Importance of Glyphosate Determination

Glyphosate, a broad-spectrum systemic herbicide, was introduced in 1974 by Monsanto under the trade name Roundup®. Glyphosate (N-(phosphonomethyl)glycine or 2-[(hydroxy-oxidophosphoryl)methylamino]acetic acid) is the largest selling agrochemical in the world and is marketed under dozens of trade names by many different manufacturers. Glyphosate is used for vegetation control of perennial and annual plants, broad-leaf weeds, grasses, woody plants, and aquatic weeds, as well as grain desiccation to increase harvest yield. The introduction of genetically modified crops resistant to Glyphosate (i.e. Roundup Ready®) has caused an increased use of Glyphosate, allowing farmers to control weeds without harming their crops. The emergence of Glyphosate-resistant weeds has also caused increases in frequency and volume of applications of Glyphosate in combination with other herbicides. Due to its widespread use, Glyphosate has become ubiquitous in the environment and food supply.

Glyphosate can adsorb to soil and is highly water soluble, which can cause surface and ground water contamination from run-off, soil erosion, and leaching especially after heavy rainfall. The long-term impact on the environment and human health are growing concerns. In March 2015, the World Heath Organization's International Agency for Research on Cancer classified Glyphosate as "probably carcinogenic in humans" (category 2A). Some studies show a correlation between exposure to Glyphosate-based herbicides and non-Hodgkin's Lymphoma in humans and others show evidence of Glyphosate causing cancers in laboratory animals.

In the European Union, the combined maximum residue level (MRL) for Glyphosate and its relevant metabolites in drinking water is 0.1 ng/mL. In February 2016, the U.S. Food and Drug Administration announced it will be testing the US food supply for Glyphosate.

The Eurofins Abraxis Glyphosate ELISA Assay can be performed in about 2 hours and requires only a few milliliters of sample.

Performance Data

Test sensitivity: The Glyphosate ELISA has an estimated detection limit (90% B/B_0) of 0.05 ppb (µg/L). The middle of the test (50% B/B_0) is approximately 0.5 ppb. Determinations closer to the middle of the calibration curve give the most accurate results.

Test reproducibility: Intra and inter assay: < 17%

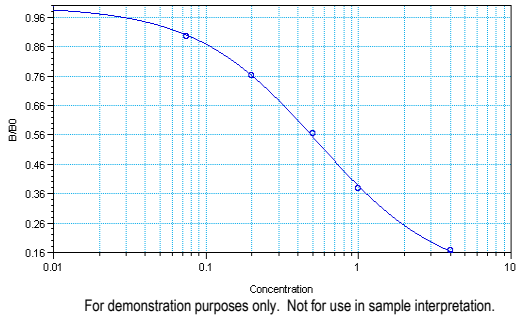
Recoveries:

Level (ppb)	% Recovery
0.25	102
0.50	105
1.00	103
2.00	106

Specificity: The cross-reactivity of the Glyphosate ELISA for various related analogues expressed as the least detectable dose (LDD) or 90% B/B_0 and as the dose required for 50% inhibition (50% B/B_0) are as follows:

Compound	LDD (ppb)	50% (ppb)
Glyphosate	0.05	0.5
Glyphosine	50	3000
Glufosinate	2000	70,000
AMPA	35,000	> 1,000,000
Glycine	> 10,000	> 1,000,000

Standard Curve:



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

General Limited Warranty: Eurofins Abraxis warrants the products manufactured by the Company, against defects and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. **Eurofins Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose.**

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Glyphosate ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Glyphosate in Water Samples

Product No. 500086

1. General Description

The Eurofins Abraxis Glyphosate ELISA Plate Kit is an immunoassay for the quantitative and sensitive screening of Glyphosate in water samples. This test is suitable for the quantitative and/or qualitative screening of Glyphosate in groundwater, surface water, well, and tap water samples (refer to section C, Sample Collection and Handling). For soil, crop, and food sample applications, contact Eurofins Abraxis for the appropriate technical bulletin and/or matrix validation guidelines. Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard and control solutions in the test kit contain small amounts of Glyphosate. The Derivatization Reagent Diluent is Dimethyl Sulfoxide (DMSO). In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of these solutions with skin and mucous membranes. If these reagents come in contact with skin, wash thoroughly with water.

3. Storage and Stability

The Glyphosate ELISA Kit should to be stored in the refrigerator (2-8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the last day of the month as indicated by the expiration date on the box. Consult state, local, and federal regulations for the proper disposal of all reagents.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Glyphosate by polyclonal antibodies. The sample to be tested is derivatized (please refer to Section D, Test Preparation) and then added to microtiter wells coated with goat anti-rabbit antibodies. A rabbit anti-Glyphosate antibody solution is added to the wells with the derivatized samples and allowed to incubate for 30 minutes. The Glyphosate enzyme conjugate is then added and a competitive reaction occurs between the Glyphosate, which may be present in the sample, and the enzyme labeled Glyphosate for the binding sites of the rabbit anti-Glyphosate antibodies bound by the goat anti-rabbit antibodies immobilized on the microtiter plate. The reaction is allowed to continue for 60 minutes. 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 Glyphosate 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 Glyphosate ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, 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 up to 10,000 ppm were found to have no significant effect on the Glyphosate ELISA results: nitrate, phosphate, sulfate, sodium fluoride, calcium, magnesium, copper, zinc, iron, and sodium thiosulfate. Manganese up to 100 ppm, humic acid up to 10 ppm, and sodium chloride up to 1M also had no significant effect on the Glyphosate ELISA results.

Solvents commonly used to extract pesticides from soil or plant matrices, such as methanol and acetone, were found to be acceptable for use at concentrations up to 100% with the Glyphosate ELISA.

Samples containing gross particulate matter should be filtered (refer to Section C, Sample Collection and Handling). Samples, which have been preserved with monochloroacetic acid or other acids, should be neutralized (pH ~ 7) prior to testing.

Standards, control, and samples must be derivatized prior to each analysis with the Glyphosate ELISA kit (See Section D, Test Preparation).

Mistakes in handling the test can also 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, exposure to direct or indirect sunlight during the substrate reaction, or extreme temperatures (lower than 10°C or higher than 30°C) during the test performance.

As with any analytical technique (GC, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.

A. Reagents and Materials Provided (*Additional quantities available for purchase, contact Eurofins Abraxis)

1. Microtiter plate coated with a secondary antibody (anti-rabbit), in a re-sealable aluminum pouch with desiccant.
2. Glyphosate Antibody Solution, 6 mL
3. Glyphosate Conjugate Solution, 6 mL
4. Glyphosate Standards (6): 0, 0.075, 0.20, 0.5, 1.0, 4.0 ppb, 2 mL each
5. Control at 0.75 ± 0.2 ppb, 2 mL
6 Diluent/Zero Standard (Sample Diluent)*, 30 mL
7. Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section D)
8. Substrate (Color) Solution (TMB), 16 mL
9. Stop Solution, 12 mL (handle with care)
10. Assay Buffer*, 125 mL
11. Derivatization Reagent*, 3 vials, 100 µL each
12. Derivatization Reagent Diluent*, 3 vials, 4 mL each

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

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-300 µL), stepper pipette (10-300 µL), or electronic repeating pipette with disposable plastic tips (capable of delivering 50-1000 µL)
3. Disposable glass test tubes or vials
4. Parafilm or microtiter plate cover slip
5. Microtiter plate washer (optional)
6. Microtiter plate reader (wave length 450 nm)
7. Deionized or distilled water
8. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section D)
9. Paper towels or equivalent absorbent material
10. Timer

C. Sample Collection and Handling

Collect water samples in glass or plastic sample containers. Drinking water samples should be treated with ascorbic acid (0.1 mg/mL) immediately after collection to remove residual chlorine. Samples, which have been preserved with monochloroacetic acid or other acids, should be neutralized (pH ~ 7) prior to testing.

Samples containing gross particulate matter should be filtered prior to analysis using any of the following syringe filters: Environmental Express 0.2 mm PES (PN SF020E), Pall Acrodisc® 0.2 mm PVDF (PN 4450), Whatman™ 0.2 mm Anotop™ 25 Plus (Cat. No. 6809-4022), or Environmental Express 1.2 mm Glass Fiber (PN SF012G).

Store samples refrigerated for up to 2 weeks. For storage periods greater than 2 weeks, samples should be stored frozen.

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. A multi-channel, stepping, or electronic repeating pipette is recommended for adding the enzyme conjugate, antibody, substrate (color), and stop solutions in order to equalize the incubation periods across the entire microtiter plate. 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. The standard solutions, control, antibody, conjugate, substrate (color), and stop solutions are ready to use and do not require any further dilutions.
3. Dilute the Wash Buffer (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL) add to 400 mL of deionized or distilled water.
4. The stop solution must be handled with care as it contains diluted H_2SO_4.
5. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag with desiccant and zip-locked closed.
6. After analysis, store the remaining kit components in the refrigerator (2-8°C).
7. **Derivatization of Standards, Control, and Samples (must be performed prior to each analysis)**:
   a. Dilute the Derivatization Reagent with 3.5 mL of Derivatization Reagent Diluent. Vortex to mix thoroughly. ***Note: Diluted Derivatization Reagent must be used within 8 hours of preparation. If additional samples are to be analyzed more than 8 hours after dilution, discard the vial, and a new vial of Derivatization Reagent should be diluted for use.***
   b. Label single glass test tubes or vials for standards, control, and samples.
   c. Pipette 250 µL of standard, control, or sample into appropriately labeled glass test tube/vial.
   d. Add 1 mL of Assay Buffer to each test tube/vial. Vortex to mix.
   e. Add 100 µL of the diluted derivatization reagent to each tube/vial. **Vortex each tube immediately after addition of diluted reagent until no swirling lines are present.**
   f. Incubate at room temperature for 10 minutes.
   g. Derivatized standards, control, and samples are ready to be analyzed. Procede to Assay Procedure, Section F, Step 1. **Note: Discard derivatized standards, control, and samples after use. Do not use for re-analysis.**

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-Std5: Derivatized Standards
Contr.: Derivatized Control
Samp1, Samp2, etc: Derivatized Samples

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

F. Assay Procedure

1. Add **50 µL of the derivatized standards, control, or samples (see Section D, Test Preparation)** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended
2. Add **50 µL of the antibody solution** to the individual wells successively using a multi-channel pipette, stepping, or electronic repeating 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 60 seconds. Be careful not to spill the contents. Incubate the strips for **30 minutes** at room temperature.
3. Remove the covering and add **50 µL of the enzyme conjugate solution** to the individual wells successively using a multi-channel pipette, stepping, or electronic repeating 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 60 seconds. Be careful not to spill the contents. Incubate the strips for **60 minutes** at room temperature.
4. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. ***Blot the inverted plate after each wash step*** on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
5. Add **150 µL of substrate (color) solution** to the individual wells successively using a multi-channel pipette, stepping, or electronic repeating 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 **20-30 minutes** at room temperature. Protect the strips from sunlight.
6. Add **100 µL of stop solution** to the wells in the same sequence as for the substrate (color) solution using a multi-channel pipette, stepping, or electronic repeating pipette.
7. Read the absorbance at 450 nm using a microtiter plate ELISA photometer within 15 minutes after the addition of the stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ 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 $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Glyphosate concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control and samples will then yield levels in ppb of Glyphosate by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Eurofins Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Glyphosate than standard 1 (0.075 ppb) should be reported as containing < 0.075 ppb of Glyphosate. Samples showing a higher concentration than standard 5 (4.0 ppb) should be reported as containing > 4.0 ppb of Glyphosate or must be diluted using Diluent/Zero Standard (Sample Diluent) and re-analyzed to obtain accurate results. The concentration of the positive control provided should be 0.75 ± 0.2 ppb.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards. Samples with lower absorbances than a standard will have concentrations of Glyphosate greater than that standard. Samples with higher absorbances than a standard will have concentrations of Glyphosate less than that standard.

As with any analytical technique (GC, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.