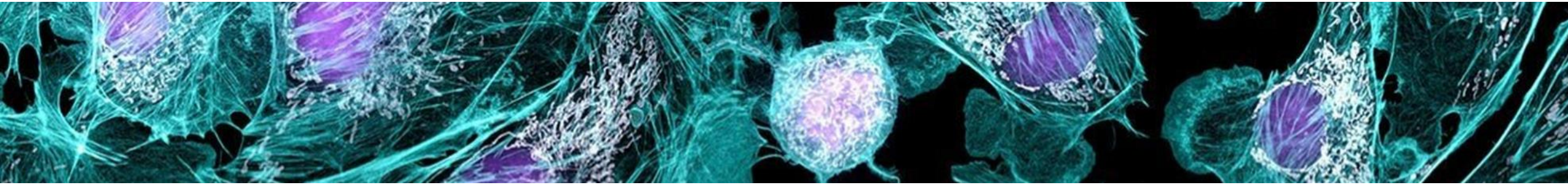
Advanced Cell Biology and Biotechnology

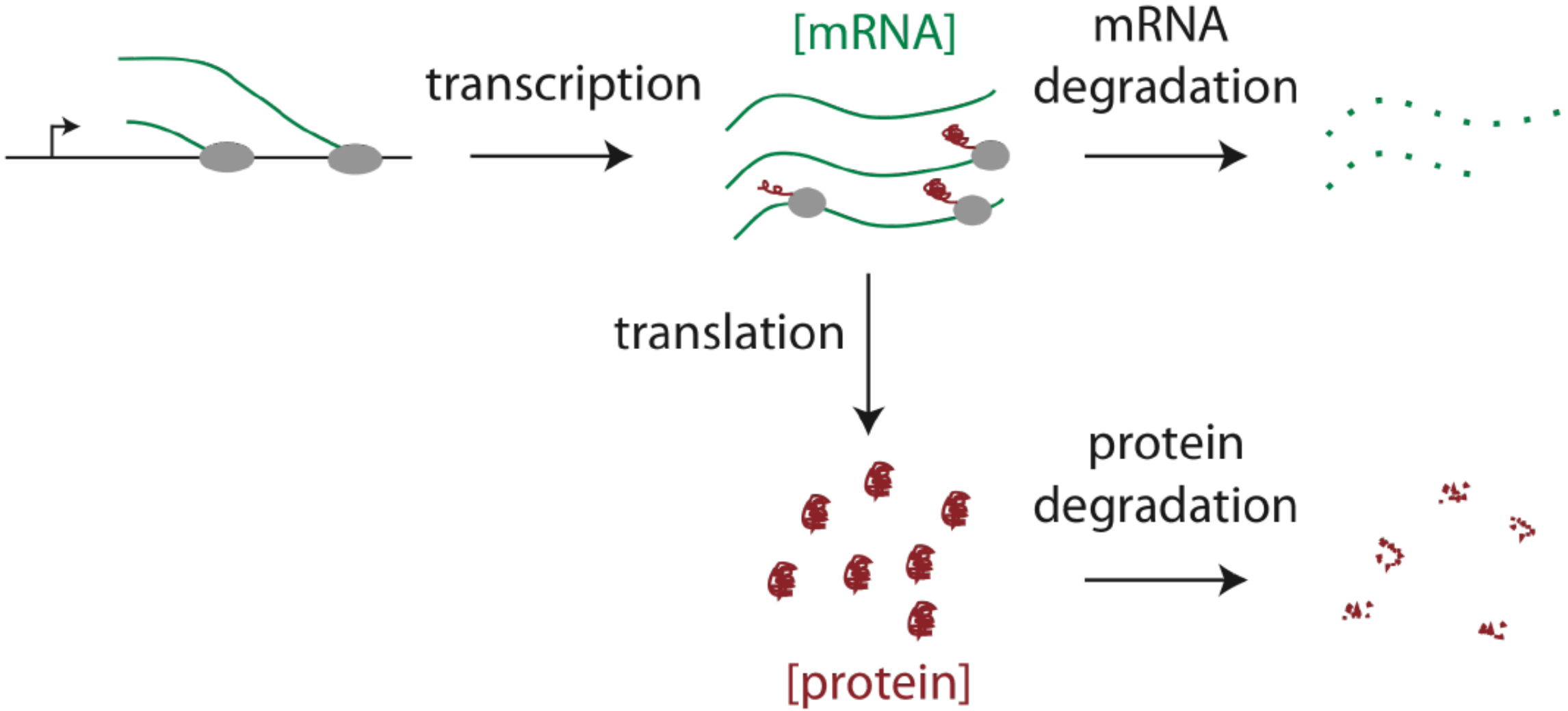
ACBB 2021/22

…the lecture is about to begin…

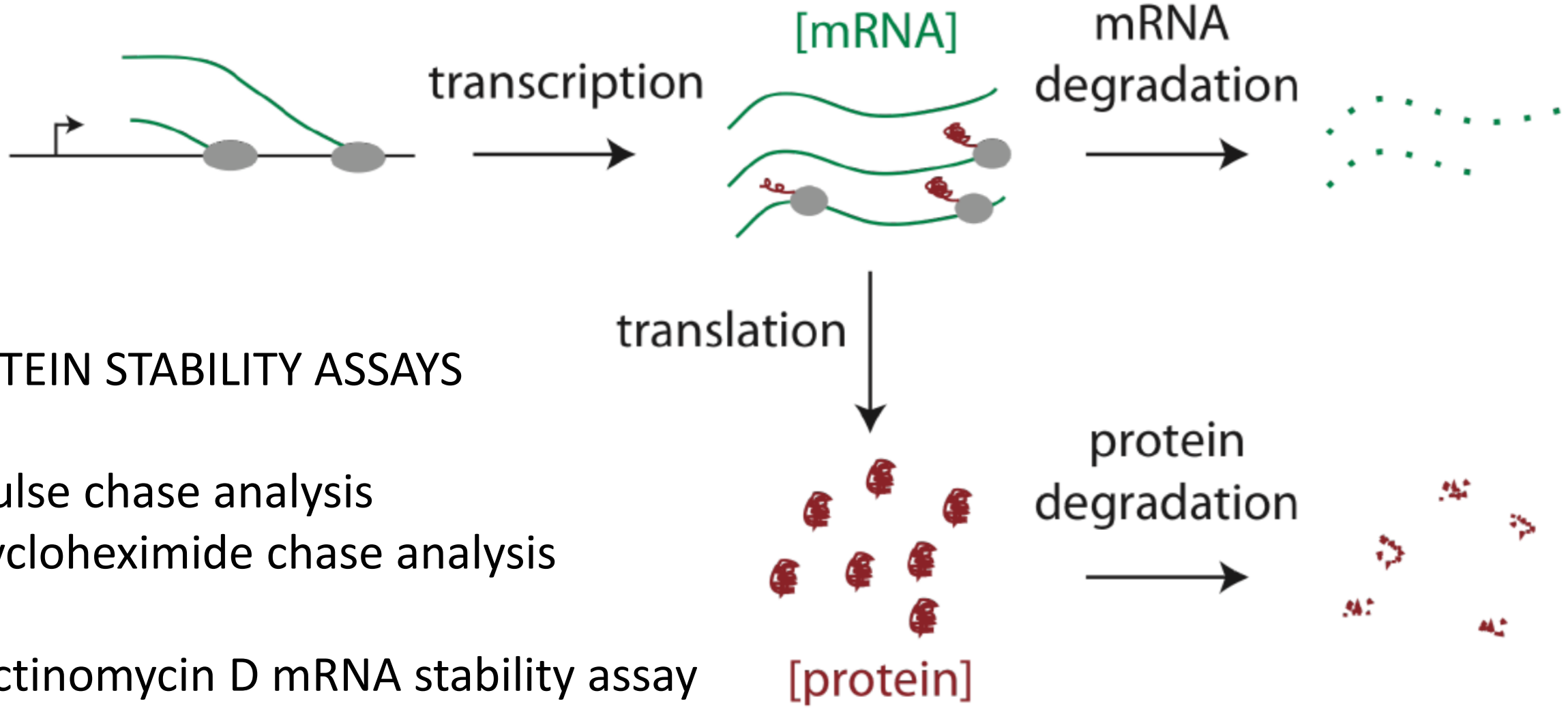


- How can you explain if mRNA does not change, while protein amount increases or decreases?
- Or RNA expression changes, while protein amount does not change?
- Why protein expression after siRNA treatment is not immediately switched off?

Protein stability and kinetics of protein degradation



Protein stability and kinetics of protein degradation

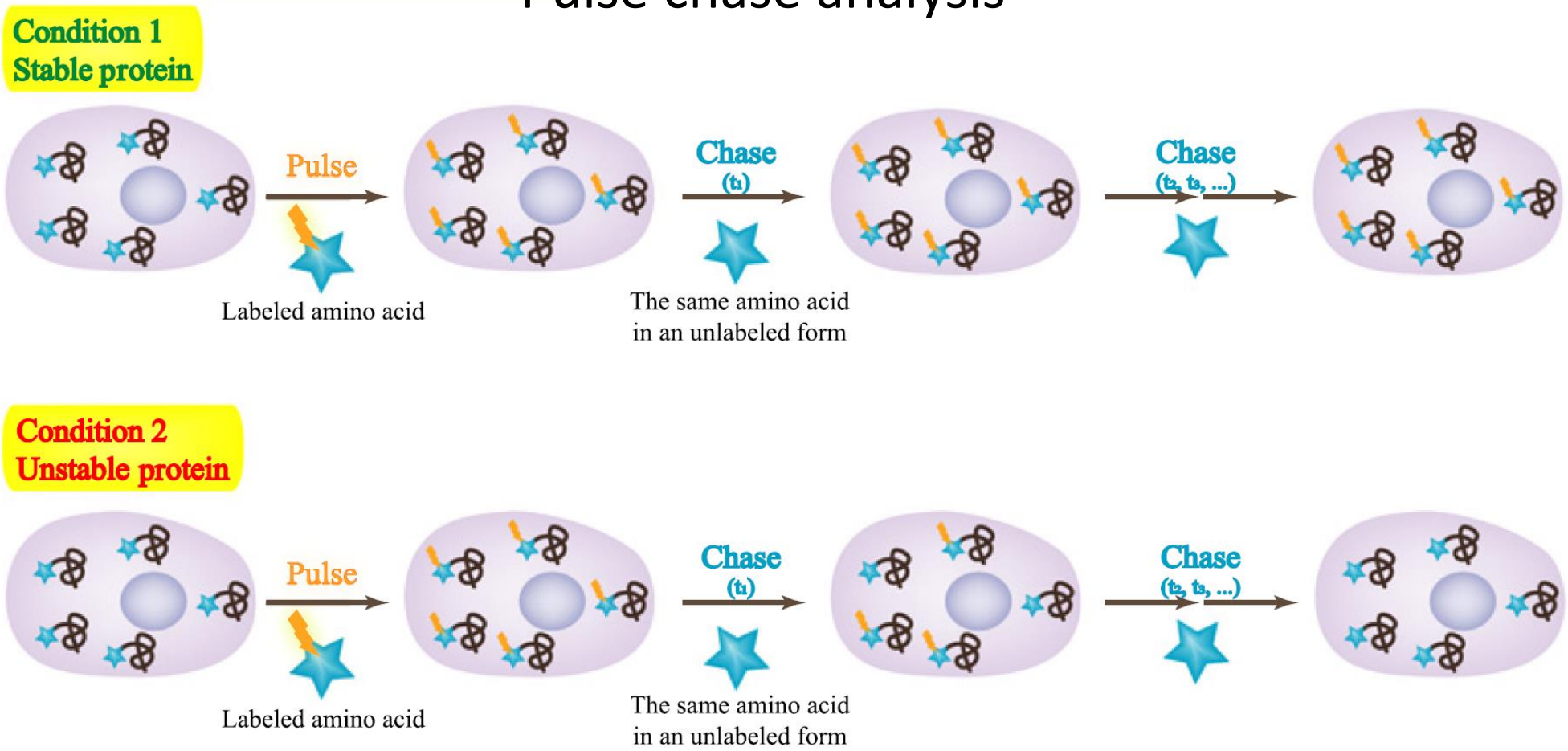


PROTEIN STABILITY ASSAYS

- pulse chase analysis
- cycloheximide chase analysis

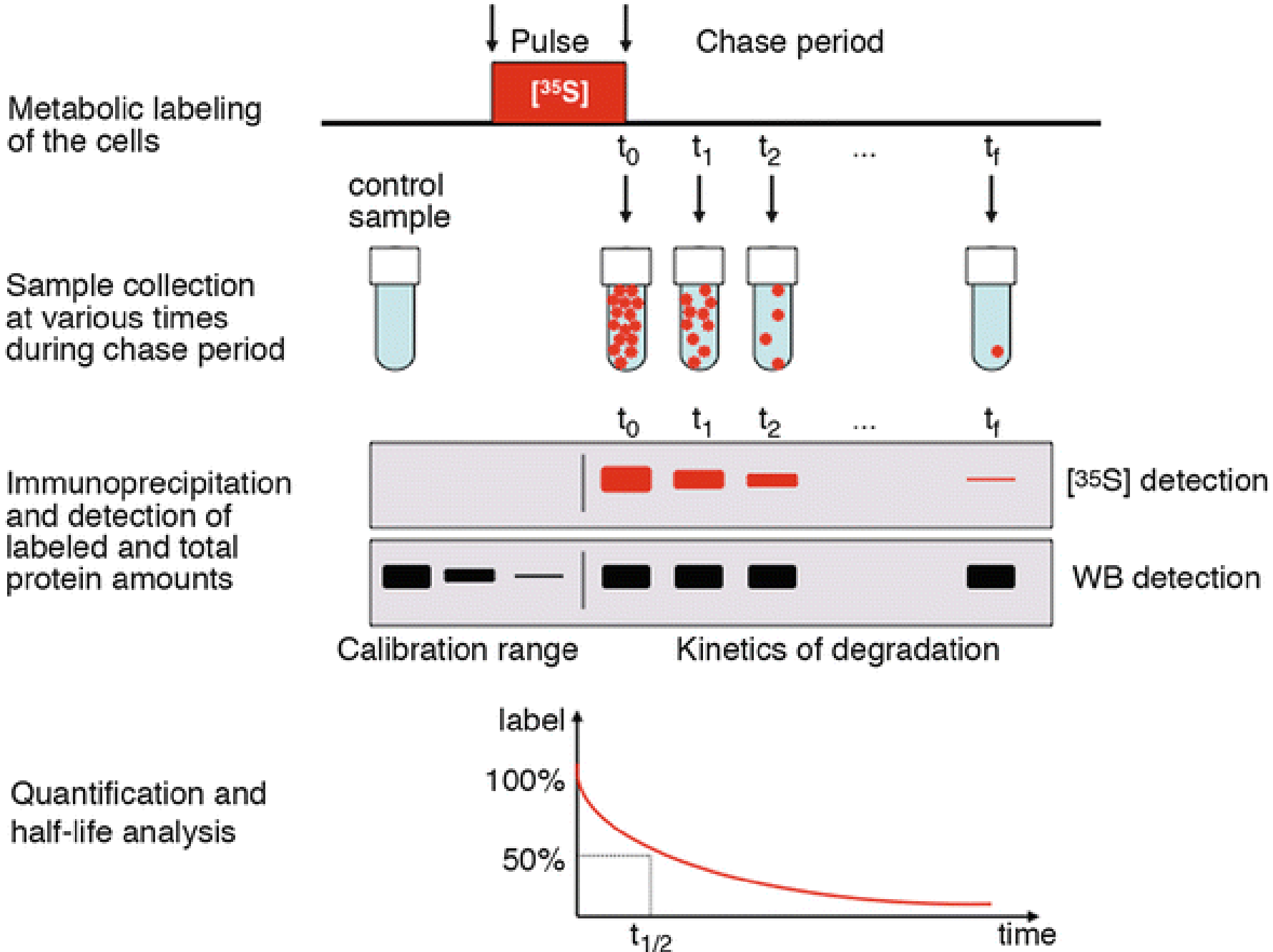
- Actinomycin D mRNA stability assay

Pulse chase analysis

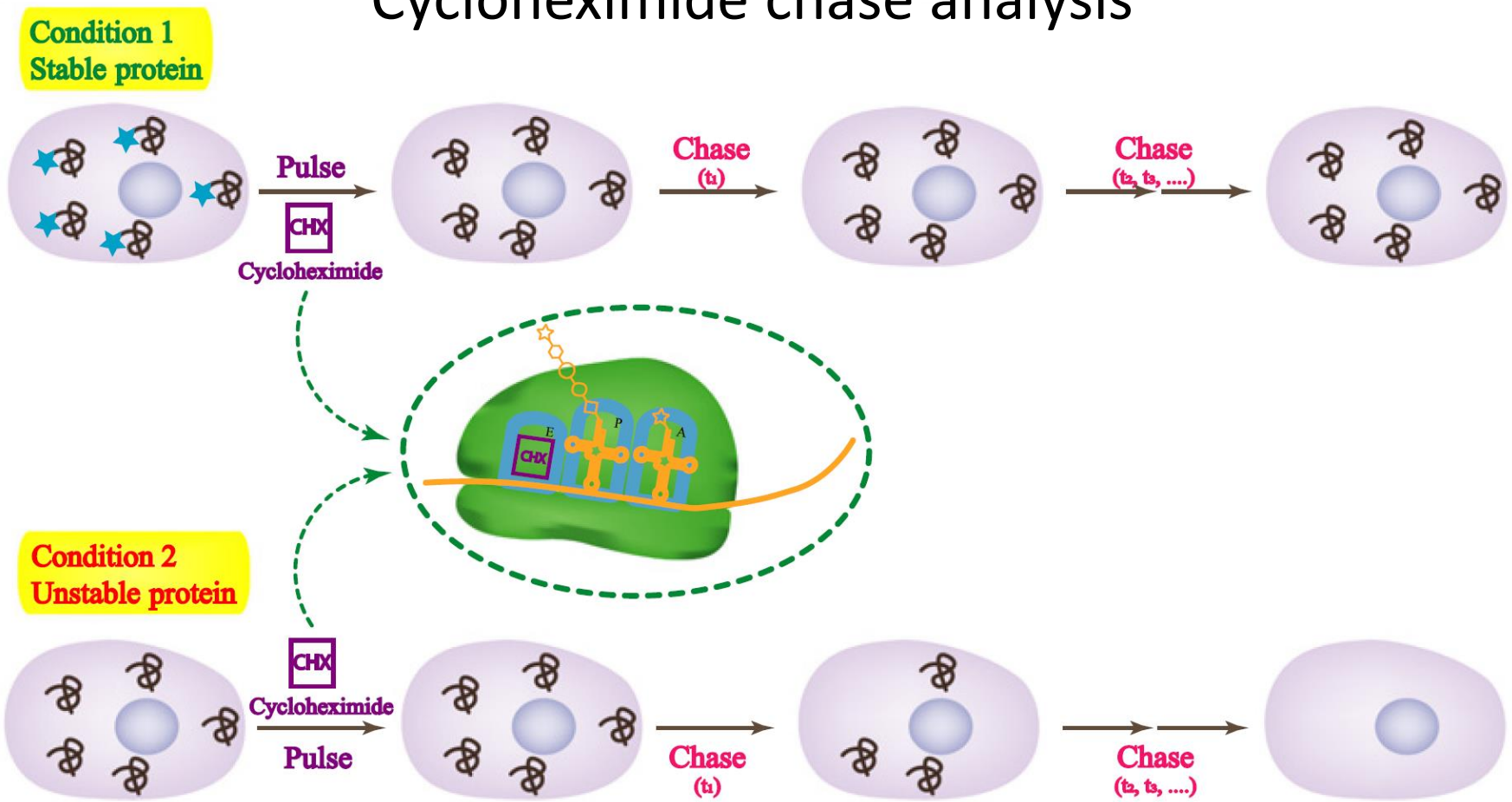


Pulse-chase analysis involves labelling of all proteins by culturing cells in the 'pulse phase' medium containing a radioactive labeled amino acid and subsequently following the proteins in 'chase phase' medium containing the nonradioactive labeled form of the amino acid; the rate of degradation is determined by comparing the levels of radioactivity of tested protein with respect to a control stable protein.

Pulse-chase analysis



Cycloheximide chase analysis



In **CHX-chase analysis**, the administration of a **global protein translation inhibitor** (such as cycloheximide, CHX) to block protein translation is performed (CHX-pulse phase).
This is followed by examining the reduction in the levels of a target protein 'chase' via SDS-PAGE and WB.
The generated degradation curves can be normalized through monitoring, in parallel, the abundance of a control stable endogenous protein (for instance, tubulin, or actin).

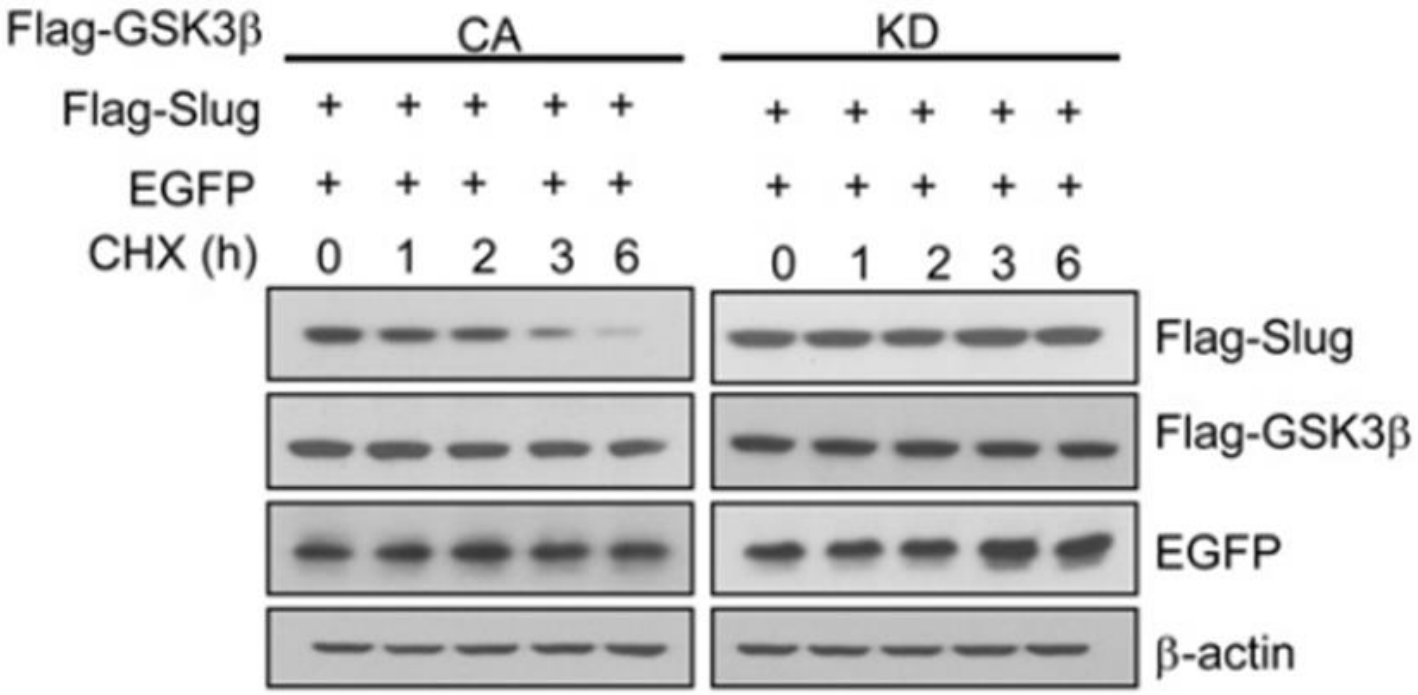
Cycloheximide

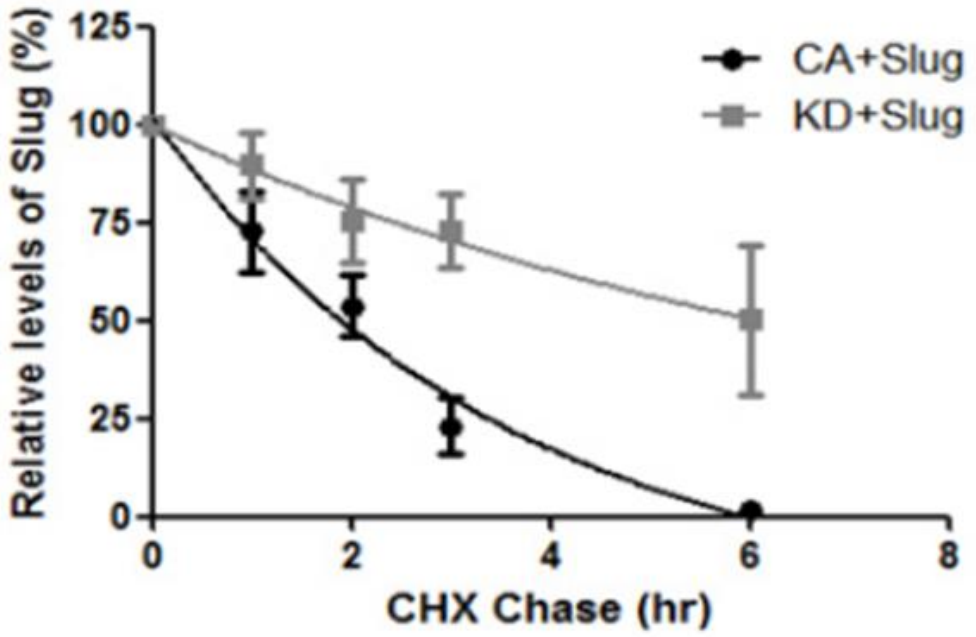
Comparison of protein stability in eukaryotic cells has been achieved by **cycloheximide**, which is an inhibitor of protein biosynthesis due to its prevention in translational elongation.

It is broadly used in cell biology in terms of determining the half-life of a given protein and has gained much popularity in cancer research.

It is suitable for analyzing **protein stability** over a relatively short time course (i.e. up to 24 hours). Over longer time courses **cycloheximide**, a global inhibitor of translation, is toxic to cells.

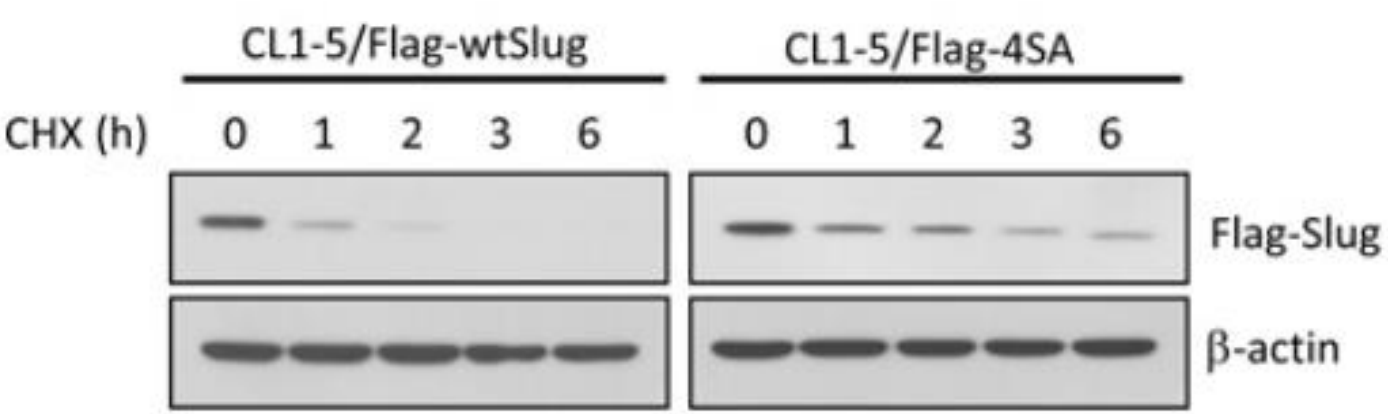
GSK3β (Glycogen synthase kinase 3 beta) phosphorylates Slug and modulates its protein stability

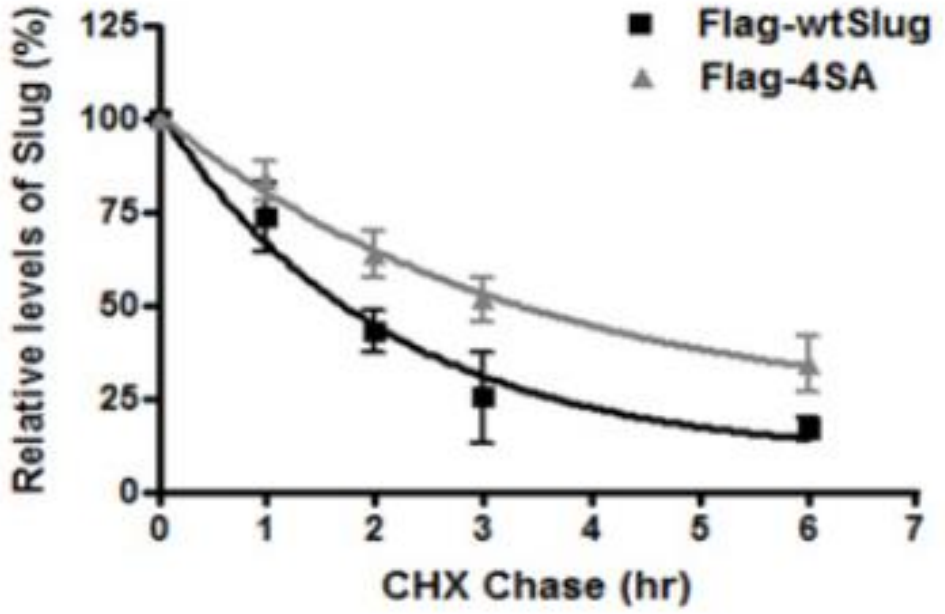




H1299 cells were transfected with the **Flag-Slug** construct together with the indicated **Flag-GSK3β**-expressing plasmids. A plasmid encoding EGFP was used as a negative control for transfection efficiency. Twenty-four hours after transfection, the cells were treated with 300 μg ml^{-1} cycloheximide (CHX). At the indicated time points, lysates were prepared and Western blotting analysis was performed with antibodies specific for the indicated proteins (left panel). The Slug band intensity was normalized to EGFP and then normalized to the t=0 controls (right panel).

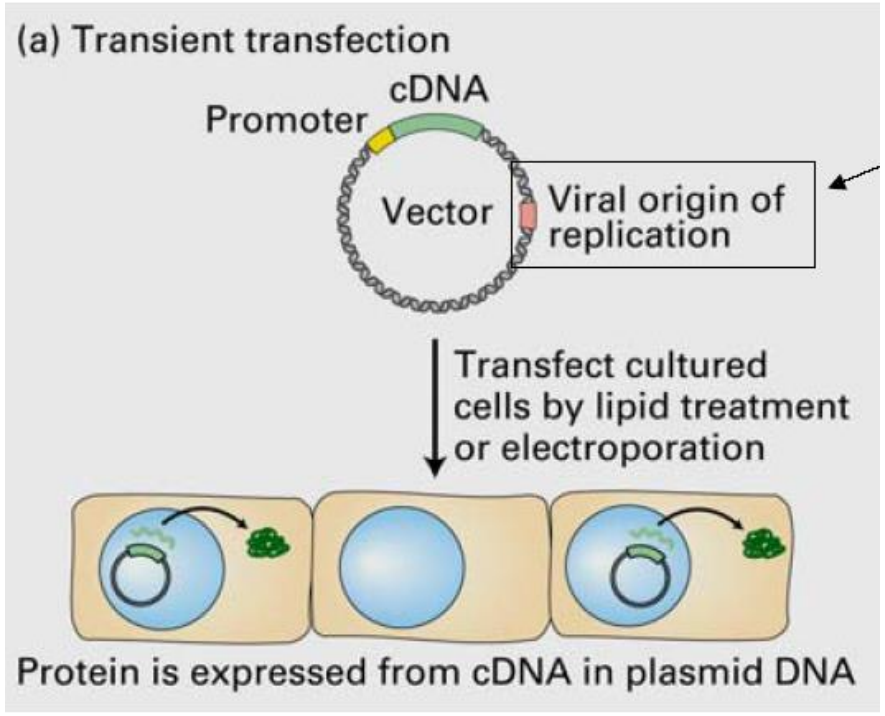
GSK3β phosphorylates Slug and modulates its protein stability

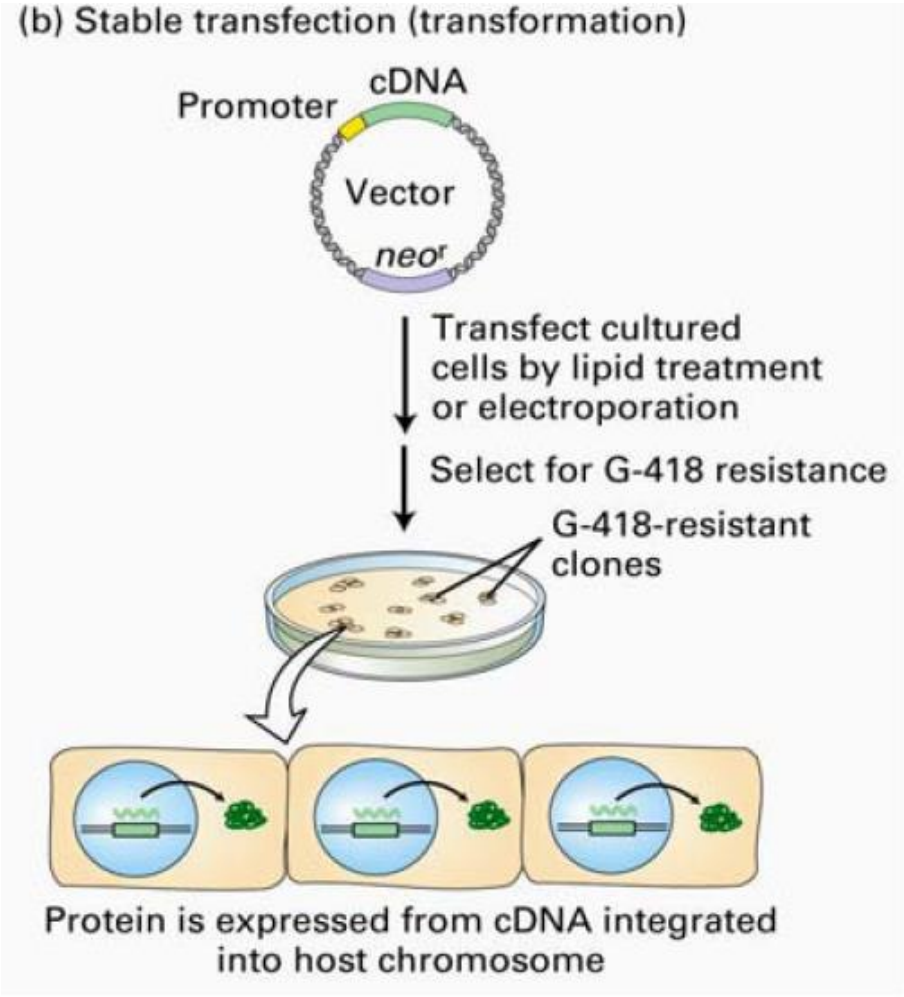




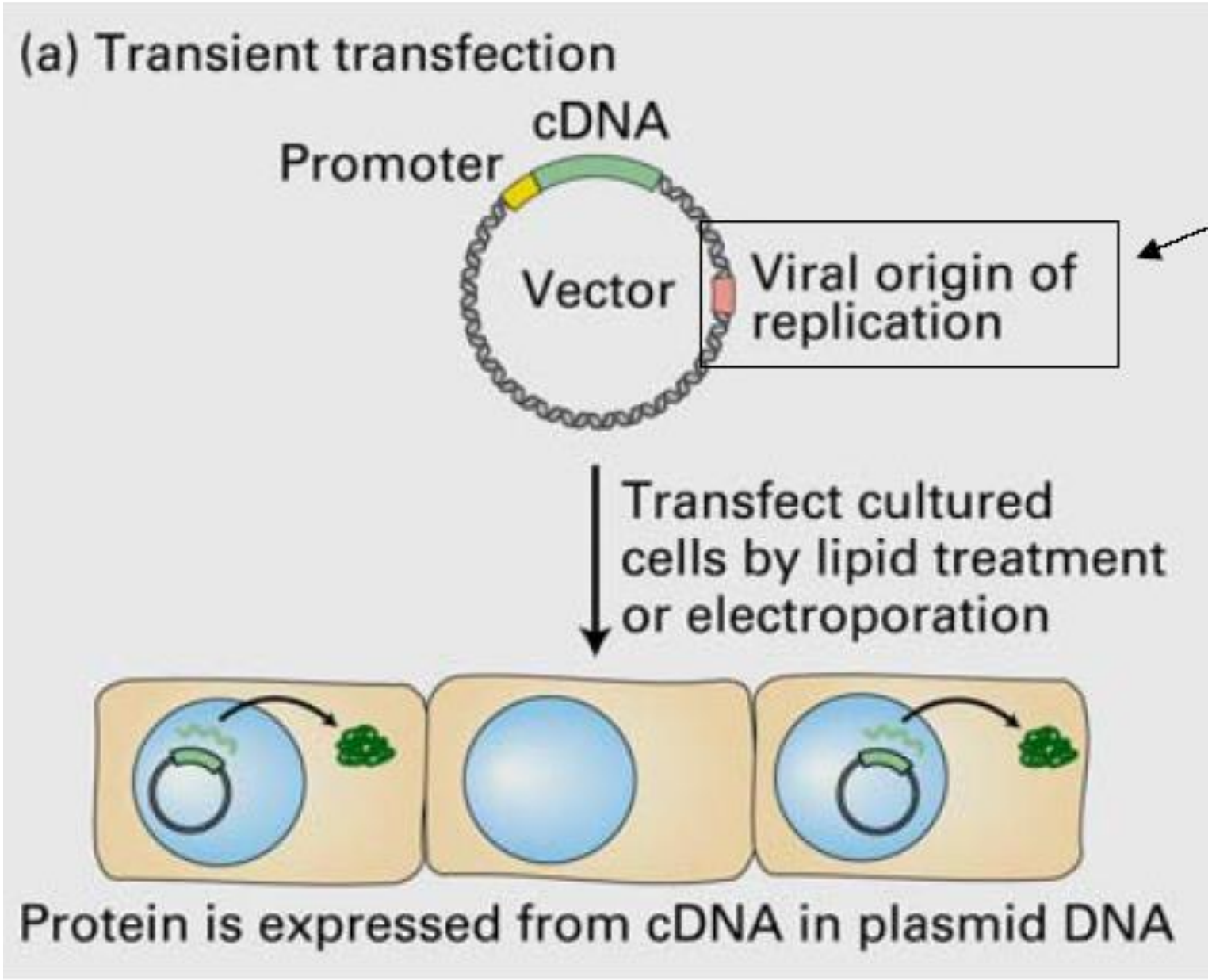
CL1-5 cells were stably transduced with viruses expressing Flag-Slug-WT or Flag-Slug-4SA for 24 h. Cycloheximide treatment and Western blotting (left panel) were conducted as in the previous panel. The Slug band intensity was normalized to actin and then normalized to the t=0 controls (right panel). Data are shown as mean ± s.e.m. in three different experiments (n=3).

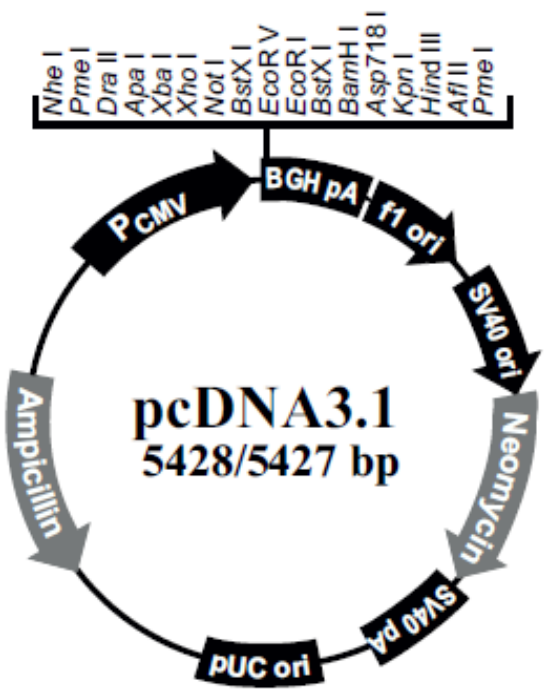
Transient versus Stable Cell Transfection

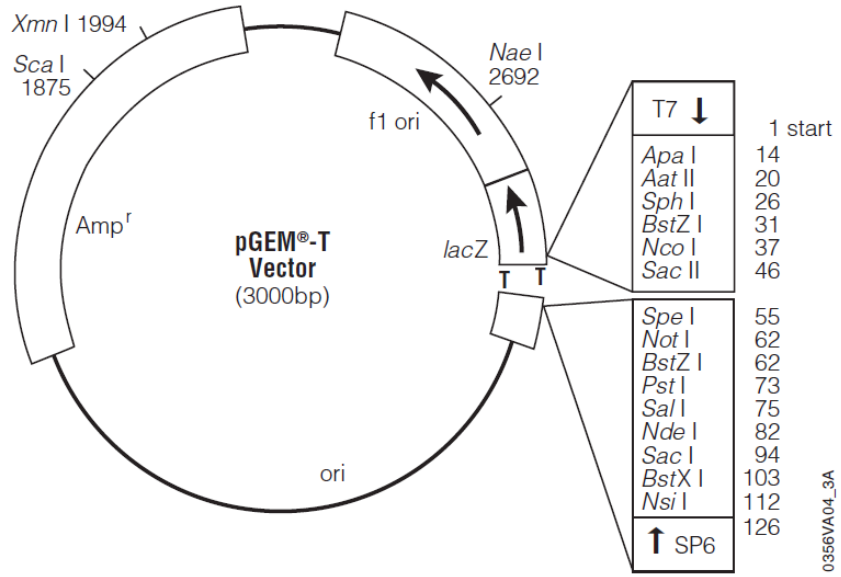


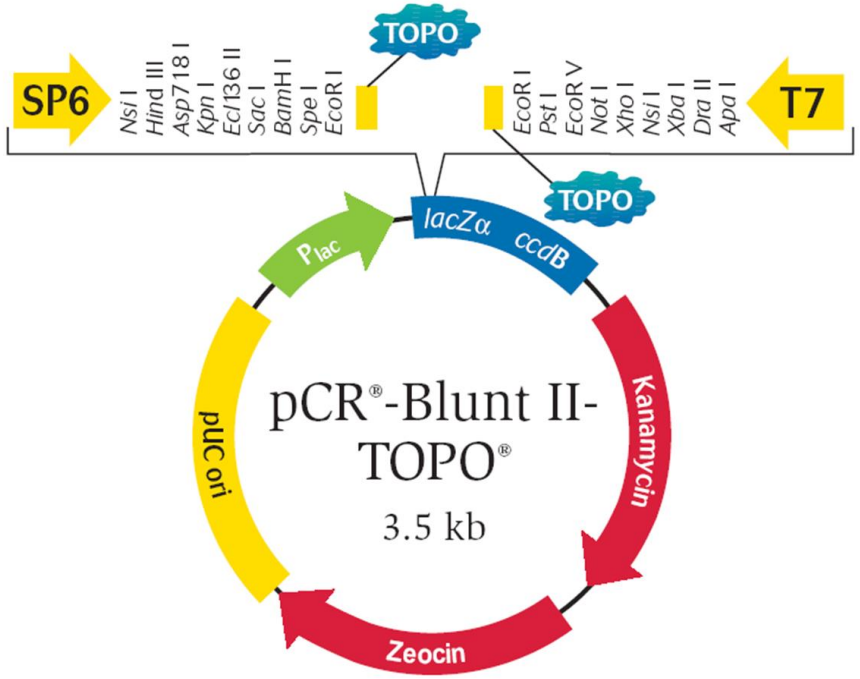


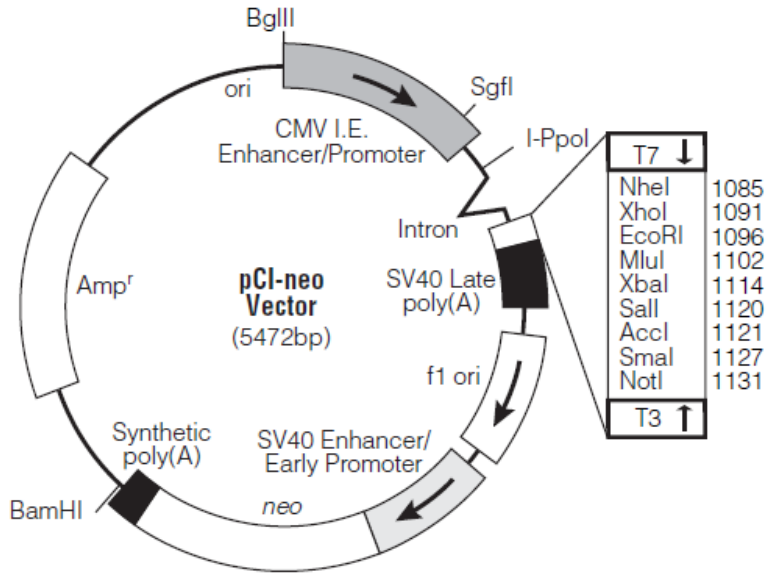
TRANSIENT TRANSFECTION of CELLS

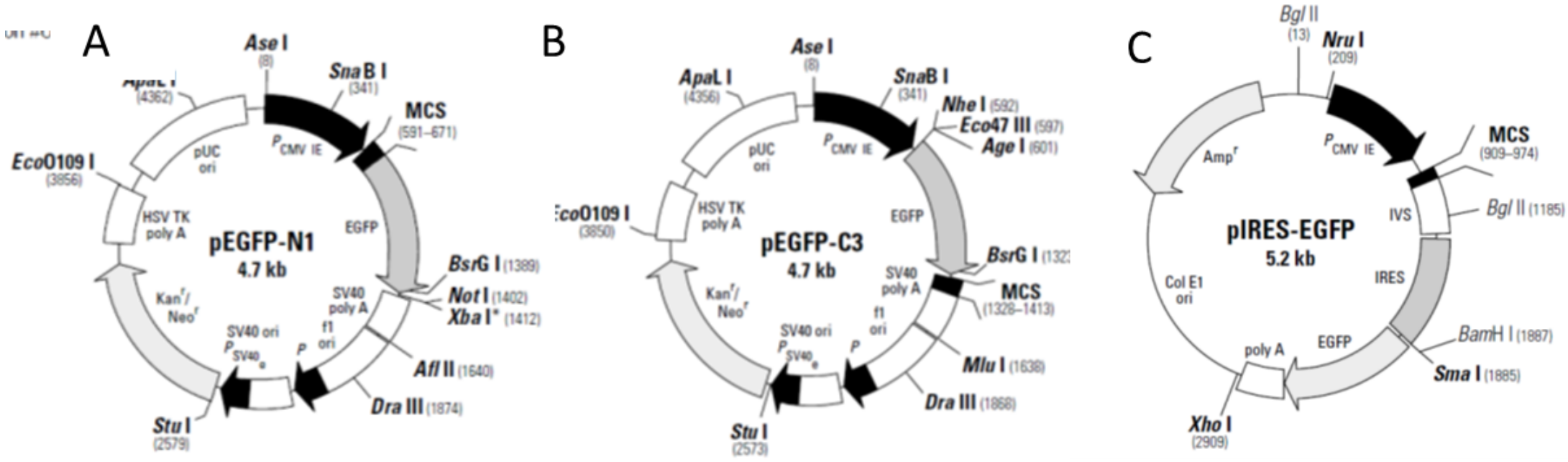












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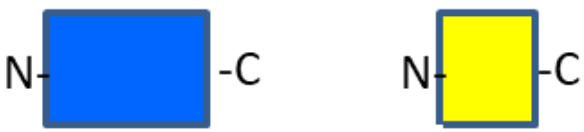
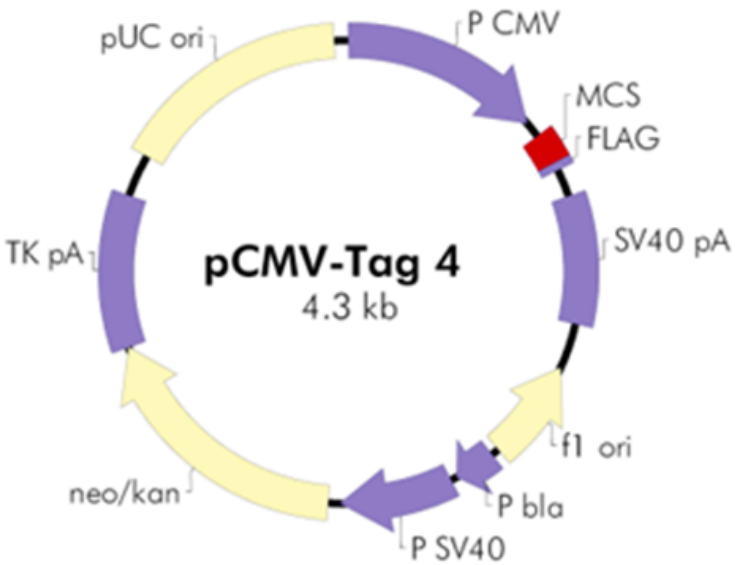
1-

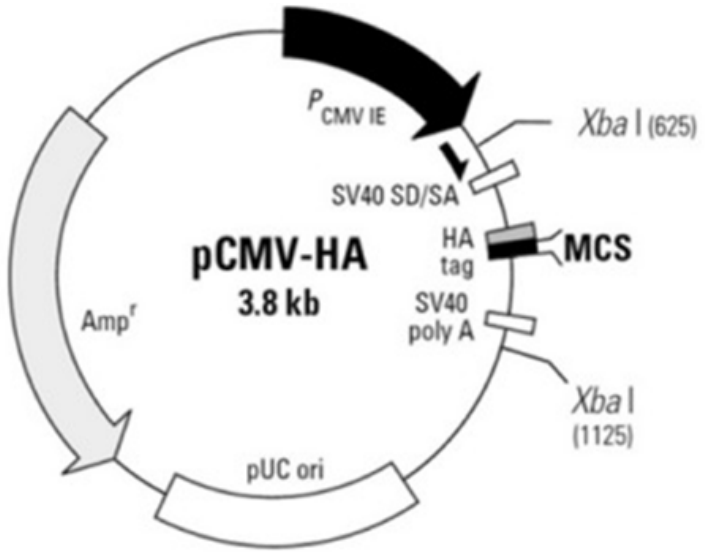


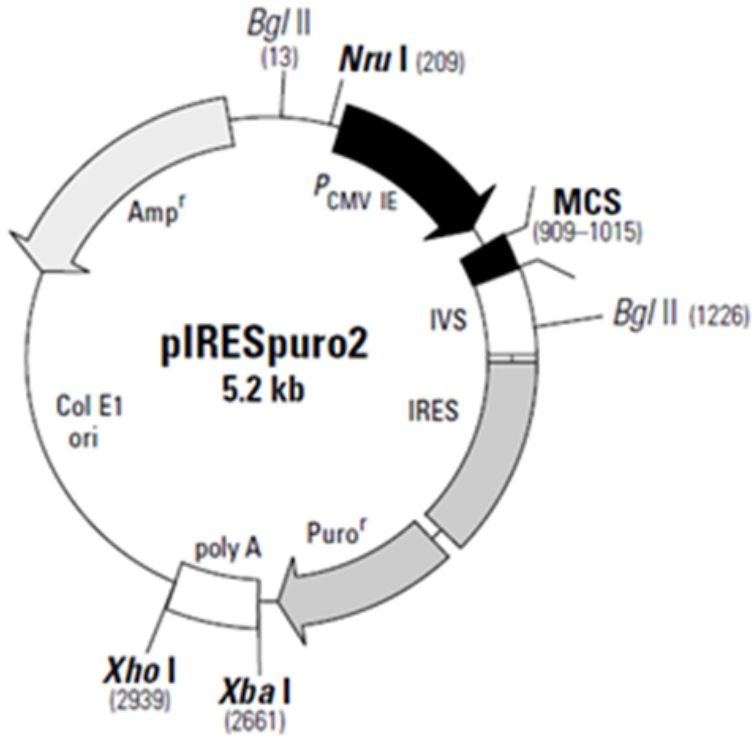
2-

PROTEIN OF INTEREST

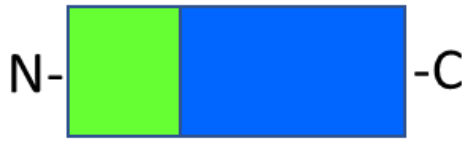
EGFP







PROTEIN OF INTEREST
FLAG



PROTEIN OF INTEREST
HA

PROTEIN OF INTEREST
PUROMYCIN RESISTANCE

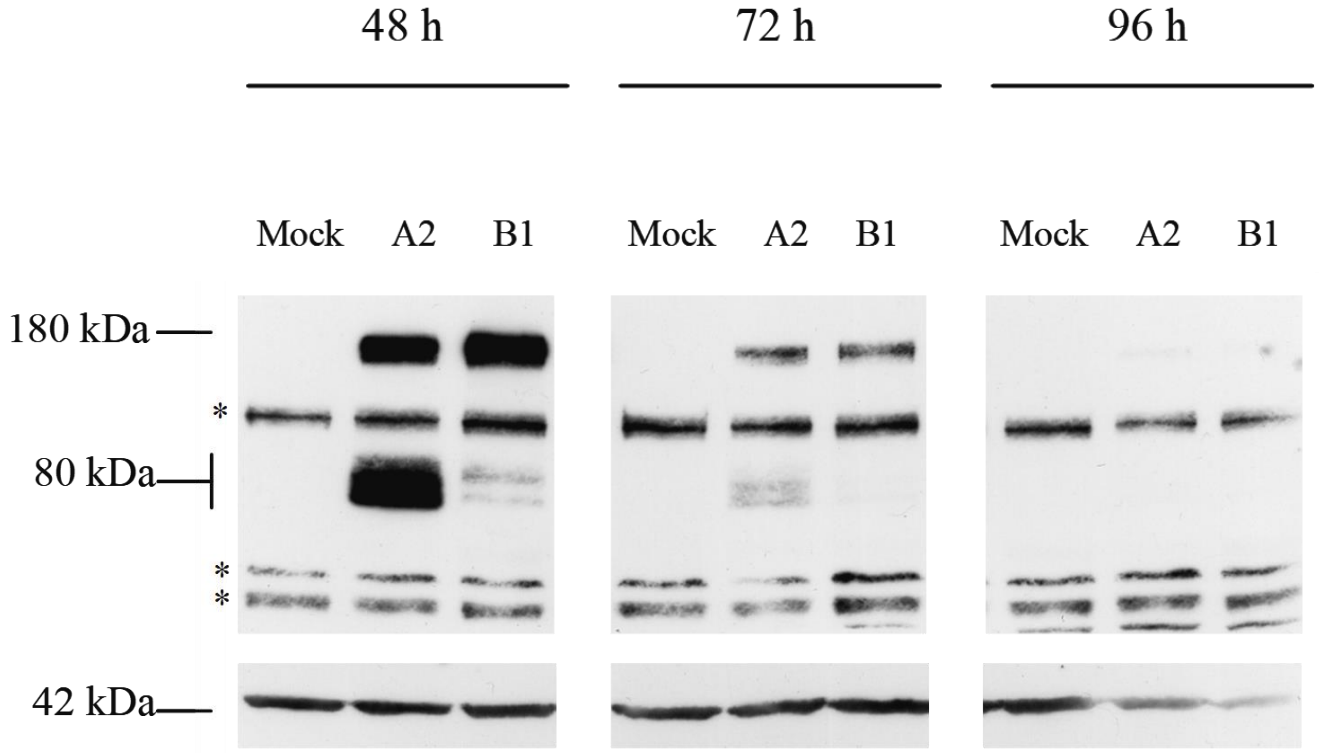
TRANSIENT TRANSFECTIONS

- transiently transfected cells express the foreign gene
- DNA does not integrate into genome
- the foreign gene will not be replicated
- these cells express the transiently transfected gene for a finite period of time, usually several days, then the foreign gene is lost through cell division

not interested in biological response	→	transient transfection of COS cells
interested in **short term** biological response	→	transient transfection of specific cells

TRANSIENT CELL TRANSFECTION

Total cell lysate



WB anti ErbB4

WB anti actin

What could be the “mock” control in a transfection?
When do you suggest to carry out the experiment?
(48, 72, or 96 hours after transfection?)

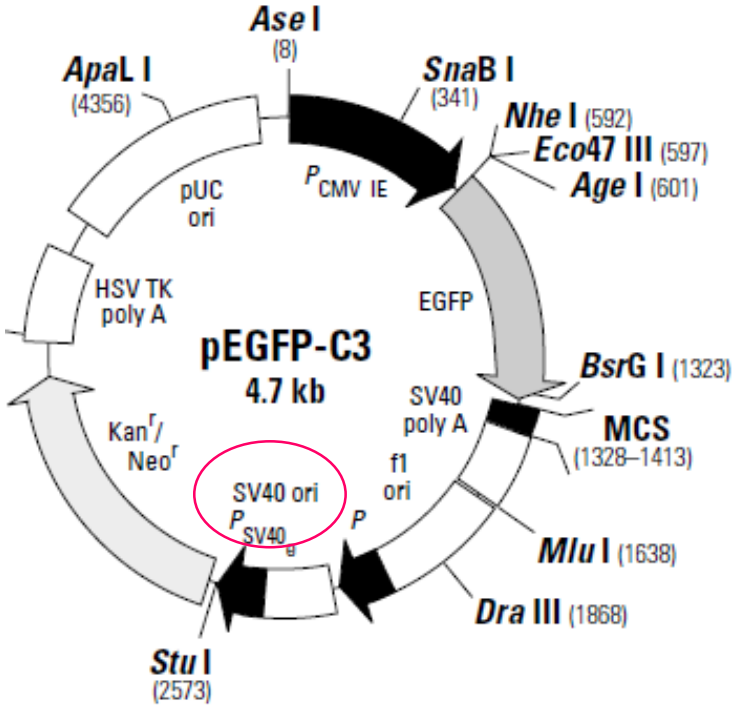
COS cells

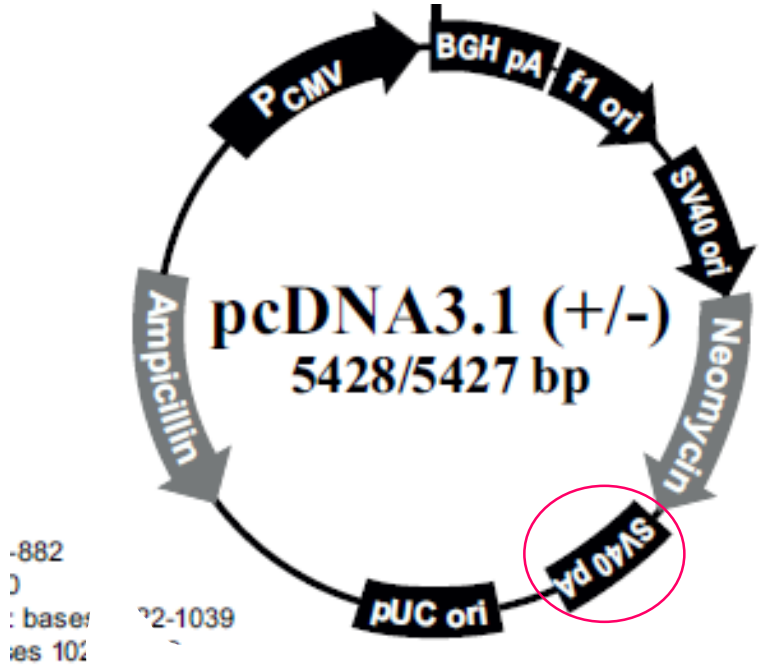
Organism	*Cercopithecus aethiops*
Tissue	kidney
Cell Type	CV-1 cell line was derived from the kidney of the African green monkey. COS cells are obtained by immortalizing CV-1 cells with a version of the **SV40 virus that can produce large T antigen** but has a defect in genomic replication.
Morphology	fibroblast –like cells

This is an African green monkey kidney fibroblast-like cell line **suitable for transfection by vectors requiring expression of SV40 T antigen**.
This line contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40°C, and supports the replication of pure populations of SV40 mutants with deletions in the early region. The acronym "COS" is derived from the cells being **C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic material. Two forms of COS cell lines commonly used are COS-1 and COS-7.

SV40 large T antigen (Simian Vacuolating Virus 40 TAg) is a hexamer protein that is a dominant-acting oncoprotein derived from the polyomavirus SV40.

SV40 large T-antigen is a product of an early gene transcribed during viral infection by SV40, and is involved in **viral genome replication** and regulation of host cell cycle.

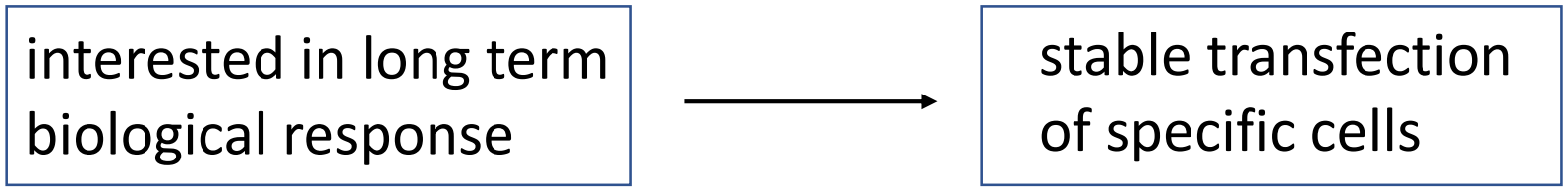




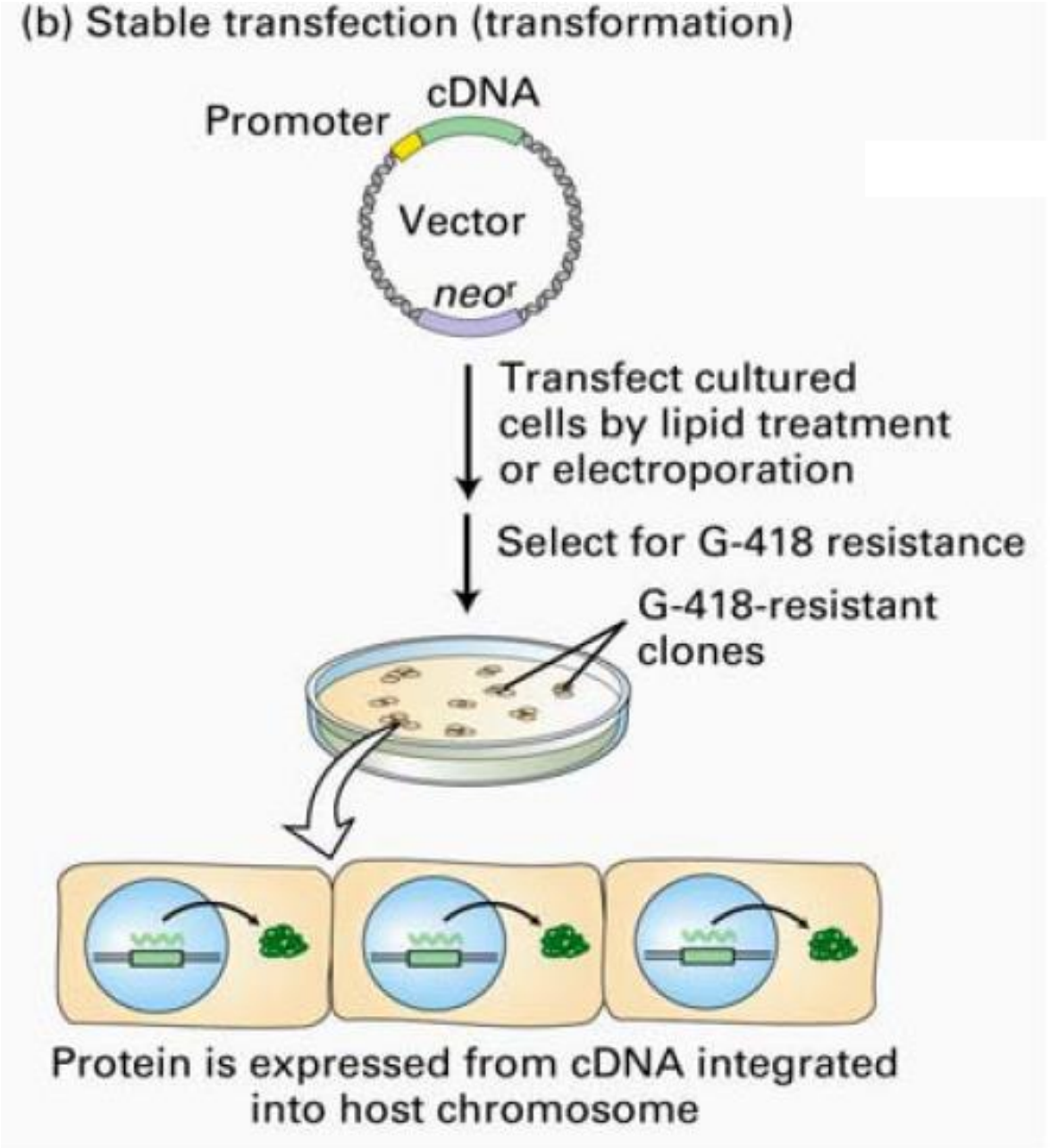
- **SV40 origin of replication** (**SV40 ori**) allows autonomous (as an **episome**) replication in mammalian cells expressing the **SV40 large T-antigen**, such as COS cells.

STABLE CELL TRANSFECTIONS

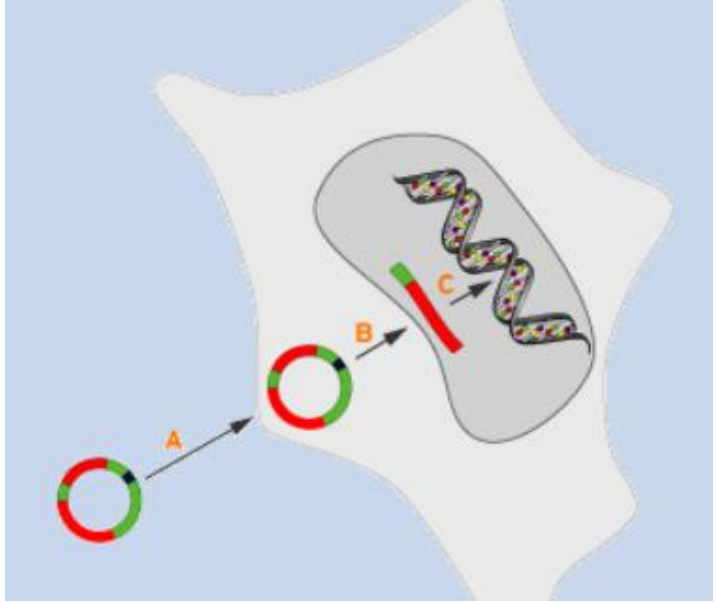
- stable transfection begins with a transient transfection
- in a very small proportion of transfected cells, the foreign gene is integrated into the cell genome and is therefore replicated
- descendants of these transfected cells will express the new gene, resulting in a **stably transfected cell line**
- a common selection method is to cotransfect the new gene with another gene for antibiotic resistance (such as the neomycin resistance gene, or puromycine resistance) and then treat the transiently transfected cells with the appropriate antibiotic for selection (such as geneticin or G418 or puromycin)
- only the stably transfected cells with resistance to the antibiotic will survive in longterm cultures, allowing for the selection and expansion of the desired cells.



STABLE CELL TRANSFECTION



STABLE TRANSFECTION of CELLS



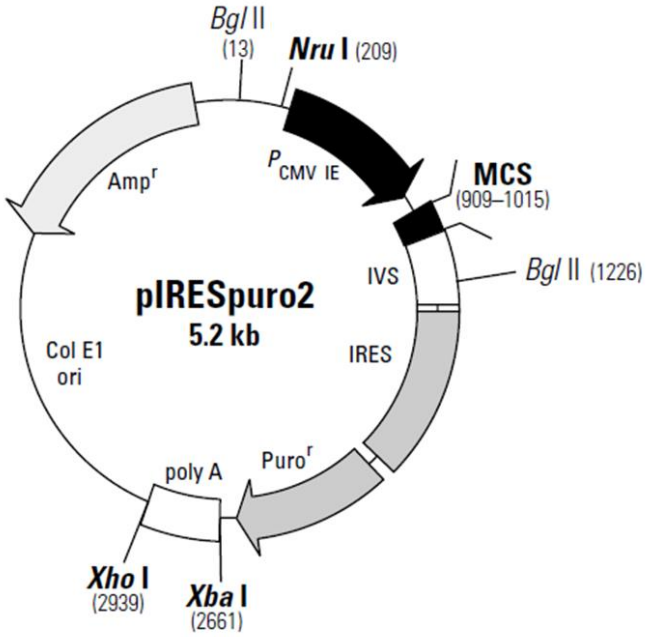
• when drug selection is used, cells are maintained in nonselective medium for 1–2 days post-transfection, then in selective medium containing the drug

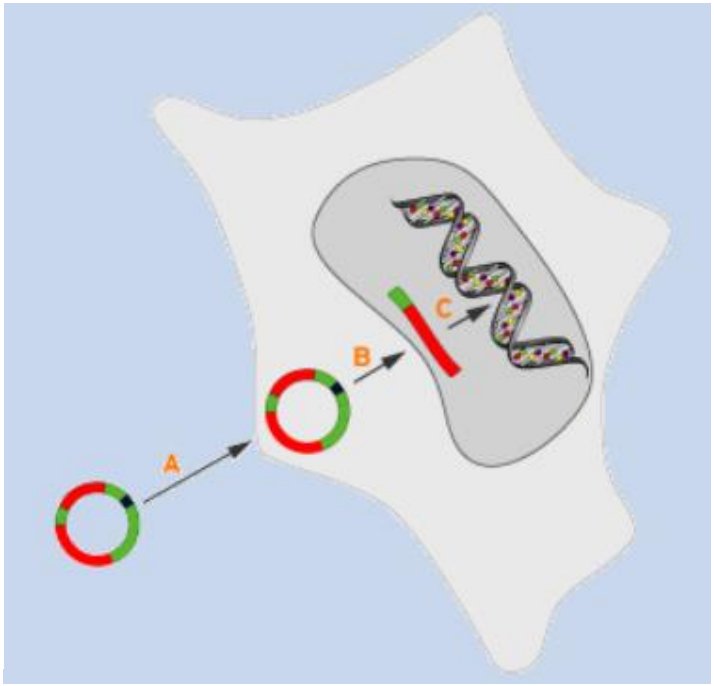
• the use of selective medium is continued for 2–3 weeks, with frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized

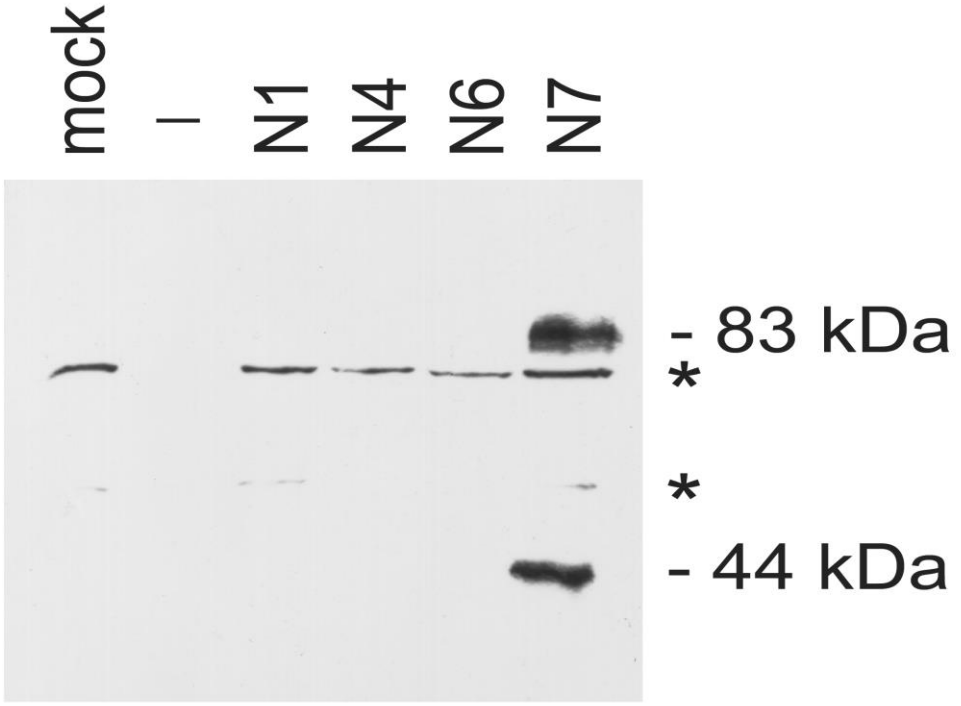
• individual colonies can be isolated by cloning cylinders, selected and transferred to multiwell plates for further propagation in the presence of selective medium

• individual cells that survive the drug treatment expand into clonal groups that can be individually propagated and characterized

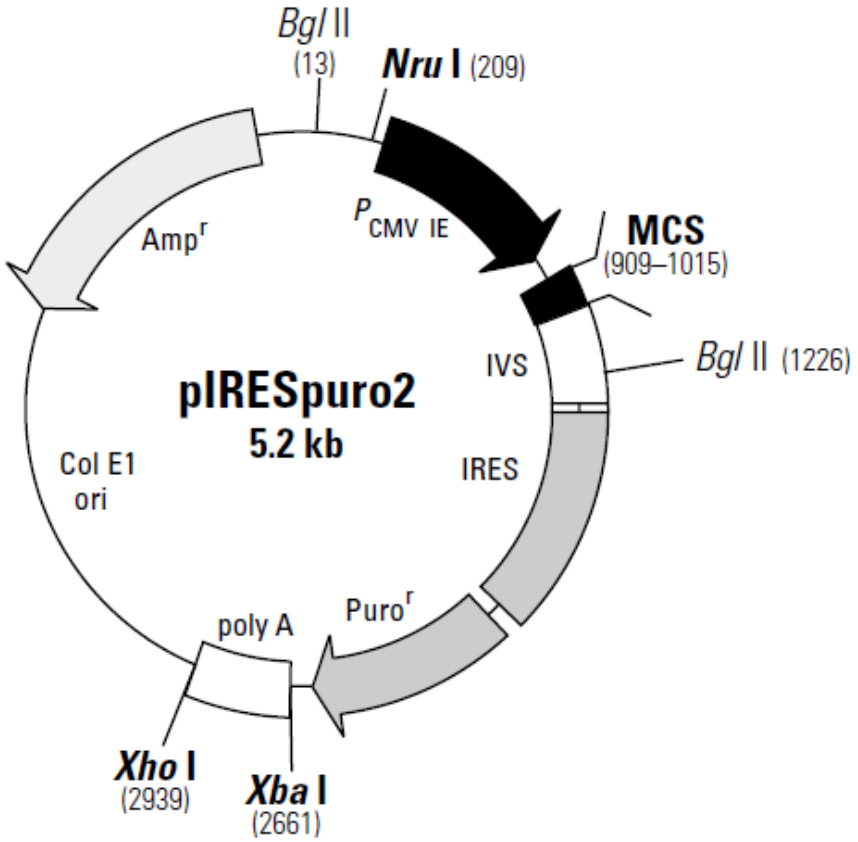
STABLE CELL TRANSFECTION

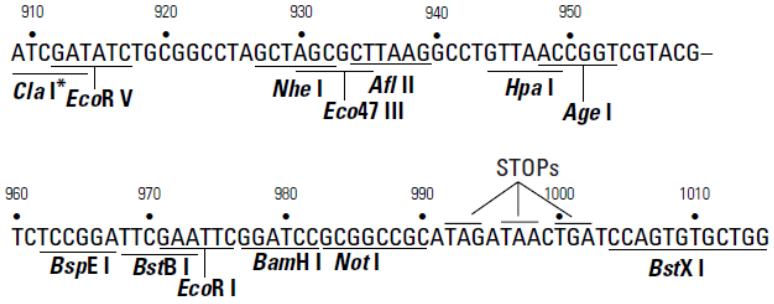






- why some stable clones do not express the exogenous protein?





• pIRESpuro2 contains **the internal ribosome entry site (IRES)** of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA.

• After **selection with puromycin**, nearly all surviving colonies will stably express the gene of interest, thus decreasing the need to screen large numbers of colonies to find functional clones.