miRNA
Perché?

- Rappresentano uno dei maggiori sistemi regolatori per famiglie di geni nelle cellule eucariotiche.
- Molti mRNA presentano più siti di legame per miRNA che sono il bersaglio per uno o più miRNA.
- I miRNA sono coinvolti un vasto campo di processi biologici quali: sviluppo, differenziamento cellulare, proliferazione, apoptosi……
- Sono implicati pure nella patogenesi di malattie umane quali: cancro, malattie metaboliche, disordini neurologici, malattie infettive……

miRNA
Perché?

✓ La deregolazione dei miRNA nei tumori può coinvolgere delezioni o amplificazioni

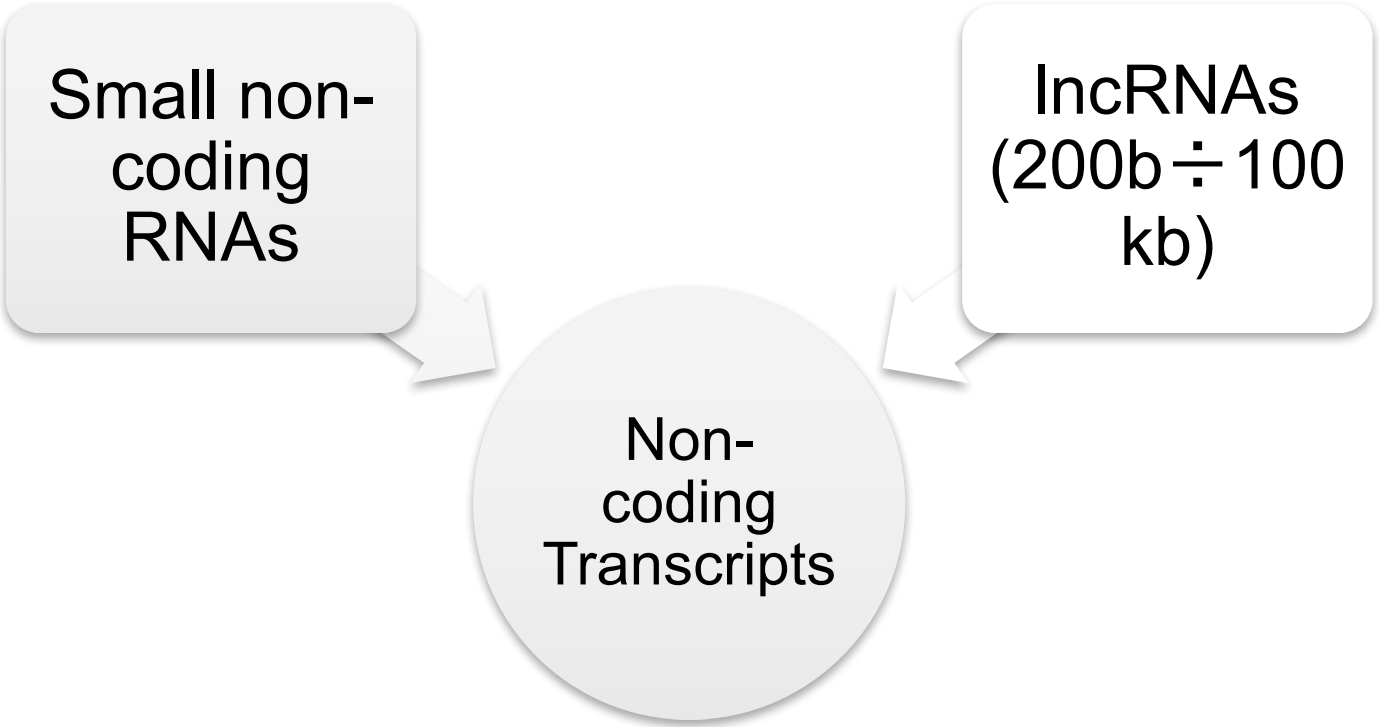
✓Il pattern dei miRNA è tessuto specifico

✓ Ci possono essere mutazioni nei geni miRNA

✓Molti miRNA sono nello stesso DNA policistronico e vengono processati assieme

✓ Ogni miRNA può avere bersagli multipli

✓I miRNA possono funzionare da tumor suppressor o oncogeni dipendentemente dal contesto cellulare.

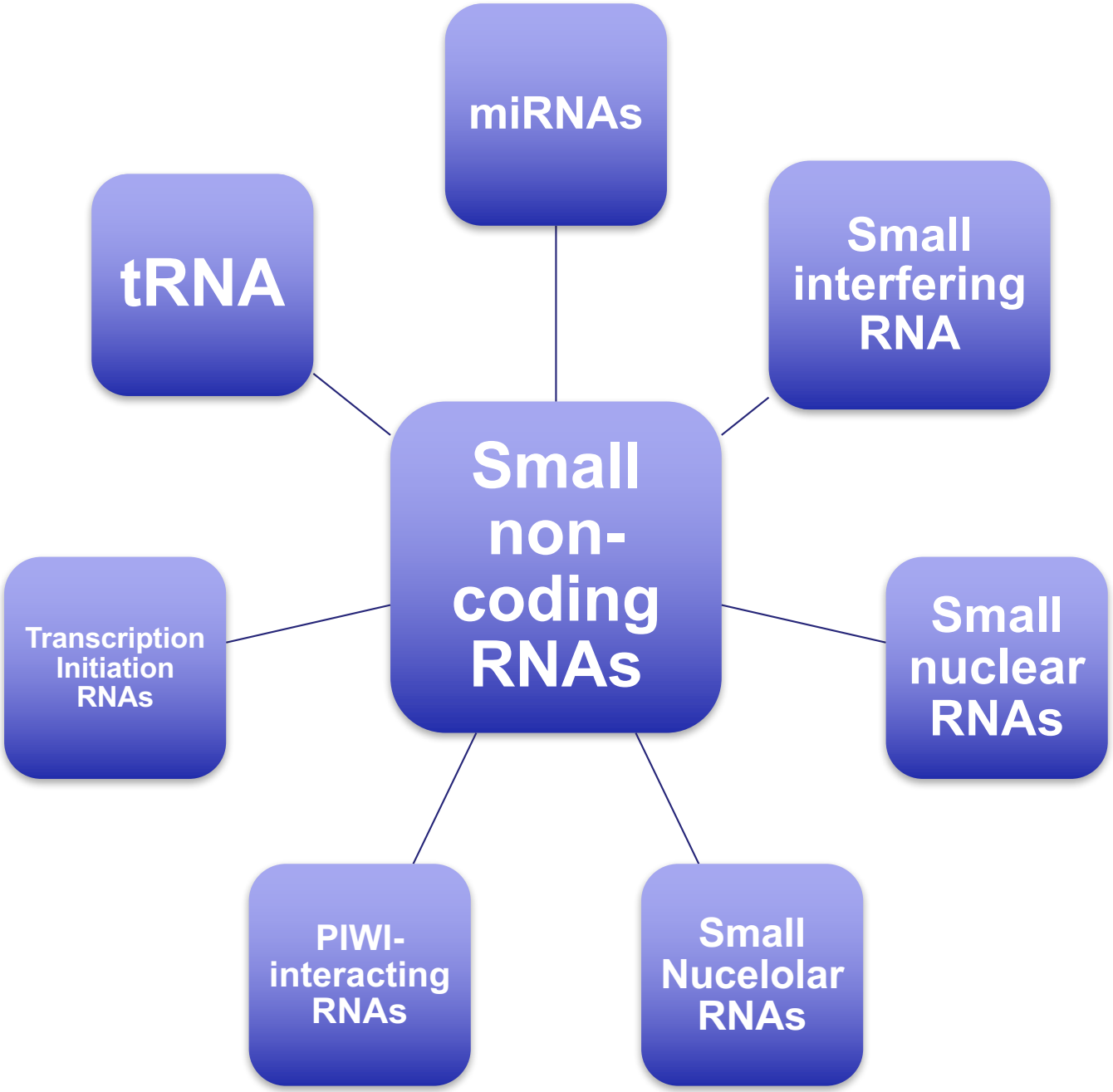


In numbers

- ✓ 21.000 protein- coding genes
- ✓ 9.000 small RNAs
- ✓ 10.000-32.000 long non-coding RNAs
- ✓ ~ 11.000 psedudogenes

Pandolfi 2014

Small non-coding RNAs-How many?



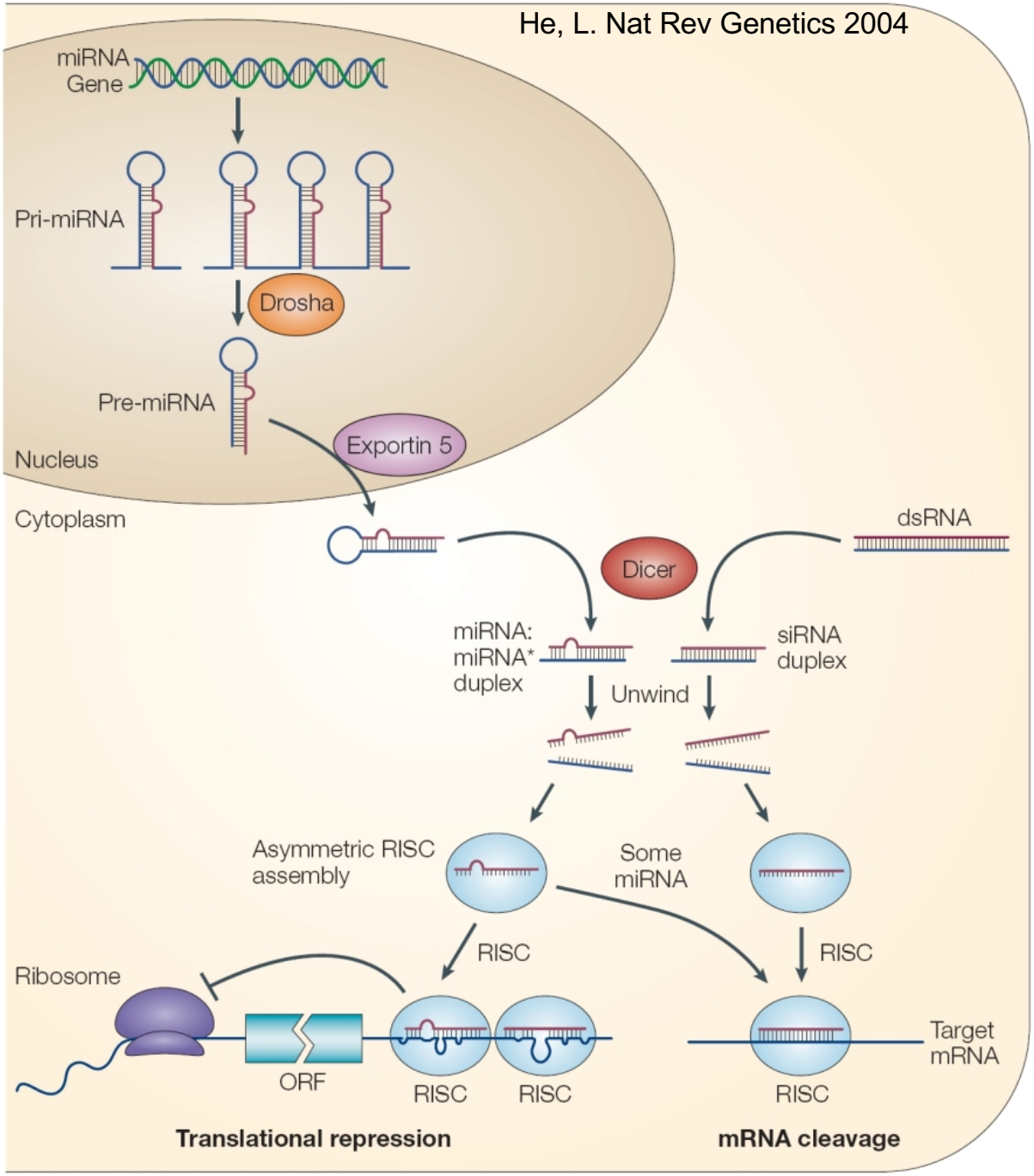
RNA-RNA CROSSTALK:

1. *New layers of gene regulation*
2. *Interactions between diverse RNA species*
3. *There are numerous miRNA-binding sites on a wide variety of RNA transcripts*

ceRNA Hypothesis

✓**All RNA transcripts that contain miRNA –binding sites can communicate with and regulate each other by competing endogenous RNAs.**

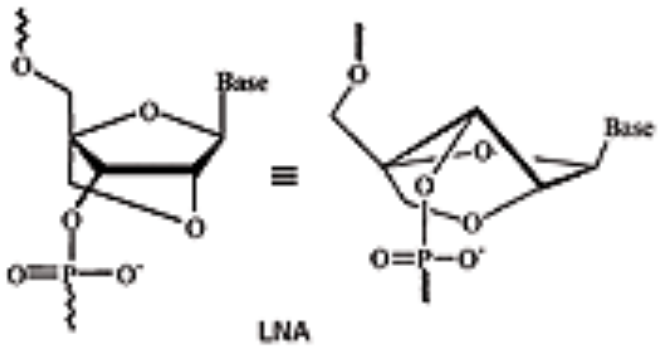
✓**miRNA competition thus extends beyond the non-coding transcriptome and confers an additional non-protein coding function to protein-coding mRNAs .**



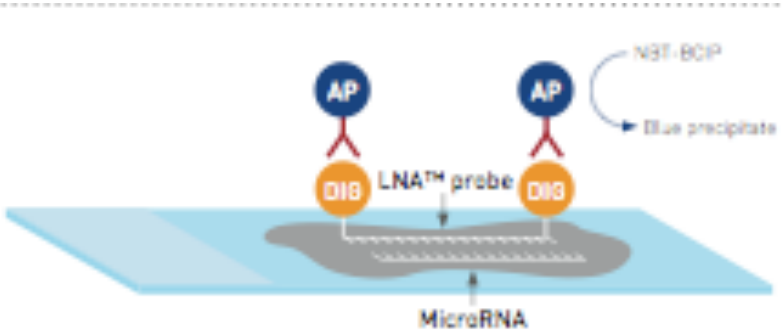
- ✓ Mature miRNA are generally long 21-25 nucleotide
- ✓ Most miRNAs seem to bind to the target 3'UTR
- ✓ Complementarity is imperfect
- ✓ Roles of targeting are not completely understood.

miRNA
Come?

- ☺ Le molecole di miRNA sono troppo piccole per analizzarle con una qPCR convenzionale, composta da primer sense, antisense e sonda TaqMan.
- ☺ Ci sono attualmente diversi sistemi disponibili in commercio per analizzarli che spaziano fra la RT-PCR alla ibridazione in situ impiegando sonde LNA.
- ☺ Le basi LNA hanno una modifica alla struttura del ribosio che lega la base alla posizione C3′-endo, favorendo una geometria a doppia elica RNA tipo A.



Saggio miRCURY LNA



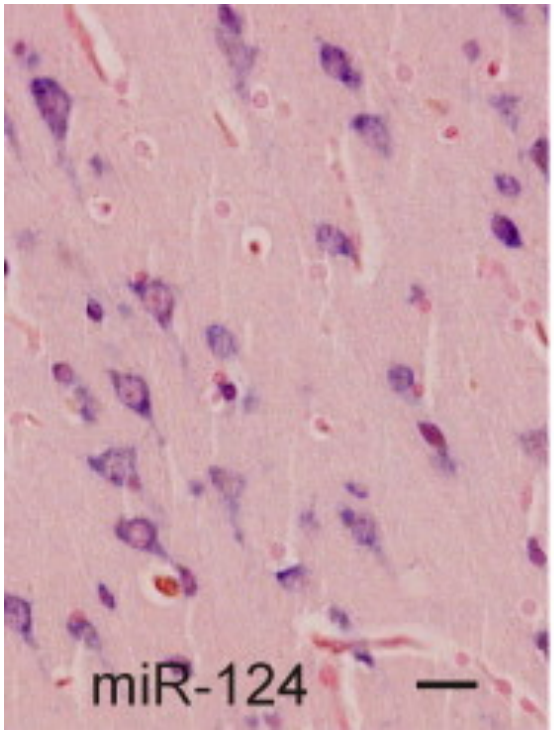
Impiega sonde LNA doppiamente marcate con la digossigenina

È ottimizzato per i tessuti FFPE e richiede 1 giorno di esecuzione

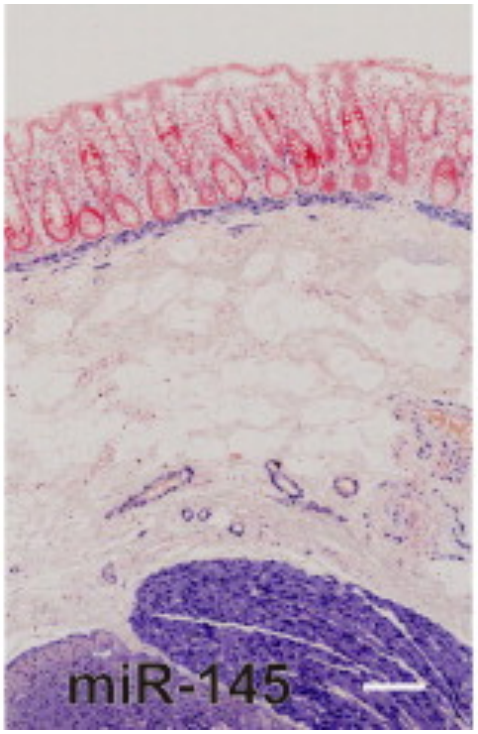
Fasi:

1. Smascheramnto dei miRNA con Proteinasi K
2. Ibridazione con le sonde
3. La digossigenina viene riconosciuta da un anticorpo anti- DIG coniugato con la fosfatasi alcalina (AP). AP converte i substrati solubili NBT e BCIP in H_2O un precipitato insolubile in alcol e H_2O NBT-BCIP (blu scuro). La controcolorazione consente una miglior risoluzione istologica.

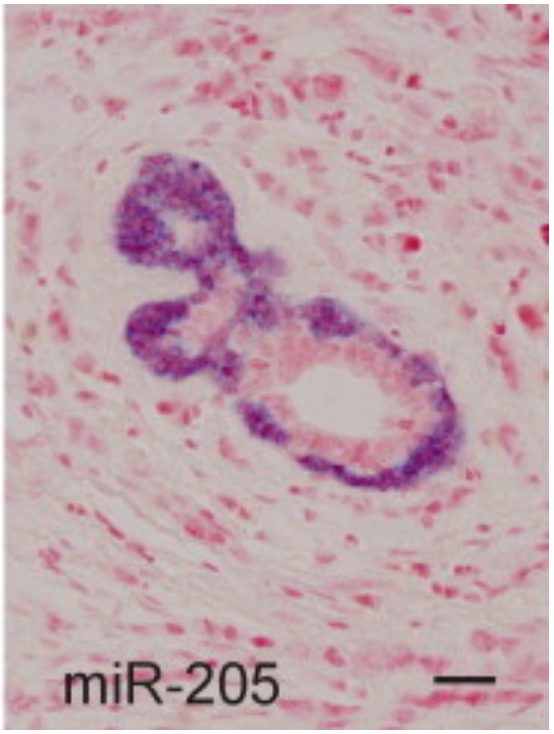
Saggio miRCURY LNA



Brain



Normal Colon

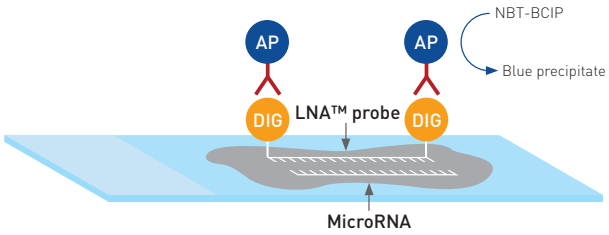


Breast Cancer

Jørgensen S, Baker A, Møller S, Nielsen BS. Robust one-day *in situ* hybridization protocol for detection of microRNAs in paraffin samples using LNA probes. MethodsVolume 52, Issue 4, December 2010, Pages 375-381.

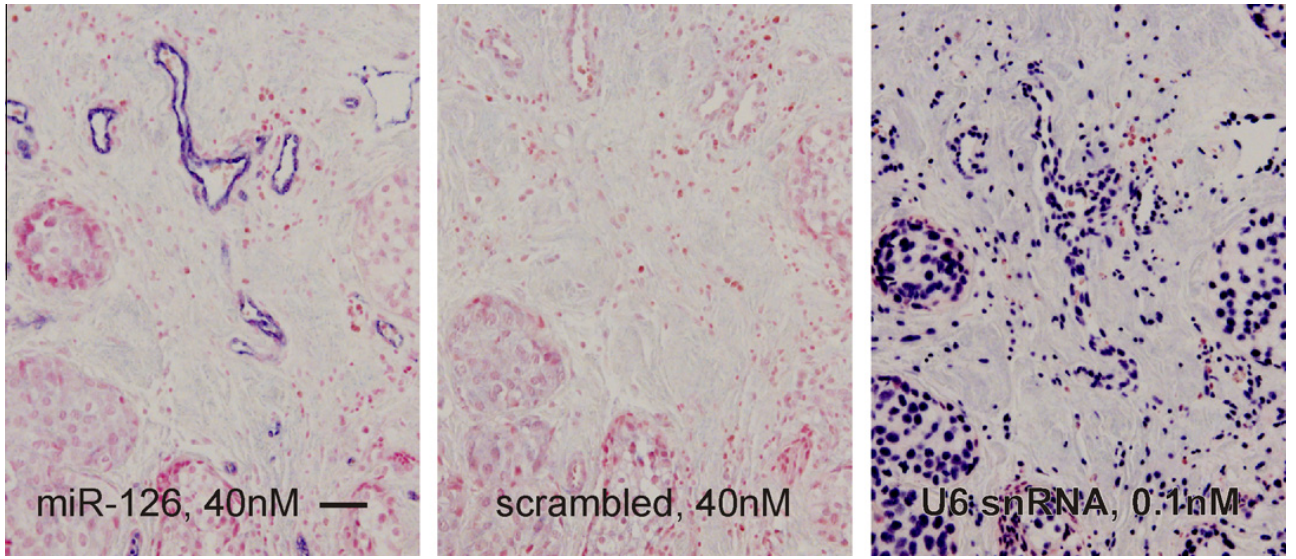
ISH for mRNA and miR detection

- ✓ Morphology
- ✓ Sensitivity
- ✓ miRs have high degree of similarity between the sequences. Some micro- RNA family members vary by a single nucleotide.
- ✓ Use LNA-enhanced oligonucleotides.
- ✓ The use of LNA™ in probes enables highly sensitive detection and analysis of the short miRNA sequences, but also mRNA.
- ✓ The use of LNA probe allows a better control of Tm even in AT rich sequences and for short stretches (miR)



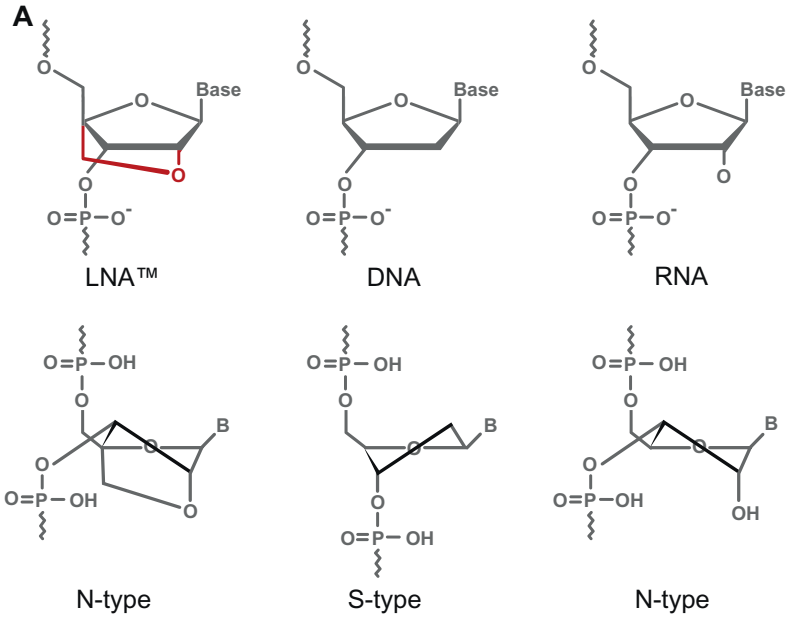
miRCURY LNA™ microRNA ISH
Exiqon

Methods 52 (2010) 375–381



Why LNA?

1. High affinity RNA
2. Ribose ring is “locked” in the ideal conformation for Watson–Crick binding.
3. As a result, LNA™ oligonucleotides exhibit thermal stability when hybridized to a complementary DNA or RNA strand.
4. For each incorporated LNA monomer, the melting temperature (Tm) of the duplex increases by 2° C–8° C (Figure A-2).
5. LNA oligonucleotides can be made shorter



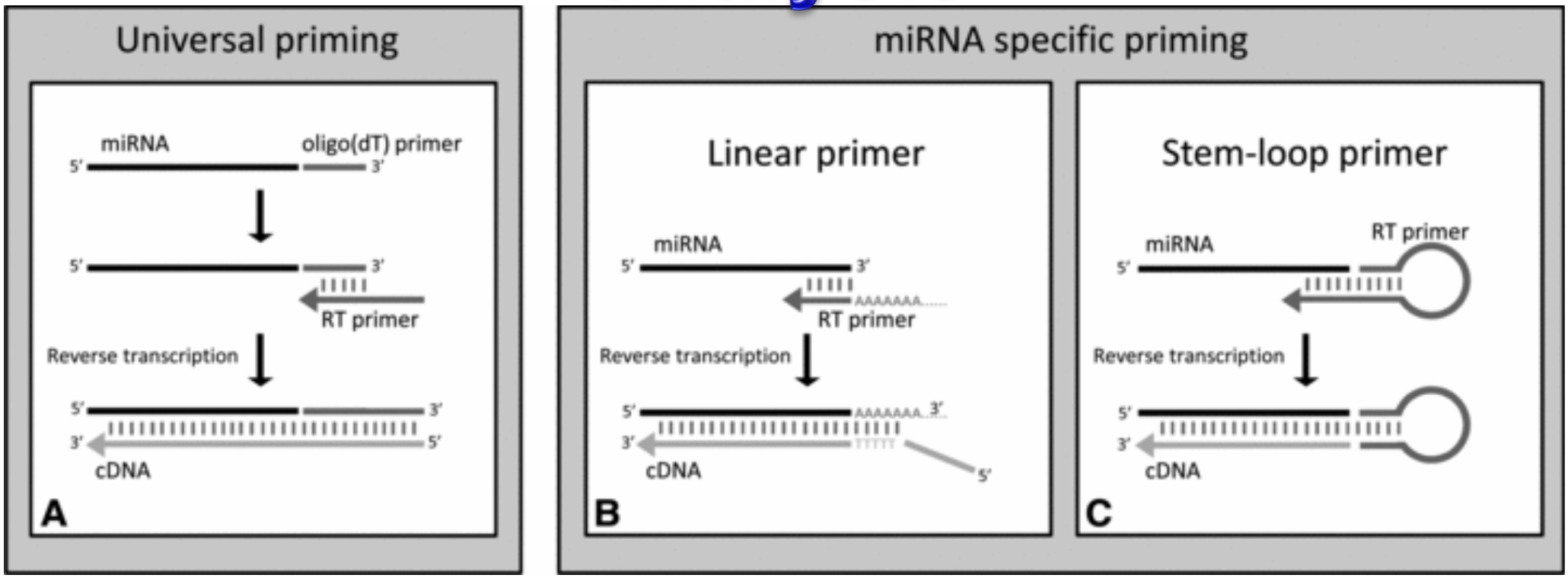
B

Probe \ Target	Perfect match 3′-acgaccac-5′	Single mismatch 3′-acggccac-5′	Δ T_m
DNA 8-mer 5′-tgctggtg-3′	T_m = 35° C	T_m = 25° C	10° C
LNA™ 8-mer 5′-TGCTGGTG-3′	T_m = 71° C	T_m = 45° C	26° C

Figure A-1 Structure and conformations of nucleotides (A) and the effect of LNA™ on melting temperature of duplexes (B).

From: G.J. Nuovo (Eds) In Situ Molecular Pathology and Co-expression analyses (2013)

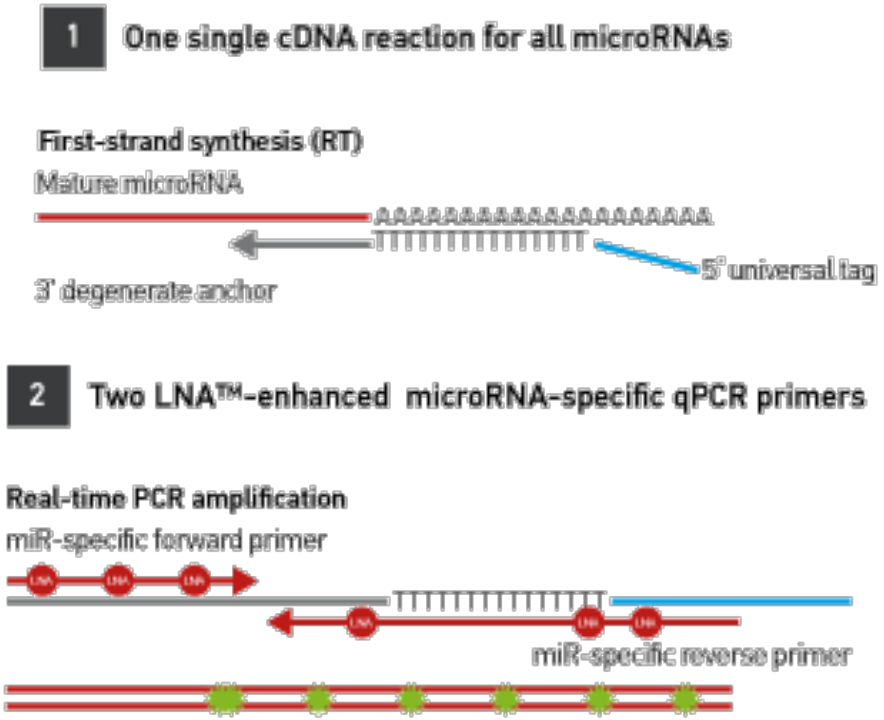
Variability of RT for miRNA analysis



Journal of Cellular and Molecular Medicine
Volume 16, Issue 4, pages 683-690, 16 APR 2012 DOI: 10.1111/j.1582-4934.2011.01467.x
http://onlinelibrary.wiley.com/doi/10.1111/j.1582-4934.2011.01467.x/full#f2

PCR based miRNA assays

miRCURY LNA™ Universal RT microRNA PCR



• – LNA™ primers are shorter, so both primers can be specific to the microRNA, enabling single nucleotide discrimination

•**Unrivalled sensitivity** – *T*m normalized LNA™ primers for accurate detection even of AT-rich microRNA from just 1 pg total RNA

TaqMan Assay