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|---|---|
| | FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM<br>*Standard Operating Procedure* |

| | | | |
|---|---|---|---|
| Title: DNA Extraction and Quantification in Preparation for Whole Genome Sequencing | | | |
| SOP #: see Wiki | Revision: 04 | Revision Date: 10/27/2016 | Effective Date: 10/27/2016 |
| Author: Sarah Beno and Sophia Harrand | | Approved by: fill in after Martin OK's | |

# ***DNA Preparation for Whole Genome Sequencing***

## FILE NAME: WGS_SOP.doc

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# SECTION 1 INTRODUCTION

## 1.1 Purpose

The purpose of this document is to set forth **standard** guidelines for genomic DNA extraction from multiple genera, and the steps to quantify and submit this DNA in 96-well skirted plates for Nextera XT library preparation and Whole Genome Sequencing on the Illumina platform at the Cornell BRC/CORE and at the ADHC.

## 1.2 Scope

This SOP applies to the Food Safety Lab and the Milk Quality Improvement Program.

## 1.3 Definitions

**260/280 ratio**: The ratio of: the wavelength at which DNA & RNA absorb light, over the wavelength at which aromatic amino acids absorb light. A spectrophotometric measure of nucleic acid purity.
**260/230 ratio**: The ratio of: the wavelength at which DNA absorbs light, over the wavelength at which organic contaminants (e.g., guanidine hydrochloride) absorb light. A spectrophotometric measure of nucleic acid purity.

## 1.4 Safety

Many of the microorganisms used in our lab (e.g., *Salmonella* and *L. monocytogenes*) are BSL-2 pathogens. If you are using this SOP with human pathogens, appropriate protective equipment should be worn and appropriate protective measures need to be taken. All waste from BSL-2 organisms must be treated appropriately.

# SECTION 2 MATERIALS

- **DNA extraction kit:** Qiagen QiAmp, Qiagen DNAEasy, or MO BIO PowerSoil kits can be used, depending on what has been optimized for the organism from which you are extracting DNA.
- **Tris-HCL, pH = 8:** for preparing lysozyme solution, and eluting, storing and diluting DNA during all downstream processes **when using Qiagen Extraction Kits**. You must prepare this (recipes are available in any of the lab library reference manuals). Note: MO BIO kits have their own elution buffer (also a 10mM Tris solution) that should be used for elution, and potentially for all downstream processes, also.
- **EDTA, pH = 8:** for preparing lysozyme solution **when using Qiagen Extraction Kits**. You must prepare this (recipes are available in any of the lab library reference manuals).

- **NanoDrop** Spectrophotometer: for 260/280 & 260/230 ratios (to check nucleic acid purity) prior to dsDNA quantifiation.
- **Qubit®**

  Qubit 3.0 CORE/BRC: Fluorometer & Assay Kit (dye, buffer and equipment are all available and housed at the Cornell Biotechnology CORE/BRC): for quantifying dsDNA prior to sample submission.

  Qubit 2.0 Stocking: Fluorometer & Assay Kit (reagents and tubes are in our lab, the Qubit 2.0 is in the Dando lab). Reagents are Qubit dsDNA High Sensitivity (HS) Assay kit for 100 (Invitrogen, Cat. No. Q32851) or 500 (Invitrogen, Cat. No. Q32854) assays. The High Sensitivity Kit (HS) is designed to be accurate for initial sample concentrations from 10 pg⁄µl to 100 ng⁄µl.
- **Skirted 96-well plates**: for prep and submission to Cornell Biotechnology CORE/BRC.

# SECTION 3 PROCEDURES

## 3.1. Extraction of genomic DNA

*DNA extraction is performed using the QIAmp DNA Mini Kit; for detailed information on DNA extraction for WGS, please consult protocol* "8.3.13-Genomic_DNA_Extraction_Qiagen".

***NOTE**: We did not use the Qiagen DNA Easy Kit for development of this protocol; if DNA extraction of your organism is optimized with the DNA Easy kit, you should be cautious of potential incompatibilities, even though the solutions and processes are similar between the Qiagen kits.*

*Alternatively, the MO BIO "PowerSoil®" DNA Isolation Kit (see link in Reference Section) can be used, but an RNase step should be added at the first step; tech support mentions 1uL of a 25ug/mL, but the Qiagen 100ug/mL RNase A can be used with no ill effects.*

## 3.2 NanoDrop measurement of nucleic acid quality

*See* "8.3.10-Quantification of Purified DNA by NanoDrop" *for instructions on using the NanoDrop. Briefly, initiate with $dH_2O$, blank with your elution solution (e.g., 10mM Tris HCl), place 1-1.5uL of your sample on the pedestal, apply magnet and click "Measure". Once the 'clicks' stop, your 260/280 and 260/230 ratios will display, along with the concentration and a curve of your sample's optical reading. Besides the numbers, the curves are important in determining sample quality: running only **12 samples at a time** makes it easier to resolve between your curves when you print and evaluate your graph.*

NOTE: The minimum reliable concentration on the NanoDrop is **10ng/uL**. Anything lower may also mean your ratios are unreliable. Using published sources and anecdotal evidence, we have determined that **260/280 ratios between 1.7-2.1 (optimal ratio is 1.8), and 260/230 ratios between 1.4-2.4 (optimal ratio is 2-2.2)** produces good results. We also typically use Elution 2 for our downstream processes, unless Elution 1 returns better ratios. If your ratios deviate greatly from acceptable criteria, you may need to perform DNA extraction again (see Troubleshooting Section). Make sure that you mix your DNA 5 times by pipetting up and down (if 50uL elution volume have pipette set to 40uL) before measuring with Nanodrop.

## 3.3 Qubit quantification

*Though not used here, additional SOPs for quantification of dsDNA exist: "Quant iT broad range dsDNA Assay SOP" and "Pico Green dsDNA Assay SOP". Use at your own discretion.*

### 3.3.1 Qubit quantification at CORE/BRC

Precise quantification of double-stranded DNA is performed on the Qubit 3.0 Fluorometer in Rm 140 Biotech. Email Jennifer Mosher (jdm323@cornell.edu) at least 2 days ahead of the time you want to quantify, since it will take anywhere from 2-6 hours, depending on the number of samples and people present (approximately 2 hours with 3 people measuring 96 samples) – PLEASE let them know when you are coming! You need to bring your own 10uL (or 2.5uL), 200uL and 1000uL micropipettes, corresponding filter tips, a 15mL Falcon tube (to prepare Master Mix), nitrile gloves, and all your samples **diluted to 10ng/uL** (calculated from the NanoDrop concentration), for a **final volume of 20uL** each (use $C_1V_1 = C_2V_2$). Also bring a pen, fine-tipped Sharpie, and a large Eppendorf tube rack. Clear-sided, 0.6mL sample tubes are provided at the BRC/CORE.

**Set-Up**

Once there, follow the instructions posted for starting the instrument. Use the "High Sensitivity" assay and reagents. You do not need to calibrate it, but if desired, you can prepare and run the standards, though it is not necessary, as the BRC does both. You will need to prepare a Master Mix (fluorescent dye and buffer) at a ratio of [1uL dye + 199uL Buffer per sample; e.g., 24uL dye + 4,776uL Buffer for 24 samples]. **DO NOT prepare more than 24 samples' worth** of the Master Mix, as the dye is light-sensitive. Vortex the mixture and make sure there are no bubbles. **The dye must not be exposed to light for long periods of time (>30min)**. The standards, dye and buffer are all at the CORE facility; if there are reagents missing from Room 140, ask one of the techs and they will get you a new tube.

**Assay Preparation**

Handle the tubes as little as possible, do not touch the sides, and never with bare fingers. For each sample: make sure they are at room temp, vortex your DNA, add 2uL of your DNA to 198uL of the prepared Master Mix in the clear 0.6mL Eppendorf tubes. Vortex and spin down briefly to make sure there are no bubbles. Incubate at room temperature for 1-2 minutes, then follow the screenshots below. Record the displayed concentration in your WGS spreadsheet; there is no way to print the results. The Qubit will store numeric data and build graphs, if desired, but that is beyond the scope of the present needs.

**Note:** Incubate the samples for the appropriate amount of time after mixing them with the working solution (2 minutes for the Qubit® DNA and RNA assays, 15 minutes for the Qubit® protein assay).

1. In the *Read standard* screen, touch **Run samples**


2. In the *Sample volume* screen, select the sample volume and units:
   a. Touch the + or – buttons on the wheel to select the sample volume added to the assay tube (between 1 and 20 µL).
   b. From the dropdown menu, select the units for the output sample concentration.

3. Insert a sample tube into the sample chamber, close the lid, and then touch **Read tube**. The reading takes approximately 3 seconds.


4. The software displays the results.

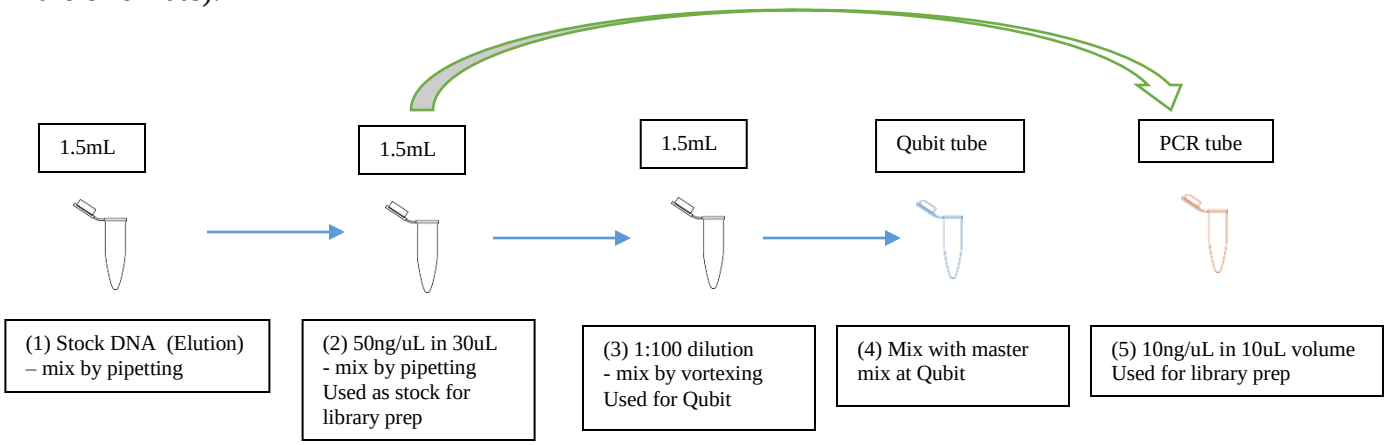

To read multiple samples for the same assay:

1. Remove the current sample, and insert a new sample.
2. To change the sample volume, swipe **right** or press the **left arrow**.
3. Touch **Read tube**.
4. Repeat step 2 and step 3 (above).

### 3.3.2 Qubit quantification at Stocking using Qubit 2.0

Precise quantification of double-stranded DNA is performed on the Qubit 2.0 Fluorometer in the Dando lab. Please bring the reagents and tube from our lab, as well as pipettes (we want to avoid cross contamination). The reagents are stored in Pedro's friend. The Qubit dye is light sensitive and should be covered in aluminum foil at all times. Both the dye and the tubes are stored in the shelf under the PFGE 37°C water bath. Furthermore, you need to bring your own 10uL, 20uL, 200uL and 1000uL micropipettes, corresponding filter tips, a 15mL Falcon tube (to prepare Master Mix), nitrile gloves. You should have a set of samples **diluted to 50ng/uL** (calculated

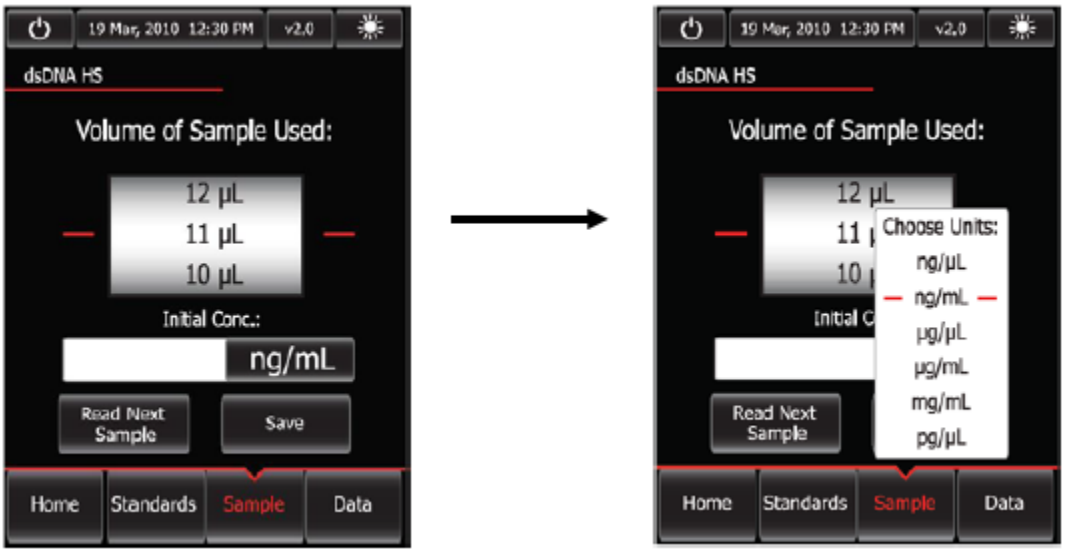
from the NanoDrop concentration), for a **final volume of 30uL** each (use $C_1V_1 = C_2V_2$). This will be used when preparing DNA for WGS submission. Prepare **1:100** dilutions using Tris-HCl (final concentration **0.5ng/uL**) and bring the 1:100 dilution sample to the Qubit. Store all DNA dilutions at 4C as freeze thawing cycles can reduce the quality of your DNA. Also bring a pen, fine-tipped Sharpie, and a large Eppendorf tube rack.
Note: Before you prepare dilutions make sure you mix your stock DNA by pipetting gently up and down (do not vortex as you don't want to shear your DNA). The mixing step is important to get a more accurate reading. The 1:100 dilution can be mixed by vortexing as you will not use it for the library prep. Furthermore, if the Qubit read is very high and outside of the range, prepare a higher dilution of your stock DNA (don't further dilute your 1:100 dilution as it will increase the error rate).




**Set-Up**
Once there, follow the instructions posted for starting the instrument. Use the "High Sensitivity" assay and reagents. You will need to prepare a Master Mix (fluorescent dye and buffer) at a ratio of [1uL dye + 199uL Buffer per sample; e.g., 24uL dye + 4,776uL Buffer for 24 samples]. **DO NOT prepare more than 24 samples' worth** of the Master Mix, as the dye is light-sensitive. Vortex the mixture and make sure there are no bubbles. **The dye must not be exposed to light for long periods of time (>30min) and vortex before adding to master mix**. Prepare your two standards by setting up two clear 0.6mL Eppendorf tubes and load 190uL of your master mix and add 10uL of standard #1 in your first tubes and 10uL of standard #2 in your second tube. Vortex your tubes for 2-3sec. Incubate all tubes for a minimum of 2 minutes at room temperature and read within 1 hr. The short time frame is required to minimize the exposure of the reagent to light.
**NOTE:** Ensure that the assay is performed entirely at room temperature. Do not hold the assay tubes in your hand before a measurement, because holding the tubes warms the solution and results in a low reading. Make sure there are no air bubbles in the tube before the read. Wear gloves at all times.

**Qubit 2.0 Operation**

Touch the screen to activate the device. When prompted to choose the sample type, select "DNA". When prompted to choose the assay type select "dsDNA Broad Range" or "dsDNA High Sensitivity" depending on the assay used. When asked whether new standards are available, select "yes". Insert Standard 1 and push "read". Insert Standard 2 and push "read". **NOTE:** If the instrument is unable to read the standards, start the process over by preparing new standards and samples using the instructions outlined in step. Insert the first user sample and push "read." Once the initial read is complete, push "calculate stock concentration" and select 10 µ on the volume roller wheel, in the Dilution Calculator Screen (**the units should be ng/µL** when proceeding with Nextera XT library prep).




Hit "calculate stock concentration" after each read and press save if you wish to save your data on a flash drive. Alternatively, record the displayed concentration in your WGS spreadsheet; there is no way to print the results.
**NOTE:** The "Save" button can be pushed multiple times without creating duplicate entries.

Proceed to the next user sample and repeat steps until all samples have been read.

### 3.4.1 Preparing Sample Plates for Submission to BRC

Dilute your samples to **0.2-1 ng/uL** each in 10mM Tris-HCl and load them into a 96-well plate, making sure to add the correct sample to the correct well. Prepare the dilution directly in the 96-well plate wells, as this minimizes loss (you are working with very small volumes!). You will need to submit a spreadsheet with the Sample Name, the Final Concentration and the Well Number with your plate to the BRC. The 96-well plate should be filled column-wise (A1, B1, C1 etc.). We have had success submitting at **1 ng/uL**, but you can choose to submit at any

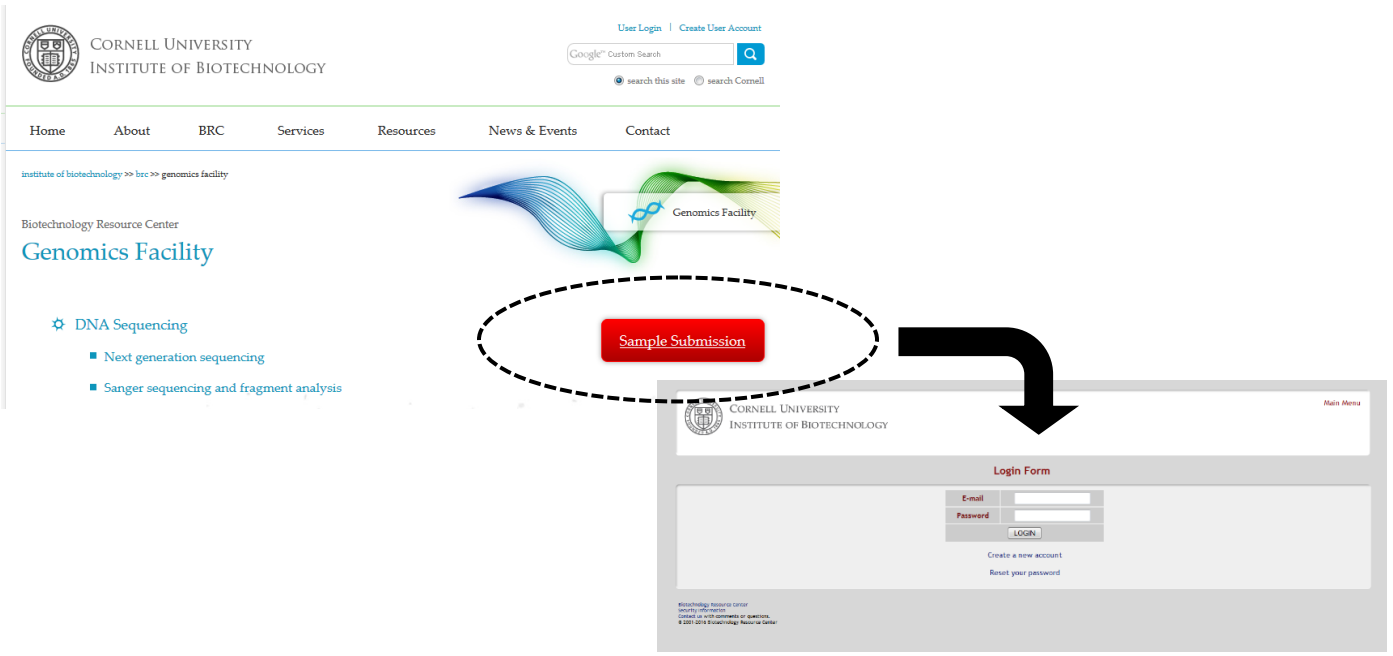
concentration within the specified range. If any sample is 0.2ng/uL< **x** <1ng/uL, you can submit it with that concentration, but make sure that is noted clearly in your submission form, and that you've discussed with someone at the CORE/BRC. If a significant number are <1ng/uL, check with the CORE/BRC to see if you should dilute everything to a lower concentration, as this may affect library prep.

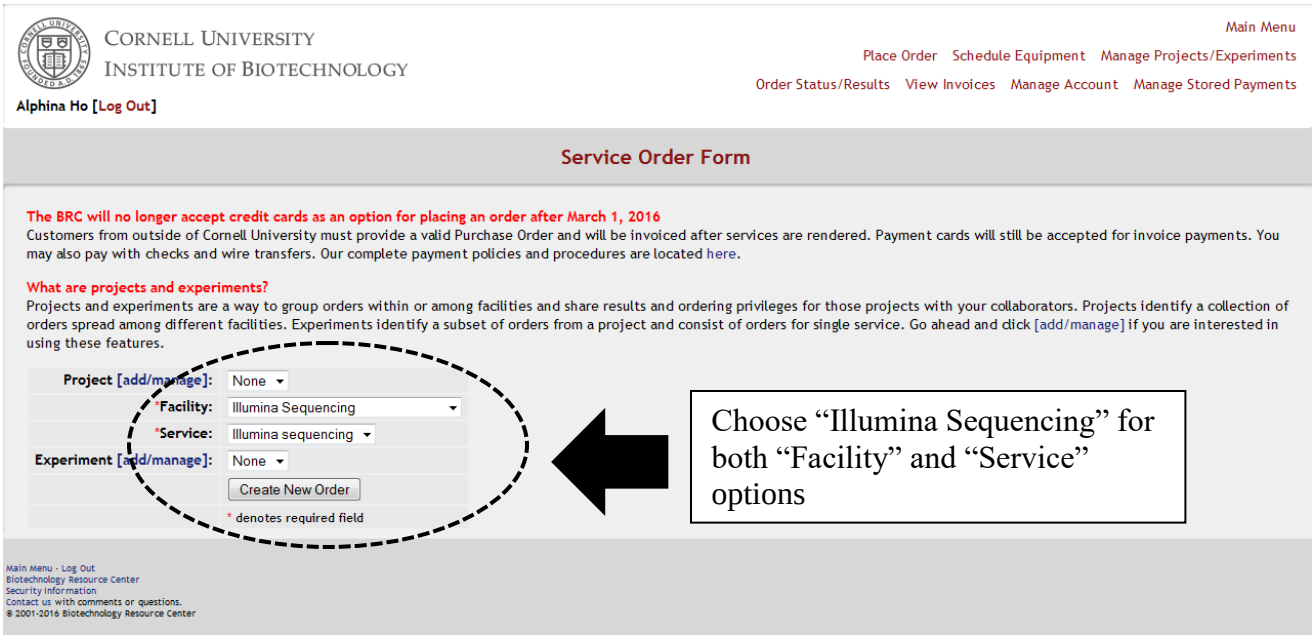
### 3.4.2 Preparing Sample Plates for Submission at ADHC

Dilute your samples to **10 ng/uL** each in 10mM Tris-HCl and load them into a 96-well plate (or PCR tubes depending on the number of samples), making sure to add the correct sample to the correct well. Prepare the dilution directly in the 96-well plate wells, as this minimizes loss. You will need to submit a spreadsheet with the Sample Name, the Final Concentration and Nanodrop 260/280 ratio. The 96-well plate should be filled column-wise (A1, B1, C1 etc.). At the ADHC the samples will be serial diluted to 2ng/uL and 0.2ng/uL. You can prepare your 10ng/uL dilution the day before and store at 4°C. If you wish to further prepare the dilutions only do so just before submission.

## 3.5 Submitting Plates to BRC

Go to http://www.biotech.cornell.edu/brc/genomics-facility, click "Sample Submission" and log in:

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Institute of Biotechnology
Alphina Ho [Log Out]
Main Menu
Place Order Schedule Equipment Manage Projects/Experiments
Order Status/Results View Invoices Manage Account Manage Stored Payments
Service Order Form
The BRC will no longer accept credit cards as an option for placing an order after March 1, 2016
Customers from outside of Cornell University must provide a valid Purchase Order and will be invoiced after services are rendered. Payment cards will still be accepted for invoice payments. You may also pay with checks and wire transfers. Our complete payment policies and procedures are located here.
What are projects and experiments?
Projects and experiments are a way to group orders within or among facilities and share results and ordering privileges for those projects with your collaborators. Projects identify a collection of orders spread among different facilities. Experiments identify a subset of orders from a project and consist of orders for single service. Go ahead and click [add/manage] if you are interested in using these features.
Project [add/manage]: None
*Facility: Illumina Sequencing
*Service: Illumina sequencing
Experiment [add/manage]: None
Create New Order
* denotes required field
Choose "Illumina Sequencing" for both "Facility" and "Service" options
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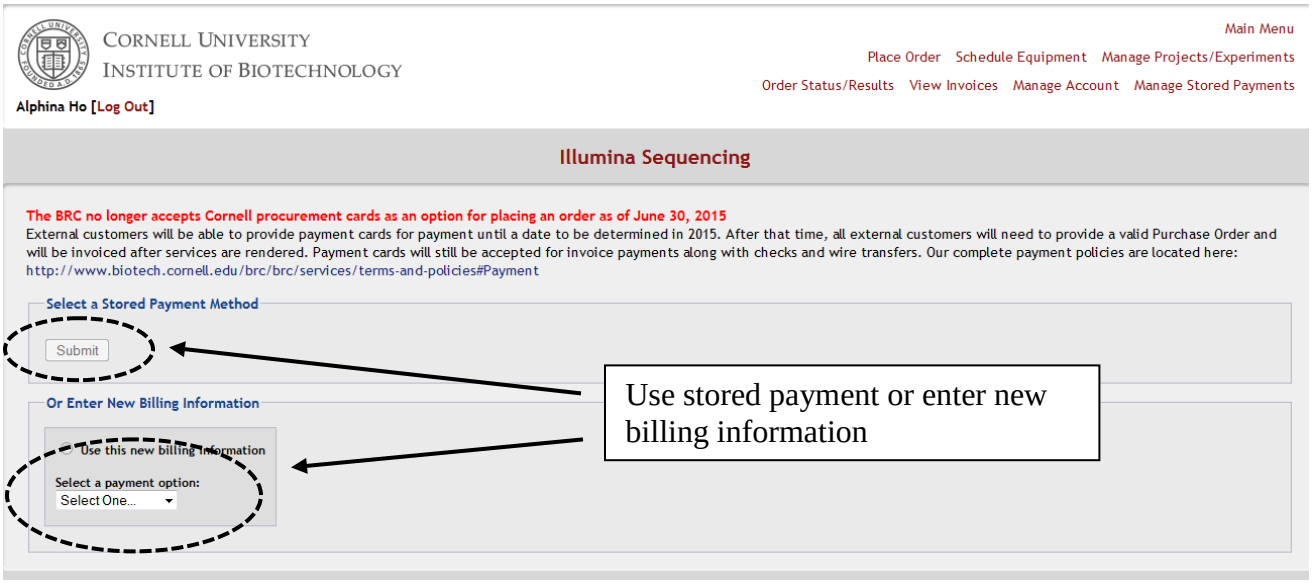
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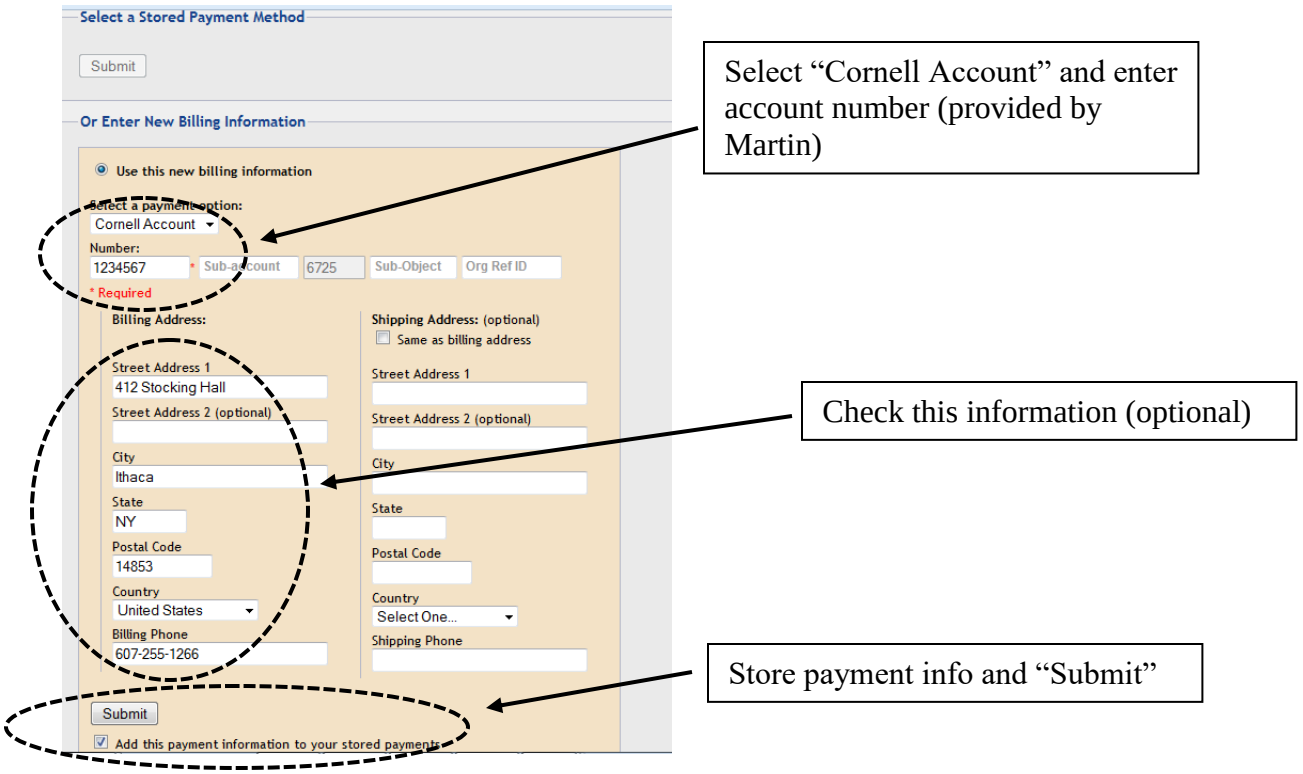
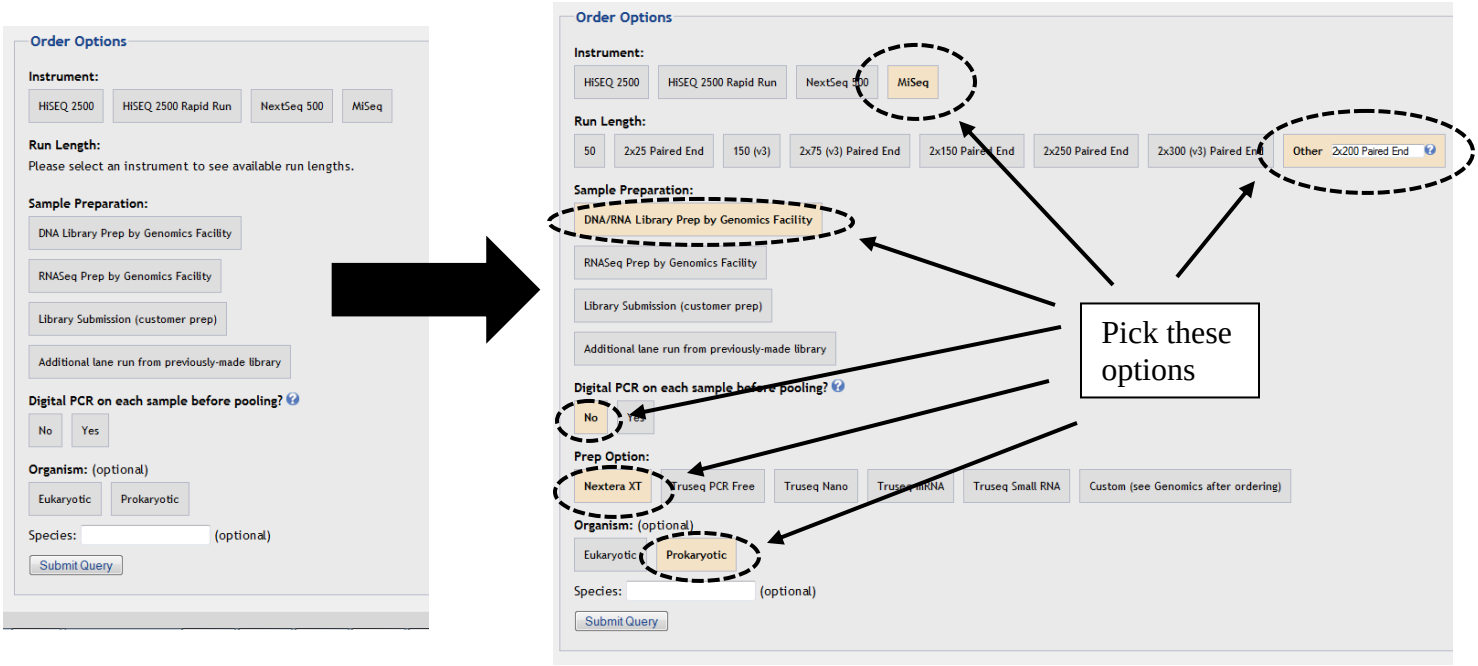
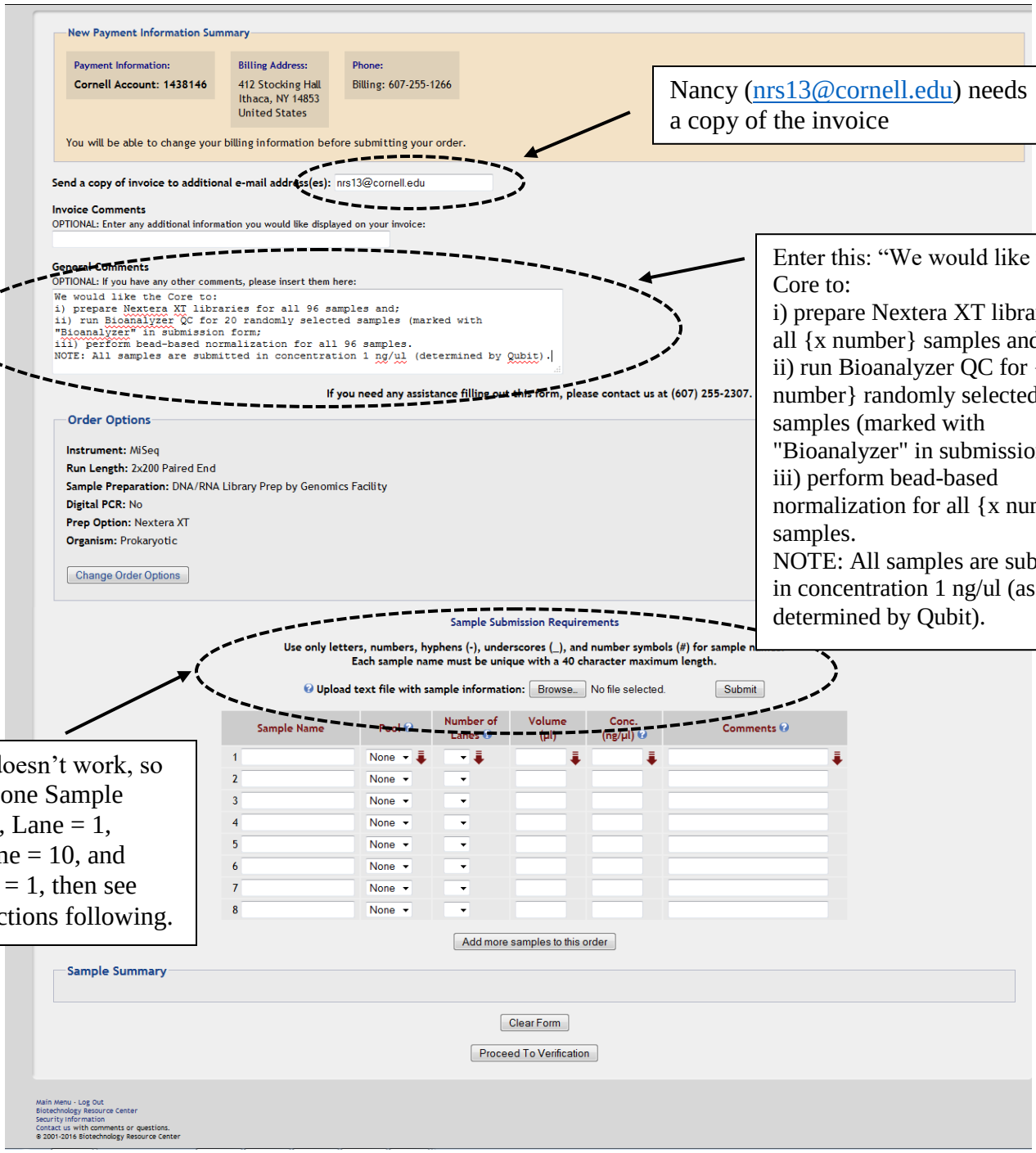
Main Menu
Place Order Schedule Equipment Manage Projects/Experiments
Order Status/Results View Invoices Manage Account Manage Stored Payments
Illumina Sequencing
The BRC no longer accepts Cornell procurement cards as an option for placing an order as of June 30, 2015
External customers will be able to provide payment cards for payment until a date to be determined in 2015. After that time, all external customers will need to provide a valid Purchase Order and will be invoiced after services are rendered. Payment cards will still be accepted for invoice payments along with checks and wire transfers. Our complete payment policies are located here:
http://www.biotech.cornell.edu/brc/brc/services/terms-and-policies#Payment
Select a Stored Payment Method
Submit
Or Enter New Billing Information
Use this new billing information
Select a payment option:
Select One...
Use stored payment or enter new billing information




Fill out the form (left) to look like the on the right (the Instrument and Run length may vary depending on what type of a sequencing run you decide for; other options stay the same):

Fill out next page as below:




Nancy (nrs13@cornell.edu) needs a copy of the invoice

Enter this: "We would like the Core to:
i) prepare Nextera XT libraries for all {x number} samples and;
ii) run Bioanalyzer QC for {y number} randomly selected samples (marked with "Bioanalyzer" in submission form;
iii) perform bead-based normalization for all {x number} samples.
NOTE: All samples are submitted in concentration 1 ng/ul (as determined by Qubit).

This doesn't work, so fill in one Sample Name, Lane = 1, Volume = 10, and Conc. = 1, then see instructions following.

*Make sure* you specify you want the CORE lab to **prepare the Nextera XT libraries** and what type of quality control (QC) steps they should perform. You should also let the CORE laboratory know you would like them to perform **bead-based normalization**, which is part of

the Nextera XT library preparation protocol. The alternative is digital PCR, which results in substantial additional costs.

**YOU MUST SUBMIT AN .xls FILE via email** to Jennifer Mosher (jdm323@cornell.edu) [please cc Peter Schweitzer (pas48@cornell.edu)] with: Sample Names with corresponding 96-well plate well numbers; Pool {A}; Lane {1}; Volume submitted; Concentration submitted. Indicate which samples should be run on BioAnalyzer in the comments column. The CORE/BRC **NEEDS** these, minimum (see example below), but you can add extra information (e.g., Qubit concentration) if desired.

| | Order #: 10359865 | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Sample** | **Pool** | **Lane** | **Concentration (Qubit; ng/ul)** | **Volume (ul)** | **Comments** | **Position in 96 well plate** |
| 1 | FSL W10-0001 | A | 1 | 1 | 10 | | A1_plate1 |
| 2 | FSL W10-0002 | A | 1 | 1 | 10 | | B1_plate1 |
| 3 | FSL W10-0003 | A | 1 | 1 | 10 | | C1_plate1 |
| 4 | FSL W10-0004 | A | 1 | 1 | 10 | | D1_plate1 |
| 5 | FSL W10-0005 | A | 1 | 1 | 10 | | E1_plate1 |
| 6 | FSL W10-0006 | A | 1 | 1 | 10 | | F1_plate1 |
| 7 | FSL W10-0007 | A | 1 | 1 | 10 | | G1_plate1 |
| 8 | FSL W10-0008 | A | 1 | 1 | 10 | Bioanalyzer QC | H1_plate1 |
| 9 | FSL W10-0009 | A | 1 | 1 | 10 | | A2_plate1 |
| 10 | FSL W10-0010 | A | 1 | 1 | 10 | | |
| 11 | FSL W10-0011 | A | 1 | 1 | 10 | | |
| 12 | FSL W10-0012 | A | 1 | 1 | 10 | | D2_plate1 |
| 13 | FSL W10-0013 | A | 1 | 1 | 10 | | E2_plate1 |
| 14 | FSL W10-0015 | A | 1 | 1 | 10 | Bioanalyzer QC | F2_plate1 |
| 15 | FSL W10-0014 | A | 1 | 1 | 10 | | G2_plate1 |
| 16 | FSL W10-0016 | A | 1 | 1 | 10 | | H2_plate1 |
| 17 | FSL W10-0017 | A | 1 | 1 | 10 | | A3_plate1 |
| 18 | FSL W10-0018 | A | 1 | 1 | 10 | | B3_plate1 |
| 19 | FSL W10-0019 | A | 1 | 1 | 10 | | C3_plate1 |
| 20 | FSL W10-0020 | A | 1 | 1 | 10 | | D3_plate1 |

Once you have completed and submitted the order, you will receive an order number. **Write this Order Number on your plate**, and use it as your subject line when you send Jennifer the email, and make sure it is on every hardcopy form you bring down to the BRC. When you take samples to the BRC, make sure they have the correct library, QC, sequencing run and sample concentration information.

Create a submission .xls file containing the order number, date of submission and names of people involved in organizing a run in a file header. This file should be saved in a subfolder of a "WGS" folder on the BoorWiedmannLab server, together with all QC files and basic sequence QC information and location of fastq.gz files received after the sequencing run is completed.

Make sure all samples and files are in the correct format: "FSL {user ID}-{4-digit number}".

### 3.6 Quality Control and BioAnalyzer

**For HiSeq runs, randomly select 10% of your samples for Bioanalyzer QC** after the tagmentations step. If 90% of these samples pass the QC control (i.e., the majority of fragments are **700-1200 bp** long, there is a **single peak** in a size distribution curve, and **curves look smooth**), you can let the CORE lab know they can proceed with library preparation. If samples pass the Bioanalyzer QC, the Core laboratory will proceed with Nextera XT library preparation and will perform bead-based normalization for all samples prior to library pooling.

If more than 10% of samples fail the Bioanalyzer QC, perform Bioanalyzer QC for **ALL** samples and **repeat** the DNA extraction and tagmentation steps for samples of poor quality. **DO NOT PROCEED with sequencing if a large percentage of your samples fail!** If a low percentage fail Bioanalyzer, you can exclude them from the current sequencing run and proceed with fewer samples. However, this may affect the cost ($$) of the sequencing run, so there are other factors to consider when deciding whether or not to proceed.

## SECTION 4 TROUBLESHOOTING

Problems potentially encountered with this procedure include:

1. **The 260/230 Ratio is too low:** Most of the time, this is due to guanidine contamination (guanidine compounds are part of the binding solution and absorb at 230nm). Qiagen recommends re-running the entire extraction procedure from the start, but there is a shortcut: re-bind the DNA to the Qiagen columns and re-wash the DNA. *Only attempt this if you have the option to re-extract the DNA if it does not work – you could completely lose all your DNA!* Start from the addition of Buffer AL and repeat the protocol from that step onward, using 700uL (vs. 500uL) AW1 and AW2 each time the DNA is washed (once with AW1 and twice with AW2). This has resulted in decreased absorbance at 230nm.

## SECTION 5 REFERENCES

1. QiAmp DNA Spin Mini Kit Manual: https://www.qiagen.com/resources/download.aspx?id=67893a91-946f-49b5-8033-394fa5d752ea&lang=en

2. MO BIO PowerSoil® DNA Isolation Kit Manual: https://mobio.com/media/wysiwyg/pdfs/protocols/12888.pdf

3. NanoDrop User Guide: http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf

4. Qubit® dsDNA HS Assay Manual: https://tools.thermofisher.com/content/sfs/manuals/qubit_3_fluorometer_man.pdf