

True lab QRT-PCR protocol 01/05

This protocol uses the Stratagene Brilliant QPCR kit (cat # 600548 or 600552) with SYBR green to estimate the expression level of a specific transcript (CG####) from Drosophila pupal RNA preparations (15-30 pupae per sample). CG#### levels are normalized by dividing by the expression level of the "housekeeping" gene transcript RPII-215.

no-RT reactions (no reverse transcriptase) are used to control for amplification of background genomic DNA in the RNA samples.

Standards are amplifications of known amounts of a specific PCR product (DNA) that is roughly the same length as the experimental amplicon of interest. Reverse transcriptase (RT) is not needed for standard reactions.

RNA prep kits used: Stratagene cat # 400800 or 400805, Qiagen cat # 74104

RNA sample concentrations must be measured on a spectrophotometer and diluted to a ~100 ng/ul working concentration prior to QRT-PCR.

basic protocol:

- (1) mix standard master mix and dispense into standard wells
- (2) dispense standard DNA
- (3) mix experimental master mix and dispense into experimental wells
- (4) dispense RNA samples into experimental wells
- (5) mix housekeeping gene master mix and dispense into appropriate wells
- (6) repeat step (4) in housekeeping gene wells
- (7) repeat steps (3)-(6) with no RT experimental and housekeeping controls
- (8) use an extra aliquot of experimental master mix plus 3 ul water for no template experimental control (NTC)
- (9) repeat step (8) for housekeeping gene no template control (NTC)
- (10) spin plate down and place in RT-PCR thermal cycler

note: it is advantageous to organize plate vertically as shown below so that each column can be capped immediately after it is set up

thermal cycling program:

- (1) 50C 20 minutes (reverse transcriptase step)
- (2) 94C 10 minutes (kills RT and activates Taq polymerase in Stratagene kit)
- (3) 94C 30 sec
- (4) 55C 30 sec
- (5) 72C 30 sec
- (6) fluorescence read 11 sec
- (7) repeat steps (3)-(6) for 39 more cycles
- (8) 95C 30 sec
- (10) 55C 30 sec
- (11) ramp to 95C with fluorescence read every 0.1 degree for thermal profile calculation
- (12) end

each experimental reaction

3	ul	RNA template (300 ng total RNA)		
0.375	ul	reference dye dilution		
0.25	ul	forward primer (25 pmol) [$\$=100$ pmol/ul]		
0.25	ul	reverse primer (25 pmol)[$\$=100$ pmol/ul]		
12.5	ul	QRT-PCR master mix		
0.125	ul	RT-MIX		
8.5	ul	dH2O		
25	ul	TOTAL VOLUME		

20 CG### experimental reactions (17 + NTC + waste)

0.0	ul reference dye dilution	
0.0	ul forward primer (25 pmol) [\$=100 pmol/ul]	
0.0	ul reverse primer (25 pmol)[\$=100 pmol/ul]	
0.0	ul QRT-PCR master mix	
0.0	ul RT-MIX	
0.0	ul dH2O	
DISPENSE		0 ul per rxn

20 RPII-215 experimental reactions (17 + NTC + waste)

7.5	ul reference dye dilution	
5.0	ul forward primer (25 pmol) [\$=100 pmol/ul]	
5.0	ul reverse primer (25 pmol)[\$=100 pmol/ul]	
250.0	ul QRT-PCR master mix	
2.5	ul RT-MIX	
170.0	ul dH2O	
DISPENSE		22 ul per rxn

19 CG### no-RT reactions

0.0	ul reference dye dilution	
0.0	ul forward primer (25 pmol) [\$=100 pmol/ul]	
0.0	ul reverse primer (25 pmol)[\$=100 pmol/ul]	
0.0	ul QRT-PCR master mix	
0.0	ul dH2O	
DISPENSE		0 ul per rxn

19 RPII-215 no-RT reactions

7.1	ul reference dye dilution	
4.8	ul forward primer (25 pmol) [\$=100 pmol/ul]	
4.8	ul reverse primer (25 pmol)[\$=100 pmol/ul]	
237.5	ul QRT-PCR master mix	
163.9	ul dH2O	
DISPENSE		22 ul per rxn

standard mix for 6

2.3	ul	reference dye dilution	
1.5	ul	forward primer (25 pmol) [\$=100 pmol/ul]	
1.5	ul	reverse primer (25 pmol)[\$=100 pmol/ul]	
75.0	ul	QRT-PCR master mix	
57.8	ul	dH2O	
DISPENSE		23 UL PER RXN	
ADD		2 UL STANDARD DNA TO EACH REACTION	

REFERENCE DYE

make dilution for 450 rxns and throw out rest		
0.3375	ul \$ ref dye	
168.4125	ul dH2O	

example plate configuration

	1	2	3	4	5	6	7	8	9	10	11	12
A	Canton[S] CG####	017B CG####	42A CG####	Canton[S] RPII-215	017B RPII-215	42A RPII-215	Canton[S] CG#### no-RT	017B CG#### no-RT	42A CG#### no-RT	Canton[S] RPII-215 no-RT	017B RPII-215 no-RT	42A RPII-215 no-RT
B	Oregon[R] CG####	019A CG####	t[1] CG####	Oregon[R] RPII-215	019A RPII-215	t[1] RPII-215	Oregon[R] CG#### no-RT	019A CG#### no-RT	t[1] CG#### no-RT	Oregon[R] RPII-215 no-RT	019A RPII-215 no-RT	t[1] RPII-215 no-RT
C	Bahamas[3] CG####	038B CG####	t[2] CG####	Bahamas[3] RPII-215	038B RPII-215	t[2] RPII-215	Bahamas[3] CG#### no-RT	038B CG#### no-RT	t[2] CG#### no-RT	Bahamas[3] RPII-215 no-RT	038B RPII-215 no-RT	t[2] RPII-215 no-RT
D	w[1118] CG####	20A CG####	t[3] CG####	w[1118] RPII-215	20A RPII-215	t[3] RPII-215	w[1118] CG#### no-RT	20A CG#### no-RT	t[3] CG#### no-RT	w[1118] RPII-215 no-RT	20A RPII-215 no-RT	t[3] RPII-215 no-RT
E	19282 CG####	27A CG####	t[5] CG####	19282 RPII-215	27A RPII-215	t[5] RPII-215	19282 CG#### no-RT	27A CG#### no-RT	t[5] CG#### no-RT	19282 RPII-215 no-RT	27A RPII-215 no-RT	t[5] RPII-215 no-RT
F	05K CG####	37C CG####		05K RPII-215	37C RPII-215		05K CG#### no-RT	37C CG#### no-RT		05K RPII-215 no-RT	37C RPII-215 no-RT	
G	.02 ng standard	.002 ng standard	.0002 ng standard	.00002 ng standard	.000002 ng standard	CG#### NTC	RPII-215 NTC					
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