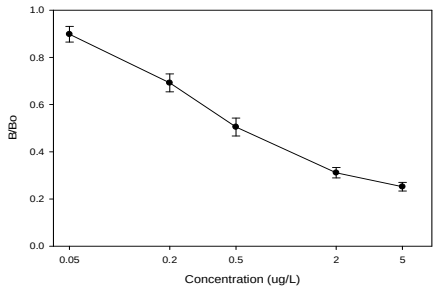
Importance of Cotinine Determination in Water

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. Cotinine has been proposed as an effective marker for tracing surface water pollution from sewage effluents from wastewater treatment plants and septic systems.

Performance Data

Test sensitivity: The estimated minimum detectable concentration, based on 90% B/Bo, is 0.045 ppb (µg/L) in water, 4.5 ppb (µg/L) in urine, and 9.0 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 Cotinine and not with other non-related compounds tested:

Cross-reactivities:		
	Cotinine	100% (per definition)
	trans-3'-hydroxycotinine	61%
	Nicotine	1%
	N-formylnornicotine	<1%
	Nornicotine	<0.1%

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

For ordering or technical assistance contact:

Abraxis LLC
54 Steamwhistle Drive
Warminster, PA 18974
Tel.: (215) 357-3911
Fax: (215) 357-5232
Email: info@abraxiskits.com
WEB: www.abraxiskits.com

R042011

Cotinine ELISA (Microtiter Plate)

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

Product No. 515565

1. General Description

The Abraxis Cotinine ELISA is an immunoassay for the quantitative and sensitive detection of Cotinine. This test is suitable for the quantitative and/or qualitative screening of Cotinine in water, urine, and saliva samples. 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 Cotinine 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. 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 Cotinine by specific antibodies. Cotinine, when present in a sample, and a Cotinine-HRP analogue compete for the binding sites of rabbit anti-Cotinine antibodies in solution. The Cotinine antibodies are then bound by a second antibody (goat anti-rabbit) 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 Cotinine 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 Cotinine 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 Cotinine Assay results for water samples: aluminum oxide, calcium chloride, calcium sulfate, manganese sulfate, magnesium sulfate, magnesium chloride, sodium chloride, phosphate, sodium thiosulfate, sodium nitrate, sodium fluoride up to 10,000 ppm; copper chloride, zinc sulfate, ferric sulfate 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.

Working Instructions

A. Reagents and Materials Provided

1. Microtiter plate coated with a second antibody (goat anti-rabbit)
2. Standards (6): 0, 0.05, 0.2, 0.5, 2.0, 5.0 ng/mL
3. Antibody Solution (rabbit anti-Cotinine), 6 mL
4. Cotinine-HRP Conjugate Solution, 6 mL
5. Diluent/zero, 25 mL, use to dilute samples
6. Wash Solution (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation, section C
7. 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) or stepper pipette with disposable plastic tips (50-250 µL)
3. Vortex mixer
4. Timer
5. Paper towels or equivalent absorbent material
6. Microcentrifuge tubes
7. Glass vials with Teflon-lined caps
8. Microtiter plate reader (wave length 450 nm)

C. 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 (4-8°C).
3. The standard, control, antibody, enzyme conjugate, 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. 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.

D. 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 which may cause matrix interferences in the assay.

1. Add 1.98 mL of Sample Diluent to an appropriately labeled glass vial with a Teflon-lined cap.
2. Add 20 µL of urine sample. Vortex thoroughly.
3. Analyze as sample (Assay Procedure, step 1).

The Cotinine concentration contained in urine samples is determined by multiplying the ELISA result by the dilution factor of 100. 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 3.98 mL of Sample Diluent to an appropriately labeled, clean glass vial with a Teflon-lined cap.
5. Add 20 µL of the supernatant (from step 3). Vortex thoroughly.
6. Analyze as sample (Assay Procedure, step 1).

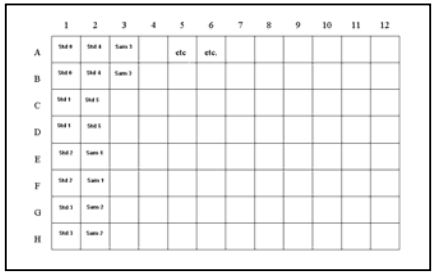
The Cotinine concentration contained in saliva samples is determined by multiplying the ELISA result by the dilution factor of 200. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further, in Sample Diluent, and re-analyzed. The supernatant (step 3) can be frozen for later analysis, but should be re-centrifuged to ensure that any additional precipitate which may form is removed.

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

Sam1, Sam2, Sam3, etc.: Samples



F. Assay Procedure

1. Add 50 µL of the 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 µL of the enzyme conjugate solution to the individual wells successively using a multi-channel pipette or a stepping pipette.
3. 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 circular motion on the bench top for 30 seconds. Be careful not to spill the contents. Incubate the strips for sixty (60) 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. Incubate 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 pipette or a stepping 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 a vertical linear (y) axis versus the corresponding Cotinine concentration on horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Cotinine 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 Cotinine than standard 1 (0.05 ppb) should be reported as containing < 0.05 ppb of Cotinine. Urine samples showing a lower concentration of Cotinine than standard 1 should be reported as containing < 5 ppb of Cotinine. Saliva samples showing a lower concentration of Cotinine than standard 1 should be reported as containing < 10 ppb of Cotinine. 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 calibrators. Samples with lower absorbances than a calibrator will have concentrations of Cotinine greater than the concentration of that calibrator. Samples which have higher absorbances than a calibrator will have concentrations of Cotinine less than that calibrator.

H. Recovery in Matrix Samples

Water Samples

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

Spike Level (ppb)	Average Recovery (ppb)	Std. Dev.	% Recovery
0.25	0.235	0.017	94%
1.00	1.122	0.144	112%
2.00	2.062	0.336	103%

Urine Samples

Urine samples were collected from various people with and without cotinine exposure: group 1, non-smokers without second-hand or third-hand exposure, and group 2, non-smokers with second-hand and third-hand exposure/active smokers. Samples were evaluated both unspiked and Cotinine spiked:

Type/Spike Level (ppb)	Average Recovery (ppb)	Adjusted Recovery (ppb)	Adjusted % Recovery
Non-smoker(No exposure) at 0	nd		
Non-smoker(No exposure) at 25	28.0	28.0	112%
Smoker or ambient exposure 0	31.4		
Smoker or ambient exposure 25	54.3	22.9	92%

Saliva Samples

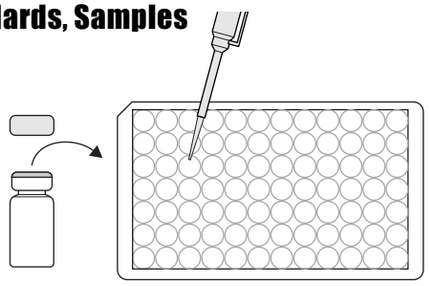
Saliva samples were collected from various people with and without cotinine exposure: group 1, non-smokers without second-hand or third-hand exposure, and group 2, non-smokers with second-hand and third-hand exposure/active smokers. Samples were evaluated both unspiked and Cotinine spiked:

Type/Spike Level (ppb)	Average Recovery (ppb)	Adjusted Recovery (ppb)	Adjusted % Recovery
Non-smoker(No exposure) at 0	nd		
Non-smoker(No exposure) at 100	102.4	102.4	102%
Smoker or ambient exposure 0	886.4		
Smoker or ambient exposure 100	1000.2	113.8	114%

Cotinine Plate, Detailed ELISA Procedure

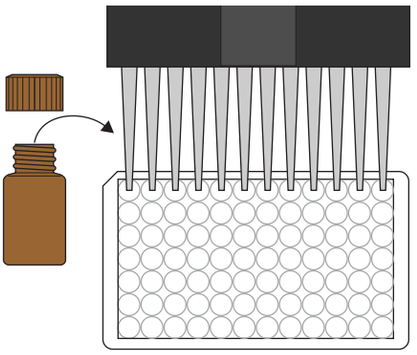
1. Addition of Standards, Samples

Add 50 uL 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.



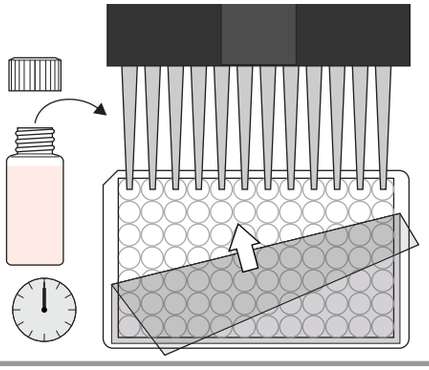
2. Addition of Enzyme Conjugate

Add 50 uL of the enyzme conjugate to the individual wells successively using a multi- channel pipette.



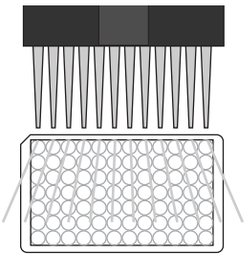
3. Addition of Antibody Solution

Add 50 uL of the anti-Cotinine 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 60 min at room temperature.

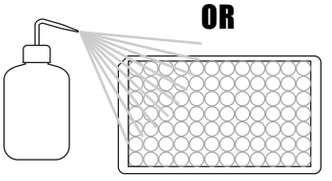


4. Washing of Plates

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 uL 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.

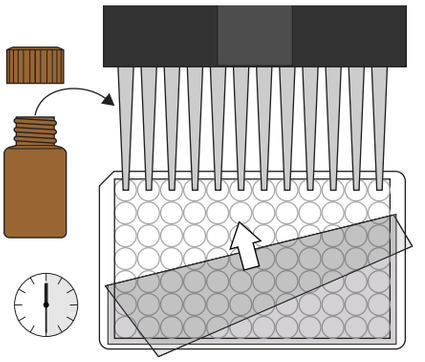


OR



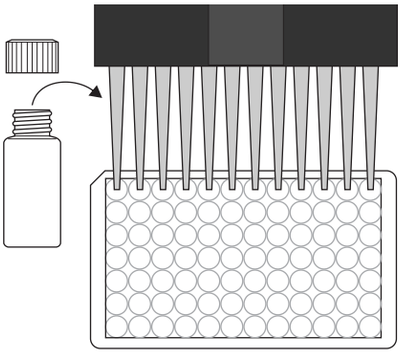
5. Addition of Substrate/Color Solution

Add 100 uL 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 30 min at room temperature.



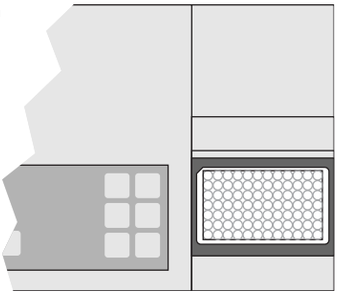
6. Addition of Stopping Solution

Add 100 uL 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.

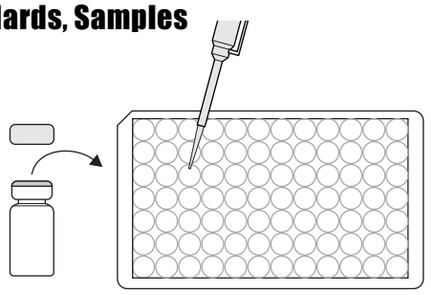


For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

Cotinine Plate, Concise ELISA Procedure

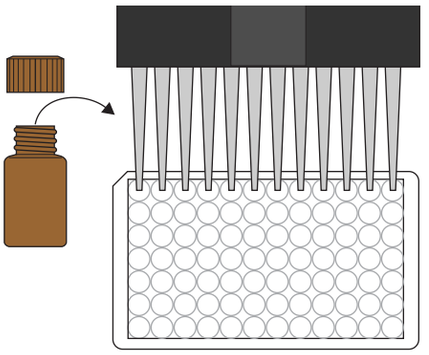
1. Addition of Standards, Samples

Add 50 uL of standard solutions, control or samples.



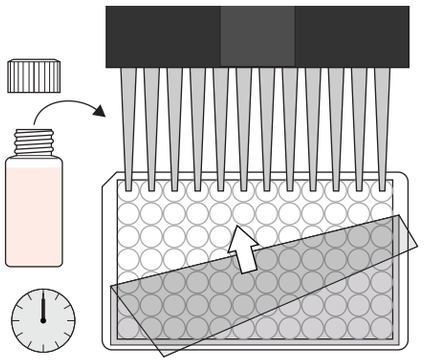
2. Addition of Enzyme Conjugate

Add 50 uL of the enyzme conjugate.



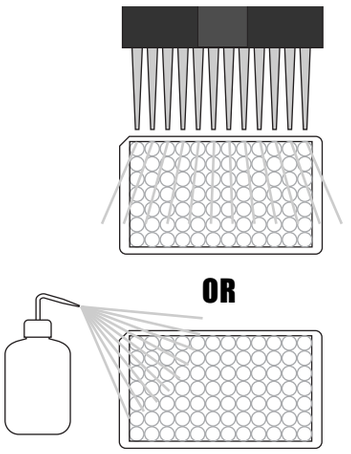
3. Addition of Antibody Solution

Add 50 uL of antibody solution. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 60 minutes at room temperature.



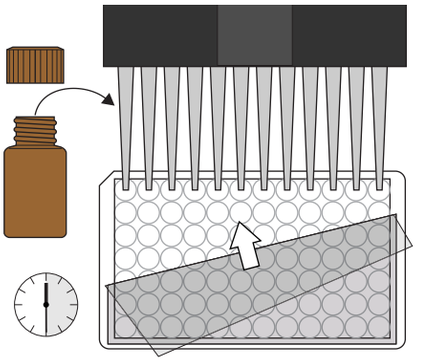
4. Washing of Plates

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



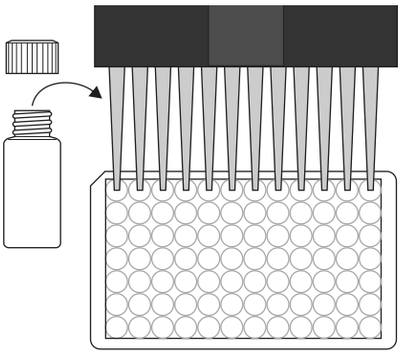
5. Addition of Substrate/Color Solution

Add 100 uL of substrate/color solution. Incubate 30 minutes at room temperature and away from direct sunlight.



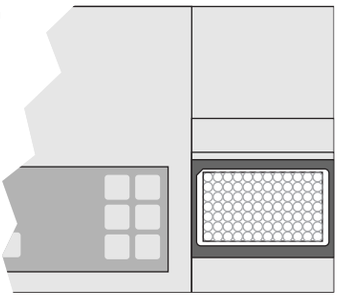
6. Addition of Stopping Solution

Add 100 uL of stop solution.



7. Measurement of Color

Measure color at 450 nm. Calculate results.



For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com