
Welcome

The science behind a SYBR Green assay

Ursule Dumont and Gilbert Robic

The world leader in serving science

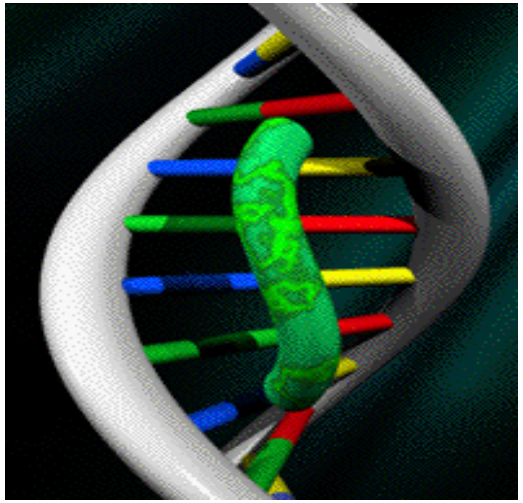
Agenda

1. Challenges in the SYBR Green workflow
2. Design a high quality SYBR Green assay
3. Summary and Conclusion
4. Q&A

Introduction

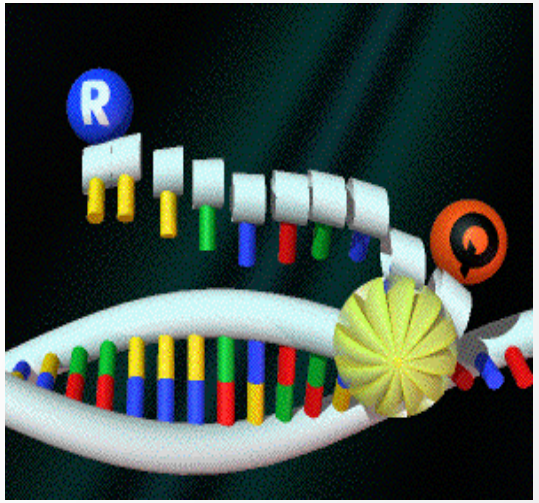
Real-Time PCR chemistries

SYBR™ Green dye



Binds double-stranded DNA

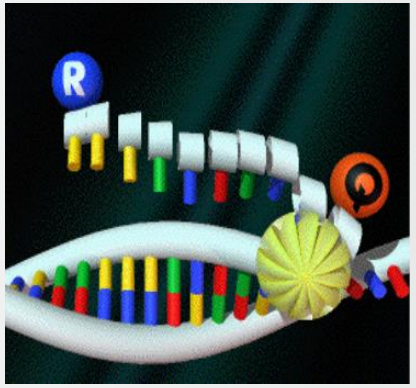
TaqMan® probes



Fluorescent labelled probes

Taqman probe :

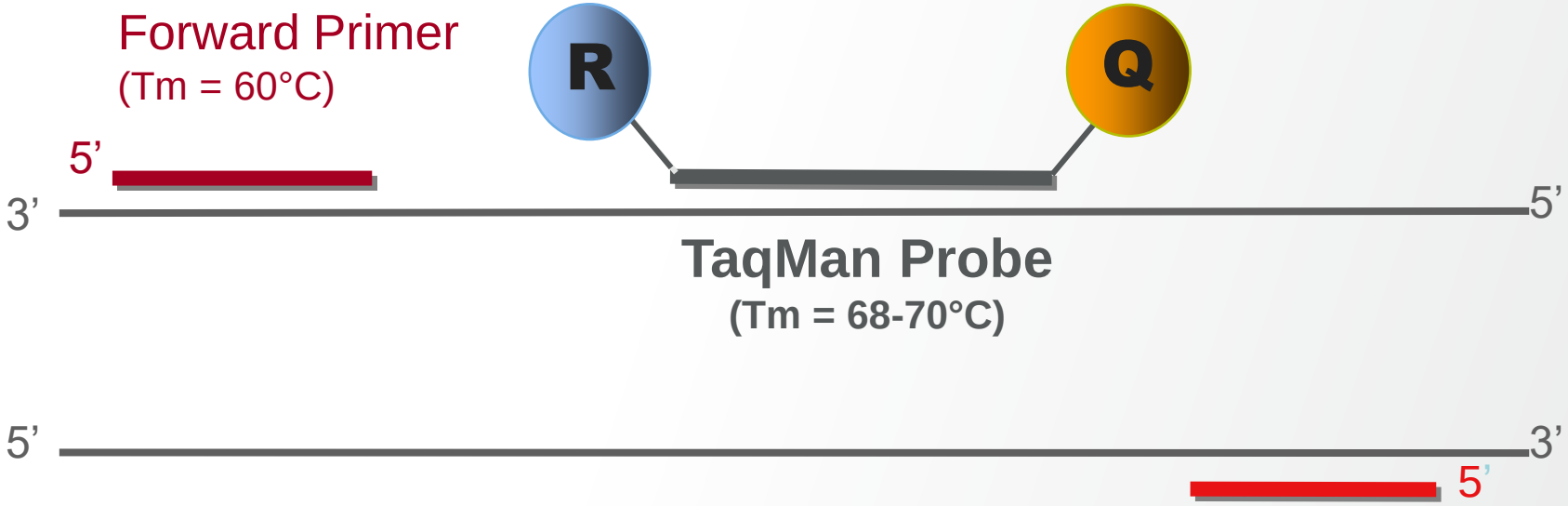
- This method uses 2 principles:
  - FRET Technology
  - 5'- nuclease activity of the Taq polymerase



Reporter Dyes:
FAM™, VIC™, NED™

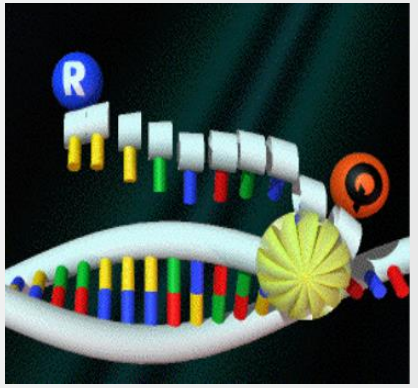
Quencher:
TAMRA™/NFQ…

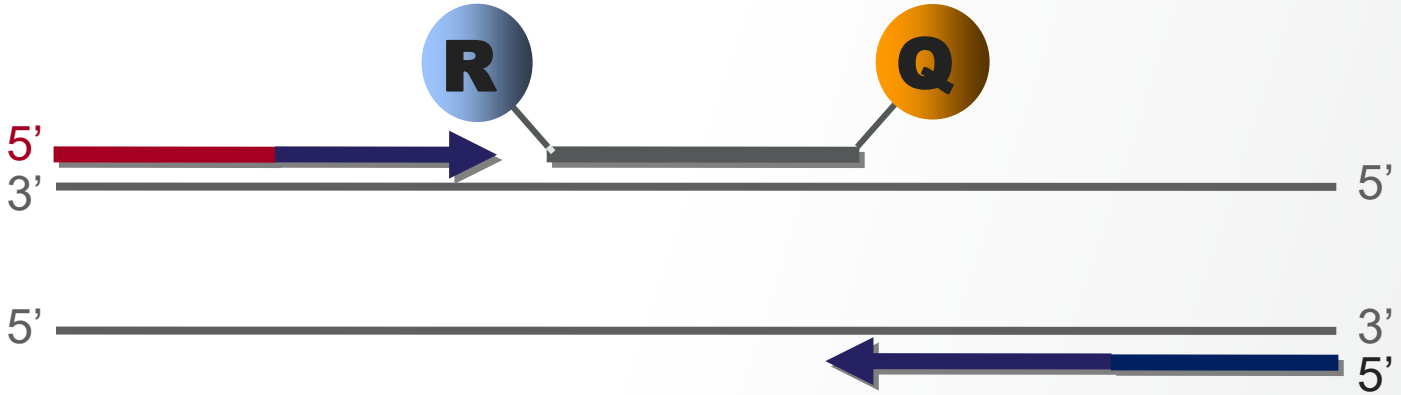
Forward Primer
(Tm = 60°C)



Taqman probe :

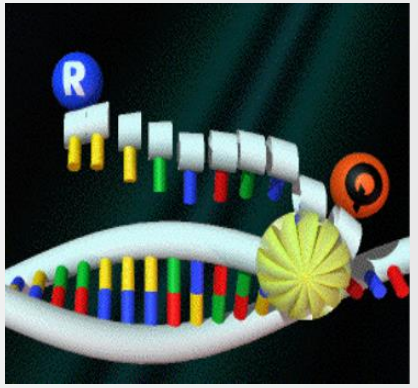
- Displacement of probe by polymerase: fork-like structure signal for cleavage

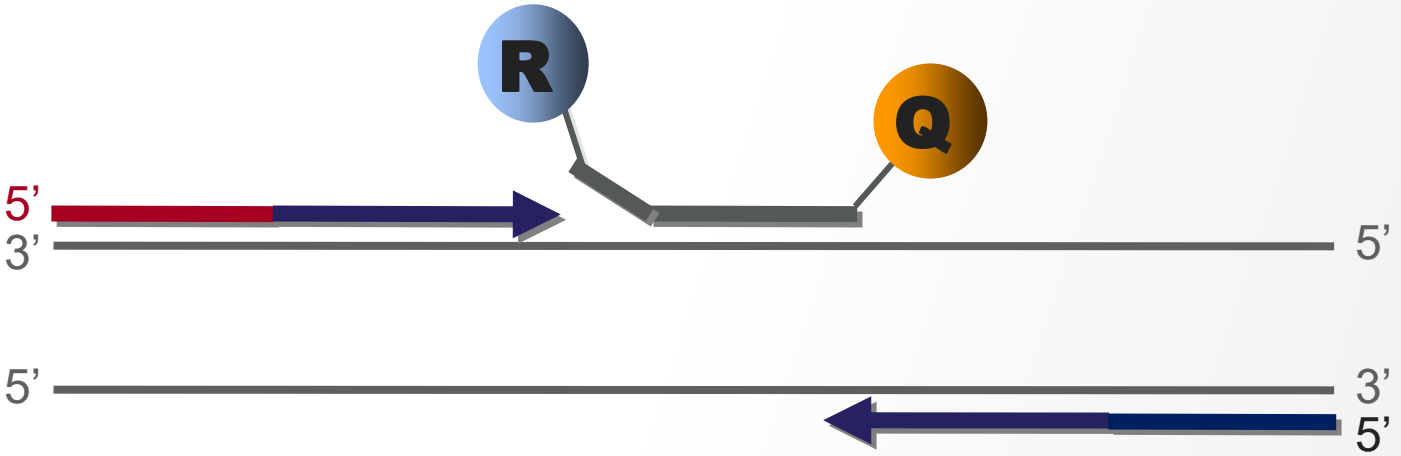




Taqman probe :

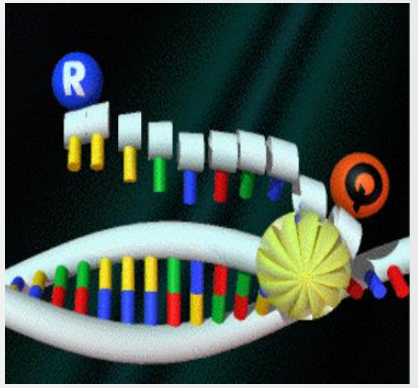
- Displacement of probe by polymerase: fork-like structure signal for cleavage

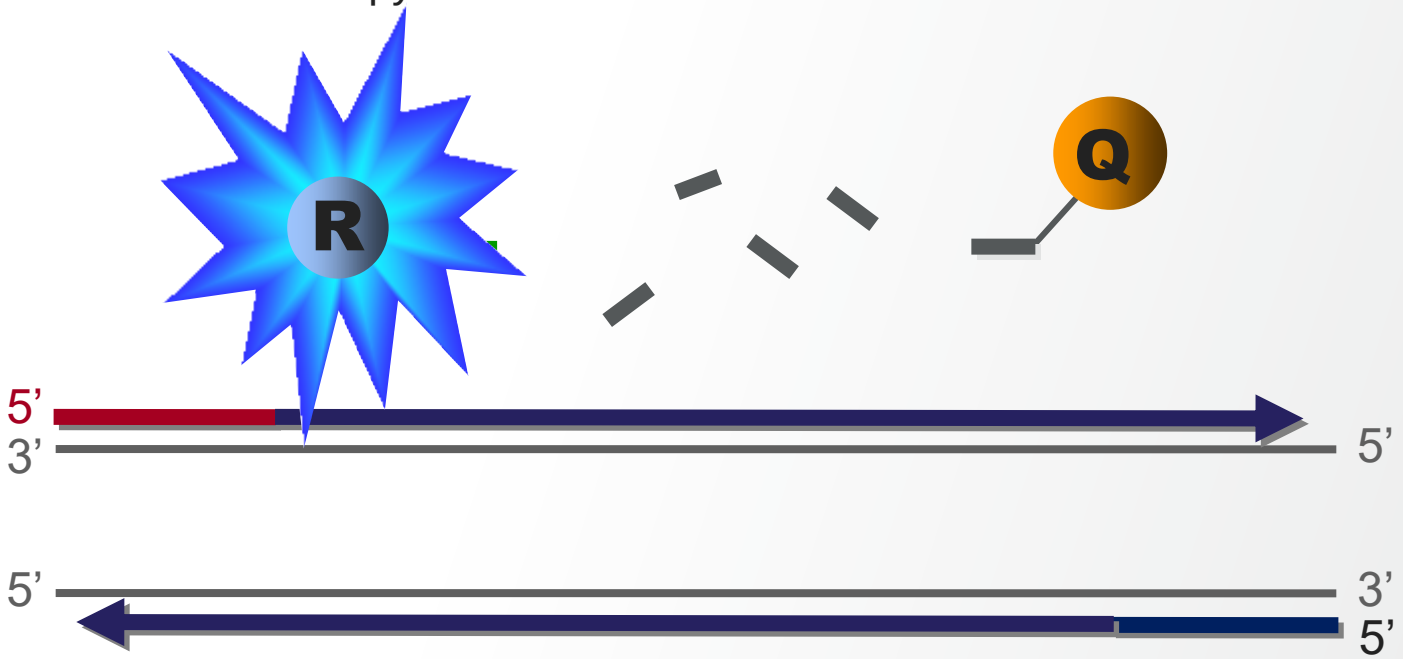




Taqman probe :

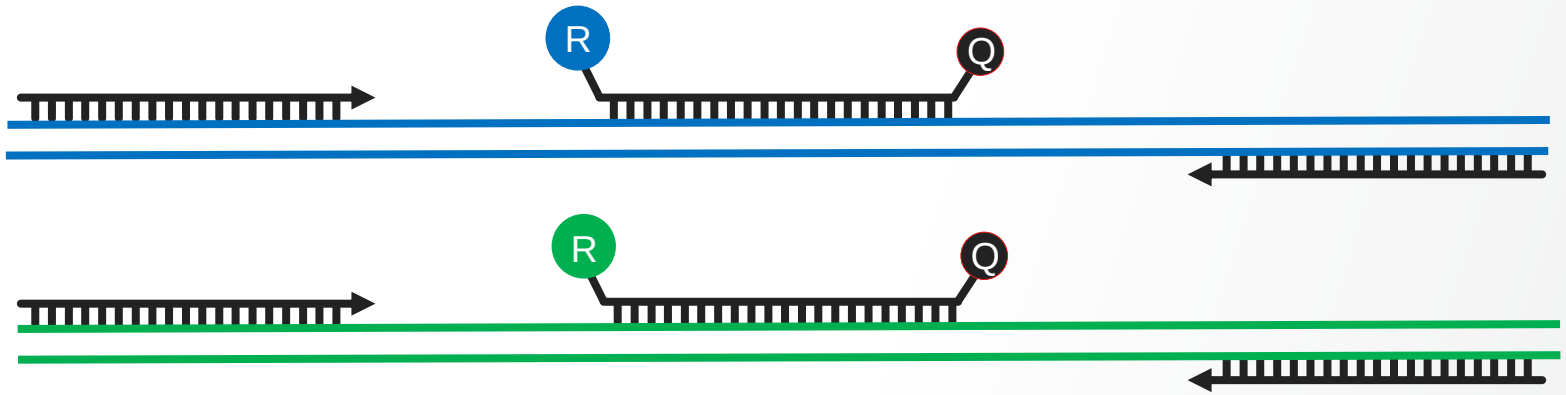
- Polymerization completed
- Signal generation
  - Strongly proportional to product number
  - One reporter signal for each new DNA copy





Benefits of Using TaqMan Assays

- **Universal run conditions** (either fast or standard)
- **100% efficient** – no need to check efficiency
- **Specific** (does not detect off-targets or gDNA)
- **Multiplexing**: analyze more than one target in one reaction

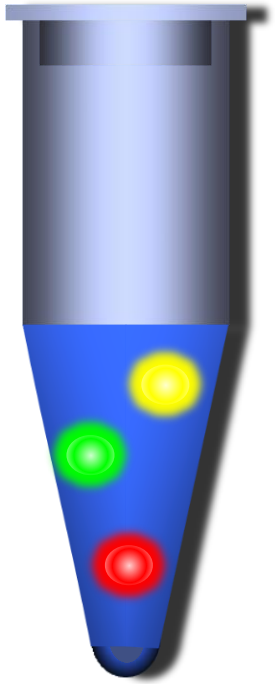


Target 1 (e.g. FAM)

Target 2 (e.g. VIC)

Suffix “-plex”

- The suffix “-plex” is used to categorize real-time PCR experiments based on the number of assays per well.



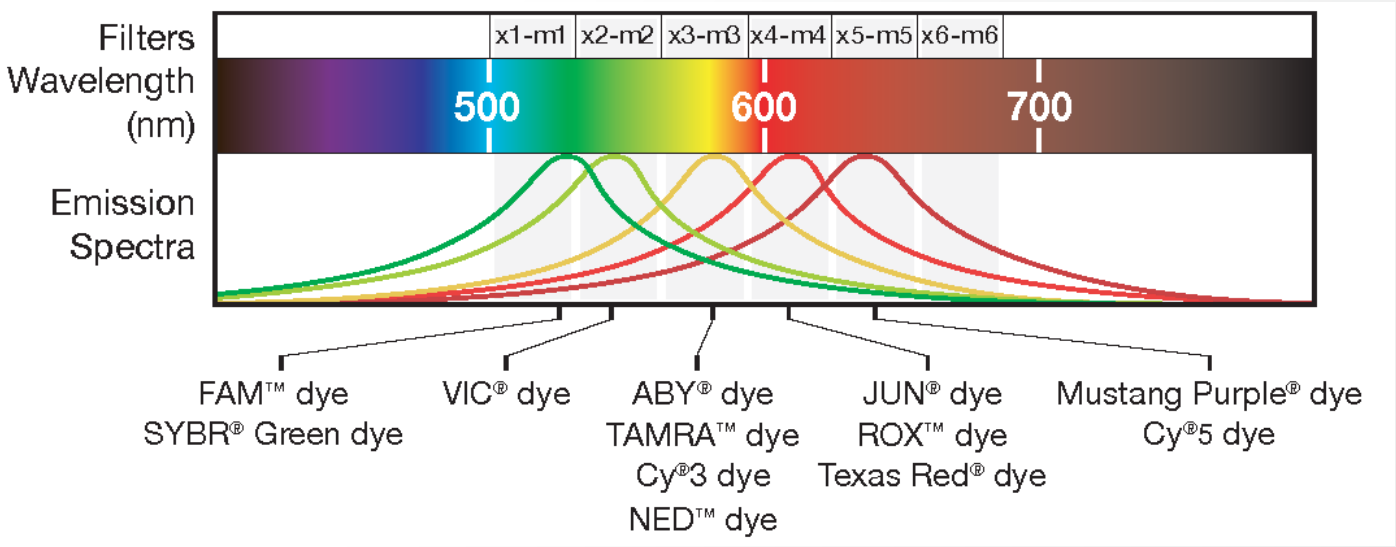
- **Singleplex:** One gene target detected per well.

- **Multiplex:** More than one gene target detected per well.

	1	2	3
A	Sample 1 Normalizer Target 1	Sample 1 Normalizer Target 1	Sample 1 Normalizer Target 1
B	Sample 2 Normalizer Target 1	Sample 2 Normalizer Target 1	Sample 2 Normalizer Target 1

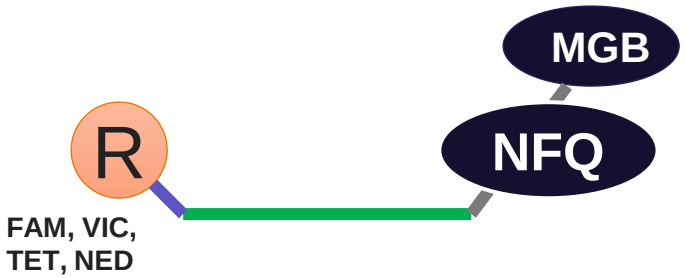
Which reporter dyes to choose?

FAM	530 nm
VIC	554 nm
JOE™	554 nm
NED	576 nm
TAMRA, Cy3®, ABY™	582 nm
ROX™, Texas Red, JUN™	610 nm
Cy5 ®, Mustang Purple™	650 nm



Which quenchers to choose?

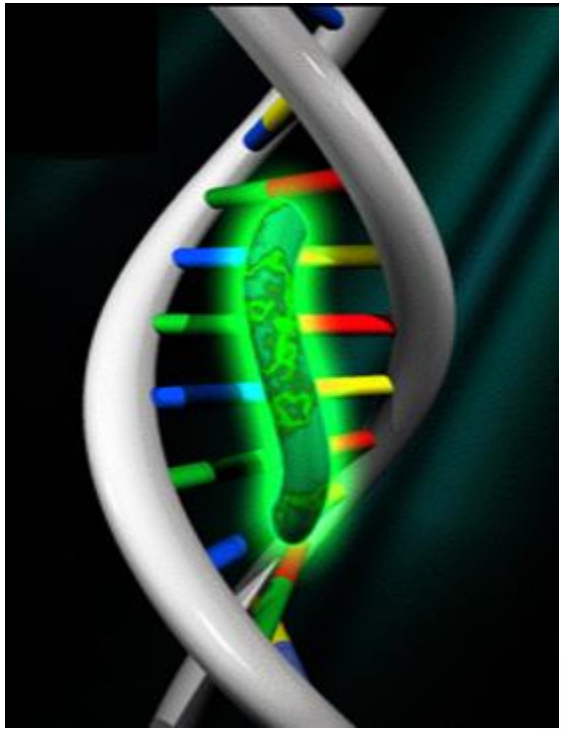
TAMRA*: (1994 -)*
Fluorescent probe

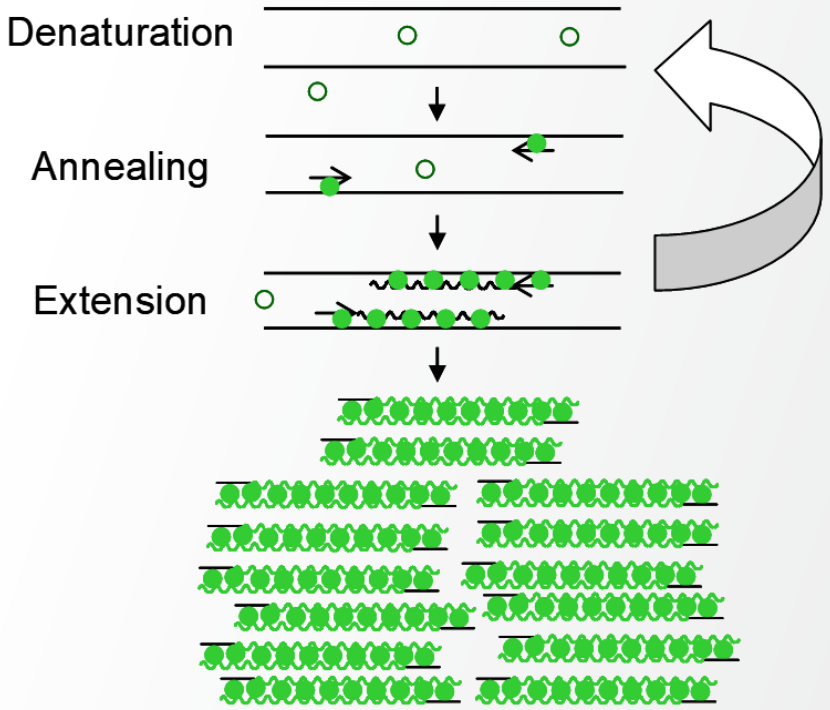


MGB-NFQ: (1999 -)
Non fluorescent probe which allows for shorter probe design

QSY® quencher (2009 -)
QSY is a new non-fluorescent quencher that can be used for designing Custom TaqMan probes. QSY custom probes can be substituted for 3' TAMRA probes without redesigning the probe sequence.

Signal generation with SYBR Green





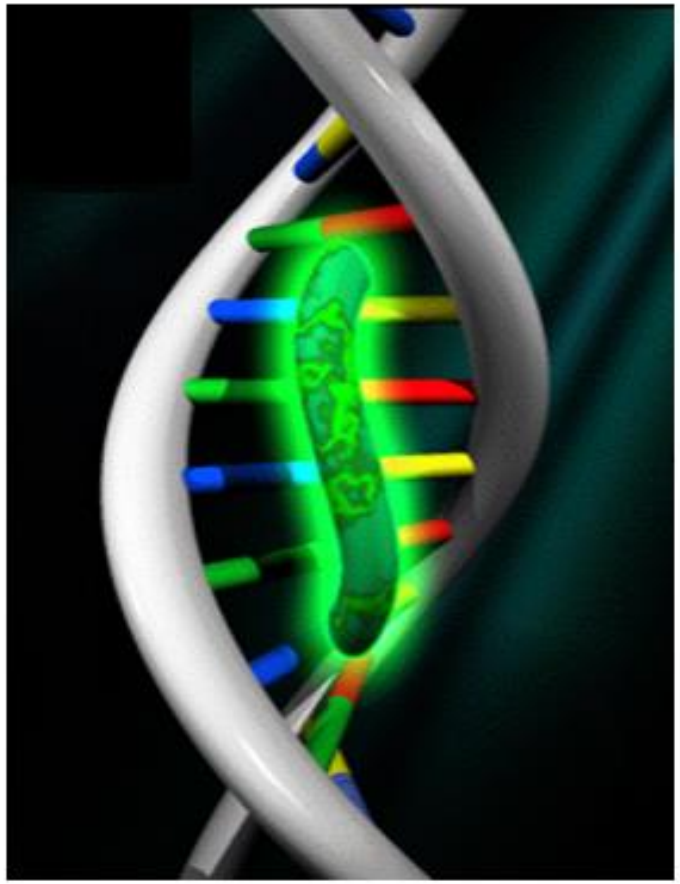
SYBR Green fluoresces upon binding to minor groove of dsDNA product

⇩

Fluorescent signal is proportional to the amount of amplified DNA

Challenges in the SYBR Green workflow

What could be the main challenge of SYBR Green?



SYBR Green binds non-specifically to **any** double stranded DNA

⇩

Signal could originate from non-specific products

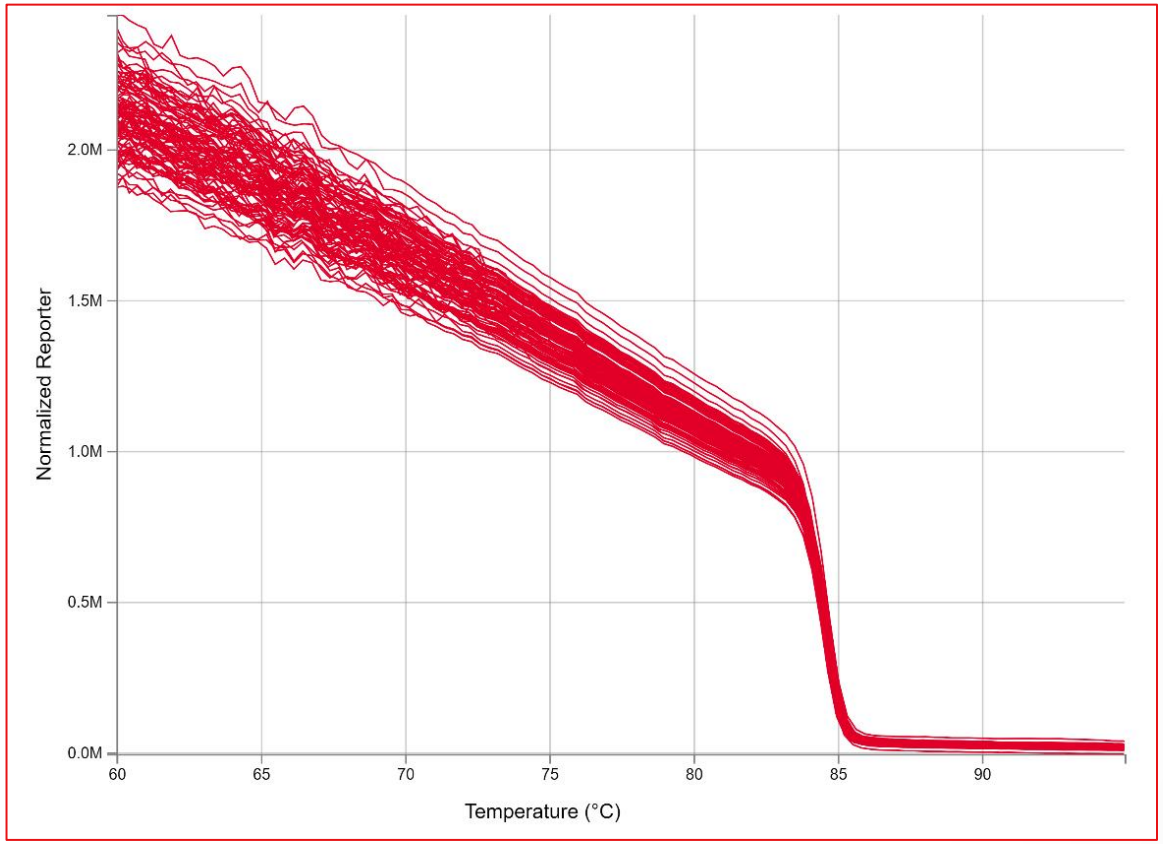
⇩

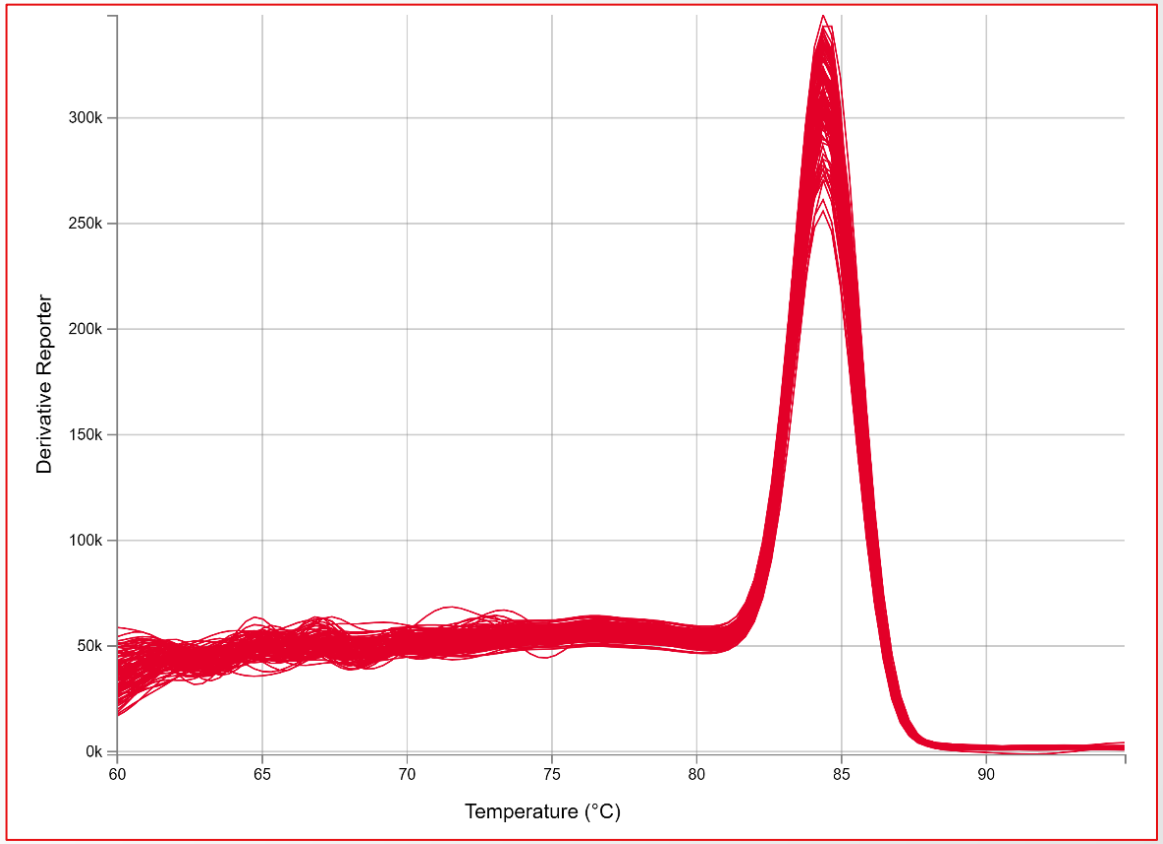
Inaccurate results

What could be the main challenge of SYBR Green?

Challenge: non-specific PCR products result in confounding SYBR Green signal.

Melt curve must be generated to exclude the existence of non-specific PCR products.



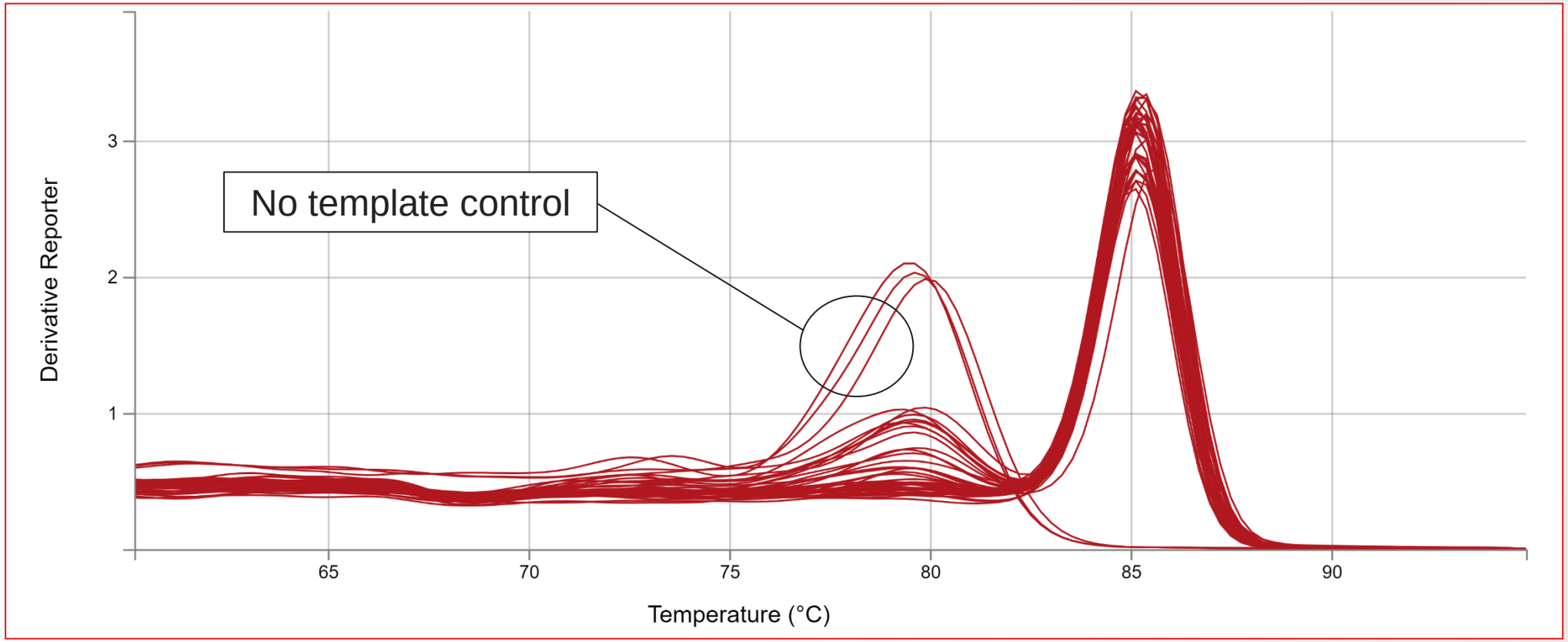


Keep in mind:

The acquisition of a melt curve adds extra time to your run protocol

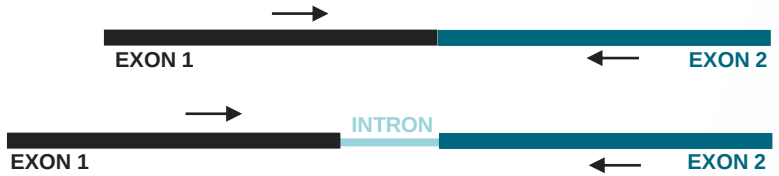
What could be the main challenge of SYBR Green?

- **Primer-dimer formation** is a common source of non-specific signal



What could be the main challenge of SYBR Green?

- Primer-dimer formation
- **Non-specific amplification**
  - Incorrect target in transcriptome.
  - Primers bind to correct sequence but detect both gDNA and cDNA (where gDNA has higher Tm, due to a short intron).



cDNA → Expected peak

gDNA → 2nd peak at higher Tm

  - <u>Important:</u> design and optimalization of primers (bio informatics)

Design a high quality SYBR Green assay

Requirements for a high quality SYBR Green experiment

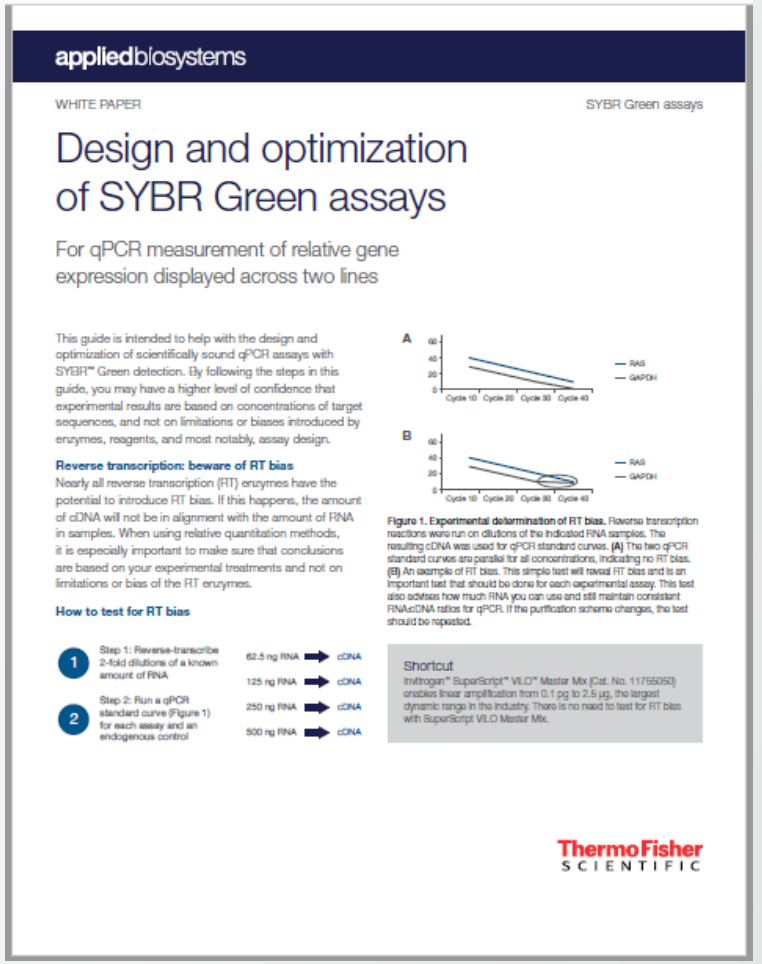
- ✓ **Step 1 : Bioinformatics:**
  Design specific primers for your target

- ✓ **Step 2 : Assay validation:**

- ✓ **Step 3 :** Test the **efficiency** of your primers **--** Achieve high **reproducibility**, **fold discrimination** and determine **sensitivity** over different sample types

- ✓ **Step 4 : Normalization steps:**

- ✓ **Apply** in your workflow and avoid contamination



appliedbiosystems

WHITE PAPER

SYBR Green assays

Design and optimization of SYBR Green assays

For qPCR measurement of relative gene expression displayed across two lines

This guide is intended to help with the design and optimization of scientifically sound qPCR assays with SYBR™ Green detection. By following the steps in this guide, you may have a higher level of confidence that experimental results are based on concentrations of target sequences, and not on limitations or biases introduced by enzymes, reagents, and most notably, assay design.

Reverse transcription: beware of RT bias

Nearly all reverse transcription (RT) enzymes have the potential to introduce RT bias. If this happens, the amount of cDNA will not be in alignment with the amount of RNA in samples. When using relative quantitation methods, it is especially important to make sure that conclusions are based on your experimental treatments and not on limitations or bias of the RT enzymes.

How to test for RT bias

1. Step 1: Reverse-transcribe 2-fold dilutions of a known amount of RNA
   62.5 ng RNA → cDNA
   125 ng RNA → cDNA
   250 ng RNA → cDNA
   500 ng RNA → cDNA
2. Step 2: Run a qPCR standard curve (Figure 1) for each assay and an endogenous control

A

B

Figure 1. Experimental determination of RT bias. Reverse transcription reactions were run on dilutions of the indicated RNA samples. The resulting cDNA was used for qPCR standard curves. **(A)** The two qPCR standard curves are parallel for all concentrations, indicating no RT bias. **(B)** An example of RT bias. This simple test will reveal RT bias and is an important test that should be done for each experimental assay. This test also advises how much RNA you can use and still maintain consistent RNA:cDNA ratios for qPCR. If the purification scheme changes, the test should be repeated.

Shortcut

Invitrogen™ SuperScript™ VILO™ Master Mix (Cat. No. 11755050) enables linear amplification from 0.1 pg to 2.5 µg, the largest dynamic range in the industry. There is no need to test for RT bias with SuperScript VILO Master Mix.

ThermoFisher SCIENTIFIC

Step 1 : Bioinformatics – Primer Design

- ✓ **Qualify a sequence**
  - ✓ Select a max ~200 bp sequence
  - ✓ Try to be smart in choosing: splicing, Exon-Exon boundary(ensemble.org)
  - ✓ Mask the SNP's in the sequence (bioinfo.ebc.ee/snpmasker/)

- ✓ **Primer design**
  - ✓ Design multiple assays using a primer design software (free or paid)
  - ✓ Check specificity on species BLAST all multiple options for design (blast.ncbi.nlm.nih.gov/)
  - ✓ Order your primers as sequence detection primers at Thermo Fisher Scientific

Good sequence quality is the key for a good assay design!

Primer design

Design recommendations are identical for primers used in TaqMan® probe or SYBR® Green I dye based assays

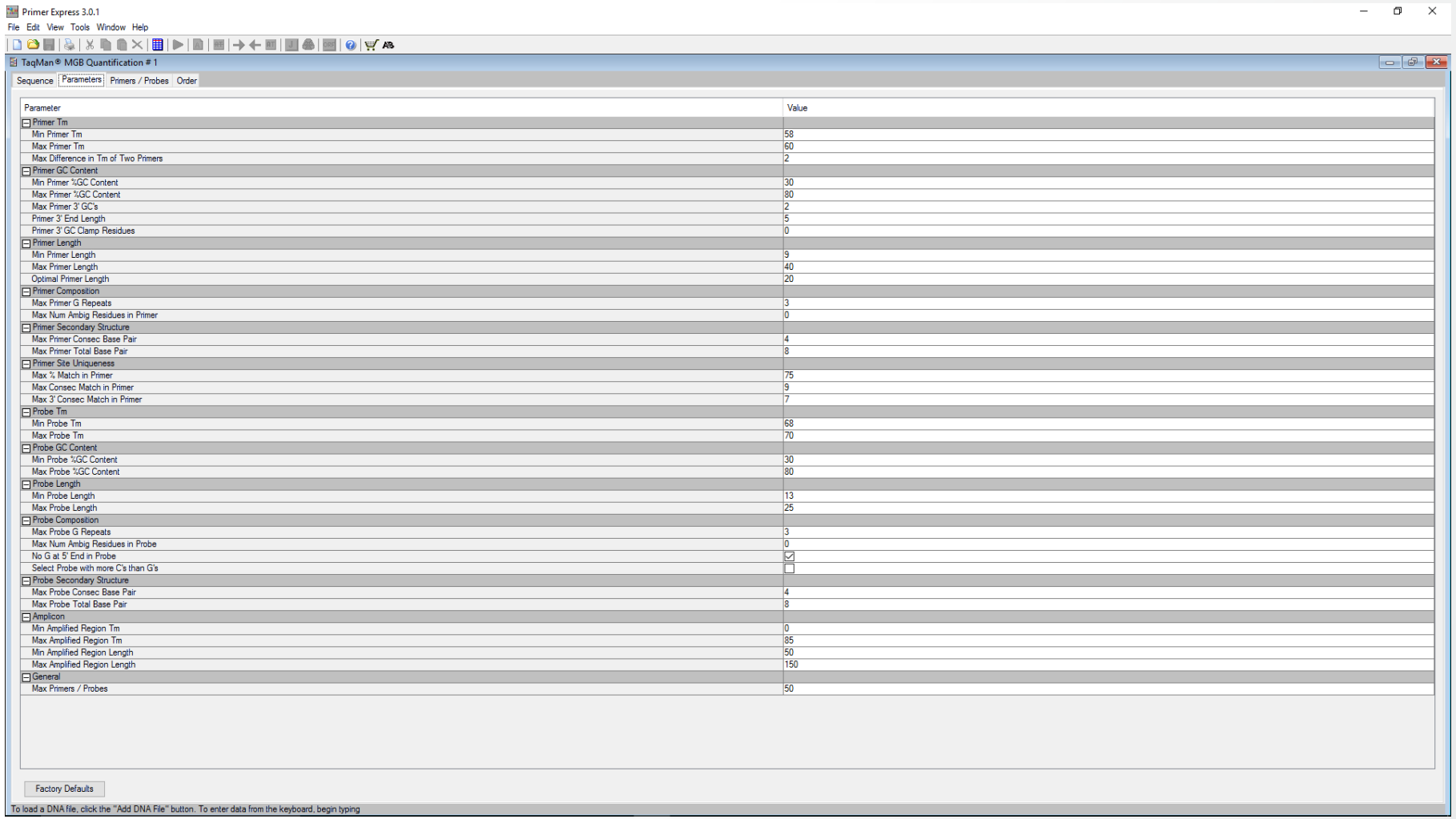
- ✓ Tm: 58 – 60 °C
- ✓ ΔTm maximally 2 °C to avoid unspecific initiation

Primer design

- ✓ Avoid runs of an identical nucleotide (especially G's)

- ✓ The five nucleotides at the 3´ end should have no more than
  - two G and/or C bases

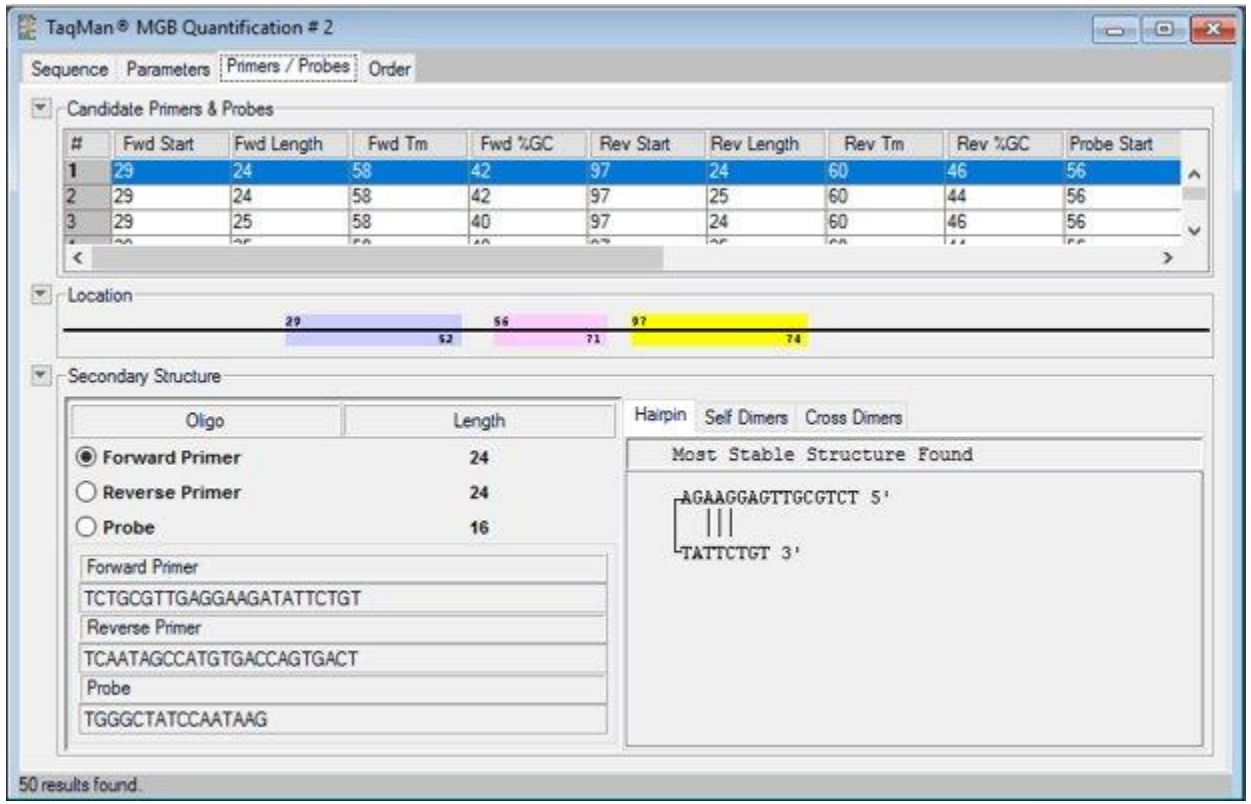
Design Parameters in Primer Express Software 3.0.1

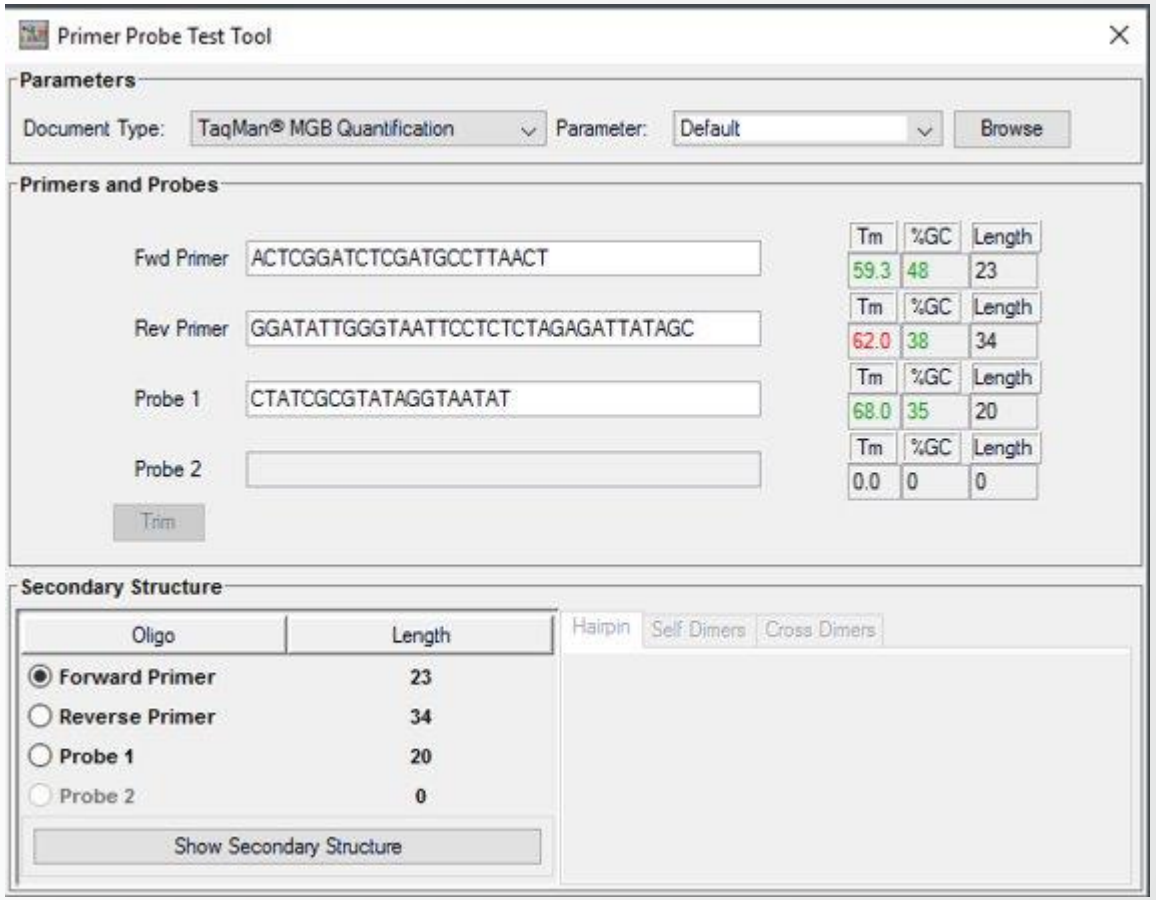


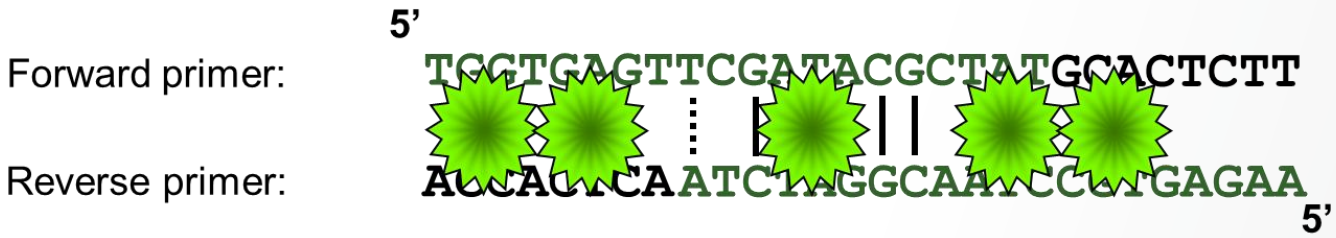
Parameter	Value
Primer Tm	
Min Primer Tm	58
Max Primer Tm	60
Max Difference in Tm of Two Primers	2
Primer GC Content	
Min Primer %GC Content	30
Max Primer %GC Content	80
Max Primer 3' GC's	2
Primer 3' End Length	5
Primer 3' GC Clamp Residues	0
Primer Length	
Min Primer Length	9
Max Primer Length	40
Optimal Primer Length	20
Primer Composition	
Max Primer G Repeats	3
Max Num Ambig Residues in Primer	0
Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
Primer Site Uniqueness	
Max % Match in Primer	75
Max Consec Match in Primer	9
Max 3' Consec Match in Primer	7
Probe Tm	
Min Probe Tm	68
Max Probe Tm	70
Probe GC Content	
Min Probe %GC Content	30
Max Probe %GC Content	80
Probe Length	
Min Probe Length	13
Max Probe Length	25
Probe Composition	
Max Probe G Repeats	3
Max Num Ambig Residues in Probe	0
No G at 5' End in Probe	☑
Select Probe with more C's than G's	☐
Probe Secondary Structure	
Max Probe Consec Base Pair	4
Max Probe Total Base Pair	8
Amplicon	
Min Amplified Region Tm	0
Max Amplified Region Tm	85
Min Amplified Region Length	50
Max Amplified Region Length	150
General	
Max Primers / Probes	50

more on this later...

Design Parameters in Primer Express Software 3.0.1

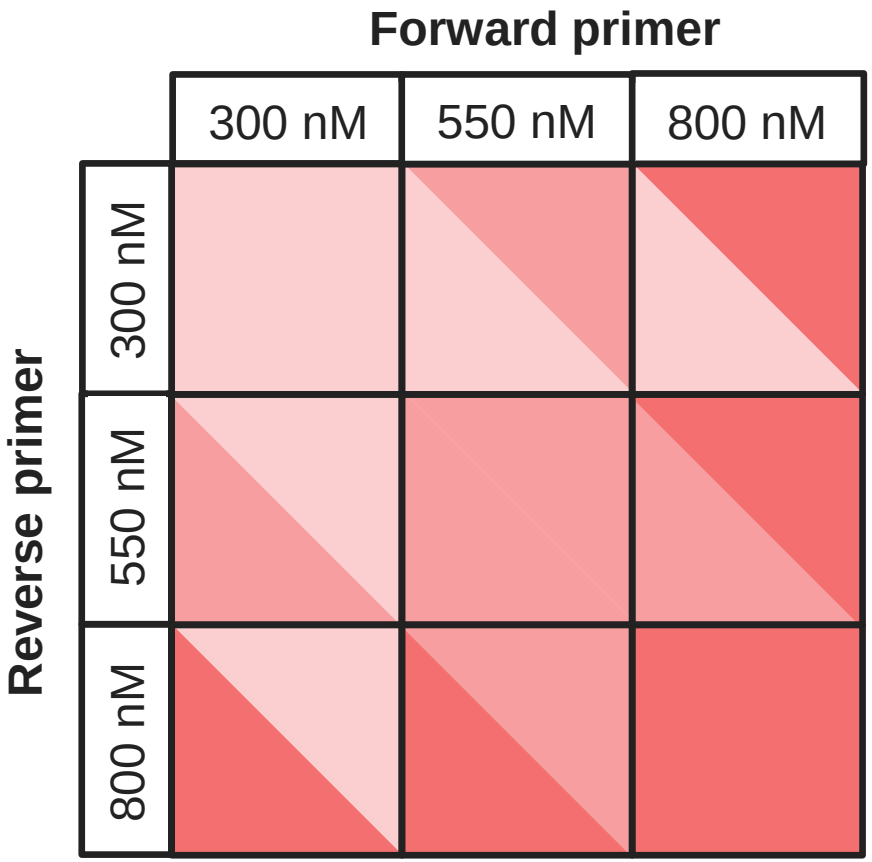






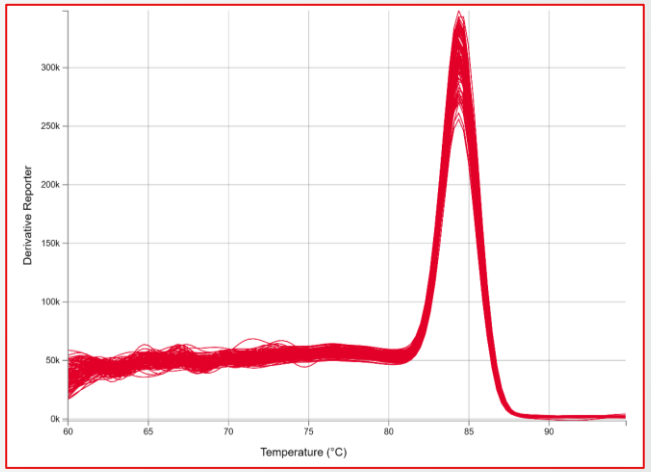
Step 2 : Primer validation :

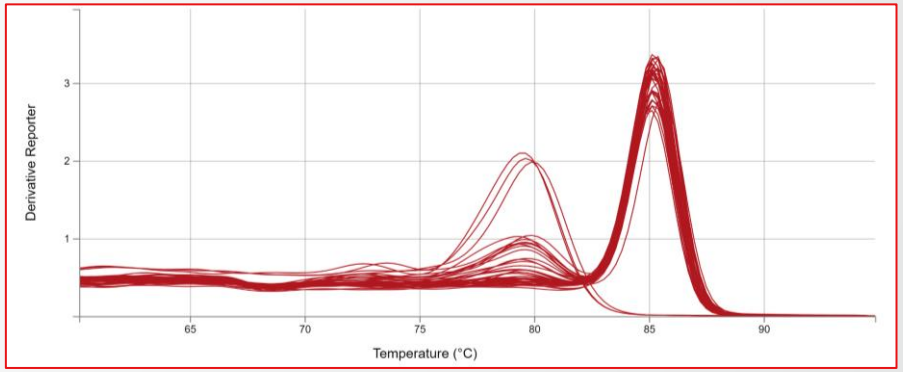
Different primer concentrations need to be tested to identify the best combinations:
high efficiency but no **primer-dimer formation**.



Choose the combination that

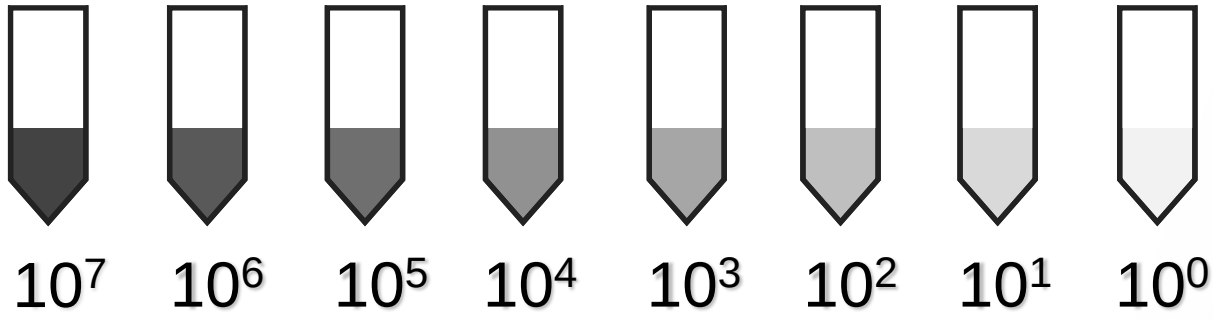
- ✓ doesn‘t show primer-dimer formation in the negative control..
- ✓ results in the lowest Ct value for the samples.



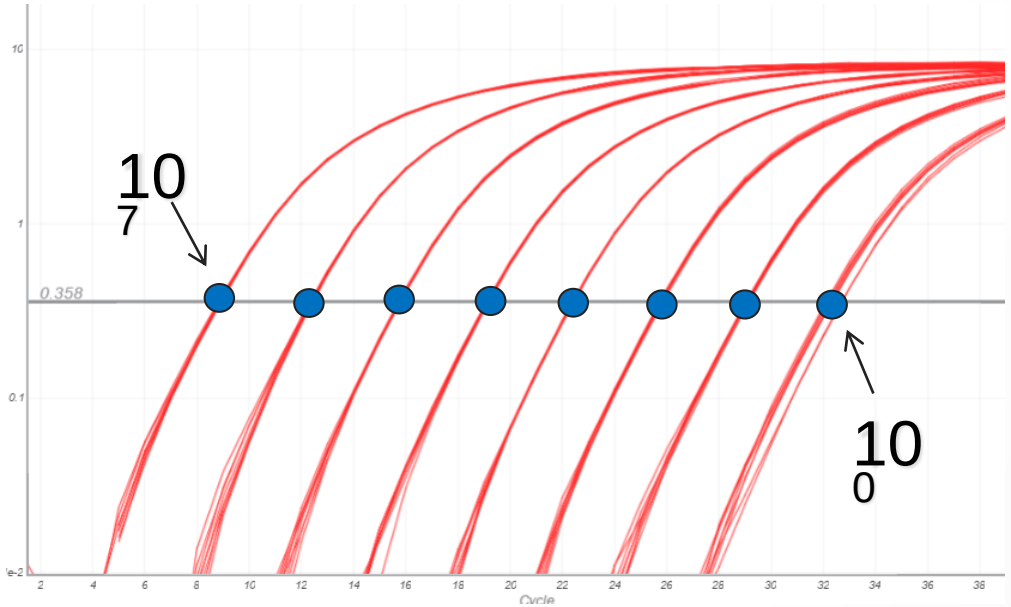


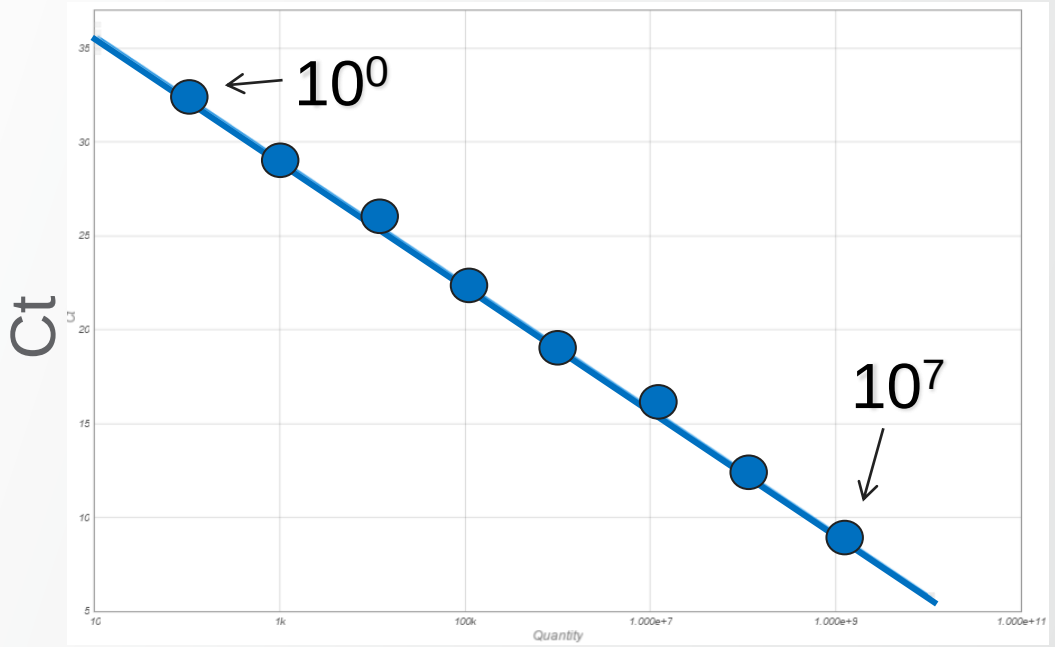
Tag Talk Video - How to optimize qPCR using SYBR Green assays

Step 3 : Assay efficiency



Dilution factor needs to be known



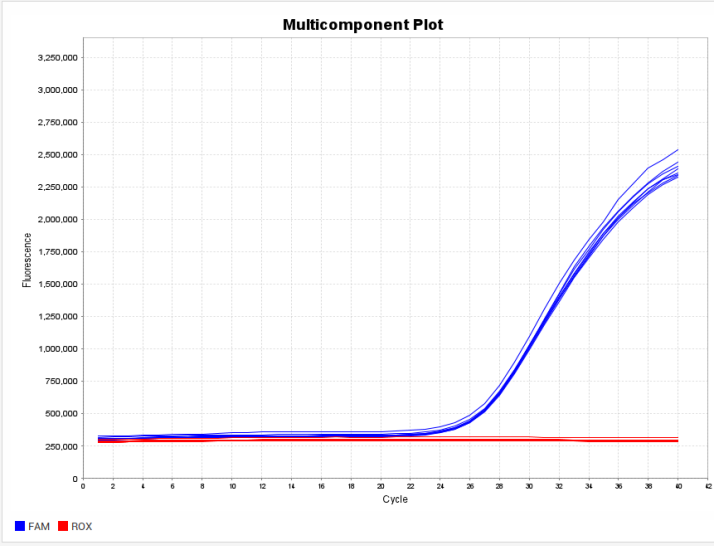


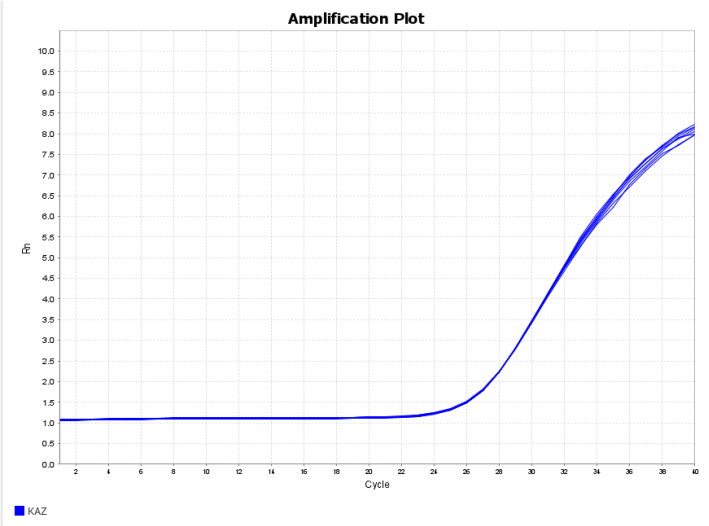
Quantity

Step 4 : Normalization strategies

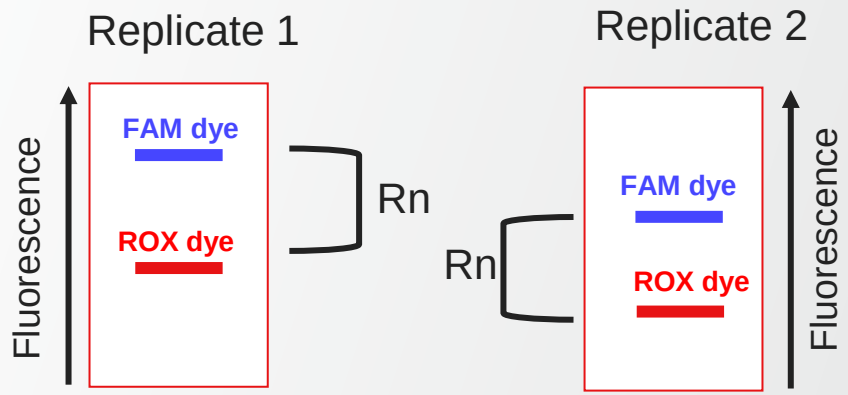
What is a passive reference ?

ROX normalization example

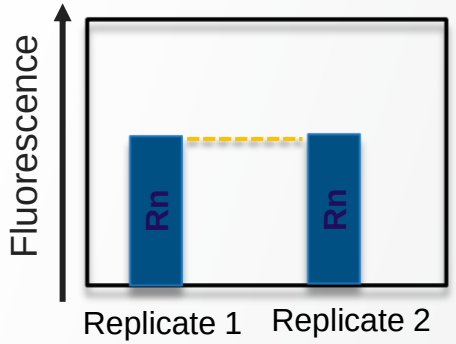




Before normalization



After normalization



$$Rn = \frac{\text{Reporter signal}}{\text{ROX signal}}$$

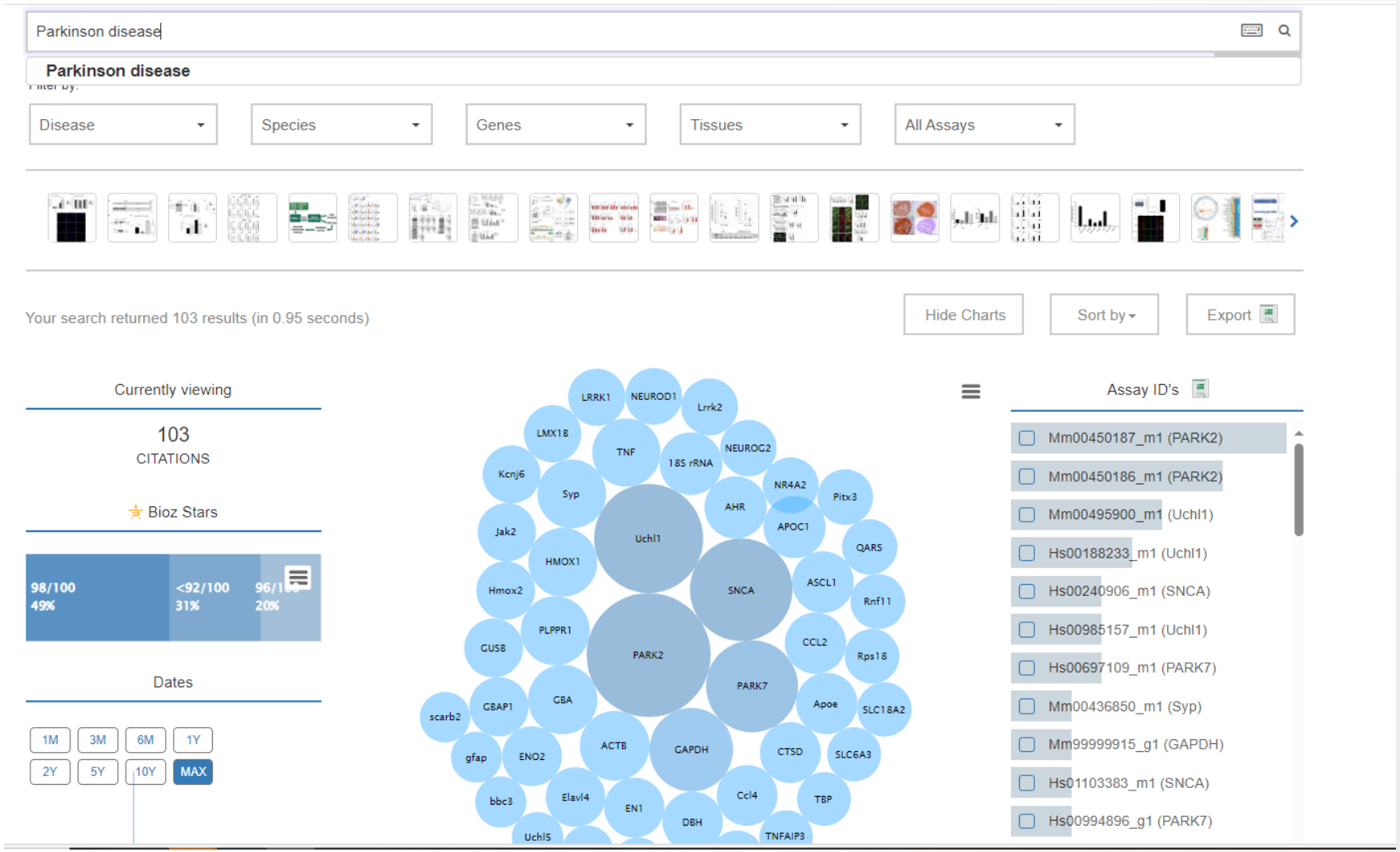
Endogenous controls

- Endogenous normalizers are genes naturally present in each sample and have a constant expression level in all samples used in that study.
- Endogenous normalizers are commonly used for samples derived from cells or cellular tissues.
- The EC normalizes for RNA input measurements errors and RT efficiency variations

Universal endogenous controls don't exist, they need to be evaluated for each study

Uses controls

- **Endogenous control (EC) for gene expression**



[TaqMan Endogenous Controls | Thermo Fisher Scientific - FR]

Uses controls

- TaqMan™ Array Endogenous Controls Plates 96-well (Human, rat and mouse)

Format 32+ Custom Format 32 Plus Candidate Endogenous Control Genes Set

	1	2	3	4	5	6	7	8	9	10	11	12
A	MC	CEC1	CEC2	CEC3	MC	CEC1	CEC2	CEC3	MC	CEC1	CEC2	CEC3
B	1	2	3	4	1	2	3	4	1	2	3	4
C	5	6	7	8	5	6	7	8	5	6	7	8
D	9	10	11	12	9	10	11	12	9	10	11	12
E	13	14	15	16	13	14	15	16	13	14	15	16
F	17	18	19	20	17	18	19	20	17	18	19	20
G	21	22	23	24	21	22	23	24	21	22	23	24
H	25	26	27	28	25	26	27	28	25	26	27	28

MC = Manufacturing Control (18S)

CEC = Candidate Endogenous Control Gene (GAPDH, HPRT, GUSB)

#	Assay IDs	Gene
1	Hs99999901_s1	18S rRNA
2	Hs99999905_m1	GAPDH
3	Hs99999909_m1	HPRT1
4	Hs99999908_m1	GUSB
5	Hs99999903_m1	ACTB
6	Hs99999907_m1	B2M
7	Hs00609297_m1	HMBS
8	Hs00183533_m1	IPO8
9	Hs99999906_m1	PGK1
10	Hs99999902_m1	RPLP0
11	Hs99999910_m1	TBP
12	Hs99999911_m1	TFRC
13	Hs00824723_m1	UBC
14	Hs00237047_m1	YWHAZ
15	Hs99999904_m1	PPIA
16	Hs00172187_m1	POLR2A
17	Hs00201226_m1	CASC3
18	Hs00355782_m1	CDKN1A
19	Hs00153277_m1	CDKN1B
20	Hs00169255_m1	GADD45A
21	Hs00206469_m1	PUM1
22	Hs00197826_m1	PSMC4
23	Hs00426752_m1	EIF2B1
24	Hs00362795_g1	PES1
25	Hs00245445_m1	ABL1
26	Hs00152844_m1	ELF1
27	Hs02596862_g1	MT-ATP6
28	Hs00608519_m1	MRPL19
29	Hs00198357_m1	POP4
30	Hs01102345_m1	RPL37A
31	Hs00265497_m1	RPL30
32	Hs00734303_g1	RPS17

[TaqMan™ Array Human Endogenous Controls Plate, Fast 96-well (thermofisher.com)]

Uses controls

- **How to validate a EC ?**

Relative quantification analysis module : Select candidate or try other candidates if you don‘t not get acceptable results

Endogenous

Candidate Control	Target	Score
☑	ACTB	2.55
☑	GAPDH	2.745
☑	GH1	3.039
☑	LIPC	5.201

Research

Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes

Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman

Address: Center for Medical Genetics, Ghent University Hospital 1K5, De Pintelaan 185, B-9000 Ghent, Belgium.

Correspondence: Frank Speleman. E-mail: franki.speleman@rug.ac.be

Published: 18 June 2002

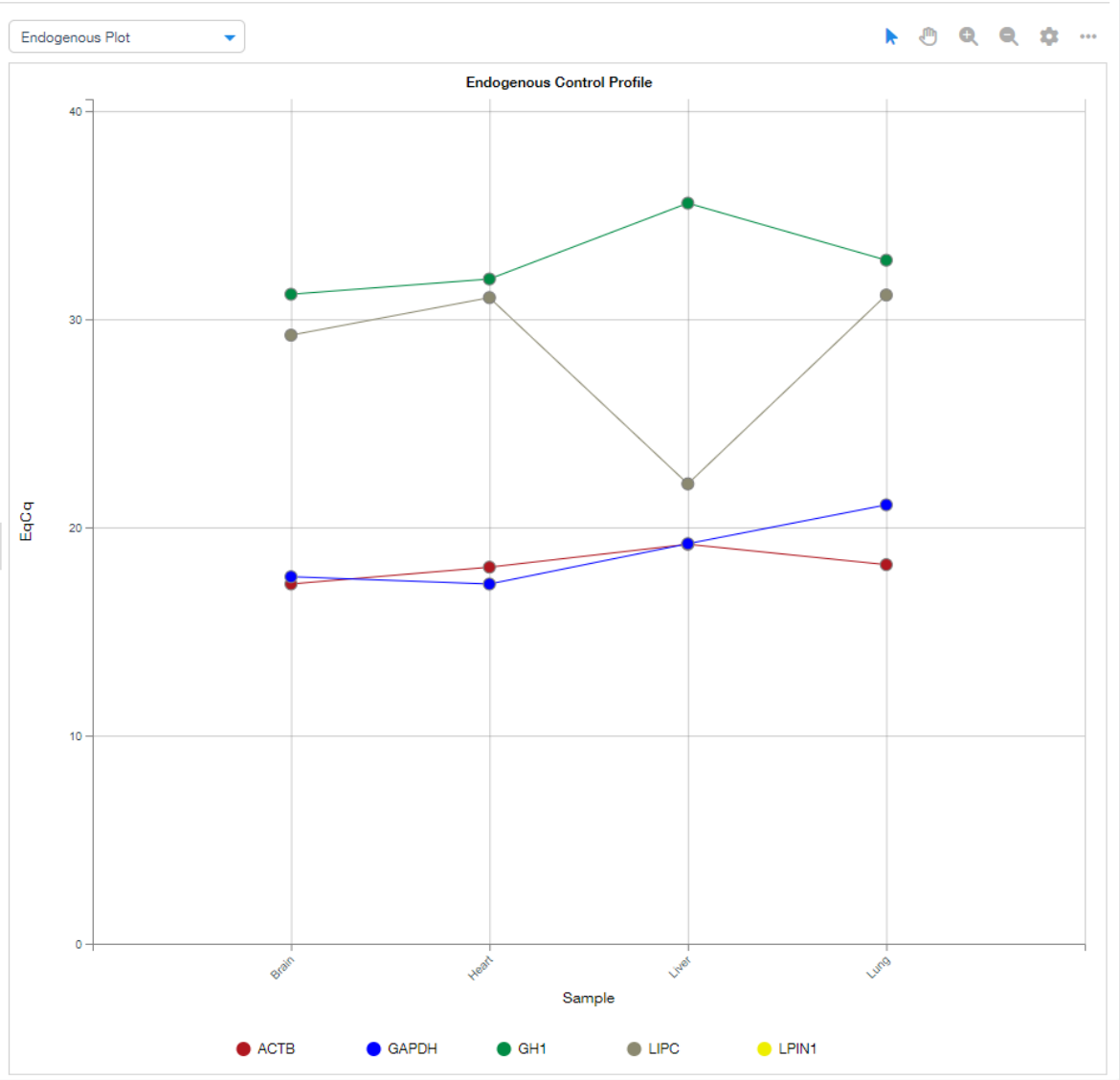
Genome Biology 2002, **3(7)**:research0034.1–0034.11

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2002/3/7/research/0034

(Print ISSN 1465-6906; Online ISSN 1465-6914)

Received: 20 December 2001
Revised: 10 April 2002
Accepted: 7 May 200

geNorm



Summary & Conclusion

Summary & Conclusion

- A **good SYBR Green workflow** requires your attention:
  - Design and optimization of your assays
  - Determine the efficiency and analysis method
  - Normalization strategies
  - Melt curve analysis

We care about your SYBR Green experiments and can help you along the way

- We can provide you with the **support** needed to create your assay
- We offer the **tools and products** you need in the different steps of the workflow
- Be aware of any **challenges** you can find in the design and workflow

PowerTrack™ SYBR™ Green Master Mix Overview

Ease of use and Flexibility at a Competitive Price

For SYBR Green gene expression, performance seekers looking for broad dynamic range and minimize pipetting errors, PowerTrack SYBR Green Master Mix offers

- TRACKING dye to reduce pipetting errors
- BROAD primer Tm and concentration allows flexibility and minimal optimization
- SUPERIOR specificity & sensitivity
- TIGHT reproducibility
- UNIVERSAL instrument compatibility

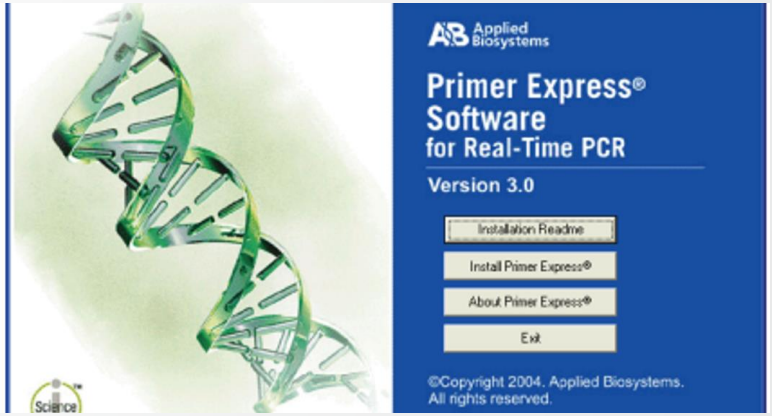
Reproducibility -- Specificity -- Sensitivity -- Compatibility -- Price

Exclusive offer for SYBR Green workflow

Leave your contact details with us and receive exclusive benefits till summer of 2024

- PowerTrack SYBR Green Master Mix
  - Free sample program
  - Discounted prices within the LUMC

- Sequence detection primers
  - 68% discount till 31st of May 2024
  - Same price independent from number of base pairs



- Primer Express license
  - 50% off till 29th of March 2024 (€816,-)
  - 1 license to use on more than 99+ devices

Q&A