shRNA experiment design

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Core Manager

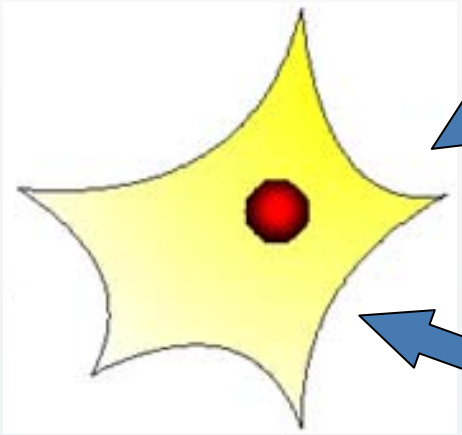
Comparison of siRNA and shRNA

- ✓ **Structure**
  - **siRNA (19-27 nts)**

  - **shRNA (19-27 nts)**

- ✓ **Advantage vs. disadvantage**
  - **cost**
  - **delivery efficiency**
  - **duration of knockdown**
  - **renewability**

Long dsRNAs trigger non-specific silencing in mammalian

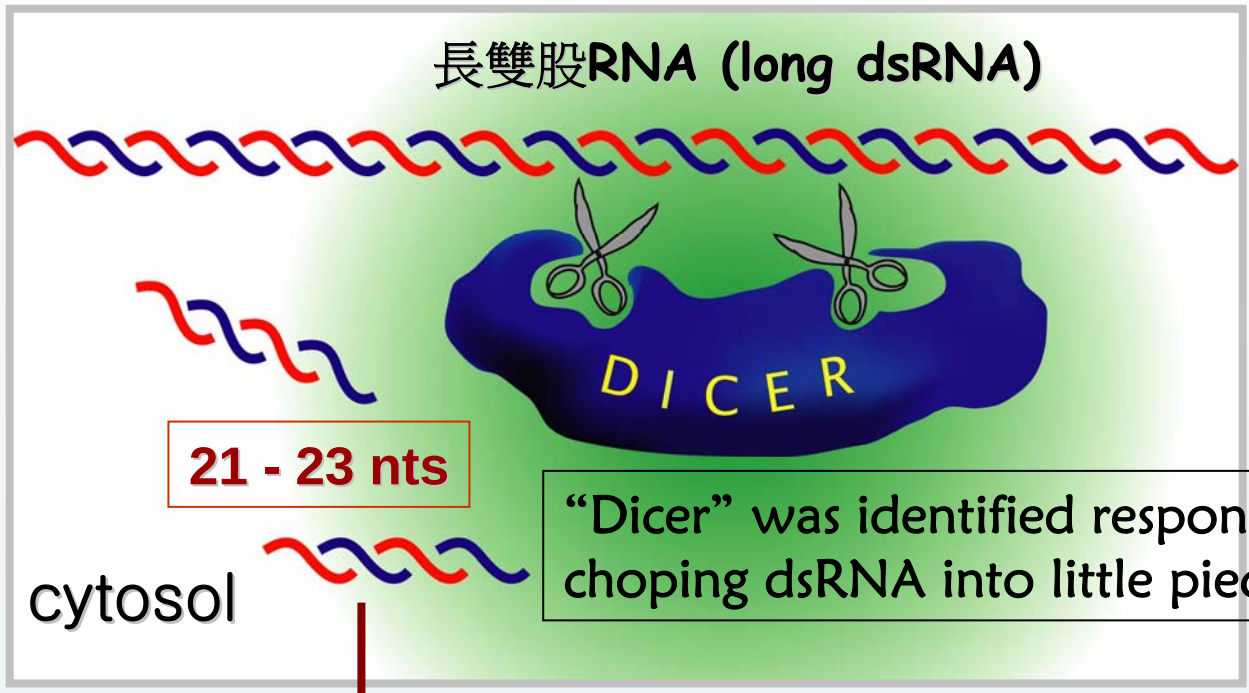


dsRNA
- ✓ C. elegans
- ✓ Drosophila

In mammal?

IFN response in mammalian system---global gene silencing

RNAi Effective Molecule



small-interfering RNA; siRNA

Nature. 2000;404:293-6; Nature. 2001;409:363-6

Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells

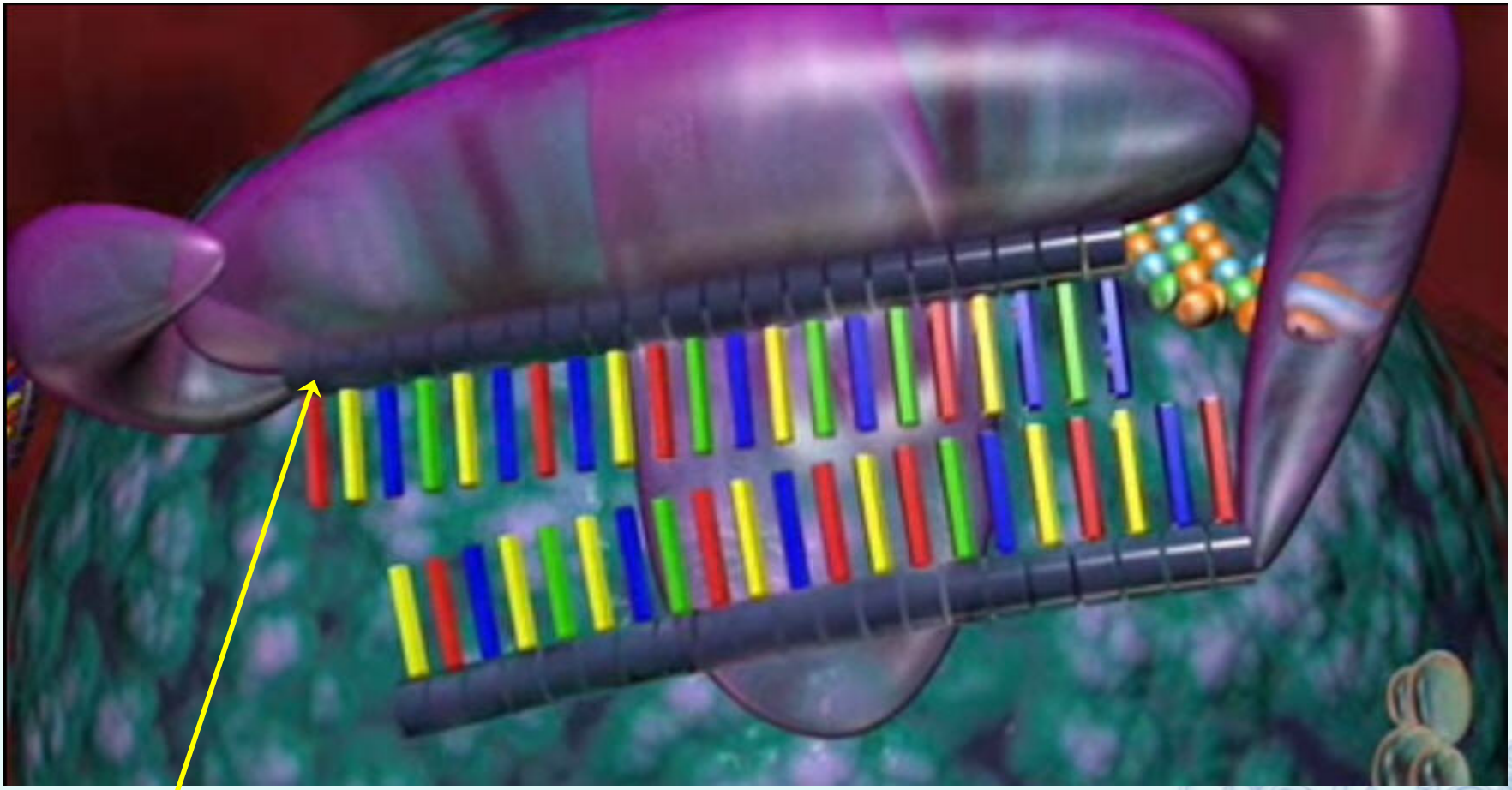
Sayda M. Elbashir[1], Jens Harborth[2], Winfried Lendeckel[1], Abdullah Yalcin[1], Klaus Weber[2] and Thomas Tuschl[1]

Department of Cellular Biochemistry; and Department of Biochemistry and Cell Biology, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

Worked with Dharmacon to offer this know-how to the public

Nature **411**, 494-498 (24 May 2001)

Strand bias selection during upload of siRNA

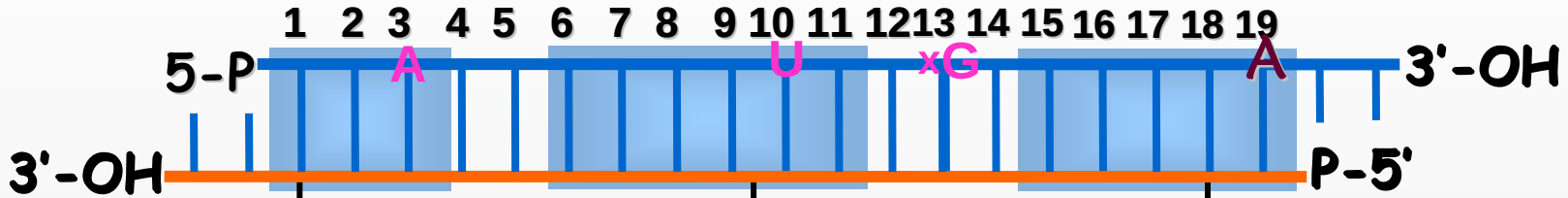


Guide/ antisense strand

http://www.pbs.org/wgbh/nova/sciencenow/3210/02.html

siRNA design: from empirical to rational

19-nt duplex region



High thermal stability of the 5' sense strand (SS) blocks incorporation of SS into RISC. ***G or C is preferred.***

Low thermal stability of the 5' anti-sense strand (AS) promotes Incorporation of AS into RISC. ***AU rich is suggested.***

Low stability in this region enhances RISC/AS-mediated cleavage of mRNA and promote RISC complex release. ***U at position 10 at SS is recommended.***

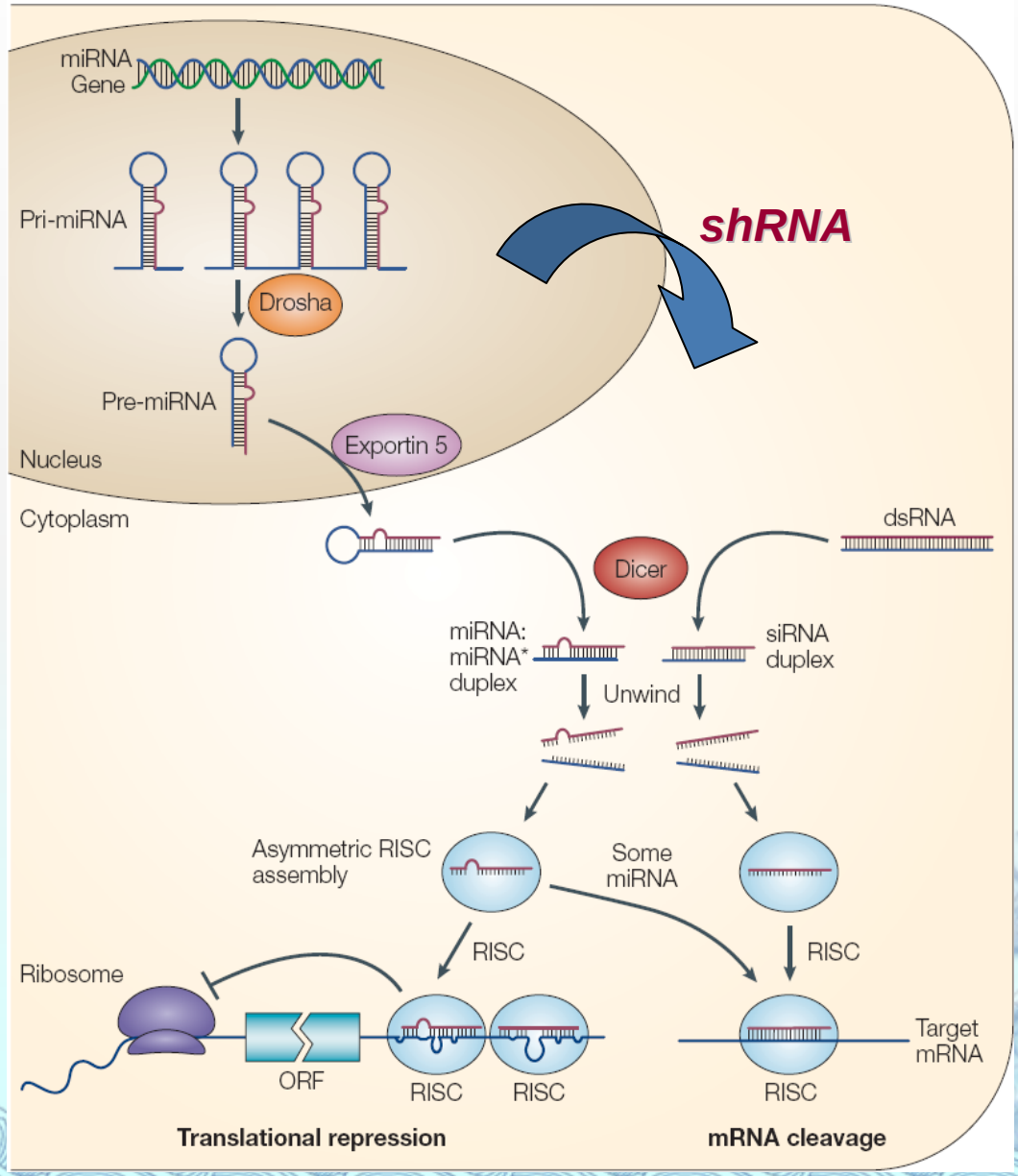
— Sense strand — Antisense strand

Renolds et al. 2003

Other considerations

- ✓ no high GC content (35-65%);
- ✓ no inverted repeat sequence;
- ✓ no consecutive 3 Gs or 3 Cs;
- ✓ no consecutive 4 Ts if use pollll promoter;
- ✓ mRNA secondary structure?

Biogenesis of mi/siRNAs

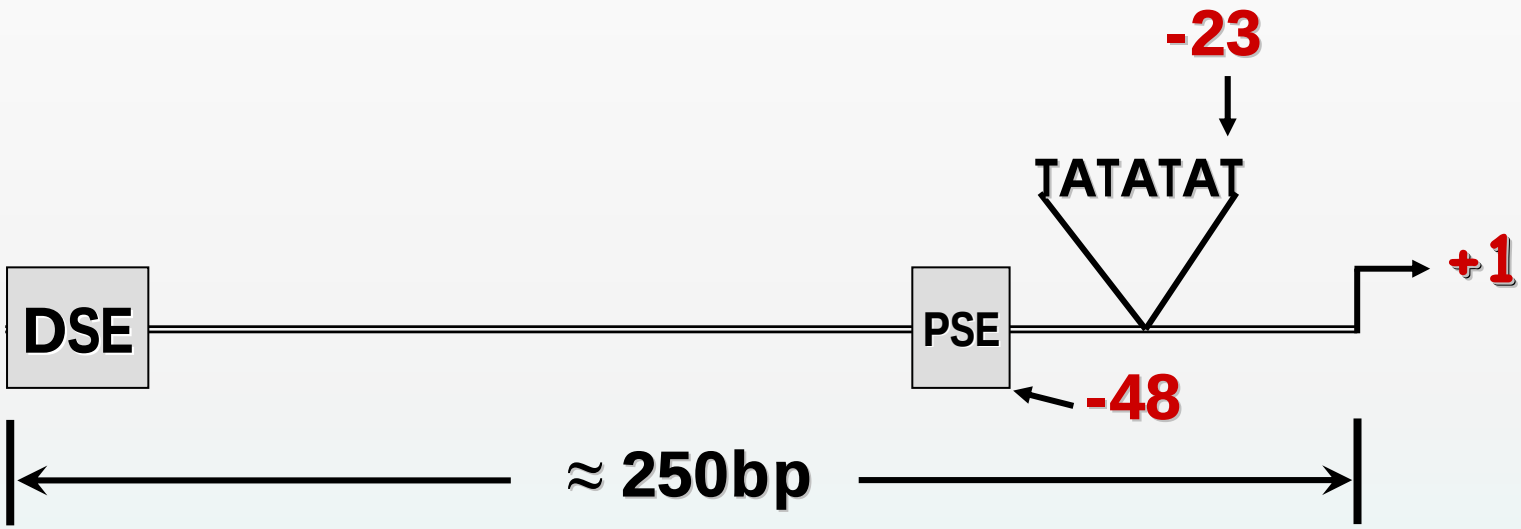


Nature Review: 5: 522, 2004

Expression of Hairpin RNA (shRNA) Using Pol III Promoters

- ✓ **Transcription initiation of DNA-dependent RNApol III promoters (U6 or H1) are well characterized. RNApol III transcription uses a well-defined termination signal (TTTTT) and the products have no extra sequence.**
- ✓ **Transcription from these promoters is very efficient in various tissues.**

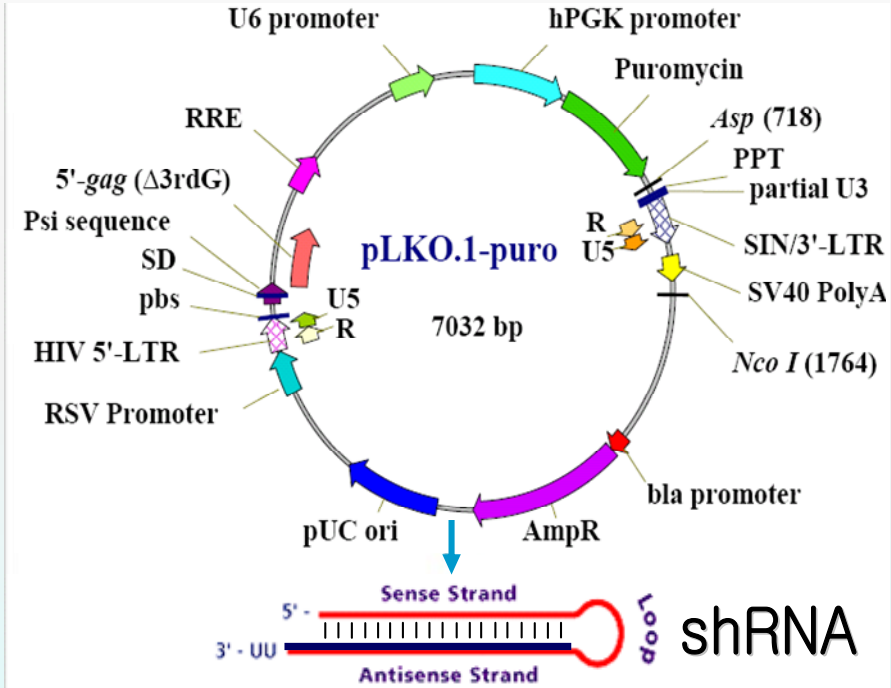
Configuration/Structure of hU6 Promoter



In vivo synthesis of siRNA through shRNA

siRNA

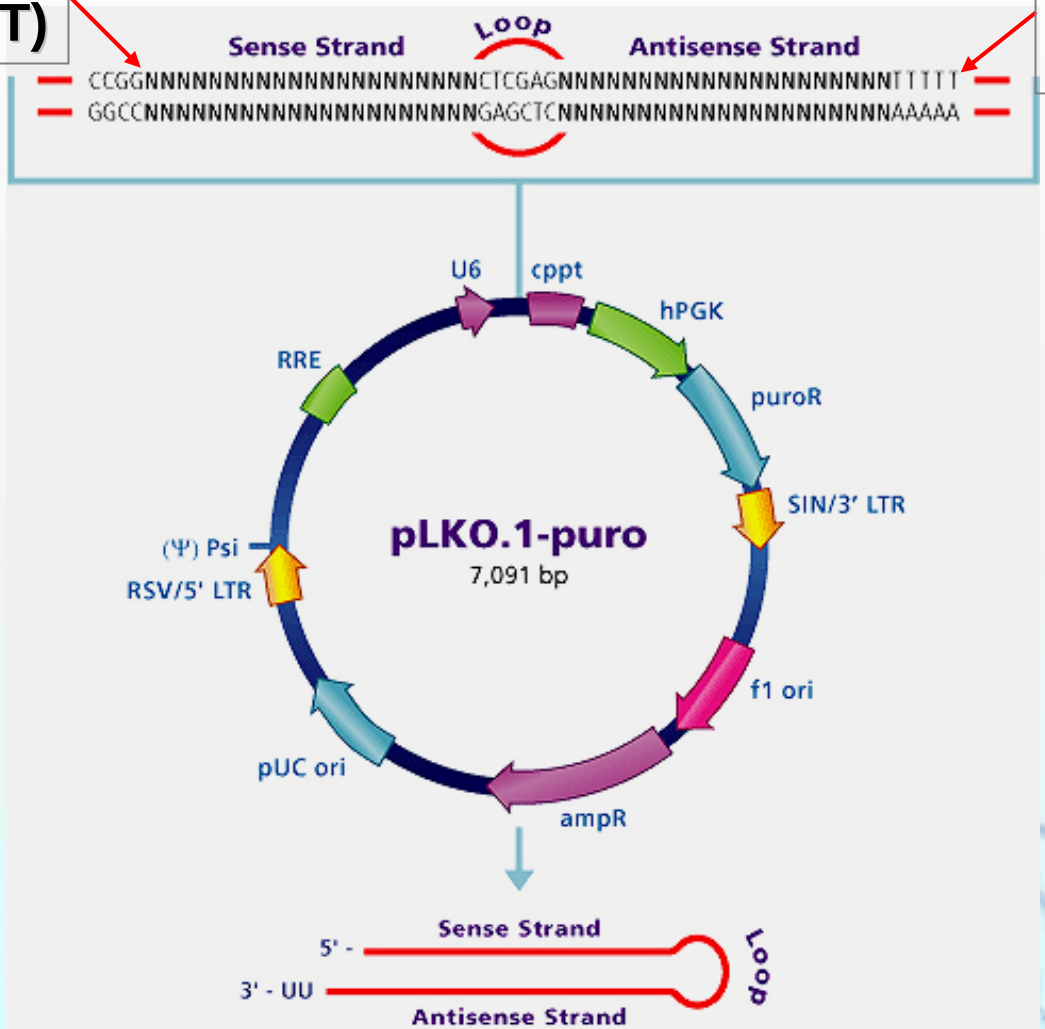
RNAi machinary



Vector Used by TRC/RNAi Core

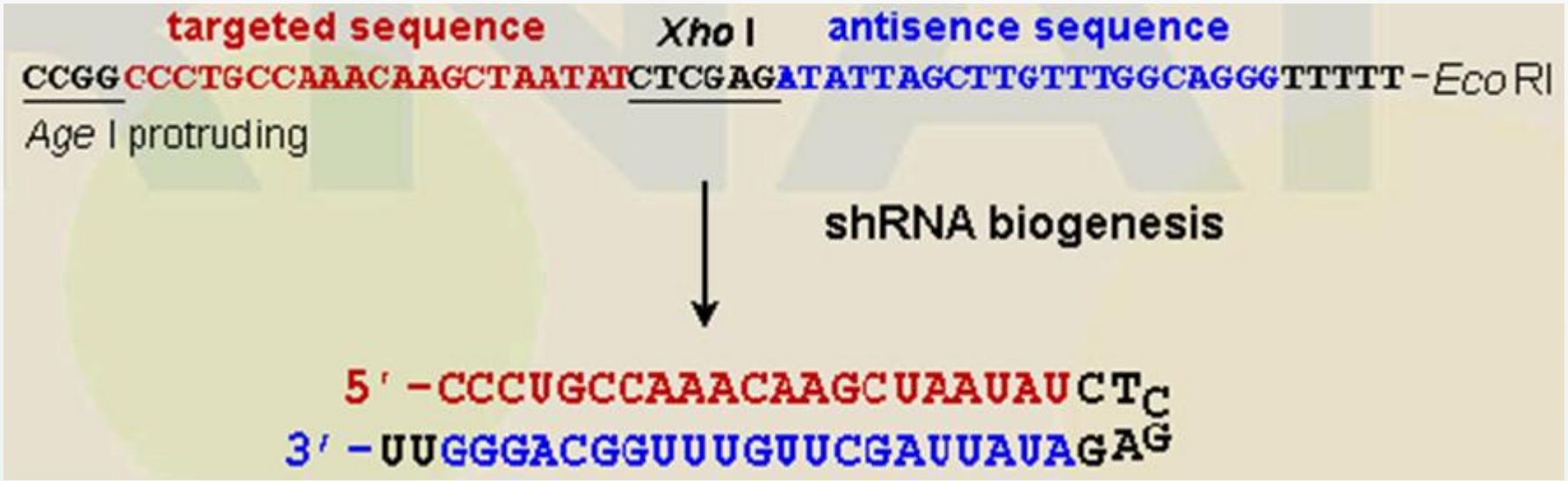
AgeI (ACCGGT)

EcoRI (GAATTC)



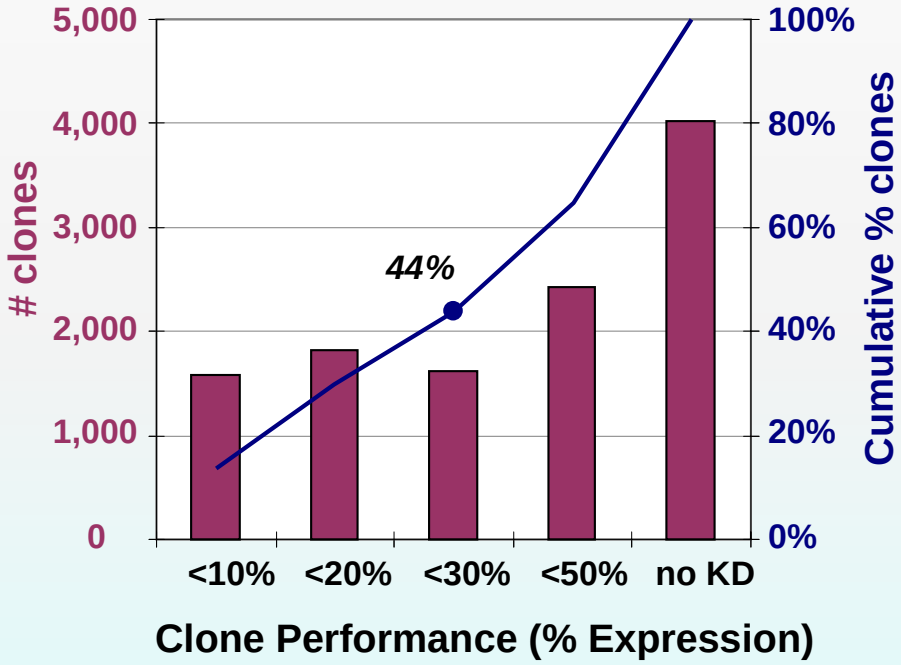
http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Functional_Genomics_and_RNAi/Product_Lines/shRNA_Library.html

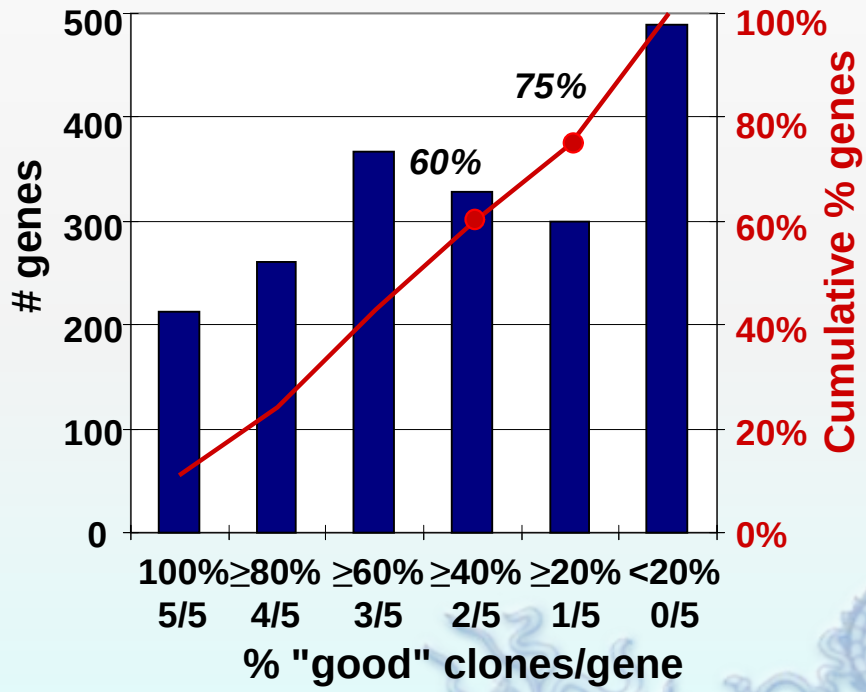
shRNA Structure of TRC RNAi



Library performance-I

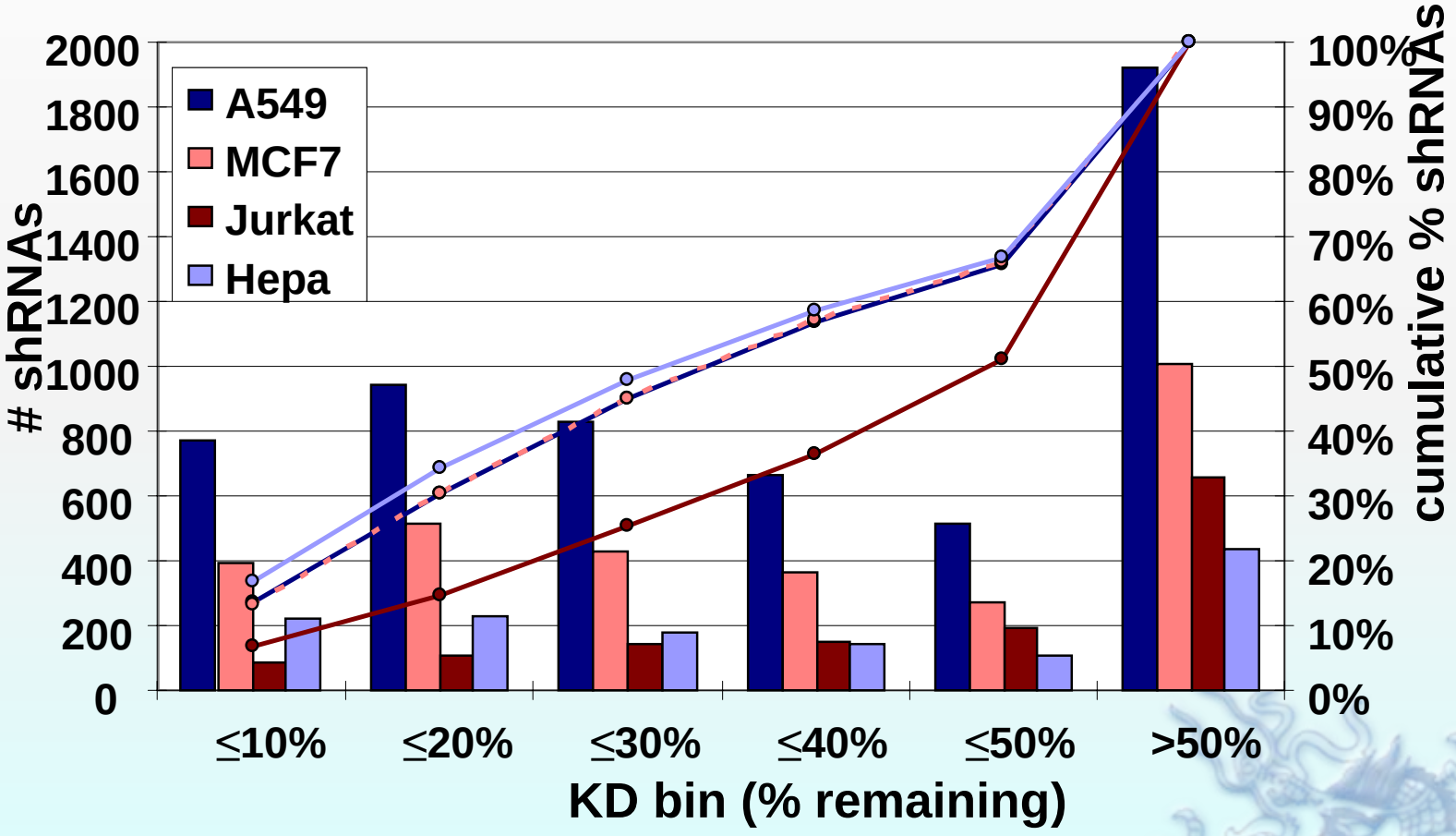
(11,466 TRC shRNAs targeting 1,956 genes)



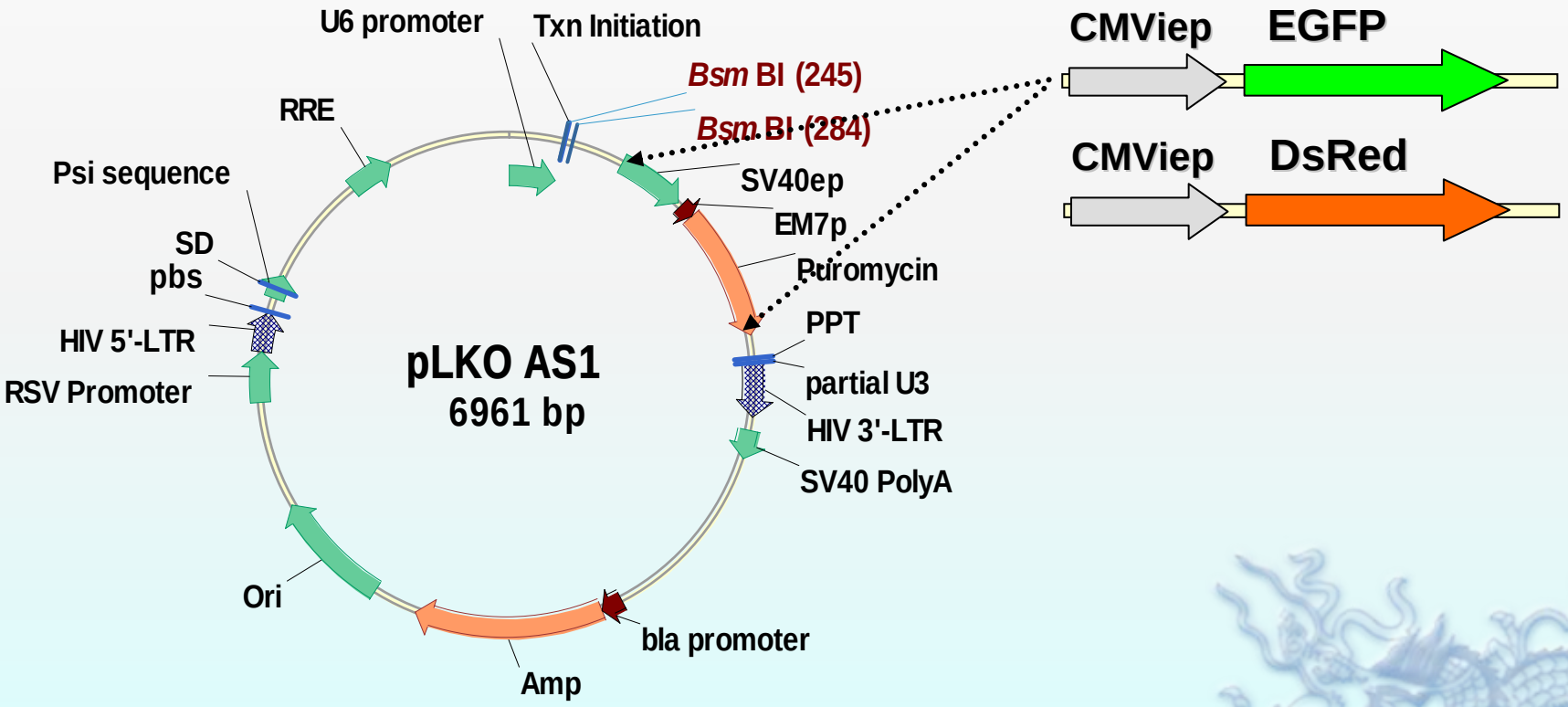


Library performance-II

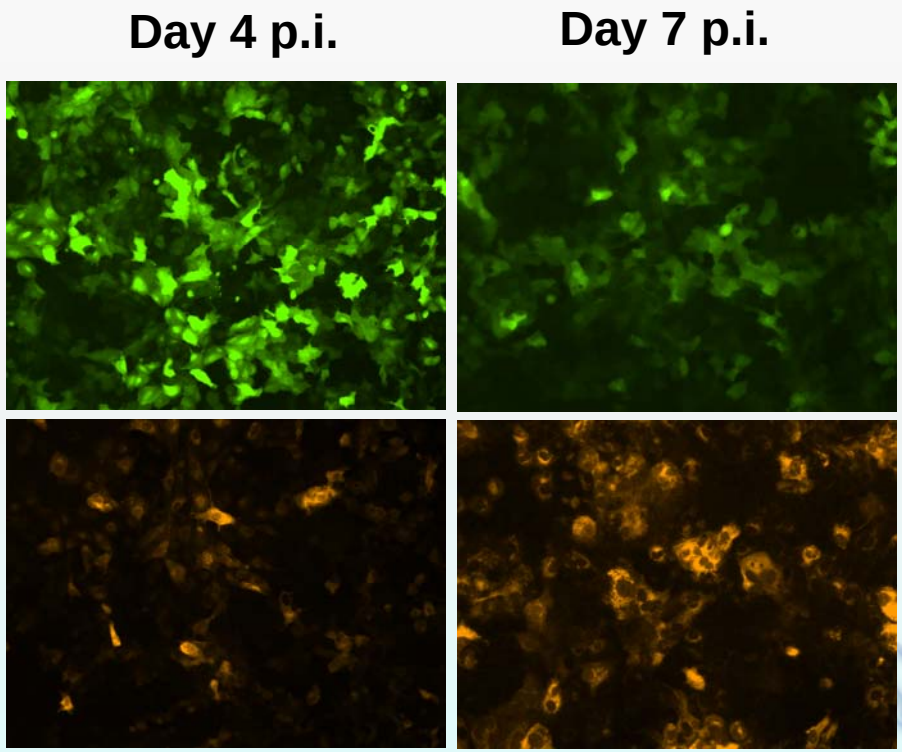
(11,466 TRC shRNAs targeting 1,956 genes)



shRNA vector provided by RNAi Core



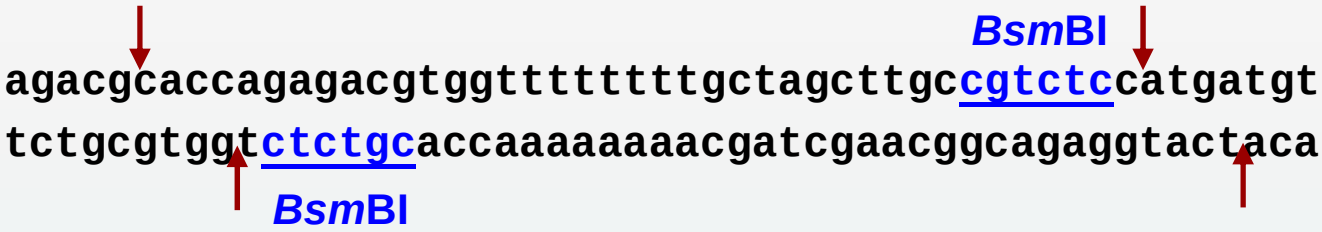
shRNA Lentiviral transfer vectors co-expressing fluorescence protein



How to Insert miRNA or shRNA into pLKO_AS1 Vector

A PCR-Based Cloning Method:

*Bsm*BI-digested pLKO_AS1 vector will produce two 5'-protruding ends with different sequences.



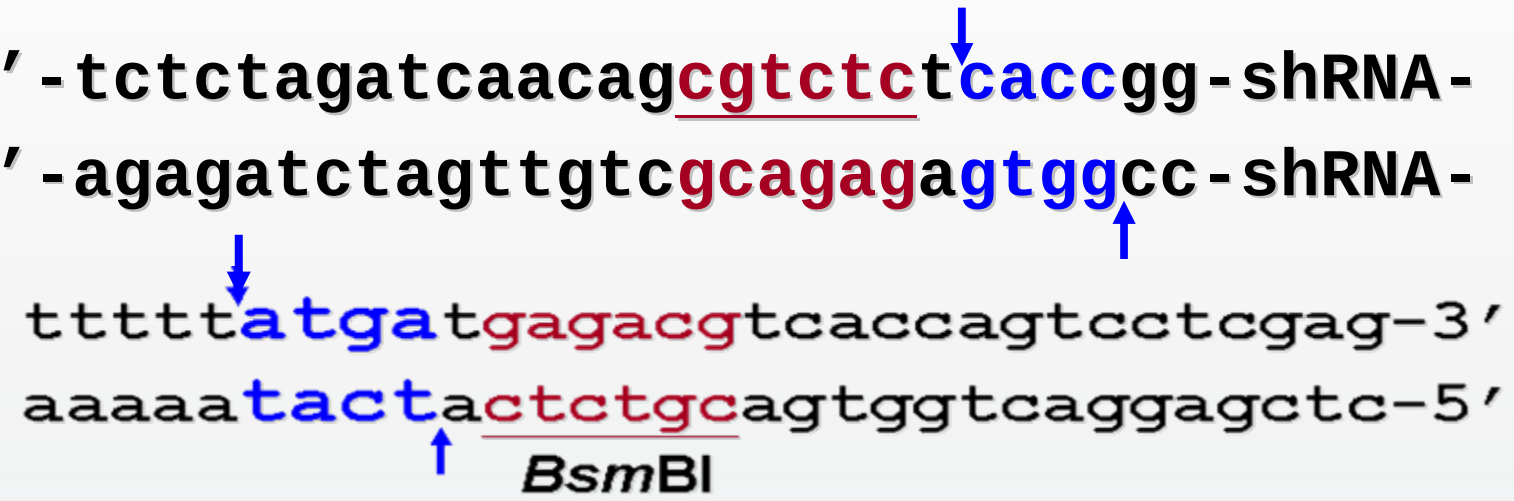
***Bsm*BI digestion**

```
agacg      caccagagacgtggttttttttgctagcttgccgtctcc          atgatgt
tctgcgtgg      tctctgcaccaaaaaaaacgatcgaacggcagaggtact          aca
```

Vector left arm | **Replaced by shRNA** | **Vector right arm**

How to Design Oligonucletides for PCR

shRNA sequence followed by TTTTT is flanked with common 5' and 3' end sequences that include two *Bsm*BI recognition sites (*Bsm*BI-restricted PCR fragments will ligate to *Bsm*BI-restricted pLKO_AS1 vector.



shRNA: antisense sequence – loop – sense sequence

Loop sequence: `tgagtagattagcaat`

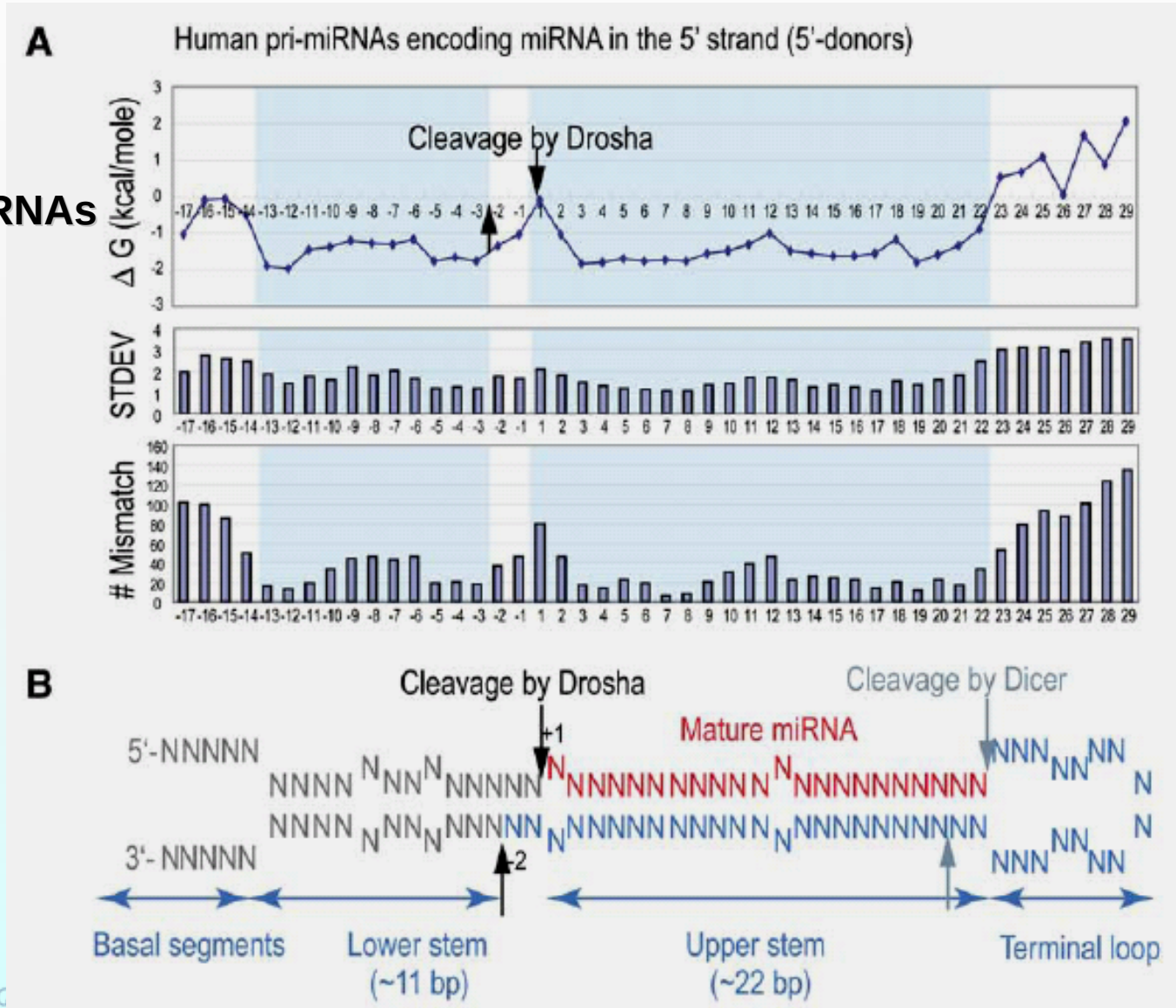
Primer design:

mir-all/F: 5′-tctctagatcaacagcgtctc-3′

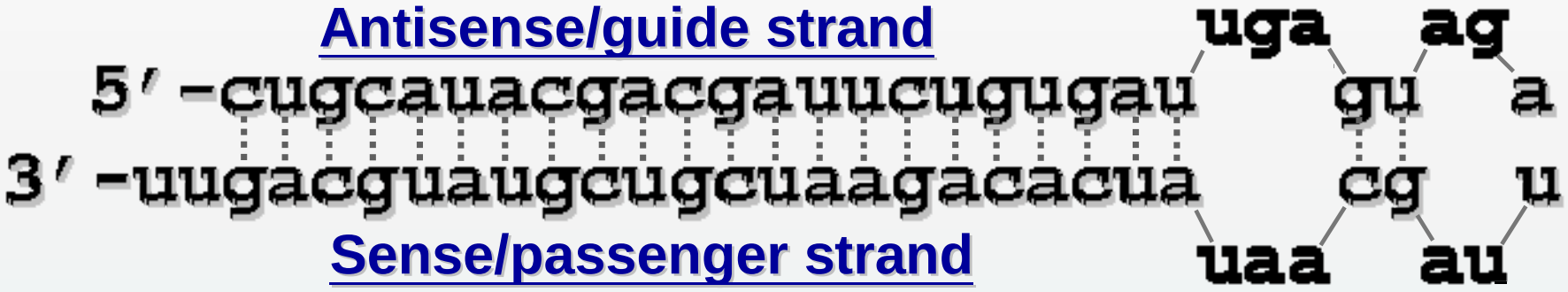
mir-all/R: 5′-ctcgaggactggtgacgtctc-3′

Thermodynamic Stability Profiling of Human pri-miRNAs – 5'-Donors

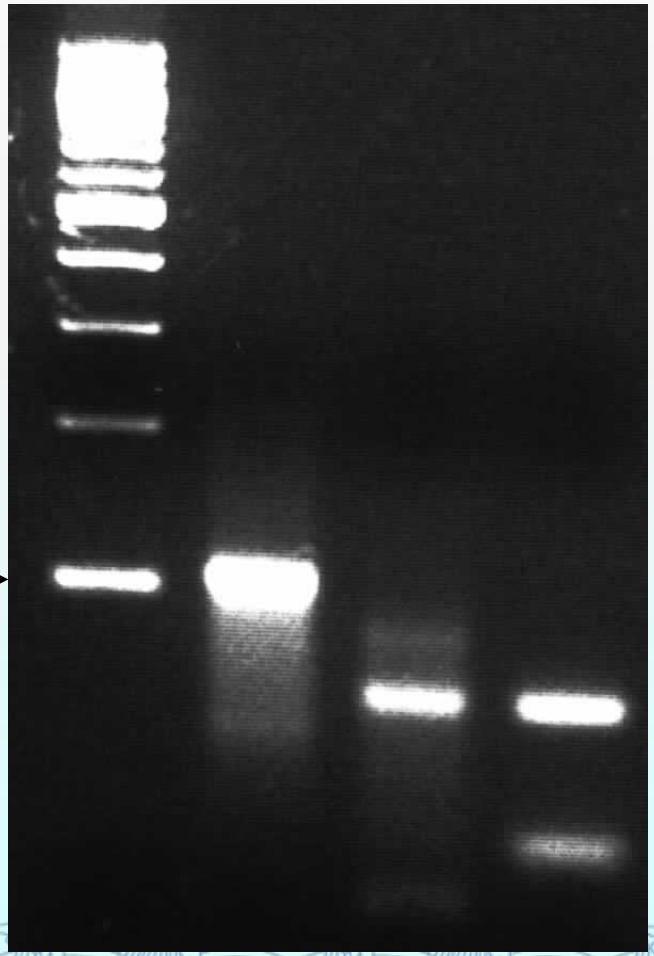
157 pri-miRNAs



shRNA structure with hmiRNA loop sequence



Agarose Gel Analysis of PCR Products Amplified from Hairpin Containing Insert



1. PCR products
2. *Bsm*BI-digested
3. Purified products (3% agarose)

Tips

Agarose:

NuSieve 3:1 agarose

DNA Purification Kit:

Qiagen MinElute Gel Extraction Kit

How to Insert miRNA or shRNA into pLKO.1 Vector (annealing method)

Oligo: 100μM sense & antisense (purified by PAGE)

Annealing:

sense	20μl	
antisense	20μl	
10X buffer	4.5μl	
↓		
95^{0}C	3min	1 cycle
80^{0}C	10min	1 cycle
decrease to 4^{0}C slowly		(0.01^{0}C / sec)

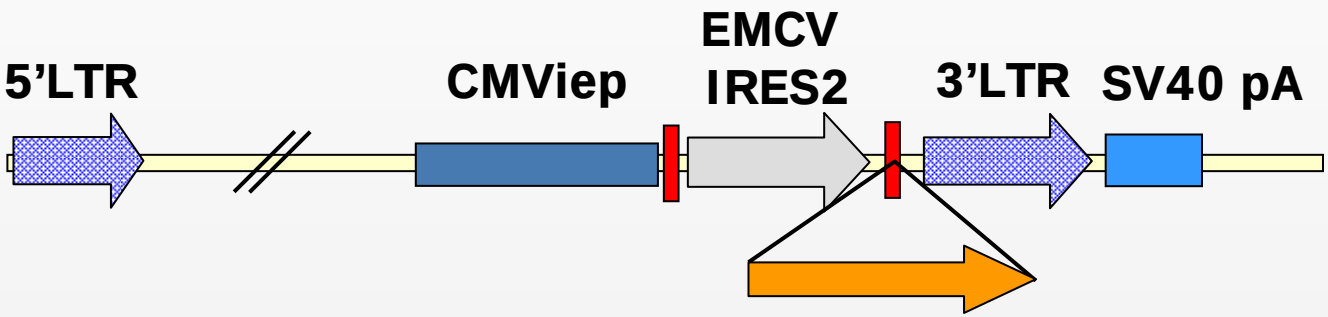
10X Annealing Buffer:
1M K-acetate
0.3M HEPES-KOH pH7.4
20mM Mg-acetate

↓

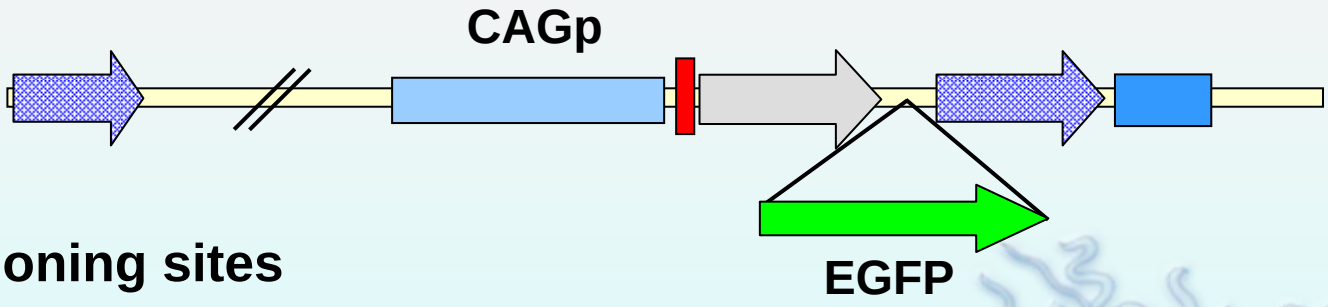
take 2μl of annealed oligos plus 50ng *AgeI*/*Eco*RI-restricted and pLKO.1 vector to perform ligation and transformation.

Maps of lentivirus-based cDNA expression transfer vectors

pLKO_AS2



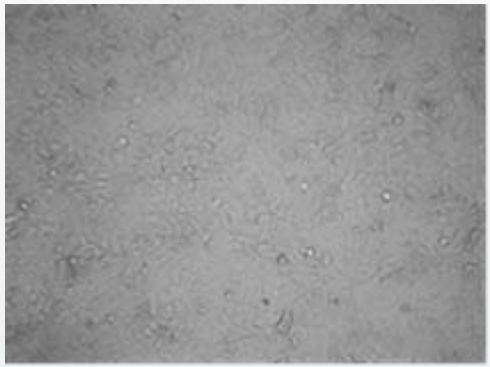
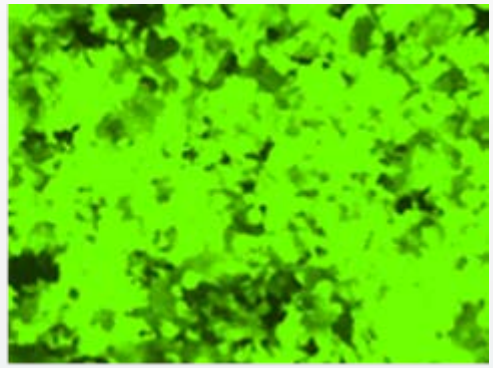
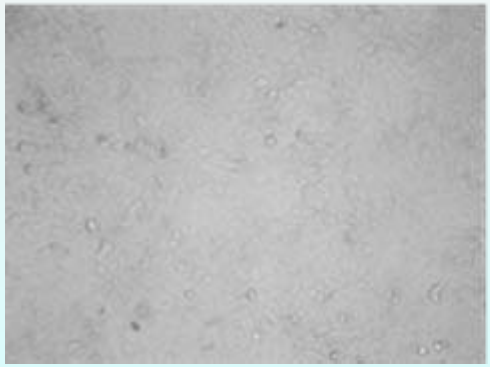
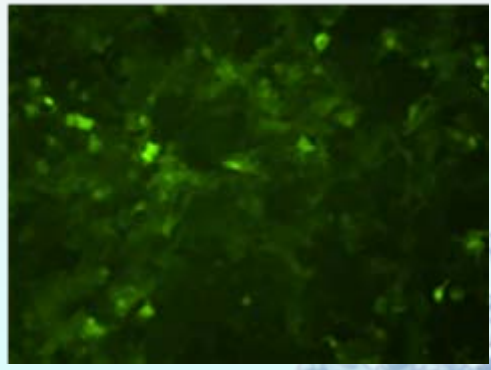
pLKO_AS3.1.EGFP3'



| = multiple cloning sites

CAG promoter: a chimeric promoter of CMV enhancer and beta actin promoter

GFP as a sorting marker for establishing stable line in transduced cells

	Phase	EGFP
AS.3.1.EGFP5’		
AS.3.1.EGFP3’		

Thank You!