sequencing (and other PCR-based approaches) are starting to give way to next generation sequencing (discussed later), particularly when analysis of large genes or multiple genes is required. Still, 36 years after its Nobel-worthy invention by Frederick Sanger, Sanger sequencing is still considered the "gold standard" for sequence determination.

- *Pyrosequencing*: This approach takes advantage of the release of pyrophosphate when a nucleotide is incorporated into a growing DNA strand. Like Sanger sequencing, it is performed on PCR products using a single sequencing primer, but instead of terminator nucleotides it involves cycling individual nucleotides (A, C, T, or G) one at a time into the reaction. If one or more nucleotides are incorporated into the growing strand of DNA, pyrophosphate is released and participates in a secondary reaction involving luciferase that produces light, which is measured by a photo detector. Pyrosequencing is most often used when testing for particular sequence variants and is more sensitive than Sanger sequencing, allowing for detection of as little as 5% mutated alleles in a background of normal alleles. For this reason, it may be used to analyze DNA obtained from cancer biopsies, in which tumor cells are often "contaminated" with large numbers of admixed stromal cells.
- *Single-base primer extension.* This is a useful approach for identifying mutations at a specific nucleotide position (e.g., an oncogenic mutation in codon 600 of the *BRAF* gene). An interrogating sequencing primer is added to the PCR product, which binds just one base upstream of the target. Differently colored terminator fluorescent nucleotides are also added (corresponding to the normal and variant bases), and a single base polymerase extension is performed. The relative amounts of normal/variant fluorescence are then detected (Fig. 5-29). Like pyrosequencing, this technique is very sensitive, down to approximately 1% to 2% mutated alleles, with the obvious disadvantage of only producing one base pair of sequence data.
- *Restriction fragment length analysis.* This simple approach takes advantage of the digestion of DNA with endonucleases known as restriction enzymes that recognize and cut DNA at specific sequences. If the specific mutation is known to affect a restriction site, then the amplified PCR product may be digested, and the normal and mutant PCR products will yield fragments of different sizes. These can be identified as different bands following electrophoresis. Needless to say, this approach is considerably less comprehensive than direct sequencing but remains useful for molecular diagnosis when the causal mutation always occurs at an invariant nucleotide position.
- *Amplicon length analysis.* Mutations that affect the length of DNA (e.g., deletions or expansions) can be easily detected by PCR. As discussed earlier, several diseases, such as the fragile X syndrome, are associated with alterations in trinucleotide repeats. Figure 5-30 reveals how PCR analysis can be used to detect this mutation. Two primers that flank the region containing the trinucleotide repeats at the 5′ end of the *FMR1* gene are used to amplify the intervening sequences. Because there are large differences in the number of repeats, the size of the PCR products obtained from the DNA of normal individuals, or those with a premutation, is quite different and can be easily distinguished by gel electrophoresis. An important caveat is that this technique will fail if a trinucleotide repeat expansion is so large that it is beyond the amplification capacity of conventional PCR, a situation that is commonly seen in some trinucleotide repeat disorders. In such a case, Southern blot analysis of genomic DNA must be performed (see "Southern Blotting").
- *Real-time PCR.* A variety of PCR-based technologies that use fluorophore indicators can detect and quantify the presence of particular nucleic acid sequences in "real time" (i.e., during the exponential phase of DNA amplification rather than post-PCR). It is most often used to monitor the frequency of cancer cells bearing characteristic genetic lesions in the blood or in tissues (e.g., the level of *BCR-ABL* fusion gene sequences in patients with CML), or the infectious load of certain viruses (e.g., HIV, EBV). It can also be used to detect somatic point mutations in oncogenes such as KRAS and BRAF, an approach that has the advantage of avoiding the need for post-PCR analysis.

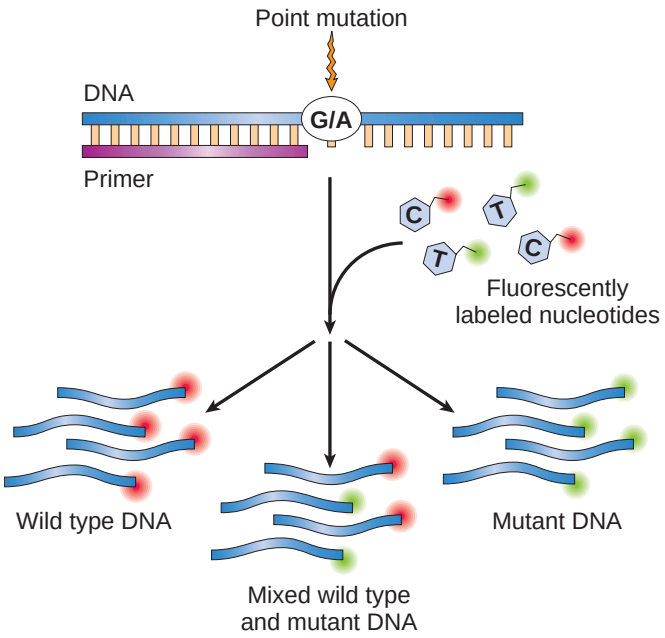

Figure 5-29 Single-base extension analysis of a PCR product, using a primer to interrogate a single base position. Nucleotides complementary to the mutant and wild-type bases at the queried position are labeled with different fluorophores, such that incorporation yields fluorescent signals of varying intensity based on the ratio of mutant to wild-type DNA present.

Molecular Analysis of Genomic Alterations

A significant number of genetic lesions involve large deletions, duplications, or more complex rearrangements that are not easily assayed using standard PCR methods. Such genomic-scale alterations can be studied using a variety of hybridization-based techniques.