



High Sensitivity
SALIVARY COTININE
Quantitative
ENZYME IMMUNOASSAY KIT



For Diagnostic In-Vitro Use

Item No. 1-2112, (Single) 96-Well Kit;
1-2112-5, (5-Pack) 480 Wells



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Intended Use

The Salimetrics® Cotinine Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the in vitro quantitative measurement of salivary Cotinine. This kit may be used to measure primary or secondhand exposure to nicotine. This kit is not intended to diagnose nicotine poisoning. Salimetrics has not validated this kit for serum or plasma samples, however a validated urine protocol is available upon request.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

Since primitive times, tobacco leaves have been processed and used by humans to deliver nicotine to the central nervous system. Historically, the preferred route of nicotine administration has varied from snuff, chew, and inhalation of smoke from burned tobacco leaves to contemporary methods such as transdermal patches, chewing gums, and smokeless inhalers. Regardless of delivery route, nicotine has addictive properties that cause the user to continue use despite efforts to quit. When tobacco leaves are smoked in cigarettes, nicotine is absorbed and distributed in the body within seconds. Metabolism is mainly by oxidation to Cotinine and nicotine-N-oxide. Volumes of literature document the negative economic impact and health consequences of nicotine exposure. The costs to individuals and societies associated with nicotine exposure have led to widespread public health interventions to curb nicotine use.

The detection of exposure to tobacco smoke by measurement of Cotinine is the preferred method. Nicotine is not considered a valid marker of smoking status due to its relatively short half-life (approximately 2 hours). By contrast, Cotinine has an average half-life of 17 hours, and blood levels closely reflect the dose of nicotine absorbed from tobacco smoke. Saliva samples are easier to obtain, however, and saliva levels are highly correlated and used interchangeably with blood levels (1).

Many of the commercially available assays for salivary Cotinine are qualitative. They return "positive" or "negative" determinations with respect to tobacco/nicotine exposure. However, many studies show that levels of Cotinine in saliva show large inter-individual differences. The sources of these differences include factors related to intrinsic and extrinsic predispositions that affect the physiology of nicotine metabolism, the dose of nicotine present in the source, and health behaviors relevant to how nicotine is used (e.g., cigarette vent blocking, duration and frequency of puffs) (2). There is a clear advantage to providing inexpensive, accurate,



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quantitative, and noninvasive means of validating nicotine use, measuring the immediate physiological consequences of individual differences and intra-individual change in users' behaviors and determining secondhand tobacco smoke exposure. Salimetrics has designed this assay to provide the medical community with a highly sensitive and reliable means to do so.

Cotinine levels in biologic fluids have been measured by chromatographic (GC or HPLC – sometimes coupled with mass spectrometry) and immunoassay methods. Chromatographic methods have the advantage of higher specificity and sensitivity, (1) but EIA Cotinine results have shown near perfect agreement with GC/MS confirmation of smoking status (3). Immunoassay methods also use smaller sample volumes than chromatography methods and they do not require extractions or other manipulations of the samples, making them easier to use in large-scale epidemiological studies and avoiding the need for specialized laboratories (4,5). Levels, however, may be higher with EIA since metabolites of Cotinine, such as 3-OH-Cotinine, are also measured (1).

Test Principle

This is a competitive immunoassay kit. Cotinine in standards and samples compete with Cotinine conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Cotinine Enzyme Conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of Cotinine Enzyme Conjugate detected is inversely proportional to the amount of Cotinine present in the sample (6).



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Safety Precautions

Read Safety Data Sheets before handling reagents.

Hazardous Ingredients

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing call a physician.

The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.

Safety Data Sheets are available by contacting Salimetrics at support@salimetrics.com (See www.salimetrics.com for alternative contact options).



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General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and enzyme conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
- When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
- When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
- The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
- Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
- When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.
- Use care when removing adhesive plate cover, in order to avoid spilling or cross contamination of wells after incubation at 37°C.
- We recommend saving all reagents until data analysis has confirmed a successful run to facilitate troubleshooting if necessary.
- Prior to sample addition, please label each strip to assure plate orientation and sample order when data is acquired on plate reader

Storage

All unopened components of this kit are stable at 2-8°C until the kit's expiration date.



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pH Indicator

Cotinine values from samples with a pH ≤ 3.5 or ≥ 9.0 may be inaccurate. A pH indicator in the Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the Assay Diluent, acidic samples will turn yellow and alkaline samples will turn purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Samples with a pH ≤ 3.5 or ≥ 9.0 should be recollected.

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial. Collection protocols/methods are available online at www.salimetrics.com or upon request.

Record the time and date of specimen collection.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes and freeze at or below -20°C within 4 hours of collection. (Samples may be stored at -20°C for up to 6 months.) For long term storage, refer to the Salimetrics Collection and Handling Advice Booklet.

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at $1500 \times g$ for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Re-freeze saliva samples as soon as possible after adding to the assay plate. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Saliva samples for smoking subjects ONLY should be diluted for this assay. See Procedure for details.



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Materials Supplied with Single Kit

	Item	Quantity/Size
1	Microtitre Plate	1/96 well
2	Cotinine Standard 200 ng/mL in a saliva-like matrix. Contains: Cotinine, buffer, preservative.	1 vial / 500 µL
3	Cotinine Controls High, Low, in a saliva-like matrix. Ready to use. Contains: Cotinine, buffer, preservative.	2 vials / 250 µL each
4	Cotinine Enzyme Conjugate Concentrate. Dilute before use with assay diluent. (See step 7 of Procedure.) Contains: Cotinine conjugated to HRP, preservative.	1 vial / 75 µL
5	Assay Diluent Contains: phosphate buffer, pH indicator, preservative.	1 bottle / 60 mL
6	Cotinine Antiserum Contains: rabbit anti-Cotinine antibody, buffer, preservative.	1 bottle / 15 mL
7	Wash Buffer Concentrate (10X) Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
8	TMB Substrate Solution Non-toxic, ready to use.	1 bottle / 25 mL
9	Stop Solution	1 bottle / 12.5 mL
10	Adhesive Plate Covers	2



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Materials Needed But Not Supplied

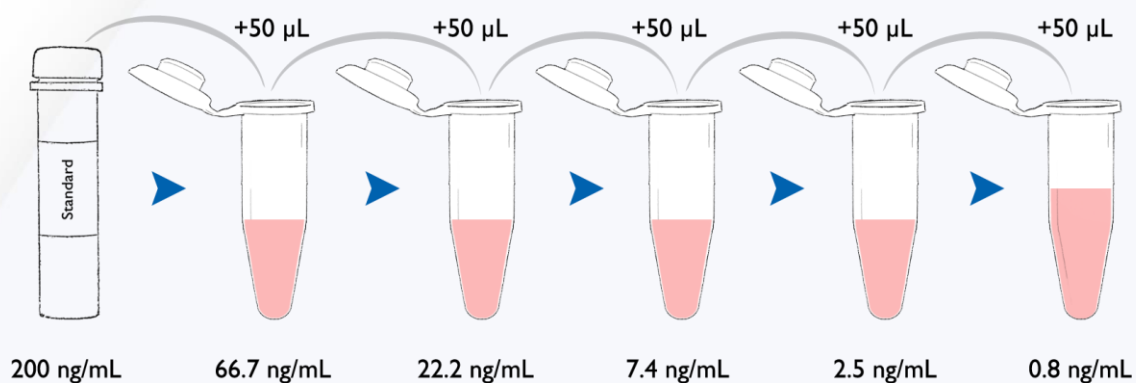
- Precision pipette to deliver 10 μL , 20 μL , 50 μL , 90 μL , and 100 μL
- Precision multichannel pipette to deliver 50 μL , 100 μL , and 200 μL
- Vortex
- 37°C Microplate incubator/shaker with 0.08-0.17 inch orbit capable of operating at 500 rpm
- Plate reader with 450 nm and 620 to 630 reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 15 mL
- Small disposable polypropylene tubes for dilution of standard and some samples
- Pipette tips
- Serological pipette to deliver up to 15 mL
- Centrifuge capable of 1500 x g



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Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 15 mL of Assay Diluent used in Step 7 (conjugate dilution) to come to room temperature.
- Bring microtitre plate to room temperature before use. ***It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.***
- Prepare 1X wash buffer by diluting Wash Buffer Concentrate (10X) 10-fold with room-temperature deionized water (100 mL of Wash Buffer Concentrate (10X) to 900 mL of deionized water). ***Dilute only enough for current day's use and discard any leftover reagent.*** (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
- Prepare serial dilutions of the Cotinine Standard as follows:
 - Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
 - Pipette 100 μL of Assay Diluent into tubes 2 through 6.
 - Serially dilute the standard 3X by adding 50 μL of the 200 ng/mL standard (tube 1) to tube 2. Mix well.
 - After changing pipette tips, remove 50 μL from tube 2 to tube 3. Mix well.
 - Continue for tubes 4, 5, and 6.
 - The final concentrations of standards for tubes 1 through 6 are, respectively, 200 ng/mL, 66.7 ng/mL, 22.2 ng/mL, 7.4 ng/mL, 2.5 ng/mL, and 0.8 ng/mL.



Procedure

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 Std	200 Std	Ctrl-H	Ctrl-H								
B	66.7 Std	66.7 Std	Ctrl-L	Ctrl-L								
C	22.2 Std	22.2 Std	SMP-1	SMP-1								
D	7.4 Std	7.4 Std	SMP-2	SMP-2								
E	2.5 Std	2.5 Std	SMP-3	SMP-3								
F	0.8 Std	0.8 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	*Blank	*Blank	SMP-6	SMP-6								

*Blank. Use is optional.

Step 2: Set your microplate incubator/shaker to 37°C.

Step 3: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Step 4: Pipette 15 mL of Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 7.

Step 5: Sample Preparation

- Known Smokers ONLY: Dilute saliva samples 10X in Assay Diluent using 10 µL saliva to 90 µL Assay Diluent.
- Non-Smokers: Run saliva samples straight.

Step 6:

- Pipette 20 µL of standards, controls, and saliva samples into appropriate wells.
- Pipette 20 µL of Assay Diluent into 2 wells to serve as the zero.
- Pipette 120 µL of Assay Diluent into 2 Blank wells, if used.



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Step 7: Dilute the Enzyme Conjugate 1:300 by adding 50 μL of the conjugate to the 15 mL tube of Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 100 μL to each well using a multichannel pipette.

Step 8: Pipette 100 μL of Cotinine Antiserum into all wells, except the Blank wells (if used), using a multichannel pipette.

Step 9: Place adhesive cover provided over plate. Mix plate in a ***preheated 37°C*** microplate incubator/shaker continuously at 500 rpm for 1.5 hours at 37°C.

Step 10: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 μL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 11: Add 200 μL of TMB Substrate Solution to each well with a multichannel pipette.

Step 12: Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 25 minutes.

Step 13: Add 50 μL of Stop Solution with a multichannel pipette.

Step 14:

- Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow.

Caution: Spillage may occur if mixing speed exceeds 600 rpm.

- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 620 to 630 nm is recommended.)



Quality Control

The Salimetrics' High and Low Cotinine Controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the Blank wells (if used) from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. Samples with Cotinine values greater than 200 ng/mL (or >2,000 ng/mL after multiplying by the dilution factor of 10) should be diluted with Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

A new Standard Curve must be run with each full or partial plate.

Typical Results

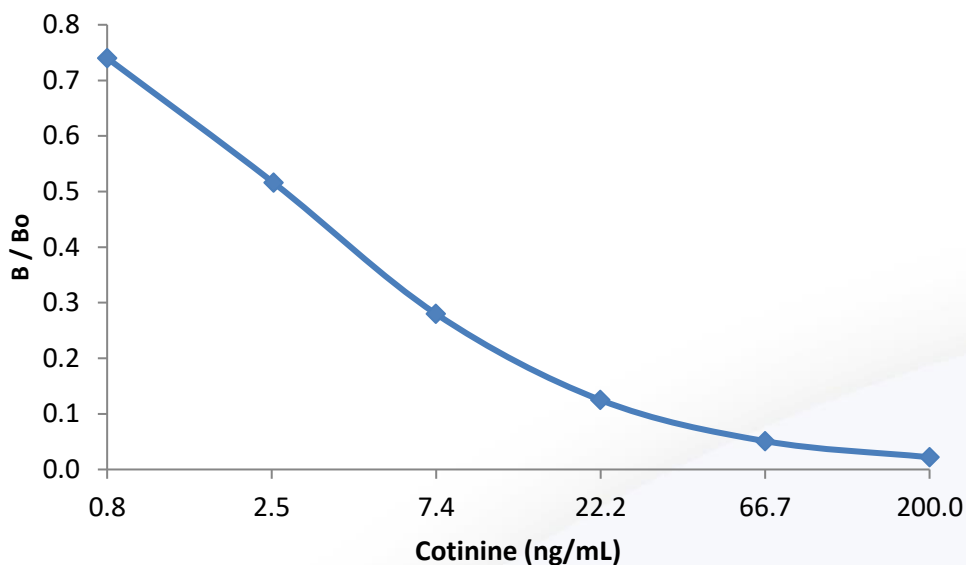
The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	Cotinine (ng/mL)
A1,A2	S1	0.045	0.037	0.022	200
B1,B2	S2	0.096	0.088	0.051	66.7
C1,C2	S3	0.222	0.214	0.125	22.2
D1,D2	S4	0.489	0.481	0.280	7.4
E1,E2	S5	0.893	0.885	0.516	2.5
F1,F2	S6	1.278	1.270	0.740	0.8
G1,G2	Bo	1.724	1.716	NA	NA
H1,H2	Blank	0.008	NA	NA	NA



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Example: Cotinine 4-Parameter Curve Fit



Limitations

- Samples with Cotinine values greater than 200 ng/mL (or >2,000 ng/mL after multiplying by the dilution factor of 10) should be diluted with Assay Diluent and rerun for accurate results. To obtain the final Cotinine concentration, multiply the concentration of the diluted sample by the dilution factor.
- A pH value should be obtained on samples that appear yellow or purple after the diluted conjugate solution is added and the plate is mixed (Step 7). Samples with pH values ≤ 3.5 or ≥ 9.0 should be recollected.
- See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodium azide are unsuitable for this assay.

Example Ranges

Measurement of Salivary Cotinine in Smokers and Non-smokers Using the Salimetrics EIA

Group	N	Mean (ng/mL)	Standard Deviation (ng/mL)	Range (ng/mL)
Adult Smokers	21	206.33	123.47	47.87 - 586.39
Non-smokers	10	0	0	NA

The Salimetrics EIA is able to distinguish smokers from non-smokers with a high level of accuracy.



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Salimetrics High Sensitivity Salivary Cotinine Quantitative Enzyme Immunoassay discriminates smokers from non-smokers and differentiates primary from secondary smoke exposure (7).

Table 1: Salivary Cotinine levels (ng/mL) in smoking and non-smoking mothers and their 6-month old infants

Mother's Self-Reported Status: Smokers (n=27)		
Group	Mean	Standard Deviation
Number of cigarettes smoked in prior 48 hours by mother	13.11	12.00
Mother's salivary Cotinine (ng/mL)	252.27	178.81
Infant salivary Cotinine (ng/mL)	10.96	9.08
Mother's Self-Reported Status: Non-Smokers (n=20)		
Group	Mean	Standard Deviation
Number of cigarettes smoked in prior 48 hours by mother	0.0	NA
Mother's salivary Cotinine (ng/mL)	0.91	1.43
Infant salivary Cotinine (ng/mL)	2.26	3.30

Notes:

1. Smoking status determined by number of cigarettes smoked in the past 48 hours, "0" = non-smoker, "> 3" = smoker.
2. Independent sample t-test comparing smoking and non-smoking groups, $p < 0.001$.
3. Only 1 infant of a smoking mother had received breast-milk in prior 7 days.

Comparison of Cotinine Measurement by LC-ES/MS/MS to EIA (unpublished data)

(n=40)	Cotinine Results by LC-ES/MS/MS*	Cotinine Results by Salimetrics EIA
LC-ES/MS/MS Pearson Correlation (p-value)	--	0.90 (0.00)
Salimetrics Cotinine EIA Pearson Correlation (p-value)	0.90 (0.00)	--
Total Hrs. Exposed[^] Pearson Correlation (p-value)	0.39 (0.01)	0.48 (0.00)

*Liquid Chromatography Electrospray Ionization Tandem Mass Spectroscopy

[^] Self-reported hours of exposure to secondhand smoke



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HS Salivary Cotinine Quantitative EIA Kit Performance Characteristics

Precision

The intra-assay precision was determined from the mean of 10 replicates each.

Saliva Sample	N	Mean (ng/mL)	Standard Deviation (ng/mL)	Coefficient of Variation (%)
1	10	5.49	0.25	4.5
2	10	52.35	4.50	8.6
3	10	105.21	6.16	5.9
4	10	495.47	32.04	6.5

The inter-assay precision was determined from the mean of average duplicates for 8 separate runs.

Saliva Sample	N	Mean (ng/mL)	Standard Deviation (ng/mL)	Coefficient of Variation (%)
L	8	6.07	0.55	9.04
H	8	102.23	4.30	4.21

Recovery

Three saliva samples containing different levels of an endogenous Cotinine were spiked with known quantities of Cotinine and assayed.

Saliva Sample	Endogenous (ng/mL)	Added (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1	3.26	5	8.26	8.22	99.6
1	2.96	50	52.96	60.45	114.1
1	3.22	100	103.22	102.27	99.1
2	0.00	500	500.00	470.32	94.1
3	17.02	5	22.02	20.69	94.0
3	17.02	50	67.02	64.77	96.6



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Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 ng/mL level. The minimal concentration of Cotinine that can be distinguished from 0 is 0.15 ng/mL.

Sample Dilution Recovery

Two samples were serially diluted with Assay Diluent and assayed.

Saliva Sample	Dilution Factor	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1			120.27	
	1:2	60.14	56.87	94.6
	1:4	30.07	27.12	90.2
	1:8	15.03	14.00	93.1
	1:16	7.52	6.77	90.0
	1:32	3.76	3.50	93.2
	1:64	1.88	1.82	96.7
2			538.34	
	1:2	269.17	278.40	103.4
	1:4	134.59	139.68	103.8
	1:8	67.29	67.27	100.0
	1:16	33.65	33.34	99.1
	1:32	16.82	16.91	100.5
	1:64	8.41	9.24	109.8

Cross Reactivity

Nicotine	0.0293%		Nicotinamide	ND
Nicotinic acid	ND		3-OH-Cotinine*	24.82 %

*3-OH-Cotinine is a metabolite of Cotinine.



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Seller's Limited Warranty

"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties."

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