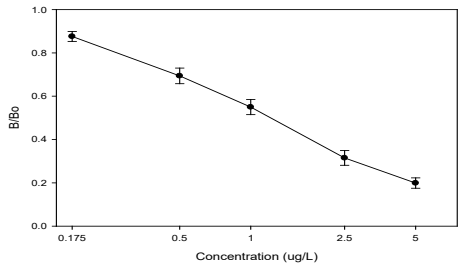
Importance of Caffeine Determination

Most of the household water in developed nations is treated before it is allowed to enter the environment. Accidental release can occur, however, resulting in untreated wastewater entering into streams, lakes, rivers, and other bodies of water. Identifying waste water treatment plant (WWTP) and septic system failures can be difficult, especially when these failures occur in remote or unmanned locations.

Traditionally, the quality of drinking and recreational waters have been ascertained using indicator bacteria. These bacterial tests require approximately 24 hours to complete and do not discriminate between human and animal sources. One potential solution is to analyze surface waters for changes in marker compounds. Such testing would require shorter analysis times and, due to the nature of the chemicals, be human specific. Caffeine, possibly the most widely consumed drug in the world due to its extensive use in beverages (coffee, tea, soft drinks), food (chocolate, dairy products), and pharmaceuticals, has been proposed as an effective marker for tracing surface water pollution from sewage effluents from wastewater treatment plants and septic systems (1).

Performance Data

Test sensitivity: The estimated minimum detectable concentration, based on 90% B/Bo, is 0.150 ppb (µg/L) in water, 300 ppb (µg/L) in urine, and 75 ppb (µg/L) in saliva.



Test reproducibility: Coefficients of variation (CVs) for standards: <15%, for samples: < 20%.

Selectivity: The assay exhibits very good cross-reactivity with Caffeine and not with other non-related compounds tested:

Cross-reactivities:		
	Caffeine	100% (per definition)
	1, 7-Dimethylxanthine	60%
	Theophyline	5%
	Theoxanthine	3%
	Xanthine	<0.1%
	1-Methyluric acid	<0.1%

Samples: Parallel sample analysis using this ELISA and GC methods showed a good correlation.

References: Buerge, I.J.; Poiger, T.; Müller, M.D.; Buser, H.-R. Caffeine, an Anthropogenic Marker for Wastewater Contamination of Surface Waters. *Environ. Sci. Technol.* **2003**, *37*, 691-700.

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ABRAXIS® Caffeine ELISA Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Caffeine in Contaminated Samples

Product No. 515575

1. General Description

The ABRAXIS® Caffeine ELISA is an immunoassay for the quantitative and sensitive detection of Caffeine. This test is suitable for the quantitative and/or qualitative screening of Caffeine in water, urine, and saliva samples (see Sample Preparation, Section C). If necessary, positive samples can be confirmed by HPLC, GC/MS, or other conventional methods. **Note:** This assay is intended For Research Use Only.

2. Safety Instructions

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

3. Storage and Stability

The ABRAXIS® Caffeine ELISA should 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 proper disposal of all reagents.

4. Test Principle

The test is a direct competitive ELISA which is based on the recognition of Caffeine by specific antibodies. Caffeine, when present in a sample and a Caffeine-HRP analogue compete for the binding sites of mouse anti-Caffeine antibodies in solution. The Caffeine 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 Caffeine 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 ABRAXIS® Caffeine ELISA, Possible Test Interference

Due to the high variability of compounds that might be found in water, urine, and saliva samples, test interferences caused by matrix effects cannot be completely excluded.

Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. The presence of the following substances were found to have no significant effect on the Caffeine Assay results for water samples: aluminum oxide, calcium chloride, calcium sulfate, manganese sulfate, magnesium sulfate, magnesium chloride, sodium chloride, phosphate, sodium thiosulfate, sodium nitrate up to 10,000 ppm; copper chloride, zinc sulfate, ferric sulfate, sodium fluoride up to 1,000 ppm; humic acid up to 10 ppm; salt water up to 50%.

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, and extreme temperatures during the test performance (lower than 10°C or higher than 30°C). The assay procedure should be performed away from direct sunlight.

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

6. Working Instructions

A. Materials Provided

1. Microtiter plate coated with a second antibody (goat anti-mouse)
1. Standards (6): 0, 0.175, 0.5, 1.0, 2.5, 5.0 ng/mL
3. Antibody Solution (mouse anti-Caffeine), 6 mL
4. Caffeine-HRP Conjugate Solution, 6 mL
5. Diluent/zero, 25 mL, use to dilute samples
6. ABRAXIS® Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation, Section D
7. Substrate (Color) Solution (TMB), 12 mL
8. Stop Solution, 12 mL

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

1. Micro-pipettes with disposable plastic tips (50-250 µL)
2. Multi-channel pipette (50-250 µL), stepper pipette (50-250 µL), or electronic repeating pipette with disposable plastic tips
3. Deionized or distilled water
4. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section D)
5. Vortex mixer
6. Timer
7. Paper towels or equivalent absorbent material
8. Microcentrifuge tubes
9. 4 mL glass vials with Teflon-lined caps
10. Microtiter plate reader (wave length 450 nm)

C. Sample Preparation

Water Samples

Water samples do not require any additional sample preparation prior to analysis. Proceed to Assay Procedure, Step 1.

Urine Samples

Urine samples must be stored frozen to avoid bacterial growth.

1. Add 980 µL of Sample Diluent to an appropriately labeled 4 mL glass vial with a Teflon-lined cap.
2. Add 20 µL of urine sample. Vortex thoroughly. The sample is then at a 1:50 dilution.
2. Add 975 µL of Sample Diluent to a second appropriately labeled 4 mL glass vial with a Teflon-lined cap.
3. Add 25 µL of the 1:50 diluted sample (produced in Step 2). Vortex thoroughly. The sample is then at a 1:2000 dilution.
4. Analyze the 1:2000 diluted sample (Assay Procedure, Step 1).

The Caffeine concentration contained in urine samples is determined by multiplying the ELISA result by the dilution factor of 2000. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further, in Sample Diluent, and re-analyzed.

Saliva Samples

Saliva samples must be frozen immediately after collection and stored frozen to avoid bacterial growth and to precipitate compounds which may cause matrix interferences in the assay.

1. Allow samples to thaw completely.
2. Add 1 mL of sample to an appropriately labeled microcentrifuge tube.
3. Centrifuge for 10 minutes at 1300 rpm. Pipette the supernatant into an appropriately labeled vial or tube.
4. Add 980 µL of Sample Diluent to an appropriately labeled, clean 4 mL glass vial with a Teflon-lined cap.
5. Add 20 µL of the supernatant (from Step 3). Vortex thoroughly. The sample is then at a 1:50 dilution.
6. Add 900 µL of Sample Diluent to a second appropriately labeled 4 mL glass vial with a Teflon-lined cap.
7. Add 100 µL of the 1:50 diluted sample (produced in Step 5). Vortex thoroughly. The sample is then at a 1:500 dilution.
8. Analyze the 1: 500 diluted sample (Assay Procedure, Step 1).

The Caffeine concentration contained in saliva samples is determined by multiplying the ELISA result by the dilution factor of 500. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further, in Sample Diluent, and re-analyzed. The supernatant (Step 3) can be frozen for later analysis, but should be re-centrifuged to ensure that any additional precipitate which may form is removed.

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the antibody, enzyme conjugate, substrate, and stop solutions in order to equalize the incubations periods of the standard solutions and the samples on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination. Read and understand the instructions and precautions given in this insert before proceeding.

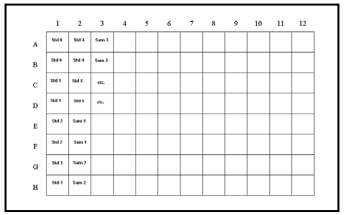
1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (2-8°C).
3. The standard, antibody, enzyme conjugate, substrate, and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the ABRAXIS® 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 and mix thoroughly. The diluted solution is then used to wash the microtiter wells.
5. The stop solution must be handled with care as it contains diluted H_2SO_4.

E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually. 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, 0.175, 0.5, 1.0, 2.5, 5.0 ppb)

Sam1, Sam2, Sam3, etc.: Samples



F. Assay Procedure

1. **Add 50 µL of the standards 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 µL of antibody solution** to the individual wells successively using a multi-channel, 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 bench top for 30 seconds. Be careful not to spill the contents. **Incubate the strips for 30 minutes at room temperature.**
3. **Add 50 µL of the enzyme conjugate solution** to the individual wells successively using a multi-channel, 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 bench top for 30 seconds. Be careful not to spill the contents. **Incubate the strips for 45 minutes at room temperature.**
4. After incubation, 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 four times using the diluted wash buffer.** 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 100 µL of substrate (color) solution** to the wells using a multi-channel, stepping, or electronic repeating pipette. I**ncubate the strips for 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, stepping, or electronic repeating pipette.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after stopping the reaction.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-parameter (preferred), Logit/Log, or alternatively point to point). 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 Caffeine concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Caffeine by interpolation using the standard curve.

The concentrations of the samples are determined using the standard curve run with each test. Do not use values from a previous analysis. Water samples showing a lower concentration of Caffeine than standard 1 (0.175 ppb) should be reported as containing < 0.175 ppb of Caffeine. Urine samples showing a lower concentration of Caffeine than standard 1 should be reported as containing < 350 ppb of Caffeine. Saliva samples showing a lower concentration than standard 1 should be reported as containing < 87.5 ppb of Caffeine. Samples showing a higher concentration than standard 5 (5.0 ppb) must be diluted and re-analyzed to obtain accurate results

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 Cotinine greater than the concentration of that standard. Samples which have higher absorbances than a standard will have concentrations of Caffeine less than that standard.

H. Recovery in Matrix Samples

Water Samples

Surface water samples with no detectable Caffeine were spiked with Caffeine at various levels and analyzed:

Spike Level (ppb)	Average Recovery	Std. Dev.	% Recovery
0.5	0.536	0.094	107%
1.0	1.002	0.071	100%
4.0	3.676	0.212	92%

Urine Samples

Urine samples were collected from people with varying levels of Caffeine consumption: group 1, no Caffeine containing foods, beverages, and/or supplements ingested, and group 2, Caffeine containing foods, beverages, and/or supplements ingested. Samples were evaluated both unspiked and Caffeine spiked:

Type/Spike Level (ppb)	Average Recovery (ppb)	Adjusted Recovery (ppb)	Adjusted % Recovery
No Caffeine Ingestion	nd		
No Caffeine Ingestion at 2000	2130	2130	107%
Caffeine Ingestion	9073		
Caffeine Ingestion at 2000	11157	2084	104%

Saliva Samples

Saliva samples were collected from people with varying levels of Caffeine consumption: group 1, no Caffeine containing foods, beverages, and/or supplements ingested, group 2, moderate intake of Caffeine containing foods, beverages, and/or supplements, and group 3, high intake of Caffeine containing foods, beverages, and/or supplements. Samples were evaluated both unspiked and Caffeine spiked:

Type/Spike Level (ppb)	Average Recovery (ppb)	Adjusted Recovery (ppb)	Adjusted % Recovery
No Caffeine Ingested	nd		
No Caffeine Ingested at 300	361	361	120%
Moderate Caffeine	679		
Moderate Caffeine at 300	1005	305	102%
High Caffeine	4767		
High Caffeine at 2500	7598	2802	112%