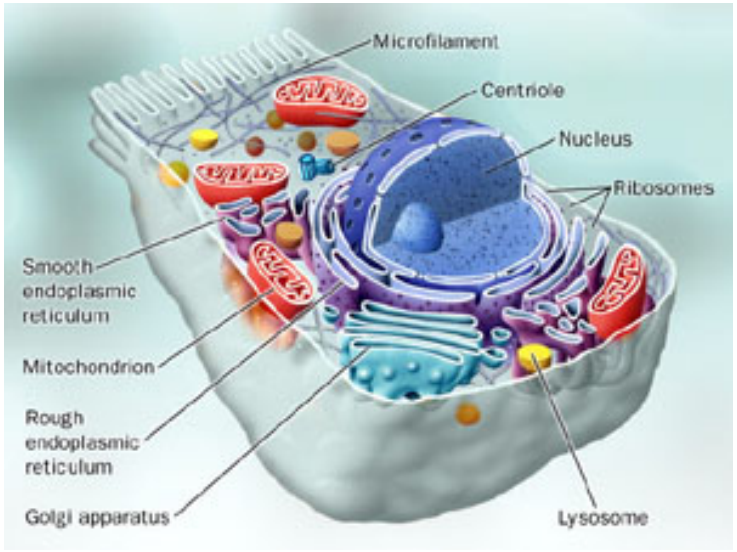
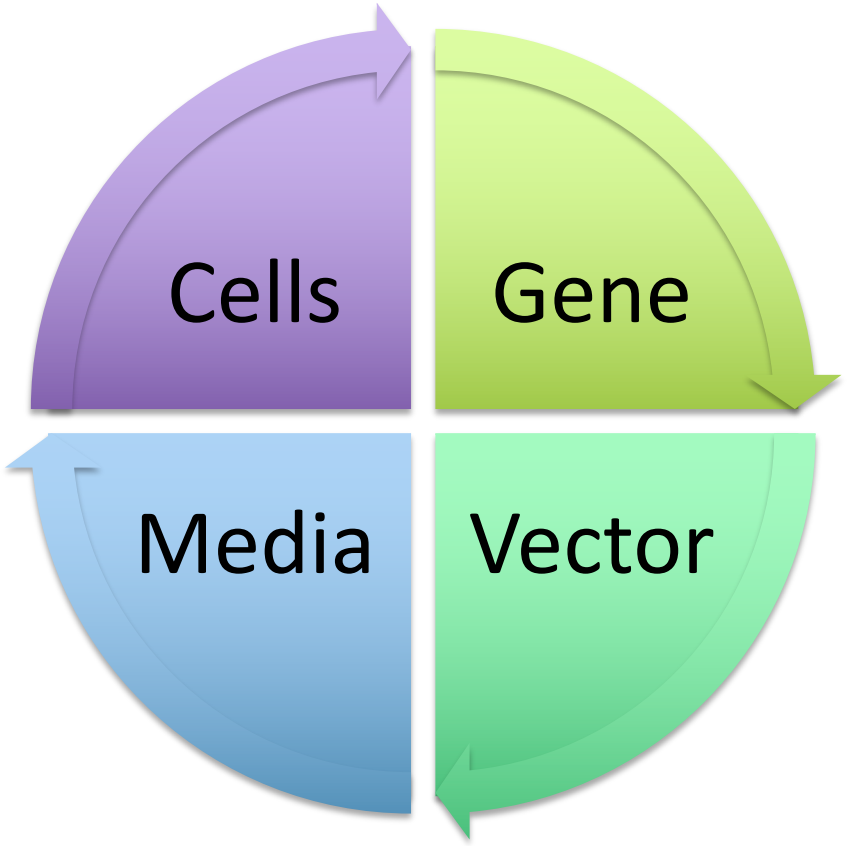
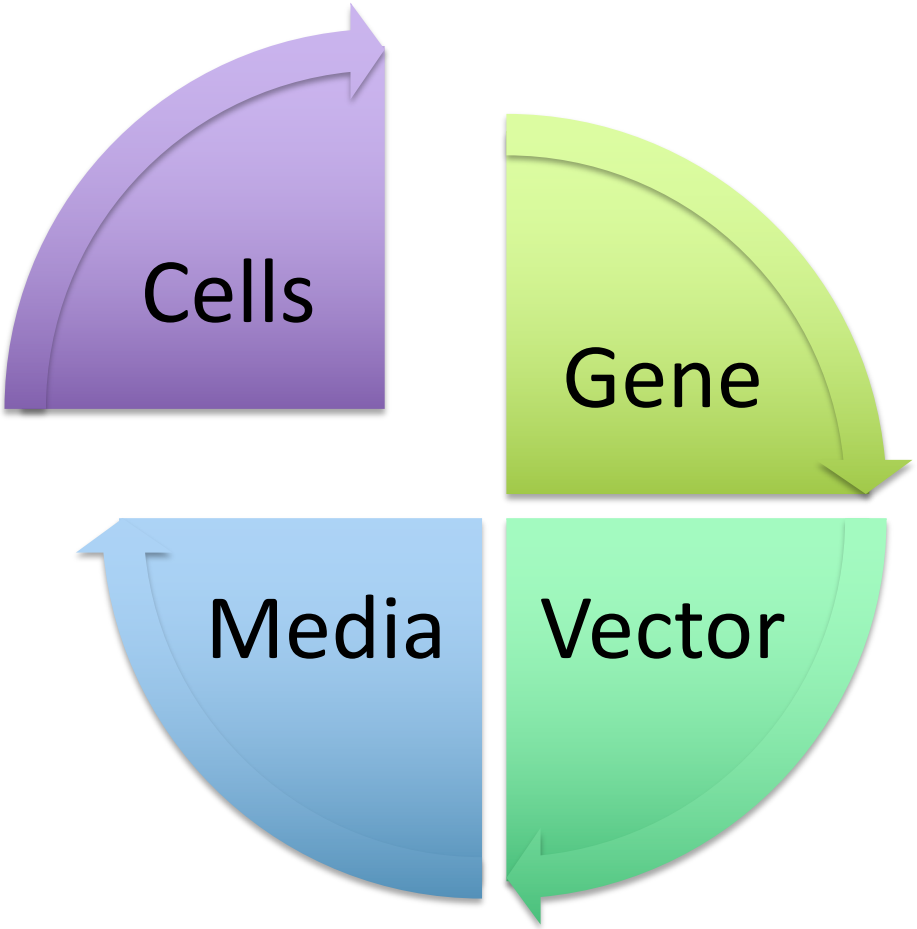
Reproducible Production of Proteins in mammalian cells



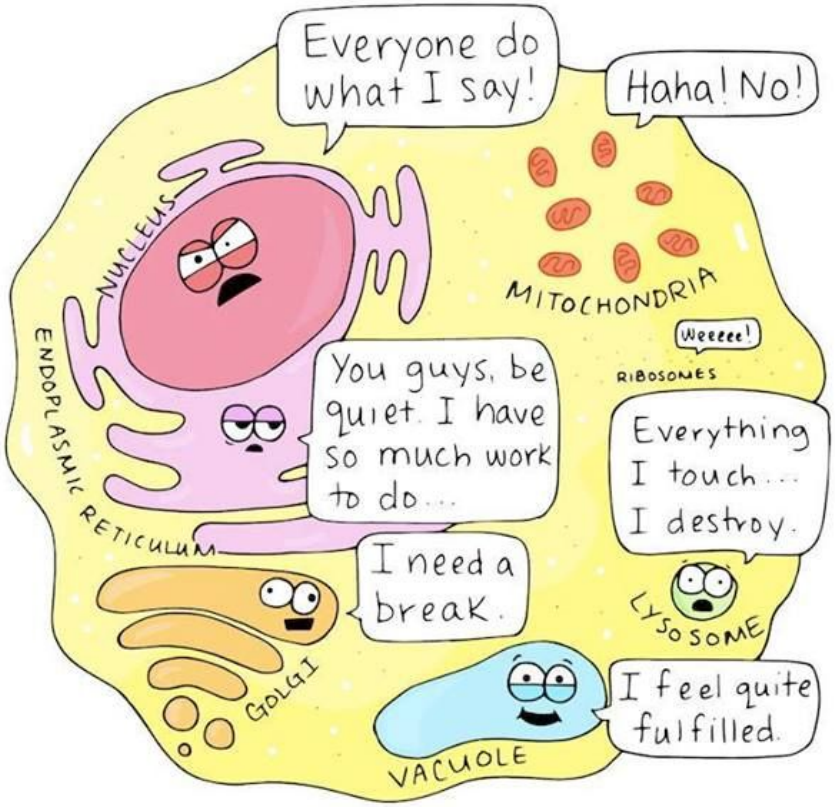
Main influencing factors



Reproducibility pitfalls



Optimal cell function is essential

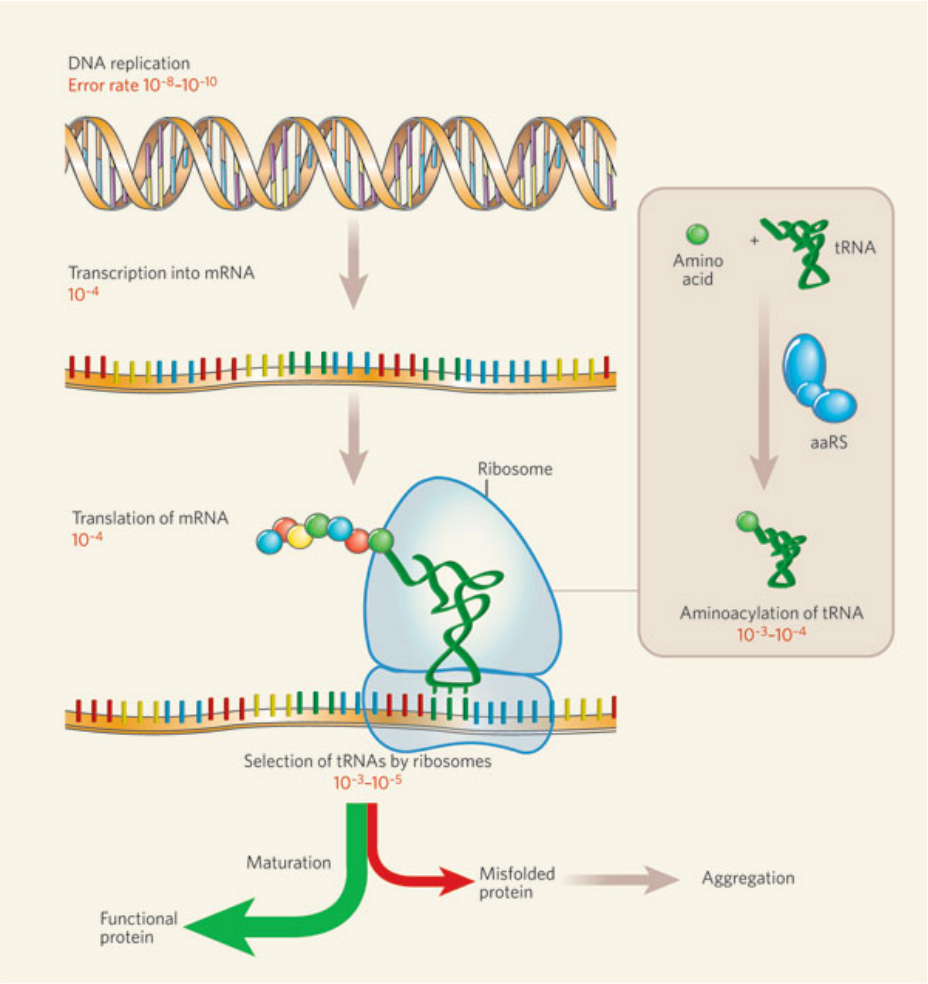


If organelles could talk.

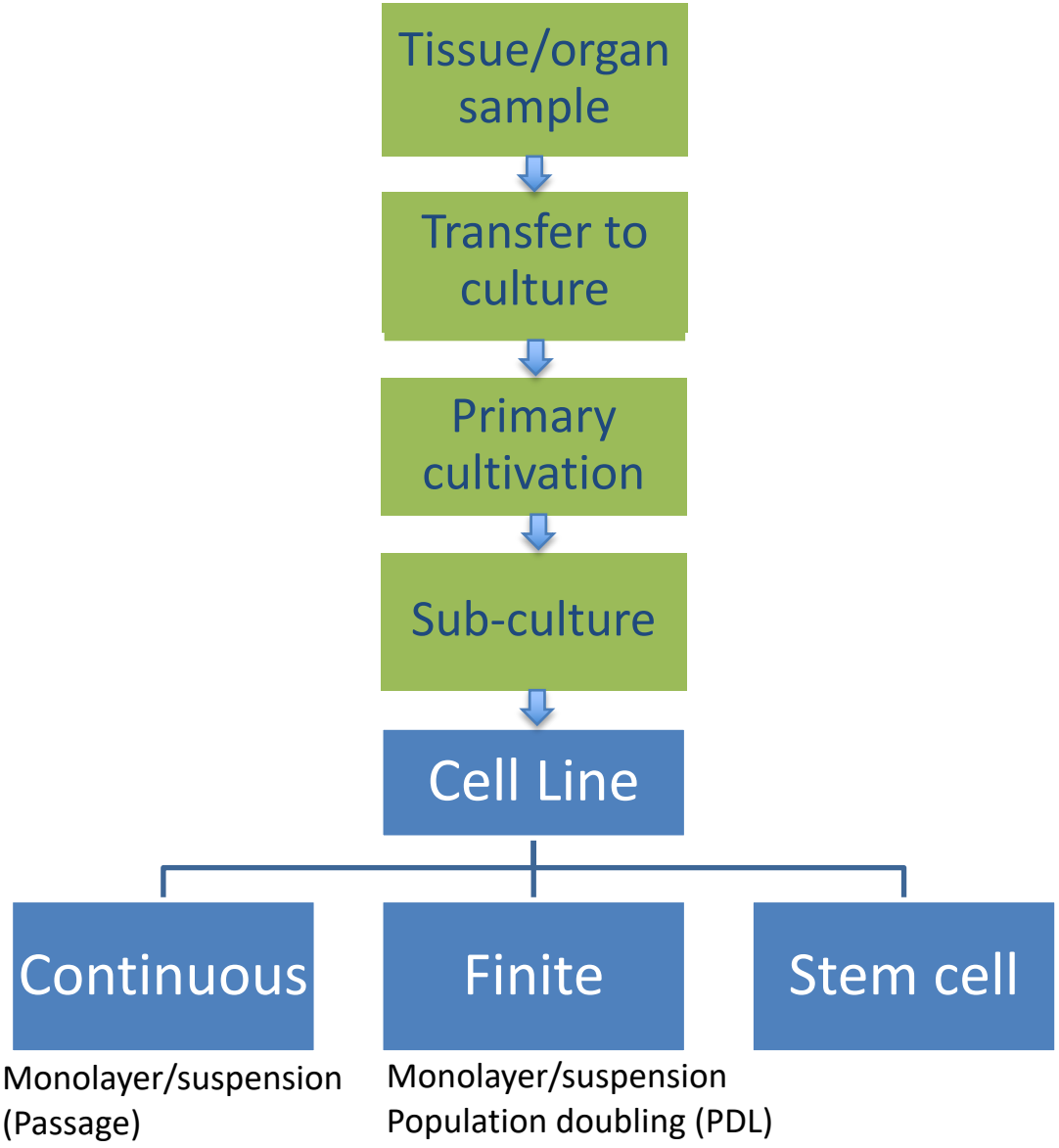
Beatrice the Biologist

The protein pathway in the host machinery

- Transcription
- Translation
- Modification
- Folding
- Localization
- Interactions
- Degradation



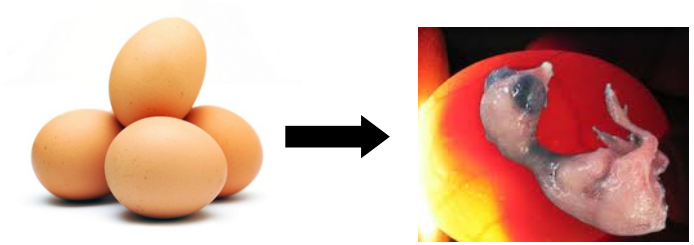
Cell Line generation

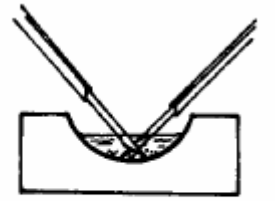


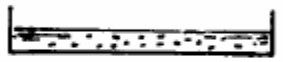
Primary cell culture

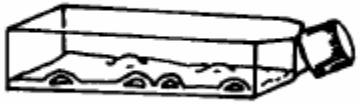
Chicken embryo fibroblasts

9-11 days fertilized eggs







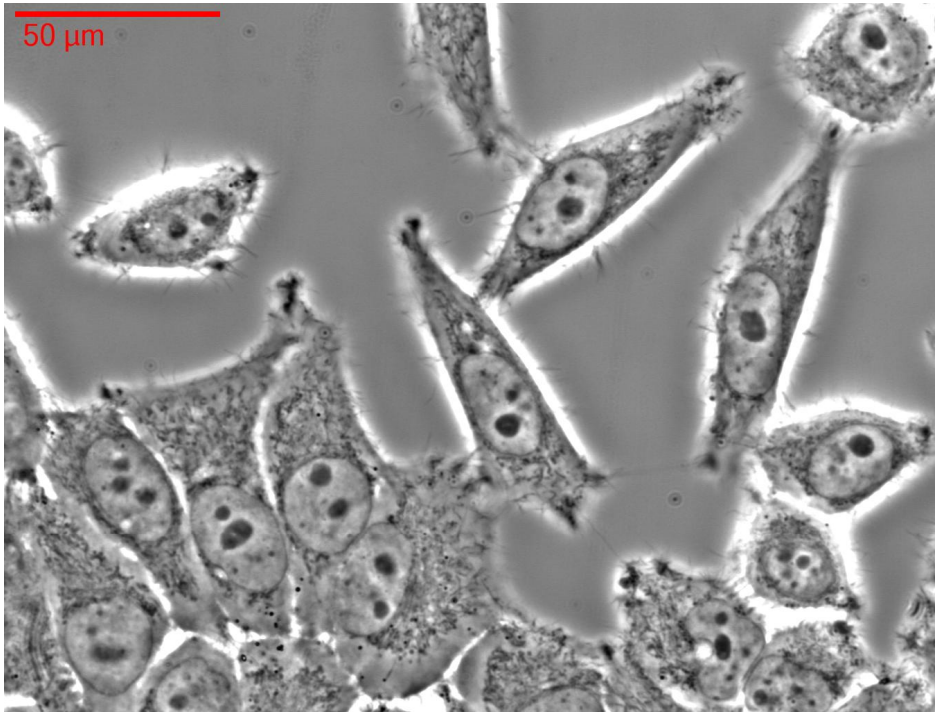


Tumor cells lines

Isolated from naturally transformed growths

Genome might be unstable or contain deletions in chromosomes

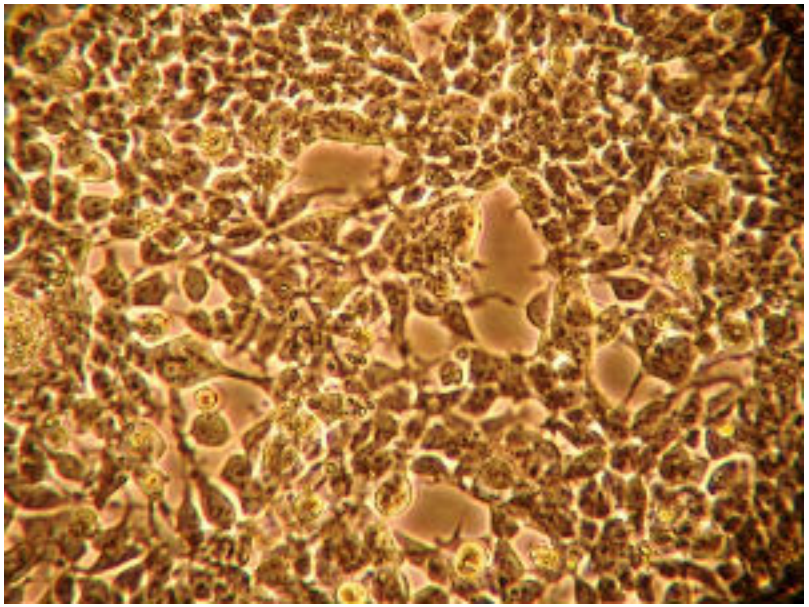
Cells are mostly at low level of differentiation



HeLa cells

Immortalized cell lines:

SV40 Large T-Ag oncogene:
Inactivates tumor suppressor protein p53
(Might cause genomic abnormalities)



HEK293 Cells

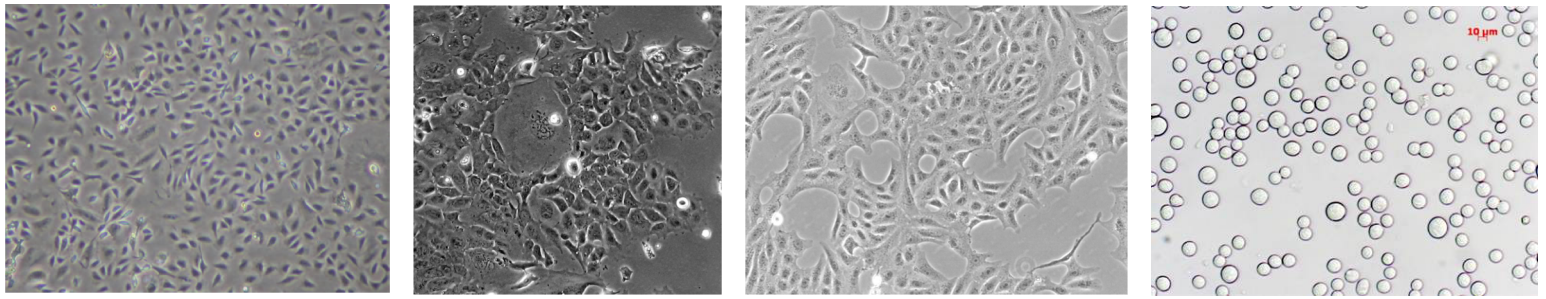
E7 oncoprotein of HPV: for epithelial cells immortalization

hTERT: Telomerase catalytic subunit (prolonging TC life)

EBNA of EBV: immortalization of B-cells

Commonly used Production Cell lines

- CHO-K1 Chinese Hamster Ovary cells
- HEK-293 Human Embryonic Kidney cells
- VERO African Green Monkey kidney cells
- PER.C6 Human retina cells



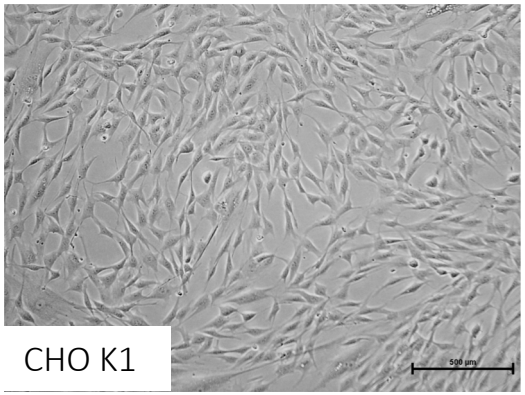
Cell Growth characteristics

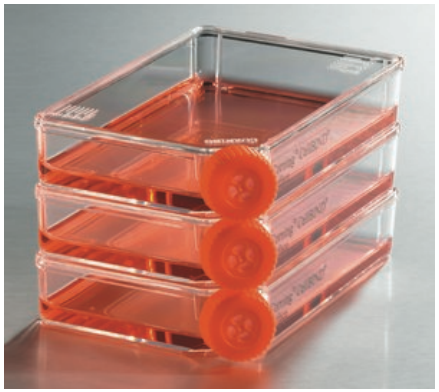
Growth Characteristics	Normal	Tumor
Proliferative life span	finite	continuous
Density dependent inhibition of growth	present	absent
Growth factor requirements	high	low
Anchorage dependence	present	absent
Adhesiveness	high	low

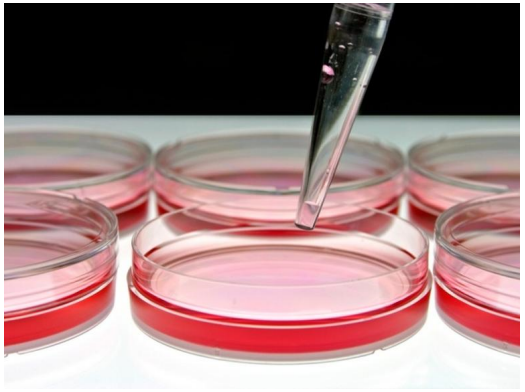
Terms:

- Split ratio- dilution ratio of a cell culture at subculture
- Passage number- number of times that the culture has been re-seeded
- Generation number- number of doublings that a cell population has undergone (PDL)

Monolayer culture





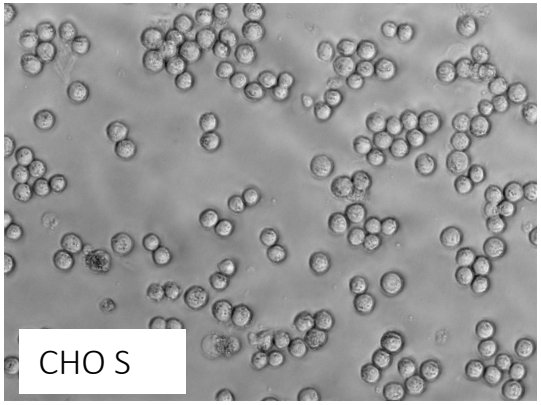


plates

flasks

Multi well plates

Suspension culture



Microcarriers suspension culture

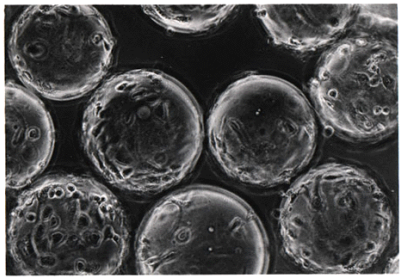


Figure 4: Vero cells cultured on Cytodex microcarriers

CO_2 Incubator

- CO_2 (usually 5%)
- Humidity) 90-95% (
- Temperature (37°C)
- Aseptic conditions (water, surfaces)
- Circulated/Non Air
- Filtered/Non

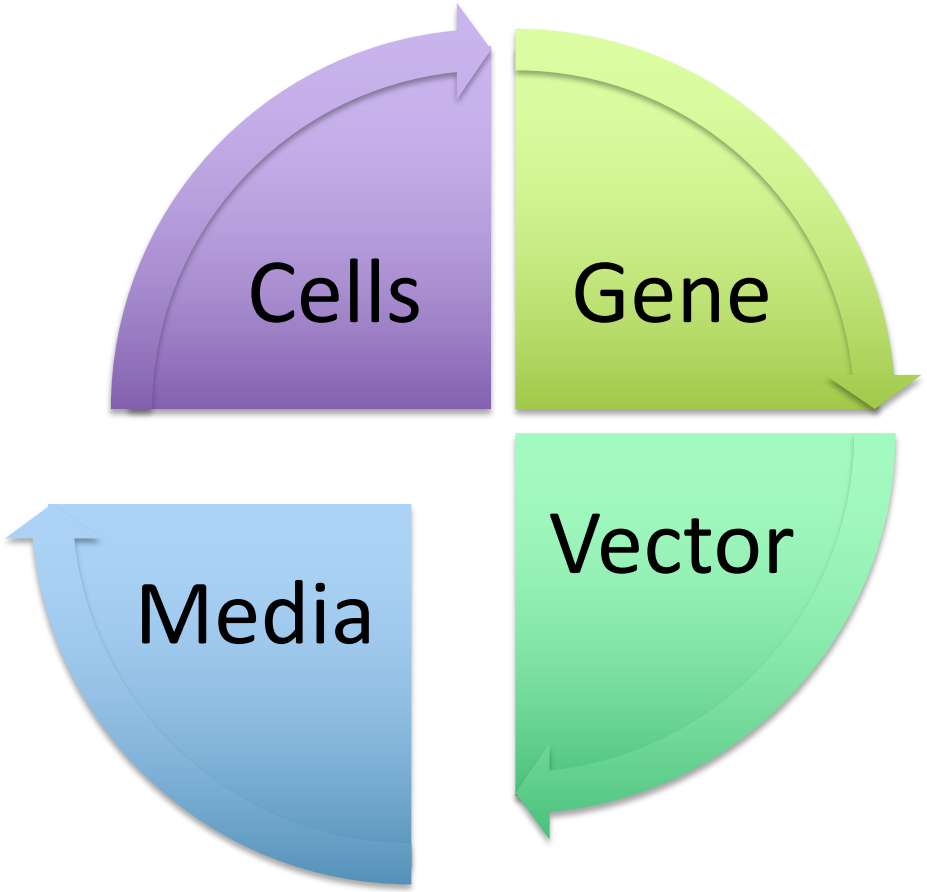
Cell origin and culturing

- Use certified cells
- Document culturing procedures (SOP)
- Bank large stocks at early passage
- Split cells on time and don’t let them overgrow
- Do not exceed recommended passage
- Routinely test for mycoplasma

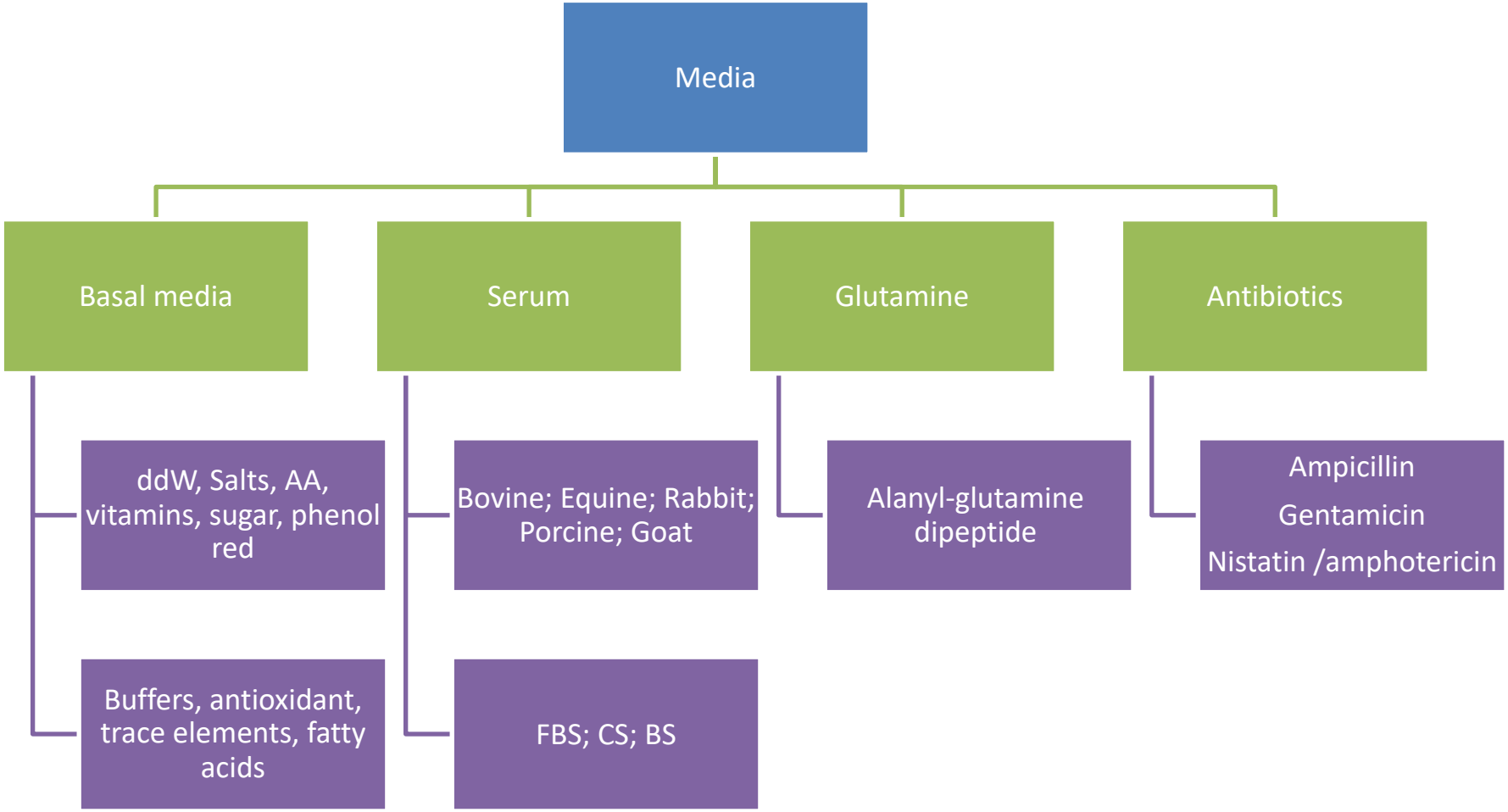
Cell Culture Banks

Collections	Website
American Type Culture Collection (ATCC)	www.atcc.org
CellBank Australia	www.cellbankaustralia.com
Coriell Cell Repository	http://ccr.coriell.org
Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)	www.dsmz.de
European Collection of Animal Cell Cultures (ECACC)	www.phe-culturecollections.org.uk/
Health Science Research Resources Bank (HSRRB), Japan	www.jhsf.or.jp/English/index_e.html
Japanese Collection of Research Bioresources (JCRB)	http://cellbank.nihs.go.jp
NIH Stem Cell Unit	http://stemcells.nih.gov/research/nihresearch/scunit/
RIKEN Gene Bank	http://en.brc.riken.jp
UK Stem Cell Bank (UKSCB)	www.ukstemcellbank.org.uk/
WiCell	www.wicell.org

Reproducibility pitfalls



Media for animal cell culture

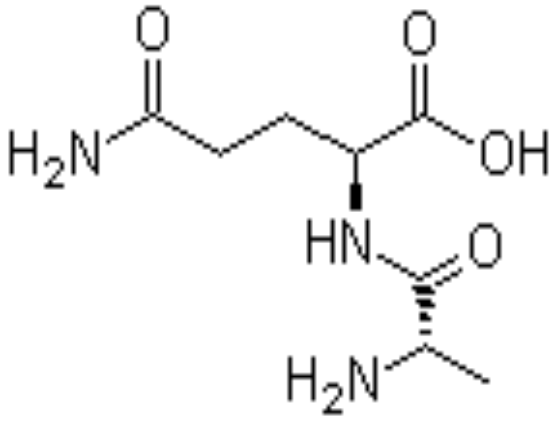


Media for animal cell culture

Glutamine stability

Glutamine can break down non-enzymatically into ammonia and pyroglutamate (pyrrolidonecarboxylic acid) in liquid media .

The ammonia is toxic to cells and the use of Alanyl-glutamine is recommended.

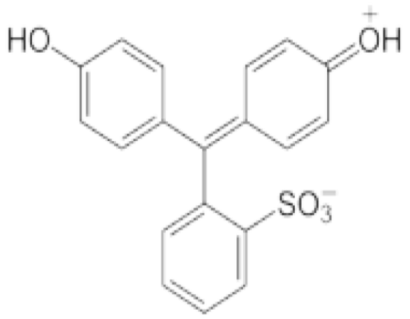


Alanyl-glutamine dipeptide

Media for animal cell culture

– **Phenol red**

- **Estrogenic-like activity!**



$C_{19}H_{14}O_5S$

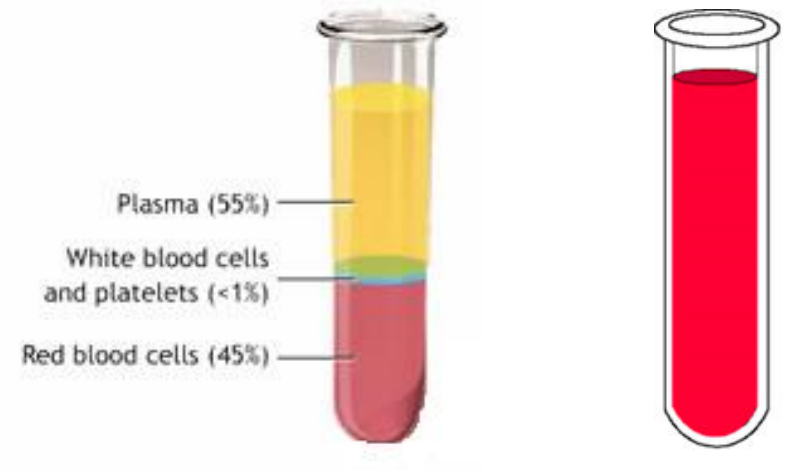
Phenol red	
below pH 6.6	***above pH 8.0***
6.6 ↔	**8.0**

The optimal physiological pH for mammalian cell cultures is usually considered to be pH 7.2–7.4, and pH 6.0 for insect cells.

Serum

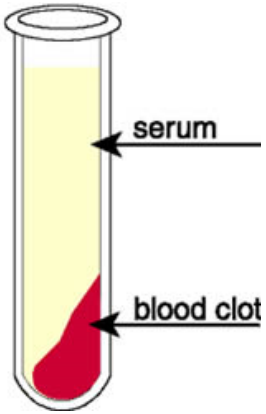
Plasma:

liquid component of blood after removing blood cells



Serum:

liquid component of clotted blood



Serum-free media

Disadvantages of serum

- Contamination (mycoplasma, viruses)
- Ethical issues (FBS)
- Undefined biological material
- Quality differences between batches
- Regulation issues

Basic formulation: Basal medium with the addition of:

- Albumin
- Insulin
- Transferrin
- Selenium

Effects of Media Formulations

Animal Components in media

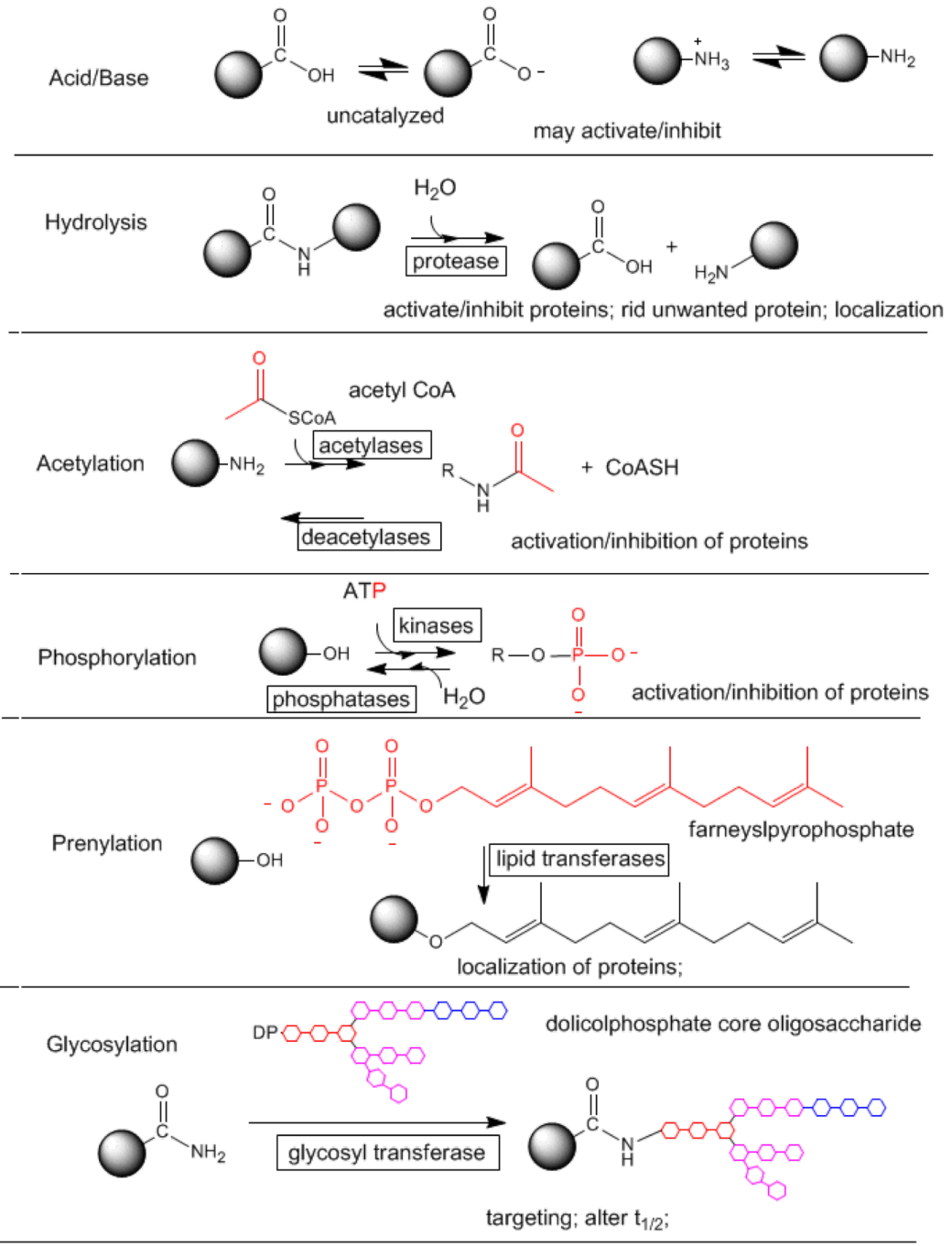
Contaminations

Batch and Lot Variations

Variability in cells and recombinant proteins (PTMs)

Modifications affected by cell health

- Proteolytic processing of the polypeptide
- Glycosylation and carbohydrate trimming
- Phosphorylation via cell-receptor-protein interactions
- Targeting & localization by fatty acid acylation
- Assembly of multimeric proteins



Controlled formulations

- ## Reduced serum medium

Basal formulation enriched with insulin, transferrin, nutrients and animal-derived factors to reduce supplemental serum requirement.

- ## Serum-free medium (SFM)

Basal formulation containing a broad range of additives and protein factors (which may be animal-derived). Does not require serum to support cellular function

- ## Protein-free medium (PFM)

Does not contain supplemental polypeptide factors. May contain various peptide fragments derived from enzymatic or acid hydrolysis of proteins obtained from animal or plant sources.

- ## Chemically-defined medium (CDM)

Usually protein-free, comprised exclusively of well characterized, low molecular weight constituents.

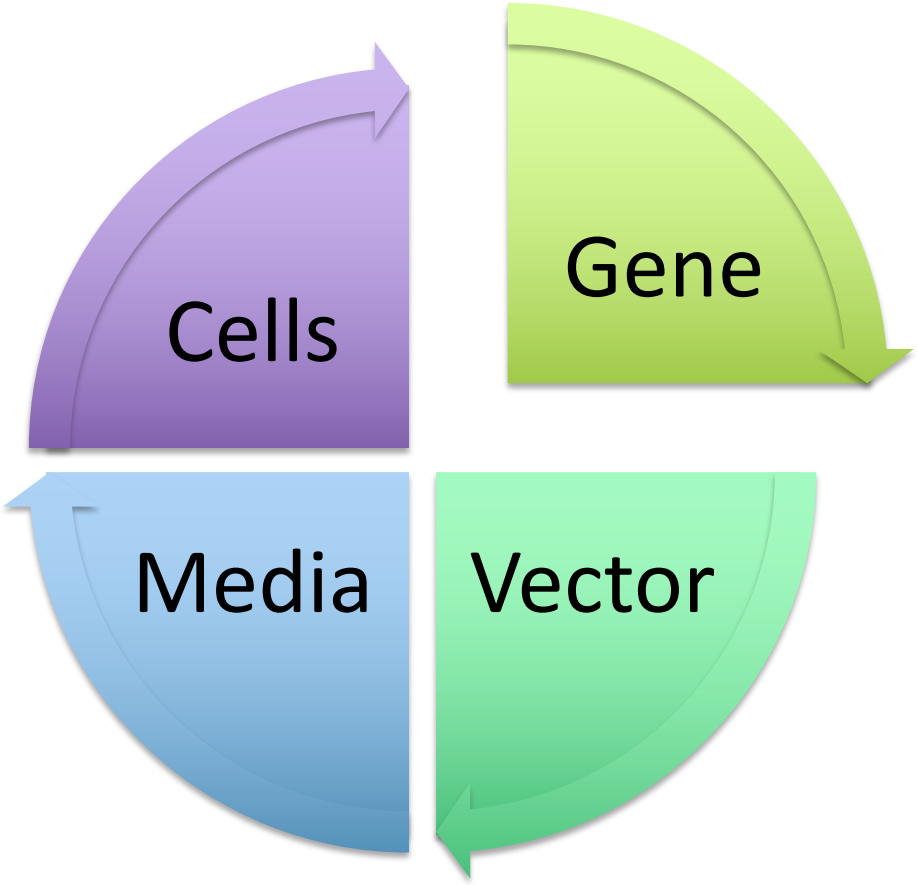
Cell dissociation solutions

- Crude trypsin (contains chemotrypsin, elastase)
- Crystalline trypsin
- Non-enzymatic (chelators)
- Plant enzyme (papain, ficin)
- Non-animal proteases (collagenase, dispase)
- Recombinant trypsin

Media Dos and Don’ts

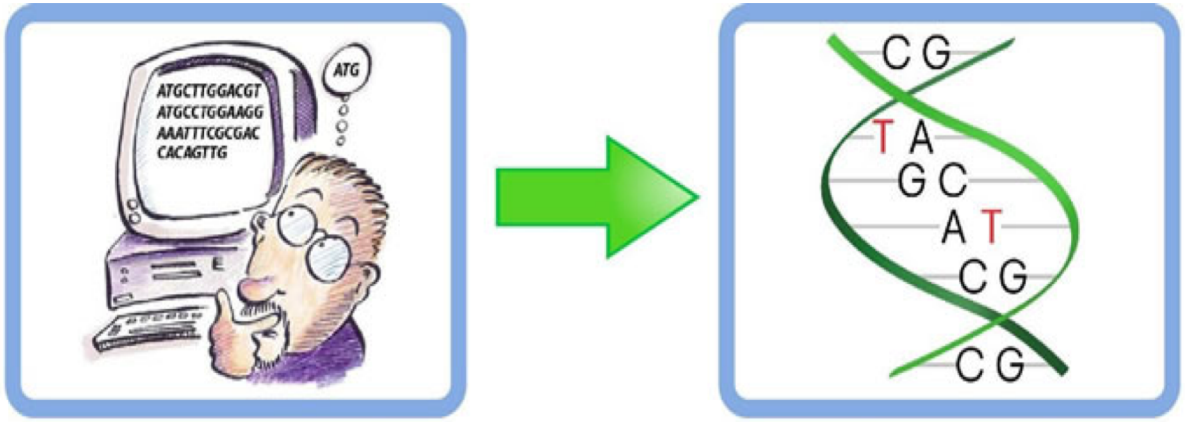
- **Do not expose media to light**
- **Do not use beyond expiration date**
- Document catalogue and lot numbers (SOP)
- Use same serum lot after testing
- Document additives cat and lot numbers (antibiotics, Gln, AA supplements, etc..)

Reproducibility pitfalls



Cloning of GOI

- Codon Optimization
- Signal-Peptide Optimization
- Domain Selection



Codon optimization is not always an advantage

Published in final edited form as:
Trends Mol Med. 2014 November ; 20(11): 604–613. doi:10.1016/j.molmed.2014.09.003.

PLOS ONE

Codon Optimization of the Human Papillomavirus E7 Oncogene Induces a CD8$^+$ T Cell Response to a Cryptic Epitope Not Harbored by Wild-Type E7

Felix K. M. Lorenz, Susanne Wilde, Katrin Voigt, Elisa Kieback, Barbara Mosetter, Dolores J. Schendel, Wolfgang Uckert

Molecular Therapy
Methods & Clinical Development
Original Article

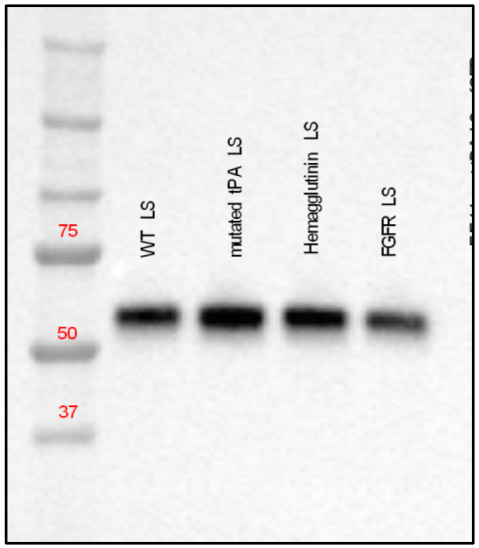
AMERICAN SOCIETY of GENE & CELL THERAPY

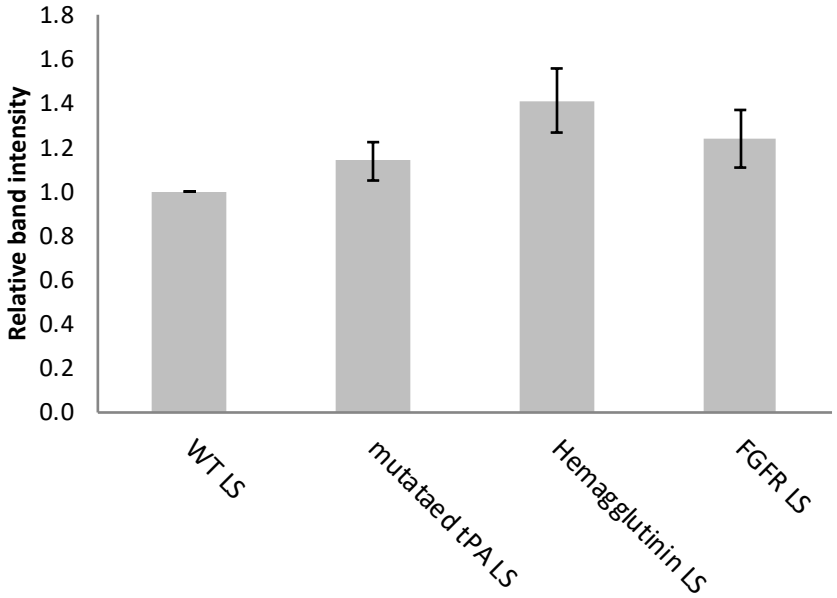
Codon Optimization Leads to Functional Impairment of RD114-TR Envelope Glycoprotein

Eleonora Zucchelli,[1,2,3] Monika Pema,[1,3] Anna Stornaiuolo,[1] Claudia Piovan,[1] Cinzia Scavullo,[1] Erica Giuliani,[1] Sergio Bossi,[1] Stefano Corna,[1] Claudia Asperti,[1] Claudio Bordignon,[1] Gian-Paolo Rizzardi,[1] and Chiara Bovolenta[1]

[1]MolMed S.p.A., 20132 Milano, Italy; [2]Great Ormond Street Institute of Child Health, University College London, London WC1N 1EH, UK

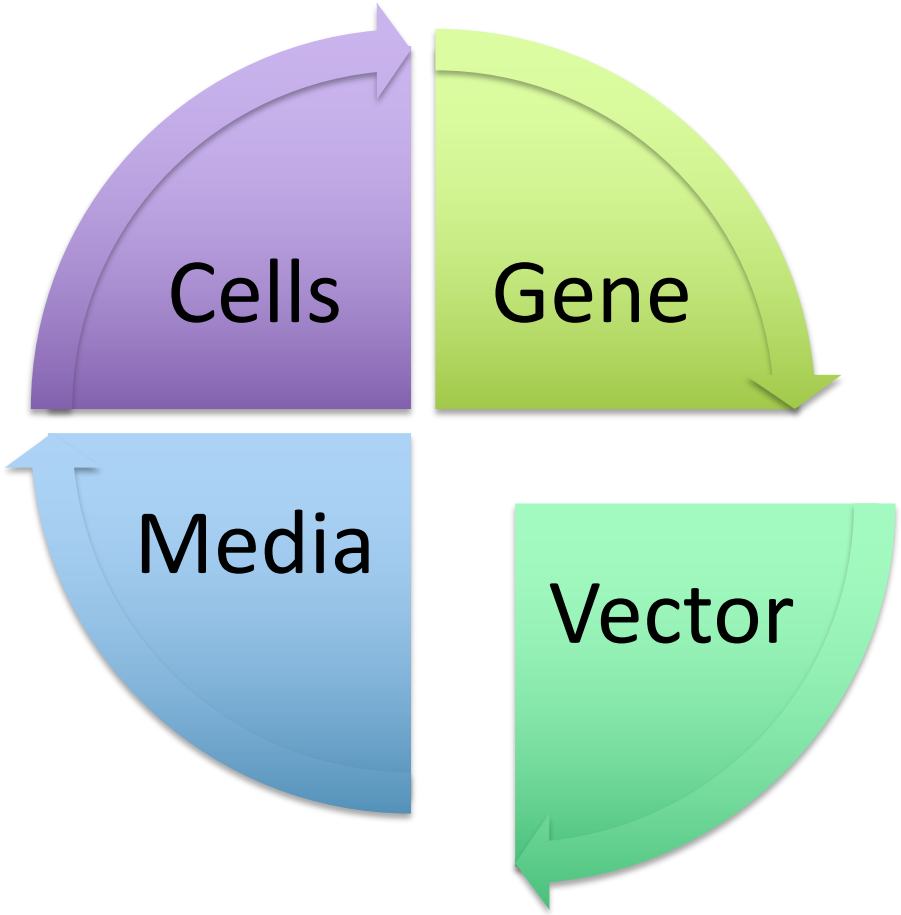
Signal peptide Optimization and UTR





CM samples from HEK293T/17 cells transfected with 60kDa secreted protein were loaded on TGX 4-20% Stain-free gel.
Bands intensity was calculated relative to intensity of wt LS. N=4. Average +/- SEM.

Reproducibility pitfalls

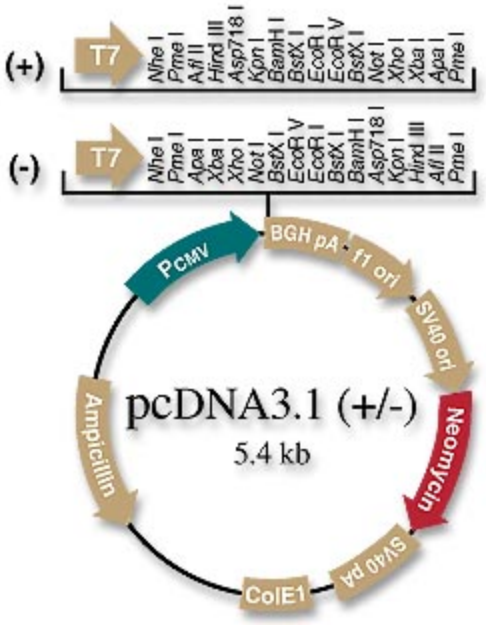


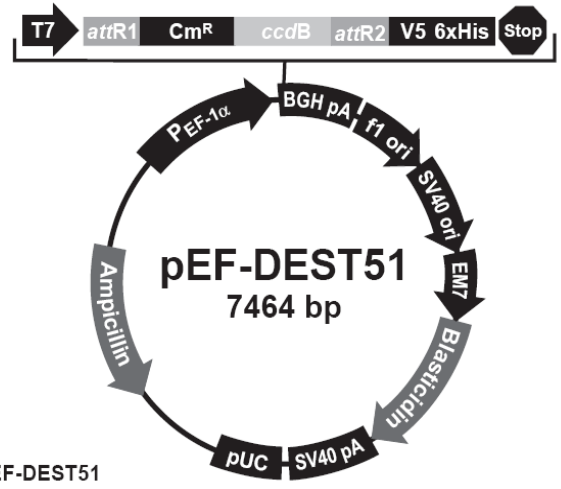
Expression vectors for transient and stable expression:

Common Promoters:

CMV: Cytomegalovirus promoter

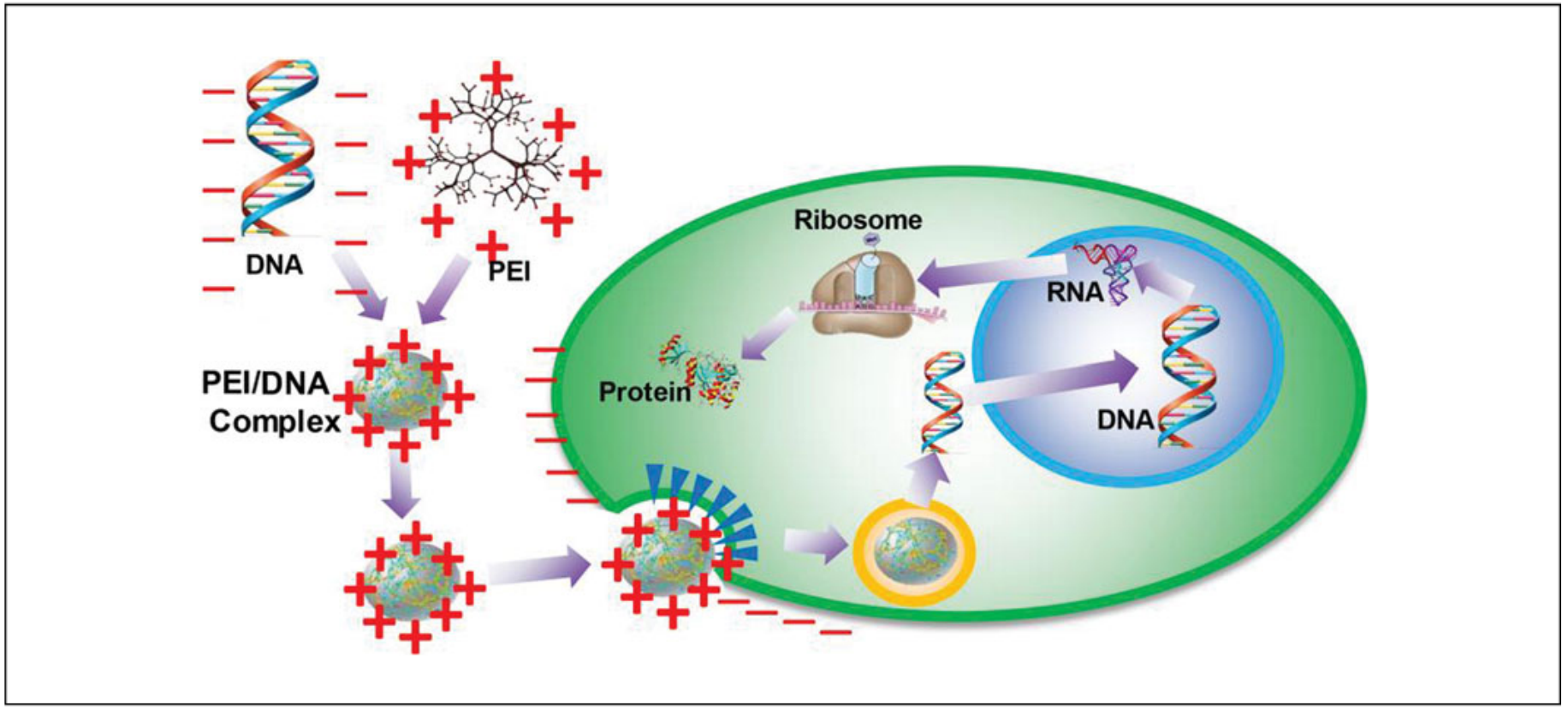
EF: Elongation Factor promoter





f pEF-DEST51

Transfection of plasmid DNA into cells

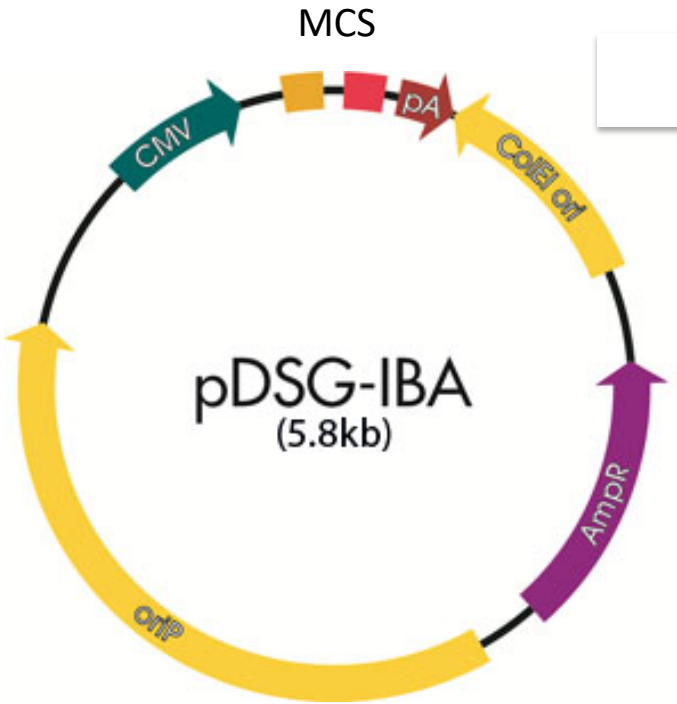


Schematic overview of PEI-Based transfection

EBNA based transient expression systems

MEXi-293E (HEK293) cells containing the EBNA1 gene for episomal proliferation of oriP vectors (IP FREE!!)

Can transiently express protein for over 1 month!



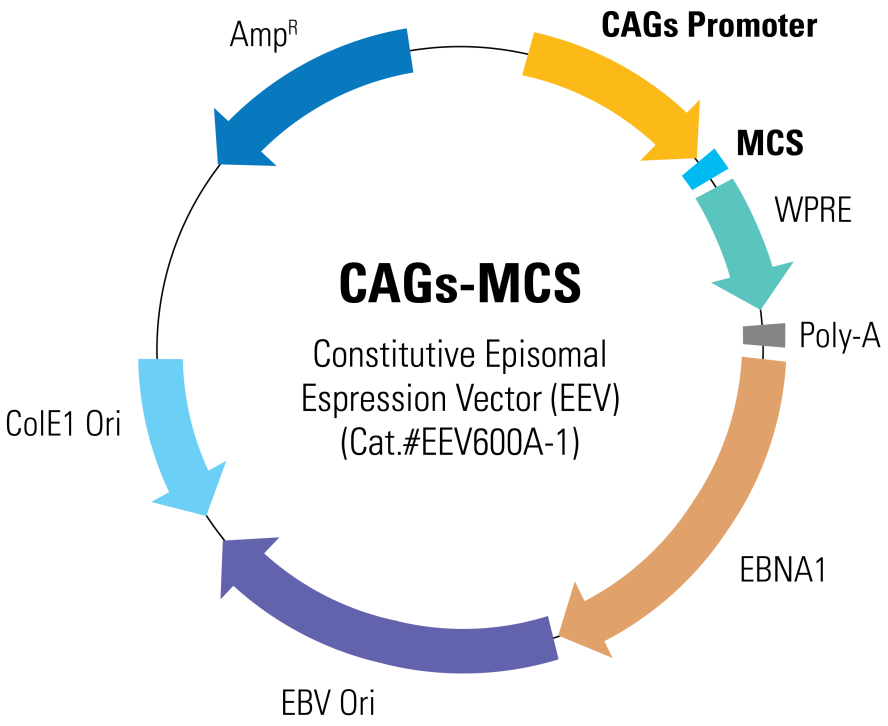
Other options (not as robust):

Freestyle 293 (Thermo)

Expi293 (Thermo); HEK-293T

https://www.iba-lifesciences.com/mexi-technology.html

EBV-based non-integrating vector for gene expression in HEK293 cells



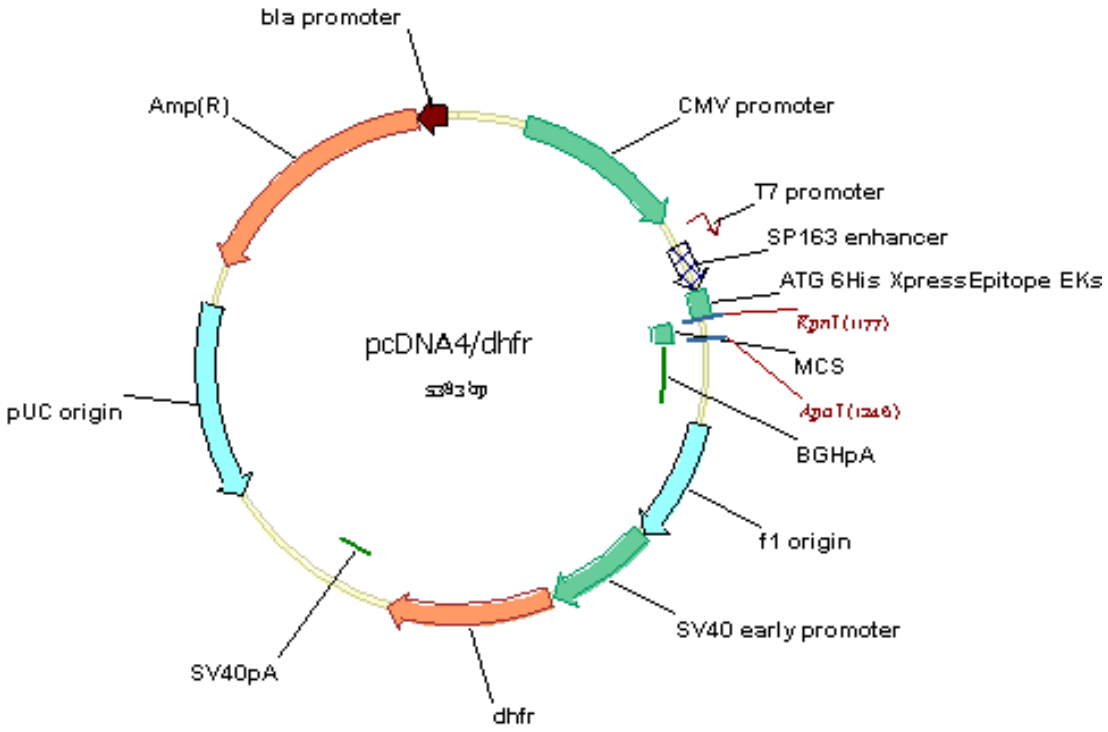
CAG promoter: (**C**) the cytomegalovirus (CMV) early enhancer element,(**A**) the promoter, the first exon and the first intron of chicken beta-actin gene,(**G**) the splice acceptor of the rabbit beta-globin gene

System BioSciences (SBI) ™

https://www.systembio.com

Stable Expression: DHFR amplification by MTX for commercial production of proteins in CHO cells

CHO cells lack endogenous DHFR gene, allowing amplification of the integrated vector under MTX selection.

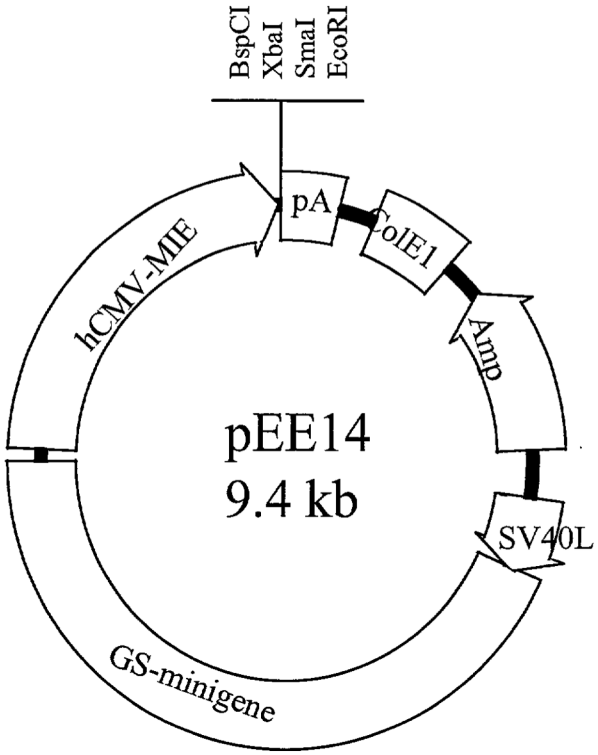


DNA amplification vectors for enhanced production

- Cytomegalovirus promoter
- A weaker SV40 promoter for the Glutamine synthetase (GS) gene
- Host cell lines, CHO and B-lymphocytes
- MSX: a competitive inhibitor of GS is used for selection of resistant cells in which DNA amplification of the vector occurs

Another genome amplification system: adenosine deaminase (ADA) based

pEE14 expression vector



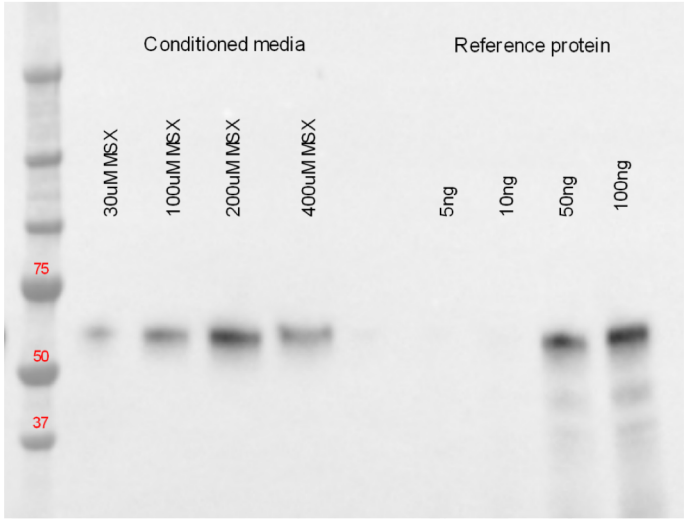
Lonza

Effects of amplification rounds and MSX concentration on expression levels

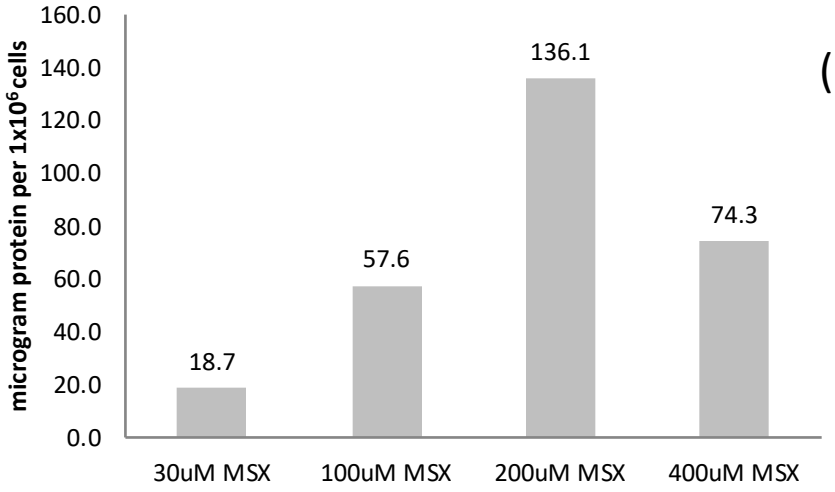
Stable expression in CHO cells

a. CM samples from stable cells growing at increasing concentrations of MSX were loaded on TGX 4-20% Stain-free gel.

b. Absolute amount of protein was calculated based on the band intensity of the reference protein.



(a)



(b)

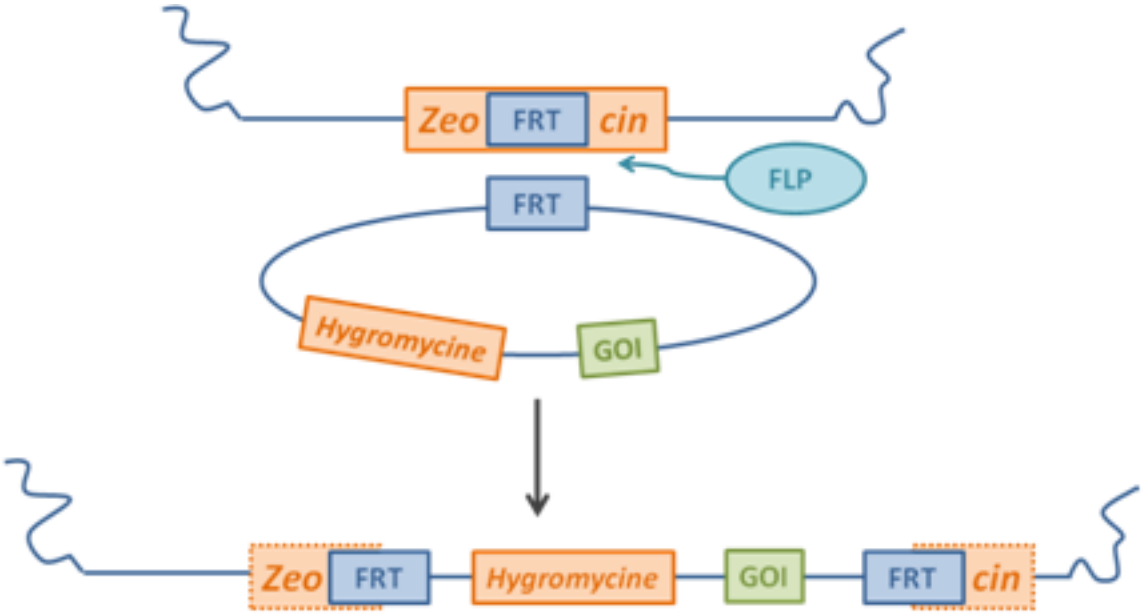
Transient expression in HEK293T/17 cells: ~1mg/ml ($1x10^7$ cells)

Transient expression in CHO-K1 cells: ~150 mg/ml

Stable expression in CHO-K1 cells in 200µM MSX: ~1.4mg/ml

The Flip-In System

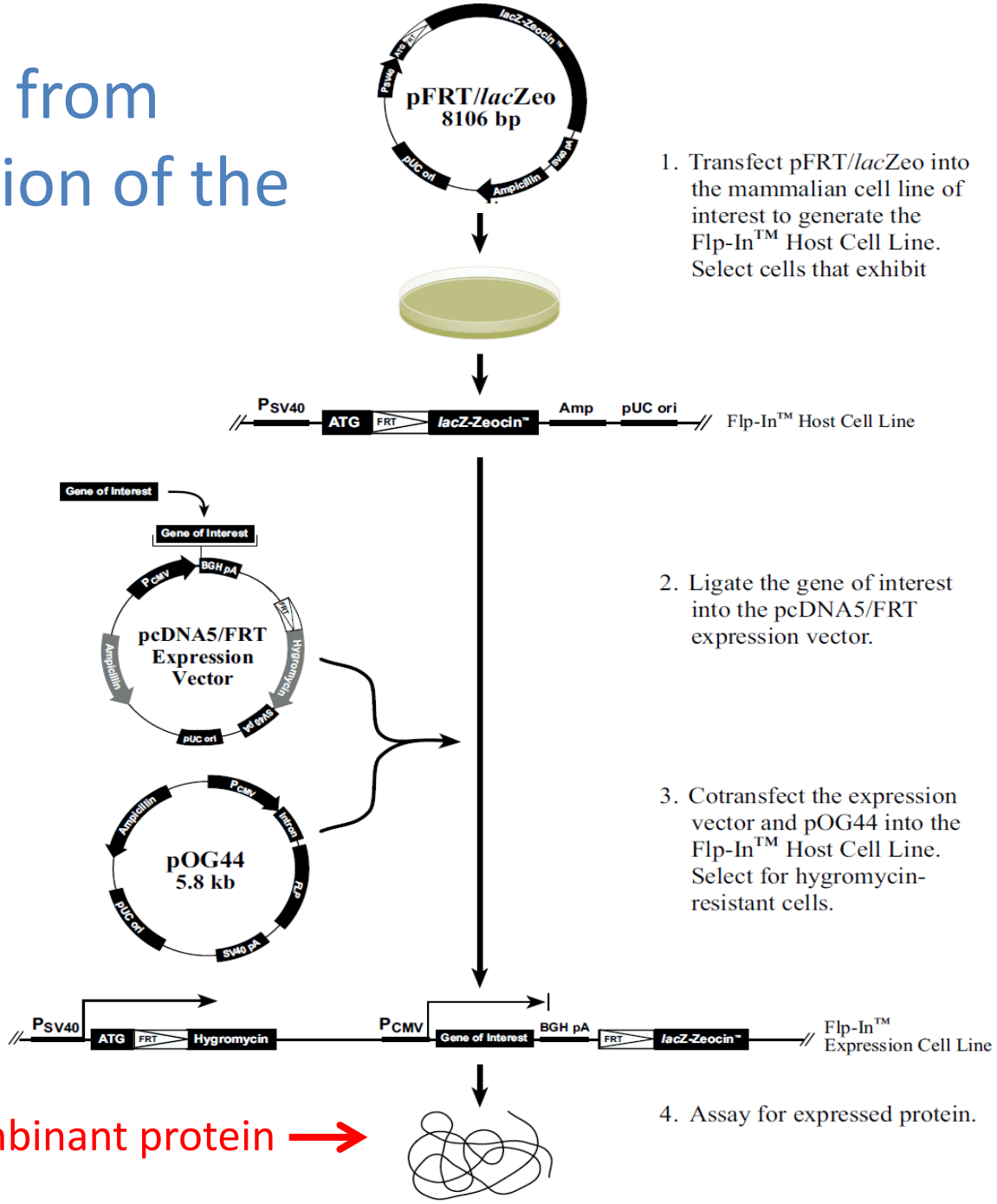
Saccharomyces cerevisiae-derived DNA recombination system



FRT: Flp Recombination Target

Flp-mediated recombination, (Craig, 1988; Sauer, 1994).

Starting from production of the cell line

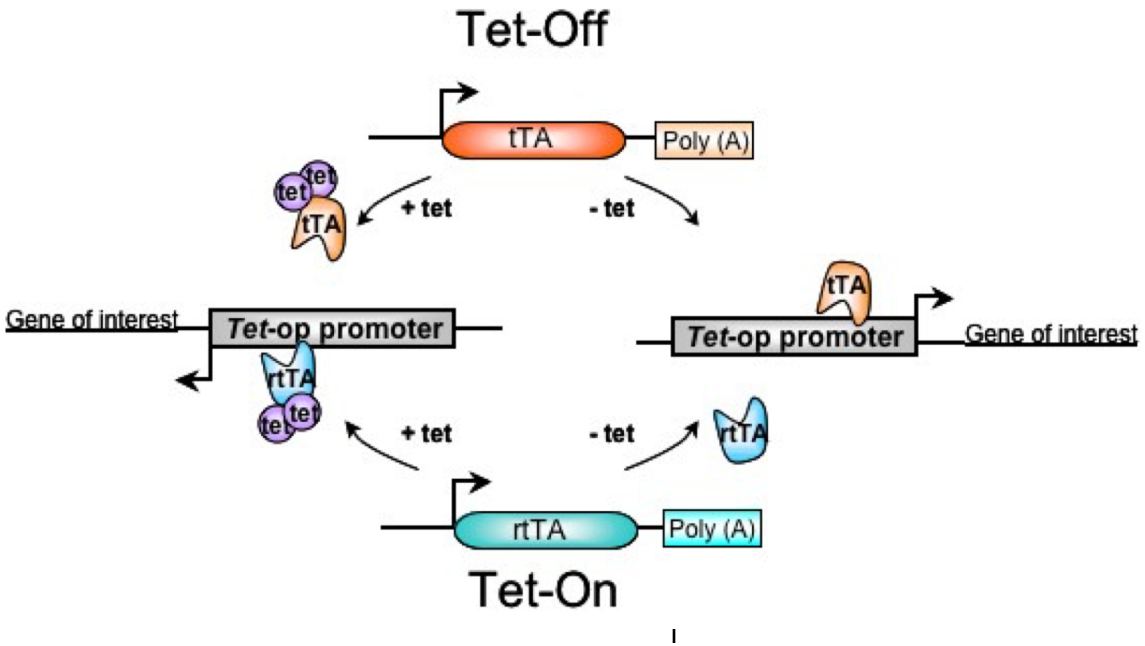


The recombinant protein →

The Tet off / Tet-On system

Recombinant tetracycline-controlled transcription factor (either tTA for TET-Off or rtTA for TET-On) binds to the *Tet*-op promoter, driving the expression or controlling the inhibition of the target gene.

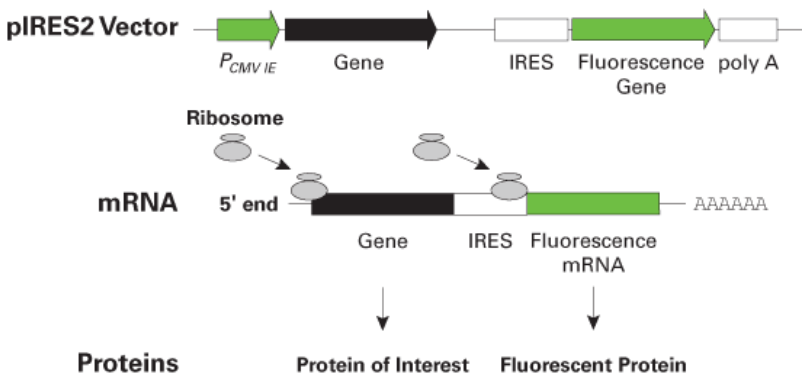
Gene expression is regulated by the presence or absence of tetracycline or one of its derivatives such as Doxycycline. Tetracycline binds directly to the transcription factors.

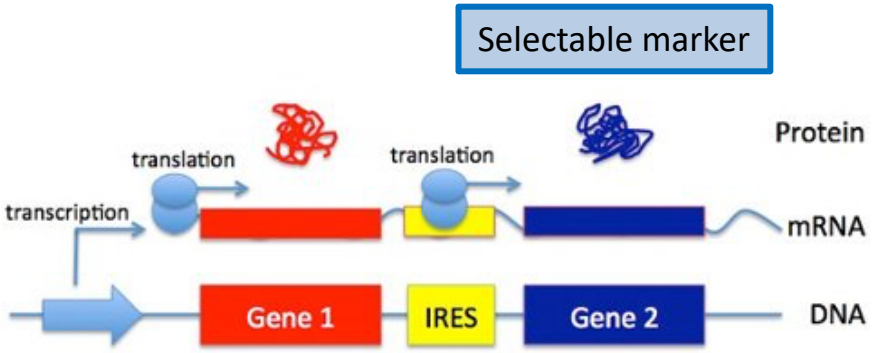


Tet-Off system: tetracycline prevents the tTA transcription factor from binding DNA at the promoter. Gene expression is inhibited in the presence of tetracycline.

Tet-On system: tetracycline binds the rtTA transcription factor and allows it to bind DNA at the promoter. Gene expression is induced in the presence of tetracycline.

IRES vectors:
Expression 2 proteins from the same construct

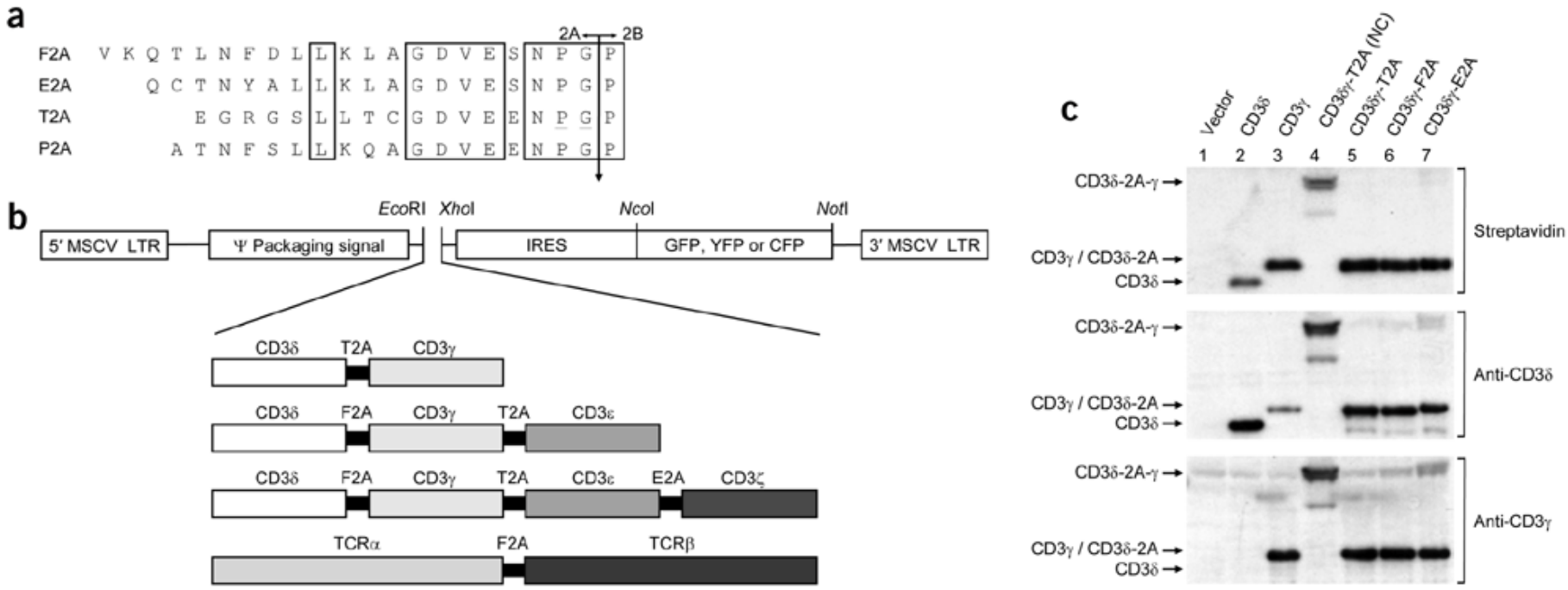




- Downstream ORF will always express to a lesser extent

Self-cleaving 2A peptides for efficient translation of four genes.

originated from viruses such as picorna and foot & mouth, used to generate

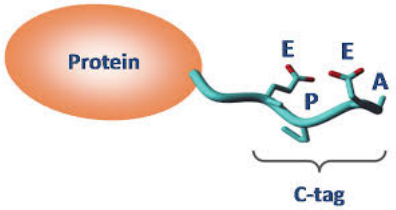


Through a ribosomal skip mechanism, the 2A peptide impairs normal peptide bond formation between the 2A glycine and the 2B proline without affecting the translation of 2B

Nature Biotechnology 22, 589 - 594 (2004)

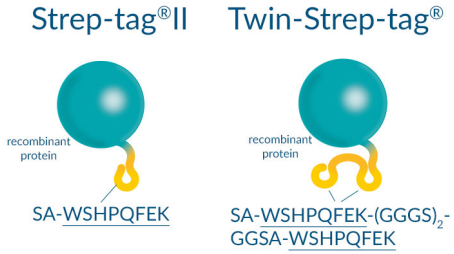
Mammalian affinity purification tags

C-tag (EPEA) C-term tagging for protein interactions and complexes identification (CaptureSelect resin ThermoFisher)

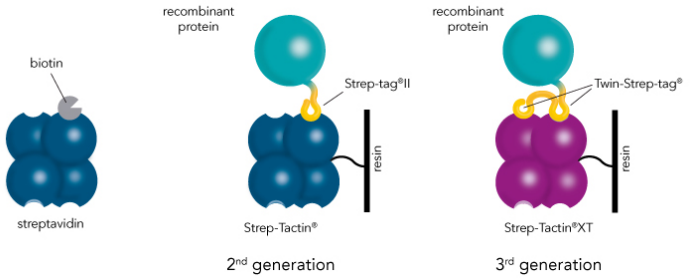


Active in the presence of urea and guanidine HCl, Used to isolate complexes from cytoplasm or periplasmatic fractions. Mild elution at neutral pH with MgCl or propylene glycol.

Strep Tag N or C-term tags for high affinity purification with Strep-Tactin (IBA)



Used mainly for secreted proteins. Biotin Blocking solution (BioLock) to block biotin in conditioned media



And: when using His tag, prefer x10 and not x6

Transient expression in mammalian cells

Pros:

- Suitable for quick screening of expression
- Better productivity for proteins which affect cell growth
- More suitable for expression of toxic proteins
- Possible expression scale up in suspension
- Shorter procedure

Cons:

- Batch to batch variability due to DNA and cell condition
- Over expression may lead to non homogeneous PTMs
- Difficult to scale up
- Might be more costly for scale up: DNA and reagents
- Extended regulatory requirements

High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells Jäger et al. BMC Biotechnology (2013)

Stable expression in mammalian cells

Pros:

- More suitable for therapeutics regulations
- Cells can be banked at early passage for reproducibility
- Improved adaptation to suspension growth
- Suitable for secreted proteins
- Cost effective in the long run

Cons:

- Long procedure
- Not suitable for toxic and growth affecting targets
- Low productivity

High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells Jäger et al. BMC Biotechnology (2013)

Regulation requirements for biologics:

- Full sequenced gene
- Biologics production-certified cell line
- Establishing viable cell bank
- Serum-free (animal free?) media formulations
- Product analysis : PTMs homogeneity
- Activity, stability, aggregations, immunogenicity, contaminants

Leading to more efficient scale up

Spinner flasks can be used to grow cells in CO2 incubator, on magnetic plates. (0.5-3L)

Medium Scale: 5-10L Bioreactors

Large Scale: 50-200L Hyclone/Sartorius/etc. Bioreactors

suspension or adherent on polycarbonate beads to increase cell/volume ratio.

Questions? Common problems?

Lentivirus expression