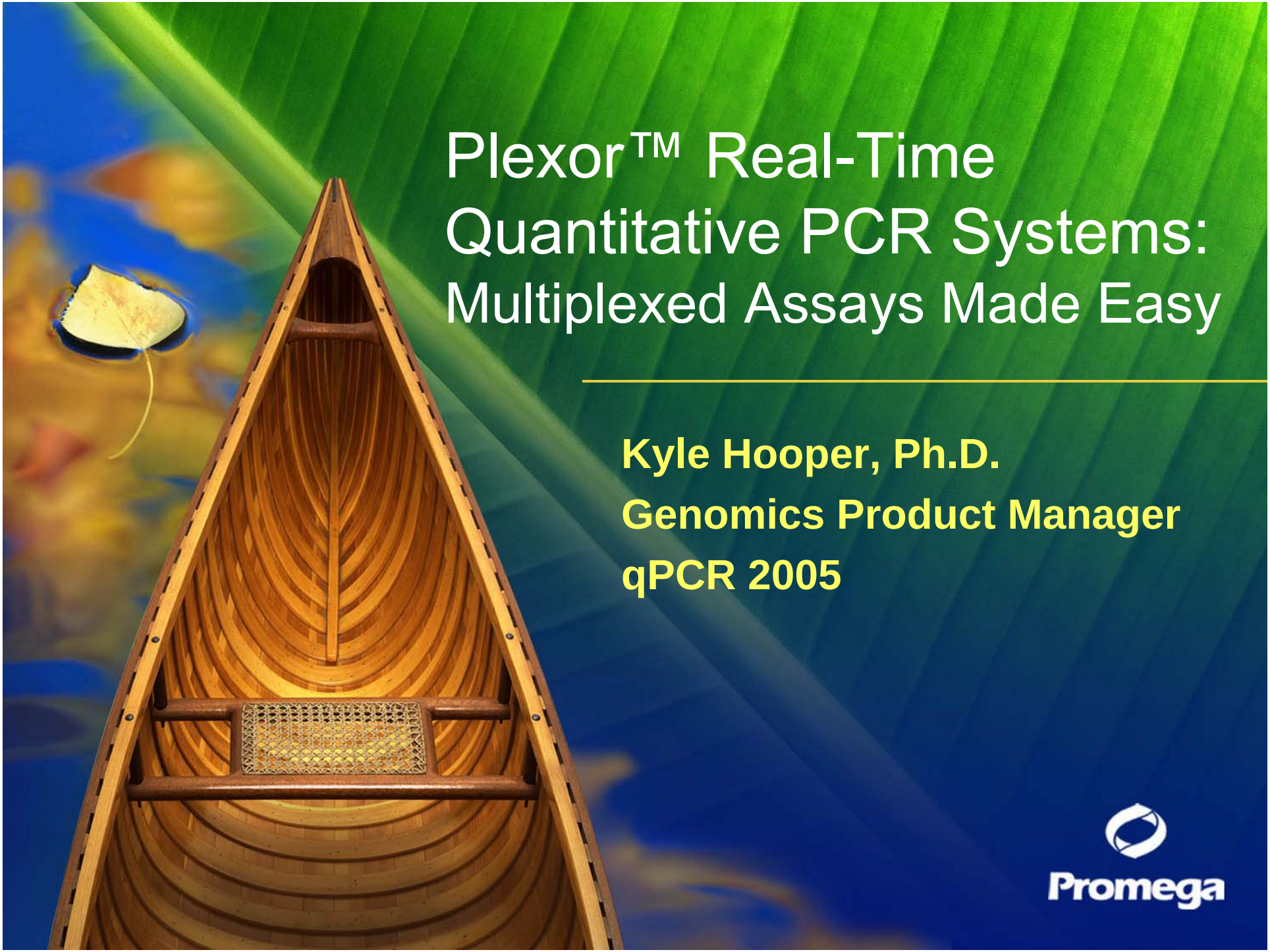


Plexor™ Real-Time Quantitative PCR Systems: Multiplexed Assays Made Easy

Kyle Hooper, Ph.D.
Genomics Product Manager
qPCR 2005

Promega

Plexor™ Systems Real-Time qPCR Systems: Multiplexed Assays Made Easy

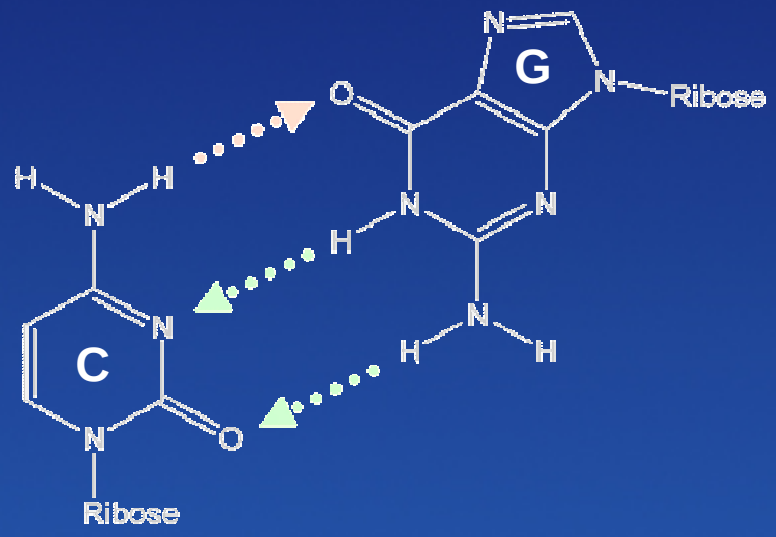
- Plexor Technology
- Plexor Reagents
- Applications of Plexor Technology
  - Gene Expression Analysis
  - SNP Genotyping
- Conclusions

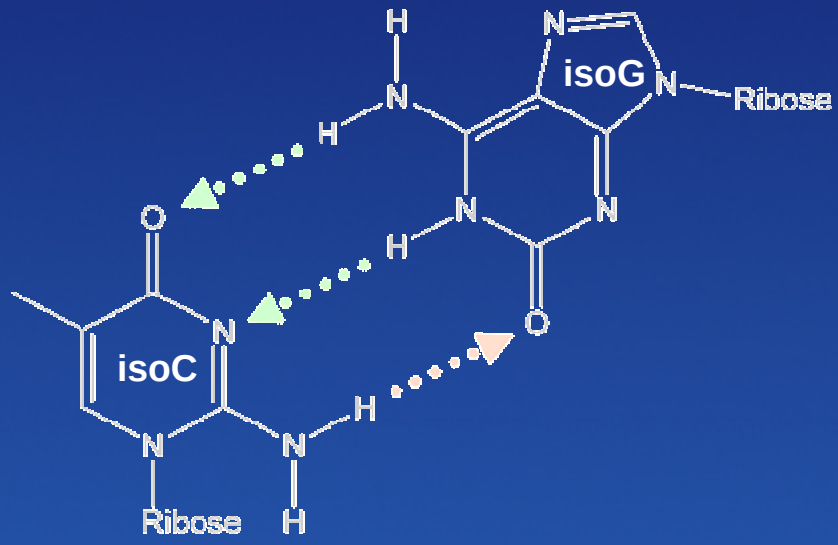
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Plexor™ Technology
Specificity from novel base-pairing
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Novel Base-Pairing Drives Plexor™ Technology

- Designed and licensed from EraGen Biosciences
- IsoC & IsoG dNTPs incorporated by DNA Polymerase
- IsoC & IsoG bases do not base pair with ACGTU

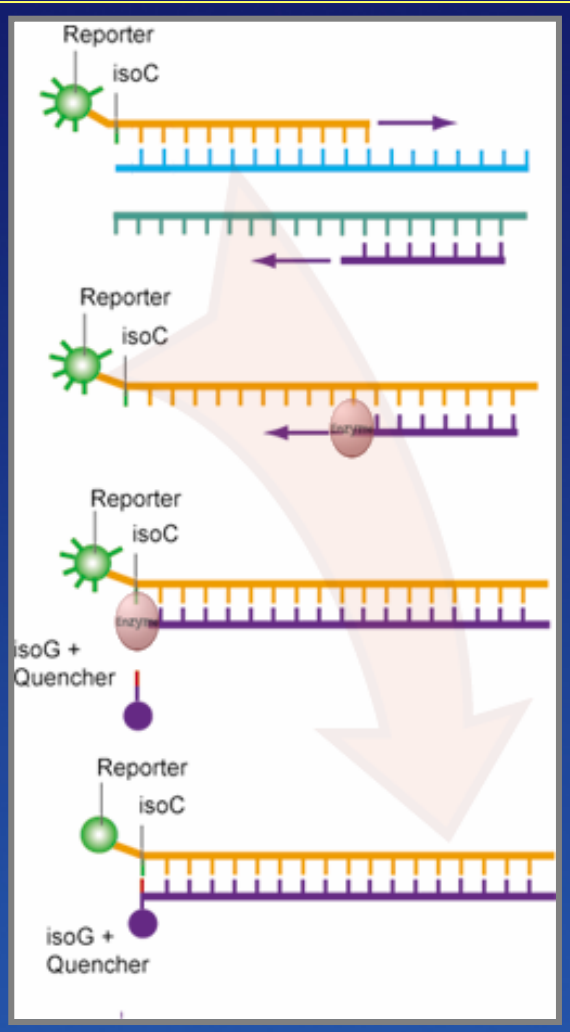




Johnson, S.C., *et al.* (2004) *Nucleic Acids Res.* **32**, 1937-41.

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Application of Novel IsoG & IsoC Bases



- Two primer method
- Oligo primer synthesized with
  - 5’ novel base (isoC)
  - Fluor: FAM, JOE, HEX, ROX, whatever is compatible with your instrument
- Amplification master mix
  - Standard dNTPs
  - Dabcyl-labeled complementary iso-dGTP

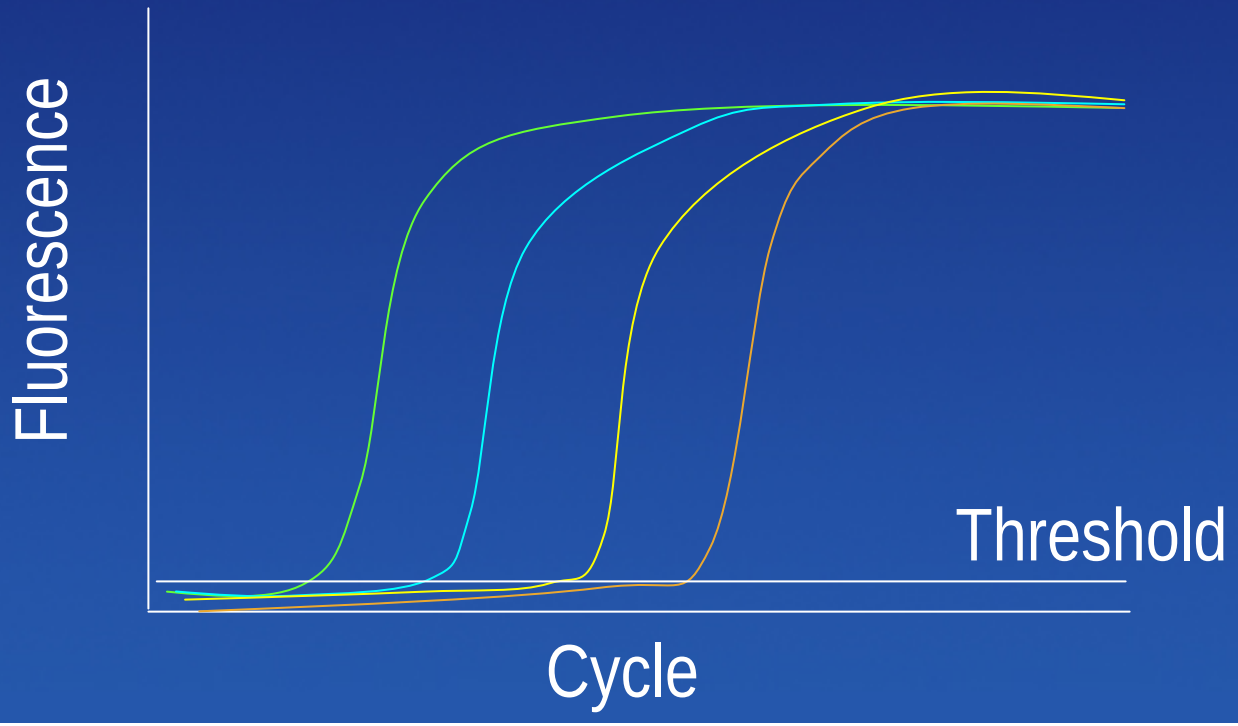
Dabcyl-iso-dGTP incorporated opposite isoC, quenching fluorescence!

Sherrill, C.B., *et al.* (2004) *J. Amer. Chem. Soc.* **126**, 4550-6.

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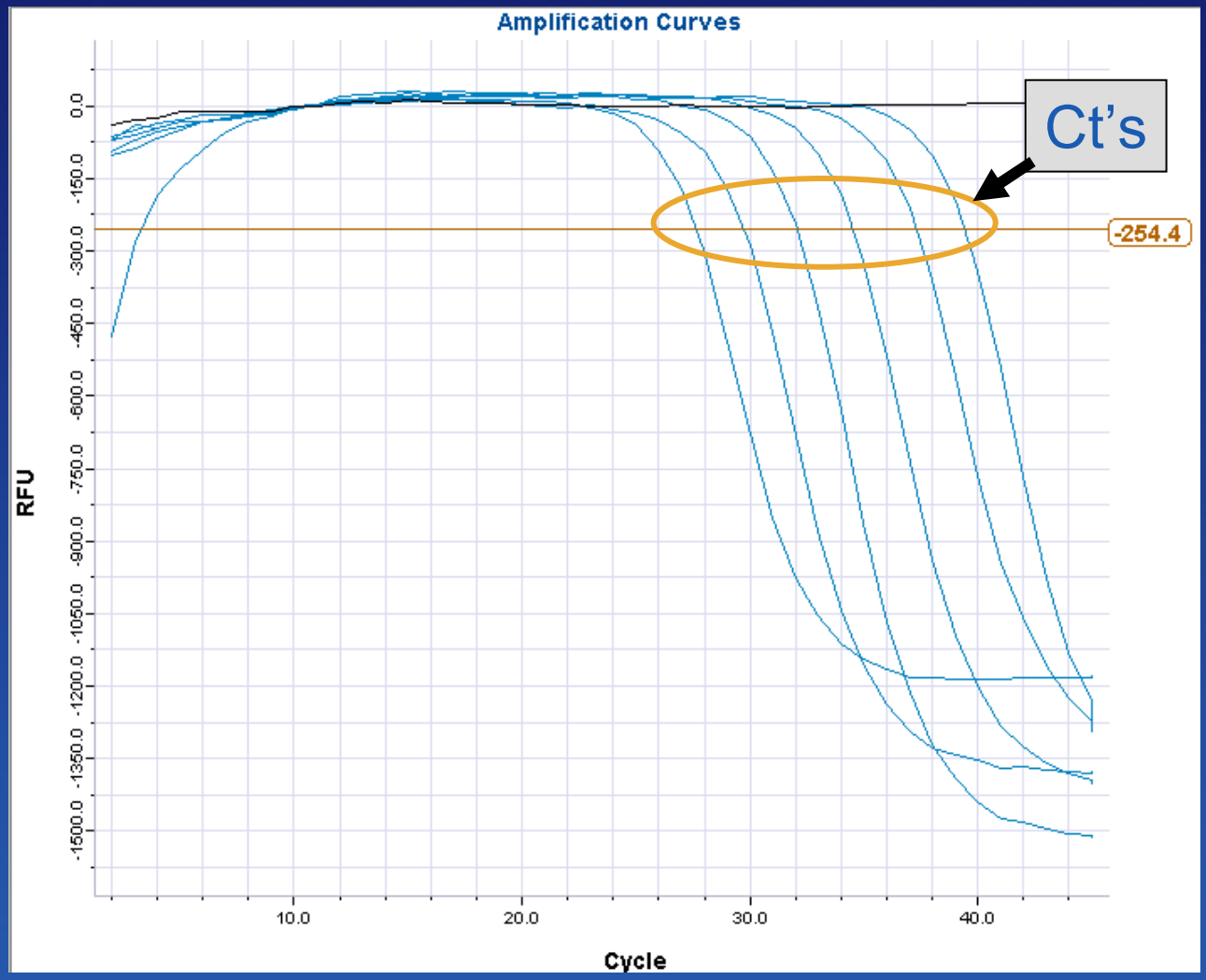
Yes, It's Different…

- Existing methods
  – gain of fluorescence



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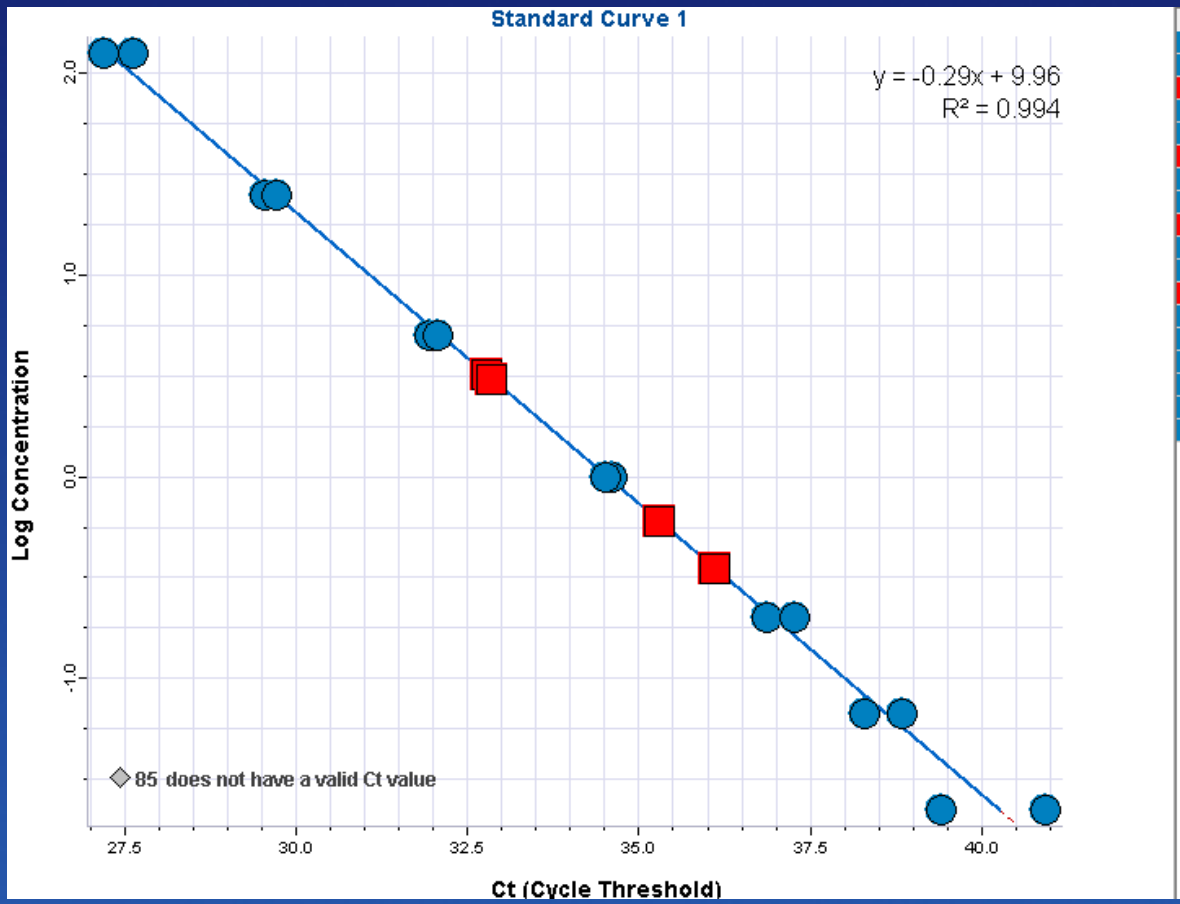
Different…Yet Familiar



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C_t vs. Concentration

- You get the same data as gain of fluoresence methods

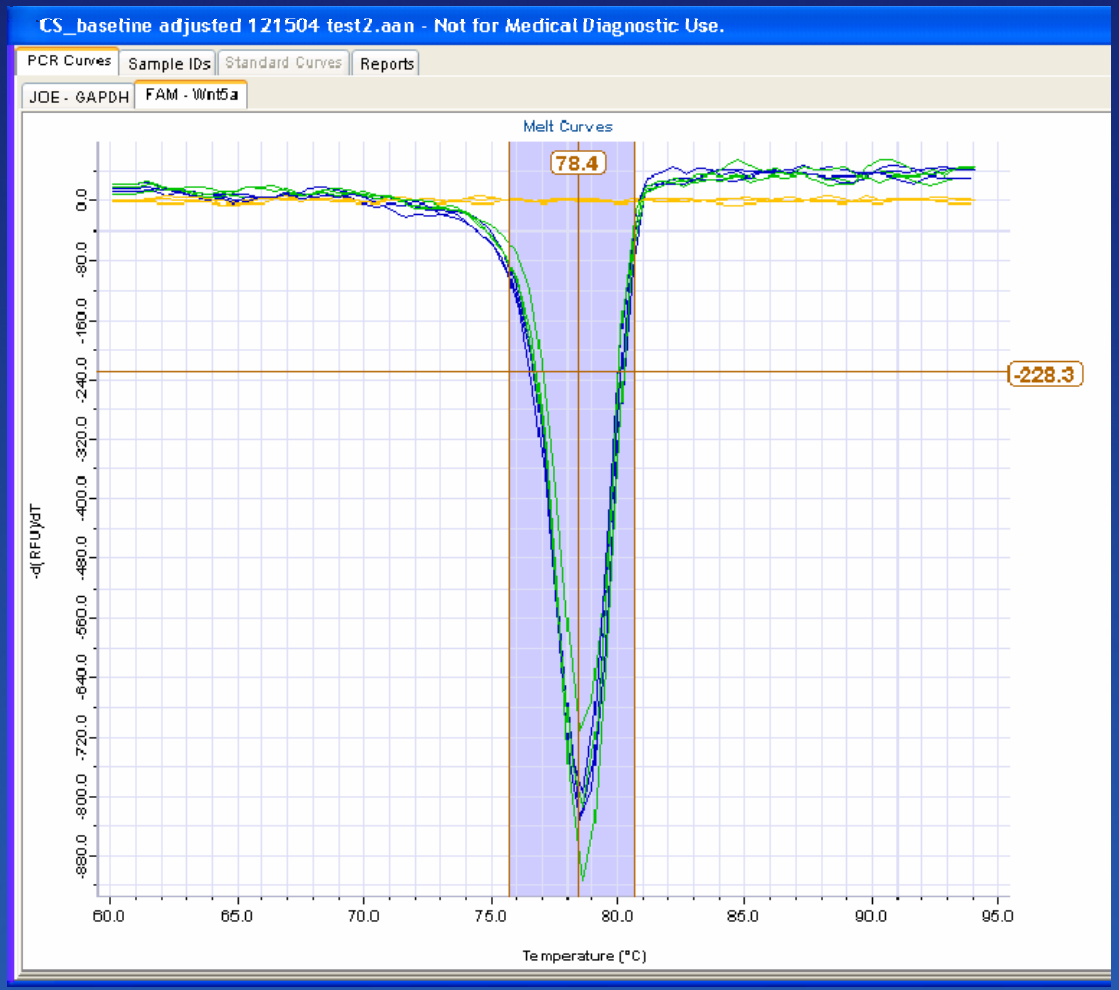


Sample ID	Ct	Tm	Conc
1	27.2	78.3	1.2E02
2	27.6	78.7	1.2E02
3	36.1	80.1	3.5E-01
13	29.5	78.7	2.5E01
14	29.7	78.5	2.5E01
15	32.8	80.5	3.2E00
25	31.9	79.2	5.0E00
26	32.1	79.2	5.0E00
27	35.3	80.5	6.0E-01
37	34.6	79.2	1.0E00
38	34.5	79.2	1.0E00
39	32.9	80.5	3.0E00
49	36.9	79.0	2.0E-01
50	37.3	80.2	2.0E-01
61	38.3	79.5	6.7E-02
62	38.8	79.3	6.7E-02
73	40.9	80.2	2.2E-02
74	39.4	80.2	2.2E-02

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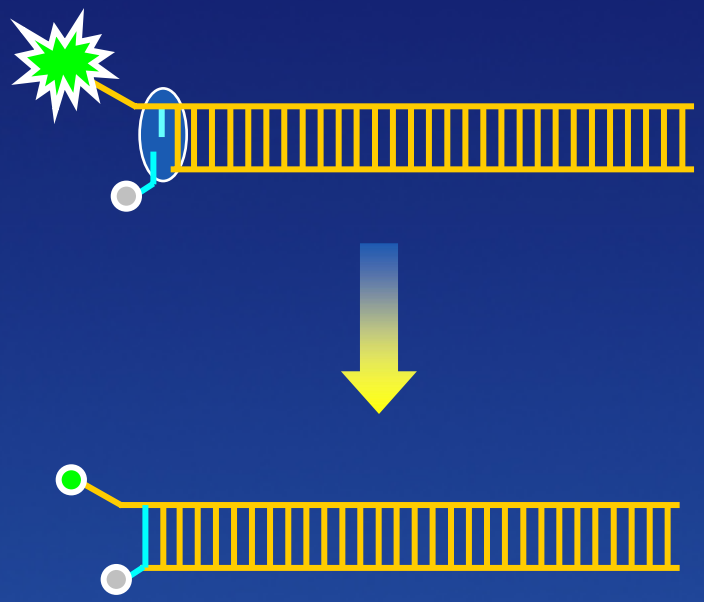
Melt Curves

- Confirmation of specificity



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Dabcyl-iso-dGTP quenches by proximity



- Interaction governed by iso-dC in primer
- Dabcyl-iso-dGTP incorporated opposite any iso-dC

Dabcyl iso-dGTP makes multiplexing possible!

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Can Dabcyl Quench Red Shifted Dyes?

Dye	% Quenching	Emission Peak
FAM	62%	518nm
TET	53%	536nm
HEX	71%	554nm
ROX	64%	606nm
LC640	48%	640nm
Cy5	35%	667nm

Table 1 in: Sherrill, C.B., *et al.* (2004) *J. Amer. Chem. Soc.* 126, 4550-6.

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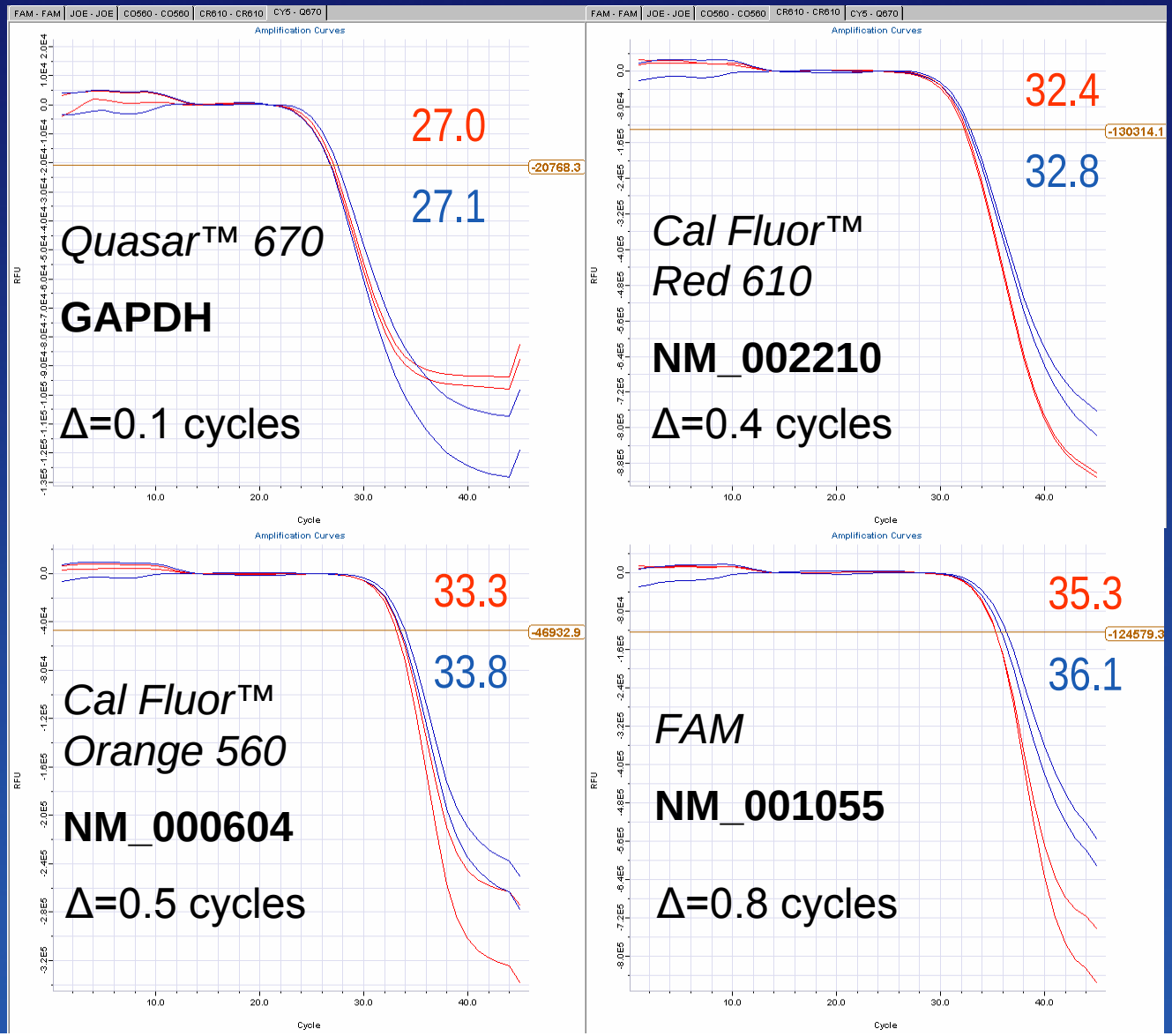
4-Color Multiplexing

Experimental details

- Four primer sets
- Biosearch Technologies dye sets
- All targets are human cDNA
  - Plexor™ Two-Step qRT-PCR System
- Performed on the ABI® 7500

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2-Step qRT-PCR — 4-Color Multiplexing



Red = Monoplex
Blue = 4-plex

ABI® 7500

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The Plexor™ Reagents
qPCR
Two-Step qRT-PCR
One-Step qRT-PCR
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Plexor™ Systems

- qPCR System
  - For real-time quantitation from genomic DNA, SNP genotyping
  - For 2-step qRT-PCR methods with your cDNA
- 2-step qRT-PCR System
  - ImProm-II™ RT Reagents for cDNA synthesis
  - Plexor Master Mix for qPCR from cDNA template
- 1-step qRT-PCR System
  - Combines ImProm-II™ RT with Plexor Master Mix for qRT-PCR directly from RNA template

Promega

Plexor™ Reactions

- 25µl standard reaction
- 2X Plexor Master Mix contains:
  - Enzyme (no 5´ exo activity)
  - High performance buffer
  - Proprietary primer-dimer inhibitor
  - dATP, dCTP, dGTP, dTTP and Dabcyl-iso-dGTP

For amplification of the DNA sequence of interest

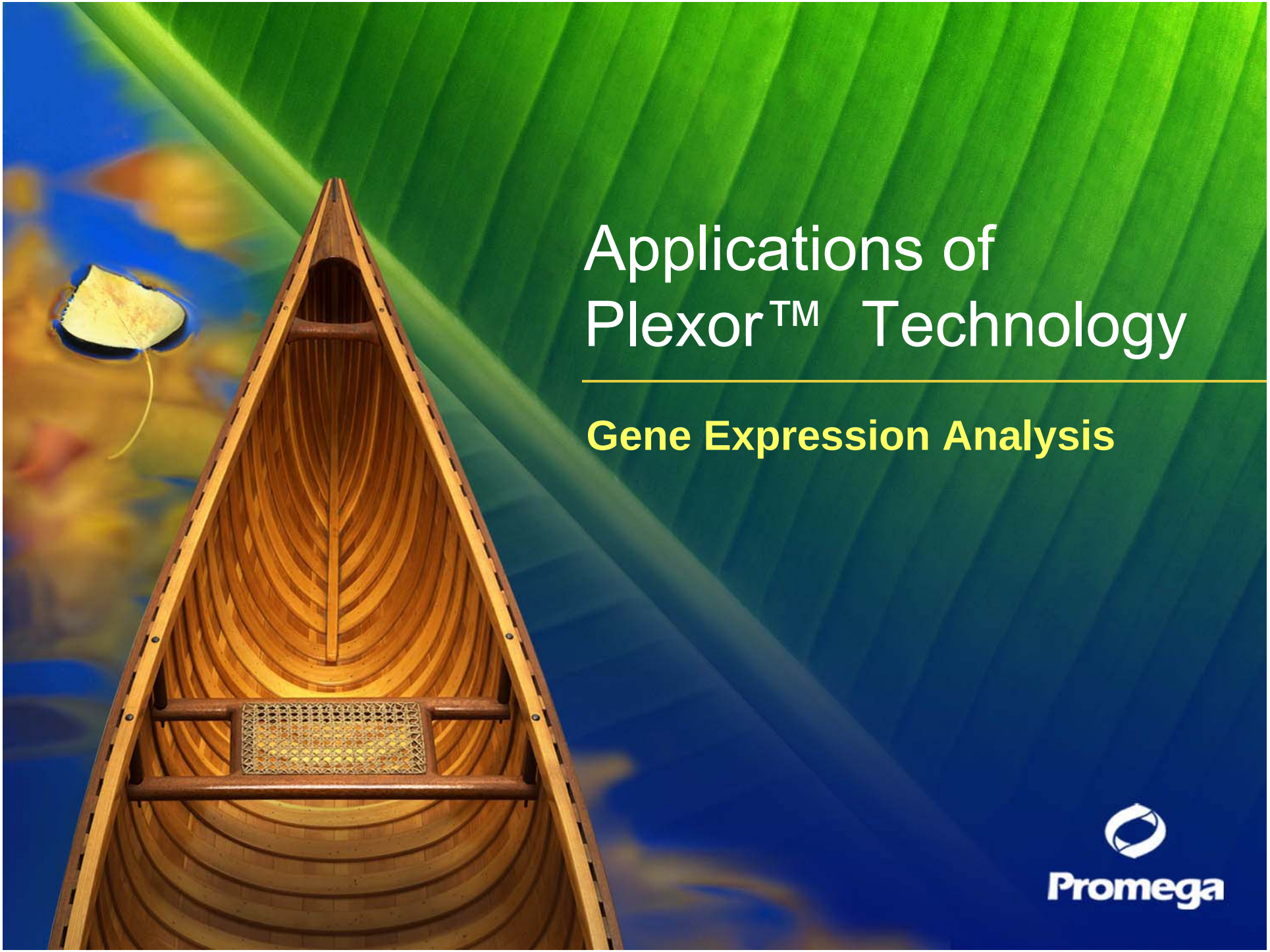
For incorporation opposite the iso-dC in the Plexor Primers

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What you need…

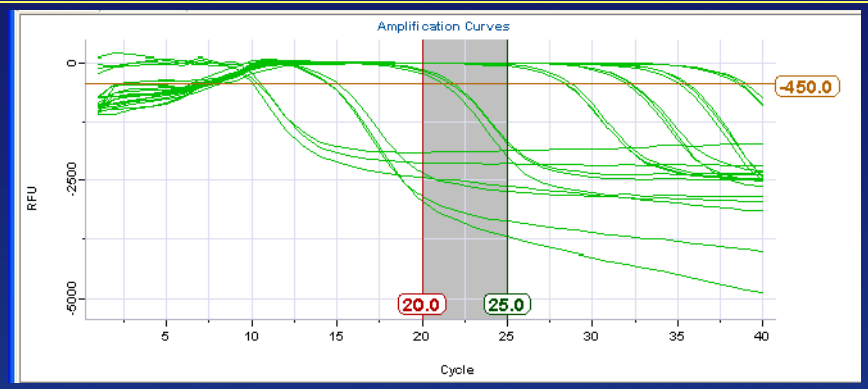
- Plexor™ Primer Design Software
  - Engineered for multiplex assays
  - Free web access
- Plexor™ Primer Sets
  - BioSearch Technology
  - Eurogentec
  - Intergrated DNA Technologies (IDT)
- Real-Time Instrument
  - Existing instruments capable of detecting more than one fluor
  - ABI, Roche, etc.
- Plexor™ Analysis Software
  - Analyzes quenching data from a variety of instruments
  - Free download

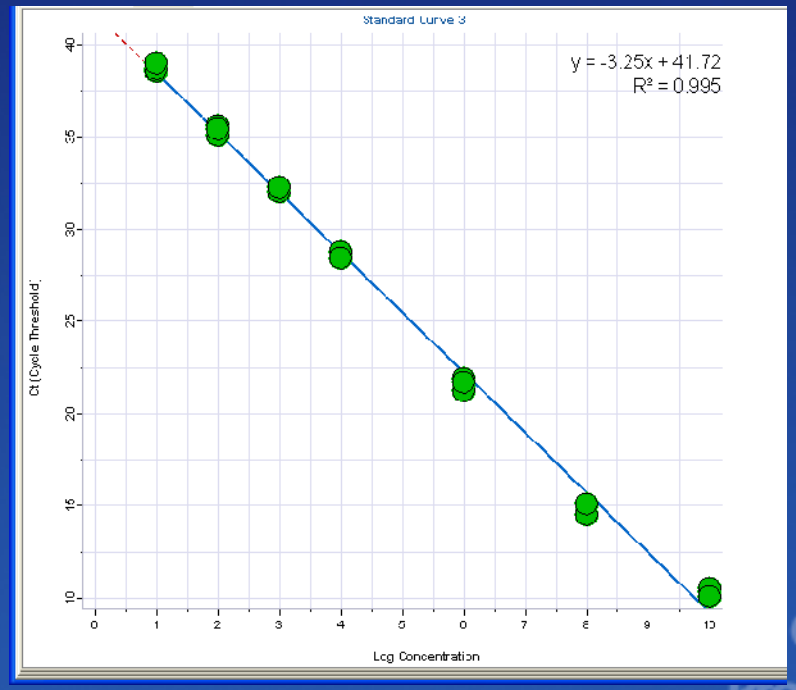
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Applications of
Plexor™ Technology
Gene Expression Analysis
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Large Dynamic Range…Monoplex Reactions

- Detection of Kanamycin 1.6kb synthetic transcript RNA (93 bp amplicon)
  - Dilutions in background of 10ng human total RNA
  - Kanamycin (FAM) primers
- Dynamic Range: 10^1 to 10^{10} copies



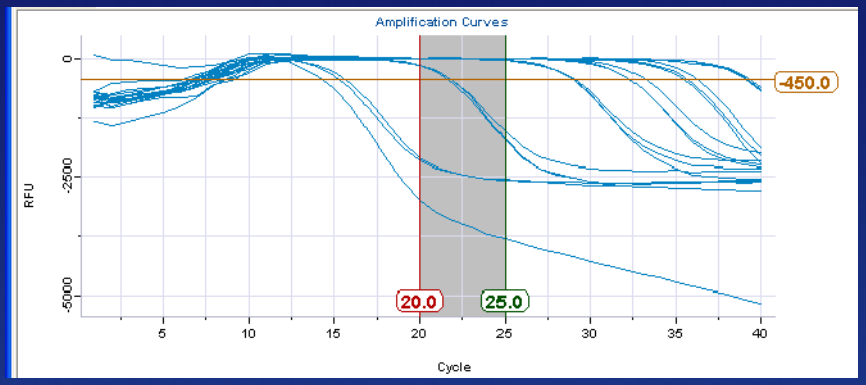


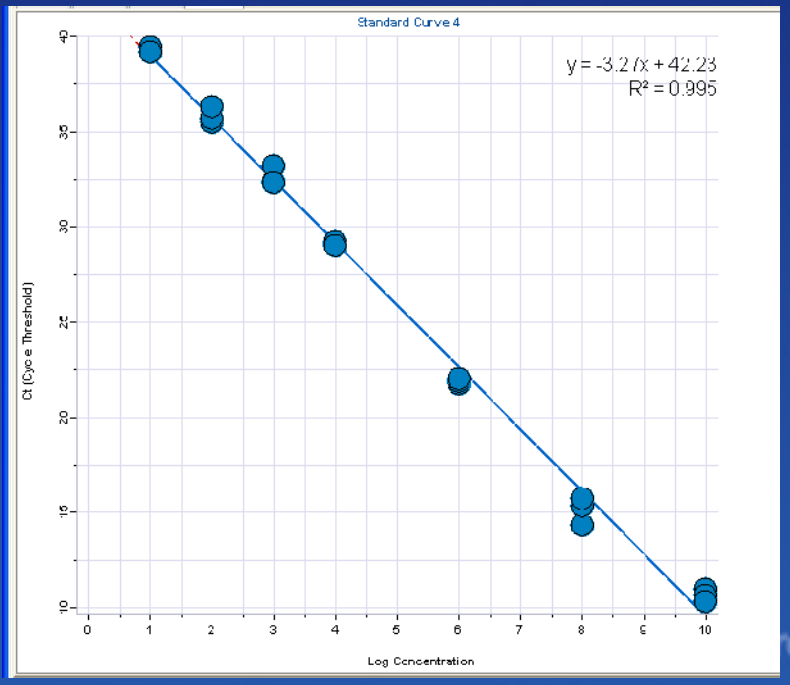
Plexor™ One-Step qRT-PCR System

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Large Dynamic Range…Duplex Reactions

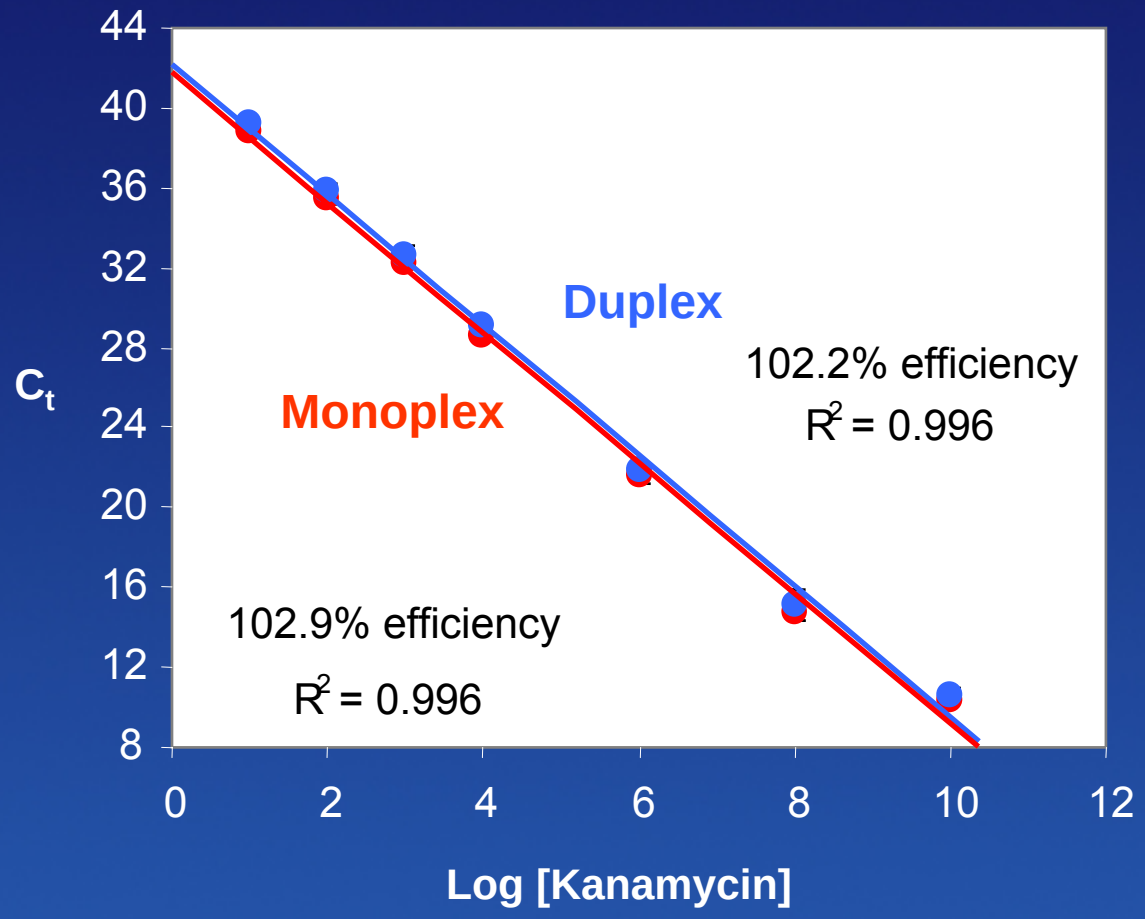
- Detection of Kanamycin 1.6kb synthetic transcript RNA
  - Dilutions in background of 10ng human total RNA
  - Kanamycin (FAM) primers
  - GAPDH (JOE) primers
- Dynamic Range: 10^1 to 10^{10} copies





Plexor™ One-Step qRT-PCR System

Consistent C_t Values in Monoplex & Duplex



- Detection of synthetic target in background of 10ng human total RNA
- Dynamic range from 10^1 to 10^{10}
- C_t Values consistent in monoplex and duplex reactions

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Quantitation in Multiplex

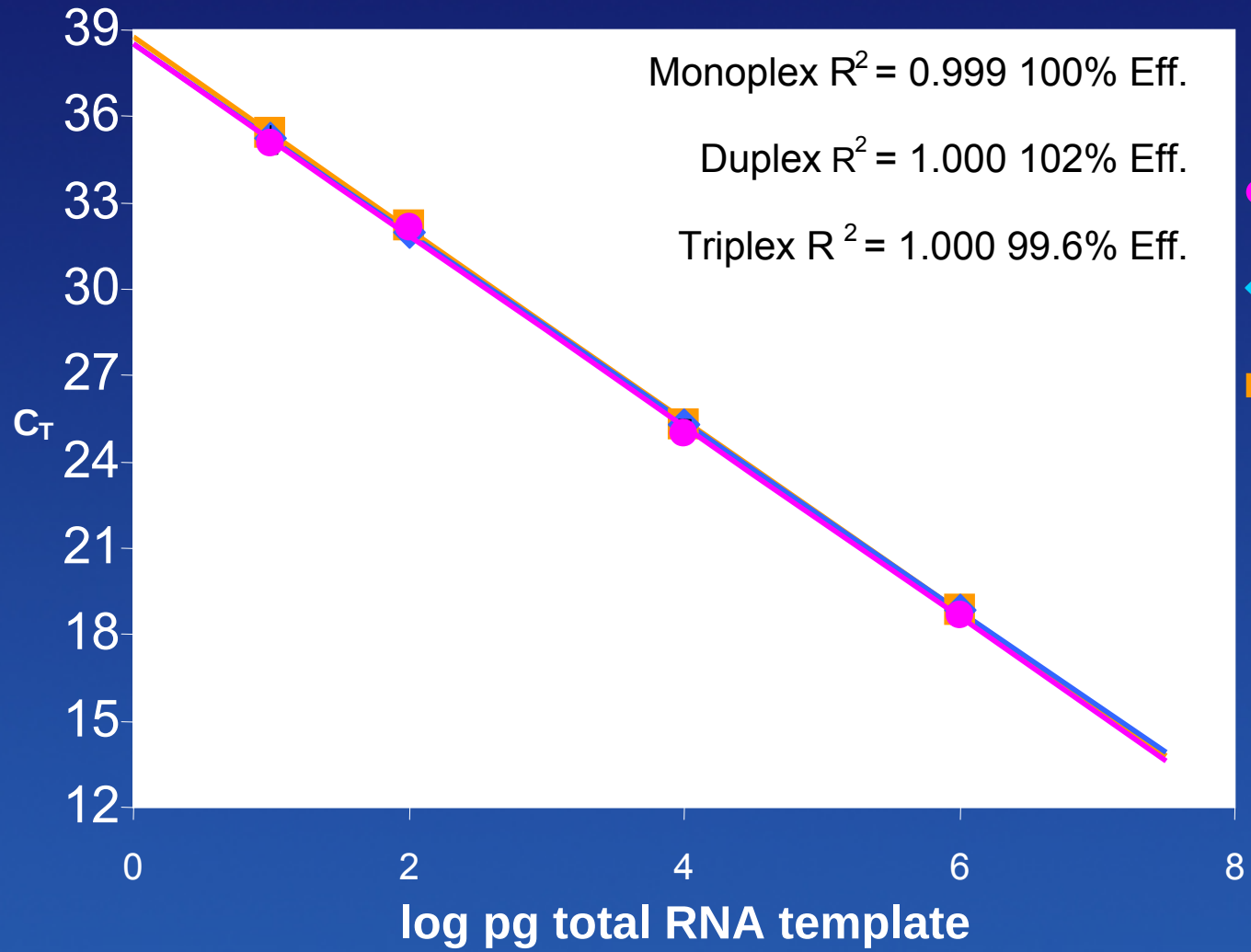
Experimental Details

- Three primer sets designed to multiplex together with Plexor™ Primer Design Software
- Biosearch Technologies dye sets
- All targets are human total RNA
  - Plexor™ One-Step qRT-PCR System
- Performed on the ABI® 7500

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Quantitation in Multiplex

Fibroblast Growth Factor Receptor 1 (FGFR1)



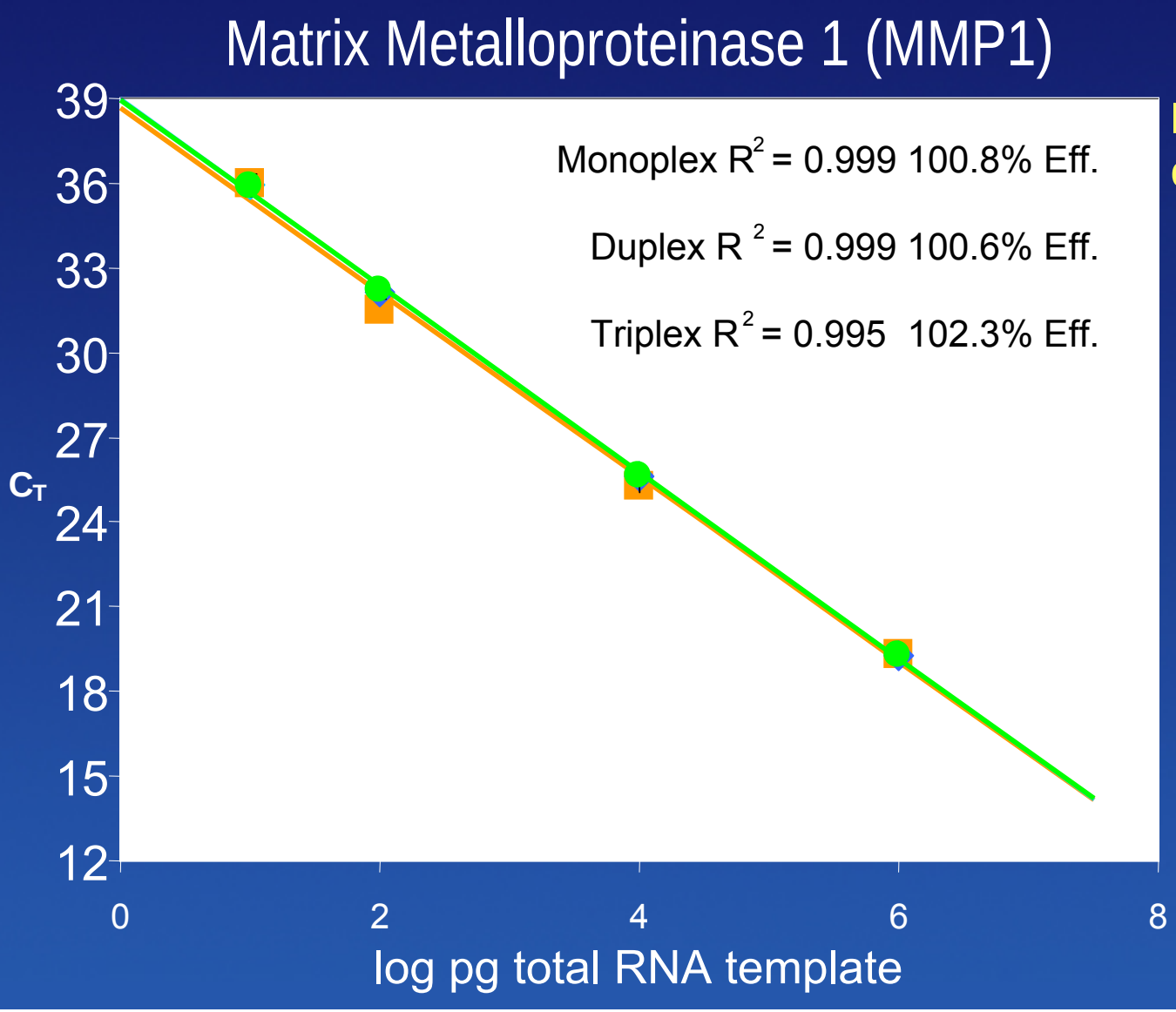
Plexor™ One-Step qRT-PCR System

- FGFR1
- FGFR1 + GAPDH
- FGFR1 + MMP1 + GAPDH

FAM Channel

Promega

Quantitation in Multiplex



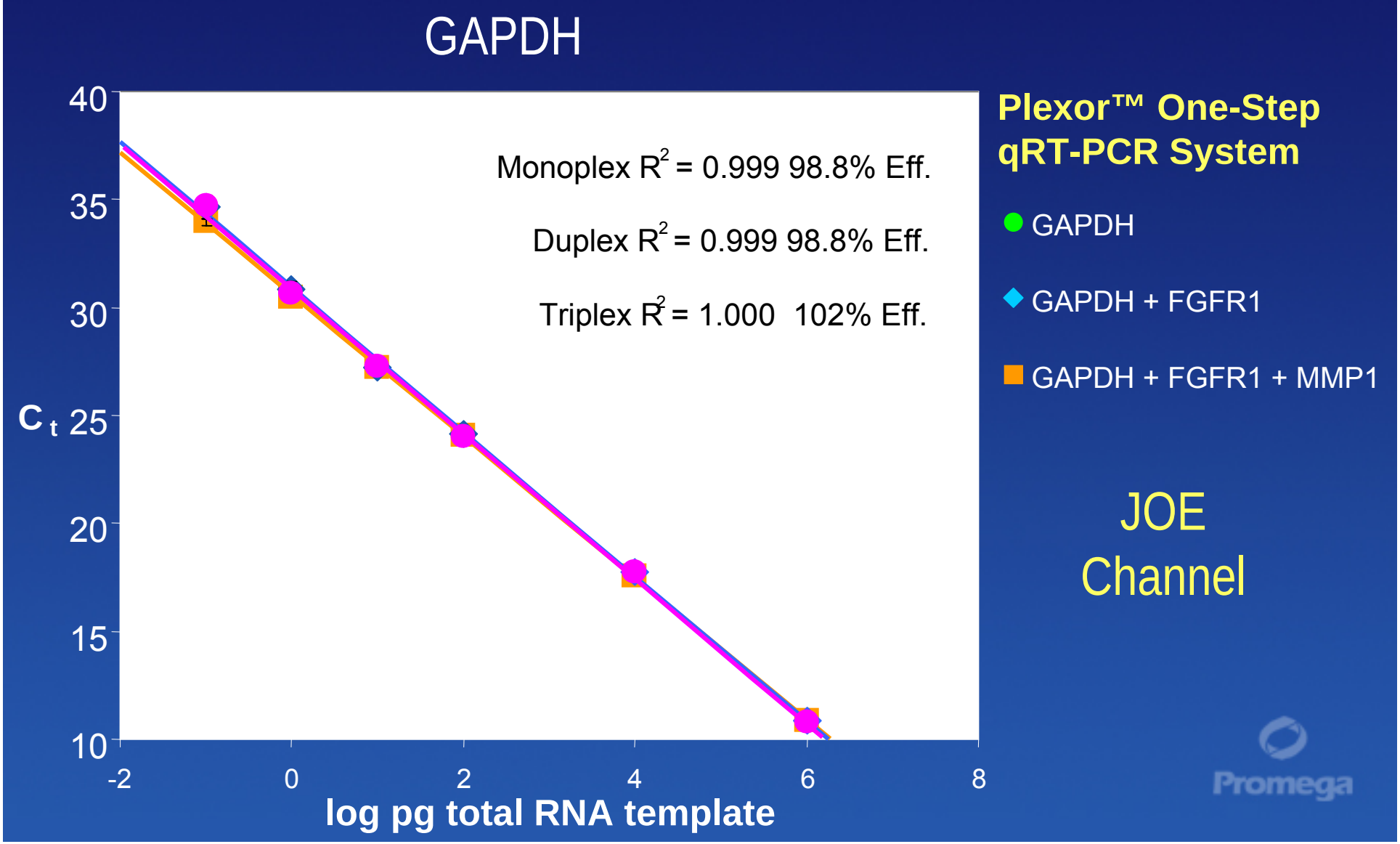
Plexor™ One-Step qRT-PCR System

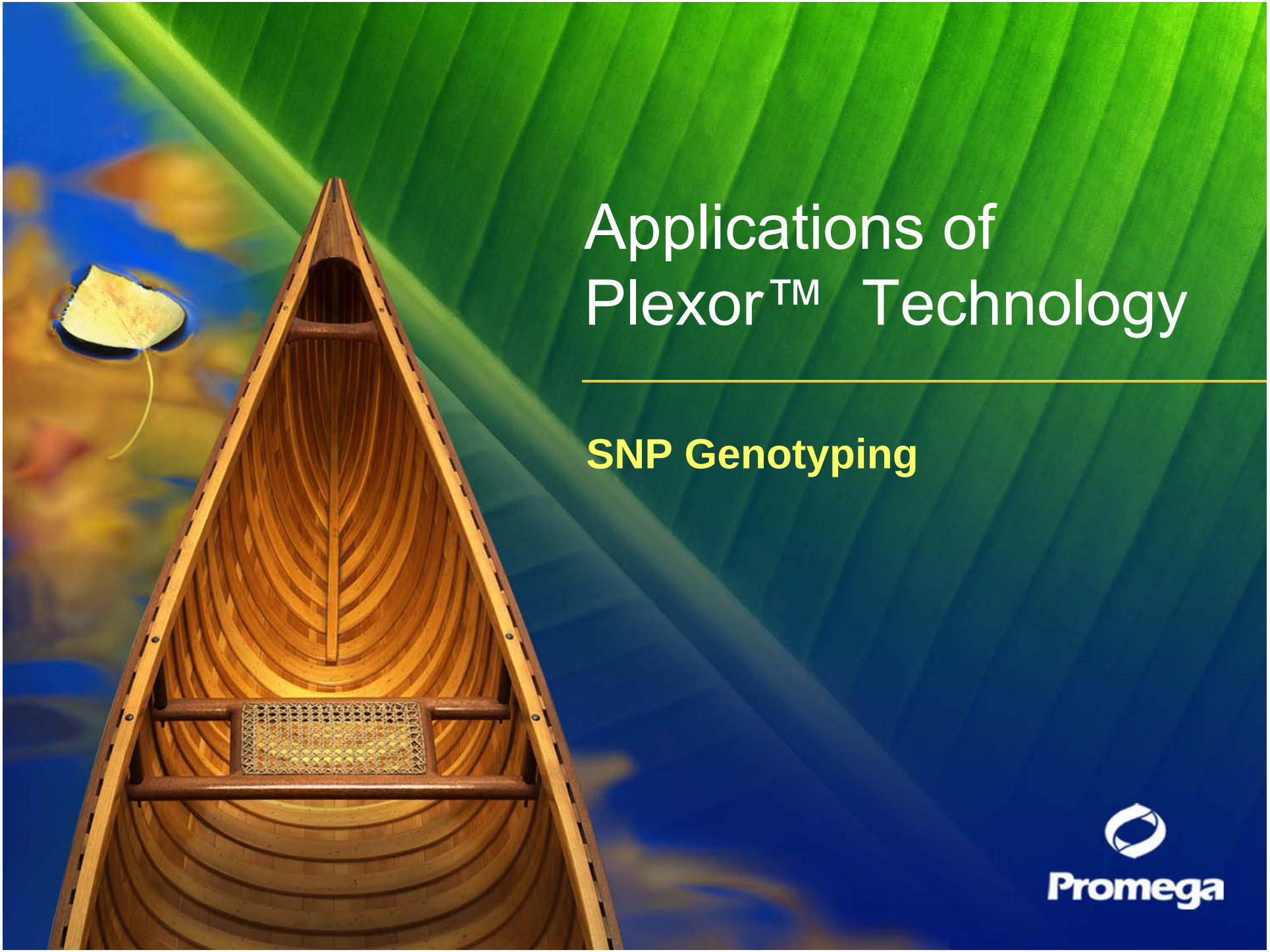
- MMP1
- MMP1 + GAPDH
- MMP1 + FGFR1 + GAPDH

CalFluor™ Red 610 Channel

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Quantitation in Multiplex



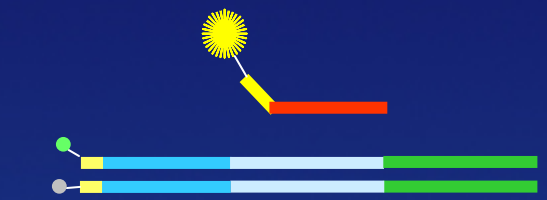

Applications of
Plexor™ Technology
SNP Genotyping
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SNP Genotyping with Plexor™ Technology

- Based on 3 primer allele specific PCR
- Each allele specific primer labeled with a different fluor
- Genotype determination is by endpoint melt curve analysis

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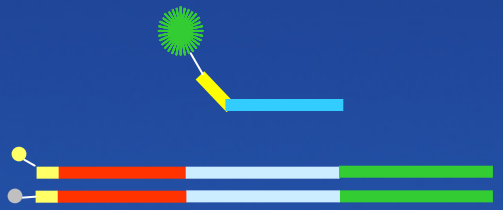
Genotyping by Melt Curve Analysis



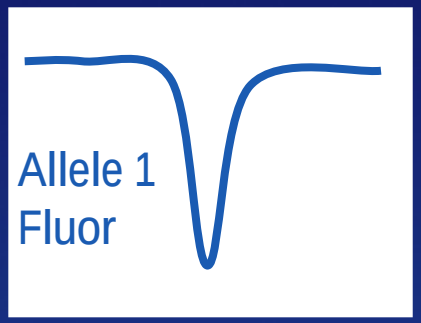
Homozygous Allele 1



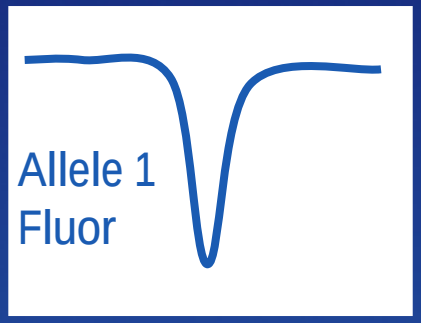
Heterozygous

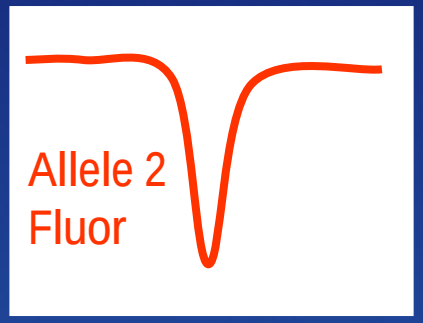


Homozygous Allele 2







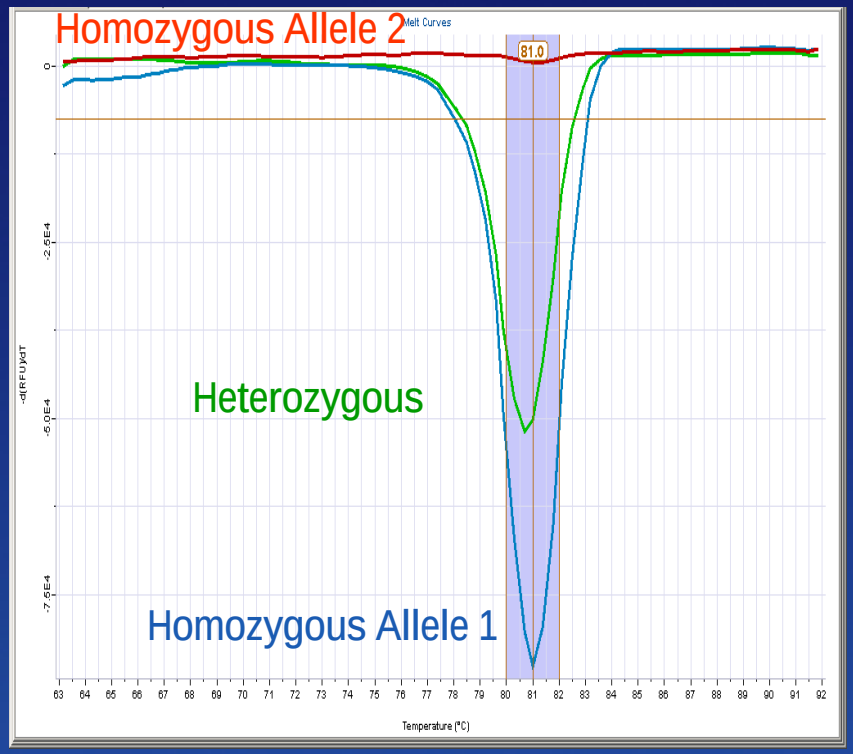


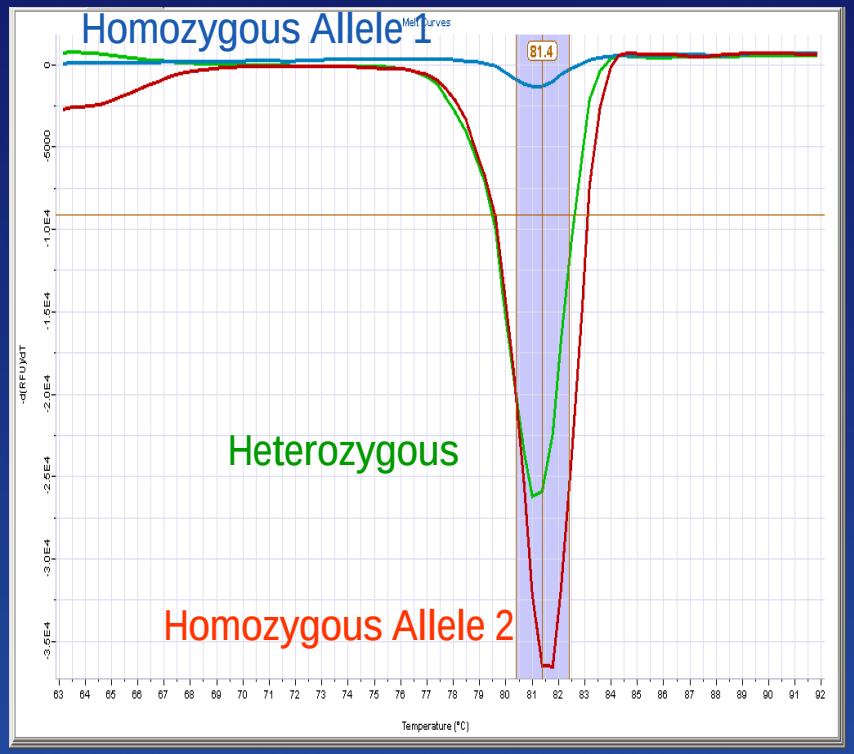




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Genotyping- Hemochromotosis HFE H63D





Allele 1 Primer
FAM Channel

Allele 2 Primer
CalFluor™ Orange 560
Channel

Plexor™ qPCR System

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Summary

- Two primer method allows easy multiplexing
- High correlation of C_t values between monoplex and multiplex (less than 1 C_t change)
- Accurate quantitation of lower copy messages in multiplex with high copy messages
- Three Primer method for closed tube genotyping (thermal melt)

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Acknowledgements

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Yi Wu

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Amanda Glebs
Julie Heger
Dave Leland
Jen Romanin
Michael Ma

Quality Assurance

Carol Lindsay
Brian McNamara

EraGen Biosciences

Michael Ho
Scott Johnson
Jim Prudent
Mike Moser
Mary Holland
Regina Reynolds

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More Questions?

For more information, please contact:

plexor@promega.com

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Legal disclaimer

The PCR process, which is the subject of European Pat. Nos. 201,184 and 200,362 owned by Hoffmann-LaRoche*, is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

*The above primary European Pat. Nos. 201,184 and 200,362 will expire on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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