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Nucleic Acids Research Group 2003 Study:
Evaluation of Multiple Taqman® Assay Designs: What Did We Learn from Looking at 32 Assays Made for Mouse IFNγ?

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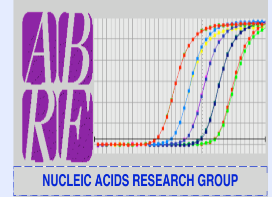


Abstract

The Nucleic Acids Research Group (NARG) of the Association of Biomolecular Resource Facilities (ABRF) invited researchers to participate in an empirical study to define the parameters required to make an optimal 5'-nuclease (Taqman¨) real-time PCR assay. New assay design can be one of the major rate-limiting steps to those new to this technology in rapidly acquiring data from their genes of interest. Although a large number of pre-made assays can be purchased from Applied Biosystems and Qiagen, it is much more cost effective to make your own assays if a large number of samples need to be run with each assay. There are general guidelines available concerning assay design. However, exactly how important each of these parameters are has not been studied in an empirical manner. Further, there may be as yet unknown factors that should be taken into account during assay design.

The purpose of this study was to give a large number of investigators an opportunity to design what they feel will be an optimal primer/probe set for a common transcript and then have them tested empirically for efficiency. Each participant was asked to provide the sequence of a pair of primers and a probe within a slightly reduced coding region of the mouse IFNg transcript. Members of the NARG synthesized the primers and probes, and tested the reagents for each assay using a recombinant plasmid containing a partial mouse IFNg cDNA as template. The results for each assay will be posted on the ABRF web site and published. Entries have been identified by a user designated code and are completely anonymous.

Research Plan

- Participants were asked to submit two primers and probe sequences for what they thought was an optimal assay for the mouse IFNγ transcript.
- Members of the NARG also submitted assays in an effort to test some of the common rules for assay design.
- Primers and probes were synthesized in different NARG laboratories with in house QC.
- The assays were tested for functionality by a real-time PCR test that utilized a plasmid construct (bearing most of the mouse IFNγ coding region). The linearized DNA clone was used as a synthetic template to generate standard curves covering a 6-log range from $3.4x10^6$ to $3.4x10^1$ molecules in duplicate. All assays were run in 96-well plates on an ABI 7700 using Tecan and Biomek robotic workstations to setup the assays. Each reaction contained 25 µl total volume.

Methods

FRET Probe Synthesis

All TaqMan™ probes were synthesized on a single ABI 394 DNA synthesizer using standard phosphoramidite chemistry. Probes were synthesized starting with a BHQ-1 CPG (Biosearch Technologies, Novato, CA.) and labeled at the 5' end with 6-FAM (Glen Research, Sterling, Virginia). Probes were purified to greater than 90% purity by reverse-phase HPLC, quality of probe purification was verified by capillary electrophoresis on a Beckman PACE/MDQ system.

Primer Synthesis

Primers were synthesized at the Penn State University Nucleic Acid Facility using the MerMade 12 DNA Synthesizer (Bioautomation, Plano TX) using 50 nmole scale columns obtained from Biosearch Technologies (Novato, CA). Primers were cleaved from the CPG support, deprotected and dried down before use.

Real-Time Assay Reaction conditions:

400 nM primers/100 nM probe; 1X PCR Buffer (Invitrogen); 5 mM $MgCl_2$; 200 µM dNTPs; 125 nM ROX (Invitrogen); 1.25 U Taq Polymerase (Invitrogen)

7700 Cycling conditions:

95ºC, 1', (95ºC, 12", 60ºC, 30'') 40 cycles

Results

Summary of Real-Time Assay Results

Assay #	ID #	Primers & Probes	Tm Forward Primer	Tm Reverse Primer	Forward Primer %GC	Reverse Primer %GC	Amplicon Size	#G/C last 5- Forward Primer	#G/C last 5- Reverse Primer	Δ %G/C Primers	Terminal G or C- Primers	Probe G/C	Probe - Av Primer	Av ΔRn	Slope	Y-axis Intercept	Av Ct 3.4E6 Molecs	Assay quality, 1/(-slope*Ct), higher is better
1	GLS1	1,51,101	59.1	60.4	50.0	55.0	72	2	2	5.0	0	0.5	9.8	2.23	-3.42	36.3	14.1	0.0207
2	GLS2*	2,52,102	61.6	62.0	41.7	47.6	92	1	2	5.9	1	0.3	9.4	2.41	-3.43	36.1	13.7	0.0212
3	4618	4,54,104	65.7	64.6	50.0	50.0	77	2	2	0.0	1	0.7	-1.5	2.14	-3.94	40.3	14.7	0.0173
4	6016	6,56,106	59.4	59.5	50.0	55.6	71	3	3	5.6	1	0.6	9.8	2.74	-3.33	34.8	13.2	0.0228
5	0166	7,57,107	61.8	61.0	44.0	64.7	101	3	3	20.7	2	0.6	9.5	3.25	-3.45	35.5	13.2	0.0220
6	3303	8,52,102	59.9	62.0	35.7	47.6	129	1	2	11.9	2	0.3	10.3	2.19	-3.40	36.2	14.1	0.0208
7	2412	9,59,109	64.7	61.4	50.0	55.0	96	1	2	5.0	0	0.7	7.5	2.75	-3.44	39.7	17.4	0.0167
8	1645	10,60,110	61.1	61.1	40.0	45.5	85	2	2	5.5	0	0.4	9.4	2.42	-3.37	35.6	13.5	0.0219
9	7952	11,61,111	61.1	61.4	60.0	57.9	69	3	3	2.1	2	0.8	9.1	2.89	-3.22	34.3	13.2	0.0236
10	8532	12,62,112	N/A	N/A	61.1	58.8	65	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	795B	13,63,113	61.9	62.4	45.5	66.7	201	2	3	21.2	1	1.6	7.6	1.63	-3.48	35.9	13.3	0.0216
12	795D	13,64,114	61.9	62.4	45.5	66.7	201	2	3	21.2	1	1.6	8.1	1.87	-3.39	35.3	13.2	0.0223
13	795C	14,64,115	60.6	59.6	55.0	33.3	79	2	0	21.7	1	1.2	11.7	2.18	-3.47	36.4	14.1	0.0204
14	6288	16,66,117	N/A	N/A	50.0	60.0	113	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
15	3762	17,67,118	61.6	59.5	41.7	40.0	107	1	3	1.7	0	0.4	13.8	1.97	-3.44	36.0	13.5	0.0215
16	8085	18,68,119	58.6	59.9	31.0	52.4	93	1	2	21.4	0	2.5	10.3	1.85	-3.28	36.3	15.2	0.0201
17	8086	19,69,120	56.0	61.1	25.8	45.8	90	2	1	20.0	1	2.5	11.3	1.55	-3.47	36.7	14.0	0.0205
18	8087	20,70,121	55.9	63.9	26.7	45.8	90	2	1	19.1	1	2.0	3.7	1.60	-3.40	37.0	14.7	0.0200
19	GLS3	21,51,101	60.0	60.4	47.6	55.0	197	4	2	7.4	1	0.4	9.4	1.50	-3.55	38.3	15.4	0.0183
20	GLS4	22,51,101	58.9	60.4	50.0	55.0	193	3	2	5.0	0	0.4	10.0	1.78	-3.48	37.5	14.9	0.0192
21	GLS5	23,51,101	60.1	60.4	44.4	55.0	181	3	2	10.6	1	0.4	9.3	1.49	-3.47	36.9	14.5	0.0200
22	GLS6	24,51,101	59.4	60.4	36.7	55.0	177	1	2	18.3	0	0.4	9.7	1.80	-3.42	36.8	14.5	0.0202
23	GLS7	25,51,101	59.2	60.4	58.8	55.0	73	4	2	3.8	0	0.4	9.8	2.06	-3.33	38.0	16.3	0.0184
24	GLS8	1,71,101	59.1	60.2	50.0	55.0	71	2	2	5.0	1	0.4	9.9	2.11	-3.46	36.3	13.7	0.0212
25	GLS9	1,72,101	59.1	59.2	50.0	63.2	106	2	3	13.2	1	0.4	10.5	2.01	-3.38	35.8	13.6	0.0217
26	GLS10	1,73,101	59.1	60.1	50.0	63.2	108	2	3	13.2	1	0.4	10.0	2.03	-3.29	35.3	13.7	0.0222
27	GLS11	1,74,101	59.1	60.1	50.0	58.5	127	2	3	8.5	1	0.4	10.0	1.81	-3.35	35.2	13.2	0.0225
28	GLS12	1,75,101	59.1	59.6	50.0	64.7	140	2	3	14.7	0	0.4	10.3	1.79	-3.42	35.6	13.4	0.0217
29	7935	26,76,122	58.6	59.2	45.5	50.0	90	2	2	4.5	1	1.3	9.1	1.93	-3.41	36.1	13.6	0.0215
30	8088	27,77,123	67.2	61.5	52.0	44.0	94	4	2	8.0	1	1.0	14.0	0.60	-3.32	38.2	16.2	0.0187
31	1188	28,78,124	56.0	55.2	40.9	40.9	91	3	2	0.0	0	0.6	13.0	2.82	-3.38	35.6	13.5	0.0219
32	3034	29,79,125	58.5	58.4	50.0	45.0	129	3	3	5.0	2	0.4	9.8	3.13	-3.39	34.5	12.6	0.0234

Table 1. Key physical data for each of the assays tested and the results for each following real-time PCR analysis.

* Assay from Overbergh, *et al*. J. Biomol. Tech., **14**:33-43, 2003

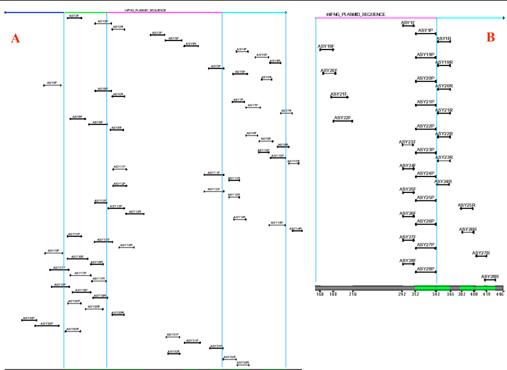


Fig. 1- A map of all the assays in the study in relation to the mIFNγ gene sequence. The maps were made using Sequencher software (Gene Codes Corp., Ann Arbor, MI). The cloned portion of the mIFNγ gene used as a template for this study was from bases 1 to 446. Panel A shows the locations of unique assays 2-18 and 29-32. The first 3 blue vertical lines (L-R) represent the positions of intron/exon junctions, the fourth the end of the template used in the study. Panel B shows the locations of assays 1 and 19-28 which share the same probe.

Fig. 2- A a single base deletion in the probe

Probe-ATGCATTCATGAGTATTGCC**a**AGTTTGAGGTC

Tm of region up to the **a**= 57.2˚C; whole probe= 70.2˚C

Assay #11 Missing the 'a' in probe sequence
Ct 3.4E6 = 13.3
Av ΔRn = 1.63
Slope = -3.48
Y-intercept = 35.9

Assay #12 Complete sequence
Ct 3.4E6 = 13.2
Av ΔRn = 1.87
Slope = -3.39
Y-intercept = 35.3

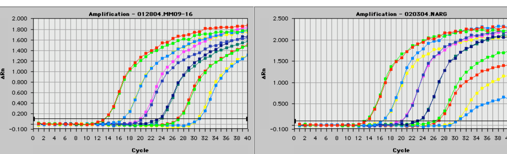
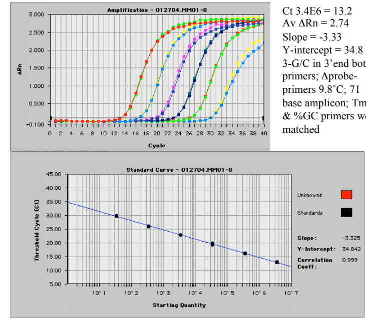
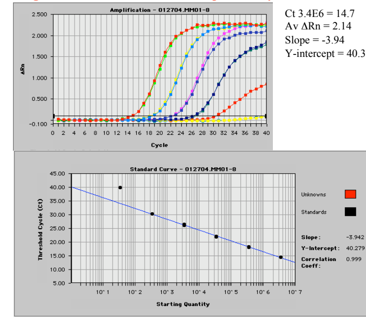


Fig. 3- Example of an optimal Assay- #4



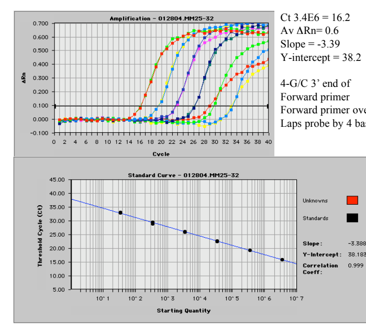
Ct 3.4E6 = 13.2
Av ΔRn = 2.74
Slope = -3.33
Y-intercept = 34.8
3-G/C in 3'end both primers; Δprobe-primers 9.8˚C; 71 base amplicon; Tm & %GC primers well matched

Fig. 4- Probe with Tm lower than primers by 1.5 ˚C- #3



Ct 3.4E6 = 14.7
Av ΔRn = 2.14
Slope = -3.94
Y-intercept = 40.3

Fig. 5- Example of a sub-optimal Assay- #30



Ct 3.4E6 = 16.2
Av ΔRn= 0.6
Slope = -3.39
Y-intercept = 38.2

4-G/C 3' end of Forward primer
Forward primer over Laps probe by 4 bases

How do you determine a good assay?

The slope of an assay has historically been used as the yardstick for measuring how well the assay is performing. From the slope one can determine the efficiency of the assay. Obviously, the more efficient the PCR, the better the assay. However, the slope does not totally define how well an assay is performing. We have found that the Ct for a fixed mass of template is also an important barometer of how well an assay is performing.

One of us (ATY) has come up with a mathematical relationship to predict how Well an assay is working encompassing both the slope and Ct values.

Assay Quality = 1/(-slope x Ct)

Using this method, the two best assays were #9 and #32; 0.0236 & 0.0234
The two worst assays were #3 and #7; 0.0173 & 0.0167, resp. (Table 1)

Conclusions

1- The Taqman assay is incredibly robust. Assays that had physical problems all worked, some remarkably well. Examples:
Assays 10 and 14 - the reverse primers were outside of the template sequence and did not work; this was comforting.

Assay 7- only 10 bases of the reverse primer were on the template. This explains the high Ct.

Assay 30- the forward primer overlaps the probe by 4 bases (Fig. 5)

Assays 11 & 12- a base was removed in the probe for Assay 11(Fig. 2). Both worked about the same.

2- There is a direct correlation between the length of the probe and the ΔRn (R^2 0.81). For best results probes over 30 bases in length should be avoided.

3- To test the effect of assay length, 10 new primer sets were designed around the probe of Assay #1. We found that amplicons up to 196 bases worked very well. There was no direct correlation between assay length nor distance of the primer from the probe in how well an assay performed (Fig. 1B).

4- There were no strict correlations between primer sequence structure and how well an assay performed. Three assays, 9, 24 and 30 had 4 G/Cs in the last 5 bases on the 3' end of one primer and they ranked at the bottom using the formula described above.

Acknowledgements

We gratefully acknowledge all the participants of this study. We would like to acknowledge the hard work of Mark LaMere and Jessica Hoffman of Trudeau Institute, Glen Miller and Emmanuelle Nicolas of FCCC, Josef Limor of the CDC, Ying Wang and Jun Ying of UTHSC-Houston and Ashley Price, Michael Porzio and Kelly Sitch of Pennsylvania State University, 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.

Announcements

NARG Research Group Presentation!

Monday, March1 4:00-5:00 pm

Room A106

The data in this study will be posted at the ABRF.org web site under Nucleic Acids Research Group. Please submit poster requests to sadams@trudeauinstitute.org