REV. 01/2024

DNA Sanger Sequencing
Not Purified PCR product quantition

As many of you know, the DNA Sanger Sequencing Service receives materials already diluted to a specific concentration, so the way a sample's concentration is evaluated is a critical step.

While it is appropriate to estimate a plasmid's concentration using Qubit, NanoDrop or a spectrophotometer, not-purified PCR fragments contain free primers that will influence the values you obtain using these methods, thus they are not indicated for this purpose.

The best and cheapest way to estimate the concentration of a not-purified PCR fragment is by agarose gel electrophoresis.

Amount Estimation

In order to obtain an accurate estimation of the DNA concentration, it is essential that the amount of DNA loaded on the gel is comparable to the amount of a known standard in a way that similarly intense bands are observed.

Figure 1 shows 3 not-purified PCR fragments of different size and unknown concentration. The concentration of each band is estimated by comparing the intensity of the band of interest with the intensity of the marker's band closest in size.

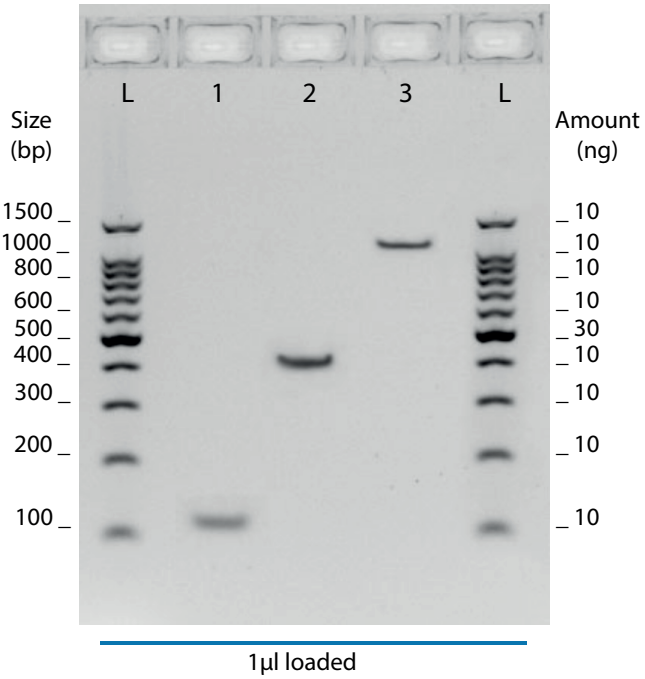

Figure 1: 1 µl of a 100bp DNA Ladder from Promega (L) was loaded on 2% agarose gel next to 3 different not purified PCR fragments (1,2,3) of varying size and unknown concentration (1 µl loaded in each case). Sample concentration is estimated based on band intensity. Sample 1 - 10 ng/µl, Sample 2 - 10 ng/µl, Sample 3 - 10 ng/ul.

Sequencing Conditions

In the table below are summarized the requirements for DNA sequencing of not-purified PCR fragments.

Please note that the Ready-To-Go format is not possible with not-purified PCR fragments as the primers still present in the sample would interfere with the sequencing reaction.

Sample Requirements (Standard Format Only)		
Sample size	**Sample concentration**	**Volume to be provided**
Not purified PCR product	20-50 ng/ ul	15 ul

cogentech

DNA Sanger Sequencing

REV. 01/2024

DNA Sanger Sequencing
Dealing with HairpinLoop

In recent years, the use of RNA interference (RNAi) has emerged as a powerful tool for the study of gene function in mammalian cells. Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNA), which are used to silence the expression of specific target genes, contain RNA sequences that form tight hairpin loops. Hairpin loops are also present in single guide RNAs (sgRNAs) used for CRISPR/CAS9 genome editing.

The corresponding DNA sequences (for example cloned in expression plasmids) form less stable hairpin loops than RNA sequences but still can pose steric hindrance for elongation of the DNA Polymerase used in Sanger Sequencing Protocols.

Sequencing hairpin loops

In line with these theoretical considerations, our experience shows that DNA sequencing through hairpin structures can be quite tricky. Efficient denaturing of all DNA molecules in the Sequencing reaction might not be easily obtained using the standard Sequencing protocol. In those cases where denaturation and thus resolution of the hairpin structure is not complete, the elongation of the DNA Polymerase might fail. The result could be a total failure or an incomplete Sequence read.

Our Facility developed a specific protocol to solve the problem of "difficult templates" containing hairpin loops, which is based on a mixture of BD v3.1/dGTP and DMSO/Betaine. This protocol is now routinely used in our facility.

However, in some cases, when the hairpin loop is very tight, it is necessary to **reduce the complexity of the template** to resolve the structure. Reduced complexity can be obtained by either:

- linearization of the plasmid
- amplification of the target regions by PCR
- specific restriction digest to break the loop, as shown in Figure 1 taken from D.C. Ducat et all; Biotechniques. 2003 Jun; 34(6): 1140–1144.

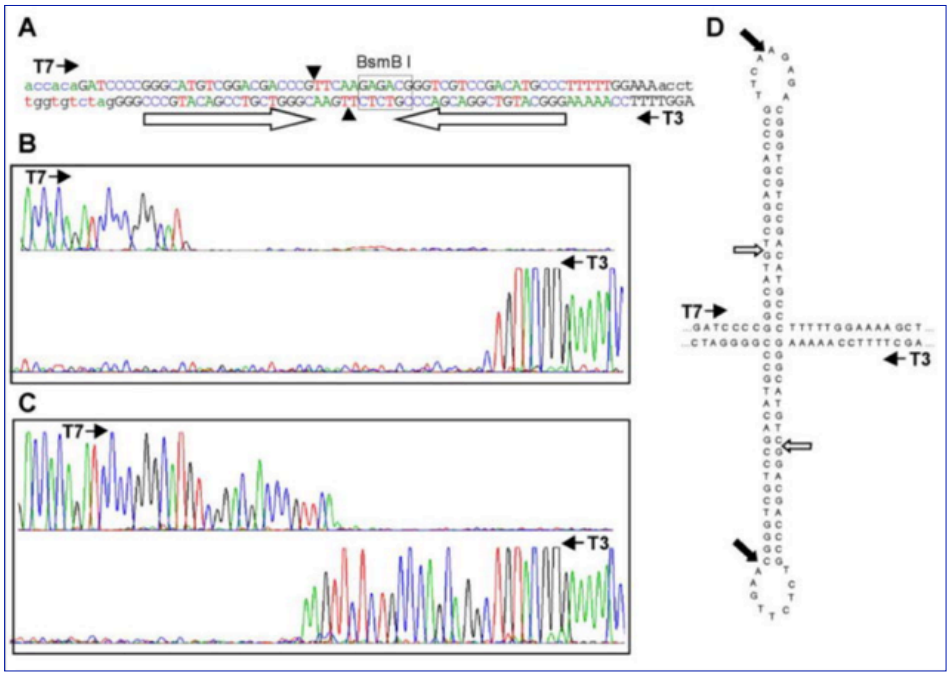

Figure 1. *Sequence analysis of the pSUPER-BRG1 siRNA expression plasmid. (A) Capital letters indicate the sequence of the DNA insert and lower case letters indicate flanking sequence from the pSUPER vector. Open arrows mark the inverted repeat sequences. A BsmB I recognition site (boxed) directs cleavage at sites indicated by arrowheads within the intervening "loop" region. Filled arrows indicate the direction of sequencing reactions using T7 and T3 primers. (B) DNA sequencing chromatograms of the undigested pSUPER-BRG1 plasmid using T7 and T3primers. (C) DNA sequencing chromatograms of the pSUPER-BRG1 plasmid after digestion with BsmB I. (D) DNA secondary structure predicted to occur within the siRNA encoding region due to sequence complementarity of the inverted repeats. Positions of premature termination of sequencing reactions are indicated with open arrows. Solid arrows indicate run-off termination of sequencing reactions at the template ends following digestion with BsmB I.*

For "difficult" templates containing hairpin loops it is especially important to provide us with **clean and correctly concentrated templates** to avoid addition of further challenges to the sequencing reaction.

In addition, we suggest that you indicate in the **"chemistry"** field of the request form whenever you submit a "difficult" template and that you write in the **"Comments"** field if you are using plasmid coding for shRNA, siRNA or sgRNA, so we can better analyze your results.

REV. 01/2024

DNA Sanger Sequencing
Direct Sequencing of PCR Products

Direct PCR fragments sequencing is a common method to sequence genomic regions.
However, direct PCR sequencing is rarely successful unless you spend some time to create a good template.
How? Please, take a look to these tips.

Tips

1. If the PCR **primers** will also be the sequencing primer(s), make sure they **match our design conditions**.
   They should have:
   - Tm between 50 and 65 degrees
   - regions that cannot form secondary structures or promote dimer formation
   - G/C anchor at the 3' (at the 5' end is optional)
   - GC content of about 30-70%
   - at least 18-20 bp
   - annealing site at about 20-30 bp upstream of the region of interest
2. Load the PCR product into an **agarose gel**, to check for both size and specificity.
   It happens to create unexpected bands during PCR step if:
   - only one of your primers anneal to the sample, at both forward and reverse orientation. If you use the PCR primers also to sequence this PCR reaction, you will have a **failure** with one primer and a double signal with the other one;
   - PCR primer(s) anneal (also) to specific sites. The resulting bands could have the same size (extra bands are not distinguishable from the right band on an agarose gel*) or different sizes (extra bands are distinguishable from the right band only if their amount is detectable by agarose gel**).

   If you sequence these PCR products with the same primes used for the PCR amplification, you will have:
   - a diffuse, low-level **background** of illegitimate products **in both forward and reverse reactions**, that sometimes continues even longer than your fragment size (see **Figure 1a and 1b**);
   - **interfering peaks** for the **first nucleotides**, if a small, illegitimate product is present.

**If the presence of an unexpected band in your template is suggested only after sequence it, you can:*
- *cut the PCR fragments with enzyme(s) and look for the correct generated bands, to verify the data;*
- *use (semi)nested primers to re-amplify the PCR product, to confirm its identity and contextually eliminate any illegitimate products (we can directly sequence this new product).*

***If extra band(s) are visible on agarose gel (as a smear or sharp multiple bands), it is necessary to:*
- *optimize the PCR condition (by varying MgCl2 and/or Tm);*
- *design new primers.*

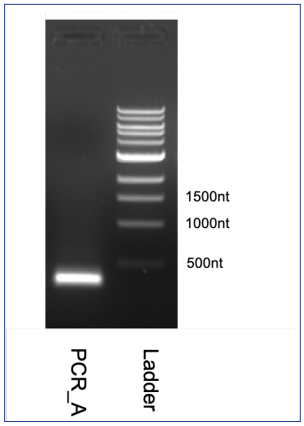

Figure 1a. *shows an 2% agarose gel loaded with 5ul of PCR_A product and 500ng (5ul) of 1Kb ladder NEB. No extra bands are visible*

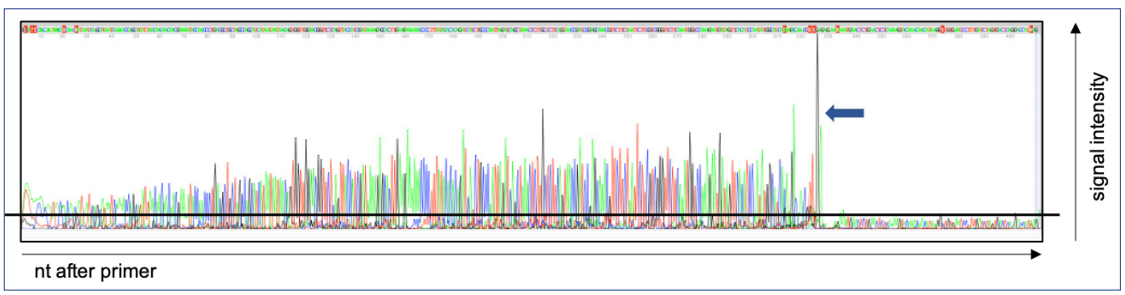

Figure 1b. *shows the electropherogram after PCR_A sequencing. The blue arrow shows the end of the major amplified fragment. The peaks with low signal intensity suggest the presence of a less abundant, longer aspecific band in the PCR_A, unless not distinguible from the expected one on agarose gel analysis. The horizontal black line indicates the backgound's intensity threshold*

REV. 01/2024

DNA Sanger Sequencing
Direct Sequencing of PCR Products

If the two primers are known to produce more than one band (for example if you want to amplify the region of genes that have also pseudogenes or isoforms), you can:

- **gel-elute** the PCR band you need to sequence, if the extra bands have different size;
- **clone** the PCR product in a plasmid and sequence many clones, if the extra bands show the same dimension.

3. Evaluate the **concentration** of the PCR product. Remember that you can use the spectrophotometer only with PCR purified by its primers. Otherwise, please see our 'Not Purified PCR product quantition' brochure: we take care of the extra primers removal by EXO I-SAP treatment.

Although the DNA sequencing reactions perform well within a range of concentration, an extra dilution could be necessary in those cases in which the PCR reaction shows the presence of inhibitors. It is possible to deduce this problem after sequencing: we will help you in doing an accurate troubleshooting to dilute the inhibitors (see **Figure 2a and 2b**).

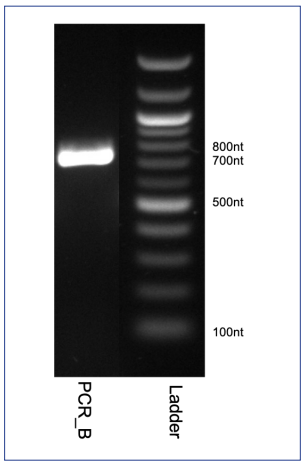

Figure 2a. *shows an 2% agarose gel loaded with 5ul of PCR_B product and 250ng (5ul) of 100BP ladder NEB.*

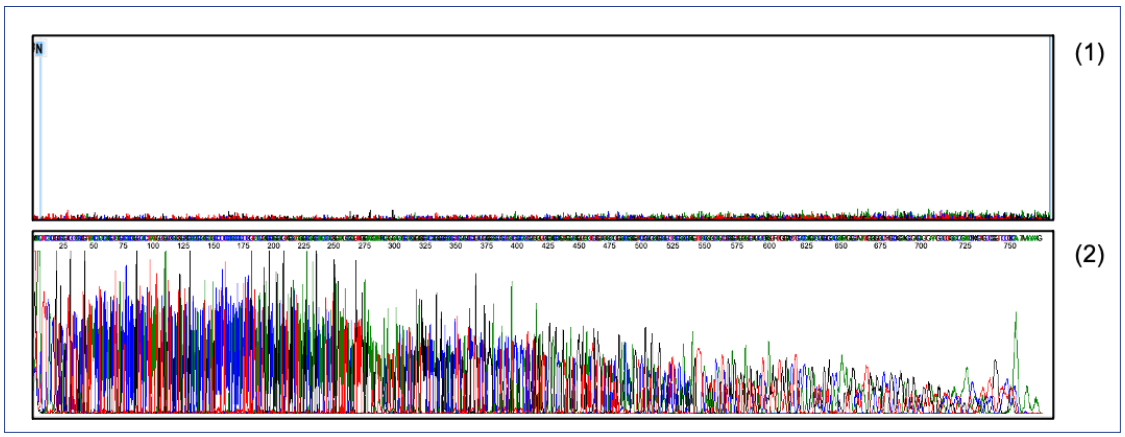

Figure 2b. *shows the electropherogram after PCR_B sequencing, in standard condition (1) and after dilution (2).*