

### **Verification of Copy Number Aberrations (CNAs)**

To verify copy number gain or loss based on the outcome of the aCGH analysis, several quantitative methods can be employed: PCR, QPCR and FISH to name a few.

**PCR verification of CNAs:** RPCI BAC clones featured on arrays have unique markers that can be utilized to PCR confirm copy number changes in the test sample. These markers are found on the publicly available genomic databases (UCSC, NCBI and Ensemble) and include gene sequences, ESTs, STSs and miscellaneous unique sequences. A query using the suspected BAC clone(s) address (ie. RP11-237F24) will readily provide marker information contained within the BAC and allow selection of appropriate PCR primers. PCR verification is not sensitive enough to pick up one copy gain or loss, but is certainly useful for homozygous deletions and significant amplification. FISH verification on interphase nuclei or metaphase chromosomes is recommended for the minimal CNAs.

- 1 To verify a CNA using a PCR based assay in a test sample DNA, a multiplex PCR is setup to include a control marker and the suspected CNA marker. The amplicons for these markers should differ at least 100bp in size to adequately separate them on a gel. It is often advantageous to select a PCR primer that allows simultaneous verification of the CNA and a candidate gene residing within a BAC.
- 2 Sample DNA (1 microliter ~50ng) is transferred to a thin-walled PCR tube containing marker specific primers, Platinum Taq polymerase (Invitrogen), and PCR reaction buffer. The 25 $\mu$ l amplification reactions are carried out in a MJ Research Tetrad Thermalcycler using standard conditions of 30 cycles at 95 $^{\circ}$ C (denaturing), 56 $^{\circ}$ C (annealing), and 72 $^{\circ}$ C (extension), following a 2-minute pre-incubation at 95 $^{\circ}$ C.
- 3 After amplification, the products are loaded, separated and analyzed for the appropriate size bands on 2.5% agarose gels. Gel images are stained with SYBR Green I (BioWhittaker) and captured on Typhoon 8600 imaging system (Amersham). Copy number gains can be estimated using the ImageQuant software (Amersham), while the deletions do not demonstrate a PCR product.

**Quantitative-PCR (QPCR) verification of CNAs.** As with conventional PCR analysis, primer sequences are obtained from the genome databases and utilized on an ABI7900 sequence detection system (Applied Biosystems) using the QuantiTect SYBR Green PCR kit (Qiagen Inc.). The initial copy number of a sample is determined by analyzing the cycle-to-cycle change in fluorescence signal as a result of the amplification of template during PCR. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number. Increase in real-time fluorescence is measured and relative fold changes calculated using the  $2^{-\Delta\Delta C_t}$  method.(Livak and Schmittgen 2001) This approach is more expensive than conventional PCR, but has greater sensitivity and is quantitative. The complete protocol is included in the QuantiTect SYBR Green PCR kit.

[http://www1.qiagen.com/literature/handbooks/PDF/PCRAndReverseTranscription/KitsAndEnzymes/PCR\\_QT\\_Probe/1028037HBQuantiTectProbePCR.pdf](http://www1.qiagen.com/literature/handbooks/PDF/PCRAndReverseTranscription/KitsAndEnzymes/PCR_QT_Probe/1028037HBQuantiTectProbePCR.pdf)

For both assays, a cytogenetically normal human DNA is used as a reference control. DNA can be selected from the Coriell cell repository healthy normal control collection.

**FISH validation of CNAs:** Slides of metaphase chromosomes prepared from cultured cells or interphase tumor nuclei isolated from tissue samples, can be used as FISH targets for CNA validation using a modification of the methods described previously.(Chernova and Cowell 1998;Matsui et al. 1979)

1. DNA from the implicated RPCI BAC are isolated using standard alkaline lysis procedures and can be utilized as FISH probes by labeling with digoxigenin-11-dUTP using the DIG-Nick Translation Mix (Roche) or with Biotin-16-dUTP using the Biotin-Nick Translation Mix (Roche) as described.(Sait et al. 2002)
2. The hybridized probes are detected using anti-digoxigenin-fluorescein Fab fragments (Roche) or Avidin-Rhodamine (Roche).
3. Slide preparations are viewed using a fluorescent microscope (Nikon) with the images captured and adjusted for signal intensity with Easyfish software (ASI). For each sample, twenty-five nuclei are scored, with an additional twenty-five examined in cases where greater than 2 of 25 nuclei demonstrate a different number of FISH signals.