Genomics Core
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Web: http://www.biotech.cornell.edu/brc/genomics-facility
Revised: December 2015

DNA Sequencing Handbook

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526 Campus Road
147 Biotechnology Building
Ithaca, NY 14853
607-254-4857
Monday -Thursday: 8:00AM-4:30PM
Friday: 8:00AM-4:00PM

Description

DNA Sequencing is performed using the Applied Biosystems Automated 3730xl DNA Analyzer. We use Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. We routinely provide up to 900 bases per reaction, providing the template is of high quality.

Please note that our web page supersedes all written material. Please check our web site often for the most current information
http://www.biotech.cornell.edu/brc/genomics-facility
Revised: March 2016

Sample Submission

1. Orders will only be accepted using our online ordering system
All requests for sequencing must be submitted electronically via our web based user interface, which can be reached from our web site: http://www.biotech.cornell.edu/brc/genomics-facility If you wish to use a Purchase Order to pay for sequencing, we require a hard copy of the PO before we can process your samples.

2. Premixed samples or universal primers only
Samples utilizing custom primers must have the template DNA and primer premixed (please be sure to place only one primer in each tube with the template DNA). We will not mix your custom primers with your template DNA. We offer M13F, M13R, T7HT, and T3HT universal primers. If you wish to use our universal primers, please submit the template DNA and indicate which universal primer you require on the electronic sample submission form. There will be an additional $1 fee per sample tube for internal customers and $1.60 fee per sample tube for external customers applied for this service. Please note that you must supply a separate tube of the DNA for each universal primer that you wish to use.

Automated DNA sequencing on capillary format instruments requires that the template be of higher purity than manual sequencing. Please prepare your samples carefully. Inadequate DNA cleanup is the most common cause of poor sequencing results. Please provide us with the correct amount of sample, as indicated below, for your sample type. We require these amounts so that we can perform a rerun if needed and to ensure that our robot has an adequate volume from which to pipet.

Preparation of premixed samples

Plasmids: Place 1ug of plasmid DNA and 8 pmole of primer in the specified tubes; bring up to **18µl** with H2O or low concentration (10mM) pH9 Tris. Do not use TE.

PCR: For PCR products, place the required amount of PCR product and 8 pmole of primer in the specified vials and bring up to **18µl**. To determine the required amount of PCR product to add, use the following formula:

- ➔ #base pairs/5.0 = amount of PCR product in ng that we need. For example: for a 250bp PCR product. 250bp ÷ 5.0 = 50ng of DNA + 8 pmole primer in 18µl (Note: The maximum PCR product concentration is 100ng/µl).

Preparation of samples for universal primer sequencing, added by the Genomics Facility.

Add the same amount of DNA as indicated above, but only bring up to a volume of **10µl**. We will add the appropriate amount of universal primer to the tube.

3. Sample vial requirement

We accept samples in two formats:

Tube submission policies:

1. Tubes: 500ul standalone screw top vials. USA Scientific (catalog number 1405-9700 to 1405-9706 select color, 1405-9799-mixed color caps). These are the tubes the lab orders and uses. Picture example and prefered labeling method can be found on page 3 of this handbook.
2. Alternatively, if you have large batches of samples, you may submit samples in 96 or 384 well plates. We prefer to receive large numbers of samples in plates, rather than in individual tubes, as it allows us to process the samples more efficiently.

Plate submission policies:

1. Any PCR style plate can be used for full services Sanger Sequencing (template and primer submissions). We recommend when shipping your plates at room temperature to use strip caps on the top of your plate as seen here. The uses of adhesive seals is not recommend and can cause contamination due to sample leakage
2. A total of 94 samples can be submitted in the 96 well plate. The last 2 wells, H12 andG12 on the 96 well plate must be left empty for controls.
3. Fill your samples down the columns as followed. Sample 1-A1, Sample 2-B1, Sample 3-C1 and so one. Fill the following column Sample 9-A2, Sample 10-B2, Sample 11-C2 and so on throughout the entire plate. The last 2 wells, H12 andG12 on the 96 well plate must be left empty for controls.
4. The plate **must** contain all premixed samples or all DNA samples that are to be sequenced with the SAME universal primer.
5. You may use our Batch Upload feature on our online ordering system to submit your sequencing order. You will fine detailed instructions for submitting the electronic portion of your order there.

4. New Sample for each sequence.

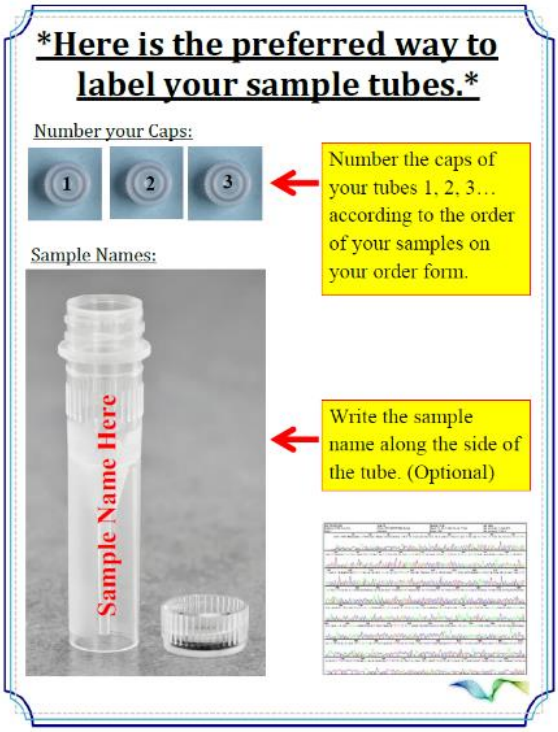
Please submit new samples with each order. Samples will be discarded one week after they are processed and it has been determined that they do not need to be rerun.

5. Sample Drop off and Mailing

Samples may be dropped off in room 147 in Biotechnology Building from 8:00 AM to 4:30 PM Monday through Thursday and 8:00 AM to 4:00 PM on Fridays and may also be sent via FedEx or US Mail. Our mailing address is **526 Campus Road, 147 Biotechnology Building, Cornell University, Ithaca NY 14853**. Please note that there is no delivery on Saturdays or holidays and we have received numerus broken tubes and plates, so please cushion and seal your plates before mailing

Customers from the Weill Medical College and Memorial Sloan Kettering Cancer Center in NYC may submit their samples via their Fed Ex drop box. The Office of Sponsored Programs manages the drop box, so please direct all questions regarding the drop box to them.

The office can be reached at 212-746-6020. Please review the following guidelines. http://www.med.cornell.edu/research/research_support/core_facilities/dna_seq.html Specifically, to protect tubes from breaking in transit sample tubes should be wrapped in appropriate packaging. The requester's name, contact number, lab location and **order number** should appear on the outside of the package.



6. Sample Names
Sample names are limited to fifteen characters, consisting of only letters, numbers and dashes. No spaces, periods, or symbols are allowed.

Numbering Caps:
It would be a big help to us if you could number the caps of your sample tubes for future orders. Please do this so that the first sample you entered on your on-line order form is numbered as 1, the second sample is numbered as 2, the third sample is numbered as 3... and so on. This will allow us to check your order into our system much more quickly.

You do not have to name your samples as numbers, continue to name them whatever you like. If you would like to write the sample name on the tube please do so down the side of the tube.

Billing Information
http://www.biotech.cornell.edu/brc/brc/services/terms-and-policies#Payment

All customers must submit a valid Cornell Account number and Purchase Order number to which we can bill the sequencing. The Weill medical college will need to submit a purchase order, we are not yet able to use your Weill account numbers. Service will not be performed until we have a valid number to which we can charge. If you wish to use a Purchase Order, we need a hard copy of the Purchase Order before we can perform your sequencing. You may fax or email the PO to us at 607-254-4847 or genomics@cornell.edu.

Please see our web site for the current prices: http://www.biotech.cornell.edu/brc/genomics/services/price-list#

Your Sequencing Results

Distribution
Our usual turnaround time is 1-2 days. You can track the progress of your samples from our web site: https://cores.lifesciences.cornell.edu/userdev/ When your results are ready, an email will be sent to you pointing you to our secure web site where your electropherograms and text files can be viewed, printed, and downloaded. See **Appendix 1** for more detailed information on your results.

File Storage
After results are no longer on the web, files can be accessed again by contacting us with your order number. A $5 internal and an $8 external fee per order is associated with data retrieval, so please remember to download and save your results as soon as they are ready.

Data generated by the BRC is made available to the core user for a minimum of 30 days. Except as noted for specific services, storage and backup of the core users' data is the core users' responsibility. Beyond 30 days, any data still stored by any BRC core may be retrieved by request for an additional fee. No guarantee is made about the availability of data after 30 days.

Analyzing Your Data
Always look at the electropherogram, not just the text file. The sequencing software calls the strongest signal (highest peak) at any location. However, if the noise level is high, weaker signals may not be distinguishable from the background noise, resulting in questionable calls. Be sure that each peak is clearly stronger than any background at that site.

The BioResource Center Computing Facility http://www.biotech.cornell.edu/node/137 can also provide software and support for primary sequence analysis, which includes the use of confidence values for individual base calls.

Rerun Policy
All samples that fail (meaning there is no readable sequence) will be rerun automatically at no additional charge, using the original samples. If you would like any other sample rerun, you must resubmit the sample as a new order.

Plasmid Sequencing

DNA Quality
The most important factor for successful DNA sequencing is the quality of DNA used. Automated sequencing with Taq polymerase is very sensitive to trace amounts of salts, ethanol, proteins, and other contaminants. DNA that sequences well manually may not be pure enough for automated sequencing. We recommend that you use a commercial kit to prepare your DNA, such as those made by Qiagen and Promega. Be sure to follow the directions exactly and do not overload columns. Note that the purification outcome is dependent on both the amount of DNA and the volume of liquid applied to the column. We suggest that you stay well below the recommended volumes and quantities.

-Your DNA should meet the following tests for purity:
The A260 /A280 ratio must be ~1.8. Values less than 1.7 give less than optimal results.
You should also evaluate the purity of your DNA on an agarose gel.

DNA Concentration Determination
The easiest way to determine DNA concentration is to measure the absorbance at 260. 1 OD ~ 50 ug/mL DS DNA. We have a Nano-drop available for your use, free of charge, located at 140 Biotech. If you use the Nano-drop, we recommend that you at a minimum double the concentration when submitting your samples. The Nano-drop detects not only your template but all contaminates and usually reads the concentration at **2-5x the actually value**.

Difficult Templates

The AmpliTaq FS enzyme offers improved sequencing through difficult templates, such as those with high G+C content, homopolymer regions, and secondary structures. However, it cannot always sequence these regions effectively. If you are having trouble with a high GC template and our standard reaction cannot solve the problem, we can run the reaction with a different chemistry (the dGTP Kit). This is not a rerun, but will be considered a new reaction and additional charges will apply. Secondary structure is the hardest problem to overcome and often the solution is to sequence from the other end. We add 5% Betaine to every sequencing reaction in an attempt to eliminate secondary structure. Other options are also available, so please contact us if you are having problems with secondary structure.

PCR Product Sequencing
PCR products must be purified before performing automated sequencing to remove PCR primer carryover and excess dNTPs. We recommend purifying PCR products with a commercial product available from Qiagen, Promega, and other companies. Often, the desired PCR product is contaminated with other amplification products, and gel purification is necessary. We recommend that you run your sample out on a gel and cut out and purify the band of interest.

Internal primers seem to work more reliably than the PCR primers, as even gel purified PCR products may contain more than one product. Using an internal primer specific to the desired product results in less interference from secondary sequences.

Phage Lambda, Cosmid and BAC Sequencing
Utilizing lambda, cosmid, and BAC DNA is less time consuming than subcloning into smaller plasmids. Cosmid and BAC sequencing is becoming more popular and the success rate is increasing. Please note on the online order form if your DNA is phage, cosmid, lambda, or BAC and we will use a different chemistry to help get a better signal. Please note that samples that require special conditions may take a couple of extra days to process, and will cost an extra $5 per reaction.

Guidelines for Primer Design

Specificity

Primer length should be 17-25 nucleotides.
Be sure to choose a primer whose sequence is in your vector. Also be sure that there is only one binding site for your primer.

The primer should match the template exactly.
Near the 3' end an exact match is essential, especially the last 8 bases. When designing a primer from a sequence obtained from the DNA Sequencing Facility remember that sequence data beyond 500 bases is more likely to have errors than the first 50-500 bases. Unless you have sequence information from the opposite strand or overlapping data from another sequence, be conservative and choose your primer in the safer region, preceding base 500. Degenerate primers are not recommended.

Estimated Melting Temperature (Tm)

Primers for cycle sequencing should have a Tm of 50-70°C, with the best at 55-65°C.

Please be aware that we add Betaine to each reaction, which may lower both the Tm and annealing temperature of your primer. Our thermocycling protocol anneals at 50°C and extends at 60°C. If the Tm of your primer is on the low side, please consider redesigning a longer primer. When the Tm is too low, the primer may anneal incorrectly or not at all. A high Tm can be OK if there are not long strings (>3) of Gs or Cs that can bind quickly, often incorrectly, and very tightly. Your G+C content should be approximately 50%.

Be aware that primer design software packages calculate Tms based on some theoretical model that does not always yield actual experimental Tms. Base stacking and nearest neighbor models give the most accurate theoretical Tms. However, we have found that two fairly simple equations can give useful results.

1. The McConaughy equation (Biochemistry 8: 3289-3295, 1969) modified for cycle sequencing:
   $$Tm = 60 + 41(G + C)/L - 500/L \quad \text{where } L = \text{length of primer}$$

2. The Wallace equation (Nucleic Acids Research 6: 3543-3557, 1979):
   $$Td = 2(A + T) + 4(G + C)$$ (This is actually dissociation temperature.)

Remember that all calculated Tms are only estimates. They are meant only as starting points and do not guarantee success. We recommend that you avoid the extremes and choose a Tm between 55-65°C, if possible.

The Tm of the 5' end should be similar to the Tm of the 3' end.
A quick way to determine the Tm at each end of the primer is to count the number of A/T bases and C/G bases within 6 nucleotides of each end. Choose the primer with the most similar numbers. This will help ensure that the primer anneals flat with the template strand.

Primer Sequence

Avoid primers that can form hairpin loops or primer-dimers. Also avoid stretches of more than 2 identical bases (especially C or G), particularly at the 3' end. This can cause slippage or mismatch during annealing, resulting in a bulge in the primer/template hybrid which could prevent the polymerase from priming.

Universal Primer Available for DNA Sequencing

These primers can be requested for an additional $1.00 internal and $1.60 external cost per reaction.

*These high temperature (HT) versions match many common vectors and are recommended for cycle sequencing. Be sure to check the sequence against your vector, as these primers differ from the commonly used T7bs and T3 primers.

Tm 1 Estimated using the McConaughy equation
Tm 2 Estimated using the Wallace equation

Primer name	Length	Sequence (5' to 3')	Tm1	Tm2
Forward:				
M13F (-21)	18	TGTAAAACGACGGCCAGT	53	54
T7HT*	22	GTAATACGACTCACTATAGGGC	56	64
Reverse:				
M13 Rev	16	AACAGCTATGACCATG	47	46
T3HT*	20	AATTAACCCTCACTAAAGGG	51	56

Plasmid-Universal Primer Compatibility

Please be sure your plasmid samples are compatible with our universal primers before submitting samples for sequencing with our primers.

Additional Services

Fragment Analysis (FA)

You can determine the size of your DNA fragments, such as microsatellites (STRs), AFLPs, and RAPDs, and do mutation analysis such as SSCPs, using a fluorescence based detection system on our Applied BioSystems 3730xl DNA Analyzer. Please see the Fragment Analysis home page http://www.biotech.cornell.edu/node/557 for more information.

Real Time PCR

We now have the ABI ViiA™ 7 Real-Time PCR System. This instrument will determine copy number of your target gene and also gene identification based on fluorescent signaling. If you have question on using this machine please see our real time home page http://www.biotech.cornell.edu/node/559 for more information.

Ready to Load (RTL) Sequencing

User-prepared DNA sequencing can be run by the CLC staff on the ABI 3730xl capillary DNA sequencers. The charge for analysis is for plates only. More information on this service can be found here:
http://www.biotech.cornell.edu/node/562

Appendix 1: Sample Comments

Ran Well: The sequencing reaction was successful and your sample was able to be read out to the appropriate length.

Failed: A failure means that no priming occurred, the lane on the gel was empty, and there are no results. One likely explanation is a mismatch between template and primer, or a problem with the primer (Tm too low or primer forming primer-dimers, hairpin loops, or other secondary structures). Other possible causes include poor quality templates, incorrect template or primer concentrations, or contaminated templates.

Homopolymer: A homopolymer is a long stretch of a repeated base or several bases. Homopolymers frequently cause the polymerase to slip, seriously affecting the downstream sequences. The easiest solution is to sequence from the other direction or use a different primer. You may also try using different sequencing chemistry such as the dGTP kit, which we can provide upon request.

Noisy or Deteriorated: A noisy sequence has high background noise, which is seen as low peaks under major peaks on the electropherogram. The noise interferes with the real sequence and causes the software to miscall or not call bases. A sequencing may be noisy for many reasons, but most often has to do with inferior DNA quality. If there are any contaminants left over after the DNA prep, they can interfere with the reaction. Contaminants include protein, salt, ethanol, isopropanol, PEG, etc. The more contaminated your DNA is, the more noise you will see. To fix this, make sure you follow the guidelines of the purification protocol you are using. Take too many shortcuts may hinder your data. Check the 260/280 ratio of your DNA to ensure it is clean. A sequence that deteriorates starts out well but will slowly decline in quality or signal strength. The most likely cause of this is low DNA concentrations. Be sure your DNA is at the requested concentrations.

Drop Off: Sequences that drop off usually do so because the template is a PCR product, although secondary structure can also cause a drop off. If your template is a PCR product, the drop off point is the end of your product. If the drop off is due to secondary structure, you may wish to try using a different sequencing chemistry, such as the dGTP kit or Hairpin protocol, which can be provided upon request. This chemistry is sometimes successful at getting through areas with secondary structure.

Overlap: Overlapping sequences are caused by two different sequencing reactions occurring in the same tube. Both sequences cannot be distinguished from one another. Overlaps can be caused by presence of two plasmids within one sequencing reaction, incomplete PCR cleanup, two priming sites within the template, or two different prims in the sequencing reaction. To solve this problem be sure that each sequencing reaction contains a single plasmid and a single primer. If your template is a PCR product be sure the PCR product is gel purified to ensure only one band is present.