

Frequently Asked Questions

DNA Sequencing Questions

Q: How much DNA do I need?

Please see the sample submission page on our website at <http://www.cabnr.unr.edu/genomics/sample.htm>

Q: How should I quantitate my DNA?

It's essential to quantitate the DNA. Many labs use either a spectrophotometer or the NanoDrop to quantitate their DNA samples. Both of these techniques will measure total nucleic acids present (including RNA) and consequently clean DNA preps are vital. We quantitate DNA samples by fluorimetry, using PicoGreen, and because it measures only the double stranded DNA, we often get lower values than a spectrophotometer or a Nanodrop. If you do not have access to a reliable instrument for quantitation, or your sequencing is failing due to insufficient template DNA, it may be beneficial to compare your quantitation technique to the Picogreen for accuracy. If you look at your DNA on agarose gels then once you have got your sequencing to work, it's possible to just estimate the DNA concentration from the agarose gel. Don't try this until you have successfully sequenced your DNA, and remember that if you are going to clean up the DNA you need to look at it on the gel AFTER you clean it up, not before.

Q: How much primer do I need?

Please see the sample submission page on our website at <http://www.cabnr.unr.edu/genomics/sample.htm>

Q: Are primers provided by the Nevada Genomics Center?

Yes, we have 100ul aliquots of the following primers at 2uM that we offer at no charge to our in-house customers. We will add them for customers who are off campus. Please be sure to check these sequences against your vector before you use them. There is more than 1 version of many of these primers!

Primers provided by NGC	
Name	Sequence
M13(-21)F	TGTA AACGACGGCCAGT
M13(-46)R	GAGCGGATAACAATTTACACAG
T3	ATTAACCCTCACTAAAGGGA
T7	TAATACGACTCACTATAGGG

SP6	ACGATTTAGGTGACACTATAG
polyT mix	TTTTTTTTTTTTTTTTTTTTTTTTTT(A/C/T/G)

Q: What type of tube should I use and how do I send sequencing samples?

Please see 'sample format' on the sample submission page on our website at <http://www.cabnr.unr.edu/genomics/sample.htm>

Q: How do I submit a sequencing request?

You will need to establish an account in our dnaTools server before you will be able to submit a job request. If the lab PI already has a dnaTools account then any new user from the lab can create an account from the [Create Login Account for dnaTools](#) page and selecting the PI from the drop down list. If your lab does not have an account on dnaTools then please contact us first by [email](#) with all of the following information:

Lab PI	Billing	Your Information
Name	Name	Name
Email	Email	Email
Phone #	Phone #	Phone #
Mailing address	Fax #	Mailing address
	Mailing address	

Once you have a dnaTools account there are two options for submitting a sequencing request: you may use either the [Enter DNA Sequencing Requests](#) link or you may [Upload and Import](#). Both procedures are described in detail in the PDF instructions for using dnaTools that are found on the Submissions page of the NGC website <http://www.ag.unr.edu/genomics/protocols.html>.

Q: When will I get my data?

Average turnaround time for sequencing and fragment analysis is 2 to 4 working days after the job request has been entered on dnaTools AND the samples have been received. If we need to do other steps, e.g. PCR cleanup, enzyme digest, then it may take longer.

Q: How do I view and download my sequencing data?

Log into dnaTools and to view or download your DNA Sequencing Results select the Download DNA Results link. Two tables are displayed, so you can choose the data you want to view either by order number or by the run plate number. After you make your selection click "submit". The next page allows you to either download or view your sequencing results. Complete instructions on viewing or downloading your data can be found in the PDF instructions for using dnaTools

that are found on the Submissions page of the NGC website
<http://www.ag.unr.edu/genomics/protocols.html>.

If you wish to access old data that is on the Finch server, then you need to log onto the Finch server using your assigned login name and password. Open either the sequencing folder list or the genotyping folder list. If you click on the folder name you will get a summary of various statistics for the folder and you can choose the download icon (near top right of page) to download all the files in the folder. If you click on the number of samples, then you will get a list of the sample names. You may need to sort by date (click on the blue 'date' header) to order the samples. There will be a summary for each sample with: trim length, number of Q20 bases, screen positive (E.coli sequence), short insert and pass/fail. To see an individual sample, click on the sample name.

Q: What are Q20 values?

The base calling program used in conjunction with the ABI Prism 3730 DNA analyzer is Phred, a program developed by Dr. Phil Green and Dr. Brent Ewing. Phred reads DNA sequencer trace data, calls bases, assigns quality values to the bases, and writes the base calls and quality values to output files. After calling bases, Phred evaluates the trace surrounding each called base using four or five quality value parameters to quantify the trace quality. Quality scores range from 4 to about 60, with higher values corresponding to higher quality. The quality scores are logarithmically linked to error probabilities, as shown in the table below.

Phred quality score	10	20
Probability that the base is called wrong	1 in 10	1 in 100
Accuracy of the base call	90%	99%

It has been shown that Phred's error probabilities are very accurate. The most commonly used method is to count the bases with a quality score of 20 and above, thus the "Q20" value, which indicates an accuracy of 99% for the base called.

References:

Brent Ewing, LaDeana Hillier, Michael C. Wendl, and Phil Green. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. 1998. Genome Research 8:175-185.

Brent Ewing and Phil Green. Base-calling of automated sequencer traces using phred. II. Error probabilities. 1998. Genome Research 8:186-194.

Q: How do I view my sequencing data after I've downloaded it?

To view chromatograms in .ab1 files you will need to download the freeware version of FinchTV from the Geospiza website <http://www.geospiza.com/finchtv/index.htm>. FinchTV is a viewer for the sequencing electropherograms. If you have downloaded the text string of sequence you can open the files in either Microsoft Word or Excel. For complete instructions on the various downloading options available the files that you will generate refer to the PDF instructions for using dnaTools that are found on the Submissions page of the NGC website <http://www.ag.unr.edu/genomics/protocols.html>.

Q: How many bases of sequence should I get?

We use 36 inch capillaries on our ABI Prism 3730 DNA analyzer which will give you 650-750 bases of Q>20.

Q: What does the Nevada Genomics do to check that the capillaries are all functioning on the sequencer?

The dnaTools server provides a summary to the administrator of all plate average Q20 values and sequence pass rates, as well as a visual representation of the quality of sequences obtained from each capillary, in order to allow tracking of the array quality.

Q: What controls does the Nevada Genomics Center use for the sequencing reaction?

Each plate has a pGEM control run in one well. This control allows monitoring of the Big Dye master mix, the thermocycler run, the clean-up process, and the 3730 run.

Q: Why did my sequencing reactions fail?

The primary reason samples fail is template concentration. Look at the signal strength of the failed sample to see if it is low (less than 100). To view the signal strength for a run/file, first select "View" for that file, then once the file opens, select "Chromat Info", scroll to the bottom of the table and you will see "Average intensity" – that is the signal strength. We have found that low signal strength frequently occurs due to insufficient template. We suggest that you check your method of quantifying the template DNA to insure it accurately tells you how much double stranded DNA is present.

The primer also plays a vital role in successful sequencing. A low signal strength and failed sequencing may be seen if there was no or very poor priming. The simplest method to test the priming efficiency is to run a PCR reaction using the primer and see if you get product. Sequencing can also fail if the primer lies down in more than spot on the template DNA, in which case double sequencing will result.

Sequencing depends not only on the quantity of template DNA, but also upon the quality. It is essential to the success of the sequencing reaction that the DNA be purified using a method that gives good quality DNA. For plasmids we are using Qiagen R.E.A.L. kits. It is important that your sample contain only a single template as multiple templates will give unreadable double sequencing. Contamination with RNA or protein will affect sequencing, so clean-up your sample to remove any contaminants. Sequencing will not occur in the presence of various chemical contaminants, primarily EDTA. Your template DNA and primer should be in water, never in TE, if you wish to have efficient sequencing.

To prepare a PCR product for sequencing a spin column or filter cleanup can be used if there is only one PCR product. When the PCR reaction yields more than one product, and the primer used for sequencing is the same as one of those used to generate the PCR products that primer will sequence every PCR product present and the result will be unreadable sequence. In this case the PCR products should be separated on an agarose gel and the band of interest excised from the gel. Kits are available to purify the DNA from the agarose (but you must wash ALL the salt out). A second option for multiple PCR products is to sequence with an internal primer which will only see the band of interest. Be aware that spin column or filter cleanup of PCR products does not remove all of the PCR primers and these will carry over into the sequencing reaction. If the PCR yield is high and the PCR product size is greater than 300 base pairs, the effect of these PCR primers in the sequencing reaction is negligible. However, for small PCR products, with low yield, the PCR primers can generate significant sequence, and agarose gel purification is recommended.

If we can be of any further assistance in trouble shooting failed sequencing, please feel free to contact us.

Q: Will my samples be rerun if the results are not good?

NGC checks the data from all plates. If any problem appears to be an instrument or chemistry failure, the plate will be rerun at no charge. If the client wishes samples to be rerun, NGC will rerun them. If there is no improvement, then the client will be charged for the run, but if the sample gives a better result – an improvement of more than 100 in the Q>20 value – then NGC will not charge for the rerun. If NGC sees an obvious problem with the sample, eg high salt concentration, G/C rich region, double priming, then we will inform the researcher and suggest remedies.

Fragment Analysis / Genotyping Questions

Q: How do I submit a fragment analysis request?

You will need to establish an account in our dnaTools server before you will be able to submit a job request. If the lab PI already has a dnaTools account then any new user from the lab can create an account from the [Create Login Account for dnaTools](#) page and selecting the PI from the drop down list. If your lab does not have an account on dnaTools then please contact us first by [email](#) with all of the following information:

Lab PI	Billing	Your Information
Name	Name	Name
Email	Email	Email
Phone #	Phone #	Phone #
Mailing address	Fax #	Mailing address
	Mailing address	

Once you have a dnaTools account there are two options for submitting a fragment analysis request: you may use either the [Enter Fragment Analysis Request](#) link or you may [Upload and Import](#). Both procedures are described in detail in the PDF instructions for using dnaTools that are found on the Submissions page of the NGC website <http://www.ag.unr.edu/genomics/protocols.html>.

Q: How do I retrieve fragment analysis data from the Finch server?

Log into dnaTools and to view or download your Fragment Analysis Results select the Download Fragment Analysis Results link. Two tables are displayed, so you can choose the data you want to view either by order number or by the run plate number. After you make your selection click "submit". The next page allows you to either download or view your results. Complete instructions on viewing or downloading your data can be found in the PDF instructions for using dnaTools that are found on the Submissions page of the NGC website <http://www.ag.unr.edu/genomics/protocols.html>. Once you have downloaded the data you will need to extract or unzip the files. You can then open GeneMapper or PeakScanner and import the files.

If you wish to access old data that is on the Finch server, then you need to log onto the Finch server using your assigned login name and password. Open the Genotyping folder list, and click on your folder name. You will see a list of 25 of your sample names. If you have more than 25 samples, click on "all" to list them all, or "more", if you don't want to download all of them ('more' will double up the number listed each time). Click on the down arrow above the column of boxes to the left of the file names to select the whole list and 'unclick' some of the boxes if too many are listed. Scroll to the bottom of the page and select a download method and OK. Whatever method you choose the files will download as a zipped folder and you will need to use winzip to unzip the files. You can then open GeneMapper or PeakScanner and import the files.

Q: What dyes can I use to label my primers for fragment analysis?

The 3730 sequencer runs filter set G5, which detects 6-FAM (blue), VIC (green), NED (yellow), PET (red) and LIZ (orange) for the size standard.

Q: Can I use any other dyes?

Yes, and no. A number of people have primers labeled with HEX which is green in filter set D. We can run HEX on the 3730 but in addition to green there will also be a yellow peak. It is important not to run a multiplex with HEX and NED, or you will run the risk of confusing the yellow from the HEX with the yellow from the NED. There will also be a lower signal for HEX which means that you will have needed a good PCR reaction.

Q: Which primer do I label?

Whichever you like, but for microsatellites only label one from each pair.

Q: How do I know what dilution to send for microsatellite PCR products?

Each new set of primers has to be tested to find the optimal dilution. The first time you try out a new set of primers, or multiplexed primers, send us the undiluted PCR product (we need about 5ul), and we will determine the dilution that needs to be made for our instrument. After that, you can make the dilution and send 1ul of the diluted PCR product.

Q: How can I get a good signal from every sample on the plate?

In order to get all the samples on a plate on scale, they must all have about the same amount of PCR product in them. The only way to ensure that is to normalize your DNA before you do any PCR reactions.

Q: How do I normalize my DNA?

Determine the DNA concentration for every sample on the plate. Calculate how many ng you will need to get, say 100ng total DNA and transfer that amount from each well to a new plate. Dry it down. Resuspend all the samples in 20ul water. They will now all be at 5ng/ul. You can then add 4ul to each PCR reaction and know that each reaction has 20ng template DNA.

Q: Normalizing seems like a lot of trouble. Is it really necessary?

YES!!! It's a couple of hours spent at the beginning when you are in a hurry to get results, but it's many hours saved later (not to mention money) when you are trying to analyze data, and some of it is off scale and some has dropped out, and then you must cherry pick your way through the plate and redo the PCR on those that didn't work.