



**microRNA Quantification by  
Real-time PCR**

**Nicole Schonrock**

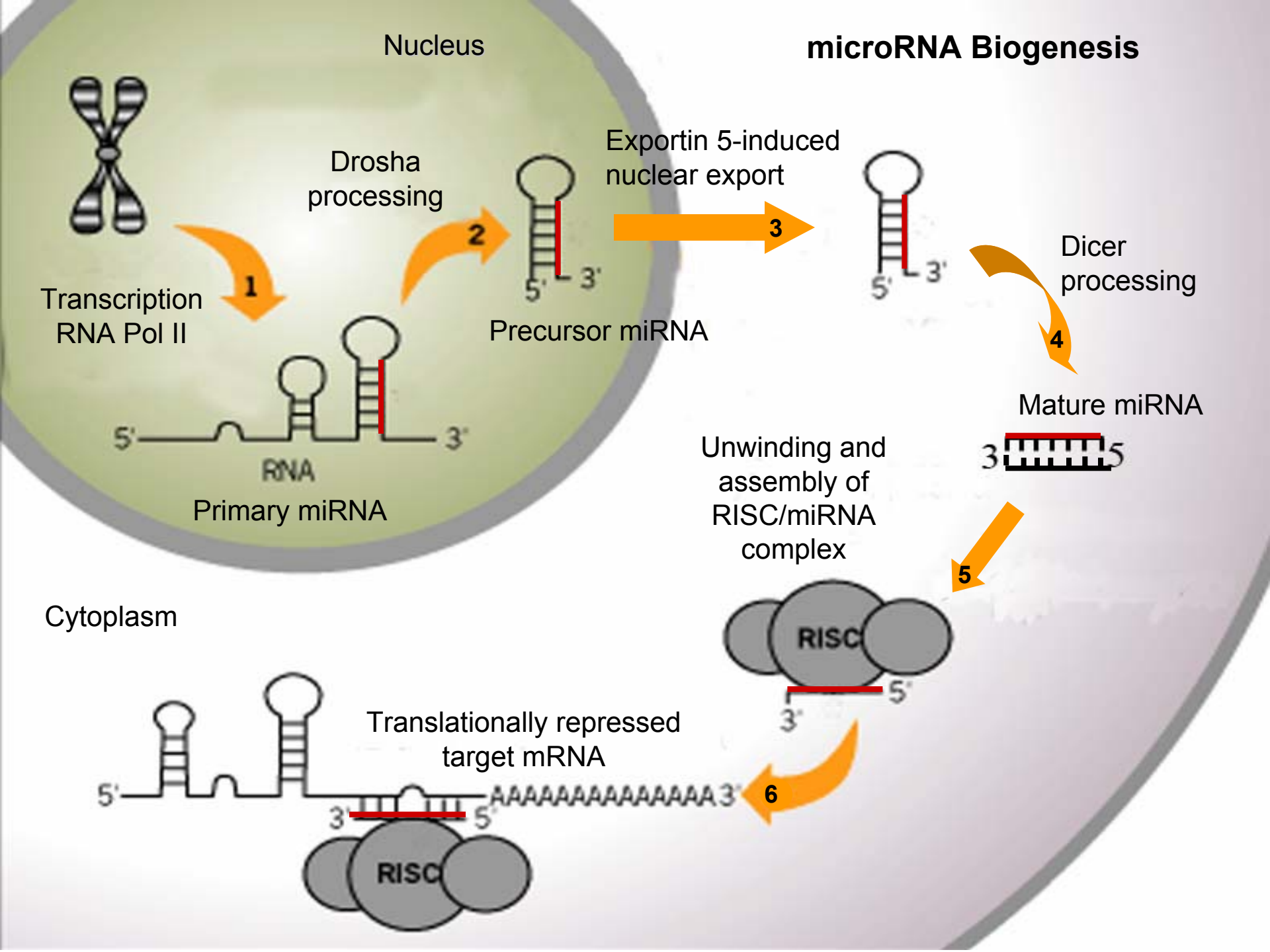
**Brain and Mind Research Institute**

**Alzheimer's and Parkinson's Disease Laboratory**



## Overview

- 1) Preparation for microRNA Real-time PCR
- 2) Which method/kit to use and when
- 3) Primer design for microRNA amplification
- 4) Which normalizer to use
- 5) Which SYBR Green based fluorescent dye works best
- 6) Kit comparisons





## Considerations when working with microRNAs

miRNAs are small 19-25 single-stranded non-coding RNA molecules

hsa-miR-197 : 5'-UUCACCACCUUCUCCACCCAGC-3'

miRNA family members can be very similar eg let-7 family:

```
mmu-let-7a: UGAGGUAGUAGGUUGUAUAGUU
mmu-let-7b: UGAGGUAGUAGGUUGUGUGGUU
mmu-let-7c: UGAGGUAGUAGGUUGUAUGGUU
mmu-let-7d: AGAGGUAGUAGGUUGCAUAGUU
mmu-let-7e: UGAGGUAGGAGGUUGUAUAGUU
mmu-let-7f: UGAGGUAGUAGAUUGUAUAGUU
mmu-let-7g: UGAGGUAGUAGUUUGUACAGUU
mmu-let-7i: UGAGGUAGUAGUUUGUGCUGUU
```

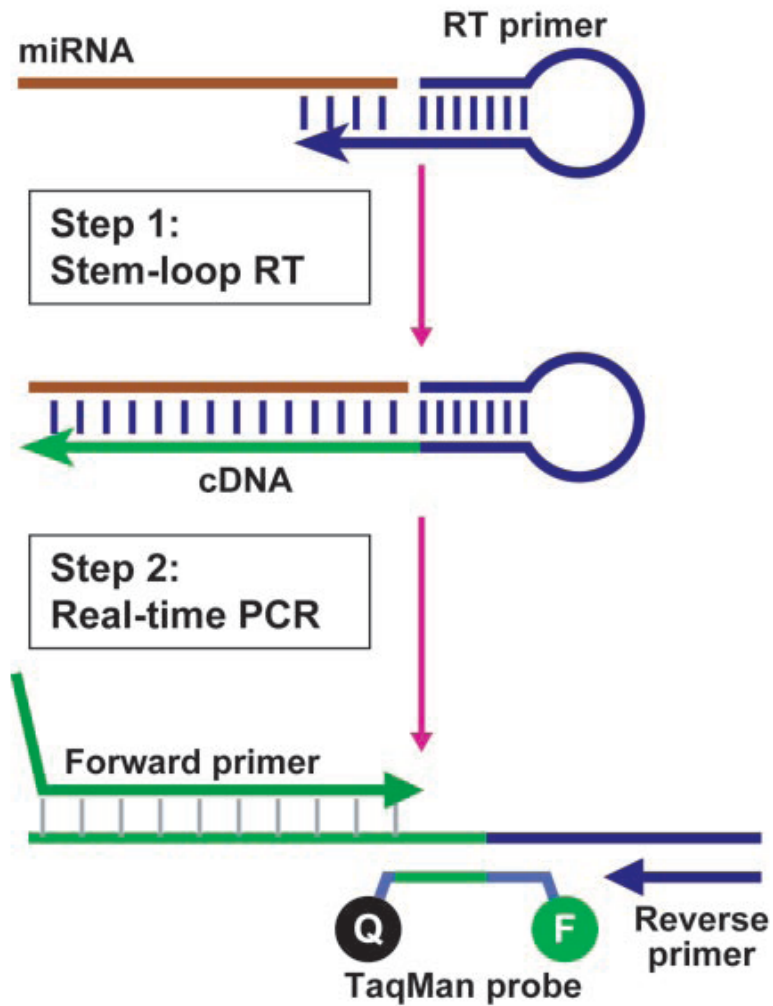
RNA Extraction Method Must Retain Small RNAs

- Trizol (Invitrogen)
- mirVANA miRNA Isolation Kit (ABI)
- miRNeasy (Qiagen)

Small RNA enrichment is Not necessary!



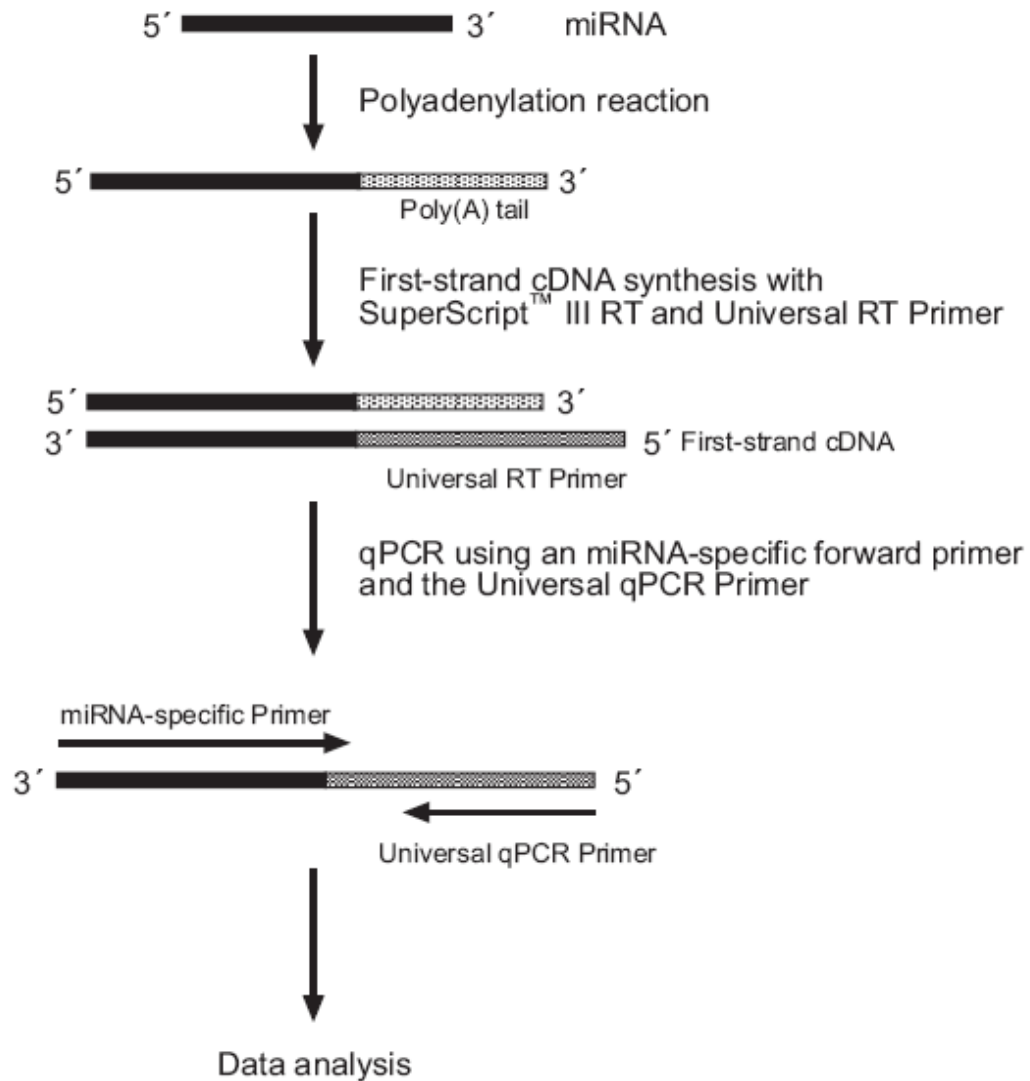
## Taqman microRNA Assays (ABI)





## NCode miRNA QPCR (Invitrogen)

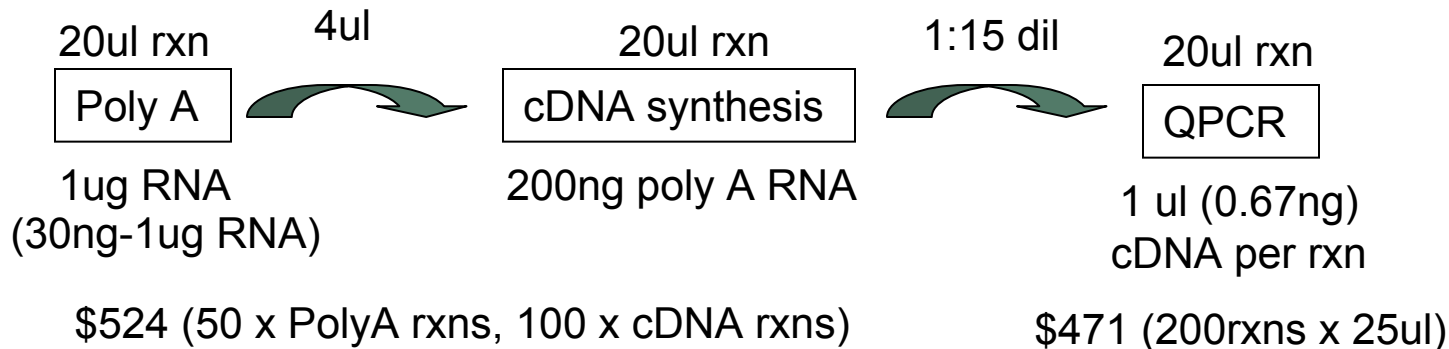
## High-Specificity miRNA QRT-PCR Kit (Stratagene)



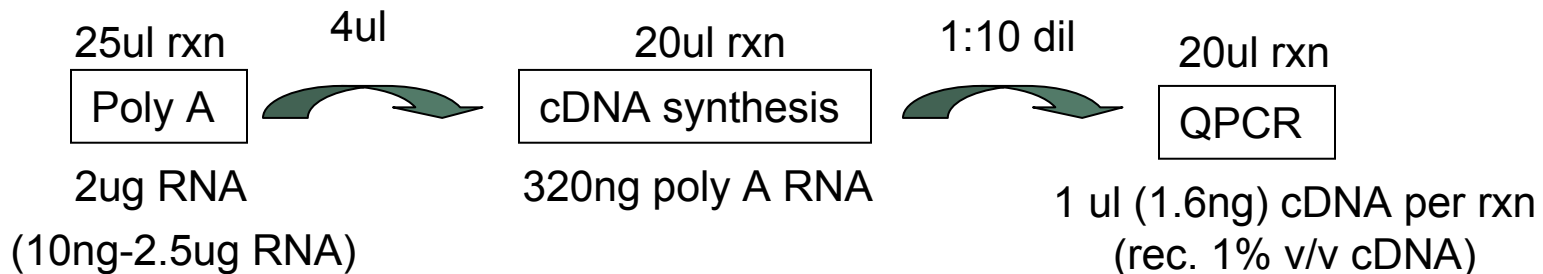


## Preparing cDNA for microRNA Real-time PCR

### Stratagene miRNA QRT-PCR Detection Kit:



### Invitrogen NCode miRNA cDNA synthesis and qRT-PCR Kit:



\$599 (50 x PolyA rxns, 100 x cDNA rxns, "unlimited" QPCR rxns)



## microRNA Primer Design and Cycling Conditions

**mmu-miR-467b\*:** 5'-AUAUACAUAACACACACCAACAC-3'  
miR-467b primer: 5'-ATATACATACACACCAACAC-3'  
T<sub>m</sub>=53.7°C

**mmu-miR-714:** 5'-CGACGAGGGCCGGUCGGUCGC-3'  
miR714 primer: 5'-CGACGAGGGCCGGTCGGTCGC-3'  
T<sub>m</sub> = 82.2°C with strong secondary structure



Primers with a high GC content can be truncated by 3-4 bases on the 3' end

Primers are used at a final concentration of 125nM

Cycling conditions:

10min / 95°C

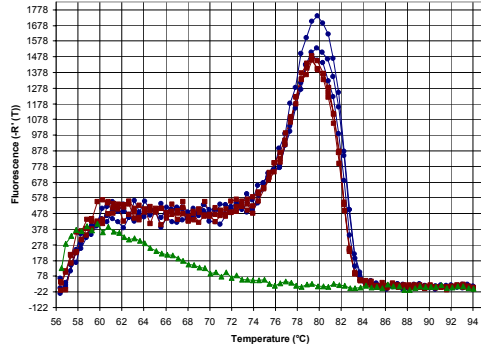
10sec / 95°C  
15sec / 60°C  
20sec / 72°C } 40-45 cycles

Melt Curve!

# microRNA Dissociation curves for Stratagene and Invitrogen Kits

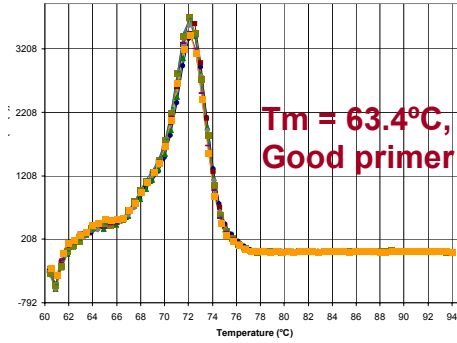
## Stratagene (EvaGreen)

Dissociation curve for snoRNA202



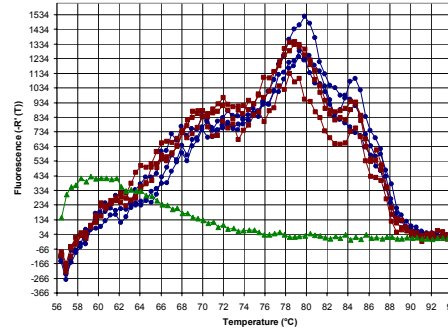
## Invitrogen (SYBR)

Dissociation curve for snoRNA202



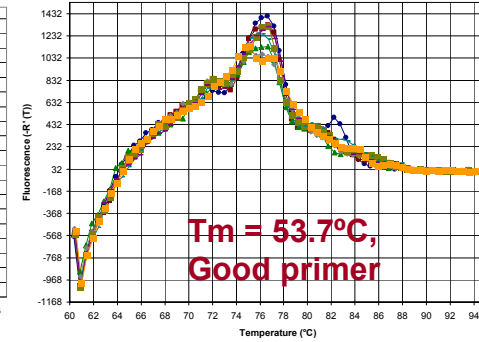
## Stratagene (EvaGreen)

Dissociation curve for miR467b

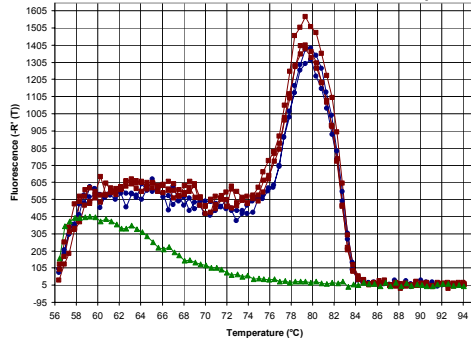


## Invitrogen (SYBR)

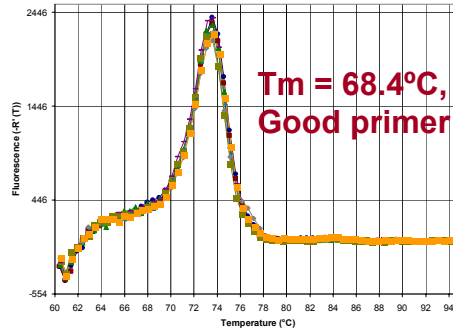
Dissociation curve for miR467b



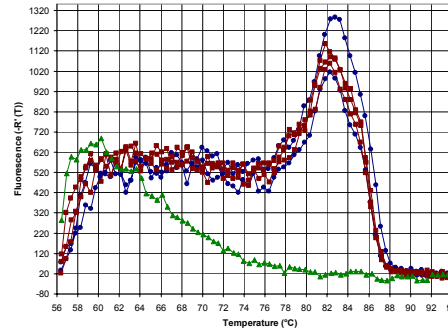
Dissociation curve for miR409-5p



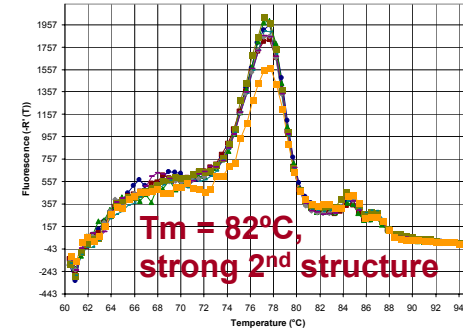
Dissociation curve for miR409-5p



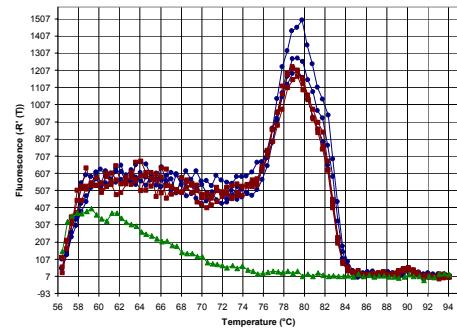
Dissociation curve for miR714



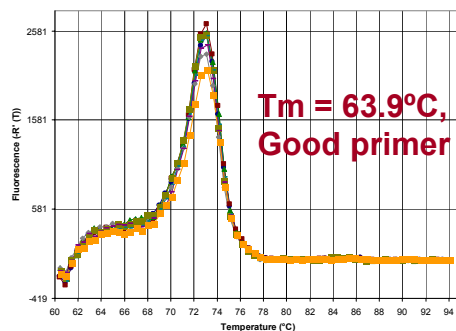
Dissociation curve for miR714



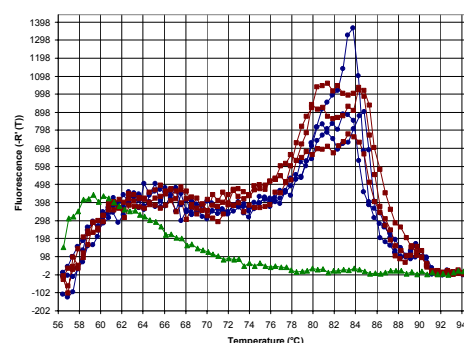
Dissociation curve for miR323



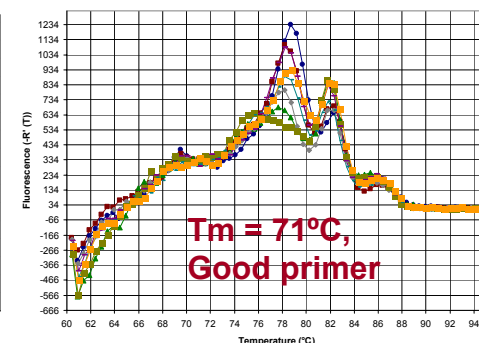
Dissociation curve for miR323



Dissociation curve for miR197



Dissociation curve for miR197

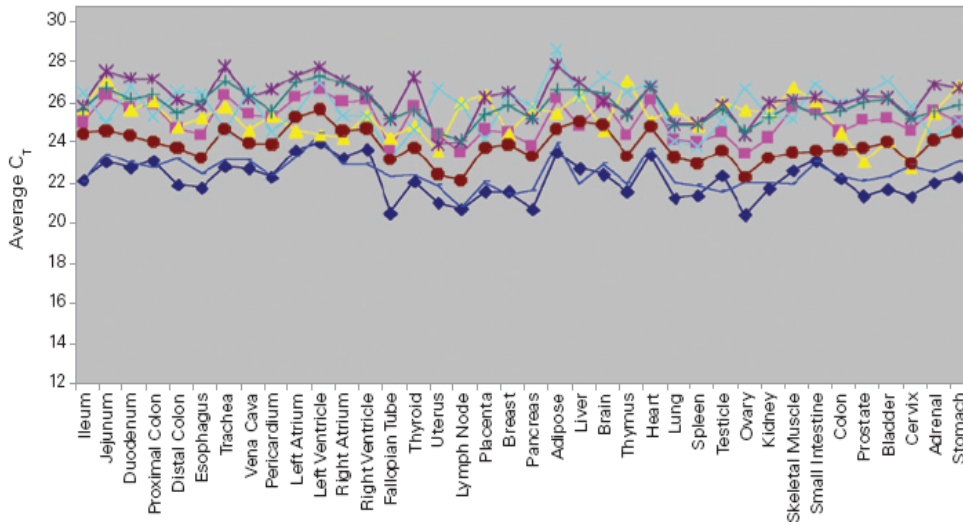




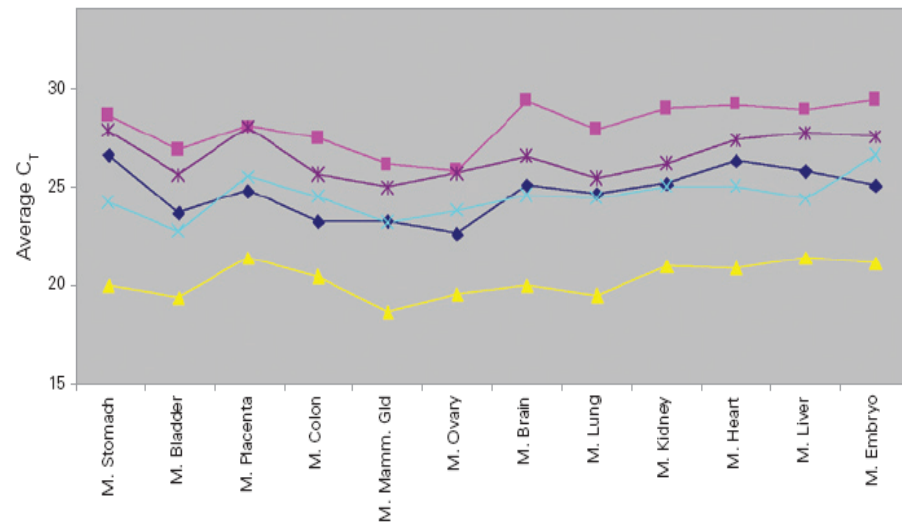
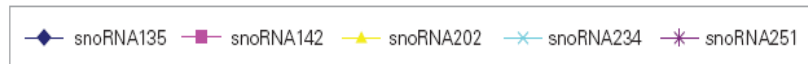
# What do I use as a Normalizer?

ABI – Application Note: “Endogenous Controls for Real-Time Quantitation of miRNA using TaqMan microRNA assays”.

Human tissue



Mouse tissue



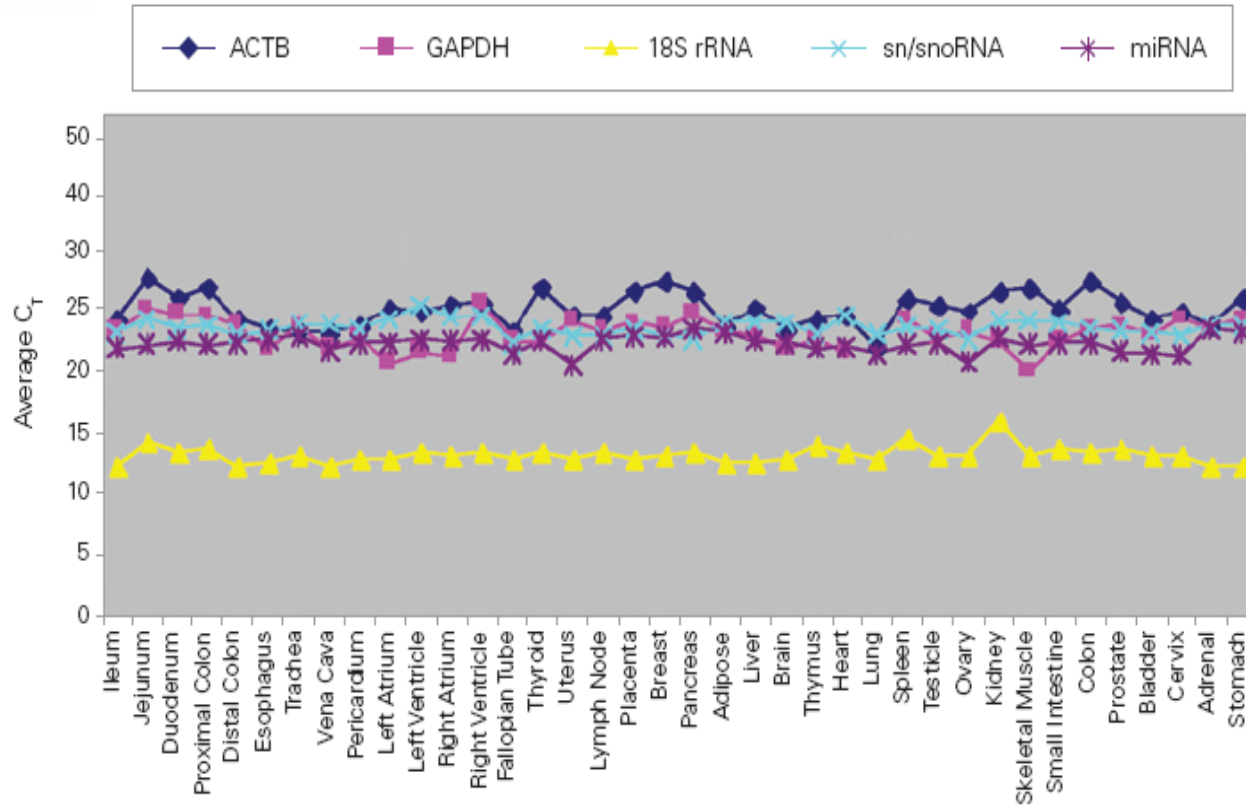
Human - RNU48, RNU44, U47, RNU6B, hsa-miR-26b, hsa-miR-92, hsa-miR-92N.

Mouse - snoRNA202, snoRNA234



# Comparing snoRNA to other used Normalizers

Human tissue



**mmu-snoRNA202:** GCUGUACUGACUUGAUGAAAGUACUUUUGAACCCUUUUC CAUCUGAUG  
 snoRNA202 primer: CTTTTGAACCCTTTTCCATCTG

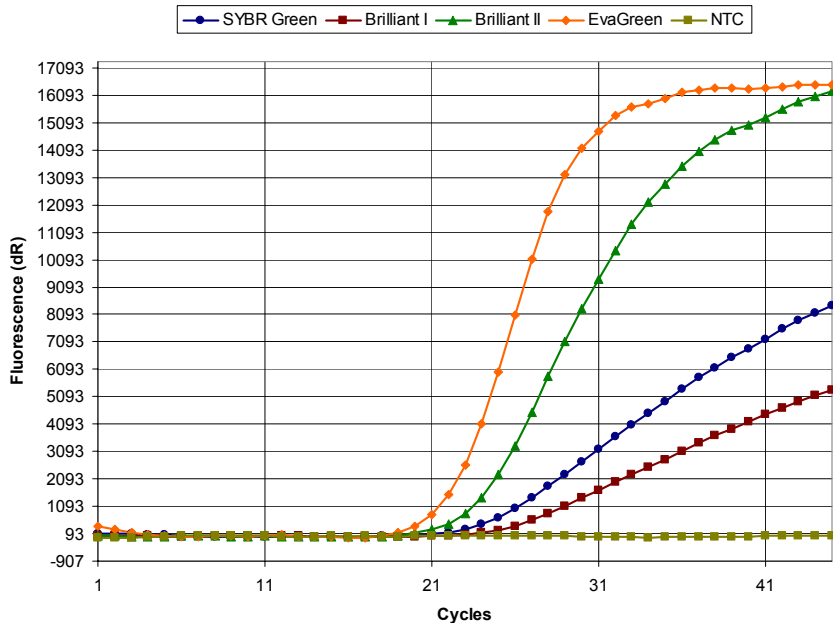
# Testing Different Master Mixes

Used Stratagene Kit with snoRNA202 primers to test different master mixes:

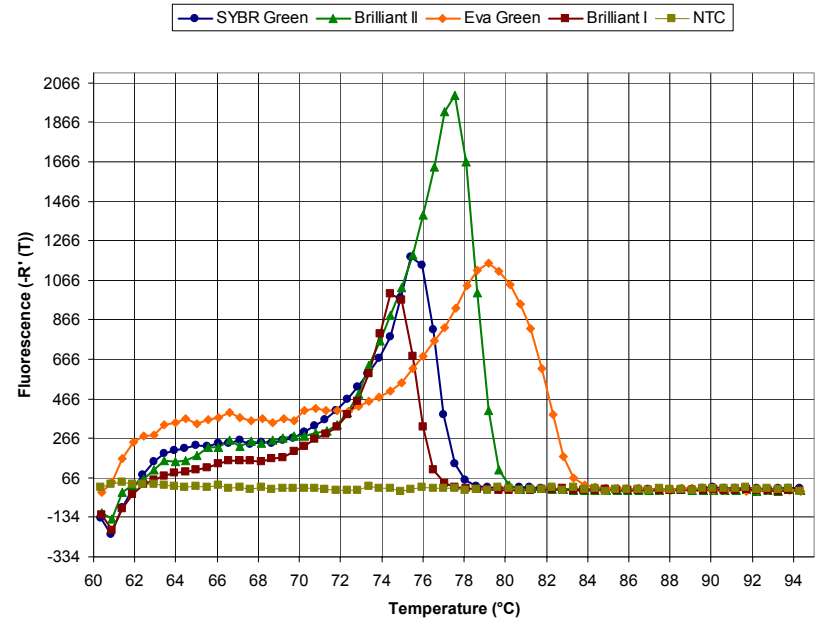
- SYBR Green (ABI) = 75.96°C
- Brilliant I (Stratagene) = 74.97°C
- Brilliant II (Stratagene) = 77.68°C
- EvaGreen (Stratagene) = 79.68°C

Although the amplicon should be the same the different master mixes produce slightly different melt curves.

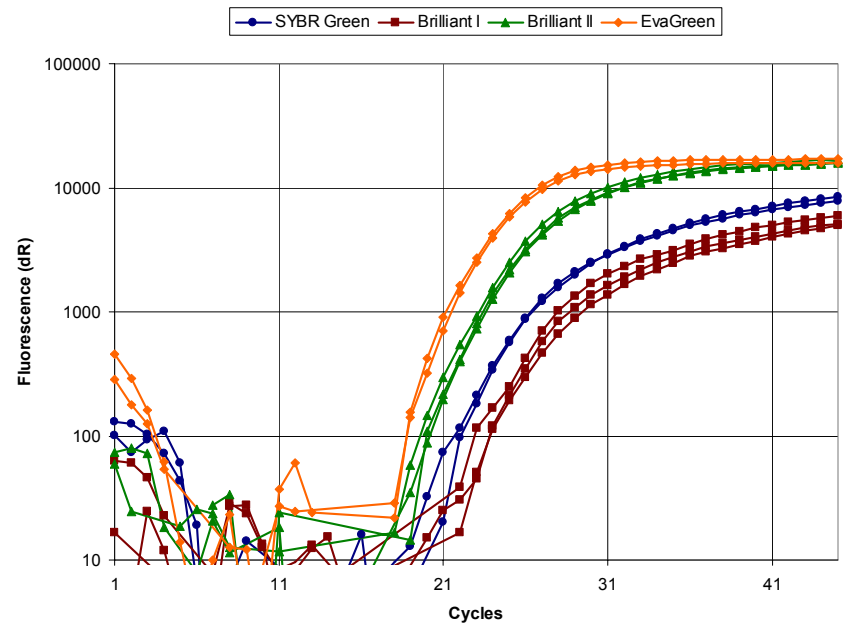
## Amplification plots for snoRNA202 (Linear)



## Dissociation Curve for snoRNA202



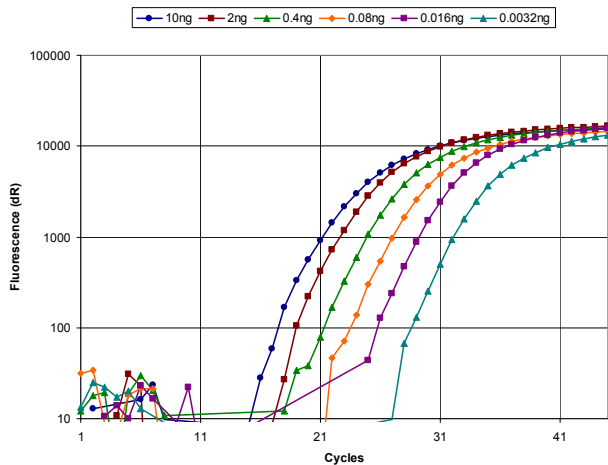
## Amplification plots for snoRNA202 (Log)



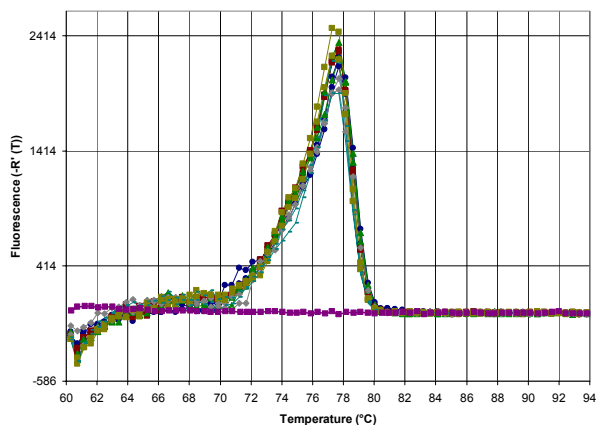
# Testing sensitivity of Invitrogen vs Stratagene Kits via cDNA dilution series

snoRNA202 primers on a 1:5 dilution series of WT cDNA using Brilliant II (20ul)

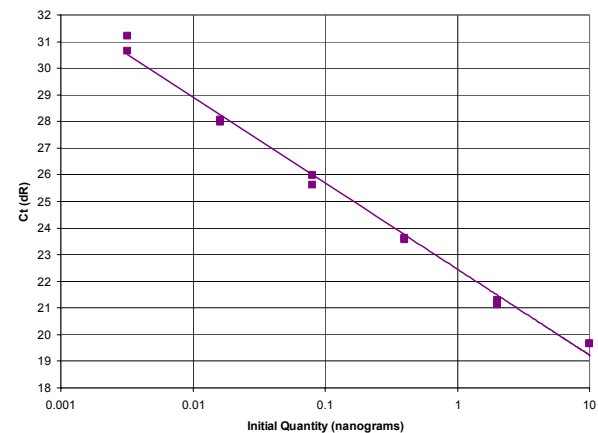
### Amplification Plot for snoRNA202 (Stratagene)



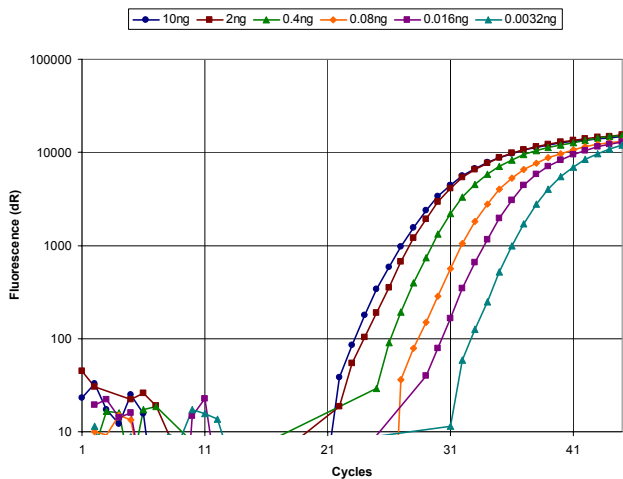
### Dissociation curve for snoRNA202 (Stratagene)



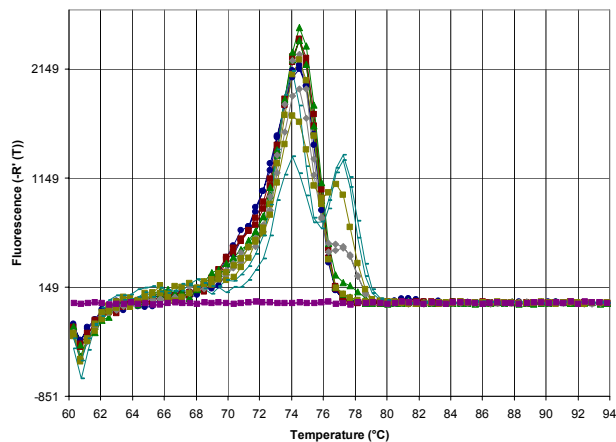
### Standard curve for snoRNA202 (Stratagene)



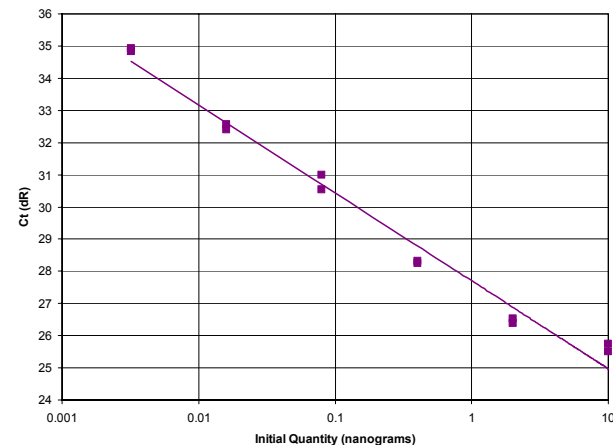
### Amplification Plot for snoRNA202 (Invitrogen)



### Dissociation curve for snoRNA202 (Invitrogen)

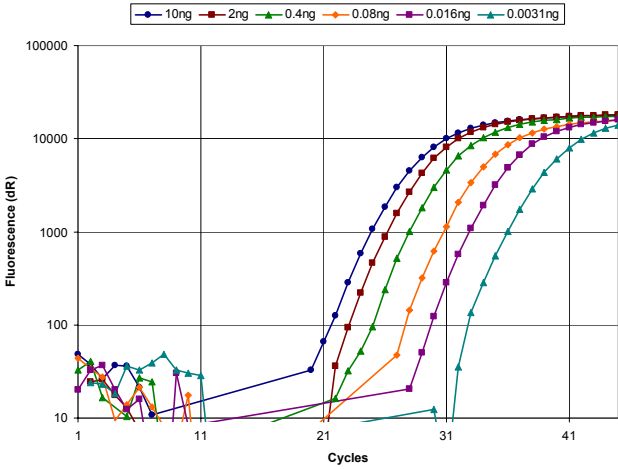


### Standard curve for snoRNA202 (Invitrogen)

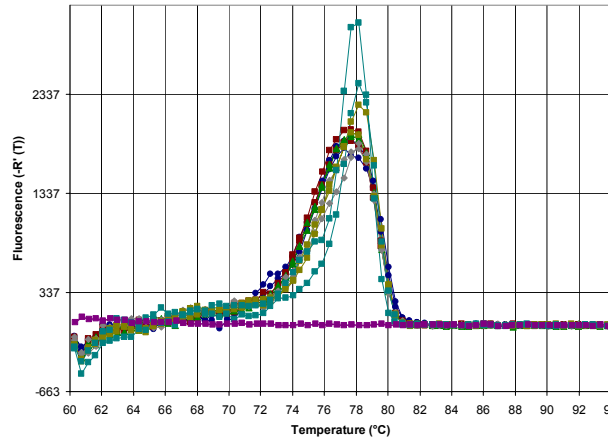


# miR409-5p primers on a 1:5 dilution series of WT cDNA using Brilliant II (20ul)

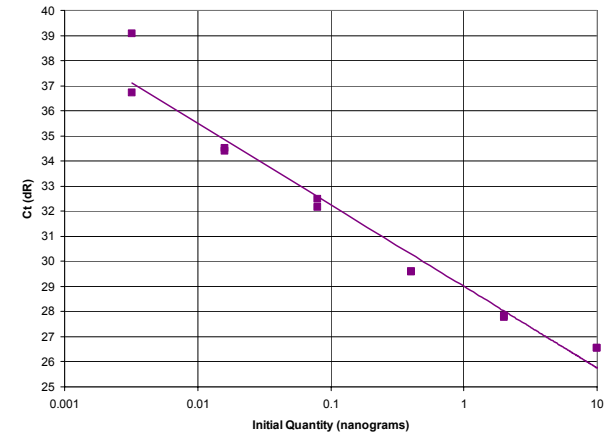
## Amplification Plot for miR409-5p (Stratagene)



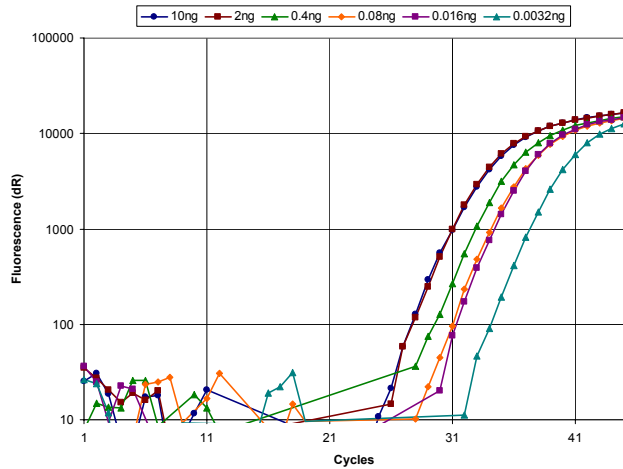
## Dissociation curve for miR409-5p (Stratagene)



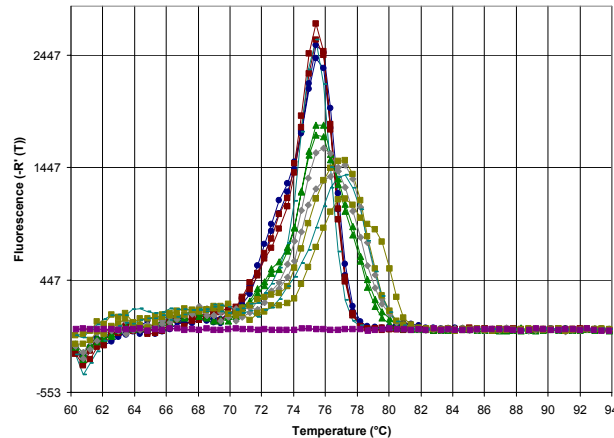
## Standard curve for miR409-5p (Stratagene)



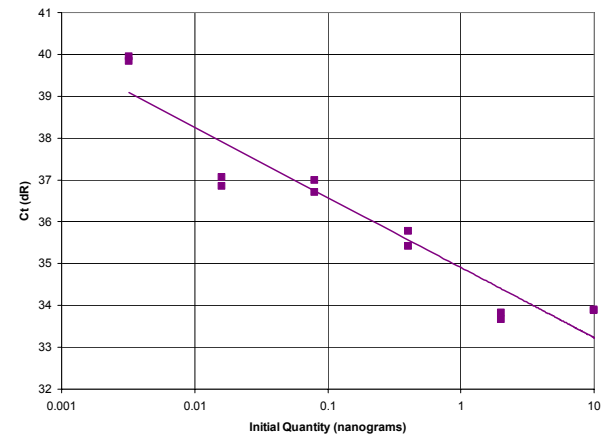
## Amplification Plot for miR409-5p (Invitrogen)



## Dissociation curve for miR409-5p (Invitrogen)



## Standard curve for miR409-5p (Invitrogen)





## Conclusions

- Invitrogen and Stratagene Kits provide cost effective method for real-time PCR for many different miRNAs per sample.
- Stratagene Kit seems more sensitive than Invitrogen
- Dynamic range seems greater for Stratagene Kit
- EvaGreen dye from Stratagene is the most sensitive for miRNA assays followed by Brilliant II
- Stratagene kit very limiting in the number of QPCR reactions per kit.

# Acknowledgements

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**Denise Nergenu**



**Brain and Mind Research Institute**

**Alzheimer's and Parkinson's Disease Laboratory**