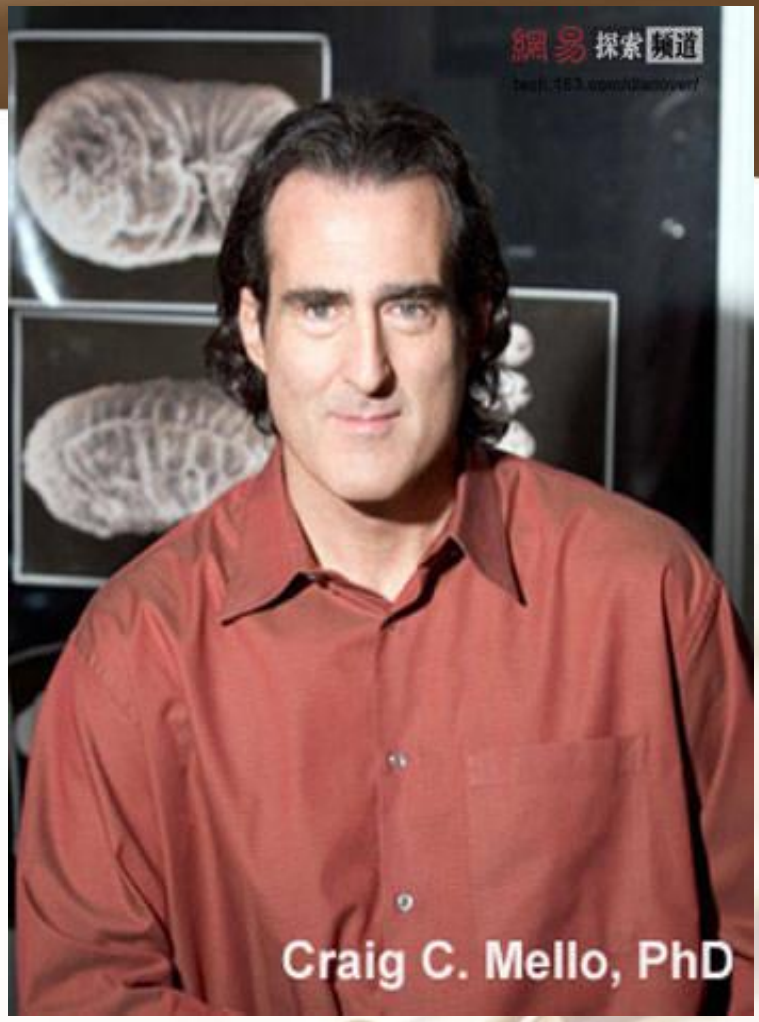
Company
LOGO

RNA interference (RNAi)

Jinqiang Hu

30th Oct, 2012


網易 探索頻道
Craig C. Mello, PhD

RNAi dsRNA induces homologous mRNA degradation, leading to repression of gene expression, is post transcriptional gene silencing (PTGS) phenomenon, is protection mechanism which by organism during evolution resists virus infection, defends repeated sequence, genome unstability caused by mutation..

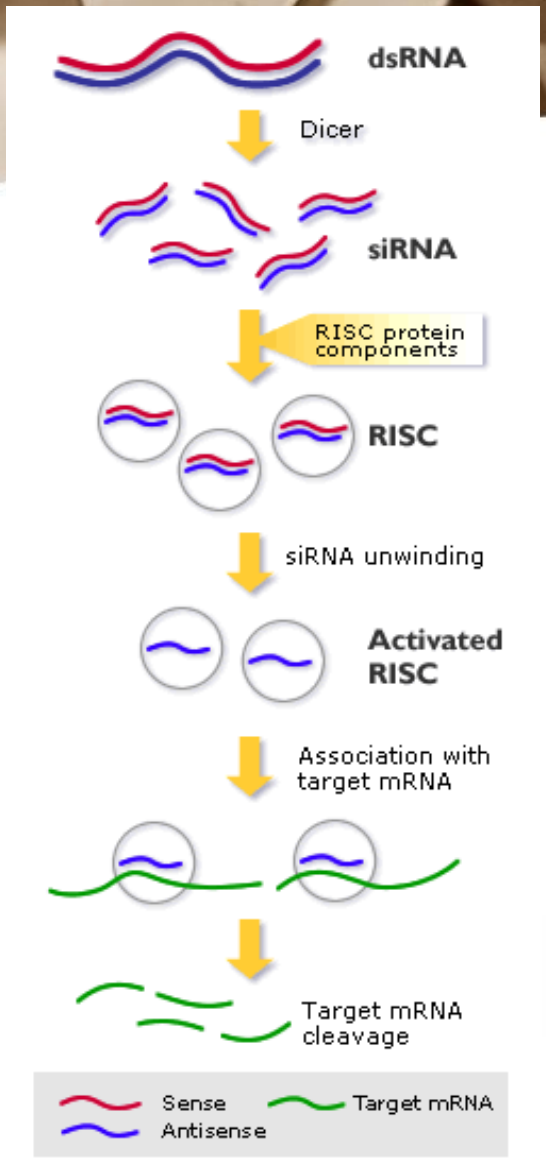
1. **Biological implication of RNAi mainly maintain genome stability ,represses transgenic expression and protects genome from invading by exogenous nucleotides.**

The phenomenon of RNA inference - universality

Table 1 Gene-silencing mechanisms in different species

	Organism	Phenomenon	Initiator event	Genes involved
Fungi	*Neurospora*	Quelling	Transgenes	*qde-1*, *qde-2*, *qde-3*
Plants	*Arabidopsis*, *Nicotiana*, *Petunia*	Post-transcriptional gene silencing, co-suppression	Transgenes, viruses	*sgs*, *ego*
	Arabidopsis	Transcriptional gene silencing	Transgenes	*ddm*, *som*, *hog*, *sil*
Invertebrates	*Paramecium*	Homology-dependent gene silencing	Transgenes	?
	Hydra	RNAi	dsRNA	?
	T. brucei	RNAi		?
	Planaria	RNAi		?
	Drosophila	RNAi Co-suppression	dsRNA Transgenes	 Polycomb group
	C. elegans	RNAi	dsRNA	*mut-2*, *mut-7*, *rde-1*, *rde-2*, *rde-3*, *rde-4*
		Gene silencing	Transgenes	*Polycomb group*, *mut-7*, *rde-2*, *rde-3*
Vertebrates	Zebrafish	RNAi	dsRNA	?
	Mouse	RNAi	dsRNA	?

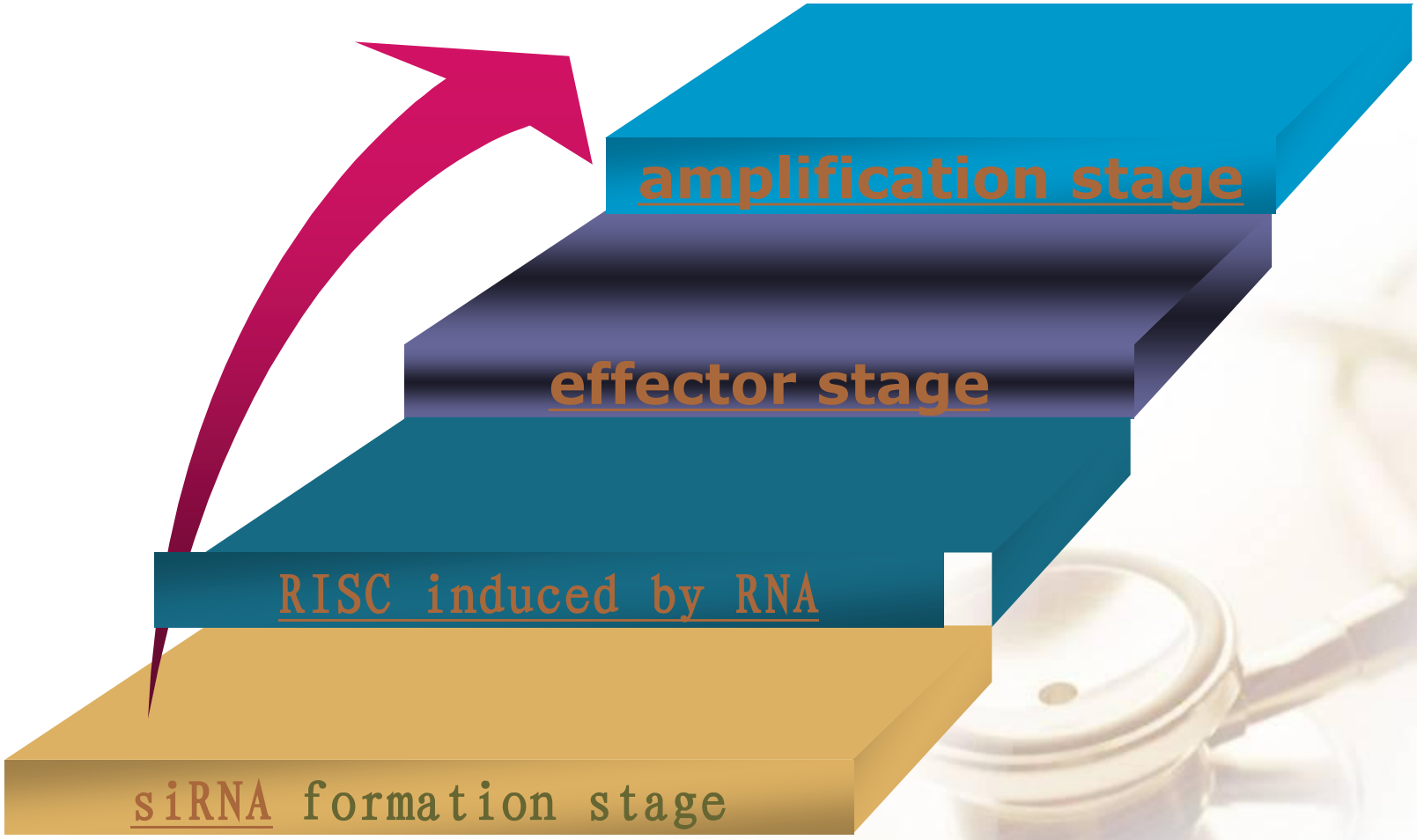
The Mechanism of RNA Interference (RNAi)



- Long double-stranded RNAs (dsRNAs; typically >200 nt) can be used to silence the expression of target genes in a variety of organisms and cell types (e.g., **worms, fruit flies, and plants**).
- In **mammalian cells**, introduction of long dsRNA (>30 nt) initiates a potent antiviral response, exemplified by nonspecific inhibition of protein synthesis and RNA degradation. The mammalian antiviral response can be bypassed, however, by the introduction or expression of siRNAs.

- www.ambion.com
- www.genscript.com/rnai.html
- www.promega.com
- www.invivogen.com
- www.irisgenetics.com

RNAi mechanism of action



RNAi mechanism of action

formation stage of siRNA

- This stage requires Rde-1、Rde-4 and dsRNA specific Dicer to participate
- protein edcoded by Rde-1 recognizes and directs the combination of dsRNA and Dicer. Then Dicer unwinds dsRNA and clearage it into RNA (small interfering RNA,siRNA with 21-25nt.Dicer is suited in the cytoplasm.
- Nowadays,for siRNA in 21-25nt with overhanging 2-3nt at the 3’ -end and phosphorylation at 5’ -end, the effector induction by RNAi is the most strong.
- Dicer plays a role in the dimeride form. Due to difference of molecular size in Dicer from different species, size of siRNA spliced by dsRNA is species Specific

RISC induced by RNA

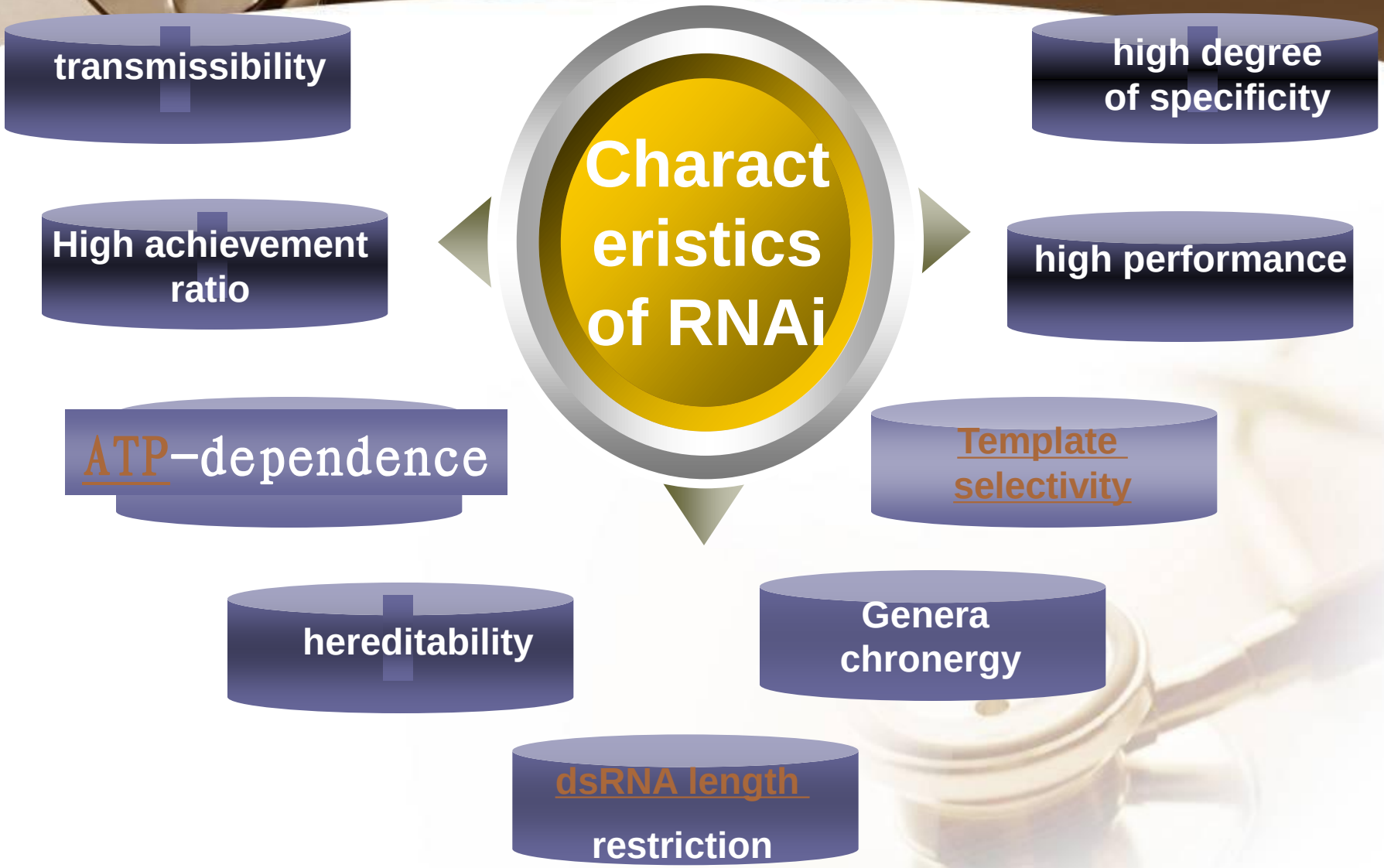
- Combination of formed enzymes specific for siRNA and RNAi such as AGO-2,MUT-7,RED-1,PAZ protein and DNA-RNA helicase formed RISC , has sequenc-specific nucleotide endonuclease, exonuclease and unwindase activities and can specifically degrade target mRNA homologus to siRNA.

Effector stage

- siRNA guides RISC to combine with homologous mRNA, under ATP and unwindase (Rde-3,MUT-6,MUT-14),makes siRNA chain dissociation and makes RISC precursor with 250×10^3 change into active form with 100×10^3.Meanwhile, unwindase catalyses homologous exchange of mRNA and sense-chain of siRNA.Nuclease cuts mRNA 7-10 nt close to 5'-end of double-Stranded region formed by mRNA and antisense RNA,which results in specifically inbihit gene expression.

Amplification stage

- Acting one chain of siRNA as primer, target mRNA as template, under RdRP (RNA-dependent RNA Ploymerase), target mRNA was amplified and produced novel secondary siRNA. These siRNA can counteract target mRNA, which not only increases copy number of siRNA, but also change specific ssRNA into dsRNA.RdRP is an also important inductor, recognizing normal and abnormal RNA. Trangene and viral siRNA trigger reaction process under RdRP. In addition,two mechanisms for RNAi amplification effect exist: （1） Dicer cleavage long dsRNA into primary siRNA, which is dependent on length of dsRNA; (2） siRNA was applied many times under enzymes and can produce further scale effect.


Charact
eristics
of RNAi
transmissibility
high degree
of specificity
High achievement
ratio
high performance
ATP-dependence
Template
selectivity
hereditability
Genera
chronergy
dsRNA length
restriction

Characteristics of RNAi

High degree of specificity

Sometimes, one nucleotide change of siRNA can decease greatly the effect of blocking target gene. Brummelkamp et al have used RNA(small hairpin,shRNA expressed by vector to block the CDH1 gene of MCF-7, result showed just one pair of nuceotide mutation of shRNA can not suppress CDH1 gene expression. Of whichi,base site at the center and second base from the bottom of 3’-end play an important role.

Characteristics of RNAi

High performance

Within cell,RNAi pathway was amplified,in lower animal, inhibition ratio of target gene is more than 90%. Somebody even believe that only one copy of dsRNA in one cell could reach the scope of blocking target gene.

Template selectivity

RNAi just takes action to exons but not introns.No obvious effection was found if dsRNA is promoter of some certain gene.Besides, the inhibition efficiency of RNAi is not high for target gene with stable and/or rich expression.

Characteristics of RNAi

Genera chronergy

Irie et al discovered that, in lower organism,RNAi continuously exists and however maitains for sometime in mammalian. Effect of RNAi is generally most obvious at 2-3d after injection of dsRNA and 1-2 d later, abundance of mRNA can recover the level which at RNAi was not injected.

Characteristics of RNAi

Length restriction

Dicer can bind dsRNA ranging 200 from 500nt.shorter of substrate segment, weaker of Dicer activity, implicating that RNAi is dependent on dsRNA length

Hereditability

Presently, lots of proofs have confirmed that， in lower organism, RNAi can passage.RNAi heredity of elegans needs Rde-1 and Rde-2 to initiate

Characteristics of RNAi

transmissibility

RNAi effect can diffuse between cells.Recently,Van et al reported that sid-1 gene can encode one protein with 11 times across membrane, which is associated with this phenomenon.

ATP-dependency

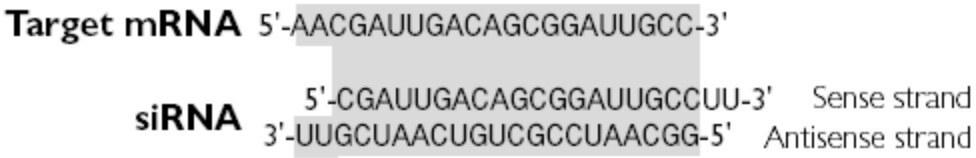
In sample without ATP， RNAi phenomenon decreased or disappeared,indicating that RNAi is ATP-dependent process, which is related to the fact that enzyme cut reaction of Dicer and RISC must require energy provide by ATP

High achivement ratio

RNAi has been used for functional analysis of elegans genomewide. of which 50%-80% sequence selection is effective and 12.9%-27% gene blocking can produce obvious anormal phenotype

siRNA design

- 21-23nt
- 2-nt 3' overhangs (**UU overhangs**)
- G/C content: 30-50%.
- No basepair mismatch



- BLAST : eliminate any target sequences with significant homology to other coding sequences.
- design and test 3–4 siRNA sequences
- http://www.ambion.com/techlib/misc/siRNA_finder.html

Five Ways to Produce siRNAs

- **In vitro:**

  in vitro preparation of siRNA

  introduced directly into mammalian cells by transfection, electroporation, or by another method.

1. **Chemical synthesis**
2. **In vitro transcription**
3. **Digestion of long dsRNA by an RNase III family enzyme (e.g. Dicer, RNase III)**

Five Ways to Produce siRNAs

In vivo:

the transfection of DNA-based vectors and cassettes that express siRNAs within the cells.

- **4.** Expression in cells from an siRNA expression plasmid or viral vector
- **5.** Expression in cells from a PCR-derived siRNA expression cassette

Company
LOGO

1. Chemical synthesis

Chemical Synthesis

- high quality, chemically synthesized siRNAs on a custom basis.
- the large yield of high purity siRNA obtained.
- most expensive

- **Best for:**

  Studies that require large amounts of a defined siRNA sequence

- **Not suitable for:**

  Screening siRNA sequences (cost prohibitive), long term studies

Company
LOGO

2. In Vitro Transcription

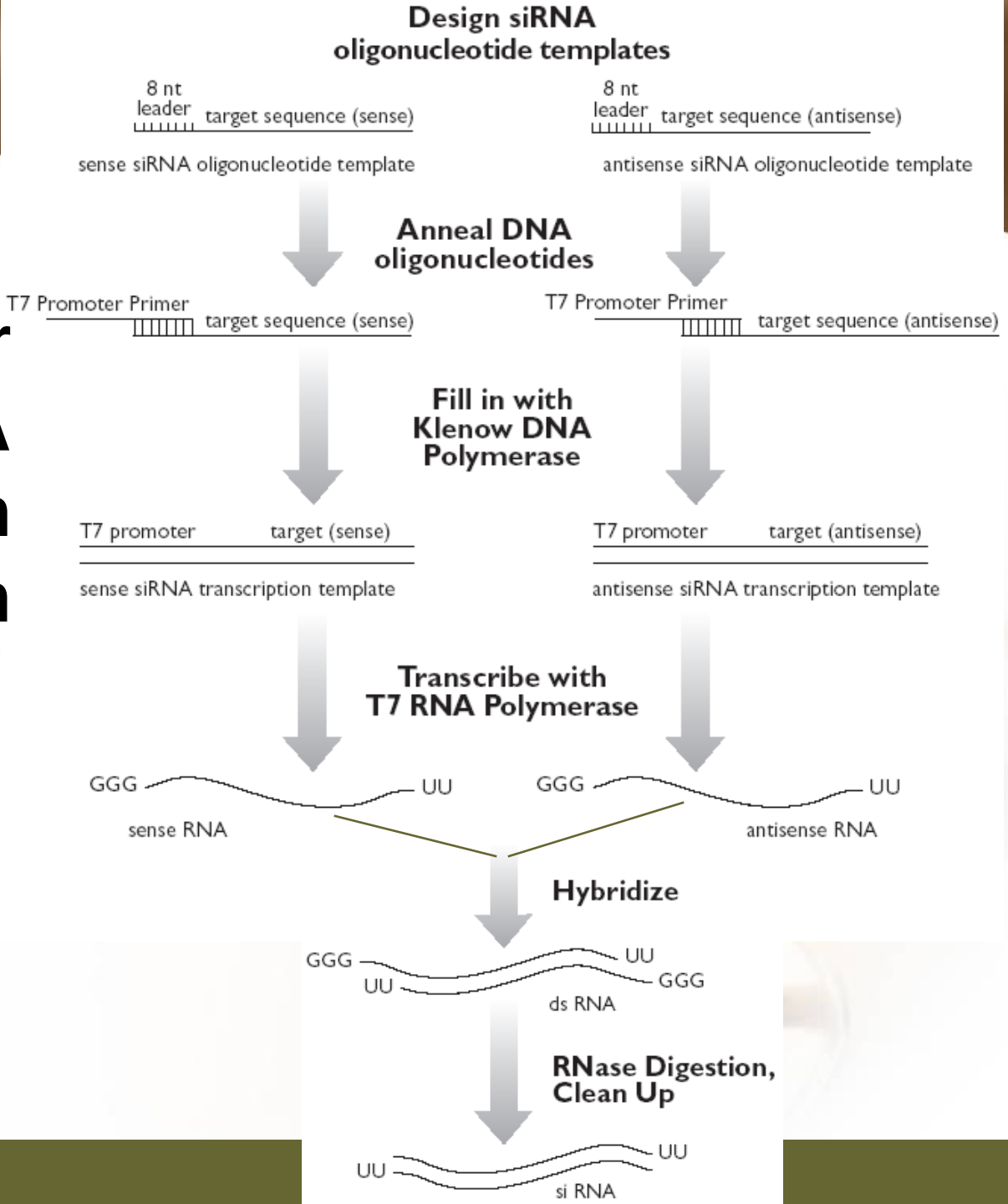
In Vitro Transcription

- **Relative cost per gene: Moderate**
- **little hands on time**
- **Relative ease of transfection**
- ***Best for:***
  Screening siRNA sequences or when the price of chemical siRNA synthesis is an obstacle
- ***Not suitable for:***
  Long term studies or studies that require large amounts of a single siRNA sequence

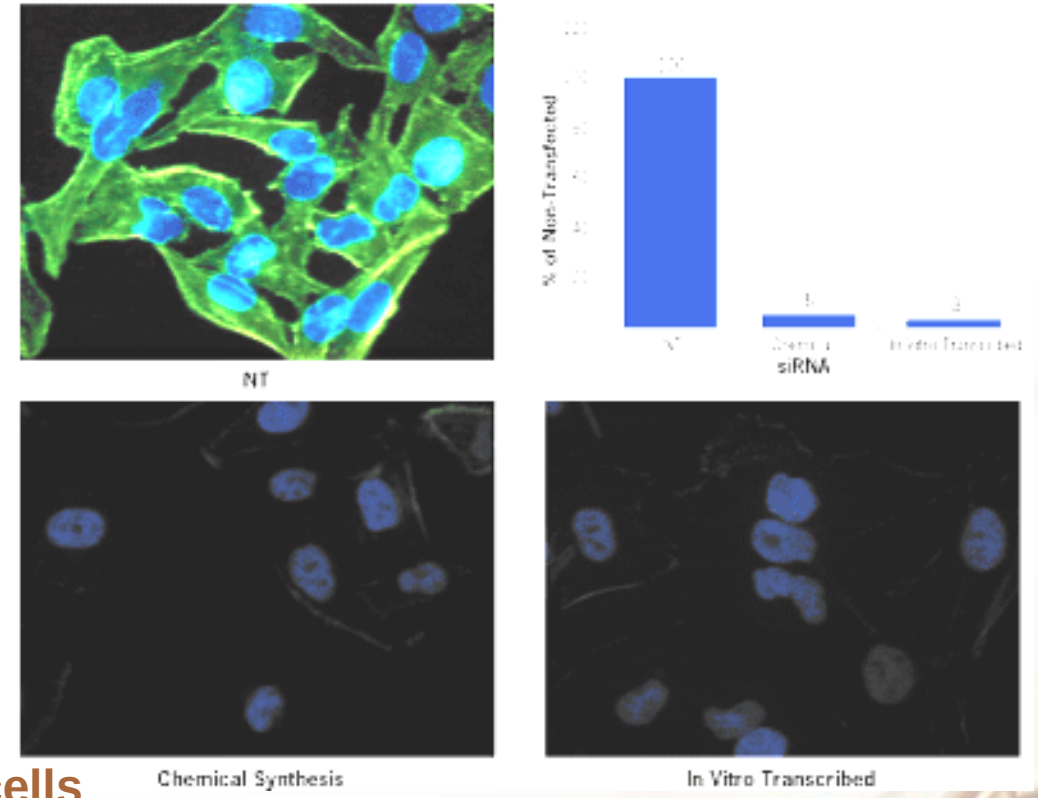
A problem

- In vitro transcription using T7 RNA polymerase requires that the first 2 nucleotides of the RNA transcript be GG or GA to ensure efficient synthesis (Milligan 1987).
- Requiring a **GG or GA** at the 5' ends of both the sense and antisense strands of an siRNA in addition to the required 3' terminal UU greatly reduces the number of potential target sites for siRNA experiments.
- This constraint essentially eliminates in vitro transcription as a viable option for preparing siRNAs.

Silencer siRNA Construction Kit --Ambion



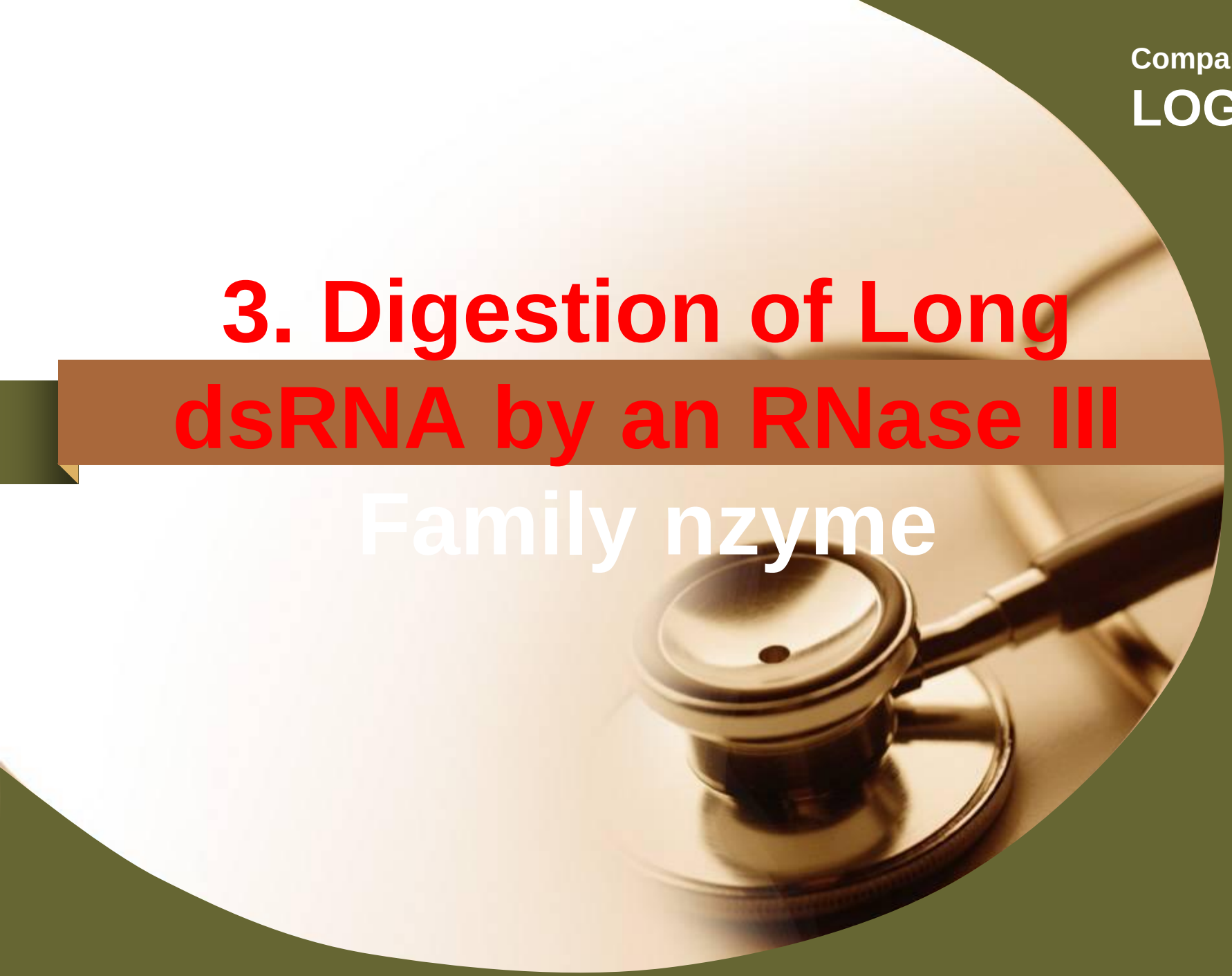
Use of Chemically Synthesized and in Vitro Transcribed siRNAs to Induce β-Actin Gene



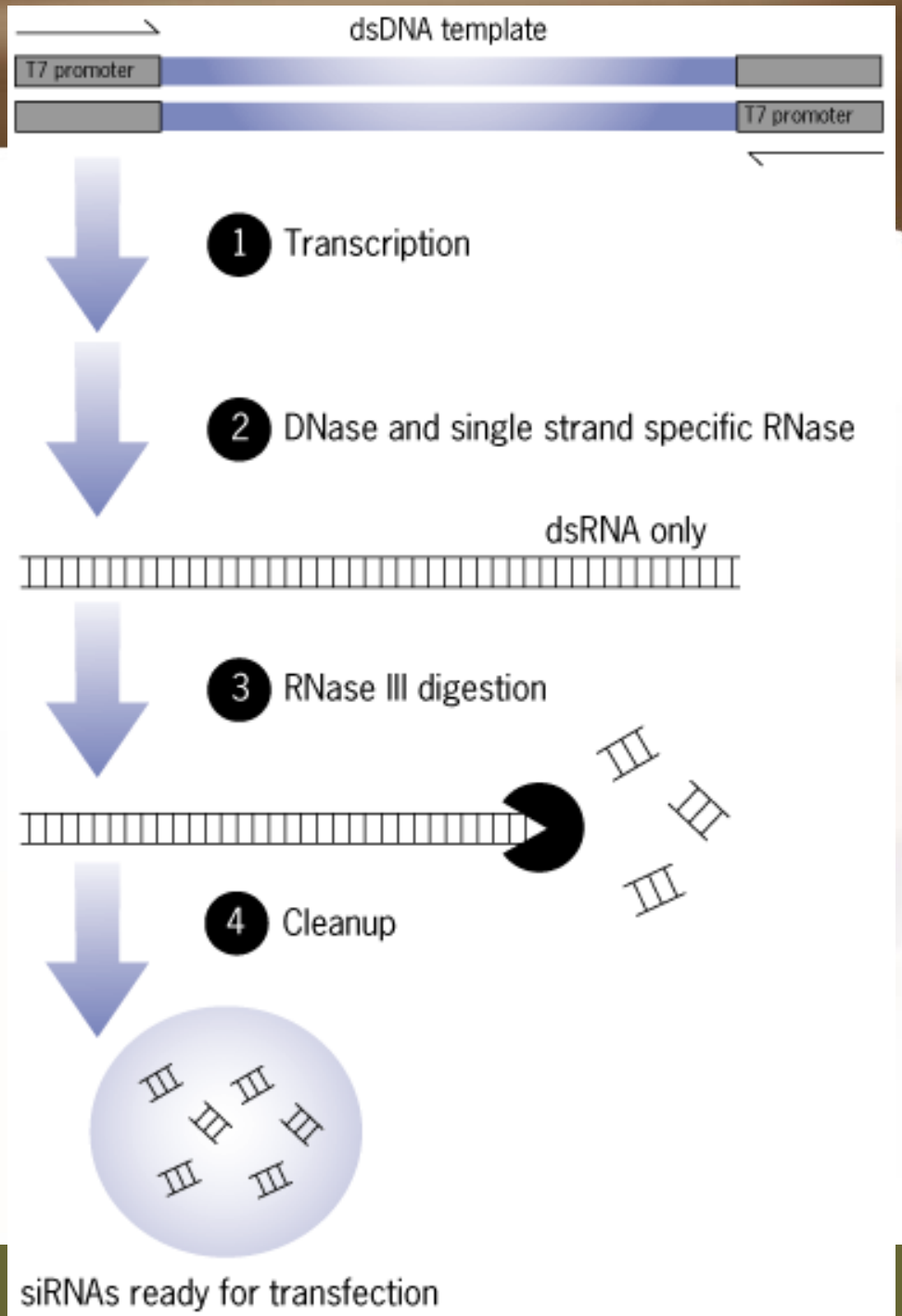
- HeLa cells
- siRNA preparation:
- chemical synthesis (Ambion)
- in vitro transcription(Ambion's Silencer™ siRNA Construction Kit)
- Transfection: siPORT™ Lipid (Ambion) w/ a 75 nM siRNA

Company
LOGO

3. Digestion of Long dsRNA by an RNase III Family nzyme



- long dsRNA: 200–1000 nt (in vitro transcription)
- digest in vitro with RNase III (or Dicer)
- remove any undigested dsRNA
- Transfection

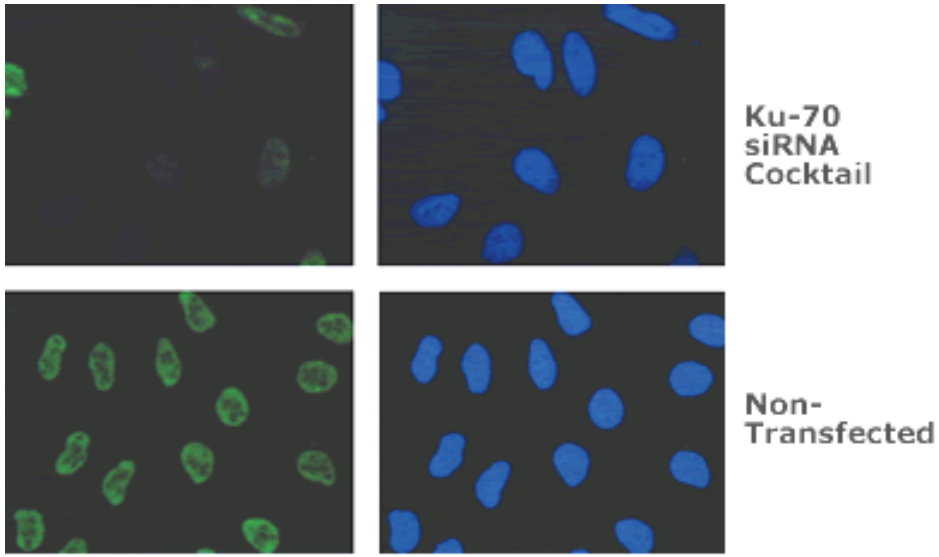


- no need to design and test several siRNA sequences before an effective one can be identified
- the theoretical potential for nonspecific silencing effects

- ***Best for:***

  Fast and inexpensive analysis of loss of function phenotypes
- ***Not suited for:***

  Long term studies or studies that require a single, defined siRNA sequence



- 200 nt of the Ku-70 mRNA
- HeLa cells

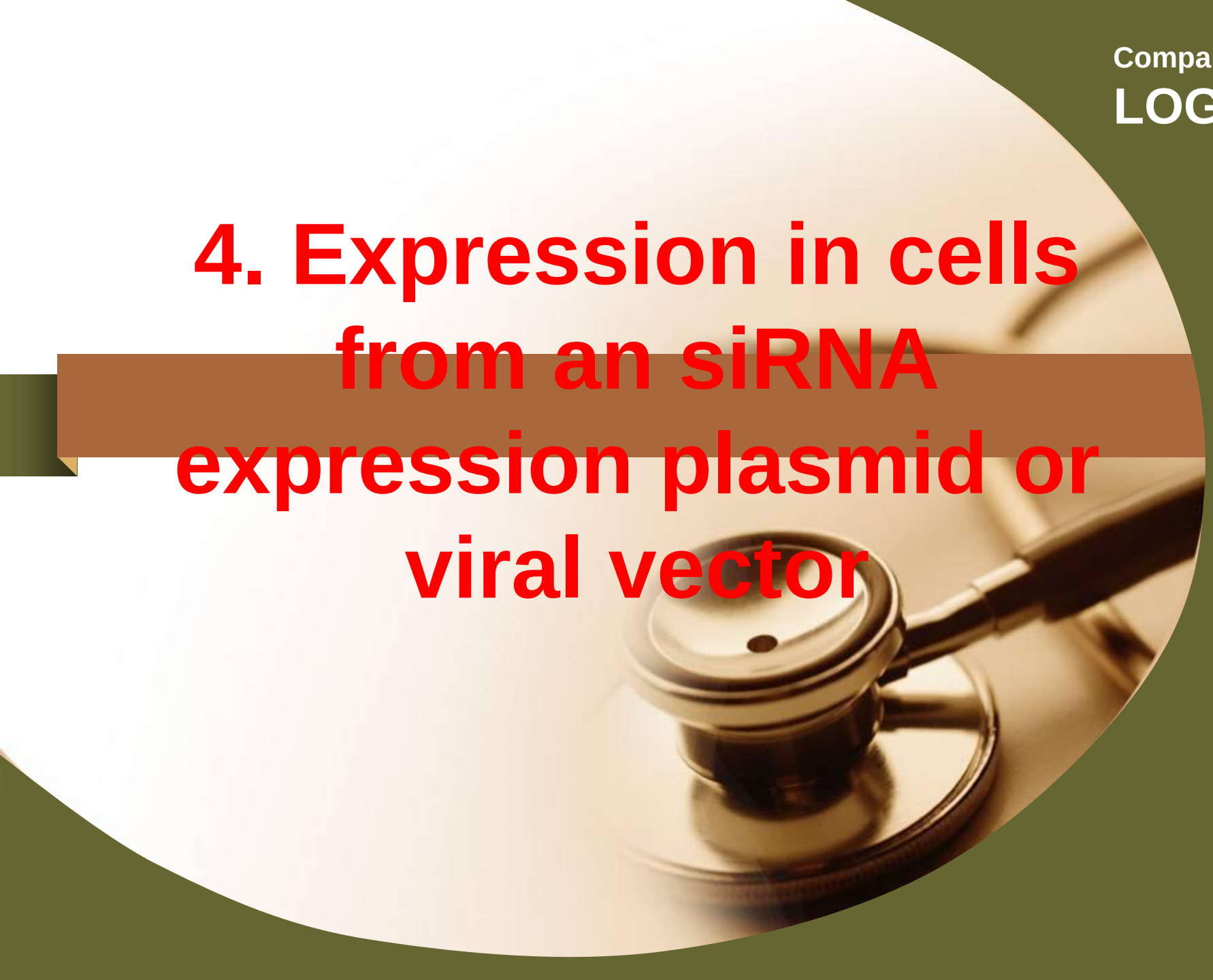
In Vivo Expression

- no need to work directly with RNA

- **4.** Expression in cells from an siRNA expression plasmid or viral vector
- **5.** Expression in cells from a PCR-derived siRNA expression cassette

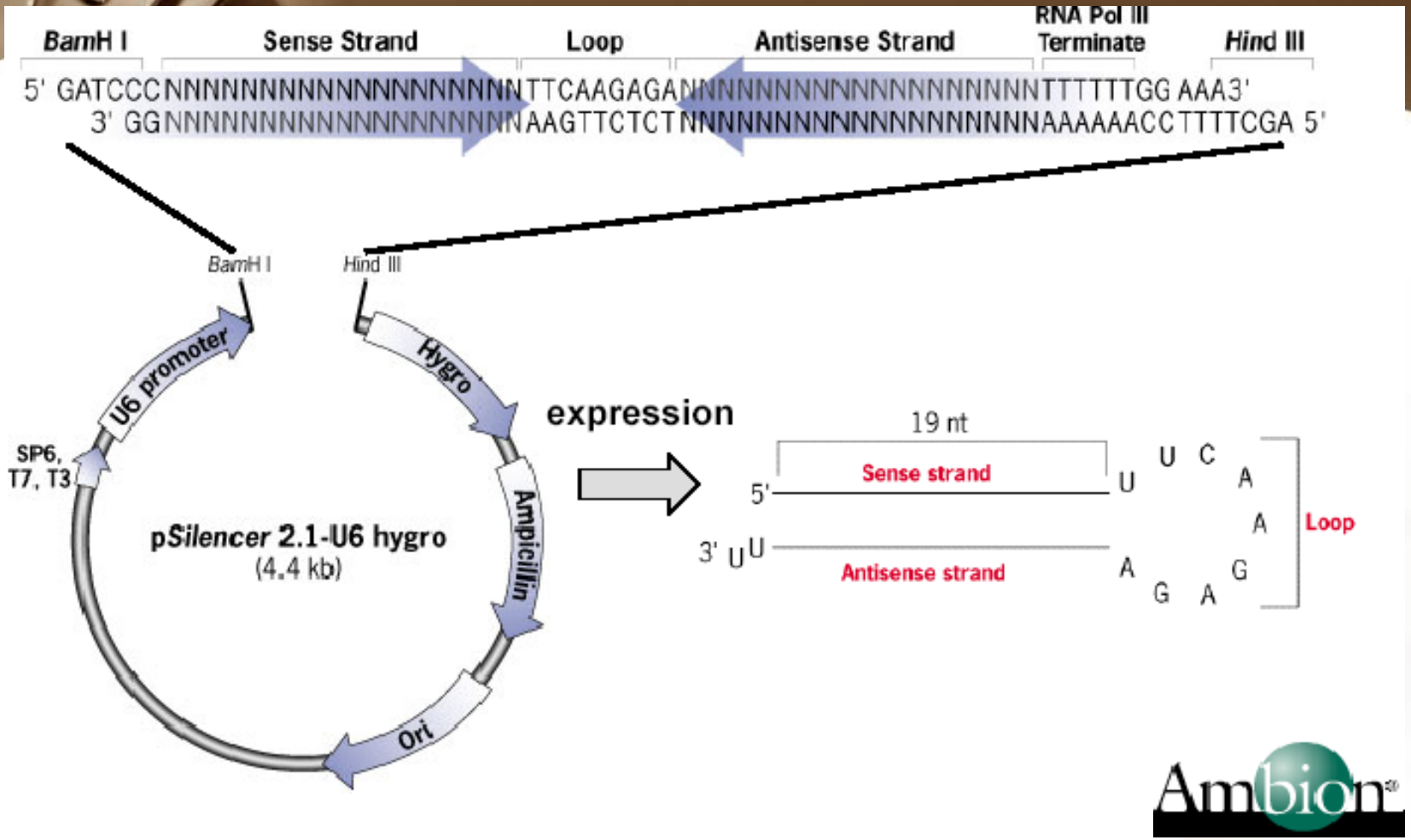
Company
LOGO

4. Expression in cells from an siRNA expression plasmid or viral vector



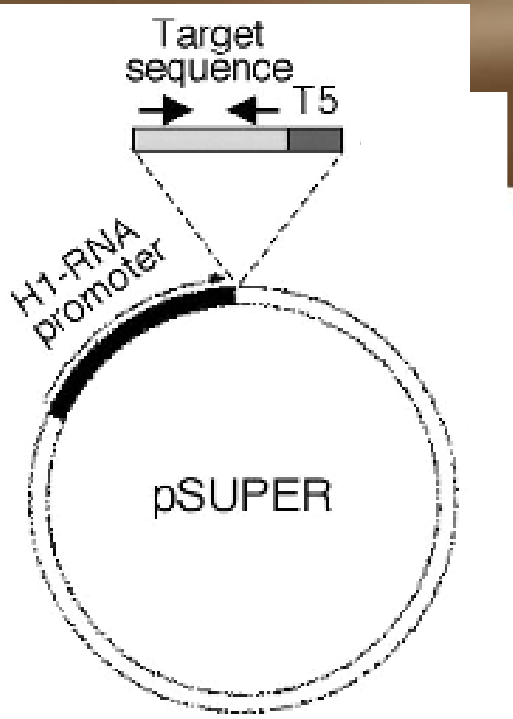
siRNA Expression Vectors

- **RNA polymerase III (pol III) :**
  human U6 promoters
  mouse U6 promoters
  the human H1 promoter
- **RNA pol III was chosen to drive siRNA expression because it naturally expresses relatively large amounts of small RNAs in mammalian cells and it terminates transcription upon incorporating a string of 3–6 uridines.**


BamH I
Sense Strand
Loop
Antisense Strand
RNA Pol III Terminate
Hind III
5' GATCCCNNNNNNNNNNNNNNNNNNNTTCAAGAGANNNNNNNNNNNNNNNNNNNTTTTTTGGAAA3'
3' GGNNNNNNNNNNNNNNNNNNNAAGTTCTCTNNNNNNNNNNNNNNNNNNNAAAAAACCTTTTCGA 5'
BamH I
Hind III
U6 promoter
Hygro
SP6, T7, T3
Ampicillin
pSilencer 2.1-U6 hygro
(4.4 kb)
Ori
expression
19 nt
Sense strand
Antisense strand
5'
3'
U
U
C
A
A
Loop
A
G
A
G
U
U
A
G
A

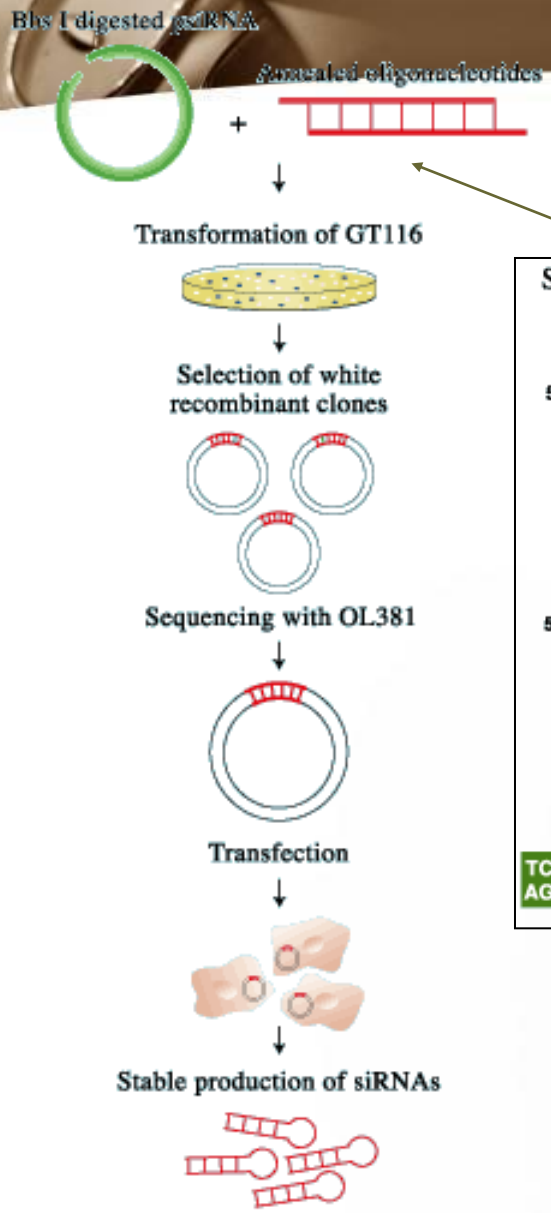
pSUPER

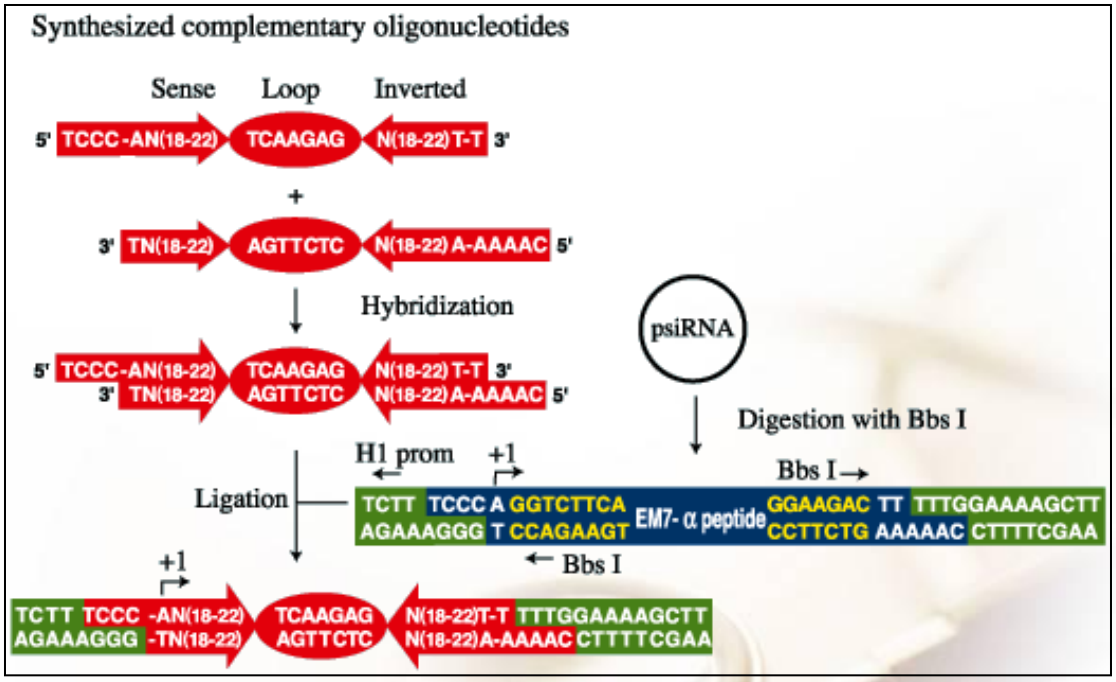
---------- Thijn R. Brummelkamp,René Bernards,Reuven Agami. A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. ***Science***, Vol. 296, 550-553, April 19, 2002



- polymerase-III H1-RNA gene promoter: produces a small RNA transcript lacking a poly-adenosine tail
- well-defined start of transcription
- a termination signal consisting of five thymidines in a row (T5).
- the cleavage of the transcript at the termination site is after the second uridine yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides (nt).

psiRNA


Bbs I digested psiRNA
Annealed oligonucleotides
+
Transformation of GT116
Selection of white recombinant clones
Sequencing with OL381
Transfection
Stable production of siRNAs


Synthesized complementary oligonucleotides
Sense
Loop
Inverted
5' TCCC-AN(18-22) TCAAGAG N(18-22) T-T 3'
+
3' TN(18-22) AGTTCTC N(18-22) A-AAAAC 5'
Hybridization
5' TCCC-AN(18-22) TCAAGAG N(18-22) T-T 3'
3' TN(18-22) AGTTCTC N(18-22) A-AAAAC 5'
psiRNA
Digestion with Bbs I
Ligation
H1 prom
+1
Bbs I
TCTT TCCC A GGTCTTCA EM7- α peptide GGAAGAC TT TTTGGAAAAGCTT
AGAAAGGG T CCAGAAGT CCTTCTG AAAAAC CTTTTCGAA
Bbs I
+1
TCTT TCCC -AN(18-22) TCAAGAG N(18-22)T-T TTTGGAAAAGCTT
AGAAAGGG -TN(18-22) AGTTCTC N(18-22)A-AAAAC CTTTTCGAA

1. Synthesis of two complementary oligonucleotides and hybridization

Both oligonucleotides are designed such that the first four bases create 5' overhangs compatible with Bbs I (TCCC for the sense strand and AAAC for the antisense strand). In the sense strand, the 5' overhang is followed by an A (transcription initiation point of the human H1 promoter), then the target sequence of 18-22 mer, 5 to 7 bases for the spacer region, and the inverted 18-22 mer sequence. The sense strand ends with TT to reconstitute the T5 terminator sequence.

2. Ligation into psiRNA linearized with Bbs I

Digestion with Bbs I liberates the lacZ cassette and creates uncompatible cohesive ends. This increases the number of recombinant clones with an insert in the proper orientation.

3. Transformation of E. coli GT116 strain

GT116 is an engineered *E. coli* strain compatible with hairpin structures.

4. DNA extraction and sequencing of the siRNA insert.

The psiRNA has been optimized so that analysis of only 5 white transformed colonies is sufficient to obtain the expected siRNA.

siRNA Expression Vectors

1. more effective than synthetic siRNA
2. Very stable and easy to handle:
3. Stable cell line can be established:
4. Inducible system can be established:
5. Unlimited supply: once a DNA construct is made, you will have unlimited supply of siRNA.
6. Cost-effective:
7. One big obstacle : it takes a lot of time and trouble to make the DNA constructs.

siRNA Expression Vectors

- long term studies and antibiotic resistance markers

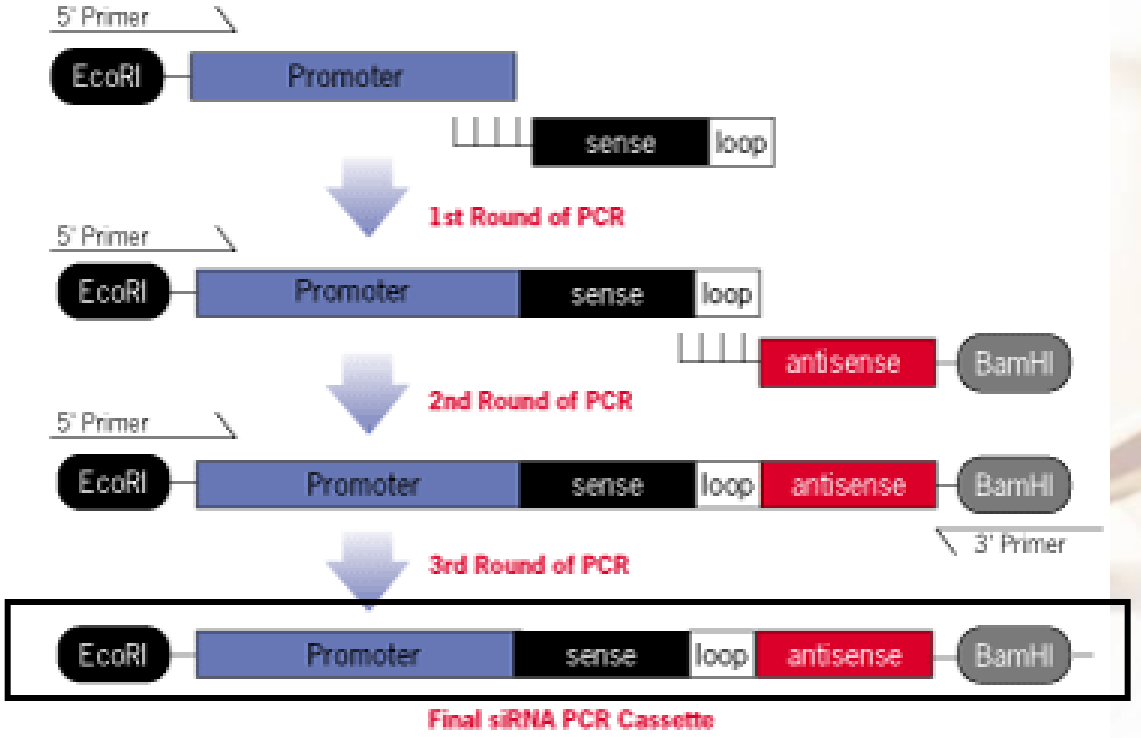
- ***Best for:***
- Long term and other studies in which antibiotic selection of siRNA containing cells is desired
- ***Not suitable for:***
- Screening siRNA sequences (note: screening siRNA sequences is possible, but is time and labor intensive with vectors)

Company
LOGO

5. siRNA Expression Cassettes (SECs)

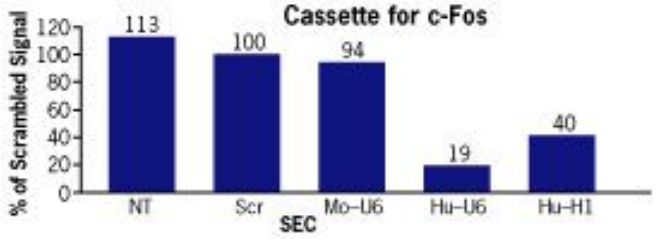
siRNA Expression Cassettes (SECs)

- PCR-derived siRNA expression templates
- PCR product introduced into cells directly — without first being cloned into a vector.
- Castanotto et al., (2002) *RNA* 8:1454

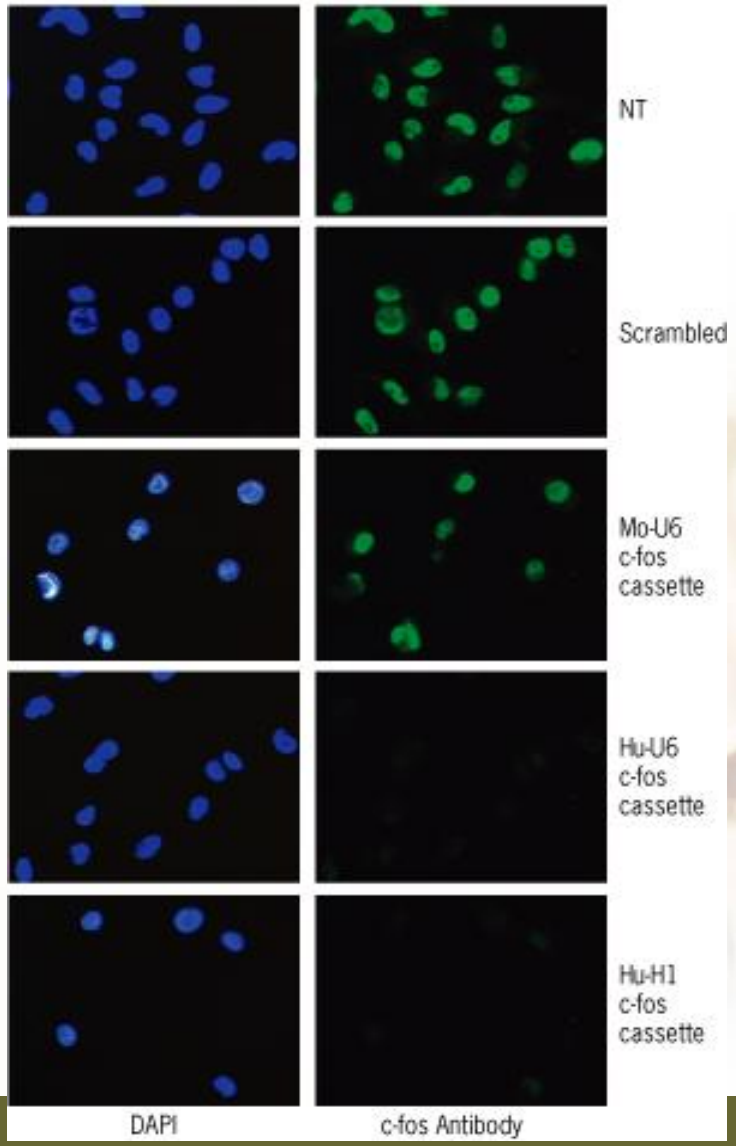


- Rapidly prepare siRNA expression cassettes by PCR
- no cloning, plasmid preps or sequencing necessary
- Quickly test different siRNA sequences and promoters before cloning into a vector
- Avoid costly siRNA synthesis

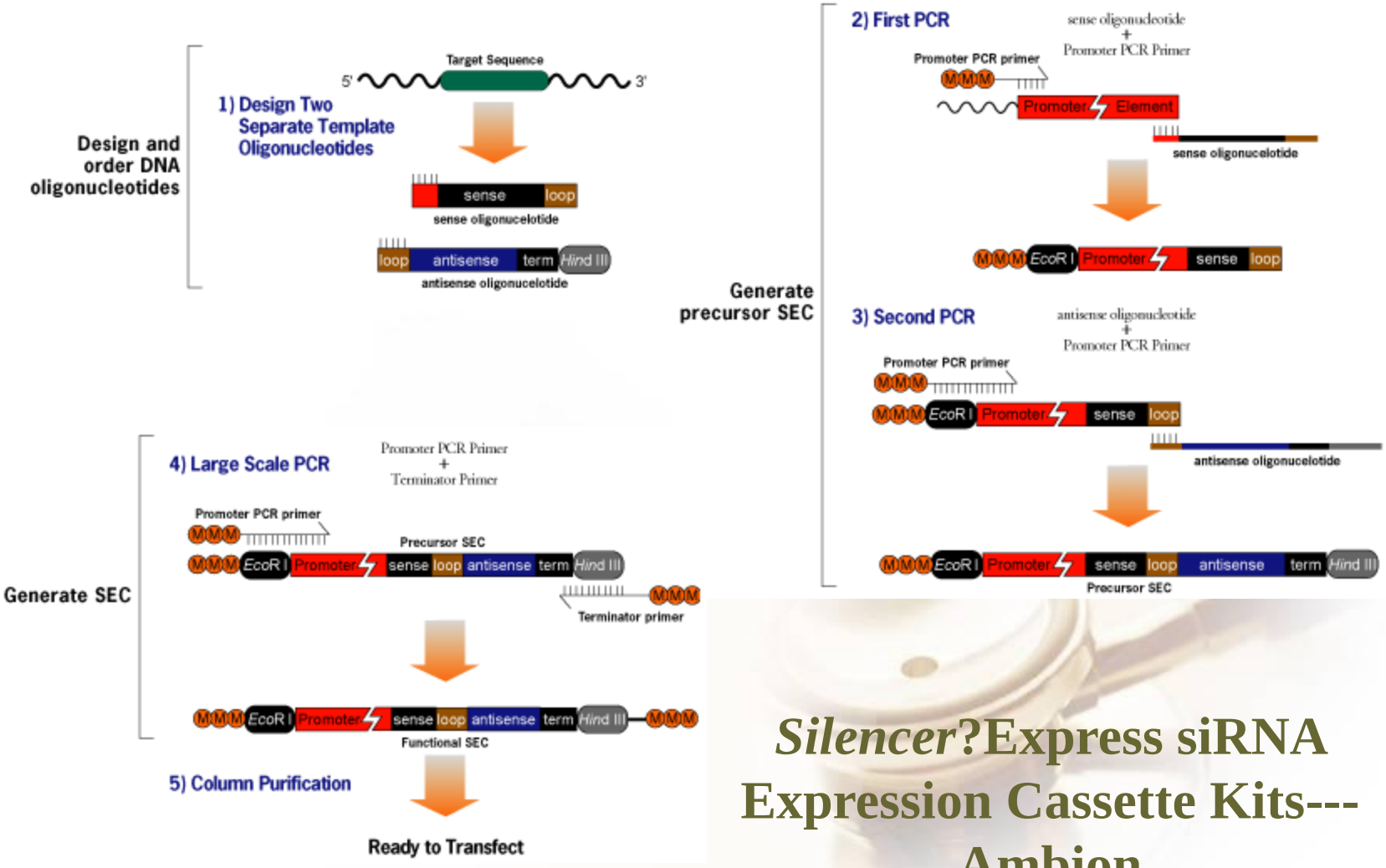
Variable Reduction in Target Gene Expression



- promoters :
  mouse U6 (Mo-U6),
  human U6 (Hu-U6),
  human H1 (Hu-H1)
- c-fos gene
- HeLa cells
- a negative control siRNA (scramble)



Schematic of Preparing SECs using Two siRNA Template Oligonucleotides

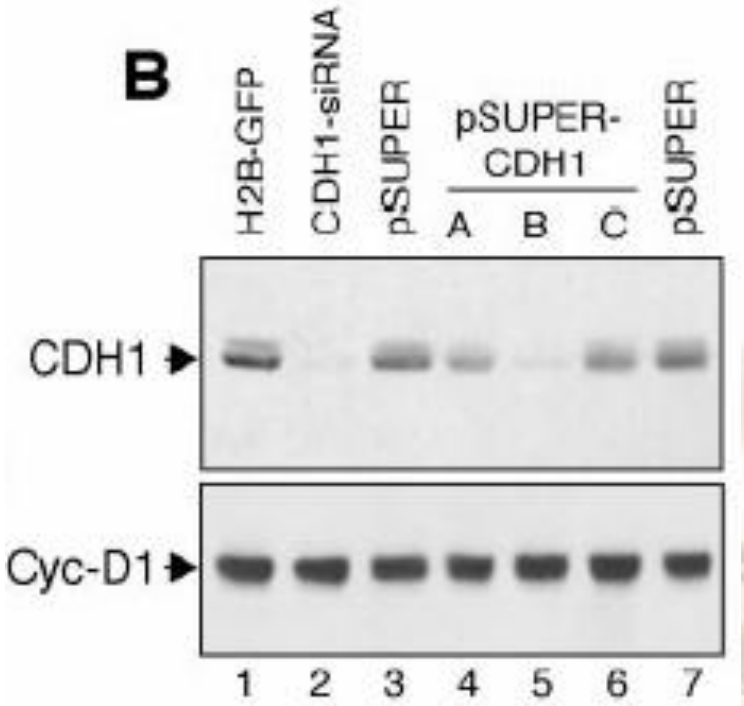


***Silencer*?Express siRNA Expression Cassette Kits---Ambion**

Length and Sequence of the Loop Linking Sense and

Loop Size (# of Nucleotides)	Specific Loop Sequence	Reference
3	AUG	4
3	CCC	7
4	UUCG	5
5	CCACC	7
6	CTCGAG	2
6	AAGCUU	2
7	CCACACC	7
9	UUCAAGAGA	6
23	Not reported	9


A)
5′-UGAGAAGUCUCCCAGUCAG C A G A
3′-UUACUCUUCAGAGGGUCAGUC U C G
B)
5′-UGAGAAGUCUCCCAGUCAG U U C A A
3′-UUACUCUUCAGAGGGUCAGUC A G A G
C)
5′-UGAGAAGUCUCCCAGUCAG U U C
3′-UUACUCUUCAGAGGGUCAGUC A G


B
H2B-GFP
CDH1-siRNA
pSUPER
pSUPER-CDH1
A
B
C
pSUPER
CDH1
Cyc-D1
1
2
3
4
5
6
7

Company
LOGO

Summary

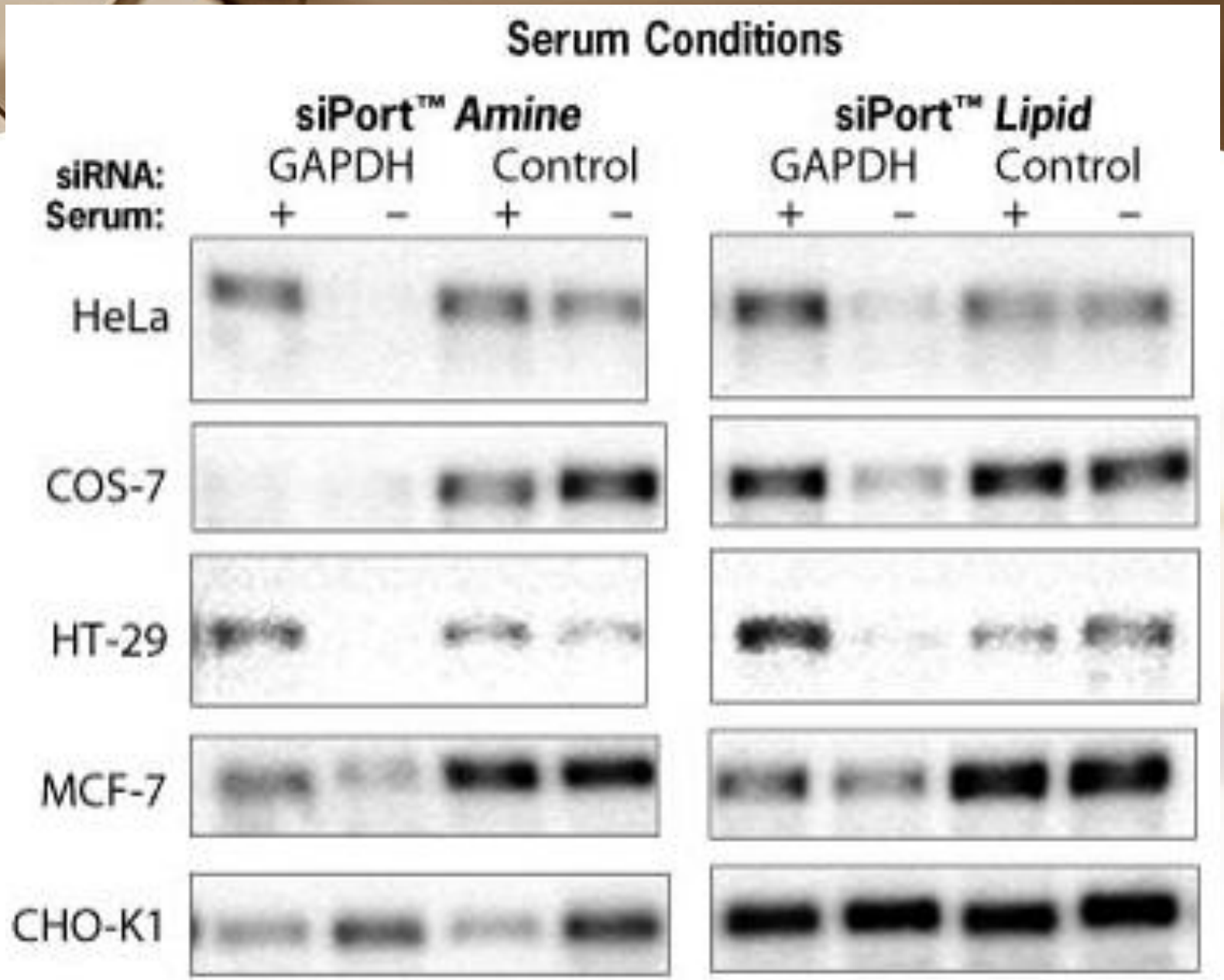
	Chemical synthesis	RNase III digestion of dsRNA	siRNA Expression Vector	PCR Expression Cassette	In vitro transcription
Requirements	2 21-mer RNA oligos	Transcription template (200-800 bp region flanked by T7 promoters)	2 55-60-mer DNA oligos	2 ~55-mer DNA oligos	2 29-mer DNA oligos
Turnaround time (total preparation/synthesis time)	4 days to 2 weeks*	1 day + transcription template preparation time	5+ days + DNA oligo	~ 6 hours + DNA oligo	24 hours + DNA oligo
Hands on time	Little to none*	Moderate	High	Moderate	Moderate
Testing to find optimal siRNA sequence	Required	Not needed	Required	Required	Required
Ability to label siRNA (i.e., for analyzing siRNA uptake or localization by fluorescence microscopy)			No	No	Yes
Relative ease of transfection	Good		Fair	Good	Good
Selectability (i.e, antibiotic selection)		No			
Useful for long term studies	No	No	Yes, with selection	No	No
Ability to scale up synthesis	Yes	Limited	Yes	Limited	Limited
Monitor transfection efficiency of entire population	No	No	Yes	No	No
Relative cost per gene (not including labor)	High	Low	Moderate	Moderate	Moderate
*Depends on purification/deprotection options selected and format (e.g., annealed and ready to transfect versus single strands supplied lyophilized)					

- **Chemical and IVT synthesis:**
  - Allows rapid screening of multiple targets
  - High siRNA homogeneity and purity
-
- **RNase III mediated hydrolysis:**
  - Eliminates the need for screening of target site
  - Overcomes variability in silencing by synthetic/IVT siRNA
  - Cost-effective for functional genomic studies
- **PCR based siRNA expression cassettes:**
  - Ideal for screening siRNA sequences prior to cloning in a vector
  - Rapid and inexpensive procedure
- **Vector based in vivo expression:**
  - Permits long-term and stable gene silencing
  - Possibility to use inducible/repressible markers
  - Use of viral vectors

Company
LOGO

Optimize Transfection of siRNAs for RNAi

- **Cell confluence:** For most adherent cells, the optimal confluence for transfection is 30-70%.
- **Choice of transfection agent.**
- **Determine the optimal volume of transfection agent.**
- **Quality and Quantity of siRNA:**
  chemically synthesized siRNA: 1-100 nM
  in vitro transcribed siRNA: 0.1 to 10 nM for
- **Effect of serum on transfection.**


Serum Conditions
siPort™ Amine
siPort™ Lipid
siRNA:
Serum:
GAPDH
Control
GAPDH
Control
+ − + −
+ − + −
HeLa
COS-7
HT-29
MCF-7
CHO-K1

RNAi application

research of Human fucntional gene

Application in clinic-gene therapy

tumor

Infectious diseases

Other diseases

Company
LOGO

Thank You !