Preparing PCR Product

When the template is a PCR-amplified product rather than cloned DNA, the basic sequencing process does not change. As in all DNA sequencing procedures, the quality of the data depends substantially on the quality of the starting DNA template. For high quality direct PCR sequencing, it is important to optimize all steps involved in PCR prior to sequencing. These steps include preparation of the starting template, the primer design used for amplification, and the cycling protocol. In general, the more initial PCR optimization, the less need for stringent post-PCR cleanup.

General Recommendations

Although the PCR tubes do not need to be sterilized or siliconized, it is recommended that the tubes be autoclaved, especially when dealing with small quantities of starting DNA template, as many copies of a sequence can be generated from very small amounts of starting DNA. To avoid contamination, use pipet tips that contain a physical barrier to prevent entrance of aerosols into the barrel. Use reagents that are guaranteed to be PCR grade. Most PCR protocols are performed with volumes from 25ul to 100ul. Volumes larger than 100ul are usually not recommended but if they are used, longer incubation times may be needed for adequate thermal equilibration. Use both a positive and negative control in any PCR experiment.

PCR Enzymes

To eliminate nonspecific products in PCR amplification, ABI recommends the use of AmpliTaq Gold DNA Polymerase (a modified version of AmpliTaq DNA Polymerase) in generating PCR templates for sequencing. This enzyme is designed as a "Hot Start PCR". It is completely inactive prior to cycling and is activated only when incubated at 95 C° for 9-12 minutes. This allows PCR setup on many samples to be performed at room temperature without concern for extension at misprimed sites. The hot start allows the DNA template to become thoroughly denatured prior to polymerase activity, eliminating the formation of non-specific products resulting from mis-priming and/or primer-dimer formation. This increase in sensitivity and specificity usually translates to good sequence data.

Primers for PCR Amplification

The sequences of the primers used for PCR amplification can have a major effect on the specificity and sensitivity of the reaction. When choosing two PCR amplification primers, the following guidelines should be considered:

- Primers should be typically 20-30 bases long.
- Primers should not contain bases complementary to themselves or with each other; complementarity at the 3' and 5' ends should especially be avoided; a 3'end

complementarity may promote the formation of an artifactual product referred to as a "primer-dimer"

- The melting temperatures™ should be 50-70 C°, so that high annealing temperatures can be used.
- if possible, primer pairs with similar Tm should be created.
- A 40% – 60% G+C content is often recommended for both primers; for primers with a G+C content much less than 40%, the primer sequence might need to be extended beyond 20 bases to keepthe melting temperature above the recommended lower limit of 50 C°.
- Avoid long runs of a single base, as this may cause secondary hybridization on targets that happen to contain the complementary motif.
- The 3' end should contain a G or C to stabilize this end.
- Avoid designing primers that have secondary structures.
- Use primer design computer programs when designing custom primers, they are good predictors of internal relationships.

Post PCR Clean-up

If the PCR conditions are fully optimized, and most of the primers and dNTP's are used up during amplification, it is possible to carry out direct sequencing, in other words, the PCR products do not need to be purified prior to sequencing. However, to obtain the cleanest sequencing data, it is usually recommended to clean up the PCR products. This is especially true with "dye terminator" cycle sequencing reactions (what RTSF uses). If primers are carried over from PCR, they can act as extension primers, resulting in the generation of an additional set of dye-labeled sequencing fragments making data interpretation difficult or impossible. Carryover of dNTP's will also affect the balance of the sequencing reaction, so their removal is desirable. The clean-up procedure should remove proteins, unincorporated nucleotides, and residual primers that can interfere with the sequencing reactions. Several commercial kits are available to purify PCR-amplified fragments if they are to be used for sequencing. Centricon-100 Micro-Concentrator columns (P/N N930-2119) are recommended by ABI, although they may not work as well for PCR products <125bases. Any method that removes dNTPs and primers should work. Post-cleanup PCR prducts should be examined on an agarose gel. If the products smear, or if more than one band is present, high quality sequence data is usually not obtained. Gel purification of the fragment can be accomplished using a low melting temperature agarose (SeaPlaque or SeaPrep from FMC BioProducts, Rockland, ME) and the desired product recovered using electroelution or other standard methods. After clean-up, the quantity of the PCR product should be verified. Inaccurate quantitation may lead to problems in the sequencing reaction. The PCR product should be diluted in water to the appropriate concentration.

Primer Selection for PCR Sequencing

The guidelines for primer selection for PCR sequencing are the same as for sequencing cloned DNA templates.