General Instructions for Sequencing DNA (01Mar2018)

1. **Apply**: request user name & password so that you can access the Genomics Facility website.

2. **‘Self’ or ‘Full’ Service?:** Choose to submit completed reactions for electrophoresis, or to submit DNA templates for Full-Service DNA sequencing. If the latter, consult Template Submission Requirements.

3. **Clone or directly Sequence PCR product?**
   a) With PCR templates, sequence data is unreadable (due to strand-slippage) after pure poly-“single-base” regions (> ~10 bases); either clone or sequence in both directions.
   b) Unless a PCR product runs as a single band, it must be gel-purified or cloned.
   c) With purified plasmid DNA, stretches of pure poly-“single-base” regions are of minimal concern, unless the homopolymers are extremely long (e.g., ~30-40 bases).

4. **Prepare DNA for sequencing** (Note: eliminating ethanol prior to sequencing is critical).
   a) Plasmid preparations: also consult “Plasmid Prep” document on website.
   b) Commercial spin-columns: do final spin (1-5 min) after final discard of flow-through fluid; consider incubating **eluted** samples at 60°C (open caps; ~10 min) to eliminate residual EtOH.
   c) Some other possibilities: **EtOH precipitation**; PEG precipitation; ExoSAP-IT (PCR products).

5. **Volume of sample DNA per reaction**:
   a. ‘More’ is not necessarily ‘better’: it’s better to err on the side of too little DNA than too much as (1) **excessive template** can greatly shorten sequence read length and (2) more DNA volume also means greater chances of including compounds that will adversely affect reactions.
   b. **Nanograms/rxn**: Ideally, total DNA in a reaction should be ~2-6 ng for PCR products (~750 bp) and 25-200 ng for plasmid DNA (~5 kb, including insert); scale accordingly for other sizes of templates.
   c. **PCR products**: Residue from commercial kits may give false A260 readings, even on a Nanodrop.
      i. **Option A**: run equivalent of 1 µl & 3 µl of purified samples in a clean 2% gel; if 1-µl band is barely visible and 3-µl band is faint (but distinct), use 1-3 µl of DNA in sequencing reaction.
      ii. **Option B**: purify enough PCR product for valid A260 reading (subtracting any ‘false’ reading values); make serial-dilution reference photo (down to ~1 ng in a clean 2% gel) to compare with samples.
   d. **Plasmid DNA**: Take A260 readings of at least several samples and dilute the DNA accordingly.
      i. Minimize variation: always grow bacteria for same length of time and process same volume.
      ii. Minimize growth time: yields better quality DNA and better sequencing results.

6. **Prepare sequencing reactions** (in Dec. 2017, BigDye costs us $800 for a “100-rxn” tube [~800 ul]).
   a) **+ Control**: including ≥1 p-ctrl (up to 3-5% of total samples) is **STRONGLY recommended**.
   b) **Sample organization**: The ABI 3130xl sequencers inject samples in **batches of 16** (2 columns X 8 rows [A-H]), so arrange templates on plate accordingly to minimize the number of ‘columns’ required.
   c) **Faster results**: Consolidating samples of similar desired read length into sets of 16 can reduce run time.
   d) **BigDye volume** (10-µl rxn): Standard ABI recommendation for 20-µl reactions is 8 µl BigDye; however, excellent results can be generated with much less than 4-µl BigDye in 10-µl reactions.
      i. 0.5 µl/rxn: For the vast majority of templates, 0.5 µl of BigDye/rxn provides an excellent balance between cost savings and ensuring robustness of the sequencing reaction.
      ii. 0.2-0.4 µl/rxn: Exercise caution when choosing these levels of BigDye. Signal intensity, read lengths and sequence quality will typically be similar to results from reactions that use 0.5 µl of BigDye; however, reactions done with very low BigDye levels are more sensitive to conditions that can adversely affect sequencing reactions (e.g., difficult-to-sequence motifs, poorly-cleaned templates, and templates with excessive levels of interfering salts).
      iii. 0.1 µl/rxn: Signal intensity will be substantially lower with 0.1 µl/rxn (vs. ≥0.2 µl). Thus, read lengths will typically be <700 bp; however, for standard nt composition templates, at least 500-bp of good quality reads can be expected on a routine basis.
      iv. These low levels of BigDye require that reaction volumes not exceed 10-ul; otherwise, signal strength and read length will be reduced.
   e) **Sequencing Buffer**: you MUST replace ‘lost’ volume of BigDye with same volume of 2.5X Sequencing Buffer (a Mg-Tris solution); otherwise, reaction quality will suffer (e.g., for 0.5-ul BigDye reactions, use 3.5 ul of 2.5X Sequencing Buffer for a combined volume of ‘4-ul’ in your 10-ul reactions).
   f) **Aliquoting BigDye stocks**: BigDye begins to degrade after 5-10X freeze-thaw cycles. So, because these reaction sizes yields ≥1600 reactions from 1 tube of BigDye Terminator v3.1, you must vigorously vortex new stock tubes and aliquot them into volumes that match your typical sequencing needs.
7. **Run sequencing reactions** (see ‘Sequencing Tips’ for difficult templates).
   a) Typical conditions: 95°C initial denaturation (2’); 25X [95°C (10 s); 50°C (5 s); 60°C (4’ [but, typically, 2’ will be more than sufficient time])]; 20°C hold (10°C hold, if reactions will not be removed upon completion).
   b) Upon completion, either freeze or immediately clean the samples (at a minimum, refrigerate the samples).
8. **Clean the sequencing reactions** (see pdfs on Genomics Facility website)
   a) **Ethanol precipitation** (with EDTA) is inexpensive, simple, and generates good quality data.
   b) Commercial columns are ok, but expensive; some brands may reduce signal strength.
   c) ‘Sephadex’ columns: reuse leads to unincorporated dye terminator peaks in data; to minimize signal loss, dilute completed reactions to 20 μl prior to loading on column.
   d) CleanSEQ by Agencourt: Simple & effective (yielding extremely clean DNA and exceptional sequence reads), but expensive. **Please see us prior to using this method.**
   e) BigDye® X Terminator™: Simple & effective (retaining all sizes of sequenced fragments), but expensive; you MUST inform us if you use this method.
9. **Resuspend reactions** (URGENT: do NOT leave open-cap tubes in thermal cycler with block cooler than RT).
   a) After drying reactions, add 15 μl of ABI Hi-Di formamide (no other formamide is acceptable); see links on web site ‘Request Form’ for other options).
   b) Seal samples; lightly vortex; and, briefly centrifuge (store at 4°C or freeze).
10. **Submit sequencing request online** at Genomics Facility website.
   a) Log in, select correct entry link for your ‘type’ of sequencing, and fill out basic information.
   b) Download Excel spreadsheet to input sample names and desired read lengths; upload file.
11. **Physically submit samples.**
   a) In 96-well plates or 0.2 ml STRIP tubes (stored in a 0.2 ml 96-place rack); if using tubes, please ensure that the caps can be removed easily. Do NOT submit samples in individual tubes, as these are more likely to be mishandled such that your sample is contaminated or lost.
   b) Label plates: Submission #, PI name; and, Submitter name (for tubes, label “T-I” – “T-X”).
   c) Place samples in “**Samples To Be Processed**” box in the Genomics Facility refrigerator (Rm. A628).
12. **Download sequencing results from the Genomics Facility website.**
   a) **Time** required for a standard run module: ~2 hr for a single run (16 samples); ~6 hr for a half-plate (48 samples); and, ~12 hr for a full plate (96 samples).
   b) Typically, results are available within 1-3 working days of submission.
   c) When your sequences are ready, your submission will include a link to a zipped ‘**Results File**’ (~10 Mb for 96 samples); eventually, result files are archived and disappear from your view.
13. **Analyze your data.**
   a) "**Peaks**" in your electropherograms might not equal ‘good’ data; use **Sequence Scanner** (ABI freeware) to assess basecall signal strength & quality.
   b) We can sometime improve your data by reanalyzing it with the 3130XL’s Sequencing Analysis Software; however, we will perform such analyses only after a specific request.
   c) Results are available as text files and electropherograms.
      i. Text files are accessible with DNASTar, BioEdit, or Notepad.
      ii. Electropherograms are accessible with freeware such as:
         1. **Sequence Scanner** by ABI (provides access to raw data, signal intensity, & more!);
         2. **BioEdit** – an excellent alignment tool; and,
         3. **Chromas LITE**.
14. **Sequencing woes?** Check for solutions at our **SCIENCE AID CENTER**, or contact the Genomics Facility (8-7106, genomics@lsu.edu).
15. **Bookkeeping:** About every 3 months, P.I.s are given a summary of their usage of the Genomic Facility with respect to processed sequencing samples and supplies.
16. **Further Website info:** **Documents for Equipment and Protocols** – ABI 3130XL Genetic Analyzers
   a. **Sequencing Flowchart**
   b. **Plasmid Preparation**
   c. **EtOH Precipitation**