Fast Protocols & Multiplexing

Cynthia Potter, Eppendorf UK


Accelerate your reality
NEW!

Mastercycler ep *realplex*

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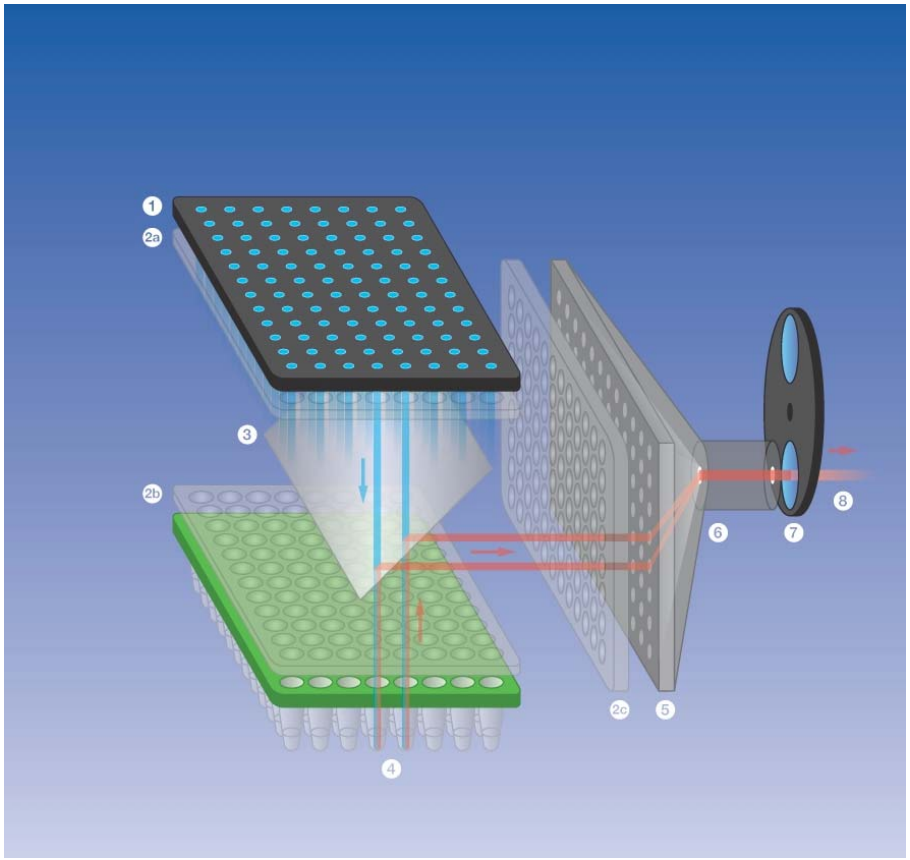
Mastercycler ep *realplex* - Optics

- Optic Module: 96 LED Array and channel PMT Detector



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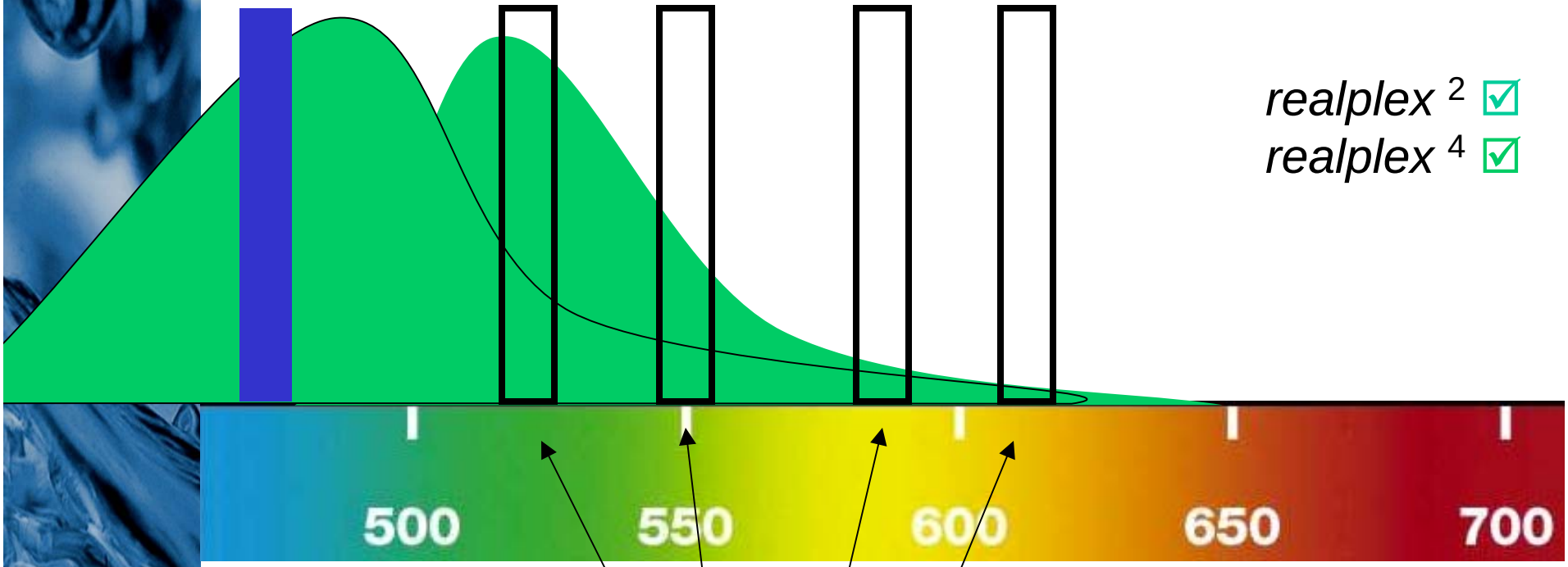
Fluorescent Dyes - e.g. FAM

***realplex* excitation light (470 nm)**

FAM: Excitation 494 nm
Emission 518 nm

realplex ^{2} ☑
realplex ^{4} ☑



Emission filters realplex ^{4}

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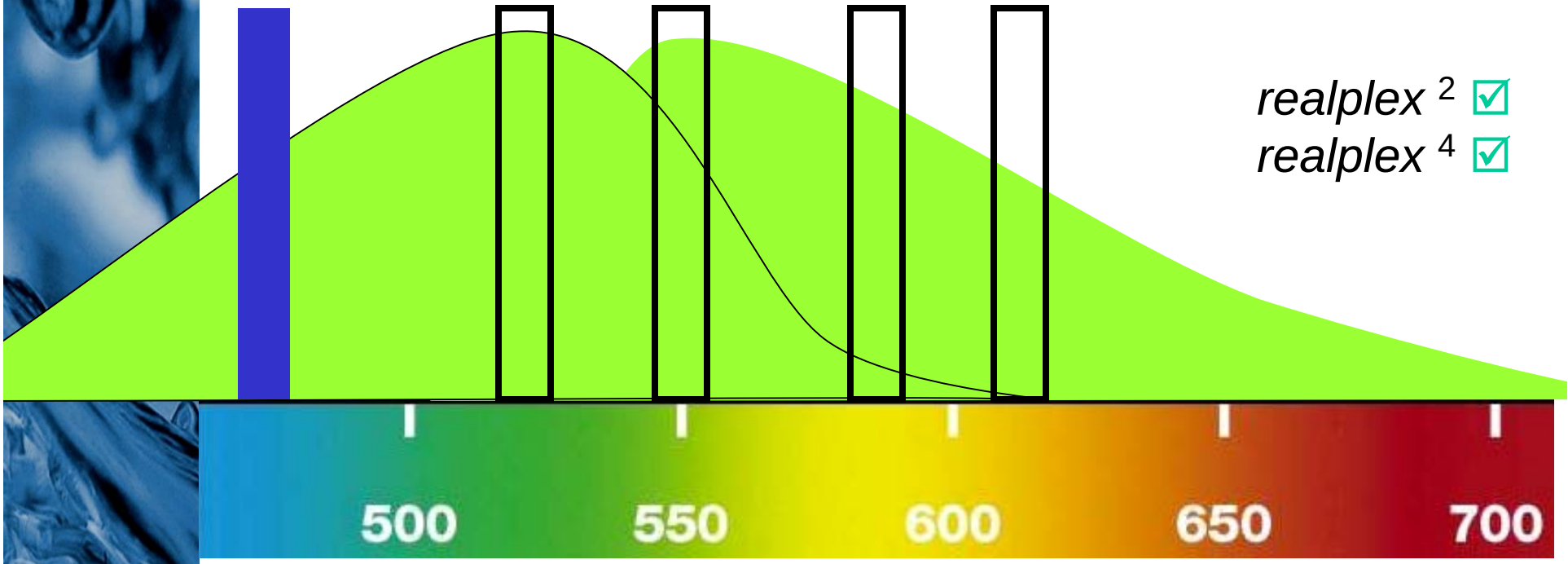
Fluorescent Dyes - e.g. JOE

JOE: Excitation 520 nm
Emission 548 nm

realplex [2] ☑
realplex [4] ☑



…and CalFluor Orange 560?

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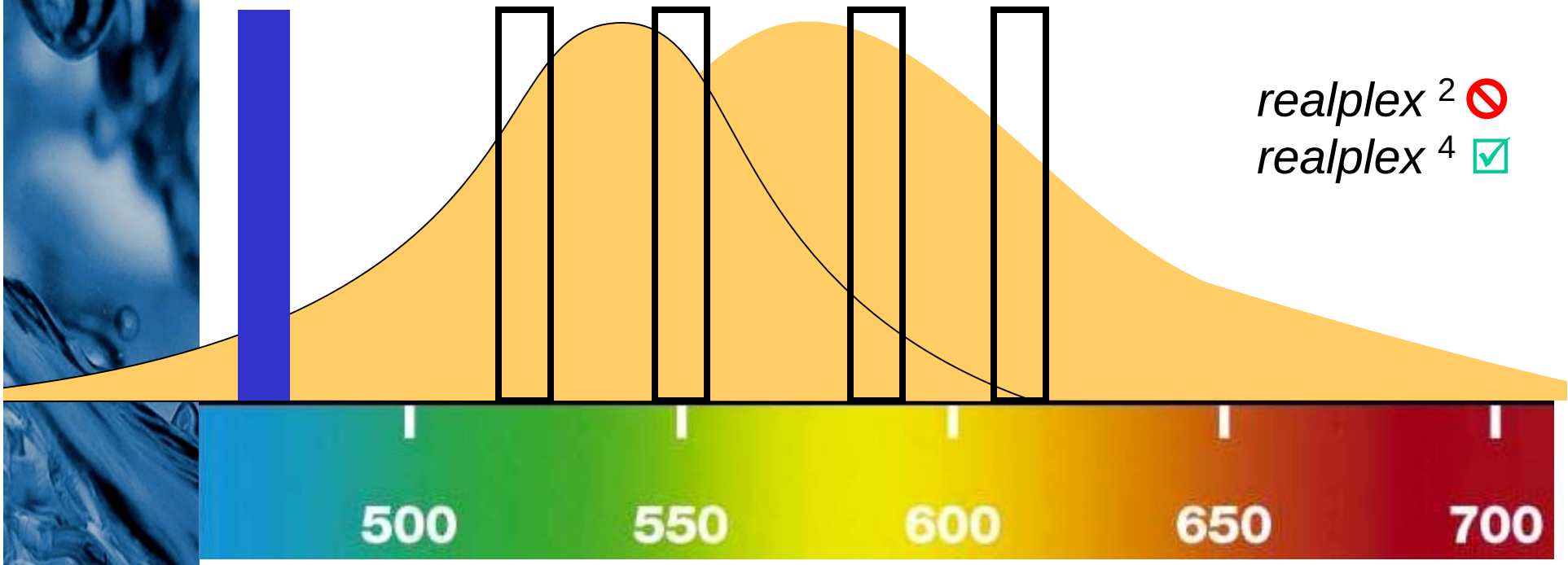
Fluorescent Dyes - e.g. TAMRA

TAMRA: Excitation 540 nm
Emission 568 nm

realplex ^{2} ⊘
realplex ^{4} ☑



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Four months before UK launch...

EpiStem, experts in epithelial, tumour and stem cell biology based in Manchester, wanted a demo

- EpiStem designed probes for their primer pairs for 4 target genes and a reference gene.
- We ordered them from Biosearch Technologies along with calibration dyes

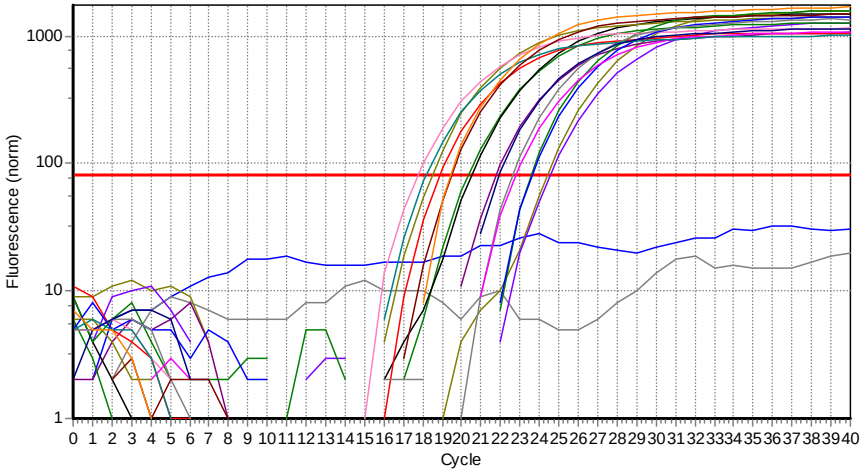
What can Cynthia do in 15 days?

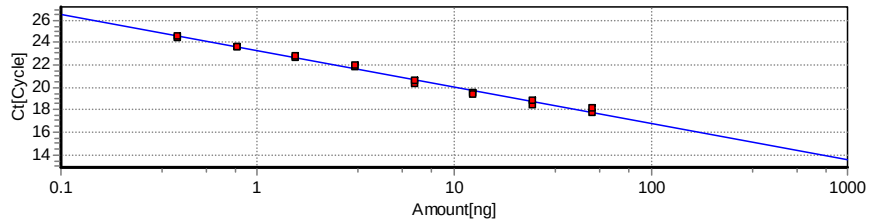
- EpiStem's 4 target genes and reference working efficiently with SYBR Green, with fast protocols
- Determine which two target genes and Ref gene are in range of each other and can quantify the 4 patient samples
- The 2 target genes picked, get the probes working
- Use gradient function to determine optimal anneal temp for all three
- Compare 20uL reactions with 10uL and 5uL using epMotion for setups
- Duplex Ref Gene with each target
- Triplex Ref Gene and 2 target genes

SYBR Green for Ref Gene & G1-4

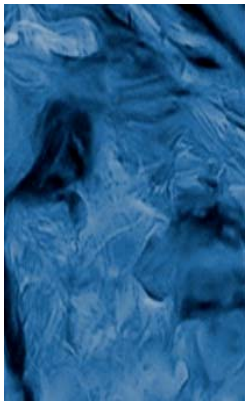
Using Eppendorf RealMasterMix and SYBR Green on Twin.Tec plates...

SYBR Green for Ref Gene





Pos	Name	Ct SYBR	Amount SYBR	Target SYBR
H1	Std_Ref	24.59	0.3906 ng	Ref
H2	Std_Ref	24.44	0.3906 ng	Ref
G1	Std_Ref	23.66	0.7813 ng	Ref
G2	Std_Ref	23.60	0.7813 ng	Ref
F1	Std_Ref	22.81	1.563 ng	Ref
F2	Std_Ref	22.65	1.563 ng	Ref
E2	Std_Ref	21.97	3.125 ng	Ref
E1	Std_Ref	21.81	3.125 ng	Ref
D2	Std_Ref	20.57	6.25 ng	Ref
D1	Std_Ref	20.40	6.25 ng	Ref
C2	Std_Ref	19.51	12.5 ng	Ref
C1	Std_Ref	19.45	12.5 ng	Ref
B1	Std_Ref	18.88	25 ng	Ref
B2	Std_Ref	18.49	25 ng	Ref
A2	Std_Ref	18.16	50 ng	Ref
A1	Std_Ref	17.76	50 ng	Ref
A11	NTC	-	-	Ref
A12	NTC	-	-	Ref



Threshold detection parameters:	
Threshold	82 (Automatic Noise
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.236
Y-Intercept	23.26
Efficiency	1.04
R^2	0.992

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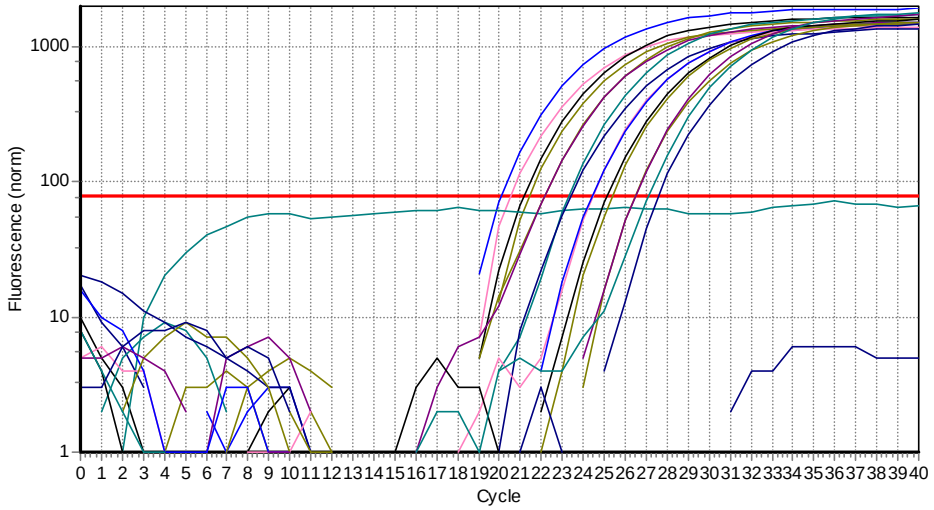
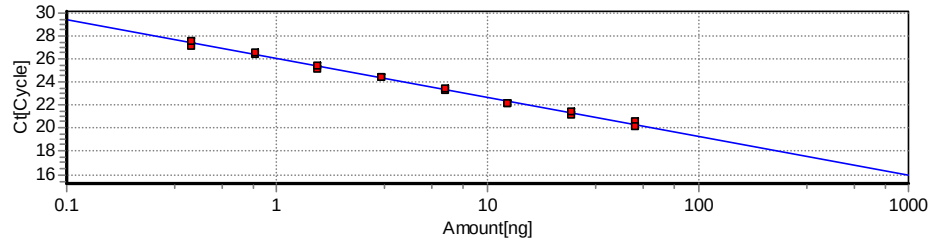
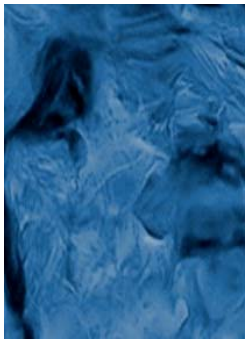
SYBR Green for G1





Pos	Name	Ct SYBR	Amount SYBR	Target SYBR
H4	Std_G1	27.60	0.3906 ng	Gene 1
H3	Std_G1	27.13	0.3906 ng	Gene 1
G4	Std_G1	26.52	0.7813 ng	Gene 1
G3	Std_G1	26.50	0.7813 ng	Gene 1
F4	Std_G1	25.41	1.563 ng	Gene 1
F3	Std_G1	25.15	1.563 ng	Gene 1
E3	Std_G1	24.50	3.125 ng	Gene 1
E4	Std_G1	24.46	3.125 ng	Gene 1
D4	Std_G1	23.45	6.25 ng	Gene 1
D3	Std_G1	23.35	6.25 ng	Gene 1
C4	Std_G1	22.20	12.5 ng	Gene 1
C3	Std_G1	22.18	12.5 ng	Gene 1
B4	Std_G1	21.49	25 ng	Gene 1
B3	Std_G1	21.22	25 ng	Gene 1
A3	Std_G1	20.59	50 ng	Gene 1
A4	Std_G1	20.14	50 ng	Gene 1
B11	NTC	-	-	Gene 1
B12	NTC	-	-	Gene 1



Threshold detection parameters:	
Threshold	79 (Automatic Noise
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.368
Y-Intercept	26.04
Efficiency	0.98
R^2	0.995

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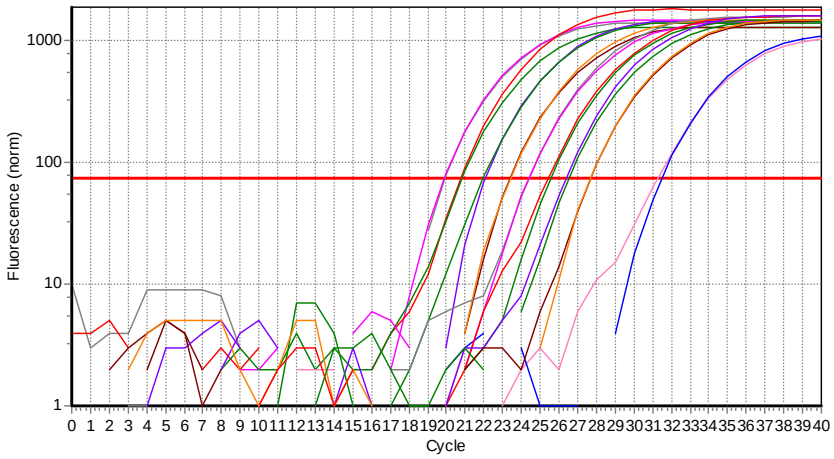
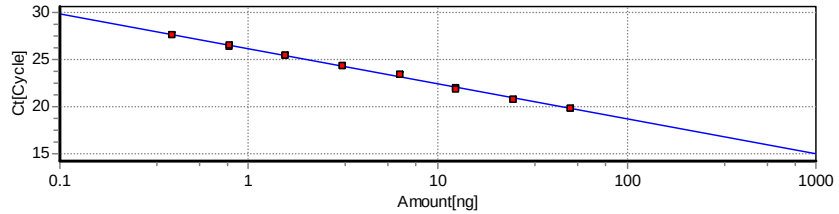
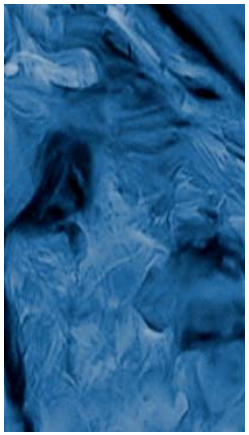
SYBR Green for G2





Pos	Name	Ct SYBR	Amount SYBR	Target SYBR
C12	NTC	31.48	0.03779	Gene 2
C11	NTC	31.25	0.04373	Gene 2
H5	Std_G2	27.71	0.3906 ng	Gene 2
H6	Std_G2	27.68	0.3906 ng	Gene 2
G6	Std_G2	26.55	0.7813 ng	Gene 2
G5	Std_G2	26.42	0.7813 ng	Gene 2
F6	Std_G2	25.57	1.563 ng	Gene 2
F5	Std_G2	25.45	1.563 ng	Gene 2
E5	Std_G2	24.44	3.125 ng	Gene 2
E6	Std_G2	24.41	3.125 ng	Gene 2
D6	Std_G2	23.46	6.25 ng	Gene 2
D5	Std_G2	23.42	6.25 ng	Gene 2
C5	Std_G2	22.11	12.5 ng	Gene 2
C6	Std_G2	21.98	12.5 ng	Gene 2
B6	Std_G2	20.86	25 ng	Gene 2
B5	Std_G2	20.79	25 ng	Gene 2
A5	Std_G2	19.95	50 ng	Gene 2
A6	Std_G2	19.92	50 ng	Gene 2



Threshold detection parameters:	
Threshold	75 (Automatic Noise
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.717
Y-Intercept	26.19
Efficiency	0.86
R^2	0.998

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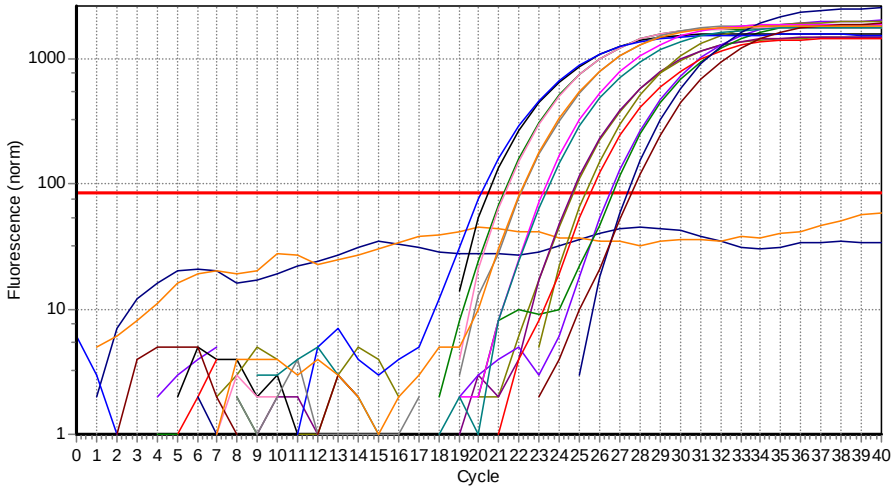
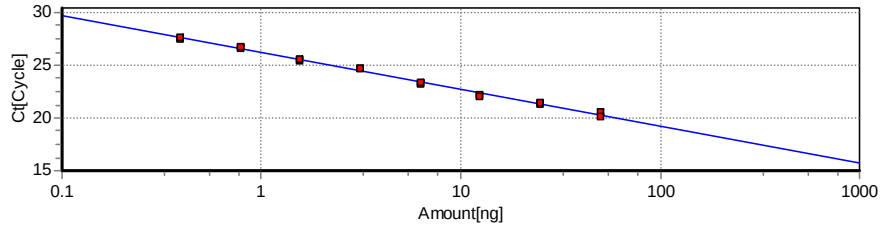
SYBR Green for G3





Pos	Name	Ct SYBR	Amount SYBR	Target SYBR
H7	Std_G3	27.60	0.3906 ng	Gene 3
H8	Std_G3	27.40	0.3906 ng	Gene 3
G7	Std_G3	26.67	0.7813 ng	Gene 3
G8	Std_G3	26.53	0.7813 ng	Gene 3
F7	Std_G3	25.57	1.563 ng	Gene 3
F8	Std_G3	25.34	1.563 ng	Gene 3
E7	Std_G3	24.72	3.125 ng	Gene 3
E8	Std_G3	24.67	3.125 ng	Gene 3
D8	Std_G3	23.37	6.25 ng	Gene 3
D7	Std_G3	23.21	6.25 ng	Gene 3
C8	Std_G3	22.12	12.5 ng	Gene 3
C7	Std_G3	22.08	12.5 ng	Gene 3
B8	Std_G3	21.36	25 ng	Gene 3
B7	Std_G3	21.28	25 ng	Gene 3
A8	Std_G3	20.50	50 ng	Gene 3
A7	Std_G3	20.17	50 ng	Gene 3
D12	NTC	-	-	Gene 3
D11	NTC	-	-	Gene 3

Threshold detection parameters:	
Threshold	86 (Automatic Noise
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.481
Y-Intercept	26.16
Efficiency	0.94
R^2	0.995

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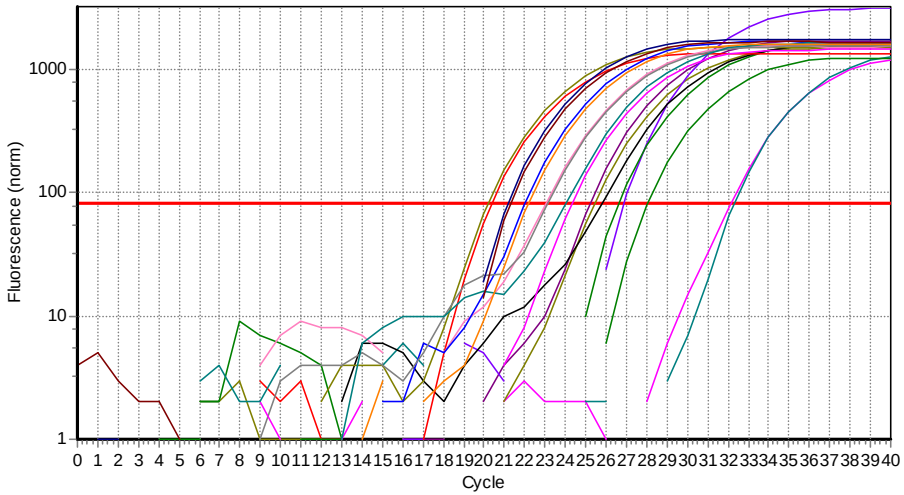
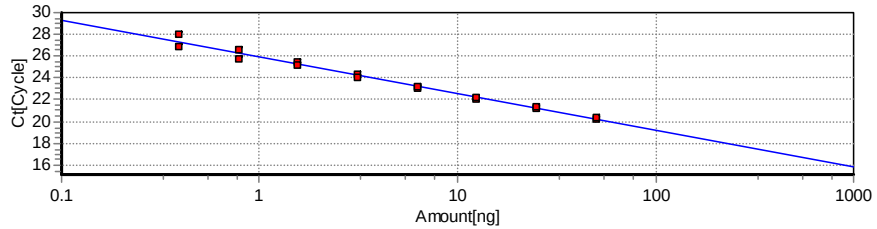
SYBR Green for G4





Pos	Name	Ct SYBR	Amount SYBR	Target SYBR
E12	NTC	32.27	0.01311	Gene 4
E11	NTC	32.11	0.01466	Gene 4
H10	Std_G4	28.02	0.3906 ng	Gene 4
H9	Std_G4	26.88	0.3906 ng	Gene 4
G10	Std_G4	26.63	0.7813 ng	Gene 4
G9	Std_G4	25.81	0.7813 ng	Gene 4
F10	Std_G4	25.46	1.563 ng	Gene 4
F9	Std_G4	25.22	1.563 ng	Gene 4
E9	Std_G4	24.35	3.125 ng	Gene 4
E10	Std_G4	24.06	3.125 ng	Gene 4
D10	Std_G4	23.15	6.25 ng	Gene 4
D9	Std_G4	23.07	6.25 ng	Gene 4
C10	Std_G4	22.22	12.5 ng	Gene 4
C9	Std_G4	22.05	12.5 ng	Gene 4
B10	Std_G4	21.35	25 ng	Gene 4
B9	Std_G4	21.22	25 ng	Gene 4
A9	Std_G4	20.43	50 ng	Gene 4
A10	Std_G4	20.25	50 ng	Gene 4

Threshold detection parameters:	
Threshold	82 (Automatic Noise
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.368
Y-Intercept	25.93
Efficiency	0.98
R^2	0.985

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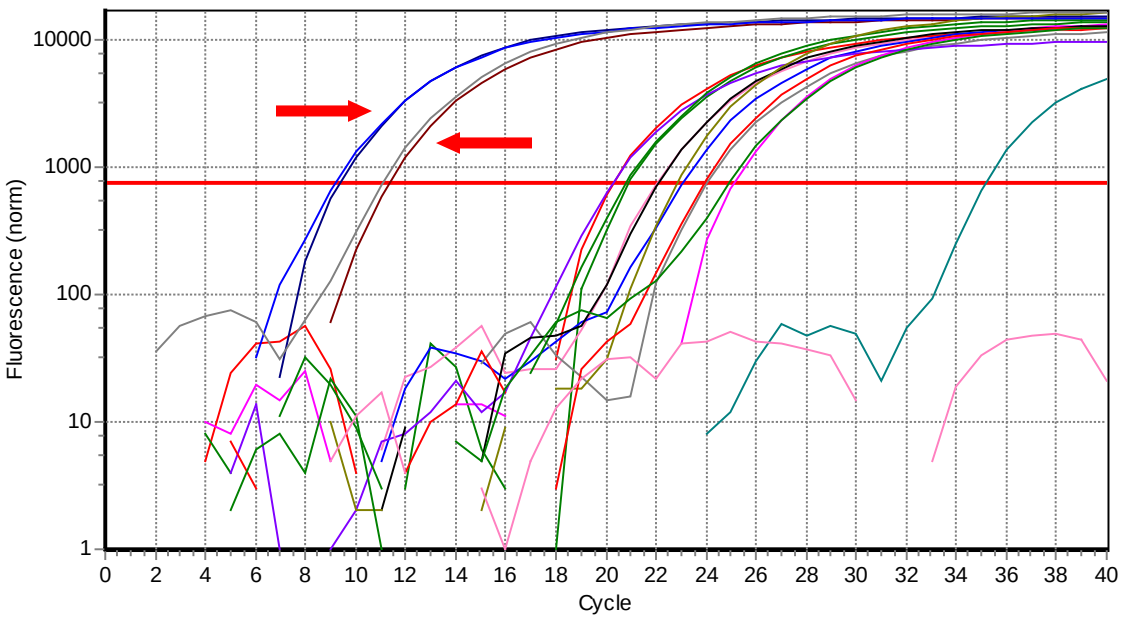
SYBR Green runs repeated

Runs repeated using Eurogentec SYBR Green mastermix on AbGene plate:

- Cts virtually the same
- Run time was 55 minutes doing 2-step:
  10 min, (15s@95/30s@56.9) 40x = 55 min

If activation time is taken off and initial denature time of 20s is inserted, 45 min run with very conservative cycling protocol

SYBR Green for Ref Gene + Samples



Threshold detection parameters:	
Threshold	755 (Automatic Nois
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.226
Y-Intercept	23.33
Efficiency	1.04
R^2	0.993

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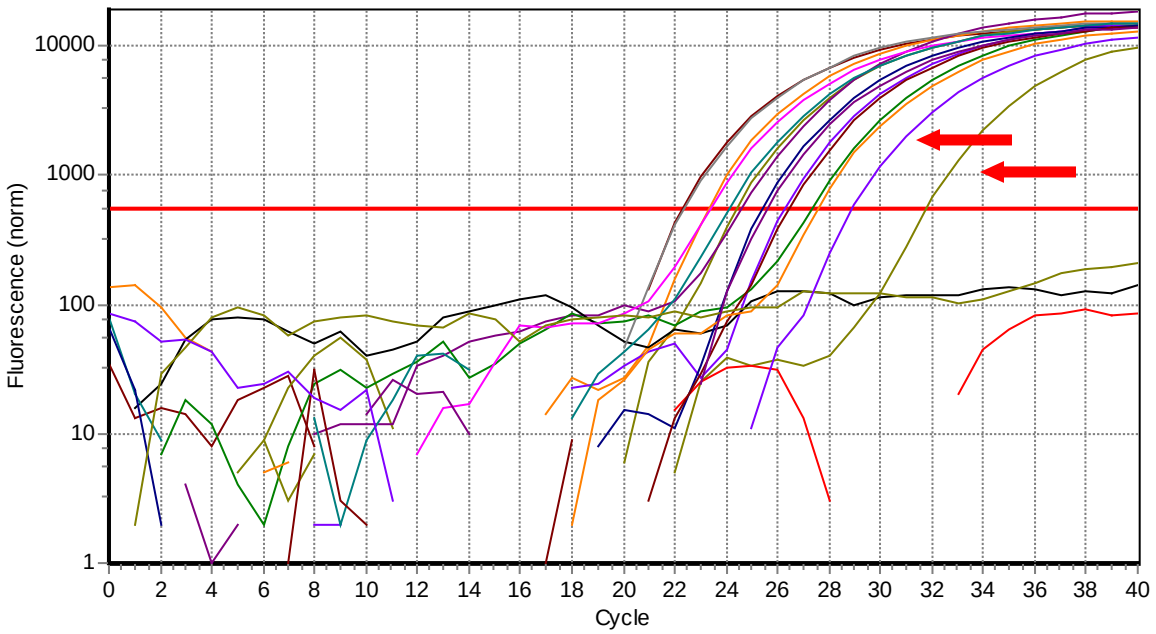
SYBR Green for G1 + Samples



Threshold detection parameters:	
Threshold	545 (Automatic Nois
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.372
Y-Intercept	25.62
Efficiency	0.98
R^2	0.982

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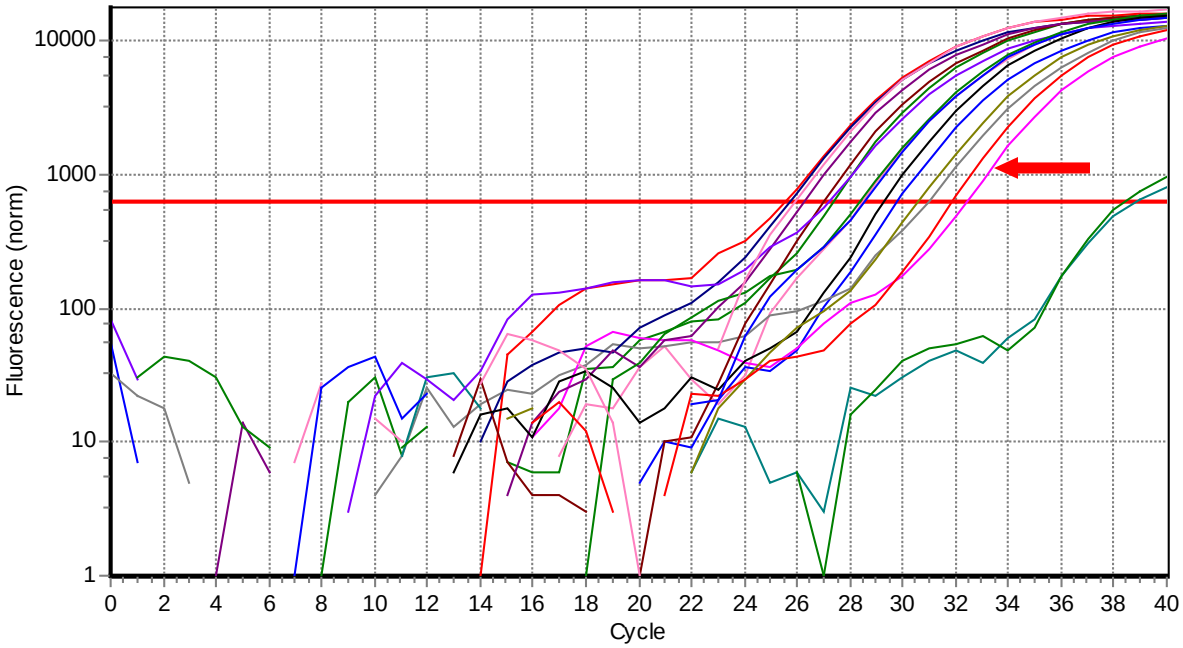
SYBR Green for G2 + Samples



Threshold detection parameters:	
Threshold	629 (Automatic Nois
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-4.059
Y-Intercept	30.02
Efficiency	0.76
R^2	0.987

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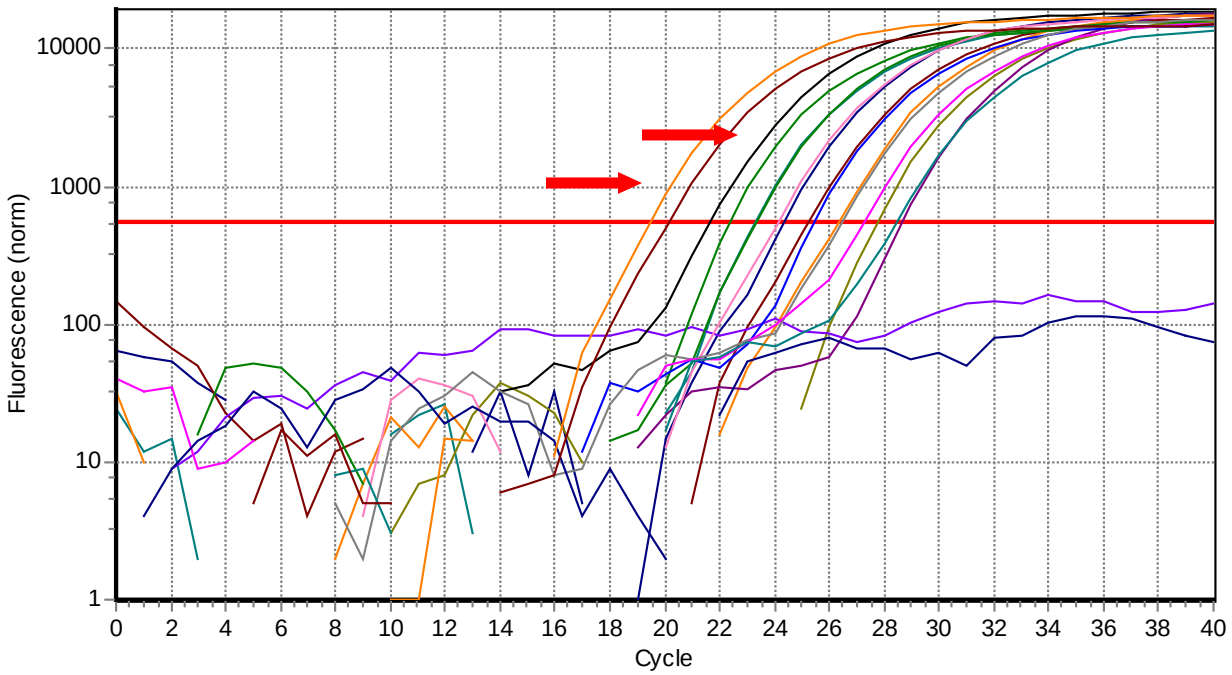
SYBR Green for G3 + Samples



Threshold detection parameters:	
Threshold	551 (Automatic Nois
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.531
Y-Intercept	26.77
Efficiency	0.92
R^2	0.995

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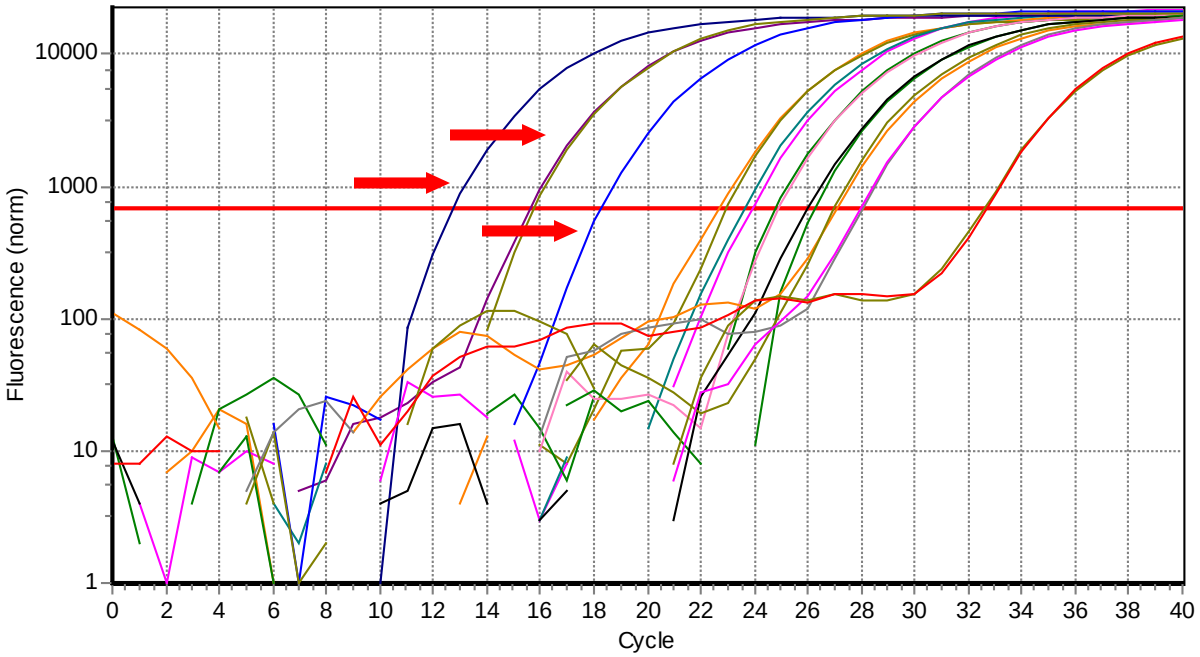
SYBR Green for G4 + Samples



Threshold detection parameters:	
Threshold	691 (Automatic Nois
Baseline settings	automatic
Drift correction	OFF
Standard curve parameters:	
Slope	-3.519
Y-Intercept	26.31
Efficiency	0.92
R^2	0.995

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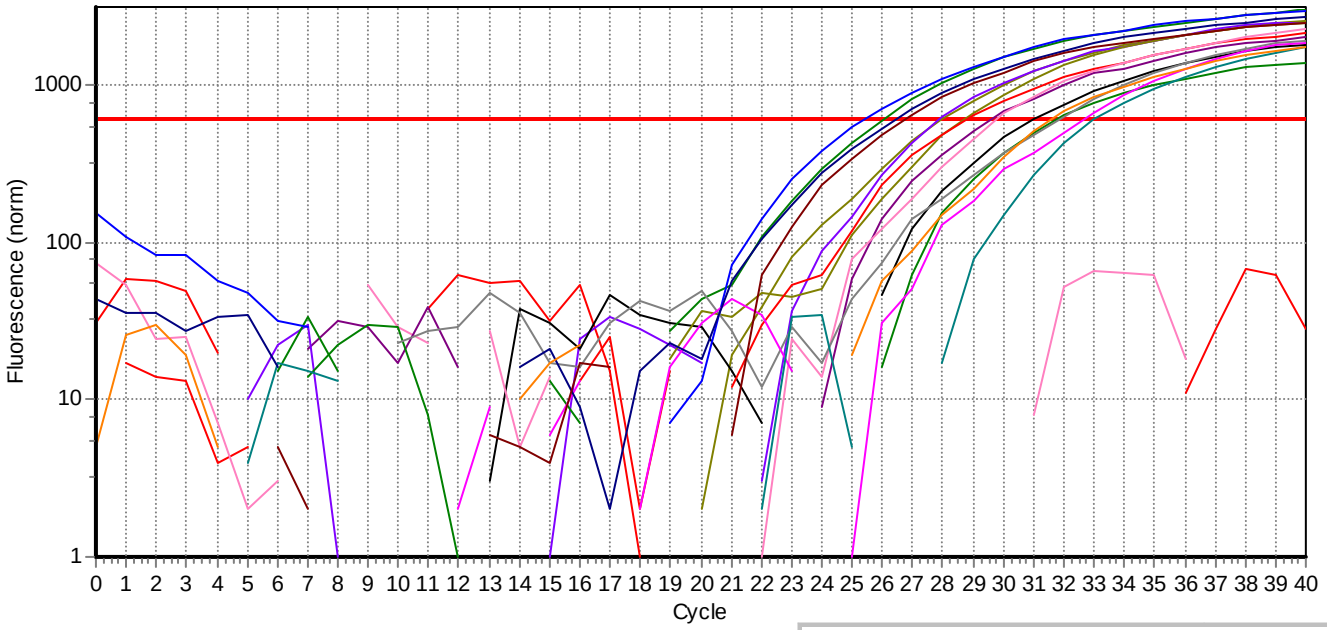
G1-CalFluor Orange 560 (CFO560)/BHQ1



Standard curve parameters:	
Slope	-3.420
Y-Intercept	31.61
Efficiency	0.96
R^2	0.984

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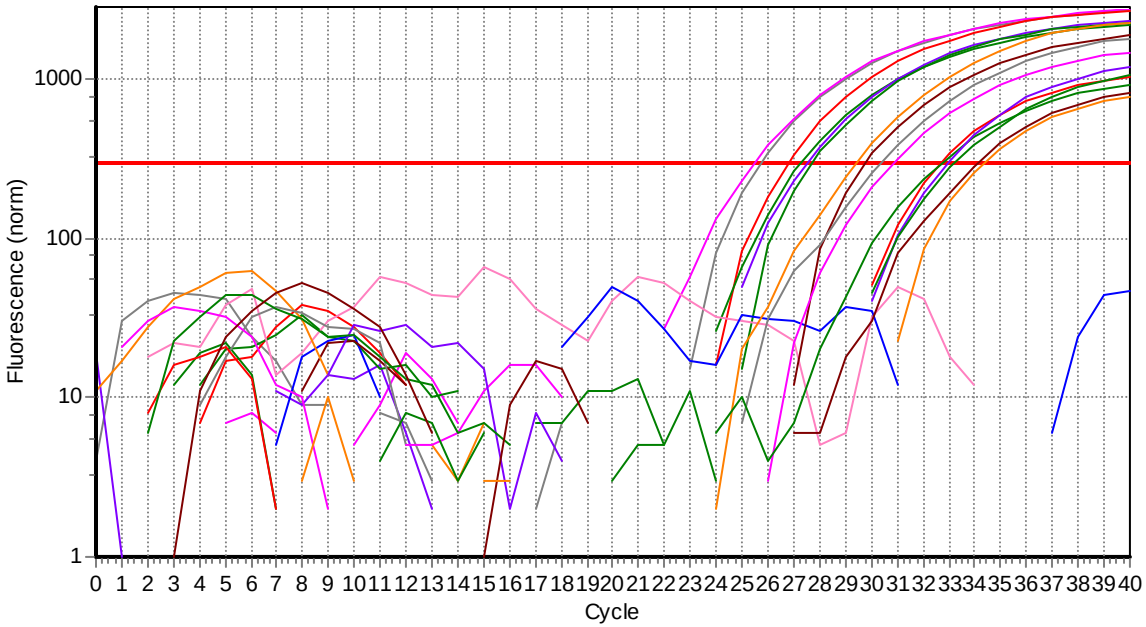
G2 - FAM/BHQ-1



Standard curve parameters:	
Slope	-4.240
Y-Intercept	32.83
Efficiency	0.72
R^2	0.983

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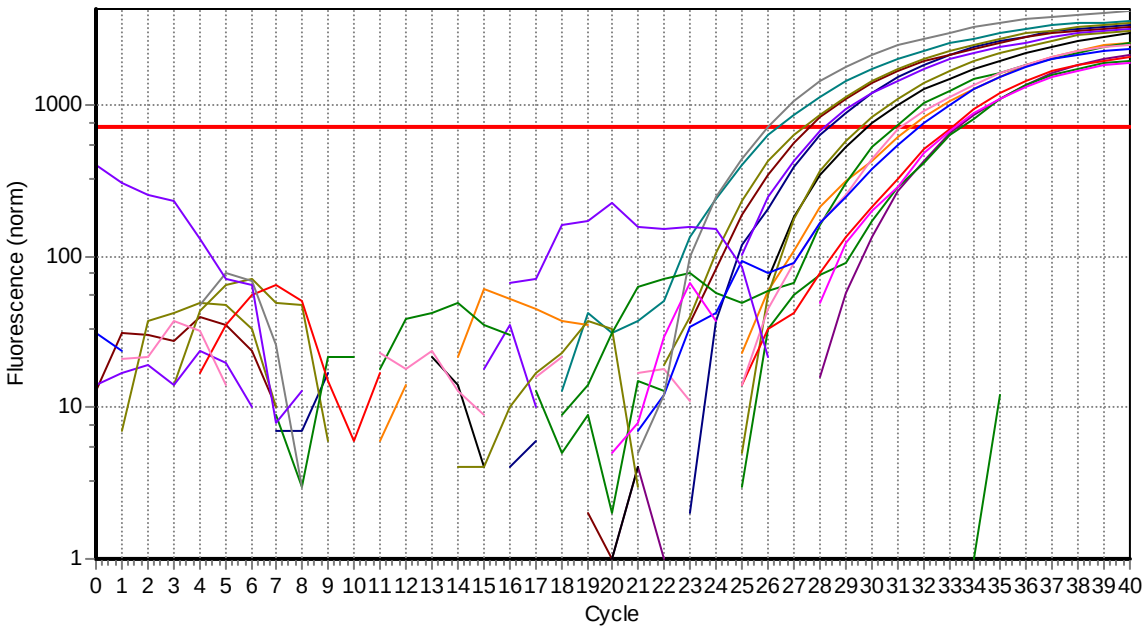
G3-CalFluor Orange 560 (CFO560)/BHQ1



Standard curve parameters:	
Slope	-3.567
Y-Intercept	32.41
Efficiency	0.91
R^2	0.987

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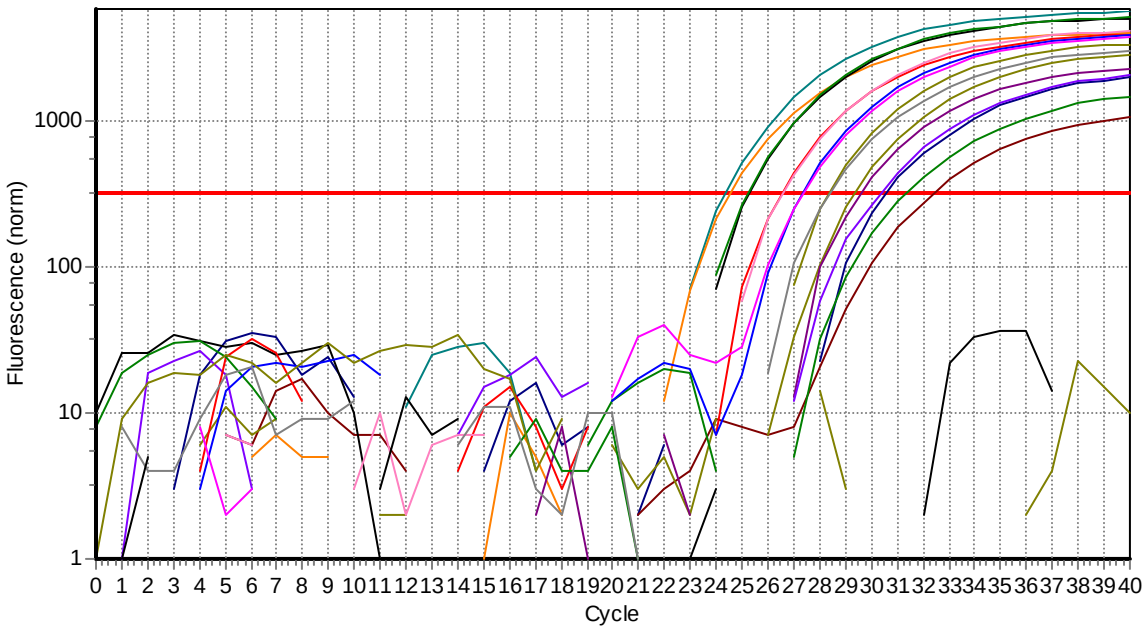
G4 - FAM/BHQ-1



Standard curve parameters:	
Slope	-3.450
Y-Intercept	30.19
Efficiency	0.95
R^2	0.989

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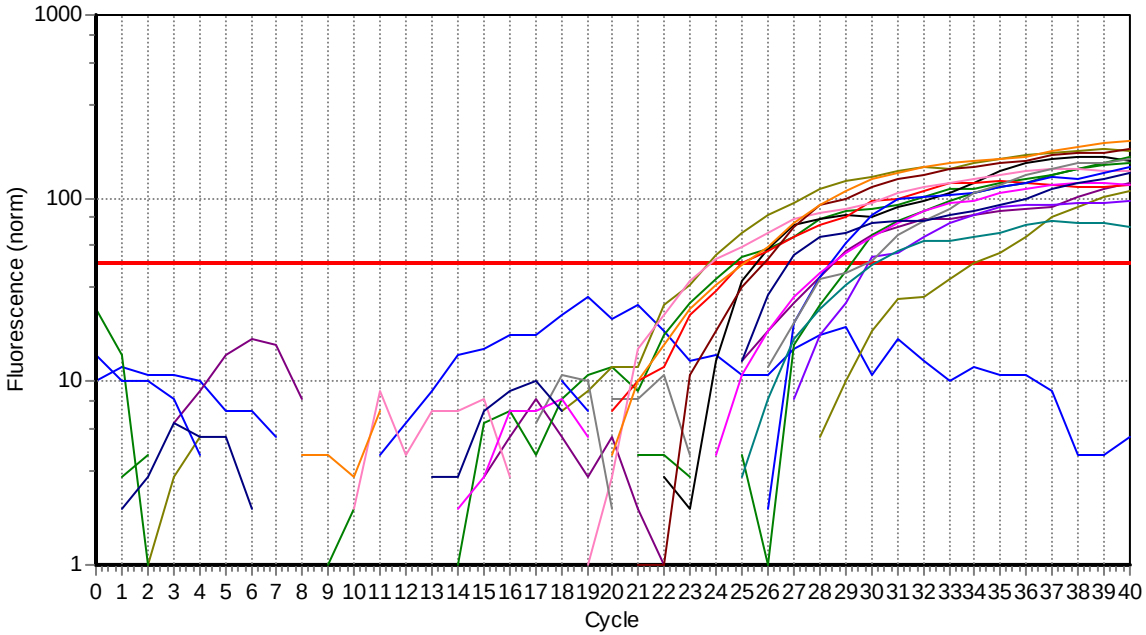
Ref Gene - TAMRA/BHQ-2



Standard curve parameters:	
Slope	-2.787
Y-Intercept	29.26
Efficiency	1.28
R^2	0.488

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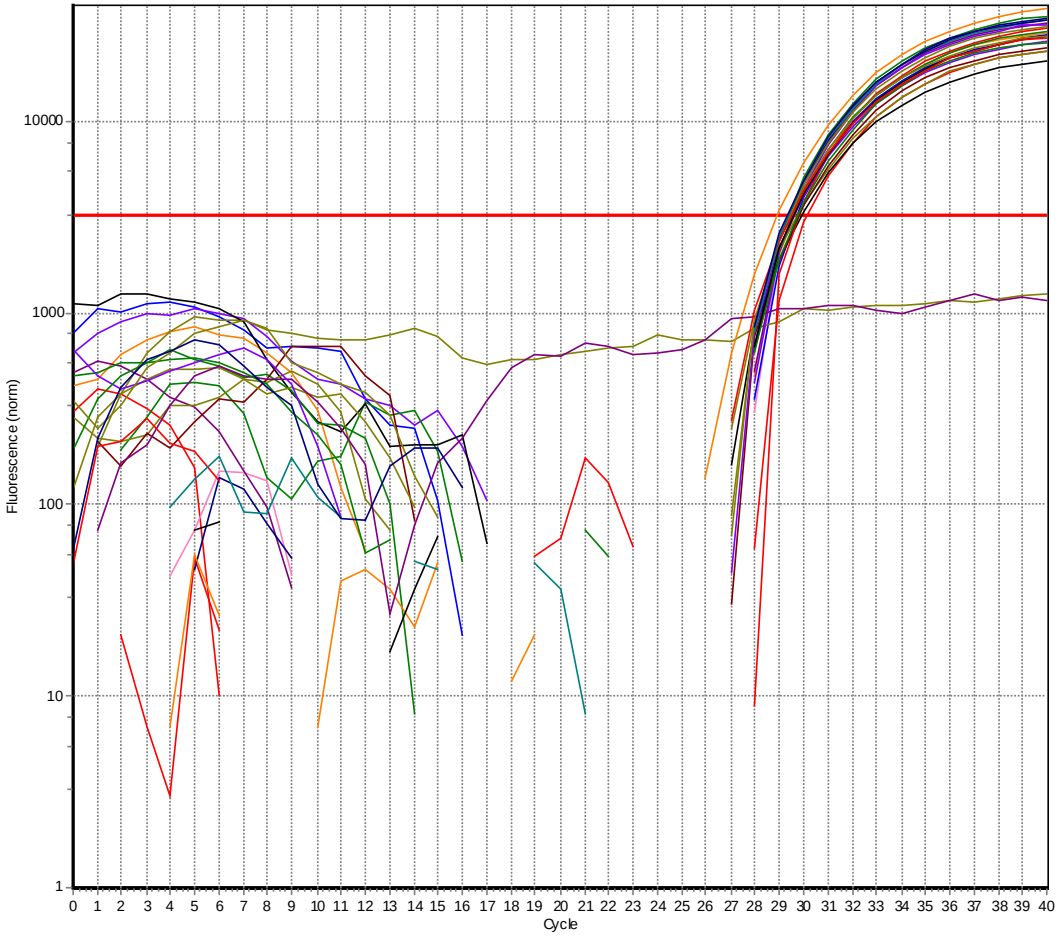
Summary so far...

- G1 and G2 need a different Ref Gene than this one
- G3, G4 & Ref Gene are in range of each other and can be reliably quantified, if samples are diluted 2:125
- TAMRA/BHQ-2 needs to be optimized
- G3 efficiency = 91%
- G4 efficiency = 95%

What to do next...

- Use gradient function to determine optimal anneal temp for all three (Ref Gene, G3 & G4)
- Compare 20uL reactions with 10uL and 5uL using epMotion for setups
- Duplex combinations regardless of inadequate Ref Gene since it is assumed another Ref Gene can be found for G1 and G2
- Triplex TAMRA/BHQ-2 Ref Gene with G3 (CFO560/BHQ-1) and G4 (FAM/BHQ-1), if duplex reactions indicate that there is no lowering of efficiency

Gradient - G4 (FAM)



Pos	Name	Ct FAM
G1	NTC	-
H1	NTC	-
H2	G4	28.91
H5	G4	29.32
H4	G4	29.38
G2	G4	29.40
H3	G4	29.42
H6	G4	29.43
H11	G4	29.49
H12	G4	29.50
G4	G4	29.53
G7	G4	29.54
G5	G4	29.58
G10	G4	29.58
H7	G4	29.60
G6	G4	29.63
H8	G4	29.63
G8	G4	29.73
G12	G4	29.74
H9	G4	29.76
G3	G4	29.80
H10	G4	29.82
G11	G4	29.92
G9	G4	30.15

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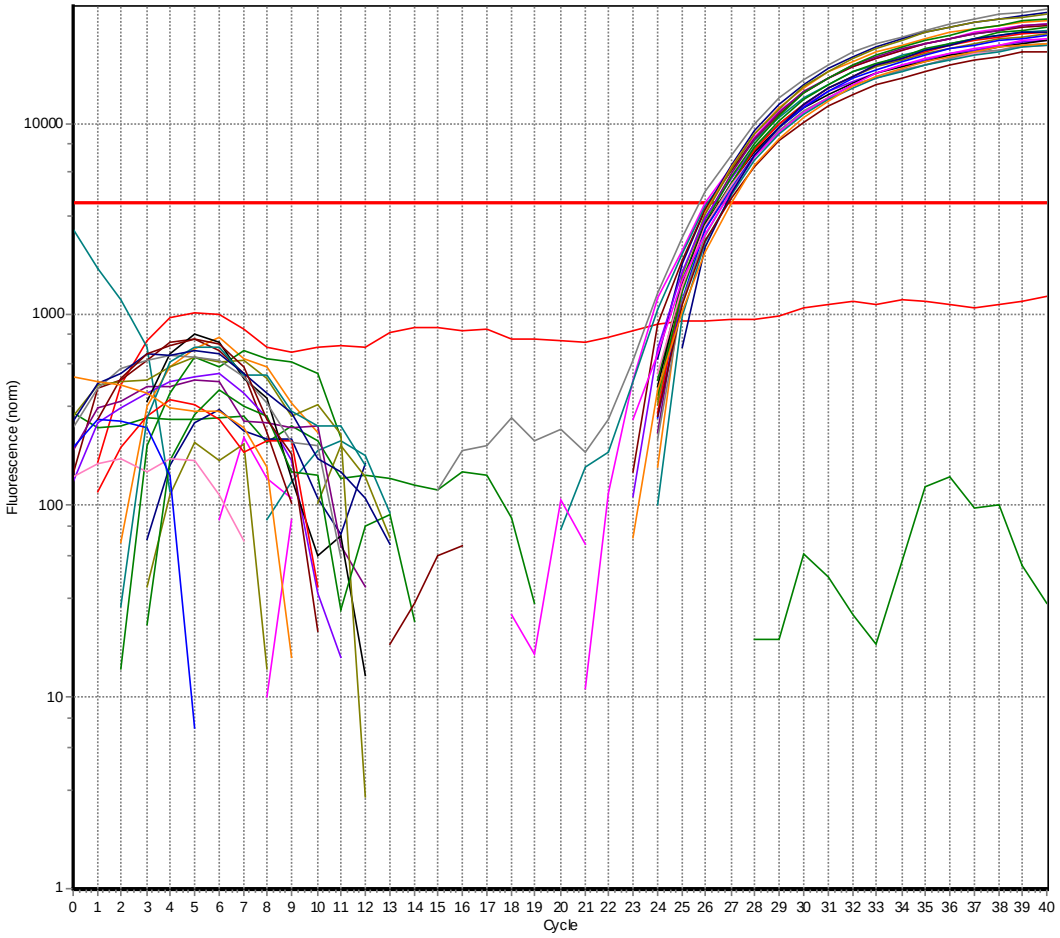
Gradient - Ref Gene (FAM)



Pos	Name	Ct FAM
A1	NTC	-
B1	NTC	-
A12	Ref -FAM	25.78
B10	Ref -FAM	26.04
A5	Ref -FAM	26.11
B8	Ref -FAM	26.16
B5	Ref -FAM	26.20
B7	Ref -FAM	26.24
B9	Ref -FAM	26.25
B6	Ref -FAM	26.37
B3	Ref -FAM	26.39
A11	Ref -FAM	26.44
A2	Ref -FAM	26.53
A7	Ref -FAM	26.56
A6	Ref -FAM	26.57
B12	Ref -FAM	26.57
B4	Ref -FAM	26.59
A8	Ref -FAM	26.65
A4	Ref -FAM	26.73
A10	Ref -FAM	26.74
A3	Ref -FAM	26.82
B11	Ref -FAM	26.83
B2	Ref -FAM	26.90
A9	Ref -FAM	27.04

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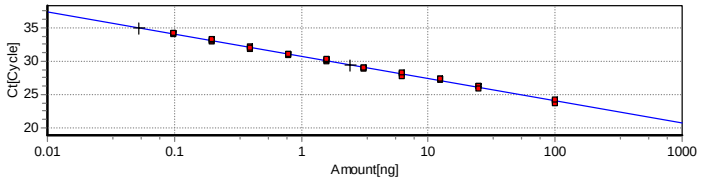
In touch with life

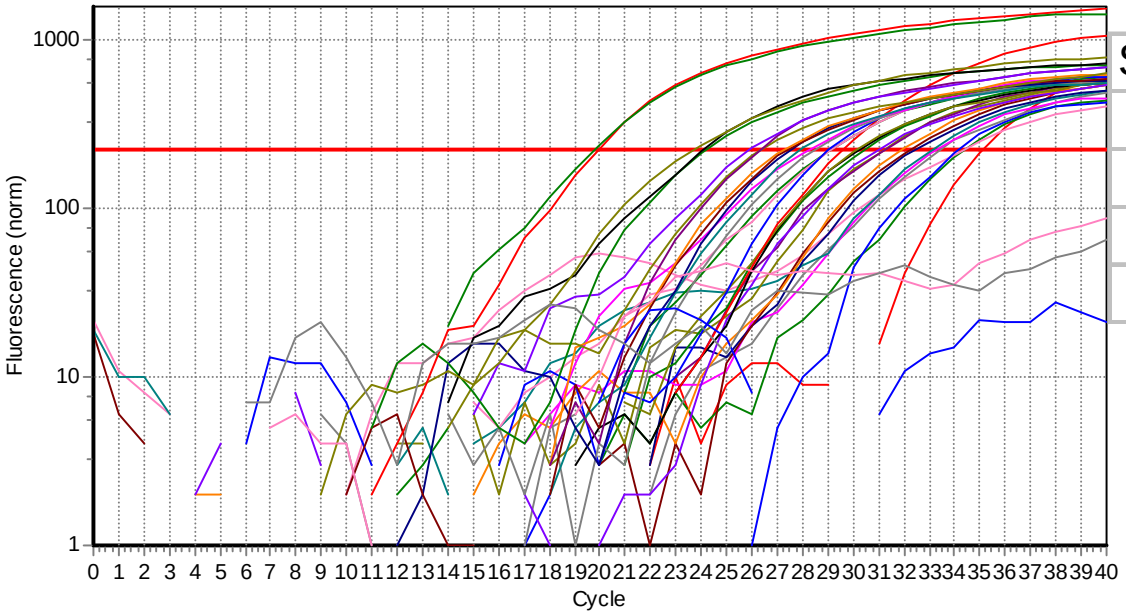
Gradient

- Ref Gene, G3 & G4 were not designed with multiplexing in mind
- All runs set to optimal anneal for Ref Gene since it will be present in all multiplexing reactions

TAMRA/BHQ-2 - Ref Gene - single

- Re-calibration with dye from Biosearch
- Mg+2 added to self-adjusting Mg+2 buffer to final concentration of 6.25mM
- [TAMRA/BHQ-2] = 500nM





Standard curve parameters:	
Slope	-3.353
Y-Intercept	30.81
Efficiency	0.99
R^2	0.996

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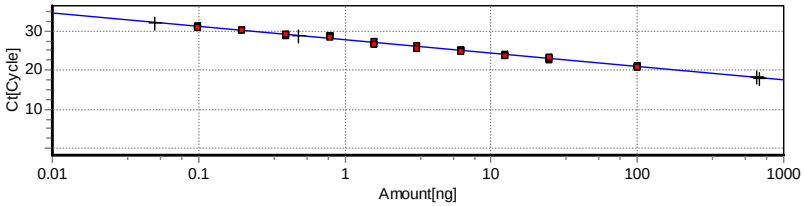
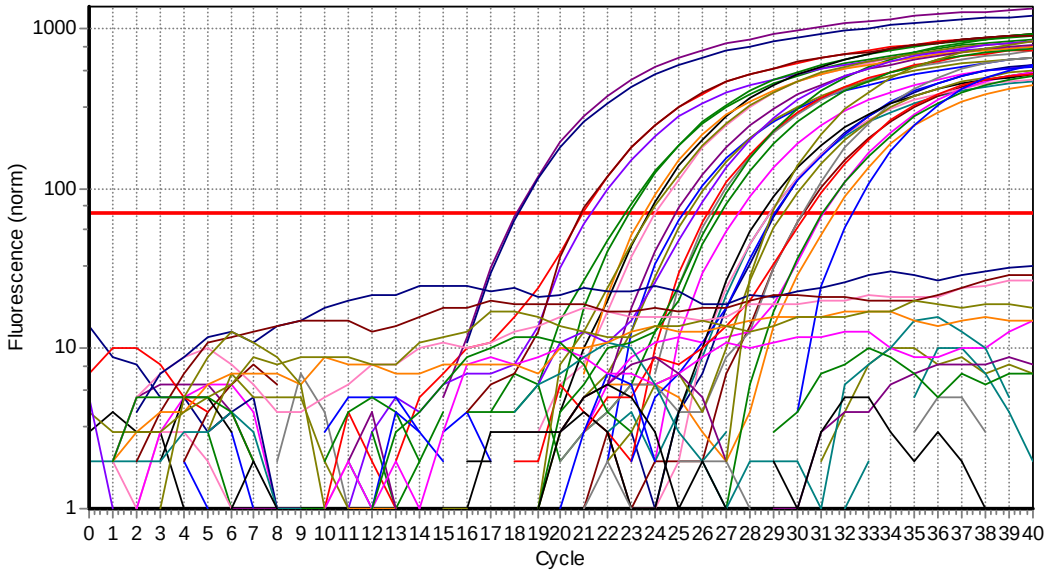
FAM/BHQ-1 - Ref Gene - single

- No additions to RealMasterMix Probe
- [FAM/BHQ-1] = 200nM





Standard curve parameters:	
Slope	-3.440
Y-Intercept	27.84
Efficiency	0.95
R^2	0.990

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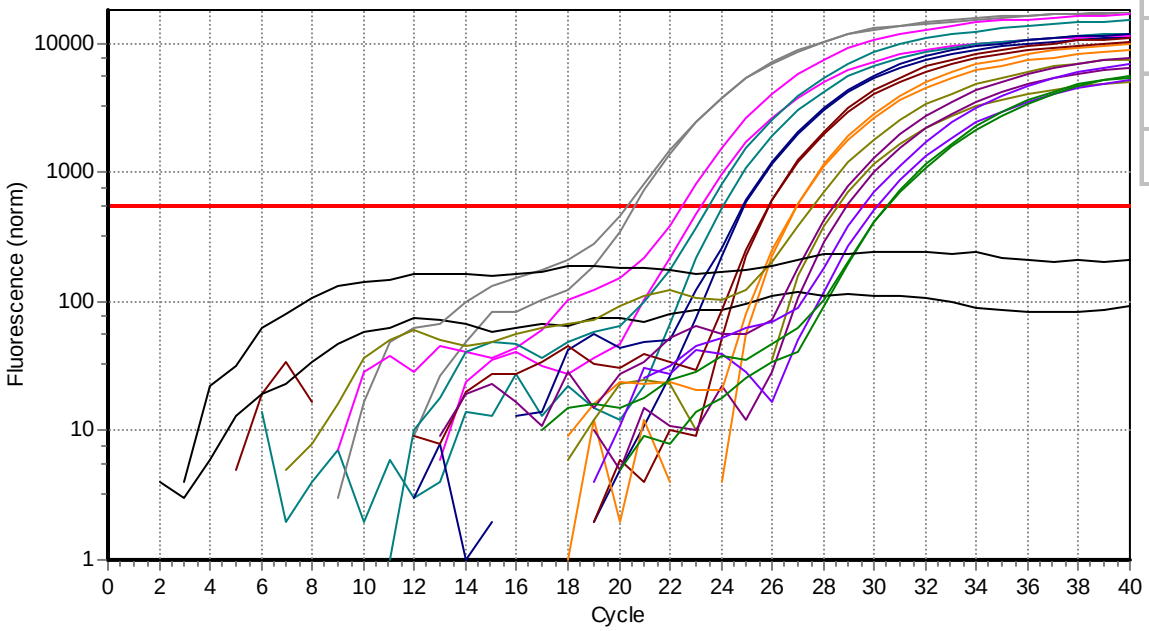
FAM/BHQ-1 - Ref Gene in duplex w/G3

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- [FAM/BHQ-1] = 200nM



Standard curve parameters:	
Slope	-3.349
Y-Intercept	27.45
Efficiency	0.99
R^2	0.990

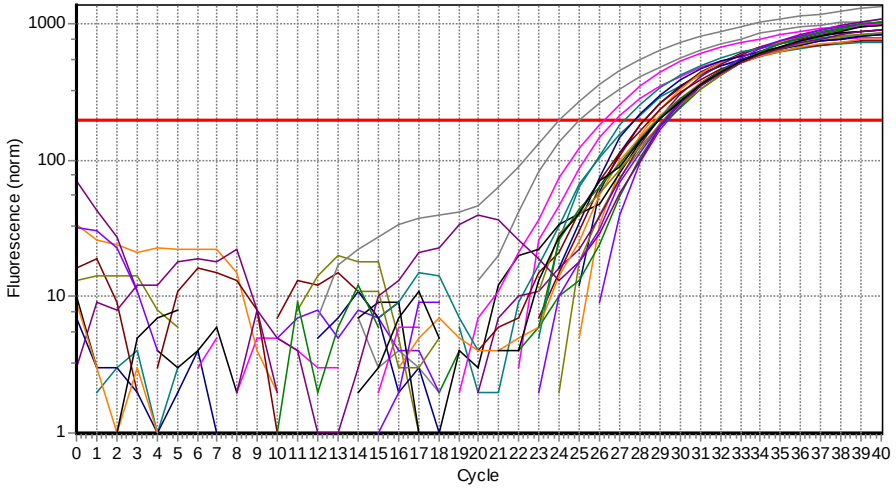
If only G3-CFO looked as good...

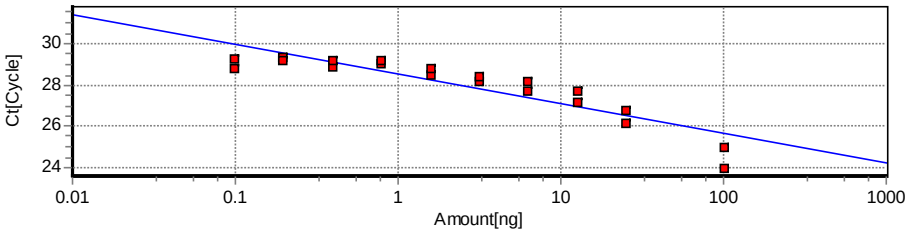
eppendorf
In touch with life

G3-CFO560/BHQ-1 - duplex w/Ref Gene

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- [CFO560/BHQ-1] = 300nM

Standard curve parameters:	
Slope	-1.430
Y-Intercept	28.54
Efficiency	4.01
R^2	0.808





…not too promising, maybe G4 will be better...

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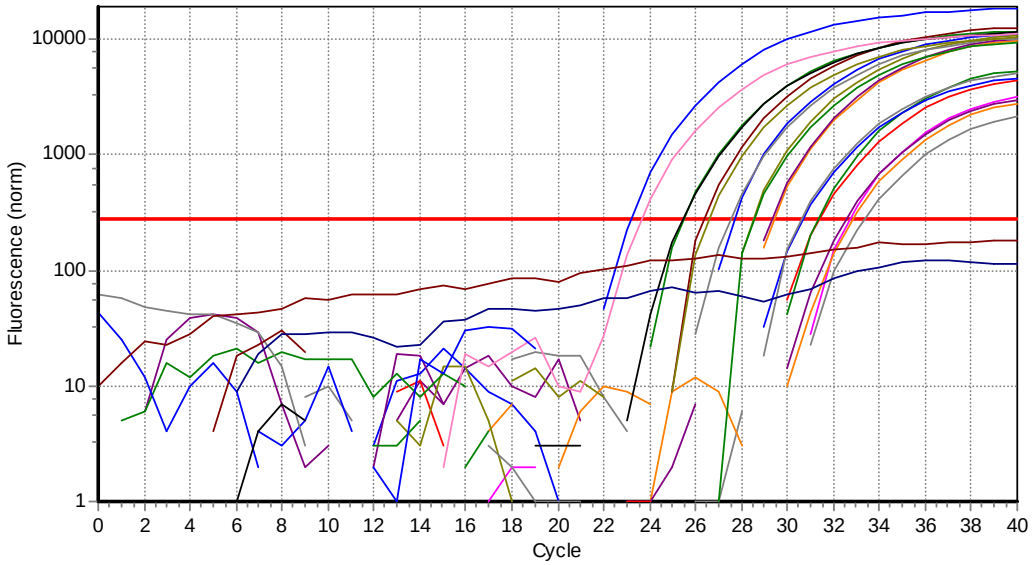
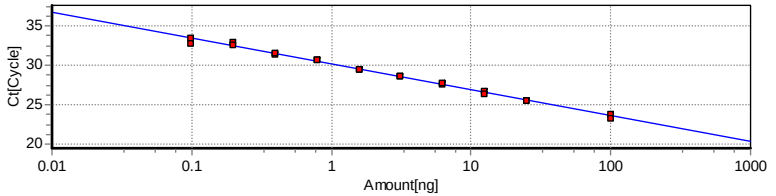
In touch with life

G4 FAM/BHQ-1 - duplex w/Ref Gene

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- [FAM/BHQ-1] = 200nM





Standard curve parameters:	
Slope	-3.274
Y-Intercept	30.12
Efficiency	1.02
R^2	0.995

...WOOHOO!!

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In touch with life

G3 & G4 duplexes with Ref Gene

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- G4 [FAM/BHQ-1] = 200nM,
  G3 [CFO560/BHQ-1] = 300nM,
  Ref [TAMRA/BHQ-2] = 500nM

Ref + G4 optimized

Ref + G3 indicates that G3 needs redesigning
or perhaps primers must be optimized

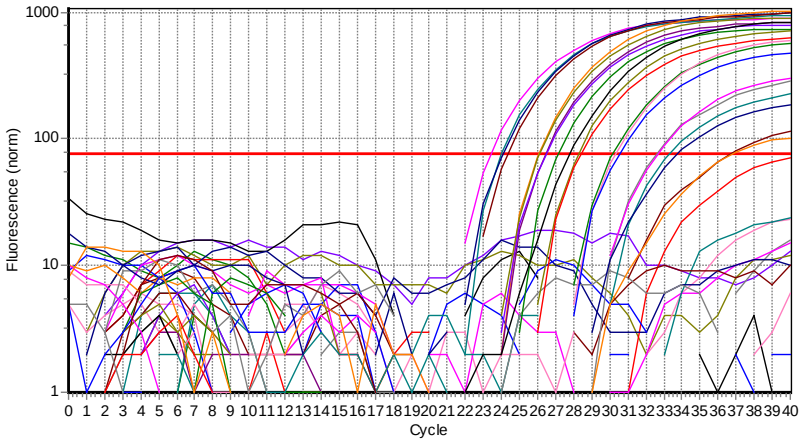
Perhaps G4 can be optimized further...

Eppendorf real-time PCR System

G4: Reaction volume

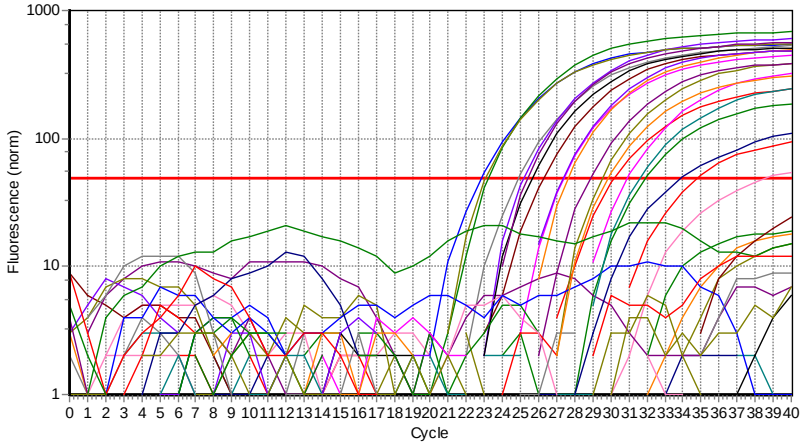
10uL

Standard curve parameters:	
Slope	-3.996
Y-Intercept	31.74
Efficiency	0.78
R^2	0.980



5uL

Standard curve parameters:	
Slope	-4.008
Y-Intercept	30.81
Efficiency	0.78
R^2	0.940



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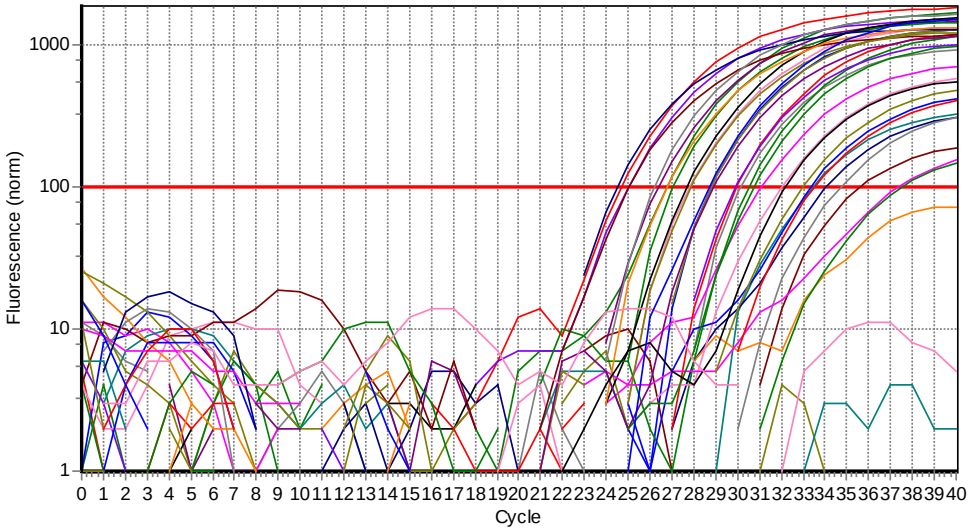
G4: Reaction volume

20 uL

Standard curve parameters:	
Slope	-3.869
Y-Intercept	32.16
Efficiency	0.81
R^2	0.968



Need to check this epMotion method

G4 has been 95% before

These 3 runs are, at least, uniformly mediocre across 3 volumes

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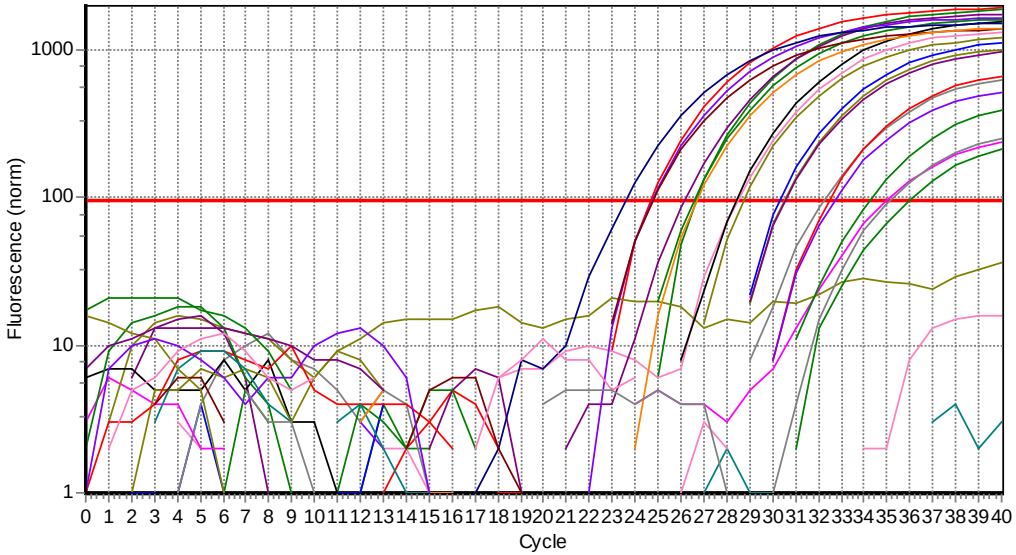
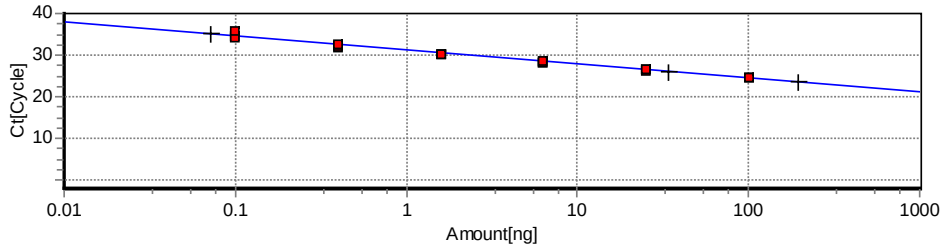
G4: Fast protocols

2:00@95, (15s@95/30s@58.1) 40x = 53 minutes





[FAM/BHQ-1] = 200nM
[Mg+2] = 6.25mM

Standard curve parameters:	
Slope	-3.374
Y-Intercept	31.33
Efficiency	0.98
R^2	0.988

Note: 58.1 is optimal anneal for Ref Gene not G4, so this could be better

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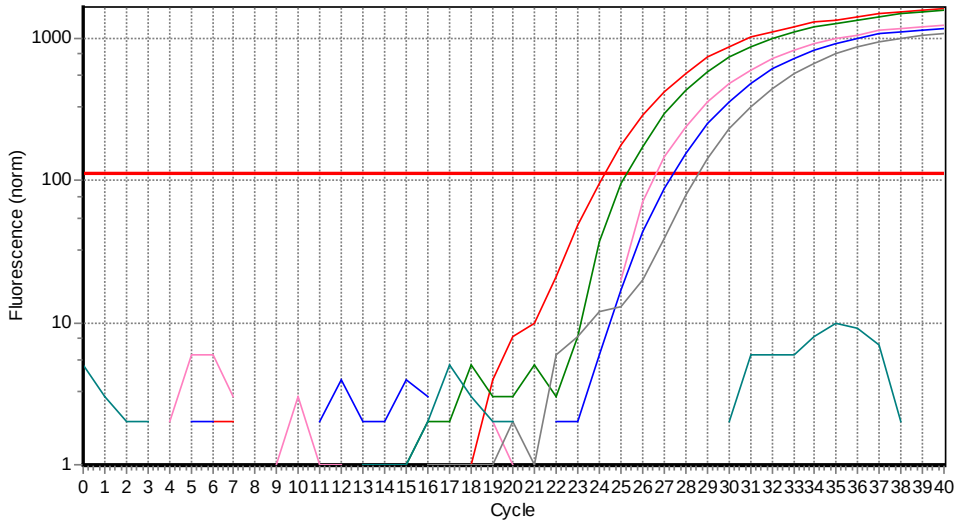
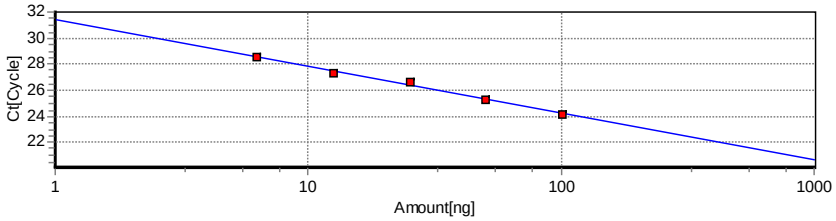
G4: Fast protocols

2:00@95, (5s@95/10s@58.1) 40x = 30 minutes

[FAM/BHQ-1] = 200nM



Standard curve parameters:	
Slope	-3.594
Y-Intercept	31.45
Efficiency	0.90
R^2	0.995



What would it be with 6.25mM Mg+2 and optimal anneal temp?

…too bad I ran out of time!

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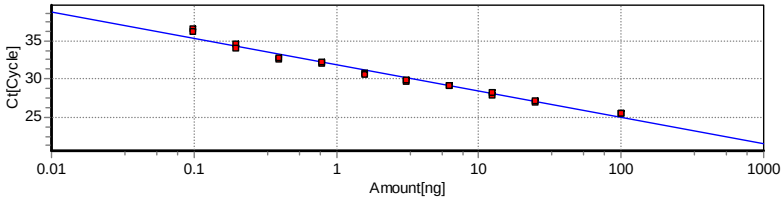
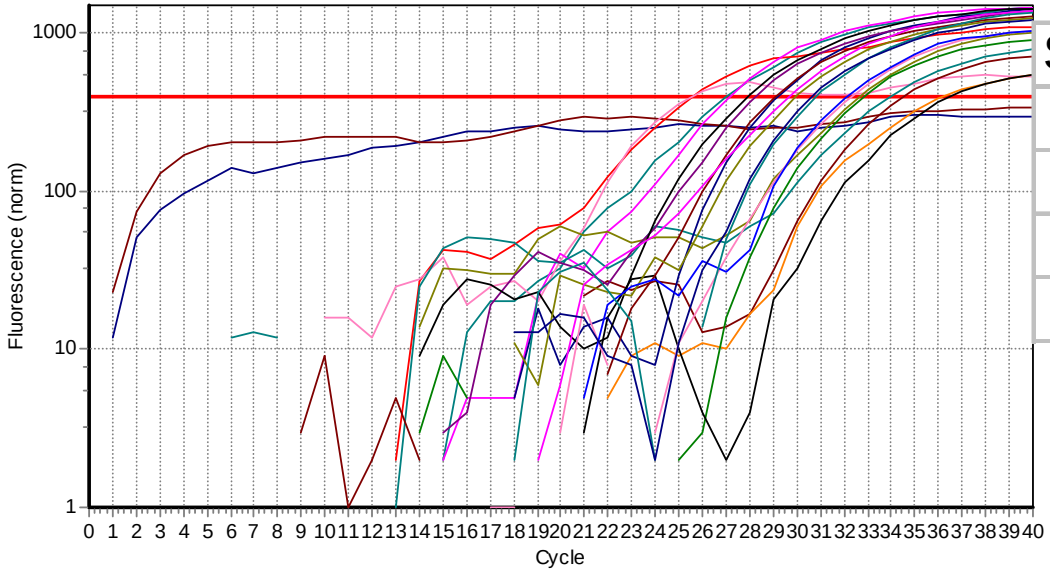
Ref TAMRA/BHQ-2 - duplex G4-FAM

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- [TAMRA/BHQ-2] = 500nM





Standard curve parameters:	
Slope	-3.428
Y-Intercept	31.86
Efficiency	0.96
R^2	0.978

About as efficient as FAM/BHQ-1 version of probe in duplex

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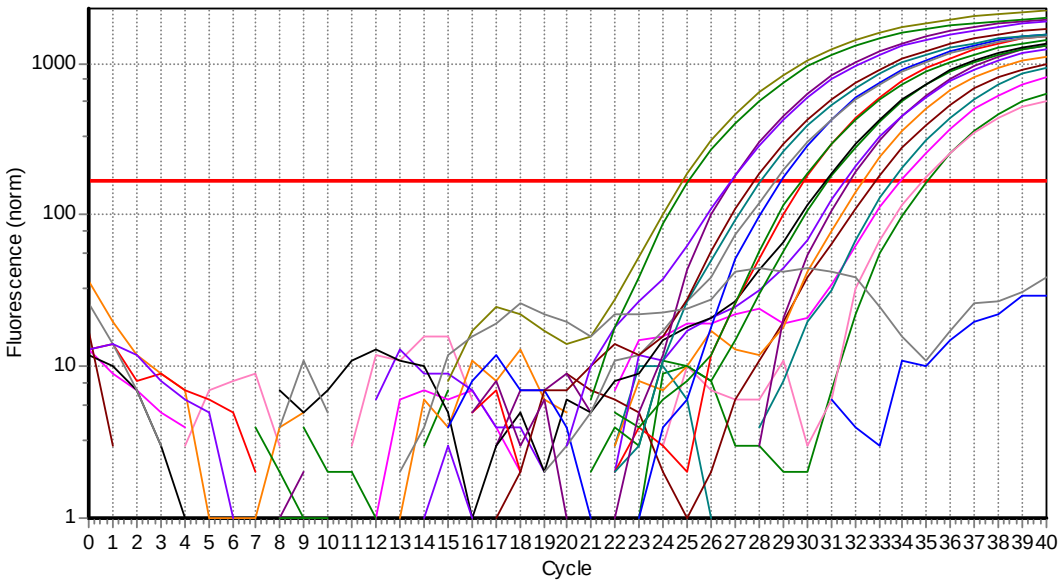
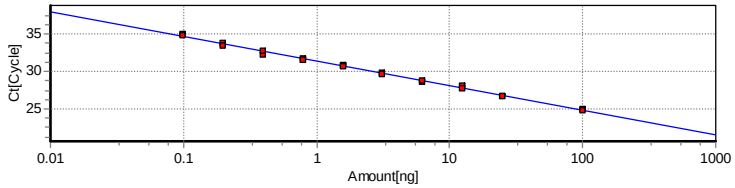
In touch with life

G1-CFO560/BHQ-1 duplex with G2

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- [CFO560/BHQ-1] = 300nM





Standard curve parameters:	
Slope	-3.267
Y-Intercept	31.41
Efficiency	1.02
R^2	0.997

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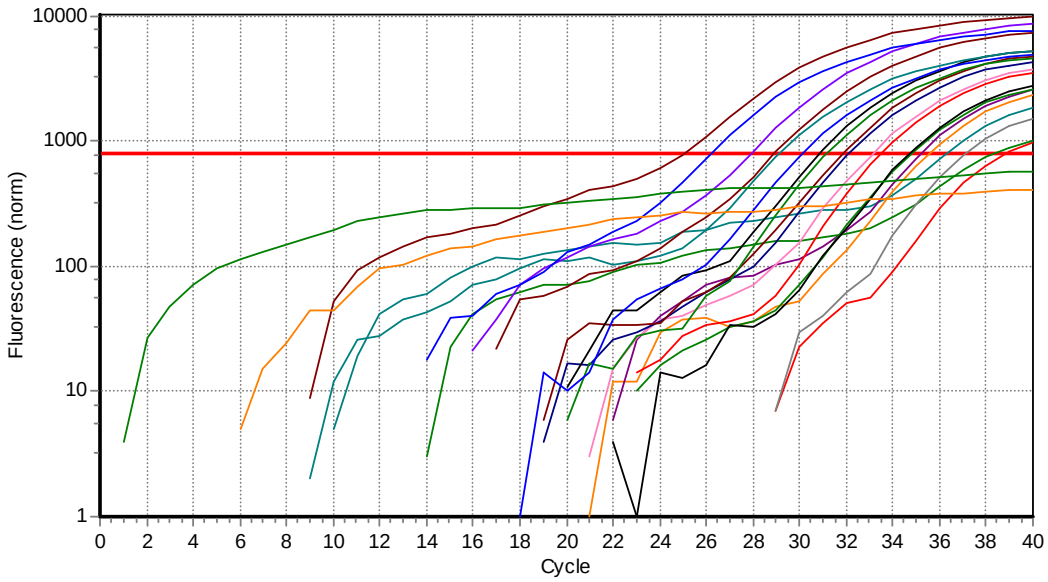
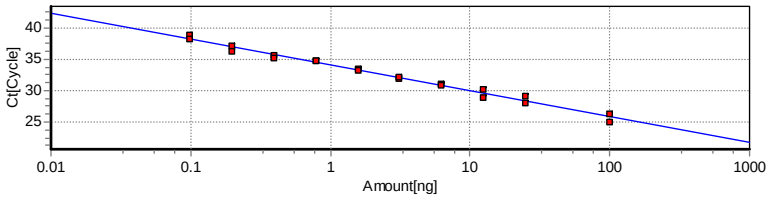
G2-FAM/BHQ-1 duplex with G1

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]
- [FAM/BHQ-1] = 200nM





Standard curve parameters:	
Slope	-4.122
Y-Intercept	34.06
Efficiency	0.75
R^2	0.987

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G2 + G1 duplex summation

- Additions to RealMasterMix Probe: +500uM dNTPs, +1.25U HotMaster Taq, +1.25mM Mg+2 [6.25mM]

G1

Standard curve parameters:	
Slope	-3.267
Y-Intercept	31.41
Efficiency	1.02
R^2	0.997

G2

Standard curve parameters:	
Slope	-4.122
Y-Intercept	34.06
Efficiency	0.75
R^2	0.987

G1 & G2 need a different Ref Gene, but the decision has to be made whether to keep G2.

G2 has had an efficiency of 76% (SYBR) and 72% (FAM/BHQ-1), with additions to mix it stays at 75%. One could say it is reproducibly average

Conclusions

- Limitations experienced in multiplexing were due to chemistry, and were target specific. If targets were designed with multiplexing in mind, it probably wouldn't be difficult
- Calibrating for new dyes, like CalFluor Orange 560 is easy, too
- 40 cycle runs are 45 minutes with very conservative protocols, so I could get lots of runs completed in a day
- Using epMotion went from luxury to absolute necessity
- Short protocols simply need more time to optimize, very encouraging
- 10uL and 5uL reactions are very encouraging, too

Thanks!!

EpiStem: Trish Hurley, Ph.D & Emma Grimes

- Rafal Grzeskowiak, Ph.D, epMotion Application Specialist, Eppendorf AG, Hamburg
- Everyone at Biosearch Technologies, especially Ben Sowers

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