Metagenomics Protocol (Team 7)

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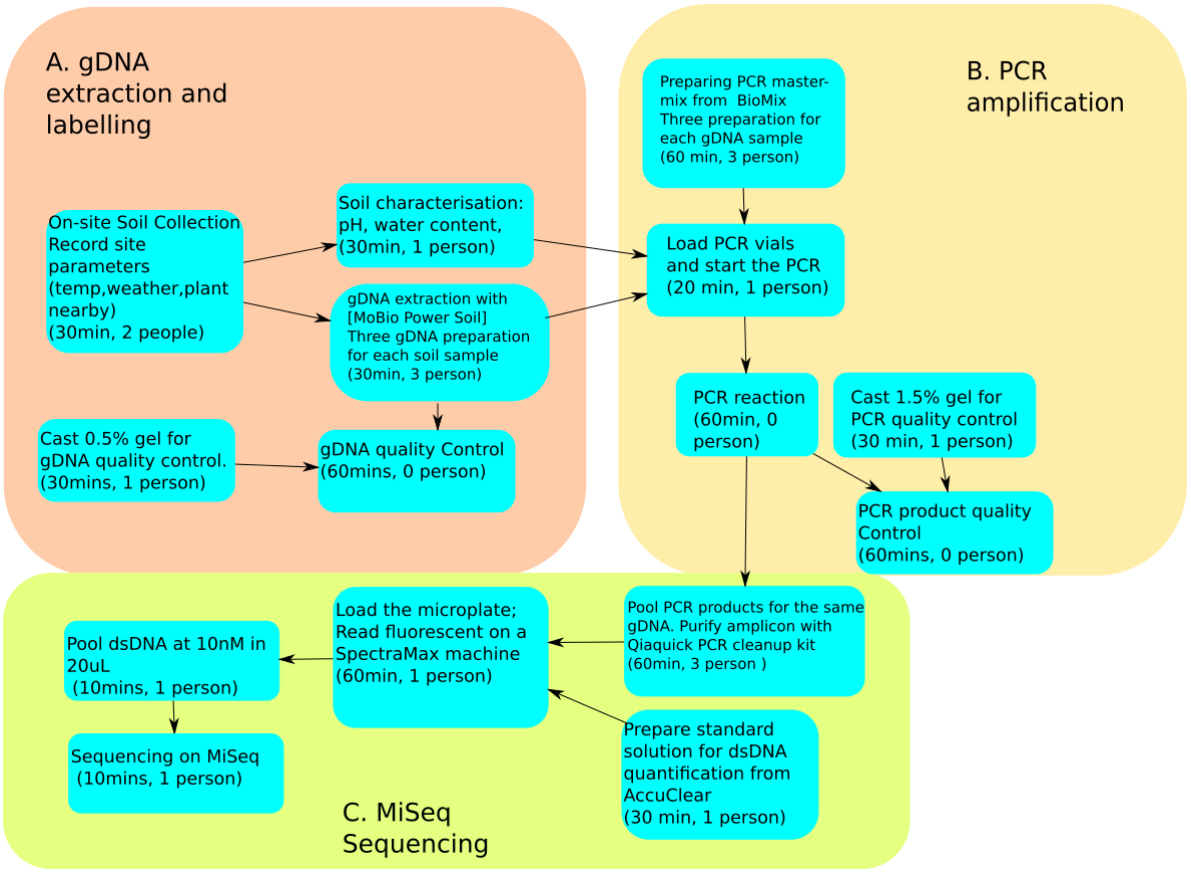
Overview:

This protocol outlines the activity undertook on Jan.31, 2017. Sample was taken at Gordon Square.

The protocol specify a reproducible workflow to produce equimolar multiplexed DNA sample for sequencing on illumina platform, from gDNA extracted from soil samples.



A1.Soil Collection and characterisation (1 hr)

1. Extract soil at 0.2m depth with a bulb planter or whatever tool.
2. Transfer 8cm^3 of soil into a clean Falcon tube.
3. Take a picture of the collection site with a marker on it.
4. Note the GPS coordinate and error.
5. Record the weather of nearest site(Bloomsbury in our case) using BBC weather.
6. Measure pH and soil water content using a 3-in-1 soil tester at the depth where you collect the soil and jot it down.
7. Alternative to step 6 :
    i. pH: Dissolve 1.0g of the collected soil in 1ml of water (100%), water-bath it at 37 degree celsius for 5min. Spin down the soil at 10,000xg for 60s and measure the pH of solution using a pH paper. Place a pH paper dipped with ddH2O as a control.
    ii. Soil moisture : Weigh 2.0g of soil. Dry the soil in the microwave/oven for 5 minutes and reweigh the sample again. Calculate water content as:

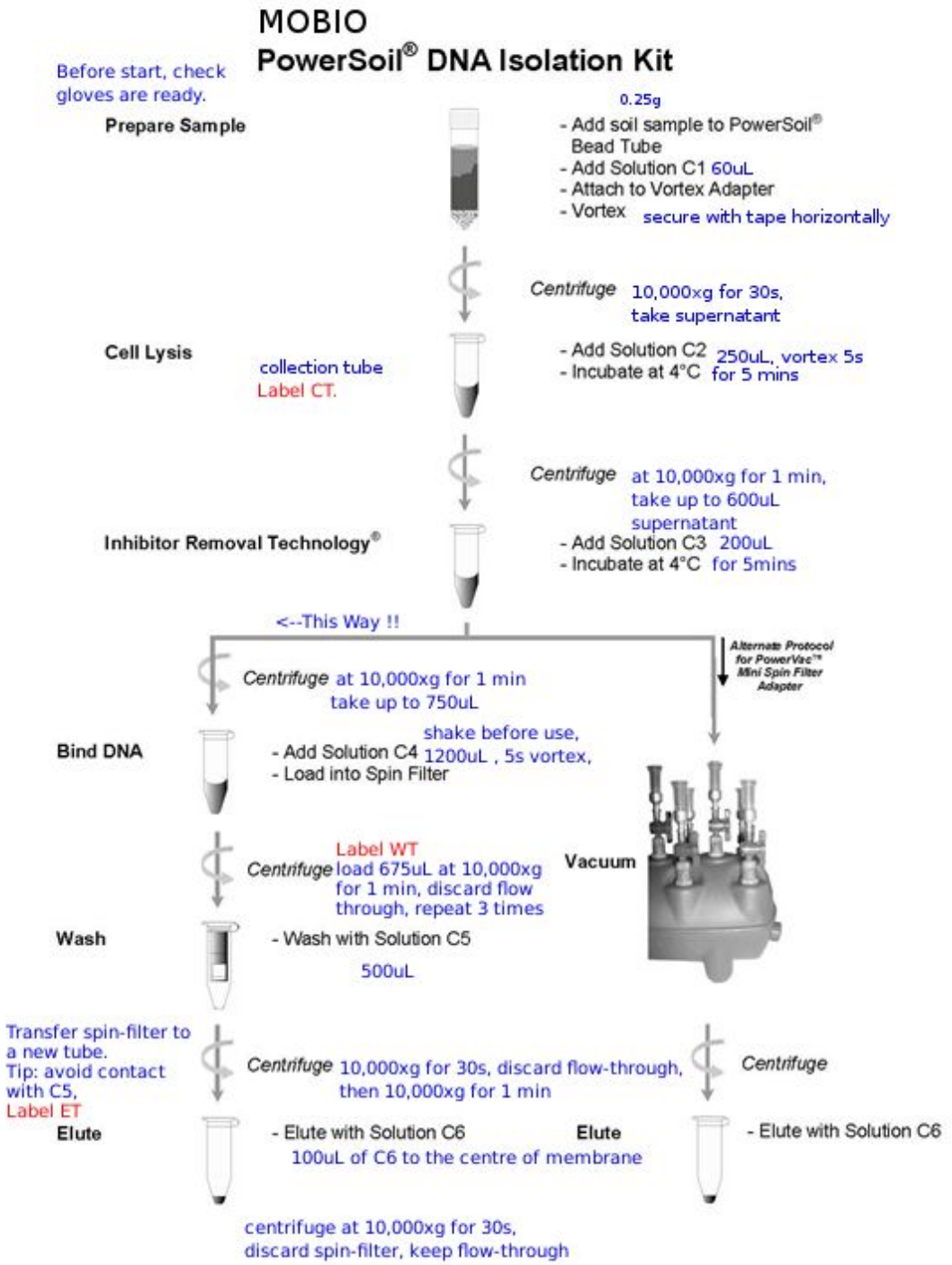
    Water Content = (Wet mass - Dry mass)/wet mass x 100%

8. Fill in the table below:

Parameter	Value
Depth	0.1m
Temperature, humidity, UV /Weather station if applicable	
pH	
Water content	
Data and time	
GPS coordinates	

A2.gDNA extraction (30 minutes)

Three gDNA extractions should be made from each soil sample. For each gDNA extraction, do the following:



1. Add 0.25g of soil sample to powerbead tubes and gently vortex.
2. Heat solution C1 to 60°C to dissolve any precipitate formed in the solution.
3. Add 60 µl of solution C1 to powerbead tubes and vortex.
4. Secure powerbead tubes horizontally and vortex at max speed for 10 minutes.
5. Centrifuge tubes at 10,000g for 30 seconds at room temperature.
6. Transfer supernatant to the provided 2ml collection tube.
7. Add 250 µl of Solution C2 and vortex for 5 seconds.
8. Incubate the collection tube at 4°C for 5 minutes.
9. Centrifuge the tubes at 10,000g for 1 minute at room temperature.
10. Transfer 600 µl of supernatant to a clean 2ml collection tube.
11. Add 200 µl of Solution C3 and vortex briefly.
12. Incubate at 4°C for 5 minutes.
13. Centrifuge tubes at 10,000g for 1 minute at room temperature.
14. Transfer 750 µl of supernatant to a clean 2ml Collection Tube.
15. Shake Solution C4 and add 1.2ml of Solution C4 to the supernatant and vortex for 5 seconds.
16. Load approximately 675 µl of supernatant into a Spin Filter and centrifuge at 10,000g for 1 minute at room temperature.
17. Discard the flow-through and repeat step 16.
18. Discard the flow-through and load the remaining supernatant into the Spin Filter and centrifuge at 10,000g for 1 minute at room temperature.
19. Add 500 µl of Solution C5 into the Spin Filter and centrifuge at room temperature for 30 seconds at 10,000g.
20. Discard the flow-through from the 2ml collection tube.
21. Centrifuge at 10,000g for 1 minute at room temperature.
22. Carefully place Spin Filter in a clean 2ml Collection tube. Avoid splashing any Solution C5 onto the Spin Filter.
23. Add 100 µl of Solution C6 to the centre of the white filter membrane.
24. Centrifuge at room temperature for 30 seconds at 10,000g.
25. Discard the Spin Filter and keep the solution.
26. The resultant ~100µl solution is a gDNA sample of the soil sample used. Designate a sampleID for it as appropriate.

A3.gDNA Quality Control (30 minutes loading, 1 hour running gel and 15 minutes recording)

1. Casting a 0.5% agarose gel following:
    a. Weigh 0.5g(1.0g) of agarose powder.
    b. Dissolve the agarose powder in 100mL(or 200mL for big tank)of 1x TAE buffer in an erlenmeyer flask.
    c. Stuff the flask opening with 2 pieces of kimwipes/Leave the cap loose.
    d. Microwave for 30 seconds then swirl, microwave for another 30seconds
    e. Swirl and make sure there is no undissolved agarose floaties. Otherwise, microwave shortly again.
    f. Let it cools on bench for 3 minutes before operation.
2. While gel is cooling, prepare the gel tray by:
    a. Secure the open ends by rubber dams.
    b. Place a comb near one end of the tray.
3. When the gel has cooled down where the bottle is cooled enough to be held be bare hands. Or else run the flask under tap water while swirling the gel.
    a. Add 10µl(or 20µl for big tank) of 10mg/mL EtBr.
    b. Swirl the bottle to mix.
    c. Dispose the tip into EtBr waste.
4. Pour the gel slowly into the gel tray.
    a. Tip around the tray to evenly distribute the gel.
    b. Flame any bubble using a lighter, especially around the comb.
    c. Let the gel settle on a level surface for 30 minutes.
5. Set up the electrophoresis as the following
    a. Place the gel tray into a horizontal gel tank.
    b. Remove the rubber stopper.
    c. Fill the tank with 1xTAE buffer until the buffer surface is 2mm above the gel.
    d. Place the tank to an undisturbed region near the power supply.
6. Load each well according to the table, store the table safely with an alias :
    a. Add 3uL of loading dye to 15uL of sample
    b. Pipette the mixture into the well.

Well No.	Content
1	1kb plus DNA Ladder
2	gDNA SampleID:xxx
...	Repeat as appropriate

7. Start the electrophoresis by:
    a. Connect the electrodes to the power supply
    b. Set the voltage to 95V (for small tank, 140V for large tank),
    c. Cover the tank with lid.
8. Turn on the power and run the gel for 1 hour, or until the frontline reaches the end of gel.
9. Wearing gloves, take off the lid and transfer the gel tray to a UV reader.
10. Inspect the gel and take a digital photo with the workstation. Store the photo safely with an alias

B1.PCR Amplification (10 minutes hands-on + 92minutes PCR)

Three PCR reactions are prepared for each gDNA sample. In addition, a positive control reaction with known 16s rDNA should be prepared for each gDNA sample.

Label 3 PCR vials with your initials and group number on top and on side.

1. Prepare 75µl of master mixture accordingly:

Reagent	Master mix volume (x3)	Dilution factor
Biomix buffer (containing dNTP, Mg2+,Taq pol, 2x Taq buffer)	37.5µl	2-fold
50mM Magnesium Chloride	1.5µl	50-fold
10µM stock solution of forward primer and reverse primer	3.8µl of each	200-fold
ddH2O(double-distilled)	25.3µl	n/a
gDNA sample	3µl	25-fold
Total Volume	75µl	

2. Pipette 25µl of master mixture into each of 3 PCR vials.
3. Secure the PCR vial and put it into the PCR machine.
4. Set the machine to run the following cycle.
    a. Initial denaturing of 94°C for 1 minute
    b. Then 35 cycles of:
        i. 94 °C for 15 seconds
        ii. 50 °C for 45 seconds
        iii. 72 °C for 30 s
    c. FInal extension of 72°C for 5 minutes
5. In "PCR quality control", pool 3 reactions (3*25µl) and take a ~5µl of sample to load to gel,
6. Proceed to the purification protocol with the rest of the PCR product.
7. The primer sequence:
    a. Forward primer (515fB 56+19=75mer):
    AATGATACGGCGACCACCGAGATCTACACGCT XXXXXXXXXXX TATGGTAATT GT
    GTGYCAGCMGCCGCGGTAA
    b. Reverse primer(806rB 36+20=56mer):
    CAAGCAGAAGACGGCATACGAGAT AGTCAGTCAG CC GGACTACNVGGGTWTCTAAT
    c. The amplicon is approximately 385bp long.

B2.PCR Quality Control (20 minutes pre-hand-on, 1 hour running gel and 10 minutes post-hand-on)

1. Casting a 1.5% agarose gel following:
    a. Weigh 1.5g(3.0g) of agarose powder
    b. Dissolve measured agarose powder in 100mL(or 200mL for big tank) of 1x TAE buffer in an erlenmeyer flask.
    c. Stuff the flask opening with 2 pieces of kimwipes.
    d. Microwave for 30 seconds and then swirl. Microwave for another 30 seconds.
    e. Swirl and make sure there is no undissolved agarose floaties. Otherwise, microwave shortly again.
    f. Let it cool under flow water until touchable (~1.5 minutes) before operation.
2. While gel is cooling, prepare the gel tray by:
    a. Secure the open ends by rubber stoppers.
    b. Place a comb near one end of the tray.
3. When the gel has cooled down where the bottle is cooled enough to be held be bare hands. Or else run the flask under tap water while swirling the gel.
    a. Add 10µl(or 20µl for big tank) of 10mg/mL(1%) EtBr.
    b. Swirl the bottle to mix.
    c. Dispose the tip into EtBr waste.
4. Pour the gel slowly into the gel tray.
    a. Tip around the tray to evenly distribute the gel.
    b. Flame any bubble using a lighter, especially around the comb.
    c. Let the gel settle on a level surface for 30 minutes.
5. Set up the electrophoresis as the following
    a. Place the gel tray into a horizontal gel tan.
    b. Fill the tank with 1xTAE buffer until the buffer surface is 2mm above the gel.
    c. Place the tank to an undisturbed region near the power supply.
6. Load each well according to the table, store the table safely with an alias :
    a. Add 3uL of loading dye to each of sample, then pipette the mixture into the well.

Well No.	Sample volume	Content
1	15µl	1kb plus DNA Ladder
2	5µl	gDNA sampleID:xxx
...	...	repeat as appropriate

7. Start the electrophoresis by:
    a. Connect the electrodes to the power supply
    b. Set the voltage to 95V (for small tank, 140V for large tank),
    c. Cover the tank with lid.
8. Turn on the power and run the gel for 1 hour, or until the frontline reach the end of gel.
9. Wear gloves and take off the lid and transfer the gel tray to a UV reader.
10. Inspect the gel and take a digital photo with the workstation. Store the photo safely with an alias

C1.Amplicon Purification (60 minutes) Kit:QIAGEN QIAquick PCR cleanup kit

One purfication should be prepared from the pooled PCR reactions for a single gDNA sample.

QIAGEN Qiaquick® PCR purification

>Start with 70µl of PCR product in a PCR vial
>Add ethanol (98%-100%) to solution PE (check label for volume)
>solution PB should contain 1:250 diluted pH indicator 1. The indicator should be added to PB only (e.g.:20 µl pH Indicator I to 30 ml PB)
>Centrifugation are carried out at 17,900xg (13,000rpm) on benchtop centrifuge at room temp.

Load:
>Add 100 µl of solution PB to 70µl PCR sample in the vial.
>Transfer the solution to a 2mL tube and add 250µl PB.
>If solution is not yellow (e.g: reddish), add 10µl of 3M NaAc and mix well until it appears yellow (pH<7.5)

Bind:
> Load a QIAquick spin column to a new 2ml collection tube, label WT.
> Add the sample to the column. > Centrifuge for 45s. (DNA is now bound to column)
>Discard flow-through.

Wash:
>Add 0.75mL of solution PE to the column to wash.
> Centrifuge for 45s.
> Discard flow-through
> Centrifuge for 60s (to remove ethanol)

Elution:
>Transfer the column to a new 1.5ml microcentrifugation tube, label ET.
>Add 50µl solution EB to the centre of column membrane.
>Centrifuge for 60s.

Verbal version:

Preparation:

1. Start with 70µl PCR sample in a 2mL tube.
2. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
3. All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature (15–25°C).
4. Add 1:250 volume pH Indicator I to Buffer PB (i.e., add 120 µl pH Indicator I to 30 ml Buffer PB or add 600 µl pH Indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH Indicator I indicates a pH of ≤ 7.5. (Add pH Indicator I to entire buffer contents. Do not add pH Indicator I to buffer aliquots.)

Actual:

1. Add 100µl of Buffer PB to 70µl of the PCR sample in a vial and mix, transfer to a 2mL tube and add 250µl of buffer PB.
2. If pH Indicator I has been added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 45s.
5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 45s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 60s.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min
10. Proceed with the 48µl eluted purified amplicon to quality-control gel and to dsDNA quantification.

C2.dsDNA Quantification Protocol (20 mins)

Estimate PCR product concentration:

Product length: (806-515+1)+56+36=384mer

0.5uM of primer ⇒ 0.5uM product

200uM of nucleotides ⇒ 200/384/2=0.26uM product

Maximum product concentration: 0.26uM

Maximum conc: 260nM*(384*660/1E6)=66ng/ul (Educated guess)

Use the following procedure to set up the assay:

1. Allow all components to reach room temperature before using.

Tip: AccuClear Nano dye is provided in DMSO, which can freeze during storage at 4°C. All kit components can be placed in a 37°C water bath for rapid warming. Allow solutions to cool to room temperature before using.

2. To minimize reagent loss in the cap, before removing the required volume, shake or vortex each component well, and then centrifuge vials briefly.

3. AccuClear Nano buffer is supplied at 20X. Dilute the buffer to 1X with deionized water on the day of use.

4. For the Bulk kit, use the dsDNA standards that are provided. To prepare a set of standards for the Explorer kit, dilute the 25 ng/µL dsDNA standard in the 1X AccuClear Nano buffer as shown in Table below.

Note: Prepare the standards fresh the day of the assay. Volumes can be scaled as necessary.

Note: When following the assay protocol that is described here, the linear range of the assay is determined to be between 34 pg/well and 250 ng/well. Depending on the microplate reader and assay volume, accuracies of 0.1 pg/well or less might be obtainable.

Std.	Final Conc.	Std. Volume	1X AccuClear Nano Buffer Volume
A	25 ng/µL	100 µL of 25 ng/µL AccuClear Nano Standard	None
B	10 ng/µL	40 µL of 25 ng/µL AccuClear Nano Standard	60 µL
C	3 ng/µL	12 µL of 25 ng/µL AccuClear Nano Standard	88 µL
D	1 ng/µL	10 µL of 10 ng/µL of C	90 µL

5. Prepare the working solution as follows on the day of the assay: Dilute the AccuClear Nano dye 1:100 in 1X AccuClear Nano buffer in a plastic container (do not use glass), and then vortex or shake to mix well.

Note: Because 200 µL of working solution is required for each standard and sample that is to be tested, volumes can be scaled as required. Prepare only as much working solution as you plan to use within 24 hours.

6. For each dsDNA standard or unknown DNA sample that is to be tested do the following: Add 10 µL of the standard or sample to a well in a black, 96-well microplate, and then add 200 µL of the working solution and mix.

Note: To test samples in triplicate, prepare three separate wells for each dsDNA standard and three separate wells for each unknown DNA sample. (Are we doing triplicates??)

7. Incubate the microplate at room temperature for 5 minutes in the dark.

8. Measure fluorescence in a fluorescence microplate reader with excitation at 468 nm and emission at 507 nm. The flowing table shows Microplate Reader Setup with SoftMax Pro Software.

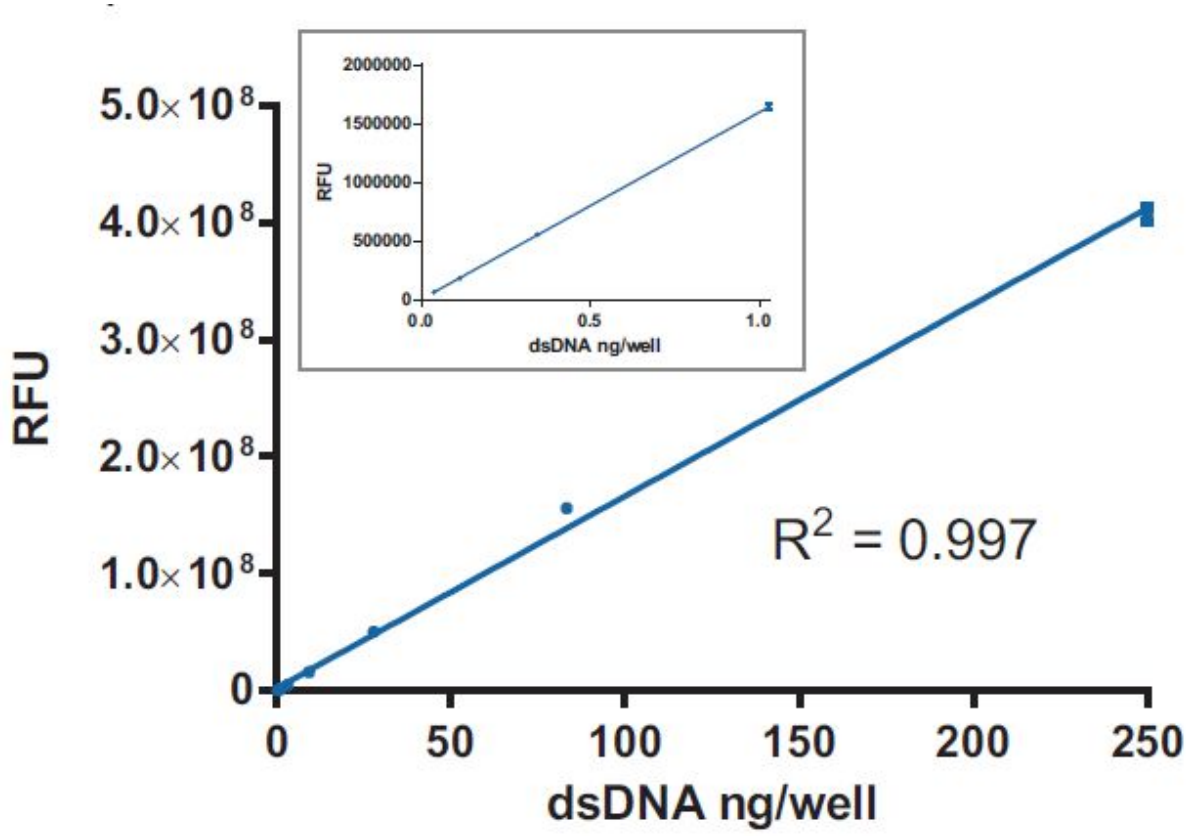
Parameter	Setting
Read mode	Fluorescence
Ready type	Endpoint
Wavelengths	Excitation: 468 nm with 9 nm bandwidth Emission: 507 nm with 15 nm bandwidth
PMT and Optics	PMT Gain: Automatic Flashes per read: 10 Read from top

9. Use the standard curve to calculate the concentrations of the unknown DNA samples. See the following example.

Note: Because the fluorescence signal decreases over time after the DNA and dye are combined—an approximately 15% decrease after 3 hours and an approximately 30% decrease after 6 hours—Molecular Devices recommends that you measure fluorescence within 1 hour of setting up the assay.

Note: If the fluorescence of any of the unknown samples exceeds the linear range, then further dilute the sample and use 10 µL of the diluted sample to do the assay. For consistency, use the same volume of sample in all the wells.

The first step in data analysis is generation of a DNA standard curve that is used to calculate the concentration of unknown DNA samples. A standard curve is generated by plotting the fluorescence values for the DNA standards on the Y-axis and the standard DNA concentrations on the X-axis. A linear curve fit is applied using the drop-down list in the graph section of SoftMax Pro Software. From the standard curve, concentrations of unknown DNA samples are interpolated. A preconfigured assay protocol in the Protocol Library of SoftMax Pro Software enables automatic graphing of standards, and calculation of unknown DNA sample concentrations from the standard curve. The figure below is an example standard curve.



R^2 is the regression coefficient.

C3.Pooling

- Convert the weight of dsDNA in 10 DNA sample into concentration of DNA amplicons in nM using the formula below:

$$Concentration\ of\ DNA\ amplicons\ in\ nM = \frac{Concentration\ in\ ng/\mu l}{\left(\frac{660g}{mol} \times 385\right)} \times 10^6$$

(385 is the expected average length of the amplicon)

- Dilute the amplicon library using Resuspension Buffer (RSB) or 10mM Tris pH 8.5 to 10nM.
- Aliquot 5µL of diluted DNA from each library and mix the aliquots for pooling libraries with unique indices.
- The pooled mixture is ready to be sequenced on MiSeq platform.