BioSpotlight

Blocking and Melting: the Double Life of an Oligo

The detection of low levels of DNA sequence mutations in a background of mostly wild-type DNA is important for diagnostic tests of early-stage cancer and prenatal diseases. While standard genotyping methods like sequencing are not sensitive enough to detect variant alleles at <10% of wild-type levels, greater sensitivity can be obtained using PCR assays. This is achieved by specific amplification of the mutant allele relative to wild-type DNA using a primer whose 3' end matches the mutant allele, as is done in the amplification refractory mutation system and mutant allele-specific method. Detection sensitivity is limited, however, by background amplification of the wild-type allele due to low-level binding of the mutant-specific primer to wild-type allele. In the method of allele-specific competitive blocker PCR (ACB-PCR), this background can be suppressed by combining the mutant allele-specific primers with blocking probes that specifically suppress amplification of the wild-type allele, but when detection below 0.1% is attempted, false positives occur. In this issue, researchers in the laboratory of C. Wittwer at the University of Utah (Salt Lake City, UT) have increased the sensitivity of the qPCR

version of ACB-PCR to $\geq 0.001\%$ by enlisting the wild-type blocking probe to also function as a probe for melting analysis after completion of ACB-qPCR amplification. By carrying out asymmetric PCR amplification using an excess of reverse primer and the blocking probe relative to the mutant-specific primer, greater specificity could be obtained, though with a decrease in PCR efficiency that was compensated for by increasing total cycles. In addition, the asymmetric amplification produces more of one strand, whose melting signature can then be determined using the excess of the wild-type blocking probe. This prevents false positives due to primer dimers and nonspecific PCR products, even in the presence of a 10^5 -fold excess of wild-type DNA. The method is rapid; works on any thermocycler with melting analysis capability; and dual hybridization or molecular beacon probes can also be used as the blocking probes.

See “Rare allele enrichment and detection by allele-specific PCR, competitive probe blocking and melting analysis” on page 311.

Vaccinia Virus Vehicles

Vaccinia virus (VACV) has the ability to incorporate large DNA inserts and grow in a wide variety of cell lines, making it a popular delivery vehicle for expression of foreign proteins in mammalian cells. One drawback to the more widespread use of VACV has been the need for time-consuming procedures in preparing and isolating the recombinant viruses. Standard methods require generation of a plasmid or PCR product carrying the protein-coding sequence of interest flanked by viral sequences, transfection of this plasmid, homologous recombination with the replicating viral DNA, and finally, isolation of the recombinant viruses. Since only 0.1% of virions undergo the desired recombination, the final isolation of recombinant VACV often requires a series of selection steps. While the population of viral recombinants can be enriched by inclusion of antibiotic resistance genes, this requires several rounds of passage and the marker is often retained in the final virus. Other methods—such as transient dominant selection using mycophenolic acid or thymidine kinase negative and positive selection—require specialized cells for virus isolation, serial passage, or use of potentially mutagenic agents that can affect host cells. To circumvent these issues and simplify recombinant VACV isolation, White et al. of Arizona State University (Phoenix,

Schematic representation of the coumermycin-PKR system used to rapidly select vaccinia virus.

AZ) describe a new method in the current issue that uses coumermycin—an antibiotic capable of inhibiting bacterial DNA gyrase, which is essential for cell division—and a coumermycin-sensitive VACV. Coumermycin is relatively innocuous to eukaryotic cells, so special cells are not required for growth or virus purification. The parental virus, VACVΔE3L::GyrB-PKR, expresses the coumermycin dimerization domain of *Escherichia coli* gyrase B fused to the

catalytic domain of mammalian dsRNA-dependent protein kinase, PKR. The gyrase domain binds coumermycin in a 2:1 ratio, facilitating dimerization of the PKR kinase domain and activation of endogenous PKR, thereby inhibiting protein synthesis. Homologous recombination then replaces the GyrB-PKR region with a protein of interest, leaving recombinants resistant to the effects of coumermycin while replication of the parental virus is inhibited. The authors demonstrate that the VACVΔE3L::GyrB-PKR system requires fewer rounds of selection passage, eliminates the selection marker, allows for growth in a variety of cell types, and uses an antibiotic that is not toxic to eukaryotic cells, making the system an advantageous option when selecting for recombinant VACV.

See “Use of a negative selectable marker for rapid selection of recombinant vaccinia virus” on page 303.

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BioTechniques 50:277 (May 2011)
doi 10.2144/000113660

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