

Real-Time Quantitative PCR (QPCR) for LOH Analysis in Primary Tumors and Derivative Cell Lines.

Essential to the validation efforts of all models developed under the MMHCC is the availability of reliable assays that monitor the status of known oncogene and tumor suppressor genes as well as candidate loci in emerging tumors. Such validation efforts determine whether genetic alterations, classical for certain human tumor types, are also present in the mouse tumor counterpart. Several standard methodologies are available for targeted molecular profiling including in situ detection for RNA or protein, Southern blotting, semi-quantitative PCR, among others. Relative to these standard assays, real-time quantitative PCR assays are superior in that they are highly quantitative, while being extremely efficient in time and material. The recent advances in QPCR have significantly improved its reliability and reproducibility. However, major hurdles to widespread use remain the time, expense and technical expertise required for optimization of the assay for each target sequence.

Gene deletion and amplification are common events in tumor genesis and progression. The major advantages of this method include the fact that respective markers do not have to be heterozygous to be informative, analysis is rapid and tumor DNA acts as its own control for copy number determination.

Relative Quantitation Utilizing the Comparative CT method

The assays described in the handouts utilize single tube multiplex PCR to quantify gene dosage. Single tube multiplex PCR reactions contain primers and probes specific for the experimental target and endogenous reference standard.

Relative quantitation utilizes a calibrator. A calibrator is a DNA sample of known copy number for experimental and endogenous reference. A calibrator sample is included on every quantitation PCR run. The assays were developed using wild type 129 SvEv genomic DNA as a calibrator.

The copy number of an experimental gene normalized to an endogenous reference and relative to a calibrator sample is given by:

$$2^{-\Delta\Delta Ct} *$$

Ct: Cycle threshold

* See User Bulletin #2

All calculations are found in ABI user bulletins #2 and #5. Ratios are determined based on these calculations. **Calls are made based on the final ratios. Ratios may vary between assays. Confirmation by PCR or Southern Blot analysis should be done for the first three to four hundred samples to insure accuracy of the calls.**

Recommended readings

- User Bulletin #2, ABI PRISM 7700 Sequence Detection System
- User Bulletin #5, ABI PRISM 7700 Sequence Detection System
- TaqMan Gold RT-PCR Kit, protocol
- Heid C. A., Stevens J., Livak K., Williams P. M.; Real Time Quantitative PCR; Genome Research 1996, 6:986-994
- Gibson U. E. M., Heid C. A., Williams P.M.; A Novel Method for Real Time Quantitative RT-PCR; Genome Research 1996, 6:995-1001

Readings 1, 2, and 3 available on PE Applied Biosystems website
www.pebio.com

The following are real-time QPCR assays for the determination of gene copy number changes. Additional assays will be made available as they are developed.

ApoB Molecular Beacon Assay

Primer and Probe Sequences

ApoB accession Number: X15191

Amplicon: Start 762 bp; End 842 bp. Size: 80 bp

ApoB 762 F ATC-TCA-gCA-CgT-ggg-CTC

ApoB 842 R TCA-CCA-gTC-ATT-TCT-gCC-TTT-g

ApoB 794 P JOE-CgC-gAT-g-CCA-ATg-gTC-ggg-CAC-TgC-TCA-A-CAT-CgC-g-QSY (dark quencher)

Instrument: PE Applied Biosystems 7700, Sequence Detection System

Thermal Cycling Conditions:

Temperature	Time	# of Cycles
95° C	3 minutes	1
95° C	15 Seconds	40
60° C	1 minute	40

Reagents	Concentration	Total uL per reaction
Taq Buffer	10X	5 uL
ddH ₂ O	-----	23.4 uL
MgCl ₂	25mM	8 uL
dNTPs	100mM	0.1 uL
ApoB 762 F (10uM)	300nM	1.5 uL
ApoB 842 R (10uM)	300nM	1.5 uL
ApoB 794 P (10uM)	200nM	1 uL
Taq 2000	-----	0.5 uL
DNA 10ng/uL	50ng	5 uL

C-myc Molecular Beacon Assay

Primer and Probe sequences

C-myc Accession Number: L00038

Amplicon: Start 582 bp; End 657 bp. Size: 75 bp

C-myc 582 F TTT-CTg-ACT-CgC-TgT-AgT-AAT-TCC-A

C-myc 657 R CTC-TgC-ACA-CAC-ggC-TCT-TC

C-myc 614 P FAM-CCg-gAT-g-ACA-gAg-ggA-gTg-AgC-ggA-Cgg-TTg-CAT-CCg-g-QSY (dark quencher)

Instrument: PE Applied Biosystems 7700, Sequence Detection System

Thermal Cycling Conditions:

Temperature	Time	# of Cycles
95° C	3 minutes	1
95° C	15 Seconds	40
60° C	1 minute	40

Reagents	Concentration	Total uL per reaction
Taq Buffer	10X	5 uL
ddH ₂ O	-----	23.4 uL
MgCl ₂	25mM	8 uL
dNTPs	100mM	0.1 uL
C-myc 582 F (10uM)	300nM	1.5 uL
C-myc 657 R (10uM)	300nM	1.5 uL
C-myc 614 P (20uM)	200nM	0.5 uL
ApoB F (10uM)	300nM	1.5 uL
ApoB R (10uM)	300nM	1.5 uL
ApoB P (10uM)	200nM	1 uL
Taq 2000	-----	0.5 uL
DNA 10ng/uL	50ng	5 uL

INK4a Molecular Beacon Assay

Primer and probe sequences

INK4a accession number: U79633

Amplicon: Start 17 bp; End 93 bp. Size: 76 bp

INK4a 17AF ACg-TTC-ACg-TAg-CAg-CTC-TTC-Tg

INK4a 93 R Cgg-gCg-ggA-gAA-ggT-AgT

INK4a 51 P FAM-CAC-ggT-g-CAg-ATT-CgA-ACT-gCg-Agg-ACC-C-CAC-CgT-g-QSY (dark quencher)

Instrument: PE Applied Biosystems 7700, Sequence Detection System

Thermal Cycling conditions:

Temperature	Time	# of Cycles
95° C	3 Minutes	1
95° C	15 Seconds	40
60° C	1 Minute	40

Reagents	Concentration	Total uL per reaction
Taq Buffer	10X	5 uL
ddH ₂ O	-----	23.4 uL
MgCl ₂	25mM	8 uL
dNTPs	100mM	0.1 uL
INK4a 17AF (10uM)	300nM	1.5 uL
INK4a 93 R (10uM)	300nM	1.5 uL
INK4a 51 P (20uM)	200nM	0.5 uL
ApoB F (10uM)	300nM	1.5 uL
ApoB R (10uM)	300nM	1.5 uL
ApoB P (10uM)	200nM	1 uL
Taq 2000	-----	0.5 uL
DNA 10ng/uL	50ng	5 uL

P53 Molecular Beacon Assay

Primer and Probe Sequences

P53 accession Number: X00879

Amplicon: Start 50 bp; End 140 bp. Size: 110 bp

P53 50 F CTg-TgC-AgT-TgT-ggg-TCA-gC

P53 140 R ACC-TCC-gTC-ATg-TgC-TgT-gA

P53 86 P FAM-CCg-gAT-g-ggA-gCC-gTg-TCC-gCg-CCA-T-CAT-CCg-g-QSY (dark quencher)

Instrument: PE Applied Biosystems 7700, Sequence Detection System

Thermal Cycling Conditions:

Temperature	Time	# of Cycles
95° C	3 minutes	1
95° C	15 Seconds	40
60° C	1 minute	40

Reagents	Concentration	Total uL per reaction
Taq Buffer	10X	5 uL
ddH ₂ O	-----	23.4 uL
MgCl ₂	25mM	8 uL
dNTPs	100mM	0.1 uL
P53 50 F (10uM)	300nM	1.5 uL
P53 140 R (10uM)	300nM	1.5 uL
P53 86 P (10uM)	200nM	1 uL
ApoB F (10uM)	300nM	1.5 uL
ApoB R (10uM)	300nM	1.5 uL
ApoB P (10uM)	200nM	1 uL
Taq 2000	-----	0.5 uL
DNA 10ng/uL	50ng	5 uL

PTEN Molecular Beacon Assay

Primer and Probe sequences

PTEN accession number: NM_008960

Amplicon: Start 1295 bp; End 1374 bp. Size: 79 bp

PTEN 1295 F ACA-ATC-ATg-TTg-CAg-CAA-TTC-AC

PTEN 1374 R CCg-ATg-CAA-TAA-ATA-TgC-ACA-AA

PTEN 1323 P FAM-CCg-gAT-g-AgC-Tgg-AAA-ggg-ACg-gAC-Tgg-TgT-AA-CAT-CCg-g-QSY
(dark quencher)

Instrument: PE Applied Biosystems 7700, Sequence Detection System

Thermal Cycling Conditions:

Temperature	Time	# of Cycles
95° C	3 minutes	1
95° C	15 Seconds	40
60° C	1 minute	40

Reagents	Concentration	Total uL per reaction
Taq Buffer	10X	5 uL
ddH ₂ O	-----	23.4 uL
MgCl ₂	25mM	8 uL
dNTPs	100mM	0.1 uL
PTEN 1295 F (10uM)	300nM	1.5 uL
PTEN 1374 R (10uM)	300nM	1.5 uL
PTEN 1323 P (20uM)	200nM	0.5 uL
ApoB F (10uM)	300nM	1.5 uL
ApoB R (10uM)	300nM	1.5 uL
ApoB P (10uM)	200nM	1 uL
Taq 2000	-----	0.5 uL
DNA 10ng/uL	50ng	5 uL