

ADDENDUM C

To assure clarity and consistency as to the basis for U.S. certification regarding TCK, the following sampling and testing procedures, as referenced in the Agreement on U.S.-China Agricultural Cooperation, will be used on shipments of U.S. wheat sold to the PRC.

Sampling

Each wheat lot will be sampled in accordance with existing procedures prescribed for all export lots of grain as specified in the United States Grain Standards Act (7 USC 71 et seq.), regulations thereunder, and Grain Inspection, Packers and Stockyards Administration's (GIPSA) Federal Grain Inspection Service (FGIS) Grain Inspection Handbook.

A representative sample will be obtained from the running grain stream on a specified interval (every 15-20 seconds) with a diverter-type (D/T) mechanical sampler approved by FGIS after the final elevation of the wheat prior to loading at the export elevator.

For the purposes of determining lot quality and uniformity, FGIS inspects "sublot" samples, normally representing up to 1,640 metric tons (depending on the average hourly loading rate of the elevator). A portion of each sublot sample will be combined to make a composite sample representing each lot loaded aboard the vessel. The composite sample will be reduced to 1,000 grams using approved equipment. The 1,000-gram composite sample will be divided in half: one for the TCK analysis, and one to be held on file for 90 days. FGIS will apply a USDA-FGIS seal to the sample for analysis and send it by overnight delivery to the designated FGIS or FGIS-approved laboratory for analysis.

Sample Assay

From each 500-g composite a sample 50-g sample will be drawn using an official divider and analyzed for TCK. Spores will be isolated from the 50-g sample using minor modifications of standard ISTA seed-wash protocols that involve washing, sieving, centrifuging, resuspending and examining pelleted spores and extraneous material under a microscope. The 50-g sample will be swirled with 100 ml of Tween-20 water in a 500 ml flask for 5 minutes, then poured through a 53 μ m nylon screen. The entire filtrate containing any teliospores will be centrifuged for 5 minutes at 600 X g and the supernatant removed. The pellet will be resuspended in 250-500 μ l Shear's mounting medium, depending upon the amount of debris. The number of teliospores observed in some samples may make visual counting of all spores impractical, therefore, a statistical method for estimation of teliospore concentration was developed.

a. Teliospore Count and Identification

The number of drops (D) in a sample suspension must be determined. While some variation may exist, the number of drops must be accurately measured. There must also be at least 6 drops in the sample suspension. If not, the sample must be prepared again to meet this requirement.

The first slide will be prepared with one drop of the sample suspension and a second slide with two drops, using separate covers so that they can be completely isolated. Up to 3 additional slides will be prepared with three isolated drops each, or until the sample

suspension is totally exhausted. Debris on the slide should be in a single thin layer for ease of observation. The slides will be scanned with a microscope at 100X- 400X. Obviously broken spores, i.e., less than 75 percent of a spore, will not be counted.

The number of spores on the first slide of one drop (C1) will be counted and the number of spores for the sample computed as:

$$T1 = C1 * D$$

If $T1 > 70$, $T1$ will be reported as the total number of spores for the sample. If $T1 \leq 70$, the number of spores in the two drops on the second slide will be counted and added to the total number of spores on the first slide (C2). The number of spores for the sample will then be computed as:

$$T2 = (C2 * D)/3$$

If $T2 > 50$, $T2$ (rounded to integer value) will be reported as the total number of spores for the sample.

If $T2 \leq 50$, then the number of spores on the third slide will be counted, the total number of spores on all three slides (C3) will be summed, and the number of spores for the sample will be calculated as:

$$T3 = (C3 * D)/6$$

If $T3 > 30$, $T3$ (rounded to integer value) will be reported as the total number of spores for the sample.

If $T3 \leq 30$, then the number of spores on the fourth and fifth slides will be counted, the total number of spores on all 5 slides (C4) will be summed, and the number of spores for the sample will be calculated as:

$$T4 = (C4 * D)/ 12$$

$T4$ (rounded to integer value) is reported as the total of spores for the sample.

If there are less than 12 drops in the suspension, the total count of spores on all slides will be recorded as the total. If no teliospores are found in the first 12 drops, the remainder of the sample will be examined and the number of teliospores will be recorded.

Between sample extractions, all extraction glassware and sieves should be washed and soaked in 33% bleach solution for 15 minutes, then thoroughly rinsed again and stored covered and away from airborne contamination. (Note: The purpose of the bleach is to bleach out all of the pigment from any spores that may be trapped on the filter. If a colorless spore is observed in a sample, it will not be counted.)

b. Spore Identification

The method jointly developed by USDA's Agricultural Research Service (ARS) and the PRC will be utilized. Identification of *T. controversa* teliospores will be based on confirmation of reticulum depth where reticulations greater than 0.95 μ will be considered TCK and those equal to or less than 0.95 μ are *T. tritici* (PRC/ARS, unpublished).

A microscope equipped with video camera and monitor will be used to scan the slides at 100X. When a spore is encountered, the scope is to be switched to oil immersion at 1000X to observe the teliospore. Broken spores will not be measured. The spore will be centered and the image redirected to the monitor.

The focus is adjusted on the periphery of the spore so that the endospore wall becomes a sharp black line. Using a 15-cm plastic rule, the depth of the reticulum is measured from the center of this dark line to the top of the clearest reticulum "spike". Four measurements will be taken from each spore, with points chosen that closely correspond to points on a compass.

c. Logistics Index

$$\text{Index} = e^{a+bR}/1 + e^{a+bR}$$

$$a = -10.6914 \quad b = 11.6214$$

$$R = \text{reticulum} \quad e = 2.71828$$

The mean of the four measurements will be calculated and converted to micrometers using the optical conversion factor determined by the microscope/monitor system being used. The Logistics Index value can be determined using the Logistics Index equation above or Table 1 to determine the corresponding index for the reticulum depth. The index can be interpreted as the probability that a teliospore is TCK. In general, an index value over 0.5 (reticulum depth = 0.95 μ) would indicate that the spore is TCK.

Table-1. Logistics Indices for the identification of *Tilletia controversa* (TCK)

<u>Reticulum (μm)</u>	<u>Logistics Index</u>	<u>Reticulum(μm)</u>	<u>Logistics Index</u>
0.05	0.000	1.00	0.717
0.10	0.000	1.05	0.819
0.15	0.000	1.10	0.890
0.20	0.000	1.15	0.935
0.25	0.000	1.20	0.963
0.30	0.001	1.25	0.979
0.35	0.001	1.30	0.988
0.40	0.002	1.35	0.993
0.45	0.004	1.40	0.996
0.50	0.008	1.45	0.998
0.55	0.013	1.50	0.999
0.60	0.024	1.55	0.999
0.65	0.042	1.60	1.000
0.70	0.072	1.65	1.000

0.75	0.122	1.70	1.000
0.80	0.199	1.75	1.000
0.85	0.307	1.80	1.000
0.90	0.442	1.85	1.000
0.95	0.586	1.90	1.000

Logistic indices values of 0.586 or above are considered TCK

d. Absolute and Relative Indices

When evaluating an entire sample, a maximum of 30 spores will be measured. The Absolute Index will be calculated by summing the Logistics Index values and can be interpreted as the expected number of TCK spores in a fixed sample size. The Relative Index is the average of the Logistics Indices and is interpreted as the proportion of TCK spores in a fixed sample size.

Certification

Each shipment will be certified by FGIS. The testing laboratory will notify the FGIS field office that performed the original sampling of the analysis results. This field office will issue an official export inspection certificate with the following statement.

"Tilletia controversa Kuhn spores (exceed/do not exceed) 30,000 per 50 grams of sample."