

Example instructions

ABRF NARG 06 Study: Priming Strategies for Real-time RT-PCR

Reagents provided

Reagent	Amt/tub	unit	Resusper	Conc	unit
hReference RNA Template	4000	ng	40	100	ng/ μ l
Random Primer	125	μ mole	50	2.5	μ M
Oligo dT	125	μ mole	50	2.5	μ M
hGUS R primer for RT	125	μ mole	50	2.5	μ M
h GUS F primer	1000	μ mole	50	20	μ M
hGUS R primer	1000	μ mole	50	20	μ M
hGUS Probe 5'FAM 3' BHQ1	200	μ mole	10	20	μ M
hTBP R primer for RT	125	μ mole	50	2.5	μ M
hTBP F primer	1000	μ mole	50	20	μ M
hTBP R primer	1000	μ mole	50	20	μ M
hTBP Probe 5'FAM 3' BHQ1	200	μ mole	10	20	μ M

All other reagents provided by user.

Expt outline

2 genes	hGUS	hTBP			
5 priming methods	hexamers (RH)	oligo dT (dT)	primer (SP)	RT + dT	primer (NP)
Triplicate samples					
Choice of chemistry	Taqman and/or SYBR Green				
RNA (cDNA)/well	100	ng			
Reference RNA @	100	ng/ μ l			
Reference RNA /well for RT reaction	2	μ l = 200 ng			
RT volume	10	μ l			
RT RP concentration	500	nM			
RT oligo dT concentration	500	nM			
RT SP concentration	500	nM			
RT RP+ oligo dT concentration	250	nM	each		
All PCR primers	400	nM			
Both PCR probes	100	nM			
PCR volume	25	μ l			
Amount of cDNA/PCR reaction	2.5	μ l = 50 ng			

Reverse transcribe each well. A sample protocol is given below but you can use whatever you routinely use.

For example:

Total volume of RT reaction = 10 μ l 2 μ l RTP + 8 μ l RT mix

Reagent	Conc.	Unit	1X	Unit	Amt(1X)
5X RT buffer	5	X	1	X	2
DEPC-H ₂ O	****		****		2
DTT	0.1	M	10	mM	1
RNase Inhibitor	40	U/ μ l	1	U/ μ l	0.25
hReference RNA	100	ng/ μ l	20	ng/ μ l	2
dNTP mix	10	mM	500	μ M	0.5
RTP	2.5	μ M	500	nM	2
Reverse Transcriptase	200	U/ μ l	5	U/ μ l	0.25
Total					10

RT Master Mix

Need enough for 3 wells x 5 RTP=15, make enough for 18.

RT mix includes everything but the RT primer						
Reagent	Conc.	Unit	1X	Unit	Amt(1X)	Amt x
5X RT buffer	5	X	1	X	2	36
DEPC-H ₂ O	****		****		2	36
DTT	0.1	M	10	mM	1	18
RNase Inhibitor	40	U/ μ l	1	U/ μ l	0.25	4.5
hReference RNA	100	ng/ μ l	20	ng/ μ l	2	36
dNTP mix	10	mM	500	μ M	0.5	9
Reverse Transcriptase	200	U/ μ l	5	U/ μ l	0.25	4.5
Total					8	144

- 1) Add 8 μ l of RT mix to all wells (tubes).
- 2) Add 2 μ l Random Hexamers to 3 wells. E.g., Row A (1-3)
- 3) Add 2 μ l oligo dT to 3 wells. E.g., Row A (4-6)
- 4) Add 2 μ l hGUS Specific Primer to 3 wells. E.g., Row A (7-9)
- 5) Add 2 μ l hTBP Specific Primer to 3 wells. E.g., Row A (10-12)
- 6) Add 1 μ l Random hexamer +1 μ l oligo dT to 3 wells. E.g., Row B (1-3)
- 7) Add 2 μ l No Primer (water) to 3 wells/gene/chemistry- E.g., Row B (4-6)

Put the plate in a thermocycler and run as follows:

25C	10			min	Incubate
42C	50			min	Reverse transcribe
70C	5			min	Inactivate

While doing RT, make up PCR mix.

Place RT on ice, add PCR mix

Taqman assay

Prepare one Master Mix for BGUS and one for TBP as indicated below.

Prepare enough for tubes =

22.5 μ l each gene
25 μ l total volume

For each gene, need enough for 3 wells x 1 gene x 1 chemistry x 5 RTP=15 + 3 NTC, make enough for 20.

Vol (μ l) to add

Reagent	Conc.	Unit	1X	Unit	Amt (1X)	Amt x
RNAse free water					8.88	177.5
2X Master Mix	2	X	1	X	12.5	250
Forward primer	20	μ M	400	nM	0.5	10
Reverse primer	20	μ M	400	nM	0.5	10
Probe 5'FAM 3' BHQ1	20	μ M	100	nM	0.125	2.5
Total					22.5	450

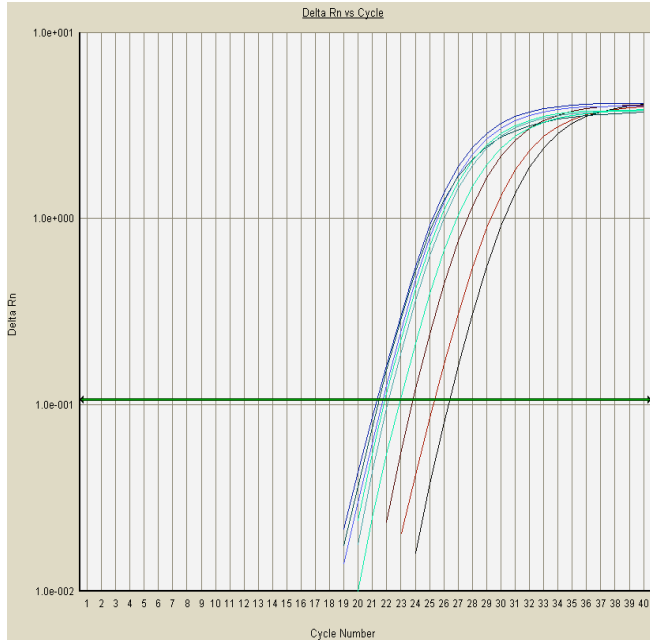
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- 8) Aliquot 22.5 μ l assay master mix into each of 18 wells in PCR plate for each gene as indicated in the PCR set-up sheet.
- 9) Add 2.5 μ l of RT mix from each reverse transcription to the wells for both genes as indicated in the PCR set-up sheet.
One aliquot will be removed from each RT mix for the BGUS assay and one for the TBP, i.e. 5 μ l of the 10 μ l RT will be used.
- 10) Run on 7700 (or whatever instrument is in your lab) using usual settings.

50C	2 min	UNG inactivation	
95°C	10 min	Taq activation	
40 cycles of			
95°C	15 sec	PCR	
60°C	1 min		

Example of how to report result:

Gene	RT primer	Well	Ct	Mean	St. Dev.
hGUS	RP	A1	25.3		
hGUS	RP	A2	25.2		
hGUS	RP	A3	25.7	25.39	0.30
hGUS	oligo dT	A4	21.3		
hGUS	oligo dT	A5	21.7		
hGUS	oligo dT	A6	21.5	21.49	0.21
hGUS	SP	A7	21.4		
hGUS	SP	A8	22.1		
hGUS	SP	A9	21.8	21.77	0.36
hGUS	ψ + oligo c	A10	21.9		
hGUS	ψ + oligo c	A11	22.9		
hGUS	ψ + oligo c	A12	22.1	22.29	0.56
hGUS	⊖ RT prim	B1	22.8		
hGUS	⊖ RT prim	B2	26.3		
hGUS	⊖ RT prim	B3	23.5	24.22	1.85
hGUS	NTC	B4	40.0		
hGUS	NTC	B4	40.0		
hGUS	NTC	B4	40.0	40.00	0.00



hTBP	RP	A1	29.0		
hTBP	RP	A2	29.1		
hTBP	RP	A3	29.2	29.12	0.10
hTBP	oligo dT	A4	27.0		
hTBP	oligo dT	A5	26.1		
hTBP	oligo dT	A6	26.7	26.62	0.47
hTBP	SP	A7	25.0		
hTBP	SP	A8	25.1		
hTBP	SP	A9	25.3	25.12	0.17
hTBP	ψ + oligo c	A10	26.7		
hTBP	ψ + oligo c	A11	28.7		
hTBP	ψ + oligo c	A12	27.1	27.49	1.06
TBP	⊖ RT prim	B1	32.8		
TBP	⊖ RT prim	B2	30.4		
TBP	⊖ RT prim	B3	31.2	31.45	1.20
TBP	NTC	B4	40.0		
TBP	NTC	B4	40.0		
TBP	NTC	B4	40.0	40.00	0.00

