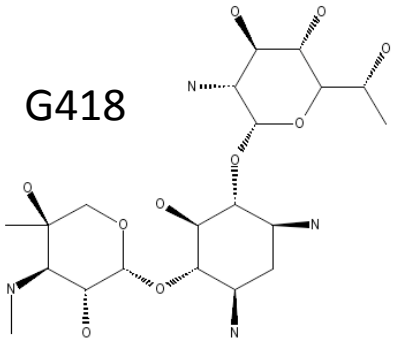
Transfection

Overview

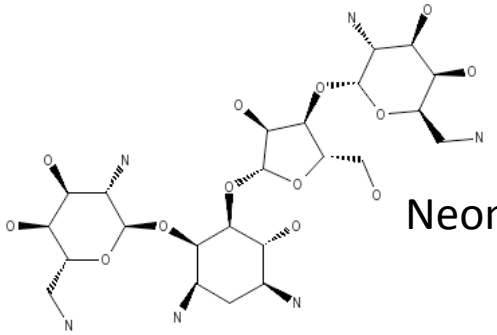
- Antibiotics
- Transfection reagents
- Cell specific kits
- Approaches to optimization

Antibiotics

G418



Neomycin



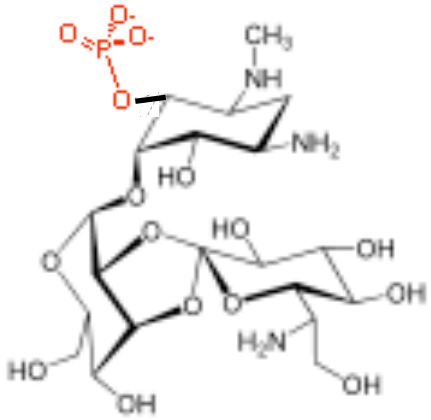
Neomycin

Mechanism of action	Aminoglycoside that specifically binds the 80S ribosome complex to inhibit protein synthesis
Resistance gene	*APH (3´)II* (from Tn601(903)) aminoglycoside phosphotransferase 3´ (II) *APH (3´)I* (from Tn5) aminoglycoside phosphotransferase 3fi(I)
Selection time	3-7 days for cells in log phase
Concentration	100 - 5,000 µg/mL G418

Q: Why not just use neomycin?

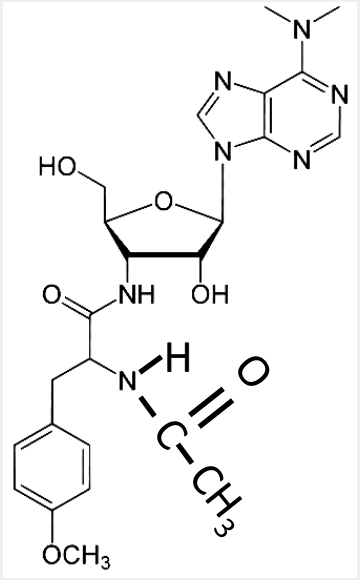
A: The neomycin resistance gene *aph* confers resistance to neomycin, kanamycin, and G418 (Geneticin) differently in eukaryotes and procaryotes. Bacteria without the gene are sensitive to neomycin, kanamycin and G418. Eukaryotes without the gene are resistant to kanamycin and neomycin but sensitive to G418. So, for selection of resistant eukaryotes you have to use G418, and for selection of prokaryotes you might use either neomycin or kanamycin which are cheaper.

Hygromycin



Mechanism of action	Inhibits protein synthesis at the translocation step on the 80S ribosome and causes misreading of the mRNA
Resistance gene	*hph*, encodes hygromycin-B-phosphotransferase.
Selection time	7-14 days
Concentration	100-1000 ug/ml

Puromycin

Mechanism of action 	An aminonucleoside antibiotic that inhibits protein translation. Puromycin has a structure similar to the tyrosinyl aminoacyl-tRNA. Thus, it binds to the ribosomal A site and participates in peptide bond formation, producing peptidyl-puromycin. However, it does not engage in translocation and quickly dissociates from the ribosome causing a premature termination of polypeptide synthesis.
Resistance gene	*Pac,* encodes a puromycin N-acetyl transferase. PAC inactivates puromycin by acetylating the amino position of its tyrosinyl moiety.
Selection time	2-7 d
Concentration	1-100 ug/ml

Zeocin

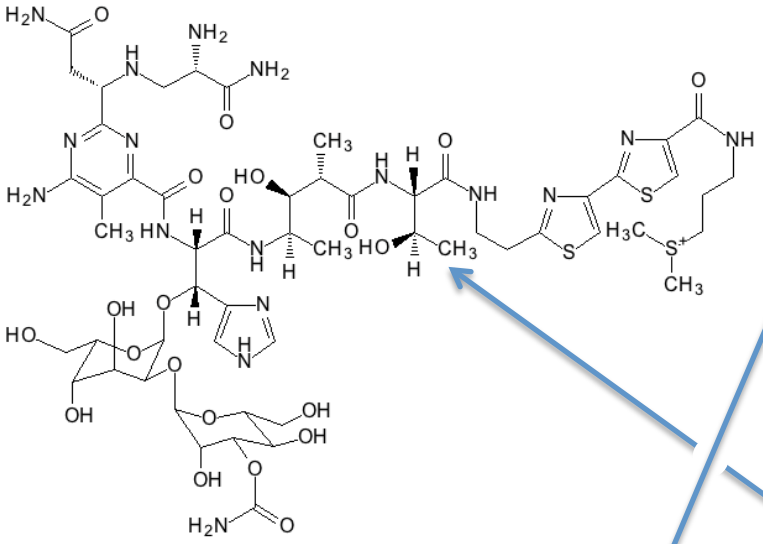
Mechanism of action	Zeocin is a formulation of phleomycin D1, a basic, water-soluble, copper- chelated glycopeptide. Zeocin bound to Cu^{2+} is inactive. When it enters cells it loses its Cu^{2+} becoming activated. Activated Zeocin then binds double-stranded DNA and inhibits DNA synthesis, resulting in cell death.
Resistance gene	*Sh ble* encodes a Zeocin binding protein that prevents Zeocin from binding and cleaving DNA.
Selection time	7-14 d
Concentration	50-1000 ug/ml

BLEOCIN

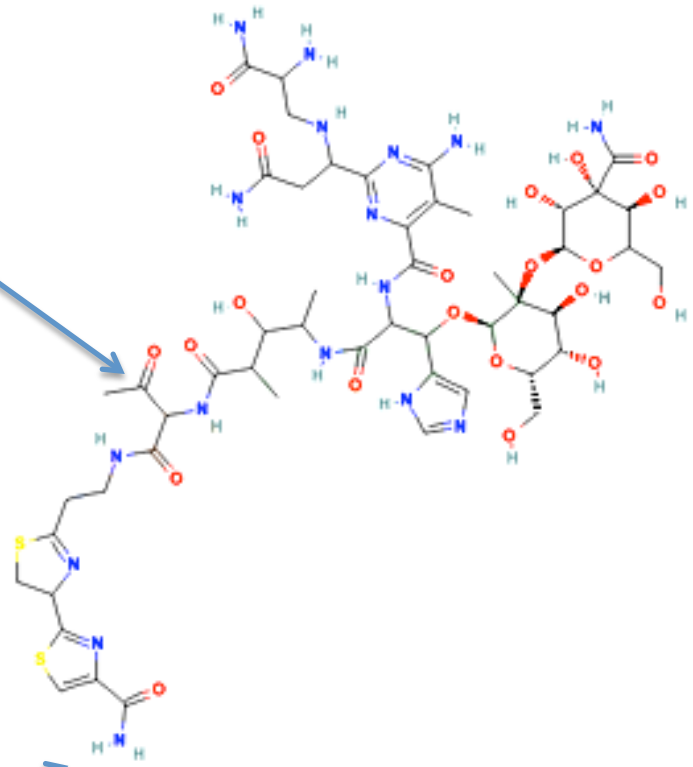
Mechanism of action	Acts by cleavage of double-stranded DNA, resulting in cell death. Unlike Zeocin, BLEOCIN is activated with bound to iron and oxygen (ferric peroxide). BLEOCIN is derived from bleomycin.
Resistance gene	*ble* I assume encodes a BLEOCIN binding protein that prevents BLEOCIN from binding and cleaving DNA
Selection time	5-15 d
Concentration	4-100 ug/ml

Said to be 8-fold more potent than Zeocin.

BLEOCIN is derived from bleomycin:



Zeocin is derived from phleomycin:



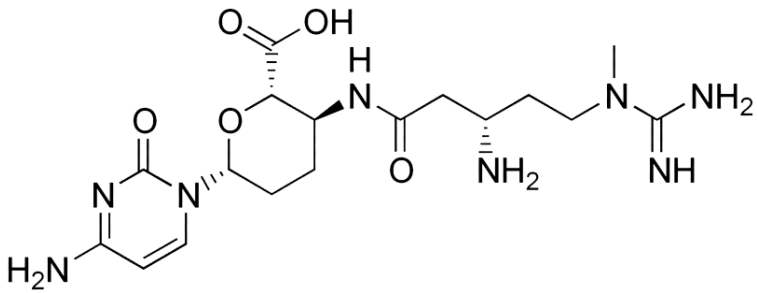
Blasticidin S

Mechanism of action	Nucleoside antibiotic that inhibits protein synthesis
Resistance gene	*bsd* or *bsr*, blasticidin S deaminase
Selection time	10-14 d
Concentration	2-20 ug/ml

Some blasticidin vectors:

InvivoGen
- pBLAST, pUNO, pDUO, pMOD-bsr

Invitrogen
- pcDNA6, pIB



Other things to think about...

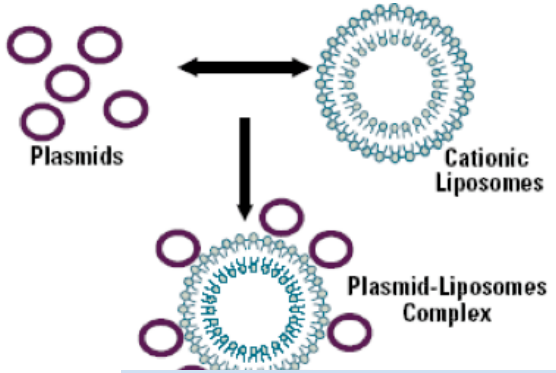
- Keep cells in log phase
  Cells will die faster if not overcrowded
- Prepare antibiotics fresh
  Just because G418 and hygromycin are stored in a liquid form at 4C, don't make 500 ml of G418 or hygromycin media and use for 2 months because its shelf life in media is not the same as the shelf life of the concentrated solution in storage buffer.
- Mix antibiotics before plating
  Don't plate cells then add antibiotic later because that may create transient high local concentration that stresses cells
- Don't use too much antibiotic
  Some antibiotics like G418 are acidic, so using too much G418 will cause cell death of resistant cells.

Transfection Reagents

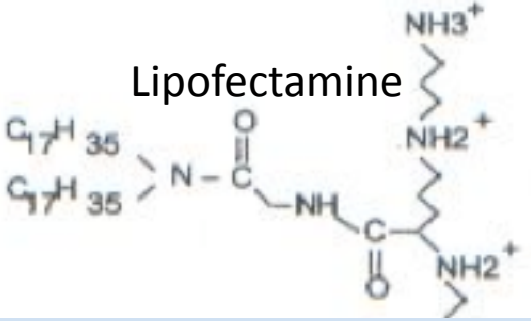
Classes of transfection reagents

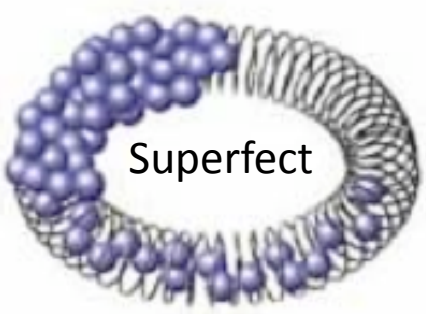
- Liposomal
- Non liposomal
  - Calcium Phosphate
  - “other non-liposomal”
  - Polyethylenimine
- Electroporation
- Magnetic Assisted Transfection
- Sonoporation

Liposomal concepts



Lipofectamine





Positively charged liposomal-DNA complexes interact with negatively charged proteoglycans on the cell surface and enter the cell via endocytosis. The product should create a liposomal-DNA particle with an ideal density that will be heavy enough to settle onto the cells but will be small enough for efficient cell entry.

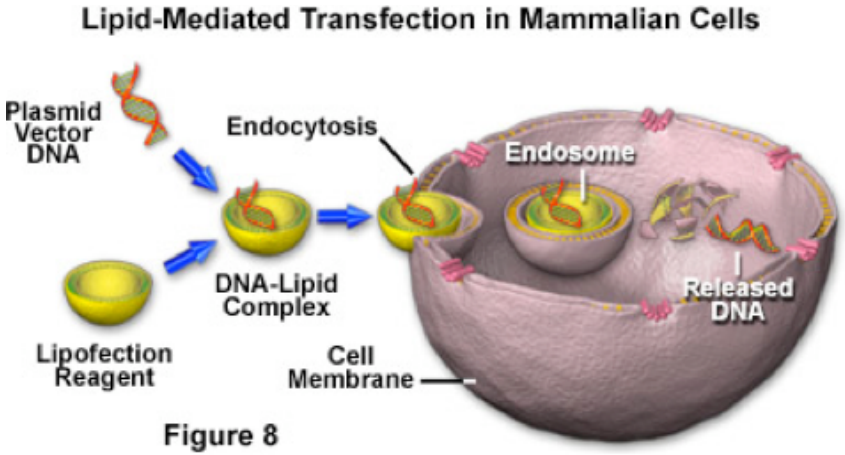
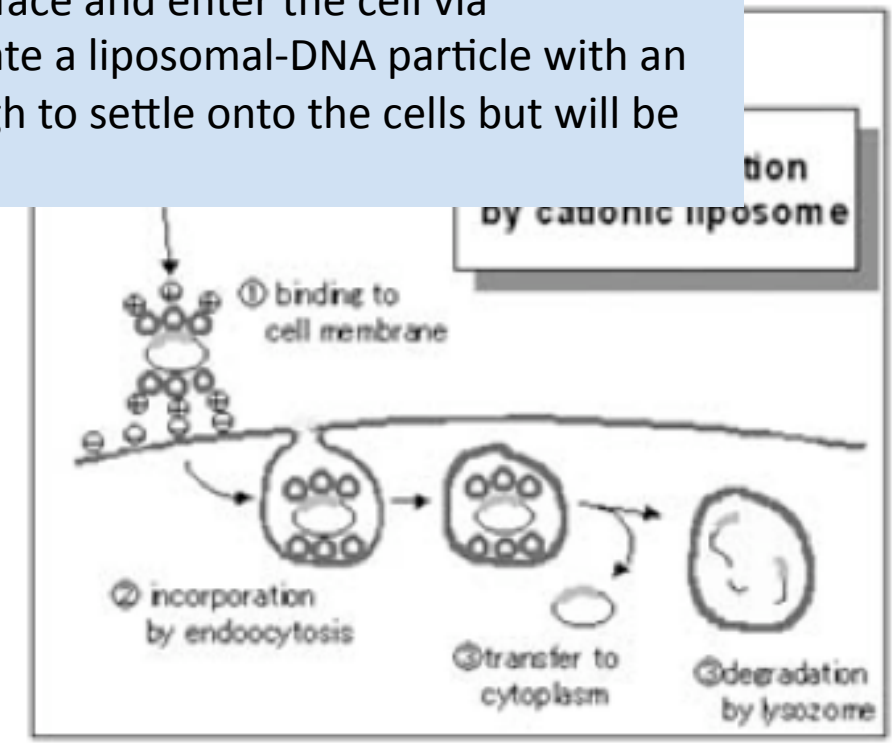


Figure 8



Liposomal

34 products

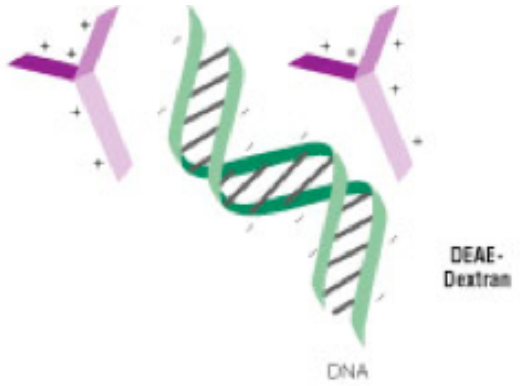
SureFECTOR	B-Bridge International
UniFector	B-Bridge International
PlasFect	Bioline
Nupherin	BIOMOL
Metafectene	Biontex
TriFECTin	Integrated DNA Technologies
Lipofectamine 2000 CD	Invitrogen
Lipofectamine	Invitrogen
Lipofectamine LTX	Invitrogen
Optifect	Invitrogen
PLUS	Invitrogen
LyoVec	InvivoGen
HiFect	Lonza Cologne AG
N-Blast	Neuromics
N-Fect	Neuromics
N-Fect Neuro	Neuromics
P-Fect	Neuromics
TransPass D1	New England Biolabs
EcoTransfect	OZ Biosciences
DreamFect	OZ Biosciences
Tfx	Promega
TransFast	Promega
Transfectam	Promega
Superfect	Qiagen
DOSPER	Roche
DOTAP	Sigma-Aldrich
ESCORT I-IV	Sigma-Aldrich
CalFectin	SignaGen Laboratories
GenJet	SignaGen Laboratories
LipoD293	SignaGen Laboratories
LipoJet	SignaGen Laboratories
PolyJet	SignaGen Laboratories
Gene Transfer	Wako Chemicals USA, Inc.
HMG-1,2 Mixture	Wako Chemicals USA, Inc.

17 vendors

Biocompare's list

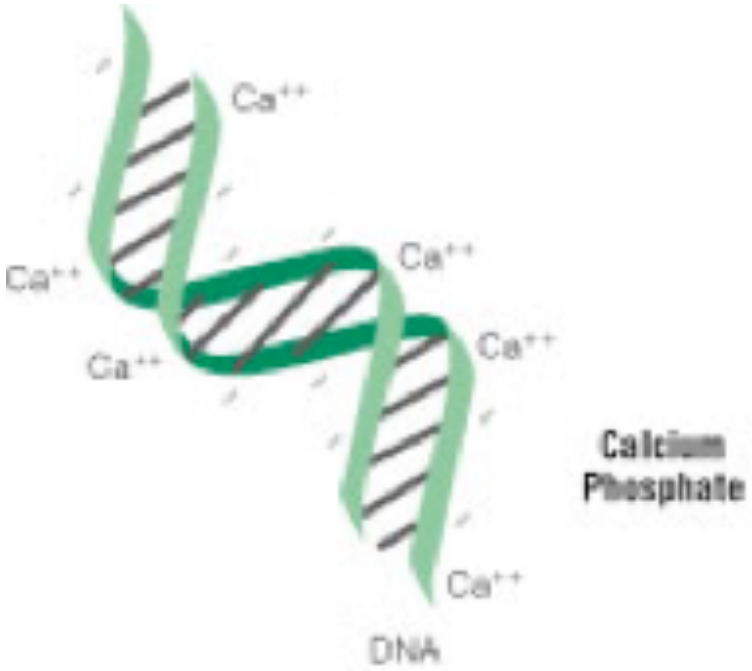
Non-Liposomal Kits

DNotion	5 Prime
GeneJammer	Agilent
SatisFection	Agilent
Transfection MBS	Agilent
CellPhect	GE
Polybrene Infection	Millipore
Transient Expression	Millipore
TransIT	Mirus Bio Corporation
TransPass	New England Biolabs
Fecturin	Polyplus
jetPRIME	Polyplus
ProFection-DEAE Dextran	Promega
FuGene Non-liposomal	Roche
DEAE-Dextran	Sigma-Aldrich



Calcium Phosphate Kits

Mammalian Transfection Kit	Agilent
Calcium Phosphate Transfection Kit	Invitrogen
Mammalian Cell Transfection Kit	Millipore
ProFection	Promega
Calcium Phosphate Transfection Kit	Sigma-Aldrich



HEPES-buffered saline solution (HeBS) containing phosphate ions is combined with a calcium chloride solution containing the DNA to be transfected. When the two are combined, a fine precipitate of the positively charged calcium and the negatively charged phosphate will form, binding the DNA to be transfected on its surface. The suspension of the precipitate is then added to the cells to be transfected (usually a cell culture grown in a monolayer). The cells take up some of the precipitate, and with it, the DNA.

Polyethylenimine (PEI)

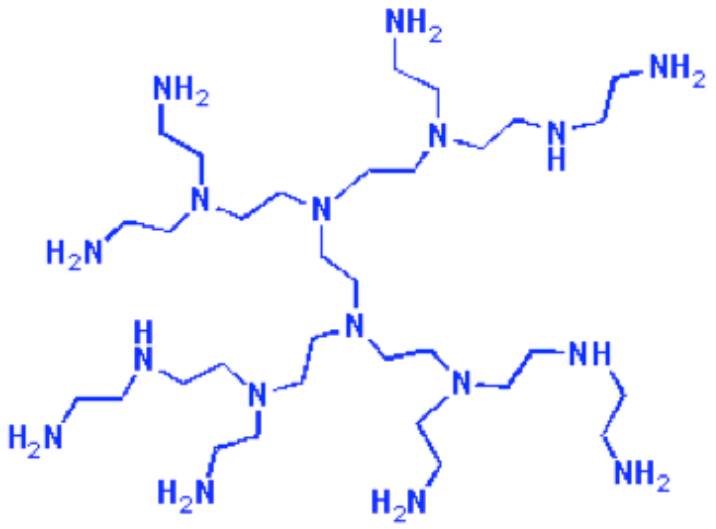
Polyethylenimine-Transferrinfection	Bender MedSystems
jetPRIME	Polyplus
jetPEI	Polyplus
Polyethylenimine MAX	Polysciences, Inc.

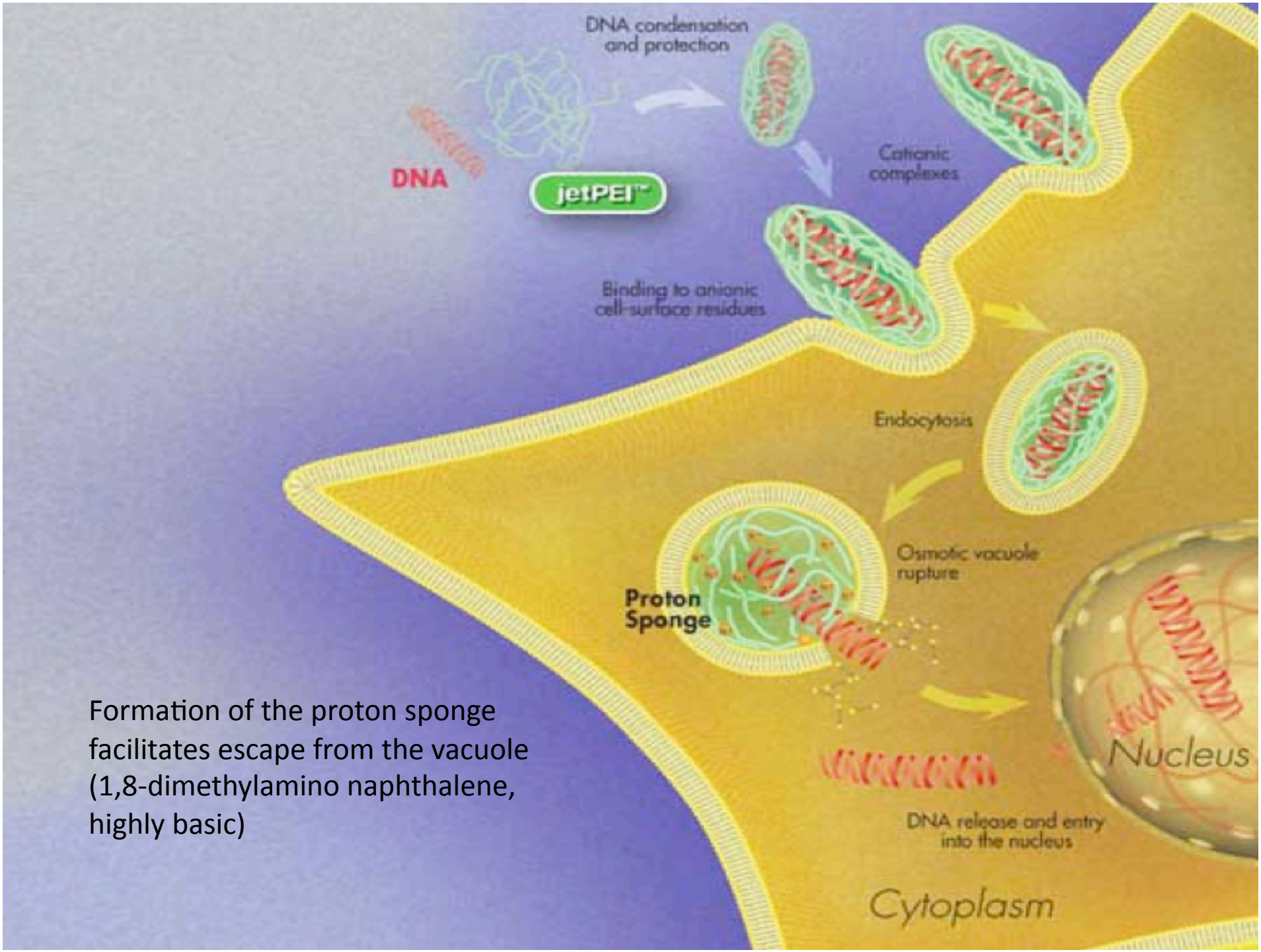
Enhanced endosomal escape

jetPEI is linear PEI which facilitates nuclear translocation (branched PEIs or liposomal complexes do not).

These are useful for delivery of siRNAs in vivo by tail vein injection.

Hyperbranched Polyethylenimine





Formation of the proton sponge facilitates escape from the vacuole (1,8-dimethylamino naphthalene, highly basic)

Cell line specific kits

HEK293		
	TransIT-293	Mirus Bio Corporation
	293fectin	Invitrogen
	TransPass COS/293	New England Biolabs
Neuro2a		
	GeneJet Neuro2a	SignaGen Laboratories
COS		
	TransIT-COS	Mirus Bio Corporation
	GenJet COS	SignaGen Laboratories
	TransPass COS/293	New England Biolabs

The following two are not specific kits but are references made by the vendors to how the reagents are optimized for these cell lines.

PC12		
	GenPORTER 2	Genlantis
SH-SY5Y		
	siLentFect Lipid Reagent for RNAi	Bio-Rad

Magnetic Assisted Transfection

This method associates DNA with magnetic nanoparticles. The resulting molecular complexes are then transported into cells supported by an appropriate magnetic field.

Said to expose 100% of cells to a dose of vector.

Sources:	Magnetofection	OZ Biosciences
	Matra-A Reagent	Neuromatics
	MATra-A Reagent	IBA GmbH
	MA Lipofection Enhancer	IBA GmbH

Nucleofector Technology

Also known as Nucleofection

Sold by Lonza as many many different fairly expensive kits specific for the cell type, such as spp. specfic astrocytes, neurons, fibroblasts, epithelial, etc.
Requires use of the “Nucleofector”

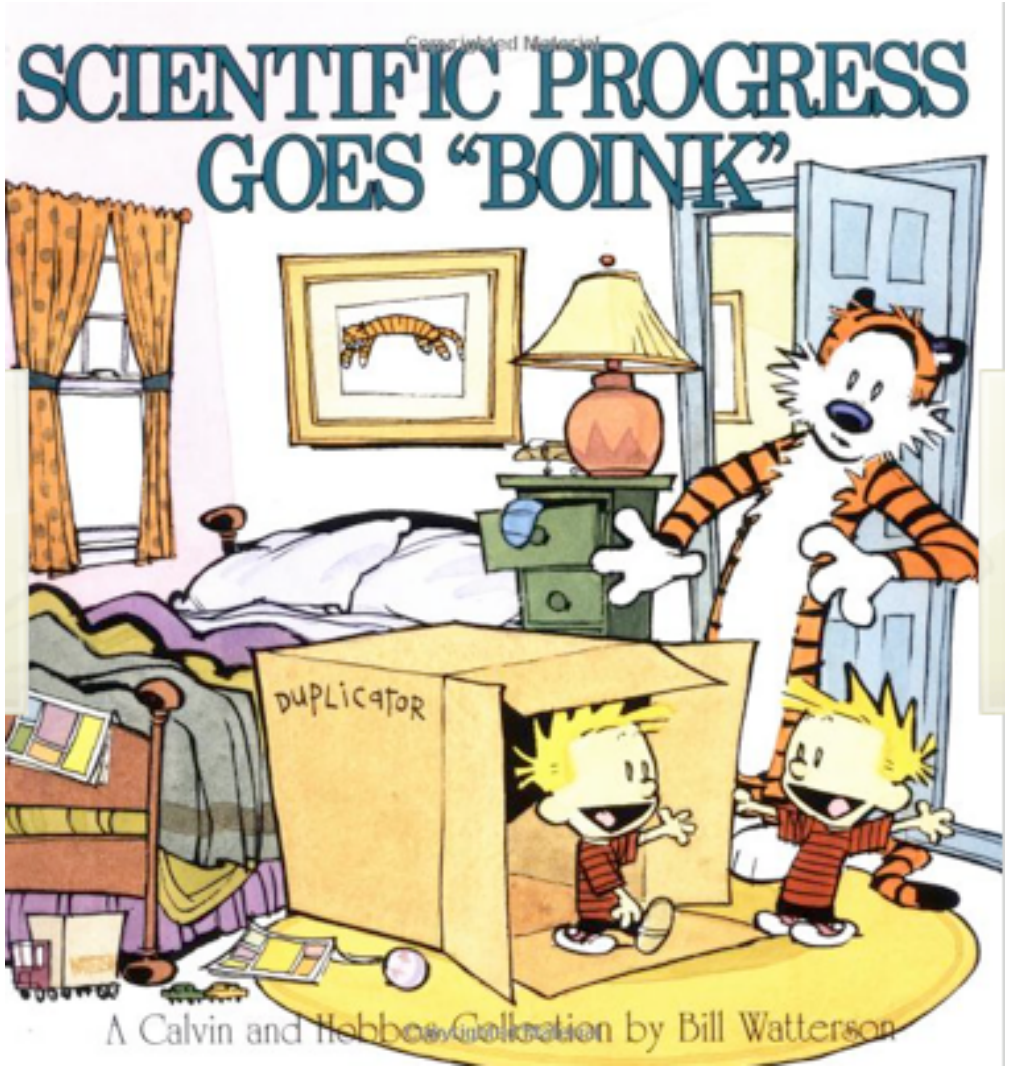
Nucleofector

Sonoporation

Sonication used to transfect large plasmids, or anything else..
Said to be superior to electroporation.

Offered by SONIDEL LTD.
SONIDEL STK-10 Transfection Kit
For use with the SONIDEL SP100

SONIDEL SP100


SCIENTIFIC PROGRESS GOES "BOINK"
DUPLICATOR
A Calvin and Hobbes Collection by Bill Watterson

Notes on optimizations

How to optimize

- Transfect with different conditions
- Assess success
- Vector choices
  - Green Fluorescent Protein
    - Assess fluorescence on microscope
  - Luciferase
    - Assess using luciferase assay and a microplate reader
  - LacZ
    - Assess using a color reaction catalyzed by β-galactosidase
  - A plasmid expressing your experimental protein
    - Assess by Western blotting

LacZ

- Transfected MDCK cells with superfect reagent
- Varied total amount of DNA
- Varied total amount of Superfect
- Varied Superfect/DNA ratio
- Assess with LacZ assay

- I would do this qualitatively, but now we could assess with the microplate reader.

Example of a real experiment I did on 6/15/98 using MDCK cells

Well #	µg DNA pCMV-LacZ	µl DNA	µl optimem	µl Superfect	Ratio Sup/DNA	µl growth media	Result
1	0.3	1	74	0.6	2	400	
2	0.3	1	74	1.2	4	400	
3	0.3	1	74	1.8	6	400	
4	0.5	1.7	73.3	1	2	400	
5	0.5	1.7	73.3	2	4	400	
6	0.5	1.7	73.3	3	6	400	
7	1.0	3.3	71.7	2	2	400	
8	1.0	3.3	71.7	4	4	400	+++++
9	1.0	3.3	71.7	6	6	400	
10	1.5	5	70	3	2	400	++++
11	1.5	5	70	6	4	400	+
12	1.5	5	70	9	6	400	

Plate cells in 12-well plate so that they will be 70% confluent by the next day. Mix DNA + optimem, add superfect, incubate 10 minutes during which time you rinse cells once with warm PBS (later I stopped doing the rinse), after 10 min add growth media to reaction and add to cells, incubate 3 hours, wish with PBS 3 times, add warm growth media. After 48 hours stain for β-galactosidase.

Histochemical staining for β-galactosidase

Fixing cells in 4% paraformaldehyde for 10 minutes at room temperature

Wash with PBS 3x

Add Histochemical Reaction Mixture

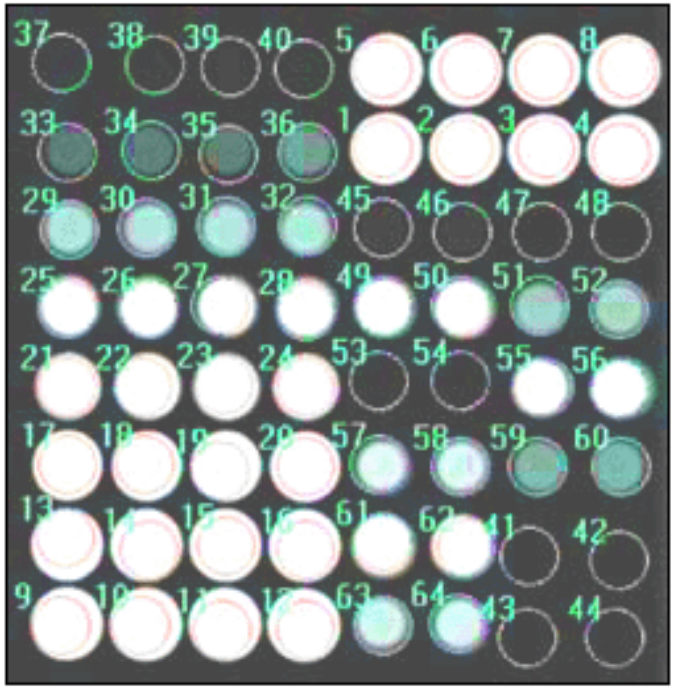
Incubate at 37C for 1-4 hours and monitor for blue color formation

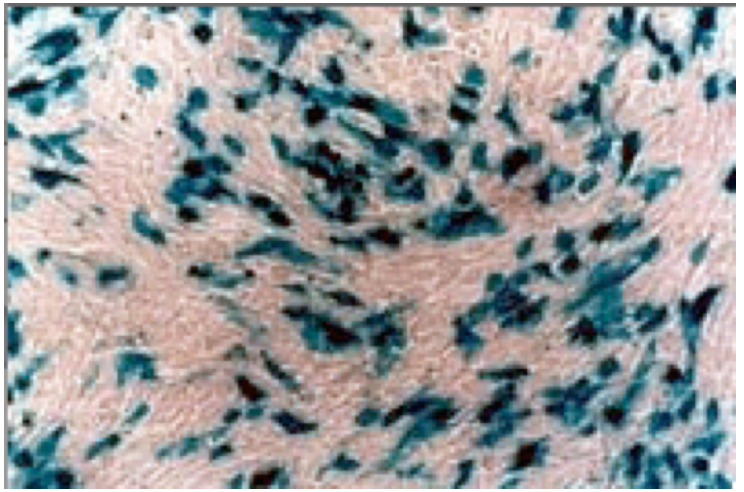
Histochemical Reaction Mixture

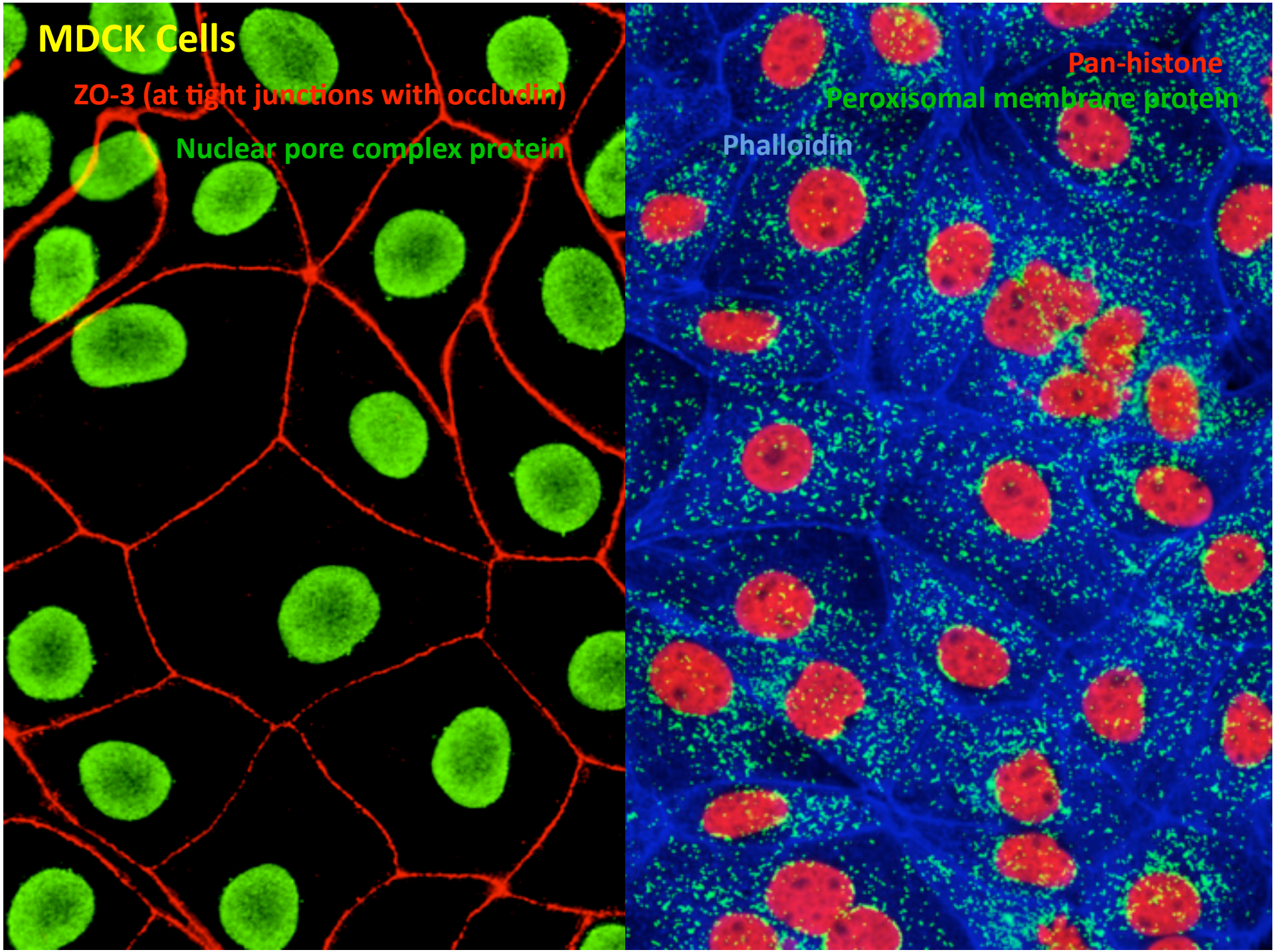
4.11 g potassium ferricyanide [$K_3Fe(Cn)_6$] (5mM final)
0.53 g potassium ferrocyanide [$K_4Fe(Cn)_6$] (5mM final)
5 ml 100 mM $MgCl_2$ (2mM final)
25 ml 10x PBS (1x final)
250 ml H_2O
Add X-gal just before use to a final concentration of 1 mg/ml

(for 12 ml reaction mixture add 120 µl 100 mg/ml X-gal)

MarkerGene™ Chemiluminescent lacZ ß-Galactosidase Detection Kit






MDCK Cells
ZO-3 (at tight junctions with occludin)
Nuclear pore complex protein
Pan-histone
Peroxisomal membrane protein
Phalloidin

Luciferase

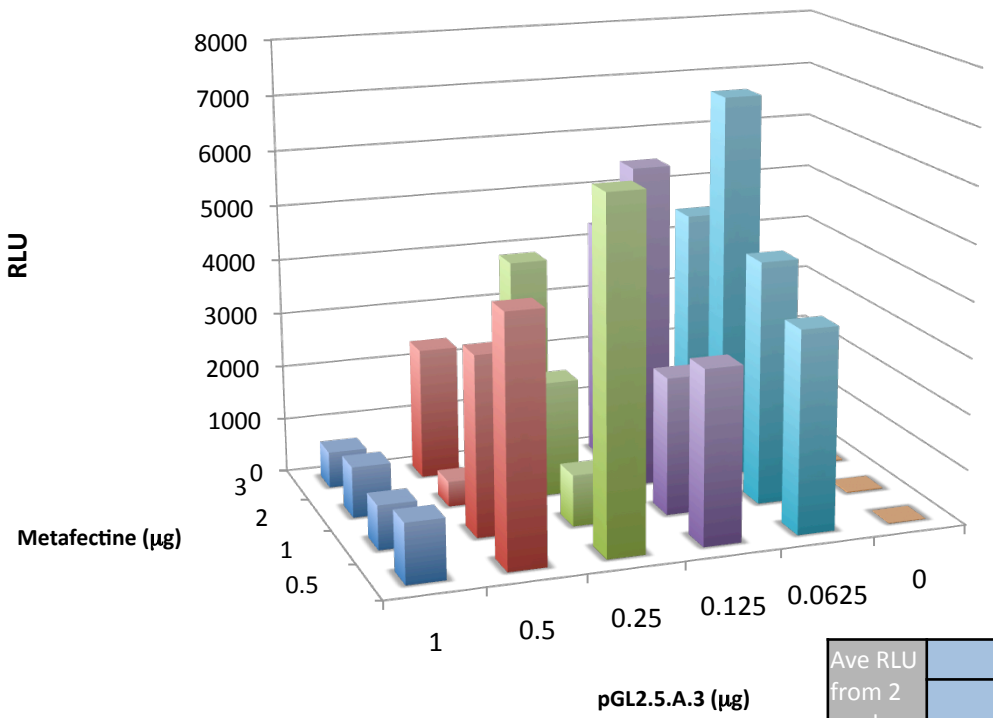
Transfection of HEK293 with pGL2b.5.A.3

24-well dish

		pGL2b.5.A.3 (ug)					
		1	0.5	0.25	0.125	0.0625	0
Metafectine (ul)	3	1:3	1:6	1:12	1:24	1:48	-
	2	1:2	1:4	1:8	1:16	1:32	-
	1	1:1	1:2	1:4	1:8	1:16	-
	0.5	1:0.5	1:1	1:2	1:3	1:4	-

Mix DNA and 15 ul serum free media. Add Metafectine and incubate 20 minutes. Add 300 ul cells in growth media. Assay for luciferase after 48 hours.

Results in next slides



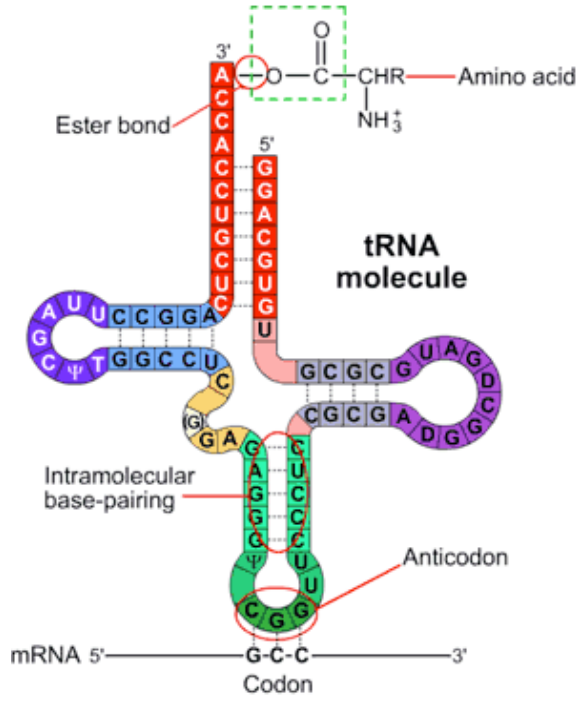
Ave RLU from 2 reads		pGL2b.5.A.3 (ug)					
		1	0.5	0.25	0.125	0.0625	0
Metafectine (ul)	3	622	2394.5	3877	4345	4475	17
	2	923	456.5	2084	5847.5	7003	22
	1	798.5	3251	924	2489.5	4408	17.5
	0.5	1078	4444.5	6270.5	3139.5	3653.5	11

Standard Deviations (single transfections read in duplicate)					
50.24542344	3.535533906	226.27417	39.59797975	28.28427125	2.828427125
11.3137085	44.54772721	114.5512986	208.5965005	240.4163056	1.414213562
33.23401872	43.84062043	31.11269837	10.60660172	91.92388155	4.949747468
155.5634919	28.99137803	300.520382	296.2777413	395.2726907	1.414213562

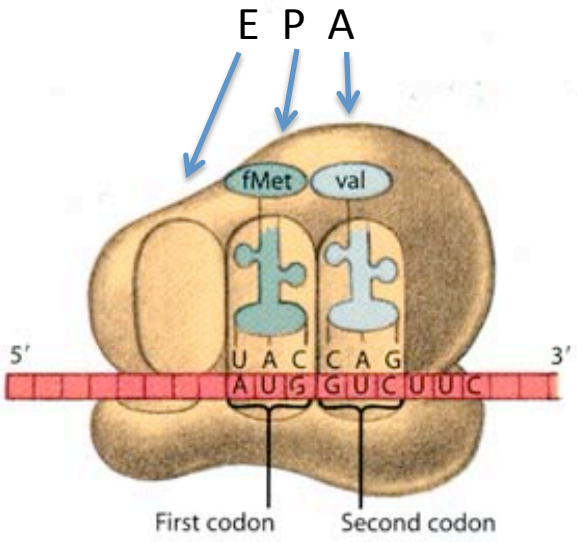
Many antibiotics act on protein translation by ribosomal binding

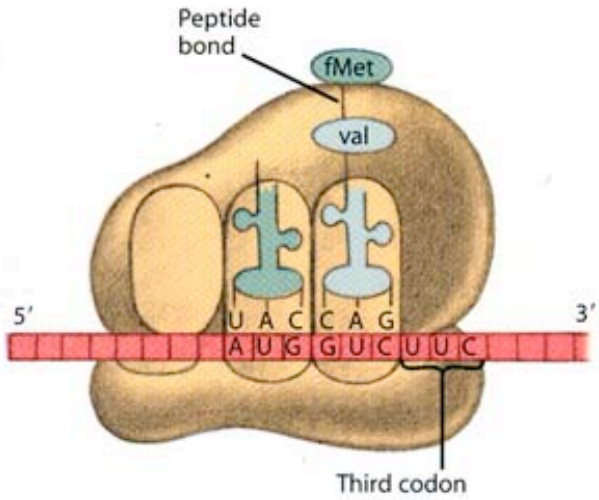
extras…

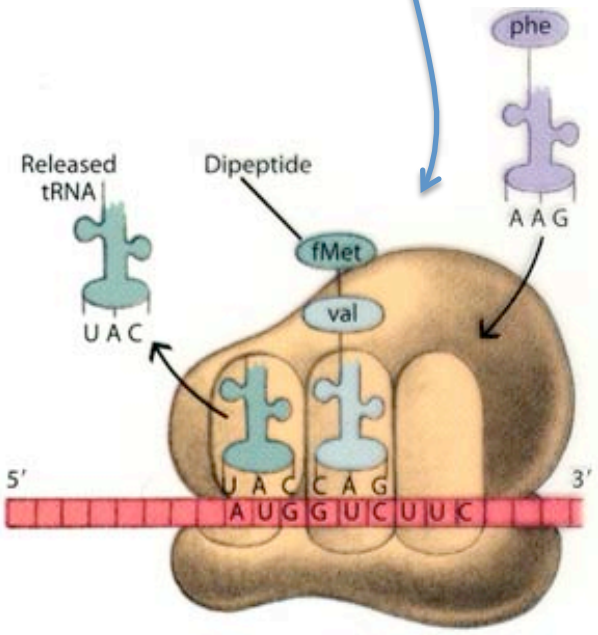
Puromycin fits
In the ‘A’ site.

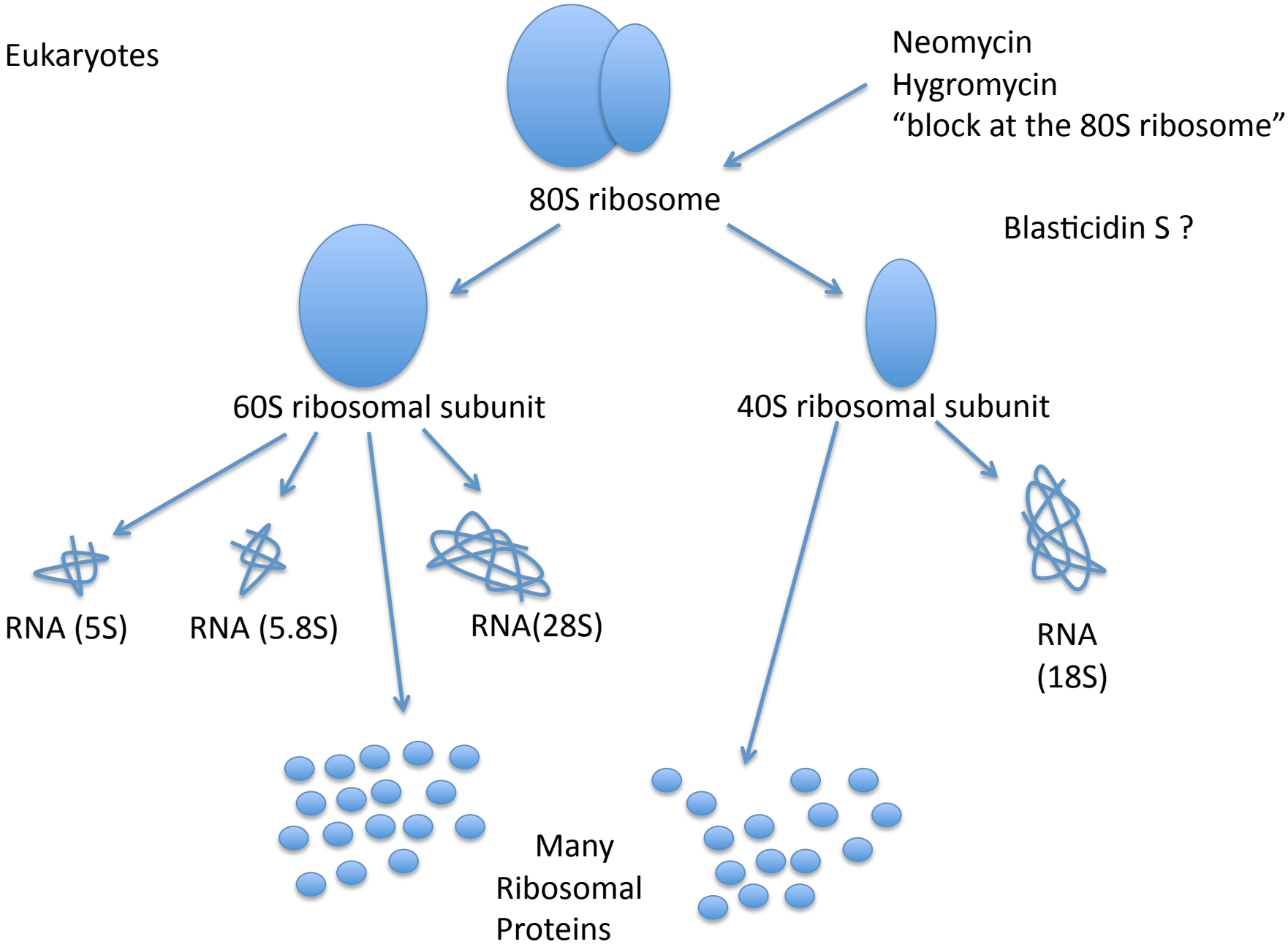


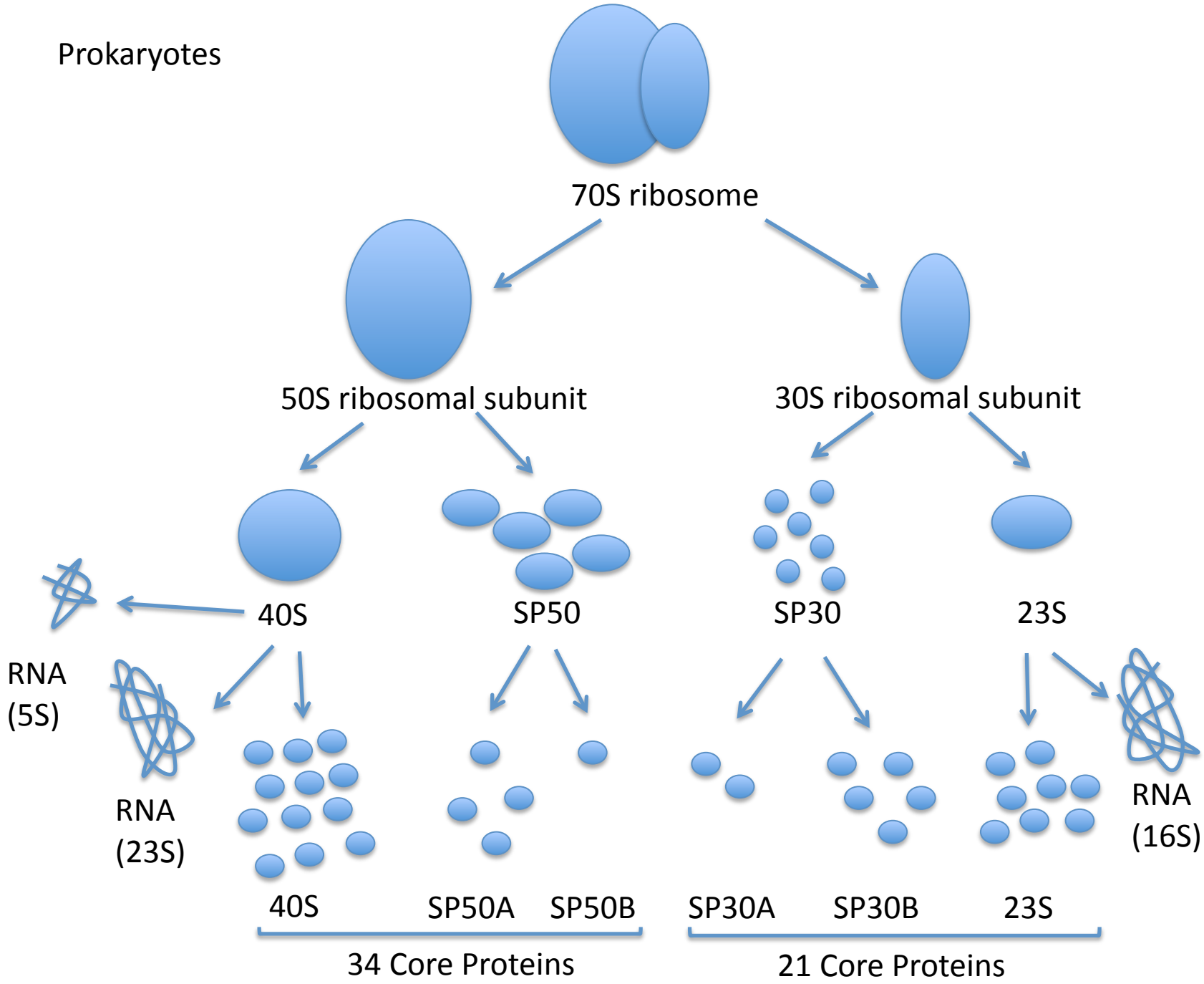
Drugs inhibiting
elongation factors
block production of the
elongating peptide








Eukaryotes
Neomycin
Hygromycin
“block at the 80S ribosome”
80S ribosome
Blasticidin S ?
60S ribosomal subunit
40S ribosomal subunit
RNA (5S)
RNA (5.8S)
RNA(28S)
RNA
(18S)
Many
Ribosomal
Proteins


Prokaryotes
70S ribosome
50S ribosomal subunit
30S ribosomal subunit
40S
SP50
SP30
23S
RNA
(5S)
RNA
(23S)
RNA
(16S)
40S
SP50A
SP50B
SP30A
SP30B
23S
34 Core Proteins
21 Core Proteins