

## Standard Operating Procedure for Hazardous Chemicals

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**Building and rooms:** Davison Life Sciences Building, Lab B310

<b>Chemical(s)</b>	Ethanol, ethylenediamine tetraacetic acid (EDTA), sodium acetate.
<b>Process</b>	<b>Sequencing</b>
<b>Specific Hazards</b> <i>referred to MSDSs for more detailed information</i>	<u>Ethanol</u> is flammable, and may be harmful upon skin/eye contact, ingestion or inhalation.
<b>Personal protective equipment</b>	Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist
<b>Engineering/ventilation controls</b>	<input checked="" type="checkbox"/> chemical fume hood or well ventilated areas <input checked="" type="checkbox"/> emergency shower and eyewash accessible
<b>Special handling procedures and storage requirements</b>	<input checked="" type="checkbox"/> flammable cabinet, Rm <u>B310</u> . <input checked="" type="checkbox"/> Store all the reagents on bench top, 4°C or -20°C accordingly.
<b>Spill and accident procedures</b>  <i>for hazardous chemicals only</i>	<u>Skin exposure</u> : Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes. <u>Eye exposure</u> : Wash eyes for > 15 minutes. For both cases, seek medical attention immediately.
	<u>Small</u> (< 2L): Absorb with vermiculite or spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	<u>Large</u> (> 2L): Evacuate the room, notify PIs and call 2-5801 to request emergency spill assistance from the Environmental Safety Division.
<b>Waste disposal</b>	Collect and label as hazardous waste according to the SOP for Hazardous Waste Disposal.
<b>Special approval</b>	No special authorization needed after SOP training & reading MSDSs.
<b>Prepared by</b>	Name/date: Kate Tay, 9/8/2010
<b>Reviewed by</b>	Name/date: C-J Tsai, 9/10/2010

## BigDye™ Sequencing Reaction

**Before sequencing, all DNA templates should be QC-checked by gel electrophoresis and Nanodrop.  
NO EXCEPTION!**

### Plasmid template:

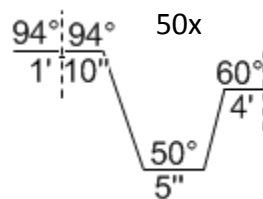
- \* Plasmid size (vector + insert) < 10kb ~200 ng
- \* Plasmid size (vector + insert) > 10kb ~500 ng

### Cleaned PCR product as template:

- \* 100 – 1000 bp 20 ng
- \* > 1kb 50 ng

In dim light, mix the following and short spin to collect the content

plasmid (see above)	1 $\mu$ l
5 $\mu$ M Primer *	0.75 $\mu$ l
2.5x Seq buffer *	3.5 $\mu$ l
BigDye *	0.5 $\mu$ l
s/w *	4.25 $\mu$ l
	<u>10.0 <math>\mu</math>l</u>



\* It is advised to prepare a master mix for multiple reactions (buffer + BigDye + water) to minimize pipetting error and reagent waste. If only one primer is used, it can be added to the master mix. Otherwise, make another mater mix with (primer + water), or with (primer + template + water).

## Dye Reaction Purification

\* Work in dim light

\* Prepare **Fresh** (1:1) 0.5M EDTA pH8 plus 3M NaOAc (both autoclaved)

1. Add 1  $\mu$ l of **Fresh** EDTA:NaOAc, mix by pipetting
2. Add 40  $\mu$ l **Ice-cold** 95% EtOH (HQ EtOH; use multichannel pipette)
3. Mix & transfer to a BarCode 96-well Seq plate, cover and seal w/ aluminum foil
4. Incubate at -20°C, 20 min
5. Fill out the Excel sample sheet (Barcode\_Tsai\_Y-M-D.xls), and export the first sheet to .txt  
Sample names CANNOT include any unusual characters (-\*/.#). Use underscore \_ for any spacing
6. Before **11 am** attached & email to...
  - a) [orders@ibl.ors.uga.edu](mailto:orders@ibl.ors.uga.edu) & [3730@ibl.ors.uga.edu](mailto:3730@ibl.ors.uga.edu), copy CJ and Kate
  - b) Sub: **Genetics RtR plate pickup, sample sheet attached**
7. Centrifuge: **1500 rcf**, 45 min or more , 10°C
8. Turn the plate upside down to empty the liquid over paper towels
9. Centrifuge: **300 rcf**, 1 min, 10°C (w/ KimWipe at bottom)
10. Evaporate EtOH by drying in the hood for ~15 min
11. Seal w/ Al foil, wrap the sample sheet around the plate with a robber band. Bring it to the GGF fridge in the Genetics office **BEFORE 11 am**

## Sequence data analysis notes

1. You **MUST** examine each and every chromatogram to determine if sequencing was successful. Scan through the end, as some sequence read terminates prematurely. Good sequence should reach ~700 nt. Take notes for reads < 300 nt. Text file is **NOT** an acceptable measure to judge sequencing success (lousy sequence could still generate text output, albeit meaningless). If YES, go to step 2; if NO, go to step 7
2. Retrieve the sequence (text file), identify vector sequence, the start of your insert (and PCR primer, if applicable) and insert orientation etc. Good quality sequence usually starts ~50 bp downstream from your sequencing primer, so you will not find the sequencing primer.
3. Create a copy the sequence for editing in a word file, along with the “original” one as a reference. Trim the vector sequences. Reverse-complement antisense sequences. Have your template sequence (from JGI poplar genome, GenBank etc, if available), perform a multiple sequence alignment, and copy the result to the word file. Check all mismatches and determine whether they represent random errors or true sequence variations (if occurring in all sequenced clones, such as species-specific SNPs).
4. For indels (insertions or deletions), examine **chromatograms** for the corresponding positions. Mismatches are more frequent toward the end of the sequence read or within polymers (e.g., AAA, GGGG). Ambiguous calls (Ns) should also be checked. Make manual correction to the “edited copy” (with notes) in the word file. Some sequence errors are generated during computer interpretation of the chromatogram (that produces the text file), due to irregular spacing, spike contaminants etc. Correct these as well.
5. Many sequence variations across species are synonymous substitutions. For constructs intended for over-expression (in bacteria or plants), translate the DNA sequence and do an alignment with the deduced protein sequences (along with the template). Select the most accurate clone(s) to continue. Sequence more, if errors are numerous or when a frame-shift is encountered.
6. If you don't have a template sequence (cloning a new gene), or if your sequence does not match with the template, Blast the sequence against the GenBank database (try both BlastX and BlastN). You might have sequenced an “empty” vector, or contamination. We have occasionally seen cross-contamination from neighboring wells, most likely during sequencing reaction clean-up's.

### Troubleshooting:

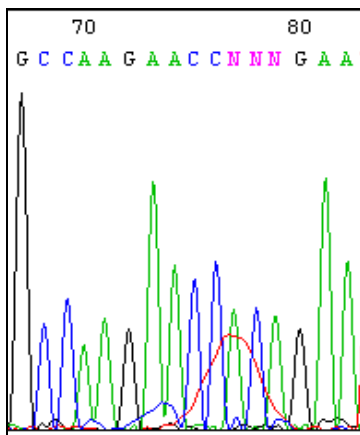
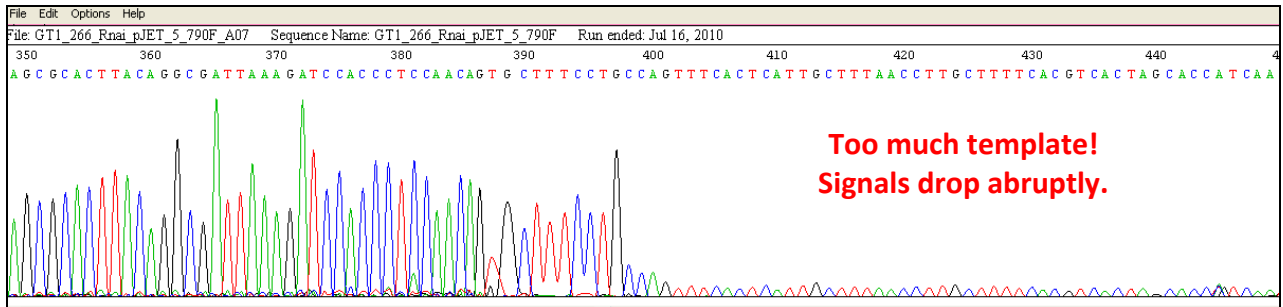
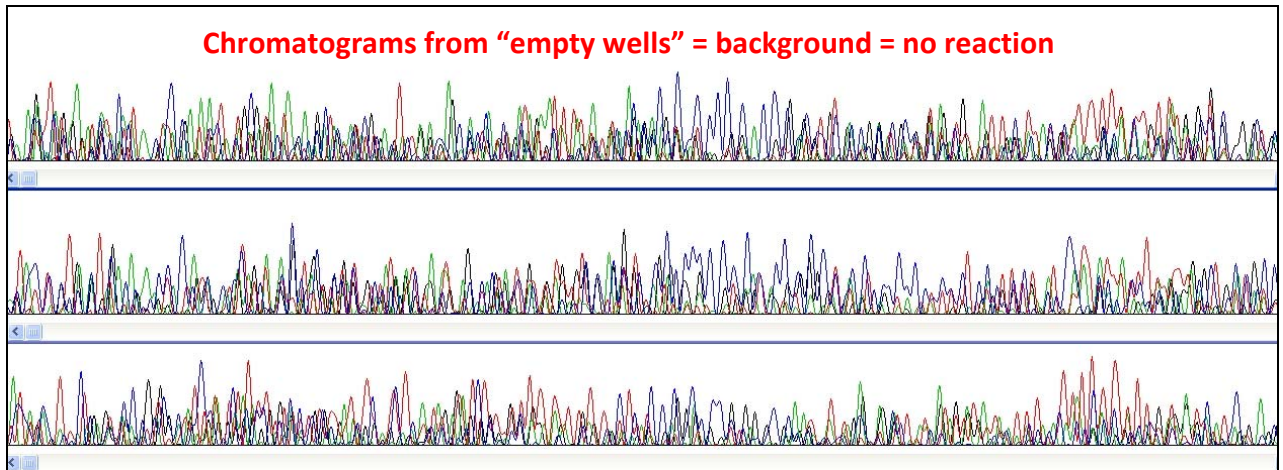
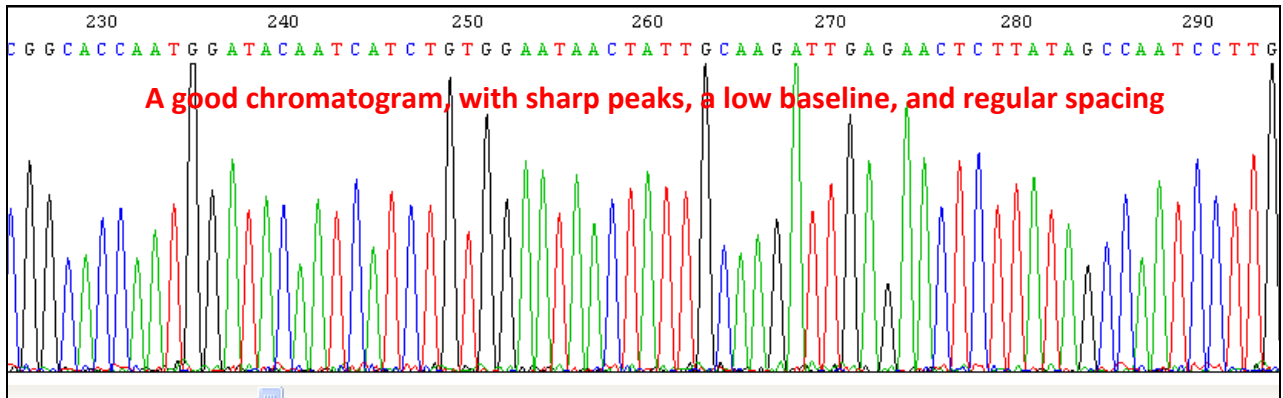
7. Huge peaks initially, short read, signals drop abruptly: too much template (consumed all dNTPs and dyes during initial amplification).
8. Noisy signals, no discernible peaks: too little template, sub-par template quality or failed reactions.

Check other samples sequenced in the same plate to see if there is a systematic error. If other reactions work fine, then the problem is likely sample-specific. If all reactions failed, check common reagents (sequencing buffer etc). We have found that the ABI buffer works better than home-made one for binary (large) plasmid sequencing. Replace the NaOAc stock regularly (make small stock volume).

Check reactions that contain the same template as well as those that contain the same primer to determine if there is a problem with a specific template or primer. Multiple freeze-thaw cycles can lead to bad primers. Make new (and small) aliquots and refresh them regularly.

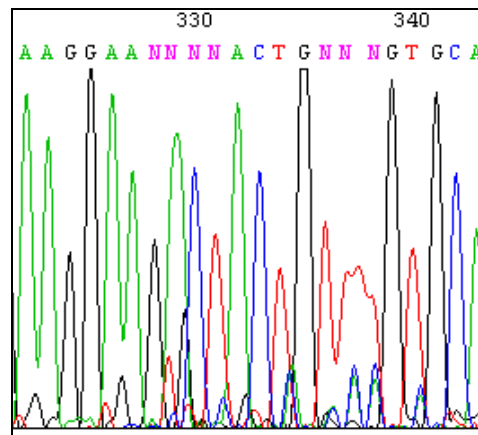
Check your plasmid/PCR product on a gel. Samples with high-background (white smear, stuck in wells, etc) or a high level of RNA contamination should be re-prepped.

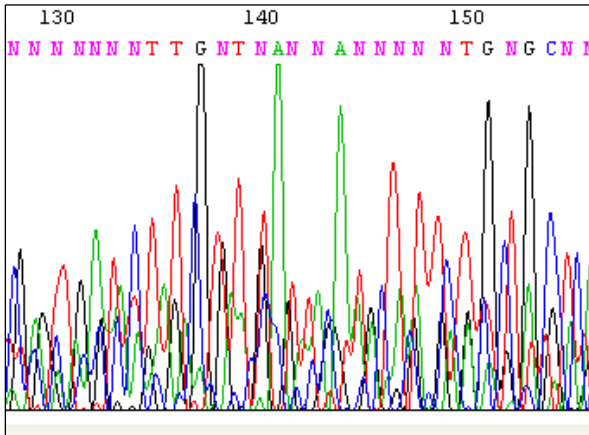
**Example chromatograms for troubleshooting:**



**Both panels show dye carry-over (red traces), leading to errors in base-calling.**

**The sequence on the left panel should be CCACAGA, and on the right panel, AAGACTACTGTCCGT**

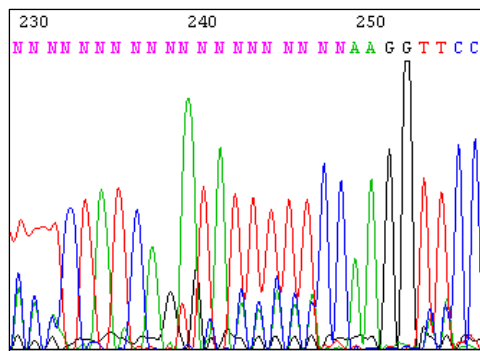
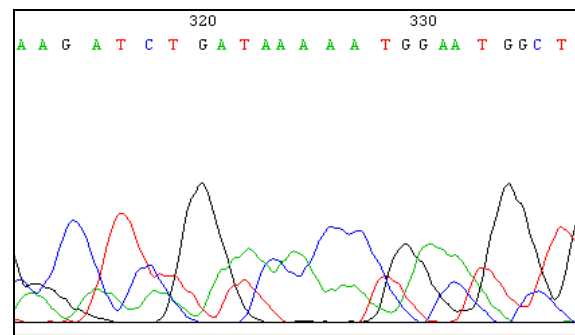
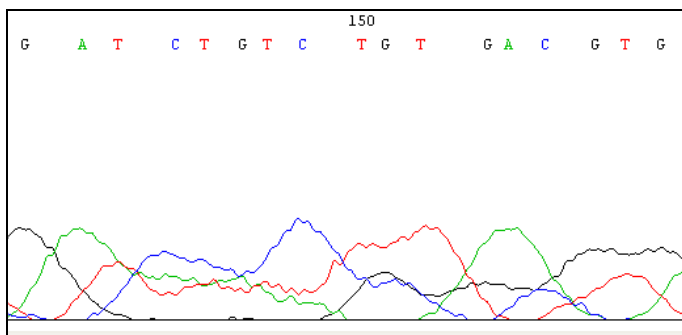




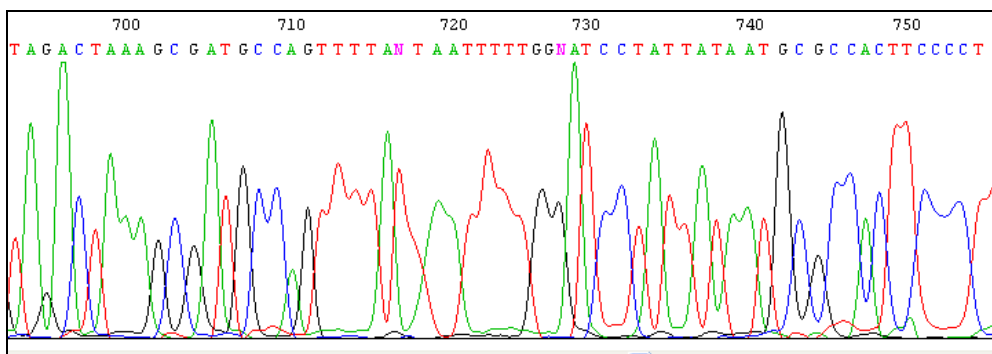
**A few examples of bad sequences:**

**Left:** there were a few clear peaks, but overall background was very high. Sequence data is NOT trustworthy. Likely due to poor template quality or failed reaction

**Below:** broad and low peaks in the early part of the reads when signals should have been stronger. Sequence data is NOT trustworthy. Likely due to low template amount.



**Higher baseline level, although signals were good. The red traces masked some of the signals. Sequence data may be useful if you have a template sequence to refer to (subcloning confirmation).**



**Toward the end of the sequence read, peaks tend to get broader in shape and polymers are difficult to call. Sequence data may be useful if you have a template sequence to refer to.**