~Lentivirus production~

May 30, 2008

Jing-Ying Huang

RNAi core R&D group member

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.
- So, is there any other doubts?

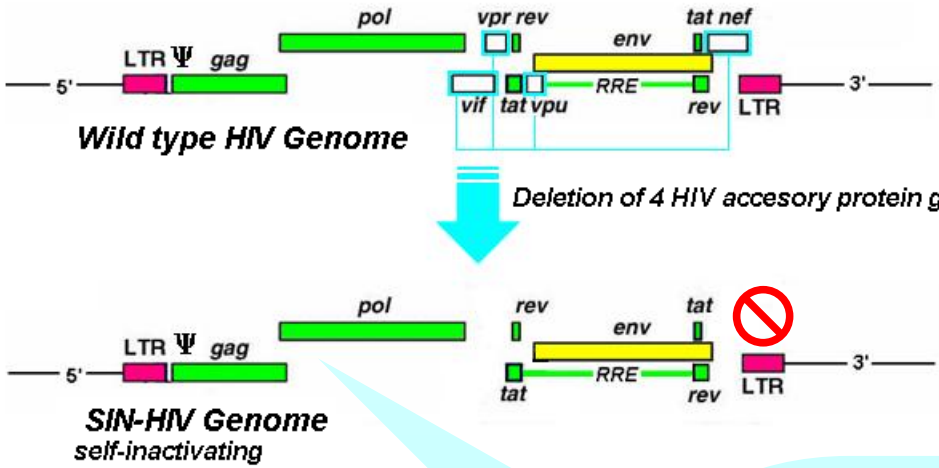
Jing-Ying Huang
RNAi Core R&D Group Member 2008

Self-inactivated Lentiviral Vector

- Replication incompetent.
- Wild-type HIV and SIN-Lentiviral vector in comparison.
- Inserted sequence can only express drug resistant gene and shRNA, none of wild-type HIV protein would be expressed.
- The risks are
  - Recombinant virus that is replication competent.
  - shRNA can target tumor-suppressor gene.
  - Random insertion could damage tumor-suppressor gene.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

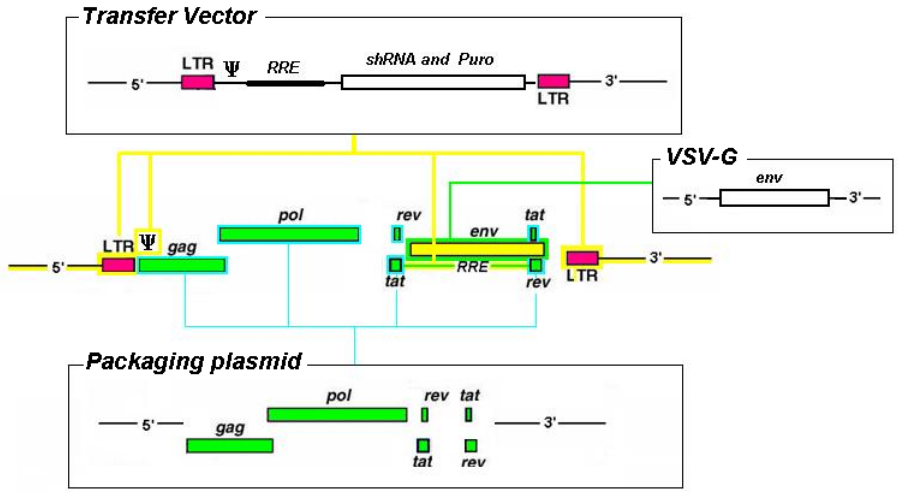
The SIN-HIV vector



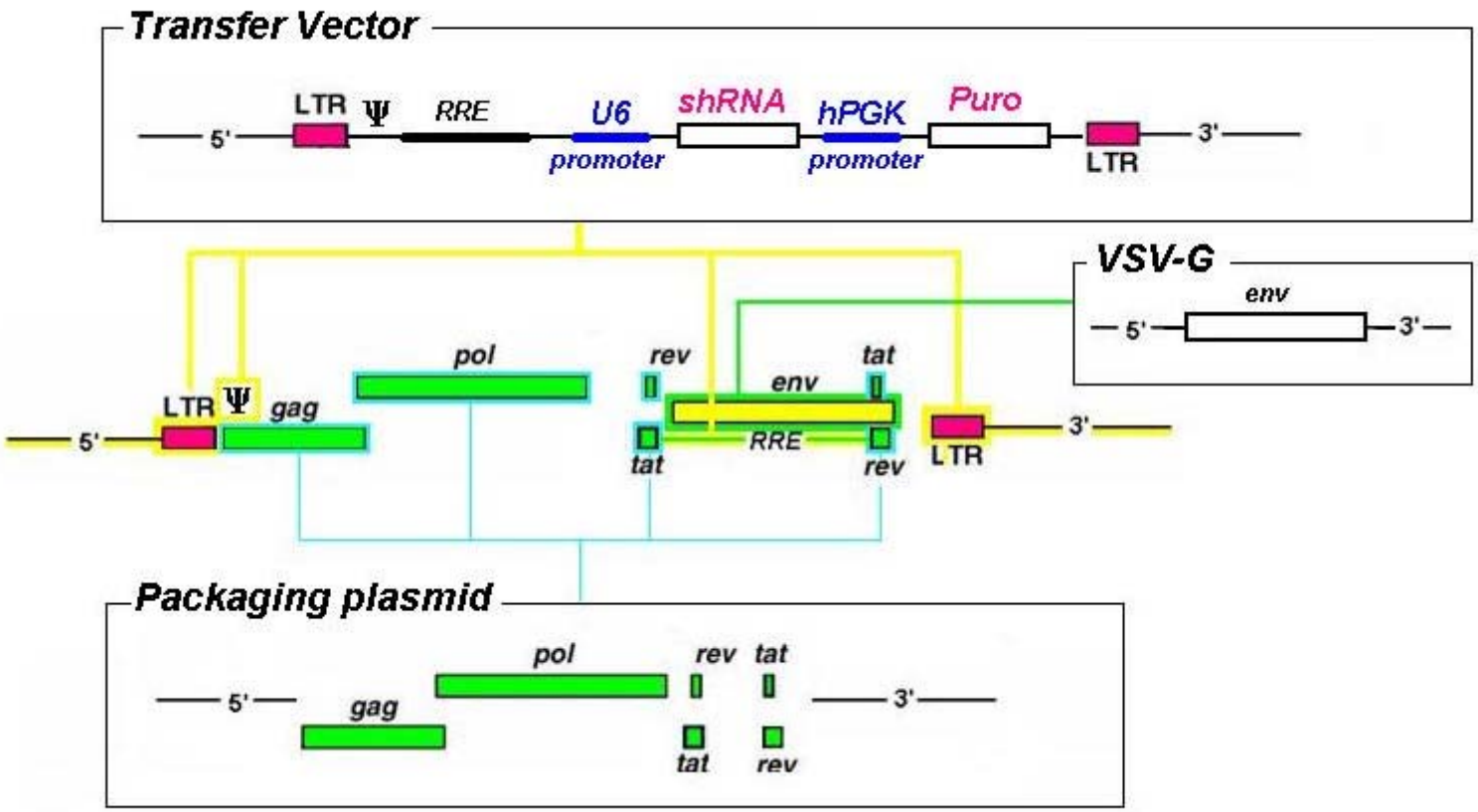
Remove genes that are dispensable in virus genome replication

3'LTR enhancer part (U3 region) was deleted, render it replication-incompetent.

The remaining indispensable genes were than separated into three plasmids to prevent formation of infectious virus.



Jing-Ying Huang
RNAi Core R&D Group Member 2008



Jing-Ying Huang
RNAi Core R&D Group Member 2008

Ψ Packaging signal

For Ψ-containing sequence to be packed into virion.

5' and 3' LTR

For host chromosome insertion, contains cellua promoter region.

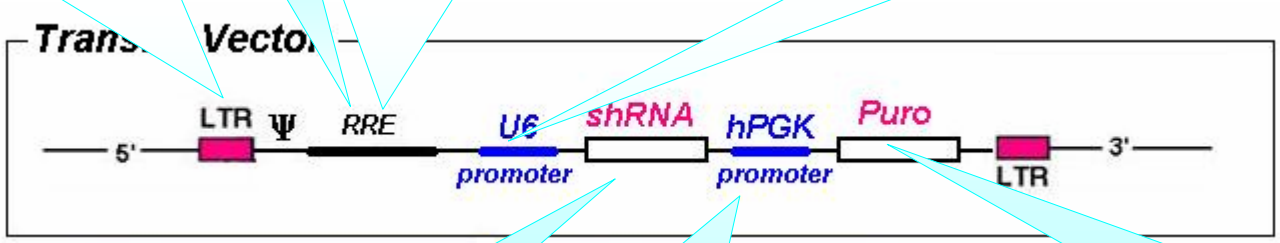
RRE Rev-responsive element

Bind with rev allowing the mRNA to be packed instead of spliced.

U6 promoter

promoter for RNA polymerase III

For shRNA to be transcribed by RNA polymerase III, generating no-capped no tail shRNA.



shRNA

Contains sense-loop-antisense total length round 57~60.

hPGK promoter (promoter for RNA polymerase II)

The phosphoglycerate kinase 1 gene is expressed ubiquitously in human tissues, therefore, use the hPGK promoter to ensure the exrpession of puromycin resistant gene after successful transduction.

Puro (puromycin resistant gene)

Serve as an indicator of successful transduction. It allows cell to survive under puromycin selection condition.

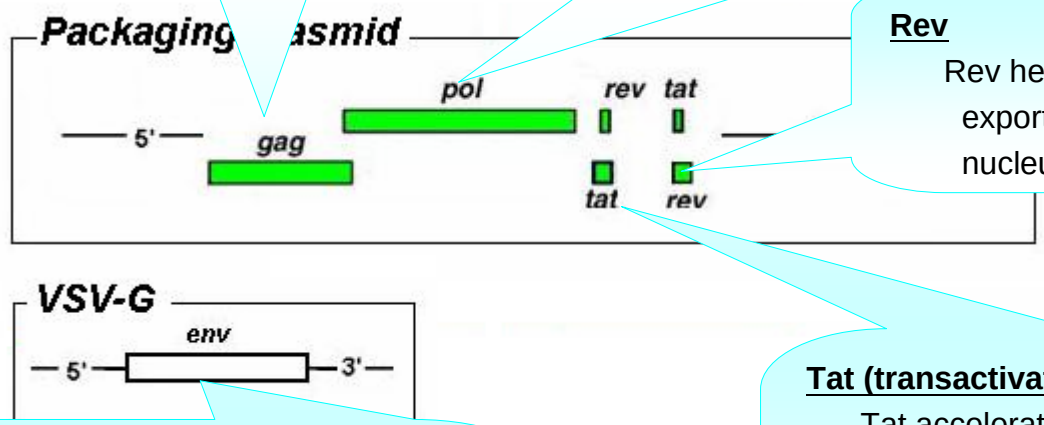
Jing-Ying Huang
RNAi Core R&D Group Member 2008

Gag

Encodes structure proteins needed to form **virus particle** (**CA** caspid protein and **NC** nucleocapsid protein) and **pre-intergration complex** （**MA** matrix protein ）

Pol

Encodes **RT** **reverse transcriptase** which reverse transcribe the transfer vector into cDNA, then the **IN** **intergrase** form **preintegration complex** with the cDNA and **matrix protein**, subsequently enter the nucleus and integrate the transfer vector cDNA into host genome.



Rev

Rev help intact mRNA to be exported from the cell nucleus.

Tat (transactivator)

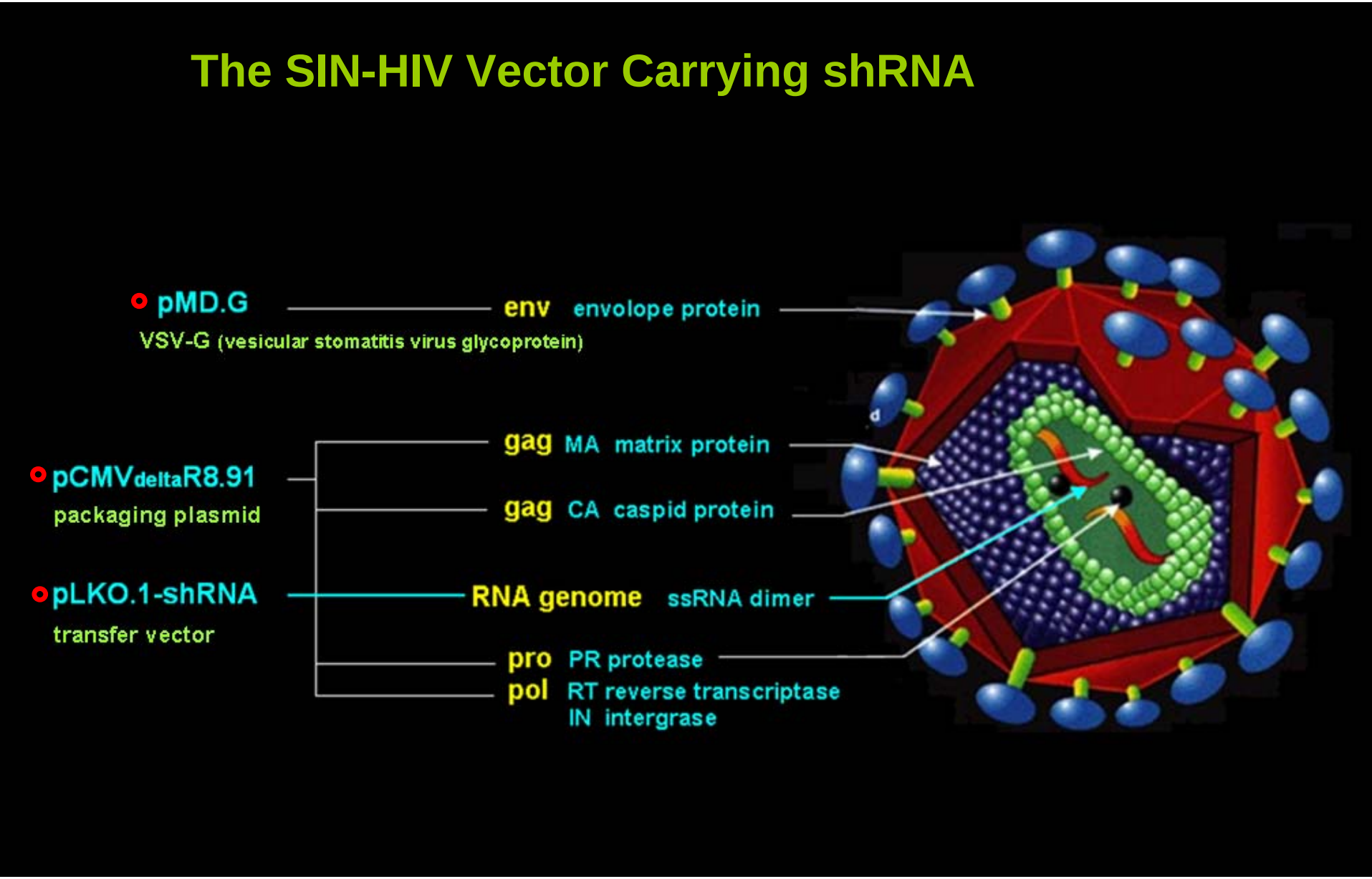
Tat accelerate the virus production by binding to the first 59 nucleotide TAR (Transactivator Acitve Region) of the virus genome thus encouraging the transcription of the remainder of the genetic code.

VSV-G

Encodes vesicular stomatitis virus glycoprotein, a viral membrane protein from vesicular stomatitis virus which has a broad spectrum of host and is able to infect almost all cell types.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

The SIN-HIV Vector Carrying shRNA



Jing-Ying Huang
RNAi Core R&D Group Member 2008

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.
- So, is there any other doubts?

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Advantages of TRC Lentivirus-based shRNA library

- Broad host range from insect, fish to mammal.
- Being able to transduce non-dividing cells. Integrate into host genome.
- Bearing drug selection marker, exclude the interference of non-transduced cells.
- Provide at least 5 different shRNA constructs for each gene.
- Much more economic than commercially available ones.

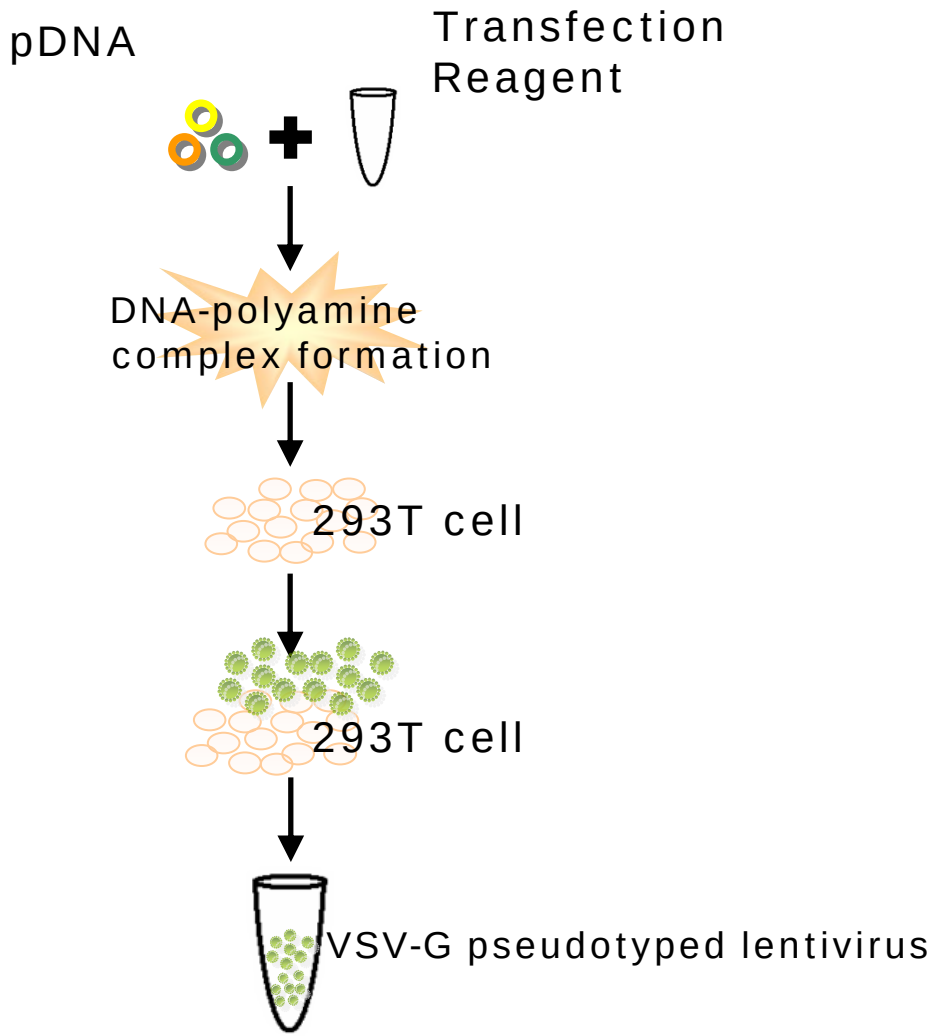
Jing-Ying Huang
RNAi Core R&D Group Member 2008

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.
- So, is there any other doubts?

Jing-Ying Huang
RNAi Core R&D Group Member 2008

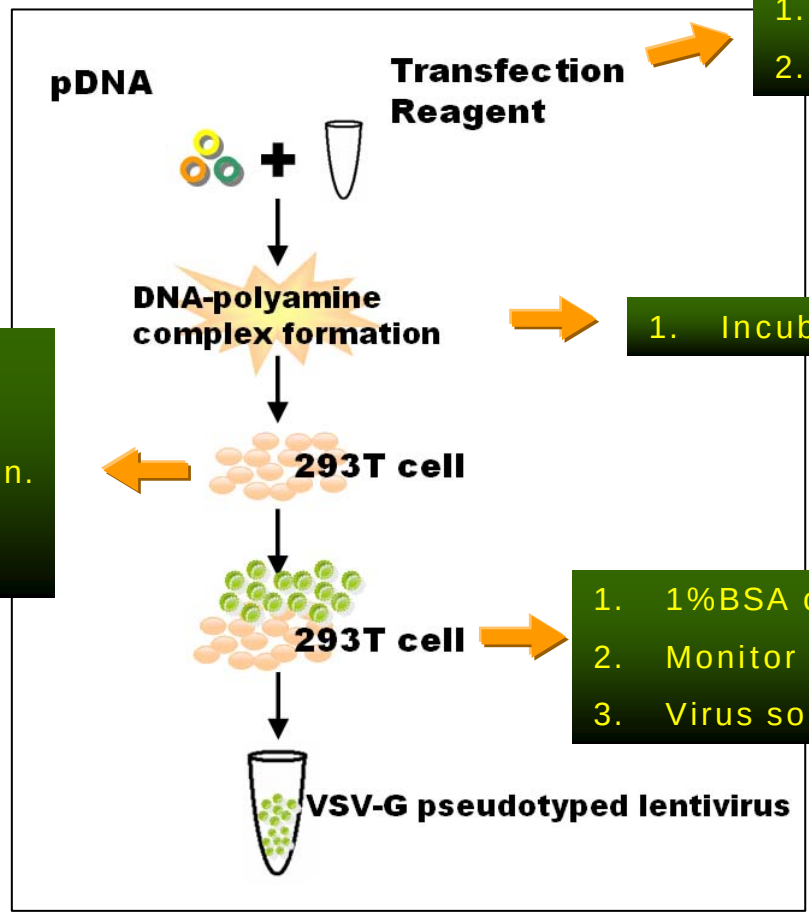
VSV-G psuedotyped Lentivirus Production



Jing-Ying Huang
RNAi Core R&D Group Member 2008

VSV-G psuedotyped Lentivirus Production

1. pDNA ratio
2. pDNA quality
3. Positive controls



1. Storage
2. Mix before use

1. Incubation time is critical

1. Passage number
2. Cell health condition
3. Confluence at transfection.
4. Low antibiotics con. at transfection.

1. 1%BSA containing medium
2. Monitor transfection efficiency.
3. Virus soup collection time point.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Special Notification-1

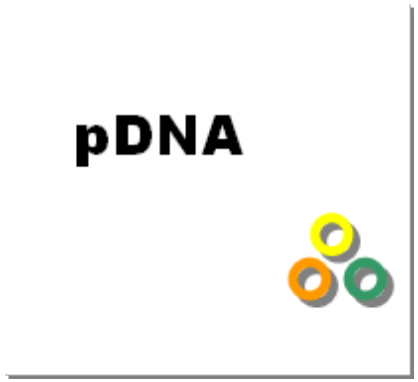
< pDNA ratio >

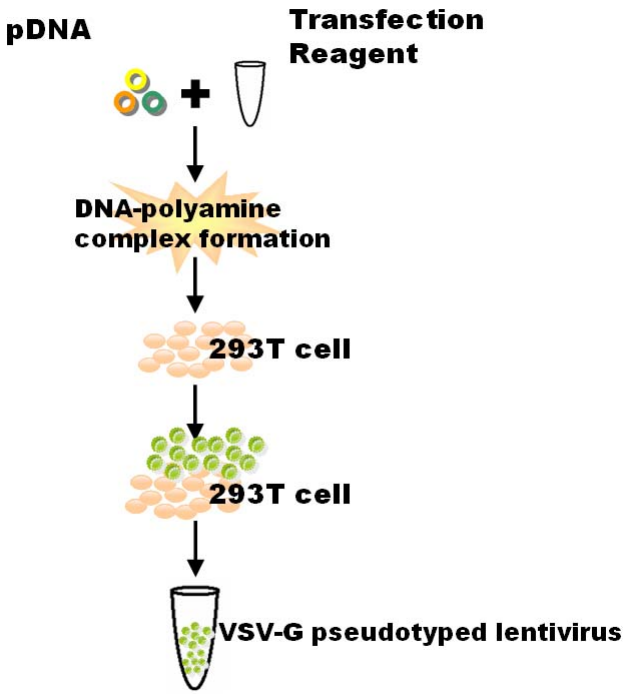
shRNA plasmid	pLKO.1-hairpin vector ------	10X
packaging plasmid	pCMV-△R8.91 (gag, pol, rev, tat) ----------	10X
envolope plasmid	pMD.G-- (VSV-G) ----------	1X

< pDNA quality >

1. Salt and phenol contamination would decrease transfection efficiency.

2. Endotoxin can lead to decreased virus titer.





< Positive controls >

1. Transfection efficiency control
   ~ pAS2.EGFP.puro ~

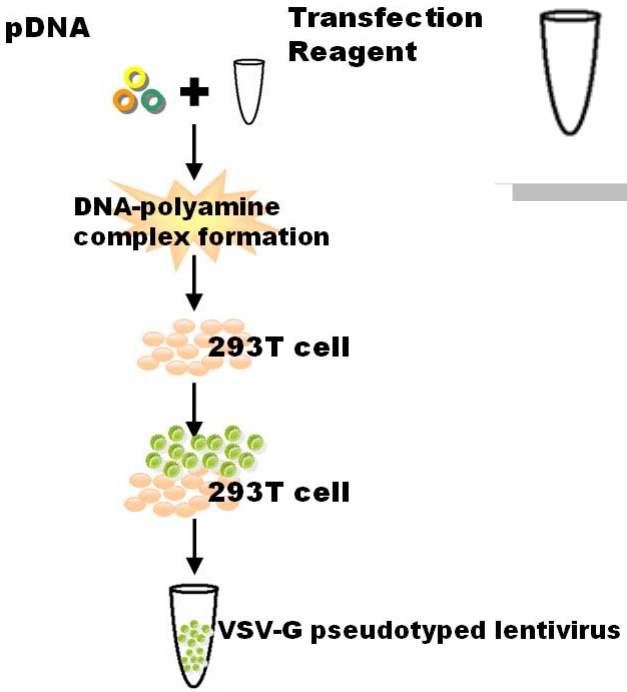
2. Successful virus production control
   ~ pLKO.1 (shLuc) & pAS2.EGFP.puro ~

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Special Notification-2

< Storage >

Mirus LT-1 **should be frozen in -20℃,**
while *Fugen 6* should be store at 4℃.



< Mix before use >

Make sure the reagent is homogeneous, otherwise this will result in low transfection efficiency.

< OPTI-MEM >

Use reduced serum OPTI-MEM in complex formation step to minimize interference.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

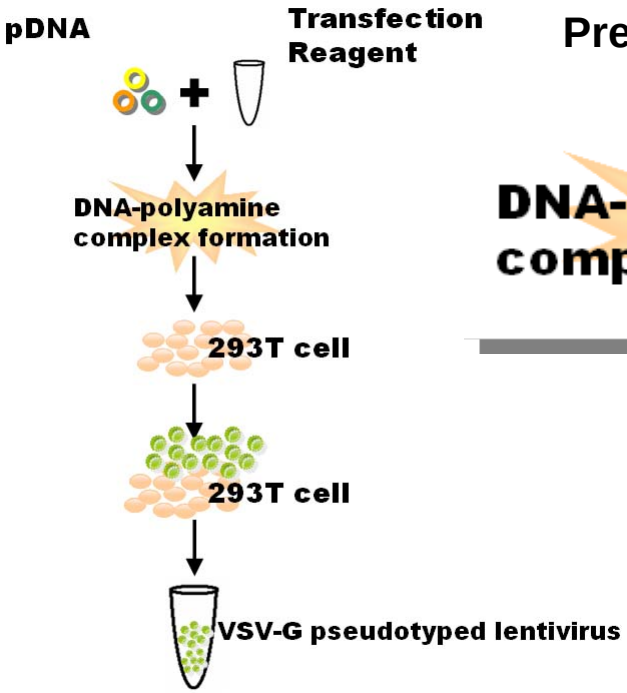
Special Notification-3

< Two-step mix of DNA and transfection reagent >

(Premix-1) pDNA and OPTI-MEM, mix thoroughly.

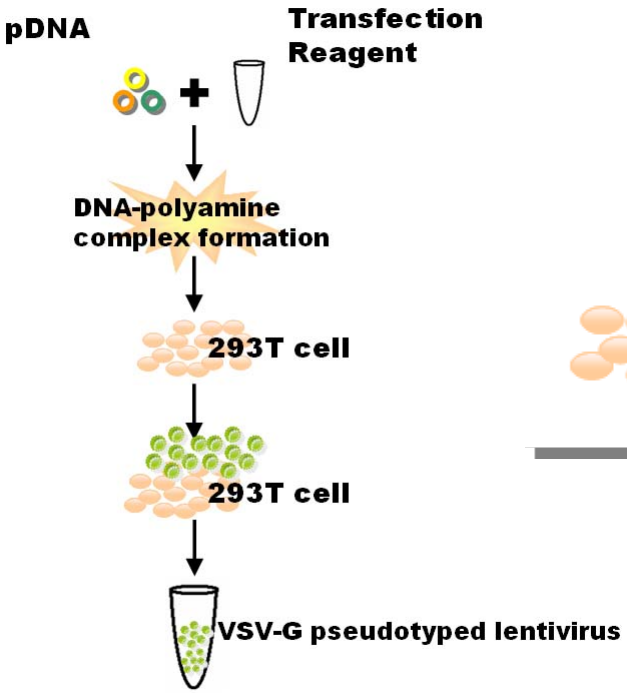
(Premix-2) LT-1 and OPTI-MEM, mix thoroughly.

Premix-1 + Premix-2 → RT incubate for 20 mins.



Jing-Ying Huang
RNAi Core R&D Group Member 2008

Special Notification-4



< Cell passage number >

No more than p10

< Cell health condition >

No overgrow, most cell show good adherent condition.

< Seeding Cell >

Cell confluence should be around 50~70% at transfection day.

< Seeding Cell >

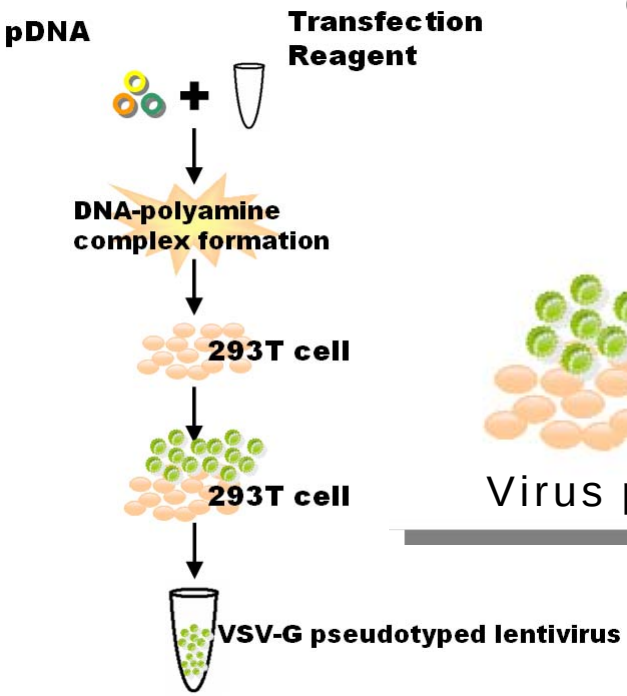
Use low-antibiotic medium : 0.1XP/S, 10% FBS, DMEM.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Special Notification-5

< 1% BSA containing medium >

1) Empirically, virus titer is positively correlated with BSA concentration.



Virus production

< Virus soup collection time point >

1) Change to 1% BSA medium 16 hrs post transfection.

2) Collect virus soup 40 & 64 hrs post transfection.

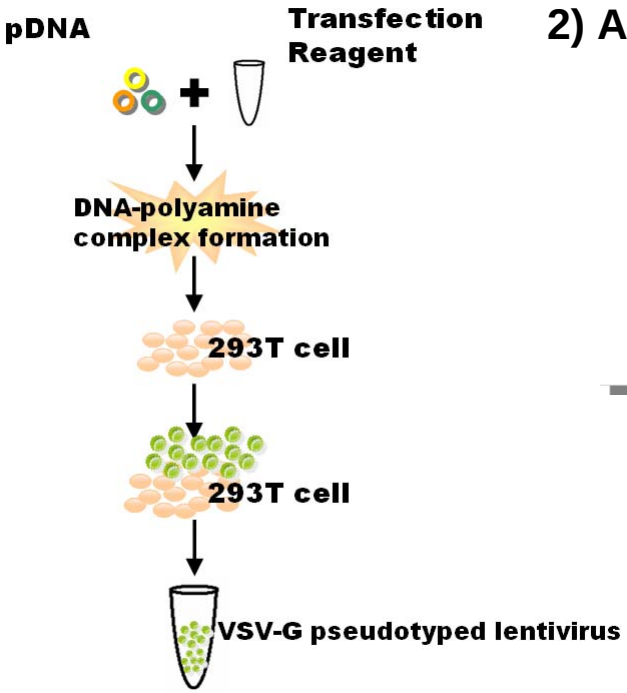
< Monitor transfection efficiency & Cell viability >

1) Monitor EGFP well, make sure there is no problem in transfection procedure.

2) shRNA targeting gene might be important survival factor for 293T cell

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Special Notification-5



< Virus storage >

1) Store at -80 ℃.
2) Avoid repeated freeze and thaw.

Virus soup collection

< Virus titering using A549 following RNAi Core titering protocol>

1) If shLuc virus is successfully produced, then the whole process is no problem.

2) Low virus titer trouble shooting.

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Virus Production in Different Scale

average titer ~ 1×10^7 RIU/mL

2×10^4	Seeding number	8×10^5
96-well	Well format	6 cm dish
100 ng	pCMV-Δ8.91	2.25 ug
10 ng	pMD.G	0.25 ug
100 ng	pLKO	2.25 ug
7 uL	OPTI-MEM	250 uL
0.6 uL	LT-1	15 uL
14.4 uL	OPTI-MEM	250 uL
340 uL	Virus soup volume	10 mL

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.
- So, is there any other doubts?

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Causes of Low Virus Titer

Technical Causes

① **293T cell**
- ① Cell confluence
- ② Cell health condition
- ③ Passage number

② **Transfection process**
- ① Wrong pDNA ratio
- ② Wrong DNA-LT1 ratio

③ **pDNA quality**
- ① Salt / organic solvant incomplete removal
- ② Endotoxin contamination
- ③ Nicked pDNA

④ **Transfection media contain antibiotics**

⑤ **Viral supernatant harvested too early or too dilute**

⑥ **Fail in Virus titering**

Intrinsic Causes

① **shRNA knockdowned gene is crucial in the following process during virus production:**
- ① 293T survival
- ② Virus packaging process

② **shRNA knockdowned gene is crucial in the following process during virus titering:**
- ① A549 survival

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.
- So, is there any other doubts?

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Materials:

1) Packaging plasmids from NRC (pDNA)→ competent cell
2) shRNA clones from NRC (glycerol stock)
3) Transfection grade plasmid DNA preparation gear
4) 293T cell for virus packaging
5) Transfection reagent
    1) Mirus LT-1
    2) OPTI-MEM
6) Cell-culture grade BSA
7) 293T culture medium (1× P/S, 10% FBS, DMEM)
8) 293T culture medium (0.1× P/S, 10% FBS,DMEM)
9) 293T culture medium (1% BSA, 1× P/S, 10% FBS, DMEM)

Jing-Ying Huang
RNAi Core R&D Group Member 2008

< DAY1 >	< DAY2 >	< DAY3 >	< DAY4 >	< DAY5 >
Seeding cell	**Transfection**	**Change media**	**Virus soup collection-1**	**Virus soup collection-2**
! No more than p10	! Cell confluence around 50~70%	! 16 hrs post transfection	! 40 hrs post transfection	! 64 hrs post transfection
	! Low antibiotic medium	! 1% BSA medium	! Monitor transfection efficiency	! Store virus soup at -80℃.
	! Transfection control & Production success control		! Monitor Cell survival	! Centrifugation removal of cell debris is optional

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Test Transduction Condition in Parallel

Search literature for

1. infection condition
2. gene expression level

Transduction condition setup:

- Cell seeding number
- Puromycin killing curve determination
- Polybrene toxicity / transduction efficient con. determination
- Centrifugation or incubation overnight
- Standard curve construction using A549 and your experimental cell line → get converting factor
- use fine-tuned condition for your experiment
  - RNAi machinery competition control
    shLuc or shLacZ (choose from CTR01 plate)
  - Use the same MOI while end-points are going to be compared in parallele.

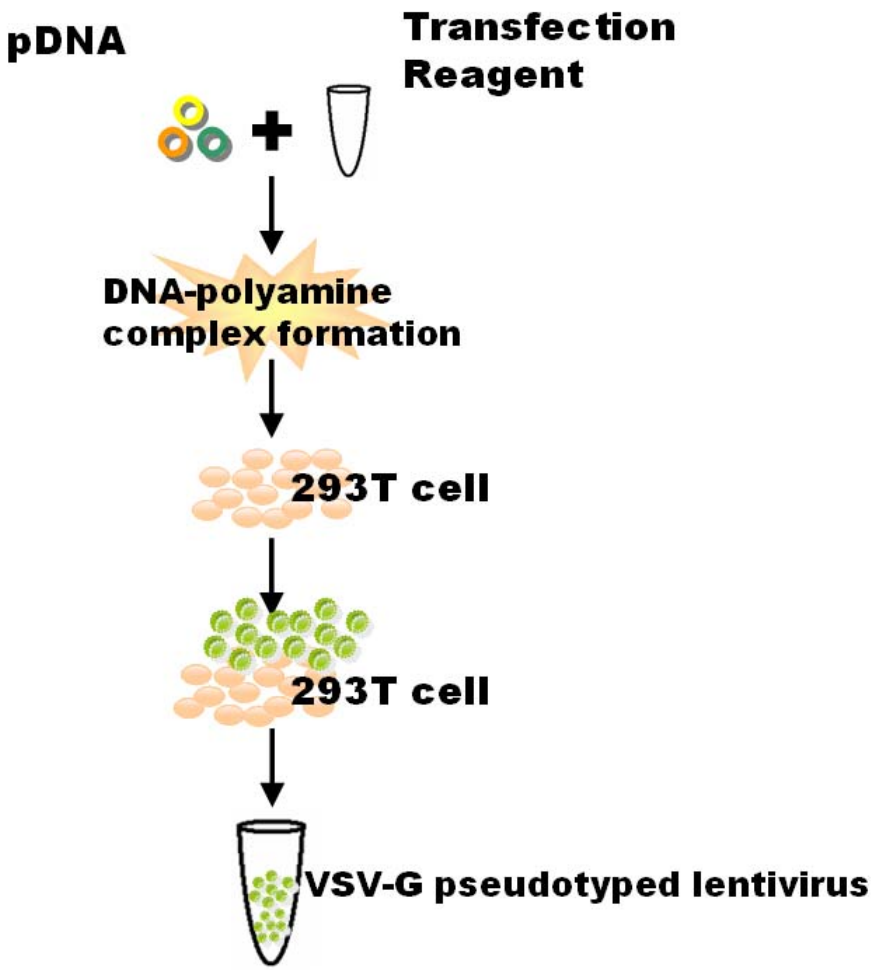
Jing-Ying Huang
RNAi Core R&D Group Member 2008

Lentivirus Production Session

- Lentivirus!!!
  Is it health threatening to lab technician?
- What's so good about this RNAi library?
- How to produce VSV-G pseudotyped lentivirus?
- Trouble shooting – the causes of low titer.
- Efficient way to set up lentivirus production system in your lab.
- So, is there any other doubts?

Jing-Ying Huang
RNAi Core R&D Group Member 2008

Thank you for your attention



Jing-Ying Huang
RNAi Core R&D Group Member 2008

User workflow

Biosafety poster session

Understanding how to operate lentiviral vectors safely.

RNAi core workflow poster session

Genes of interest

↓

Search database for shRNA clones targeting those genes

↓

Sent application form

↓

Get shRNA clones in the form of bacteria glycerol stock

↓

Get shRNA clones in the form of bacteria glycerol stock

Amplify bacteria

↓

pDNA extraction

↓

Virus production session

Virus production

↓

Virus titer determination session

Virus titering on A549

↓

Converting virus titer to your cell line

↓

Lentiviral-based shRNA knockdown experiment (Candidate subset screening)

↓

shRNA experiment design session

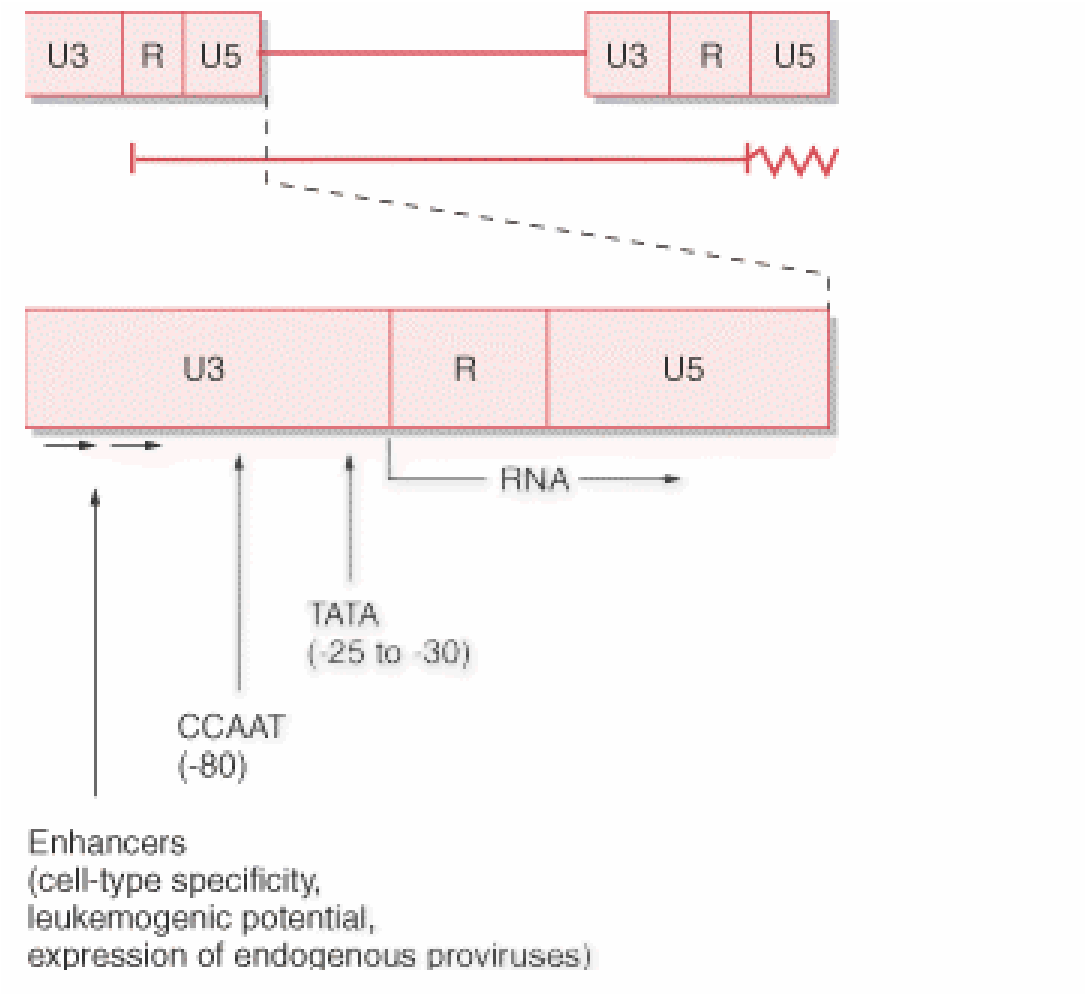
Confirm study by rescue experiment using lentiviral-based cDNA expression

↓

Customized shRNA sequence design

Jing-Ying Huang
RNAi Core R&D Group Member 2008

LTR: Long terminal repeat



Jing-Ying Huang
RNAi Core R&D Group Member 2008