PCR Samples Purification Hints

Purpose

At Segolip, We endeavor for a seamless sequencing experience with our clients.

The outlined hints are therefore targeted to help our clients to generate high quality PCR Products for direct sequencing at the SegoliP Unit.

Introduction

It is quite possible to directly sequence a PCR product without first cloning the fragment. Indeed, there are some distinct advantages to this approach. However, you need to be aware of some of the drawbacks as well. Direct PCR sequencing is rarely successful unless you spend some time ensuring that you aren't falling into one of the many traps. This document will explain how to get sequence directly from a PCR product with a reasonable chance of success.

Getting the right product: Optimise your PCR

To get the sequence you want from a PCR product you must first ensure that you have amplified the correct sequence, and that your PCR product is of sufficient quantity for sequencing. The best way to achieve this is to optimize the PCR. Optimisation entails many things, including:

(1) Correct design of your PCR primers.

(2) Ensuring the template DNA is of sufficient quality and quantity.

(3) Adjusting the concentrations of particular reaction components.

(4) Adjusting the PCR cycling parameters.

There are many documents available on the web describing PCR optimization. We recommend the following: http://www1.qiagen.com/literature/brochures/pcr/

Confirm the existence of your product

1. Do not submit a PCR product for sequencing before confirming the existence of your product on an agarose gel.
2. Stain your Agarose gel with Gel red for visualization of your products under UV light.

   All but the smallest products should be visible.

Purification of the PCR product: you must remove all residual PCR primers and unincorporated nucleotides etc.

1. All PCR products must be purified. Do not give them to us straight out of the thermal cycler. Excess primers, salts, or Taq polymerase, interfere with the sequencing reaction. Purify the PCR product with an appropriate purification method.
2. There are many ways to purify a PCR reaction prior to sequencing. There are Several purification kits available in the market for this purpose. We recommend the QIAquick PCR Purification Kit (cat#28104 or #28106) **if** your PCR product appears as a single band on an agarose gel.

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3. However, if your PCR product appears as multiple bands on an agarose gel, you **must** gel-purify the desired band. Multiple bands within the sequencing template may give unreadable sequence. Some extraneous bands may appear low intensity to you, but could easily ruin the sequencing run. Gel-purification not only eliminates illegitimate PCR products it also removes the extraneous primers and nucleotides etc. For gel-purification we recommend you use a QIAquick Gel Extraction Kit (cat#28704 or #28706).
4. Do an additional wash! We recommend that you do one extra wash during the kit purification process, whether or not it is an option in the kit protocol.
5. Elute your DNA template in very clean dH_2O (autoclaved, deioinised, distilled water). *You can purchase high quality water for elution, e.g. Nuclease-Free Water (cat# P1193) from Promega.* **Do not elute with TE! EDTA chelates the magnesium ions required for our enzyme to work**. Sequencing primers that are stocked in TE can be diluted with dH_2O if the dilution factor is >5.

If the PCR primer(s) will also be used as sequencing primer(s), make sure they match our conditions

1. You may be able to adjust your PCR conditions to optimize reactions but unfortunately, we cannot do that at SegoliP Unit. Samples are processed in large numbers at a time, and we must use consensus conditions throughout.
2. Please make sure your primer(s) are appropriately designed for automated sequencing. The ideal annealing temperature for Taq polymerase in the BigDye sequencing Chemistry is 50^{o}C.
3. Ensure the primers have little tendency for self primer-dimer formation. See http://seqcore.brcf.med.umich.edu/doc/dnaseq/primers.html for complete information on primer design for DNA sequencing.

Double-check the template concentrations

1. PCR products are generally smaller and thus more effective sequencing templates than plasmids. Consequently, they don't need to be as concentrated as plasmid templates. However, sufficient material must be used to obtain good sequencing data.
2. Quantify your PCR product with a Nanodrop spectrophotometer. Alternatively, you can run a small quantity of your product on an analytical agarose gel and compare the band with DNA markers of known amounts. After estimating the concentration, dilute your PCR product to the appropriate concentration in very clean dH_2O (autoclaved, deioinised distilled water). *You can purchase high quality water for dilution, e.g. Nuclease-Free Water (cat# P1193) from Promega.*
3. **Note**: Concentrations are very important in attaining the desired results! Please don't submit samples that are not within the recommended concentrations! Dilution of multiple samples with different concentrations takes up too much time and this may affect the turnaround time to getting your results.

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The amount of template needed to sequence a PCR product depends on the size of the PCR fragment:

Template size	Amount needed (per reaction)	Volume needed (per reaction)*
100-200 bp	25-30 ng	1 μl
200-500 bp	30-100 ng	1 μl
500-1000 bp	100-250 ng	1 μl
1000-2000 bp	250-500 ng	1 μl
> 2000 bp	500-600 ng	1 μl

*Please submit at least 10 μl in case the sequencing reaction needs to be repeated. *Keep in mind that the above numbers are guidelines, and that some trial and error may be needed to optimize your sequencing reaction.*

Summary

- Optimize your PCR.
- Check quality of product on an agarose gel.
- Purify the product (gel purify if the product contains multiple bands). Elute in water.
- Determine concentration of the purified product.
- Dilute the product in water to the appropriate concentration.

Other useful links

The QIAGEN Guide to Template Purification and DNA Sequencing

Direct Sequencing of PCR Products

PCR Optimization - Reaction Conditions and Components

Qiagen Guide to Template Purification and DNA Sequencing