ABRAXIS® Glyphosate ELISA Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Glyphosate in Wheat, Whole Oats, Oat Groats, Yellow Peas and Red Lentils

Product No. 500089

1. General Description

The ABRAXIS® Glyphosate ELISA Plate Kit is an immunoassay for the quantitative and sensitive screening of Glyphosate in Wheat, Whole Oats, Oat Groats, Yellow Peas and Red Lentils. Test results requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional validated methods.

2. Intended Use and Validation

The ABRAXIS® Glyphosate ELISA is intended for the quantitation of glyphosate by trained laboratory or field personnel. It is validated for the determination of glyphosate in durum wheat, whole oats, oat groats, dried yellow peas, and dried red lentils in the range ~20-400 µg/kg and certified as AOAC *Performance Tested Method*℠ 072104.

3. Safety Instructions

Personal protective equipment is recommended (eye protection, laboratory coat and gloves). The calibration standard and control solutions in the test kit contain small amounts of glyphosate. The Derivatization Reagent contains 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.

4. Storage and Stability

The ABRAXIS® Glyphosate ELISA Kit should be stored in the refrigerator (2-8°C). The solutions must be allowed to reach room temperature (17-27°C) before use. Reagents may be used until the expiration date on the box. Consult state, local, and federal regulations for the proper disposal of all reagents.

5. Test Principle

The test is a direct competitive ELISA based on the recognition of glyphosate by polyclonal antibodies. The test portion is solubilized, and the extract is derivatized (please refer to Section D, Test Preparation) and added to microtiter wells coated with goat anti-rabbit antibodies. A rabbit anti-glyphosate antibody solution is added to the wells with the derivatized extracts and allowed to incubate for 30 minutes. The glyphosate enzyme conjugate is then added, and a competitive reaction occurs between the derivatized glyphosate, if present, and the enzyme-labeled glyphosate for the binding to 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 test portion. The color reaction is stopped after 25 minutes, and the color intensity is measured using an ELISA reader. The glyphosate concentration is determined using the standard curve constructed with each assay.

6. Limitations of the ABRAXIS® Glyphosate ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in cereal grains and legumes have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in cereal grains and legumes, test interferences caused by matrix effects cannot be completely excluded. The presence of the following substances up to 10,000 mg/kg were found to have no significant effect on the ABRAXIS® Glyphosate ELISA results: nitrate, phosphate, sulfate, sodium fluoride, calcium, magnesium, copper, zinc, iron, and sodium thiosulfate. Manganese up to 100 mg/kg, humic acid up to 10 mg/kg, and sodium chloride up to 1 M also had no significant effect on the ABRAXIS® Glyphosate ELISA results.

Sampling error associated with 0.5 g test portions can be reduced by taking a larger test portion or running replicate (2, 3 or more) test portions and averaging replicate test results. This is especially true for testing concentrations near the limit of quantification (LOQ). The replicate test portions must be randomly selected from >500 g analytical sample,

The temperature range for the test method is important. The assay sensitivity deteriorates above 30°C.

Extracts containing gross particulate matter should be filtered (refer to Section C, Sample Collection, Handling, and Preparation).

Calibration standards, control, and test portion extracts must be derivatized prior to each analysis (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 (Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectroscopy / Mass Spectroscopy (LC-MS/MS), etc.), test results indicating potential regulatory action should be confirmed by an alternative validated method.

7. Working Instructions

A. Reagents and Materials Provided (*Additional quantities available for purchase, contact Gold Standard Diagnostics)

1. Microtiter plate coated with a secondary antibody (anti-rabbit), in a re-sealable aluminum pouch with desiccant.
2. ABRAXIS® Glyphosate Antibody Solution, 6 mL
3. ABRAXIS® Glyphosate Conjugate Solution, 6 mL
4. ABRAXIS® Glyphosate Standards (6): 0, 0.075, 0.20, 0.5, 1.0, 4.0 µg/kg, 2 mL each
5. ABRAXIS® AOAC Glyphosate Control, 2 mL, refer to Certificate of Analysis for lot-specific acceptable range
6. Diluent/Zero Standard (Sample Diluent)*, 2 x 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. 50 x 15 mL Centrifuge tubes with Styrofoam rack
12. 45 x 2 mL Microcentrifuge vials
13. 108 x Glass Test Tubes*, 3 packs of 36
14. Derivatization Reagent*, 3 vials, 100 µL each
15. Derivatization Reagent Diluent*, 3 vials, 4 mL each

16. Instructions for use and warranty booklet
17. Certificate of Analysis for AOAC ABRAXIS® Glyphosate Control

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 (50-1000 µL), or electronic repeating pipette with disposable plastic tips (capable of delivering 50-1000 µL)
3. Analytical balance
4. Weighing boats
5. Spatula or disposable scoopulas
6. Microcentrifuge capable of 10,000 rpm, VWR model 1814 or equivalent
7. Vitamix Professional Series 750 Blender for large scale grinding at 500 grams
8. Serological pipettes, 5 mL or 10 mL
9. Rotator, Glas-Col model 099A DD 4512 or equivalent
10. Vortex mixer, VWR Vortex Genie 2 or equivalent
11. ABRAXIS® Glyphosate Sample Diluent (provided in the kit; additional available for purchase: PN 500082)
12. Parafilm or microtiter plate cover film
13. Microtiter plate washer (optional)
14. Microtiter plate reader (wavelength 450 nm; differential 630nm)
15. Mesh 20/sieve 850 µm (0.85 mm) metal or nylon fiber
16. Deionized water
17. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section D)
18. Collection container to hold 1 kg size sample, for example 11” x 11” resealable bag
19. Paper towels or equivalent absorbent material
20. Timer
21. Tube holder for all types

Contact Gold Standard Diagnostics for additional help with automation.

C. Sample Collection, Handling, and Preparation (Oats, Wheat, Pulses)

Representative Sampling: To assure that both the supplier and the customer obtain similar test results, a sample representing the entire sampling unit must be taken.
When selecting a primary sample from large amounts (tons) of a food material such as in a granary, shipping, or distribution facility, it is very important to take multiple small increments (≥30), selected from all parts of the material, preferably when the material is being transferred. The final primary sample should be of sufficient mass (≥ 1kg) to represent the variations in distribution of glyphosate within the material. For larger amounts of material and larger variations in source of product, select more increments and a larger primary sample.
(See free sampling guidance: GOOD Samples: https://www.aafco.org/Publications)
Laboratory samples must be ground before extraction to produce accurate results. Grind the entire laboratory sample (≥500 g) as described below with the specified grinder. Use proper technique to minimize the potential for sample-to-sample contamination due to carryover during sample preparation.
Cover the wells with parafilm or cover film and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds, taking care not to spill the contents.
Analysis should be performed with the ABRAXIS® Glyphosate Plate ELISA Kit as soon as possible after derivatization. Note: Discard derivatized standards, control, and test solutions after use. Do not use for re-analysis. Prepared extract solutions are stable for up to five days at room temperature in plastic microcentrifuge tubes before being diluted and analyzed.

D. Laboratory Sampling and Extraction of Glyphosate:

1. Grind laboratory sample of at least 500 g in the blender, starting at variable speed 1 and gradually increasing to speed 7 or 8. Continue grinding at speed 7 or 8 for 60 sec, producing a fine powder yielding >95% by weight of particles that pass through a 20-mesh sieve (gentle agitation, smaller than 0.85 mm). Transfer the entire ground material to a resealable bag and seal. For whole oats, regrind the material trapped on the sieve for an additional 3 minutes and add to previously ground material.
2. Tumble the ground material in the bag manually for at least 1 minute.
3. Weigh a 0.5 ± 0.01 g test portion of the now finely ground grain/pulse material into a 15 mL centrifuge tube.
4. Add 10 mL deionized water to the tube (1:20 dilution).
5. Vortex vigorously for 15 seconds and place tubes on rotator at 40 rpm for 10 ± 1 minutes.
6. Remove tubes from rotator and allow the solids to settle for at least 2 minutes (extract solution).
7. Transfer 1.5 mL to 2.0 mL supernatant extract to an appropriately labeled microcentrifuge vial.
8. Centrifuge for 5 minutes at ~8000 x g. Make sure the centrifuge is properly balanced.
9. To a new labeled 4 mL glass vial, add 800 µL of Sample Diluent.
10. Add 200 µL of the supernatant from Step 8 to the Sample Diluent in the vial (1:5 dilution), cap, and vortex. This is the test solution. Continue to Section D.

E. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the extracts 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. Use only the reagents and calibration standards from one package lot for any test, as they have been adjusted in combination.

1. Allow the microtiter plate, reagents, and test solutions to reach room temperature (22 ± 5 °C) before use.
2. The standard solutions, control, antibody, conjugate, substrate, 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 resealed in the foil bag with desiccant.
6. After analysis, store the remaining kit components in the refrigerator (2-8°C).
7. Derivatization of Standards, Control, and Test Solutions (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 tests will be performed more than 8 hours after dilution, discard the vial, and dilute a new vial of Derivatization Reagent.
    b. Label single disposable glass test tubes for standards, control, and test solutions.
    c. Pipette 250 µL standard, control, or test solution into an appropriately labeled glass test tube.
    d. Add 1 mL Assay Buffer to each test tube. Vortex to mix.
    e. Add 100 µL diluted Derivatization Reagent to each test tube. A repeater pipet is useful for this step. Vortex each tube immediately after addition of diluted reagent until no swirling lines are present.
    f. Incubate at room temperature (22 ± 5°C) for 10 ±1 minutes.

g. Derivatized calibration standards, control, and test solutions are ready to be analyzed. Proceed to Assay Procedure, Section F, Step 1. **Note: Discard derivatized standards, control, and test solutions after use. Do not use for re-analysis.**

F. Working Scheme

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

Std 0-Std 5: Derivatized Calibration Standards
Contr.: Derivatized Control
Samp1, Samp2, etc.: Derivatized Test Solutions

	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										

G. Assay Procedure

1. **Add 50 µL derivatized calibration standards, control, or test solutions** (see Section D, Test Preparation) into the wells of the test strips according to the working scheme given. Analyze at least in duplicate.
2. **Add 50 µL Antibody Solution** to the individual wells successively using a multi-channel pipette, stepping pipette, or electronic repeating pipette. Cover the wells with parafilm or cover film and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds, taking care not to spill the contents. Incubate the strips for 30 minutes at room temperature (22 ± 5 °C).
3. Remove the covering and **add 50 µL Conjugate Solution** to the individual wells successively using a multi-channel pipette, stepping pipette, or electronic repeating pipette. Cover the wells with parafilm or cover film and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds, taking care not to spill the contents. **Incubate the strips for 60 minutes at room temperature (22 ± 5 °C)**.
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**. Use at least 250 µL 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. Alternatively, a plate washer can be used.
5. **Add 150 µL Substrate Solution** to the individual wells successively using a multi-channel pipette, stepping pipette, or electronic repeating pipette. Cover the wells with parafilm or cover film and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds, taking care not to spill the contents. **Incubate the strips for 25 minutes at room temperature (22 ± 5 °C)**. Do not place the strips in sunlight.
6. **Add 100 µL Stop Solution** to the wells in the same sequence as for the Substrate Solution using a multi-channel pipette, stepping pipette, or electronic repeating pipette.
7. Read the absorbance at 450 nm using a microtiter plate ELISA photometer within 15 minutes after the addition of Stop Solution. Note: Discard derivatized calibration standards, control, and test solutions after use. Do not use for re-analysis.

H. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter Logistic Regression).
The coefficient of variance (%CV) for the standard absorbance values must be <10%. If outside this range, repeat the assay. The concentration of the positive control provided should be within 20% of the value determined in the certificate of analysis. If outside this range, repeat the assay. The concentration of glyphosate in the test portions is determined using the standard curve performed with each test. For grains and pulses, the ELISA results must be multiplied by the dilution factor of 100. Responses below the matrix LOQ concentration should be reported as < LOQ µg/kg of glyphosate (see table below for each matrix LOQ). Responses above the highest standard (Standard 5 = 4.0 µg/kg) should be reported as > 400 µg/kg or extracts can be diluted further with Sample Diluent, derivatized, and analyzed to obtain an accurate quantitative result.

Matrix	LOQ_{est}, µg/kg	n	LOQ_{ACTUAL}, µg/kg	s_r, µg/kg	RSD_r, %
Durum wheat	14	10	14.3	2.31	16.1
Whole oats	18	19/20[a]	18.4	2.96	16.1
Oat groats	14	10	23.0	3.90	17.0
Dried yellow peas	13	10	12.1	1.88	15.5
Dried red lentils	23	10	14.9	2.92	19.6

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

Importance of Glyphosate Determination

Glyphosate, a broad-spectrum systemic herbicide, was introduced in 1974 by Monsanto (now Bayer) 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 has caused an increased use of glyphosate, allowing farmers to control weeds in Roundup Ready® crops resistant to the herbicide. 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 Health 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.
Current regulations in the U.S. set the tolerance level for glyphosate residues at 30 mg/kg for grains and 8.0 mg/kg for dried peas (2). In the EU, Maximum Residue Limits (MRLs) are currently set at 10 mg/kg for dried lentils, dried peas, and wheat, and at 20 mg/kg for oats. In the current market climate, however, health concerns have created a desire for determination of lower residual concentrations in the range of 10-40 µg/kg, 1000-fold below current regulations.

The ABRAXIS® Glyphosate ELISA Assay can be performed in about 2 hours.

Performance Data

Test sensitivity: The ABRAXIS® Glyphosate ELISA has an estimated detection limit (90% B/B_0, signal corresponding to 90% signal achieved in the absence of analyte) of 0.05 µg/kg, corresponding to 5 µg/kg in matrix. The middle range of the test (50% B/B_0) is approximately 0.5 µg/kg, corresponding to 50 µg/kg in matrix. Determinations closer to the middle of the calibration curve give the most accurate results.

			ELISA			LC-MS/MS[a]			Relative
Matrix	Level	n	Mean, µg/kg	s_r, µg/kg	RSD_r, %	Mean, µg/kg	s_r, µg/kg	RSD_r, %	Recovery, %
Durum wheat	Bkgd[c]	5	6.26	1.27	20	5.25	0.293	5.6	ND[b]
	Low	5	41.1	3.46	8.4	52.9	6.89	13	78
	Med	5	119	14.8	12	133	7.99	6.0	89
	High	5	308	37.4	12	388	12.1	3.1	79
Whole oats	Bkgd	5	8.55	1.79	21	9.93	0.723	7.3	ND
	Low	5	14.1	2.01	14	21.3	1.47	6.9	ND
	Med	5	184	26.2	14	223	12.7	5.7	82
	High	5	259	19.7	7.6	371	10.5	2.8	70
Groats	Bkgd	5	7.96	1.34	17	6.51	0.284	4.4	ND
	Low	5	75.2	10.7	14	58.6	6.02	10	128
	Med	5	123	4.77	3.9	122	4.36	3.6	101
	High	5	387	19.5	5.1	400	58.1	15	97
Dried yellow peas	Bkgd	5	6.72	1.11	17	4.00	0.370	9.3	ND
	Low	5	37.4	9.19	25	53.7	3.84	7.1	70
	Med	5	99.5	2.87	2.9	103	4.11	4.0	97
	High	5	354	21.6	6.1	451	21.4	4.7	79
Dried red lentils	Bkgd	5	4.68	2.51	54	0.795	0.294	37	ND
	Low	5	24.0	0.84	3.5	28.3	0.922	3.3	85
	Med	5	57.6	1.98	3.4	67.3	3.84	5.7	86
	High	5	371	15.9	4.3	472	11.0	2.3	79

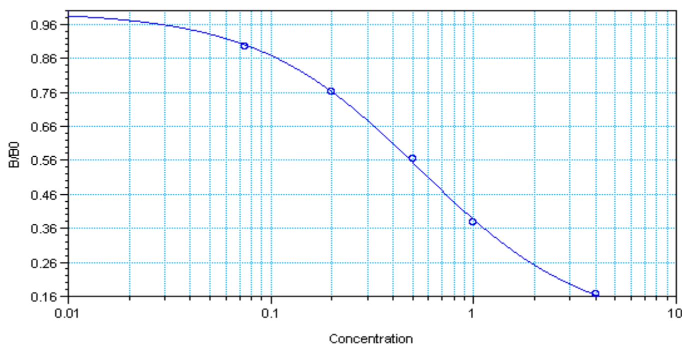
[a]LC-MS/MS was performed an independent laboratory.
[b]ND = Not determined. These results are below the limit of quantitation.
[c]Bkgd = Matrix with very low level glyphosate or interference.

Test reproducibility: The standard curve R^2 must be ≥ 0.98. The absorbance Coefficient of variation (CVs) for standards should be ≤10% and for the samples and Control should be ≤15%. The Control should be within its acceptable range and Standard 0 absorbance value should be between 0.8 - 3.000.

Specificity: The cross-reactivity of the ABRAXIS® Glyphosate ELISA to potentially interfering compounds is as follows. Relative recovery and bias were calculated for each spiked test portion in comparison to the unspiked test portion. Relative recovery ranged from 90 to 113% and bias ranged from -11 to 15 µg/kg:

Potential Interferent	Concentration, µg/kg	ELISA result, µg/kg	Relative Recovery, %	Bias, µg/kg
None	-	110	-	-
Aminomethyl phosphonic acid (AMPA)	100	121	110	11
Dicamba	1000	107	98	-2.5
Dicamba	10,000	103	94	-6.6
Glufosinate	100	113	103	3.0
Glyphosine	10	110	100	0.4
Glyphosine	50	105	96	-4.8
Glycine	100	99.2	90	-11
Atrazine	10,000	112	102	2.4
2,4-Dichlorophenoxyacetic acid	10,000	116	105	5.9
$CaCl_2$	10,000,000	124	113	14
$CaCl_2$	20,000,000	125	113	15
$FeSO_4$	100,000	111	101	0.9
$FeSO_4$	500,000	114	104	4.2

Calibration Standard Curve:



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