

MICB ABI PRISM 310 SEQUENCING GUIDE

SEQUENCING OF PLASMID DNA

Plasmid DNA Preparation

Introduction:

- I have always used the classic "Alkaline Lysis" miniprep method to isolate plasmid DNA. (*See below*)
- If you prefer miniprep kits, then any commercial plasmid prep kit should be fine. ABI recommends Qiagen purification kits like the Qiagen QIAprep Spin Miniprep Kit (50) Cat.#27104 for plasmid minipreps. If you are using the Qiagen QIAprep Spin Mini-prep Kit **ALWAYS BOIL** the isolated DNA prior to quantitation. We have noticed that Qiagen Miniprep DNA is sometimes somewhat "insoluble" and boiling the sample resolves this problem. I have also successfully used Sigma's GenElute Plasmid Purification MiniPrep Kit, Product Code PLN-10 (10) or PLN-70 (70 purifications). No matter which method you use, remember to dissolve your sample DNA in ddH₂O and not TE as EDTA inhibits the ABI sequencing reaction.
- Finally, always use 100% ethanol for precipitations. Impurities in 95% ethanol will inhibit the sequencing reaction.

Alkaline Lysis Method (DD & DT Modifications)

Reagents Needed: Lysis Buffer 6.25 ml 2M Tris pH8 25mM 10 ml 0.5M EDTA 10mM 50 ml 0.5M Glucose 50mM 433.75 ml double distilled water Store refridgerated. NaOH / SDS 4 ml 5M NaOH 0.2M 2 ml 20% SDS 0.2% 94.67 ml double distilled water (prepare fresh, DO NOT place on ice)	Potassium Acetate Solution 300 ml 5M KAc 57.5 ml Glacial Acetic Acid 142.5 ml double distilled water Store refridgerated Phenol:Chloroform:Isoamyl Alcohol (25:24:1) buffer saturated Sigma-Aldrich Cat# P-3803 Chloroform:Isoamyl Alcohol (24:1) Sigma-Aldrich Cat# C-0549 or prepare RNaseA (Ribonuclease A) Sigma-Aldrich Cat# R-4875
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Preamble:

The solutions used in this mini-prep procedure are those of the large scale plasmid prep. In this mini-prep procedure various steps are combined and shortened to speed up the procedure. This method works very well to OK with most but NOT ALL plasmid / bug combinations. BEWARE! (ul = microlitre)

Procedure:

- Using a fresh overnight culture of the plasmid containing bacteria, add 1.5 ml of this culture to an eppendorf tube.
- Pellet the bacteria by microcentrifugation for 1 min and then aspirate off the culture media.
- Add 200 ul of the LYSIS BUFFER and resuspend the pellet by vortexing. Make sure that the pellet is totally resuspended.
- Add 200 ul of the NaOH / SDS solution and mix by inverting until the solution becomes clear.
- Add 200 ul of POTASSIUM ACETATE solution and vortex until a white precipitate forms.
- Microcentrifuge for 5 min at top speed (>12,000 rpm). Remove the liquid into a fresh eppendorf tube.
- Add 500 ul of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) and vortex vigorously.
- Microcentrifuge for 5 min and then very carefully remove 500 ul of the upper aqueous phase into a fresh eppendorf tube. Be particularly careful NOT to remove any of the organic interphase. Remember..... don't get greedy, leave behind 100 ul of the aqueous layer, there is plenty of DNA in the first 500 ul.
- To the aqueous layer add 500 ul of Chloroform:Isoamyl Alcohol (24:1) vortex vigorously, microcentrifuge for 2 minutes and remove 450 ul of the top aqueous layer into a fresh eppendorf tube. (This step will remove any contaminating phenol).
- Precipitate out plasmid DNA from the 450 ul aqueous layer by adding 500 ul of isopropyl alcohol and allowing the sample to sit at room temperature for 5 mins.
- Pellet plasmid DNA by microcentrifugation for 5 mins.
- Aspirate the isopropyl alcohol, recentrifuge briefly and reaspirate any trace amount of alcohol. Air dry the plasmid DNA pellets at room temperature for 20-60 mins.
- Redissolve the plasmid DNA in 50 ul of double distilled water. Add 1 ul RNase A (10mg/ml) to remove residual RNA.
- Quantitate DNA and prepare sequencing samples.

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Primer Selection

- Most commercially designed plasmid vectors have sequencing primers flanking the MCS (multiple cloning site) . The most common primers are listed below. Note that primers vary slightly by manufacturer.

T7 (17-mer) (Stratagene)	5' AATACGACTCACTATAG 3'
T7 (22-mer) (Stratagene)	5' GTAATACGACTCACTATAGGGC 3'
T3 (17-mer) (Stratagene)	5' ATTAACCTCACTAAAG 3'
T3 (20-mer) (Stratagene)	5' AATTAACCTCACTAAAGGG 3'
SP6 (Gibco-BRL)	5' ATTTAGGTGACACTATAG 3'
M13 forward (Stratagene)	5' GTAAAACGACGGCCAG 3'
M13 forward (Gibco-BRL)	5' CCCAGTCACGACGTTGTAAAACG 3'
M13 reverse (Stratagene)	5' GGAAACAGCTATGACCATG 3'
M13 reverse (Gibco-BRL)	5' AGCGGATAACAATTCACACAGG 3'
M13 (-20) (Stratagene)	5' GTAAAACGACGGCCAGT 3'

- If you need to prepare your own sequencing primer then YOU WILL NEED TO CONFIRM that your primer is suitable ie that it does not form primer-dimer pairs, that it does not self compliment, that it's Tm is not too low etc. This is best done using a primer analysis program. For more info see http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm and try their FASTPCR demo program. In general these are the preferred characteristics of your primer:

- 1)18-30 nt in length
- 2)40-60% GC content
- 3)Tm between 50°C-70°C
- 4)No primer-dimer pair formation particularly at the 3' end of the primer
- 5)Confirm that the primer is unique within your sequence

Remember that even though your primer meets all the above criteria THERE ARE NO GUARANTEES it will work. The ultimate test is the sequencing.

- If your sequencing primer is NOT ideal then you can try to increase the primer concentration in the sequencing reaction from 5 pmol to 10 pmol. You may also request a modified annealing temperature, Ta.

Sequencing Sample Preparation

TIPS:

1. If your plasmid preparation includes a phenol/chloroform extraction be careful to remove all traces of phenol and chloroform as these will inhibit the sequencing reaction.
2. Amount of plasmid DNA to use depends on the size of the plasmid. The larger the plasmid being sequenced the smaller the moles of plasmid present. Therefore use the following as a guideline:

plasmid size 2-3Kb	use 150 ng
plasmid size 3-5Kb	use 200 ng
plasmid size 5-8Kb	use 300 ng
plasmid size > 8Kb	use 400 ng
3. Pay attention to your pipetter. Monitor the pipetting to confirm that the amounts look correct.
4. Sequencing data will start 20-50+ nucleotides from the 3' end of the primer site.

Total volume of plasmid DNA and primer must be 3 ul. This should be placed in a 200 ul thin-walled PCR tube. The tube should be labeled with your initials and a sequential number ON THE SIDE of the tube. The tube and a completed SEQUENCING REQUEST FORM should then be brought to the Sequencing Room ON6042. Place the request form in the holder at the door and the tube in the RED rack located in the freezer.

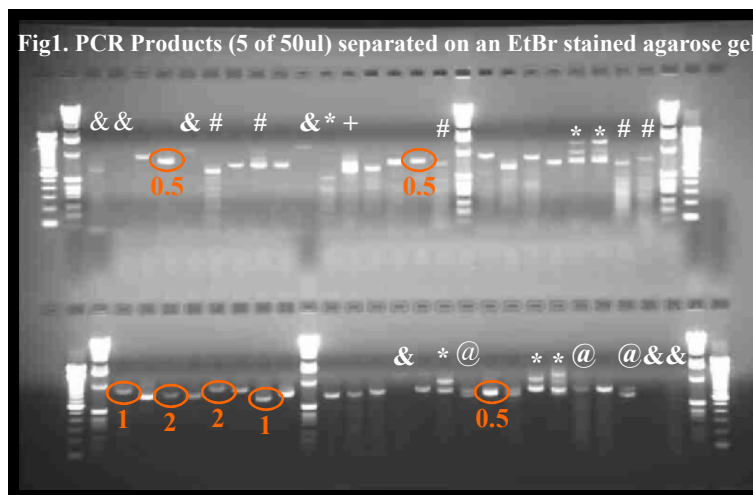
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SEQUENCING OF PCR PRODUCT DNA

PCR Product Analysis

Sequencing from PCR products is usually more problematic than sequencing from a plasmid. None-the-less by using a clean PCR product and a good primer excellent sequence will be obtained. The most important aspect of PCR product sequencing is to ensure that the PCR product is a pure, single band of sufficient quantity. This can be done by running part of the product on an 1-3% agarose gel, staining with ethidium bromide (EtBr) to view the PCR DNA (Fig 1). This gel should be loaded carefully as it will later be used to predict the relative amount of purified product that will be used for sequencing. You should avoid sequencing product where:

- 1) Multiple PCR product are observed (eg lanes labelled * in Fig 1)
- 2) Significant artifactual smears are observed (eg lanes labelled # in Fig 1)
- 3) Where the PCR product band is diffuse (eg lanes labelled @ in Fig 1)
- 4) Where the PCR product band is very faint (eg lanes labelled & in Fig 1)
- 5) One band – two products? (eg lanes labelled + in Fig 1)



Cleaning Up PCR Products

Gel Elution:

The “classic” method of purifying PCR products is to do GEL ELUTION. The band of interest is excised, PCR DNA separated from the agarose, ethidium bromide removed and purified PCR DNA reprecipitated. Commercial kits like Qbiogene’s Clean-Gene II (Cat. # 1001-400) are available for this purpose. Note that there is a size limit.

Alternatively, the PCR DNA band can be eluted into a well containing 5x TBE that has been cut out in front of the PCR DNA band. Ethidium bromide is then extracted from the PCR DNA using isoamyl alcohol and purified PCR DNA is then reprecipitated.

In both cases the procedure is relatively long and the amount of PCR DNA must be relatively large; however, if a PCR fragment MUST be sequenced and multiple bands are present then this may be the only way. Where multiple bands are NOT a problem then a quicker more efficient method of purifying the PCR DNA for sequencing is the ExoSAP-IT kit.

ExoSAP-IT Kit (US78200) for rapid efficient clean-up of PCR DNA:

from Amersham Pharmacia Biotech [Available from the MICB BioBar]

ExoSAP-IT Procedure:

To 5 ul of your PCR product add 1 ul *Exonuclease I* and 1 ul *Shrimp Alkaline Phosphatase*. Incubate at 37°C for 15 min and then 80°C for 15 min. You would then use 0.5-2 ul of this ExoSAP purified PCR product for sequencing. The amount is dependent on the intensity of the PCR product band on the ethidium bromide stained gel (5ul of a 50ul rxn). (See circled samples in Fig 1. Numbers below indicate microlitres (ul) ExoSAP purified PCR product to be used for sequencing)

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Sequencing Primer Selection

Q– Can I sequence using the same primers used to generate the PCR product?

A– Ideally you would want to use a nested primer distinct from the PCR primer; however, in many cases using the same primer will give good sequence. The primer (particularly its 3' end) must be able to bind the PCR product DNA strongly to initial elongation. If the end of the PCR product DNA obscured by tertiary folding and if the primer can not bind well to the template under sequencing conditions, then no sequence will be obtained. REMEMBER that elongation parameters in sequencing could be significantly different from those of the PCR. Ultimately the quality of the DNA, the quality of the primer and the sequencing elongation parameters will determine whether satisfactory sequence will be obtained.

Primer Selection

- If you need to prepare your own sequencing primer then YOU WILL NEED TO CONFIRM that your primer is suitable,- that it does not form primer-dimer pairs, that it does not self compliment, that its T_m is not too low etc. This is best done using a primer analysis program. For more info see http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm and try their FASTPCR demo program. In general these are the preferred characteristics of your primer:

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Remember that even though your primer meets all the above criteria THERE ARE NO GUARANTEES it will work. The ultimate test is the sequencing.

- If your sequencing primer is NOT ideal then you can try increase the primer concentration in the sequencing reaction from 5 pmol to 10 pmol. You may also request a modified annealing temperature, T_a .

Sequencing Reaction Setup

TIPS:

1. Purify your PCR DNA (see Page 1 of 3 Cleaning up PCR products)
2. Amounts of purified PCR DNA to use depend on the size. The larger the PCR product being sequenced the smaller the moles present. Therefore use the following as a guideline:

PCR product size 100-200bp	use 1-3 ng
PCR product size 200-500bp	use 3-10 ng
PCR product size 500-1000bp	use 5-20 ng
PCR product size 1000-2000bp	use 10-40 ng
PCR product size >2000bp	use 20-50 ng
ExoSAP-IT cleaned PCR product	use 0.5-2ul (See Fig.1 & ExoSAP-IT on page 1 of 3)

3. Pay attention to your pipetter. Monitor the pipetting to confirm that the amounts look correct.
4. Sequencing data will start 20-50+ nucleotides from the 3' end of the primer site.

Total volume of PCR DNA and primer must be 3 ul. This should be placed in a 200 ul thin-walled PCR tube. The tube should be labeled with your initials and a sequential number ON THE SIDE of the tube. The tube and a completed SEQUENCING REQUEST FORM should then be brought to the Sequencing Room ON6042. Place the request form in the holder at the door and the tube in the RED rack located in the freezer.

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BASICS ON ELECTROPHEROGRAMS

Overall Profiles

The three principle sequencing profiles are shown below.

Figure 1: Good Sequence Profile

Strong, but not excessive, peaks initially gradually decreasing in height.

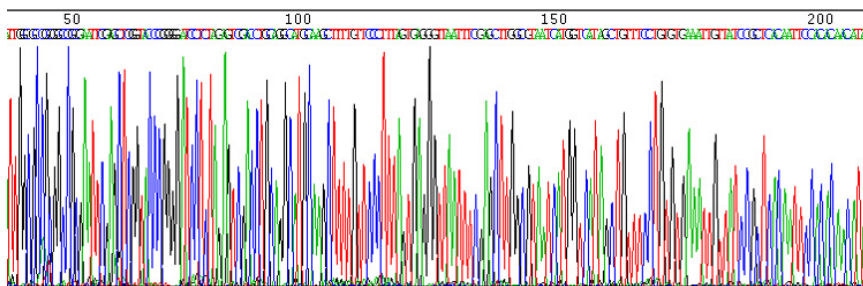


Figure 2: Too Much DNA Sequence Profile

Excessive peaks initially with a rapid decline after some 130 bp. The excessive initial peaks will tend to obscure the start of the sequence.

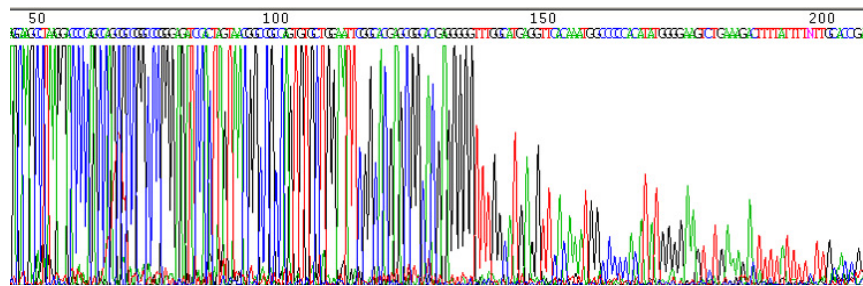
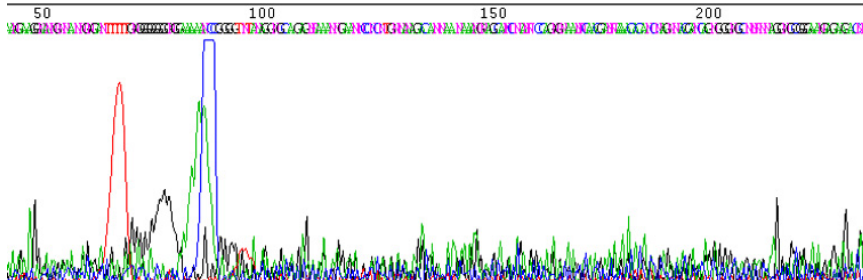


Figure 3: Failed Sequence Profile

Failure may be due to any of the factors listed:

1. Too little DNA template
2. Poor or degraded primer
3. Poor reaction setup and execution
4. Poor loading of sample on the sequencer
5. Problem with the sequencer

*For EXTERNAL samples we control for 3-5



T- Artifact

With the BIGDYE reaction mix there is a propensity to have a large T (red-peak) artifact in the 200-230 nt region. This artifact can be removed with repeated ethanol precipitations. The presence and intensity of the T-artifact peak is a good indicator of how well or poorly ethanol is being removed during ethanol purification of elongated sequencing fragments.

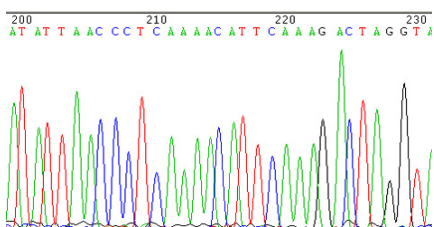


Figure 4: No T- artifact

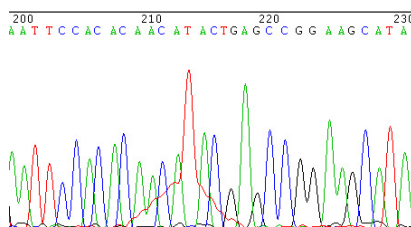


Figure 5: Small T- artifact

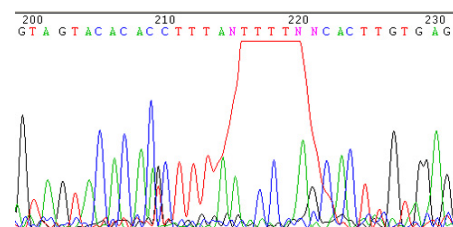


Figure 6: Large T- artifact

Assessing Ambiguous (N) Nucleotide

If tertiary structure causes specific sequence fragments to run faster or slower than predicted, the ABI software will generate an N.

This problem can easily be seen on the electropherogram and the correct nucleotides can be manually assigned.

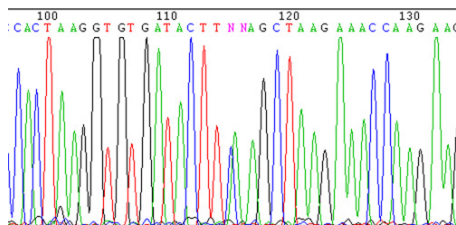


Figure 7: TNNAG is really TCAAG

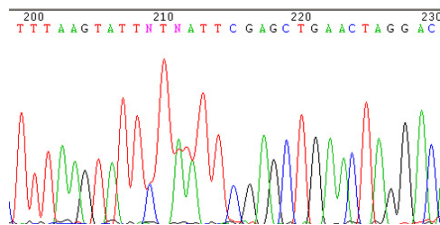


Figure 6: T- artifact obscured sequence ATTCTAATTC

SEQUENCING REQUEST FORM MICB RM ON6042

Name: _____ Phone #: _____ Account to Charge _____
Investigator: _____ Dept. _____ Bldg. _____ Rm# _____
Date Submitted (mm/dd/yy): ___ / ___ / ___ E-mail _____

TUBE LABEL _____

DNA Template: Single-Stranded DNA (50ng) Double-Stranded DNA (200ng) PCR Product (50ng)
Required Sequence:* 250 bases or less 250-350 bases maximum

For our use only. Do not complete: Liquid in Tube Too much Template T-artifact Other _____
Completed ___ / ___ / ___ Prep by: _____ Status: failed <200 200-300 300-400 >400

TUBE LABEL _____

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**Help us conserve machine time*