

## Importance of Pyraclostrobin Determination

Pyraclostrobin is a broad-spectrum fungicide which is used in the treatment and prevention of fungal diseases in plants. It is used worldwide in both agricultural (cereal grains, fruits, vegetables) and non-agricultural settings (flowers, grass).

Humans may be exposed to Pyraclostrobin through direct physical contact with treated plants or ingestion of contaminated foods or drinking water. Pyraclostrobin is not readily biodegradable and drinking water sources can be contaminated through run-off. It is very toxic to aquatic organisms. In humans, Pyraclostrobin can cause eye damage, is a respiratory and skin irritant, and can be absorbed through the skin. Numerous incidents of accidental exposure resulting in illness have been reported, including one in which 27 farm workers were exposed through drift from aerial application of Pyraclostrobin on an adjacent field.

Many countries have established Acceptable Daily Intake (ADI) levels for Pyraclostrobin. The European Union and Australia have established an ADI of 0.03 mg/kg of body weight. Many countries have also established regulatory limits on the levels of chemical residues on many individual foods. These levels vary depending on country and food type.\* For example, the European Union (EU) has established a maximum residue limit (MRL) of Pyraclostrobin for oranges at 1 mg/kg, while the United States (US) has placed the limit at 2 mg/kg. Many foods and drinks have no established MRL. For example, although wine grapes have a Pyraclostrobin MRL of 2 mg/kg in both the EU and the US, no MRL has been established for the wine made from those grapes. Pesticide content in wine has become an area of concern, as grapes are one of the most highly pesticide treated foods. A study by the United States Department of Agriculture (USDA) released in 2012 evaluated pesticide use (insecticides, fungicides, herbicides, and other chemicals) on 21 fruit crops in California in 2011 and found that 70% of the land used to grow grapes was treated with fungicides (51% for grapes used for raisins, 85% for table grapes, and 73% for wine grapes).<sup>Δ</sup> Pyraclostrobin was one of the most frequently applied fungicides used for all grapes (20%), table grapes (52%), and wine grapes (20%), as well as for apricots (50%), sweet cherries (41%), and plums (6%). The monitoring of water sources and food products for fungicide residues is an important step in ensuring the health and safety of consumers.

\*For listings of foods for which MRLs have been established: for the European Union, search "EU Pesticides Database – Pesticides," select "Pesticides," scroll to and select "Pyraclostrobin" and "All" then select "Search Current MRL" and for the United States, search "40 CFR 180.582 – Pyraclostrobin; Tolerances for Residues."

<sup>Δ</sup>For full text of study, search "California Agricultural Chemical Use Fruit Crops 2011."

The Pyraclostrobin ELISA allows for the analysis of 42 samples in duplicate determination. Less than 1 mL of sample is required. The test can be performed in less than 2 hours.

## Performance Data

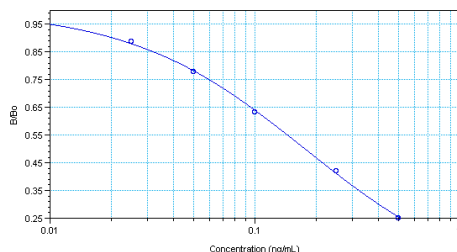
Test sensitivity:

The limit of quantitation for Pyraclostrobin (90% B/B<sub>0</sub> calculated from the average of 23 calibration curves) is approximately 0.020 ng/mL. The middle of the test (50% B/B<sub>0</sub> calculated from the average of 23 calibration curves) is approximately 0.175 ng/mL. Determinations closer to the middle of the calibration curve give the most accurate results.

Test reproducibility:

Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Standard Curve:



For demonstration purposes only. Not for use in sample interpretation.

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# Pyraclostrobin ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Determination of Pyraclostrobin in Water and Wine Samples



Product No. 500705

## 1. General Description

The Abraxis Pyraclostrobin ELISA is an immunoassay for the quantitative and sensitive screening of Pyraclostrobin. This test is suitable for the quantitative and/or qualitative screening of Pyraclostrobin in water or wine samples. Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

## 2. Safety Instructions

The standard solutions in the test kit contain small amounts of Pyraclostrobin. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

## 3. Storage and Stability

The Pyraclostrobin ELISA should be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20–25°C) before use. Reagents may be used until the expiration date on the box.

## 4. Test Principle

The test is a direct competitive ELISA based on the recognition of Pyraclostrobin by specific antibodies. Pyraclostrobin, when present in a sample, and a Pyraclostrobin-HRP analogue compete for the binding sites of the mouse anti-Pyraclostrobin antibodies in solution. The Pyraclostrobin antibodies are then bound by a second antibody (goat anti-mouse) immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Pyraclostrobin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

## 5. Limitations of the Pyraclostrobin ELISA, Possible Test Interference

Although many organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects cannot be completely excluded.

The presence of the following substances were found to have no significant effect on the Pyraclostrobin assay results: calcium sulfate, magnesium chloride, magnesium sulfate, sodium chloride, and aluminum oxide up to 10,000 ppm; ferric sulfate, manganese sulfate, potassium phosphate, sodium fluoride, sodium nitrate, sodium thiosulfate, and zinc sulfate up to 1,000 ppm; calcium chloride and copper chloride up to 100 ppm; humic acid up to 10 ppm.

Samples containing methanol must be diluted to a concentration of 10% methanol to avoid matrix effects.

Mistakes in handling the test can cause errors. Possible sources for such errors include: inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures (lower than 10°C or higher than 30°C) during the test performance.

The Abraxis Pyraclostrobin ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring some regulatory action should be confirmed by an alternative method.

#### A. Reagents and Materials Provided

1. Microtiter plate (12 X 8 strips) coated with a secondary antibody, in a resealable aluminum pouch
2. Pyraclostrobin Calibrators/Standards (6): 0, 0.025, 0.05, 0.10, 0.25, 0.5 ng/mL (ppb), 1 mL each
3. Antibody Solution (mouse anti-Pyraclostrobin), 6 mL
4. Pyraclostrobin-HRP Conjugate Solution, 6 mL
5. Wash Solution (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section C)
6. Sample Diluent, 25 mL
7. Substrate (Color) Solution (TMB), 16 mL
8. Stop Solution, 12 mL (handle with care)

#### B. Additional Materials (not delivered with the test kit)

1. Micro-pipettes with disposable plastic tips (50-200  $\mu$ L)
2. Multi-channel pipette (50-250  $\mu$ L) or stepper pipette with disposable plastic tips (50-250  $\mu$ L)
3. Microtiter plate reader (wave length 450 nm)
4. Container with 500 mL capacity (for 1X diluted wash solution, see Test Preparation, Section C)
5. Deionized or distilled water
6. Methanol
7. Glass vials with Teflon lined caps
8. Paper towels or equivalent absorbent material
9. Timer
10. Tape or parafilm

#### C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. In order to equalize the incubation periods on the entire microtiter plate, a multi-channel pipette or a stepping pipette is recommended for adding the enzyme conjugate, antibody, substrate, and stop solutions. Please only use the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Allow the microtiter plate, reagents, and samples to reach room temperature before use.
2. Remove the number of microtiter plate strips required from the aluminum pouch. The remaining strips are stored in the aluminum pouch with the desiccant (tightly sealed) in the refrigerator (4-8°C).
3. The standard solutions, conjugate, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Wash Solution (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water and mix thoroughly.
5. The stop solution must be handled with care as it contains diluted H<sub>2</sub>SO<sub>4</sub>.

#### D. Sample Preparation

##### Water Samples

Water samples should be collected in glass sample containers. Immediately upon collection, water samples should be preserved with methanol at a ratio of 1 mL of methanol per 9 mL of water. For example, a 9 mL sample of water added to a sample container should have 1 mL of methanol added.

The Pyraclostrobin concentration contained in water samples is determined by multiplying the ELISA result by the dilution factor of 1.1. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in sample diluent and re-analyzed.

##### Wine Samples

In order to eliminate matrix interferences, wine samples must be diluted in deionized or distilled water prior to analysis:

1. Add 8 mL of deionized or distilled water to an appropriately labeled glass vial.
2. Add 25  $\mu$ L of wine sample to the vial.
3. Vortex thoroughly.
4. Diluted sample is ready to analyze (Assay Procedure, step 1).

The Pyraclostrobin concentration contained in wine samples is determined by multiplying the ELISA result by the dilution factor of 320. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in deionized or distilled water and re-analyzed.

#### E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 5: Standards  
(0; 0.025; 0.05; 0.1; 0.25; 0.5 ppb)

Samp1, Samp2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	etc.									
B	Std 0	Std 4	etc.									
C	Std 1	Std 5										
D	Std 1	Std 5										
E	Std 2	Samp1										
F	Std 2	Samp1										
G	Std 3	Samp2										
H	Std 3	Samp2										

#### F. Assay Procedure

1. Add 50  $\mu$ L of the **calibrator/standard solutions or samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add 50  $\mu$ L of **antibody solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 30 minutes at room temperature.
3. Add 50  $\mu$ L of **conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 30 minutes at room temperature.
4. Remove the covering and decant the contents of the wells into a sink. Wash the strips **three times** using the **diluted washing buffer solution**. Please use at least a volume of 250  $\mu$ L of washing buffer for each well in each washing step. Remaining buffer in the wells should be removed by patting the inverted plate dry on a stack of paper towels.
5. Add 150  $\mu$ L of **substrate (color) solution** to the wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 20 minutes at room temperature. Protect the strips from direct sunlight.
6. Add 100  $\mu$ L of **stop solution** to the wells in the same sequence as for the substrate solution.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of stopping solution.

#### G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the %B/B<sub>0</sub> for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the %B/B<sub>0</sub> for each standard on the vertical linear (y) axis versus the corresponding Pyraclostrobin concentration on the horizontal logarithmic (x) axis on graph paper. %B/B<sub>0</sub> for samples will then yield levels in ppb (or ng/mL) of Pyraclostrobin by interpolation using the standard curve; results for water samples are determined by multiplying the concentration determined from the curve by the dilution factor of 1.1, results for wine samples are determined by multiplying the concentration determined from the curve by the dilution factor of 320. Samples showing a lower concentration of Pyraclostrobin than standard 1 (0.025 ppb) should be reported as containing < 0.0275 ppb of Pyraclostrobin for water samples or < 8 ppb for wine samples. Samples showing a higher concentration than standard 5 (0.5 ppb) should be reported as containing > 0.55 ppb for water samples or > 160 ppb for wine samples. If a quantitative result is necessary, samples must be diluted further with the appropriate sample diluent and re-analyzed.