Protocols for environmental samples

Modified and compiled by Joy D. Van Nostrand 10-22-09

These protocols are for use with environmental samples (soils, sediments, and filters) for eventual use with the functional gene array (GeoChip). Please check with Liyou or Joy V. with any questions on these protocols.

DNA preparation order:

Important Reminders

- Remember to label all solutions and include the date and your initials – this includes water.
- Clean up common areas (scales, hoods, gel box area, etc.) after you are finished.
- Dispose of chloroform waste in the waste bottle located in the fume hood. When this bottle is full it needs to be taken down for pickup and a new bottle set up – check with the lab manager on the proper protocol.
- Leave pipettes, tubes, and other items that have chloroform residue on them in the fume hood overnight to evaporate the chloroform. The next day, throw these items away or place them at the dish washing station – do not just leave them in the hood!
- Remember to log equipment use (centrifuges, scanner, Tecan, etc.). Logs will be located next to or on the equipment.
- Some pieces of equipment (Tecan) have a reservation log. Remember to reserve them in advance and if you are unable to use it when scheduled remove your reservation so that others can use the instrument.

DNA Extraction from Bacterial Communities

Two buffers are available to use in DNA extractions: PIPES and Tris. There are slight differences in the protocols for each buffer – these are noted below.

PIPES buffer generally results in a cleaner DNA extract but the DNA is slightly sheared or damaged so the DNA should not be purified using gel extraction (the DNA smears when run on a gel). The column purification method should be used instead.

Tris buffer generally produces a less clean DNA extract, especially for soils with high levels of humic acid. The DNA is in larger fragments and remains in a compact band when run on a gel, so gel purification can be used with this method. Gel purification is ideal for soil or sediment samples with high DNA yields. Gel purification is also appropriate for this method if samples have low DNA yield, such as groundwater samples.

Solutions:

Extraction buffer
6.8 mL 1 M NaH_2PO_4 (*monobasic*)
93.2 mL 1 M Na_2HPO_4 (*dibasic*)
Combine phosphate sol., pH to 8.0 with NaOH, continue with remaining ingredients
200 mL 0.5 M EDTA, pH 8.0
100 mL 1 M Tris-HCl
300 mL 5 M NaCl
100 mL 10% CTAB [*for **filter samples**, leave CTAB out]*
Bring to 1 L with DI water, **if CTAB is left out; bring to 900 mL with DI water**

(Final concentrations: 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1 M EDTA, 0.1 M Tris-HCl, 1.5 M NaCl, 1% CTAB)

Other Chemicals Needed:

Proteinase K
10 mg mL^{-1} (*store at -20C*)

20 % SDS (*pre-made*)

70 % Ethanol (*cold, store at -20C*)

2-Isopropanol

0.5 M EDTA, pH 8.0

1 M Tris-HCl

5 M NaCl

Chloroform:isoamyl alcohol (24:1)

Combine the chloroform and isoamyl alcohol and store in a dark or foil covered bottle and keep it in the flammables cabinet below the fume hood.

*[For **filter samples**:* 10% CTAB (*store at room temperature – heat to redissolve*)]

Other Notes:

The Oak Ridge tubes used in this protocol should not be autoclaved. If the tubes are autoclaved, the DNA pellet does not form a tight pellet and can be difficult to see. To clean the tubes, rinse them in DI water after use and then boil for 20 min in DI water. Once cooled, rinse the tubes with 70% EtOH and dry.

Protocol:

*For **soils and sediments**, skip to step 8*

(1) If not already, place **filters** in a 50 mL conical centrifuge tube. Add 5 mL of extraction buffer (without CTAB).

(2) Shake the tubes vigorously (or vortex the tube) for several minutes to remove the biomass.

(3) Transfer liquid to a fresh centrifuge tube.

(4) If the filters are large tear them up after the first wash so that it is easier to remove biomass. (*Placing the filters onto a Petri dish makes tearing them easier or use scissors to cut the filters – make sure to preclean the scissors. Then place the filter pieces back into the original centrifuge tube*). Repeat steps 1-3 twice (total of 3 washes). Remove as much liquid as possible from the tube.

(5) Check the pH of the liquid using pH paper. The pH should be ~pH 8. If it is too low, add NaOH to bring the pH up.

(6) Centrifuge the collected liquid 5000 x *g* for 20 min to pellet cells [*use the floor model Sorvall RC5C Plus with the conical tube adaptor (SLA-600TC)*].

(7) Transfer supernatant to a fresh tube, use 1 mL of the supernatant to resuspend the pellet.

(8) For **soils or sediments**, weigh out 5 g of sample into a sterile mortar. *Only take out one sample at a time to minimize DNA degradation from being at room temperature.* [For **filter**, add resuspened pellet to the mortar]. Add 2 g of sterile sand to the mortar. *More sand can be added if needed.* Add liquid N_2 to the mortar to cool the sand and mortar. *Be generous with the N_2.*

(9) Add more N_2 to cover the sand/sample and begin grinding once the N_2 has evaporated. Try to contain sample to a small area of the mortar. Grind until the sample starts to thaw. If possible, keep the sample frozen while grinding (this can be easy if the soil is dry and sandy). *But if the sample is difficult to grind when frozen, you can allow it to thaw, but make sure to add 1-2 mL of extraction buffer to the sample. The buffer with inhibit degradation of the DNA when it warms.*

(10) Repeat freezing and grinding twice (3 times total).

(11) While the sample is still frozen scrape the sides of the mortar with a spatula to collect the sample in the center of the mortar (add more N_2 if necessary to keep the sample frozen). Transfer this to the supernatant collected previously (for **filter samples**) or to a fresh tube (for **soils and sediments**) using a spatula.

<u>At this point the sample can be kept frozen (-80 °C) until ready to proceed with DNA extraction</u>

(12) Remove samples from freezer and thaw. For **soil and sediment samples**, add 16.5 mL Extraction Buffer (with CTAB) and proceed to step 14. *The total volume of buffer should be 16.5 mL, so if you added buffer during the freeze-grinding, remember to subtract that amount from the added at this step.*

(13) For **filter samples**, add 1.5 mL 10% CTAB (final concentration, 1%) to the thawed and warmed samples, mix gently.

(14) For **Tris buffer** add 61 μL proteinase K (10 mg mL^{-1}), mix gently. For **PIPES buffer** skip to step 16.

(15) Incubate at 37 °C for 30 min (*keep in a 37 °C water bath and invert every 5-10 min*)

(16) Add 1.83 mL 20 % SDS, mix gently

(17) Incubate at 65 °C for 2 h for **TRIS buffer** with gentle inversion every 15-30 min. Incubate at 65 °C for 1 h for **PIPES buffer** with gentle inversion every 15-30 min.

(18) Centrifuge 20 min, 3600 x *g* at 25 °C (*use the benchtop centrifuge*). Increase speed to 6000 x *g* if samples are dirty.

(19) Transfer liquid to a 50-mL conical centrifuge tube, avoiding the white surface layer. (*Pour the liquid slowly into the new tube, the surface layer will remain in the tube – if the surface layer breaks apart, you can use a pipette)*

(20) Add 6 mL extraction buffer containing CTAB (or 5.5 mL extraction buffer without CTAB plus 0.55 mL 10 % CTAB) to the remaining sand pellet and mix.

(21) Add 0.67 mL 20 % SDS, mix gently

(22) Incubate at 65 °C for 15 min

(23) Centrifuge 10 min, 3600 x *g* at 25 °C

(24) Collect supernatant and combine with previous supernatant, avoiding the surface layer as in step 17

(25) Extract supernatant with an equal volume of isoamyl:chloroform (1 part isoamyl, 24 parts chloroform) for 5-10 min by continuous inversion (*the rotator located in the fume hood can be used to continuously mix the samples*)

(26) Centrifuge at 3700 x *g*, 20 min – use the benchtop centrifuge *(the **orange** or **purple** capped conical tubes can withstand this speed).*

(27) Collect the supernatant into a fresh conical tube.

(28) Repeat steps 23-25, except transfer the supernatant into an oak ridge tube (*Use the translucent oak ridge tubes. Pay attention to the final volume of supernatant transferred*.)

(29) Add 0.6 volume of 2-isopropanol (*very important that exactly 0.6 volume is added)*

(30) Incubate at -20 or -80 °C overnight. *The cold will help the DNA to precipitate.*

(31) Remove the tube from the freezer and warm in a 37 °C water bath. Make sure the sample is warm and all precipitates have dissolved before proceeding. *Warming the sample prior to centrifugation will dissolve any mineral precipitates that may have formed overnight.*

(32) Centrifuge 15,000 x *g* (RCF) for 20 min at 25 °C *(Make sure the centrifuge is at RT – if it is too cold, mineral precipitates in the sample will be allowed to form).* Immediately after centrifugation, transfer the supernatant to a fresh tube (*keep the supernatant until you know whether DNA is present*).

(33) Wash the pellet with 1 mL ice-cold 70 % ethanol, if no pellet is visible, centrifuge 15,000 x *g*, 5 min

(34) Allow the pellet to dry 10-15 min.

(35) Dissolve the pellet with 50-500 µL nuclease-free water pre-warmed to 50 °C (*start with 50 µL, if pellet is larger, use a larger volume).* At this point DNA can be stored at -20 °C or -80 °C (long-term storage). For sediment samples, keep the volume as low as possible and immediately begin gel purification.

(36) **Optional**: Measure DNA concentration of filtered samples using the nanodrop and/or run a gel of the DNA (~1 µL)
Keep in mind that the nanodrop is probably not very accurate at this point. Possible contaminants in the DNA (e.g., humics) have absorbance values over the 230-280 nm range. Based on the curve, 260/280 ratio, and gel image, decide whether purification is necessary. If you have questions about the DNA quality or quantity, please discuss with Liyou before proceeding.

If DNA is from a site that has or is expected to have high microbial activity, check to see if there is a high amount of RNA. If so, this should be removed using an RNase protocol before proceeding. The presence of large amounts of RNA may interfere with subsequent steps.

Note: Extracted DNA should be purified as soon after extraction as possible As the extracted DNA ages, it degrades, which will make purification difficult.

For **soils and sediment** samples extracted with **TRIS buffer**, proceed to "Gel Purification for Soils and Sediments", p. 7.

Gel Purification for Soils and Sediments

(Liyou Wu, Meiying Xu)

Note: It is important that gel purification be done using freshly extracted DNA. As the extracted DNA ages, it degrades resulting in a DNA smear on the gel rather than to clean band. Extracted DNA should be purified as soon after extraction as possible.

Prepare a 0.5 % low melting point agarose gel in 1X TAE (100-150 mL) with 5 µl of 10 mg mL^{-1} EtBr per 100 mL of gel. Use the white/clear combs for large volume samples. This will take ~ 2 h to solidify. The wells from these combs have ~80 µL volume, run ~ 60 µL of sample per well.

Mix **FRESH** DNA with sufficient loading buffer (20X) so the buffer is 2X. Run the DNA sample slowly (e.g. 50V, 6-8 h; 30V, 14 h) to get good resolution and prevent gel deformation.

Excise bands, using a long wave length UV wand to visualize the band, and place the slice (~ 1 mL) in a 15 ml purple capped conical tube (VWR Ultra-High Performance Centrifuge Tubes # 21008-103).
Note: When the concentration of the DNA is high and most of the DNA is high molecular weight, the high molecular weight band may be darker or less intense than expected and the middle of the band may be wider, but the edges of the band will be bright.

Melt the agarose gel at 65 °C until the gel slice has completely melted (may take 5-10 min). To help the gel melt, invert the tube a few times every 2-3 min during incubation.

Add an equal volume of 65 °C water (for example, add 1 ml of water to 1 ml of molten gel) and mix. Cool the gel at RT for 2 min, add an equal volume (e.g., 1 ml + 1 ml = 2 ml) of cold, buffer-saturated phenol, invert several times or use the rotator to mix the sample, put on ice for 5 min, and centrifuge at 8000 rpm for 5 min at RT. If the phases are not well separated, centrifuge an additional 10-15 min.
Note: The phenol removes the gel from the aqueous phase and allows any DNA within the gel matrix to be released back into the aqueous phase.

Transfer the aqueous layer (top layer) to a fresh tube. Back extract the organic phase with 0.8 volume of fresh TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to 1 volume of phenol (e.g. add 200 µl of TE to each 250 µl of molten gel), mix put on ice for 5 min, and re-spin (8000 rpm, 5 min). Remove the new aqueous layer and combine with the first round aqueous layer.

Add an equal volume of chloroform:isoamyl alcohol [24:1 (v/v)] to the twice extracted aqueous phase and rotate (or invert) 5 min. Centrifuge at 8000 rpm for 5 min at RT. Transfer the aqueous (top) layer to a new tube. If the phases are not well separated, remix the sample and repeat centrifugation. Repeat extraction if necessary.

Add an equal volume of sec-butanol, mix 5 min on rotator (or invert by hand) and then centrifuge at 8000 rpm for 5 min at RT. Remove the butanol layer (**upper** layer) and discard. You will note that the aqueous volume has decreased.

Repeat the butanol extraction at least 3 times (adjusting the volume of butanol to equal the volume of the current aqueous phase). Continue the butanol extractions until the aqueous volume has been reduced to ~400-500 µL. You will usually need 4 extractions to reduce the volume sufficiently. *Use weight (1 mL=1 g) for a more accurate determination of the volume (important in the next step.*
Note: Do not reduce the volume to less than ~400 µL or all of the DNA will be lost.

Add 1:10 (v/v) of 3 M NaOAc (pH 5.2) and 2-times the volume of cold 100 % ethanol (for example, for 400 µl of purified product add 40 µl NaOAc and 800 µl ethanol), and incubate at -20 °C overnight. *If you can see the DNA precipitating you do not need to incubate overnight,*

Remove the tube from the freezer and centrifuge 12,000 rpm for 30 min, wash the pellet with 95 % ethanol. Dry the pellet and resuspend it in a small volume (e.g. 50-100 µl to start) of TE (10mM Tris-HCl, pH 8.0, 1 mM EDTA). *If you have a large amount of visible DNA you can loop out the DNA instead of centrifuging.*

Check DNA quality with nanodrop. Ideally, the 260/280 = 1.8; 260/320 > 1.7. Repeat purification if necessary.

Proceed with amplification, p. 10.

DNA purification using <u>Wizard® DNA Clean-Up System</u>

(1) For **ground water** *(or samples with very low DNA concentrations)* purify approximately half the sample.

(2) Bring volume of the sample up to 50-100 µL with nuclease-free H_2O

(3) Add 100 µL Direct Purification Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton® X-100)

<u>Direct Purification Buffer</u> (100 mL)
2.5 mL 2 M KCl
1 mL 1 M Tris-HCl
150 µL 1 M $MgCl_2$
100 µL Triton X-100

(4) Mix 1 mL resin with DNA by inversion, incubate at room temperature 30 min, mixing every 10 min. *Make sure the resin is well-mixed prior to use.*

(5) Attach the minicolumn to the syringe barrel (both provided with the kit). Add DNA/resin mix to the barrel. Apply a vacuum to draw the sample through the minicolumn. Once all the samples have passed through the column, release the vacuum.

(6) Transfer 2 mL 80 % isopropanol to the column and apply a vacuum. Continue to apply a vacuum for 30 s after the wash solution has been pulled through the column (*no more than 30 s*). Remove the syringe barrel and place minicolumn into a microcentrifuge tube.

(4) Centrifuge the minicolumn for 2 min 10,000 x *g* to remove excess isopropanol.

(5) Transfer the minicolumn to a new microcentrifuge tube and place 100 µL pre-warmed (80 °C) nuclease-free water to the minicolumn. Incubate at 80 °C for 10 min (*you can keep the microcentrifuge and minicolumn in a hotplate set at 80 °C).*

(6) Centrifuge the minicolumn for 20 s, 10,000 x *g* to elute the DNA

(7) Repeat steps 5 and 6 with an additional 100 µL of water

(7) Check the concentration and purity with nanodrop.
If the 260/280 ratio is too low (<1.8), repeat purification process.
If the color of the sample is too dark, repeat the purification – *the DNA extracts should be colorless.*
If the 260/230 ratio is low (<1.7), proceed with the desalting protocol.

(8) Store DNA at -20 ℃ or -80 ℃

Desalting Protocol

If the 260/230 ratio is low after purification (<1.7), the desalting protocol can be used to remove contaminants (most likely guanidine from the resin in the purification kit).
If you are concerned about the quantity of DNA you have, please discuss with Liyou prior to desalting. Another option is to desalt only half of your DNA and see how much DNA is recovered.

(1) Precipitate the Wizard purified product with 2.5 vol of 100 % ice cold ethanol and 1:10 volume of 3 M NaOAc (pH 5.2). Incubate the samples overnight at -20 ℃. *If you can see the DNA precipitating you do not need to incubate overnight.*

For 100 μL purification product add 250 μL ethanol and 10 μL NaOAc

(2) Centrifuge 30 min at 13,000 x *g* to pellet the DNA

(3) Decant the supernatant and wash the pellet with 1 mL 70 % ethanol (cold). *Keep the supernatant until you are sure that DNA has been recovered.*

(4) Centrifuge the pellet 10 min at 13,000 x *g*

(5) Decant the supernatant

(6) Dry the pellet for ~30 min (may take longer). *Make sure to not over dry the sample.*

(7) Resuspend the pellet in nuclease free water

(8) Some impurities may still be present, which will not dissolve – possibly from the purification filter. Centrifuge the samples after dissolving and transfer the supernatant to a fresh tube.

(9) Check DNA using nanodrop
260/230 ratio should be $\geq$ 1.7

(10) Store DNA at -20 ℃ or -80 ℃

At this point the optimum numbers are:
260/280 -- 1.8
260/230 – $\geq$1.7

Protocol DNA Amplification Using TempliPhi Amplification Kit
(For use with FGA)

Amplification should be used for samples with low concentrations of DNA.

Try to amplify all samples within a group at the same time. If there are too many samples, make sure you amplify replicates at the same time. This will limit day-to-day variations.

Success of amplification and subsequent steps greatly increases with the use of high quality DNA.

Spermidine (2.4 mM) has been aliquoted out and is stored at -80 °C. Dispose of any of the remaining aliquot. This will limit potential DNA contamination of the spermidine and minimize freeze/thawing.

Materials

Purified DNA

TempliPhi Amplification Kit

Sample buffer: contains random hexamers and is used for re-suspending DNA

Reaction buffer: contains salts and deoxynucleotides and is adjusted to a pH that supports DNA synthesis

Enzyme mixture: contains phi29 DNA polymerase and random primers in 50 % glycerol

Additives

Single-Stranded DNA binding Protein (SSB) (*stored at -20 ^{o}C*)

Spermidine: 2.4 mM stock solution

Precautions to limit background DNA

- **Use the dedicated amplification hood and the dedicated pipettes (stored in the hood)**
- **UV irradiate all tubes and water in the Stratalinker for 10 min**
- **Use new, unopened boxes of filtered tips for each experiment**
- **Place all items needed in the hood (tubes, tips, gloves, ice bucket, etc.) and turn on UV light for 20-30 min prior to start**
- **Remember to clean hood and UV it for 10-15 min after each experiment**

Procedure:

IMPORTANT: keep all reagents on ice, and keep all components at 0-4 ^{o}C until ready for amplification

(1) Transfer 10 µl aliquots of sample buffer to an appropriate reaction tube or micro-well plate.

(2) Transfer DNA (up to 5 µl) to the sample buffer. Use the same volume of DNA for all samples. Ideally, 100 ng of DNA should be used, but lower amounts can be used. If the original concentration of a sample is very low, the DNA can be concentrated.

(3) Mix DNA and buffer thoroughly and incubate at RT 10 min.

(4) While DNA and buffer are incubating, prepare Templiphi premix:
In a separate tube, for each Templiphi reaction combine:
   a) 10 µl of reaction buffer
   b) 0.6 µl enzyme mixture
   c) 1.25 µl SSB (final concentration: ~260 ng/µl)
      1. *Current SSB stocks are 5 µg/µL*
      2. *If the SSB stock is a lower concentration, increase the volume to reach ~260 ng/µL.* ***Do not add more than 2 µL because this dilutes the remaining components.***
   d) 1 µl spermidine (2.4 mM stock) (final concentration: 0.1 µM)

(5) Transfer 12.85 µl of the Templiphi premix to the DNA/buffer mixture.

(6) Remember to run a negative control to check for background DNA. Using the steps outlined above the negative control should be much lower than the samples.

(7) Incubate the reaction at 30 ^{o}C for 10 hours.

(8) Heat-inactivate the enzyme by incubating the reaction at 65 ^{o}C for 10 min and then cool at 4 ^{o}C.

(9) Run a gel of the amplified samples (no more than 2 µl) to make sure all the samples amplified. *The gel should have a slight "smear" of DNA at the top of the gel.*

(10) Measure the amount of amplified DNA product using the picogreen protocol. *The presence of primers and dNTPs in sample results in inaccurate measurements when using the 260/280 ratio.*

Troubleshooting:

If no or poor amplification occurs, try decreasing the amount of sample volume used to dilute out any inhibitors that may be present.

Serial dilution (2-3 dilution steps) of the sample can be used to 'wash' the DNA. Serial dilution can also be used if the DNA quality is above the recommended thresholds, but the amplification results are poor.

Labeling of amplified microbial community DNA (for FGAII)

Combine (Solution 1):

Random primer [octamers, 2.5x (750 μg/mL)] 20 μL

Target or control DNA — For amplified DNA, add all the DNA (20 μL)
For unamplified DNA, add 3-5 μg DNA

If necessary, bring volume up to 35 μL with water (*may not need to add water*)
Incubate at 99.9 °C for 5 min (*use thermocycler*)
Immediately chill tubes on ice

Combine (Solution 2): *Create a master mix to be added to Solution 1*

dNTP mix [5 mM, (dTTP 2.5 mM)] 2.5 μL

[Using **100mM** dNTPs:
5 μl each of dA/G/CTP
2.5 μl of dTTP
82.5 μl DEPC Water]

CyDye (25 nM) [for samples, use Cy-5] 1 μL

CyDyes are light sensitive – dispense in the dark room. This can be added last

Klenow (40 U μL^{-1}) 2 μL

Water — enough to bring volume up to 50 μL when combined with Solution 1

Total volume (solution 1 + solution 2): 50 μL

Add Solution 2 to Solution 1

Incubate at 37 °C for 3 hrs, followed by 3 min at 95 °C. *Labeled DNA can be kept at 4 °C in the thermocycler if labeling is done overnight, or proceed to purification.*

Purify labeled DNA with Qiagen QIAquick Kit as specified by the manufacturer; elute the DNA using water or EB buffer

Check CyDye incorporation using nanodrop

Minimum dye incorporation: pmol ≥ 50 (pmol/μL * total μL)

[*for example*: Labeled DNA eluted with 100 μL of EB buffer and the pmol/μL is 0.8; 100*0.8=80 – labeling is fine, proceed with hybridization]

Calculations:

pmol of dye incorporated = Cy3 OD_{550} x (total volume of probe)/0.15
= Cy5 OD_{650} x (total volume of probe)/0.25

Specific activity = (ng of target x 1000)/(pmol dye incorp x 324.5)

Please keep track of the labeling efficiency (pmol/μL and SA) and hybridization results so that we can determine if the threshold efficiency should be increased.

Dry using a Speed Vac

Program: Temp. 45 °C, Run 45 min, Vacuum (Level) 5.1 (preset)
Press "Auto Run"

Pellet should be dry at the end of the run

GeoChip Slides

Ask Joy if you need GeoChip slides.

***IMPORTANT:** The array is printed on the **back** of the slide (the barcode is on the "front"). The arrays are printed on the back because the seals in the Tecan's hybridization chamber hit the barcode, so a good seal is not made and sample and buffers can leak out, ruining the array.

When you receive a box of array slides – check the box for the date the arrays were printed and to see if the arrays were UV-linked. Ask if you are unsure whether UV-linking has been done.

If the slides were not UV-linked, UV-crosslink them prior to starting a hybridization

UV radiation: 600 mJ (*use the Stratalinker*)

1. Turn power on
2. Place slides in a single layer in the Stratalinker (array side up)
3. Press "Energy"
4. Enter 6000 (μJoules x 100)
5. Press Start
6. Stratalinker will beep when crosslinking is complete, once the beep ends, remove slides

Slides can now be used for hybridizations

Scanning Hybridization Slides

(1) Sign in on the computer next to the scanner using your IEG username and password

(2) Open "ScanArray Express" by double-clicking the icon

(3) Click on the necessary laser(s) [Laser 1 (630 nm) for Cy-5 and Laser 3 (543) for Cy-3, upper right] to turn them on and let them warm up. A 15 min countdown appears – the lasers only need to warm up 5 min, so you can start scanning before the 15 min have passed.

(4) Insert slide, array side up, into the scanner (slot in front). There will be slight resistance as you push the slide in. It will stop at the edge of the barcode. *If you have not inserted a slide into the scanner before **please** have someone show you the first time so the array or scanner is not damaged.*

(5) Click on the "Configure" button (to the left of the screen), then click "scan protocols". *This will allow you to optimize the setting for your arrays. You only need to do this with your first array. The settings will be saved so you can use the same settings for all of your arrays.*

1. A list of protocols will appear – chose the one you want by double-clicking on the name. *If you have questions about which protocol to use or would like to create your own, check with Joy or Liyou*
2. Click on the gray button next to "tools", then on the "Run" button next to "Quick Scan".
3. Change the scan resolution (top of the box) to 30 then click "start". *This box shows the PMT gain (%) and the laser power (%). Generally, we keep the laser power at 90 and change PMT.*
4. The array will be brought into the scanner.
5. If the lasers have not been on for at least 15 min, a message will appear that the lasers have not warmed up, do you want to scan anyway. The lasers only need 5 min to warm up (according to the technical rep), so you can click "yes" if the lasers have been on for at least 5 min.
6. Change the palette to rainbow by clicking on the palette button (lower right) and then double click on rainbow. *This will allow you to compare the Cy-5 and Cy-3 labeling results.*
7. This is a quick, low resolution scan that will allow you to change the PMT (lower right). Increase or decrease the number, then look at the scanning image to determine if that is the appropriate setting. *A higher PMT results in brighter probes, but more background.*
8. If you are scanning both Cy-5 and Cy-3 then, you will need to set the PMT for both (*click on "Scan next fluor" on the bottom right*). *Remember to change the palette!*

9. Once you are satisfied with the results, you can click "stop" (upper left) or you can let it complete the scan to check the scan area and make sure the entire array is being imaged.
10. A box will appear showing the changes made to the protocol and asking if you want to save the changes – click "yes"
11. If you want to change the scan area, click on the button next to "scan area", you can increase or decrease the scan area using the white box.
12. Click "finish" – this will save the changes you made.
13. Click "close"

(6) To do the real scan, click "Scan" (upper left)

(7) Make sure the program shown under "scan protocol" (in the second box) is the one you want. *The program that was last used is listed by default.* If you need to change the program, double-click the name to see the complete listing of programs. Double-click the one you want – *this should be the same protocol you did for the "Quick Scan"*.

(8) Click "Start" – *If you just did the quick scan, a message will pop up asking if you want to save the image, click "No"*.

(9) **Important:** Change the "palette" to rainbow (palette button is in the lower right corner). If scanning more than one dye, you will need to change the palette for both. *You can only change the palette once scanning has begun and the palette needs to be changed before scanning is complete.*

(10) Once the scan is finished, save the images to the server (in "Zhoulab" there is a folder "Microarray (image)" that should contain a folder for each person in the lab – you can create one if there is not one for you there. You can save the images to the computer temporarily if you have to, but that computer does not have much memory so you will need to move the files to the server as soon as possible.

1. Click the tab at the top of the image for Cy-5 or Cy-3 in order to save that image (*each dye must be saved separately*)
2. Click "File" (upper left) and then "Save As"
3. Save the image as a TIFF and a JPEG. *The TIFF will be used for image analysis and the JPEG can be used in papers or presentations.*
4. Normally, the image name includes the dye, the date, and some type of sample identification information (for example: Cy5_102306_FRC1) – *use a labeling system that works for you, but make sure you can identify what each image is*.

(11) Once you are done with all the scans, turn the lasers off by clicking on the boxes, close the program, and log out of the computer.

(12) Remember to log your use of the scanner in the log book