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Quantifying short RNA molecules in a regulatory environment

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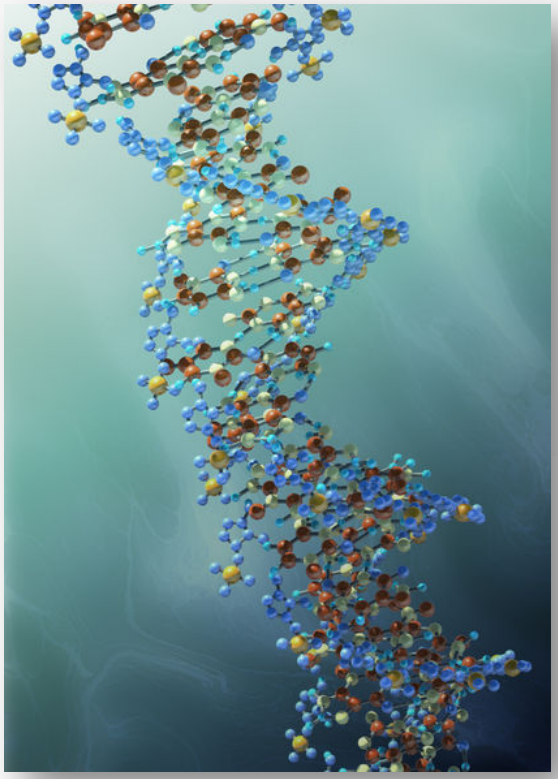
Summary

- Nucleic acids as drugs
- Short RNA extraction
- RT-qPCR
- Validation
- Important considerations



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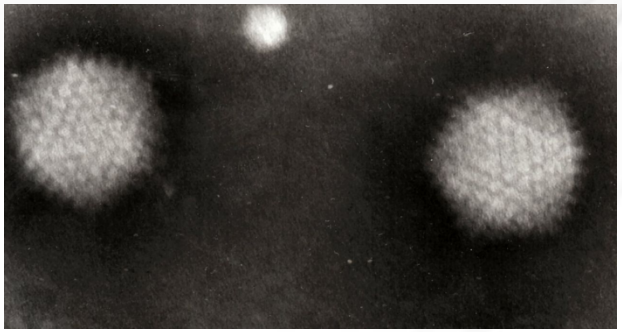
Nucleic acids as drugs

- **Drugs based on DNA**
  - Plasmids, viral DNA (e.g. adenovirus)
  - Synthesis of proteins



- **Drugs based on RNA**
  - RNA vaccines, siRNA, miRNA
  - Synthesis of proteins and blockage of transcription

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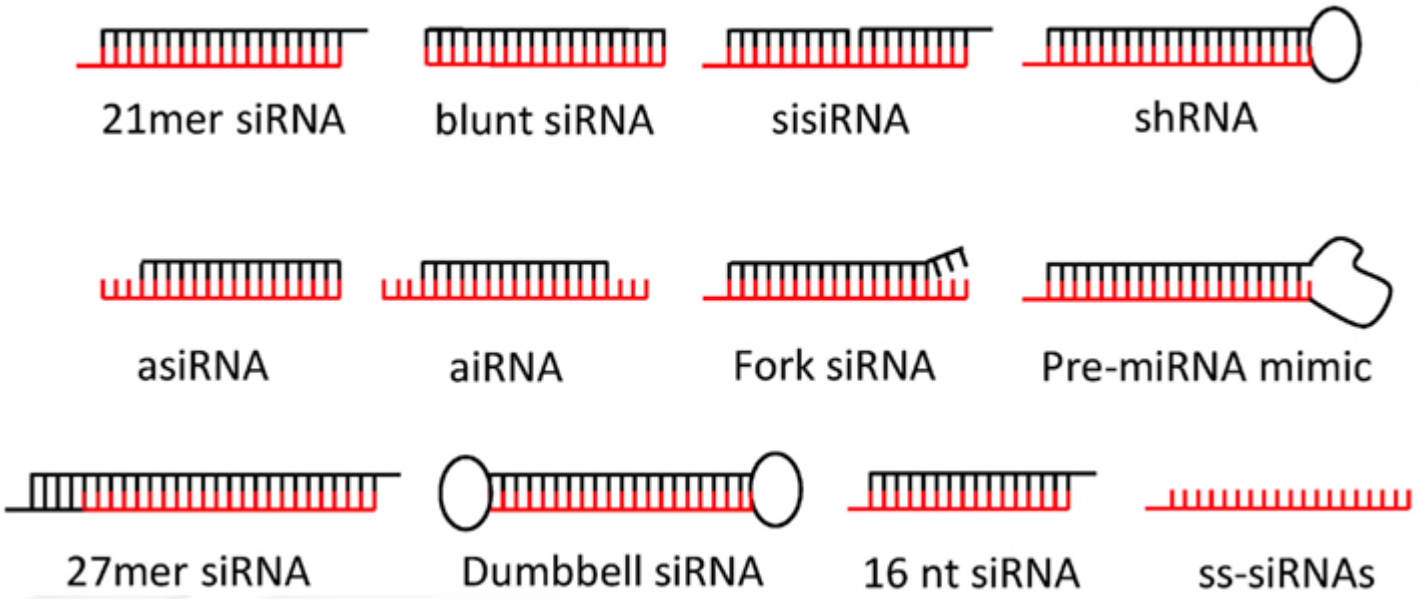
Short RNA

- Approximately 20 nucleotides with a net negative charge
- Usually double stranded RNA that is processed within the cell that mediates sequence-specific RNA degradation.



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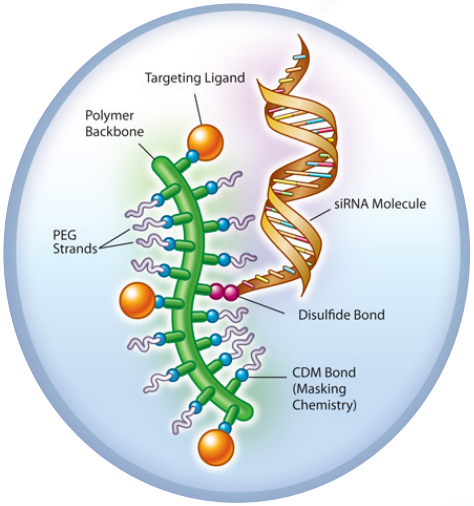
Short RNA administration

- Must enter cell crossing negative charged lipid bilayer
- Unstable in the blood stream when unprotected.
- Delivery systems:
  - various lipid nanoparticles (tissue specific?)
  - conjugated to polycarbonates with cell targeting ligands
  - viral vectors
  - cationic peptides
- Subcutaneous delivery of naked RNA



DPC from Arrowhead Research Corp.

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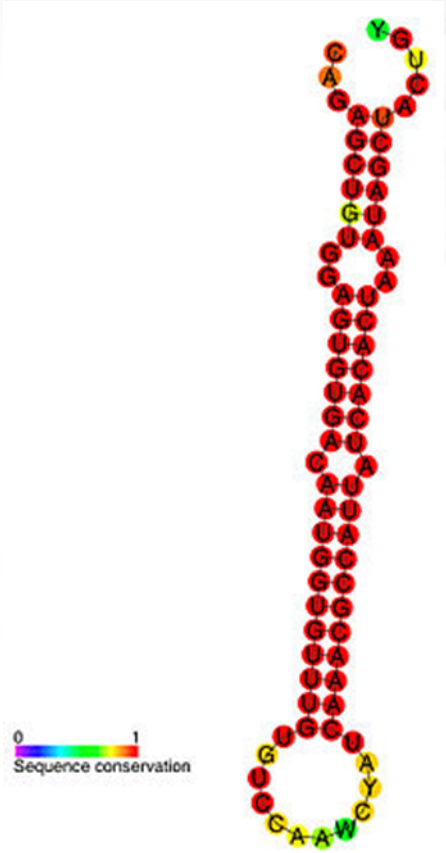
Quantification of DNA and RNA molecules

- **DNA/RNA extraction**
  - Plasma, serum or whole blood?
  - Variety of kits available
  - Remember extraction efficiency and stability
- **Quantification**
  - qPCR or RT-qPCR
  - Many kits/enzyme systems available.
- **Specific for short RNA**
  - Extraction from plasma (whole blood spun twice)
  - Qiagen RNeasy kits perform best
  - RNA is inherently unstable
- **Quantification**
  - Specific RT-qPCR with stem loop or LNA primers.



miR-122
source Wikipedia

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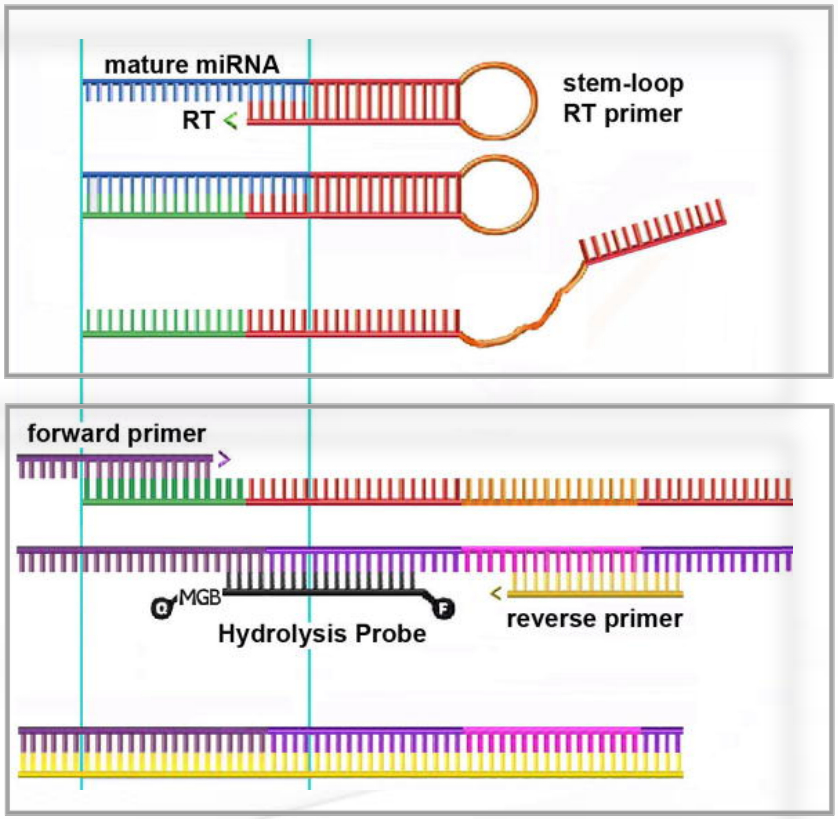
Short RNA quantification – stem loop primers

RT



TaqMan PCR

Adapted from Kramer. Curr Protoc Mol Biol. 2011

- PCR requires a minimum of 40 nt to amplify
- Stem loop primers used in RT to increase length of cDNA
- PCR forward primers designed with overhang
- High specificity and sensitivity

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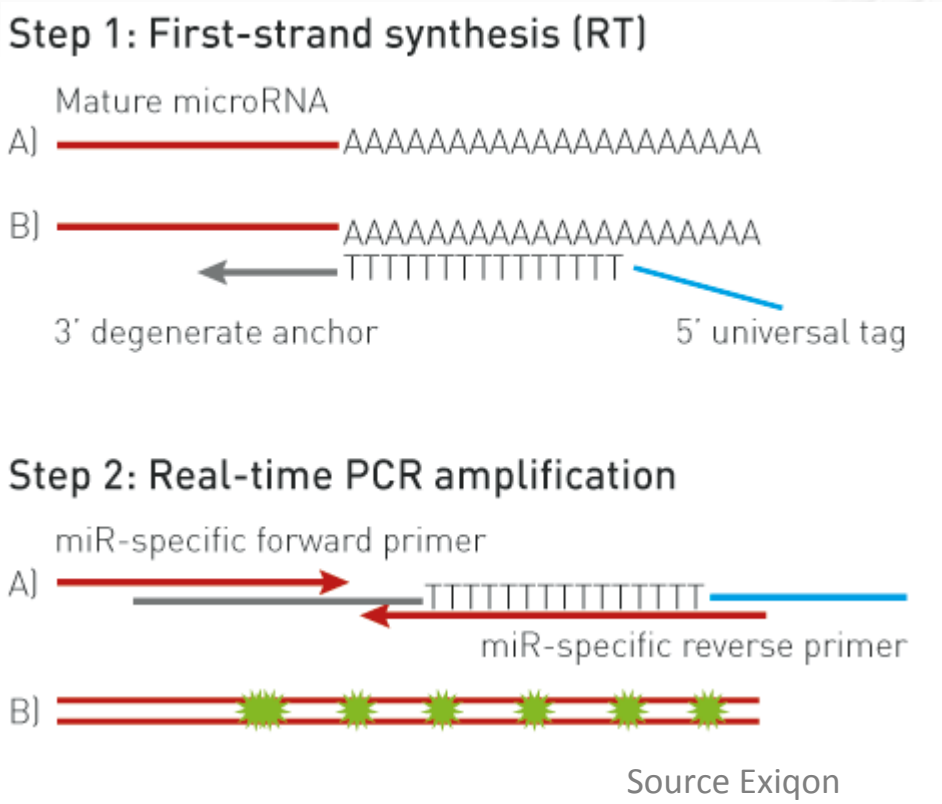
Short RNA quantification - LNA

- Locked nucleic acid PCR from Exiqon
- High-affinity RNA analogues in which the ribose ring is “locked” in the ideal conformation for Watson-Crick binding.
- qPCR with SYBR green
- High specificity and sensitivity



Source Exiqon

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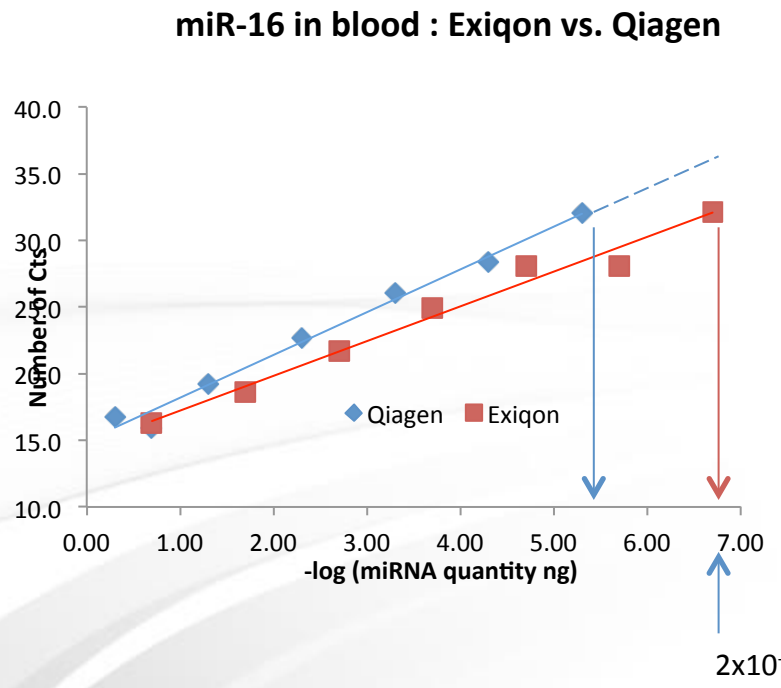
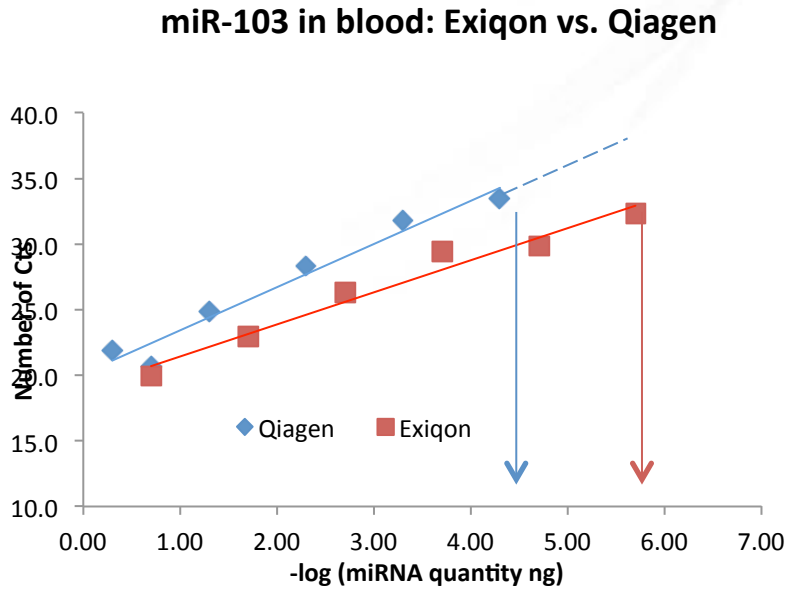
Increased sensitivity of specialised kits

- Exiqon LNA RT-qPCR versus standard RT-qPCR
- Substantial differences in sensitivity (10-15 fold)
- Exiqon limit of detection at $2x10^{-7}$ ng miRNA





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Validation of RT-qPCR

- A validation of a qPCR method should adhere to the principles of GLP

- The following parameters must be addressed:
  - Specificity
  - Dynamic range and detection limits
  - qPCR efficiency
  - Matrix effects
  - RNA Extraction efficiency
  - RNA stability

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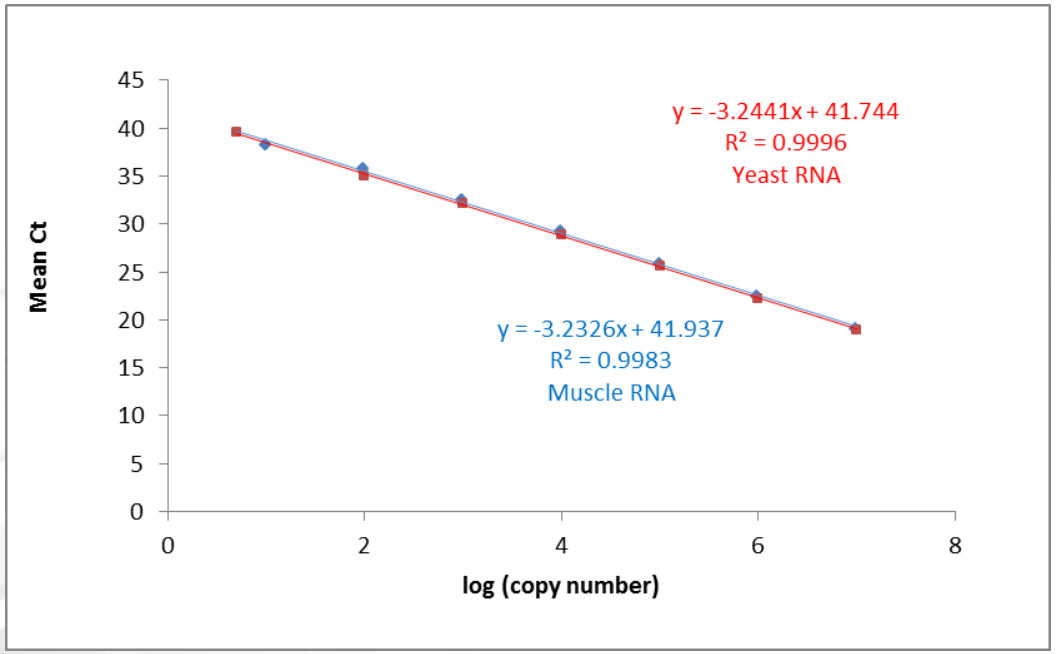
Validation of RT-qPCR

- **Specificity**
  - Perform RT-qPCR in RNA background
    - Either host background or Yeast RNA
    - NTC should be undetermined
    - Require good efficiency of standard curve



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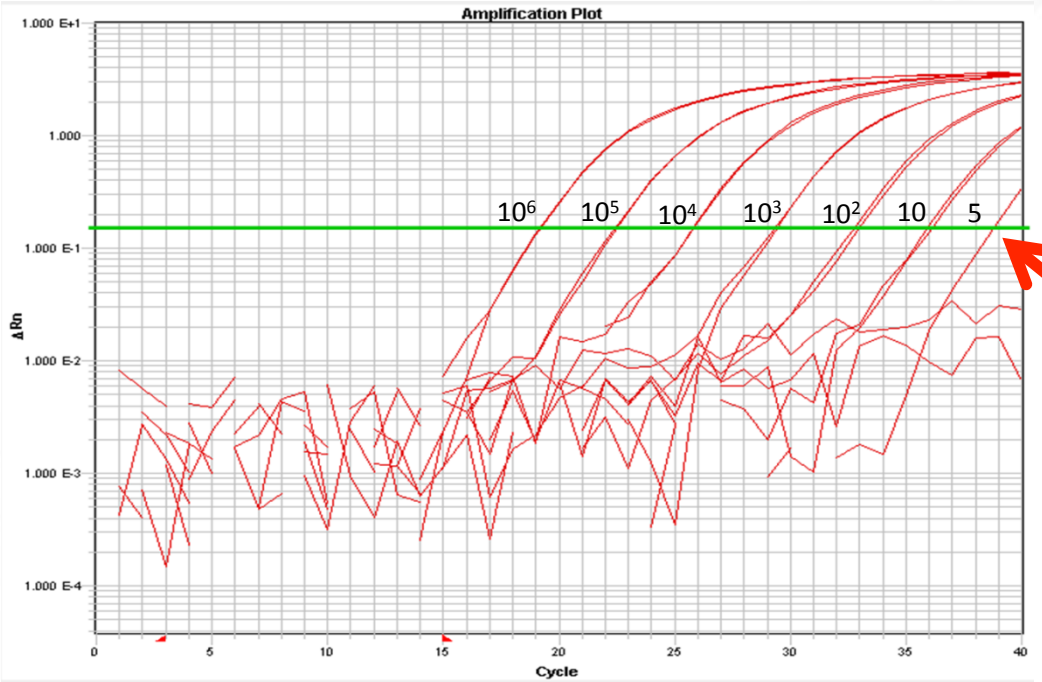
Validation of RT-qPCR

- **Dynamic range**
  - Standard curve reproduced 4 times over minimum 2 days
  - qPCR efficiency
    - Slope -3.3 +/- 10%
  - Quantification limits
    - ΔCt <0.7 between duplicates
  - Detection limit
    - LLOD should be >NTC



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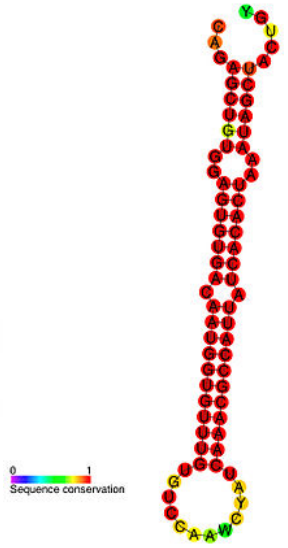
Validation of RT-qPCR

- **Matrix effects (PCR inhibition)**
  - To avoid false negatives
  - qPCR of exogenous ANTP from *Drosophila*
  - Accept if >50% of nominal copies

- **Extraction efficiency**
  - To avoid false negatives
  - Spike the test item between 10^4 or 10^6 copies into matrix
  - Acceptable if > 50% recovery

- **Stability**
  - Is the test item still there after 2 months storage?
  - Spike the test item between 10^4 or 10^6 copies
  - Stable if > 50% recovery



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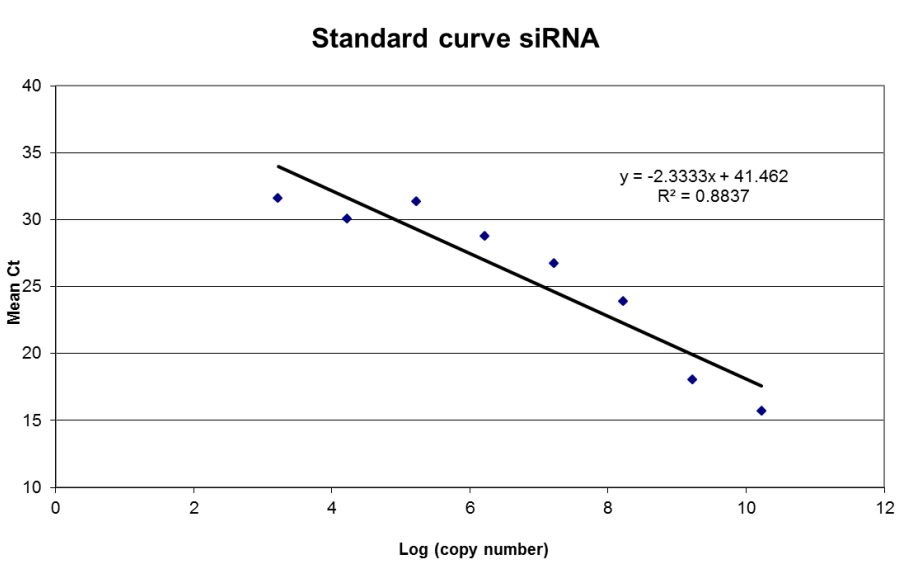
Special considerations

- Avoid high concentration preparations of test item (increased likelihood of aerosol contamination)
- Is your stem loop configured?
- Transferability of method…
- Decontaminate regularly
- Use low binding lab ware
- For stability periods
  – expect the unexpected



Example of plastic binding on siRNA quantification

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