Design and Optimization of real-time qPCR Assays Suitable for Publication

qPCR 2005
Weihenstephan

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Topics

* Tips for primer and probe design - all assay types
* Optimization protocol for probe-based assays - Taqman
* Optimization protocol for primer-based assays - SYBR Green I
* Publication of real-time qPCR assays

Assay Design Tips

* The most important step in real-time qPCR assay design is to know as much as possible about the transcript or gene of interest
* Obtain the refseq sequence (NM_xxxxxx) from the NCBI
* Perform a sequence comparison using BLAST to look for related splice variants, allelic variants or other sequences that share sequence motifs
* Align all related sequences and find regions that are unique to your target sequence
* Look up genomic information (NCBI) and look for splice junctions

Assay Design Tips

- Choose a software package that you are comfortable using
  - Assays with Primers or Primers & Probe:

    Primer Express - Applied Biosystems, comes with their instruments

    RealTimeDesign - Biosearch Technologies, free over the web (Poster P43)

    Beacon Designer - Premier Biosoft, comes with Stratagene instruments

    Primer3 - MIT, free over the web

    Lightcycler Probe Design Software - Roche Applied Science, LightCycler assays
  - Assays with Primers Only:

    LUX Designer - Invitrogen, LUX assays, free over the web

    PrimeQuest - IDT, free over the web

    MacVector/DS Gene- Accelrys; Vector NTI- InforMax/Invitrogen; DNAStar-DNASTAR; Oligo- Molecular Biology Insights, big bucks

Assay Design Tips

* Primers

1- Primer concentrations for SYBR Green I assays are lower (50 -100 nM) than for probe-based assays (300 -400 nM)- will affect the primer Tm

2- The primer Tm values should be kept the same or at most +/- 1°C

3- Primers usually work best when there are 2-3 G/Cs in the last 5 bases on the 3' end, 4-5 G/Cs on the end give poor results

4- PCR amplicons for probe-based assays are made as short as possible; those for SYBR Green I assays are between 200-250 bases so that primer dimers can be seen easily on a melt curve

5- Choose assays that have more than two possible primers that can be used in combinations for assay optimization

Assay Design Tips

* Probes

  1- Fluorescein-based reporter dyes (FAM, JOE, Cal Gold, Vic, etc.) should not be conjugated to a G residue

  2- Maximum Taqman probe length is 30 bases for optimal quenching, other probe-based assays can use longer oligos

  3- To keep probes and amplicons short, lower the primer Tm to 55°C or even 50°C for low G/C organisms- MGB probes often not necessary

  4- More Cs than Gs if there is an large imbalance in number- make the complementary probe

  5- No more than 3-Gs in a row

  6- Probe Tm should be 8-10°C higher than the primers

Example 1 - hS100P Assay - Taqman

One forward primer and two reverse primers tested

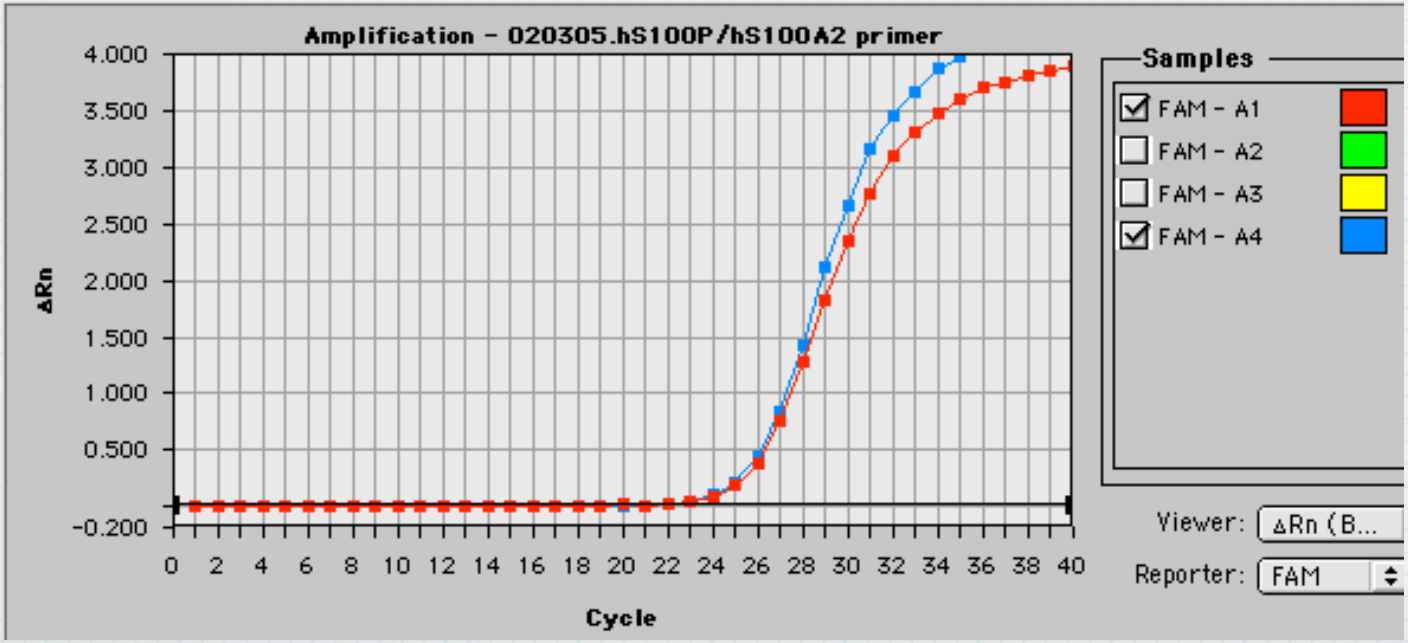
AAATTGCTCAAGGACCTGGAC - 187(+)

AAATTGCTCAAGGACCTGGACGCCAATGGAGATGCCCAGGTGGACTTCAGTGAGTTCATCGTGTTCGTGGC

TTTAACGAGTTCCTGGACCTGCGGTTACCTCTACGGGTCCACCTGAAGTCACTCAAGTAGCACAAGCACCG

256(-) -CACTCAAGTAGCACAAGCACC

257(-) -CTCAAGTAGCACAAGCACCG

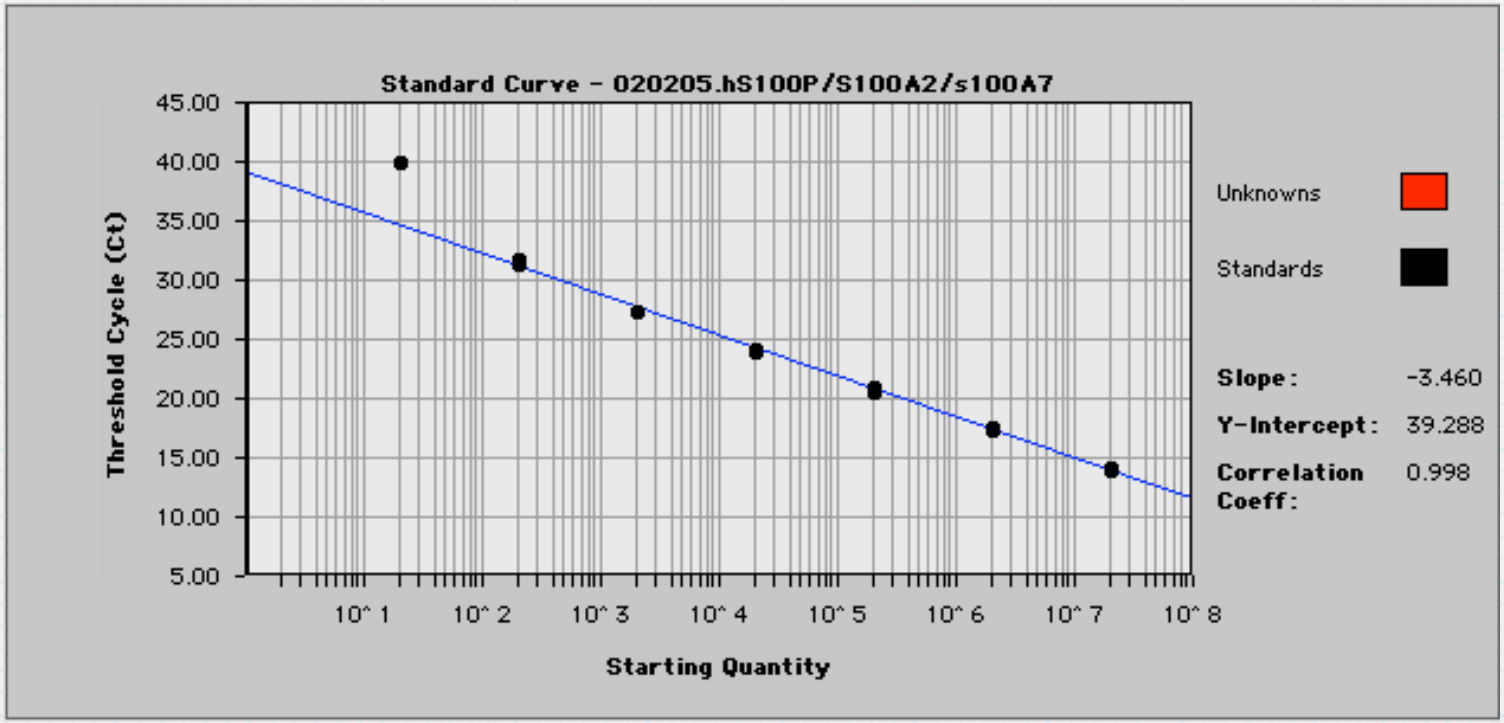


Ct values

187/256 = 24.2

187/257 = 24.0

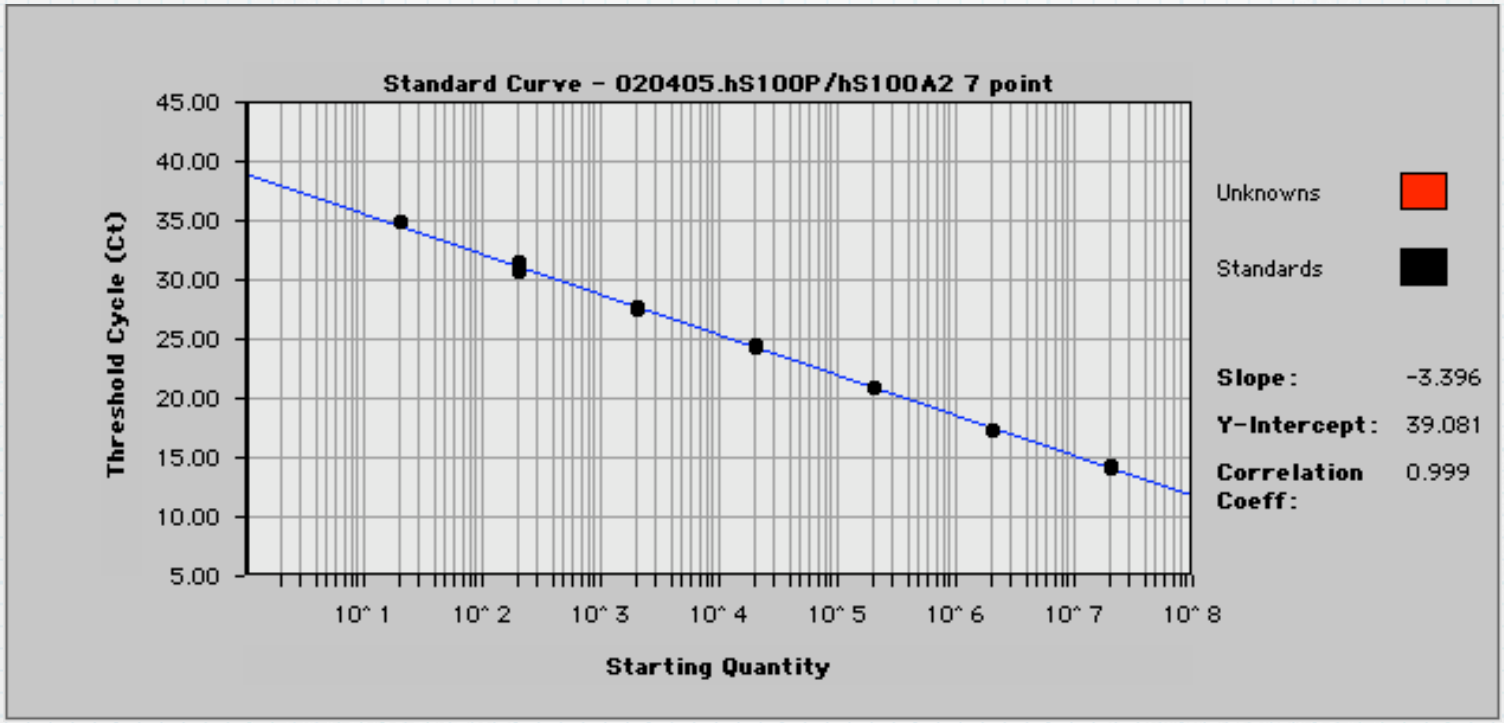
Example 1 - hS100P Assay



AAATTGCTCAAGGACCTGGAC- 187(+)

CCACGAACACGATGAACTCAC - 256(-)

Example 1 - hS100P Assay



AAATTGCTCAAGGACCTGGAC- 187(+)

GCCACGAACACGATGAACTC - 257(-)

Example 1 - hS100P Assay

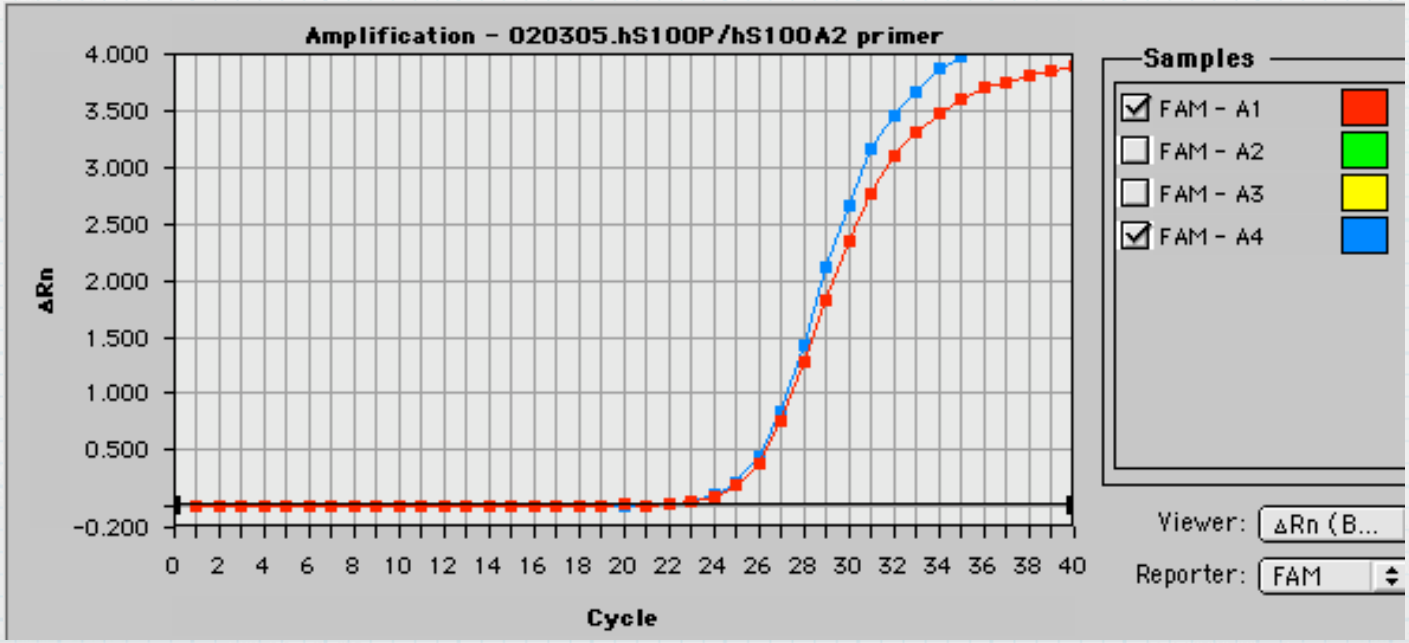
Final Taqman assay- 187(+), 257(-), 209(+)FAM/BHQ1
Efficiency = 97%
Lowest quantifiable value = 20 molecules

AAATTGCTCAAGGACCTGGAC- 187(+)

AAATTGCTCAAGGACCTGGACGCCAATGGAGATGCCCAGGTGGACTTCAGTGAGTTCATCGTGTTCGTGGC

TTTAACGAGTTCCTGGACCTGCGGTTACCTCTACGGGTCCACCTGAAGTCACTCAAGTAGCACAAGCACCG

257(-) -CTCAAGTAGCACAAGCACCG



Example 2- hCyclophilin B - SYBR Green I

Two forward primers and two reverse primers tested

GCAGGCAAAGACACCAACG - 446(+)

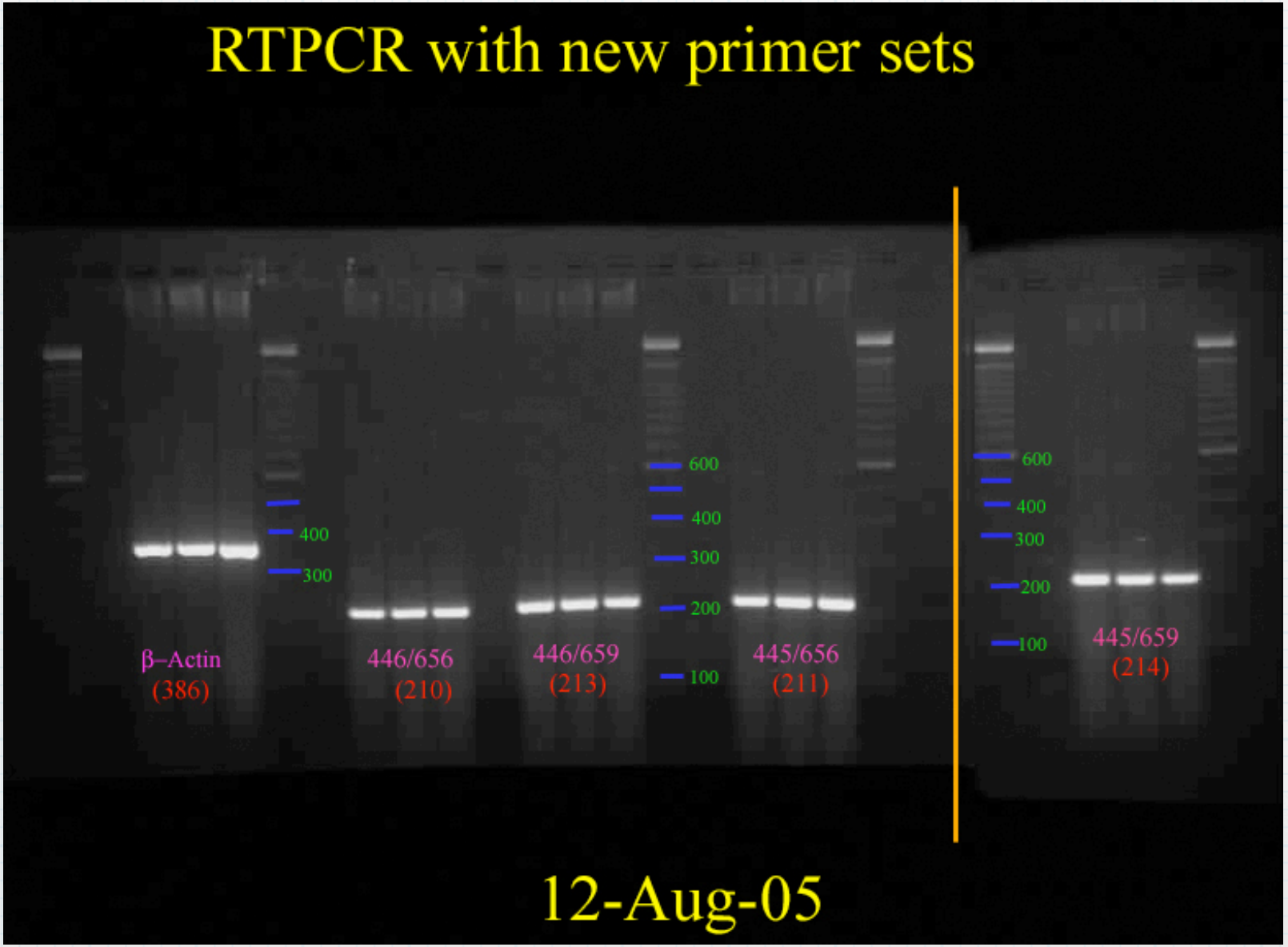
CGCAGGCAAAGACACCAAC - 445(+)

CGCAGGCAAAGACACCAACGGCTCCCAGTTCTTCATCACGACAGTCAAGACAGCCTGGCTAGATGGCAAGCA
TGTGGTGTTTGGCAAAGTTCTAGAGGGCATGGAGGTGGTGCGGAAGGTGGAGAGCACCAAGACAGACAGCCG
GGATAAACCCCTGAAGGATGTGATCATCGCAGACTGCGGCAAGATCGAGGTGGAGAAGCCCTTTGCCATCG

656(-) - GTGGAGAAGCCCTTTGCCA

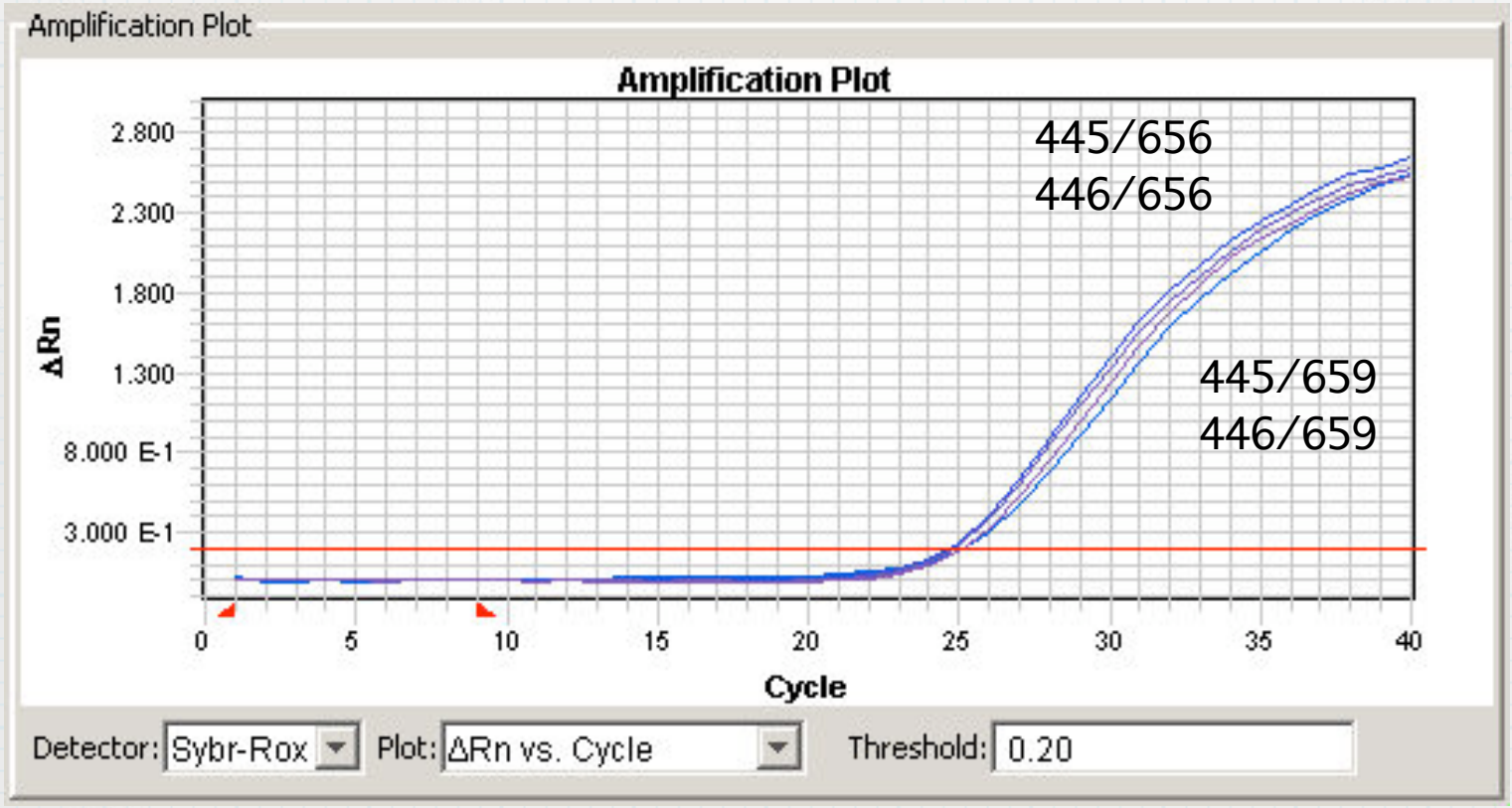
659(-) - GAGAAGCCCTTTGCCATCG

Example 2- hCyclophilin B SYBR Green I



Example 2- hCyclophilin B SYBR Green I

All 4 primer combinations at the same template concentration (purified PCR product)



Ct values

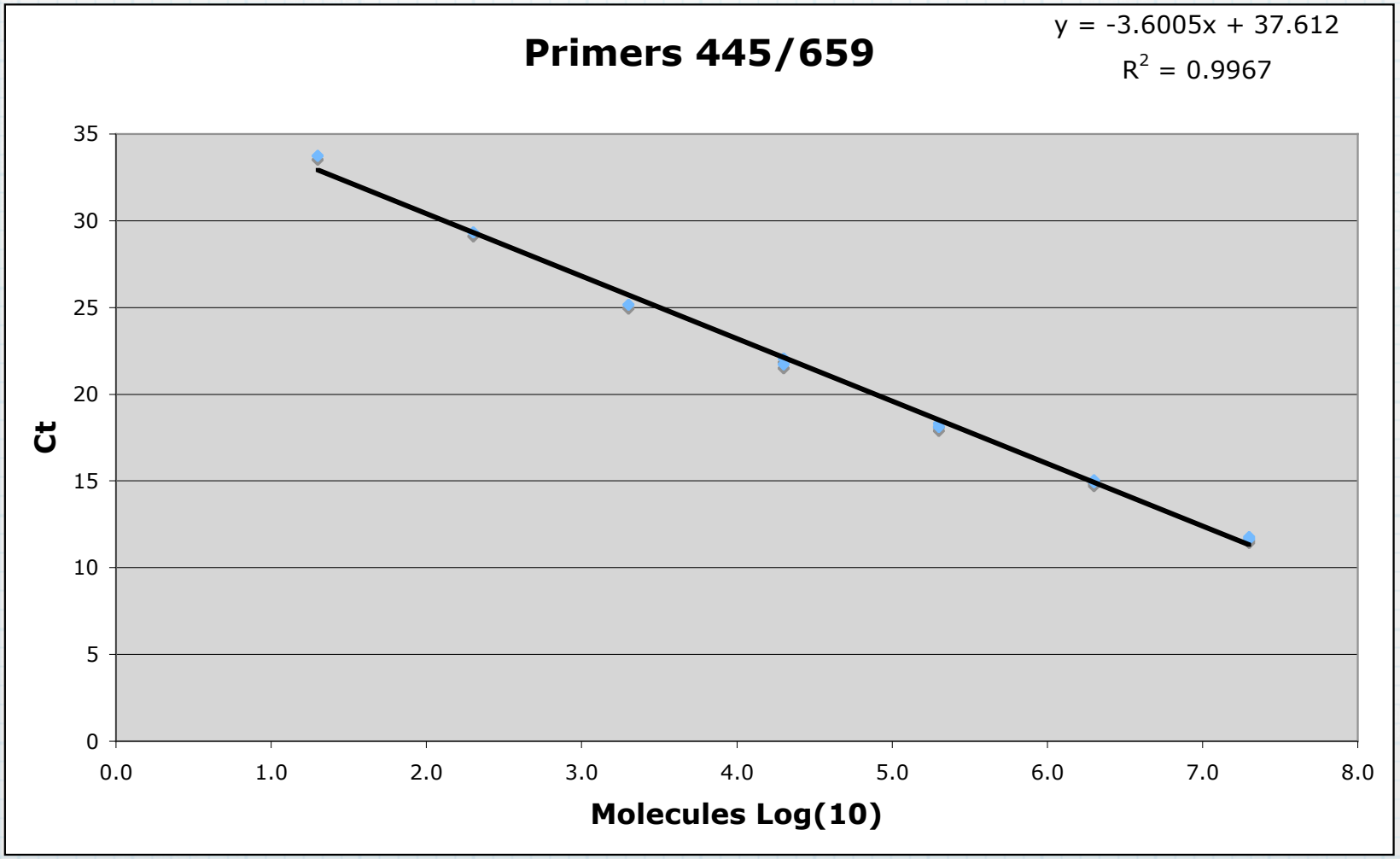
445/656= 24.4
446/656= 24.8
445/659= 25.2
446/659= 25.2

A very small difference can be seen, but measurable

Example 2- hCyclophilin B SYBR Green I

CGCAGGCAAAGACACCAAC - 445(+)
CGATGGCAAAGGGCTTCTC - 659(-)

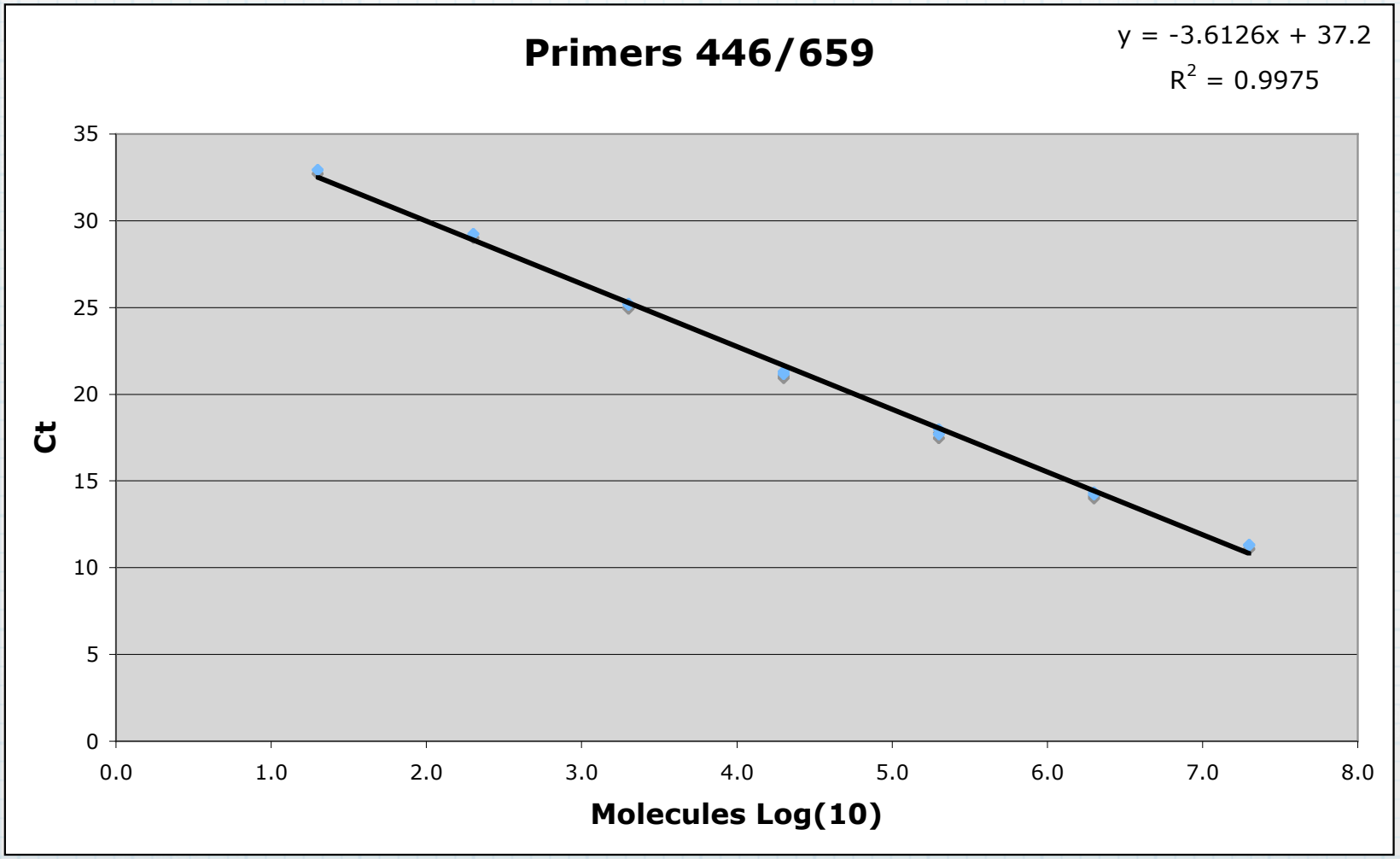
Efficiency = 88%



Example 2- hCyclophilin B SYBR Green I

GCAGGCAAAGACACCAACG - 446(+)
CGATGGCAAAGGGCTTCTC - 659(-)

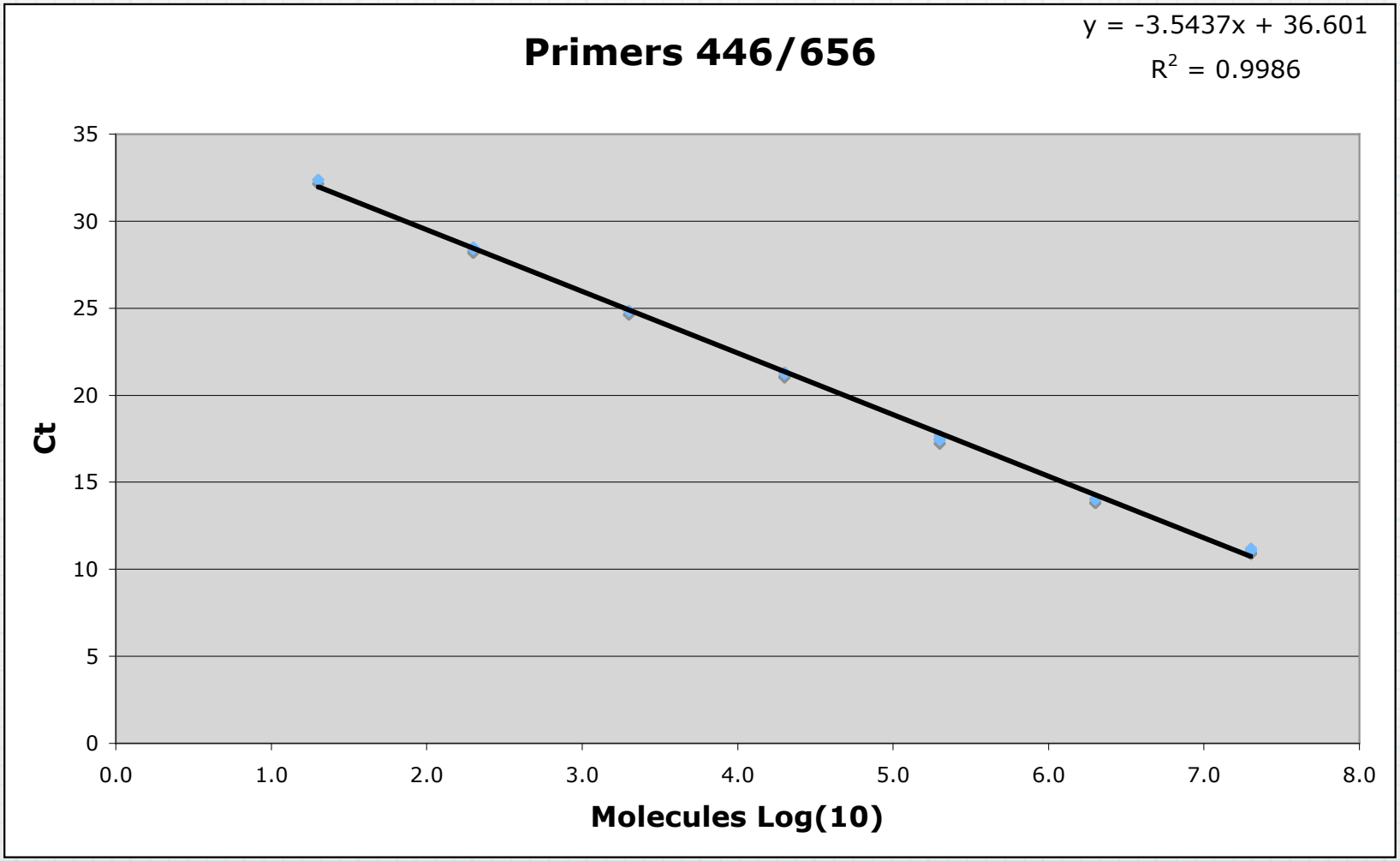
Efficiency = 89%



Example 2- hCyclophilin B SYBR Green I

GCAGGCAAAGACACCAACG - 446(+)
TGGCAAAGGGCTTCTCCAC - 656(-)

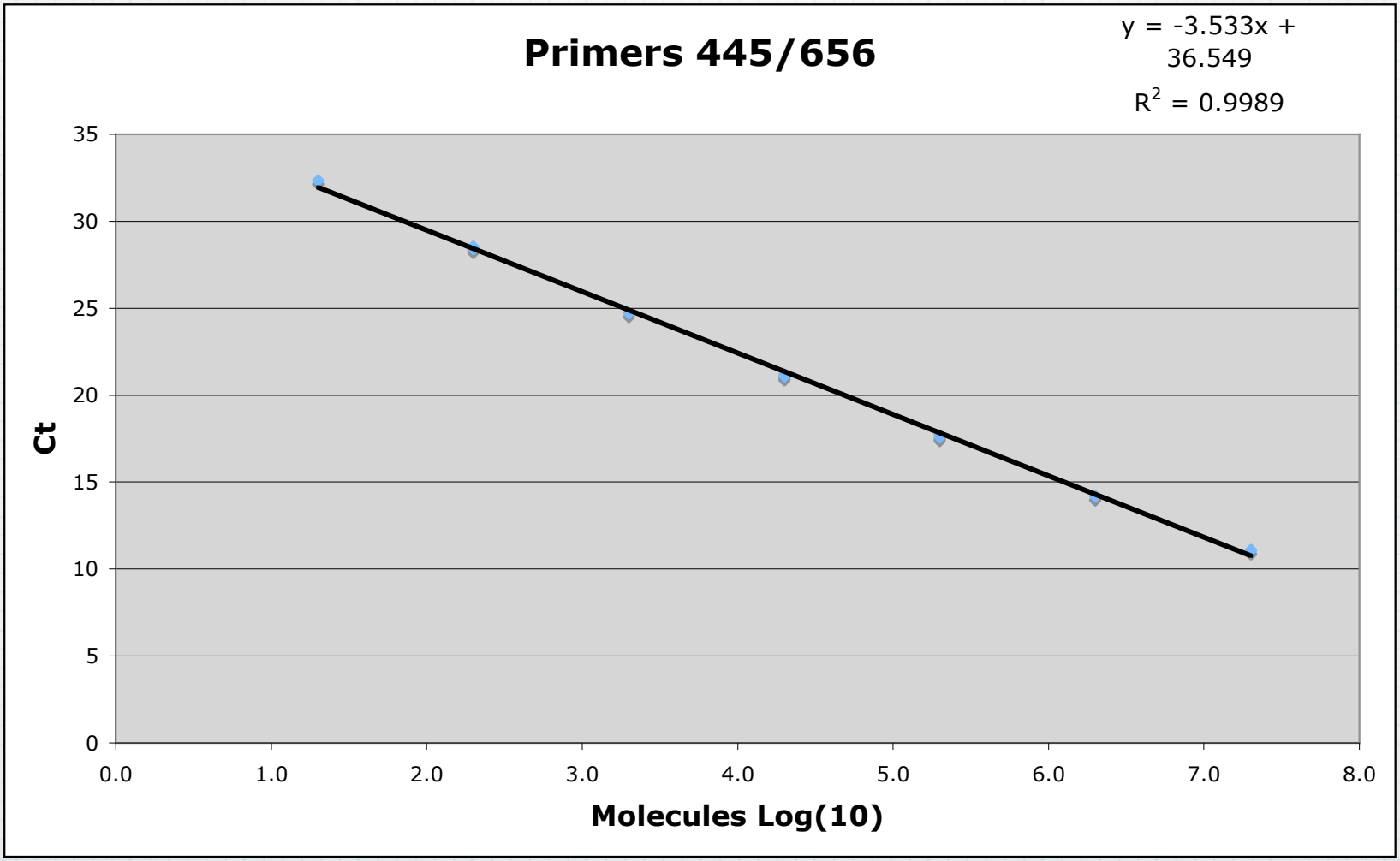
Efficiency = 91%



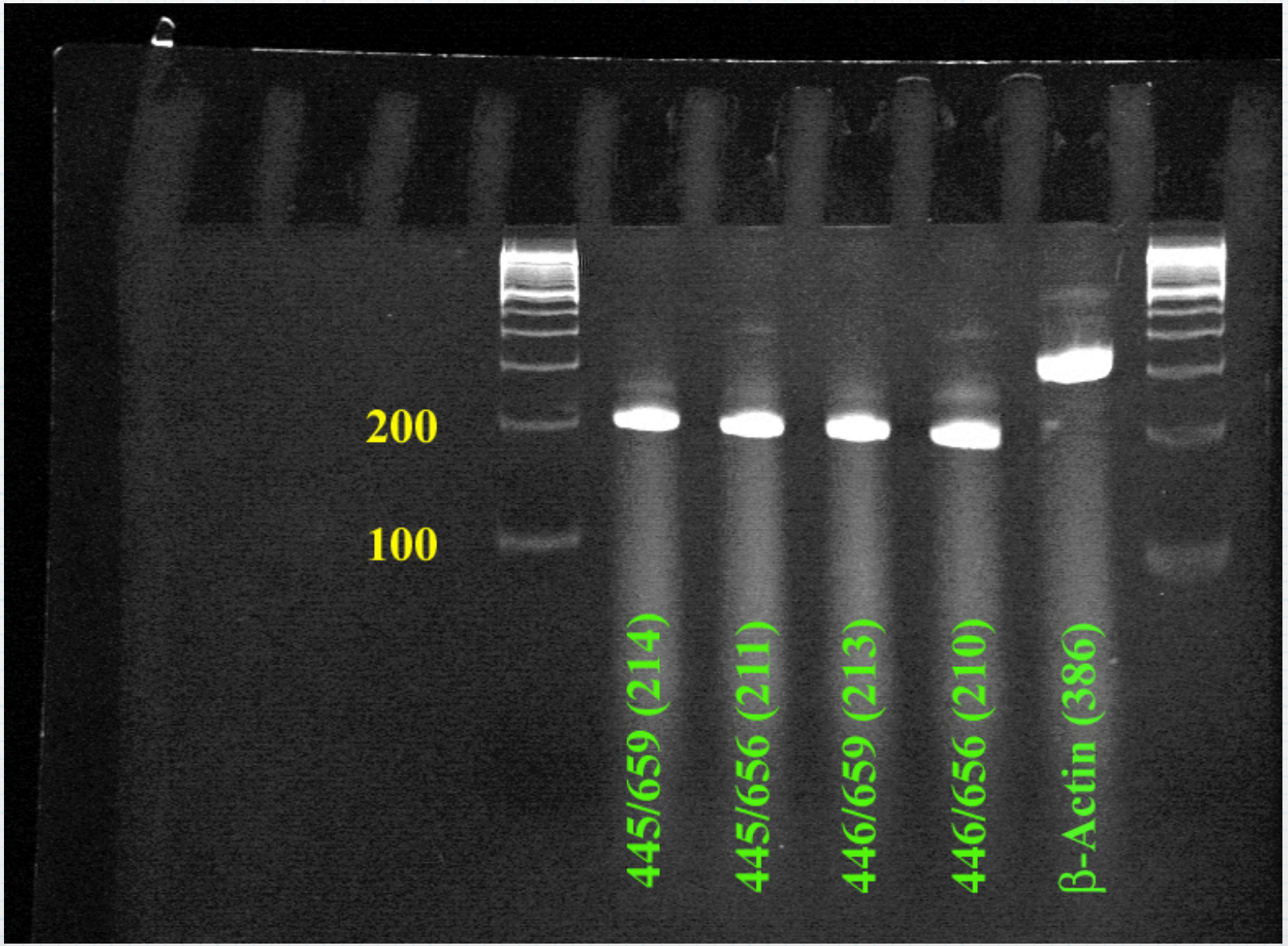
Example 2- hCyclophilin B SYBR Green I

CGCAGGCAAAGACACCAAC - 445(+)
TGGCAAAGGGCTTCTCCAC - 656(-)

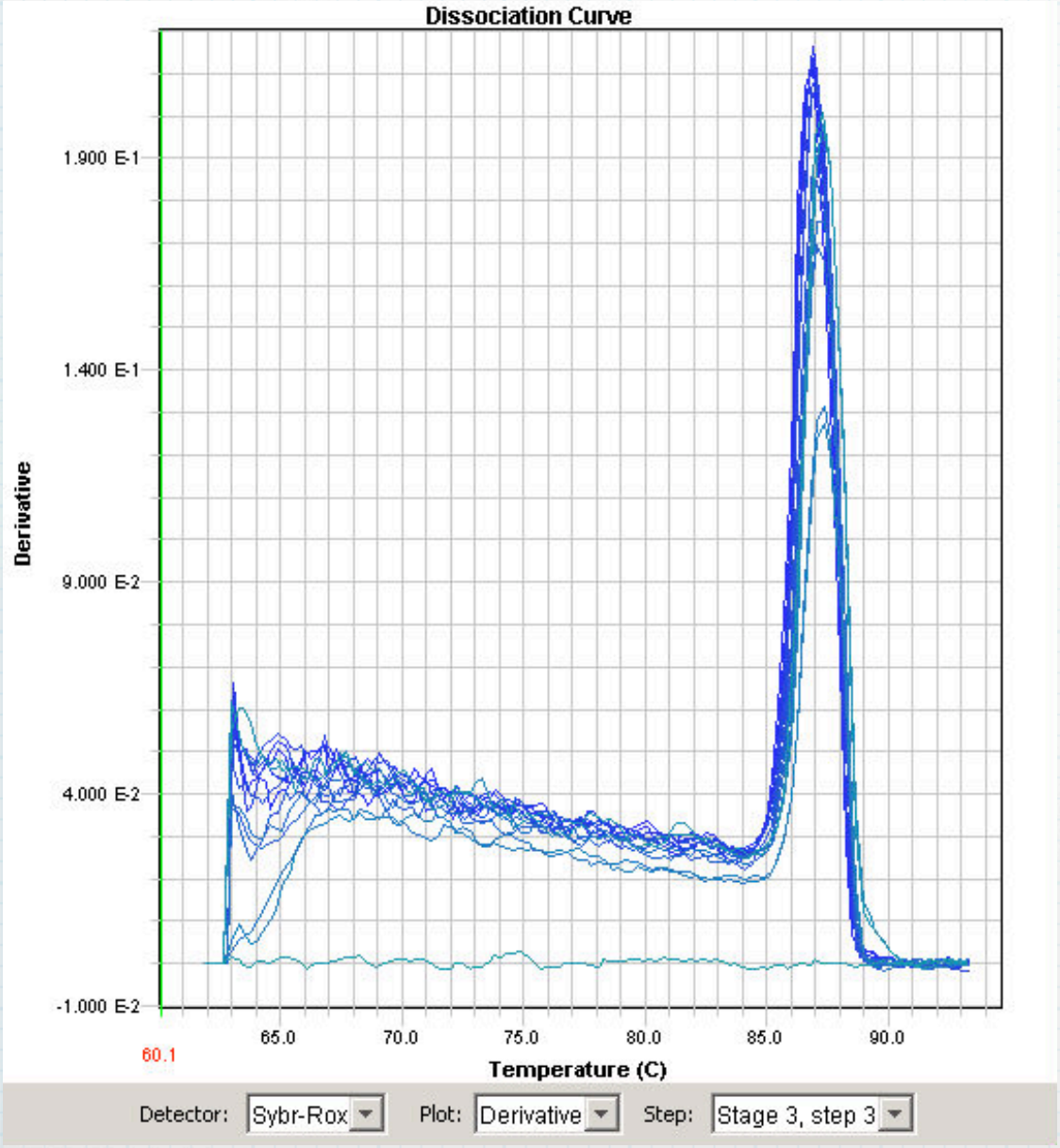
Efficiency = 92%



Example 2- hCyclophilin B SYBR Green I



Example 2- hCyclophilin B SYBR Green I



445(+)/656(-)

No sign of primer-dimers

Example 2- hCyclophilin B - SYBR Green I

Final SYBR Green I assay primer pair- 445(+)/656(-)

Assay efficiency = 92%

Lowest Quantifiable Value = 20 molecules

CGCAGGCAAAGACACCAAC - 445(+)

CGCAGGCAAAGACACCAACGGCTCCCAGTTCTTCATCACGACAGTCAAGACAGCCTGGCTAGATGGCAAGCA
TGTGGTGTTTGGCAAAGTTCTAGAGGGCATGGAGGTGGTGCGGAAGGTGGAGAGCACCAAGACAGACAGCCG
GGATAAACCCCTGAAGGATGTGATCATCGCAGACTGCGGCAAGATCGAGGTGGAGAAGCCCTTTGCCATCG

656(-) - GTGGAGAAGCCCTTTGCCA

Using Real-Time Assays Responsibly
Minimum Requirements for Publication

* Accession number of the transcript or gene
* The sequence of primers & probe (if used) with numbers relating to the accession sequence

  or Manufacturer & catalogue number of assay
* The PCR efficiency of the assay
* The lowest quantifiable limit of the assay

An Example of a Table for Publication

Table I

Details for the human β-Actin real-time PCR assay.

Accession number: NM_001101

Average assay efficiency: 97%

Lowest quantifiable value: 20 molecules

Primers:
997(+) CCCTGGCACCCAGCAC
1067(-) GCCGATCCACACGGAGTAC

Taqman Probe:
1020(+) FAM- ATCAAGATCATTGCTCCTCCTGAGCGC -BHQ1

Synthetic DNA template (sDNA- 71 bases):
CCCTGGCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTA
CTCCGTGTGGATCGGC

Synthetic RNA template (sRNA- 151 bases):
CGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATCCCT
GGCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCC
GTGTGGATCGGCATCGAATTCCTGCAGCCCGGGGGATCCA

Summary

- The design of new real-time qPCR assays can be accomplished by following a few simple steps
- Validating each new assay is critical
- Validated assays yield excellent results forever, bad assays are a nightmare forever
- Publication of real-time qPCR data has to include information about the performance of each assay

The Important Folks

* Ms. Xiaoying Wang - ace technician
* Ms. Mary Sobieski - ace technician
* Mr. Thomas Merritt - graduate student
* Ms. Nancy Shipley - research coordinator

A Quick Texas Tour

Central North America







The Texas you are expecting to see- What you get in West Texas ya'll






Downtown Houston
1) UTHSC-Houston
2) UT-MD Anderson Cancer Center
3) Baylor College of Medicine
4) Rice University
Texas Medical Center - Houston