

Importance of Caffeine Determination

Most of the household water in the civilized world is treated before it is allowed to enter the environment but mishaps can occur and untreated water can escape into streams, etc. It's not always easy to identify when failure of WWTPs has occurred or when surface waters are contaminated from septic systems, especially if it was an accident or occurred in a remote or unmanned location. One way around this is to analyze the surface waters regularly to detect changes in the amounts of marker compounds.

Traditionally the quality of drinking and recreational water is ascertained using indicator bacteria. The traditional tests that analyze for the bacteria requires approximately 24 hours to complete and do not discriminate between human and animal sources. One potential solution is to use human wastewater chemicals such as caffeine, which would require shorter analysis times and are human specific.

One such compound is caffeine. Caffeine is perhaps the most widely consumed drug in the world because of its extensive use in beverages (coffee, tea, soft drinks), food (chocolate, dairy products), and pharmaceuticals. It has been proposed to be an effective marker for tracing surface water pollutants, which come from human wastewater due to sewage effluents from either wastewater treatment plants (WWTPs) or septic systems (1).

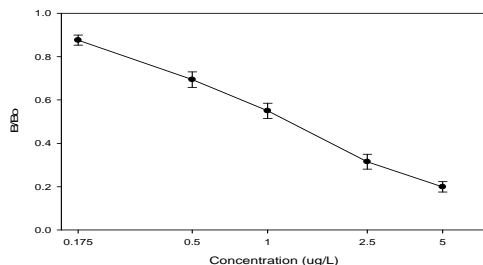
Performance Data

Test sensitivity: The detection limit for this assay is 0.150 ppb (ug/L)

Test reproducibility: Coefficients of variation (CVs) for standards: <13%, 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%
	Theophylline	5%
	Theoxanthine	3%
	Xanthine	<0.1%
	1, Methyluric acid	<0.1%



Samples: A sample correlation between the ELISA and GC methods showed a good correlation.

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R081108

Caffeine ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Determination of
Caffeine Water Samples

Product No. 515575



1. General Description

The Abraxis Caffeine ELISA is an immunoassay for the quantitative and sensitive detection of Caffeine in water samples. A pre-sample concentration is not required. If necessary, positive samples can be confirmed by HPLC, or other conventional methods.

2. Safety Instructions

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 the skin, wash with water.

3. Storage and Stability

The Caffeine ELISA should be stored in the refrigerator (4–8°C). Solutions should be allowed to reach room temperature (20–25°C) before use. Reagents may be used until 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 that allows the detection of Caffeine. It 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 in the 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 the 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 Caffeine ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in water 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 water samples, test interferences caused by matrix effects can't be completely excluded. The presence of the following substances up to 10,000 ppm were found to have no significant effect on the Caffeine Assay results: aluminum oxide, calcium chloride, calcium sulfate, manganese sulfate, magnesium sulfate, magnesium chloride, sodium chloride, phosphate, sodium thiosulfate, sodium nitrate. 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 can be: Inadequate storage conditions of the test kit, wrong 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 sun light.

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

A. Materials Provided

1. Microtiter plate coated with a second antibody (goat anti mouse).
2. Standards (6): 0, 0.175, 0.50, 1.0, 2.5, 5.0 ng/mL.
3. Antibody solution (mouse anti-Caffeine), 6 mL
4. Caffeine-HRP, 6 mL
5. Diluent/zero, 25 mL. Use to dilute samples with concentration above 5 ppb.
6. Wash Solution 5X Concentrate, 100 mL
7. Color Solution (TMB), 12 mL
8. Stop Solution, 12 mL

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the antibody, enzyme conjugate, substrate solution, and the stop solution in order to equalize the incubations periods of the standard solutions and the samples on 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. 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 (4-8°C).
3. The standard, control, antibody solution, enzyme conjugate, substrate and stop solutions are ready to use and do not require any further dilutions.
4. The wash solution is a 5X concentrated solution and needs to be diluted with deionized water. In a 1L container dilute the 5X solution 1:5 (i.e. 100 mL of the 5X wash solution plus 400 mL of deionized water). The diluted solution is used to wash the microtiter wells.
5. The stop solution has to be handled with care as it contains diluted H₂SO₄.

C. Assay Procedure

1. Add 50 µL of the standard solutions, control or samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL 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 rapid circular motion on the bench top for about 30 seconds. Be careful not to spill contents. Incubate the strips for thirty (30) minutes at room temperature.
3. Add 50 µL of the enzyme 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 rapid circular motion on the bench top for about 30 seconds. Be careful not to spill contents. Incubate the strips for forty five (45) minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips four times using the 1X washing buffer solution. Please use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 100 µL of substrate/color solution to the wells using a multi- channel pipette or a stepping pipette. The strips are incubated for 20 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 or a stepping pipette.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after stopping the reaction.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-parameters, Logit/Log or alternatively point to point). For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the %B/B₀ 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/B₀ for each standard on a vertical linear (y) axis versus the corresponding Caffeine concentration on horizontal logarithmic (x) axis on graph paper. %B/B₀ for controls and samples will then yield levels in ppb of Caffeine by interpolation using the standard curve.

The concentrations of the samples are determined using the constructed standard curve (do not use a previously stored curve). Samples showing a lower concentration than 0.175 ppb of Caffeine are considered to be negative. Samples showing a higher concentration than standard 5 (5.0 ppb) must be diluted to obtain more accurate results.

E. 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) or stepper pipette with plastic tips (50-250 µL)
3. Reagent reservoir for multichannel pipettes
4. Microtiter plate washer (optional)
5. Microtiter plate reader (wave length 450 nm)
6. Shaker for microtiter plates (optional)

F. Working Scheme

The microtiter plate consists of 12 strips of 8, 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

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

Sam1, Sam2, Sam3, etc.: Samples

Recovery

Four (3) groundwater samples, were spiked with various levels of Caffeine and then assayed using the Abraxis Caffeine Assay. The following results were obtained:

Amount of	Recovery		
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Caffeine	Mean	S.D.	
Recovery			
Added (ppb)	(ppb)	(ppb)	(%)
0.50	0.536	0.094	107
1.00	1.002	0.071	100
4.00	3.676	0.212	92

Precision

The following results were obtained:

Control	1	2	3
Replicates	3	3	3
Days	3	3	3
n	9	9	9
Mean (ppb)	0.516	0.996	3.876
% CV (within assay)	7.9	5.5	3.6
% CV (between assay)	12.8	10.2	
6.8			

Sensitivity

The Abraxis Caffeine ELISA has an estimated minimum detectable concentration, based on 90% B/B₀ of 0.150 ppb.

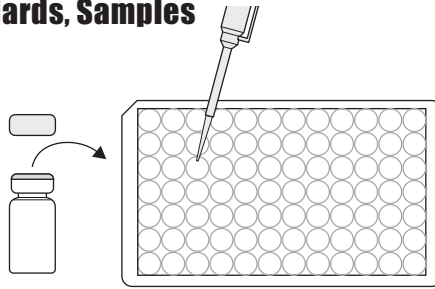
G. References

- (1) Buergle, Ignaz, J. Poigner, Thomas, Markus D., and Buser, Hans-Rudolph. (2003). Caffeine, an Anthropogenic Marker for Wastewater Contamination of Surface Waters. *Environ. Sci. Technol.* 37, 691-700.

Caffeine Plate, Detailed ELISA Procedure

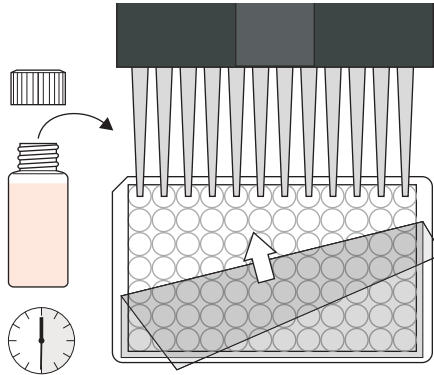
1. Addition of Standards, Samples

Add 50 μ L of the standard solutions, control or samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.



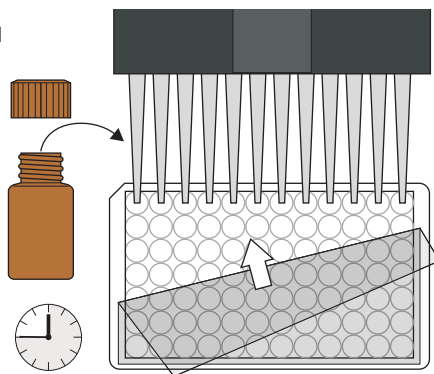
2. Addition of Antibody Solution

Add 50 μ L of the Caffeine 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 rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 30 min at room temperature.



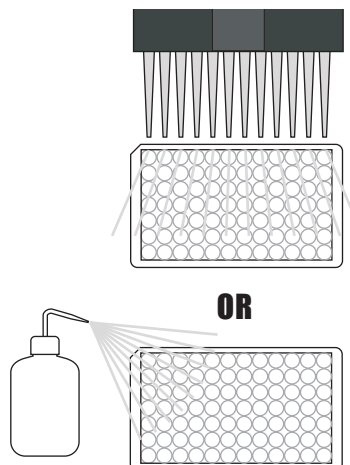
3. Addition of Enzyme Conjugate

Add 50 μ L of the enzyme conjugate to the individual wells successively using a multi-channel pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 45 min at room temperature.



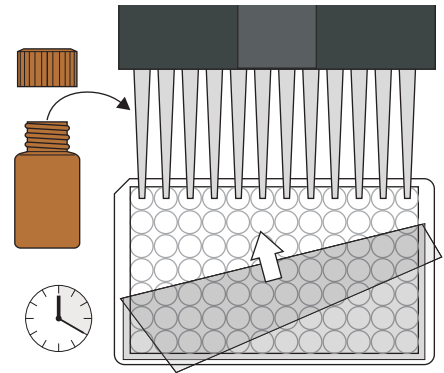
4. Washing of Plates

After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips four times with a multi-channel pipette or wash bottle using the diluted 1X washing buffer solution. Please use at least a volume of 250 μ L of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels. Repeat steps an additional three times.



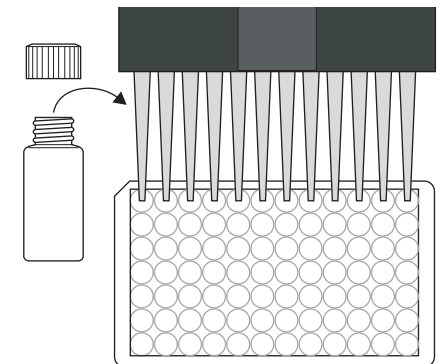
5. Addition of Substrate/Color Solution

Add 100 μ L of substrate/color 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 rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 20 min at room temperature.



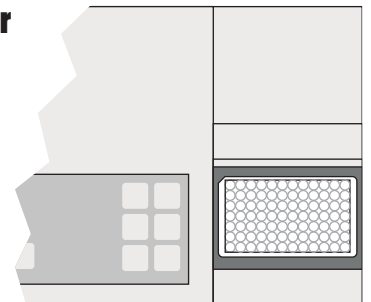
6. Addition of Stopping Solution

Add 100 μ L of stop solution to the wells in the same sequence as for the substrate solution using a multi-channel pipette or a stepping pipette.



7. Measurement of Color

Read the absorbance at 450 nm using a microplate ELISA reader. Calculate results.

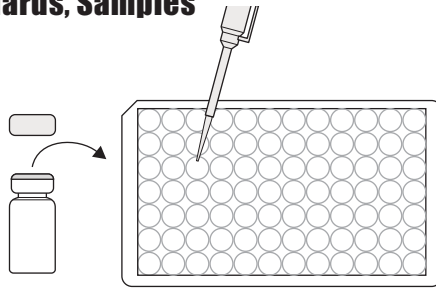


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Caffeine Plate, Concise ELISA Procedure

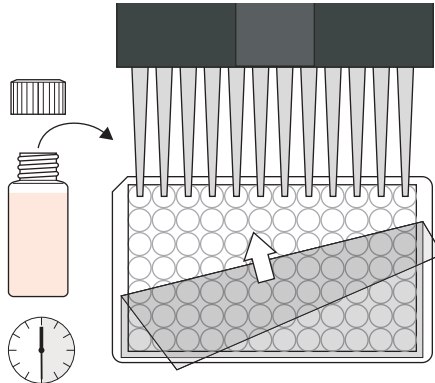
1. Addition of Standards, Samples

Add 50 μ L of standard solutions, control or samples.



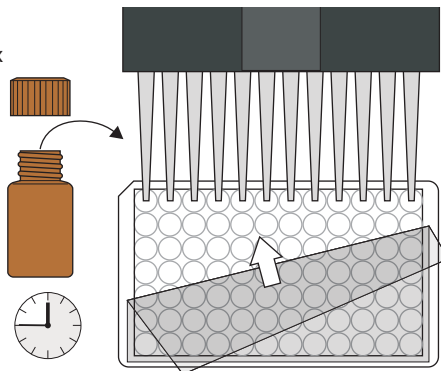
2. Addition of Antibody Solution

Add 50 μ L of antibody solution. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 30 minutes at room temperature.



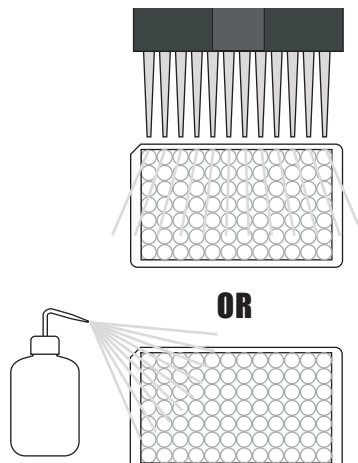
3. Addition of Enzyme Conjugate

Add 50 μ L of the enzyme conjugate. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 45 minutes at room temperature.



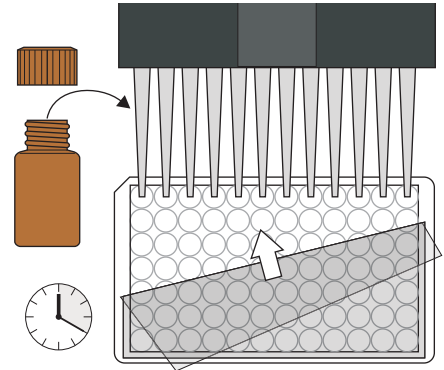
4. Washing of Plates

Wash the plates four times with 250 μ L of diluted 1X washing buffer.



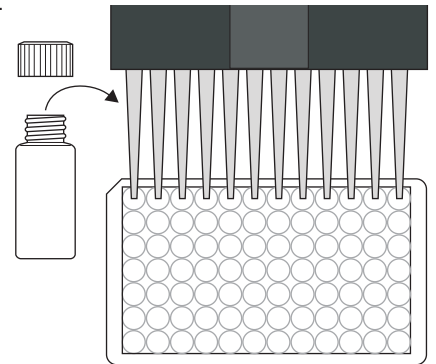
5. Addition of Substrate/Color Solution

Add 100 μ L of substrate/color solution. Incubate 20 minutes at room temperature and away from direct sunlight.



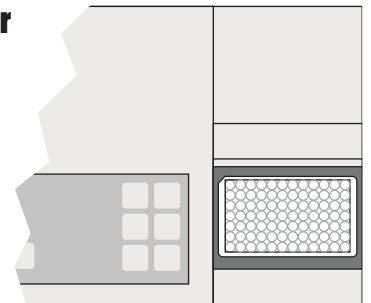
6. Addition of Stopping Solution

Add 100 μ L of stop solution.



7. Measurement of Color

Measure color at 450 nm.
Calculate results.



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