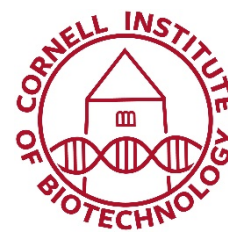


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Sanger Sequencing Handbook **FULL SERVICE**

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This document (“Sanger Sequencing Handbook FULL SERVICE”) explains all the steps you need to take to prepare and submit your samples if you want the **Genomics Facility to perform both the BigDye reaction and the sequencing.**

The information provided assumes you want to sequence a PCR product, a plasmid, a BAC, a phage lambda or a cosmid.

If you need only the sequencing service and intend to perform the BigDye reaction yourself, please consult an alternative document: “Sanger Sequencing Handbook
READY TO LOAD”

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Step 1. Determine if you are going to use tubes or plates

Depending on how many samples you submit, there are different options:

- For less than 16 samples, samples can be submitted in tubes.
- For 16 samples or more, we recommend that you submit the samples in plates.

PROS of plate submission:

- Cheaper, with our "Plate Pricing" discount available when submitting 71 samples or more.
- For less than 71 samples, tube price will apply but we will perform failure re-runs. (We don't provide failure reruns when Plate Pricing is applied for 71 samples or more.)
- For any number of samples, plates are checked in and processed faster.

Step 2. Order the right tubes, plates and cover

We accept samples in two formats: plates or tubes.

a. Tubes:

Please use one of the following 500ul standalone screw top vials:

- USA Scientific: item number 1405-9799.
- VWR: 16466-052 (Tubes with caps).
- VWR: 16466-036 (Tubes only); 16466-084 (Caps only).
- Fisher Scientific: catalog number 50-476-676.



DO NOT USE Fisher: 02-681-333, Fisher: 02-682-559 and Fisher: 14-755-274.

These tubes cause problems with the robotics we use and your samples may not be pipetted properly.

If you have tubes that you think are compatible, please email us a picture.

b. Plates:

If you have large batches of samples, we recommend that you submit your samples in 96 well plate. This allows us to process the samples more efficiently and get your results earlier. You can use any type of PCR-type plate.

c. Covers:

- If you will ship plates by mail: we recommend that you use strip caps and NOT adhesive seal. They can leak and lead to sample contamination.
- If you will personally deliver plates: no need for strip caps. Cover the plate with an adhesive seal.

Step 3. Prepare your DNA

1. Purify your DNA

One of the most important factors 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. Inadequate DNA cleanup is the most common cause of poor sequencing results. Clean your samples carefully!

There are several methods to purify your PRC product (remove PCR primer carryover and excess dNTPs). Here are a few that are commonly used:

- Purification Column kit (Qiagen or Promega): Be sure to follow the directions exactly. The purification outcome is dependent on the amount of DNA and the volume of liquid applied to the column. Do not overload your columns. We suggest that you stay well below the recommended volumes and quantities.
- ExoSAP-IT purification kit (Thermo Fisher): If you use this method, plan to **use a Qubit to quantify your DNA**. Do not use a NanoDrop to estimate your sample concentration after an ExoSAP-IT purification, it yields unreliable measurements.
- Gel cutting: Sometimes, the desired PCR product is contaminated with other amplification products, and gel purification is necessary. If this is the case, we recommend that you run your sample out on a gel and cut out and purify the band of interest.
- AMPure XP beads (Beckman Coulter): This method is not the cheapest, but it yields very good results.

Important: Even gel purified PCR products may contain more than one product. If you think you may have more than one PCR product in your samples, using internal primers instead of the PCR primers may be a solution. Using an internal primers specific to the desired product result in less interference from secondary sequences.

2. Evaluate your DNA purity

The absorbance ratio A260 /A280 ratio of your sample must be equal to 1.8 or greater. Values lower than 1.7 lead to low quality results or even failed sequencing.

3. Evaluate your DNA integrity

We recommend you evaluate the integrity of your DNA on an agarose gel. If your DNA appears highly degraded, the sequencing has a higher chance of failing.

Step 4. Determine your DNA concentration

Once you have determined that your samples are clean and your DNA is of good quality, you will need to know how much DNA you have in your tubes to know what volume you need to provide for the sequencing reaction.

Besides low DNA quality, the main reason sequencing fails is the wrong concentration of DNA template in the template + primer mix.

The easiest way to determine DNA concentration is to measure the optical density (OD) at 260.

An OD₂₆₀ of 1.0 roughly corresponds to 50 ug/mL double-stranded DNA.

There are three ways you can easily measure OD₂₆₀: using a NanoDrop, using a Qubit (or another fluorescence method), using a combination of both.

If you use the NanoDrop alone:

The NanoDrop tends to overestimate the DNA concentration. We therefore recommend that you assume a real concentration 4X lower than the instrument reading. The facility has a NanoDrop available for your use, free of charge, located at 152 Biotech.

Example:

The instrument reads 100 ng/uL. You will assume you actually have 25 ng/uL in the tube.

If you use the Qubit alone:

The Qubit or other florescent method for quantification tend to be highly accurate. The facility hosts a Qubit. If you want to use it, contact us to receive a short training. You will also have to buy your own kits.

If you use a combination Qubit + NanoDrop:

To benefit from the Qubit accuracy but saving on measurement fees, you can measure a few samples with both instruments. You will assume the “correct” measurement is the one given by the Qubit and calculate a “correction factor” for the NanoDrop. You will then measure all subsequent samples with the NanoDrop and apply the correction factor to the each NanoDrop measurement.

Example:

For tube 1, Qubit reads 50 ug/uL ; Nanodrop reads 125 ug/uL (2.50 times higher than Qubit)

For tube 2, Qubit reads 38 ug/uL ; Nanodrop reads 91 ug/uL (2.40 times higher than Qubit)

For tube 3, Qubit reads 39 ug/uL ; Nanodrop reads 102 ug/uL (2.55 times higher than Qubit)

From these three measurements, we can estimate a correction factor of approximately 2.5 for this particular set of samples. For all other tubes of this batch, you can estimate concentration with the NanoDrop alone dividing all readings by 2.5.

Step 5. Determine volumes of DNA template and primers

We offer two options for sequencing primers:

1. You can mix your DNA templates and primers yourself (generally using the same primers you used for the PCR).
2. You can bring us your DNA templates and request us to add universal primers. If you choose this option, see Appendix 2 at the end of this document for how to proceed.

Before you proceed, remember:

- You need to bring your primer + template mix to a total volume of 18 uL.
- Your mix will contain your template DNA and only one of your primers (either forward or reverse) in each tube or well.

Instructions if you are sequencing plasmids (or PCR products >4kb)

- **Step 1.** Place **1 ug** of plasmid DNA in the tube or plate well.
- **Step 2.** Add **25 pmole** of primer. (Skip this step if the Genomics Facility adds universal primers.)
- **Step 3.** Bring up to **18 µL** with **H₂O** or **10 mM pH9 Tris** if you are doing your own primer+DNA mix.

Use Water. Do not use TE. The EDTA in TE acts as a chelating agent capturing the Mg²⁺ needed as a cofactor for the polymerase in our cycle sequencing reaction. This will inhibit your reaction and could cause it to fail completely. We recommend re-suspending your DNA in ddH₂O.

Instructions if you are sequencing PCR products

- **Step 1.** Place **the right amount** of DNA in the tube or plate well. The amount of DNA you need depends on the length of your PCR product. To determine the amount of DNA (PCR product) you need to add, divide the number of base pairs to sequence by 5. The result is the amount of PCR product (in ng) needed in 18uL volume.

Example:

You want to sequence a 250 bp PCR product.

250 bp ÷ 5 = 50ng of DNA.

You need 50ng of DNA.

If your 250 bp PCR product has a concentration of 6ng/uL

50 ng ÷ 6 = 8.3uL of PCR product (DNA) template

You need to add 8.3 uL of DNA template.

- **Step 2.** Place the required amount of PCR product and **25 pmole** of primer in tubes or plates. (Skip this step if the Genomics Facility adds universal primers.)

Example:

Your primer has a concentration of 5pmole/uL.

You will need 5uL of primer in each well or tube.

- **Step 3.** Bring up to **18 µL** with **H₂O** or **10 mM pH9 Tris** if you are doing your own primer+DNA mix.

Use water. Do not use TE. The EDTA in TE acts as a chelating agent capturing the Mg²⁺ needed as a cofactor for the polymerase in our cycle sequencing reaction. This will inhibit your reaction and could cause it to fail completely. We recommend re-suspending your DNA in ddH₂O.

Example 1:

You added 8.3 uL (50ng) of DNA + 5 uL (5 pmole) primer

Your volume is currently 13.3 uL.

You need to bring your volume to 18 uL.

You need to add $18 - 13.3 = 4.7$ uL H₂O

Example 2:

For a 550 bp PCR product at 16 ng/uL, and a primer at 10 pmole/uL

You need to add $550/5=110$ ng of DNA; with a concentration of 16 ng/uL, you need $110 \div 16 = 6.9$ uL of template.

You will add 2.5 uL of primer (at 10 pmole/uL) + 8.6uL H₂O.

Example 3: DNA concentration is too low

For a 400 bp PCR product at 1.5ng/uL, and a primer at 25pmole/ uL

You need to add $400/5=80$ ng of DNA; with a concentration of 4 ng/uL, you would need $80 \div 4 = 20$ uL of template.

*You are well above 18uL total. In this case, **you need to concentrate your sample** before you can use it to prepare the sequencing mix.*

Example 4: very long PCR product

For a 4 kb PCR product or above, follow the instruction of plasmid sequencing (and add 1 ng of DNA + 25 pmole of primers.)

Special case: Phage lambda, Cosmid and BAC sequencing

Please send **500 ng/uL – 1 ug/uL** of template DNA and double the primer recommendation (50pmole primers). 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 (\$8 for external pricing).

Step 6. Fill up your plates or tubes with the volumes you calculated

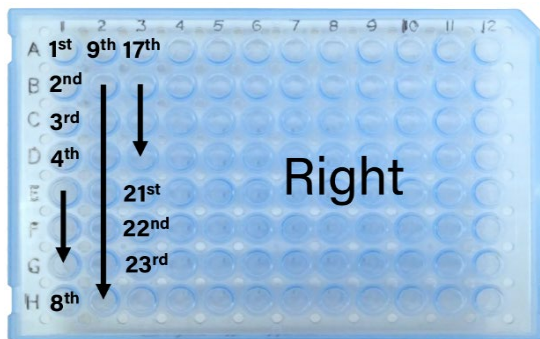
If you use plates

- Fill your samples down the columns.
- The last 2 wells, H12 and G12, must be left empty for controls.
- If you accidentally skip a well, add it to your order so that your samples are not shifted.

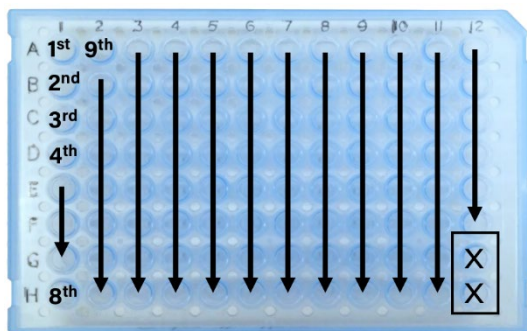
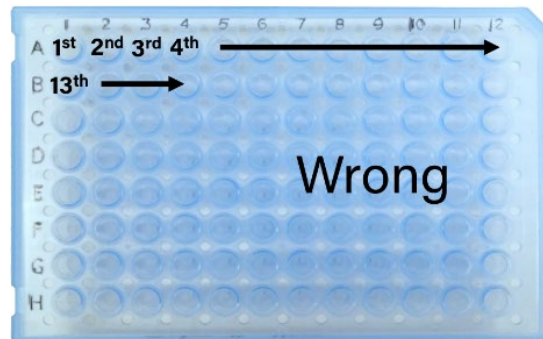
Example:

Your first sample goes in A1, your second sample in B1, your third samples in C1 and so one. When you are done with the first column, you will fill the next column. Sample 9 will be in A2, sample 10 in B2, sample 11 in C2 etc. If you have 23 samples, your 23rd sample is in G3.

You can put up to 94 samples in a 96-well plate, leaving the last two wells empty.



Example with 23 samples



Example with 94 samples



If you use tubes

- Please, do **write a number on the caps of your sample tubes**. Use the numbers in the same order you will enter your samples in the online ordering system. This will allow us to process your order much more quickly.
- In addition, you can write your sample name on the side of the tube.

Step 7. Place your order online

All requests for sequencing must be submitted online. You can access our online ordering system by clicking “Submit samples” on [our website](#).

We cannot process your samples if your order is not in our online ordering system.

Important comments:

- a) 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.
- b) Do not forget to check the “Plate pricing” box if you require it. You can also add it in the comment as a precaution. Plate pricing and other pricing details are available online.
- c) Sample names are limited to fifteen characters, consisting of only letters, numbers, and dashes. No spaces, periods, or symbols are allowed.
- d) If you would like us to run your samples using dGTP or as a hairpin sample, please specify in the comments section of the order:
 - “Please use dGTP kit”
 - “Please use hairpin protocol”
- e) If you plan to use the Batch Upload feature on our online ordering system, we recommend you read the instructions for batch upload available on our website before login in.
- f) External customers will need a Purchase Order for payment. Please send us a copy of the PO at brc_payment@cornell.edu. We cannot start processing your samples until we receive a copy of the PO. Email is preferred over a paper copy; it will expedite the process.

Step 8. Send your samples or bring them to our facility

There are different methods to send you samples to sequencing.

1. **In person.** You can come and drop your samples off in room 147 in Biotechnology Building. Our work hours are written on the first page of this document.
2. **By FedEx or UPS.** Avoid USPS because USPS packages are not delivered directly to the lab. Our mailing address is written on the first page of this document. Please note that there is no delivery on weekends or holidays. Also, note that we have received numerous broken tubes and plates, so please cushion and seal your plates before mailing.
3. **Fed Ex drop box.** This option is available for customers from the Weill Medical College and Memorial Sloan Kettering Cancer Center in NYC. The Office of Sponsored Programs manages the drop box, so please direct all questions regarding the drop box to them.

Whether you send or bring your samples, don't forget to write your name, contact number, lab location and **order number (that you obtained through the online system)** on the outside of the plate and package.

If you have any question, our contact information is on the first page of this document.

Step 9. Receive and analyze your results

Our usual turnaround time is 1-2 business days.

You can track the progress of your samples from our [ordering online system](#)

When your results are ready, we will send you an email pointing you to our secure website where your electropherograms and text files can be downloaded.

The results may come with some comments, which are listed in **Appendix 1**.

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.

We also strongly recommend that you load the AB1 file into a program, and not rely uniquely on the picture provided by the facility.

To this end, Thermo Fisher Scientific offers a [cloud-based data storage and data analysis software](#).

The [Bioinformatics Facility](#) can also provide software and support for primary sequence analysis.

Step 10. Store your data

Data will be available for 30 days. After this, your results will no longer be available on your account. If you want to access your files, contact 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.

Note on DNA storage:

All samples will be discarded two weeks after they are processed.

If you want to re-sequence a sample you previously send us for sequencing, you will need to send new samples for *each new order*.

The only exceptions are:

- If sequencing failed (there is no readable sequence). In this case, we will automatically rerun using the original samples. We do not charge for rerun due to sequencing failure, and therefore a sample rerun is not considered as a new order
- If you are dissatisfied with your results and wish to order a re-run, we will use the DNA that we already have. Only if the sample re-run is better than the original, there will be no charge for the re-run. For more details, see Sanger Sequencing Troubleshooting.

Appendix 1: Sample Comments

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

Low signal strength: A sample with low signal strength could mean one of several things, but likely there was no DNA or far less DNA than was necessary for the reaction. Miscalculating the primer concentration or priming failure can also cause low signal strength. Check your chromatogram for text at the top: "Signal G:131 A:140 T:98 C:78". These numbers should ideally be between 500 and 2000 for best sensitivity and low background noise. Signals between 50 and 150 SOMETIMES give reasonable data and sometimes give poor data. With low signal you will definitely see baseline noise that would not trouble you on a high signal strength sample.

Failed: A failure means that no priming occurred, the well on the plate 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 (for C or G repeats only), 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. Contaminants left over after the DNA prep (protein, salt, ethanol, isopropanol, PEG, etc) can interfere with the reaction. To fix this, make sure you follow the guidelines of the purification protocol you are using. 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 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 primers 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.

Appendix 2: The Genomics Facility add universal primers

You can bring your DNA templates and request us to add universal primers. (Note that we do not add your custom primers, **only universal primer.**)

We offer the choice between four universal primers: M13F, M13R, T7HT¹, and T3HT¹.

| Primer name | Length | Sequence (5' to 3') | Tm1 ² | Tm2 ³ |
|--------------------|--------|------------------------|------------------|------------------|
| Forward M13F (-21) | 18 | TGTAAACGACGGCCAGT | 53 | 54 |
| Forward T7 HT | 22 | GTAATACGACTCACTATAGGGC | 56 | 64 |
| Reverse M13 Rev | 16 | AACAGCTATGACCATG | 47 | 46 |
| Reverse T3 HT | 20 | AATTAACCCTCACTAAAGGG | 51 | 56 |

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.

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

Note that we cannot mix both customer and universal primer in the same order. If you want to use both, you have to place two separate orders.

When it's time to fill your plate or your tubes, follow all the steps in "Step 4. Mix your DNA template and primers" with two exceptions:

1. You will bring your DNA samples to a **total volume of 10 uL** instead of 18 uL.
2. The tubes or plate you give us contain **ONLY** your template DNA. Therefore, you will follow the instructions to fill the plates or tubes, but you will skip the step where you add the custom primer.

¹HT: high temperature. These 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.

² Tm1 Estimated using the McConaughy equation.

³ Tm2 Estimated using the Wallace equation.