

Critical Parameters and Troubleshooting

BAC clone selection

The BACs selected for array fabrication must be judiciously chosen for a number of reasons. The reagent cost alone, from selection to printing, approaches \$15.00 per clone. Selection of redundant, discrepant or multi-site clones will dilute the overall array resolution, and significantly increase the overall costs. Investigators must be aware that the human genome databases which align and map the RPCI-11 BAC clones to the sequence, is an evolving database with updates and 'new builds' that may change a BACs position or inclusion as a unique clone. It is advisable to choose BACs that map to unique positions from two different databases (i.e. UCSC, NCBI and ensemble), and are confirmed by FISH or sequence alignment of both BAC ends.

BAC clone preparation

High quality, high molecular weight BAC DNA preparations are critical for successful LM-PCR amplifications. We have found that alkaline lysis, phenol:chloroform based procedures do not provide suitable templates for MseI digestion and adapter ligation. Our experience indicates that the Qiagen plasmid DNA isolation kit, or other similar column-based chemistries, works best with the LM-PCR procedure described. It is important not to shear the DNA during preparation and storage, as this will have an effect on the quality of LM-PCR products. Major causes of DNA damage include repeated freeze/thawing, introduction of nucleases and over manipulation of the DNA.

Printing solutions

The correct final concentration of One-Phor-All Buffer Plus is important for optimal MseI digestion and subsequent ligation of primers. All buffers and reagents should be prepared fresh and used before the date of expiration. The process should proceed as quickly as possible from MseI digestion through LM-PCR. The LM-PCR and second round PCR products can be safely stored for further processing at 4C. PCR products are adequately resuspended in 20% DMSO and eventually equilibrate to a ~25% final DMSO concentration upon storage. The products are very difficult to resuspend in DMSO concentrations of 30% or higher, and result in precipitates and large diameter spots when printed on glass slides. The DMSO printing solutions are denatured, have low evaporation rates and can be stored at 4C for several years. Each second round PCR amplification of LM-PCR products can generate enough product to print over 3,500 slides in triplicate (using the slides, pins and arrayer described above).

Quality of arrays

An extremely critical parameter for optimal BAC aCGH analysis is the quality of the arrayed slides. It is important to use slides that allow the uniform deposition and retention of printing material, have low autofluorescent characteristics and the ability to maintain their chemical properties over several lots. It is best to use slides that do not require lengthy post-print processing, as any residual from this processing will be detected as artifactual signal while scanning the image. When etching identifiers on un-barcoded slides, be careful not to introduce glass chards on the surface as they will scratch the glass and also prevent adequate sealing to the hybridization chamber/coverlip. Etching should

be performed after the slides are spotted, with any chards gently removed by a compressed air stream. It is recommended to store the BAC arrayed slides in a desiccated environment, at room temperature out of direct light. The printing parameters are different for every slide type, arrayer, printing solution and pin combination used. It is important to optimize these parameters and adjust them as necessary. The most critical parameters include: relative humidity, temperature, pin speed, contact time and pitch. Generally speaking, smaller spots are achieved by decreasing humidity, contact time and pitch while increasing temperature and pin speed

Specimen preparation

The sample used to prepare the test DNA should be of high molecular weight. Short nucleic acids strands are problematic for microarray studies in that it is difficult to incorporate enough fluorescent molecules for the signal to be detected. In addition, the standard fluorescent labeling technique described can bias certain nucleic acid fragments over others. When using DNA from FFPE or improperly stored samples, it is recommended that the degree of DNA degradation be assessed using gel electrophoresis. Typically, the greater the percentage of low molecular weight DNA (<10kb), the greater the noise observed in the aCGH analysis. It is also important to use DNA that contains at least 50% cells carrying a suspected genetic imbalance, as contaminating normal tissue or heterogeneity in the sample lead to decreased sensitivity. Collection of tissue from tumor blocks by Laser Capture Microdissection (LCM) can remove normal cells and allow a more focused analysis of the disease site. It is also important to label at least 500ng of DNA to achieve appropriate signal intensity across all elements. Lower amounts can be used, but the number of spots that do not meet an appropriate signal:noise threshold will increase, resulting in fewer analyzable clones. If the DNA yield is <500ng, amplification of sample is an appropriate option.

Hybridization

Hybridization to the BAC arrays is most effective when the hybridization cocktail is circulating. This can be accomplished by automated hybridization stations or rocking/rotating incubators. Repeat-element regions are blocked during the hybridization by Cot-1 DNA and Yeast tRNA, but must be monitored for effectiveness. If the repeat-element regions are not adequately blocked, the resultant aCGH profile will have suppressed copy number ratios, potentially masking real genomic aberrations. A good indicator of effective blocking is to gauge how the BACs representing the X and Y chromosomes perform as part of the sex-mismatch hybridization. If the DNA samples co-hybridized are female:male, then the resulting female aCGH profile should theoretically show 2 copies of X and no copies of Y (except for BACs representing the pseudoautosomal regions) . Significant deviation from the expected copy number ratios can indicate inadequate blocking or other hybridization related events that need to be addressed.

Washing and Scanning

Good fluorescent signals with low surrounding background is a critical feature for spot identification in aCGH. It is very important to proceed through the washing and scanning steps quickly to avoid decay in signal strength. Fluorescent dyes can be difficult to work

with in extreme environmental conditions (high ozone) and also have photobleaching properties that necessitate quick signal measurements. If the slides dry out at anytime during the hybridization or wash process, the resultant image is usually affected with severe background. In addition, any impurities/sediment in the wash solutions can leave artifacts that eliminate many spots on the imaged array. Care should be taken not to handle the slides with bare hands or powdered gloves, as the residual oils and powder are easily detected by conventional laser scanners. Laser power or 'gain' should be adjusted to give consistent, raw background levels of 50-150. Attempts to artificially increase signal strength by adjusting the laser usually results in lower signal:noise ratios.