D

Karnal Bunt

Appendix D

Laboratory Procedures

Contents

Introduction and Background

The purpose of the laboratory section is to describe procedures for examining wheat grain samples for the presence of *Tilletia indica* infected grain (i.e., bunted kernels) and seed samples for the presence of *T. indica* teliospores. Grain analysis laboratories normally process grain samples from fields, bins, or national surveys for the presence of bunted kernels. Seed laboratories process seed samples and perform extraction procedures to determine the presence of *T. indica* teliospores.

In program areas where bunted kernel analysis precedes seed analysis for spores, seed lots from regulated areas must be negative for bunted kernels prior to being considered for spore testing, and seed samples may also be processed at grain labs for the detection of bunted kernels. However, because of contamination potential, a different subsample of that seed lot must be submitted for spore analysis than the one processed for bunted kernel analysis and that subsample should not enter grain analysis laboratories unless it is sealed in nalgene bottles and is unopened. Some program areas analyze seed samples first for spores, then do a bunted kernel analysis on positive spore lots.

Grain and seed analysis laboratories must be physically separate and personnel working in grain laboratories (or field personnel) should not enter a seed analysis laboratory without proper decontamination safeguards.

The details of these procedures may be modified to individual laboratory needs; however, basic analysis methods and contamination safeguards should not be deviated from. Some seed analysis laboratories may wish to do the spore wash first and then the analysis of bunted kernels from the remaining dry sample afterward, and that is permissible.

Be very careful to maintain the identity of all grain and seed samples, with careful, accurate labeling.

The techniques for extracting teliospores by selective sieving were developed by Gary Peterson and Morris Bonde[1], United States Department of Agriculture, Agricultural Research Service (USDA, ARS), Ft. Detrick, MD. Plant Protection and Quarantine (PPQ) Plant Pathologist Ted Boratynski developed the grain inspection machine for bunted kernel analysis. Arizona Department of Agriculture Plant Pathologist Ron Ykema and others at the State Agriculture Laboratory in Phoenix, Arizona assisted in refinement of all the techniques described here and in the preparation of this manual. Joel Floyd, as PPQ Area Identifier in Nogales, AZ, wrote the teliospore and bunted kernel identification sections.

1 Peterson, G. L. and M. R. Bonde. 2000. Size-selective sieving for detecting teliospores of *Tilletia indica* in wheat seed samples. Plant Disease 9:999-1007.

Grain Analysis Methods

Laboratories for bunted kernel detection should be in an enclosed area and equipped with the supplies listed below. A process for logging in samples received should be in place. The area designated for grain analysis must be physically separate from the seed (spore) analysis laboratory.

Background

This subsection describes three methods used to identify wheat kernels infected with *Tilletia indica*, the causal agent of Karnal bunt. These infected kernels are called bunted kernels. Included are specific procedures for decontamination of samples and facilities.

Important

Seed samples tested for bunted kernels and/or spores must not utilize the same subsamples.

Obtaining Samples

The laboratory manager will specify which types and the priority of samples to be examined for bunted kernels. If assigned the task of detecting bunted kernels, **do not enter any seed analysis areas.**

Automated Methods

The automated methods are preferred for analyzing grain samples for bunted kernel detection. There are two types of automated methods in use. One involves optical high speed sorting technology and the other employs the use of a grain inspection machine. The optical scanner, where available, is used for both samples from the regulated area and National Survey samples. When grain inspection machines or high speed optical sorting machines are not available, the manual method can be used.

High Speed Optical Sorting Method

Supplies

Laboratory personnel will need the following supplies to examine wheat kernels for Karnal bunt using (Satake®) optical sorting technology:

- Bags, paper
- Bags, plastic
- Beaker, glass or plastic, 600-mL
- Bleach (6%), 1 gallon
- Bottle, spray
- Boxes, slide holder

- Brush, fine tip applicator
- Brushes, paint
- Cover slips, glass
- Dust masks
- Fluorescent paint/dye
- Forceps
- Gloves, latex
- Illuminators, microscope
- Labels for slides
- Lab towels, disposable
- Ladder
- Lamp, magnifying
- Markers, permanent, fine-point, black
- Microscopes, compound
- Microscopes, dissecting
- Nail polish, clear
- Needle probes
- Paper towels
- Parafilm
- Petri dish, plastic disposable
- Razor blades
- Reinforcements, white
- Shear's mounting medium (see **Preparing Shear's Mounting Medium** for preparation)
- Slides, glass
- Slides, identification aid
- Tape, packing
- Tape, quarantine
- Tissue, toilet
- Trash can, small
- Tub, small
- Tubs, 18-gallon/sample storage
- Ultra-violet light
- Vacuum cleaner, large shop type
- Vacuum cleaner, small portable

Procedure

The protocol for the high speed optical sorting method (using the Satake® Scanmaster II) follows:

NOTICE

Before opening front panel ensure that the ejectors are turned off. Failure to do so could result in damage to the ejectors!

At the beginning of the day and about every four hours thereafter:

Step 1—
Make sure the machine is on for at least 30 minutes before use and that the valve for the compressor hose is at the "ON" position.

Step 2—
Check the light levels. Both should be at about 12.0–14.4 V (Setup 1 Menu).

Step 3—
Select the MEDIUM Preset (Setup 3 menu). Be sure to press RECALL after selecting the correct product. When you go back to the Main menu, make sure the proper Preset is showing at the top of the screen.

Step 4—
Spike the sample with 3-13 fluorescent QC kernels. Recovery of spiked kernels will be done using the manual seed inspection machine.

Step 5—
Weigh the rejects. Based on a 4-lb sample, rejects will run between 180g–320g, depending on the color of the wheat and the number of darkened kernels.

Step 6—
Adjust the DARK TRIP as needed to achieve the appropriate percentage of rejects (10-15% depending on variety). Light colored samples require higher trip rates. Red winter wheat trip rates are between 784 and 796. Durum wheat trips are between 844 and 864.

Step 7—
Open and clean the machine. Check the feeder, chute, and sorting chamber for kernels.

For each sample:

Step 1—
Clean the sample as needed.

Step 2—
Select the correct pre-set based on sample color and variety.

Step 3—
Run the sample.

Step 4—
Open and clean the machine. Check the feeder, chute, and sorting chamber for remaining kernels.

Repeat Steps 1 through 4 for each sample.

To shut down the sorter:

Step 1—
Press the "X" in the upper right hand corner of the screen. This should get you back to the computer "desktop."

Step 2—
Press "START" in the lower left hand corner.

Step 3—
Press "SHUTDOWN."

Step 4—
A window will appear that says "What do you want the computer to do?" The "SHUTDOWN" command should already be selected. Press "OK."

Step 5—
When the screen says "It is OK to turn off your computer," press the power switch on the upper left side of the sorter. Do NOT shut down the sorter without going through this process.

Step 6—
Turn off the air supply valve.

Grain Inspection Machine Method

Supplies

Laboratory personnel will need the following supplies to examine wheat kernels for Karnal bunt using the grain inspection machine method:

- Beakers, glass, 600-mL
- Bleach, 6 percent solution
- Cover slips, glass (any size)
- Dazzlens, glass cleaner
- Dish, petri, plastic disposable, 50 x 9 mm
- Dust mask or respirator

- Forceps, specimen, stainless steel, large tip, small tip
- Gloves, latex, small, medium, and large
- Grain inspection machine
- Kimwipes XL
- Labels, white paper
- Microscope, compound
- Microscope, dissecting
- Nail polish, clear, toluene/formaldehyde free
- Paint brushes
- Plastic bags, 12 x 12 inch, zip-lock
- Shear's mounting medium (see **Preparing Shear's Mounting Medium** for preparation)
- Slides, microscope, 3" x 1"
- Tape, labeling
- Tech Spray, inert dusting gas, or vacuum with hose attachment
- Vials, plastic
- Worksheets, Karnal bunt kernel analysis, in-house

Preparing the Workstation

Designated bunted kernel readers must wear clothing that will prevent sample contamination and protect them from harmful chemicals.

Step 1—Put on protective clothing.

Put on a dust mask or respirator, and latex gloves as needed. If the sample shows pink or violet dye, wear a dust mask when examining the grain. The dye shows that the grain has been treated with pesticide.

Step 2—Prepare the grain inspection machine.

Turn the feeder rheostat control knob on the grain inspection machine counter clockwise to the OFF position (see **Figure D-1** for a diagram of a grain inspection machine). Turn ON the power switch located on the control panel. Place a clean petri dish and a pair of clean forceps on the work surface to the right side of the feeder trough. Open the acrylic lid above the grain hopper.

Analyzing the Sample

Step 1—Pour the sample into the hopper.

Obtain the sample to be tested and record the Karnal bunt sample number of the worksheet. Carefully open the sample bag(s) without tearing. Being careful not to over-fill, pour a portion of the sample into the grain hopper and close the acrylic lid.

Step 2—Activate the vibrating mechanism.

Turn ON the light switch. Turn ON the feeder rheostat control knob (vibrator switch). Adjust the grain hopper discharge opening and the feeder rheostat control until you get the desired feed rate. Only one level of grain should cover the bottom of the feeder trough as it vibrates, permitting observation of the kernels from all sides.

Important

Be careful to empty the grain bin cup frequently to avoid spillage. You may wish to pour only a cup full of sample at a time into the grain hopper.

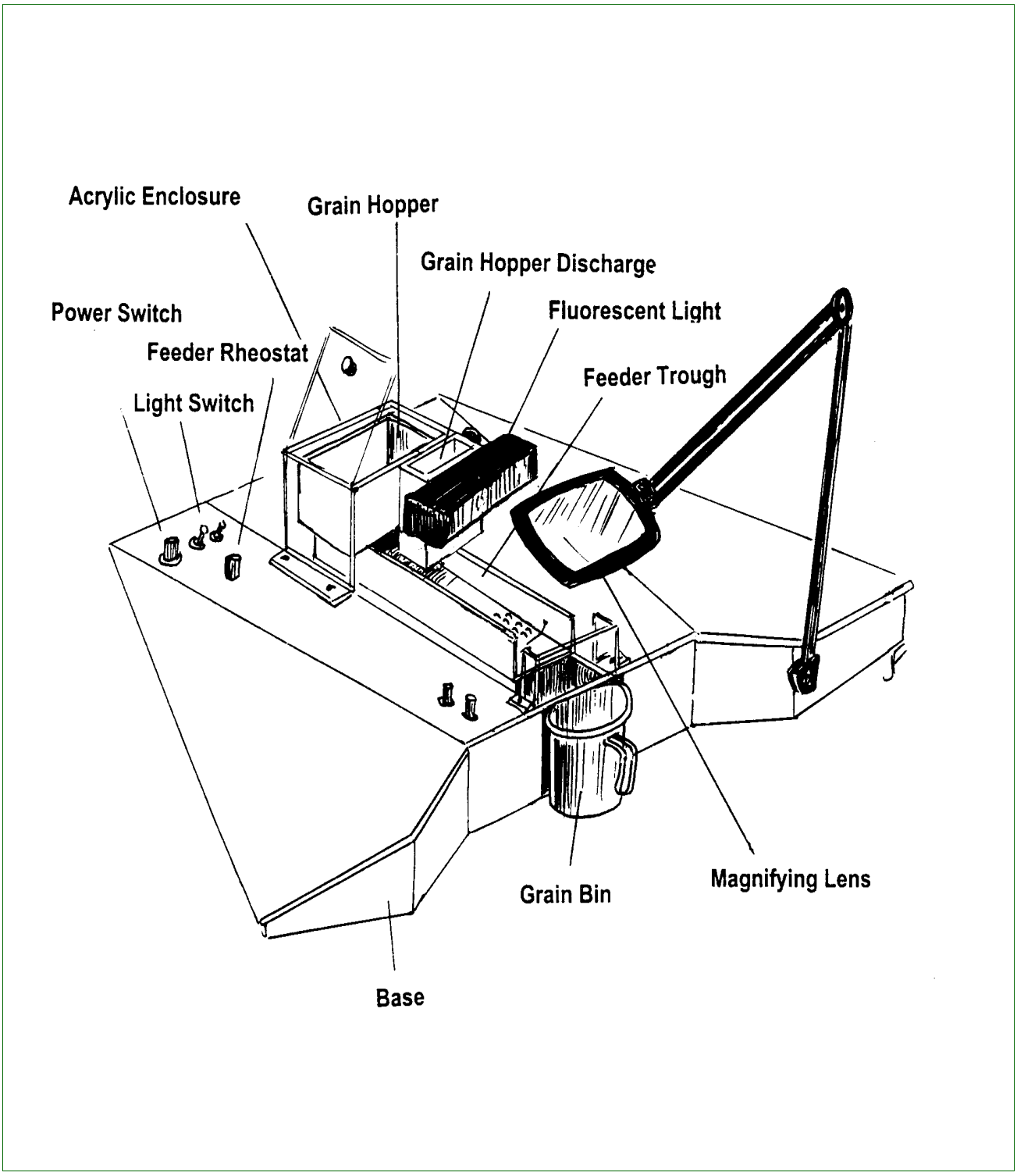

FIGURE D-1: Grain Inspection Machine

Step 3—Check for bunted kernels.

When you observe a suspect bunted kernel, turn down the feed rate or the rheostat control and use forceps to move the kernel to the petri dish. Take the suspect bunted kernels to the dissecting microscope and examine for the presence of teliospores. Place all of the bunted kernels into a clean plastic vial or in a sealed petri dish and prepare a paper label to include in the vial with the Karnal bunt number, your initials, and the date. Using a black marker label the outside of the vial/petri dish. Follow identification authority verifier's training procedures. If teliospores are present, prepare a microscope slide using Shear's mounting medium. Seal the cover slip with nail polish. Review the slide using the compound microscope, verifying the characteristics of the *T. indica* teliospore. Have an identifier with identification authority verify the presence of *T. indica* on the kernels found in a sample. Decontaminate the forceps with bleach and dispose of or disinfect the used petri dish.

Step 4—Record results.

Record your results on the appropriate worksheet. For positive results, record the total number of bunted kernels found in the sample. Place all positive samples in the storage area for positive samples. Place all negative samples in the storage area for negative samples, or dispose of as per project guidelines.

Step 5—Clean the inspection machine.

Clean the grain inspection machine as follows: Turn OFF the power switch and the light switch. Remove the seed bin cup and tilt the unit to permit any loose kernels to fall out through the seed bin cup area. Use inert dusting gas to blow any kernels from the unit. Return the unit to its upright position. Clean the magnifying lens with Dazzlens and Kimwipes. Periodically open the top of the machine and use paint brushes, or a vacuum with a hose attachment, or high pressure air to clean kernel pieces and dust from the inside of the machine.

Step 6—Clean the area and remove protective clothing.

When all of the samples have been examined, sweep up any spilled grain and dispose of it in a disposal bag in the area. Remove all protective clothing and place in the disposal bag.

Quality Control Procedures

The following are quality control procedures for detection of *Tilletia indica* bunted kernels.

As part of Quality Control (QC) procedures, samples are spiked with QC kernels, *Tilletia indica* bunted kernels, to measure efficacy of the Satake Optical Sorter and grain inspection machine personnel. These QC kernels are actual bunted kernels painted with a dye that fluoresces under UV light. Collected data will include the number of QC kernels added to the sample and the number of recovered QC kernels. The laboratory supervisor will use this data to keep the project director informed concerning validity of the screening procedures, and to provide feedback to lab personnel.

- Each lab will have a vial containing 16 bunted kernels, painted with fluorescent paint, with which to "spike" the QC sample.
- The 4-lb QC sample consists of previously tested negative grain and 3-13 QC kernels determined by the random number generator.

Facilities Equipped with Satake Optical Sorter Technology

Step 1—
Use the random number generator to determine the number of bunted kernels with which to spike the QC sample.

Step 2—
Place the bunted kernels into the QC sample and run through the sorter at the lowest acceptable Dark Trip (784).

Step 3—
Look at the QC sample "discard container" with the UV light to ascertain that all of the QC kernels were ejected.

- If all of the QC kernels are found, proceed to next step.
- If any QC kernels are missing, look at clean portion container with the UV light to determine if the sorter missed any QC kernels. If it is determined that QC kernels were missed, raise the Dark Trip one unit (i.e. 784 to 788) and return the QC kernels to the QC sample and run again. Repeat this process until all QC kernels are ejected into the discard portion container. This will be the Dark Trip value to use for one shift (every four hours). Run a QC sample for each shift.

Step 4—
Replace all QC Kernels into discard portion. Have each lab technician working in the lab look at the discard using a manual inspection station (bunt machine) until he or she finds one bunted kernel.

Step 5—
Remove and secure all bunted kernels from the QC sample into a safe container and safeguarded in an appropriate location.

- Appropriate worksheets will be provided for the daily recording of all QC results (see Figure D-3).

Field Facilities without Satake Optical Sorter Technology

Step 1—
The laboratory supervisor should spike each 4-lb QC sample with 3-13 fluorescent QC kernels. Determine the number of kernels per sample by using a random number generator.

Step 2—
Inspect the sample using the manual grain inspection machine until all the QC kernels are found.

Step 3—
Record quality control information (see Figure D-3). If all QC kernels are recovered, no further action is required. If all QC kernels are not recovered, screen the 4-lb sample using the portable UV light to insure the indicated number of QC kernels were present.

Manual Method

Laboratory personnel will need the following supplies items to examine wheat kernels for Karnal bunt using the manual method:

Supplies

- Bags, disposal, 12" x 24"
- Beaker, glass, 600-mL
- Bleach, 6 percent solution
- Bottle, spray, 1-L, hardware store
- Boxes, cardboard
- Cover slips, glass (any size)
- Dish, petri, plastic disposable, 50 x 9 mm
- Dish pans, plastic, department store
- Forceps, specimen, stainless steel, large tip
- Gloves, latex, small, medium and large
- Labels, white paper
- Lamp, magnifying
- Microscopes, dissecting and compound
- Nail polish, clear, toluene/formaldehyde free
- Shear's mounting medium (see Preparing Shear's Mounting Medium for preparation)

- Slides, microscope, 3" x 1"
- Storage units
- Towels, lab
- Vials, plastic
- Wash basin, double sided
- Worksheets, Karnal Bunt Kernel Analysis, in-house

Preparing the Workstation

Designated bunted kernel readers must wear clothing that will prevent sample contamination and protect them from harmful chemicals.

Step 1—Put on protective articles.

Put on a dust mask or respirator, and latex gloves as needed. If the grain samples show pink or violet dye, wear a dust mask when examining the grain. The dye indicates that the grain has been treated with pesticide.

Step 2—Set up items.

Take one 600-mL beaker, one petri dish, one large forceps, and one plastic tray. Set these items up at your workstation and use the appropriate worksheet for recording data.

Analyzing the Sample

Step 1—Scoop grain into the beaker.

From the sealable plastic bag holding the grain sample, scoop approximately 500 mL of grain and pour it into the beaker. Take the sample to your workstation for examination.

Step 2—Spread the sample.

Spread a portion of the sample throughout the plastic tray in approximately one layer of grain. Do a visual overview of the sample in the tray, looking for grain that shows a darkening on one end or obvious deterioration of the grain tissue.

Step 3—Check for bunted kernels.

When you observe a suspect bunted kernel, use forceps to move the kernel to the petri dish. Take the suspect bunted kernels to the dissecting microscope and examine for the presence of teliospores. Place all of the bunted kernels into a clean plastic vial or a sealed petri dish and prepare a paper label to include in the vial with the Karnal bunt number, your initials, and the date. Using a black marker label the outside of the vial. Follow identification authority verifier's training procedures. If teliospores are present, prepare a microscope slide using Shear's mounting medium. Seal the coverslip with nail polish. Review the slide using the compound microscope, verifying the characteristics of the *T. indica* teliospore. Have an identifier with identification authority verify the presence of *T. indica* on the kernels found in a sample. Decontaminate the forceps with bleach and dispose of or disinfect the used petri dish.

Step 4—Record results.

Record your results on the appropriate worksheet. For positive results, record the total number of bunted kernels found in the sample. Place all positive samples in the storage area for positive samples. Place all negative samples in the storage area for negative samples, or dispose of as per project guidelines.

Step 5—Clean the work area.

Clean and disinfect the work area with bleach solution.

Identifying *Tilletia indica*

Early stages of infection first appear at the germ end of the wheat kernel. These symptoms are usually not visible when the grooved side is facing down. Therefore, an effective technique for the manual method is to turn many seeds over to look for symptoms.

The first visible stage of Karnal bunt infection is a swollen area that appears gray or dark at the germ end forming a triangular shaped area on either side of the groove (see illustrations **A2**, **B1**, and **B2** on the laminated insert titled **Symptoms of Karnal Bunt (*Tilletia indica*) and Black Point**). This is the thin pericarp, or outer epidermal layer, of the seed coat through which a darkening is visible and is evidence of a mass of teliospores beneath (the sorus). This swollen area of the pericarp may already be ruptured or can easily be punctured with a teasing needle, exposing many dark brown teliospores.

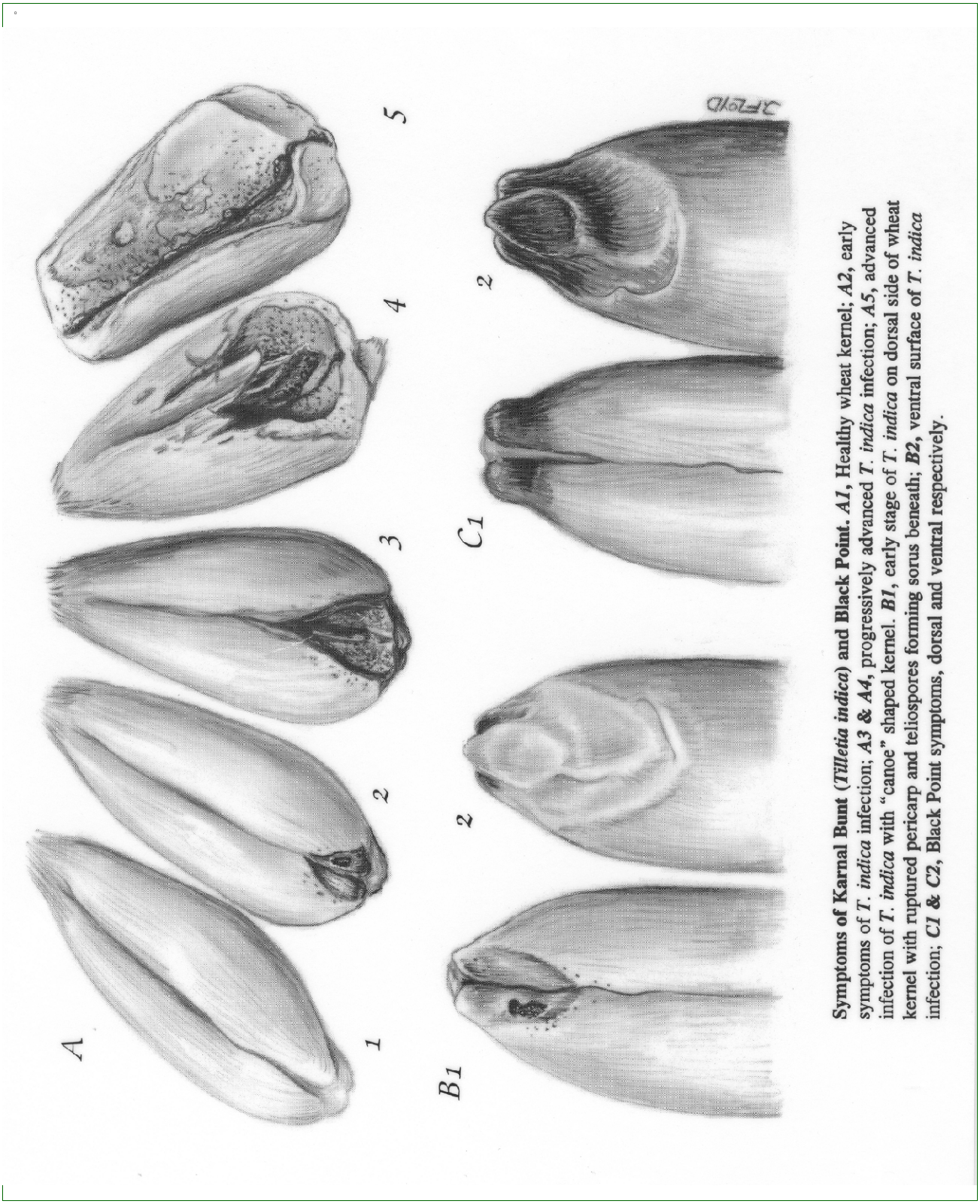

FIGURE D-2: Symptoms of Karnal Bunt (*Tilletia indica*) and Black Point

Karnal bunt teliospores, large by fungal spore standards, are easy to see with a hand lens. However, to verify Karnal bunt symptoms from a seed sample, viewing the infected kernels under a dissecting microscope will be necessary to see if teliospores are present. They appear as hundreds or thousands of small, granulate, shiny dark brown to black spheres, resembling microscopic ball bearings or buckshot. They are contained within the area of the seed that the fungus has consumed, together forming the sorus.

Refer to the laminated insert. More advanced infection (**A3** and **A4**) in the wheat kernel manifests itself as a blackening along the groove, progressively consuming more of the inner portions of seed tissue. A very advanced stage of infection results in the wheat kernel taking on the appearance of a boat or dugout canoe (**A5**).

Black point is a disease of non-quarantine importance caused by a variety of fungal species (*Alternaria* spp. and *Helminthosporium* spp.). The superficial symptoms of Karnal bunt have been confused with those of black point, but close examination reveals distinct differences. While both diseases give the germ end of the wheat a darkened appearance, black point is actually more of a darkening or discoloration of the pericarp (**C1** and **C2**, laminated insert). Upon examination under a dissecting microscope, no spores, nor anything that resembles teliospores, are visible. Also, the darkening caused by black point organisms may extend farther from the tip of the seed and be more general in its occurrence on the seed, often appearing on the ventral and dorsal surfaces. Black point shows no obvious erosion of the seed tissue as in Karnal bunt.

Other smut diseases such as common bunt (*T. laevis* or *T. tritici*) may occur in the samples. Consult with a bunted kernel identifier to verify.

To make a positive identification for any spore, making a microscope slide will be necessary to view the spores under high magnification.

Storing Positive Kernels

Step 1—Place bunted kernels in a plastic vial or petri dish.

Place all of the bunted kernels from the same sample in a plastic vial or petri dish. Using a black marker, label the vial with the sample number, your initials, and the date on the outside of the vial, and inside the vial on a white label.

Step 2—Verify presence of *Tilletia indica*.

Have an identifier with identification authority for the bunted kernel verify the presence of *T. indica* on the kernels found in a sample. Return all negative grain to its original packaging. Also return the positive sample (minus any bunted kernels) to its original packaging and place it in the positive storage area. Verify the presence of *T. indica* teliospores on the kernel by making a slide and storing all positive slides with proper labels. Retain voucher slides for a project-specified time in a secure storage container.

Step 3—Record findings.

Record your findings, initials, and date on the appropriate analysis worksheet (Figure D-3). Have the verifier initial in the appropriate space for the positive samples verified.

Step 4—Take care of used equipment.

Place all labware and instruments into the dirty bin for decontamination. Use new gloves, labware, and lab tools for each new sample. Continue with the next sample.

Decontaminating Labware and Instruments

Follow this procedure to decontaminate labware and instruments used in manual or automated methods:

Take all dirty labware and lab tools to a designated area for decontamination. As a precaution, keep labware "wet" until decontamination with the bleach solution. Soak all labware and lab tools in a 30 percent bleach solution for 15 minutes, rinse with clear water, dry, and return to the bunt area for the next use.

After examining all the samples, or at the end of a shift, decontaminate the area. Decontamination includes these activities:

- Bleach all workstation surfaces (hoods, chairs, tables, etc.).
- Make sure no dirty labware or instruments remain in the area.
- Sweep the floor and mop with 30 percent bleach solution.

When decontamination is complete, dispose of all shoe covers, hair covers, coveralls, and gloves into the biohazard container. Dispose of all waste during the examination process into the biohazard container. Turn off all hoods and lights when finished.

Important

Handle all samples as if positive to help prevent contamination.

Seed Analysis Methods

Background

The purpose of seed analysis laboratories is to determine the presence or absence of Karnal bunt, *T. indica*, teliospores in submitted wheat seed samples. For a seed lot to be considered negative, five replications must be performed and all five tests must be negative for the presence of *T. indica* teliospores. Seed lot samples with only spore fragments that cannot be measured can be resampled and run a second time.

Setting Up the Laboratory

Whether laboratory personnel process a few samples per day or a hundred, the same minimum facility requirements are necessary. By meeting these requirements, each laboratory will have the flexibility to use its available resources, while still maintaining a national standard. The seed analysis laboratory must have three basic areas:

- A reception area
- A dirty room
- A clean room

Combining functions performed in the dirty room and the clean room into one room is acceptable under the following conditions:

- Samples for processing come from a non-Karnal bunt area.
- No positive samples have been in the laboratory.

Once a suspect positive sample is encountered, these rooms and functions need to be separate. Also, workers should always process a negative control wheat sample with each group of samples once any samples entering the laboratory test Karnal bunt positive.

Important

Field sample personnel or individuals entering the grain analysis laboratory are not allowed entry into the seed analysis laboratory without proper decontamination safeguards. Showering and changing clothes is usually sufficient.

Reception Area

The reception area, a place for initial delivery of samples and decontamination of packaging, must be outside the laboratory facilities, whether literally outside or in a designated building, loading dock, or shed. This area can also be used for storing the remaining sample not used in the laboratory evaluation. The area must have access to running water.

Dirty Room

The dirty room is an area for opening dry seed samples, adding extraction solution, and transferring or weighing samples. The primary concern is to prevent dry, loose teliospores from becoming airborne and contaminating other samples and the external surfaces of the extraction equipment. The minimum equipment required is a clean air station or biological containment hood, to prevent direct air movement and ventilation from this room into the clean room. Between each sample, workers must wipe down all working surfaces with detergent and 30 percent bleach (6 percent sodium hypochlorite). Technicians must change gloves between samples and not wear contaminated garments into clean areas.

Clean Room

No sample should enter the clean room unless it is in liquid suspension. Workers moving from the dirty room into the clean room should have separate lab coats. Activities performed in the clean room are sample extraction, slide preparation, extraction ware decontamination, and microscopy. Laboratory personnel must decontaminate the extraction table surfaces between sets of samples. Workers must clean up any spills before they dry and spray the area of any spill with 30 percent bleach. After extracting samples on trays, remove and decontaminate the trays.

Receiving Samples

Background

This subsection describes procedures used to receive seed samples at approved laboratories for the analysis for *Tilletia indica*. Included are specific procedures for decontaminating the seed samples before they enter the laboratory, and log-in procedures.

Supplies

Laboratory personnel will need the following supplies to receive the samples:

- Bleach, (sodium hypochlorite), 6 percent
- Cardboard boxes
- Centrifuge tubes
- Chain-of-custody seals (if appropriate)
- Container to hold bleach solution
- Coveralls
- Dish pans, plastic, department store
- Garden hose with nozzle, hardware store
- Karnal bunt spore analysis worksheets, in-house designed
- Lab coats
- Latex gloves
- Plastic disposal bags, 12" x 24"

- Safety glasses
- Secure storage units for samples
- Spray bottle, 1-liter, hardware store
- Utility cart

Sample Decontamination

When samples arrive at the laboratory, make sure to receive them at a designated check-in/decontamination area. This area must be physically separate from the laboratory facility where samples are processed. A wash basin and hose setup is necessary at this location. Use the following procedure to check in and decontaminate the samples before bringing them inside the laboratory. Wear gloves, safety glasses, and a lab coat or coveralls during this procedure.

Step 1—Check samples.

Check each sample against the accompanying paper work to ensure matching sample number. Check all seals and samples for integrity.

Step 2—Decontaminate samples.

Decontaminate the sample containers using Table D-1 as a guide:

TABLE D-1: Decontamination of Samples

If the samples (or subsamples) are in:	Then:
Plastic bottles	1. REMOVE the bottles from the sample bag. 2. TIGHTEN the lids to ensure a water tight seal. 3. SUBMERGE each bottle in a 30 percent bleach solution prepared fresh daily. 4. After 15 minutes, RINSE the bottles with tap water from a garden hose. 5. GO to **Step 3.**
Plastic bags	1. MAKE sure the bags are sealed tightly. 2. FILL a spray bottle with 30 percent bleach solution, prepared fresh daily. 3. SPRAY the bags with the bleach solution and ALLOW the solution to remain on the bags for 15 minutes. 4. After 15 minutes, RINSE the bags with tap water from a garden hose. 5. GO to **Step 3.**

Step 3—Log-in samples.

Take the paperwork and samples, if necessary, into the sample receiving area of the laboratory for log-in.

Important

Never take any samples into the laboratory before decontaminating them.

Sample Log-In

Accurate sample log-in is very important. Be very careful to maintain each sample's identity.

Step 1—List the sample number on the worksheet.

List the assigned sample number on the analysis worksheet, and label sample containers with the assigned laboratory number. Place samples for spore analysis on a utility cart after logging-in and labeling them. Enter a "Quality Assurance (QA) Negative" laboratory control on the worksheet as well. Make sure to include on the cart a label for the control (for assembly in the weighing room). Use positive laboratory controls occasionally to prove the laboratory's ability to detect and identify positive samples.

Step 2—Label the centrifuge tubes.

Label centrifuge tubes with laboratory sample numbers for each sample in each set. Place the tubes in a disposable tube rack supplied with the tubes, and label the rack with the set number. Place the samples, worksheet, and centrifuge tubes on a utility cart and deliver these items to the weighing area or the solution addition area.

Reserve Sample Storage

Using approved sample journals, label excess sample bags and bottles with correct laboratory sample numbers. Sign and date any broken seals. Reseal the samples and dispose of used gloves in a biohazard bag at the decontamination area. Group samples by box number and place them in cardboard boxes in a secure storage area. Identify the boxes appropriately.

Extracting Samples

Background

This section describes procedures used at approved laboratories to extract teliospores of *Tilletia indica* from wheat or other crop samples. Included are specific procedures for preparation of extraction solution and preparation of Shear's mounting medium. Also included are procedures used to prepare extraction containers and measure out samples, and an alternative method for adding extraction solution to premeasured bottled samples. This section also details the extraction technique itself.

Supplies

Laboratory personnel will need the following supplies to process the samples:

- Aprons, rubber, 45" x 35"
- Bags, biohazard, 38" x 48"
- Bags, biohazard, 12" x 24"
- Balance, analytical
- Beakers, glass, 2-L and 1-L

- Beakers, 600 mL
- Bleach (6 percent sodium hypochlorite)
- Bottle, glass, 1-L with ground glass stopper
- Bottle, spray, plastic, 1-L, hardware store
- Bottles, HDPE wide mouth, 250-mL, plastic
- Bottles, wash, 500-mL
- Box, broken glass disposal
- Bulb, pipette
- Carboy, 20-L with spigot
- Cart, glassware
- Cart, utility, stainless steel
- Centrifuge, minimum 1,000 r/min required
- Citric acid monohydrate ($C_6H_8O_7.1H_2O$)
- Cups, disposable, paper, 5-oz
- Cups, disposable, paper, 3-oz
- Cups, portion, paper, 1-oz
- Cylinders, graduated, 25-mL, 250-mL, 500-mL, 1-L
- Deionized water
- Detergent
- Dissecting microscope, linen tester, or 10X magnifying glass
- Dust masks
- Ethyl alcohol, 95 percent, anhydrous
- Flasks, Erlenmeyer, graduated, 500-mL
- Funnels, glass, 60-mm ID top x 13-mm OD stem
- Funnels, glass, 100-mm ID top, 18-mm OD stem
- Glasses, safety
- Gloves, gauntlet, sizes 9, 10, and 11
- Gloves, latex, surgical
- Gloves, nitrile, sizes 8, 9, 10, and 11
- Glycerol
- Hood, biosafety with HEPA filter
- Hoods, fume
- Inverted light microscope, equipped with ocular micrometer

- Isopropyl alcohol, 70 percent
- Karnal Bunt Sieve Log
- Karnal Bunt Sieve Usage Log
- Labels, 1" x 2", 3 up x 10, Avery, 5160
- Liner, bench (substitute for plastic trays)
- Magnifying glass, illuminated
- Mixer, vortex, thermolyne
- Parafilm
- Pipette tips, 200-1000 µL, for Eppendorf adjustable pipettor
- Pipette tips, repeating
- Pipettes, Mohr, 1-mL and 10-mL
- Pipettor, adjustable, Eppendorf, 100-1000 µL
- Pitcher, 3000-mL
- Potassium acetate ($KC_2H_3O_2$)
- Rack, test tube
- Scoop, lab, stainless steel
- Shaker, orbital, thermolyne
- Sieve, nylon, 20 µm pore with square openings, 3" ID x 50 mm high, USDA[2]
- Sieve, nylon, 53 µm pore with square openings, 3" ID x 50 mm high, USDA[2]
- Silicone sealant
- Sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
- Stage micrometer
- Stir bars, magnetic, 1½" x ½"
- Stirrer, magnetic
- Syringes, 10 ml
- Tape, lab, blue, 1"
- Tape, lab, red, ½"
- Tape, yellow stretchy, lab safety
- Terri-wipes
- Timers

2 See attachment for construction of the sieves.

- Trays, plastic, 18" x 12" x ½" deep (substitute for bench liners)
- Tubes, centrifuge, disposable, plastic, conical, 15-mL
- Tubs, disinfectant
- Tubs, self-draining and holding
- Tween 20 or wetting agent

Preparing the Extraction Solution

Prepare the extraction solution in a separate, clean room. **Never bring grain samples into this room.**

Step 1—Fill a carboy with deionized water.

Wearing safety glasses and latex gloves, fill a 20-L carboy with 18 L of deionized water.

Step 2—Add detergent.

Using a graduated cylinder, add 12 mL Tween 20 detergent or wetting agent to the carboy. Rinse the cylinder several times with deionized water into the carboy.

Step 3—Label the carboy.

Place a strip of red lab tape at the 18-L mark and label as Tween 20 solution. Cap tightly and roll the carboy on a utility cart to make sure that the Tween is well dispersed in the water.

Preparing Shear's Mounting Medium

After centrifuging aqueous extracts of the sample and decanting the supernatant, re-suspending the remaining pellet in mounting fluid is necessary. Shear's mounting medium, the standard mounting medium for smut fungi, provides excellent buffering capacity. The fluid maintains the structural integrity of the Karnal bunt spores, while limiting evaporation. Additionally, the glycerol in the Shear's fluid maintains a refractive index of 1.47 at 27°C, which allows the effective transmission of light by the compound microscope. Shear's mounting fluid preserves sample extracts for future microscopic evaluation. You can use the procedure for preparation below, or purchase the mounting fluid from a commercial source.

Preparing 1 L of 0.2 M Na_2HPO_4 solution

Step 1—Weigh anhydrous Na_2HPO_4.

Wearing safety glasses and latex gloves, weigh 38.39 g of anhydrous Na_2HPO_4 into a tared 2-L beaker on a 3 decimal balance.

Step 2—Add deionized water.

Using a graduated cylinder, add 1 L of deionized water to the beaker and stir with a magnetic stirring bar until all granules are dissolved.

Step 3—Transfer the solution.

Using a funnel, transfer the solution to a 1-L glass stoppered bottle and label as 0.2 M Na_2HPO_4 with the date of preparation and your initials.

Preparing 1 L of 0.1 M citric acid solution

Step 1—Weigh citric acid monohydrate.

Weigh 21.01 g of citric acid monohydrate into a tared 2-L beaker on a 3 decimal balance.

Step 2—Add deionized water.

Using a graduated cylinder, add 1 L of deionized water to the beaker and stir until all granules are dissolved.

Step 3—Transfer the solution.

Using a funnel, transfer the solution to a 1-L glass stoppered bottle and label as 0.1 M citric acid solution with the date of preparation and your initials.

Preparing 600 mL of Shear's Mounting Medium

Step 1—Transfer Na_2HPO_4.

Using a pipette, transfer 19.45 mL of 0.2 M Na_2HPO_4 into a 1-L beaker.

Step 2—Transfer citric acid.

Using a 1-mL pipette, transfer 0.55 mL of 0.1 M citric acid solution into the same beaker.

Step 3—Add potassium acetate and deionized water.

Add 6 g of potassium acetate and 280 mL of deionized water to the beaker.

Step 4—Stir to dissolve solids.

Stir with a magnetic stirring bar until all solids are dissolved and the solution is uniform in appearance.

Step 5—Add glycerol and ethyl alcohol.

Continue stirring and add 120 mL of glycerol and 180 mL of 95% ethyl alcohol to the beaker.

Step 6—Transfer the solution.

After the solution is uniform in appearance, use a funnel to transfer to a 1-L glass stoppered bottle. Label as Shear's mounting medium, and include the date of preparation and your initials.

Step 7—Clean glassware.

Clean all glassware with lab detergent and rinse with tap water followed by deionized water. Place pipettes in the pipette washer with tips up.

Optional Method for Preparing Extraction Flasks

Prepare and label an Erlenmeyer flask containing extraction solution for each sample that requires weighing or measuring out before extraction. Carry out the following procedure in the room designated for preparing extraction solution:

Step 1—Fill flasks with extraction solution.

Wearing safety glasses and latex gloves, fill 500-mL flasks to the 100 mL mark with extraction solution directly from the 20-L carboy. Place two 3-oz disposable paper cups over the mouth of each flask immediately after adding the solution.

Step 2—Label flasks.

Label one flask for each sample in the set to be extracted. Place the flasks for that set on the top shelf of a utility cart in the order listed on the worksheet and move it to the sample weighing area.

Step 3—Place worksheet and tubes on cart.

Place the spore analysis worksheet and labeled centrifuge tubes on the top shelf of the cart. Place the samples in order from left to right on the second shelf of the cart and take the cart to the room designated for measuring out the samples.

Weighing and Measuring the Samples

It is preferable to have 50-g pre-measured samples arrive at the laboratory in sealed nalgene bottles that are disinfected with chlorine solution before entering the laboratory. Use these procedures when larger, unmeasured, or dirty samples arrive at the seed testing laboratory.

Take samples that require weighing to the sample weighing area. It is recommended that this procedure be carried out in a biosafety hood fitted with a HEPA filter. Generally, the only samples of this type are bin sweepings or other “dirty” samples that contain mostly dirt and chaff and very little grain. **Measure most other samples by volume rather than by weight.** Consult your supervisor if weighing or measuring unusual sample matrices or sample types is necessary. **Wear safety glasses, latex gloves, and a lab coat or overalls.**

Sanitizing the hood

Before weighing or measuring any samples, first sanitize the hood thoroughly.

Step 1—Turn on power.

Turn on power to the hood, light, and balance.

Step 2—Sanitize the hood.

Thoroughly sanitize the interior of the hood (including walls, glass door, and ceiling) by spraying with 30 percent bleach solution. This also includes the balance and the area under the balance. Let the bleach stand for 15 minutes.

Step 3—Spray hood again.

Spray the hood again with the bleach solution and wipe dry with terry wipes. Dispose of used terry wipes in the biohazard bag.

Step 4—Clean bleach residues from hood.

Spray the entire hood with 70 percent isopropyl alcohol and wipe dry. Doing this will help to clean bleach residues from hood surfaces, particularly the glass window of the hood. Dispose of used terry wipes in the biohazard bag.

Weighing the samples

For samples that are very dirty and contain very little sample by weight, measure them out by weighing at least a 2.0 g portion of sample, up to 50 g, on an analytical balance. Wear a dust mask when handling dirty and dusty samples.

Step 1—Take a sample from the cart.

From the cart containing the sample set to be weighed, take a sample and make sure that the sample number and the number on the container of extraction solution are identical. Place the sample and the corresponding container in the hood. Pull down the hood door to allow only enough space to move sample containers in and out of the hood.

Step 2—Put on gloves.

Put on a new pair of latex gloves; place one of the two paper cups on the balance and tare it.

Step 3—Mix the sample.

Mix the sample well by inverting and rolling thoroughly. Open the sample container and use one paper cup to scoop out 2.0 g ± 0.1 g of sample into the cup on the balance. Transfer excess sample that has not touched the hood or balance back to the sample container.

Step 4—Transfer the contents.

Transfer the contents of the paper cup to the sample container and seal the opening with parafilm.

Step 5—Close the sample container.

Close the sample container and place the cups in the palm of one gloved hand and fold that glove over them. Now place this glove in the palm of the other hand and repeat. Dispose of the used materials in the biohazard bag.

Step 6—Remove the sample from the hood.

Remove the sample and the container from the hood and place the container back on the cart. Return the sample to the storage area.

Step 7—Repeat procedure for additional samples.

For additional samples, repeat **Steps 1 through 6** using new gloves for each sample. Deliver the cart to the extraction lab after weighing all samples. Return sample bags to the storage area.

Step 8—Maintain cleanliness.

Always maintain cleanliness of the weighing area. If any sample falls on the balance or the hood floor, do **not** place in any sample container. Dispose of the sample with the gloves and cups at the end of the weighing cycle. If any sample falls on the hood or balance, sanitize the area of the spill before weighing the next sample. Wipe up the spilled sample and dispose of it in a biohazard bag. Spray the area where the sample fell and the surrounding area with the bleach solution. Wipe up the bleach solution and dispose of the dirty gloves and terry wipes in the biohazard bag.

Step 9—Resanitize the hood and balance.

Resanitize the hood and balance after each sample.

Measuring the samples

Measuring the samples by volume is preferable to measuring by weight. Measure by volume these types of samples:

- Samples that do not arrive premeasured in plastic bottles
- Samples that require additional analyses

Measure these samples into clean glass or new plastic sample containers.

Step 1—Measure the samples.

The procedure is the same as for weighing samples in the previous section, except that this procedure involves measuring the samples by scooping three 1-oz cupfuls into a container (may be different for other types of crops). Use the paper portion cups for this purpose and then dispose of them in the biohazard bag.

Step 2—Take plastic bottles by cart.

When measuring the samples into plastic bottles, take them by cart to add extraction solution before taking them to the extraction lab. See **Adding Extraction Solution** below.

Step 3—Measure control wheat.

Also, measure control wheat into containers using the above procedure. Seal these containers of control wheat and store them in the solution addition room for later use in extraction sets as needed.

Step 4—Resanitize the hood.

When finished weighing or measuring samples for the day or at the end of a shift, resanitize the hood.

Adding Extraction Solution

When an extraction set consists of premeasured samples in plastic bottles, add the extraction solution to the bottles in the hood in the solution addition room. Take only decontaminated samples into this room. Wear safety glasses and latex gloves throughout this procedure.

Step 1—Turn on power and sanitize the hood.

Turn on power to the hood and sanitize the hood as described in the subsection Sanitizing the hood. Pull down the hood door to allow only enough space to move sample bottles in and out of the hood.

Step 2—Check samples.

Before adding solution to any of the bottles, first check to make sure that each sample on the utility cart has a matching sample number listed on the worksheet. The bottles should also appear to contain approximately 50 g of sample for wheat; other crops may be different. If any bottle appears to contain too much or too little sample, call it to the attention of your supervisor before measuring a new sample from the bag of grain retained in storage.

Step 3—Add extraction solution.

For each sample, place bottle(s) in the hood and put on a new pair of latex gloves. Add extraction solution from the carboy on the counter outside the hood to a clean, disposable paper cup. Fill to a level equal to the mark on the reference cup next to the carboy. This should be approximately 100 mL of solution. Place the cup of solution in the hood and open the sample bottle. Add the solution to the bottled sample. Tighten the bottle cap and place the sample back on the cart. After completing a sample, place the cup(s) in the palm of one gloved hand and fold that glove over it. Now place this glove in the palm of the other hand and repeat. Dispose of the gloves and cup in the biohazard bag. Repeat this procedure for each sample on the cart, using a new pair of gloves and cup for each sample. Do not reuse paper cups. Once extraction fluid has been added to the sample containers, samples **must** be extracted within 30 minutes.

Step 4—Resanitize the hood.

When finished weighing or measuring samples for the day or at the end of a shift, resanitize the hood.

Extracting the Samples

The clean room must have sufficient counter space (approximately 15 ft) to carry out the extractions. The lab must also have a shaker table, a chemical fume hood for bleach baths, and sinks to rinse glassware. A separate counter top is necessary to support the centrifuge station.

Extract the samples in a clean room, always wearing safety glasses. As an added precaution, wear protective clothing such as a rubber apron and heavy rubber gloves when decontaminating glassware with bleach solution. Wear latex gloves when extracting samples and change the gloves between samples.

Setting up for extraction

Step 1—Position bench liner or plastic trays.

Place the laboratory bench liner on the bench top with the plastic side down. To prevent confusion when processing multiple samples, use lab tape to mark out lanes approximately 30 cm wide. Another approved method is to use individual plastic trays (18" x 12" x ½") for each sample. Plastic trays will contain spills, and tubes cannot roll into the wrong lane. The trays also work well at smaller labs that do not have room to set up multiple lanes.

Step 2—Lay out beaker and sieve sets.

Place one 600-mL beaker toward the rear of each lane and set a 20 µm pore nylon sieve on top of it. Place another 600-mL beaker in front of the first beaker and set a 53-µm pore nylon sieve on top of that beaker. Lay out as many beaker and sieve sets as there are samples to extract, and place centrifuge racks between each set. Place small glass funnels (60 mm ID) in the center of the bench, along with pitchers and wash bottles containing tap water. When using the plastic tray option, keep all the extraction equipment for one sample in that sample's tray. Assure that flasks, beakers, and centrifuge tubes are properly labeled with corresponding sample numbers.

Step 3—Prepare bleach baths.

Prepare bleach baths by filling large disinfectant tubs inside fume hoods (or in a separate room) with 30 percent bleach solution. The bleach solution baths need to contain a sufficient volume of solution to completely submerge the glassware being decontaminated.

Extracting the samples

Step 1—Shake samples.

Place sample bottles or flasks containing extraction solution on an orbital shaker. Secure the containers in the shaker so that the fit is appropriate for the type of container used. Shake samples for 5 minutes at 200 r/min. Do not allow samples to remain in the extraction solution for longer than 30 minutes due to release of starch granules. While samples are shaking, remove the centrifuge tubes that arrived with the samples from the disposable rack and place one per lane on the bench top. Place the disposable centrifuge tube rack and worksheet in the center of the bench. Sanitize the utility cart with 30 percent bleach solution before returning it to the sample receiving area.

Step 2—Place samples with tubes.

After shaking the samples, place each in the lane/tray with the tube that corresponds to that sample. Double check to make absolutely sure that tubes and sample bottles have matching sample numbers.

Step 3—Pour sample into 53-µm sieve[3].

For each sample, invert the bottle or swirl the flask to re-suspend the grain and debris. Immediately pour the contents into the 53-µm sieve, collecting the filtrate in the 600-mL beaker. Add an additional 100 mL of water to the container, swirl or recap and shake the bottle, and then pour into the sieve to rinse the grain. Take care to avoid splashing the sample solution during sieving procedures. Reextract samples if cross-contamination occurs. Rinse a minimum of twice, preferably three times, tilting the sieve on top of the beaker to aid draining. Place the empty extraction container on the glassware cart.

Step 4—Pour filtrate into 20-µm sieve[3].

After the sieve has drained, discard the grain into a small disposal bag, and place the used sieve on a glassware cart. Pour the filtrate from the beaker into the 20-µm sieve, collecting the filtrate in the second 600-mL beaker. Add an additional 100 mL of water to the first beaker, swirl, and pour through the sieve. Use a wash bottle to rinse down the side walls of the beaker, and pour this rinsing into the sieve as well. Rinse a minimum of twice, preferably three times. Place the used beaker on the glassware cart.

The sieves are manufactured with a ring to make them stackable as an option for using them together on one beaker.

Step 5—Rinse the nylon screen.

Once the 20-µm sieve has drained, tilt the sieve on top of the beaker and use the wash bottle to rinse down the sides and upper portion of the nylon screen. Tip the sieve over the 50-mL beaker at a 45° angle and use a wash bottle with a steady stream to rinse debris into the beaker. Repeat this rinse twice. Debris will pool in the lower portion of the sieve.

3 Follow quality assurance checks for sieves.

Step 6—Rinse debris into the centrifuge tube.

Open the centrifuge tube and place it upright in the centrifuge tube rack. Use a wash bottle to rinse debris from the 20-µm sieve into the tube. You may also use a small glass funnel to facilitate this transfer. To assure that all tubes have the same amount of liquid, fill the tubes with liquid to just below the screw cap threads. Recap the tube and return to the disposable centrifuge rack. Pour the extraction filtrate into a 20-L carboy containing ½ gallon of bleach. Place filtrate from samples treated with fungicide in a separate carboy. Place used glassware and sieve on the glassware cart. As an added precaution against contamination by airborne spores, soak all used labware in a water bath when not in use, until decontamination.

Step 7—Repeat procedure for each sample.

Repeat **Steps 3 through 6** for each sample in the extraction set. If extracting multiple samples becomes necessary, processing all samples through one step before proceeding to the next step is most efficient. **Be sure to remain undistracted while doing this.** Take care not to mix samples or confuse the extraction steps.

Step 8—Notify your supervisor in case of spills.

In case of a sample spill, notify your supervisor to decide if a second extraction of the sample is necessary. If so, discard the sample and show the result for that sample as “L” on the worksheet with your initials and date. Record all lab accidents in the lab notebook kept at the centrifuge station. If a sample spills on the bench liner, replace it with a clean liner. If any sample contacts the bench surface, spray a 30 percent bleach solution on the area to decontaminate it, and wipe with a clean towel.

Step 9—Transfer glassware and sieves to bleach bath.

Immediately transfer all used glassware and sieves from the glassware cart to a bleach bath and soak them for 15 minutes. Do not allow sieves to remain in the bleach bath for more than 15 minutes, as this will rapidly degrade the nylon screens. Rinse glassware and sieves thoroughly with hot tap water. Hang glassware on a rack to drain and place sieves in self-draining plastic tubs. Discard plastic sample bottles after decontaminating them in the bleach bath.

Step 10—Centrifuge the tubes.

After preparing all of the tubes for the set, take them to the centrifuge station and centrifuge at 1,000 r/min for 5 minutes. Pour off the supernatant and re-suspend the pellet by pipetting 500 µl of Shear’s mounting medium into the tube. Recap and vortex a few seconds to mix the pellet with the Shear’s fluid. Record the set information in the centrifuge log book before delivering the centrifuge tubes and worksheet to the microscope area.

Cleaning Up the Extraction Lab

Clean up the clean room by following these steps:

Step 1—Remove the bench liner.

At the end of each extraction series or as necessary, remove the bench liner and discard in a large biohazard bag along with the small bags of extracted grain and other potentially contaminated materials. Tie these bags shut with lab tape. Place in red biohazard containers in a designated hazardous waste area. When using plastic trays, removing and discarding bench liners is not necessary.

Step 2—Dispose of the filtrate.

Pour the bleached sample filtrate from the carboy down a sink drain as necessary. After filling any carboys containing filtrate from seed treated with pesticide, pour the contents into a labeled hazardous waste container.

Step 3—Sanitize surfaces.

Spray all bench tops, carts, and other potentially contaminated surfaces with 30 percent bleach solution. Wipe all these surfaces down at the end of each extraction series. Sweep and mop the floor in the clean room with bleach water at the end of each extraction series as well.

Fumes from chlorine bleach solutions can be hazardous if inhaled. Make sure the area is properly ventilated and consider having personnel use a respirator during disinfection.

Step 4—Sanitize pitchers and wash bottles.

Soak all pitchers and wash bottles in 30 percent bleach solution for at least 15 minutes at the end of each shift. Take care to rinse these items thoroughly before using them again.

Preparing Slides

Background

This section describes procedures used to prepare sample slides for microscopic examination for *Tilletia indica* teliospores. Included are specifications for lab garments and set-up of the working area. The section also identifies priorities in the processing of samples. Topics include receiving sample tubes, preparing slides, filling out slide labels and data sheets, preparing quality assurance slides, handling spills, and cleaning up. Keep the slide preparation area separate from other sample processing areas.

Supplies

Laboratory personnel will need the following supplies to prepare the slides:

- Applicators, cotton-tipped
- Bags, biohazard 38" x 48"

- Bags, biohazard 12" x 24"
- Beakers, 150-mL, 250-mL, and 400-mL
- Bleach (6 percent sodium hypochlorite)
- Bottles, plastic, narrow mouth
- Bottles, plastic, spray
- Box, microscope slide
- Boxes, glass disposal, floor
- Bulbs, rubber pipette
- Cabinet, storage
- Caps, bouffant, 21"
- Centrifuge, minimum 1,000 r/min required
- Cleanser, skin, antimicrobial
- Coats, lab, polyethylene, medium, large, and extra large
- Cover slips, microscope, 22 x 50 mm
- Cytoseal 60, low viscosity
- Flasks, Erlenmeyer, 500-mL and 1,000-mL
- Fume absorber
- Gloves, latex, medium and large
- Graduate, transfer pipettes, polyethylene, 5-mL
- Labels, multi purpose, white
- Petroleum jelly (100 percent)
- Pipettes, Pasteur, disposable, 9-inch
- Pipettes, transfer, Padl-Pet 0.5-mL
- Scissors
- Shear's mounting medium
- Slide warmer
- Slides, microscope, 3" x 1"
- Teri Reinforced Towels
- Tray, slide, aluminum or plastic

Store all supplies in a designated Karnal bunt cabinet. Designate a clean area specifically for slide preparation. Clean all surfaces in this area with a 30 percent bleach solution before setting up the work area.

Setting-Up the Working Area

Prepare the slides under a fume extractor or canopied laminar flow hood to minimize exposure to chemicals. Keep a supply of Shear's solution on hand. During the process of slide making, Shear's solution is necessary to dilute dense samples or to add liquid under the cover slip as needed.

Step 1—Disinfect surfaces.

Before making any slides, disinfect surfaces with 30 percent bleach solution from a spray bottle.

Step 2—Fill plastic bottles.

Fill separate plastic bottles with Cytoseal and Shear's solution. Label the bottles.

Step 3—Fill a container with bleach.

Fill a container half full with 30 percent bleach solution for disposal of Pasteur pipettes.

Step 4—Add bleach to a plastic bottle.

Label a large plastic screw-cap bottle with "Quarantine Material." Add 200 mL of 30 percent bleach. Place the bottle by the centrifuge. Use this bottle to decant supernatant from centrifuged samples.

Step 5—Use new supplies for each sample.

Use new slides, cover slips, and pipettes for each sample. Check supplies and restock if needed. Place plastic pipettes, Pasteur pipettes, and padl-pets in separate, clean beakers.

Step 6—Turn on the slide warmer.

Under a hood place one layer of reinforced towel. On top of the towel, place a colored sheet of paper (throw away between samples). Turn on the slide warmer to approximately 58°C.

Identifying Sample Priority

Sample processing priority will vary by region. Laboratory managers will establish sample priorities based on program needs. Contact the laboratory manager if sample priority is in question.

Processing Sample Tubes

Step 1—Count tubes and data entries.

Count the tubes and data entries on the sheet. Make sure that the sample number matches the data sheet number, along with the set number. Report discrepancies to the laboratory manager.

Preparing the Slides

Step 1—Extract the pellet.

Use a new piece of paper, pipettes, gloves, slide(s) and cover slip(s) for each sample. Use clean pipette bulbs for each sample if not using cotton-plugged Pasteur pipettes. Move the sample tube to be prepared to the front of the rack. Put on new gloves. Use a Pasteur pipette with a pipette bulb to extract the whole pellet at the bottom of the tube.

Squeeze the pipette bulb before inserting into the tube. Slowly release the pressure on the pipette bulb to extract the pellet. Avoid extracting excess supernatant to reduce the number of slides required per sample pellet. **However, transferring the entire pellet to slides is important.**

Step 2—Homogenize dense samples.

When processing dense, muddy, or thick samples, introduce the Pasteur pipette into the sample tube and homogenize the sample by pressing the pipette bulb several times. Homogenize the sample between each slide. Maintain pressure on the pipette bulb and remove the pipette from the tube. Hold the pipette at approximately a 45° angle above the slide and expel two or three small drops onto the slide depending on the sample density. For dense samples, dispense two drops of sample onto the slide and using a plastic pipette, place a drop of Shear's mounting medium in the middle. Doing this will help produce a nonlayered, uniform slide.

Step 3—Spread the sample.

Use the end of the Pasteur pipette to spread the sample to a small rectangle. Avoid placing excess sample on the slide to eliminate seepage, or preparing a slide that will be too dense to read easily. Use more slides if necessary.

Step 4—Place a cover slip on the slide.

Return the tube with Pasteur pipette to the sample rack or pipette bucket. Place a cover slip over the drops on the slide. Do this by holding the cover slip above the slide at approximately a 45° angle and then touching the drop nearest the edge of the slide with the edge of the cover slip. A padl-pet pipette may help to move the opposite side of the cover slip down slowly until it touches the slide. This may help to reduce air under the cover slip. Remove air bubbles only by tapping lightly on the cover slip. Do **not** lift the cover slip to remove air bubbles.

Step 5—Position the cover slip.

Leave a ¼ inch margin between the cover slip and the slide edge opposite the label. Place the Pasteur pipette in 30 percent bleach solution. Cap the sample tube and discard the tube only after making the label.

Step 6—Seal the cover slip.

Seal the slides if you are unable to read them immediately. All positive voucher slides must be sealed for storage.

Dispose of the padl-pet pipette in a disposal bag. Seal the cover slip to the slide after preparing all of the slide(s) from a sample. Using a plastic pipette, extract the needed amount of Cytoseal. Seal the long sides of the cover slip, then the narrow sides to the slide. Release the Cytoseal (or clear nail polish) slowly, running the pipette along the edge of the cover slip. Avoid making a pass over an area more than once, but inspect the perimeter of the cover slip for missed or poorly sealed areas. Touch up with Cytoseal as needed. Discard excess Cytoseal from the plastic pipette into a plastic screw cap bottle for disposal. Keep containers of Cytoseal and Shear's mounting medium closed when not in use. Remove gloves and discard along with sample tubes, colored paper, and plastic pipettes into the biohazard bag.

Making Slide Labels and Filling Out the Data Sheet

Make slide labels only for the current sample. **Never** use correction fluid on slide labels; make a new label.

Step 1—Prepare slide labels.

Prepare the label from top to bottom using a permanent ink pen as instructed in Table D-2.

TABLE D-2: Preparation of Slide Labels

Description	Slide Label (Example)
Sample Code	CG-96-172
Number of slides prepared	1/3, 2/3, 3/3, etc.
Set Number	Set 3052
Person who read the slide	Reader Initials:
Authorized person who verified the positive finding of *Tilletia indica* or person who completed negative reread	Ver. Initials:
Date the sample was read	Date:

Step 2—Label the slides.

Affix the label(s) to the slide(s). Record the number of slide(s) prepared for the sample in the comment section of the data sheet.

Step 3—Warm the slides.

Keeping the slide horizontal, place it on the warmer for 5 to 15 minutes. Transfer slides to a slide tray. Repeat the slide preparation for each sample in the rack.

Step 4—Complete data sheet.

After completing a data sheet of samples, count the number of slides prepared and compare it with the total listed on the data sheet. Initial "Slide Preparer" section on the data sheet.

Step 5—Place tray and data sheet on counter.
Place the tray and data sheet on the counter marked for reading. After readers read the slides and record the results on the data sheet, they should return each slide to its appropriate sequential location in the slide tray.

Handling Spills

Cover all spills of supernatant or sample with 30 percent bleach solution and a reinforced towel. Let soak for 15 minutes. Wipe up the bleach and rinse with water. Discard towels in a biohazard bag.

Cleaning Up

Discard all used disposables, clean slide-making area with 30 percent bleach solution, and replace supplies. Turn off the extraction hood and slide warmer.

Examining Slides for Karnal Bunt

Background

This section describes procedures used for the microscopic examination for and identification of teliospores that may be *Tilletia indica*. A compound microscope is necessary to examine the slides. Included are procedures for selecting, examining, and identifying Karnal bunt. The section also includes procedures for handling positive slides, negative slides, and record keeping. Microscopic identification requires a room with sufficient counter top and chair space to hold multiple microscope stations.

Selecting a Slide

Select a slide based on the priority established by the laboratory manager. Before taking a slide, place your initials in the "Slide Taken By" box and record the slide number in the "Slide" box of the *Tilletia indica* Spore Analysis Worksheet (Figure D-6).

Examining the Slide

Examine the slide using the 10X objective and 10X ocular. Begin at one corner of the cover slip, including the area on the edge of the cover slip containing slide sealant. Scan the entire slide, either vertically or horizontally, left to right or right to left. Be consistent in your scanning procedure and be aware that the image is reversed. Take care to remember your direction if interrupted during a scan. A simple method is to place a pen or pencil on the table pointed in the direction of the scan at the time of interruption.

Identifying Karnal Bunt Teliospores

After carefully examining the *Tilletia indica* reference slides, and recognizing the variability in mature and immature spores, look for similar objects. Refer also to the laminated color illustration titled **Karnal Bunt Teliospores, *Tilletia indica* Mitra.** Upon finding a suspect spore, change the objective to 40X and look for the following characteristics:

Mature Teliospores

- Spherical shape with an average diameter of 39 µm and a range of 24–47 µm. Atypical spores that are broken and/or irregularly shaped may be present.
- Color light brown to near black
- Smooth clear or yellowish sheath enveloping the spore that is visible when focusing on cross section of the spore
- Distinctive truncate projections that are visible on the circumference of the spore when focusing through a cross section and dark brown spots in the middle of the spore when focusing on the surface of the spore

Immature Teliospores Combined with Mature Teliospores

- Similar shape but often smaller than mature teliospores
- Color yellow to lighter brown
- More distinct, finer ornamentation than mature teliospores

Any of the above characteristics will suggest a suspect find of *Tilletia indica* teliospores.

Verifying Teliospores

Refer suspect teliospores (intact, broken, or fragmented) to the appropriate authority for verification.

If identified as *T. indica*, the verifier will do the following:

1. Circle the position of the verified teliospore using a permanent marker.
2. Print his or her initials on the slide label and in the "Verified By" column of the *Tilletia indica* Spore Analysis Worksheet.

If the teliospores are indicative of *T. indica*, but are undersized or comprised of fragments less than 50 percent, the verifier will follow the steps above and in addition will do the following:

3. Record an asterisk (*) in the "# of Spores" column.
4. Add the statement "undersized spores" or "fragments" to the "Comments" column on the *Tilletia indica* Spore Analysis Worksheet (Figure D-6).

Counting Teliospores as Positive

A seed sample will be considered positive for *T. indica* if at least one spore is found equal to or exceeding 33 µ in diameter and possessing morphological characteristics consistent with *T. indica* and not other *Tilletia* species (i.e., *T. walkeri*, *T. horrida* syn. *T. barclayana*). All teliospores used to make a positive determination for a sample must be verified by an identifier with identification authority for the *T. indica* teliospore in absence of the host.

Keeping Records

Maintaining accurate records of samples is extremely important. Always record information in ink. **NEVER** use correction fluid on laboratory documents, slide labels, or sample labels. If a correction is necessary, draw a line through the incorrect entry, enter the correct entry, and place your initials and date beside the correct entry.

Tilletia indica Microscopy Log Sheet

Keep your own record of the samples and their counts using the *Tilletia indica* Microscopy Log Sheet (Figure D-7). Record the sample #, set #, and 1st/2nd reads. Use the Sample # section to record slide number, if there are multiple slides. For example, "1 of 2 slides." Record spore coordinates in the Comments section.

Slide labels

After completing a slide, place your initials **and** the date on the slide label.

Tilletia indica Spore Analysis Worksheet

Return the slide to the appropriate slide holder. Record the following data on the *Tilletia indica* Spore Analysis Worksheet (Figure D-6):

- Your initials
- The date

Have the verifier who confirmed the first *T. indica* teliospore record his or her initials under the *Confirmed By* section.

Interpreting Laboratory Results

A laboratory result, whether positive or negative, stands on its own. Repeating a test does not necessarily invalidate or validate the previous test unless the results are the same.

Rating on the Karnal Bunt Spore Analysis Worksheet

After a slide reader reads the slides and documents the results on the worksheet, the laboratory manager will rate the results using the following codes (only one rating is necessary per sample):

- **P** for **positive**. Used when at least one spore of *T. indica* is confirmed for the sample.
- **N** for **negative**. Used when no *T. indica* spores are confirmed for the sample.
- **X** for **retest**. Used when the verifier cannot make a positive identification.
- **L** for **laboratory accident**. Used when a sample is spilled, mislabeled, etc. Retesting is required.
- **R** for **redundant**. Used when there are multiple test results for the same sample, if a rating of P, X, or L is used. One test is rated and the others are rated with an R.

Quality Control

The following are recommended minimum procedures for quality control purposes:

- Have a different reader do a second examination of every 5th negative slide for beginners. Experienced readers should have every 10th negative slide re-read.
- Include slides of known negative and positive controls to test readers and verifiers.

Training Technicians and Verifiers

Background

To assure accuracy and efficiency when laboratory personnel are processing samples, technicians need to be appropriately trained and successfully complete documented procedures. Technicians should complete the training program before actually processing real samples.

Bunted kernel verifiers should receive appropriate training, including identification and equipment usage. Spore verifiers, as well, should have training in examining isolates of *Tilletia indica*, and should meet certain educational and experience requirements. Slide readers should receive all laboratory procedure training, including gross teliospore identification and slide preparation. Written training records for all analysts covering all procedures will be maintained at the laboratory.

Important

Do **not** allow technicians involved in bunted kernel analysis or field personnel to enter the laboratory areas where seed analysis is taking place.

Suggested training content for all laboratory personnel

- Presenting general orientation about Karnal bunt
- Identifying and explaining the purpose of processing areas
  - Setting up the reception (sample receiving) area
  - Setting up the dirty rooms
  - Setting up the clean rooms
- Receiving samples
  - Maintaining supplies
  - Decontaminating samples
  - Logging-in samples
  - Storing reserve samples

- Extracting samples
  - Maintaining supplies
  - Preparing Shear's mounting fluid
  - Preparing sample containers
  - Weighing and measuring samples
  - Adding extraction solution
  - Extracting the samples
  - Cleaning up the extraction lab
- Preparing slides
  - Maintaining supplies
  - Setting up the working area
  - Establishing priority and processing sample tubes
  - Preparing the slides
  - Making labels and data sheets
  - Handling spills and cleanup
- Examining slides for Karnal bunt
  - Selecting and examining slides
  - Identifying Karnal bunt teliospores
  - Verifying teliospores (for spore verifiers)
  - Counting teliospores, if appropriate
  - Reading positive slides
  - Keeping records and interpreting laboratory results
- Examining wheat kernels for Karnal bunt (automated and manual methods)
  - Maintaining supplies
  - Processing samples
  - Preparing the workstations
  - Identifying *Tilletia indica* from bunted kernels
  - Storing samples
  - Decontaminating equipment and cleaning up

Selecting and Training Bunted Kernel Verifiers

Background

The Karnal bunt bunted kernel verifier must be able to positively identify *Tilletia indica* Mitra, the causal agent of Karnal bunt, in or on the wheat grain kernel to the exclusion of other fungi. The bunted kernel verifier serves as the final authority for the identification of Karnal bunt from grain samples.

Requirements

The bunted kernel verifier should have work experience in microscopy, or other work experience in an agricultural or biological field. The verifier must pass the bunted kernel identification course given by a PPQ Identifier.

Job Description

The incumbent is responsible for verifying the identification of *T. indica* bunted kernels found during Karnal bunt screening to the exclusion of other fungi. This identification will be based on a visual examination of infected grain. Symptoms must be verified with a microscopic examination of Karnal bunt teliospore gross morphological characteristics.

Training

Kernel verifiers need to complete the following training activities:

- Receive introduction and background information on Karnal bunt and training and testing from a PPQ Plant Pathology Identifier.
- Learn about *Tilletia indica*, specifically:
  - Life cycle
  - Initial infection
  - Morphology of symptoms at various stages of infection
  - Teliospore morphology
- Recognize other fungi that infect wheat kernels:
  - Black point symptoms (*Helminthosporium sativum*, *Alternaria* sp.)
  - Common bunt (*T. laevis*, *T. tritici*) kernel infection/teliospore morphology
  - Dwarf bunt (*T. controversa*) kernel infection/teliospore morphology
- Learn slide making and record keeping procedures.
- Observe grain examination machine demonstration and practice runs with known infected samples.
- Examine specimens.

Identification Authority

After the incumbent completes the required training and shows the ability, through testing or correct identifications, to identify *T. indica* bunted kernels in Karnal Bunt Project samples, designated USDA/APHIS personnel (PPQ Plant Pathology Identifiers) will grant identification authority for use on the Karnal Bunt Project.

Selecting and Training Spore Verifiers

The Karnal bunt teliospore verifier must be able to positively identify *Tilletia indica* Mitra, the causal agent of Karnal bunt, to the exclusion of other fungi. The verifier serves as the final authority for identification of Karnal bunt.

Requirements

The verifier should hold a college degree in mycology, plant pathology, or other related fields. The verifier should have work experience with microscopy, fungal taxonomy, and morphology, or course work in plant pathology and mycology and work related experience in plant pathology and mycology. Knowledge or experience in plant pathology is desirable but not a requirement. For those without a degree in plant pathology or a related field, the requirement should be a foundation in mycology or plant pathology with recent work experience in that area (for example, PPQ Port Identifier or backup, in plant pathology).

Job Description (the incumbent is responsible for these duties)

1. Verifies the identification of *T. indica* teliospores found by microscopists during Karnal bunt screening to the exclusion of other fungi.
2. Distinguishes by microscopic examination other species of *Tilletia* that could be encountered (for example, *T. walkeri* and *T. horrida*).
3. Using established guidelines on spore size, makes determinations of when to send “suspect spores” to National Mycologist for final determination.
4. Confirms wheat kernels infected by *T. indica*.

Training

Training will consist of microscopic examination of several isolates of *T. indica*. Depending on their age and condition, teliospores will exhibit significant variation in size, shape, and color. Verifiers will frequently find immature and fragmented spores.

Karnal bunt teliospore verifiers will complete the following training activities:

- Analyze slides of *T. indica*, *T. horrida* (rice smut), and *T. walkeri*.
- Review microscope slides of actual samples to familiarize themselves with the other fungi commonly found associated with wheat and pollen grains frequently encountered.

- Scan a minimum of 10 training slides of actual wheat samples, with known numbers of teliospores. At least one of these slides will be a negative control. The purpose of this training slide exercise is to familiarize the verifiers with the microscopist's tasks, not for them to become skilled microscopists. For the verifier to qualify as a reader, the verifier must meet the requirements for readers described in the previous subsection, Training Technicians.
- Examine wheat kernels macroscopically and learn how to positively confirm a bunted kernel caused by *T. indica*.

Retesting

A system of retesting verifiers should be in place. This should include examining slides with various wheat smuts, particularly variations of *Tilletia* species.

Identification Authority

After the candidate completes the required training and shows the ability to identify *T. indica* teliospores and bunted kernels in Karnal Bunt Project samples, designated USDA personnel (PPQ National Mycologist or designee) will grant identification authority for use on the Karnal Bunt Project.

Preparing New Sieves for Use

Background

New sieves generally arrive at the laboratory by mail from the USDA manufacturer. These sieves should arrive pre-labeled with the mesh size (20 or 53µ), month and year of manufacture, and unique serial number. Each size should be uniquely color coded. Upon receipt of newly manufactured sieves, it is the laboratory's responsibility to ensure the quality of each sieve prior to use.

Because sieves have a limited life, quality control measures require replacement at prescribed intervals. It is important to anticipate future needs for sieve replacement well in advance of when new sieves are required.

Step 1—Log the date received.

For each new sieve, log the date received at the laboratory and that sieve's unique serial number into the Karnal Bunt Sieve Log.

Step 2—Examine the sieve.

Using a microscope equipped with an ocular micrometer, verify the quality and mesh size of each sieve.

- Examine the entire mesh under the microscope to look for tears, holes, or gaps in the mesh (recommended at 40x).

- Measure the mesh pore size with an ocular micrometer at any location where a gap in the mesh appears, and additionally at five or more separate random locations across the mesh (recommended at 200x).
- Examine the silicone seals. If the silicone seal is imperfect and can be repaired, touch up with silicone; otherwise it is unusable. After a silicone application, do not place the sieve into service for 24 hours.

If you find no damage, the mesh size is correct, and the silicone seal is intact, the sieve can go into service following step 3 below. If the sieve is unusable due to damage, improper mesh size, or an unsalvageable silicone seal, the sieve cannot be used, and must be destroyed. The person performing the verification enters the results into the Karnal Bunt Sieve Log. When a sieve is destroyed, log the date and your initials into the Karnal Bunt Sieve Log sheet.

Step 3—Place the sieve into use.

Each sieve that passes the examination can be placed into use. Log the date into use on the Karnal Bunt Sieve Log. Then soak the sieve in soapy water for 15 minutes and rinse with clear water. After rinsing, soak the sieve in 30 percent bleach solution for an additional15 minutes and rinse once more in clear water. Log the serial numbers of the sieves that are now ready for use into the spaces across the top row of the Karnal Bunt Sieve Usage Log. These sieves can now be used for sample extraction.

Maintaining Sieves

Background

To ensure that intact sieves are used to extract samples, inspect the sieves before each use.

Step 1—Record the sieve's use.

Record every use of each sieve into the Karnal Bunt Sieve Usage Log. In the column below each sieve's serial number on the log sheet, enter the sample/sub-sample number that the sieve is used to extract. Before performing an extraction, visually inspect every sieve to be used for any tear in the mesh or other imperfection, such as damaged silicone. If you find a sieve to be unusable, destroy it. Record the date, your initials, and the reason destroyed on Karnal Bunt Sieve Log sheet. Also record your initials and the date destroyed at the bottom of the column for that sieve's serial number on the Karnal Bunt Sieve Usage Log.

Step 2—Recheck the sieve.

After ten uses, recheck each sieve for quality as in Step 2 above. Record your initials and the date of damage check on the Karnal Bunt Sieve Usage Log in the space below the 10th extraction set entry. If you find the sieve to be usable, soak it in warm soapy water for 15 minutes and rinse in clear water before further use. If you destroy the sieve at this point, record the action in both the Karnal Bunt Sieve Log and Sieve Usage Log. Perform these rechecks after the tenth and twentieth uses. Take each sieve out of service and destroy it after **30 uses**, regardless of the sieve's condition at that point.

Ordering Sieves

Sieves are manufactured at the USDA, APHIS, PPQ Aircraft and Equipment Operations Center in Mission, Texas. If you need replacement sieves, fax a request including the number of sets required, the requested date of delivery, and delivery information.

Contact the Operations Center at the following numbers:

- Fax: (956) 580-7276
- Phone: (956) 580-7270

Forms

Each laboratory location will develop forms used for recording results of bunted kernel and teliospore analyses. Assure that the appropriate levels of information collection are included on each data collection form. Refer to samples of such forms on the following pages.

KARNAL BUNT KERNEL ANALYSIS WORKSHEET

BOX #	SAMPLE #	KERNELS #	KERNELS IDENTIFIED BY	KERNELS DATE	KERNELS CONFIRMED BY	COMMENTS	DATA ENTERED

DATA ENTRY CONFIRMED BY (NAME/DATE): _____

FIGURE D-3: Karnal Bunt Kernel Analysis Worksheet

KB - Quality Control (QC) Worksheet for Bunted Kernels

Sample Number	Sample Positive? Y / N	# of QC Kernels Found in Primary Screening	# of QC Kernels Found in Secondary Screening (UV)	# of QC Kernels Assigned to Sample	Reader Name

FIGURE D-4: Karnal Bunt Quality Control (QC) Worksheet for Bunted Kernels

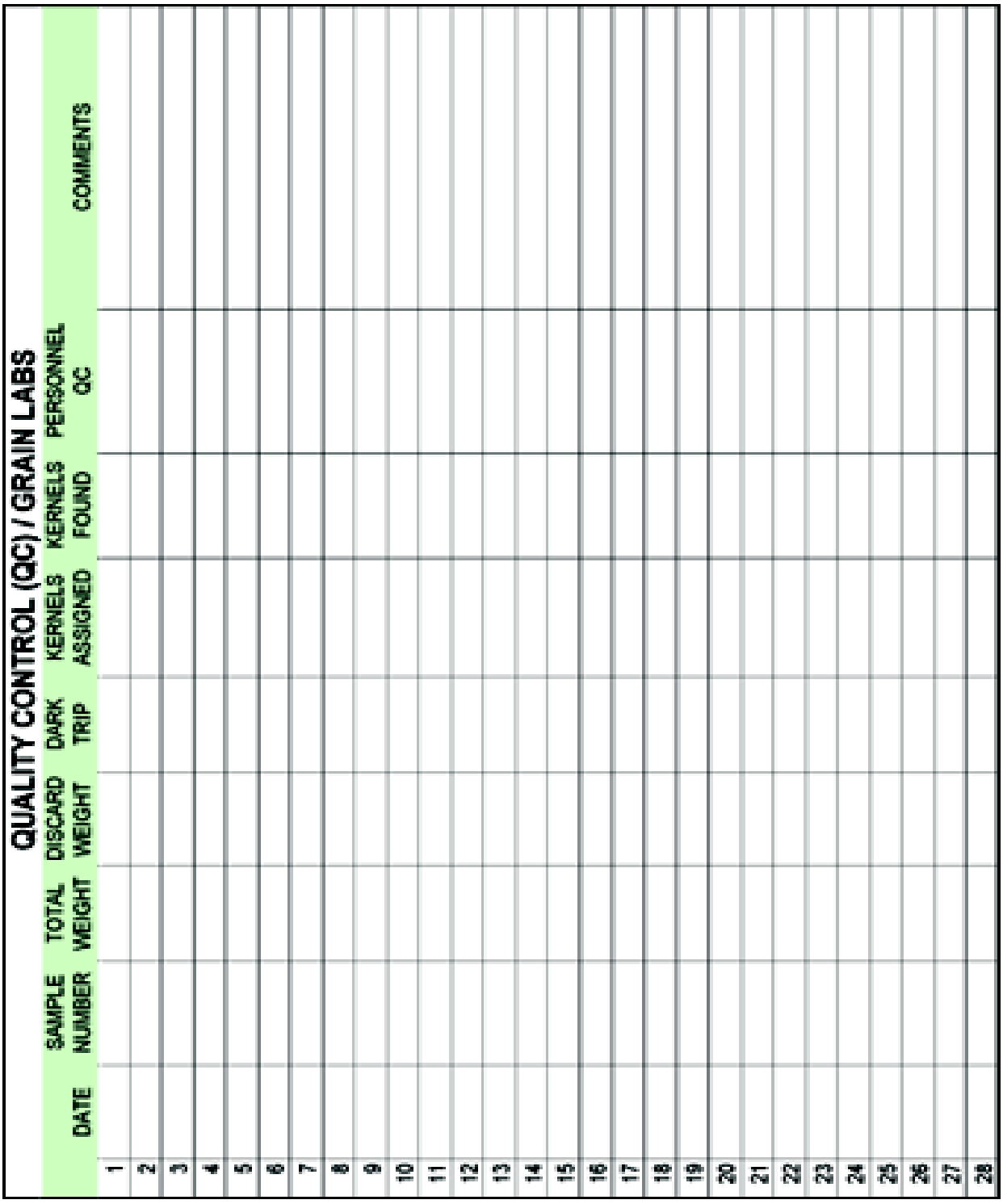

FIGURE D-5: Quality Control (QC) Grain Labs

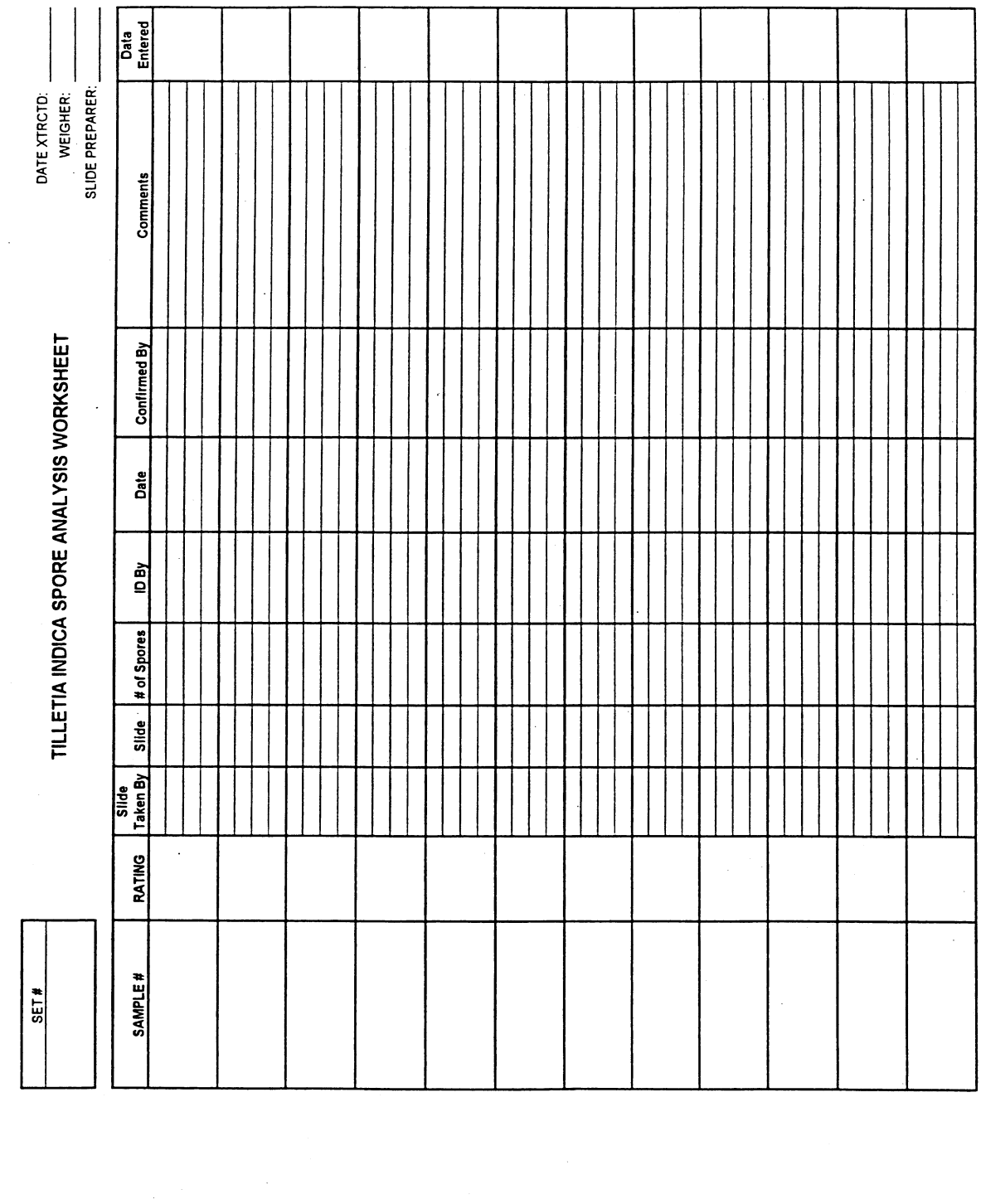

FIGURE D-6: *Tilletia indica* Spore Analysis Worksheet

Tilletia indica Microscopy Log Sheet

Identifier: _____

Date: _____

Shift: _____

Sample #	Set #	# of Spores	1ST/2ND	Comments

FIGURE D-7: ***Tilletia indica*** **Microscopy Log Sheet**