Lentivirus Titer Determination

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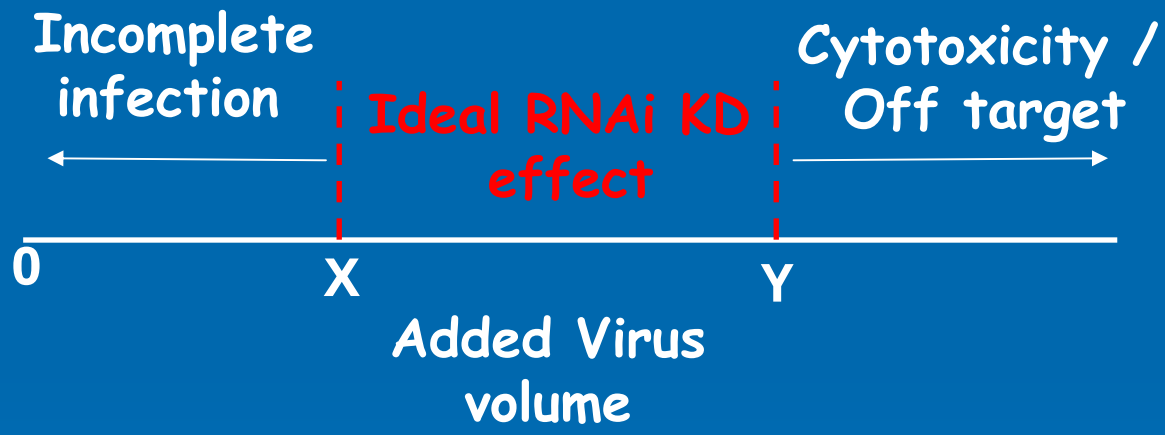
May.,2008

Why do we need to get virus titer ?

RNAi knockdown vs. Viral dose



Lentiviral stock titration-Protocol

Seeding cell

↓

Virus infection / transduction
Serial diluted virus incubate with cells
(mix with polybrene and Spin)

↓

Proper Antibiotic selection (optional)

↓

Virus titer (TU/ml) determination

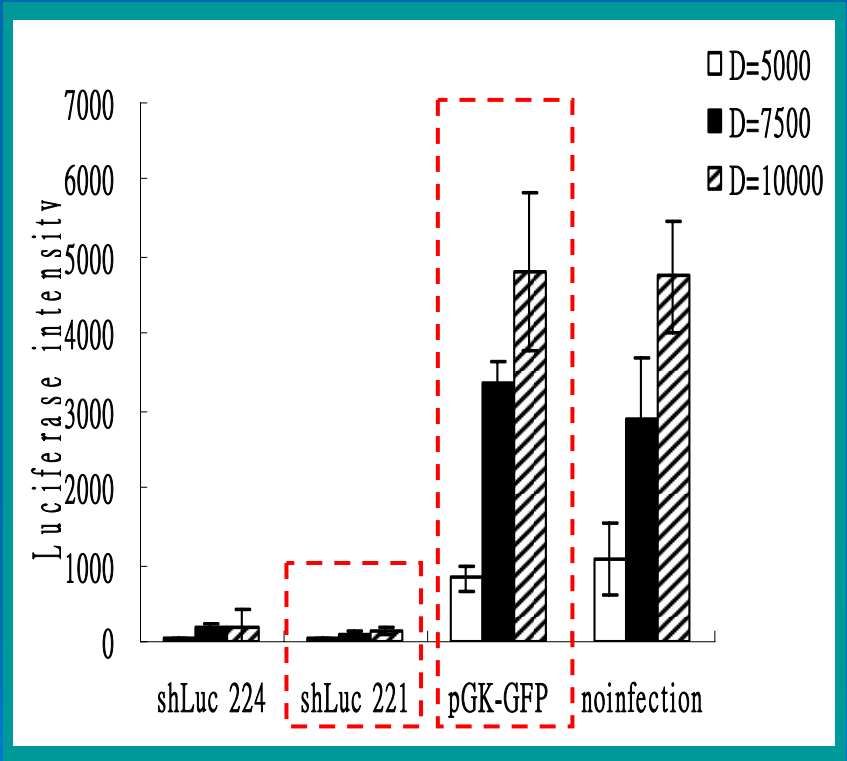
Optimization of lentiviral transduction

- Lentiviral transduction should be optimized for each cell line and cell-based assay.

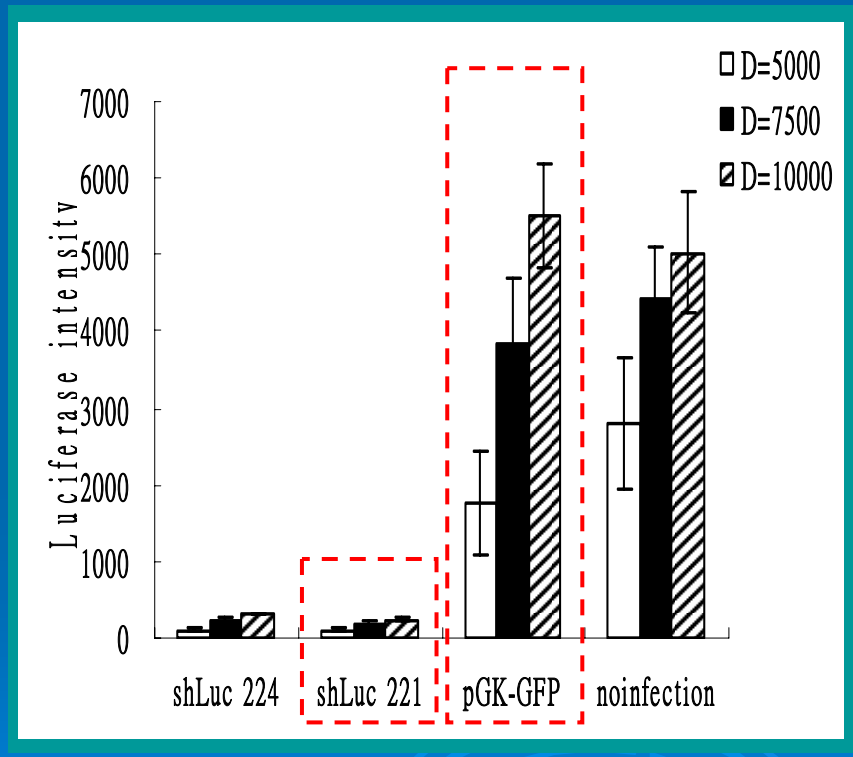
- Parameters needed to be test before starting infections to determine the optimal conditions for a given experiment
  - Cell seeding density
  - Antibiotic concentration
  - Time-course

Optimize transduction method

–Cell seeding density/ Time-course



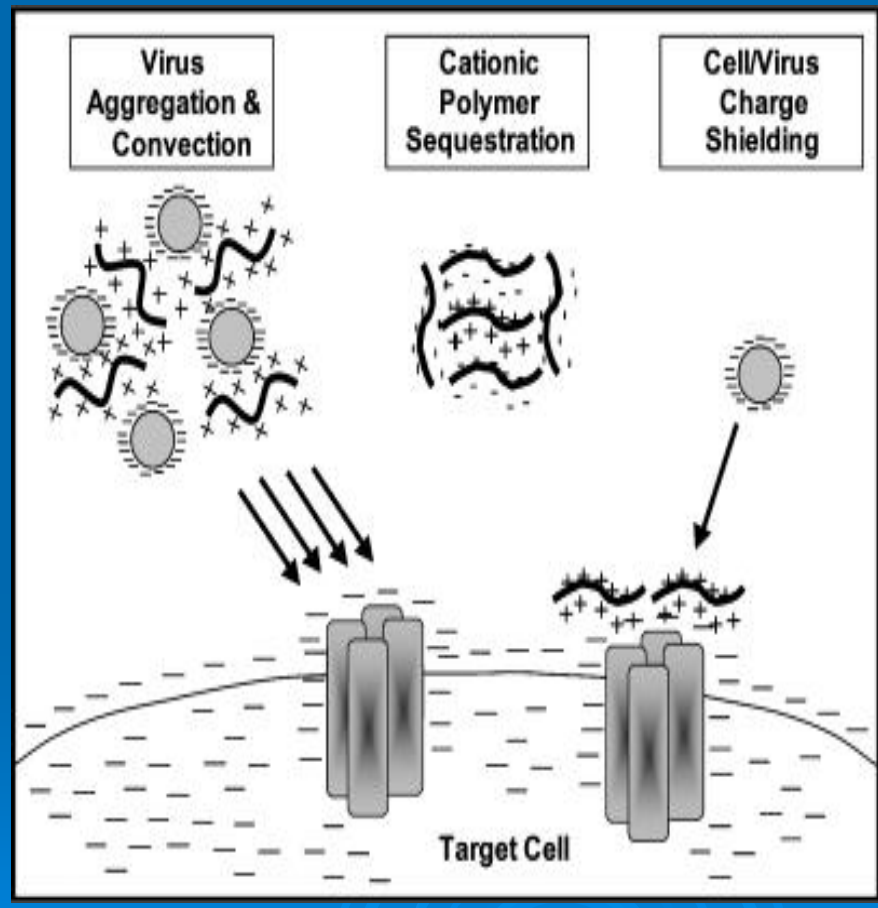
4 days (post-infection)



6 days (post-infection)

Optimize transduction method
- Use of polybrene to enhance transduction

- Polybrene (hexadimethrine bromide) mediated transduction of lentivirus can enhance transduction efficiency
- Determine experimental cell's sensitivity to polybrene. (range from 0-10 ug/ml)
- Protamine sulfate could be an alternative to enhance transduction efficiency.



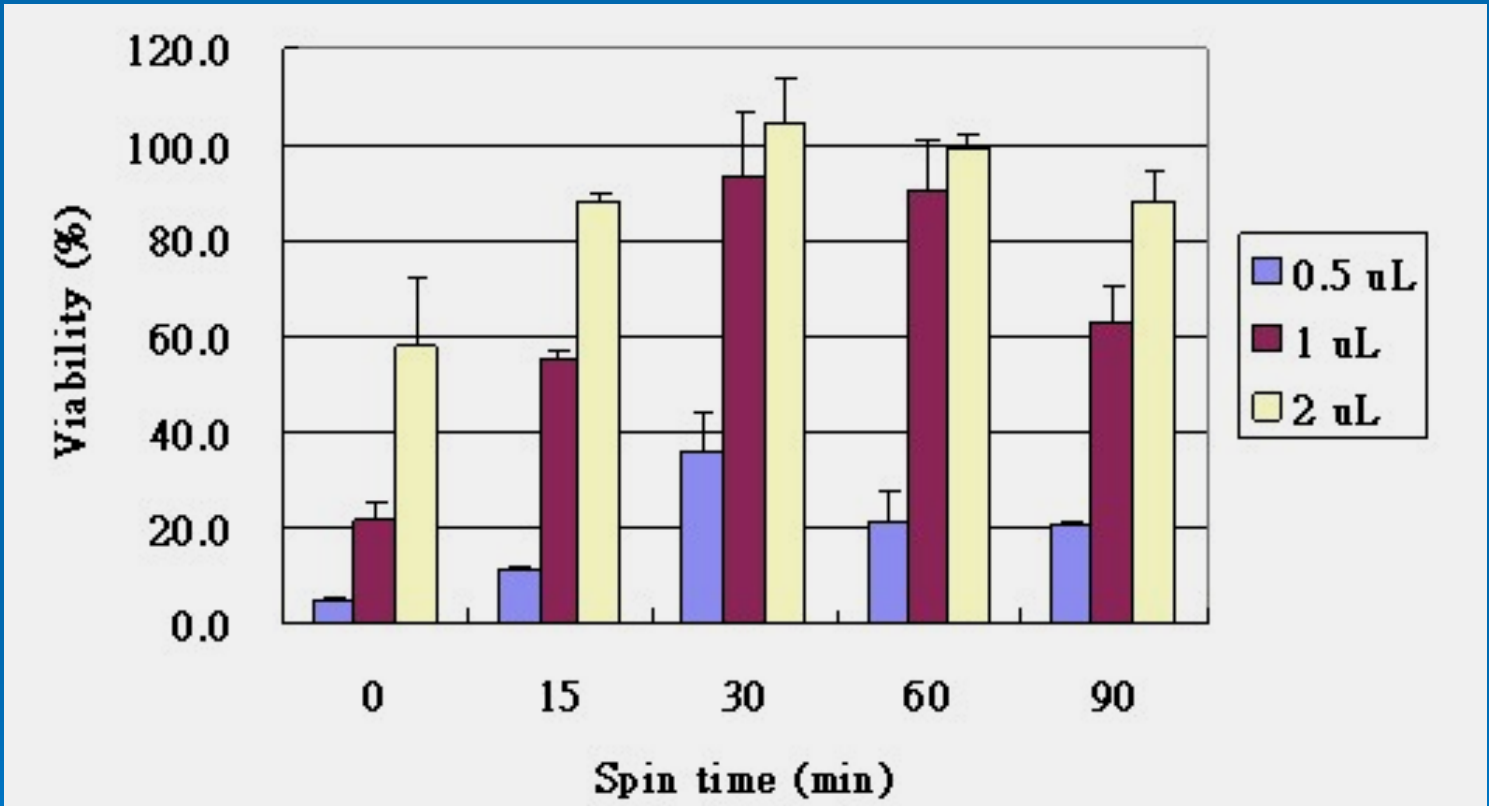
Howard *et al* (2004). *Biophysical Journal* **86**: 1234–42.

Optimize transduction method

- Perform spin infection or just leave overnight without spin

- Spin infection condition:
  - Speed : 1170g
  - Time : 30 ~90 min. at 37℃ or RT
  - Remove virus immediately after spin and replace with fresh medium

Optimize transduction method
- Spin infection

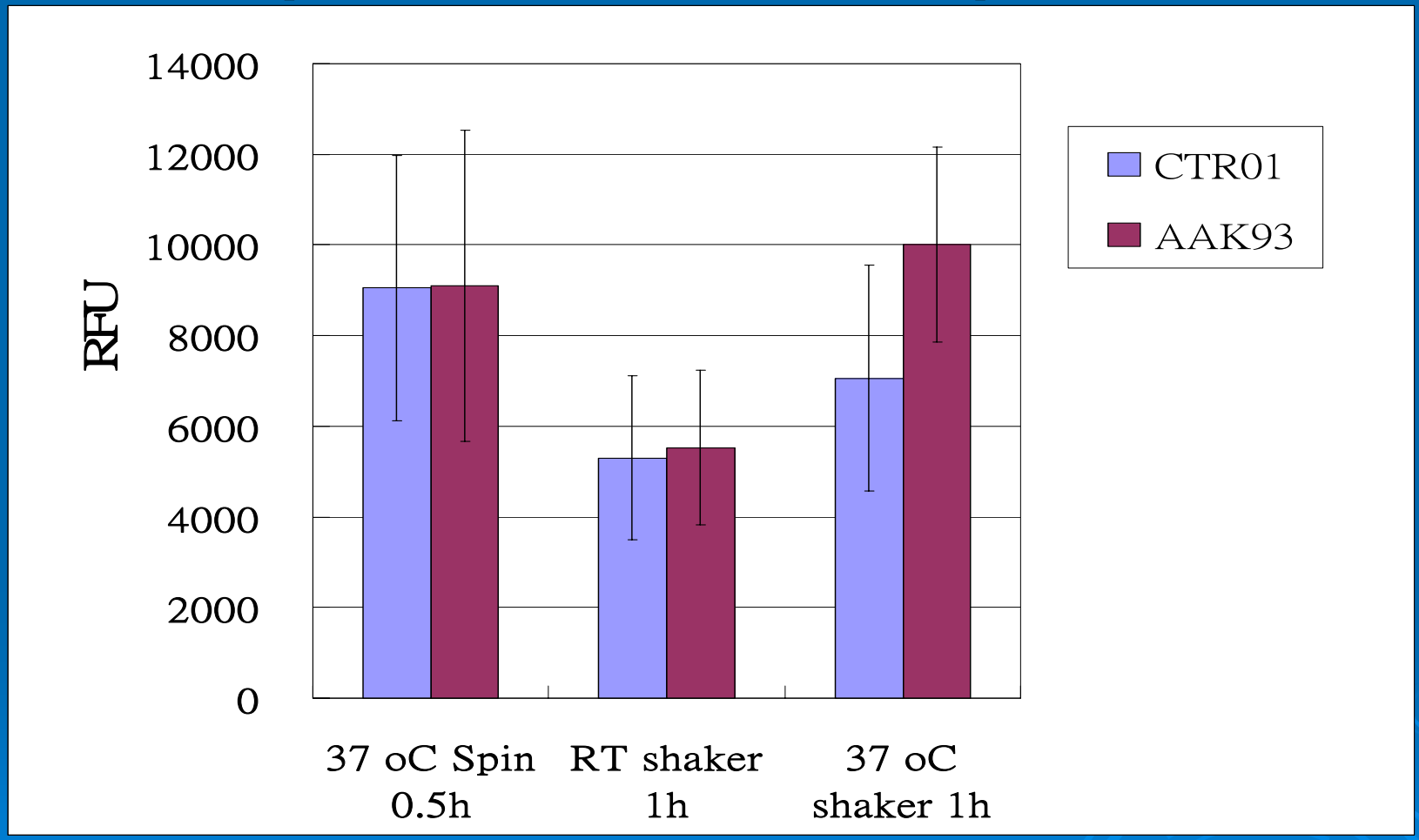


A549 cell, 1170g, 37°C

Polybrene : working concentration 8μg/ml

Optimize transduction method

- Spin-infection vs. non-spin infection

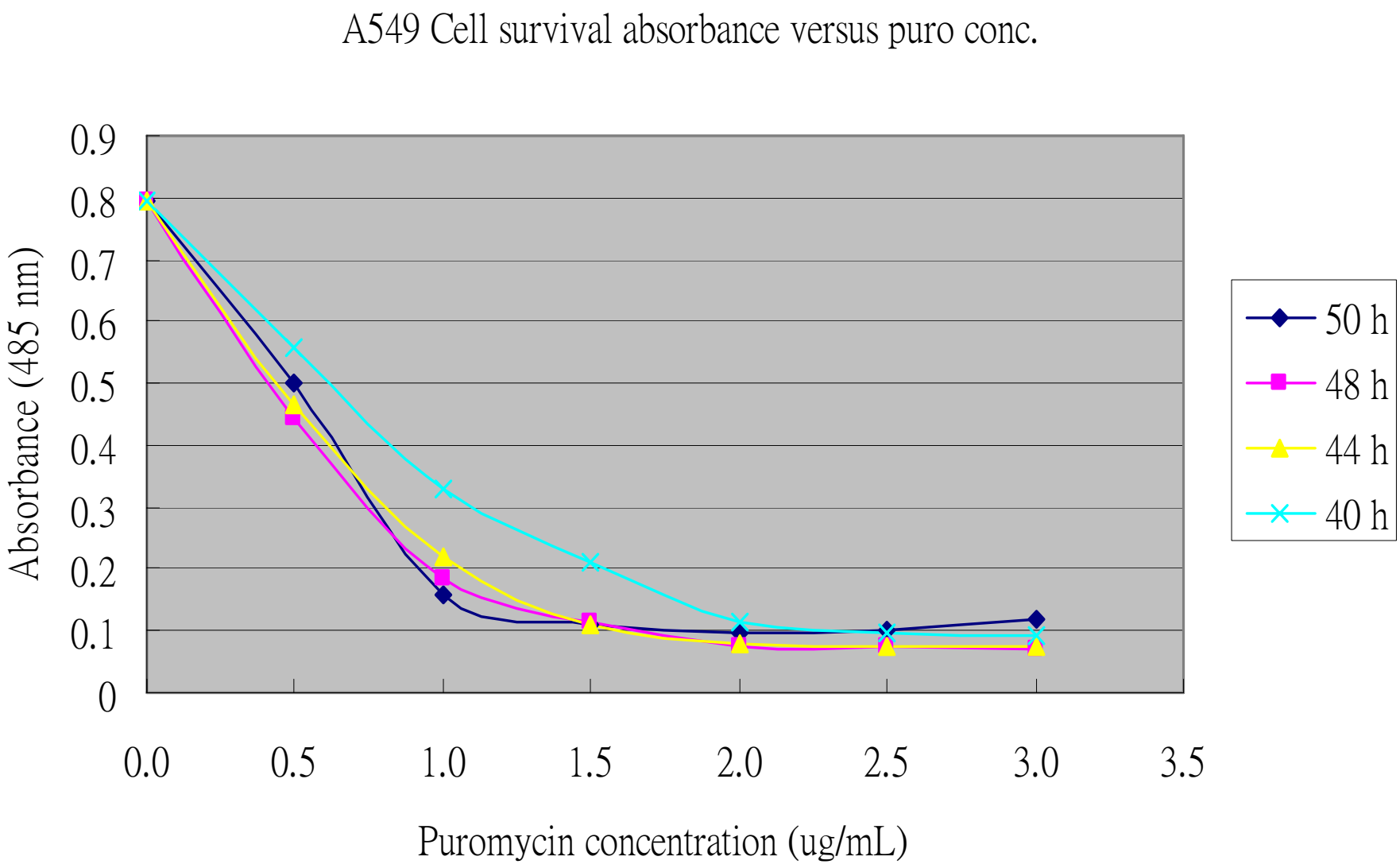


A549 cell

Polybrene : working concentration 8μg/ml

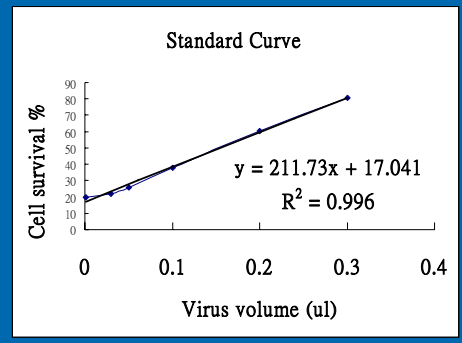
Optimize transduction method

-Antibiotic (puromycin) concentration

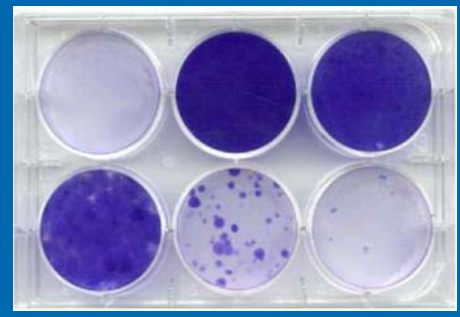


Virus stock titration- Methods

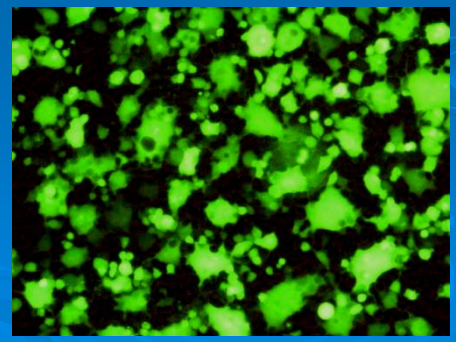
- Relative virus titer determination
  - Relative infection unit (RIU)/ml
  - Puromycin selection / cell viability assay.



- End point dilution or limiting dilution method
  - Colony formation unit (CFU)/ml
  - Puromycin selection / colonies counting



- GFP expressed detection
  - Transduction Unit/ml or TU/ml
  - FACS analysis



Relative virus titer determination

Day 1 Seed A549 cells 6000/well at 96 well plate

Day 2 Prepare virus dilutions
Add diluted virus to cells in media containing polybrene
Centrifuge cells

Day 3 *24 hours after virus addition:* Change media with culture media containing puromycin

Day 5 *48 hours after puromycin addition:*
Remove media; replace with phenol red free media containing 10% MTS.
After 40-50 minutes incubation, record the absorbance at 490 nm using plate reader.
Define the liner range to determine the relative titer

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After 40-50 minutes incubation, record the absorbance at 490 nm using plate reader.
Define the liner range to determine the relative titer

Make standard viruses as follows: (Use a new tip for each dilution)

Dilution number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Volume (µl) corresponding to stock virus in per 5µL transduction	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
Media added (µl)	**75**	**30**	**40**	**60**	**30**	**40**	**60**	**40**	**60**	**100**	**100**	**67**
shLuc#221 virus stock or previous diluted virus added (µl)	**75**	**120**	**120**	**120**	**120**	**120**	**120**	**120**	**120**	**100**	**100**	**100**
Total volume (µl)	150	150	160	180	150	160	180	160	180	200	200	167
Removed volume to next well/ dilution (µl)	**120**	**120**	**120**	**120**	**120**	**120**	**120**	**120**	**100**	**100**	**100**	**0**
Remaining volume for transduction (µl)	30	30	40	60	30	40	60	40	80	100	100	167

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Define the liner range to determine the relative titer

Value in each well represents stock virus in per 5μL infection (shLuc#221)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
C	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
D	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
E	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
F							Tested #1	Tested #2				
G							Tested #1	Tested #2				

Relative virus titer determination

Day 1 Seed A549 cells 6000/well at 96 well plate

Day 2 Prepare virus dilutions
Add diluted virus to cells in media containing polybrene
Centrifuge cells 1170g 30 min. at 37℃
then return to incubator

Day 3 *24 hours after virus addition:* Change media with culture media containing puromycin

Day 5 *48 hours after puromycin addition:*
Remove media; replace with phenol red free media containing 10% MTS.
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D	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
E	2.5	2	1.5	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.03
F							Tested #1	Tested #2				
G							Tested #1	Tested #2				

Mock

Relative virus titer determination

Day 1 Seed A549 cells 6000/well at 96 well plate

Day 2 Prepare virus dilutions
Add diluted virus to cells in media containing polybrene
Centrifuge cells

Day 3 *24 hours after virus addition:* Chang media with culture media containing puromycin

Day 5 *48 hours after puromycin addition:*
Remove media; replace with phenol red free media containing 10% MTS.
After 40-50 minutes incubation, record the absorbance at 490 nm using plate reader.
Define the liner range to determine the relative titer

Raw MTS data

Standard	2.00	1.50	1.00	0.80	0.60	0.40	0.30	0.20	0.10	0.05	0.03	0.00
B	1.3501	1.3608	1.2888	1.2416	1.1512	1.0561	0.855	0.585	0.322	0.2071	0.1529	0
C	1.3356	1.3216	1.2918	1.293	1.1451	1.09	0.9395	0.5973	0.3437	0.2137	0.1461	0
D	1.3283	1.36	1.3505	1.282	1.2211	1.0839	0.9994	0.6413	0.3342	0.2035	0.1361	0
E	1.3741	1.2942	1.3319	1.307	1.2275	1.1451	0.9733	0.7213	0.3409	0.2203	0.1458	0
Control	1.3971	1.3963	0.1066	0.1046	1.29	1.29						
Average	1.29											
CV %	1.50	2.42	2.31	2.20	3.72	3.41	6.67	9.69	2.88	3.51	4.76	#DIV/0!

Relative virus titer determination

Day 1 Seed A549 cells 6

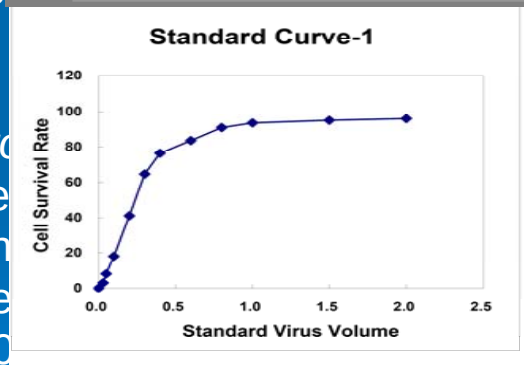
Day 2 Prepare virus dilut
Add diluted virus t
containing polybre
Centrifuge cells

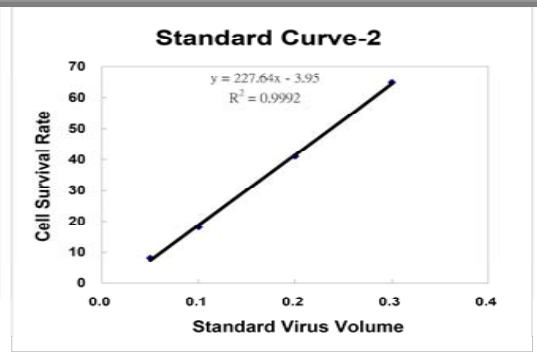
Day 3 *24 hours after viru*
media with culture
puromycin

Day 5 *48 hours after purc*
Remove media; re
free media contain
After 40-50 minute
absorbance at 490
Define the liner range to determine the relative titer

Cell survival percentage

Standard	2.00	1.50	1.00	0.80	0.60	0.40	0.30	0.20	0.10	0.05	0.03	0.00
B	96.39	97.22	91.64	87.99	80.99	73.62	58.04	37.13	16.76	7.86	3.66	-8.18
C	95.27	94.18	91.88	91.97	80.51	76.25	64.59	38.08	18.44	8.37	3.14	-8.18
D	94.70	97.16	96.42	91.12	86.40	75.77	69.23	41.49	17.71	7.58	2.36	-8.18
E	98.25	92.06	94.98	93.05	86.89	80.51	67.21	47.69	18.22	8.88	3.11	-8.18
average	96.15	95.16	93.73	91.03	83.70	76.54	64.77	41.10	18.12	8.18	3.07	0.00





Standard virus titer

12954 /uL

Relative virus titer determination

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l survival percentage

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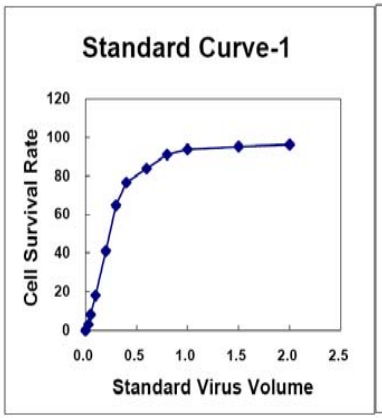

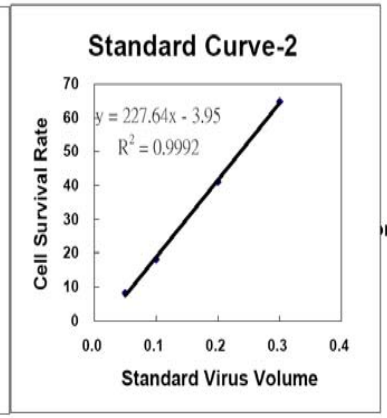

Standard virus titer

6000*0.6477/0.3

1.30E+04 /uL

test virus #1

volume 0.1 ul

Cell survival % 18.44

nvert to standard virus volume 0.0984 uL

0.0984/0.1*1.3E+04

test virus #1 titer 1.27E+04 /uL

End point dilution or limiting dilution method

Day 1	Seed A549 cells 2X10 ^{5}/well at 6 well plate
Day 2	Prepare virus dilutions Add diluted virus to cells in media containing polybrene Centrifuge cells
Day 3	*24 hours after virus addition:* Change media with culture media without puromycin
Day 4	*48 hours after virus addition:* Change media with culture media containing puromycin
Day 5-14	Replace medium with fresh medium containing puromycin every 3–4 days.
Day 14	Remove the medium and gently wash each well with 3ml PBS once. Add 1 ml crystal violet solution and incubate 10 minutes at room temperature.

End point dilution or limiting dilution method

Day 1 Seed A549 cells 2X10 ^{5}/well at 6 well plate

Day 2 Prepare virus dilutions
Add diluted virus to cells in media containing polybrene
Centrifuge cells

2 mL 10-fold serial dilutions ranging from 10-2 to 10-6 in 15 ml conical vials.

Mix gently by inverting the tubes 10 times.

Day 3 *24 hours after virus addition:* Change media with culture media without puromycin

Day 4 *48 hours after virus addition:* Change media with culture media containing puromycin

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Add diluted virus to cells in media containing polybrene
Centrifuge cells

Add 1 ml culture medium containing polybrene to one well as a mock control.

Add 1ml of each of diluted virus to the remaining wells of the plate.

Day 3 *24 hours after virus addition:* Change media with culture media without puromycin

Day 4 *48 hours after virus addition:* Change media with culture media containing puromycin

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Add 1 ml crystal violet solution and incubate 10 minutes at room temperature.
Count the blue-stained colonies

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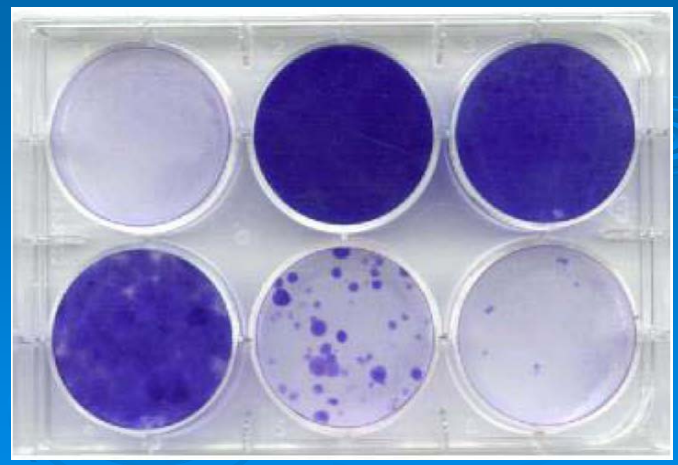


End point dilution or limiting dilution method

Titer of the lentiviral stock was determined by: Number of clonies × Folds of dilution (transducting units/ml; TU/mL).

Example: The colony counts were shown at table,

Dilution	Mock	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
number of colony	No colonies	UD*	UD	UD	38	4

*UD: undeterminable

Thus, the titer of the lentiviral stock is 3.9×10^6 TU/ml (*i.e* . average of 3.8×10^5 and 4×10^6)

Critical Factors Affecting Virus Titer

- Storage time for lentivirus stock.
  - -80°C, > 6 months → re-titration
- Number of freeze/thaw cycles.
  - Virus titer decreases by 5-10% with each freeze/thaw cycle
- Storage condition of lentivirus stock.
  - Aliquot & store at -80°C temperature

How to Convert Virus Titer on different cell lines

$$TU_N = TU_O \times (TU_R \div TU_S)$$

TU_N=換算後,相對在實驗細胞株之Lentivirus-X的RIU

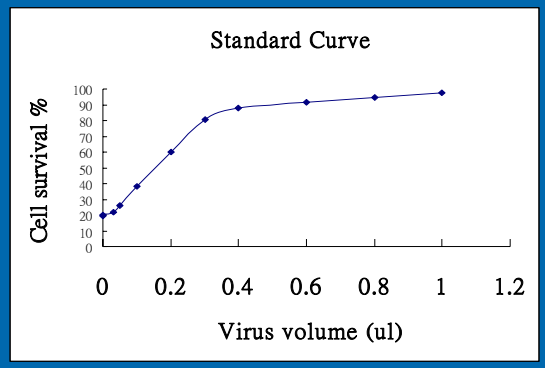
TU_O=利用A549細胞測量之Lentivirus-X RIU (由RNAi Core 提供)

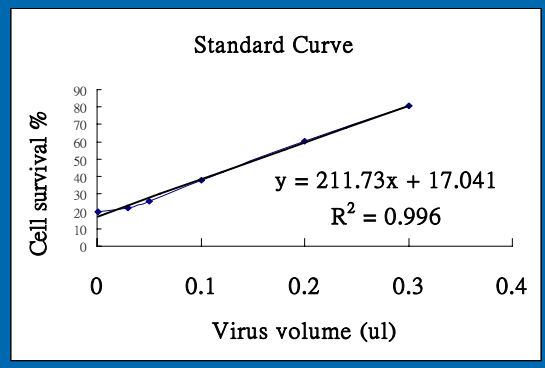
TU_R=利用實驗細胞株測量所得的標準病毒的RIU

TU_S=利用A549細胞測量所得的的標準病毒的RIU (由RNAi Core 提供)

How to Convert Virus Titer on different cell lines

A549

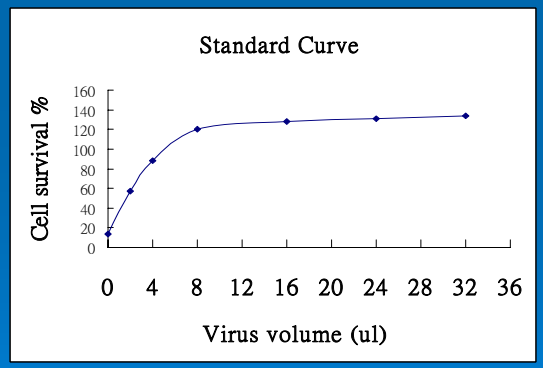


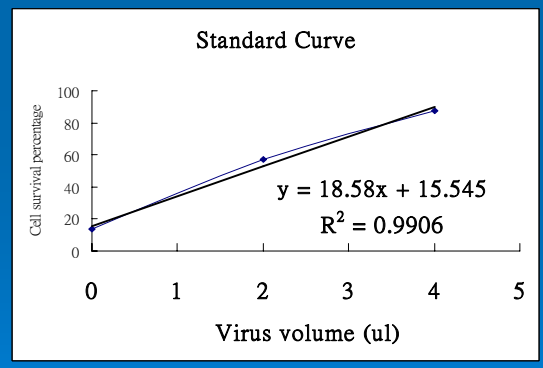


shLuc virus
1x10^4 RIU/ul

Lentivirus X1…Xn
3x10^4 …9x10^3 RIU/ul

S





shLuc virus
1x10^3 RIU/ul

Lentivirus Xn
? RIU/ul

How to Convert Viruses Titer on different cell lines

$$TU_N = TU_O \times (TU_R \div TU_S)$$

$3x10^3$ $3x10^4$ $1x10^3$ $1x10^4$

TU_N=換算後,相對在S細胞之Lentivirus-X1的RIU

TU_O=利用A549細胞測量之Lentivirus-X1的RIU (由RNAi Core 提供)

$3x10^4$ RIU/ul

TU_R=利用S細胞株測量所得的標準病毒的RIU

$1x10^3$ RIU/ul

TU_S=利用A549細胞測量所得的的標準病毒的RIU (由RNAi Core 提供)

$1x10^4$ RIU/ul

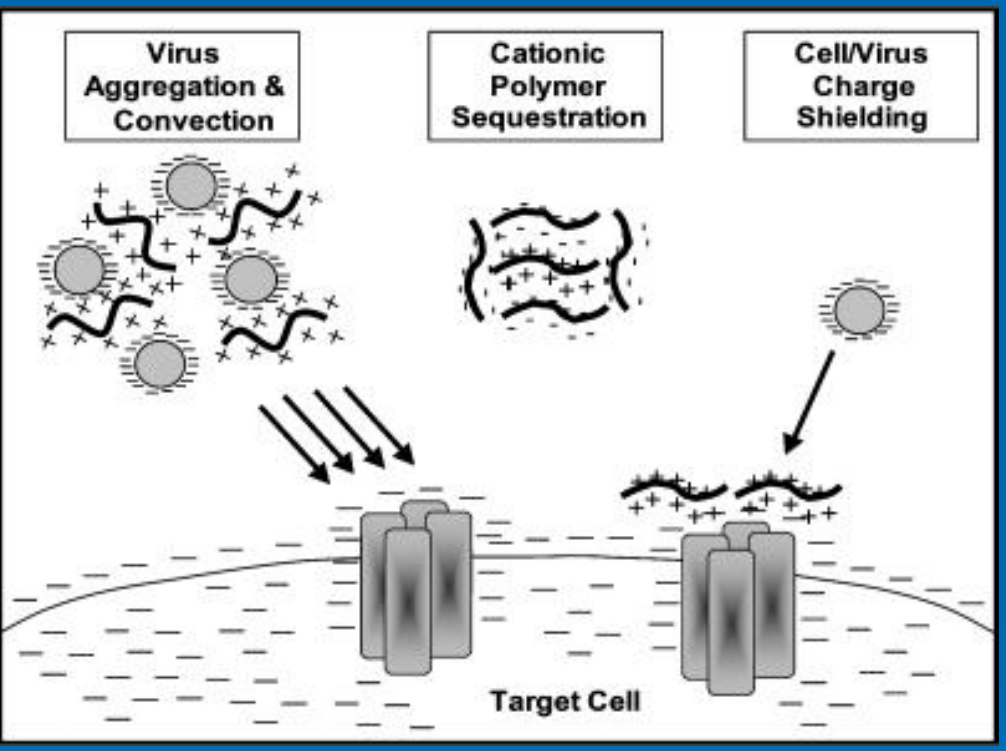
Thank You

Protamine sulfate

- Protamine sulfate is a small cationic protein that binds and precipitates DNA. It may be used for the removal of DNA from protein samples or the purification of DNA binding proteins.
- Protamine sulfate may also be used as an alternative to polybrene in retroviral mediated gene transfer.
- Protamine sulfate is a drug that reverses the anticoagulant effects of heparin by binding to it.
- Protamine was formerly isolated from the sperm of various fish, but is now produced through recombinant biotechnology.
- It is a highly cationic peptide.
- It binds to heparin to form a stable ion pair which does not have anticoagulant activity.
- This complex is then removed and broken down by the reticuloendothelial system.

- Cornetta, K., and Anderson, W. F., Protamine Sulfate as an Effective Alternative to Polybrene in Retroviral-mediated Gene-transfer: Implications for Human Gene Therapy. J. Virol. Methods, **23(2)**,187-194 (1989).

Polybrene



- **Polybrene** (hexadimethrine bromide) is a cationic polymer used to increase the efficiency of infection of certain cells with a retrovirus in cell culture.
- Polybrene acts by neutralizing the charge repulsion between the virions and cell surface.
- It has other uses, including a role in protein sequencing.
- Howard E. Davis, Matthew Rosinski , Jeffrey R. Morgan and Martin L. Yarmush, *et al* (2004). "Charged Polymers Modulate Retrovirus Transduction via Membrane Charge Neutralization and Virus Aggregation.". *Biophysical Journal* **86**: 1234–42.