Document version 1.3, October 2004

DNA Sequencing Instructions

PLEASE NOTE: Before deciding to sequence by yourself you should consider to make use of a (commercial) DNA sequencing service. The price for such a service is currently constantly falling and is already yet a real alternative to in-house sequencing. The following text in this document just treats the *Taq*-cycle DyeTerminator procedure, because this is the most flexible approach. However, in some circumstances other procedures (e.g. DyePrimer) might be the better solution.

Criteria for cycle sequencing are more stringent than manual sequencing and many other molecular biological procedures. The success of sequencing reactions depends critically on having **high purity template** in the **correct amount**. That means template DNA should be free of proteins, RNA, genomic DNA, EDTA and other salts. The most important contaminants are bacterial proteins and cell wall carbohydrates, and organic solvents (ethanol, propanol, etc.).

Please follow these recommendations for **checking concentration** and purity of your template DNA:

- Measure DNA sample absorbance at 260 and 280 nm in a spectrophotometer. Nucleic acids have their peak absorbance at 260, with a 260/280 ratio of 1.8 for DNA. Estimate DNA concentration from OD_{260}, i.e. concentration (µg/ml) = OD_{260} X dilution factor X 50. Remember that OD260 can have contributions from DNA as well as contaminants.
- Run a sample of DNA on an agarose gel with a quantitative standard (e.g. lambda *Hind* III digest, where amount of DNA in each band is known). By comparing intensities of template band with comparable size bands in standard lane, estimate concentration of DNA. Compare this estimate to the OD_{260} estimate of DNA concentration. If OD_{260} estimate is significantly higher than gel estimate, it indicates contamination with RNA or RNA fragments. An RNase treatment, followed by phenol/chloroform extraction and precipitation would be recommended.

Sample and primer amounts for a cycle sequencing reaction for different types of templates are given below (Tab. 1):

Template Type	Size (kb)	Template amount/rxn	Primer amount/rxn	# Cycles
PCR product	0.1-2	10 ng per 100 bp	5-10 pmol	25
Plasmid	2-15	200-500 ng	5-10 pmol	25
Phage/Cosmid/P1	20-100	500-1000 ng	15-20 pmol	30
BAC	200	1000-2000 ng	20-25 pmol	30

Tab. 1: Template and primer amounts for a cycle sequencing reaction.

- **Template concentration:** Cycle sequencing reactions are made up in a final volume of 10-20 µL. If your DNA is not concentrated enough, you need to concentrate your template in a vacuum centrifuge.

For **preparation of the cycle sequencing reaction mixtures** (ABI BigDye Terminator v3.0/3.1 Ready Reaction Cycle Sequencing Kit, ABI order number 4390244) for 96-well reaction plates or microcentrifuge tubes it is referred to the following table (Tab. 2):

For each reaction add the following reagents to a separate tube	
Reagent	**Quantity**
Terminator Ready Reaction Mix	See table 3
Template	See table 1
Primer	See table 1
5x Sequencing Buffer*	See table 3
Deionized water**	*q.s.*
Total volume	See table 3

Tab. 2: Cycle sequencing reaction mixture.

* 5x Sequencing Buffer (ABI, order number 4305605), alternatively you may produce this buffer by your own. 5x Sequencing Buffer: 400 mM TrisHCl pH 9.0 and 10 mM $MgCl_2$. The pH of the 1M stock Tris buffer should be adjusted to 9.0 by SLOW, DROP-BY-DROP addition of either concentrated HCl or glacial acetic acid. This is necessary in order to minimize the amount of salt created in the buffer. If you overshoot the buffer pH, discard the buffer and repeat the procedure. Be sure the pH meter and electrode are in good working order.
** Merck water for chromatography (order number 115333.1000) is highly recommended.

In order to save money, the amount of the **Terminator Reaction Cycle Reaction Mix** used for each reaction is usually reduced. A **1:8 dilution** should work for all sorts of templates on the new ABI capillary Genetic Analyzers (BACs not yet tested). In order to compensate for a reduced buffering capacity, 5x Sequencing Buffer should be added to the reactions at higher dilutions. Table 3 summarizes our recommendations:

Terminator Reaction Mix	**Dilution**	**5x Sequencing Buffer**	**Total Volume**
8 µL	1:1	-	20 µL
4 µL	1:2	-	10 µL
2 µL	1:4	1.0 µL	10 µL
1 µL	1:8	1.5 µL	10 µL
0.5 µL	1:16	1.8 µL	10 µL

Tab. 3: Dilution of Terminator Ready Reaction Mix.

Place the tubes containing 10 or 20 µL total reaction volume in a thermal cycler (**cycler programme**) and repeat the following for 25-30 cycles:

- Rapid thermal ramp* to 96 ºC
- 96 ºC for 10 seconds.
- Rapid thermal ramp to the primer annealing temperature**
- Primer annealing temperature ºC for 10 seconds.
- Rapid thermal ramp to 60 ºC
- 60 ºC for 4 minutes.

- Finally, thermal ramp to 4 ºC and hold until ready to purify.
- Spin down the contents of the tubes in a microcentrifuge.

* Rapid thermal ramp is 1 ºC/second.
** If the annealing temperature of the primer is > or = 60 ºC, a two step reaction may be performed.

Sequencing products must be purified to remove non incorporated DyeTerminators. Use CentriSep Spin columns* (ABI order number 4017662) for reactions in 1.5 mL tubes. For reactions in 96-well plates use either the Montage SEQ_{96} Sequencing Reaction Cleanup Kit** (Millipore order number LSKS09624, 96 reactions **must** be done in parallel) or use the MultiScreen HV plates*** (Millipore order number MAHVN4550, up to 96 reactions **can** be done in parallel) for DyeTerminator removal. Dry the samples in a vacuum centrifuge for 10-15 minutes.

* according to the manufactures instructions (with only one exception, please let the column hydrate for 2 hours).
** according to the manufactures instructions (you need to have a vacuum filtration system and the correct manifold [Millipore order number SAVM38401). Please deliver the samples in a Micro-amp Optical 96-well plate (Applied Biosystems, order number N8010560).
*** according to the manufactures instructions (you need to have a centrifuge for 96-well plates and must buy separately from Amersham Sephadex G-50 Superfine). Use Micro-amp Optical 96-well plates (Applied Biosystems, order number N8010560) or Thermo-Fast 96 Detection Plates (Abgene, order no.: AB-1100).
N.B.: To clean the glass plates of vertical gel electrophoresis machines (e.g. ABI 377) use fluorescent dye free towels, e.g. large roll of Kleenex soft (extra-soft) from Kimberly-Clark (order no.: 7201).

Template Preparation and Purification

PLEASE NOTE: These are just our recommendations, feel free to chose your protocol and your supplier best suited for your applications!!!

PCR product cleaning

If the PCR product is a single band, purify directly with PCR purification columns. If there are multiple bands, run products on low melting point or standard agarose gel, excise the desired band and purify DNA using purification columns.

a. 1.5 mL tube format

e.g. for direct purification: **QIAquick PCR purification Kit** (QIAGEN, order number: 28106 for 250 spin columns).
e.g. for direct purification: **ExoSAP cleaning**, 1 U Exonuclease I (New England Biolabs, order number: M0293) and 1 U Shrimp Alkaline Phosphatase (USB Amersham, order number: E70092) per 5µL PCR reaction (incubate for 30 min at 37 °C and then at 80 °C for 15 min), alternative use the ExoSAP-IT Kit from USB Amersham (order number: US78200).
e.g. for gel extraction: **QIAquick Gel Extraction Kit** (QIAGEN, order number: 28704 for 50 spin columns).

b. 96-well plate format

e.g. **MultiScreen$_{96}$ PCR Plates*** (Millipore, order number: MANU03010 for 10 plates).
*you need to have a vacuum filtration system and a special manifold (Millipore, order number: MAVM0960R). Up to 96 samples can be done in parallel. The optional washing step is according to our experience necessary to remove the primers completely
e.g. **MinElute 96 UF PCR Purification Kit*** (QIAGEN, order number 28051).
*you need to have a vacuum filtration system and a special manifold
e.g. **ExoSAP cleaning:** 1 U Exonuclease I (New England Biolabs, order number: M0293) and 1 U Shrimp Alkaline Phosphatase (USB Amersham, order number: E70092) per PCR reaction. Rather cheap and especially interesting alternative method for small PCR products (< 200 bp).
e.g. **AMPure*** (Agencourt, order number 000130 for 1666 reactions).
*you need to have a SPRI Magnet Plate (order number 000219 for 96-well plate format)

Sequencing product cleaning

a. 1.5 mL tube format

e.g. **CentriSep Spin columns*** (Applied Biosystems, order number 4017662).
*according to the manufactures instructions (with the only exceptions, please let the column hydrate for 2 hours and use Merck water for chromatography (order number 115333.1000))
e.g. **Ethanol precipitation method*.**
*according to ABI User Bulletin April 11, 2002; Document P/N 4333020 (by using this method you will loose approximately the first 20 bases behind the sequencing primer)

b. 96-well plate format

e.g. **Montage SEQ_{96} Sequencing Reaction Cleanup Kit*** (Millipore, order number LSKS09624, 96 reactions **must** be done in parallel).
*according to the manufactures instructions (you need to have a vacuum filtration system and the correct manifold [Millipore order number SAVM38401)
e.g. **MultiScreen HV plates*** (Millipore, order number MAHVN4550, up to 96 reactions **can** be done in parallel).
*according to the manufactures instructions (you need to have a centrifuge for 96-well plates and must buy separately from Amersham Sephadex G-50 Superfine)
e.g. **CleanSeq*** (Agencourt, order number 000121 for 800 reactions).
*you need to have a SPRI Magnet Plate (order number 000219 for 96-well plate format)
e.g**. DyeEx 96 Kit*** (QIAGEN, order number 63181).
*using the optimized protocol for the ABI PRISM 3100, (you need to have a centrifuge for 96-well plates)
e.g. **Ethanol precipitation method*.**
*according to ABI User Bulletin April 11, 2002; Document P/N 4333020, (you need to have a centrifuge for 96-well plates, by using this method you will loose approximately the first 20 bases behind the sequencing primer)

Special Laboratory Equipment needed

- **Minimal**
  - Thermocycler
  - Desktop centrifuge (e.g. Eppendorf 5415 with rotor F-45-24-11 for 1.5/2 mL reaction tubes)
- **Optional** (for 96-well plate processing)
  - 96-well plate shaker
  - 96-well plate centrifuge (e.g. Eppendorf 5810 with rotor A-4-62-MTP for 96-well plates) **or**
  - Vacuum centrifuge (e.g. Eppendorf Concentrator 5301 with rotor A-2-VC for 96-well plates)
  - Vacuum filtration system and special manifolds
  - SPRI Magnet Plate

Sequencing Resources

- **MWG Sequencing Results Guide**. **URL:** http://ecom2.mwgdna.com/improve_results_seq/improve_results.html
- **Ridom TraceEdit.** Ridom TraceEdit is a DNA trace editor and viewer, which is available free for Microsoft Windows, MacOS X and UNIX platforms. Ridom TraceEdit displays the chromatogram files from Applied Biosystems automated sequencers and files in the Staden SCF format. Incorrect base calls can be edited and saved. **URL:** http://www.ridom.de/traceedit/