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### More than Movement – Ant Seed Dispersal Inhibits Phytopathogenic Fungi

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More than movement – ant seed dispersal inhibits phytopathogenic fungi

by

Charlene Gray

An Abstract of a Thesis

in

Biology

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

December 2015

Buffalo State College

State University of New York

Department of Biology

ABSTRACT OF THESIS

Seed dispersal by ants is a cosmopolitan mutualism involving thousands of plant species. Ants gain a food reward (elaiosomes) from the plant seed, and ant-mediated plants gain several presumed benefits, including dispersal away from parent plants and placement in 'safe site' microhabitat. Higher plant germination and survival in ant nest soils has been attributed to higher levels of nutrients, aeration, and soil moisture than surrounding soils, but evidence in support of these benefits is inconsistent. Ants secrete anti-microbial compounds that inhibit microbial pathogens. I explore the possibility that ants transfer anti-microbial properties to the soil they inhabit, and there is some evidence that the anti-microbial body chemistry may benefit plants. Hence, an additional benefit of ant-mediated seed dispersal might be that myrmecochores (ant-dispersed plants) gain pathogenic fungal protection from anti-microbial ant secretions. If so, I expect that seed-dispersing ants inhibit plant pathogenic (phytopathogenic) fungi. I first re-analyzed published data to determine whether ant-occupied soil contained fewer phytopathogenic fungi. I then conducted two experiments to test for anti-fungal effects on plant pathogen fungi. In the first, I used assays of known phytopathogenic fungi (*Alternaria alternata* and *Fusarium oxysporum*) with ant soaked hexane. I measured the zone of inhibition on agar plates and calculated area to compare the effect of the treatments. In the second experiment, I conducted a metagenomic-sequencing analysis to determine fungal diversity in ant nests as compared to control soils. The reanalysis of published data showed plant pathogenic fungal diversity decreased within ant nests but not control soil. I found no evidence of direct

pythotopathic inhibition in the fungal assays, but I also found that the methodology did not have enough power to detect effects. The metagenomic analysis did confirm significantly less fungal diversity in ant-colonized than control soils. More importantly, the colonized soils contained significantly less diversity of plant pathogenic fungi than control soils. These results suggest ant presence in soils reduces fungal diversity and that ant-mediated seed dispersal provides a heretofore unexplored benefit to myrmecochores: fungal pathogen protection. The added benefit of fungal protection strengthens the evidence for myrmecochores dependency on their dispersal partner, and provides a more consistent explanation for why plants recruit better in ant nest soil. Pathogenic fungal protection may be a key evolutionary benefit to the worldwide phenomenon of ant-mediated seed dispersal.

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## CHAPTER I:

### LITERATURE REVIEW

Ant-mediated seed dispersal (myrmecochory) occurs worldwide as thousands of plant species produce seeds with ant-attracting appendages (Lengyel et al. 2009). Myrmecochory presumably provides ants a food resource and, in return, ants provide plants with dispersal services (Hanzawa et al. 1988, Giladi 2006, Ness et al. 2009). The benefits of ant dispersal for plants may include distance dispersal (i.e., colonization), a reduction in the maladaptive density of seedling aggregation around parent plants, and the placement of seeds in a nutrient-enhanced 'safe site' from rodents and fire (Giladi 2006, Warren and Giladi 2014). However, ants only move seeds an average 1-2 meters (Lengyel et al. 2009), and research is mixed whether ants provide 'safe sites', with most benefits seemingly contingent upon study system and location (Giladi 2006, Warren and Giladi 2014).

#### *Effects of ant handling*

Approximately 90% of plants die during recruitment (seed and seedling survival) [Fenner and Kitijima 1999] and many losses are attributed to fungal pathogens (Blaney and Kotanen 2001, Kotanen 2007, Warren and Bradford 2011). Indeed, fungal pathogens appear a stronger selective force on plants than interspecific competition (Moles and Westoby 2004). Warren and Bradford (2011) confirmed that fungal pathogens shape the woodland distributions of two myrmecochorous plants— suggesting that fungal attack might be a considerable selective force on herbaceous plant populations. A potential benefit of seed handling by ants may be inadvertent protection from fungal pathogens. For example, tropical myrmecochore and non-myrmecochore ants “clean” seeds by removing fruiting bodies from the seed coat (Guimaraes and Cogni 2002), and seeds that are cleaned by ants have improved germination rates and are less likely to be

infected by plant fungal pathogens (Oliveira et al. 1995, Leal and Oliveira 1998). Furthermore, seeds cleaned by ants suffer less fungal attack than both non-cleaned seeds and seeds cleaned by hand (Ohkawara and Akino 2005). The seeds handled by ants have increased germination rates (Leal and Oliveira 1998), and the seeds have lowered fungal attack (Oliveira et al. 1995). Given that seed germination rates increase (Oliveira et al. 1995, Leal and Oliveira 1998) and seed fungal infections decrease (Ohkawara and Akino 2005) in seeds cleaned by ants, and given that ants produce antimicrobial secretions that can be controlled by grooming (Fernandez-Marin et al. 2006, Yek and Mueller 2011), ants may pass on their antimicrobial secretions to the seeds they handle.

Ants likely convey fungicide through their body chemistry: out of all species of insects, only ants have metapleural glands (Bot et al. 2002). Metapleural glands secrete a waxy, acidic substance (Beattie et al. 1985) that protects ants from microbial pathogens such as fungi (Beattie et al. 1986, Bot et al. 2002). Protection from microbial pathogens is essential in eusocial insects like ants that reside in moist, dense living conditions with little genetic diversity (Bot et al. 2002, Stow and Beattie 2008). Metapleural gland secretions once were thought to disperse passively, flowing from the gland and spreading across the ant cuticle with no deliberate effort by the ants (Beattie et al. 1986, Yek and Mueller 2011). Recent investigation has shown that ants actively groom their metapleural glands, and increased grooming leads to increased metapleural output (Yek and Mueller 2011). Moreover, grooming behavior increases with increased exposure to pathogens (Fernandez-Marin et al. 2006).

A second possibility of ant effects on soil fungi is passive transfer of fungal protection through the ant nest environment. Seeds returned to ant nests have higher germination and seedling survival rates than non-ant dispersed plants (Hanzawa et al. 1988). Such improvement

has been attributed to soil enrichment through ant midden (Wagner and Jones 2004, Wagner et al. 2004), but another possibility is that ants modify soil fungal communities through their metapleural excretions. Zettler et al. (2002) found less fungal diversity in ant-colonized soils, investigating both an invasive species (*Solenopsis invicta*) and a native seed-disperser (*Aphaenogaster texana carolinensis*). Warren and Bradford (2012) also found reduced wood-rot fungi in wood colonized by *Aphaenogaster rudis*. These results suggest that anti microbial compounds limit specific fungal species. However, Zettler et al. (2002) isolated fungal colonies picked at random from diluted sample cultures, which may underestimate microbial diversity, and (Nelson 2012) found greater fungal diversity in ant-colonized soil soils.

If ant metapleural secretions inhibit pathogenic fungi in ant-colonized soil, then phytopathogenic fungal protection might be an important component of ant-mediated seed dispersal. Given that plant survivorship increases with protection from fungal pathogens (Blaney and Kotanen 2001, Kotanen 2007, Warren and Bradford 2011), and that ants produce fungicidal compounds (Beattie et al. 1986, Bot et al. 2002), plants in North American (N.A.) woodlands may gain a heretofore unexplored benefit from myrmecochory: fungal pathogen protection. I test this hypothesis using ants from the *Aphaenogaster* complex, the most abundant ant species in the northeastern United States (Lubertazzi 2012, King et al. 2013), and the keystone seed dispersers of myrmecochores in N.A. woodlands (Ness et al. 2009). First, I re-analyzed published data from Zettler et al. (2002) by investigating differences in plant pathogen fungi diversity between ant nest soil and control soils. I then conducted two experiments, the first examining ant effects on pathogenic fungal growth *in vivo*, the second examining the effects of ant presence on metagenomic fungal community diversity from soil samples collected in the field.

## CHAPTER II

### REANALYSIS OF ZETTLER et al. (2002)

#### **Introduction**

Ant chemical defenses reduce their microbial pathogens (Beattie et al. 1985, Beattie et al. 1986, Veal et al. 1992, Nascimento et al. 1996, Bot et al. 2002), but also may indirectly impact non-target microbes. Zettler et al. (2002) compared fungal species in ant-colonized and control soils using red imported fire ants (*Solenopsis invicta*) and a native seed dispersing ants (*Aphaenogaster texana*). The objective was to compare fungal species richness, diversity and composition between ant-colonized and control soils. The results indicated greater abundance of fungi within ant nests soils, especially for *S. invicta*, but ant-colonized soil contained less fungal species diversity compared to control soils. The study did not distinguish between fungal types, however. If ant seed retrieval benefits plants through fungal protection, creating a pathogenic 'safe-site', I predict less phytopathogenic fungi within ant-colonized soil. I tested this hypothesis by reanalyzing Zettler et al. (2002) data by categorizing phytopathogenic or non-pathogenic fungal species.

#### **Methods**

Zettler et al. (2002) measured fungal abundance by the average number of colony forming units (cfu's) that grew in cultures. Twenty colonies were picked randomly from each plate and were cultured on potato dextrose agar slants, with a total of 100 random isolates from each soil type. The cfu's were identified morphologically, and Zettler et al. (2002) estimated the relative sample frequency of each soil type (percent of samples with fungal species) and the relative colony frequency of each soil type (percent of total fungal colonies present). In order to compare fungal



plant pathogens in the different soil types, I researched the pathogenicity of fungi found in Zettler et al. (2002) soil fungi list by genus (Valera et al. 2005, Sun et al. 2008, Balaji et al. 2009, Gazis and Chaverri 2010, Dean et al. 2012) and categorized fungi as plant pathogenic or non-pathogenic.

Differences in the frequency of samples containing phytopathogenic fungi, the abundance of phytopathogenic fungal species within samples and the number of isolated fungal colonies (measured in colony forming units, cfu g<sup>-1</sup>) categorized as phytopathogenic were analyzed as a function of soil type (*A. texana*-colonized, *S. invicta*-colonized or control soil) using analysis of variance (ANOVA) models and Tukey's Honest Significant Difference *post hoc* tests in the R statistical package (Miller 1981, Yandell 1997). I considered coefficients with *p* values  $\leq 0.05$  significant, and I considered coefficients with *p* values  $\leq 0.10$  marginally significant.

## Results:

The frequency of fungal pathogen presence within samples differed significantly by soil type ( $DF = 2$ ,  $SS = 1.412$ ,  $MS = 0.706$ ,  $F\text{-value} = 3.097$ ,  $P\text{-value} = 0.0543$ ) with fungal pathogens occurring less frequently in *A. texana* and *S. invicta* soils (Fig. 1a). Fungal pathogen abundance differed marginally significantly by soil type ( $DF = 2$ ,  $SS = 2180$ ,  $MS = 1090$ ,  $F\text{-value} = 2.580$ ,  $P\text{-value} = 0.086$ ), but only *S. invicta* soil differed in abundance of phytopathogenic fungi from control soil (Fig. 1b). The number of isolated fungal colonies (measured as colony forming units, cfu g<sup>-1</sup>) that were identified as phytopathogenic, differed significantly by soil type ( $DF = 2$ ,  $SS = 48.500$ ,  $MS = 24.250$ ,  $F\text{-value} = 3.377$ ,  $P\text{-value} = 0.042$ ) with *A. texana* and *S. invicta* soils containing marginally significantly fewer phytopathogenic fungal colonies than control soils (Fig. 1c).

**Discussion:**

Ant occupation appears to reduce soil fungi diversity, including plant pathogens. The metapleural gland secretions from ants act as an important defense against pathogens that attack ant colonies, and studies show that the secretions reduce fungi hyphael growth and spore germination (Beattie et al. 1985, Beattie et al. 1986, Bot et al. 2002). Given that phytopathogenic fungi are less diverse in ant nest soils, and given that metapleural secretions reduce fungal growth (Beattie et al. 1985, Beattie et al. 1986), myrmecochore seeds may gain fungal protection in ant nests, a benefit that may provide another explanation for the dependency of myrmecochores on their ant dispersal partners. Plants dependent on ant dispersal may have evolved to take advantage of an inadvertent defense against fungal plant pathogens.

Zettler et al. (2002) identified species in isolated, random fungal colony cultures, but only approximately 17% of known fungi readily grow in culture (Hawksworth 1991) so this approach can miss underrepresented fungi from field samples (Nagano et al. 2010). In another study, Nelson (2012) suggested that ant nest soils actually host higher fungal diversity than control soils but Nelson (2012) used estimated fungal diversity based on DNA fragments rather than genomic identity. Many fungal species have similar morphological characteristics that are difficult to delineate (Lacey 1996), and it is possible to miss uncommon species (Nelson 2012). The differences in methodology between Zettler et al. (2002) and Nelson (2012) may explain the inconsistency in findings, with neither approach fully satisfactory, and suggest that a comprehensive identification of ant soil fungal communities is required to identify ant impacts on soil fungal diversity.

## CHAPTER III

### FUNGAL ASSAYS

#### **Introduction:**

Most ant species are equipped with a metapleural gland, a structure unique to ants and the contents of which are waxy and contain several acidic compounds (Beattie et al. 1985, Beattie et al. 1986, Cabrera et al. 2004). Secretions from the metapleural gland are antiseptic, an essential defense against microbial pathogens that threaten eusocial insect environments. Metapleural chemicals inhibit fungal growth and spore germination (Beattie et al. 1985, Beattie et al. 1986, Veal et al. 1992, Nascimento et al. 1996, Bot et al. 2002). I tested the effects of *Aphaenogaster* ant metapleural glands on two known fungal pathogens in order to further explore the influences on myrmecochory and a possible anti-fungal benefit from ant nests.

#### *Study species*

For this study, I used ants from the *Aphaenogaster* complex. *Aphaenogaster* ants are small, only about 4mm in length (Prior et al. 2014), but they are the most abundant ant species in N.A. woodlands (King et al. 2013), and they are keystone seed dispersers (Ness et al. 2009).

#### **Methods:**

I collected *Aphaenogaster* ants from New York State Parks (Allegheny State Park, 42°5'58.3"N 78°45'6.7"W, altitude 498 m, Zoar Valley State Park, 42°26'34.5"N 78°51'59.7"W, altitude 317 m) and private woodlands (10062 Trevett Road, Springville, NY, 42°36'21.7"N 78°43'46.8"W, altitude 328 m) during the summer of 2014. Permission to collect ants in the state parks was granted under New York State Parks permit 2013-MP-004. All collections took place under

forest cover, and I located *Aphaenogaster* ant nests by turning over rocks and coarse woody material.

I collected ant colonies with a cordless vacuum (Dewalt, Baltimore, MD, USA) and a hand held aspirator. I emptied the contents of the vacuum into separate plastic freezer bags for each colony, and I transported the colonies back to the laboratory on ice. The samples were stored at -80 °C upon arrival at the laboratory. To ensure identification accuracy, I re-identified the ants in the laboratory using a dissecting microscope and *Aphaenogaster* ants reference from a reference collection.

I also collected *Vespula maculifrons* (Eastern Yellowjacket) as a control organism as vespids share the same order, Hymenoptera, as ants, but do not have metapleural glands. I followed *V. maculifrons* foragers back to their nests at the SUNY Buffalo State College campus (42°56'1.5"N 78°52'55.7"W, altitude 183 m) and I collected them using the cordless vacuum. Following collection, I placed the cordless vacuum in a freezer (-20°C) for 24 h before removing the vespids and storing them at -80°C.

#### *Preliminary methods:*

I first crushed ant bodies using a tissue homogenizer, and I plated the ants on agar inoculated with fungi. This method released bacteria from the ant bodies (G. Pettibone, *pers. comm.*), which corrupted the results. I attempted sterilizing the ants in an autoclave at 121°C to eliminate the bacterial contamination but this experiment failed to create an effect zone around the treatment, possibly because the ant anti-microbial compounds broke down at high temperatures. For my final experimental approach, I attempted to extract the polar and non-polar molecules from the ants by placing 100 ants in 0.5 mL of water for the polar molecules and 0.5 mL of hexane for the

non-polar molecules (modified from (Revis and Waller 2004), who used 10 mL of hexane and 50 ants). The water-extracted compounds were also contaminated with bacteria from the ant bodies so I eliminated this method as well. I used the extraction with hexane for the final experimental trials.

My first attempts to inoculate new plates with fungal hyphae produced uneven fungal growth so that I could not distinguish an effect from the treatments. In order to control fungal spore distribution, I designed a new plating methodology by isolating spores from *Fusarium oxysporum* and *Alternaria alternata* (Carolina Biological, Burlington, NC, USA) and inoculating tempered agar (agar that is held in liquid form at a specific temperature), yielding an even distribution of trapped spores. This method successfully produced an even distribution of fungal spores on my agar plates, which enabled me to detect effects from the ant treatments.

#### *Fungal Assays:*

*Fusarium oxysporum* and *Alternaria alternata* cultures were stored at SUNY Buffalo State under APHIS permit P526P-14-02309. Pure cultures were maintained in the lab throughout the experiment by inoculating fresh nutrient broth (potato dextrose or centrifuged, filtered tomato juice and carboxylic acid) with fungal hyphae every 7-10 days. I grew *F. oxysporum* on potato dextrose agar plates and incubated the plates at 24 °C for ten days to promote sporulation.

*Alternaria alternata* cultures were grown on V-8 juice (filtered, centrifuged V-8 tomato juice, carboxylic acid, and agar) agar plates under a full-spectrum light with 12-hour cycles of darkness and light to promote sporulation.

Conidiophores were collected from two plates of each fungal culture using a vacuum filtration system constructed with an Erlenmeyer flask, a rubber hose connected to a vacuum, a

glass funnel with eight layers of cheese cloth, and a rubber stopper. Spores were obtained by pipetting 6 mL of sterile distilled deionized water to the overgrown fungus; the hyphae were then scraped with a sterilized metal spatula to loosen the spores. The resulting spore suspension was emptied directly onto the cheesecloth and the contents were vacuumed into a sterilized flask.

I inoculated plates with 3 mL of tempered Potato Dextrose agar (58°C) containing 2mL of spore suspension. The inoculated agar was vortexed and immediately emptied it onto the agar plates; the plate was swirled to spread the spores evenly. The treatments and controls were next applied to each plate, with a total of 6 discs per plate (Fig. 2).

One hundred ants were soaked in 0.5 mL pure hexane to extract polarized compounds from the metapleural glands (Revis and Waller 2004). Ant-handled seeds also were soaked in 0.5 mL hexane to extract any compounds the ants may have transferred to the seeds. As a control, three *Vespula maculifrons* were soaked in 0.5 mL hexane. Each solution was incubated at room temperature for one hour and vortexed for one minute following incubation. Twenty  $\mu$ L of each solution was pipetted onto sterile 6 mm paper discs, which were placed on inoculated agar plates. Twenty  $\mu$ L of pure hexane was pipetted onto a paper disc as a negative control. Twenty  $\mu$ L of Nyastatin (10,000 U/mL) was pipetted onto a sterile disc as a positive control. Each treatment was placed on a freshly inoculated plate so that treatments had as much direct contact with the trapped fungal spores as possible. A blank paper disc was placed on the plates as well as a control. A number (1-6) was designated to each treatment type.

I used a blind study to measure the effects of the treatments on fungal growth. Primary (Dr. Robert Warren) and secondary (Charlene Gray) observers measured fungal growth around treatment discs beginning 24 h (day 1) after plates were inoculated and for 5 days afterwards. We

measured area around the discs for growth using a square centimeter grid and recorded the percent growth coverage in a square centimeter.

Fungal inhibition for each species (*A. alternata* and *F. oxysporum*) as a function of treatments (ant soaked hexane, yellow jacket soaked hexane, seed soaked hexane, Nystatin, hexane, and blank discs) was analyzed using ANOVA models with Tukey's Honest Significant Difference *post hoc* tests in the R statistical package.

## Results

*Alternaria alternata* growth was significantly inhibited by treatment ( $DF = 5$ ,  $SS = 89429$ ,  $MS = 17886$ ,  $F\text{-value} = 33.550$ ,  $P\text{-value} < 0.001$ ); however, only the Nystatin treatment differed from the rest. *Fusarium oxysporum* growth also was significantly inhibited by treatment ( $DF = 5$ ,  $SS = 10062$ ,  $MS = 2012$ ,  $F\text{-value} = 74.620$ ,  $P\text{-value} < 0.001$ ), and it also only had a difference between Nystatin and the rest of the treatments. There was a significant growth interaction between fungal species and date as well as fungal species and treatment (Table 2).

## Discussion

None of the treatments except the antifungal Nystatin impacted fungal assay growth. The two fungal species grew at different rates (Table 2) and responded differently to treatments (Table 2), but there was no significant interaction between treatments of each species. I was not able to determine whether I extracted enough metapleural solution to cause an effect on fungal growth. Storing the ants at  $-80^{\circ}\text{C}$  for one to two months may have disrupted their ability to confer metapleural gland extracts. It also is possible that using hexane to extract metapleural gland contents diluted the compounds too drastically for a detectable effect. Further experiments that

include a direct measure of metapleural chemistry and concentration need to be conducted before making definite conclusions about the effects on phytopathogenic fungi. Previous metapleural gland research used large (2-8 cm) species of ants, i.e., *Myrmecia nigriscapa* (bull ants), or ants with larger bulla (a bubble-like structure filled with fluid) beneath the metapleural gland, i.e., *Acromyrmex octospinosus* (leaf cutter ants), to milk metapleural contents directly from live specimens (Beattie et al. 1985, Beattie et al. 1986, Ortius-Lechner et al. 2000). Researchers also have dissected metapleural glands from ant bodies (Cabrera et al. 2004). Milking live ants as well as extracting metapleural glands was impractical for this experiment as N.A. *Aphaenogaster* species are smaller, with a mean length of 4 mm.

## **Conclusion**

The results of this study did not support my hypothesis that ant presence inhibits fungal plant pathogen growth. There was no difference in growth among the treatments used except for the positive control Nyastatin (Table 2). There was no detectable growth on negative control discs, however there was a slight, though statistically insignificant, increase in growth on ant hexane and yellow jacket hexane treated discs.

I had no verification that the ants used in this study produced enough metapleural solution to inhibit fungal growth. Without positive results, I cannot determine whether metapleural gland extracts inhibit fungal growth using this analysis. Studying cumulative effects of ant metapleural glands on fungal growth may lead to more pronounced, definitive effects on fungal growth. This was an exploratory methodology, and whereas the analysis I used is not suitable for studying the effects of metapleural gland contents on fungal growth, the unique plating methodology may be applicable to other research projects. It is highly useful to grow



fungi evenly on agar plates at measurable densities, and future researchers could benefit from this protocol.

## CHAPTER IV

### SOIL DIVERSITY

#### **Introduction:**

*Aphaenogaster* ants appear to influence soil fungal communities (Zettler et al. 2002, Nelson 2012, Warren and Bradford 2012), but their influence on fungal diversity is unclear, and no research has conclusively identified fungal types. Given that *Aphaenogaster* is the main seed-dispersing ant genera, the most relevant impact for plant benefit would be a reduction in phytopathogenic fungi in *Aphaenogaster* nests.

Next Generation Sequencing (NGS) technologies use massively parallel processing to generate sequence data, creating multiple copies of sequence from many DNA samples that can be analyzed simultaneously (Ronaghi et al. 1996, Voelkerding et al. 2009, Hall-Mendelin et al. 2013). In the NGS process, target ‘barcode’ DNA regions from multiple species are amplified and sequenced, allowing identification of sample unknowns to genus or species level, depending on sequence library availability (Tonge et al. 2014). The capacity to detect previously underrepresented fungi with NGS technology makes classifying the fungal community structure within an ant nest possible, allowing us to detect the previously unexplored fungal communities within ant nests.

#### **Methods**

DNA was extracted from each of the six *Aphaenogaster* soil samples and the corresponding six control soils using an Axygen soil and stool extraction kit following the manufacturer's protocol (Appendix A).

Separate PCR reactions were run for each primer set with specific thermal cycle regimes and chemical reactions for each fungal genus targeted (Appendix B).

Amplicons were purified using an Agencourt AMPure XP Reagent kit (Beckman Coulter, Product # A63880, Appendix C). Many of the targeted amplified fungi were expected to have 500-1000 bp amplicons based on the primer sequence data gathered from National Center for Bioinformatics Nucleotide Basic Local Alignment Search Tool (NCBI Blastn, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sizes of the amplicons produced were checked on an Agilent TapeStation (Agilent Technologies, Waldbronn, Germany, product # G2964A, Appendix D). In addition, the amplicons were checked on a Qubit Fluorometer 2.0 for DNA concentration (Appendix E). From the multiple amplicons generated for each soil DNA sample, an approximately equimolar amplicon library was created for each sample by pooling the amplicons, with an end result of 12 sample libraries corresponding to the original 12 soil samples (Appendix F). Libraries that contained amplicons larger than 200 bp were sheared to approximately 200 bp using the Ion Shear Plus kit (Life Technologies, Product #4471269, Appendix G). All amplicon libraries were end repaired and purified (Appendix H). The final product concentration and sizes were checked on the TapeStation (Appendix D).

The library samples were prepared using an Ion Xpress Plus Fragment Library kit (Life Technologies, Product #4471269, Appendix I). Sample libraries were ligated with a specific barcode prior to sequencing (Appendix I). All barcoded samples were pooled into a final library (Appendix F) that was then amplified and purified (Appendix J).

Samples were templated on Ion Sphere Particles (ISPs) using the Ion PGM Template OT2 200 kit (Life Technologies, Product # 4480981) and the OneTouch 2 instrument (Life Technologies, Product # 4474779, Appendix K). Sequencing was performed on the Life Technologies Ion Torrent Personal Genome Machine (PGM) using a 200 base pair sequencing kit (Product #4482006) with one 314 chip (Life Technologies, Product # 4462923) for 4

barcoded soil samples, and a second 314 chip was used for the final 8 barcoded soil samples (Appendix L).

### *Biodata analysis*

A digital reference library was constructed for comparison with the sequencing results from the PGM. The genus-specific primer sequences were searched using the National Center for Bioinformatics Nucleotide Basic Local Alignment Search Tool (NCBI BLASTn, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) function to find fungal species genomes with sequences that corresponded to the primer sequences used to create the amplicons. A total of 67 fungal species were referenced in the library. The FASTA files of the genomes were copied and organized according to the primer sequences in a multi-FASTA file. The reference library was loaded onto the Ion Torrent Server and compared to the data from the PGM to find species matches.

The Torrent Suite software plugin Coverage Analysis (Technologies 2015), was used to map the sample library sequences against the digital reference library, matching sequence reads produced from my samples against the known fungal genomes organized in the digital reference library. The read depth and coverage for each fungal species was produced with the Coverage Analysis software, and the genus and species names were added according to the accession number from NCBI. Only species with a minimum of 25 whole sequence reads were included in the analysis to limit false-positive sequence alignments. The frequency of samples containing each fungal species was calculated based on their presence or absence within each sample.

Student's *t*-tests were used to test for differences between ant and control soils in total fungal species richness and pathogenic fungal species richness. I considered treatment effect sizes as small ( $d = 0.2$ ), medium ( $d = 0.5$ ) and large ( $d = 0.8$ ) as suggested by Cohen (1988).

## Results

Ant nest soil contained marginally significantly fewer fungal species than control soils ( $t = 1.548$ ,  $df = 10$ ,  $p$ -value = 0.076) [Fig. 3a], and marginally significantly fewer pathogenic fungal species in ant nest than control soils ( $t = 1.595$ ,  $df = 10$ ,  $p$ -value = 0.071) [Fig. 3b]. The first 314 chip (sample id 3, 4, 7, and 8) run had 30% of the usable reads aligned with the digital reference library, with an average coverage depth of 68.5 X (Fig. 4). The second 314 chip with the final eight samples (sample id 1, 2, 5, 6, 9, 10, 11, and 12) had 22% alignment with the digital reference library, with an average coverage depth of 48.5 X (Fig. 4). Unaligned bases were not identified and were not analyzed. A medium effect size ( $d=0.5$ ) was found for ant effects on both general and pathogenic fungal species richness.

Of the 67 species filed in the digital reference library, 60 matched sequences from the PGM sequencing data. The most common fungal species across all samples (occurring > 90% across samples) were *Alternaria alternata*, *Amyloporia carbonica*, *Coniophora puteana*, *Cortinarius malachus*, *Entoloma alpicola*, *Fibroporia radiculosa*, *Inocybe phaeoleuca*, *Neolentinus lepideus*, and *Pythium amasculinum* (Table 4). Of these species, only *Alternaria alternata* is a known seed pathogen (Table 4).

Eighty-four percent of all fungal species occurred more in control than ant nest soils, and 53% of those are plant pathogens (Table 5). Only 9% of fungal species occurred more frequently

in ant nest than control soils, and only 40% of those were plant pathogens (Table 5). Seven percent of all fungal species occurred at the same frequency in both ant nest and control soils, and 50% of those are plant pathogens (Table 5).

Seventeen percent of the fungal species were ectomycorrhizal fungi. Of these, 67% occurred more frequently in control than ant soils, whereas 22% occurred more often in ant soils (Table 6). Eleven percent of the ectomycorrhizal fungi occurred at the same frequency in ant nest soil and control soil. Nine percent of the fungal species were brown rot fungi, and all of the brown rot fungi occurred more frequently in control than ant nest soils (Table 6).

## **Discussion**

Ant presence lowered overall soil fungal diversity. More importantly for plants, ant presence lowered the diversity of phytopathogenic fungal species. The reduction of plant pathogen fungal diversity could indicate that ant anti-fungal properties inadvertently provide a major ant seed dispersal benefit to plants. Current research suggests that ant nests create a 'safe site' free of seed predators with enhanced recruitment conditions (Beattie and Culver 1983, Christian and Stanton 2004, Giladi 2006, Kwit et al. 2012); my results suggest an additional evidence for directed dispersal: fungal pathogen protection.

The passive exchange between ant body chemistry and the surrounding nest soils creates an environment defended against harmful pathogens, a characteristic essential to eusocial insects because of their dense living conditions that make them vulnerable to disease (Bot et al. 2002, Stow and Beattie 2008). The anti-fungal properties of ant nests may partially explain why germination rates are higher for seeds dispersed into ant nests (Hanzawa et al. 1988, Prior et al. 2014), with ant immune defenses inadvertently creating a safer environment for seeds. Given the

results from this experiment, fungal pathogen protection is a likely explanation for seed germination success in ant nests.

Pathogenic fungi appear to be more affected by ant presence than more benign species (Table 5), however all fungal diversity is lowered by ant presence (Fig. 3), with a few exceptions (Table 6). The presence of DNA sequences within ant soil does not necessarily imply function. It is possibly a consequence of a general ant fungicide that plants gain protection from pathogens within ant nests rather than a more interconnected, reciprocal mutualism between ant and plant, and myrmecochory is still a relationship open to a great deal of exploration and research.

Whereas this study focused on the effects of ants from the *Aphaenogaster* complex that live in NA woodlands (King et al. 2013), myrmecochory is a mutualism seen worldwide and occurring across 10,000 plant species (Lengyel et al. 2009). Why this mutualism is so frequent across many ecosystems is debatable. Some explanation may be derived from relationships between ants and nonmyrmecochorous species, particularly in the tropics. Ants acting as secondary dispersal agents for seeds first moved by birds and mammals (Roberts and Heithaus 1986) remove fruit pulp and other fruiting structures from the seeds (Leal and Oliveira 1998), which has been associated with increased germination rates (Oliveira et al. 1995, Ohkawara and Akino 2005). Anti-fungal ant handling benefits seen in nonmyrmecochores gives us insight to the coevolution of this relationship, with potential early benefits including protection from fungal attack.

Most plants die during recruitment (90%), and most seedling losses are attributed to early fungal infection (Fenner and Kitijima 1999, Blaney and Kotanen 2001, Kotanen 2007, Warren and Bradford 2011). By protecting seeds from early fungal infection, dispersal to ant nests greatly improves chances for recruitment. Fungal protection may help shape woodland plant

distributions by expanding plant access to wetter areas otherwise uninhabitable because of the prevalence of phytopathogenic fungi (Warren and Bradford 2011). Fungal protection in ant nests suggests that directed dispersal into the less pathogenic ant nests is a strong selective force for seed dispersal by ants.

Brown rot fungi, which break down woody material, also are negatively impacted by ant colonization (Table 4). *Aphaenogaster* ants, which often inhabit coarse woody debris, interfere with wood rot fungi and prevent wood decomposition (Warren and Bradford 2012). By slowing the decomposition of coarse woody debris, *Aphaenogaster* ants inadvertently influence major forest processes, including nutrient cycling and ecosystem carbon storage (Warren and Bradford 2012, Wieder et al. 2013). Such influences on nutrient and carbon cycling are great implications for *Aphaenogaster*'s role as a keystone species in N.A. woodlands.

There is still much complexity to be explored within the fungal communities in ant nests. The results presented here represent presence-absence data established with sample DNA sequencing matches to known fungal sequences. Only 30% of Chip 1 and only 22% of Chip 2 matched sequences within the digital reference library, leaving the majority of the sequences generated unidentified (Fig. 4). By creating a digital reference library, I limited the analysis to specific groups of fungi. I also was unable to determine the abundance of individual species. Future research could use Quantitative PCR methods in order to check for the abundance of species as well. By controlling DNA sample quantities, more realistic community structures can be established, enabling future researchers not only to discover what species are present in samples, but also which species are dominant in fungal communities within and out of ant nests. In addition, these data can be reanalyzed when more fungi can be barcoded and identified, giving



more information for the digital reference library. Such information will help to explain the evolutionary causes of myrmecochory.

## **Conclusion**

Ant occupation seemingly reduces soil fungi, particularly plant pathogens. These results suggest that fungal pathogen protection may be as critical a benefit of ant-mediated seed dispersal as seed movement. Whereas support is mixed for the putative benefits of seed placement in ant nests such as higher soil moisture and nutrients, fungal protection strengthens the evidence for myrmecochore dependency on their dispersal partner and may give a more consistent explanation for why plants do well in ant nests. If all myrmecochores derive some fungal protection from ants, fungal protection may be a key evolutionary benefit to the worldwide phenomenon of ant seed dispersal.

## LITERATURE CITED

- Al-Askar, A. A., K. M. Ghoneem, and Y. M. Rashad. 2012. Seed borne mycoflora of alfalfa (*Medicago sativa* L.) in the Riyadh region of Saudi Arabia. *Annals of Microbiology* **62**:11.
- Alvarado-Rosales, D., L. D. Saavedra-Romero, and A. Almaraz-Sanchez. 2008. First report of *Phytophthora cinnamomi* Rands. associated with oak (*Quercus* spp.) in Tecoaapa, Guerrero, Mexico. *Agrociencia* **42**:565-572.
- Balaji, D. S., S. Basavaraja, R. Deshpande, D. B. Mahesh, B. K. Prabhakar, and A. Venkataraman. 2009. Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus. *Colloids and Surfaces B-Biointerfaces* **68**:88-92.
- Baroncelli, R., S. Sarrocco, A. Zapparata, S. Tavarini, L. G. Angelini, and G. Vannacci. 2015. Characterization and epidemiology of *Colletotrichum acutatum sensu lato* (*C. chrysanthemi*) causing *Carthamus tinctorius* anthracnose. *Plant Pathology* **64**:375-384.
- Bashyal, B. M., and R. Aggarwal. 2013. Molecular identification of *Fusarium* species associated with bakanae disease of rice (*Oryza sativa*) in India. *Indian Journal of Agricultural Sciences* **83**:71-76.
- Bateman, G. L., G. Murray, R. J. Gutteridge, and H. Coskun. 1998. Effects of method of straw disposal and depth of cultivation on populations of *Fusarium* spp. in soil and on brown foot rot in continuous winter wheat. *Annals of Applied Biology* **132**:35-47.
- Beattie, A., and D. C. Culver. 1983. The nest chemistry of two seed-dispersing ant species. *Oecologia* **56**:99-103.

- Beattie, A. J., C. Turnbull, T. Hough, S. Jobson, and R. B. Knox. 1985. The vulnerability of pollen and fungal spores to ant secretions- evidence and some evolutionary implications. *American Journal of Botany* **72**:606-614.
- Beattie, A. J., C. L. Turnbull, T. Hough, and R. B. Knox. 1986. Antibiotic production-a possible function for the metapleural glands of ants (Hymenoptera: Formicidae). *Annals of the Entomological Society of America* **79**:448-450.
- Bjorbækmo, M. F. M., T. Carlsen, A. Brysting, T. Vrålstad, K. Høiland, K. I. Ugland, J. Geml, T. Schumacher, and H. Kauserud. 2010. High diversity of root associated fungi in both alpine and arctic *Dryas octopetala*. *BMC Plant Biology* **10**:1-12.
- Blaney, C., and P. Kotanen. 2001. Effects of fungal pathogens on seeds of native and exotic plants: a test using congeneric pairs. *Journal of Applied Ecology* **38**:1104-1113.
- Bot, A., D. Ortius-Lechner, K. Finster, R. Maile, and J. Boomsma. 2002. Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Sociaux* **49**:363-370.
- Broders, K. D., P. E. Lipps, P. A. Paul, and A. E. Dorrance. 2007. Evaluation of *Fusarium graminearum* associated with corn and soybean seed and seedling disease in Ohio. *Plant Disease* **91**:1155-1160.
- Cabrera, A., D. Williams, J. Hernandez, F. Caetano, and K. Jaffe. 2004. Metapleural- and postpharyngeal-gland secretions from workers of the ants *Solenopsis invicta* and *S. geminata*. *Chemistry & Biodiversity* **1**:303-311.
- Cardin, L., L. Vincenot, and M. H. Balesdent. 2009. First Report of *Pilidium concavum* on *Bergenia crassifolia* in France. *Plant Disease* **93**:548-548.

- Cartwright, D. K., and H. W. Spurr. 1998. Biological control of *Phytophthora parasitica* var. *Nicotianae* on tobacco seedlings with non-pathogenic binucleate *Rhizoctonia* fungi. *Soil Biology & Biochemistry* **30**:1879-1884.
- Chatterton, S., J. C. Sutton, and G. J. Boland. 2004. Timing *Pseudomonas chlororaphis* applications to control *Pythium aphanidermatum*, *Pythium dissotocum*, and root rot in hydroponic peppers. *Biological Control* **30**:360-373.
- Christian, C. E., and M. L. Stanton. 2004. Cryptic consequences of a dispersal mutualism: Seed burial, elaiosome removal, and seed-bank dynamics. *Ecology* **85**:1101-1110.
- Clemmensen, K. E., R. D. Finlay, A. Dahlberg, J. Stenlid, D. A. Wardle, and B. D. Lindahl. 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. *New Phytologist* **205**:1525-1536.
- Craft, C. M., and E. B. Nelson. 1996. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium graminicola*. *Applied and Environmental Microbiology* **62**:1550-1557.
- Crespo-Sempere, A., N. Estiarte, S. Marin, V. Sanchis, and A. J. Ramos. 2013. Propidium monoazide combined with real-time quantitative PCR to quantify viable *Alternaria* spp. *International Journal of Food Microbiology* **165**:214-220.
- Crist, T. O., and C. F. Friese. 1993. The impact of fungi on soil seeds- implications for plants and granivores in a semiarid shrub-steppe. *Ecology* **74**:2231-2239.
- Cui, B. K., and Y. C. Dai. 2013. Molecular phylogeny and morphology reveal a new species of *Amyloporia* (Basidiomycota) from China. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **104**:817-827.

- De Mares, M. C., J. Hess, D. Floudas, A. Lipzen, C. Choi, M. Kennedy, I. V. Grigoriev, and A. Pringle. 2015. Horizontal transfer of carbohydrate metabolism genes into ectomycorrhizal *Amanita*. *New Phytologist* **205**:1552-1564.
- Dean, R., J. A. L. Van Kan, Z. A. Pretorius, K. E. Hammond-Kosack, A. Di Pietro, P. D. Spanu, J. J. Rudd, M. Dickman, R. Kahmann, J. Ellis, and G. D. Foster. 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**:804-804.
- Dhingra, O. D., C. B. Maia, D. C. Lustosa, and J. B. Mesquita. 2002. Seedborne pathogenic fungi that affect seedling quality of red angico (*Anadenanthera macrocarpa*) trees in Brazil. *Journal of Phytopathology-Phytopathologische Zeitschrift* **150**:451-455.
- Fenner, M., and K. Kitijima. 1999. Seed and seedling ecology. *Functional Plant Ecology*. Marcel-Dekker, New York, NY.
- Fernandez-Marin, H., J. Zimmerman, S. Rehner, and W. Wcislo. 2006. Active use of the metapleural glands by ants in controlling fungal infection. *Proceedings of the Royal Society B-Biological Sciences* **273**:1689-1695.
- Fuerst, E. P., J. V. Anderson, A. C. Kennedy, and R. S. Gallagher. 2011. Induction of polyphenol oxidase activity in dormant wild oat (*Avena fatua*) seeds and caryopses: a defense response to seed decay fungi. *Weed Science* **59**:137-144.
- Gachango, E., L. E. Hanson, A. Rojas, J. J. Hao, and W. W. Kirk. 2012. *Fusarium* spp. causing dry rot of seed potato tubers in Michigan and their sensitivity to fungicides. *Plant Disease* **96**:1767-1774.
- Gazis, R., and P. Chaverri. 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. *Fungal Ecology* **3**:240-254.

- Giladi, I. 2006. Choosing benefits or partners: a review of the evidence for the evolution of myrmecochory. *Oikos* **112**:481-492.
- Gilmore, S., T. Grafenhan, G. Louis-Seize, and K. Seifert. 2009. Multiple copies of cytochrome oxidase 1 in species of the fungal genus *Fusarium*. *Molecular Ecology Resources* **9**:90-98.
- Goncalves, P., E. Valerio, C. Correia, J. M. G. C. F. de Aleida, and J. P. Sampaio. 2011. Evidence for divergent evolution of growth temperature preference in sympatric *Saccharomyces* species. *PLoS ONE* **6**.
- Graf, F., and I. Brunner. 1996. Natural and synthesized ectomycorrhizas of the alpine dwarf willow *Salix herbacea*. *Mycorrhiza* **6**:227-235.
- Guimaraes, P., and R. Cogni. 2002. Seed cleaning of *Cupania vernalis* (Sapindaceae) by ants: edge effect in a highland forest in south-east Brazil. *Journal of Tropical Ecology* **18**:303-307.
- Hall-Mendelin, S., R. Allcock, N. Kresoje, A. F. van den Hurk, and D. Warrilow. 2013. Detection of arboviruses and other micro-organisms in experimentally infected mosquitoes using massively parallel sequencing. *Plos One* **8**:7.
- Hanzawa, F. M., A. J. Beattie, and D. C. Culver. 1988. Directed dispersal- demographic analysis of an ant-seed mutualism. *American Naturalist* **131**:1-13.
- Hastrup, A. C. S., B. Jensen, and J. Jellison. 2014. Fungal accumulation of metals from building materials during brown rot wood decay. *Archives of Microbiology* **196**:9.
- Hawksworth, D. L. 1991. The fungal dimension of biodiversity- magnitude, significance, and conservation. *Mycological Research* **95**:641-655.

- Heine, G., G. Tikum, and W. J. Horst. 2007. The effect of silicon on the infection by and spread of *Pythium aphanidermatum* in single roots of tomato and bitter melon. *Journal of Experimental Botany* **58**:569-577.
- Hess, J., and A. Pringle. 2014. The Natural Histories of Species and Their Genomes: Asymbiotic and Ectomycorrhizal *Amanita* Fungi. Pages 235-257 in F. M. Martin, editor. *Fungi*. Academic Press Ltd-Elsevier Science Ltd, London.
- Hodges, C. F., and D. A. Campbell. 1994. Infection of adventitious roots *Agrostis palustris* by *Pythium* species at different temperature regimes. *Canadian Journal of Botany-Revue Canadienne De Botanique* **72**:378-383.
- Inglis D.O., M. S. Skrzpek, D. Liaw, V. Moktali, G. Sherlock, and J. E. Stajich. 2014. Literature-based gene curation and proposed genetic nomenclature for *Cryptococcus*. *Eukaryotic Cell* **13**:878-883.
- Kavitha, P. G., and G. Thomas. 2008. Expression analysis of defense-related genes in Zingiber (*Zingiberaceae*) species with different levels of compatibility to the soft rot pathogen *Pythium aphanidermatum*. *Plant Cell Reports* **27**:1767-1776.
- Khan, A., J. C. Sutton, and B. Grodzinski. 2003. Effects of *Pseudomonas chlororaphis* on *Pythium aphanidermatum* and root rot in peppers grown in small-scale hydroponic troughs. *Biocontrol Science and Technology* **13**:615-630.
- Kiecana, I., M. Cegiello, E. Mielniczuk, and A. Pastucha. 2014. Fungi infecting ornamental grasses and the pathogenicity of *Fusarium culmorum* (WGSsm.) Sacc. and *Fusarium equiseti* (Corda) Sacc. to selected species. *Acta Scientiarum Polonorum-Hortorum Cultus* **13**:61-75.

- King, J., R. Warren, and M. Bradford. 2013. Social insects dominate eastern US temperate hardwood forests macroinvertebrate communities in warmer regions. *Plos One* **8**.
- Kipfer, T., T. Wohlgemuth, M. G. A. van der Heijden, J. Ghazoul, and S. Egli. Growth response of drought-stressed *Pinus sylvestris* seedlings to single- and multi-species inoculation with ectomycorrhizal fungi. *Plos One* **7**.
- Kotanen, P. 2007. Effects of fungal seed pathogens under conspecific and heterospecific trees in a temperate forest. *Canadian Journal of Botany-Revue Canadienne De Botanique* **85**:918-925.
- Kwit, C., G. Marcello, J. Gonzalez, A. Shapiro, and R. Bracken. 2012. Advantages of seed dispersal for a myrmecocorous temperate forest herb. *American Midland Naturalist* **168**:9-17.
- Lacey, J. 1996. Spore dispersal - Its role in ecology and disease: The British contribution to fungal aerobiology. *Mycological Research* **100**:641-660.
- Leal, I., and P. Oliveira. 1998. Interactions between fungus-growing ants (Attini), fruits and seeds in cerrado vegetation in southeast Brazil. *Biotropica* **30**:170-178.
- Lengyel, S., A. Gove, A. Latimer, J. Majer, and R. Dunn. 2009. Ants sow the seeds of global diversification in flowering plants. *Plos One* **4**.
- Life Technologies. 2015. Torrent Suite 4.4 Analysis Report Guide. Page 53-64. Thermo Fisher Scientific.
- Lubertazzi, D. 2012. The biology and natural history of *Aphaenogaster rudis*. *Psyche*:1-11.
- Ma, M. Z., M. J. Christensen, and Z. B. Nan. 2015. Effects of the endophyte *Epichloe festucae* var. *lolii* of perennial ryegrass (*Lolium perenne*) on indicators of oxidative stress from



- pathogenic fungi during seed germination and seedling growth. *European Journal of Plant Pathology* **141**:571-583.
- Miller, R. G. 1981. Simultaneous statistical interference. Springer Series in Statistics. Springer, New York, New York.
- Moles, A., and M. Westoby. 2004. What do seedlings die from and what are the implications for evolution of seed size? *Oikos* **106**:193-199.
- Muchovej, J. J., R. M. C. Muchovej, and E. J. Goncalves. 1991. Effect of kind and method of fungicidal treatment of bean seed on infections by the VA-mycorrhizal fungus *Glomus macrocarpum* and by the pathogenic fungus *Fusarium solani* 2- temporal-spatial relationships. *Plant and Soil* **132**:47-51.
- Nagano, Y., J. S. Elborn, B. C. Miller, J. M. Walker, C. E. Goldsmith, J. Rendall, and J. E. Moore. 2010. Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis. *Medical Mycology* **48**:166-176.
- Nascimento, R. R. D., E. Schoeters, E. D. Morgan, J. Billen, and D. J. Stradling. 1996. Chemistry of metapleural gland secretions of three attine ants, *Atta sexdens rubropilosa*, *Atta cephalotes*, and *Acromyrmex octospinosus* (Hymenoptera: Formicidae). *Journal of Chemical Ecology* **22**:987-1000.
- Nelson, B. 2012. Increased fungal diversity associated with *Aphaenogaster* spp.: more evidence for keystone mutualisms. Western Carolina University, Cullowhee, North Carolina, United States.
- Ness, J., D. Morin, and I. Giladi. 2009. Uncommon specialization in a mutualism between a temperate herbaceous plant guild and an ant: are *Aphaenogaster* ants keystone mutualists? *Oikos* **118**:1793-1804.

- Ocamb, C. M., J. Juzwik, and F. B. Martin. 2002. *Fusarium* spp. and *Pinus strobus* seedlings: root disease pathogens and taxa associated with seed. *New Forests* **24**:67-79.
- Ohkawara, K., and T. Akino. 2005. Seed cleaning behavior by tropical ants and its anti-fungal effect. *Journal of Ethology* **23**:93-98.
- Okamoto, K., R. Kanawaku, M. Masumoto, and H. Yanase. 2012. Efficient xylose fermentation by the brown rot fungus *Neolentinus lepideus*. *Enzyme and Microbial Technology* **50**:96-100.
- Oliveira, P. S., M. Galetti, F. Pedroni, and L. P. C. Morellato. 1995. Seed cleaning by *Mycocepurus goeldii* ants (Attini) facilitates germination in *Hymenaea courbaril* (Caesalpinaceae). *Biotropica* **27**:518-522.
- Ortius-Lechner, D., R. Maile, E. D. Morgan, and J. J. Boomsma. 2000. Metapleural gland secretions of the leaf-cutter ant *Acromyrmex octospinosus*: new compounds and their functional significance. *Journal of Chemical Ecology* **26**:1667-1683.
- Polashock, J. J., F. L. Caruso, P. V. Oudemans, P. S. McManus, and J. A. Crouch. 2009. The North American cranberry fruit rot fungal community: a systematic overview using morphological and phylogenetic affinities. *Plant Pathology* **58**:1116-1127.
- Prior, K., K. Saxena, and M. Frederickson. 2014. Seed handling behaviours of native and invasive seed-dispersing ants differentially influence seedling emergence in an introduced plant. *Ecological Entomology* **39**:66-74.
- Reddy, M. N., and A. S. Rao. 1974. New leaf spot disease of mulberry caused by *Fusarium concolor* reinking. *Current Science* **43**:530-530.

- Revis, H., and D. Waller. 2004. Bactericidal and fungicidal activity of ant chemicals on feather parasites: An evaluation of anting behavior as a method of self-medication in songbirds. *Auk* **121**:1262-1268.
- Roberts, J. T., and E. R. Heithaus. 1986. Ants rearrange the vertebrate-generated seed shadow of a neotropical fig tree. *Ecology* **67**:1046-1051.
- Romon, P., M. Troya, M. E. Fernandez de Gamarra, A. Eguzkitza, J. C. Iturrondobeitia, and A. Goldarazena. 2008. Fungal communities associated with pitch canker disease of *Pinus radiata* caused by *Fusarium circinatum* in northern Spain: association with insects and pathogen-saprophyte antagonistic interactions. *Canadian Journal of Plant Pathology- Revue Canadienne De Phytopathologie* **30**:241-253.
- Ronaghi, M., S. Karamohamed, B. Pettersson, M. Uhlen, and P. Nyren. 1996. Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry* **242**:84-89.
- Roy, K. W. 1996. Falcate-spored species of *Colletotrichum* on soybean. *Mycologia* **88**:1003-1009.
- Sajjad Ur, R., M. H. Rasool, and M. Rafi. 2012. Penicillin production by wild isolates of *Penicillium chrysogenum* in Pakistan. *Brazilian Journal of Microbiology* **43**:476-481.
- Sarver, B. A. J., T. J. Ward, L. R. Gale, K. Broz, H. C. Kistler, T. Aoki, P. Nicholson, J. Carter, and K. O'Donnell. 2011. Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. *Fungal Genetics and Biology* **48**:1096-1107.
- Shearer, B. L., and C. E. Crane. 2014. *Phytophthora cinnamomi* disease expression and habitat suitability of soils on a topographic gradient across a coastal plain from dunes to forested peneplain. *Australasian Plant Pathology* **43**:131-142.

- Shubin, L. H., H. Juan, Z. RenChao, X. ShiRu, and J. YuanXiao. 2014. Fungal endophytes of *Alpina officinarum* rhizomes: insights on diversity and variation across growth years, growth sites, and the inner active chemical concentration. *Plos One* **9**:1-21.
- Starkey, D. E., T. J. Ward, T. Aoki, L. R. Gale, H. C. Kistler, D. M. Geiser, H. Suga, B. Toth, J. Varga, and K. O'Donnell. 2007. Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. *Fungal Genetics and Biology* **44**:1191-1204.
- Stow, A., and A. Beattie. 2008. Chemical and genetic defenses against disease in insect societies. *Brain Behavior and Immunity* **22**:1009-1013.
- Sun, B. D., H. Y. Yu, A. J. Chen, and X. Z. Liu. 2008. Insect-associated fungi in soils of field crops and orchards. *Crop Protection* **27**:1421-1426.
- Talhinhas, P., E. Goncalves, S. Sreenivasaprasad, and H. Oliveira. 2015. Virulence diversity of anthracnose pathogens (*Colletotrichum acutatum* and *C-gloeosporioides* species complexes) on eight olive cultivars commonly grown in Portugal. *European Journal of Plant Pathology* **142**:73-83.
- Tang, J. D., L. A. Parker, A. D. Perkins, T. S. Sonstegard, S. G. Schroeder, D. D. Nicholas, and S. V. Diehl. 2013. Gene expression analysis of copper tolerance and wood decay in the brown rot fungus *Fibroporia radiculosa*. *Applied and Environmental Microbiology* **79**:1523-1533.
- Thorn, R. G., J. M. Moncalvo, C. A. Reddy, and R. Vilgalys. 2000. Phylogenetic analysis and the distribution of nematography support a monophyletic pleurotaceae within the polyphyletic pleurotoid-lentinoid fungi. *Mycological Society of America* **92**:241-252.

- Tonge, D. P., C. H. Pashley, and T. W. Gant. 2014. Amplicon-based metagenomic analysis of mixed fungal samples using proton release amplicon sequencing Plos One **9**:1.
- Valera, H. R., J. Gomes, S. Lakshmi, R. Gururaja, S. Suryanarayan, and D. Kumar. 2005. Lovastatin production by solid state fermentation using *Aspergillus flavipes*. Enzyme and Microbial Technology **37**:521-526.
- Veal, D., J. Trimble, and A. Beattie. 1992. Antimicrobial properties of secretions from the metapleural glands of *Myrmica gulosa* (the Australian Bull Ant). Journal of Applied Bacteriology **72**:188-194.
- Voelkerding, K. V., S. A. Dames, and J. D. Durtschi. 2009. Next-Generation Sequencing: From basic research to diagnostics. Clinical Chemistry **55**:641-658.
- Wagner, D., and J. Jones. 2004. The contribution of harvester ant nests, *Pogonomyrmex rugosus* (Hymenoptera : Formicidae), to soil nutrient stocks and microbial biomass in the Mojave Desert. Environmental Entomology **33**:599.
- Wagner, D., J. Jones, and D. Gordon. 2004. Development of harvester ant colonies alters soil chemistry. Soil Biology & Biochemistry **36**:797-804.
- Wang, P., Y. Wang, and J. White. 2004. Species-specific PCR primers for *Pythium* developed from ribosomal ITS1 region. Letters in Applied Microbiology **38**:78-78.
- Warren, R.J., and M. Bradford. 2011. The shape of things to come: woodland herb niche contraction begins during recruitment in mesic forest microhabitat. Proceedings of the Royal Society B-Biological Sciences **278**:1390-1398.
- Warren, R. J., and M. Bradford. 2012. Ant colonization and coarse woody debris decomposition in temperate forests. Insectes Sociaux **59**:215-221.

- Warren, R. J., and I. Giladi. 2014. Ant-mediated seed dispersal: A few ant species (Hymenoptera: Formicidae) benefit many plants. *Myrmecological News* **20**:129-140.
- Wieder, W. R., B.G.B., and S. D. Allison. 2013. Global soil carbon projections are improved by modeling microbial processes. *Natural Climate Change* **3**:909-912.
- Wilman, K., L. Stepien, I. Fabianska, and P. Kachlicki. 2014. Plant-pathogenic fungi in seeds of different pea cultivars in Poland. *Arhiv Za Higijenu Rada I Toksikologiju-Archives of Industrial Hygiene and Toxicology* **65**:329-338.
- Wulff, E. G., J. L. Sorensen, M. Lubeck, K. F. Nielsen, U. Thrane, and J. Torp. 2010. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environmental Microbiology* **12**:649-657.
- Xue, C. Y., Y. Tada, X. N. Doing, and J. Heitman. 2007. The human fungal pathogen *Cryptococcus* can complete its sexual cycle during a pathogenic association with plants. *Cell Host & Microbe* **1**:263-273.
- Yaltirak, T., B. Aslim, S. Ozturk, and H. Alli. 2009. Antimicrobial and antioxidant activities of *Russula delica* Fr. *Food and Chemical Toxicology* **47**:2052-2056.
- Yandell, B. S. 1997. *Practical data analysis for designed experiments*. Chapman and Hall, London.
- Yek, S., and U. Mueller. 2011. The metapleural gland of ants. *Biological Reviews* **86**:774-791.
- Zettler, J., T. McInnis, C. Allen, and T. Spira. 2002. Biodiversity of fungi in red imported fire ant (Hymenoptera : Formicidae) mounds. *Annals of the Entomological Society of America* **95**:487-491.
- Zhang, H. Y., Q. Y. Yang, H. T. Lin, X. F. Ren, L. N. Zhao, and J. S. Hou. 2013. Phytic acid enhances biocontrol efficacy of *Rhodotorula mucilaginosa* against postharvest gray mold

spoilage and natural spoilage of strawberries. *Lwt-Food Science and Technology* **52**:110-115.

Zhong, S. B., S. Ali, Y. Q. Leng, R. Wang, and D. F. Garvin. 2015. *Brachypodium distachyon-Cochliobolus sativus* pathosystem is a new model for studying plant-fungal interactions in cereal crops. *Phytopathology* **105**:482-489.

**Table 1:** Fungal species identified by Zettler (2002) with pathogenicity of fungal species. Pathogenicity ranks are assigned based on review of available literature.

Fungal Species	Pathogenicity	Fungal Species	Pathogenicity
<i>Absidia cylindrospora</i> <i>Hagem</i>	Insect <sup>1</sup>	<i>Papulaspora byssina</i> Hotson	unknown
<i>Absidia spinosa</i> Lendn	Insect <sup>1</sup>	<i>Penicillium</i> sp. 1	Opportunistic <sup>1</sup>
<i>Aspergillus flavipes</i>	No <sup>2</sup>	<i>Penicillium</i> sp. 2	Opportunistic <sup>1</sup>
<i>Cladosporium</i> <i>cladosporioides</i>	Plant <sup>3</sup>	<i>Penicillium</i> sp. 3	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 1	Plant <sup>4</sup>	<i>Penicillium</i> sp. 4	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 2	Plant <sup>4</sup>	<i>Penicillium</i> sp. 5	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 3	Plant <sup>4</sup>	<i>Penicillium</i> sp. 6	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 4	Plant <sup>4</sup>	<i>Penicillium</i> sp. 7	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 5	Plant <sup>4</sup>	<i>Penicillium corylophilum</i> Dierckx	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 6	Plant <sup>4</sup>	<i>Penicillium herquei</i> Bainier and Sartory	Opportunistic <sup>1</sup>
<i>Fusarium</i> sp. 7	Plant <sup>4</sup>	<i>Penicillium janthinellum</i> Biourge	Opportunistic <sup>1</sup>
<i>Fusarium culmorum</i>	Plant <sup>4</sup>	<i>Penicillium rubrum</i> Sopp	Opportunistic <sup>1</sup>
<i>Fusarium dimerum</i>	Plant <sup>4</sup>	<i>Ramichloridium</i> sp. Stahel ex de Hoog	Opportunistic <sup>1</sup>
<i>Fusarium merismoides</i>	Plant <sup>4</sup>	<i>Rhizopus</i> sp	Opportunistic <sup>1</sup> secondary colonizer <sup>1</sup>
<i>Fusarium oxysporum</i> Fr	Plant, Insect <sup>1,4</sup>	<i>Rhizopus stolonifer</i>	Opportunistic <sup>1</sup> secondary colonizer <sup>1</sup>
<i>Gliocladium</i> sp. 1 Corda	Unknown	<i>Trichoderma</i> sp. 1	Plant <sup>5</sup>
<i>Gliocladium</i> sp. 2	Unknown	<i>Trichoderma</i> sp. 2	Plant <sup>5</sup>
<i>Gongronella butleri</i>	Unknown	<i>Trichoderma</i> sp. 3	Plant <sup>5</sup>
<i>Mortierella</i> sp. 1	Opportunistic <sup>1</sup>	<i>Trichoderma polysporum</i>	Plant <sup>5</sup>
<i>Mortierella</i> sp. 2	Opportunistic <sup>1</sup>	<i>Trichoderma pseudokoningii</i>	Plant <sup>5</sup>
<i>Mortierella</i> sp. 3	Opportunistic <sup>1</sup>	<i>Verticillium lecanii</i> (Zimm.) Vie'gas	unknown
<i>Mucor hiemalis</i>	Unknown	<i>Zygorhynchus moelleri</i> Vuill.	unknown
<i>Mycelia sterile</i> sp. 1	Unknown		
<i>Mycelia sterile</i> sp. 2	Unknown		

<sup>1</sup>- Sun et al. (2008), <sup>2</sup>- Valera et al. (2005), <sup>3</sup>- Balaji et al. (2009), <sup>4</sup>- Dean et al. (2012), <sup>5</sup>- Gazis and Chaverri (2010).



**Table 2:** Analysis of variance of fungal species as a function of growth rate and treatment response. There was a significant interaction between fungal species and date as well as fungal species and treatment.

	<i>df</i>	SS	<i>F</i> -value	<i>p</i> -value
Fungal species	1	25033	120.162	< 0.001
Plate	4	829	0.995	0.41
Treatment	6	78467	75.33	<0.001
Date	1	27860	133.731	<0.001
Species x Date	1	17507	84.033	<0.001
Species x Treatment	5	21025	28.052	<0.001

**Table 3:** PCR primers used for fungi DNA amplification for *Fusarium*, *Pythium*, Basidiomycota, and All Fungi with the corresponding DNA sequences. Known fungal species were targeted to improve the detection of plant pathogenic fungi.

Fungus species	Primer	Primer Sequence
Fusarium <sup>a</sup>	AHyFu Forward	CTTAGTGGGCCAGGAGTTCAATA
	AHyFu Reverse	ACCTCAGGGTGTCCGAAGAAT
	Fus-I3 Forward	TTAAAAGTATCGAAAAATCAAAAAGGTGT
	Fus-I3 Reverse	ATCTATCTCTTATTTCTTGGCTCATTGGTT
	Fus-14b Forward	CCTTTAAACTAGTACCGCAGAC
	Fus-I3 Reverse	ATCTATCTCTTATTTCTTGGCTCATTGGTT
	Pythium <sup>b</sup>	Pac1 Forward
ITS2 Reverse		GCTGCGTTCATCGATGC
Pa1 Forward		TCCACGTGAACCGTTGAAATC
ITS2 Reverse		GCTGCGTTCATCGATGC
Pa3 Forward		ATTTTTCAAACCCATTTACC
ITS2 Reverse		GCTGCGTTCATCGATGC
Pgr1 Forward		ACGAAGGTGGGCTGCATGTA
ITS2 Reverse		GCTGCGTTCATCGATGC
Phy1 Forward		GTCTGCGTCTATTTTGGATGC
ITS2 Reverse		GCTGCGTTCATCGATGC
Po1 Forward		TGCGTCTATTTTGGATGCGG
ITS2 Reverse		GCTGCGTTCATCGATGC
All Fungi <sup>c</sup>		ITS1f Forward
	5.8s Reverse	CGCTGCGTTCATCG
Basidiomycota <sup>c</sup>	ITS4b Forward	CAGGAGACTTGTACACGGTCCAG
	5.8sr Reverse	TCGATGAAGAACGCAGCG

<sup>a</sup>- Gilmore et al. (2009), <sup>b</sup>-Crespo-Sempere et al. (2013), <sup>c</sup>-Wang et al. (2004)

**Table 4:** The frequency and pathogenicity of fungal species occurring within samples. Frequency was calculated based on the number of samples with a minimum of 25 whole genome sequence alignments. Frequencies are found by the percent of all samples containing the listed fungal species, the percent of ant nest soils containing the fungal species, and the percent control soil samples containing the fungal species. Percent change is calculated by the percent of the difference in the number of samples containing the fungal species between control soils and ant soils.

Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Alternaria</i>	<i>alternata</i>	92%	83%	100%	17%	seed	(Romon et al. 2008, Al-Askar et al. 2012, Kiecana et al. 2014, Ma et al. 2015)
<i>Amanita</i>	<i>spissacea</i>	83%	83%	83%	0%		
<i>Amanita</i>	<i>umbrinella</i>	25 %	33%	17%	-100%		
<i>Amyloporia</i>	<i>carbonica</i>	92%	83%	100%	17%	non-pathogenic	(Cui and Dai 2013, Hess and Pringle 2014, De Mares et al. 2015)
<i>Articulospora</i>	<i>tetracladia</i>	50%	50%	50%	0%	non-pathogenic	(Cui and Dai 2013)
<i>Cenococcum</i>	<i>geophilum</i>	83%	67%	100%	33%		
<i>Cochliobolus</i>	<i>sativus</i>	17%	17%	17%	0%	non-pathogenic	(Kipfer et al.)
<i>Colletotrichum</i>	<i>acutatum</i>	58%	50%	67%	25%	seedling	(Zhong et al. 2015)
<i>Colletotrichum</i>	<i>capsici</i>	8%	0%	17%	100%	plant	(Polashock et al. 2009, Baroncelli et al. 2015, Talhinhas et al. 2015)

Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Colletotrichum</i>	<i>goleosporioides</i>	42%	33%	50%	33%	seed	(Dhingra et al. 2002, Polashock et al. 2009)
<i>Colletotrichum</i>	<i>truncatum</i>	8%	0%	17%	100%	seed	(Dhingra et al. 2002, Polashock et al. 2009)
<i>Coniophora</i>	<i>puteana</i>	92%	83%	100%	17%	seedling	(Roy 1996)
<i>Cortinarius</i>	<i>malachius</i>	92%	83%	100%	17%	non-pathogenic	(Hastrup et al. 2014)
<i>Cortinarius</i>	<i>vibratilis</i>	83%	83%	83%	0%	non-pathogenic	(Clemmensen et al. 2015)
<i>Cryptococcus</i>	<i>marinus</i>	75%	50%	100%	50%	non-pathogenic	(Clemmensen et al. 2015)
<i>Debaryomycea</i>	<i>subglobosus</i>	58%	50%	67%	25%	human and plant	(Xue et al. 2007, Inglis D.O. et al. 2014)
<i>Entoloma</i>	<i>alpicola</i>	92%	83%	100%	17%	non-pathogenic	(Graf and Brunner 1996)
<i>Entoloma</i>	<i>versatile</i>	58%	67%	50%	-33%	non-pathogenic	(Graf and Brunner 1996)
<i>Fibroporia</i>	<i>radiculosa</i>	92%	83%	100%	17%	non-pathogenic	(Ocamb et al. 2002, Gachango et al. 2012, Tang et al. 2013)
<i>Fusarium</i>	<i>acuminatum</i>	75%	83%	67%	-25%	seed	(Muchovej et al. 1991)

Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Fusarium</i>	<i>avenaceum</i>	58%	33%	83%	60%	seed	(Crist and Friese 1993, Fuerst et al. 2011, Gachango et al. 2012, Kiecana et al. 2014, Wilman et al. 2014, Ma et al. 2015)
<i>Fusarium</i>	<i>circinatum</i>	83%	67%	100%	33%	seedling	(Romon et al. 2008)
<i>Fusarium</i>	<i>concolor</i>	67%	50%	83%	33%	plant	(Reddy and Rao 1974)
<i>Fusarium</i>	<i>equiseti</i>	58%	33%	83%	60%	seed	(Gachango et al. 2012, Kiecana et al. 2014, Wilman et al. 2014)
<i>Fusarium</i>	<i>flocciferum</i>	58	33	83	60	non-pathogenic	

Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Fusarium</i>	<i>fujikuroi</i>	75%	67%	83%	20%	seed borne	(Starkey et al. 2007, Wulff et al. 2010, Sarver et al. 2011, Bashyal and Aggarwal 2013)
<i>Fusarium</i>	<i>gerlachii</i>	83	83	83	0	plant	(Starkey et al. 2007, Sarver et al. 2011)
<i>Fusarium</i>	<i>graminearum</i>	83	83	83	0	seed	(Broders et al. 2007, Gachango et al. 2012)
<i>Fusarium</i>	<i>heterosporum</i>	67	50	83	33	seed borne	(Ocamb et al. 2002)
<i>Fusarium</i>	<i>incarnatum</i>	67%	50%	83%	40%	seed	(Al-Askar et al. 2012 )

Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Fusarium</i>	<i>lateritium</i>	50%	33%	67%	50%	seed	(Dhingra et al. 2002)
<i>Fusarium</i>	<i>oxysporum</i>	75%	67%	83%	20%	seed	(Romon et al. 2008, Gachango et al. 2012, Kiecana et al. 2014)
<i>Fusarium</i>	<i>proliferatum</i>	42%	50%	33%	-50%	seedling	(Ocamb et al. 2002, Romon et al. 2008, Wilman et al. 2014)
<i>Fusarium</i>	<i>solani</i>	83%	67%	100%	33%	seed	(Muchovej et al. 1991, Ocamb et al. 2002, Gachango et al. 2012)
<i>Fusarium</i>	<i>tricinctum</i>	58%	50%	67%	25%	seed	(Gachango et al. 2012)

Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Hypocrea</i>	<i>jecorina</i>	67%	50%	83%	40%	non-pathogenic	Ogbebor et al. 2014
<i>Inocybe</i>	<i>griseovelata</i>	83%	67%	100%	33%	non-pathogenic	(Bjorbækmo et al. 2010)
<i>Inocybe</i>	<i>phaeoleuca</i>	92%	83%	100%	17%	non-pathogenic	(Bjorbækmo et al. 2010)
<i>Inocybe</i>	<i>splendens</i>	58%	50%	67%	25%	non-pathogenic	(Bjorbækmo et al. 2010)
<i>Kodamea</i>	<i>ohmeri</i>	58%	50%	67%	25%	non-pathogenic	(De Mares et al. 2015)
<i>Meyerozyma</i>	<i>caribbica</i>	33%	17%	50%	67%	non-pathogenic	(Shubin et al. 2014)
<i>Meyerozyma</i>	<i>guilliermondii</i>	50%	50%	50%	0%	non-pathogenic	(Muhovej et al. 1991, Ocamb et al. 2002, Gachango et al. 2012, Shubin et al. 2014)
<i>Neolentinus</i>	<i>lepideus</i>	92%	83%	100%	17%	non-pathogenic	(Thorn et al. 2000, Okamoto et al. 2012)



Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Penicillium</i>	<i>chrysogenum</i>	25%	17%	33%	50%	non-pathogenic	(Sajjad Ur et al. 2012)
<i>Pestalotiopsis</i>	<i>microspora</i>	25%	17%	33%	50%	seed	(Dhingra et al. 2002)
<i>Phylospora</i>	<i>vaccinii</i>	58%	50%	67%	25%		
<i>Phytophthora</i>	<i>cinnamomi</i>	17%	0%	33%	100%	plant	(Polashock et al. 2009)
<i>Phytophthora</i>	<i>nicotianae</i>	33%	17%	50%	67%	plant	(Alvarado-Rosales et al. 2008, Shearer and Crane 2014)
<i>Pilidium</i>	<i>concovum</i>	50%	50%	50%	0%	seed	(Cartwright and Spurr 1998)
<i>Postia</i>	<i>placenta</i>	83%	67%	100%	33%	plant	(Cardin et al. 2009)

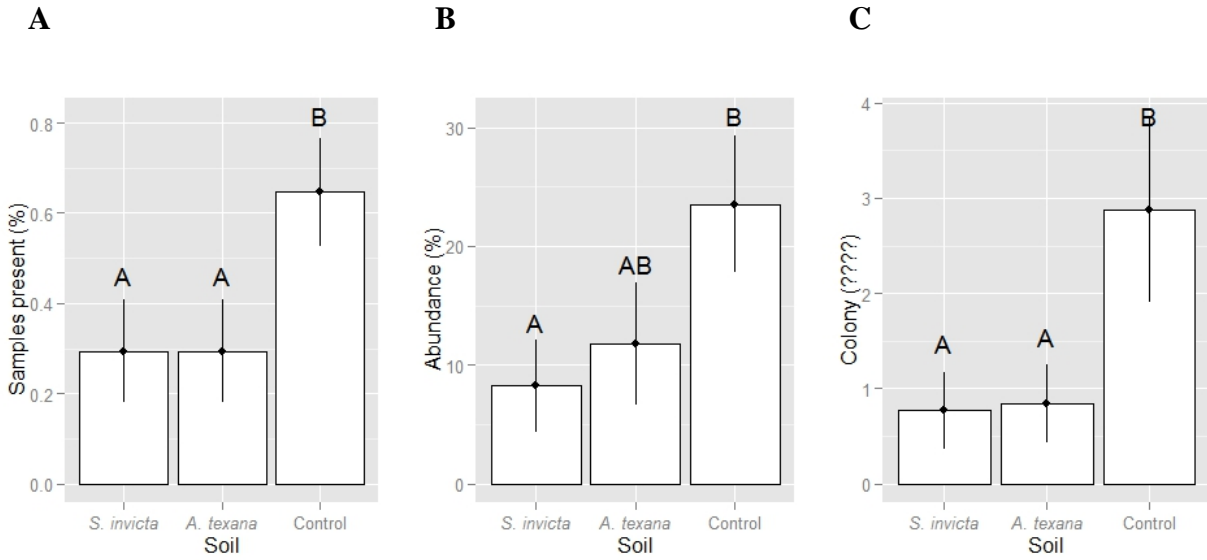
Genus	Species	Frequency in all samples	Ant soil	Control Soil	Percent change	Pathogenicity	Papers
<i>Pythium</i>	<i>amasculinum</i>	92%	83%	100%	17%		
<i>Pythium</i>	<i>aphanidermatum</i>	67%	50%	83%	33%	plant	
<i>Pythium</i>	<i>graminicola</i>	8%	0%	17%	100%	plant	(Khan et al. 2003, Chatterton et al. 2004, Heine et al. 2007, Kavitha and Thomas 2008)
<i>Pythium</i>	<i>oligandrum</i>	75%	50%	100%	50%	seedling	(Hodges and Campbell 1994, Craft and Nelson 1996)
<i>Rhizocybe</i>	<i>vermicularis</i>	25%	33%	17%	-16%		
<i>Rhodotorula</i>	<i>mucilaginoso</i>	83%	67%	100%	33%		
<i>Russula</i>	<i>delica</i>	83%	67%	100%	33%	non-pathogenic	(Zhang et al. 2013)
<i>Saccharomyces</i>	<i>cerevisiae</i>	83%	83%	83%	0%	non-pathogenic	(Yaltirak et al. 2009)
<i>Trichoderma</i>	<i>atroviride</i>	17%	0%	33%	100%	non-pathogenic	(Goncalves et al. 2011)

**Table 5:** The percent of total fungal species found in higher frequencies in the control soil samples, the ant nest soil samples, and the percent found at equal frequencies in both control soils and ant nest soils. Most fungal species, both pathogenic and nonpathogenic, occur more frequently in control soils. Within each species distribution (control, ant, both), the proportion of the species found more often in each sample type that are pathogenic or non-pathogenic is also given. The proportions of species that are plant pathogenic or non-pathogenic is calculated based on the proportion of fungal species occurring at higher frequencies that are either plant pathogenic or non-pathogenic.

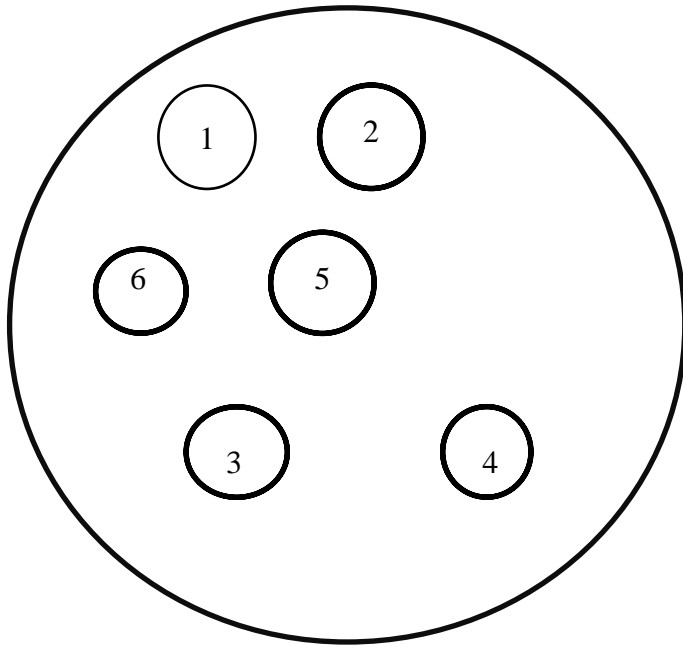
Treatment	Percent of fungal species occurring at higher frequencies (%)	Plant pathogenic (%)	Non-pathogenic (%)
Control	84	53	47
Ant	9	40	60
Both	7	50	50

**Table 6:** The percent of ectomycorrhizal fungi and brown rot fungi occurrence at higher frequencies (%) within sample types. The total occurrence of each fungal type is given followed by the proportion of each type found at higher frequencies in either control soils or ant nests soils, as well as the proportion found at the same frequencies for both control soils and ant nest soils. Percentages of sample frequencies are calculated from the total distribution of either ectomycorrhizal fungi or brown rot fungi within all fungal species identified in the samples.

Treatment	Ectomycorrhizal (%)	Brown Rot (%)
Total distribution of the fungal type within all fungal species identified in the samples	17	9
Proportion occurring more in control samples	67	100
Proportion occurring more in ant samples	22	0
Proportion occurring at same frequencies in both samples	11	0

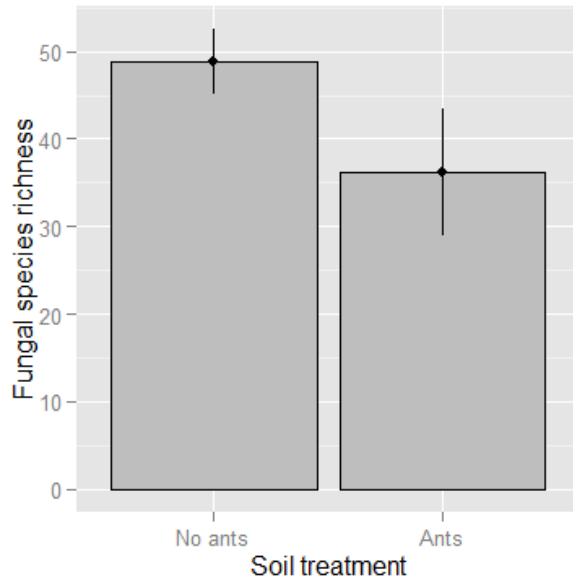


**Figure 1:** Differences in phytopathogenic soil fungi communities in Zettler (2002). A) Frequency of phytopathogenic fungi presence in ant nests soils and control soils, B) abundance of fungi within ant nests soils and control soils, and C) frequency of plant pathogen fungi colonies isolated from soil samples. Analysis was done using two ANOVA tests Letters are assigned based on significant differences between soil types.

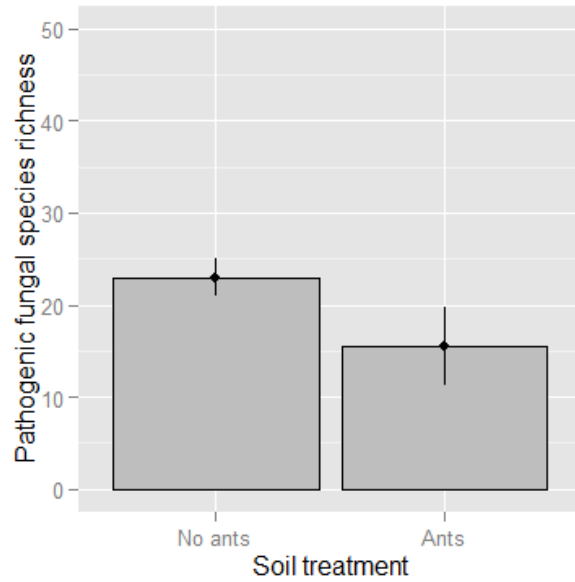


**Figure 2:** Diagram depicting the treatment disc distribution on the agar plates with plant pathogen fungal cultures. Treatments were used to test the effects of ant chemistry on plant pathogen fungal growth on agar plates.

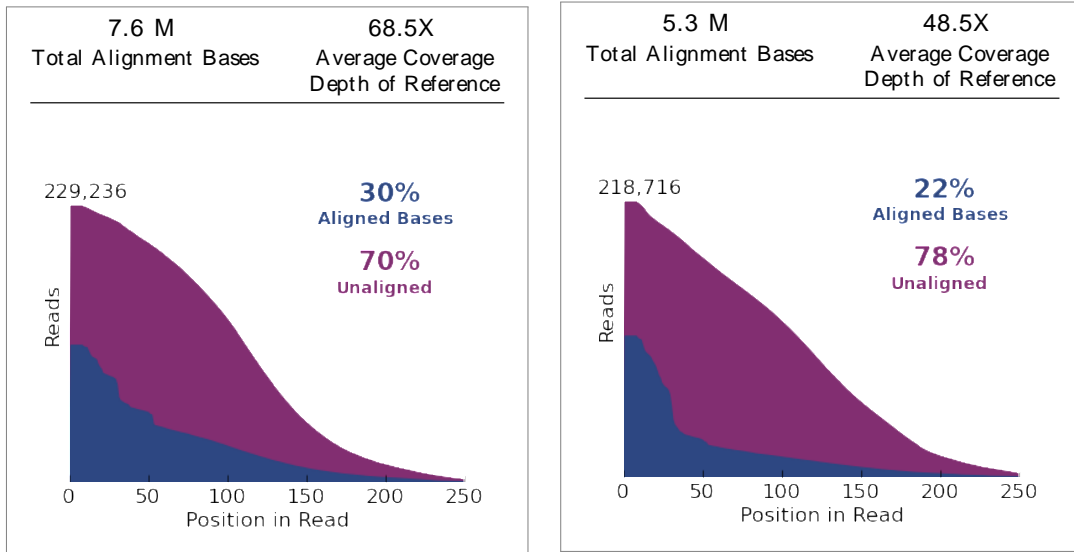
**A.**



**B.**



**Figure 3:** Fungal diversity is lower within ant nests than control soils. A) General fungal diversity in ant and control soils. B) Phytopathogenic fungal diversity in ant and control soils. There was a significant difference between ant soil and control soil fungal diversity for both the A) general fungal community and B) plant pathogen fungal community.



**Figure 4:** The percent of the generated sequences from the soil sample library analysis on the PGM aligned with the digital reference library sequences for A) Chip 1 samples (sample id 3, 4, 7, and 8) and B) Chip 2 samples (sample id 1, 2, 5, 6, 9, 10, 11, and 12).



## **APPENDICES**

## Appendix A: DNA extraction with Axygen Soil and Stool DNA extraction kit

### *Materials:*

Soil sample

Soil and Stool DNA extraction kit:

Water bath set to 95°C

Water bath set at 70°C

70% Ethanol

Ice

1. Add 150 mg glass beads and 0.25g soil sample to a 1.5mL centrifuge tube
2. Add 60uL PTL Buffer to sample
3. Vortex at a max of 13000 xg for 3-5 minutes
4. Add 60µL SL buffer to the sample
5. Vortex
6. Incubate samples at 70°C for 10 min- vortex once during incubation
7. Incubate at 95°C for 2 minutes
8. Centrifuge samples at 13000xg for 3 minutes at room temperature
9. Transfer 400 µL supernatant to a 2mL tube
10. Add 133 µL SPB buffer to the tube
11. Vortex the solution
12. Add 133 mL of IR reagent (shake bottle prior to use- cut pipette tip)
13. Vortex the solution
14. Incubate the samples on ice 5 min
15. Centrifuge the samples at 13000xg at 4°C for 5 minutes
16. Transfer the clear supernatant to 2mL tube
17. Add 50 µL SBW buffer to the tube
18. Add 20 µL MAGA1 particles to the tube
19. Add 5 µL binding enhancer to the tube
20. Mix the sample by pipetting up and down 10 times
21. Incubate samples at room temperature for 5 min
22. Place samples on magnet for 10 minutes
23. Remove and discard the supernatant
24. Remove tube from rack
25. Add 500 µL SBW buffer
26. Re-suspend by pipetting
27. Incubate at room temp for 2 min
28. Place the samples on a magnet for 5 minutes
29. Remove and discard the supernatant (while still on rack)
30. Remove from magnet
31. Add 500 µL of HSW buffer
32. Re-suspend by pipetting
33. Incubate at room temp 2 minutes
34. Place on magnet
35. Remove and discard the supernatant
36. Remove the sample from the magnet

37. Add 500  $\mu\text{L}$  70% ethanol to the tube
38. Re-suspend by pipetting
39. Incubate at room temperature for 2 minutes
40. Place on magnet 5 minutes
41. Remove and discard supernatant
42. Place on a magnet 5 minutes
43. Remove and discard supernatant
44. Dry beads by inverting on paper towel for 10 minutes after 1 minute remove remaining liquid with pipette
45. Add 50-100  $\mu\text{L}$  MB elution buffer (75  $\mu\text{L}$ )
46. Re-suspend by pipetting 20 times
47. Incubate at room temperature for 5 minutes
48. Place on magnet
49. Transfer clear supernatant (DNA) to new 1.5mL tube

### Appendix B: PCR conditions for DNA amplification

PCR conditions for all reactions. For each target group the forward and reverse primers, annealing temperature, MgCl<sub>2</sub>, dNTP, and primer concentrations are given. All PCR reactions were run on either a BioRad C1000 Touch, Techne TC-4000, or MJ Research DNA Engine PTC-200 thermal cycler. Each reaction contained 1 U of *Taq*, 1 X PCR Buffer, and approximately 12ng of template DNA.

Target group	Forward primer	Reverse primer	Annealing temp (°C)	MgCl <sub>2</sub> concentration (mM)	dNTP concentration (μM)	Primer concentration (μM)
Pythium*	Pac1	ITS2	67	2	50	0.1
	Pa1	ITS2	67	2	50	0.1
	Pgr1	ITS2	67	2	50	0.1
	Phy1	ITS2	67	2	50	0.1
	Pa3	ITS2	57	2	50	0.1
Fusarium**	AHyFu-F	AHyFu-R	55	3.5	100	0.1
	Fus-I3-F	FusI3-R	55	3.5	100	0.1
	Fus-I4b-F	Fus-I3-R	55	3.5	100	0.1
All Fungi***	5.8s	ITS1f	53	2	200	0.2
Basidiomycota***	ITS4b	5.8sr	55	2	200	0.2

\*Reaction conditions were 94°C for 2 min followed by 40 cycles of 94°C for 60 s, 67°C for 20 s, and 72°C for 5 s, followed by 72°C for 1 min, and then a hold cycle of 4°C.

\*\* Reaction conditions were 94°C for 2 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by 72°C for 5 min, and then a hold cycle of 4°C.

\*\*\*Reaction conditions were 95°C for 15 min followed by 40 cycles of 95°C for 60 s, 56°C for 30 s, and 72°C for 60 s, followed by 72°C for 5 min, and then a hold cycle of 4°C.

All PCR reactions were run on one of three Thermal Cyclers. Each reaction contained 1 unit of *Taq*, 1x 10mM PCR buffer, and approximately 12ng of template DNA.

## Appendix C: Amplicon purification

### Amplicon purification

(from pg 7 of 'Preparing long amplicon libraries requiring fragmentation using the Ion Xpress Plus Fragment Library kit user Bulletin, MAN0007044 Rev.A., [https://tools.thermofisher.com/content/sfs/manuals/MAN0007044\\_PrepAmpliconLibrFragment\\_using\\_IonXpressPlusFragKit\\_UB.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007044_PrepAmpliconLibrFragment_using_IonXpressPlusFragKit_UB.pdf), accessed 11-4-2015)

#### *Kits and materials needed:*

- Magnetic separation rack
- Agencourt AMPure XP Reagent
- Ion Plus Fragment Library Kit
- Nuclease-free water
- FRESH 70% Ethanol (70  $\mu$ L per amplicon)
- Pipettors with tips
- Timer

#### *Protocol*

1. Resuspend the Agencourt AMPure XP Reagent, and allow the mixture to come to room temperature (~30 min).
2. Prepare FRESH 70% ethanol: 70  $\mu$ L per amplicon.
3. Transfer sample from each PCR tube into a new Eppendorf low-bind tube. Add 90  $\mu$ L Agencourt AMPure XP Reagent to the sample, pipet up and down to thoroughly mix the bead suspension with the DNA and incubate the mixture at room temperature for 5 minutes.
4. Place each well or tube on the magnetic separation rack for 3 minutes or until the solution clears. Remove and discard the supernatant from each well or tube without disturbing the bead pellet.
5. Without removing the samples from the magnet, dispense 500  $\mu$ L of freshly prepared 70% ethanol into each well or tube. Incubate the samples at room temperature for 30 seconds. After the solution clears, remove and discard the supernatant without disturbing the pellet.
6. Repeat step 5 for a second wash.
7. To remove residual ethanol, keep the sample on the magnet and carefully remove any remaining supernatant with a 20 microliter pipette without disturbing the pellet.
8. Keeping the sample on the magnet, air dry the beads at room temperature for 3-5 minutes. **Do not let the pellet dry completely.**
9. Remove the tubes from the magnet, and add 15  $\mu$ L of Nuclease-free water directly to each bead pellet to disperse the beads. **If fragmenting later with Ion Shear Plus Reagents, ONLY use nuclease free water.**

10. Place the plate or tubes on a magnet for at least 1 minute. After the solution clears, transfer the supernatant containing the purified amplicons to a new tube without disturbing the pellet. **The supernatant contains the amplicon DNA. Do not discard.**
11. Optional: Store the DNA at -30 °C to -10 °C.

## Appendix D: TapeStation

### Materials:

Genomic DNA ScreenTape  
Genomic DNA Reagents  
Genomic DNA Ladder  
Genomic DNA Sample Buffer  
Loading tips  
Optical Tube 8x Strip  
Optical Cap 8x Strip  
Vortex mixer  
Volumetric pipette  
Micro- centrifuge

### Prepare Agilent TapeStation

(From Agilent Technologies, manufacturer's manual G2694-90040, [http://www.agilent.com/cs/library/usermanuals/Public/ScreenTape\\_gDNA\\_QG.pdf](http://www.agilent.com/cs/library/usermanuals/Public/ScreenTape_gDNA_QG.pdf), accessed 11-4-2015)

1. Launch the Agilent 2200 TapeStation software.
2. Load High Sensitivity D1000 ScreenTape and loading tips into the 2200 TapeStation.  
Sample Preparation High Sensitivity D1000 ScreenTape Assay
3. Allow reagents to equilibrate at room temperature for 30 min
4. Vortex mix before use
5. If running ladder, add 2  $\mu$ L High Sensitivity D1000 Sample Buffer to 2  $\mu$ L High Sensitivity D1000 Ladder
6. Add 2  $\mu$ L High Sensitivity D1000 Sample Buffer to 2  $\mu$ L DNA sample
7. Vortex using IKA vortexer and adaptor at 2000 rpm for 1 min
8. Spin down to position the sample at the bottom of the tube.

### Sample Analysis:

1. Load samples into the 2200 TapeStation.
2. Select the required samples on the controller software.
3. Click Start and specify a filename with which to save your result

## Appendix E: Quantitate purified samples with Qubit fluorometer

### Materials:

- Qubit™ assay kit
  - Standards 1 and 2
  - Qubit reaction dye
  - Qubit Wash Buffer
  - DNA, RNA, or protein samples
- Qubit® assay tubes or other appropriate 0.5 mL assay tubes
- Optional: Qubit® 2.0 USB drive for data transfer, supplied with the instrument or available separately

*Protocol (from Quick Reference Card,*

[http://www.ebc.uu.se/digitalAssets/176/176882\\_3qubitquickrefcard.pdf](http://www.ebc.uu.se/digitalAssets/176/176882_3qubitquickrefcard.pdf), accessed 11-5-2015)

1. Set up two Assay Tubes for the standards and one tube for each sample.
2. Prepare the Qubit Working Solution by diluting the Qubit reagent 1:200 in Qubit buffer. Prepare 200  $\mu\text{L}$  of **Working Solution** for each standard and sample (1 X n  $\mu\text{L}$  of reagent +199 X n  $\mu\text{L}$  buffer).
3. Prepare the assay tubes according to the table below:

	standard assay tubes	user sample assay tubes
<b>volume of working solution</b>	190 $\mu\text{L}$	180-199 $\mu\text{L}$
<b>volume of standard</b>	10 $\mu\text{L}$	-
<b>volume of user sample</b>	-	1-20 $\mu\text{L}$
<b>total volume in each assay tube</b>	200 $\mu\text{L}$	200 $\mu\text{L}$

4. Vortex all tubes for 2-3 seconds
5. Incubate the tubes for 2 minutes at room temperature
6. Insert standard 1 in the Qubit. Press read. The number displayed is the concentration of the nucleic acid or protein in the **assay tube**.
7. Repeat step 6 for standard 2.
8. Insert sample 1. Press read. The number displayed is the concentration of the nucleic acid or protein in the **assay tube**.
9. Calculate the concentration of the **original sample** by pressing Calculate Stock Conc. The Dilution Calculator screen containing the volume roller wheel is displayed. Using the volume roller wheel, select the volume of your original sample that you have added to the assay tube. Record the value displayed.
10. Use the **Excel ng to nM conversion** sheet stored under the Ion Torrent folder to determine the molar concentration.



## Appendix F: Pool the amplicons

(from pg 10-11 “Prepare Amplicon Libraries without Fragmentation using the Ion Plus Fragment Library Kit user bulletin, MAN0006846, Rev. A.

[https://tools.thermofisher.com/content/sfs/manuals/MAN0006846\\_PrepAmpliconLibr\\_using\\_IonPlusFragLibraryKit\\_UB.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0006846_PrepAmpliconLibr_using_IonPlusFragLibraryKit_UB.pdf), accessed 11-4-2015)

### *Materials:*

- Purified amplicons
- Nuclease free water

### *Protocol*

1. Calculate concentrations of each amplicon using the ng to NM conversion spreadsheet.
2. Separate large and small amplicons based on gel electrophoresis and predicted amplicon sizes.
3. Combine equimolar amounts of each amplicon stock for small and large libraries, keeping samples from different soils separate. If diluting stocks before pooling, use Nuclease free water or 10 mM Tris, pH 7.5-8.5 to prepare the diluted amplicon stocks.
4. Calculate the combined concentration of the pooled amplicons, and convert the concentration of the pooled amplicon stock to ng/ $\mu$ L.
5. Optional stopping point: store pooled amplicon stock at  $-30^{\circ}$  to  $-10^{\circ}$  C. Before use, thaw the amplicon stock on ice. To reduce the number of freeze-thaw cycles, store the amplicon stocks in several aliquots.

## Appendix G: Fragment with Ion Shear™ Plus reagents and purify

(from pg 11-14 in "Prepare Amplicon Libraries Requiring Fragmentation User Bulletin" MAN0007044 Rev. A.

[https://tools.thermofisher.com/content/sfs/manuals/MAN0007044\\_PrepAmpliconLibrFragment\\_using\\_IonXpressPlusFragKit\\_UB.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007044_PrepAmpliconLibrFragment_using_IonXpressPlusFragKit_UB.pdf))

Materials provided in the Ion Xpress™ Plus Fragment Library Kit:

- Ion Shear™ Plus 10X Reaction Buffer
- Ion Shear™ Plus Enzyme Mix II
- Ion Shear™ Plus Stop Buffer
- Low TE

Other materials and equipment:

- Nuclease-free Water
- 1.5-mL Eppendorf LoBind® tubes
- 0.2-ml PCR tubes
- 37 °C heat block/water bath
- P10–P20 and P100–P200 pipettes
- Ice
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack
- (Optional) E. coli DH10B Control DNA or Human CEPH DNA Control

Use large amplicon libraries in Fragmentation (greater than 250 base pairs) (check sizes on QUBIT)

Choose the fragmentation conditions according to the desired library size. The fragmented DNA is ready for adapter ligation. Library sizes for Ion PGM™ System sequencing

\*We adjusted our fragmentation times based on a trial run. All fragmentation times were based on approximately how many shears happened in a given time and how large our starting base pair lengths were. We fragmented all samples between 30 and 40 minutes.

Target Read Length Median Insert Size

400 bases (400-base-read library) ~410 bp

300 bases (300-base-read library) ~320 bp

200 bases (200-base-read library) ~260 bp

100 bases (100-base-read library) ~130 bp

Note: (Optional) Prepare a control sample in a separate tube. Use 1 µL (100 ng) of E. coli DH10B Control DNA for the Ion PGM™ System or 1 µL (100 ng) of Human CEPH DNA Control for the Ion Proton™ System, mixed with 9 µL of Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.

### Fragment the DNA

1. From the mass concentration calculated or determined during the pooling procedure, determine the volume for 100 ng.
2. Vortex the Ion Shear™ Plus 10X Reaction Buffer and the Ion Shear™ Plus Enzyme Mix II each for 5 seconds, pulse-spin to bring the contents to the bottom of the tubes, and place on ice.  
IMPORTANT! Thoroughly mix the Ion Shear™ Plus 10X Reaction Buffer and the Ion Shear™ Plus Enzyme Mix II individually before dispensing them in the next steps.
3. Add the following reagents in the indicated order to a 1.5 mL Eppendorf LoBind® Tube, and mix vigorously by vortexing for 5 seconds. Pulse-spin to bring the contents to the bottom of the tube.  
Note: Do not scale up the reaction volumes or prepare a master mix.

Component	Volume
Pooled amplicons: 100ng	Y $\mu$ L
Ion Shear™ Plus 10X Reaction Buffer	5 $\mu$ L
Nuclease-free Water	35 – $\gamma$
Total	40 $\mu$ L

4. Using a P10–P20 pipettor, add 10  $\mu$ L Ion Shear™ Plus Enzyme Mix II to the sample, for a total volume of 50  $\mu$ L. Proceed immediately to the next step to mix the enzyme mix with the DNA and buffer.
5. Using a P100–P200 pipette set at a 40- $\mu$ L volume, mix the reaction by rapidly pipetting up and down 8–10 times. Do not mix by vortexing and avoid creating bubbles.
6. Incubate the tube(s) in a water bath or heat block at 37°C for the indicated reaction time.
7. Add 5  $\mu$ L of Ion Shear™ Stop Buffer immediately after incubation, and mix thoroughly by vortexing for at least 5 seconds. Store the reaction tube on ice.

### *Purify the fragmented DNA*

Note: Customized for this protocol: Elute large amplicon libraries with end-repaired small libraries of the corresponding soil samples.

IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per sample) for the next steps. A higher percentage of ethanol causes inefficient washing of smaller sized molecules. A lower percentage of ethanol could cause sample loss.

1. Add 99  $\mu$ L of Agencourt® AMPure® XP Reagent (1.8X sample volume) to the sheared DNA sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture at room temperature for 5 minutes.
2. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear of brown tint when viewed at an angle. Carefully remove and discard the supernatant without disturbing the bead pellet.
3. Without removing the tube from the magnet, add 500  $\mu$ L of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20- $\mu$ L pipette without disturbing the pellet.

6. Keeping the tube on the magnet, air-dry the beads at room temperature for 3–5 minutes.
7. Remove the tube from the magnetic rack, and add 25  $\mu\text{L}$  of Low TE directly to the pellet to disperse the beads. Pipet the suspension up and down 5 times, then vortex the sample for 10 seconds to mix.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 0.2- mL PCR tube without disturbing the pellet.  
**IMPORTANT!** The supernatant contains your sample. Do not discard.
9. (Optional) Check the fragment size using 1  $\mu\text{L}$  of eluted DNA and the Agilent® TapeStation Bioanalyzer® (Appendix D)

## Appendix H: End repair and purify the pooled small amplicons

(From pg 11-12 of *Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit User Bulletin*, MAN0006846, Rev. A.  
[https://tools.thermofisher.com/content/sfs/manuals/MAN0006846\\_PrepAmpliconLibr\\_using\\_IonPlusFragLibraryKit\\_UB.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0006846_PrepAmpliconLibr_using_IonPlusFragLibraryKit_UB.pdf), accessed 11-4-2015)

### Materials

- From Ion Plus Fragment Kit:
  - 5X End repair buffer
  - End repair enzyme- orange lid
  - Low TE
- Nuclease free water
- 1.5 mL Eppendorf LoBind tubes
- Agencourt AMPure XP kit
- Magnetic separation rack
- Amplicon pool
- FRESH 70% ethanol
- Timer
- microcentrifuge

### Protocol

#### End repair

1. Use amplicon libraries smaller than 250 bp for end repair. Prepare 10-100 ng of amplicon up to 79 $\mu$ L total volume with Nuclease Free water.
2. Mix by pipetting in a 1.5 mL Eppendorf LoBind tube

Component	Volume
Pooled amplicons	79 $\mu$ L
5X End Repair Buffer	20 $\mu$ L
End repair enzyme	1 $\mu$ L
total	100 $\mu$ L

3. Incubate the end-repair reaction for 20 minutes at room temperature. Prepare 70% ethanol for purification while waiting.

#### Purify

4. Add 180  $\mu$ L of Agencourt AMPure XP Reagent to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.
5. Pulse spin and place the sample tube in the magnetic separation rack for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the pellet.

6. Without removing the tube from the magnet, dispense 500  $\mu\text{L}$  of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
7. Repeat step 6 for a second wash.
8. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20  $\mu\text{L}$  pipettor without disturbing the pellet.
9. Keeping the tube on the magnet, air-dry the beads for 3-5 minutes at room temperature.
10. Remove the tube from the magnet, and add 25  $\mu\text{L}$  of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly
11. Pulse spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, transfer the supernatant containing the eluted DNA to a new 1.5 mL Eppendorf LoBind tube without disturbing the pellet.
12. **Optional stopping point:** Store the DNA at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$ .
13. Proceed to “Fragment with Ion Shear™ Plus reagents and purify.”

## Appendix I: Ligate adaptors, nick repair, and purify

(From pg 14-15 in “Prepare Amplicon Libraries Requiring Fragmentation User Bulletin,” MAN0007044, Rev. A.

[https://tools.thermofisher.com/content/sfs/manuals/MAN0007044\\_PrepAmpliconLibrFragment\\_using\\_IonXpressPlusFragKit\\_UB.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007044_PrepAmpliconLibrFragment_using_IonXpressPlusFragKit_UB.pdf), accessed 11-4-2015)

### Materials

- From Ion Plus Fragment Library Kit:
  - 10X Ligase Buffer
  - Adaptors
  - DNA ligase
  - Nick Repair Polymerase
  - dNTP Mix
  - Low TE
- 0.2 mL PCR tubes
- Thermal cycler
- Nuclease free water
- Agencourt AMPure XP kit
- **Freshly prepared 70% ethanol**
- Magnetic separation rack

### Protocol: Ligate and nick-repair

1. In a 0.2 mL PCR tube, combine the reagents as indicated for barcoded libraries\*, and mix well by pipetting up and down.

Non-barcoded libraries		Barcoded Libraries	
Component	Volume	Component	Volume
DNA	~25 µL	DNA	~25 µL
10X Ligase Buffer	10 µL	10X Ligase Buffer	10 µL
Adapters	2 µL	Ion P1 Adapter	2 µL
		Ion Xpress™ Barcode X <sup>[1]</sup>	2 µL
dNTP Mix	2 µL	dNTP Mix	2 µL
Nuclease-free Water	51 µL	Nuclease-free Water	49 µL
DNA Ligase	2 µL	DNA Ligase	2 µL
Nick Repair Polymerase	8 µL	Nick Repair Polymerase	8 µL
<b>Total</b>	<b>100 µL</b>	<b>Total</b>	<b>100 µL</b>

<sup>[1]</sup> X = barcode chosen.

2. Place the tube in a thermal cycler and run the following program:

<b>Stage</b>	<b>Temperature</b>	<b>Time</b>
Hold	25°C	15 min
Hold	72°C	5 min
Hold	4°C	up to 1 hour

3. Transfer the entire reaction mixture to a 1.5 mL Eppendorf LoBind tube for the next cleanup step.

*Protocol: Purify the adapter-ligated and nick-translated DNA*

*Materials:*

- FRESHLY prepared 70% ethanol
- Low TE from Ion Plus Fragment Library Kit

1. **For 200-300 base read library size:** Add 120\*  $\mu\text{L}$  of Agencourt AMPure XP reagent to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse spin the tube, and incubate the mixture for 5 minutes at room temperature.
2. Pulse spin and place the tube in the magnetic separation rack for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 500  $\mu\text{L}$  of FRESHLY prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20  $\mu\text{L}$  pipette without disturbing the pellet.
6. Keeping the tube on the magnetic rack, air-dry the beads at room temperature for 3-5 minutes.
7. Remove the tube from the magnetic rack, and add 20  $\mu\text{L}$  of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind tube without disturbing the pellet.
9. **Optional stopping point:** Store the DNA at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$ .
10. Proceed to “Size select the unamplified library”\*



Check Quality and sizes using TAPESTATION (Appendix D)

*\* Specific to project design, consult user bulletin for other options.*

## Appendix J: Amplify and purify the library

(from pg 26-28 in “Prepare Amplicon Libraries Requiring Fragmentation User Bulletin, MAN0007044, Rev. A.

[https://tools.thermofisher.com/content/sfs/manuals/MAN0007044\\_PrepAmpliconLibrFragment\\_using\\_IonXpressPlusFragKit\\_UB.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007044_PrepAmpliconLibrFragment_using_IonXpressPlusFragKit_UB.pdf))

*Materials:*

- From Ion Plus Fragment library kit:
  - Platinum PCR SuperMix High Fidelity
  - Library Amplification Primer Mix
- Thermal cycler
- 0.2mL PCR tubes
- Agencourt AMPure XP Kit
- FRESHLY prepared 70% ethanol
- Magnetic separation rack

*Protocol: Amplify the library*

1. Combine the following reagents in a 1.5  $\mu$ L tube and mix by pipetting up and down.

<u>Component</u>	<u>Volume</u>
Platinum PCR SuperMix High Fidelity	100 $\mu$ L
Library Amplification Primer Mix	5 $\mu$ L
Unamplified library	25 $\mu$ L
<b>total</b>	130 $\mu$ L

2. Split the 130  $\mu$ L reaction into two 0.2mL PCR tubes, each containing approximately 65  $\mu$ L.
3. Place the tubes into a thermal cycler and run the following PCR program:

<u>Stage</u>	<u>Step</u>	<u>Temperature</u>	<u>Time</u>
Holding	Denature	95° C	5 min
8 cycles <sup>1</sup>	Denature	95°C	15 sec
	Anneal	58° C	15 sec
	Extend	70°C	1 min
holding		4 °C	hour

4. Combine previously split PCR’s in a new 1.5 mL Eppendorf LoBind tube

*Protocol: Purify the library*

1. Add 195  $\mu\text{L}$  of Agencourt AMPure XP Reagent to each sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture for 5 minutes at room temperature.
2. Pulse-spin and place the tube in a magnetic rack for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 500  $\mu\text{L}$  of FRESHLY prepared 70 % ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20  $\mu\text{L}$  pipettor without disturbing the pellet.
6. Keeping the tube on the magnet, air-dry the beads for 3-5 minutes.
7. Remove the tube from the magnetic rack, and add 20  $\mu\text{L}$  of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5 mL Eppendorf LoBind tube without disturbing the pellet. The supernatant contains the final amplified library. **Do not discard the supernatant.**
9. To remove residual beads from the eluted DNA, place the tube with the eluted DNA back on the magnetic rack for at least 1 minute, and transfer the supernatant to a new 1.5 Eppendorf LoBind Tube without disturbing the pellet.
10. Proceed to “Qualify barcoded libraries.”\*

\* Specific to project. For different projects, consult user bulletin.

Appendix D: Assess the quality of the library using TAPESTATION

OR

Appendix E: Qubit® 2.0 Fluorometer:

Example from pg 31:

- The library concentration is 450 ng/mL. The dilution factor is 450 ng/mL divided by 15 ng/mL = 30.
- Therefore, 10  $\mu\text{L}$  of library mixed with 290  $\mu\text{L}$  of Low TE (1:30 dilution) yields approximately 15 ng/mL (~100 pM)

This concentration is suitable for downstream preparation. \*NOTE\* Because the Qubit quantitation is not as precise as qPCR, when you perform the template preparation procedure, you will need to prepare 3 serial dilutions of the library at 0.5X dilution (~50pM), 1X dilution

(100pM), and 2X dilution (200pM) to ensure that one or more dilutions are in the optimal concentration range. The libraries are ready for downstream template preparation.

## Appendix K: Prepare Template Positive Ion PGM OT2 200 ISP's using the Ion One Touch

### 2

*Protocol: Set up the Ion One Touch 2 Instrument*

(from pg 17-24 in "Ion PGM Template OT2 User Guide", MAN0007220, Rev. A.

[https://tools.thermofisher.com/content/sfs/manuals/MAN0007220\\_Ion\\_PGM\\_Template\\_OT2\\_200\\_Kit\\_UG.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007220_Ion_PGM_Template_OT2_200_Kit_UG.pdf), accessed 11-4-2015)

\*Note: This step can be done ahead of time, before reactions are scheduled to take place.

*Materials:*

- From Ion PGM Template OT2 Supplies kit:
  - 2 Ion OneTouch Reagent Tubes
  - Ion OneTouch Recovery Router
  - 2 Ion OneTouch Recovery tubes
  - Ion OneTouch 2 Amplification Plate
  - 2 Ion OneTouch Sipper Tubes
- From Ion PGM Template OT2 Solutions Kit:
  - Ion OneTouch Oil
  - Ion PGM OT2 Recovery Solution

*Protocol*

1. On the instrument display, touch **Open Lid**, wait until the lid clicks open, then lift and hold the side of the centrifuge lid. **Do not lift by the tubing attached to the injector hub. Do not force the lid open.**
2. Insert a Ion OneTouch Recovery Tube into each of the slots in the centrifuge.
3. Pinch the sides of the Ion OneTouch Recovery Router then push the Recovery Router down into the center slot of the centrifuge so that the Recovery Router is seated flat and secure in the center of the rotor. Note: the Recovery Router is intentionally offset from the Recovery Tubes.
4. Close the lid of the centrifuge.

*Install the Ion OneTouch Amplification Plate using the following procedure:*

5. If there is a used Ion OneTouch 2 Cleaning Adapter on the instrument (located at the top of the instrument, round disc with 3 ports), remove and appropriately discard it.
6. Push the handle back to open the heat block. **HOT SURFACE, USE CAUTION. DO NOT USE FLAMABLE MATERIALS NEAR THIS INSTRUMENT.**
7. Obtain an Ion OneTouch 2 Amplification Plate. The disposable tubing and disposable injector are attached to the Amplification Plate. An Amplification Plate supplied in the Ion PGM Template OT2 200 Kit has "ion" written on the tab.
8. Inspect the plate port of the Amplification Plate to ensure that is straight and perpendicular to the plate. Do not disconnect tubing from the top of the plate port.

9. Insert the Amplification plate:
  - a. Hold the disposable injector , connected to the disposable tubing, in one hand and the Amplification Plate in the other hand. CAUTION: The pointed end of the injector can puncture your skin. Keep your hand away from the point of the disposable injector.
  - b. Insert the Amplification Plate into the heat block so that the single plate port aligns with the left hole of the Ion OneTouch 2 Instrument.
  - c. Pull the handle of the heat block to close the block.
  - d. Thread the disposable tubing through the Ion OneTouch DL Tubing catch (small, blue plastic arm on the shelf below the handle).
10. Install the disposable tubing in the pinch valve (metal cylinder protruding from the left side of the instrument next to the solution port).
  - a. Align the disposable tubing with the slot that runs along the bottom of the pinch valve.
  - b. Gently pull the disposable tubing upwards on both sides of the pinch valve until the disposable tubing is in the slot and secured in the round notch on each side of the pinch valve.
  - c. If necessary, adjust the disposable tubing along the notches of the open pinch valve so there is sufficient length of disposable tuning to install the disposable injector.

*Install the disposable injector using the following procedure:*

11. Before use, inspect the long metal shaft of the disposable injector. Some disposable injectors may be slightly bent, which is normal.
12. Place one hand on the centrifuge lid. Place the other hand at the top of the disposable injector , and insert the disposable injector straight into to port of the Ion OneTouch DL Injector Hub.
13. Keep your hand on the centrifuge lid, then push the disposable injector through the port until the disposable injector just stops at the base of the router.
14. Release the disposable injector.
15. Confirm automatic placement of the disposable injector above the router. Briefly press then release the spring loaded top of the Injector Hub. You should hear a click. If the Injector Hub remains in the down position, see Troubleshooting on page 59 of *Ion PGM Template OT2 User Guide*. If you raise the centrifuge lid, DO NOT hit the disposable injector against the instrument. You could damage the disposable injector. IF you damage the disposable injector, appropriately dispose of the injector, amplification plate, and tubing. Use a new disposable injector and Ion OneTouch 2 Amplification Plate.

*Install the Ion One Touch Oil*

16. If needed: Fill the appropriate Ion OneTouch Reagent Tube with Ion OneTouch Oil on the left front port.

- a. When refilling the Reagent between runs:
    - i. Remove the Reagent Tube from the instrument.
    - ii. Invert Ion OneTouch Oil bottle 3 times to mix.
    - iii. Fill the Reagent Tube half-full with Oil. Minimize bubbles.
    - iv. Insert the filled Reagent Tube into the left front port, and screw the Reagent Tube firmly into place, one-quarter turn on the instrument.
  - b. When using a new Template kit:
    - i. Use a new Reagent Tube from the kit
    - ii. Discard the used Reagent Tube and sipper Tube. Appropriately discard the residual Oil.
    - iii. Use fresh gloves to attach the Luer-Lock end of a new Ion OneTouch Sipper Tube to the left front port. Do not let the sipper tube touch any surface.
    - iv. Invert the Ion OneTouch Oil bottle 3 times to mix, then fill the Reagent tube half full with oil. Minimize bubbles.
    - v. Insert the filled Reagent Tube into the left front port, and screw the Reagent Tube firmly into place, one quarter turn on the instrument.
17. Ensure that the Recovery Solution is clear. If it is clear, move on to the next step. If the Recovery Solution is not clear, heat the bottle in a 30 °C bath until the Recovery Solution is clear.
18. Fill the appropriate Ion OneTouch Recovery Solution on the right front port.
- a. When refilling between runs:
    - i. Remove the Reagent Tube from the instrument.
    - ii. Invert the bottle of Recovery Solution 3 times.
    - iii. Add more Recovery Solution to the solution in the Reagent Tube until the tube is a quarter full. Minimize the bubbles.
    - iv. Insert the filled Reagent Tube into the right front port, and screw the Reagent Tube into the right front port and screw the Reagent Tube firmly into place, one-quarter turn on the instrument.
  - b. When using a new Template kit:
    - i. Use a new Reagent Tube from the kit.
    - ii. Discard the used Reagent Tube and Sipper Tube. Appropriately discard the Recovery Solution.
    - iii. Use fresh gloves to attach the Luer Lok end of a new Ion One Touch Sipper Tube to the right front port, do not let the sipper tube touch any surfaces
    - iv. Invert the Recovery Solution bottle 3 times, then fill the Reagent Reagent Tube a quarter full with Recovery Solution. Minimize bubbles.
    - v. Insert the filled Reagent Tube into the right front port, and screw the Reagent Tube firmly into place, one-quarter turn on the instrument.

19. Empty the waste container.
  - a. Pull the external tubing from the port of the Waste Container.
  - b. Empty the Waste Container into the appropriate receptacle
  - c. Push the tubing onto the port of the empty Waste Container.

Prepare and install the amplification solution

(p 25-32 from *Ion PGM Template OT2 User Guide*)

Materials:

- From Ion PGM Template OT2 Solutions 200 Kit (cabinet above OneTouch, Yellow label)
  - Ion PGM Template OT2 200 PCR Reagent B (blue lid)
  - Ion OneTouch Reaction Oil (small bottle)
  - Nuclease Free Water
- From Ion PGM Template OT2 Reagents 200 kit (Freezer, -30°C to -10°C, yellow label)
  - Ion PGM Template OT2 200 Reagent Mix (purple lid)
  - Ion PGM Template OT2 200 Enzyme mix (brown lid)
  - Ion PGM Template OT2 200 Ion Sphere Particles (black lid)
- From Ion PGM Template OT2 Reactions 200 kit
  - Ion PGM One Touch Plus Reaction Filter Assembly
- Provided in the Ion Control Materials 200 kit (freezer, red label)
  - *E. coli* DH10B Control 200 Library (red lid)
- Diluted library
- Microcentrifuge
- 1.5 mL Eppendorf LoBind Tubes (cabinet above OneTouch)
- Tube rack for 15 mL conical tubes
- Pipettes
- Vortexer
- Heat Block set at 75°C
- Ice

\*Remove Ion PGM Template OT2 Reagents 200 kit from the freezer. Place Enzyme mix on ice, allow Reagent mix to reach room temperature.

*Prepare the amplification solution*

1. Prepare the reagents:
  - a. Ion PGM Template OT2 200 Reagent Mix (purple lid)
    - i. Allow the reagent mix to come to room temperature before use.
    - ii. Vortex the solution for 30 seconds, then centrifuge the solution for 2 seconds. Visually inspect the solution at the bottom of the tube and verify that there is no residual precipitate. If the precipitate is visible, then see Troubleshooting on page 61 of *Ion PGM Template OT2 User Guide*.



- iii. Keep the thawed reagent mix at room temperature during use. Store the thawed reagent mix at 2-8°C.
  - b. Ion PGM Template OT2 200 PCR Reagent B (blue lid)
    - i. Vortex the reagent for 1 minute, then centrifuge the solution for 2 seconds.
    - ii. Inspect the reagent:
      - 1. If the solution is clear, then prepare the amplification solution. Keep Reagent B at room temperature.
      - 2. If the solution is cloudy or has crystals or has been accidentally stored at 2-8°C, heat the reagent for 1 minute in a heat block set at 75°C. Vortex the reagent for 1 minute, then centrifuge the solution for 2 seconds.
    - iii. Inspect the reagent. If the reagent is:
      - 1. Cloudy or has crystals, repeat steps 1-2 until the reagent is clear, then equilibrate the reagent to room temperature and prepare the amplification solution.
      - 2. Clear: Equilibrate the reagent to room temperature, then prepare the amplification solution. Store Reagent B at room temperature. Do not use the reagent if it is cloudy or has crystals.
  - c. Ion PGM Template OT2 200 Enzyme Mix (brown lid)
    - i. Centrifuge the enzyme for 2 seconds.
    - ii. Place on ice.
  - d. Ion PGM Template OT2 200 Ion Sphere Particles (black lid)
    - i. Place the suspension at room temperature. **IMPORTANT:** Use ONLY Ion PGM Template OT2 200 Ion Sphere Particles in the Ion OneTouch 2 kits with the Ion OneTouch 2 system. Do not use ISP's from other or previously used kits.
- 2. Dilute 2µL of the 100 pM library in 23 µL of Nuclease Free Water for a total volume of 25 µL. Vortex the diluted library for 5 seconds, then centrifuge for 2 seconds. Place the diluted library on ice.
- 3. In a 1.5 mL Eppendorf LoBind Tube at 15-30°C, add the following components in the designated order: (amplification solution)
  - a. Nuclease Free water, 25 µL
  - b. Ion PGM Template OT2 200 Reagent Mix, 500 µL (purple lid)
  - c. Ion PGM Template OT2 200 PCR Reagent B, 300 µL (blue lid)
  - d. Ion PGM Template OT2 200 Enzyme Mix, 50 µL (brown lid)
  - e. Diluted library, 25 µL
- 4. Vortex the solution prepared in step 3 at maximum speed for 5 seconds, then centrifuge the solution for 2 seconds.
- 5. Prepare the Ion PGM Template Sphere Particles (ISP's) (black lid) from Ion PGM Template OT2 Reagents 200 kit

- a. Vortex the Ion PGM Template OT2 200 Ion Sphere Particles at maximum speed for 1 minute to resuspend the particles.
- b. Centrifuge the ISPs for 2 seconds.
- c. Pipet the Ion PGM Template OT2 200 Ion Sphere Particles up and down to mix, then immediately add 100  $\mu\text{L}$  to the amplification solution
6. Vortex the complete amplification solution prepared in step 5 at maximum speed for 5 seconds.
7. Proceed immediately to “Fill the Ion PGM OneTouch Plus Reaction Filter Assembly”

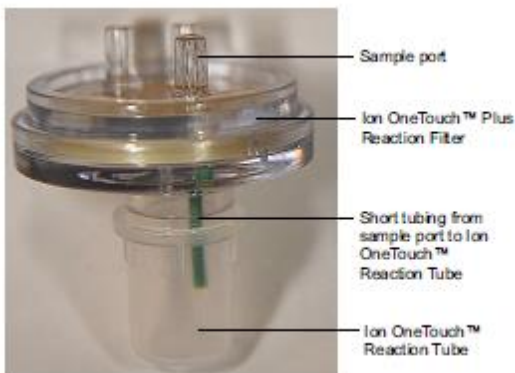
Fill and install the Ion PGM™ OneTouch Plus Reaction Filter Assembly on the Ion OneTouch™ 2 Instrument (pg

*Materials*

- From Ion PGM Template OT2 200 Reactions Kit (smaller box in cabinet above OneTouch, yellow label)
  - Ion PGM™ OneTouch Plus Reaction Filter Assembly
- 20 mL tube rack
- Pipettor, 1000  $\mu\text{L}$
- Amplification solution prepared from above

**IMPORTANT!** We recommend filling the Ion PGM™ OneTouch Plus Reaction Tube Assembly in a room dedicated to pre-PCR activities or a controlled pre-PCR hood. Do not use a reaction filter assembly from any other template preparation kit.

1. Obtain an Ion PGM™ OneTouch Plus Reaction Filter Assembly from the Ion PGM™ Template OT2 200 Kit.
2. Place the Ion PGM™ OneTouch Plus Reaction Filter Assembly into a tube rack so that the 3 ports of the Plus Reaction Filter face up.
3. Locate the sample port on the Ion PGM™ OneTouch Plus Reaction Filter Assembly. The short tubing in the Reaction Tube is connected to the sample port:



4. Add the amplification solution through the sample port:
  - a. Set a P1000 pipette to 1000  $\mu\text{L}$ , and attach a new 1000  $\mu\text{L}$  tip to the pipette.
  - b. Vortex the amplification solution at maximum speed for a full 5 seconds, then centrifuge the solution for 2 seconds. Immediately proceed to the next step.

- c. Pipet the amplification solution up and down to mix, then fill the tip with 1000  $\mu\text{L}$  of the amplification solution.
  - d. Insert the tip firmly into the sample port so that the tip is perpendicular to the Ion PGM™ OneTouch Plus Reaction Filter Assembly and fully inserted into the sample port to form a tight seal.
  - e. Slowly pipet the entire 1000  $\mu\text{L}$  of the amplification solution through the sample port, then keep the plunger of the pipette depressed.
  - f. With the plunger still depressed, remove the tip from the sample port, then appropriately discard the tip.
  - g. If necessary, gently dab a Kimwipes® disposable wipe around the ports to remove any liquid.
5. Add Ion OneTouch™ Reaction Oil (27-mL bottle) through the sample port:
- a. Set a P1000 pipette to 1000  $\mu\text{L}$ , and attach a new 1000  $\mu\text{L}$  tip to the pipette.
  - b. Fill the tip with 1000  $\mu\text{L}$  of Reaction Oil.
  - c. Insert the tip firmly into the sample port so that the tip is perpendicular to the Ion PGM™ OneTouch Plus Reaction Filter Assembly and fully inserted into the sample port to form a tight seal.
  - d. Slowly pipet 1000  $\mu\text{L}$  of the Reaction Oil through the sample port. Keep the plunger of the pipette depressed to avoid aspirating solution from the Ion PGM™ OneTouch Plus Reaction Filter Assembly.
  - e. With the plunger still depressed, remove the tip from the sample port, then appropriately discard the tip.
  - f. Set the P1000 pipette to 500  $\mu\text{L}$ , and attach a new 1000  $\mu\text{L}$  tip to the pipette.
  - g. Fill the tip with 500  $\mu\text{L}$  of Reaction Oil.
  - h. Insert the tip firmly into the sample port so that the tip is perpendicular to the Ion PGM™ OneTouch Plus Reaction Filter Assembly and fully inserted into the sample port to form a tight seal.
  - i. Slowly pipet 500  $\mu\text{L}$  of the Reaction Oil through the sample port, then keep the plunger of the pipette depressed.
  - j. With the plunger still depressed, remove the tip from the sample port, then appropriately discard the tip.
  - k. If necessary, gently dab a Kimwipes® disposable wipe around the ports to remove any liquid.
6. Invert the Ion PGM™ OneTouch Plus Reaction Filter Assembly:
- a. Keep the Ion PGM™ OneTouch Plus Reaction Filter Assembly in the tube rack, and orient the assembly so that the sample port is on your left:
- IMPORTANT!** Correct rotation of the Ion PGM™ OneTouch Plus Reaction Filter Assembly in the next step ensures minimal exposure of the short tubing in the Reaction Tube with the aqueous phase.
- b. Lift straight out the Ion PGM™ OneTouch Plus Reaction Filter Assembly from the tube rack. With the short tubing in the Reaction Tube on the left, rotate the assembly to your right until the Reaction Tube is inverted and the 3 ports of the Reaction Filter face down: (pic pg 35)
7. Firmly insert the 3 ports of the Ion PGM™ OneTouch Plus Reaction Filter Assembly into the three holes on the top stage of the Ion OneTouch™ 2 Instrument. The tab protruding from the

outer edge of the Ion PGM™ OneTouch Plus Reaction Filter Assembly fits into the front notch of the stage.

Run the Ion OneTouch™ 2 Instrument (pg 32-35)

1. Ensure that the centrifuge lid of the Ion OneTouch™ 2 Instrument is closed.
2. On the home screen, touch Run.
3. Touch the drop-down menu, then select PGM: Ion PGM™ Template OT2 200 Kit.
4. Touch Next.
5. Touch Assisted or Expert. Complete the listed tasks, if listed, then touch Next. The run begins. To cancel a run, touch Abort, then touch Yes.
6. Remove the samples  $\leq 16$  hours after **starting** the run.

Recover the Ion PGM™ Template OT2 200 Ion Sphere™ Particles

(pg 36-38)

*Materials*

- From the Ion PGM Template OT2 200 Solutions kit (Cabinet above OneTouch, yellow label)
  - Ion OneTouch Wash Solution
- 1.5 mL Eppendorf LoBind Tubes
- Pipettes
- Vortexer
- Microcentrifuge

1. At the end of the run, follow the screen prompts to centrifuge the sample.

Note: If you removed the Reaction Tubes at the end of the run before the Ion OneTouch™ 2 Instrument had spun the sample or have not processed the sample within 15 minutes, centrifuge the sample on the instrument by touching Options on the home screen, then Final Spin, then follow the screen prompts (touch Next on the next 2 screens) until the centrifugation begins. Centrifugation of the samples takes 10 minutes.

**CAUTION! ROTATION HAZARD.** Wait until rotation stops before opening. Rotating parts can cause injury.

2. Immediately after the centrifuge stops, touch Open Lid, wait until the lid opens, then remove and discard the Recovery Router.

3. Carefully remove both Recovery Tubes, and put the tubes in a tube rack. You may see some cloudiness, which is normal.

4. Remove excess Ion PGM™ OT2 Recovery Solution from the Ion PGM™ Template OT2 200 Ion Sphere™ Particles:

- a. Use a pipette to remove all but 50  $\mu\text{L}$  of the Recovery Solution from each Recovery Tube. Withdraw the supernatant from the surface and on the opposite side from the pellet. Remove any white flocculent material. Do not disturb the pellet of Ion PGM™ Template OT2 200 Ion Sphere™ Particles:



b. With a new tip and using the same tip for both tubes, resuspend the Ion PGM™ Template OT2 200 Ion Sphere™ Particles in the remaining Ion PGM™ OT2 Recovery Solution. Pipet the pellet up and down until each pellet disperses in the solution.

#### OPTIONAL STOPPING POINT :

Transfer the suspensions from both tubes into a new 1.5 mL Eppendorf LoBind® Tube for a total of 100 µL of suspension of Ion PGM™ Template OT2 200 Ion Sphere™ Particles. Add 1 mL of Ion OneTouch™ Wash Solution to the 100 µL suspension. Store the Ion PGM™ Template OT2 200 Ion Sphere™ Particles at 2°C to 8°C (**fridge**) for up to 3 days.

If the template positive ISPs were stored at 2°C to 8°C, centrifuge the ISPs at  $15,500 \times g$  (or *rcf*) for 2.5 minutes, then carefully remove all but 100 µL of supernatant. With a new tip, pipet the solution up and down to resuspend the Ion PGM™ Template OT2 200 Ion Sphere™ Particles. Proceed to step 5.

**IMPORTANT!** Do *not* store the recovered Ion PGM™ Template OT2 200 Ion Sphere™ Particles in Ion PGM™ OT2 Recovery Solution and do *not* store the recovered ISPs at -30°C to -10°C.

5. Obtain an 8-well strip from the Ion OneTouch™ ES Supplies Kit (Cabinet above One Touch). Ensure that the square-shaped tab of an 8-well strip is on the *left*:

6. Transfer the suspensions from both tubes (1 tube if stored) into Well 1 of the 8-well strip for a total of 100 µL of suspension of Ion PGM™ Template OT2 200 Ion Sphere™ Particles in the well.

*Ion PGM™ Template OT2 200 Kit Quick Reference 9*

**NOTE: Before enriching template positive Ion Sphere Particles (recovered from OneTouch) RETAIN 2µL UNENRICHED ISP's to be assessed on Qubit**

Enrich the template-positive Ion PGM™ Template OT2 200 Ion Sphere™ Particles (ISPs) with the Ion OneTouch™ ES (pg 44-51)

#### Materials:

- 1 M NaOH, prepared ahead of time
- Tween Solution (from Ion

Provided in the Ion PGM™ Template OT2 Solutions 200 Kit

- Ion OneTouch™ Wash Solution
- MyOne™ Beads Wash Solution
- Tween® Solution
- Neutralization Solution

- Nuclease-Free Water

Provided in the Ion PGM™ Template OT2 Supplies 200 Kit

- 8-well strip
- Eppendorf® LoRetention Dualfilter Tips (P300)

Provided in the Ion PGM™ Enrichment Beads (Cat no. 4478525)

- Dynabeads® MyOne™ Streptavidin C1 Beads

Prepare fresh Melt-Off Solution by combining in this order:

Order	Component	Volume
1	Tween Solution	280 µL
2	1M NaOH	40 µL

**IMPORTANT!** Prepare Melt-Off Solution as needed, but appropriately dispose of the solution after 1 day.

*Wash and resuspend the Dynabeads® MyOne™ Streptavidin C1 Beads*

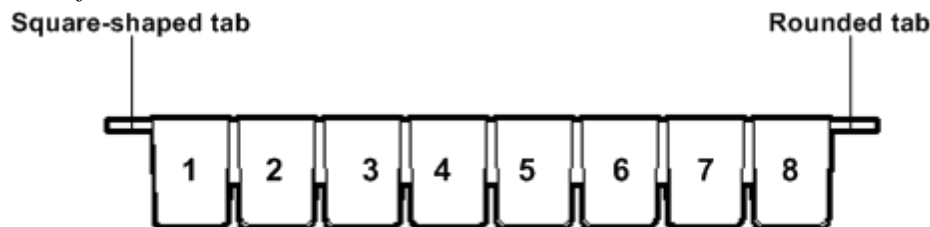
1. Vortex the tube containing the Dynabeads® MyOne™ Streptavidin C1 Beads for 30 seconds to thoroughly resuspend the beads, then centrifuge the tube for 2 seconds.
2. Open the tube, then use a new tip to pipet up and down the dark pellet of beads until the pellet disperses. *Immediately* proceed to the next step.
3. Transfer 13 µL of Dynabeads® MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind® Tube.
4. Place the tube on a magnetic separation rack (gray box, cabinet above the OneTouch) for 2 minutes, then *carefully* remove and discard the supernatant without disturbing the pellet of Dynabeads® MyOne™ Streptavidin C1 Beads.
5. Add 130 µL of MyOne™ Beads Wash Solution to the Dynabeads® MyOne™ Streptavidin C1 Beads.

Note: You add the resuspended Dynabeads® MyOne™ Streptavidin C1 Beads in the 130 µL MyOne™ Beads Wash Solution to Well 2 of the 8-well strip in step 3 of this procedure.

6. Remove the tube from the magnet, vortex the tube for 30 seconds, and centrifuge the tube for 2 seconds.

*Fill the 8-well strip*

1. Ensure that the template-positive ISPs from the Ion OneTouch™ 2 Instrument are in 100 µL of Ion PGM™ OT2 Recovery Solution and are in Well 1 of the 8-well strip (see “Recover the Ion PGM™ Template OT2 200 Ion Sphere™ Particles” on page 7). Well 1 with the ISPs is on the *left*:



Note: If the template-positive ISPs were stored at 2°C to 8°C, centrifuge the ISPs at 15,500 × g for 2.5 minutes, then carefully remove all but 100 µL of supernatant. With a new tip, pipet the

solution up and down to resuspend the Ion PGM™ Template OT2 200 Ion Sphere™ Particles. Transfer the suspension from the tube into Well 1 of the 8-well strip.

2. If you have not done so already, assess the quality of the unenriched, template-positive Ion PGM™ Template OT2 200 Ion Sphere™ Particles using the Qubit 2.0 Fluorometer.

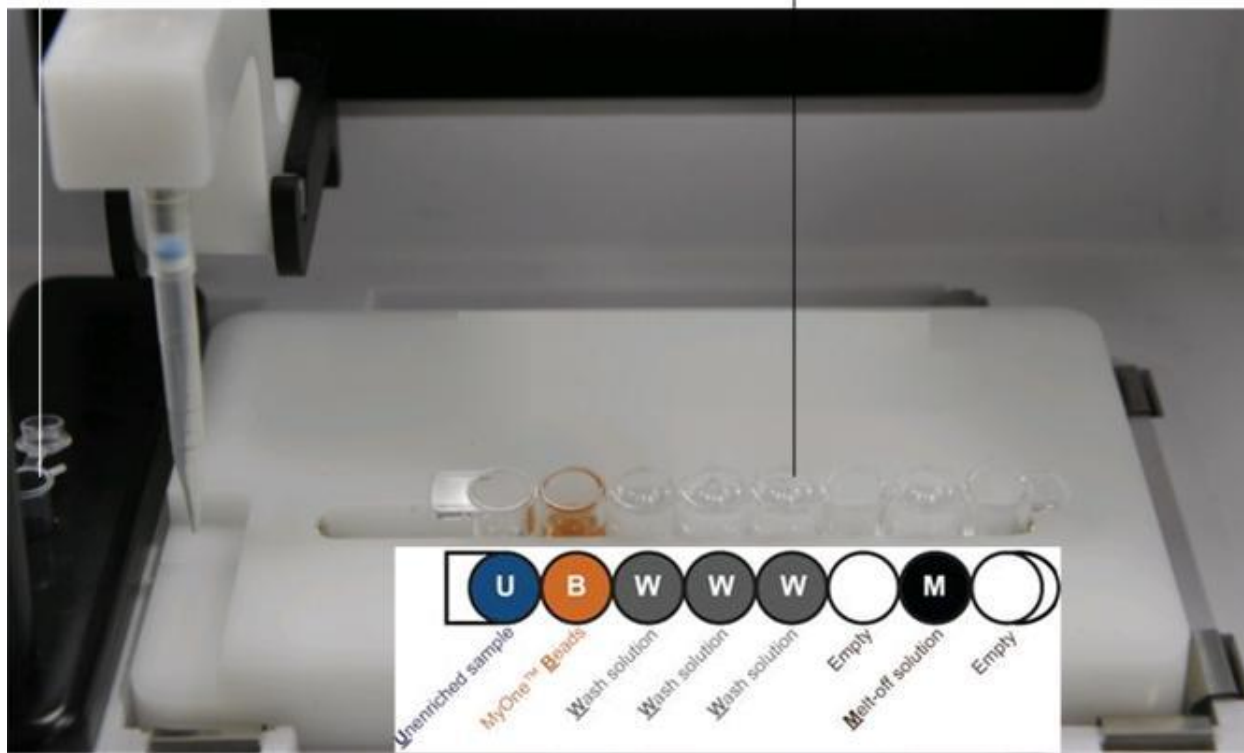
3. Fill the remaining wells as follows, then immediately proceed to step 4 (see the figure following step 4):

Well number	Reagent to dispense in well
1	Entire template-positive ISP sample (100 µL prepared in step 1 of this procedure)
2	130 µL of Dynabeads® MyOne™ Streptavidin C1 Beads resuspended in MyOne™ Beads Wash Solution
3	300 µL of Ion OneTouch™ Wash Solution
4	300 µL of Ion OneTouch™ Wash Solution
5	300 µL of Ion OneTouch™ Wash Solution
6	Empty
7	300 µL of freshly-prepared Melt-Off Solution
8	Empty

4. Confirm that the square-shaped tab is on the left, then insert the filled 8-well strip with the 8-well strip pushed all the way to the right end of the slot of the Tray:

Install 0.2-mL PCR tube  
in hole of Tip Loader

Install 8-well strip with square-shaped tab on the left and strip  
pushed to the right end of the slot



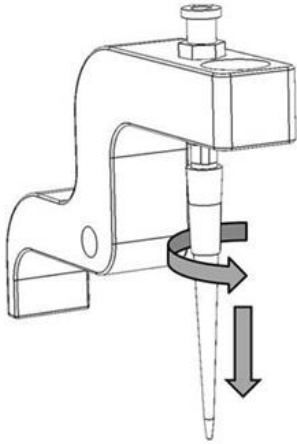
*Prepare the Ion OneTouch™ ES*

1. Load a new tip in the Tip Arm:
  - a. Place a new tip in the Tip Loader: Remove the Tip Arm from the cradle and align the metal fitting of the Tip Arm with the tip. Keeping the fitting on the Tip Arm vertical, firmly press the Tip Arm down onto the new tip until the Tip Arm meets the Tip Loader. Hold the Tip Arm to the Tip Loader for ~1 second to ensure proper installation of the tip. Lift the Tip Arm straight up to pull the installed tip from the Tip Loader tube.
  - b. Return the Tip Arm to the cradle: Tilt the Tip Arm back. Align the pins with the round notches in the cradle, then lower the Tip Arm into position. Rock the Tip Arm forward into the working position.
2. Ensure that the back/bottom end of the Tip Arm is not resting on top of the thumb screw, causing the Tip Arm to tilt forward.
3. Add 10  $\mu$ L of Neutralization Solution to a new 0.2-mL PCR tube.
4. ***Insert the opened 0.2-mL PCR tube with the Neutralization Solution into the hole in the base of the Tip Loader, as shown in the preceding photograph.***

*Perform the run*

1. Confirm that a new tip and opened 0.2-mL PCR tube with the Neutralization Solution have been loaded and that the 8-well strip is correctly loaded. Ensure that Well 1 (ISP sample) is the left-most well and that the 8-well strip is pushed to the far right position within the slot.
2. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run. Do not introduce bubbles into the solution.
3. If necessary, turn ON the Ion OneTouch™ ES and wait for the instrument to initialize: The screen displays “rdy”. The Tip Arm performs a series of initialization movements and returns to the home position (~5 seconds).
4. Press **Start/Stop**. The screen displays “run” during the run. The run takes ~35 minutes.  
Note: If necessary to stop a run, press **Start/Stop**. The instrument completes the current step, then stops the run and displays “End”. Press **Start/Stop** again to return the Tip Arm to the home position. It is not possible to restart (where you left off) after stopping a run.
5. At the end of the run, the instrument displays “End” and beeps every 60 seconds. Press the **Start/Stop** button to silence this alarm and reset the Ion OneTouch™ ES for the next run. The instrument can be left on between runs.
6. ***Immediately after the run***, securely close and remove the PCR tube containing the enriched ISPs.
7. Mix the contents of the PCR tube by gently inverting the tube 5 times.  
Note: Ensure that the 0.2-mL PCR tube has >200  $\mu$ L of solution containing the enriched ISPs. After a successful run on the instrument, the sample is in ~230  $\mu$ L of Melt-Off Solution, Ion OneTouch™ Wash Solution, and Neutralization Solution. If the tube has <200  $\mu$ L of solution containing the enriched ISPs, contact Life Technologies Technical Support.
8. Remove the used tip: While you are standing above the Tip Arm, and with the Tip Arm in its cradle, twist the tip *counterclockwise* and pull it downward to remove and discard the tip:





Assess the quality of the unenriched, template-positive Ion PGM™ Template OT2 200 Ion Sphere™ Particles using the Qubit2.0 Fluorometer (pg 66-72):

NOTE: This step can be performed while the ES is running or while the PGM is running. This is for quality control. If concerned about the quality and you are reluctant to run the PGM until you know the recovery of ISP's went well, perform this step before running the PGM.

Materials:

- Qubit 2.0 Fluorometer
- Qubit Assay Tubes
- PCR Tubes
- Qubit Easy Calculator Microsoft Excel Spreadsheet (saved under Ion Torrent).
- Ion Sphere Quality Control Kit (freezer, white label)
- Unenriched Ion PGM Template OT2 200 Ion Sphere Particles
- Ion OneTouch Wash Solution from the Ion PGM Template OT2 200 Solutions kit (yellow label, cabinet above Ion OneTouch).

1. Transfer a 2.0 aliquot of the unenriched IPSs in Well 1 to a 0.2 mL PCR Tube.
2. From the Ion Sphere Quality Control Kit, thaw the Ion Probes tube (blue lid), Annealing Buffer (white lid), and Quality Control Wash Buffer (bottle with white lid).

3. For unenriched Ion PGM™ Template OT2 200 Ion Sphere™ Particles, if not 100 µL, adjust sample volume to 100 µL with Ion OneTouch™ Wash Solution from the Ion PGM™ Template OT2 200 Kit (Part. no. 4481105) and transfer 2 µL to a 0.2-mL PCR tube.

4. Add Ion Probes to sample(s).

a. If processing one sample, add 19 µL Annealing Buffer and 1 µL Ion Probes directly to the 0.2 mL PCR tube containing the ISPs and mix well by pipetting up and down.

b. If processing more than one sample, generate an Ion Probe Master Mix:

•  $(21 \mu\text{L Annealing Buffer} * \# \text{ samples}) + (1 \mu\text{L Ion Probes} * \# \text{ samples}) = \text{total volume required}$

- Add 20  $\mu\text{L}$  of Ion Probe Master Mix to the 0.2-mL PCR tubes containing the ISPs, then mix well by pipetting up and down.

5. Load the tube(s) into a thermal cycler, then perform the following protocol to anneal the Ion Probes:

Temperature	Time
95°C	2 min
37°C	2 min

6. Remove unbound probes by washing the sample(s) three times with 200  $\mu\text{L}$  Quality Control Wash Buffer.

- Add 200  $\mu\text{L}$  Quality Control Wash Buffer to the 0.2-mL tube(s).
- Vortex properly to mix and centrifuge down at  $15,500 \times g$  for 1.5 minutes.
- Being careful not to disturb the pelleted ISPs, remove the supernatant and leave behind 10  $\mu\text{L}$ .

Note: Compare to a 10  $\mu\text{L}$  standard for reference.

- Repeat substeps a through c of step 5 two times for a total of three Quality Control Wash Buffer washes.

7. After the final wash, add 190  $\mu\text{L}$  of Quality Control Wash Buffer for a total volume of 200  $\mu\text{L}$ , mix by pipetting up and down five times and transfer the entire sample to a Qubit® assay tube.

IMPORTANT! Ensure that you measure the volumes accurately.

8. To generate a negative control, add 200  $\mu\text{L}$  of Quality Control Wash Buffer to a fresh Qubit® assay tube.

9. Read the sample(s) using the Qubit® 2.0 Fluorometer as described below:

#### *Sample measurement*

IMPORTANT! Prior to using the Qubit® 2.0 Fluorometer, ensure that the instrument is running Firmware V3.10, and the Ion\_PluginV310\_AF.qbt file has been permanently uploaded to the instrument. See section “Section 1: Upgrade the Qubit® 2.0 Fluorometer firmware and software” on page 69 for more information about managing Qubit® 2.0 Fluorometer firmware versions.

- Power on the Qubit® 2.0 Fluorometer.
- Touch Ion to access Alexa Fluor® 488 and Alexa Fluor® 647 measurement options.
- Touch AF 488 and insert the sample into the Qubit® 2.0 Fluorometer, close the lid, and touch Read.

Note: The lettering on the Read selection option changes from white to red when reading a sample. The lettering changes back to white when the reading of the sample is finished in approximately 5 seconds.

Note: If more than one sample is being processed, all samples can be read with the AF 488 setting before moving on to the AF 647 setting.

- Record the value.

Note: The data retained on the Qubit® 2.0 Fluorometer can be transferred to a USB drive. See the “(Optional) Data Transfer to USB Drive” for details. If more than one sample is being processed, all samples can be read with the AF 488 setting before moving on to the AF 647 setting.

- Touch Home, touch Ion, and then touch AF 647. Insert the sample into the Qubit® 2.0 Fluorometer, close the lid, and touch Read.

Note: The lettering on the Read selection option changes from white to red when reading a sample. The lettering changes back to white when the reading of the sample is finished in approximately 5 seconds.

6. Record the value.

**IMPORTANT!** Ensure that you read the negative control (Quality Control Wash Buffer only) in both the Alexa Fluor® 488 and Alexa Fluor® 647 settings and record the RFU values.

Templated Ion PGM Template OT2 200 ISP evaluation

1. Open the saved Qubit Easy Calculator Microsoft Excel Spreadsheet file containing the Calibration Factor specifically calculated for the Qubit 2.0 Fluorometer used.
2. Enter the raw RFU values from the Alexa Fluor 488 and the Alexa Fluor 647 Calibration Standards measurements in the appropriate fields for both the ISPs containing samples (red cells) and negative control sample (purple cells).

**Important:** The Alexa Fluor 488 value must be >100 counts to produce a valid % Templated ISPs value. If the Alexa Fluor 488 value is <100 counts, see the “Ion Sphere Quality Control Assay troubleshooting table” on page 83 of the Ion PGM Template OT2 200 Kit User guide.

*Acceptance criteria for unenriched Ion PGM™ Template OT2 200 Ion Sphere™ Particle samples*

The optimal amount of library corresponds to the library dilution point that gives Percent Templated ISPs between 10–30%. Samples that fall within the recommended range generally produce the most data; however, samples that fall outside of the recommended range can still meet the throughput specifications on the Ion chips.

The recommended optimal range is not intended to be a pass/fail criteria. The range provides guidance for the quality of the sample.

Note: If the results are outside the desired Percent Templated ISPs range, then increase or decrease the library input appropriately.

*Percent Templated ISPs Description*

<10% Sample contains an insufficient number of templated ISPs to achieve optimal loading density on the Ion Chip.

**10–30% Optimal amount of library.**

>30% Sample will yield multi-templated ISPs (mixed reads).

**See Page 83 of Ion PGM Template OT2 200 Kit User Guide for troubleshooting**

### **Maintain the Ion OneTouch™ 2 Instrument (pg 38-43)**

**IMPORTANT!** To ensure continued safe operation, visually inspect the rotor assembly and casing periodically to ensure there are no signs of cracks or other physical damage. Follow the cleaning procedure in this section to clean the Ion OneTouch™ 2 Instrument with the Ion OneTouch™ 2 Cleaning Adapter. *Always perform the cleaning procedure after every run.*

1. Determine the appropriate reagents to use for maintaining the Ion OneTouch™ 2 Instrument:
2. Ensure that there is  $\geq 20$  mL of Ion OneTouch™ Oil (*left* Reagent Tube). If not, pour oil into the Oil Reagent Tube until it is half full.
3. Remove and appropriately discard the used Ion PGM™ OneTouch Plus Reaction Filter Assembly. Remove the assembly from the instrument by grasping the *filter*.
4. Keep the Ion OneTouch™ 2 Amplification Plate in the heat block.

5. Place a 50-mL conical tube in a tube rack, then place the tube rack with the tube to the right of the instrument:



6. Firmly insert the 3 ports of the *single-use* Ion OneTouch™ 2 Cleaning Adapter into the three holes on the top stage of the Ion OneTouch™ 2 Instrument. The tab protruding from the outer edge of the Ion OneTouch™ 2 Cleaning Adapter fits into the front notch of the stage.

7. Remove the disposable injector from the Ion OneTouch™ DL Injector Hub: Place one hand on the centrifuge lid. With the other hand, firmly grip the rigid plastic connector at the top of the disposable injector, then slowly and steadily withdraw the disposable injector straight from the port of the Injector Hub.

**CAUTION! PHYSICAL INJURY HAZARD.** The pointed end of the disposable injector can puncture your skin. Keep your hand away from the point of the disposable injector.

8. Gently pull the disposable tubing downwards on the both sides of the pinch valve until the disposable tubing is out of the valve.

9. Place the used, disposable injector into an empty 50-mL conical tube in a tube rack by the instrument. The conical tube will be used for waste.

10. On the Ion OneTouch™ 2 Instrument home screen, touch **Clean**. Complete each task, then touch **Next**. After you touch **Next** on the last task, you see a progress bar, and the cleaning begins.

11. Ensure that the task in bold displays at the end of the cleaning run: “**Remove plate, injector, conical tube, and waste**”.

Note: Keep the used Cleaning Adapter on the instrument between runs.

12. Appropriately dispose of the waste in the 50-mL conical tube.

13. Remove and appropriately dispose of the used Amplification Plate, disposable injector, and tubing from the instrument:

- a. Push the handle to open the heat block.
- b. Remove the disposable tubing from the Ion OneTouch™ DL Catch.
- c. Gently pull back the Amplification Plate from the inlet and outlet holes of the instrument.
- d. Remove the Amplification Plate from the heat block, and appropriately dispose of the used amplification plate, injector, and tubing.
- e. Leave the heat block open.

**CAUTION! Hot Surface.** Use care when working around this area to avoid being burned by hot components.

14. On the instrument display, touch **Open Lid**, wait until the lid opens, then open the centrifuge lid. Wipe the residue from the centrifuge lid with a Kimwipes® disposable wipe, then close the centrifuge lid.

15. Touch **Next** to return to the home screen on the instrument.

### **Supplemental procedure**

Perform a residual volume test on the Ion OneTouch™ ES

1. Set up the Ion OneTouch™ ES. Refer to “Set up the Ion OneTouch™ ES” in the Ion PGM™ Template OT2 200 Kit.

2. Install a tip on the Tip Arm.

3. Obtain a new 8-well strip.

4. Load an 8-well strip on the Ion OneTouch™ ES:

a. Load 80 µL water or Ion OneTouch™ Wash solution into the second well (Well 2) from the square-tabbed end of the 8-well strip.

b. Load the 8-well strip into the slot of the Tray so that the square-tabbed end is to the *left* and the 8-well strip is pushed all the way to the right until it touches the end of the slot.

**IMPORTANT!** Before running the residual volume test, carefully read and familiarize yourself with step 5 of this procedure.

5. Run the residual volume test. During the test, confirm that the tip is centered with respect to the sides of the wells when moving in or out of a well.

a. Turn ON the instrument, and wait for the instrument to initialize: The screen displays “rdy”. The Tip Arm performs a series of movements and returns to the home position (~5 seconds).

b. Press **Start/Stop**.

c. Wait for the instrument to aspirate the solution from Well 2 and completely remove the tip from well 2, then *manually* push the 8-well strip to the left so that Well 4 is positioned directly under the Tip Arm.

d. Wait for the instrument to dispense the tip contents into Well 4, press **Start/Stop** to stop the test run, then press **Start/Stop** again to return the Tip Arm to the home position.

e. Place a P10 pipette at the front bottom of Well 2, aspirate the entire residual water or Ion OneTouch™ Wash Solution from the well, then estimate the residual volume.

6. Remove the used tip: With the Tip Arm in its cradle and while standing above the Tip Arm, twist the tip counterclockwise and pull it downward to remove and discard the tip (see diagram in “Sequence or store the enriched ISPs” on page 14).

**IMPORTANT!** Improper removal of tips can loosen the metal tip adapter fitting on the Tip Arm and affect instrument operation.

7. Remove and discard the used 8-well strip.

8. After performing the residual volume test, take one or more of the following actions:

Observation	Possible cause	Recommended actions
Residual volume in Well 2 is $\leq 5 \mu\text{L}$	—	Proceed to "Prepare reagents then fill the 8-well strip" on page 11.
Residual volume in Well 2 is $>5 \mu\text{L}$ <b>IMPORTANT!</b> The volume is measured from the <i>bottom</i> of the well, not from the sides.	The tip height is too high during aspiration.	Restore defaults, then calibrate the instrument. Refer to "Calibrate the Ion OneTouch™ ES" in the <i>Ion PGM™ Template OT2 200 Kit User Guide</i> .
Aspiration is irregular	This instrument out of calibration.	Restore defaults, then calibrate the instrument. Refer to "Calibrate the Ion OneTouch™ ES" in the <i>Ion PGM™ Template OT2 200 Kit User Guide</i> .
The 8-well strip lifts as the tip rises to the top of the well	The tip is angled too far forward or the tip height is set too low.	Verify that the tip is vertical and positioned directly over the notch in the calibration shelf. If the tip is positioned correctly, restore defaults, then calibrate the instrument. Refer to "Calibrate the Ion OneTouch™ ES" in the <i>Ion PGM™ Template OT2 200 Kit User Guide</i> .

## Appendix L: Ion PGM Sequencing

From Ion PGM Sequencing 200 Kit v2 User Guide, MAN0007273 Rev. 3

[https://tools.thermofisher.com/content/sfs/manuals/MAN0007273\\_IonPGMSequenc\\_200Kit\\_v2\\_UG.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007273_IonPGMSequenc_200Kit_v2_UG.pdf), accessed 11-4-2015

### Create a Planned Run (pg 14-17)

Coverage depth for a genomic DNA library with:	Ion 314™ Chip		Ion 316™ Chip		Ion 318™ Chip		Ion PI™ Chip	
	>95% of bases at 30X	>95% of bases at 500X	>95% of bases at 30X	>95% of bases at 500X	>95% of bases at 30X	>95% of bases at 500X	>95% of bases at 30X	>95% of bases at 500X
No. of amplicons per library	Approximate number of libraries per chip OR (chips per library)*							
12-24	128	8	>384	32	>384	64	>384	>384
25-48	64	4	256	16	>384	32	>384	320
49-96	32	2	128	8	256	16	>384	160
97-192	16	1	64	4	128	8	>384	80
193-384	8	(2)*	32	2	64	4	>384	40
385-768	4	—	16	1	32	2	320	20
769-1,536	2	—	8	(2)*	16	1	160	10
1,537-3,072	1	—	4	—	8	(2)*	80	5
3,073-6,144	(2)*	—	2	—	4	—	40	2
6,145-12,288	—	—	1	—	2	—	20	1
12,289-24,576	—	—	(2)*	—	1	—	10	(2)*
24,577-49,152	—	—	—	—	(2)*	—	5	—
49,153-98,304	—	—	—	—	—	—	2	—

\* Values in parentheses indicate multiple chips per library. (2) = 1 library per 2 chips.

1. Log into the Torrent Browser for the Torrent Server connected to your Ion PGM™ System.
2. Select the Plan tab, select Templates, locate the type of experiment you want to run (for example, AmpliSeq™), then select:
  - Plan Run to plan a new run using an existing template.
  - Plan New Run to plan a new run without using a template.
3. In the wizard, review each screen and make your selections. For more information about specific fields, see the Ion PGM™ Sequencing 200 Kit v2 User Guide.
4. When you have completed your selections, click on Plan Run at the end of the workflow. The plan will appear listed on the Planned Runs page under the name you specified, and be available on the Ion PGM™ Sequencer when you set up the run.

### Clean the Ion PGM™ System (pg 17-19)

\*NOTE\* If starting a new project, cleaning can be done the night before. Chlorite cleaning does not have to be done more than once between runs; if the machine has been inactive for a long period but a chlorite cleaning was performed during that period, clean the PGM with water.

Before Starting

- Weekly: Prepare a stock of 1 M NaOH by diluting 10 M NaOH with 18 MΩ water.
- Daily: Prepare 100 mM NaOH by diluting the 1 M stock in 18 MΩ water.

#### Cleaning schedule

The Ion PGM™ Sequencer requires cleaning with either 18 MΩ water or a chlorite solution every time the instrument is initialized.

Clean with: Schedule:

#### 18 MΩ water

- Daily, when instrument is in use (e.g., not necessary on weekends)
- After  $\leq 1000$  flows (e.g.,  $2 \times 200$ -base-read runs)
- If more than 27 hours but less than 48 hours have elapsed between the last cleaning/initialization and the start of a run
- If you cleaned with chlorite a week ago and have not used the instrument since then

#### Chlorite solution

- Once a week, unless the instrument has not been used since the last chlorite cleaning (in which case, clean with 18 MΩ water before using)
- If the instrument has been left with reagents for more than 48 hours (for example, over the weekend)

#### Cleaning setup

**IMPORTANT!** For all the following steps, pour the 18 MΩ water directly from the purification system into the Wash 2 Bottle. Do not use water that has been collected or stored in any other containers.

- Remove any bottles that are attached to the Ion PGM™ System. Separate cleaning bottles are provided with the instrument.
- Do not remove old sipper tubes before cleaning. The sipper tubes are used as part of the cleaning procedure.
- Ensure that an old chip is in position on the instrument before cleaning. The chip type used for cleaning/initialization can be different than the chip type used for sequencing (e.g., you can clean with a used Ion 314™ chip for an Ion 318™ Chip run).

#### **18 MΩ water cleaning**

1. Empty any remaining solution from each cleaning bottle (two 250-mL bottles and one 2-L bottle) and rinse each bottle twice with  $\sim 100$  mL of 18 MΩ water.
2. Press Clean on the touchscreen, and select the 18-MΩ water cleaning checkbox.
3. Add 250 mL of 18 MΩ water to a 250-mL cleaning bottle.
4. Rinse the outside of the W1 sipper tube on the instrument with 18 MΩ water, and attach the bottle to the W1 position. Press Next.
5. Following the touchscreen instructions, place the empty 2-L cleaning bottle in the W2 position and the empty 250-mL bottle in the W3 position. Place collection trays below the sipper tubes in the dNTP positions. Press Next to begin cleaning
6. When cleaning is complete, remove all bottles and sipper tubes from the W1, W2, and W3 positions. Press Next to return to the Main Menu and proceed to initialization. Ion PGM™ Sequencing 200 Kit v2Quick Reference 3

#### **Chlorite cleaning**

##### **Materials:**

- Chlorite cleaning tablets
- 0.22μm or 0.45 μm filter system



- Note: Prepare a stock of 1 M NaOH each week by diluting 10 M NaOH with 18 MΩ water.

1. Empty any remaining solution from each cleaning bottle (two 250-mL bottles and one 2-L bottle) and rinse each bottle twice with ~100 mL of 18 MΩ water.
2. Fill a glass bottle with 1 L of 18 MΩ water and add a PGM™ Cleaning Tablet (chlorite tablet). Allow the tablet to completely dissolve (~10 minutes).
3. When the tablet has dissolved, add 1 mL of 1 M NaOH and filter the solution using a 0.22 μm or 0.45 μm filter. Use the chlorite solution within 2–3 hours. Discard any unused solution after this time.
4. Press Clean on the touchscreen, and select the Chlorite cleaning checkbox.
5. Add 250 mL of filtered chlorite solution to a 250 mL cleaning bottle.
6. Rinse the outside of the W1 sipper tube on the instrument with a squirt bottle containing 18 MΩ water, and attach the bottle to the W1 position.
7. Following the touchscreen instructions, place the empty 2 L cleaning bottle in the W2 position and the empty 250 mL bottle in the W3 position. Place collection trays below the sipper tubes in the dNTP positions. Press Next to begin cleaning.
8. When prompted, remove the W1 cleaning bottle with chlorite solution, rinse the outside of the sipper with a squirt bottle containing 18 MΩ water, then install a clean 250 mL cleaning bottle filled with 250 mL of 18 MΩ water in the W1 position. (The second cleaning bottle is different than the one used for chlorite solution.)
9. When cleaning is complete, remove all bottles and sipper tubes from the W1, W2, and W3 positions. Press Next to return to the Main Menu and proceed to initialization.

### Initialize the Ion PGM™ System (pg 19-25)

#### IMPORTANT!

- The **first run** should be started within **1 hour** after initialization, and the **last run** must be started within **27 hours** after initialization.
  - Handle nucleotides carefully to avoid cross-contamination. Always change gloves after removing used sipper tubes and after handling concentrated dNTP stocks.
  - After four initializations, do not use the Wash 1, 2, and 3 Bottles for initialization or sequencing to avoid breakage or leaking.
  - Replace the Reagent Bottles and sipper tubes every time you initialize.
  - Make sure that you have updated the Torrent Suite and Ion PGM™ System software to Version 3.2.1 or later.

#### *Materials provided in the Ion PGM Sequencing Reagents kitv2*

- Ion PGM™ Sequencing 200 v2 dGTP
- Ion PGM™ Sequencing 200 v2 dCTP
- Ion PGM™ Sequencing 200 v2 dATP
- Ion PGM™ Sequencing 200 v2 dTTP

#### *Materials provided in the Ion PGM Sequencing Solutions kit*

- Ion PGM™ Sequencing 200 v2 W2 Solution
- Ion PGM™ Sequencing 200 v2 1X W3 Solution

#### *Other materials and equipment*

- Used chip (leave chip on the instrument during initialization)
- 18 MΩ water

- 100 mM NaOH (prepared daily)
- Ice
- 5-mL and 25-mL pipettes
- Filtered and unfiltered pipette tips and pipettes
- Vortex mixer
- Microcentrifuge

*Before initialization:*

- Remove the dNTP stock solutions from the freezer and begin thawing on ice.
- Check the tank pressure for the nitrogen or argon gas. When the pressure drops below 500 psi, change the tank.

*Prepare the Wash 2 Bottle*

**IMPORTANT!** For all the following steps, pour the 18 M $\Omega$  **water directly from the purification system into the Wash 2 Bottle**. Do not use water that has been collected or stored in any other containers.

1. Rinse the Wash 2 bottle three times with 200 mL of 18 M $\Omega$  water.
2. If your 18 M $\Omega$  water system has a spigot, extend the water spigot into but not below the neck of the Wash 2 Bottle.
3. Fill the bottle to the mold line. Volume of water will be ~2 liters.
4. Add the entire bottle of Ion PGM™ Sequencing 200 v2 W2 Solution to the Wash 2 bottle.
5. Add 70  $\mu$ L of freshly prepared 100 mM NaOH solution (not 1 M NaOH) to the Wash 2 bottle.
6. Cap the bottle and invert five times to mix, and immediately proceed through the rest of the initialization procedure.

**IMPORTANT!** Do not store the mixed Wash 2 bottle. 4 Ion PGM™ Sequencing 200 Kit v2 Quick Reference

*Prepare the Wash 1 and Wash 3 Bottles*

1. Rinse the Wash 1 and Wash 3 Bottles three times with 50 mL of 18 M $\Omega$  water.
2. Wash 1 Bottle: Add 350  $\mu$ L of freshly prepared 100 mM NaOH to the Wash 1 Bottle and cap the bottle.
3. Wash 3 Bottle: Add Ion PGM™ Sequencing 200 v2 1X W3 Solution to the 50-mL line marked on the Wash 3 Bottle and cap the bottle.

*Begin the initialization*

**IMPORTANT!** Do not remove the old sipper tubes from the dNTP ports until instructed to do so. Do not let the new sipper tubes touch any surfaces.

NOTE: Make sure you have a chip in place, NOT a cleaning chip

**IMPORTANT!** Load the bottles as quickly as possible to prevent atmospheric CO<sub>2</sub> from reducing the pH of the Wash 2 Bottle solution.

1. Confirm that the chip used to clean the Ion PGM™ System is still in place on the instrument.
2. On the main menu, press Initialize.
3. In the next screen, scan or enter the barcode on the Ion PGM™ Sequencing 200 v2 W2 Solution bottle or select the Ion PGM™ Sequencing 200 Kit v2 from the dropdown list.
4. Press Next and confirm that the cleaning chip is on the instrument and the Reagent Bottle sipper tubes and collection trays are in place. Press Next again.

5. The system will verify the gas pressure. If the gas pressure is sufficient, press Next. If the pressure is low, press Yes to re-verify. If the pressure remains low, contact Technical Support. Check the valves on the N<sub>2</sub> gas tank; there are 3.
6. Wearing clean gloves, insert a new sipper tube (long gray) into the cap in the W2 position. Do not let the sipper tube touch any surfaces.
7. Immediately attach the prepared Wash 2 Bottle and tighten the cap. Press Next.
8. Change gloves and install new sipper tubes (short gray) in the caps in the W1 and W3 positions.
9. Immediately attach the prepared Wash 1 and 3 Bottles and tighten the caps. Press Next to begin initialization (~30 minutes).

*Prepare the 50-mL Reagent Bottles with dNTP solutions*

**IMPORTANT!** In the following steps, handle the dNTPs carefully to avoid cross-contamination and keep the dNTP vials on **ice**.

1. After each dNTP stock solution has thawed, vortex to mix and centrifuge to collect the contents. Keep dNTPs on ice throughout this procedure.
2. Use the labels provided with the kit to label four new Reagent Bottles as dGTP, dCTP, dATP, and dTTP.
3. Using filtered pipette tips and clean gloves, carefully transfer 20 µL of each dNTP into its respective Reagent Bottle.

*Attach the sipper tubes and Reagent Bottles*

1. After the wash solutions have initialized, remove the used sipper tubes and collection trays from the dNTP ports.
2. Using new gloves, attach a new sipper tube (blue) to each dNTP port.
3. Attach each Reagent Bottle to the correct dNTP port and tighten until snug.
4. Follow the touchscreen prompts to complete initialization.
5. At the end of initialization, the Ion PGM™ System will measure the pH of the reagents. If there is a problem with pH, see Troubleshooting in the Ion PGM™ Sequencing 200 Kit v2 User Guide.
6. Press Next to finish initialization and return to the main menu.
7. Proceed to the appropriate sequencing protocol for your chip type.

**Sequencing Protocol—Ion 314™ Chip (pg 35-38)**

*Materials provided in the Ion PGM Sequencing Reagents kit v2*

- Sequencing primer
- Control ion sphere particles
- Polymerase

Use the following protocol with Ion 314™ Chips. For Ion 316™ or Ion 318™ Chips, see *Ion PGM Sequencing kit user guide*.

Before starting

- Prepare the template-positive ISPs.
- Thaw the Sequencing Primer on ice.
- Make sure that you have updated the Torrent Suite and Ion PGM™ System software to Version 3.2.1 or later.

**IMPORTANT!** For each initialization, the first run should be started within 1 hour after initialization, and the last run must be started within 27 hours after initialization.

**Optional:** Prepare Ion Sphere™ Test Fragments

If you are performing an installation or troubleshooting sequencing run:

1. Vortex the Ion Sphere™ Test Fragments from the Ion Controls Materials 200 Kit and centrifuge for 2 seconds before taking aliquots.
2. Add 5 µL of Ion Sphere™ Test Fragments to 100 µL of Annealing Buffer in a 0.2 mL non-polystyrene PCR tube.
3. Skip directly to Anneal the Sequencing Primer.

Prepare enriched, template-positive ISPs

1. Transfer half the volume of enriched, template-positive ISPs to a new 0.2 mL non-polystyrene PCR tube and store at 2–8°C for up to 1 week. They may be used for another sequencing run.
2. Vortex the Control Ion Sphere™ Particles and centrifuge for 2 seconds.
3. Add 5 µL of Control Ion Sphere™ Particles directly to the half-volume of enriched ISPs in a 0.2 mL non-polystyrene PCR tube.
4. Proceed directly to Anneal the Sequencing Primer.

*Anneal the Sequencing Primer*

1. Mix by pipetting up and down thoroughly. Centrifuge for 2 minutes at 15,500 × g (or rcf).
2. Carefully remove the supernatant without disturbing the pellet, leaving 3 µL in the tube.
3. Add 3 µL of Sequencing Primer and confirm that the total volume is 6 µL (add Annealing Buffer if necessary).
4. Pipet up and down thoroughly to disrupt the pellet.
5. Program a thermal cycler for 95°C for 2 minutes and then 37°C for 2 minutes, using the heated lid option.
6. Place the tube in the thermal cycler and run the program. After cycling, the reaction can remain in the cycler at room temperature while you proceed with Chip Check.

*Chip Check*

**IMPORTANT!** Do not wear gloves when transferring chips onto and off the instrument and do not place the chip directly on the bench.

Place the chip either on the grounding plate on the Ion PGM™ System or in the bucket from the Ion centrifuge adapter/rotor.

1. Remove a new chip from its packaging and label it. Save the chip package to scan the barcode.
2. Place the chip on the Ion PGM™ Sequencer grounding plate or in the Ion centrifuge adapter/rotor bucket.
3. Press Run on the main menu and follow the touchscreen prompts.
4. When prompted, ground yourself by touching the grounding pad next to the chip clamp on the instrument and replace the old chip with the new one for the experiment. (Do not wear gloves when transferring the chips on and off the instrument.)
5. When prompted, scan the barcode located on the chip package or manually enter the barcode number.
6. Press Chip Check on the touchscreen.
7. Check for leaks on the chip. If there is a leak, press the Abort button and proceed to Troubleshooting in the Ion PGM™ Sequencing 200 Kit v2 User Guide.
8. When Chip Check is complete, if the chip passes press Next. If the chip fails, open the chip clamp, re-seat the chip, and press Calibrate to retest. If the chip passes, press Next. If the chip still fails, press Main Menu and restart with a new chip.

9. Following a successful Chip Check, remove the new chip and insert a used chip in the socket and close the clamp.

10. Completely empty the waste bottle as instructed in the touchscreen.

*Bind Sequencing Polymerase to the ISPs*

1. After annealing the Sequencing Primer, remove the ISPs from the thermal cycler and add 1  $\mu\text{L}$  of Ion PGM™ Sequencing 200 v2 Polymerase to the ISPs, for a total final volume of 7  $\mu\text{L}$ .

2. Pipet the sample up and down to mix.

3. Incubate at room temperature for 5 minutes.

**Load the chip (from Ion PGM Chip Loading with the Ion PGM Weighted Chip Bucket User Bulletin, MAN0007517, Rev. 1)**

**Remove liquid from the chip**

1. Following Chip Check, tilt the chip 45 degrees so that the loading port is the lower port, as shown below.



2. Insert the pipette tip firmly into the loading port and remove as much liquid as possible from the loading port. Discard the liquid.

**IMPORTANT!** For the next steps, balance the centrifuge with a used chip of the same chip type and orientation if you are preparing one Ion PGM™ Chip at a time. Be careful to balance an upside-down chip with another upside-down chip. Mark the used chip with a laboratory marker to differentiate it from the new chip containing the sample.

3. Note the markings on the Ion PGM™ Weighted Chip Bucket indicating proper orientation in the Minifuge. Place the bucket in the MiniFuge with the side marked “In” facing toward the center of the Minifuge.

4. Place the chip **upside-down** in the bucket **with the chip tab pointing in** (toward the center of the MiniFuge), as shown below.



5. Centrifuge for 5 seconds to completely empty the chip. Remove the chip from the bucket and wipe off any liquid on the bucket.

*Load the sample on the chip*

**IMPORTANT!** When loading liquid into the chip, keep the pipette tip at a 90° angle to the chip, press the tip firmly into the circular loading port, and apply gentle pressure between the pipette tip and chip.

1. Place the Ion Chip back in the Ion PGM™ Weighted Chip Bucket and **place the bucket on a flat, stable surface such as a bench top.**
2. Following polymerase incubation, collect the entire sample into a RaininR pipette tip and insert the tip firmly into the loading port of the chip.
3. **Dial down** (rotate the calibration dial down SLOWLY) the pipette as shown below to gently and slowly deposit the ISPs at a rate of  $\sim 1 \mu\text{L}$  per second. To avoid introducing bubbles into the chip, leave a small amount of sample in the pipette tip ( $\sim 0.5 \mu\text{L}$ ).
4. Transfer the bucket back to the Minifuge, making sure the bucket is oriented correctly.
5. Place the chip in the bucket **with the chip tab pointing in** (toward the center of the Minifuge). Centrifuge for 1 minute.
6. Flip the chip so that the **chip tab is pointing out**. (Do not flip the bucket, only the chip.) Centrifuge for 1 minute.
7. Firmly tap the point of the chip tab on the bench top 2-3 times.
8. Tilt the chip at a 45-degree angle and slowly remove as much liquid as possible from the loading port by dialing up the pipette. Discard the liquid.
9. If some liquid remains in the chip, perform a 5-second quick spin with the chip tab pointing out and remove and discard any additional liquid. **Do not spin the chip upside-down.**
10. If some liquid remains in the chip after the quick spin, lightly and rapidly tap the point of the chip tab against the benchtop a few times, and remove and discard any collected liquid. Do not flush the chip.
11. Immediately proceed to “**Select the Planned Run and perform the run**” in your sequencing kit user guide or quick reference.

**Select the Planned Run and perform the run (pg 42 of Ion PGM Sequencing 200 Kit v2 User Guide, MAN0007273 Rev. 3**

[https://tools.thermofisher.com/content/sfs/manuals/MAN0007273\\_IonPGMSequenc\\_200Kit\\_v2\\_UG.pdf](https://tools.thermofisher.com/content/sfs/manuals/MAN0007273_IonPGMSequenc_200Kit_v2_UG.pdf), accessed 11-4-2015)

Select the Planned Run or enter settings manually

1. Press the Browse button next to the Planned Run field and select the name of the plan you created, then press Next.
2. The run settings will be automatically populated based on the Planned Run. Confirm that these settings are correct. Make any changes using the buttons and dropdown lists if necessary.

*Perform the run*

1. After you enter the Planned Run, press Next to verify the experimental setup.
2. When prompted by the instrument, load and clamp the chip, then press Next.
3. Visually inspect the chip in the clamp for leaks before closing the cover.
4. When the calibration is complete ( $\sim 1$  minute), the touchscreen will indicate whether calibration was successful. If calibration fails, press Abort, reseal the chip, then press Calibrate to re-calibrate. If it fails again, continue with the run and contact Technical Support after the run is complete.

5. After 90 seconds, the run will automatically begin, or press Next to begin the run immediately. Avoid touching the instrument and any of the attached bottles or tubes during a run, as it may reduce the quality of the measurements.
6. When the run is complete, the touchscreen will return to the Main Menu. You can then proceed with another run or perform a cleaning/initialization if required (see Cleaning schedule on page 2).