



**United States
Department of
Agriculture**

Marketing and
Regulatory
Programs

Animal and
Plant Health
Inspection
Service

Plant Protection
and Quarantine

Karnal Bunt Manual



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Karnal Bunt Manual

*Karnal Bunt, Tilletia indica Mitra
(Synonym Neovossia indica (Mitra)
Mundkur)*

For additional copies of the *Manual*, contact PPQ–PRIM–Manuals Unit,
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Authorization

This manual provides guidelines and actions for the management of Karnal bunt infestations. This manual supplements information contained in the following Plant Protection and Quarantine (PPQ) manuals: *Treatment, Emergency Programs, and Administrative Procedures*.

It is to be used in conjunction with other manuals when conducting program activities. The information and instructions contained in this manual are based on scientific and operational information developed since the introduction of Karnal bunt in 1996.

Deputy Administrator/Plant Protection and Quarantine
Animal and Plant Health Inspection Service

Date

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General Information

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Purpose

The information contained in this document is intended for use only for Karnal bunt programs. Use this manual for guidance in the current National Survey Program and in implementing regulatory procedures and in preventing the spread of Karnal bunt to other locations. This manual provides technical and general information needed to implement any phase of a Karnal bunt regulatory program. Specific program action is to be based on information available at that time and in accordance with local methods and procedures.

Goals and Objectives

The goal of the Karnal bunt program is to retain U. S. wheat export markets while:

- ◆ Protecting U. S. wheat production areas free of Karnal bunt
- ◆ Providing the best possible options for those who are affected
- ◆ Facilitating the movement of wheat into domestic and international markets
- ◆ Promoting the flow of pertinent disease information to reassure our trading partners about the safety of exported U. S. wheat

Orientation to Officers

The following identifies principal activities necessary for conducting a regulatory program to prevent the spread of Karnal bunt disease.

- 1.** Supervising and monitoring the cleaning and sanitizing of equipment and buildings that may have come in contact with infected grain.
- 2.** Interacting with the following:
 - A.** Farmers and ranchers
 - B.** Storage and processing facilities
 - C.** Commercial haulers of regulated articles
 - D.** Farm equipment and implement dealers
 - E.** Farm and garden supply dealers
 - F.** Custom harvesters
 - G.** Cooperators (county, state, and federal)
- 3.** Monitoring to insure that infected or contaminated grain is handled in an approved manner as outlined in protocols.
- 4.** Visiting processing establishments to verify that conditions listed on Emergency Action Notifications and Compliance Agreements are met.
- 5.** Monitoring the movement of regulated articles.
- 6.** Observing regulated area boundaries for movement of regulated articles.
- 7.** Taking samples of grain, seed, and hay for testing.

Background Information

Causal Agent

Karnal bunt (synonym partial bunt) is caused by the smut fungus *Tilletia indica* Mitra (synonym *Neovossia indica* (Mitra) Mundkur). It was first reported in 1931, infecting wheat growing near the city of Karnal in the Indian state of Haryana. Since that time, the disease has been found to occur throughout all the major wheat growing states of India, as well as in Pakistan, Iraq, and Afghanistan. More recently (1970) the disease has also been observed in the Mexican state of Sonora. The disease has also been intercepted on grain samples from Lebanon and Syria, suggesting it may be established in these countries as well. Karnal bunt is known to affect only wheat (*Triticum aestivum*), triticale (*Triticum aestivum* X *Secale cereale*), and durum wheat (*Triticum durum*). Karnal bunt was first detected in the United States in 1996.

Importance to the United States

In the United States, concern about this disease increased with the interception of an infected sample of seed at the Mexican border in 1981. Although commercial shipments of wheat from Mexico to the United States do not represent a large volume of grain, they were believed to constitute a risk. In addition, the area surrounding Ciudad Obregon in the State of Sonora is the site of a number of experimental small grain winter increase programs conducted by U.S. breeders as well as being the center for the CIMMYT (International Center for Maize and Wheat Improvement) small grain breeding program. Plots located in this area have served as a major source of seed for many U.S. wheat breeders programs.

Karnal bunt has significance as an export problem because many countries believe it to be a quarantine pest, while the United States considers it to be a quality pest.

Infection

Karnal bunt affects the heads of wheat and triticale plants. Infection occurs at anthesis when sporidia or basidiospores come into contact with the developing ovary of a host plant. Once kernels become infected, the fungal mycelium may grow throughout the endosperm. As the infected kernel matures, part of the kernel will be converted to masses of dark fetid smut spores. The disease is often referred to as partial bunt, referring to the fact that, in most cases, only a portion of the kernel is converted to teliospores and typically only a few kernels on the spike will be affected. This is in marked contrast to common bunt and loose smut, where the entire kernel is converted to a mass of teliospores and the entire spike is affected. In most cases, bunted kernels are not readily visible until the wheat is harvested.

How Karnal Bunt Spreads

This fungal disease spreads primarily by introduction of teliospores onto a field. Although infected seed planted in a field may or may not directly produce infected plants the first year, planting infected seed is the primary means of getting the spores into the soil. Infection by secondary sporidia, produced by teliospores germinating on the soil surface, occurs during the heading stages of the host plant. The greater threat of disease occurs the following years as soil is turned over, bringing these teliospores back to the surface.

The ideal conditions for infection are cool weather and rainfall or high humidity during heading. Overhead irrigation during heading time can produce excellent conditions for infection. When ideal conditions are not present, furrow or flood irrigation can produce an adequate microclimate to maintain low levels of the disease.

Although the spores may be carried on a variety of surfaces, spores and the sporidia they produce also can be windborne. Because the sporidia are fragile and can move only short distances, contaminated seeds are considered to be the major source of spread.

Losses

Losses due to Karnal bunt are attributed to the effect of the disease on grain quality. The fungus releases trimethyl-amine, a volatile compound with a characteristic fishy odor. The presence of 3% or more bunted kernels will result in flour having an objectionable odor, color, and taste. Flour made from wheat containing large numbers of heavily bunted kernels is discolored and has an unpleasant although harmless odor. All of the discoveries of Karnal bunt in the United States have resulted in infection rates of less than 1%, based on an average of 50,000 kernels per four pound sample.

Distribution

Karnal bunt currently occurs in Afghanistan, India, Iran, Iraq, Mexico, Nepal, Pakistan, and in limited areas of the United States. The pathogen has also been discovered in exported seed samples from Lebanon and Syria. In the United States, the Animal and Plant Health Inspection Service (APHIS) has designated parts of Arizona, California, New Mexico, and Texas as areas regulated for Karnal bunt. The regulated area boundaries are adjusted based on survey results.

Hosts

Hosts include *Triticum aestivum* (wheat), *Triticum durum* (durum wheat), and *Triticum aestivum* x *Secale cereale* (triticale—a hybrid of wheat and rye). Although Karnal bunt has been shown under greenhouse conditions and artificial inoculation to attack several grass species in the genera *Aegilops*, *Bromus*, and *Lolium*, as well as other species in the genus *Triticum*, the disease has never been observed on these grasses under natural conditions in the field.

Life Cycle

Teliospores are the overwintering life stage of Karnal bunt. The teliospores often require a dormancy period of at least 4–9 months.

Teliospores germinate at the soil surface by forming a promycelium. As many as 65–185 primary sporidia (infectious spores) form at the apex of the promycelium. Many secondary sporidia can be produced by budding from the primary sporidia or from mycelia.

Air currents and splashing water carry sporidia to the wheat spike. Infection occurs only during wheat heading and floral development. Germ tubes arising from the sporidia enter through stomata of the glumes and then enter the ovary through the ovary wall. Mycelia may

grow into the endosperm of the wheat kernel. Many teliospores consume and replace the infected endosperm. The harvesting process releases the teliospores to contaminate the soil or wheat seed externally.

Environmental conditions directly affect the development of the disease. Frequent rains and high relative humidity (70% or greater) at the time of heading favor disease development. Irrigation induces a higher incidence of disease by increasing humidity. High humidity lengthens the wet period, creating conditions favorable for teliospore germination and disease development. Day temperatures of 18–22.5°C (64–72°F) and soil temperatures of 17–21°C (63–70°F) at spike emergence increase the severity of Karnal bunt.

Refer to [Figure 1-1](#) for a diagrammatic representation of the Karnal bunt life cycle.

Karnal Bunt Life Cycle

Tilletia indica

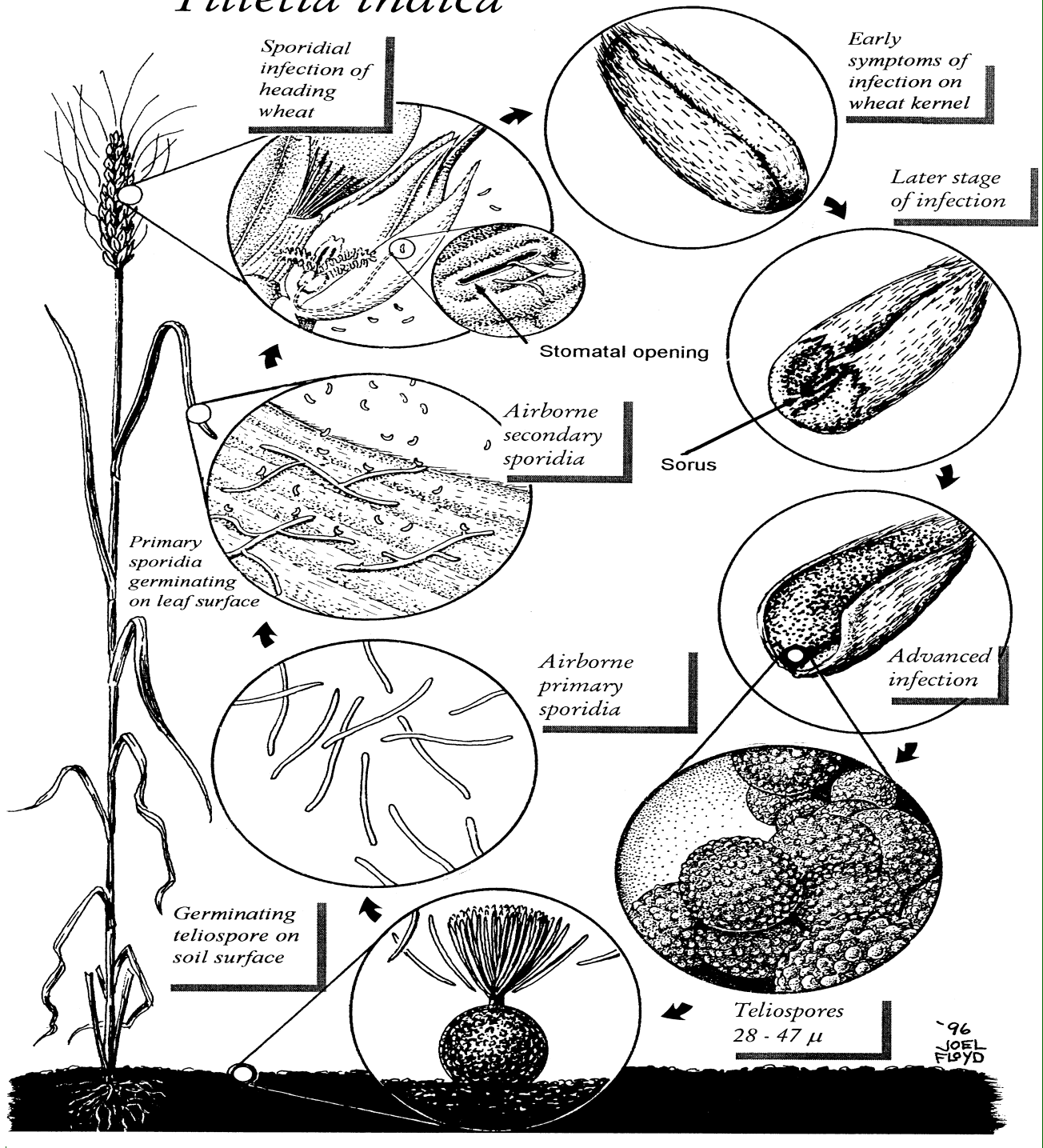


FIGURE 1-1: Karnal Bunt Life Cycle

General Information

Distribution

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Karnal Bunt

Survey Procedures

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Protocol for Sampling Grain

Sampling

Every wheat field within the regulated area, and individually regulated fields, will be sampled. Karnal bunt program personnel will obtain four pound samples at the field. Samples will then be examined for bunted kernels at the nearest lab/bunted kernel machine location. The sample will be a time sensitive sample and results will be known prior to the grain leaving the field or before grain is off loaded at a facility.

The Karnal bunt project will supply to the facility handlers maps and/or lists identifying known field numbers of all fields within the regulated areas. This information will help industry insure that regulated area grain will not be off loaded without appropriate test results.

Protocol

Draw a four pound sample prior to or at time of harvest. A sample cut by a combine will contain approximately 1,000 pounds of wheat randomly harvested from the field being tested. Obtain the sample as safely and efficiently as possible. To avoid getting grain from a previous field that was left within the combine, let the grain discharge awhile before drawing the sample.

Step 1—Collect the sample.

Use a length of PVC pipe to obtain grain as the combine discharges into a truck. A probe may be used to draw the sample from the combine or truck. The sample may also be drawn from the inspection compartment, if available, or hopper of the combine.

Step 2—Bag the sample.

Place the sample grain into a collection bag (double bagged), labeled with one of the following conventions:

- ◆ Unique field number or identification, sampler's name, producer's name, and date
- ◆ Unique identification number and accompanying sample worksheet

Properly seal the collection bag.

Step 3—Transport the sample.

Transport the sample to the nearest lab/bunted kernel machine as quickly as possible.

Personnel will process the samples as quickly and efficiently as possible and complete sample documentation. Results should be communicated as soon as known to the grower/harvester. Movement of grain will be based on a bunted kernel standard and accompanying paperwork will be completed as outlined under the heading **Certification of Grain** in Chapter 3, **Regulatory Procedures**.



Safety Hints:

1. Exercise caution around combines and field machinery to avoid injury. Do not assume the operator can see you; large machines have blind spots and field conditions can inhibit visibility.
2. Be careful climbing on and off of grain trucks and combines to take samples. Wear proper footwear.
3. Be mindful of fire hazards in field conditions.
4. Be timely and efficient in taking and reviewing samples and **DO NOT RUSH** or **SACRIFICE SAFETY**. Time is valuable to Custom Harvesters whose business is to compete with weather and harvest windows of opportunity.

Protocol for Sampling Seed Prior to Storage

Trucks may come from the fields and enter the storage facility in the usual manner. From the scale area, each truck will proceed to an assigned area for unloading. The objective is to obtain a representative sample and to maintain sanitation without cross-contamination.



Seed grown within the regulated area must be free of bunted kernels and teliospores. The seed is allowed to be planted within or outside the regulated area. It is highly recommended that all seed be tested prior to cleaning and treating.

Step 1—Draw sub-samples.

As the crop is off loaded, randomly draw three sub-samples from each truck.

Step 2—Mix the sub-samples.

Uniformly mix the random sub-samples from each truck (up to six trucks) in a sanitary bucket or bag.

Step 3—Send the samples to the laboratory.

Fields and lots identified for seed may be sampled drawing a 4 pound sample (refer to “[Protocol for Sampling Grain](#)” on [page-2-2-1](#)). Take one sample, one 250-mL bottle sample (Retain the one 250-mL sample in the event more material is needed for replication retest,) and draw five 250-mL bottles with 50 grams each.

Either of two testing sequences may be used. (1) Test a 4-pound sample for bunted kernels according to the grain sampling protocol. If the sample is negative for bunted kernels, send the five 250-mL bottles to the laboratory for spore testing. If the samples are free of teliospores of karnal bunt, allow movement as seed. (2) Send the five 250-mL bottles to the laboratory for spore testing. If the samples are free of teliospores of karnal bunt, allow movement as seed. If teliospores are found the field or lot is not eligible for use as seed and will then be tested using the four pound sample for bunted kernels. If no bunted kernels are found, the field or lot can be used as grain.

TABLE 2-1: Designation of Grain Sampled Before Storage after Laboratory Testing

If:	Then:
All five replications are NEGATIVE	The entire lot moves into storage as SEED
One or more replications have one or more spores	Handle as GRAIN

Protocol for Sampling Seed During Storage

Seed sampled during storage will consist of one composite sample per lot. The lot size will be equal to the amount of seed stored in each individual bin or broken down into individual lots containing up to 150 tons (approximately 5,000 bushels). Only one type of sampling method will be allowed for each storage facility or bin.

- ◆ The preferred method is to collect the sample while turning the grain in larger units. Many on-farm storage bins do not have the ability to turn grain, hence the need for sampling grain for spores while still in storage.

- ◆ Use probes for sampling where turning the grain is not possible. Probes used must be long enough to take samples of grain from all parts of the bin being tested.

Sample to the best method available depending upon circumstances. The objective is to get the most representative sample possible.

Draw sufficient sub-samples to provide enough composite seed to satisfy the sample size requirements. Uniformly mix all sub-samples from each bin in a sanitary bucket or bag to obtain a composite sample. Fill each of five or ten 250-mL bottles (depending on the size of the bin) with 50 grams and send to the laboratory. The bottles represent separate replications of a single, well-mixed sample from the entire lot of wheat. Also submit one or two 250-mL samples.

A description of the number of sub-samples drawn and replications submitted for each bin follows. Draw sub-samples from each bin in such a manner that the final sample is representative of the entire lot or bin being sampled. The general procedure for sampling will be as follows:

TABLE 2-2: Procedure for Sampling Seed During Storage

If there are:	Then:
1-750 tons (up to 25,000 bushels) in the bin	<ol style="list-style-type: none"> 1. Draw one sub-sample from every 25 tons (approximately 833 bushels). 2. Mix all sub-samples thoroughly. 3. Collect five 50-gram replications, one four-pound sample, and one 250-mL sample. 4. All tests must be negative for all stages of karnal bunt. The tests can be run in either of two sequences: (1) Examine the four-pound sample for bunted kernels. If the sample is negative for bunted kernels, send five 50-gram replications, and one 250-mL sample to the seed lab. (2) Send five 50-gram replications, one 250-mL and one four-pound sample to lab for teliospore test. If sample is teliospore positive it is not eligible for seed. Test one four-pound sample for bunted kernels. If Negative, permit as grain.
More than 750 tons (25,000 bushels) in the bin	<ol style="list-style-type: none"> 1. Draw one sub-sample from every 100 tons (approximately 3300 bushels). 2. Mix all sub-samples thoroughly. 3. Collect ten 50-gram replications, one four-pound sample, and one 250-mL sample. 4. All tests must be negative for all stages of karnal bunt. Examine the four-pound sample for bunted kernels. Proceed as described in (4) above, using ten 50-gram replications.

The lab will retain the 250-mL bottle sample in the event more material is needed for replication retests.

The bottles represent separate replications of a single, well-mixed sample from the entire lot of wheat. The lab will run all replications simultaneously, working toward a 24-hour turnaround. The following results are possible:

TABLE 2-3: Designation of Grain Sampled During Storage after Laboratory Testing

If:	Then:
All replications are NEGATIVE	The entire lot moves into storage as SEED
One or more replications have one or more spores	Handle as GRAIN

Protocol for Sampling Seed Following Storage

Seed in storage will receive one composite sample per lot. The lot size will be equal to the amount of seed stored in each individual bin, or the producer has the option to break down all wheat in storage into individual lots containing up to 150 tons (approximately 5,000 bushels). These individual lots will be sampled as they are removed from storage. Maintain the identity of all individual lots after taking samples. Once sample results are known, lots can be recombined according to test results. Uniformly mix all sub-samples from each bin in a sanitary bucket or bag to obtain a composite sample and fill each of five or ten 250-mL bottles (depending on the size of the bin) with 50 grams and send to the laboratory. The bottles represent separate replications of a single, well-mixed sample from the entire lot of wheat. Also submit one or two 250-mL bottle(s) filled with seed.

A description of the number of sub-samples drawn and replications submitted for each bin follows. Draw sub-samples as the seed is circulated in each bin. Take sub-samples from the grain stream at the intervals or quantities listed below. The general procedure for sampling will be as follows:

TABLE 2-4: Procedure for Sampling Seed Following Storage That Will Not Be Broken into Individual Lots

If there are:	Then:
1-750 tons (up to 25,000 bushels) in the bin	<ol style="list-style-type: none"> 1. Draw one sub-sample from every 25 tons (approximately 833 bushels). 2. Mix all sub-samples thoroughly. 3. Collect five 50-gram replications, one four-pound sample, and one 250-mL sample. 4. Examine the four-pound sample for bunted kernels. 5. If the four-pound sample is negative for bunted kernels, send five 50-gram replications, one four pound sample, and one 250-mL sample to the seed lab. All tests must be negative for the grain to qualify as seed.
More than 750 tons (25,000 bushels) in the bin	<ol style="list-style-type: none"> 1. Draw one sub-sample from every 100 tons (approximately 3300 bushels). 2. Mix all sub-samples thoroughly. 3. Collect ten 50-gram replications, one four-pound sample, and one 250-mL sample. 4. Examine the four-pound sample for bunted kernels. 5. If the four-pound sample is negative for bunted kernels, send ten 50-gram replications, one four pound sample, and one 250-mL sample to the seed lab. All tests must be negative for the grain to qualify as seed.

The lab will retain the 250-mL bottle sample in the event more material is needed for replication retests.

The bottles represent separate replications of a single, well-mixed sample from the entire lot of wheat. The lab will run all replications simultaneously, working toward a 24-hour turnaround. The following results are possible:

TABLE 2-5: Designation of Grain Sampled Following Storage after Laboratory Testing

If:	Then:
All replications are NEGATIVE	The entire lot moves into storage as SEED
One or more replications have one or more spores	Handle as GRAIN

National Survey Plan

See [Appendix H](#) for the current National Survey Plan.

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Karnal Bunt

Regulatory Procedures

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Orientation/Instruction to Officers

Regulatory actions will be initiated based on production, handling, and transportation practices, or when the program is modified by the agency to meet changing conditions. Officers must follow instructions for regulatory procedures when authorizing the movement of regulated articles. Understanding the instructions and procedures will serve as a basis for explaining procedures to persons interested in moving articles affected by the quarantine and regulations. Only authorized treatments may be used (see [“Decision Tables for Principal Activities” on page-3-3-10](#)). Specific working protocols may be tailored by individual states within these guidelines.

Regulated Articles

The list that follows is taken from 7 CFR 301.89-2 (updated March 24, 2004):

- ◆ Conveyances, including trucks, railroad cars, and other containers used to move host crops produced in a regulated area that have tested positive for Karnal bunt through the presence of bunted kernels
- ◆ Grain elevators/equipment/structures used for storing and handling host crops produced in a regulated area that have tested positive for Karnal bunt through the presence of bunted kernels
- ◆ Seed conditioning equipment and storage/handling equipment/structures that have been used in the production of wheat, durum wheat, and triticale found to contain the spores of *Tilletia indica*
- ◆ Plants or plant parts (including grain, seed, and straw) and hay cut after reaching the dough stage of all varieties of wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), and triticale (*Triticum aestivum X Secale cereale*) that are produced in a regulated area, except for straw/stalks/seed heads for decorative purposes that have been processed or manufactured prior to movement and are intended for use indoors
- ◆ *Tilletia indica* (Mitra) Mundkur
- ◆ Mechanized harvesting equipment that has been used in the production of wheat, durum wheat, or triticale that has tested positive for Karnal bunt through the presence of bunted kernels
- ◆ Any other product, article, or means of conveyance when:
 - ❖ An inspector determines that it presents a risk of spreading Karnal bunt based on appropriate testing and the intended use of the product, article, or means of conveyance; and
 - ❖ The person in possession of the product, article, or means of conveyance has been notified that it is regulated under this subpart.

Quarantine Actions

Implement the following actions in sequence:

1. Issue an Emergency Action Notification, PPQ Form 523 (EAN) to the owner or operator of each property on which Karnal bunt is confirmed by presence of bunted kernels or which is identified as a source of or recipient of infected wheat, until such time as a regulated area is established. Recipients of EANs will be required to implement treatments and handling procedures. Issue EANs and/or comparable State notifications to the property owners or managers of all establishments that are involved in the handling, moving, or processing of articles capable of spreading Karnal bunt, when these establishments are located within the area that serves any infested property. You may issue a notification pending positive identification and/or further instruction from the Deputy Administrator. EANs are usually no longer issued when the regulated area is established.
2. If necessary, the Deputy Administrator will issue a letter directing PPQ Field offices to initiate specific emergency actions under the Plant Protection Act 2000 until emergency regulations can be published in the *Federal Register*.

The Plant Protection Act 2000 provides for authority for emergency quarantine action. This authority provides for interstate regulatory action only; intrastate regulatory action is provided under State authority. However, if the Secretary of Agriculture determines that an extraordinary emergency exists and that the measures taken by the state are inadequate, USDA can take intrastate regulatory action provided that the Governor of the State has been consulted and a notice has been published in the *Federal Register*.

The Plant Protection Act also provides the Federal Government, either independently or in cooperation with States or political subdivisions thereof, farmers' associations and similar organizations, and individuals, the authority to carry out operations or measures to detect, eradicate, suppress, control, or to prevent or retard the spread of plant pests. This does not provide for trespassing on private property, but relies upon State authority and willingness to use State right-of-entry authority.

All program technology and methodology employed are determined through discussion, consultation, or agreement with the cooperating State officials.

3. The Deputy Administrator, through the National Regional Directors, will notify State cooperators of the Karnal bunt detection, actions taken, and actions contemplated. USDA and cooperators will develop and provide a description of the regulated area with support documents to the national program planning staff or its equivalent.
4. APHIS Regulatory Analysis and Development Staff (RADS) or its equivalent will publish emergency regulations under the Plant Protection Act.
5. After a reasonable time to determine the scope of the infection, a proposal to promulgate a quarantine under the Plant Protection Act will be published. The proposal will announce a date for submitting written comments, which shall be approximately 60 days after publication.
6. After receipt of written comments, a final determination specifying the action decided upon will be published in the *Federal Register*. A quarantine will be enacted if eradication has not been achieved under the Plant Protection Act.

Certification of Grain

Certify grain at the field or facility level by issuing PPQ Form 540s or local equivalent as approved by the PPQ Project Director.

TABLE 3-1: Grain Certification Decision Table

If the grain is determined to be:	Then:
Bunted Kernel Negative	◆ Use PPQ Form 540 or local equivalent as approved by the PPQ Project Director
Bunted Kernel Positive	◆ Issue PPQ Form 530 (Limited Permit) or local equivalent as approved by the PPQ Project Director for each truck load of grain ◆ Move positive grain only to an approved facility for processing

Handling Instructions for Seed Produced Inside the Regulated Area

Any wheat, durum wheat, or triticale that originates within a regulated area must be tested and found free of any stage of Karnal bunt (*Tilletia indica*) before it may be used as seed within or outside a regulated area.

Seed Storage

Place seed in new bags, or bulk containers that are new or have been certified free of Karnal bunt.

- ◆ Store all seed in Karnal bunt free facilities and transport in Karnal bunt free conveyances. One of the following conditions must be met for each lot:
 - A. Conveyances, storage facilities and planting equipment are new or have never been used to handle wheat, durum wheat, or triticale; or
 - B. Conveyances, storage facilities and planting equipment have been sanitized or cleaned with an approved treatment; or
 - C. Conveyances, storage facilities and planting equipment have never been used to hold or transport wheat that tested positive for Karnal bunt.
- ◆ Ensure that the movement of seed from the storage facility is accompanied by a Certificate (PPQ Form 540).

Handling Instructions for Seed Produced Outside the Regulated Area

The following conditions apply for seed produced outside the regulated area and intended for planting **inside** the regulated area.

- ◆ Testing for presence of Karnal bunt spores **is not required**.
- ◆ Treating seed for planting inside of the regulated area **is not required**.
- ◆ Advanced notification of movement into the regulated area is required in order to track and safeguard the identity of the seed.

Authorized Chemicals

The following chemicals are authorized to treat articles regulated for Karnal bunt:

- ◆ Methyl bromide (15 lb/1,000 ft³ For 96 hours)
- ◆ One part Ultra Clorox brand regular bleach (6 percent sodium hypochlorite; EPA Reg. 5813-50) in 3 parts water; or one part CPPC Ultra Bleach 2 (6.15 percent sodium hypochlorite; EPA Reg. No. 67619-8) in 3.1 parts water
- ◆ Pentachloronitrobenzene (PCNB) with or without Carboxin-Thiram
- ◆ Carboxin-Thiram

Approved Regulatory Treatments for Equipment and Facilities

Mechanized Harvesting Equipment

Mechanized harvesting equipment includes combines, grain buggies, etc. Equipment used in the harvesting or processing of wheat, durum wheat, or triticale that tests positive for bunted kernels will require cleaning. If disinfection is required by an inspector in addition to cleaning, the articles must be disinfected in accordance with 7 CFR 301.89-13 prior to movement from a regulated area.

Cleaning Procedure for Harvesting Machines

Use the following procedure to clean harvesting machines:¹

Step 1—Remove the header.

Step 2—Open the hopper doors.

Open the hopper doors on the bottom of the combine to allow all of the remaining wheat in the hopper below the auger to fall out.

Step 3—Blow out the debris.

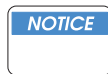
Put the combine in neutral, turn up the air pressure to maximum and run the combine up to full throttle to blow out all of the debris remaining in the system.

Step 4—Remove wheat from the return auger.

Open the return auger and run it at full throttle under maximum air pressure to remove all wheat in that portion of the system.

Step 5—Blow out remaining debris.

Use a high pressure air hose to blow any remaining debris out of the hopper and delivery system.



The inspector may require or recommend washing if necessary. Continue with the following steps for washing protocol.

Step 6—Begin the wash.

Using a high pressure wash, start at the top of the combine and work down to remove dust, debris, etc. on the machine.

Step 7—Continue the wash.

Open all inspection doors and apply the high pressure wash to remove debris.

¹ The entire process takes two people approximately 1.5 to 2 hours of steady work, depending on the type of combine used and the amount of residue left on the machinery. Under a worst case scenario (equipment breakdown, only one person to conduct cleaning, extremely dirty combine, etc.) the cleaning may take up to 3.5 hours. Small variations based on different machine types will occur. Discuss the cleaning required for a specific harvesting machine with the Project Director.

Step 8—Remove side panels.

Remove side panels from the header.

Step 9—Remove all debris from header.

Step 10—Clean the header.

Clean the header with the high pressure wash.

Conveyances That Can Be Easily Inspected

Conveyances that can be easily inspected include truck trailers, bobtails, railcars, etc. Equipment used in the harvesting or processing of wheat, durum wheat, or triticale that tests positive for bunted kernels will require cleaning. If disinfection is required by an inspector in addition to cleaning, the articles must be disinfected in accordance with 7 CFR 301.89-13 prior to movement from a regulated area.

Facilities

Facilities include silos, bins, etc. Use [Table 3-2](#) for facilities that have tested positive for Karnal bunt:

TABLE 3-2: Facility Treatments

If the facility will be used for:	Then:
Host grain storage	Clean the facility before storing host material
Host seed storage	Clean and sanitize the facility before storing host material

Cleaning is defined as the removal of all soil and plant debris.

Sanitizing is defined as using one of the following options:

Sanitizing Options

A. Sodium Hypochlorite Solution

Thoroughly wet the equipment until point of runoff with a solution of 1.5 percent sodium hypochlorite—a solution of sodium hypochlorite mixed with water applied at the rate of (1) One part Ultra Clorox brand regular bleach (6 percent sodium hypochlorite; EPA Reg. No. 5813-50) in 3 parts water; or (2) One part CPPC Ultra Bleach 2 (6.15 percent sodium hypochlorite; EPA Reg. No. 67619-8) in 3.1 parts water. Leave solution in contact with all surfaces for a minimum of 15 minutes, then thoroughly rinse surfaces with water to minimize any adverse effect on equipment. Prepare a fresh solution of sodium hypochlorite and potable water every four to five hours.

Or

B. Hot Water And Detergent

Hot water and detergent drench with a pressure of at least 30 pounds per square inch. Apply the water to all surfaces until point of runoff including penetration into cracks and crevices. At point of runoff the temperature must be a minimum of 170°F.

Or

C. Live Steam To All Surfaces

You can use live steam (212°F) to treat various harvesting and cleaning equipment, as long as the steam can directly reach all surfaces that come into contact with grain. Apply steam directly to all surfaces until the point of runoff. At point of runoff the temperature must be a minimum of 170°F.

Movement of Host Crops

Host crops include hay and straw. Before moving hay or straw within or out of the regulated area it must be inspected by USDA or State Cooperator Karnal bunt personnel. The following distinctions apply:

- ◆ Host crops cut prior to the development of the kernel (dough stage) will be considered no risk for having Karnal bunt. Host crops cut after the dough stage will be treated as grain.
- ◆ Host crops cut with fully developed kernels will be tested for the presence of bunted kernels. Take samples from bales. The host crop hay/straw will be regulated according to the results of the testing.

Host crops testing **negative** can move within and out of the regulated area if they meet all of the following conditions (refer to [Table 3-6](#)):

- ◆ The identity of the cut host crop is retained.
- ◆ The grower will be issued a Certificate for the number of bales inspected/tested free of bunted kernels.
- ◆ The number of bales noted on the Certificate for the location (field or storage area) will be allowed movement within or out of the regulated area. The Certificate must accompany any movement.

Host crop straw testing **positive** for bunted kernels may **not** be removed from the field. Host crop hay testing positive for bunted kernels can move **within** the regulated area under the following conditions:

- ◆ A grower may move host crop hay from the field of production (land owned or leased by grower) to an area owned or leased by the grower for storage.
- ◆ A grower may move host crop hay from the field of production (land owned/leased by grower) for the purpose of feeding to livestock.
- ◆ A grower must contact USDA–State Cooperator–Karnal bunt personnel prior to movement of host crop hay. A Limited Permit will be issued for movement.
- ◆ Host crop hay must be moved in a manner that safeguards the host crop hay and minimizes the potential for spreading Karnal bunt.

Host crop hay testing **positive** for bunted kernels or baled from a field that tested positive for bunted kernels can move **out of** the regulated area under the following conditions:

- ◆ The host crop hay must be destined to an approved establishment.
- ◆ Program personnel must be contacted before moving host crop hay. If movement is approved, a Limited Permit will be issued for movement to the approved establishment out of the regulated area.
- ◆ Host crop hay approved for movement out of the regulated area must be moved in a manner that safeguards the host crop hay and minimizes the spread of Karnal bunt.
- ◆ The establishment must be approved by USDA–State Cooperator–Karnal bunt personnel and under compliance to process the host crop hay in an approved manner to destroy all Karnal bunt life stages.

Mushroom Producers

Compliance Agreement Stipulations

See sample compliance agreement in [Appendix E](#).

- ◆ Wheat straw from the regulated area that tests positive must be introduced to a composting procedure that results in a pasteurized product. The composting procedure shall produce pasteurization temperatures throughout the mass of 170°F for at least 1 minute.

Decision Tables for Principal Activities

TABLE 3-3: Grain from Karnal Bunt Regulated Areas

If grain is from:	And:	And:	Then:
A field sampled for bunted kernels	Positive	For animal feed	Issue a Limited Permit (PPQ 530) for movement to facilities with a Compliance Agreement ¹
		For milling	Issue a Limited Permit for movement to facilities with a Compliance Agreement
		Going to a storage facility or to an export terminal facility	Issue a Limited Permit for movement to facilities with a Compliance Agreement ¹
	Negative	For domestic use or going to an export terminal facility ²	Issue a Certificate to any destination for non-planting purposes and export destinations ²
Storage facilities sampled for bunted kernels or storage facilities located outside the regulated area that are commingled with regulated grain	Positive	For animal feed	Issue a Limited Permit for movement to facilities with a Compliance Agreement ¹
		For milling	Issue a Limited Permit for movement to facilities with a Compliance Agreement
		Going to a storage facility or to an export terminal facility	Issue a Limited Permit for movement to facilities with a Compliance Agreement ¹
	Negative	For domestic use	Issue a Limited Permit for movement to any location for any purpose other than planting
		Going to an export terminal facility	1. Issue a Limited Permit for movement to facilities with a Compliance Agreement. 2. Allow movement to countries with no Karnal bunt certification requirements.

- 1 Conveyances for Karnal bunt positive grain must be leak proof and covered prior to moving outside the regulated area.
- 2 Crops from previously tested positive fields are not eligible for export to countries that require an Additional Declaration (AD) on federal phytosanitary certificates.

Use Either [Table 3-4](#) or [Table 3-5](#) as appropriate to your survey area.

TABLE 3-4: Seed from Karnal Bunt Regulated Areas — Option 1

If the seed tests:	And the seed tests:	And the seed is:	Then:
Negative for bunted kernels	Positive for spores	Untreated	Handle as grain
		Treated with fungicide	Bury or dispose of seed with approved methods according to label
	Negative for spores	—————▶	The seed is eligible for planting within or outside the regulated area
Positive for bunted kernels	—————▶	—————▶	The wheat is ineligible to be tested for use as seed. Handle as positive grain.

TABLE 3-5: Seed from Karnal Bunt Regulated Areas — Option 2

If the seed tests:	And the seed tests:	And the seed is:	Then:
Negative for spores	—————▶	—————▶	The seed is eligible for planting within or outside the regulated area
Positive for spores	Negative for bunted kernels	Untreated	Allow as negative tested grain
		Treated with fungicide	Bury or dispose of seed with approved methods according to label
	Positive for bunted kernels	—————▶	—————▶

TABLE 3-6: Host Crop Hay Movement Decision Table

If moving:	And:	And:	Then:
Within the regulated area	No kernels are present ¹	→	Hay cut prior to the dough stage is unregulated
	Kernels are present (collect a 4-pound sample of grain) or a survey has determined the field's status	The field or kernels test KB negative	Issue a Certificate (PPQ 540 or PPQ 527). The producer must maintain a movement log, if necessary, based on local farming practices.
		The field or kernels test KB positive	1. Send certified notification of positive field status to the owner and producer of the field. 2. No movement of straw is allowed out of the field.
Outside the regulated area	No kernels are present ¹	→	Hay cut prior to the dough stage is unregulated
	Kernels are present (collect a 4-pound sample of grain) or a survey has determined the field's status	The field or kernels test KB negative	Issue a Certificate (PPQ 540 or PPQ 527). The producer must maintain a movement log, if necessary, based on local farming practices.
		The field or kernels test KB positive	1. Send certified notification of positive field status to the owner and producer of the field. 2. Call the field manager to explain options. 3. Issue a conveyance Compliance Agreement (CA) to move host crop hay under Limited Permit (PPQ 530) to an approved processing establishment that has a CA. 4. No movement of KB-positive straw is permitted.

1 Host crop hay cut prior to the dough stage is unregulated and does **not** require an inspection. The hay is eligible for a Certificate (PPQ 540), if requested by the producer, but the certificate is not a requirement or a condition of movement.

TABLE 3-7: Cleaning and Sanitizing Decision Table¹

If item is:	Then:
Seed cleaning or reconditioning equipment	GO to Table 3-8
Grain storage facilities and conveyance equipment (bins, elevators, bags, legs, augers, railcars, etc.)	GO to Table 3-9
Combines and other harvesting equipment (thrashers, headers, grain carts, buggies, etc.)	GO to Table 3-10

1 For handling equipment within the regulated areas of AZ, CA, NM, and TX.

TABLE 3-8: Cleaning and Sanitizing Seed Cleaning or Reconditioning Equipment

If:	And:	Then:
Working in any regulated area and processed host crop material	Used in the conditioning of seed that was tested and found to contain spores or bunted kernels of <i>Tilletia indica</i> and being used in the conditioning of seed that has tested negative for the spores of <i>Tilletia indica</i> or being moved from a regulated area	Cleaning and disinfection are required in accordance with 7 CFR 301.89-13
	Used in the conditioning of seed that was tested and found negative for spores of <i>Tilletia indica</i>	Cleaning and sanitizing are NOT required; follow best management practices for that state
Transiting the regulated area	—————▶	

TABLE 3-9: Cleaning and Sanitizing Grain Storage Facilities and Conveyance Equipment¹

If:	And:	Then:
Stored or transported positive tested host material (kernels or spores)	Will be used in the production, harvest, transportation, or storage of SEED	<ul style="list-style-type: none"> ◆ Cleaning and sanitizing are required ◆ Issue Certificate (PPQ Form 540) after treatment is observed by program personnel
	Will only be used in the production, harvest, transportation, or storage of GRAIN	<ul style="list-style-type: none"> ◆ Cleaning is required; follow best management practices for that state ◆ Issue Certificate (PPQ Form 540) after treatment is observed by program personnel
Stored or transported negative tested host material; spore status unknown	Will only be used in the production, harvest, transportation, or storage of GRAIN	◆ Cleaning and sanitizing are NOT required; follow best management practices for that state
	Will be used in the production, harvest, transportation, or storage of SEED	<ul style="list-style-type: none"> ◆ Cleaning and sanitizing are required ◆ Issue Certificate (PPQ Form 540) after treatment is observed by program personnel

¹ Includes bins, elevators, bags, legs, augers, railcars, etc.

TABLE 3-10: Cleaning and Sanitizing Combines and Other Harvesting Equipment¹

If:	And:	And:	Then:
Transiting the regulated area	—————→		◆ Cleaning and sanitizing are NOT required; follow best management practices for that state
Working in the regulated area	Known to have harvested positive tested host material (bunted kernels)	Moving within or between regulated areas	◆ Cleaning required between regulated areas, optional within regulated area; follow best management practices for that state
		Leaving the regulated area	◆ Cleaning and if disinfection is determined to be necessary by an inspector, disinfected in accordance with 7 CFR 301.89-13 ◆ Issue Certificate (PPQ Form 540) after treatment is observed by program personnel
	Known to have harvested negative tested host material	Moving between regulated areas, leaving the regulated area, or crop from next field is intended to be used as seed	◆ Cleaning and sanitizing are NOT required, but optional; follow best management practices for that state ◆ Issue Certificate (PPQ Form 540) if treatment is performed

1 Includes threshers, headers, grain carts, buggies, etc.

- ◆ USDA-APHIS and State Cooperators recommend following the best management practices (BMPs) when cleaning any seed processing, seed/grain storage facilities, and harvesting equipment. The best management practices are designed by the local Cooperative Extension Service to mitigate the risk of spreading Karnal bunt and the unintentional movement of other plant pests, diseases, and noxious weeds. Some States do require cleaning and sanitizing treatments when moving from state to state. The best management practices for your state can be obtained from the local Cooperative Extension Service Office.
- ◆ Cleaning is defined as the removal of all host crop material (straw, heads, grain, etc.) from the equipment which would prevent the cross-contamination of the next field's harvest and/or the spread of bunted kernels into unregulated areas, between regulated areas.

Regulatory Records

Maintain records as necessary to carry out an effective, efficient, and responsible regulatory program. When presented with requests for information (FOIA, etc.) be sure to check with Agency legal counsel. Guidance on length of time to retain records is available from OPM.



Public Information

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Introduction

As an agency concerned about pest and disease situations that can occur or change rapidly, the Animal and Plant Health Inspection Service (APHIS) has a vital need to effectively communicate program activities to its target audiences using a wide variety of informational materials. During outbreak situations, effective and timely communication becomes even more crucial. APHIS provides onsite support when appropriate, serving along with state officials as primary liaisons with the news media to provide accurate information to stakeholders, industry, and the public.

Audiences

- ◆ Media
- ◆ State, city and county governments
- ◆ Industry/stakeholders
- ◆ Environmental groups
- ◆ General public
- ◆ Special interest groups
- ◆ Trading partners
- ◆ Congress
- ◆ Other Federal government counterparts
- ◆ Agency headquarters personnel

Goals

- ◆ To provide accurate, timely information to all identified audiences.
- ◆ To proactively inform and involve identified audiences about program activities.
- ◆ To be responsive to inquiries from various audiences about program activities.
- ◆ To create and disseminate informational materials on program activities to increase awareness.
- ◆ To communicate information to all identified audiences about program risks and risk-reducing measures.

Actions Occurring Upon Detection of Karnal bunt



Note that whether State or federal officials take the primary responsibility for the following actions will depend on circumstances and resources at the time of the outbreak.

The agency provides support to line and staff operations through Legislative and Public Affairs to address public and media concerns regarding program activities. Communication of program activities will be through the Project Director, regional offices, and staff.

Media Contact

Creating a rapport with local media results in accurate coverage of a program. To avoid conflicting and confusing statements, all outgoing information should be processed through project management. All program personnel should refer media questions to project managers.

Public Meetings/Gatherings

Public meetings/gatherings will be coordinated through SPHDs and the regional office with headquarters support.

Complaints & Concerns

Complaints and concerns directed to project managers will be communicated to SPHDs, the regional office, and headquarters.



Data Management

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Introduction

Data, mapping, and graphics will be accomplished by local, state, or federal TDY personnel with the specialized skills to perform these functions. Sources of personnel could be the Rapid Response Team (RRT), Regional TDY rosters, or State Departments of Agriculture. For assistance, contact APHIS Business Services and/or Pest Detection and Management Programs (PDMP). Basic duties include but are not limited to the following:

1. Prepares maps, overlays, charts, signs, placards, and Power Points.
2. Maintains computer equipment and other technical needs of program (fax machine, digital camera, GPS units).
3. Prepares graphics.
4. Manages data acquisition, manipulation, and retrieval.

Karnal Bunt Information System (KBIS)

The Karnal Bunt Information System (KBIS) was developed beginning in April, 2002, in a combined effort with a contractor, REI Systems; representatives of the USDA–Karnal Bunt Program offices in Arizona, California, New Mexico, and Texas; the Western Regional office in Ft. Collins, CO; and PPQ Headquarters in Riverdale, MD. The initiative was mounted in order to preserve relevant program historical data and to create a unified information collection system for the individual existing regulatory programs and record data for National Karnal Bunt Grain Survey samples analyzed by PPQ. Also included is the ability to create a new program office KBIS system in the event karnal bunt is discovered in a new area. The KBIS Lotus Notes-based system is an interim step in the creation of a larger, comprehensive Oracle-based emergency programs database in the future.

Accessing KBIS

If a computer does not have KBIS installed in Lotus Notes, it will be necessary to access the KBIS system icons on the DATA02 production server. There are three Lotus Notes databases required to support the KBIS system, including the Karnal Bunt Information System latest version, the KBIS Keyword Lookup database, and the KBIS Technical Support database. These can be installed as icons on the users Lotus Notes Desktop by clicking on File, Database, and Open. Then in the server dialogue box, select DATA/O2, and find the icons for the three KBIS databases. Clicking on them will open the databases and closing them will leave the icon on the Lotus Notes desktop for future use.

A KBIS User's Manual is available from PPQ Headquarters and copies are at each program office and the regions.

Creating a New Karnal Bunt Program Office in KBIS

When it becomes necessary to establish a new program area office, the new location program manager with a Lotus Notes connection must first define the program office.

Step 1—

Open KBIS database.

Step 2—

Click on the Program Office button from the main screen (the screen with the six buttons). This opens Program Office section of the KBIS.

Step 3—

On the left Navigator of the Program Office section, there are two main options: Reports and Menus. Click on Administration under the Menus option. This opens Administration section of the KBIS.

Step 4—

On the left Navigator of the Administration section, there are three options: Tasks, Reports, and Menus. Click on the Add Program Office under the Tasks. This creates a new program office document.

Step 5—

Fill in the fields and save the document. The new program office name will be included in the program office list.

Mapping Regulated Areas

Currently, each program office is maintaining their own mapping information that is geo-referenced. Information from the mapping programs will eventually be linked to KBIS when it becomes an Oracle based system.



Contacts

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Introduction **6-1**

Introduction

At the outset of a Karnal bunt program the following list of various groups must be informed and continually updated in all operational phases of emergency programs. Bear in mind the success of a program is heavily dependent on voluntary cooperation, assistance, and understanding from other interrelated groups. Following is a partial list of various groups which must be kept informed of and/or involved in all operational phases of an emergency program.

- ◆ Other Federal, State, County, and municipal agricultural officials
- ◆ Grower groups
- ◆ Universities and Agricultural Research Stations
- ◆ Foreign agricultural interests
- ◆ National, State, and local news media
- ◆ State and local law enforcement officials
- ◆ General public
- ◆ Public health
- ◆ Private agricultural consultants
- ◆ Public and private plant breeders
- ◆ State grain organizations
- ◆ Bureau of Indian Affairs (BIA)



Appendix A

Definitions

Best Management Practices (BMP)	Procedures and techniques designed to negate or minimize the spread of Karnal bunt.
Certificate	PPQ Form 540 or local equivalent.
Cleaning	Removing all soil and plant debris.
Compliance Agreement (CA)	A written agreement between APHIS and a person engaged in growing, handling, or moving regulated articles, in which the person agrees to comply with the provisions of 7 CFR 301.89 and any conditions imposed under that subpart.
Confirmed Detection	Positive laboratory identification of a submitted sample (specimen) of Karnal bunt disease (a bunted kernel of wheat).
Contaminated Seed	Seed from sources in which the Karnal bunt pathogen (<i>Tilletia indica</i> (Mitra) Mundkur) has been determined to exist by the presence of bunted kernels or teliospores.
Contamination	Exposure that occurs when wheat, triticale, or durum wheat grain or any building, equipment, or tools, come into contact with bunted wheat kernels.
Conveyance	Any container used to move wheat, durum wheat, triticale, or their products, including trucks, trailers, railroad cars, bins, and hoppers.
Cultivated Crop	Any crop where the soil is prepared annually to a friable state at the time of planting. No till rotation does not constitute cultivation.
Decontamination	Treatment by the application of an approved decontaminating solution or by methyl bromide fumigation of contaminated implements, material, or equipment.
Delimiting Survey	A survey conducted in the area where a confirmed detection of Karnal bunt has occurred to determine the extent of the outbreak.
Detection	Any site (1) where bunted kernels are found in the field; or (2) that has been the source of grain with bunted kernels; or (3) that has seed found positive with <i>Tilletia indica</i> .

Disease	Any prolonged disturbance in a plant that interferes with its normal structure or function.
Emergency Action Notification (EAN)	A document (PPQ Form 523) giving notice of necessary action to mitigate pest risk within a limited time frame.
Fumigation	The application of an approved fumigant (methyl bromide) to infected grain or to contaminated grain, straw, soil, tools, or buildings.
Harvesting Equipment	Mechanized equipment for harvesting purposes, e.g., combines, reapers, and balers.
Grain	Wheat, durum wheat, or triticale for consumption or processing.
Hay	Host crops cut and dried for feeding to livestock. Hay cut after reaching the dough stage may contain mature kernels of the host crop.
Host	A plant which is invaded by the fungus and from which the fungus obtains its nutrients.
Host Crops	Plants or plant parts, including grain, seed, or hay, of wheat, durum wheat, and triticale.
Infection	The establishment of the fungus within a host plant.
Infestation (infected)	The presence of Karnal bunt, or any identifiable stage of development (i.e., bunted kernels in grain, bunted kernels or teliospores in seed) of the fungus <i>Tilletia indica</i> (Mitra) Mundkur, or the existence of circumstances that make it reasonable to believe that Karnal bunt is present.
Inoculation	The transfer of a pathogen onto a host.
Inoculum	The pathogen or its parts that cause disease. The portions of individual pathogens that are brought into contact with the host.
Karnal Bunt (synonym partial bunt)	A smut disease affecting the grain of wheat, triticale, and durum wheat caused by the fungus <i>Tilletia indica</i> .
Known Infested Property	The entire field where bunted kernels were detected.

Limited Permit	A document (PPQ Form 530) in which an inspector affirms that a specified regulated article not eligible for a certificate is eligible for movement only to a specified destination and in accordance with conditions specified on the permit.
Mechanized Cultivating Equipment and Mechanized Harvesting Equipment	Mechanized equipment used for soil tillage, including tillage attachments for farm tractors — e.g., tractors, disks, plows, harrows, planters, and subsoilers; mechanized equipment used for harvesting purposes — e.g., combines, grain buggies, trucks, swathers, and hay balers.
Pathogen	Any organism that can incite a disease (<i>T. indica</i> is the pathogen that incites or causes Karnal bunt disease).
Plant	Any plant (including any plant part) for or capable of propagation.
PPQ-APHIS-USDA	Plant Protection and Quarantine, Animal and Plant Health Inspection Service, United States Department of Agriculture.
Property	A land unit under one owner or operator that is handled as a single farming or production unit.
Regulated Area	(1) An area (any definable area surrounding a three mile circular buffer from a known infected field) within a 3-mile radius from any field that has tested positive for bunted kernels. (2) Individual fields planted with spore positive seed. (3) Individual elevators under certain conditions.
Sanitize	To devitalize the pathogen by means outlined in 7 CFR 301.89-13.
Seed	Wheat, durum wheat, or triticale for propagation.
Smut	Any disease caused by fungi in the order Ustilaginales. Usually masses of dark powdery spores are present at maturity.
Sporidia	Spores produced by teliospores of smut fungi. These spores are the fragile and short lived basidiospores of the smut fungi.
Straw	The vegetative material left after the harvest of host crops. Straw is generally used as animal feed, bedding, mulch, or for erosion control.
Stubble	Standing stalks of hostplants following harvest.
Teliospore	The thick walled resting spores of smut fungi.

Tilletia indica
(synonym
Neovossia
***indica*)** The fungus that incites Karnal bunt disease in wheat, triticale, and durum wheat.

Tilling The turning of a minimum of the top 6 inches of soil.



Appendix B

Safety

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General Information

Personnel and public safety must be a prime consideration at all times. Stress safety practices in preprogram planning. Supervisors must enforce on the job safety procedures.

Pesticides authorized for use vary in toxicity. When used in accordance with label instructions, materials do not constitute a threat to people, wildlife, bees, etc. Specific safety precautions for each pesticide are listed on the label. In addition, any special precautions listed in this or specific manuals shall be observed.

Keep pesticides in closed, properly labeled containers in a dry place. Store them where they will not contaminate food or feed and where children and animals cannot reach them. When handling a pesticide, follow all precautionary labeling.

Should there be contact through spillage or otherwise, wash immediately with soap and water. Should clothing become contaminated, launder before wearing again. Refer to PPQ Treatment Manual, Section X, for additional information.

Dispose of empty pesticide containers in an approved sanitary landfill, by incineration, or by other methods approved by the Federal Environmental Protection Agency whereby they will not present a hazard or problem. Arrangements for disposal of such containers should be completed and thoroughly understood by all parties directly involved with a program before the actual start of operations. Consult PPQ Regional offices and the Pest Detection and Management Programs (PDMP) staff for pertinent information in States where operations are conducted.

When applying a pesticide, consider the potential impact of the pesticide on all components of the total environment, including humans, crops, livestock, wildlife, aquatic life, non-target insect species, and domesticated honey bees. Avoid contamination of lakes, streams, ponds, or watersheds.

You should be aware of and heed general safety considerations and those specific to the Karnal bunt project. This manual integrates safety procedures into all applicable sections. Remember that inherent hazards are involved when working in field conditions, when working with mechanized harvesting equipment, and when working with chlorine bleach.

Refer also to the APHIS Safety and Health Manual for additional information.

First Aid Suggestions

In case of accidental poisoning or as soon as any person shows symptoms of having been affected by any pesticide, do the following:

1. Remove the person to a place where there will be no further contact with the pesticide.
2. Have the person lie down and keep quiet.
3. Call a physician and provide the name and formulation of the pesticide in use and first aid given.

Keep the local Poison Control Center telephone number posted where pesticides are stored and used. The number may also be found on the inside front cover of the telephone directory. Call Chemtrec on toll free area code 800- 424-9300 for another source of assistance in the event of spills, leaks, fires, exposures, accidents, or other chemical emergencies.

Managing/Monitoring Pesticide Spills

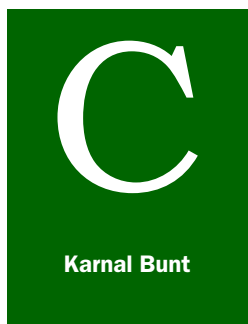
Supervisors involved in pesticide applications must be familiar with *Guidelines for Managing and Monitoring Pesticide Spills* dated March 1981. A useful reference is *The Standard Pesticide User's Guide, Fourth Edition*, by Bert L. Bohmont, Prentice-Hall, Inc., 1997. In addition, the following pesticide spill safety and clean up equipment must be present at all job sites where pesticides are stored or used:

Safety

1. First Aid Kit—Bus and Truck Kit or equivalent (GSA66545-00-5312)
2. Fire extinguisher—5 lb size for Class A, B, or C fires
3. Portable eyewash kit

Cleanup

1. Shovel, square point, D-handle
2. Large heavy duty plastic bags with ties (23)
3. Rubber boots (2 pairs)
4. Disposable coveralls (4 pairs)
5. 5 gallons of water
6. Rubber gloves (4 pairs)
7. Respirators and pesticide cartridges (2 sets)
8. Broom
9. Dust pan
10. Liquid detergent (1 pint bottle); paper towels
11. Scrub brushes (2)
12. Plastic cover or tarpaulin to cover dry spills (10' x 12')
13. Absorbent material to absorb liquid spills (sand, sawdust, vermiculite, kitty litter, etc.)
14. Portable light source



Appendix C

Technical Application Data and Chemicals

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Methyl Bromide

When fumigating contaminated elevators, storage buildings, bins, rail cars, equipment, and infected or contaminated grain, use methyl bromide at the rate of 5 pounds per 1,000 ft³ for a duration of 48 hours. Do not attempt fumigation when the temperature is below 70°F (21°C). Under normal conditions, maintain the recommended concentration of methyl bromide for 48 hours. Monitor the concentration of methyl bromide at regular intervals to ensure adequate treatment. Additional measures such as fumigant recirculation may be required in some cases.

Chlorine Bleach

Use chlorine bleach to decontaminate tools, equipment, etc., that may have come in contact with infected or contaminated grain or soil. Dilute one part Ultra Clorox brand regular bleach (6 percent sodium hypochlorite; EPA Reg. No. 5813-50) in 3 parts water; or one part CPPC Ultra Bleach 2 (6.15 percent sodium hypochlorite; EPA Reg. No. 67619-8) in 3.1 parts water. Since the working solution rapidly breaks down, prepare fresh solutions each time they are to be used. Use the chlorine bleach solution to thoroughly wet down all equipment and implements to be decontaminated. Allow the bleach solution to remain in contact with the equipment for at least 15 minutes.

Use one of the following formulations:

- ◆ Ultra Clorox brand regular bleach (EPA Reg. No. 5813-50); 6% sodium hypochlorite
- ◆ CPPC Ultra Bleach 2 (EPA Reg. No. 67619-8); 6.15% sodium hypochlorite

PCNB (Pentachloronitrobenzene)

Use PCNB with or without carboxin-thiram as a seed treatment. Treat all seed prior to use with one of the following:

1. 6.8 fl. oz. of carboxin-thiram (10% + 10%, 0.91 + 0.91 lb. active ingredient (ai/gal)) flowable liquid and 3 fl. oz. of pentachloronitrobenzene (2.23 lb. ai/gal) per 100 pounds of seed; **or**
2. 4.0 fl. oz. of carboxin-thiram (1.67 + 1.67 lb. ai/gal) flowable liquid and 3 fl. oz. of pentachloronitrobenzene (2.23 lb. ai/gal) per 100 pounds of seed; **or**
3. 6.8 fl. oz. of carboxin-thiram (10% + 10%, 0.91 + 0.91 lb. ai/gal) flowable liquid per 100 pounds of seed; **or**
4. 4.0 fl. oz. of carboxin-thiram (1.67 + 1.67 lb. ai/gal) flowable liquid per 100 pounds of seed; **or**
5. 3 fl. oz. of pentachloronitrobenzene (2.23 lb. ai/gal) per 100 pounds of seed.



Appendix D

Laboratory Procedures

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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 Bonde¹, 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.



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:



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.

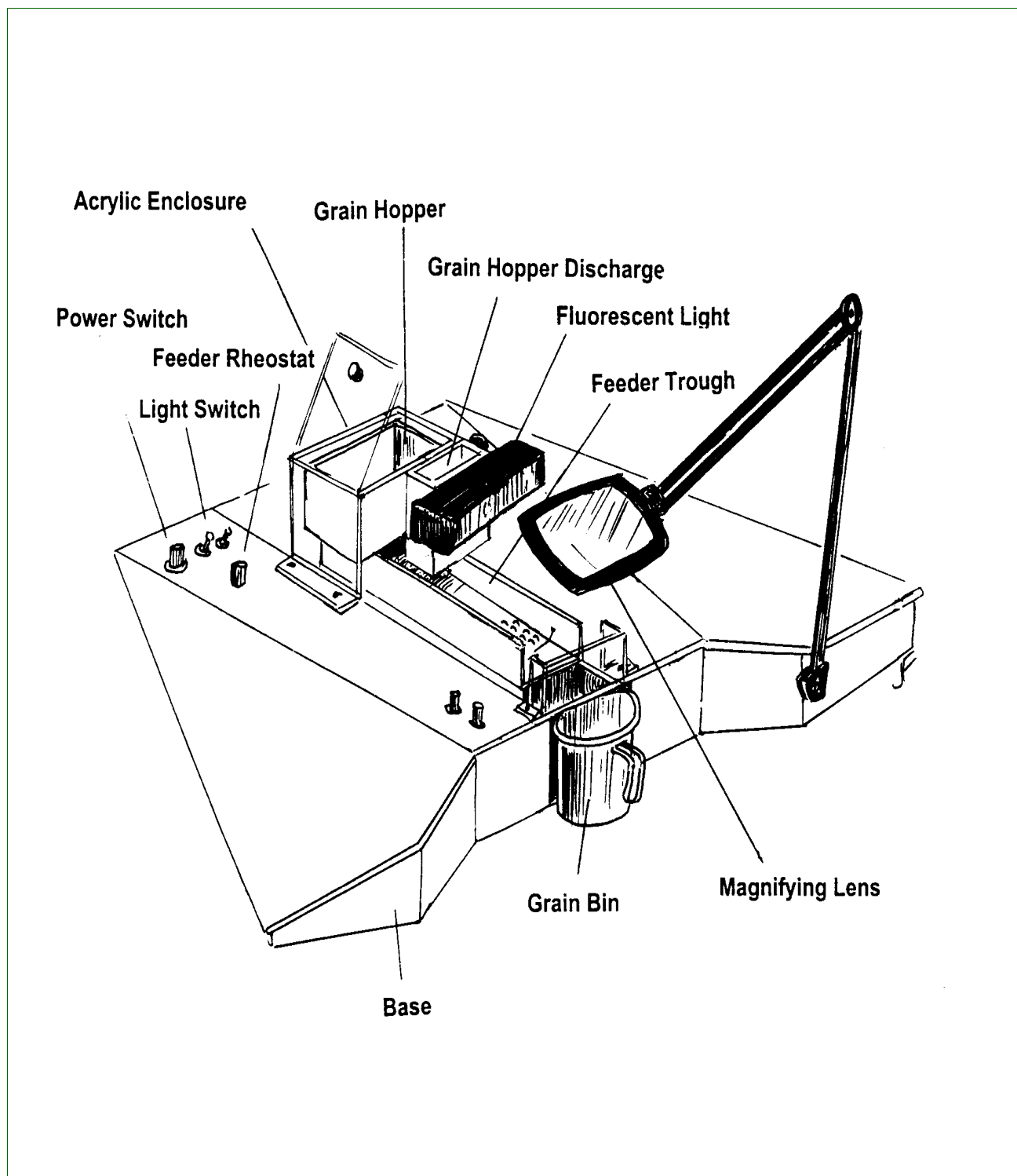


FIGURE D-1: Grain Inspection Machine

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.

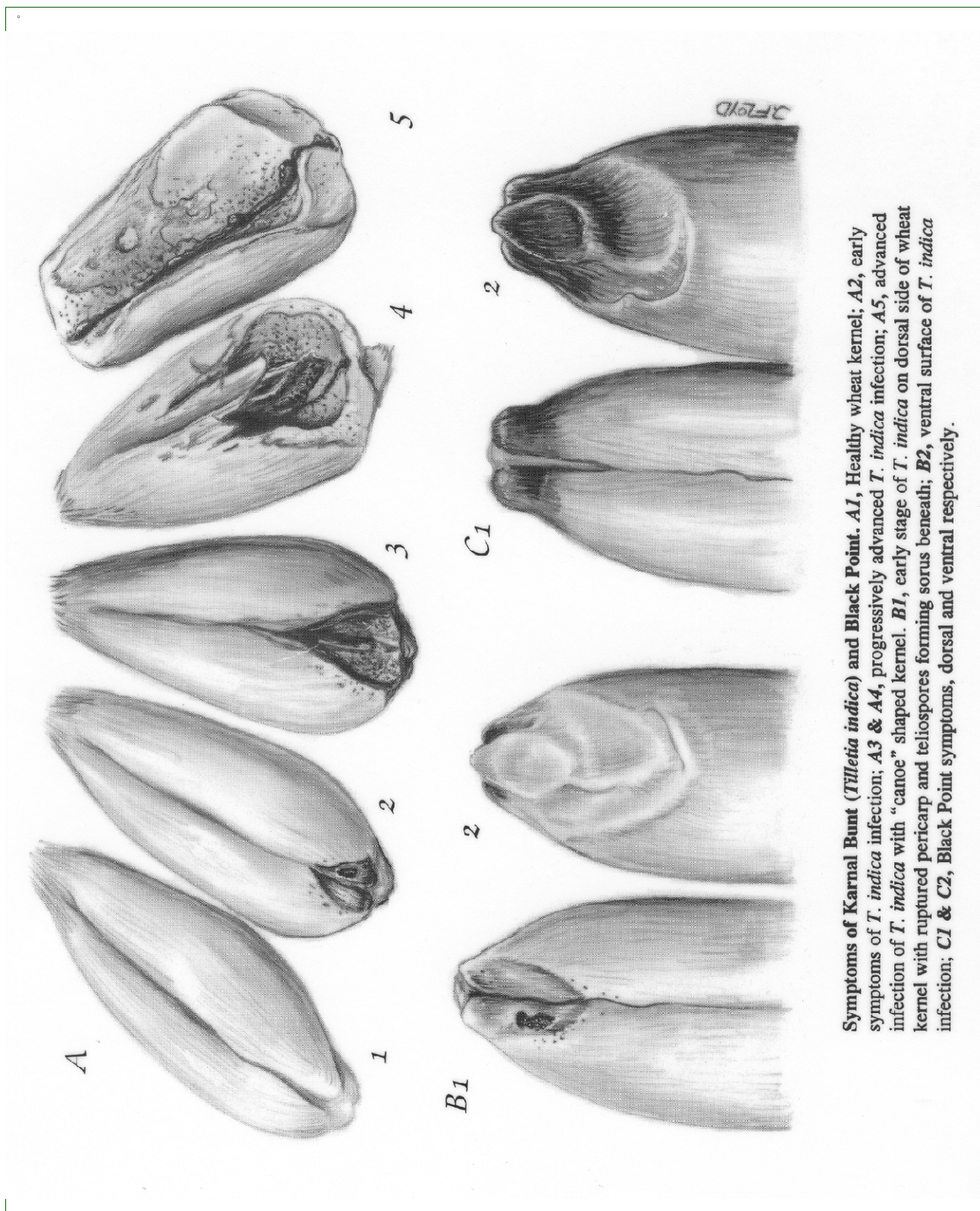
Step 5—Clean the work area.

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.



Symptoms of Karnal Bunt (*Tilletia indica*) and Black Point. *A1*, Healthy wheat kernel; *A2*, early symptoms of *T. indica* infection; *A3* & *A4*, progressively advanced *T. indica* infection; *A5*, advanced infection of *T. indica* with "canoe" shaped kernel. *B1*, early stage of *T. indica* on dorsal side of wheat kernel with ruptured pericarp and teliospores forming sorus beneath; *B2*, ventral surface of *T. indica* infection; *C1* & *C2*, Black Point symptoms, dorsal and ventral respectively.

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.



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.



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	<ol style="list-style-type: none"> 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	<ol style="list-style-type: none"> 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.



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 ($C_6H_8O_7 \cdot 1H_2O$)
- ◆ 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 μ L, for Eppendorf adjustable pipettor
- ◆ Pipette tips, repeating
- ◆ Pipettes, Mohr, 1-mL and 10-mL
- ◆ Pipettor, adjustable, Eppendorf, 100-1000 μ L
- ◆ Pitcher, 3000-mL
- ◆ Potassium acetate ($\text{KC}_2\text{H}_3\text{O}_2$)
- ◆ Rack, test tube
- ◆ Scoop, lab, stainless steel
- ◆ Shaker, orbital, thermolyne
- ◆ Sieve, nylon, 20 μm pore with square openings, 3" ID x 50 mm high, USDA²
- ◆ Sieve, nylon, 53 μm pore with square openings, 3" ID x 50 mm high, USDA²
- ◆ 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 Na₂HPO₄ solution

Step 1—Weigh anhydrous Na₂HPO₄.

Wearing safety glasses and latex gloves, weigh 38.39 g of anhydrous Na₂HPO₄ 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 $2.0 \text{ g} \pm 0.1 \text{ g}$ 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.

Adding Extraction Solution

Step 4—Resanitize the hood.

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

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- μ m sieve³.

For each sample, invert the bottle or swirl the flask to re-suspend the grain and debris. Immediately pour the contents into the 53- μ m 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- μ m sieve³.

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- μ m 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- μ m 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- μ m 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 $\frac{1}{2}$ 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 μ l 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.

Making Slide Labels and Filling Out the Data Sheet

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.

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 <i>Tilletia indica</i> 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.



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 53 μ), 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.



Important

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 additional 15 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			COMMENTS	DATA ENTERED
		#	IDENTIFIED BY	DATE		

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

QUALITY CONTROL (QC) / GRAIN LABS									
DATE	SAMPLE NUMBER	TOTAL WEIGHT	DISCARD WEIGHT	DARK TRIP	KERNELS ASSIGNED	KERNELS FOUND	PERSONNEL QC	COMMENTS	
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
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FIGURE D-5: Quality Control (QC) Grain Labs



Appendix E

Compliance Agreements

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Examples

The following examples serve as guides. Stipulations may be altered to meet local needs and/or farming practices.

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE			Hay Producer
COMPLIANCE AGREEMENT			Rev. 07/04/01
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION	
3. REGULATED ARTICLE(S): Hay cut using plants or plant parts of wheat, durum wheat, and/or triticale			
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>			
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS: HAY PRODUCERS.			
6. SIGNATURE	7. TITLE	8. DATE SIGNED	
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.	
		10. DATE OF AGREEMENT	
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program	
13. SIGNATURE			
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS	
16. SIGNATURE			

PPQ FORM 519

FIGURE E-1: Compliance Agreement for Hay Producers

HAY PRODUCERS

Compliance Agreement Stipulations

Before hay may be moved within and/or out of the regulated area it must be inspected by Karnal bunt personnel. The following distinctions will be made:

- ◆ Hay cut prior to the development of the kernel will be considered no risk for having Karnal bunt.
- ◆ Hay cut with fully developed kernels will be tested for the presence of bunted kernels. Samples can be taken from bales or from standing wheat around the edges of the field from which the hay was cut. The hay will be regulated according to the results of the testing.
- ◆ Hay cut from previously tested fields will be regulated according to the results of that previous test.

Hay testing NEGATIVE or hay cut PRIOR TO DEVELOPMENT OF KERNEL will be allowed movement under the following conditions:

Movement of hay **within and out** of the regulated area:

- ◆ Hay must be inspected/tested free of bunted kernels prior to movement from the production area.
- ◆ If hay has already been moved to a storage area inspection/testing will be done at the storage area.
- ◆ Grower will be issued a Certificate for the number of bales and/or weight inspected/tested free of bunted kernels.
- ◆ The number of bales noted on the Certificate for the location (field or storage area) will be allowed movement within or out of the regulated area. The Certificate must accompany any movement.

Hay testing POSITIVE for bunted kernels will be allowed movement under the following conditions:

Movement of hay **within** the regulated area:

- ◆ A grower may move his/her hay from the field of production (land owned/leased by grower) to an area owned by the grower for storage.
- ◆ A grower may move his/her hay from the field of production (land owned/leased by grower) for the purpose of feeding to livestock.
- ◆ A grower must contact Karnal bunt personnel prior to movement of hay. A limited permit will be issued for movement.
- ◆ Hay must be moved in a manner that safeguards the hay and minimizes the potential for spreading Karnal bunt.

Movement of hay **out of** the regulated area:

- ◆ Hay testing positive for bunted kernel or cut from a field that tested positive for bunted kernels can only move out of the regulated area if destined to an approved establishment.
- ◆ The establishment must be approved by Karnal bunt personnel and under compliance to process the hay in an approved manner so as to destroy all Karnal bunt teliospores.
- ◆ Program personnel must be contacted prior to movement of hay. If movement is approved, a limited permit will be issued for movement to the approved establishment out of the regulated area.
- ◆ Hay approved for movement out of the regulated area must be moved in a manner that safeguards the hay and minimizes the spread of Karnal bunt.

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE			Mushroom Producer
COMPLIANCE AGREEMENT			Rev. 06/24/01
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION	
3. REGULATED ARTICLE(S): Plants or plant parts of wheat, durum wheat, and/or triticale used for mushroom production.			
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>			
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS: MUSHROOM PRODUCERS.			
6. SIGNATURE		7. TITLE	8. DATE SIGNED
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.	
		10. DATE OF AGREEMENT	
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program	
13. SIGNATURE			
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS	
16. SIGNATURE			

PPQ FORM 519

FIGURE E-2: Compliance Agreement for Mushroom Producers

MUSHROOM PRODUCERS

Compliance Agreement Stipulations

5. I/We agree to the following:

- ◆ Wheat straw from the regulated area must be introduced to a composting procedure that results in a pasteurized product. The composting procedure shall produce pasteurization temperatures of 170°F for at least 1 minute.

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE			Rail Cars
COMPLIANCE AGREEMENT			Rev. 09/10/01
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION	
3. REGULATED ARTICLE(S): Rail cars transporting wheat, durum wheat, and triticale grain, mill feed, and/or associated articles.			
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>			
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS: RAIL CARS.			
6. SIGNATURE	7. TITLE	8. DATE SIGNED	
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.	
		10. DATE OF AGREEMENT	
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program	
13. SIGNATURE			
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS	
16. SIGNATURE			

PPQ FORM 519

FIGURE E-3: Compliance Agreement for Rail Cars

RAIL CARS TRANSPORTING BUNTED KERNEL POSITIVE GRAIN, MILLFEED, AND/OR ASSOCIATED ARTICLES

Compliance Agreement Stipulations

5. I/We agree to the following:

- ◆ All rail cars designated for use must pass a pre-use inspection performed by a Karnal Bunt Program inspector. Cars can be used only if he/she determines that the car is leak-proof and is enclosed or adequately covered. Cars having passed the initial inspection need not be reinspected unless the car is damaged in some way which would require an additional inspection.
- ◆ To transport regulated articles only when authorized to move under Limited Permit (PPQ Form 530).
- ◆ To present the PPQ Form 530 to the appropriate consignee representative at destination.
- ◆ To ensure that rail cars carrying regulated articles move to their approved destination by the most direct route prior to the void date on the PPQ Form 530.
- ◆ To notify the Karnal Bunt Office 24 hours in advance of any changes in movement as described in the PPQ Form 530. Approval must be obtained prior to any change movement of regulated articles.
- ◆ To immediately contact the Karnal Bunt Office in the event of a spillage or accident.
- ◆ To obtain approval in advance from the Karnal Bunt Office, if transferring of regulated grain from one rail car to another becomes necessary.
- ◆ To ensure rail cars removed from service on this project are properly cleaned in accordance with USDA protocols before being placed in service for other purposes.
- ◆ To perform treatments in accordance with the Karnal Bunt treatment protocols.

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE COMPLIANCE AGREEMENT			Trucks/Trailers
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION	
3. REGULATED ARTICLE(S): Trucks/Trailers - Transporting wheat, durum wheat, and triticale grain, mill feed, and associated articles.			
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>			
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS.			
6. SIGNATURE	7. TITLE	8. DATE SIGNED	
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.	
		10. DATE OF AGREEMENT	
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program	
13. SIGNATURE			
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS	
16. SIGNATURE			

PPQ FORM 519

FIGURE E-4: Compliance Agreement for Trucks/Trailers

TRUCKS/TRAILERS TRANSPORTING BUNTED KERNEL POSITIVE GRAIN, MILLFEED, AND/OR ASSOCIATED ARTICLES

Compliance Agreement Stipulations

5. I/We agree to the following:

Transporting Regulated Grain

- ◆ To transport regulated articles only when authorized to move under Limited Permit (PPQ Form 530).
 - ❖ The PPQ Form 530 will be issued by the inspector or designee under compliance after:
 - Trucks/trailers transporting positive grain have been determined to be leak proof and properly covered to avoid spillage of grain.
 - All debris has been removed from the exterior of the truck/trailer after loading.
- ◆ To perform treatments in accordance with the Karnal bunt treatment protocols. Refer to Cleaning and Sanitizing Protocols for approved treatments.
- ◆ To immediately contact the USDA/State Department of Agriculture Karnal bunt office in the event of a spillage or accident. Spills can be picked up and returned to the truck hauling the positive grain.
- ◆ To obtain approval in advance from the USDA/State Department of Agriculture Karnal bunt office, if transferring of regulated grain from one truck to another becomes necessary.
- ◆ To notify the USDA/State Department of Agriculture Karnal bunt office 24 hours in advance of any changes in movement as described in the PPQ Form 530. Approval must be obtained prior to any movement of regulated articles.
- ◆ To ensure that trucks/trailers carrying regulated articles move to their approved destination by the most direct route prior to the void date on the PPQ Form 530.

Discharging Regulated Grain

- ◆ To ensure that trucks/trailers are cleaned following discharge and that small quantities of debris resulting from this cleaning are:
 - ❖ Store back in the bin with bunted kernel positive grain, or
 - ❖ Buried in an approved landfill, or
 - ❖ Treated with an approved treatment.
- ◆ To clean vehicles dedicated to transporting bunted kernel positive grain (not back hauling) in accordance with attached information upon completion of transporting bunted kernel positive grain. All other vehicles transporting Karnal bunt positive grain and back hauling will be cleaned in accordance with attached information prior to loading any agricultural products.
- ◆ To provide a copy of this compliance agreement to the facilities where the trucks/trailers will be loading.

Approved Conveyances

The following license plates belong to conveyances that have been inspected and approved by the USDA/State Department of Agriculture Karnal bunt project.

_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

Cleaning and Sanitizing Protocols for Grain Bins, Elevators, Seed Plants, and Associated Equipment

Seed equipment

Grain bins, elevators, seed plants, conveyances, and associated equipment used in the storage, transportation and/or processing of wheat, durum wheat or triticale seed which had tested positive for Karnal bunt spores or kernels will require cleaning and sanitizing under the following guidelines:

- ◆ “Cleaning and sanitizing” requirements are defined as the removal of all host material followed by:

A. SODIUM HYPOCHLORITE SOLUTION

The equipment should be thoroughly wetted until point of runoff with a solution of 1.5 percent sodium hypochlorite—e.g., with a solution of sodium hypochlorite mixed with water applied at the rate of 1 gallon of household chlorine bleach (5.2 percent sodium hypochlorite) mixed with 2.5 gallons of water. Leave solution in contact with all surfaces for a minimum of 15 minutes then thoroughly rinse surfaces with water to minimize any adverse effect on equipment. Prepare a fresh solution of sodium hypochlorite and potable water every four to five hours.

NOTE: EPA approved bleach products are as follows:

- ▶ **Ultra Clorox brand bleach (EPA Reg. No. 5813-50); 6% Sodium Hypochlorite**
- ▶ **CPPC Ultra Bleach 2 (EPA Reg. No. 67619-8); 6.15% Sodium Hypochlorite**

These are the only two products that have EPA approval to be used for the sanitization of equipment.

Or

B. HOT WATER AND DETERGENT

Hot water and detergent drench with a pressure of at least 30 pounds per square inch. The water must be applied to all surfaces until point of runoff including penetration into cracks and crevices. At point of runoff the temperature must be a minimum of 170°F.

Or

C. LIVE STEAM TO ALL SURFACES

Live steam (212°F) can be used to treat various harvesting and cleaning equipment, as long as all surfaces that come into contact with grain can be directly contacted with the live steam. Apply steam directly to all surfaces until the point of runoff. At point of runoff the temperature must be a minimum 170°F.

Or

D. FUMIGATION

The storage area can be fumigated with methyl bromide at the dosage of 15 pounds/1,000 ft³ for 96 hours.

The USDA Karnal Bunt office must be notified 24 hours in advance of the sanitizing to allow monitoring of the treatment by a PPQ officer or State inspector.

Grain-only equipment

A facility, portions of a facility, associated equipment, and transportation conveyances that do not handle seed, only grain, may be approved to be cleaned and disinfected by the following manner:

Grain handling equipment (elevator legs, etc.) used to move grain between bins must be cleaned, but sanitizing is not required. The bins and other equipment must be cleaned of all kernels and particulate matter. The purpose is to clean to the point of freedom of bunted kernels, not spores. This is not approved for anything that may come into contact with seed.

Certification

- ◆ A PPQ Form 540, Certificate, or equivalent documentation will be provided stating that the treatment has met the requirements according to Karnal Bunt Project Protocol.

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE COMPLIANCE AGREEMENT			Karnal Bunt Positive Grain Dealer
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION	
3. REGULATED ARTICLE(S): Plants or plant parts of wheat, durum wheat, and/or triticale. Karnal bunt positive wheat grain (bunted kernels).			
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>			
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS.			
6. SIGNATURE	7. TITLE	8. DATE SIGNED	
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.	
		10. DATE OF AGREEMENT	
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program	
13. SIGNATURE			
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS	
16. SIGNATURE			

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FIGURE E-5: Compliance Agreement for Karnal Bunt Positive Grain Dealers

KARNAL BUNT POSITIVE GRAIN DEALER

Compliance Agreement Stipulations

5. I/We Agree To The Following:

Certificates/Permits

- ◆ To receive and safeguard PPQ Form 530 (Limited Permits).¹ These forms are accountable and must be maintained in a secure location. Only employees designated by the signatory of the Compliance Agreement are approved to handle and complete these documents. The signatory of the Compliance Agreement shall provide a list of designees to the USDA/State Department of Agriculture Karnal Bunt Office. Documents shall be completed by following the instructions that are included as an attachment to this Compliance Agreement.

Receiving Bunted Kernel Positive Grain

- ◆ To handle bunted kernel positive wheat grain from the regulated area only if it has been authorized to move under PPQ Form 530 (Limited Permit).
- ◆ To verify, upon receipt of the shipment, the identity and quantities stated on PPQ Form 530.

Storage of Grain

- ◆ To notify the USDA/State Department of Agriculture Karnal Bunt Project Office 24 hours in advance of turning/moving of stored grain.

Shipping Grain

- ◆ To notify the Consignee purchasing the grain that it is strictly prohibited to utilize this grain for the purpose of planting.
- ◆ To retain original copies of PPQ Form 530 (Limited Permit) and make them available at the request of the USDA/State Department of Agriculture Karnal Bunt Program Office, and to maintain supporting records of grain movement associated with the permit.
- ◆ To contact the USDA/State Department of Agriculture Karnal Bunt Office 24 hours in advance of each planned movement of grain to allow for the possible inspection of loads by an inspector.
- ◆ To ensure that the truck/trailer that will be loaded is covered by a compliance agreement to transport positive grain.
- ◆ To inspect each conveyance to ensure that it is leak-proof and is enclosed or adequately covered with a tarp to prevent the loss of grain in transport. Seal each truck with PPQ issued wire seals and record seal numbers on the PPQ Form 530. Attach a copy of the weight ticket for each load to the associated PPQ Form 530 - white copy to serve as a verification of the grain movement. Present the PPQ Form 530 to the appropriate consignee representative at destination.
- ◆ To immediately contact the USDA/State Department of Agriculture Karnal Bunt Office in the event of a significant spillage or accident.

¹ For guidance, see [Instructions for filling out PPQ Form 530 \(Limited Permit\)](#).

- ◆ To obtain approval in advance from the USDA/State Department of Agriculture Karnal Bunt Office if transferring of regulated grain from one conveyance to another becomes necessary.
- ◆ To ensure that all wheat kernels are removed from the conveyance following discharge and that small quantities of debris resulting from this cleaning are:
 - ❖ Collected and placed in the conveyance/bin with the positive grain, or
 - ❖ Buried under two feet of soil in an area not to be cultivated or in an approved landfill², or
 - ❖ Treated with an approved treatment.
- ◆ To remove all volunteer plants resulting from debris by cultivation or by using an herbicide.
- ◆ To retain copies of permits and other related documentation related to the transport of said regulated grain and to ensure that all documentation of the transport of the regulated wheat is made available upon request to an inspector.
- ◆ To contact the USDA/State Department of Agriculture Karnal Bunt Office prior to breaking the seals or immediately after discovering that a seal has been broken from the top loading doors of the railcars that are returned from discharge destination. This notification will allow for the monitoring of the compliance agreement by the USDA/State Department of Agriculture Karnal Bunt Project.

² Contact the USDA/State Department of Agriculture Karnal Bunt Office for information on approved establishments/facilities.

Instructions for filling out PPQ Form 530 (Limited Permit)

Block #1: Enter the date in the following format: (October 12, 2001).

Block #2: Enter the Void Date in the following format: (October 20,2001). Note: Allow a sufficient amount of time for the shipment to arrive directly to the destination to include any possible delays.

Block # 3: Enter your name/company that is selling the commodity.

Block #4: Enter the location from where the grain is being shipped.

Block #5: Enter name and address of the person/company that is purchasing the grain.

Block #6: Enter license plate number of the trailer and the issuing state.

Block #7: Enter Railcar number.

Block #8:

- A. Enter the quantity in pounds.
- B. Enter **Positive KB Wheat** and enter the seal numbers.
- C. Enter the following statement: "To designated address only."

Block #9: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Shipping Point Signatory).

Block#10: Enter the date that the shipment arrives at its destination in the following format: (October 12, 2001).

Block #11: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Receiving Point Signatory).

Distribution of copies issued by party under compliance:

- ◆ PINK COPY:
 - ❖ Goes with shipment
 - ❖ Held by consignee at destination

- ◆ YELLOW COPY:
 - ❖ Goes with shipment
 - ❖ Destination PPQ Officer picks up copy, or
 - ❖ Consignee that is under compliance maintains copy until requested by PPQ

- ◆ WHITE COPY:
 - ❖ Consignor maintains until requested by PPQ

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE Karnal Bunt Negative Grain Dealer/Broker COMPLIANCE AGREEMENT		
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION
3. REGULATED ARTICLE(S): Wheat, durum wheat, and/or triticale grain (all referred to in this document as wheat) that has been tested and found to be Karnal bunt free (no bunted kernels).		
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>		
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS.		
6. SIGNATURE	7. TITLE	8. DATE SIGNED
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.
		10. DATE OF AGREEMENT
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program
13. SIGNATURE		
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS
16. SIGNATURE		

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FIGURE E-6: Compliance Agreement for Karnal Bunt Negative Grain Dealer/Broker

KARNAL BUNT NEGATIVE GRAIN DEALER/BROKER

Compliance Agreement Stipulations

FOR REGULATED ARTICLES TESTING **NEGATIVE** FOR KARNAL BUNT

5. I/We Agree To The Following:

Certificates/Permits

- ◆ To receive and safeguard PPQ Form 530 (Limited Permits) and PPQ Form 540 (Certificates). These forms are accountable and must be maintained in a secure location. Only employees designated by the signatory of the Compliance Agreement are approved to handle and complete these documents. The signatory of the Compliance Agreement shall provide a list of designees to the USDA/State Department of Agriculture Karnal Bunt Office. Documents shall be completed by following the instructions that are included as attachments to this Compliance Agreement.



Bin-tested negative grain is eligible for a Limited Permit (PPQ Form 530).
See [Instructions for Filling out PPQ Form 530 \(Limited Permit\)](#).
Field-tested negative grain is eligible for a Certificate (PPQ Form 540).
See [Instructions for filling out PPQ Form 540 \(Certificate\)](#).

Receiving Grain

- ◆ To handle wheat from the regulated area only if it has been authorized to move under PPQ Form 540 (Certificate) or PPQ Form 530 (Limited Permit).
- ◆ To verify, upon receipt of the shipment, the identity and quantities stated on PPQ Form 540 or PPQ Form 530.

Storage of Grain

- ◆ To notify the USDA/State Department of Agriculture Karnal Bunt Project Office 24 hours in advance of turning/moving of stored grain.

Shipping Grain

- ◆ To notify the Consignee purchasing the grain that it is strictly prohibited to utilize this grain for the purpose of planting outside the regulated area. This grain can only be utilized for planting inside the regulated area after it has tested negative for karnal bunt spores and has been treated with an approved fungicide.
- ◆ To issue PPQ Form 530 (Limited Permit) or PPQ Form 540 (Certificate) on each conveyance of grain moving to another location. With this permit, grain may be moved to locations within the regulated area or to destinations outside the regulated area. This grain has been certified as free of Karnal bunt (bunted kernel free) and may be moved to any potential market (except for planting) both domestic and/or export. **Note:** many foreign countries requiring an additional declaration on the Phytosanitary Certificate (PPQ Form 577) prohibit wheat grain originating within Karnal bunt regulated areas and/or grain that is commingled with grain from regulated areas from fields that have not been tested. We advise before contracts for the exportation of grain are signed you contact the USDA/State office for the importing country's Karnal bunt export certification requirements.
- ◆ To retain original copies of PPQ Form 530 (Limited Permit) or PPQ Form 540 (Certificate) and make them available at the request of the USDA/State Department of Agriculture Karnal Bunt Program Office, and to maintain supporting records of grain movement associated with the permit or certificate.
- ◆ To obtain all Phytosanitary Certificates, for the purpose of exporting grain, from the USDA/State Department of Agriculture Karnal Bunt Project Office.

Instructions for Filling out PPQ Form 530 (Limited Permit)

Block #1: Enter the date in the following format: (October 12, 2001).

Block #2: Enter the Void Date in the following format: (October 20,2001). Note: Allow a sufficient amount of time for the shipment to arrive directly to the destination to include any possible delays.

Block # 3: Enter your name/company that is selling the commodity.

Block #4: Enter the location from where the grain is being shipped.

Block #5: Enter name and address of the person/company that is purchasing the grain.

Block #6: Enter license plate number of the trailer and the issuing state.

Block #7: Enter Railcar number.

Block #8:

- A. Enter the quantity in pounds.
- B. Enter **Wheat Grain**.
- C. Enter "Tested negative for KB." Stamp the center of the bottom two lines of Block #8 with the stamp that reads as follows: "Regulated wheat grain. Tested negative for Karnal bunt. Non-propagative domestic use or limited export only."

Block #9: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Shipping Point Signatory).

Block#10: Enter the date that the shipment arrives at its destination in the following format: (October 12, 2001).

Block #11: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Receiving Point Signatory).

Distribution of copies issued by party under compliance:

- ◆ PINK COPY:
 - ❖ Goes with shipment
 - ❖ Held by consignee at destination
- ◆ YELLOW COPY:
 - ❖ Goes with shipment
 - ❖ Destination PPQ Officer picks up copy, or
 - ❖ Consignee that is under compliance maintains copy until requested by PPQ
- ◆ WHITE COPY:
 - ❖ Consignor maintains until requested by PPQ

Instructions for filling out PPQ Form 540 (Certificate)

Block #1: Enter the date in the following format: (October 12, 2001).

Block #2: Enter the Void Date in the following format: (October 20,2001). Note: Allow a sufficient amount of time for the shipment to arrive directly to the destination to include any possible delays.

Block # 3: Enter your name/company that is selling the commodity.

Block #4: Enter the location from where the grain is being shipped.

Block #5: Enter name and address of the person/company that is purchasing the grain.

Block #6: Enter license plate number of the trailer and the issuing state.

Block #7: Enter Railcar number.

Block #8:

- A.** Enter the quantity in pounds.
- B.** Enter the Item name that is being issued a certificate.
- C.** Enter KB tested negative. Note: In the bottom two lines of block # 8 enter the following statement: "Produced in an area where Karnal bunt is not known to occur. For non-propagative use only."

Block #9: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Shipping Point Signatory).

Distribution of copies issued by party under compliance:

- ◆ WHITE COPY (PART 1): Goes with shipment to consignee
- ◆ GREEN COPY (PART 2): Consignor's Copy
 - ❖ WHITE COPY (PART 3): Consignor maintains until requested by PPQ

UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE		Feed Processor
COMPLIANCE AGREEMENT		
1. NAME AND MAILING ADDRESS OF PERSON OR FIRM		2. LOCATION
3. REGULATED ARTICLE(S): Wheat, durum wheat, triticale grain (referred to in this document as wheat) and associated equipment used to handle, transport, store, and process the wheat grain.		
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS 7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>		
5. I/WE AGREE TO THE FOLLOWING: SEE ATTACHED STIPULATIONS.		
6. SIGNATURE	7. TITLE	8. DATE SIGNED
The affixing of the signatures below will validate this agreement, which shall remain in effect until cancelled, but may be revised as necessary or revoked for noncompliance.		9. AGREEMENT NO.
		10. DATE OF AGREEMENT
11. PPQ OFFICIAL (Name and Title) Project Coordinator		12. ADDRESS Karnal Bunt Emergency Program
13. SIGNATURE		
14. STATE AGENCY OFFICIAL (Name and Title) State Entomologist		15. ADDRESS
16. SIGNATURE		

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FIGURE E-7: Compliance Agreement for Feed Processors

FEED PROCESSOR

Compliance Agreement Stipulations

5. I/We agree to the following:

Receiving Bunted Kernel Positive Grain

- ◆ To handle regulated millfeed and/or bunted kernel positive wheat grain from the regulated area or an approved facility and only if it has been authorized to move under PPQ Form 530 (Limited Permit).³
- ◆ To verify, upon receipt of the shipment, the identity and quantities stated on PPQ Form 530, (lines 1-8).
- ◆ To receive and safeguard PPQ Form 530 (Limited Permits). These forms are accountable and must be maintained in a secure location.
- ◆ To retain original copies of PPQ Form 530 (Limited Permit) and make them available at the request of the USDA/State Department of Agriculture Karnal Bunt Program Office, and to maintain supporting records of grain movement associated with the permit.
- ◆ To immediately notify the USDA/State Department of Agriculture Karnal Bunt Project Office if any discrepancies occur in the shipment, if the shipment arrives without a PPQ Form 530, if seals are broken, or if it is received after the void date or in case of emergency (no offloading of shipments with discrepancies, broken seals, or shipments arriving after void date is permitted until approved by the USDA/State Department of Agriculture Karnal Bunt Project Office).
- ◆ To ensure that conveyances (trucks/trailers) delivering contaminated wheat grain are cleaned of all grain and/or millfeed contaminants prior to leaving the facility.
- ◆ To ensure that any material resulting from cleaning or processing bunted kernel positive wheat grain is destroyed by:
 - ❖ Burial under two feet of soil in an area not to be cultivated or in an approved landfill⁴, or
 - ❖ Collection of all debris and placement in the conveyance/bin with the positive grain, or
 - ❖ Sanitizing with an approved treatment.
- ◆ To remove all volunteer plants resulting from debris by cultivation or by using an herbicide.

Storage of Bunted Kernel Positive Grain

- ◆ To ensure that regulated millfeed and/or bunted kernel positive wheat grain is not subsequently moved from the initial processing facility to another facility without advance approval from the USDA/State Department of Agriculture Karnal Bunt Project Office.
- ◆ To notify the USDA/State Department of Agriculture Karnal Bunt Project Office 24 hours in advance of turning/moving of stored grain.

³ For guidance, see [Instructions for filling out PPQ Form 530 \(Limited Permit\)](#).

⁴ Contact the USDA/State Department of Agriculture Karnal Bunt Office for information on approved establishments/facilities.

Processing Bunted Kernel Positive Grain

- ◆ To ensure that no changes occur in processing (changes that would not meet specified minimum moist heat treatment requirements) unless approved by the USDA/State Department of Agriculture Karnal Bunt Project Office.
- ◆ To ensure that all regulated millfeed, by-products, and/or positive wheat grain that is received is processed and treated with a moist heat treatment at a minimum of 170°F for at least one minute.

All entities that are under compliance are subject to 7 CFR 301.89.

Copies of the *Code of Federal Regulations* are available from the following Web site:

<http://www.gpoaccess.gov/cfr/index.html>

Penalty may be assessed for misuse of the agreement (7 USC 163).

Instructions for filling out PPQ Form 530 (Limited Permit)

Block #1: Enter the date in the following format: (October 12, 2001).

Block #2: Enter the Void Date in the following format: (October 20,2001). Note: Allow a sufficient amount of time for the shipment to arrive directly to the destination to include any possible delays.

Block # 3: Enter your name/company that is selling the commodity.

Block #4: Enter the location from where the grain is being shipped.

Block #5: Enter name and address of the person/company that is purchasing the grain.

Block #6: Enter license plate number of the trailer and the issuing state.

Block #7: Enter Railcar number.

Block #8:

- A. Enter the quantity in pounds.
- B. Enter **Positive KB Wheat** and enter the seal numbers.
- C. Enter the following statement: "To designated address only."

Block #9: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Shipping Point Signatory).

Block#10: Enter the date that the shipment arrives at its destination in the following format: (October 12, 2001).

Block #11: The signatory and designees authorized by the signatory of the Compliance Agreement sign in this block (Receiving Point Signatory).

Distribution of copies issued by party under compliance:

- ◆ PINK COPY:
 - ❖ Goes with shipment
 - ❖ Held by consignee at destination

- ◆ YELLOW COPY:
 - ❖ Goes with shipment
 - ❖ Destination PPQ Officer picks up copy, or
 - ❖ Consignee that is under compliance maintains copy until requested by PPQ

- ◆ WHITE COPY:
 - ❖ Consignor maintains until requested by PPQ

Harvesting Equipment and Conveyances

<p>FORM APPROVED OMB NUMBER 0579-0054</p>	
<p>UNITED STATES DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE PLANT PROTECTION AND QUARANTINE</p> <p style="text-align: center;">COMPLIANCE AGREEMENT</p>	<p>Public reporting burden for this collection of information is estimated to average 1.25 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the form. Send comments regarding this burden estimate or any other aspects of this collection of information, including suggestions for reducing the burden, to USDA, OIRM, Clearance Officer, Room 404-W, Washington, DC 20250. When replying refer to the OMB number and Form Number in your letter.</p>
1. NAME AND ADDRESS OF PERSON OR FIRM	2. LOCATION
	Same
3. REGULATED ARTICLE(S)	
Harvesting Equipment and Conveyances associated with wheat, durum wheat and triticale.	
4. APPLICABLE FEDERAL QUARANTINE(S) OR REGULATIONS	
7 CFR 301.89, KARNAL BUNT, <i>Tilletia indica</i>	
5. <i>I/We agree to the following:</i>	
SEE ATTACHEMENT STIPULATIONS: Harvesting Equipment & Conveyance	
6. SIGNATURE	7. TITLE
8. DATE SIGNED	
<p>The affixing of the signatures below will validate this agreement which shall remain in effect until canceled, but may be revised as necessary or revoked for noncompliance.</p>	
9. AGREEMENT NO.	
KB- 007-AZ	
10. DATE OF AGREEMENT	
04/05/2004	
11. PPQ OFFICIAL (<i>Name and Title</i>)	12. ADDRESS
13. SIGNATURE	USDA -APHIS-PPQ 3658 E CHIPMAN RD PHOENIX, AZ. 85040
14. STATE AGENCY OFFICIAL (<i>Name and Title</i>)	15. ADDRESS
16. SIGNATURE	ARIZONA DEPARTMENT OF AGRICULTURE 1688 WEST ADAMS PHOENIX, AZ 85007
<p>PPQ FORM 519 AUG 77</p>	

FIGURE E-8: Compliance Agreement for Harvesting Equipment and Conveyances

I/We agree to the following:

- ◆ Fields within the regulated area will be sampled and results known prior to the grain leaving the field or before grain is off loaded at a facility.
- ◆ Each load of **negative grain** from fields within the regulated area will be accompanied by the appropriate certificate, PPQ Form 540 or local equivalent.

Positive grain:

- ◆ To transport equipment, conveyances and/or grain from a known positive field only when authorized to move under Limited Permit (PPQ Form 530). The PPQ Form 530 will be issued by the inspector or designee under compliance after:
 - ❖ Trucks/trailers transporting positive grain have been determined to be leak proof and properly covered to avoid spillage of grain.
 - ❖ All debris has been removed from the exterior of the truck/trailer after loading.
- ◆ To perform treatments in accordance with the Karnal bunt treatment protocols. Refer to cleaning Protocols for approved treatments.
- ◆ To immediately contact the USDA/State Department of Agriculture Karnal bunt office in the event of a spillage or accident. Spills can be picked up and returned to the truck hauling the positive grain.
- ◆ To obtain approval in advance from the USDA/State Department of Agriculture Karnal bunt office, if transferring of regulated grain from one truck to another becomes necessary.
- ◆ To notify the USDA/State Department of Agriculture Karnal bunt office 24 hours in advance of any changes in movement as described in the PPQ Form 530. Approval must be obtained prior to any movement of regulated articles.
- ◆ To ensure that trucks/trailers carrying regulated articles move to their approved destination by the most direct route prior to the void date on the PPQ Form 530.
- ◆ To ensure that trucks/trailers are cleaned following discharge and that small quantities of debris resulting from this cleaning are:
 - ❖ Stored back in the bin with bunted kernel positive grain, or
 - ❖ Buried in an approved landfill, or
 - ❖ Treated with an approved treatment
- ◆ To clean vehicles dedicated to transporting bunted kernel positive grain (not back hauling) in accordance with attached information upon completion. All harvesting equipment associated with Karnal Bunt positive grain must be cleaned prior to leaving the regulated area (see Cleaning Protocol)
- ◆ To provide a copy of this compliance agreement to the facilities where the trucks/trailers will be loading upon request.

INITIAL: _____.



Appendix F

Contributors

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The aforementioned individuals were major contributors to the development, preparation, and review of the Karnal Bunt Manual. Other contributors and/or reviewers were research scientists of Agricultural Research Service and regional and staff personnel of Plant Protection and Quarantine.



Appendix G

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Appendix H

National Survey Protocol

The National Survey for Karnal bunt is conducted in each state with technical support provided to SPHDs/SPROs from the National Survey Coordinator of Pest Detection and Management Programs (PDMT). Numbers of samples taken vary depending on the amount of wheat produced within a state. The Karnal bunt protocol is available from the National Survey Coordinator for Karnal bunt on the PDMP Staff in Riverdale Headquarters. You can also download the survey plan located on the Karnal bunt Web site by clicking on the following link:

http://www.aphis.usda.gov/ppq/ep/kb/KB_Survey_Plan_FY2003.pdf

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