Metagenomics Protocol (Team 3)

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Soil Collection (1 hr)

1. Measure 0.5m from the bottom of the bulb planter with a ruler and mark it using a tape.
2. Look for a spot in Gordon Square Park that is near a plant and mark the location using a clean bulb planter.
3. Take a picture of the surrounding within 3m radius and get the GPS coordinate using a phone.
4. Jot down surrounding description of that marked spot in the table below:

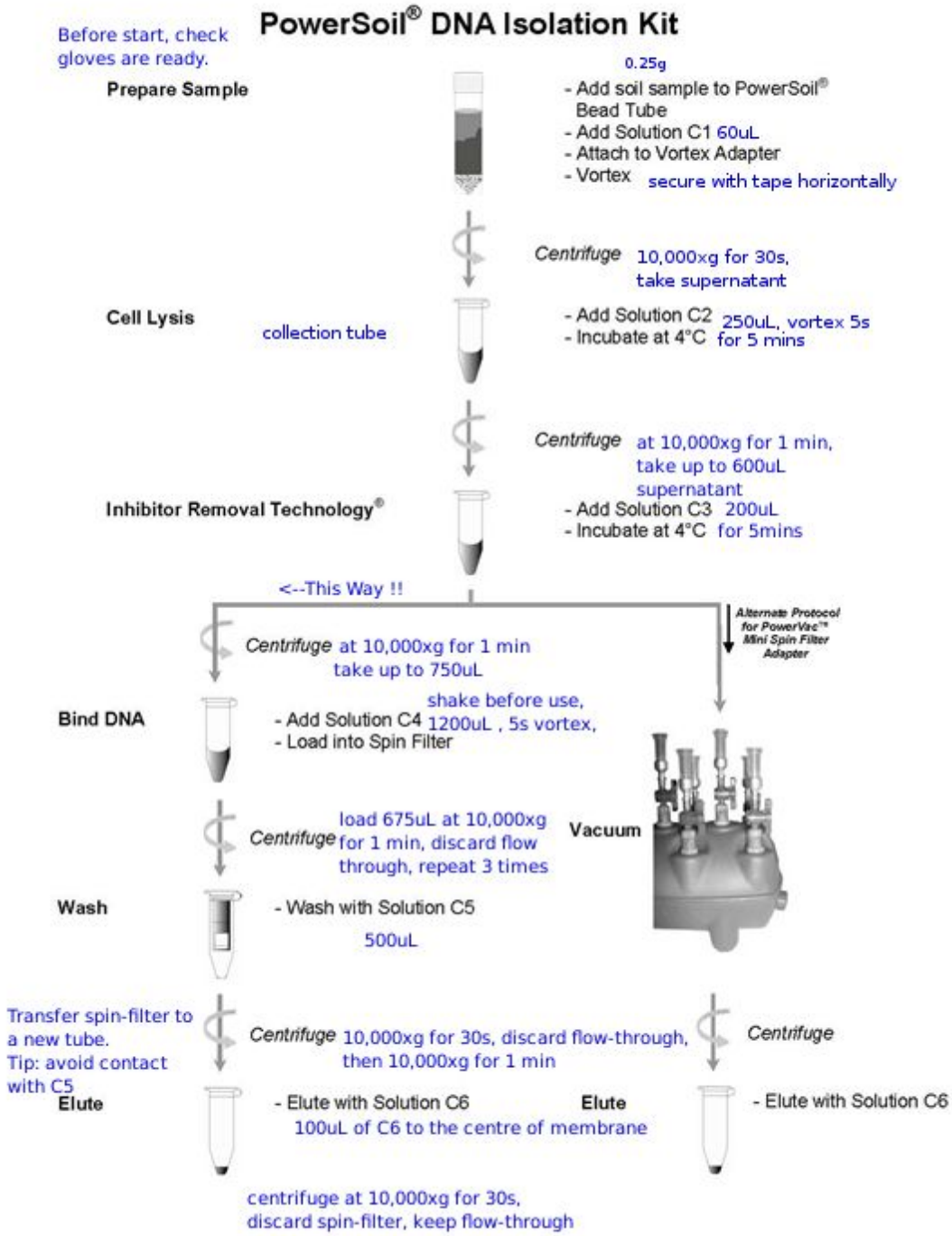
	Description
Depth (min - max)	0.5m
Temperature	
pH	
Soil moisture	
Colour of the soil	
Texture of the soil	Sandy/Smooth/Spongy/Plasticine-like
GPS coordinates	

5. With clean gloves, push bulb planter into the ground and pull it out.
6. Carefully transfer about $8cm^3$ of soil (at the depth of 1m) into the falcon tube.
7. Measure the temperature, pH and soil moisture using a 3-in-1 soil tester at the depth where you collect the soil and jot it down.
    a. **Alternative :**
        i. **Temperature : Place the thermometer into the soil at about 0.5m depth and measure the temperature.**
        ii. **pH : Dissolve 0.25g of the collected soil and measure the pH using a pH meter.**
        iii. **Soil moisture : Weigh 1g of soil. Dry the soil in the microwave/oven for 5 minutes and weigh the soil sample again. Percentage of soil moisture calculation :**

            Percentage of moisture : (Wet mass - Dry mass)/wet mass x 100%

gDNA Isolation (25 minutes)

Each person should make their own DNA extraction from 0.25g of sample.



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.
26. Take a 15µl sample for gDNA quality control. If volume is small (<22.5µl), take a 2µl sample and add 12µl ddH2O to make a 15µl sample.

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

1. Casting a 0.25% agarose gel following:
    a. Weigh 0.5g 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.
    e. Microwave for another 30seconds.
    f. Swirl and make sure there is no undissolved agarose floaties. Otherwise, microwave shortly again.
    g. 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 by:
    a. Add 3uL of loading dye to 15uL of sample
    b. Pipette the mixture into the well.

Well No.	Content
1	1kb DNA Ladder (e.g.: promega G571A, 1kb-10kb)
2	Extracted gDNA
...	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.

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

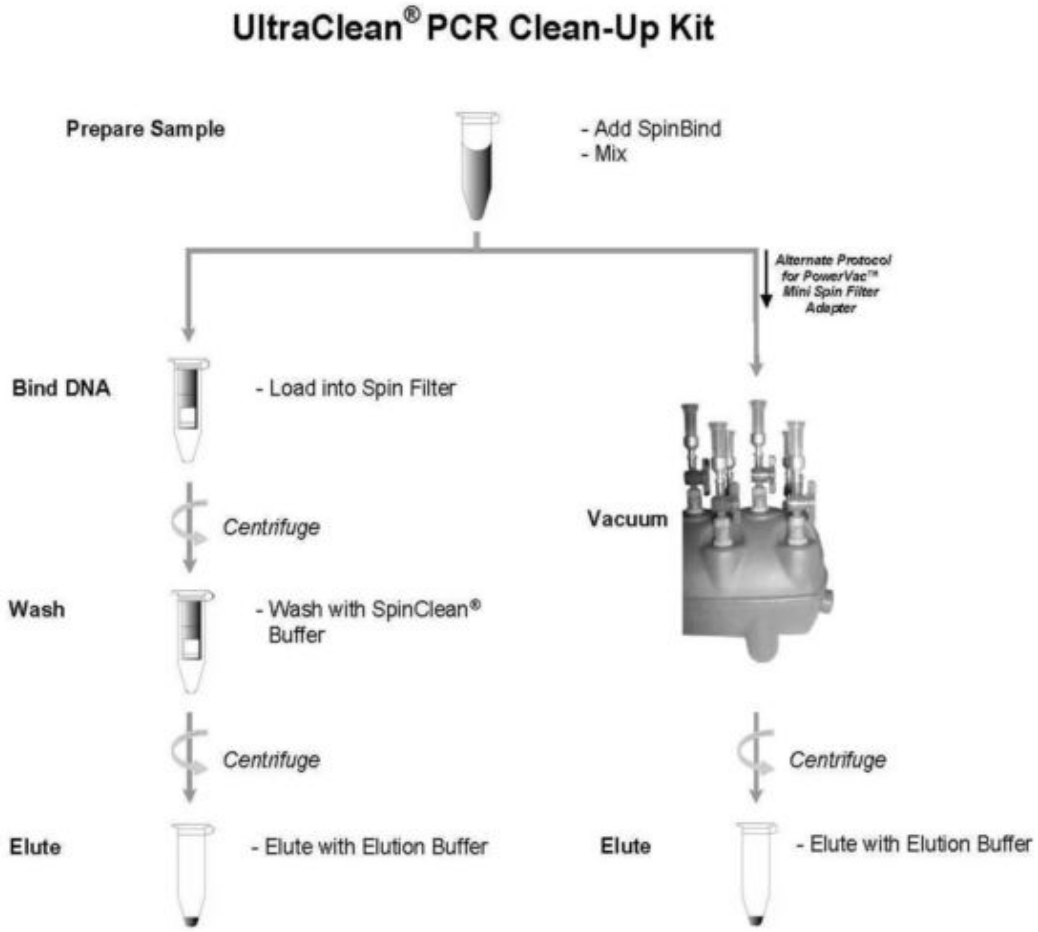
Each person should prepare 3 PCR reactions from his/her extracted DNA. In addition, positive control of known 16s rDNA (low concentration) should be prepared and added into the PCR cohort.

1. Label 3 PCR vials with your initials and group number on top and on side.
2. Prepare 67.5µl 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	n/a
10µM stock solution of forward primer and reverse primer	3.8µl of each	200-fold
ddH2O(double-distilled)	20.9µl	n/a
Total Volume	67.5µl	

3. Pipette 22.5µl of master mixture into each of 3 PCR vials.
4. Pipette 2.5µl of extracted gDNA into each of 3 PCR vials.
5. Secure the PCR vial and put it into the PCR machine.
6. 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 15 seconds **(Less stringent priming: 30 seconds)**
        iii. 72 °C for 30 s
    c. FInal extension of 72°C for 5 minutes
7. Take 5µl from one of the PCR vial to make a 500µl dilution.
8. Measure absorbance of the 500µl dilution. Calculate DNA amount as (5000*5*A260)ng
9. In “PCR quality control”, take a ~5µl of sample to load to gel, adjust the volume according to estimated DNA amount.
10. Proceed to the purification protocol with the rest of the PCR product.
11. The primer sequence:
    a. Forward primer (515fB):
    AATGATACGGCGACCACCGAGATCTACACGCT XXXXXXXXXXXX TATGGTAATT GT GTGYCAGCMGCCGCGGTAA
    b. Reverse primer(806rB):
    CAAGCAGAAGACGGCATACGAGAT AGTCAGTCAG CC GGACTACNVGGGTWTCTAAT
    c. The amplicon is approximately 385bp long.

Amplicon Purification (10 minutes)



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 3g of agarose powder
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    c. Stuff the flask opening with 2 pieces of kimwipes.
    d. Microwave for 30 seconds and then swirl.
    e. Microwave for another 30 seconds.
    f. Swirl and make sure there is no undissolved agarose floaties. Otherwise, microwave shortly again.
    g. Let it cool on bench for 3 minutes before operation.
2. While gel is cooling, prepare the gel tray by:
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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 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 by:
    a. Add 3uL of loading dye to each of sample
    b. Pipette the mixture into the well.

Well No.	Sample volume	Content
1	15µl	100bp or 1kb DNA Ladder as appropriate (e.g.: promega G571A, 100bp-1000bp)
2	15µl	Positive Control
3	5µl	Unpurified PCR product
...	...	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.
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UltraClean PCR Clean-Up Kit Protocol

1. Wear gloves all the time. Shake to mix the SpinBind before use. Add 5 volumes of SpinBind to your PCR reaction. Example: add 120 µl to a 20 µl PCR reaction.
2. Mix well by pipetting. If an oil overlay was used, you will now have two layers. The top layer is oil. (No OIL was added).
3. Transfer PCR/SpinBind mixture to a Spin Filter unit, while avoiding the transfer of oil.
4. Centrifuge 10-30 seconds at a minimum 10,000 x g (approximately 13,000 rpm) in a tabletop microcentrifuge.
5. Remove the Spin Filter basket and discard the liquid flow-through from the tube by decanting.
6. Replace the Spin Filter basket in the same tube.
7. Add 300 µl SpinClean Buffer to the Spin Filter.
8. Centrifuge 10-30 seconds at a minimum 10,000 x g.
9. Remove Spin Filter basket and discard liquid flow through by decanting then replace basket back into the same tube.
10. Centrifuge 30-60 seconds at minimum 10,000 x g.
11. Transfer Spin Filter to a clean 2 ml Collection Tube (provided).
12. Add 50 µl of Elution Buffer (10 mM Tris) solution provided or sterile water directly onto the center of the white Spin Filter membrane. The choice of using Tris or water at this point will not affect yield. DNA is more stable for storage in Tris.
13. Centrifuge 30-60 seconds at a minimum 10,000 x g.
14. Discard Spin Filter basket from the inside of the Spin Filter unit. Purified DNA is now in the 2 ml Collection Tube. The DNA will be free of all reaction components such as primers or linkers, enzyme, salt, and dNTP's. Store DNA at -20C. DNA is now ready to use.

dsDNA Quantification Protocol (20 mins)

Assay Setup

Use the following procedure to set up the assay:

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

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
E	0.3 ng/µL	10 µL of 3 ng/µL of D	90 µL
F	0.1 ng/µL	10 µL of 1 ng/µL of E	90 µL
G	0.03 ng/µL	10 µL of 0.3 ng/µL of F	90 µL
H	0.01 ng/µL	10 µL of 0.1 ng/µL of G	90 µL
I	0.003 ng/µL	10 µL of 0.03 ng/µL of H	90 µL
J	0 ng/µL	None	100 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.

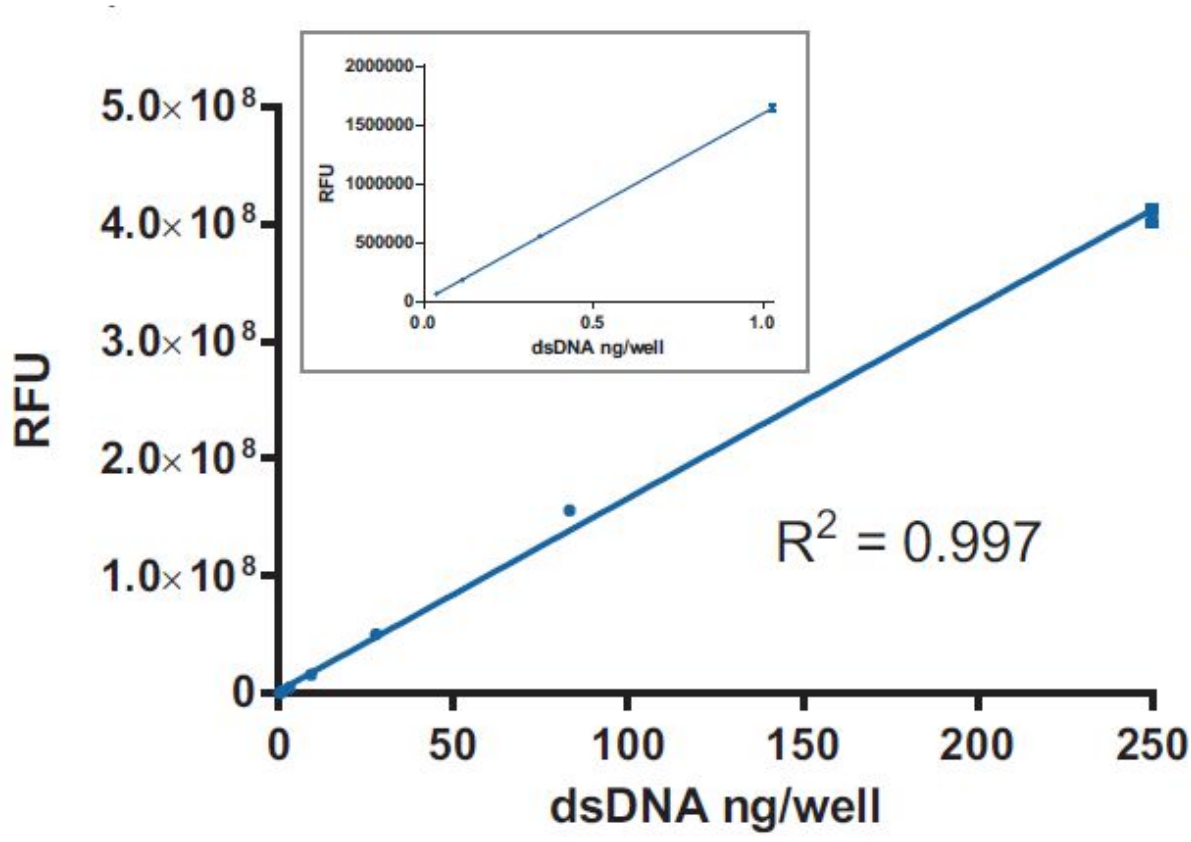
Parameter	Setting
Read mode	Fluorescence
Ready type	Endpoint
Wavelengths	Excitation: 468 nm with 9 nm bandwidth Emission: 507 nm with 15 nm bandwidth
PMT and Optics	PMT Gain: Automatic Flashes per read: 10 Read from top

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.

Pooling

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

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

- 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.