

# Evaluation of Taqman® DNA Probes: Can High Quality Syntheses be used in Quantitative Real-Time PCR Assays without Gel or HPLC Purification?

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## Abstract

Real-Time or quantitative (q)-PCR technology is of increasing importance in genomic research. The high cost of (FRET) DNA probes for experiments has long impeded the full utilization of qPCR. The commercial cost of dual-labeled probes for qPCR reactions is high because of the post-synthesis HPLC and/or gel purification steps required by limitations in the traditional synthesis chemistry. The recent availability of CPG quencher reagents to core DNA synthesis facilities has opened up the possibility that probes, when carefully prepared, may be used without extensive post-synthesis purification. This would substantially reduce the cost, making the synthesis of qPCR probes feasible and more affordable for any DNA synthesis laboratory. The NARG tested the hypothesis, that all DNA synthesis labs are able to make quality dual-labeled probes suitable for qPCR reactions without gel and/or HPLC purification, by inviting members of the DNA synthesis community to synthesize 5'-FAM, 3'-BHQ1 or -TAMRA quenched human  $\beta$ -actin probes and submit them for quality analysis. The NARG members performed quality analyses on the probes using CE, DHPLC, and PAGE. Effectiveness in Real-Time PCR experiments was determined over a five log range of standard template concentration to assess the effect on assay efficiency and sensitivity compared to highly purified probes.

## Research Plan

• Participants were asked to synthesize 5'-FAM, 3'-BHQ1 or 3'-TAMRA quenched human  $\beta$ -actin probes and submit them for quality analysis.

• The probes were analyzed for quality by three analytical methods: PAGE, CE, and DHPLC.

• The probes were assayed for functionality by a qPCR test that utilized a human  $\beta$ -Actin assay (below) and measured performance against a synthetic template to generate standard curves covering a 5-log range, either  $2 \times 10^7$  to  $2 \times 10^9$  or  $2 \times 10^6$  to  $2 \times 10^8$  molecules. 50 ng of genomic DNA was used as an unknown sample. All assays were run in duplicate on an ABI 7900 using a BioMek robot and a Tecan robot to setup the assays.

$\beta$ -Actin #2 assay: accession # NM001101 (997+) CCCTGGCACCCAGCAATGAAGATCAAGATCATTGCTCTCCCTGAGCGCAAGTACTCCTGTGGATCGGC-BHQ1/TAMRA

sDNA: (synthetic oligo 71 mer)  
 CCCTGGCACCCAGCAATGAAGATCAAGATCATTGCTCTCCCTGAGCGCAAGTACTCCTGTGGATCGGC

Reaction conditions:  
 400 nM primers/100 nM probe  
 ABI 2X Master Mix (5 mM MgCl<sub>2</sub>)

Standard cycling conditions:  
 50°C, 2', 95°C, 10', (95°C, 15", 60°C, 1') 40 cycles

## Quality Control of FRET Probes

### Summary of Probe Data

Probe ID	Label	Quencher	Purification	Y-intercept	Slope	Correlation	Delta Rn	Delta Rn (std)
NARG01	5'-FAM	3'-BHQ1	purified	1.0	1.0	0.99	1.0	0.05
NARG02	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG03	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG04	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG05	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG06	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG07	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG08	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG09	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG10	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG11	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG12	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG13	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG14	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG15	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG16	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG17	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG18	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG19	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG20	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG21	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG22	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG23	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG24	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG25	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG26	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG27	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG28	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG29	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG30	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG31	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG32	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG33	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG34	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05
NARG35	5'-FAM	3'-BHQ1	crude	1.0	1.0	0.99	1.0	0.05

Table 1. A summary of the data for submitted probes sorted by quencher and then purity as assessed by CE. Parameters shown are: purification, if any, Ct and [Rn for 50 ng genomic DNA, and [Rn for the standard curve.

### PAGE Analyses

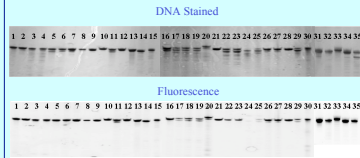


Figure 1. PAGE analyses of dual-labeled FRET probes. Top gel was stained with stains-all (Sigma), all products from the synthesis are visualized. The bottom gel was visualized with a UV light which shows only those fragments containing a fluorescent reporter dye.

### CE Analyses

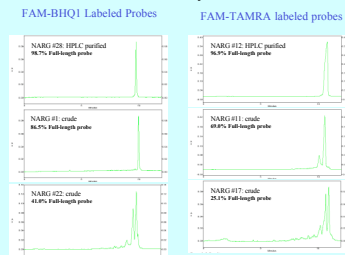


Figure 2. Separation of probes by size utilizing capillary electrophoresis. Partially conjugated probes and/or those less than full length come off first followed by full-length probes with both reporter and quencher dyes.

### DHPLC (WAVE) Profiles

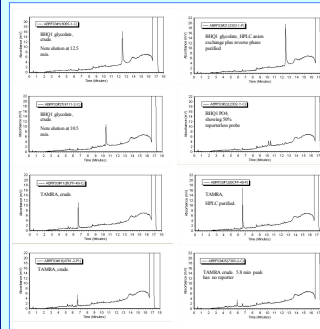


Figure 4. Denaturing HPLC profiles of Taqman probes. Analyses were performed on a WAVE™ HT (high throughput) system and a DNASp™ cartridge run at 80°C. WAVE™ automated chromatography system (TranGenomic, San Jose, USA) represents a reverse-phase ion-pair denaturing HPLC technique, and the DNASp™ cartridge consists of alkylated non-porous poly (styrene-divinylbenzene) particles. Elution was performed with a gradient of 3-20% acetonitrile in TEAA buffer.

### Amplification Curves for Selected Good and Bad Taqman Probes

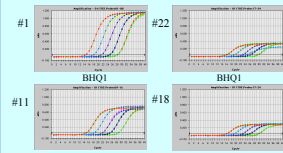


Figure 5. Comparison of the effect of good vs poor probe synthesis on the qPCR signal (delta Rn). The effect on the standard curves can be seen in Figure 3 (#'s refer to probe numbers in Table 1).

### How to Extract Data out of a Failed Probe, NARG-25

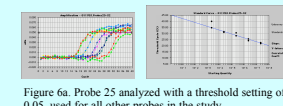


Figure 6a. Probe 25 analyzed with a threshold setting of 0.05, used for all other probes in the study.

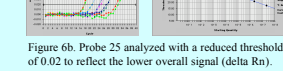


Figure 6b. Probe 25 analyzed with a reduced threshold setting of 0.02 to reflect the lower overall signal (delta Rn).

## Summary

- Total submissions = 35
- 23 BHQ1, 11 TAMRA, and 1 QSY-7
- 16 were "purified", 19 were crude
- 15 of the submissions were by labs which do not routinely synthesize FRET probes.
- Amount of full-length probe ranged from 99% to 3%
- 34 /35 probes each gave a usable standard curve down to 200 copies of template
- Ct's at 2000 copies varied from 22.5 to 24.5

## Conclusions

- Both FAM/BHQ1 and FAM/TAMRA quenched FRET probes are easy and inexpensive to make. 100% of the respondents said that they found the synthesis easy.
- qPCR is a very robust technique. Even a probe containing only 17.9% full-length probe (#3) allowed detection down to 2000 copies when used with an optimized primer pair on an oligonucleotide template. (Table 1, Figure 3).
- Purer probes gave larger delta Rn's resulting in a larger dynamic range. (Table 1, Figure 5).
- A well synthesized crude probe using either quencher could be ~ 90% pure. (Table 1: #26 and #34).
- For BHQ1 quenched probes, probes >50 % in full-length resulted in good (>0.9) delta Rn's (Table 1).
- BHQ1 quenched probes have a larger dynamic range than TAMRA quenched probes (Table 1).

## Acknowledgements

We gratefully acknowledge all the participants of this study, especially those who do not make dual labeled FRET probes on a regular basis and were brave enough to give it a try. We would like to acknowledge the hard work of Glen Miller and Emmanuelle Nicolas of FCCC, Josef Limor of the CDC, and Ying Wang of UTHSC-Houston, without whom this study would not have been possible. We would also like to thank our ABRF Executive Board *ad hoc*, Dr. Susan Hardin of the University of Houston for her guidance.

## Announcement

NARG Research Group Presentation!  
 Tuesday, February 11 4:00-5:00 pm  
 Plaza Ballroom F