Development and Evaluation of Passive Reference Candidate Compounds for Normalization of Signal in Real-Time PCR

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Purpose: to test various reference dyes (RD) in real-time PCR.

Criterion:
- RD should tighten the threshold cycle (Ct) spread (threshold & baseline set by software).
- RD fluorescence intensity should remain constant in no template control (NTC), no amplification control (NAC) & all concentrations of template.
- Reporter (FAM) fluorescence intensity should remain constant in NTC & NAC

Purpose for figures presented:
- Multicomponent View (MV) is for viewing the fluorescence intensity of each dye over all PCR cycles
- Amplification plots are for calculation of Ct and standard deviation (SD) & viewing quality of amplification

Instrument platforms utilized:
- ABI Prism 7700
- Corbett Research Rotor-Gene.

PCR conditions:
400 nM ApoB primers, 200 nM ApoB FAM/BHQ-1 probe template: Human Genomic DNA (Clontech)

Initial run: 100 ng, 10 ng, NTC

RD concentrations were adjusted so that their emission was less than the FAM emission.

Final run: 100 ng, 10 ng, 1 ng, NTC

REFERENCE DYES:

ROX dye (ROX): provided with the Stratagene Brilliant Kit (standard).

Buffer A (BuA): (ABI) this dye is in PCR buffer (standard).

3'ROX-Spacer 9 (RS9): synthesized using ROX CPG & a PEG spacer amidite. The spacer is to increase solubility & stability.

3'Cal Red-Spacer 9-Spacer 9 (CR18): synthesized using Cal Red (Texas Red) CPG & two PEG spacer amidite couplings. The double spacer is to compare to the RS9 for increase in solubility & stability.

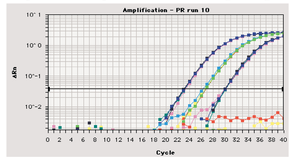
Blue 636 (B636): from MegaBases. This dye is reported to emit from 630-640 nm.

5' 6FAM-T6-3' ROX (FT6R): oligonucleotide synthesized to mimic BuA.

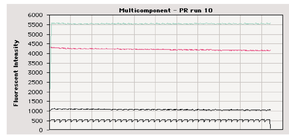
Ruthenium Complex (RuC #1, #2, and #3): various Ruthenium Complexes, which excite at 460 nm and luminesce at ~620 nm. This large Stokes' shift makes them very attractive for the ABI 7700, because it has one excitation source of 488nm

ROX
0.4 µM

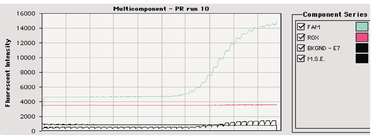
amplification plot for NTC, 1ng,10ng,100ng



MV for the NTC



MV for 100ng



Ct & SD for 1ng 10ng 100ng

Threshold: Use Threshold: .039 Suggest; Mult. * Stddev: 10.0 * .004; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

	Ct	Std Dev
FAM – C7	30.024	0.001
FAM – C8	30.072	0.001
FAM – C9	30.104	0.001
FAM – C10	29.975	0.001

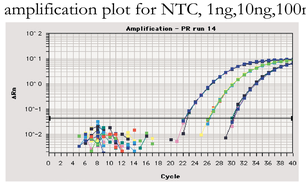
	Ct	Std Dev
FAM – D7	26.812	0.001
FAM – D8	26.735	0.001
FAM – D9	26.537	0.001
FAM – D10	26.265	0.001

	Ct	Std Dev
FAM – E7	23.302	0.001
FAM – E8	23.160	0.001
FAM – E9	23.177	0.001
FAM – E10	23.051	0.001

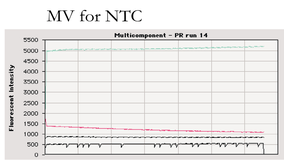
Observations: Ct spread is very small. ROX signal is very flat for all samples. FAM signal does not increase in the NTC. Large portion of each amplification plot is linear.

BuA
1X

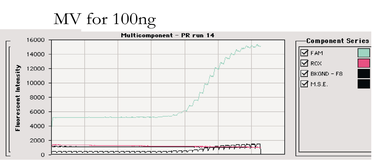
amplification plot for NTC, 1ng,10ng,100ng



MV for NTC



MV for 100ng



Ct & SD for 1ng 10ng 100ng

Threshold: Use Threshold: .043 Suggest; Mult. * Stddev: 10.0 * .004; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

	Ct	Std Dev
FAM – D5	30.238	0.007
FAM – D6	30.191	0.006
FAM – D7	29.961	0.007
FAM – D8	30.097	0.012

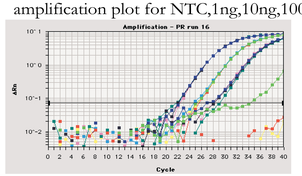
	Ct	Std Dev
FAM – E6	26.098	0.006
FAM – E7	26.143	0.007
FAM – E8	26.054	0.009
FAM – E9	26.286	0.009

	Ct	Std Dev
FAM – F7	22.282	0.004
FAM – F8	22.199	0.009
FAM – F9	22.178	0.006
FAM – F10	22.510	0.011

Observations: Ct spread is slightly larger than for ROX. Small amount of ROX drop for all of the samples. Exponential portion of amplification is not very linear.

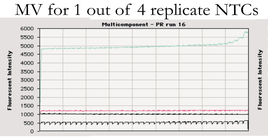
RS9
0.25 µM

amplification plot for NTC,1ng,10ng,100ng

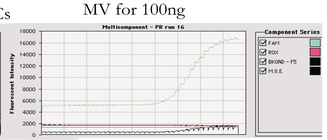


MV for the NTC (3 of 4)

MV for 1 out of 4 replicate NTCs



MV for 100ng



Ct & SD for 1ng 10ng 100ng

Threshold: Use Threshold: .072 Suggest; Mult. * Stddev: 10.0 * .007; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

	Ct	Std Dev
FAM – D5	28.020	0.007
FAM – D6	26.951	0.006
FAM – D7	29.100	0.010
FAM – D8	28.120	0.006

	Ct	Std Dev
FAM – E6	25.503	0.007
FAM – E7	25.159	0.006
FAM – E8	25.363	0.007
FAM – E9	24.698	0.007

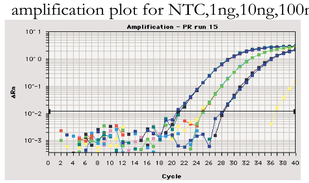
	Ct	Std Dev
FAM – F4	22.342	0.006
FAM – F5	22.137	0.007
FAM – F6	21.751	0.007
FAM – F7	22.003	0.008

Observations: The spread is about the same as BuA. There is a small increase in FAM for one of the NTC samples, but it is far past the Cts. The RS9 signal is very flat for all the samples.

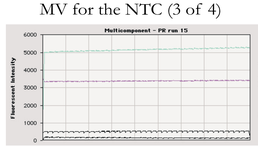
note: results are not significantly affected if the rise in the reference dye comes long after the Ct and there is no increase in the reporter signal in the NTC & NAC.

CR18
0.5 µM

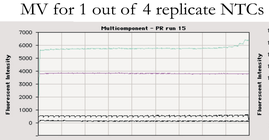
amplification plot for NTC,1ng,10ng,100ng



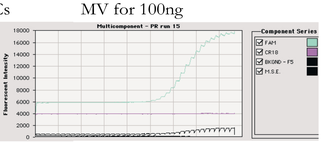
MV for the NTC (3 of 4)



MV for 1 out of 4 replicate NTCs



MV for 100ng



Ct & SD for 1ng 10ng 100ng

Threshold: Use Threshold: .012 Suggest; Mult. * Stddev: 10.0 * .001; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

	Ct	Std Dev
FAM – D5	28.224	0.001
FAM – D6	28.127	0.001
FAM – D7	28.288	0.001
FAM – D8	28.299	0.002

	Ct	Std Dev
FAM – E6	24.896	0.001
FAM – E7	25.083	0.002
FAM – E8	24.574	0.002
FAM – E9	25.079	0.001

	Ct	Std Dev
FAM – F4	21.063	0.001
FAM – F5	20.764	0.001
FAM – F6	21.237	0.001
FAM – F7	21.055	0.001

Observations: The Ct spread is about the same as ROX. There is small increase in FAM for one of the NTC samples, but it is far past the Cts. The CR18 signal is very flat for all the samples. Curve has a very linear portion as in ROX.

note: results are not significantly affected if the rise in the reference dye comes long after the Ct and there is no increase in the reporter signal in the NTC & NAC.

B636
10X

amplification plot for NTC,1ng,10ng,100ng

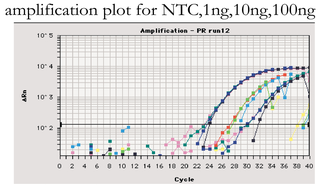
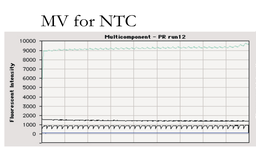
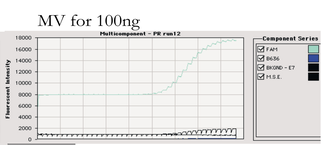


Plate Read at 3X

MV for NTC



MV for 100ng



Ct & SD for 1ng 10ng 100ng

Threshold: Use Threshold: 130.221 Suggest; Mult. * Stddev: 10.0 * 13.022; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

	Ct	Std Dev
FAM – C4	30.074	10.101
FAM – C5	29.787	11.001
FAM – C6	30.106	4.777
FAM – C7	31.622	10.914

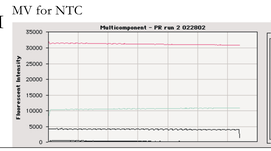
	Ct	Std Dev
FAM – D5	27.208	9.460
FAM – D6	27.513	13.025
FAM – D7	27.573	12.250
FAM – D8	28.873	47.988

	Ct	Std Dev
FAM – E6	28.071	14.990
FAM – E7	28.449	7.101
FAM – E8	23.190	25.481
FAM – E9	23.901	13.101

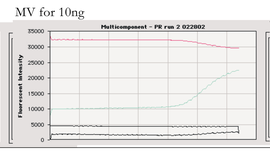
Observations: The Ct spread is very large, because the signal is very weak. The signal for running B636 at 3X (1X is recommended) is very good (see Plate Read), so 10X was expected to give a strong signal.

FT6R
0.75 µM

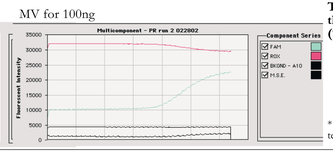
MV for NTC



MV for 10ng



MV for 100ng



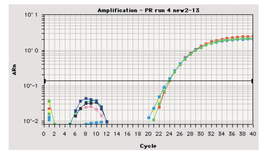
The ROX signal was more intense than BuA, but it decreased with Ct ("ROX drop" *) in real-time PCR.

* ROX drop is a common effect,that appears to be template and primer and probe dependent.

RuC1
*ca*8.0 µM

RuC2 and RuC3 are two other forms developed to avoid intercalation.

amplification plot for NTC,NAC & 100ng

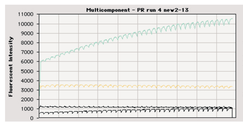


Ct and SD for 100ng

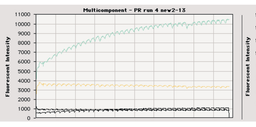
Threshold Cycle Calculation
Threshold: Use Threshold: 137 Suggest; Mult. * Stddev: 10.0 * .014; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

	Ct	Std Dev
FAM – E9	24.011	0.004
FAM – E10	23.965	0.004
FAM – E11	23.814	0.004
FAM – E12	23.712	0.004

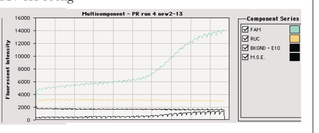
MV for NTC



MV for NAC

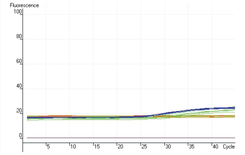


MV for 100ng



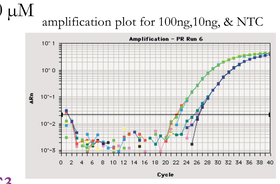
Raw data was needed to judge the 7700 data (left), so the experiment for RuC1 was repeated on a Corbett Research Rotor-Gene for NTC NAC, 10ng, 100ng and with RuC2 for NTC, 10ng, 100ng

raw RuC1 signal, where red is NTC, yellow is NAC, blue is 100ng & green is 10ng

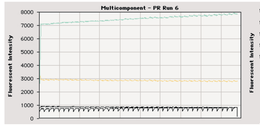


RuC2
*ca*8.0 µM

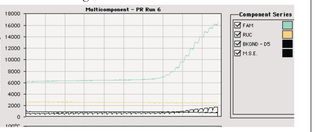
amplification plot for 100ng,10ng, & NTC



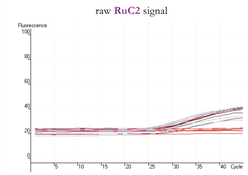
MV for NTC



MV for 10ng

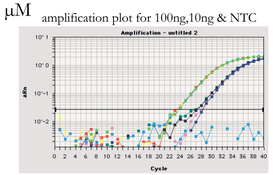


raw RuC2 signal



RuC3
*ca*8.0 µM

amplification plot for 100ng,10ng & NTC



Ct & SD for 10ng, 100ng

Threshold: Use Threshold: .027 Suggest; Mult. * Stddev: 10.0 * .003; Omit Threshold: 2.0
Baseline: Start: 3 Stop: 15
Update Calculations

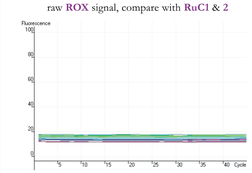
	Ct	Std Dev
FAM – C3	28.007	0.003
FAM – C4	28.217	0.003
FAM – C5	26.781	0.001
FAM – C6	27.186	0.002

	Ct	Std Dev
FAM – D3	25.215	0.002
FAM – D4	25.690	0.002
FAM – D5	25.592	0.002
FAM – D6	25.752	0.003

MV for NTC

MV for 10ng

raw ROX signal, compare with RuC1 & 2



On the 7700, the RuC signals were constant, but the FAM signals increased steadily for the NAC & NTC samples. For amplification samples, the FAM signals increased steadily from the beginning of the PCR cycles and then increased with Ct, while the RuC signals remained constant. However, on the Rotor-Gene the RuC signals increased with Ct but remained quite constant for NAC and NTC samples.

RuC1 and RuC2 performed similarly on the Rotor-Gene and RuC2 and RuC3 did not have such dramatic increases as RuC1, so RuC3 is expected to perform identically to RuC2 on the Rotor-Gene.

Conclusions:

The best reference dye was ROX for this study followed closely by CR18. This is based on Ct spread, signal reliability for many replicates of several concentrations, linearity of exponential portion of amplification curves.

Buffer A performed very well, but exhibited some ROX drop.

The CR18 performed better than the RS9. This which could be attributed to the longer spacer on the CR18, which may prevent interaction with the PCR and improve stability. RS9 performed very well--similar to Buffer A. A new compound consisting of ROX and two spacers may work as well as CR18.

Blue 636, with emission at ~645 nm, is still very interesting and warrants further study.

The difference in performance between the FRET oligos, FT6R and Buffer A, was unexpected and indicates that the dye in Buffer A is more stable for this particular assay. This merits further comparison, because this may be template or probe and primer dependent

The Ruthenium complexes, with their large Stokes' shift and long emission wavelength, show promise as reference dyes. However, more work is needed to overcome their increases in emission. Perhaps they are better suited as reporter dyes.

Acknowledgements:

We would like to thank Renee Horner for her assistance with the 7700 software. We would also like to thank Julie Vold for her assistance with running the 7700. For the use of the Rotor-Gene, we would like to thank Phenix Research Products, and especially Todd Deppe for his assistance with the software.