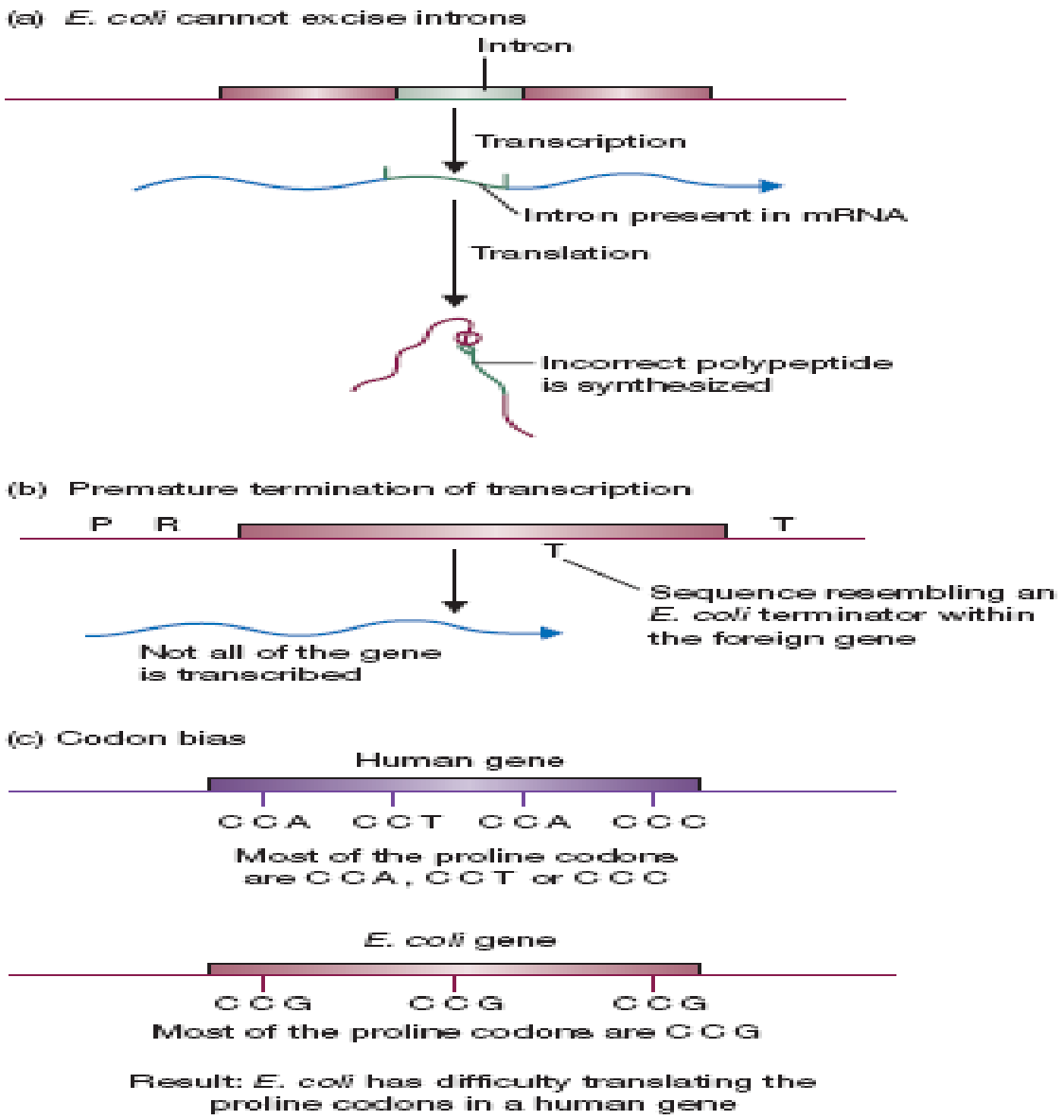
General Problems with the production of foreign recombinant protein expressed in *E.coli*

1. Problems caused by the sequence of the foreign gene
2. Problems caused by the *E.coli*

General Problems with foreign protein expressed in E.coli



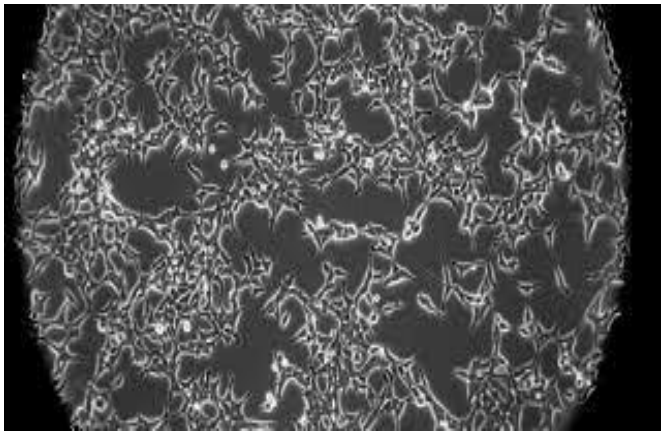
Problems caused by E.coli

- ***E.coli*** NOT always the best organism
  - *E. coli might not process the recombinant protein correctly (post translational modifications)*
    - *Correct disulfide bond formation*
    - *Proteolytic cleavage of inactive precursor*
    - *Glycosylation - addition of sugar residues*
    - *Alteration of amino acids in protein*
      - » *phosphorylation*
      - » *acetylation*
      - » *sulfation*
      - » *fatty acid addition*
  - *E. coli might not fold the recombinant protein correctly (S-S bonds, incorrect folding, inclusion bodies, inactive)-Use chaperone overexpressing strains*
  - *E. coli might degrade the foreign recombinant protein- Use protease deficient strains*

Eukaryotic Expression Systems

- *Saccharomyces cerevisiae*
- *Pichia pastoris*
- Mammalian systems
  - Transient expression:
    - Vero cells are derived from the kidney of an African green monkey, and are one of the more commonly used mammalian continuous cell lines in microbiology, and molecular and cell biology research
    - BHK (Baby hamster kidney) cells line- like BHK-21 is one of the most commonly used cell lines for the expression of biopharmaceuticals, and it is also among the top three cell types that have been most frequently used for transient expression. BHK cells are originally isolated by polyoma transformation of hamster cells and have been extensively used as substrates for virus propagation for vaccine and more generally for viral mediated expression
    - Human Embryonic Kidney Cell line- HEK293 cells
  - Long-term expression: Chinese hamster ovary and mouse myeloma cells.

HEK293 Cells



HEK293 cells are Human Embryonic Kidney cells, originally isolated and grown by Dutch biologist Alex Van der Eb in the early 1970s. They were transfected with sheared adenovirus 5 (Ad5) DNA by Frank Graham, a postdoc in Van der Eb's lab. It was his 293rd experiment, which is why they got the tag HEK293 A 4-kb Ad5 DNA fragment encoding the E1A/E1B proteins was later shown to have integrated into chromosome 19, resulting in transformation.

Incorporating the adenoviral genes into the HEK cell genome resulted in the cells becoming very efficient at producing high amounts of recombinant proteins from plasmid vectors carrying the CMV promoter region.

There are many advantages of using HEK293 cells. They are a hardy, semi-adherent, low-maintenance cell line and divide rapidly, doubling about every 36 hours. They can be utilized for both transient and stable expression, can be cultured in suspension or as a monolayer, are easy to transfect (and can be transfected via a variety of methods) and are able to produce large amounts of recombinant proteins.

HEK293 cells are used in cancer research, vaccine development, protein production, signal transduction and protein interaction studies, drug testing etc

HEK293T

- The ‘T’ in the name of this daughter cell line comes from the incorporation of the SV40 large T antigen into the HEK293 genome – this means they are able to produce large amounts of protein from plasmids vectors carrying the SV40 origin of replication.

CHO Cell lines

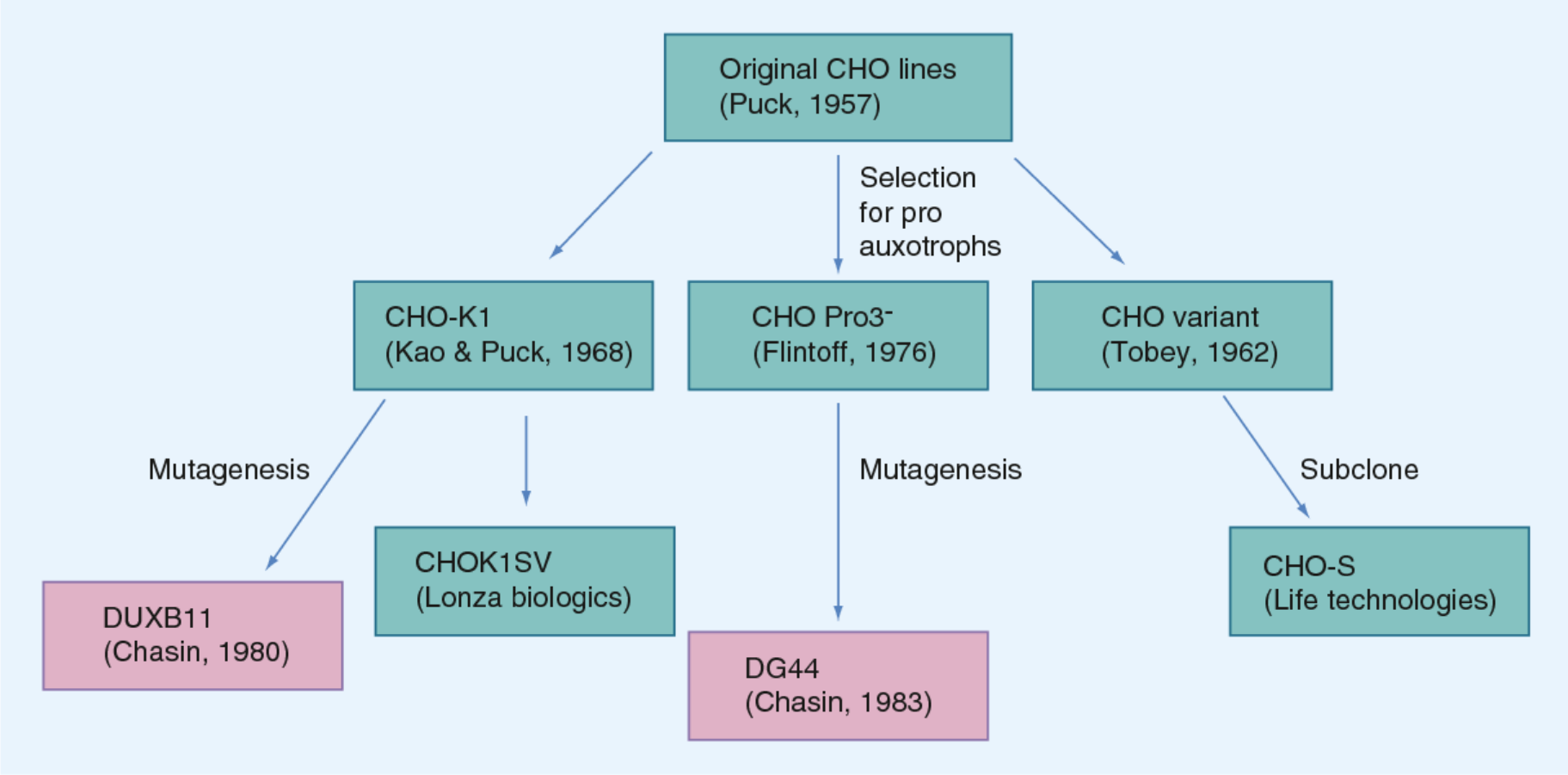


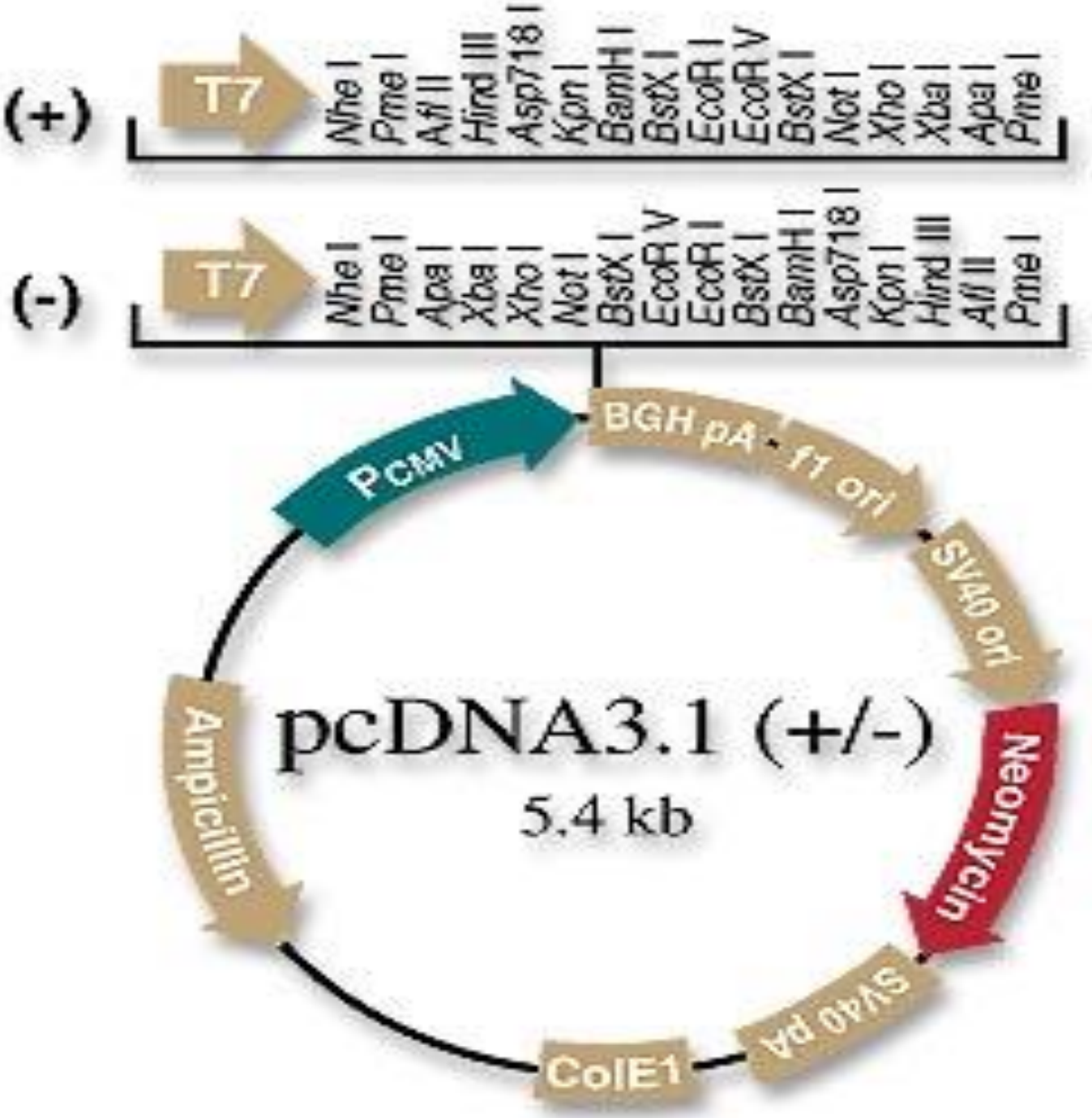
Figure 3. History and lineage of Chinese hamster ovary cell lines. Cell lines derived by mutagenesis to be DHFR

DUXB11 and DH44- A cell line deficient in DHFR activity, which requires the addition of glycine, hypoxanthine, and thymidine (GHT) in the medium for survival, allows for the implementation of a selection system based on the insertion of a cloned dhfr gene in combination with the gene of interest.

Eukaryotic Expression Vectors

- Same sorts of genetic features
  - eukaryotic promoter-generally derived from animal viruses or from highly expressed mammalian genes (SV40, cytomegalovirus (CMV), herpes simplex virus (HSV), Elongation factor 1 (EF1)
  - ori of replication (eukaryote..usually viral, SV40)
  - selectable marker (for eukaryotic cell)
  - mRNA polyadenylation signal
  - ori of replication (Plasmid)
  - selectable marker (bacteria)

https://www.synbio-tech.com/mammalian-cell-expression-vectors/


(+)
T7
Nhe I
Pme I
Afl II
Hind III
Asp718 I
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
Pme I
(-)
T7
Nhe I
Pme I
Apa I
Xba I
Xho I
Not I
BstX I
EcoR V
EcoR I
BstX I
BamH I
Asp718 I
Kpn I
Hind III
Afl II
Pme I
PCMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
ColE1
Ampicillin
pcDNA3.1 (+/-)
5.4 kb

Selectable Markers for mammalian Expression Vectors

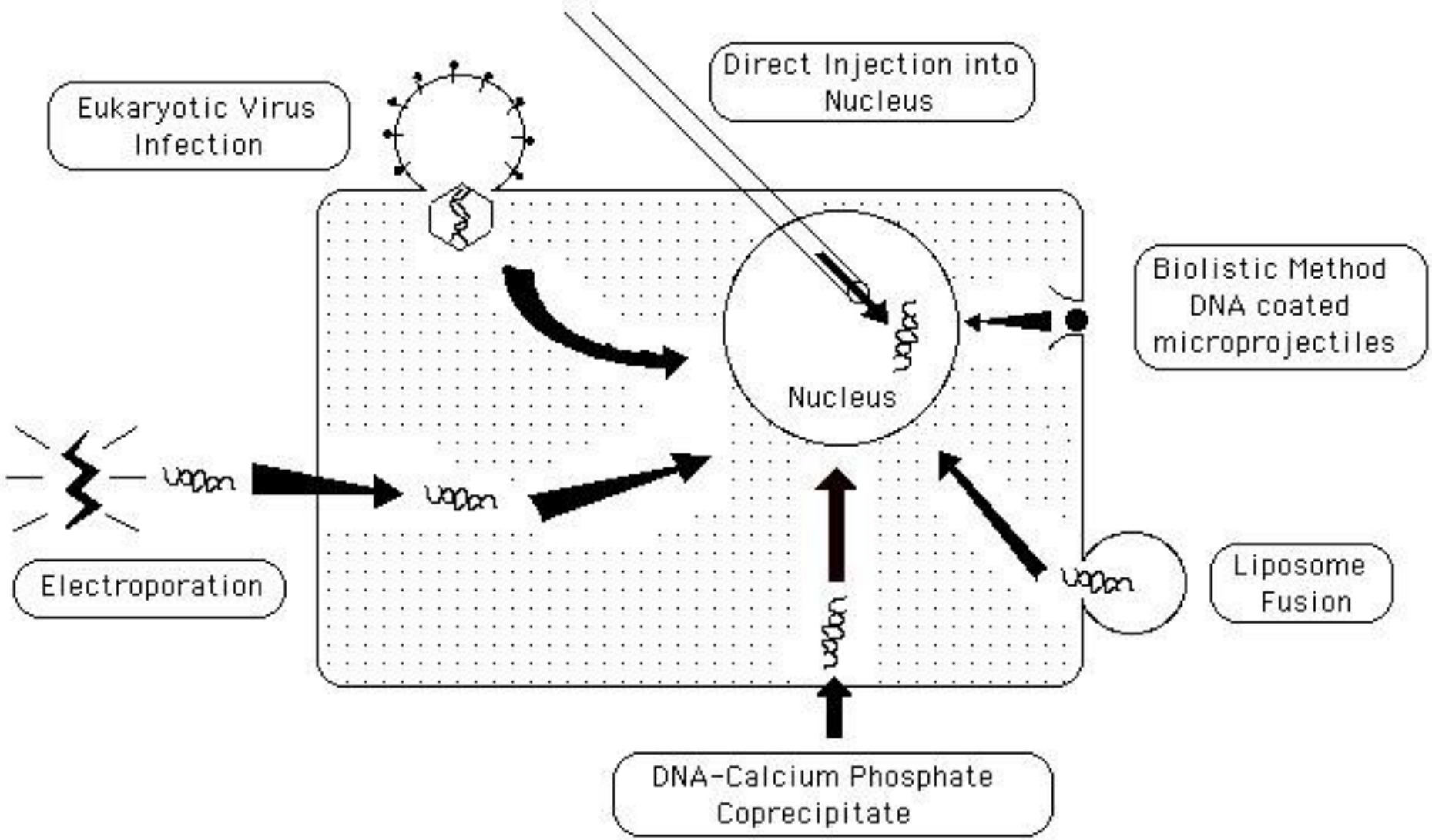
TABLE 7.7 Selective marker gene systems for mammalian cells

Selective agent	Action of selective agent	Marker gene	Action of marker gene protein
Xyl-A	Damages DNA	Adenine deaminase *(ada)*	Deaminates Xyl-A
Blasticidin S	Inhibits protein synthesis	Blasticidin S deaminases *(Bsr, BSD)*	Deaminates blasticidin S
Bleomycin	Breaks DNA strands	Bleomycin-binding protein *(Ble)*	Binds to bleomycin
G-418 (Geneticin)	Inhibits protein synthesis	Neomycin phosphotransferase *(neo)*	Phosphorylates G-418
Histidinol	Produces cytotoxic effects	Histidinol dehydrogenase *(hisD)*	Oxidizes histidinol to histidine
Hygromycin B	Inhibits protein synthesis	Hygromycin B phosphotransferase *(Hph)*	Phosphorylates hygromycin B
MSX	Inhibits glutamine synthesis	Glutamine synthetase *(GS)*	Cells that produce excess glutamine synthetase survive.
MTX	Inhibits DNA synthesis	Dihydrofolate reductase *(dhfr)*	Cells that produce excess dihydrofolate reductase survive.
PALA	Inhibits purine synthesis	Cytosine deaminase *(codA)*	Lowers cytosine levels in the medium by converting cytosine to uracil
Puromycin	Inhibits protein synthesis	Puromycin *N*-acetyltransferase *(Pac)*	Acetylates puromycin

MSX, methionine sulfoximine; MTX, methotrexate; PALA, *N*-(phosphoacetyl)-L-aspartate; Xyl-A, 9-β-D-xylofuranosyl adenine.

https://www.synbio-tech.com/mammalian-cell-expression-vectors/

Transfection Methods



Viral Vectors

Retroviruses

- Murine leukemia virus (MuLV)
- Human immunodeficiency virus (HIV)
- Human T-cell lymphotropic virus (HTLV)

DNA Viruses

- Adenovirus
- Adeno-associated virus (AAV)
- Herpes simplex virus (HSV)

Retroviruses — a class of viruses that can create double-stranded DNA copies of their RNA genomes; these copies can be integrated into the chromosomes of host cells. HIV is a retrovirus.

Adenoviruses — a class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus.

Adeno-associated viruses — a class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19.

Herpes simplex viruses — a class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores.

Viral Attributes

Viral Vector	DNA Insert Size	Maximum Titer	Cell Type	Expression	Pitfalls
Retroviral	8 kb	1×10^9	Dividing cells	Stable	Random insertion site
Lentivirus	9 kb	1×10^9	Dividing cells Nondividing cells	Stable	Random insertion site
Adenovirus	8 kb	1×10^{13}	Dividing cells Nondividing cells	Transient	Highly immunogenic
Adeno-associated virus (AAV)	5 kb	1×10^{11}	Dividing cells Nondividing cells	Stable, site-specific location	Requires helper virus to grow; difficult to remove helper virus
Herpes simplex virus	30–40 kb	1×10^9	Dividing cells Nondividing cells	Transient	No gene expression during latent infection
Vaccinia virus	25 kb	3×10^9	Dividing cells	Transient	Potential cytopathic effects

Adenoviruses

Adenoviruses are DNA viruses with broad cell tropism that can transiently transduce nearly any mammalian cell type. The adenovirus enters target cells by binding to the Coxsackie/Adenovirus receptor (CAR) (Bergelson et al.,1997). After binding to the CAR, the adenovirus is internalized via integrin-mediated endocytosis followed by active transport to the nucleus, where its DNA is expressed episomally (Hirata and Russell, 2000). Although adenoviral vectors work well for transient delivery in many cell types, for some difficult cell lines such as non-dividing cells and for stable expression, lentiviral vectors are preferred. The packaging capacity of adenoviruses is 7–8 kb.

Retroviruses

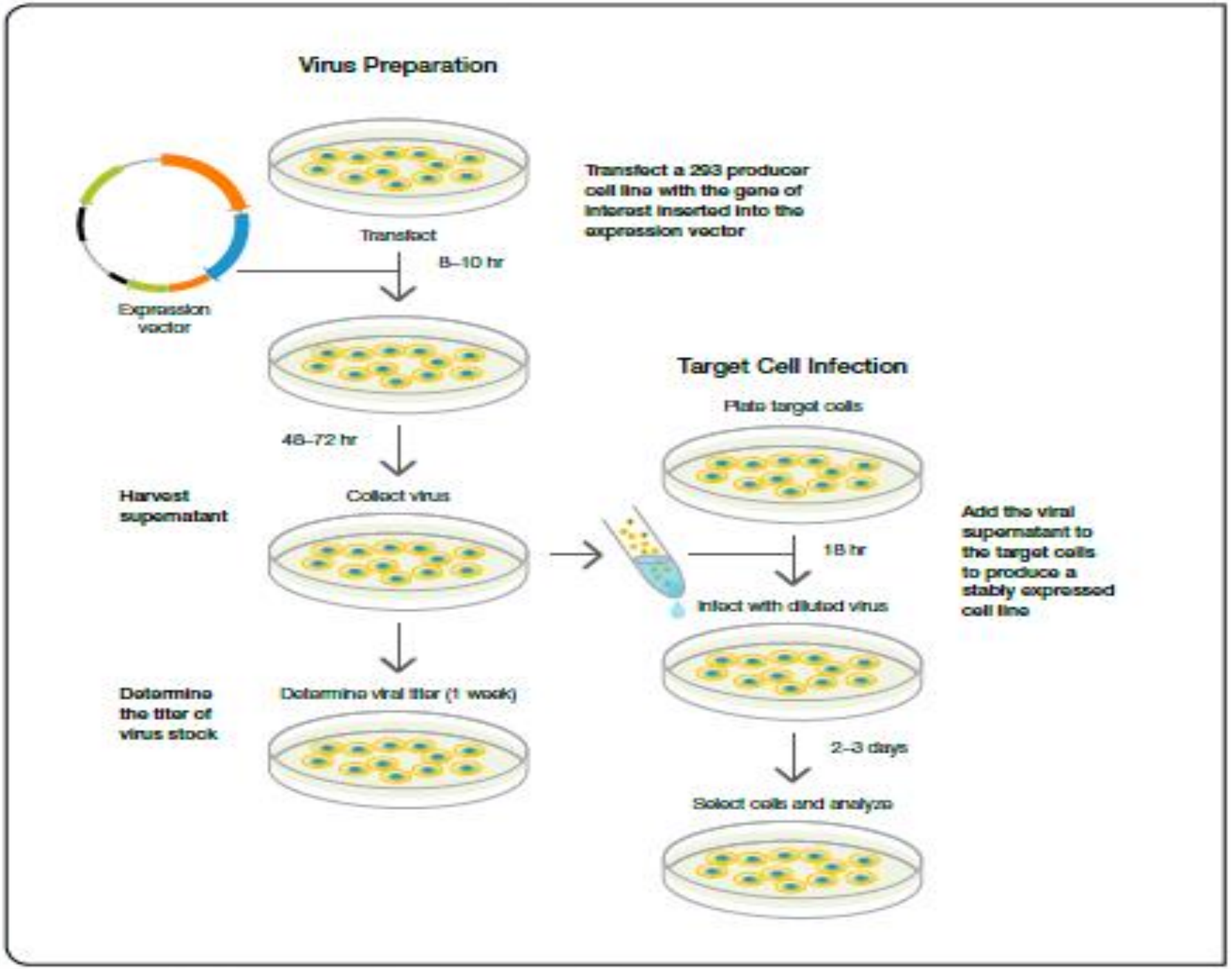
Retroviruses are positive-strand RNA viruses that stably integrate their genomes into host cell chromosomes. When pseudotyped with an envelope that has broad tropism, such as vesicular stomatitis virus glycoprotein (VSV-G), these viruses can enter virtually any mammalian cell type. However, most retroviruses depend upon the breakdown of nuclear membrane during cell division to infect cells and are thus limited by the requirement of replicating cells for transduction. Other disadvantages of retroviruses include the possibility of insertional mutagenesis and the potential for the activation of latent disease. Like adenoviruses, retroviruses can carry foreign genes of around 8 kb.

Lentiviruses

Lentiviruses are a subgroup of the retrovirus family; as such, they can integrate into the host cell genome to allow stable, long-term expression (Anson, 2004). In contrast to other retroviruses, lentiviruses are more versatile tools as they use an active nuclear import pathway to transduce non-dividing, terminally differentiated cell populations such as neuronal and hematopoietic cells.

Adeno-associated viruses

Adeno-associated viruses are capable of transducing a broad range of dividing and non-dividing cells types, but they require coinfection with a helper virus like adenovirus or herpes virus to produce recombinant virions in packaging cells. This causes difficulties in obtaining high quality viral stocks that are free of helper viruses. Furthermore, adeno-associated viruses have only limited packaging capacity of up to 4.9 kb. On the other hand, adeno-associated viruses show low immunogenicity in most cell types, and they have the ability to integrate into a specific region of the human chromosome, thereby avoiding insertional mutagenesis.


Virus Preparation
Transfect a 293 producer cell line with the gene of interest inserted into the expression vector
Transfect
8–10 hr
Expression vector
48–72 hr
Harvest supernatant
Collect virus
Determine the titer of virus stock
Determine viral titer (1 week)
Target Cell Infection
Plate target cells
18 hr
Add the viral supernatant to the target cells to produce a stably expressed cell line
Infect with diluted virus
2–3 days
Select cells and analyze

Pros and Cons

Advantages of Virus-Based Methods

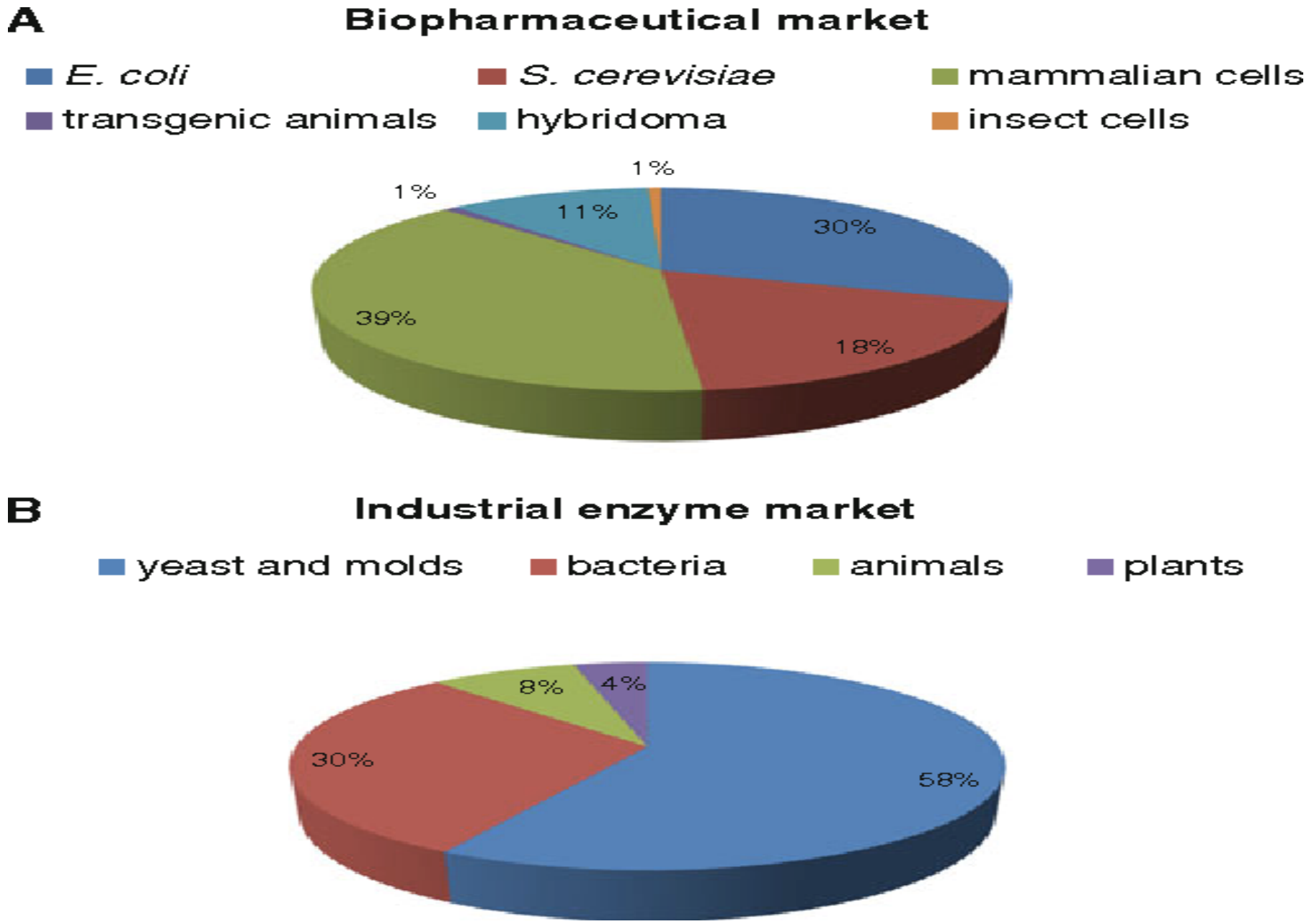
- Very high gene delivery efficiency, 95–100%
- Simplicity of infection

Disadvantages of Virus-Based Methods

- Labor intensive
- Best for introducing a single cloned gene that is to be highly expressed
- P2 containment required for most viruses
  - Institutional regulation and review boards required
  - Viral transfer of regulatory genes or oncogenes is inherently dangerous and should be carefully monitored
  - Host range specificity may not be adequate
- Many viruses are lytic
- Need for packaging cell lines

Protein Drugs Produced by Eukaryotic Cell Culture

Protein	Condition
Factor IX & VIIIc	hemophiliacs
CD4 receptor	AIDS
erythropoetin	cancer
β & γ interferons	cancer
Interleukin-2	cancer
tissue plasminogen activator	heart attack/stroke
Hepatitis B surface antigen	vaccine
monoclonal antibodies	various


A
Biopharmaceutical market
E. coli
S. cerevisiae
mammalian cells
transgenic animals
hybridoma
insect cells
1%
1%
11%
30%
39%
18%
B
Industrial enzyme market
yeast and molds
bacteria
animals
plants
8%
4%
30%
58%