

Preparing Cloned DNA

Fluorescent sequencing is a sensitive and complex procedure. Although the Quality of template DNA is one of the most important factors leading to good sequence data, many other items need careful attention. The following are guidelines for ensuring good DNA sequencing results.

Quality

The DNA sample should contain no other components except for the pure DNA molecules of interest. It should be free of residual RNA, genomic DNA, proteins, salts (10-20mM salt can inhibit a sequencing reaction!), chelating agents such as EDTA, organic solvents, dyes or any other contaminants from the media or from the cells.

Isolation

Select an isolation method that removes all of the contaminants mentioned above. The method should be reproducible, reliable and convenient. For plasmids, we have used Promega's Wizard Plus Miniprep DNA Purification System with very good results when sterile water is used in the elution step. Qiagen also provides a wide variety of products that yield excellent sequencing results. In addition to the commercially available kits, ABI has modified a mini alkaline-lysis/PEG precipitation procedure that is inexpensive and produces ample quantities of sequenceable template DNA.

Other template purification methods may also be satisfactory, but results are less consistent. These less consistent methods include the following:

- Alkaline lysis (classical)
- Alkaline lysis (no RNAase, no organic extraction)
- Boiling Mini-preps
- CsCl banding
- Some commercial glass bead/silica products

Whatever isolation method is used, redissolving the DNA in an appropriate volume of sterile water is recommended.

Growth Media

Selection of the proper bacterial culture media is important. In the enriched medium of TB, stationary phase is reached in about 8 hours and lysis starts after 16 hours. Growth in LB reaches stationary phase in about 10-12 hours and continues for 4-5 hours before cell lysis begins. Lysed cells release degraded genomic and plasmid DNA and yield plasmid DNA of reduced quality. Enriched media may also lead to elevated cell densities which may cause column or membrane

overloading. We have isolated many templates using Magnificent Broth Plasmid Growth Media (MacConnell Research, San Diego, CA, 619-452-2603). This media is a pre-mixed powder that is simply dissolved in water and autoclaved. Cells divide for up to 22 hours with no lysis or cell death occurring in that time. Whatever the media selected, it is important to provide adequate aeration.

Bacterial Host Strain

Host strain selection is important. Host strains such as DH1, DH5alpha, DH10B, and C600 are highly suitable for template purification, and consistently produce good results. Host strains MV1190 and XL1 Blue show some variability in result quality. Strain HB101 and its derivatives, including TG1, TG2 and the JM100 series, contain large amounts of carbohydrate released during lysis and generally do not work well. If these difficult strains are selected, care must be taken to remove carbohydrates (and proteins) from the crude lysates.

Quantitation

The isolated plasmid may be checked on an agarose gel (both cut and uncut) to verify that the DNA is of the predicted size. ALWAYS quantify the DNA using a spectrophotometer or fluorometer. The purity of DNA can be tested by measuring the absorbance at 260 nm and 280 nm. The ratio of 260 nm to 280 nm for pure DNA is in the range of 1.7 to 2.0. A higher ratio indicates high RNA content and a lower ratio indicates the presence of protein or phenolic impurities. The lab has a spectrophotometer which may be used by our clients.

The amount of DNA template used in a sequencing reaction will affect the quality of the data. Too much template makes data appear top heavy with strong peaks at the beginning of the run and fading as the run continues. Too little template DNA reduces the strength of the signal and the peak height (in some cases, the signal to noise ratio is so low that peaks cannot be called).

Avoid the use of a speed vacuum to concentrate the DNA. This technique will inadvertently concentrate any contaminating material present in the preparation. Ethanol precipitation (in the presence of a moderate concentration of monovalent cations) and resuspension in an appropriate volume of sterile water is preferred.

It is up to the customer to provide us with the proper DNA concentrations. We do not have time to dilute or concentrate your samples.

Clean Up of Failed Templates

- Purify the DNA by ultrafiltration through a column (i.e., Centricon 100).
- Extract DNA twice with 1 volume of chloroform or chloroform:isoamyl alcohol (24:1), removing all traces of chloroform.

- Precipitate with PEG (add 0.16 volume of 5M NaCl and 1 volume of 13% PEG; incubate on ice for 20 minutes; centrifuge at 4 C° for 20 minutes; rinse with 70% ethanol).

Difficult Templates

Occasionally we encounter template DNA which poses a problem because of its inherent properties. The following list describes the types of templates that may be difficult to sequence, and may require modifications to the standard chemistry for good results:

- Templates with a high overall GC-rich base composition (>62%)
- Templates with a high overall AT-rich base composition
- Templates with extremely AT-rich or GC-rich regions of sequence
- Templates with regions of pronounced secondary structure
- Templates with long homopolymer regions
- Templates with long repeats