The Summer Soil Institute

SOIL MICROBIOLOGY LABORATORY MODULE

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Timeline

Monday July 19, 1-5 pm:

	DNA Extraction	Fungal Counts
1:00	**TEAM 2 and 3**	**TEAM 4 and 1**
3:00	**TEAM 4 and 1**	**TEAM 2 and 3**

Tuesday July 21, 1-5 pm: ENZYME ASSAYS AND MICROBIAL RESPIRATION

	Enzyme setup	SIR setup
1:00	**TEAM 1 and 2**	**TEAM 3 and 4**
3:00	**TEAM 3 and 4**	**TEAM 1 and 2**

Enzymes will be read on the plate reader approx 1 hour after substrate addition.
CO_2 will be read on the IRGA approx 2 hour after substrate addition.

Wednesday July 21, 1-5 pm

1-1:30 Overview of real-time PCR

	qPCR	Data Analysis and Follow-up
1:30	**TEAM 4 and 2**	**TEAM 1 and 3**
2:30	**TEAM 1 and 3**	**TEAM 4 and 2**

3:30-5:00 Data Analysis

Microplate Enzyme Assay Using Fluorescence

Protocol Prepared By: Meg Steinweg (steinweg@nrel.colostate.edu) & Shawna McMahon (shawna.mcmahon@gmail.com)
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This assay is applicable to any enzyme for which there is a fluorescently-labelled (MUB or MUC) substrate:

- β-Glucosidase (BG)
- N-acetyl-β-glucoasminidase (NAG)
- Phosphatase (PHOS)

A more complete list can be found at the RCN "Enzymes in the Environment website as well as more detailed protocols: http://enzymes.nrel.colostate.edu/

Fluorescent Substrate Preparation (200 µM)

Dissolve the fluorescent substrates in deionized water according to the following list:

- BG: 4-MUB-β-D-glucopyranoside: 6.77 mg/100 mL DI H_2O
- NAG: 4-MUB-N-acetyl- β -D-glucosaminide: 7.59 mg/100 mL DI H_2O
- Phosphatase: 4-MUB phosphate: 5.12 mg/100 mL DI H_2O

Stir and use moderate heat to speed dissolution of substrates.
Aliquots: Put 11 mL aliquots into 15 mL centrifuge tubes.
Storage: Can be stored frozen (-20°C for up to two months). Thaw in warm water. Do not thaw and refreeze

Stock Standard Solution Preparation (1 mM)

Dissolve 17.6 mg of MUB in 100 mL DI H_2O (use a volumetric flask).
Dissolve 17.5 mg of MUC in 100 mL DI H_2O (use a volumetric flask).

Aliquots: Put 5 mL aliquots into 15 mL centrifuge tubes.
Storage: Can be stored frozen (-20°C for up to two months). Do not thaw and refreeze

Standard Curve

- Dilute standard stock solution (1 mM=1000 µM) to create a standard curve. Start with a 1:10 dilution of the stock solution to create a 100 µM solution. Make all further dilutions from 100 µM. Aim for 15 mL of each standard for a typical assay run with 12 samples.
  100 µM = 3 mL 1000 µM stock into 27 mL DI H_2O (1:10 dilution)
  50 µM = 15 mL 100 µM into 15 mL DI H_2O (1:2 dilution)
  25 µM = 15 mL 50 µM into 15 mL DI H_2O (1:2 dilution)
  10 µM = 12 mL 25 µM into 18 mL DI H_2O (1:10 dilution)
  5 µM = 15 mL 10 µM into 15 mL DI H_2O (1:2 dilution)
  2.5 µM = 15 mL 5 µM into 15 mL DI H_2O (1:2 dilution)

Because of background autofluorescence, a standard curve was prepared for each sample. See plate layout for clarification.

Buffer Preparation

- The buffer you select will depend on the pH of the soil. Below are the instructions for two common buffers: sodium acetate (pKa = 4.76) and Tris (pKa = 8.06).
  - Note that phosphate buffer has a pKa = 7.2 which could work for more neutral/slightly basic soils. However, high phosphate concentrations may interfere with enzyme activity so use with caution.

50 mM Sodium Acetate Buffer

1. Dissolve 30.8 g sodium acetate in 2L DI H_2O
2. Adjust the pH to match soil pH using glacial acetic acid or concentrated sodium hydroxide (~10M NaOH).
3. Bring the volume up to 4L with DI H_2O.

Storage: Buffer should be made fresh, but can be refrigerated (4°C) for up to a week.

Procedure

I. Assay Set-Up

1. Pipette 200 µL of appropriate standard into correct wells of MUB standard plate (oriented in *rows*).

2. Weigh 2.75 grams of field moist soil into blender. (the soil amount can be altered, just be sure to adjust the buffer amount as well)
   - Be sure to measure the gravimetric water content so that dry weight (d.w.) can be calculated—enzyme activity is typically presented on a g^{-1} soil d.w. basis.
3. Add 91 mL of 50 mM buffer.
4. Blend contents on high for 1 min.
5. Pour contents into a glass bowl, add stir bar and place on stir plate. Mix on low.
   - Bowl must be wide enough to accommodate the 8-channel pipette.
   - Stirring gently helps keep soil suspended and minimize variation due to different amounts of soil present in each well.
6. Pipette 800 µL of soil slurry into wells for enzyme activity measurement and standard curves (see plate layouts). Each sample should be in a separate *column*.
7. Rinse blender with DI H_2O or buffer between samples.
8. Pipette 200 µL of appropriate 200 µM substrate into correct assays wells (oriented in *rows*).
   * Record the time of substrate addition and try to start all plates/substrates as quickly as possible.

Note: Steps 1 & 8 are mostly easily performed with only six (6) pipette tips loaded on the repeat pipette (two additions across the plate for a total of 12).

II. Incubation & Analysis

1. Seal the plates with plate mats and mix by hand (invert about 10 times).
2. Place plates in appropriate incubators for required incubation period.
   **This will be determined earlier for soils your soils*
3. When incubation is complete, centrifuge plates for 3 min. at 1500 rpm (~350 x*g*).

4. Transfer 250 µL from each well into corresponding well in a flat-bottomed black 96-well plate.
5. Following manufacturer's instructions for your plate reader, measure fluorescence using the following parameters:
   Excitation Wavelength = 365
   Emission Wavelength = 450

   Additional instructions for the Tecan Infinite M200 microplate reader:
   1. Select 10 reads but no multiple reads per well.
   2. Open Excel and make sure the correct destination file is open.
      - The Tecan will put the data in a new worksheet (tab) in whatever spreadsheet is open. Label each tab or put descriptive information in each worksheet as it is generated. Information needed, sample #s, MUB or MUC sample, MUB or MUC stnd, gain of the microplate reader. If you are using numbers that don't mean anything, then do a summary sheet on each workbook that explains the numbers.
   2. Run the standard plate at optimal gain. The highest measured value should be for the 100 µM standard. Note the optimal gain determined by the machine.
   3. Set the gain manually to 5 units below the optimal gain from the standard plate for all other plates with the same soil samples.
      * The gain dramatically changes the measured values. However, it may be necessary to drop the gain even further for some samples if the value exceeds detection limits (indicated by a red OVER in the spreadsheet output). The standard plate MUST be read at the same gain as the sample plates, otherwise the values are useless.

Data Analysis & Calculations

• Plot standard curves (fluorescence on Y, standard conc. on X) in Excel for the MUB and

MUC standards for each soil sample. Calculate the slope, intercept & r2. Accept the standard

curve if r2 is >0.98.

• We often do two standard curves for each set of standards to get better resolution for high

activity and low activity enzymes, but this isn't strictly necessary as the standard curves

are quite linear.

• Low Activity: plot 0-25 µM standards

• High Activity: plot 10-100 µM standards

• Use y=mx+b to solve for unknown concentrations (x), where:

y = sample fluorescence; x = enzyme concentration; m = slope from standard curve

b = intercept from standard curve

sample enzyme concentration = (sample fluorescence – std. curve intercept) ÷ std. curve slope

• Calculating the enzyme activity:

Enzyme Concentration/Length of Incubation (h)/soil dry weight (g) = μmol activity/g soil d.w./h

Plate Layouts:

Enzyme Activity Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1+ BG	S2+ BG	S3 + BG	S4+ BG	S5+ BG	S6+ BG	S7+ BG	S8+ BG	S9+ BG	S10+ BG	S11+ BG	S12+ BG
B	S1+CB	S2+CB	S3+CB	S4+CB	S5+CB	S6+CB	S7+CB	S8+CB	S9+CB	S10+CB	S11+CB	S12+CB
C	S1+ NAG	S2+ NAG	S3+NAG	S4+ NAG	S5+ NAG	S6+ NAG	S7+ NAG	S8+ NAG	S9+ NAG	S10+ NAG	S11+ NAG	S12+ NAG
D	S1+PHOS	S2+PHOS	S3+PHOS	S4+PHOS	S5+PHOS	S6+PHOS	S7+PHOS	S8+PHOS	S9+PHOS	S10+PHOS	S11+PHOS	S12+PHOS
E	S1+ XYL	S2+XYL	S3+ XYL	S4+XYL	S5+XYL	S6+XYL	S7+XYL	S8+XYL	S9+XYL	S10+XYL	S11+XYL	S12+XYL
F												
G												
H	S1 +LAP	S2 +LAP	S3 +LAP	S4+LAP	S5+LAP	S6+LAP	S7+LAP	S8+LAP	S9+LAP	S10+LAP	S11+LAP	S12+LAP

4 and 25C Standards **Same for MUB and MUC**

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1+ std0	S2+ std0	S3+ std0	S4+ std0	S5+ std0	S6+ std0	S7+ std0	S8+ std0	S9+ std0	S10+ std0	S11+ std0	S12+ std0
B	S1+std2.5	S2+std2.5	S3+std2.5	S4+std2.5	S5+std2.5	S6+std2.5	S7+std2.5	S8+std2.5	S9+std2.5	S10+std2.5	S11+std2.5	S12+std2.5
C	S1+ std5	S2+ std5	S3+ std5	S4+ std5	S5+ std5	S6+ std5	S7+ std5	S8+ std5	S9+ std5	S10+ std5	S11+ std5	S12+ std5
D	S1+std10	S2+std10	S3+std10	S4+std10	S5+std10	S6+std10	S7+std10	S8+std10	S9+std10	S10+std10	S11+std10	S12+std10
E	S1+ std25	S2+ std25	S3+ std25	S4+ std25	S5+ std25	S6+ std25	S7+ std25	S8+ std25	S9+ std25	S10+ std25	S11+ std25	S12+ std25
F	S1+ std50	S2+ std50	S3+ std50	S4+ std50	S5+ std50	S6+ std50	S7+ std50	S8+ std50	S9+ std50	S10+ std50	S11+ std50	S12+ std50
G	S1+ std100	S2+ std100	31+ std100	S4+ std100	S5+ std100	S6+ std100	S7+ std100	S8+ std100	S9+ std100	S10+ std100	S11+ std100	S12+ std100
H												

The spaces in the enzyme activity sample plate at rows F and G are there in case other MUB based enzymes need to be measured at some point.

Plate Layouts for Summer Soil Institute:

Enzyme Activity Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Tips & Tricks

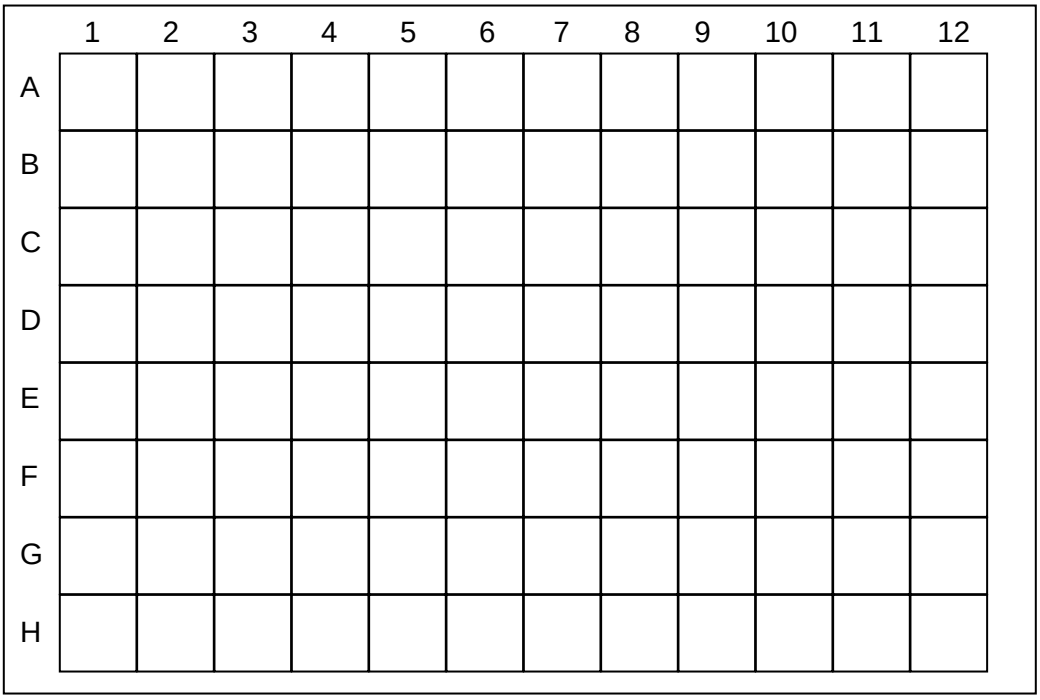
- You may need to alter the plate layout for your experiment. However, we've provided the plate layouts we use for doing all the enzymes at once.
- Because of differences in background autofluorescence, you must **prepare a standard curve for each sample** separately, hence the large standard plates.

When starting, add the standard solutions prior to slurry addition. That way when you are finished adding soil samples the standard plates are ready to be incubated right away and can be read on the microplate before the assay plate. If you don't do this then there will a time crunch because at 25C and 4C you will need to measure 2 standard plates and an assay plate at almost the same time and it takes ~10 minutes per plate, so you want them spread out a little.

Do NOT add the substrates until after all the soil slurries have been added to the plate, otherwise you'll have uneven incubation time. →these two might belong somewhere else, but are important

You can add 5 µL of 0.5N NaOH to each sample in the black well plate right before reading it on the microplate reader, it increases the intensity of fluorescence, but it is very time sensitive. If you do this you want the final well pH to be ~10

- The blank graphical representation of the plate may also be useful.



Substrate Induced Respiration

- Weigh 5- 10g soil (fresh wt.) of soil into a 50mL centrifuge tubes equipped with gas-tight lids that have rubber septa for gas sampling.

- Add 10mL yeast solution to each tube. Yeast solution should deliver 20mg yeast/g dry soil. (3g autolyzed yeast extract to 250mL ddH_2O). Other SIR protocols use glucose but I've found that yeast gives higher respiration rates and the goal of the method is to maximize CO_2 production.

- Cap tubes and shake horizontally in 20^0C room for the duration of the 4 h assay. 10 –20 minutes after sealing the tubes measure the initial (the T0 time point) headspace CO_2 concentrations. This is the TO time point. Measure headspace CO_2 concentrations two more times, approx. 2h and 4h after the T0 time point.

- Measure headspace CO_2 concentrations by injecting 5mL lab air into tube, pull out 5mL air from tube and measure cumulative CO_2 concentrations on an IRGA equipped for static CO_2 analysis. Make sure to record the time that the gas sample was taken.

- Calculate the slope of the line relating CO_2 concentrations to time. The average respiration rate (µg C-CO_2/gsoil/h) over the 4 h incubation period is an index of the SIR-responsive microbial biomass. Calculate an r^2 value for the line describing CO_2 concentrations over time to make sure the relationship is roughly linear.

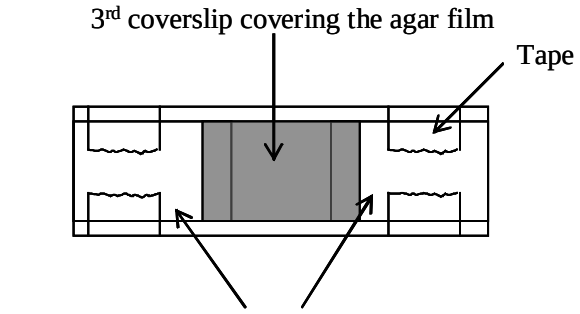
- This technique is modified from that described in: West and Sparling. 1986. *Journal of Microbiological Methods*. 5: 177-189.

Fungal Direct Counts

Materials:

1. dilution tubes containing sterile DI water
2. Waring blender with sanitized cup
3. 1-ml pipets
4. molten agar solution, 1.5%
5. water bath, ~ 45º C
6. coverslip well slides

To make, tape two coverslips of known thickness to a microscope slide, aligning the edge of each coverslip to the edge of the microscope slide. Make sure the ends of the tape do not overlap.



Coverslips taped to edge of slide to form a "well"

Procedure:

1. Add 10 g (dry weight) soil to a blender cup containing 90 ml of sterile DI water.
2. Blend on high speed setting for 1 minute.
3. Prepare a 1:100 dilution by removing a 1.0 ml aliquot from the blender cup. Transfer the aliquot to a test tube containing 9.0 ml of DI water.
3. Shake the 1:100 dilution tube, and transfer 1.0 ml of suspension to a clean test tube. Add 1.0 ml of molten agar to the same tube and mix the contents gently.
4. Add the soil solution/agar suspension dropwise to the area of the microscope slide between the two coverslips. Make sure the agar covers the area completely.
5. Press a third coverslip down onto the agar to produce an agar film of known thickness.
6. Observe fungal hyphae at 40x by phase contrast microscopy.
7. Using the ocular grid, count the number of lines intersected by the fungal hyphae.
8. Count a total of 30 fields minimum per slide (example, three transects of ten fields each).
9. Calculate meters of fungal hyphae per gram of dry soil as follows:

$$\textbf{m hyphae per gram soil} = (N/X)\,(W/V)\,(D)\,(\pi/4)$$

Where:

N = the total number of lines intersected by fungal hyphae

X = the total number of fields counted

W= the width of one ocular grid line (mm) (0.025 mm at 400x power)

V = the field volume (for my microscope = 0.01143mm^3 = area of ocular grid × thickness of one coverslip; area of ocular grid = 0.25 x 0.25 mm = 0.0625 and thickness of one coverslip is ~0.183mm)
D = the dilution factor (200)
$\pi/4$ = from equations of Olson (1950).

References:

1. Lodge, D.J., and E.R. Ingham. 1991. A comparison of agar film techniques for estimating fungal biovolumes in litter and soil. Agriculture, Ecosystems and Environment 34:131-144.

2. Olson, F.C.W. 1950. Quantitative estimates of filamentous algae. Trans. Am. Microscop. Soc. 69:272- 279.

qPCR

	µl for 25 µl rxn	Reagent concentration	Final concentration
ABgene ABsolute QPCR SYBR mix	12.5	2x	1x
BSA	2.5	5 ug/ul	0.5 ug/ul
dH2O	2.5		
Forward primer	1.25	10 uM	0.5 uM
Reverse primer	1.25	10 uM	0.5 uM
Template DNA	5	1 ng/ul	0.2 ng/ul

Master Mix for full plate:

1.25 mL master mix

125 ul of each primer

250 ul BSA

250 ul H2O

Standard conditions for Bio-Rad thermocycler:

95˚	15:00	
94˚	0:15	
48-60˚	0:30	40 x
72˚	0:30	

Melt Curve optional

Target	*Forward Primer*	*Reverse Primer*	*Annealing*
All Bacteria	Eub 338	Eub 518	53
All Fungi	5.8s	ITS 1F	53

DNA Extraction using MoBio PowerSoil Kits

Detailed Protocol (Describes what is happening at each step)

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.

   What's happening: After your sample has been loaded into the PowerBead Tube, the next step is a homogenization and lysis procedure. The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.

2. Gently vortex to mix.

   What's happening: Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the PowerBead Solution.

3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until the precipitate has dissolved before use.

   What's happening: Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will form a white precipitate in the bottle. Heating to 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. Solution C1 can be used while it is still warm.

4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.

5. Secure **PowerBead Tubes horizontally** using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. **Note:** If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

   > **Note:** *The vortexing step is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1-4 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open.*

   What's happening: The MO BIO Vortex Adapter is designed to be a simple platform to facilitate keeping the tubes tightly attached to the vortex. It should be noted that although you can attach tubes with tape, often the tape becomes loose and not all tubes will shake evenly or efficiently. This may lead to inconsistent results or lower yields. Therefore, the use of the MO BIO Vortex Adapter is a highly recommended and cost effective way to obtain maximum DNA yields.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.

7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

   > **Note**: *Expect between 400 to 500 µl of supernatant at this step. The exact recovered volume depends on the absorbancy of your starting material and is not critical for the procedure to be effective. The supernatant may be dark in appearance and still contain some soil particles. The presence of carry over soil or a dark color in the mixture is expected in many soil types at this step. Subsequent steps in the protocol will remove both carry over soil and coloration of the mixture.*

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

   What's happening: Solution C2 is patented Inhibitor Removal Technology® (IRT). It contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).

    What's happening: The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

    What's happening: Solution C3 is patented Inhibitor Removal Technology® (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

13. Transfer up to 750 µl of supernatant to a clean **2 ml Collection Tube** (provided).

    What's happening: The pellet at this point contains additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

14. Shake to mix Solution C4 before use. Add 1.2 ml of **Solution C4** to the supernatant (be careful solution doesn't exceed rim of tube) and vortex for 5 seconds.

    What's happening: Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the Spin Filters.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
    Note: A total of three loads for each sample processed are required.

    What's happening: DNA is selectively bound to the silica membrane in the Spin Filter device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

    What's happening: Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

17. Discard the flow through from the **2 ml Collection Tube**.

    What's happening: This flow through fraction is just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

18. Centrifuge at room temperature for 1 minute at 10,000 x *g*.

    What's happening: This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

19. Carefully place Spin Filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
    Note: *It is important to avoid any traces of the ethanol based wash solution.*

20. Add 100 µl of **Solution C6** to the center of the white filter membrane.

    Note: *Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris) which lacks salt.*

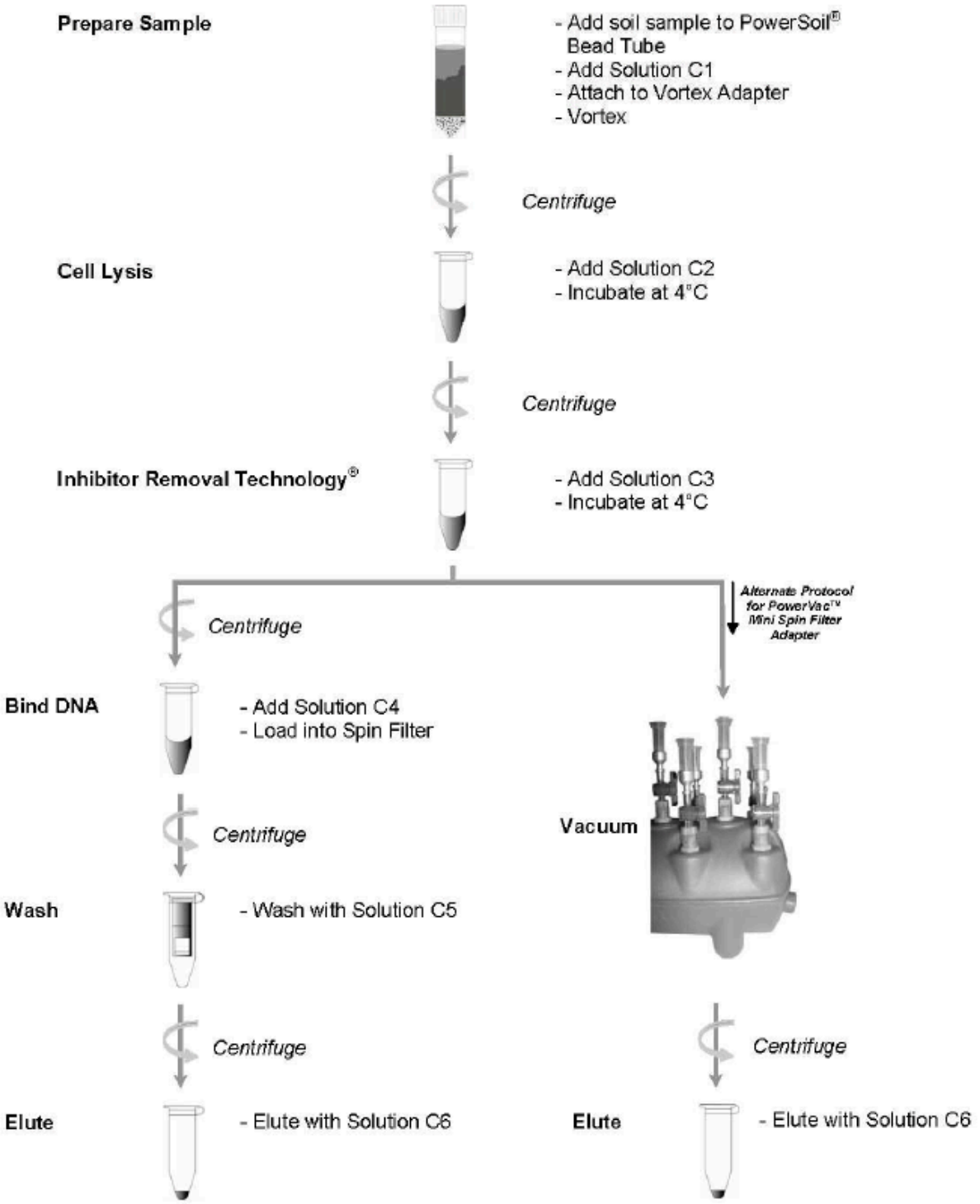
    Alternatively, sterile DNA-Free PCR Grade Water may be used for DNA elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10). Solution C6 contains no EDTA. If DNA degradation is a concern, Sterile TE may also be used instead of Solution C6 for elution of DNA from the Spin Filter.

21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

    We recommend storing DNA frozen (-20° to -80°C). **Solution C6** does not contain any EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

PowerSoil® DNA Isolation Kit



DNA quantification with Quant-it kit (Q33130 Invitrogen)

Product manual: http://probes.invitrogen.com/media/pis/mp33130.pdf

1. Make working solution (1:200 reagent to buffer)
2. Load 200 ul of solution into each well
3. Add 2 ul of standards (use appropriate range) to wells
4. Add 2 ul of DNA sample to wells
5. Read plate using Quant-it setting on plate reader.

If high precision is required, use replicate standards and samples, or increase amount of sample and standards to increase sensitivity.