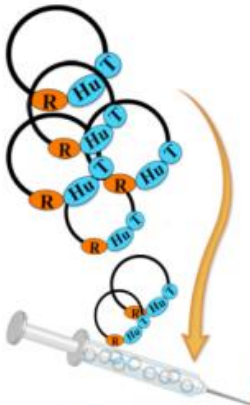
Growth of the clone

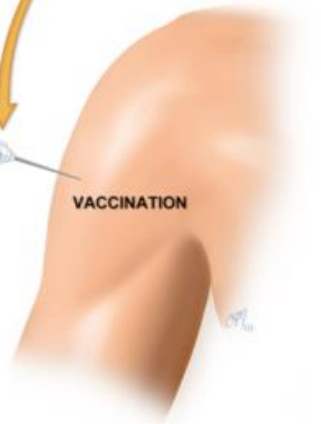
Plasmid Purification

Alkaline lysis

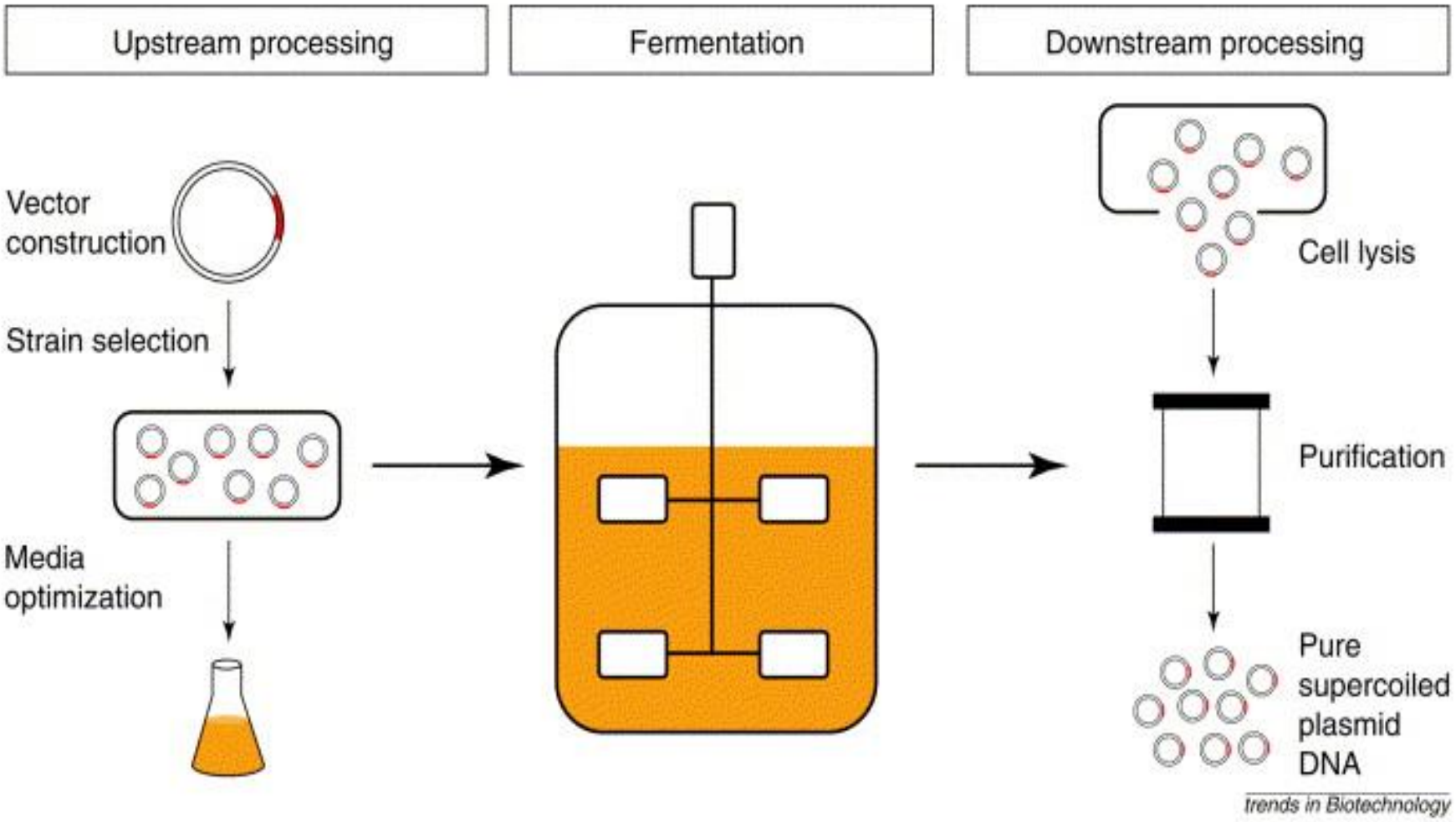
DNA vaccine Elution



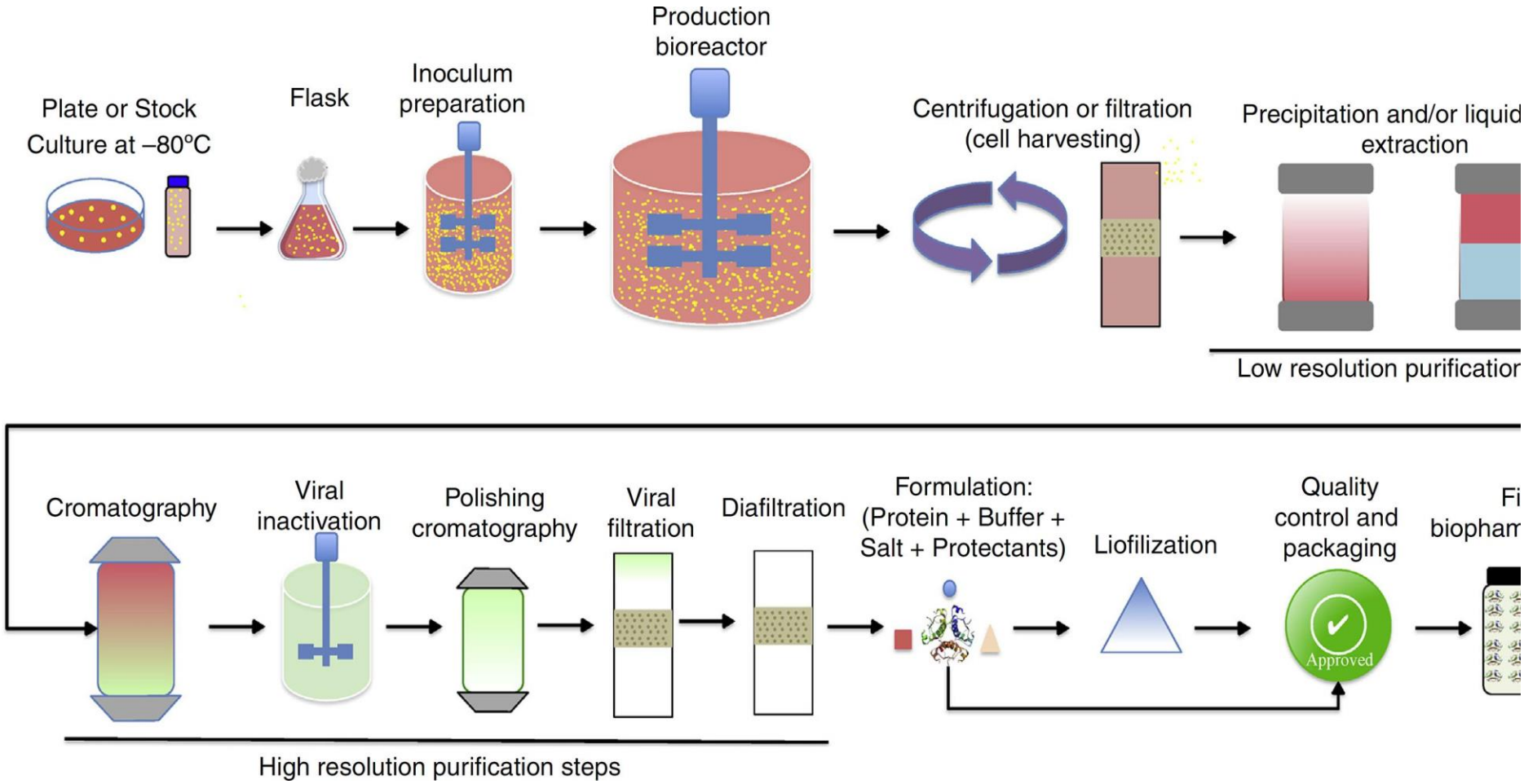




Vaccine lage scale production



Vaccine lage scale production



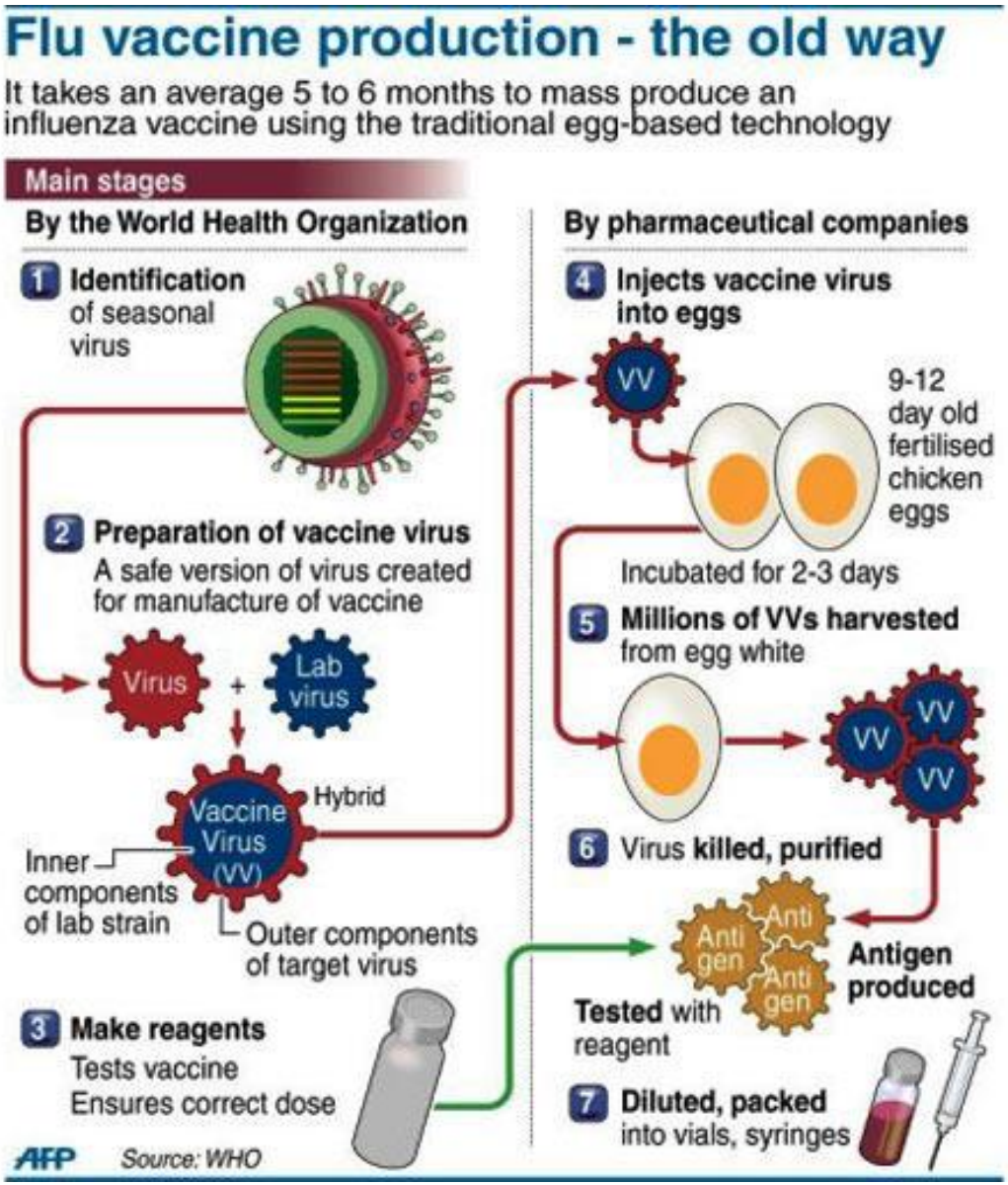
Principles of Vaccination

- A vaccine renders the recipient resistant to infection.
- During vaccination a vaccine is injected or given orally.
- The host produces antibodies for a particular pathogen.
- Upon further exposure the pathogen is inactivated by the antibodies and disease state prevented.
- Generally to produce a vaccine the pathogen is grown in culture and inactivated or nonvirulent forms are used for vaccination.

Principles of Vaccination

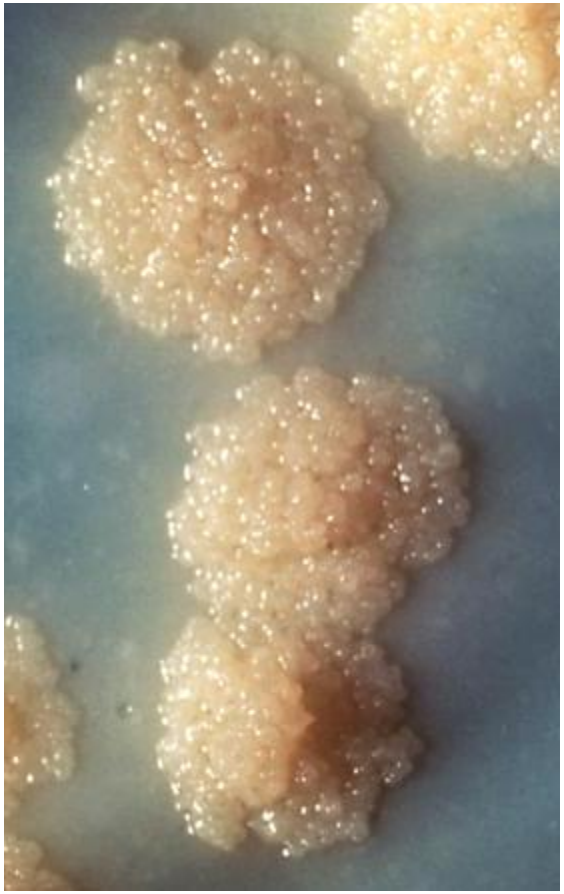
- There are many disadvantages and they include:
- Not all organisms can be cultured.
- The procedure is expensive and sometimes unsafe.
- New pathogens keep occurring.
- For some pathogens e.g. HIV vaccination is not appropriate.

Principles of Flu Vaccination



A Subunit Vaccine for M. tuberculosis

- Mycobacterium tuberculosis form lesions in the tissues and organs causing cell death. Often the lung is affected.
- About 2 billion people are infected and there are 3 million deaths/year.
- Currently tuberculosis is controlled by a vaccine called BCG (Bacillus Calmette-Guerin) which is a strain of M. bovis.
- M. bovis often responds to diagnostic test for M. tuberculosis.



Micobacterias tuberculosis

A Subunit Vaccine for M. tuberculosis

- Six extracellular proteins of M. tuberculosis were purified.
- Separately and in combinations these proteins were used to immunized guinea pigs.
- These animals were then challenged with M. tuberculosis.
- After 9-10 weeks examination showed that some combinations of the purified proteins provided the same level of protection as the BCG vaccine.

Attenuated Vaccines

- Attenuated vaccines often consists of a pathogenic strains in which the virulent genes are deleted or modified.
- **The Development of a Live Cholera Vaccine.**
- Live vaccines are more effective than a killed or subunit (protein) vaccines.
- With this in mind a live vaccine for cholera was developed.
- Cholera is characterized by fever, dehydration abdominal pain and diarrhea.
- Oral cholera vaccine more effective for adults, less effective for children under five.

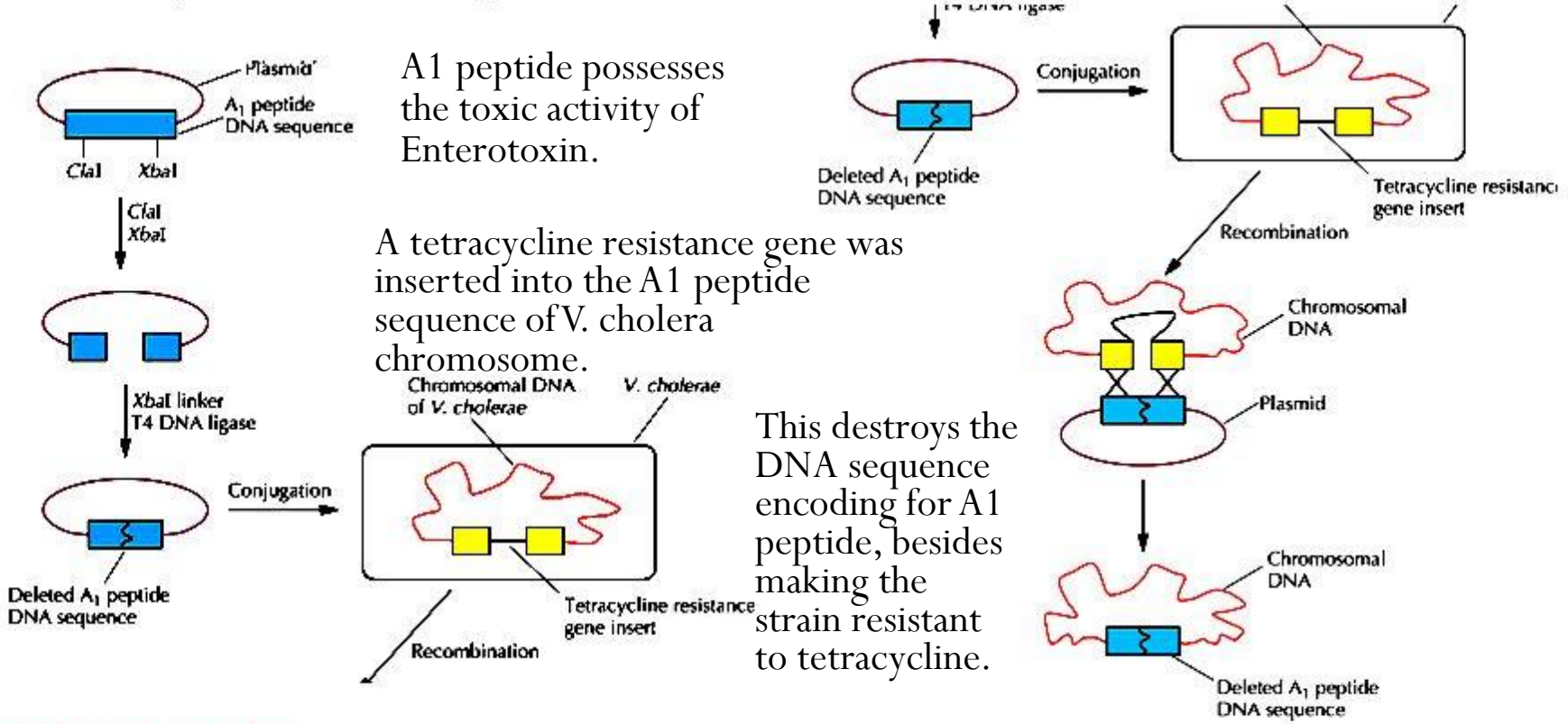
A Live Cholera Vaccine

- The causal agent of cholera is Vibrio cholerae and is transmitted through contaminated water.
- V. cholerae produces A enterotoxin with an A subunit and 5 B subunits.
- Presently the cholera vaccine consist of a phenol-killed V. cholerae and it only last 3-6 months.
- A live vaccine would be more effective.
- In the sequence of the A peptide (A enterotoxin) a tetracycline resistance gene is inserted:
  - This enterotoxin plasmid was mixed with tetracycline resistant gene.
  - By conjugation the plasmid was transferred to the strain with the tetR gene inserted into it's chromosomal DNA.

VACCINES

Attenuated Vaccines

Example -> Vaccine against Cholera



A1 peptide possesses the toxic activity of Enterotoxin.

A tetracycline resistance gene was inserted into the A1 peptide sequence of V. cholera chromosome.

This destroys the DNA sequence encoding for A1 peptide, besides making the strain resistant to tetracycline.

550 bases deleted of A1 peptide

The final result is *V. cholerae* with a 550 bp of the A peptide deleted.

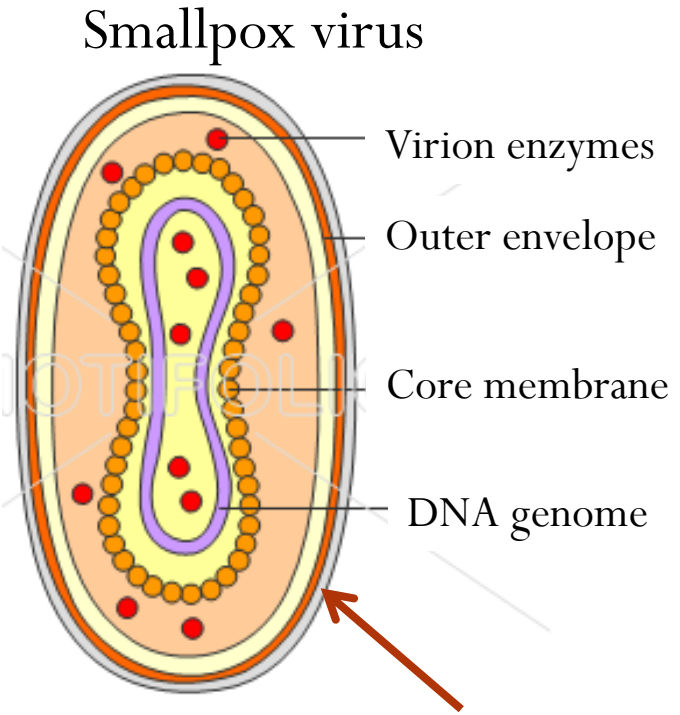
-> Currently being tested.

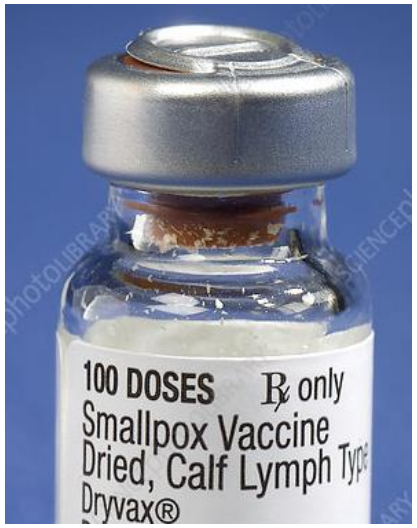
The genetically engineered V. cholera vaccine

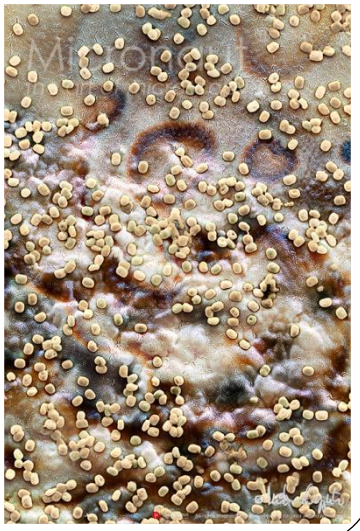
- The genetically engineered V. cholera cells with deleted A_1 peptide DNA sequence are quite stable.
- They cannot produce active enterotoxin but possess all other biochemical functions of the pathogen.
- This new strain of V. cholera is undergoing trials for its efficiency as a vaccine.
- Preliminary results indicate that this attenuated vaccine can protect about 90% of the volunteers against cholera.
- Unfortunately, the tetracycline resistant gene is easily lost and the enterotoxin activity is restored. Because of this, the new strain of V. cholera as such cannot be used as a vaccine.
- Scientists continue their work to develop a better vaccine against cholera.

Vector Vaccine

- A vector vaccine is a vaccine which is introduced by a vector e.g. vaccinia virus.
- The vaccinia virus as a live vaccine led to the globally eradication of the smallpox virus.
- The genome of the vaccinia virus has been completely sequenced.
- The virus replicates in the cytoplasm rather than in the nucleus.
- The vaccinia virus is generally nonpathogenic.

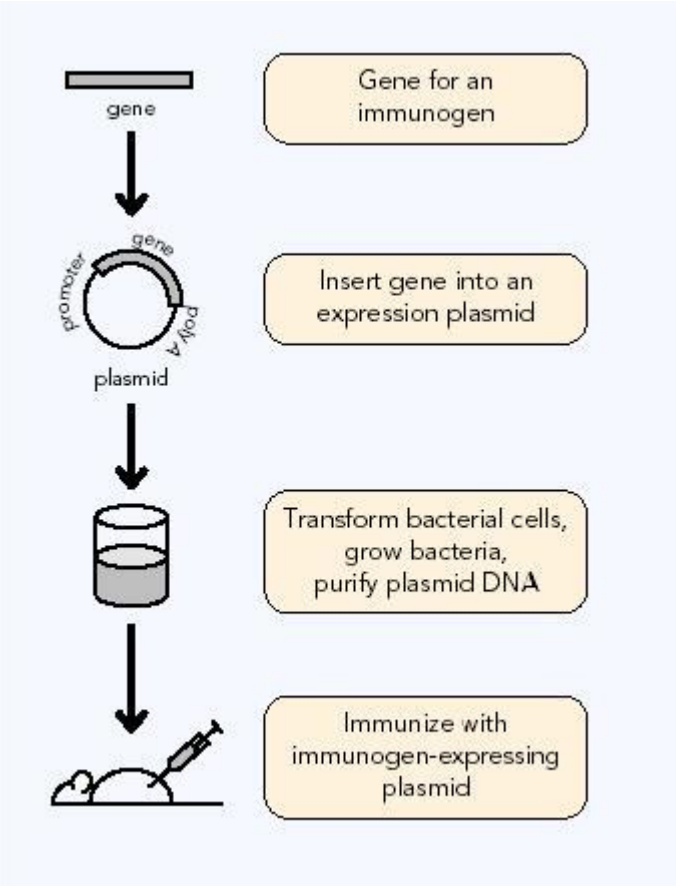


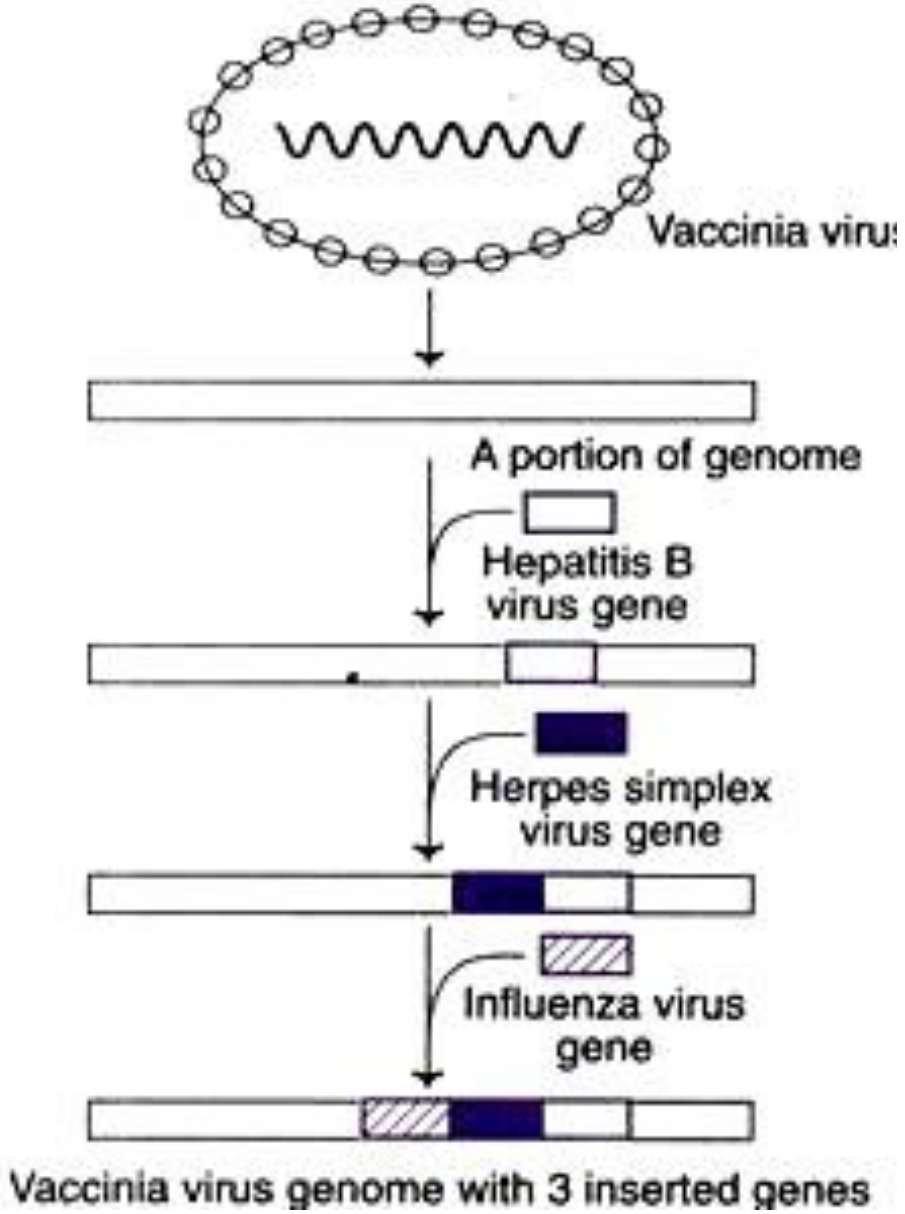


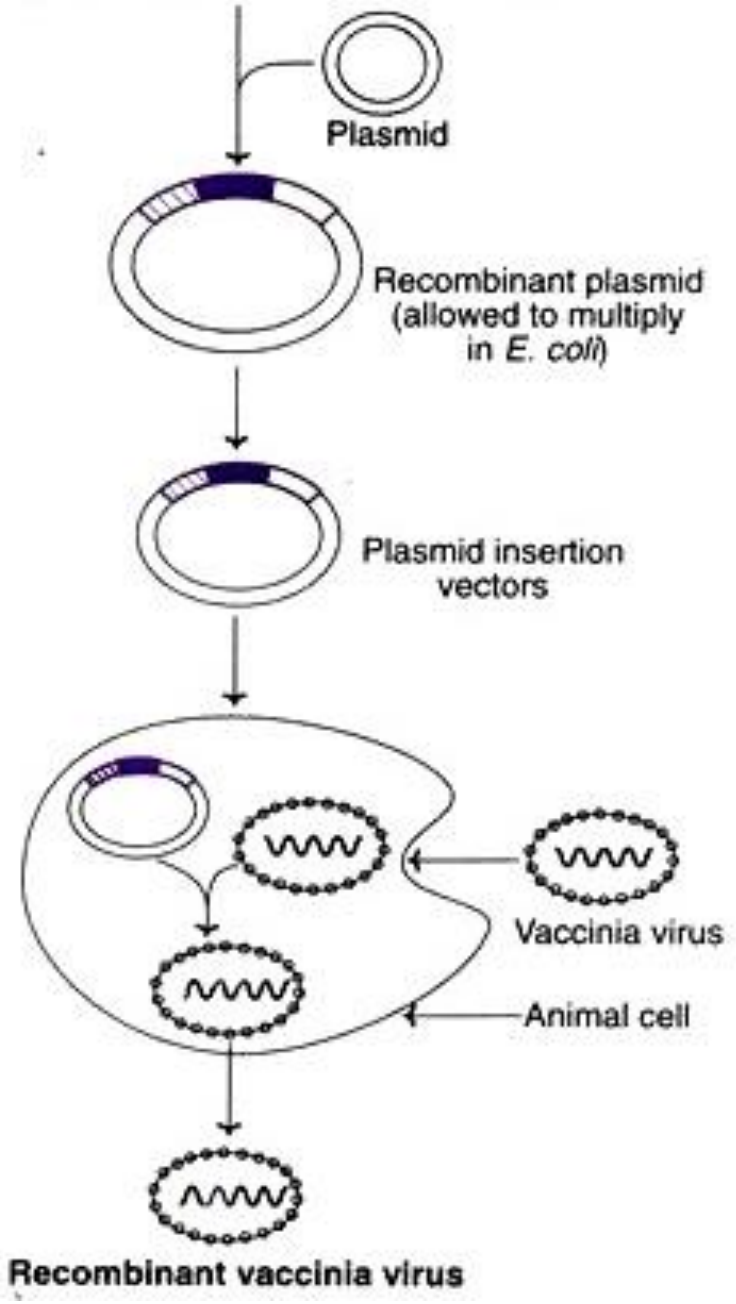


Vector Vaccine

- These characteristics makes the vaccinia virus a good candidate for a virus vector to carry gene for antigenic determinants form other pathogens.
- The procedure involves:
- The DNA sequence for the specific antigen is inserted into a plasmid beside the vaccinia virus promoter in the middle of a non-essential gene e.g. *Thymidine kinase*.
- The plasmid is used to transform *Thymdine kinase* negative cells which were previously infected with the vaccinia virus.
- Recombination between the plasmid and vaccinia virus chromosomal DNA results in transfer of antigen gene from the recombinant plasmid to the vaccinia virus.
- Thus virus can now be used as a vaccine for the specific antigen.




Vaccinia virus
A portion of genome
Hepatitis B virus gene
Herpes simplex virus gene
Influenza virus gene
Vaccinia virus genome with 3 inserted genes

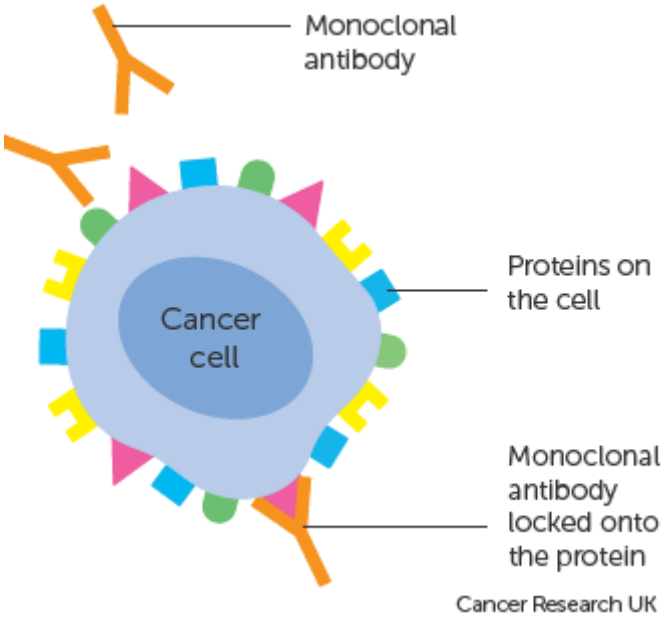

Plasmid
Recombinant plasmid (allowed to multiply in E. coli)
Plasmid insertion vectors
Vaccinia virus
Animal cell
Recombinant vaccinia virus

Monoclonal Antibodies

- There are a few of the hundreds of other biotech drugs that are either in clinical use or undergoing scientific investigation.
- Biotech vaccines undergoing investigation include vaccines for acellular pertussis (whooping cough), AIDS, herpes simplex, Lyme disease, and melanoma.
- Two new recombinant interferons are undergoing investigation: consensus interferon, for treating hepatitis C; and recombinant beta interferon 1a, for multiple sclerosis.

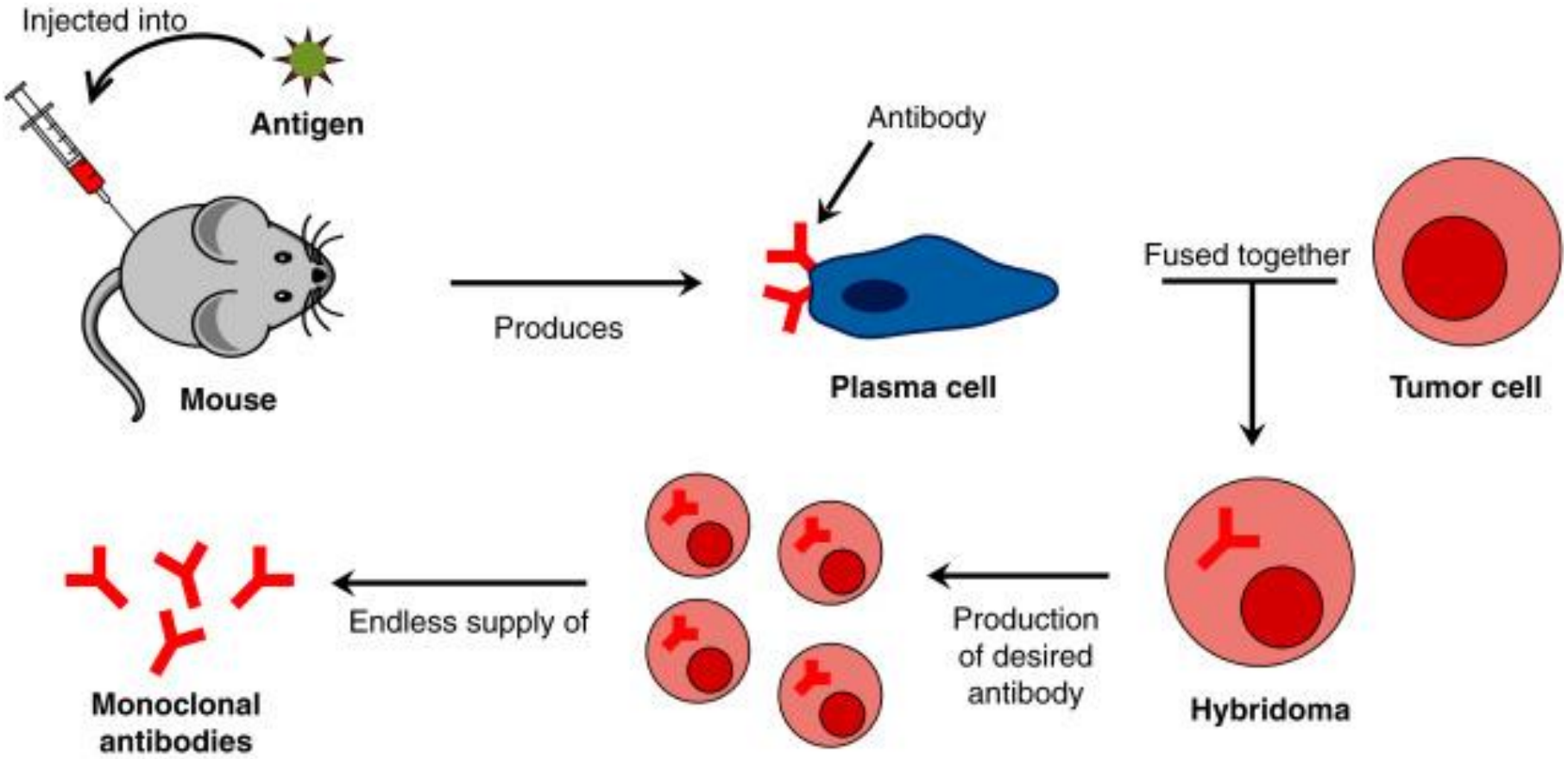
Monoclonal Antibodies

- Recombinant PTK (*Protein Tyrosine Kinase*) inhibitors may have therapeutic utility against whooping cough diseases marked by cell proliferation, such as cancer, atherosclerosis, and psoriasis.
- Recombinant human interleukin-3 is undergoing clinical investigation as an adjunct to traditional cancer chemotherapy.
- Two recombinant growth factors (cytokines that regulate cell division) are undergoing major clinical trials: recombinant human insulin, like growth factor and recombinant human platelet-derived.



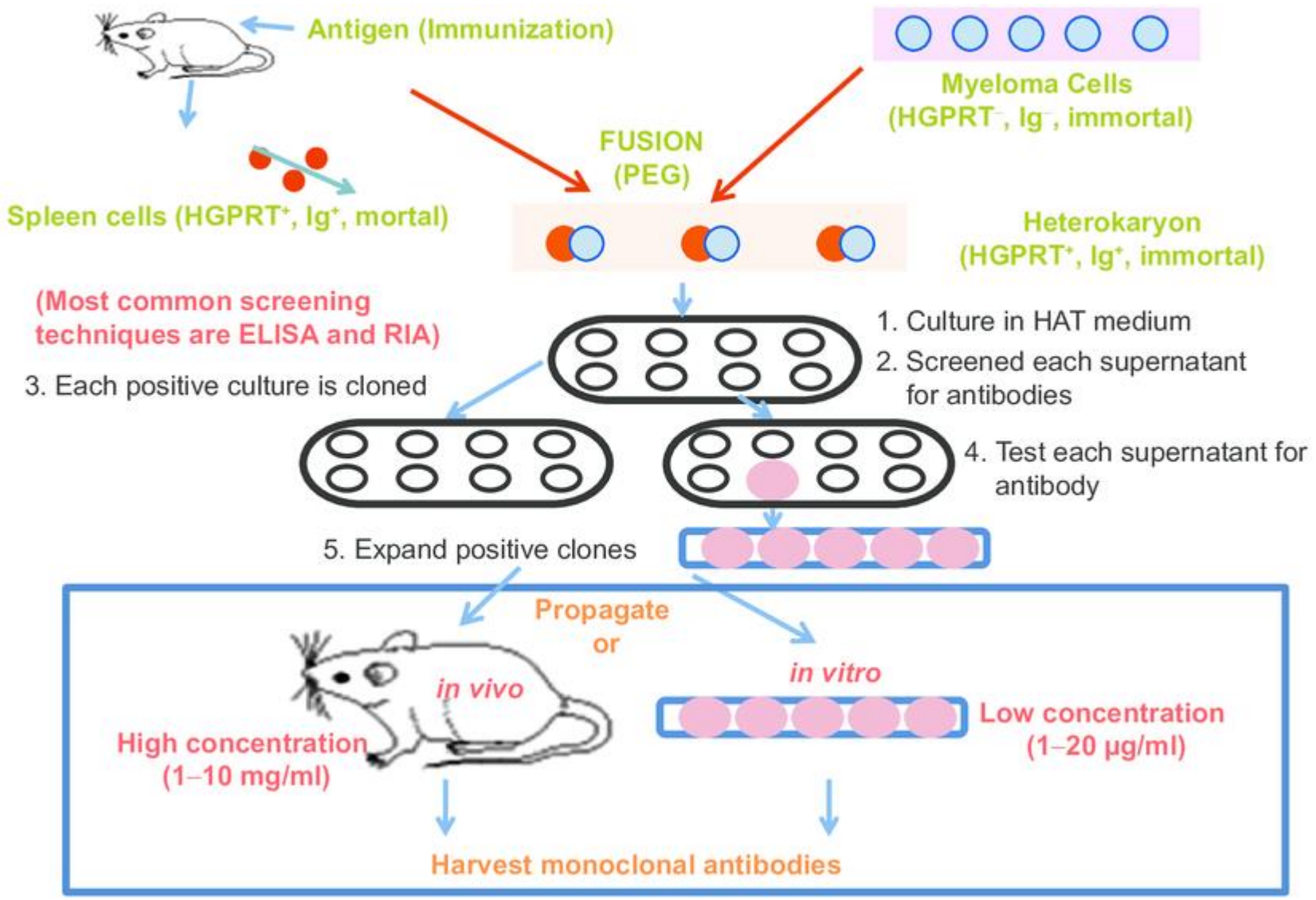
Monoclonal antibodies (mAb or moAb) are antibodies that are made by identical immune cells that are all clones of a unique parent cell.

Monoclonal Antibodies



Fusion of a myeloma (B cell which has become cancerous) with a spleen cell that is immunized with a specific antigen. The resulting hybridomas are tested for the production of a monoclonal antibodies.

Production of Monoclonal Antibodies



Production of Human Monoclonal Antibodies by E. coli

- Hybridoma cells grow relatively slow and require expensive media.
- To circumvent this problem human monoclonal antibodies are grown in E. coli.
- The produce involves: mRNA is isolated from the B cell.
- cDNA is synthesized from the mRNA by the enzyme reverse transciptase.
- Both heavy and light chains are amplified separately from the cDNA using PCR.
- The amplified products are cut with restriction enzymes and cloned into Lambda vector.

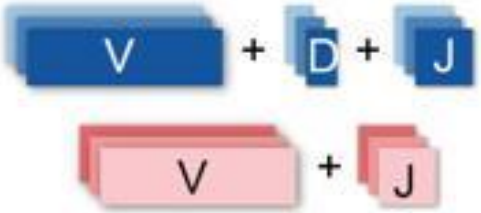
Production of Human Monoclonal Antibodies by E. coli

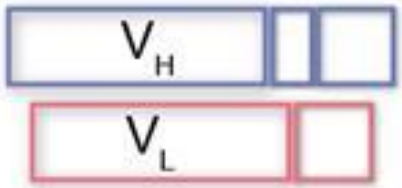
- During cloning different light and heavy chains are cloned. The DNA of one heavy and one light chain are cloned into the same vector.
- Many different combinations of H and L chains are cloned together in the same vector.
- Lambda is not useful for producing large amounts of proteins.
- The L and H chains are excised from Lambda and the recombinant plasmid transformed and cloned into E. coli.

B cell repertoire

Antibody is composed of two heavy chains (VH) and two light chains (VL).

Antibody gene

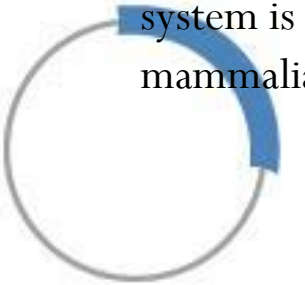




Gene Clone

Variable genes of heavy chain and light chain antibody should be cloned by PCR and designed primers.

Recombinant DNA



Can screening small antibody fragment to obtain large antibody library

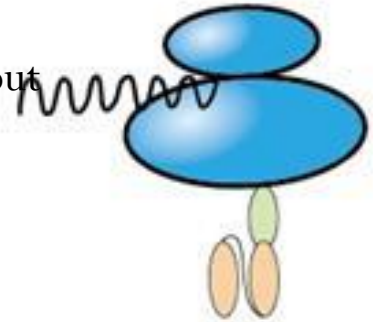
Phage display

make human antibody expression more superior because that yeast expression system is similar with mammalian cells

Yeast display

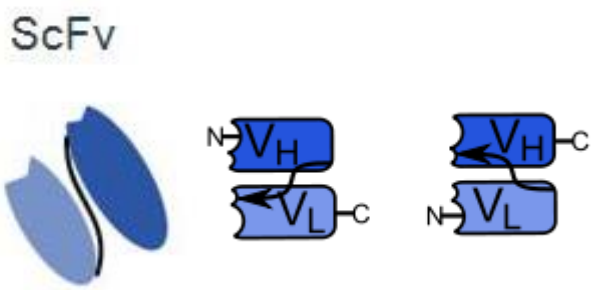
Ribosome display

can get a library of a capacity of 10^{14} without limitation of transformation efficiency



Recombinant antibodies can be constructed successfully, such as scfv antibody fragments, fab antibody fragments, and single domain antibodies.

The **antigen-binding** (Fab) fragment is a region on an antibody that binds to antigens. It is composed of one constant and one variable domain of each of the heavy and the light chain.

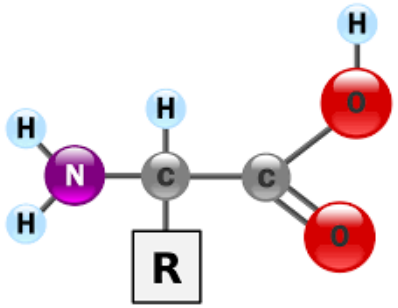


Single-chain variable fragment

An antibody fragment consisting of a single monomeric variable antibody domain. Like a whole antibody, it is able to bind selectively to a specific antigen.

These monoclonal antibodies can be used for diagnostic purposes e.g detection of HIV, therapeutically for the treatment of infection.

Biosynthesis of Amino Acids



Amino acids uses in food industry

- Flavor enhances
- Antioxidants
- Nutritional supplements

Amino acid uses in agriculture

- Feed additives

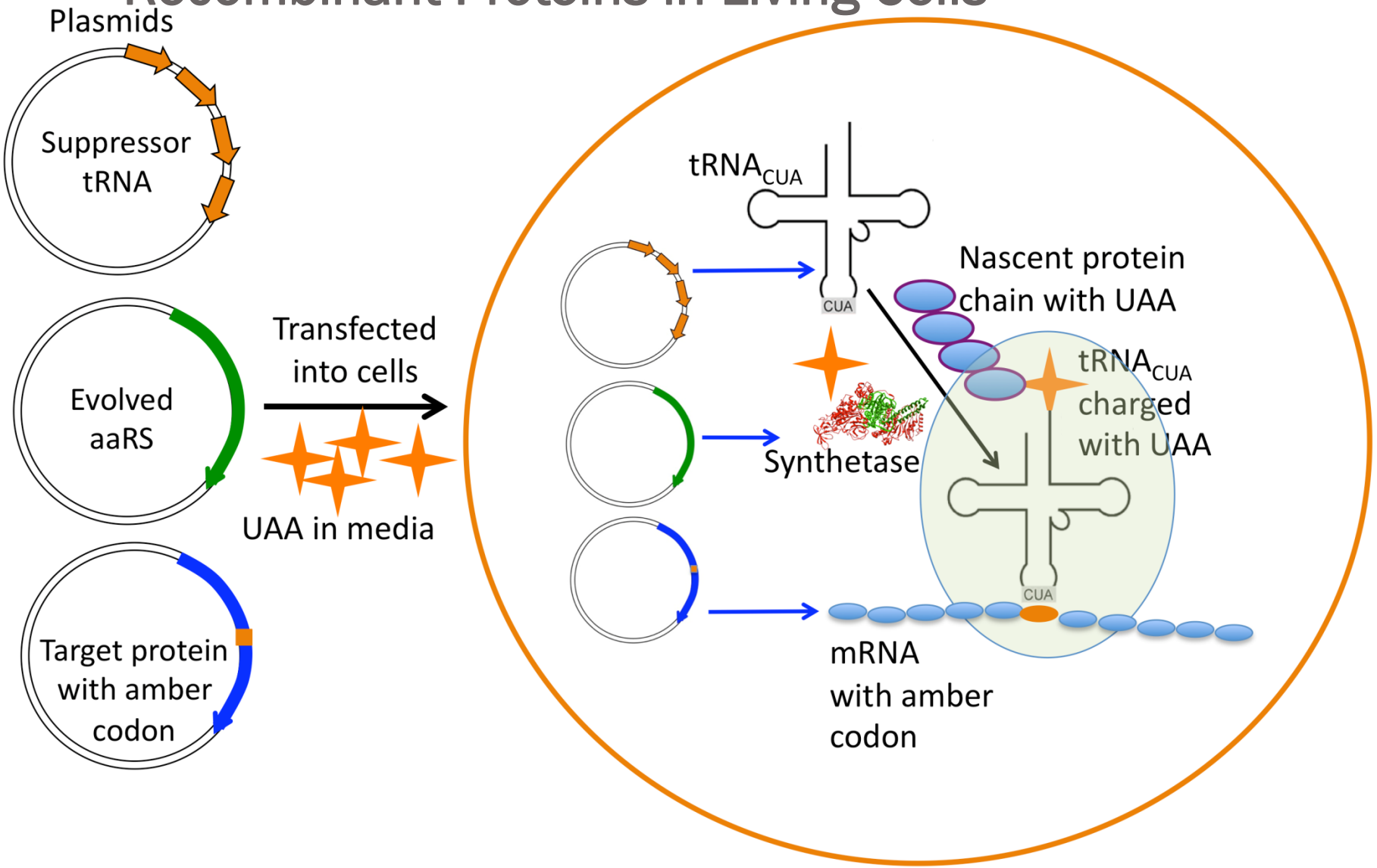
Amino acid uses in medicine

- Infusion solutions

Amino Acid uses in industry

- Starting materials for polymer and cosmetic production

Incorporating Unnatural Amino Acids into Recombinant Proteins in Living Cells



Thank you for the attention