High Sensitivity

SALIVARY COTININE

QUANTITATIVE ENZYME IMMUNOASSAY KIT

For Research Use Only

Item No. 1-2002, (Single) 96-Well Kit;

1-2002-5, (5-Pack) 480 Wells

Updated: February 26, 2014

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HIGH SENSITIVITY SALIVARY COTININE QUANTITATIVE EIA KIT

Intended Use

The Salimetrics™ cotinine kit is a competitive immunoassay designed and validated for the quantitative measurement of cotinine in saliva samples. This kit may be used to measure primary or secondhand exposure to nicotine. This kit is not for use in diagnostic procedures. It is intended only for research use in humans and some animals. (See Item No. 1-2112 for Diagnostic Cotinine Kit.) A validated urine protocol is available on 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 false 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 tobacco use. The costs to individuals and societies associated with smoking (and secondhand exposure to tobacco smoke) have led to widespread public health interventions to curb smoking behavior.

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 cigarette (or alternative source), and health behaviors relevant to how cigarettes are smoked (e.g., vent blocking, duration and frequency of puffs). (2) There is a clear need for inexpensive, accurate, quantitative, and noninvasive means of validating smoking status, measuring the immediate physiological consequences of individual differences and intra-individual change in smoking behaviors, and determining secondhand tobacco smoke exposure. Salimetrics has designed this research tool to provide biomedical researchers 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

Standards and unknowns are added to a 96-well microtiter plate along with rabbit antibodies to cotinine and cotinine linked to horseradish peroxidase (conjugate). The cotinine in standards, unknowns, and the conjugate competes for the antibody binding sites. After incubation, unbound components are washed away. Bound conjugate is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with 2-molar sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of cotinine peroxidase detected is inversely proportional to the amount of cotinine present. (6)

Storage

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

pH Indicator

A pH indicator in the assay diluent alerts the user to samples with high or low pH values. Acidic samples will turn the diluent yellow. Alkaline samples will turn the diluent purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Cotinine values from samples with a pH ≤ 3.5 or ≥ 9.0 may be artificially inflated or lowered.

Safety Precautions

- Liquid stop is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
- See 'Material Safety Information' at the end of procedure.
- A safety data sheet is available on request.

Materials Needed But Not Supplied

- Precision pipette to deliver 20 µL, 50 µL, and 100 µL
- Precision multichannel pipette to deliver 50 µL, 100 µL, and 200 µL
- Vortex
- Microplate incubator/shaker with 0.08-0.17 inch orbit capable of 500-600 rpm
- Plate reader with a 450 nm filter
- Software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable tube to hold at least 15 mL
- Small disposable tubes
- Pipette tips
- 10 mL serological pipette

Materials Supplied with Single Kit

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

Specimen Collection

Avoid sample collection 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.

Donors may collect whole saliva by tilting the head forward, allowing the saliva to pool on the floor of the mouth, and then passing the saliva through the Saliva Collection Aid (SCA), Item No. 5016.02, into a polypropylene vial. Samples from adults and children over 6 may also be collected by using the SalivaBio Oral Swab (SOS), Item No. 5001.02. Samples from younger children may be collected with the SalivaBio Children's Swab (SCS), Item No. 5001.06, and the SalivaBio Infant's Swab (SIS), Item No. 5001.08, may be used for children up to the age of 6 months. Collection protocols are available on request or online at www.salimetrics.com.

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; however, cotinine in saliva has been reported in the literature to be stable at room temperature for up to 12 days. (7) If possible, refrigerate samples within 30 minutes, and freeze at or below -20°C within 4 hours after collection. (Samples may be stored at -20°C or lower for long term storage.)

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

Freezing saliva samples will precipitate mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding, leading to falsely elevated 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. Centrifuge/ re-centrifuge saliva samples each time that they are thawed. Avoid additional freeze-thaw cycles.

Prepare samples as follows:

- Known smokers: Dilute saliva samples x 10 (10 µL saliva into 90 µL assay diluent).
- Non-smokers: Run saliva sample straight.

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 5 (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 10-fold with room-temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized water***. Dilute only the amount needed for current day's use, and discard any leftover reagent.*** (If precipitate

has formed in the concentrated wash buffer, heat to 40°C for 15 minutes to dissolve crystals. Cool to room temperature before use in assay.)

- Prepare serial dilutions of the cotinine standard as follows:
  - Label five small tubes, such as microcentrifuge tubes, with numbers 2 through 6.
  - Pipette 100 µL of assay diluent in 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.

General Kit Use Advice

- This kit uses break-apart mictotitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the sealed foil pouch with desiccant and used in the frame provided.

- The quantity of reagent provided with this kit is sufficient for three individual runs. The volume of diluent and conjugate used 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.
- When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when

adding additional reagents so that incubation time with reagents is the same for all wells.

- 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 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 68-74ºF (20-23.3ºC). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month.
- Routine calibration of pipettes is critical for the best possible assay performance.

Procedure

Step 1: Determine your plate layout. Here is a suggested layout.

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

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

Step 2: Keep the desired number of wells in the strip holder and return any remaining strips to the foil pouch. Reseal the zip-lock foil pouch with unused wells and desiccant. Store at 2-8°C.

Step 3: Pipette 15 mL of assay diluent into a disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 5.

Step 4:

- Pipette 20 µL of standards, controls, and unknowns into appropriate wells. Standards, controls, and unknowns should be assayed in duplicate.
- Pipette 20 µL of assay diluent into 2 wells to serve as the zero.
- If using NSB wells, pipette 120 µL of assay diluent into those 2 wells.

Step 5: Dilute the enzyme conjugate 1:300 by adding 50 µL of the conjugate to the 15 mL of assay diluent prepared in Step 3. (Scale down proportionally if using less than 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 6: Pipette 100 µL of antiserum into all wells, except the nonspecific binding wells (if used), using a multichannel pipette.

Step 7: Cover the plate with a plate cover. Incubate the plate on a microplate incubator/shaker for 1.5 hours at 37ºC with constant mixing at 500-600 rpm.

Step 8: 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 flipping the liquid into 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 9: Add 200 µL of TMB solution to each well using a multichannel pipette.

Step 10: Mix at 500 rpm for 5 minutes (or tap to mix) and incubate in the dark for an additional 25 minutes at room temperature.

Step 11:

- Add 50 µL of stop solution using a multichannel pipette.
- Mix on a plate rotator at room temperature for 3 minutes at 500 rpm (or tap to mix). Be sure all wells have turned yellow. If green color remains, continue mixing until green color turns to 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. (Correction at 630 nm is desirable.)

Assay Summary

1. Bring all reagents to room temperature and mix before use.
2. Bring plate to room temperature and prepare for use.
3. Prepare serial dilutions of cotinine standard.
4. Prepare tube with 15 mL of assay diluent for conjugate dilution, which will be made later.
5. Prepare 1X wash buffer.
6. Pipette 20 µL of standards, controls, and unknowns into appropriate wells.
7. Pipette 20 µL of assay diluent into two wells to serve as the zero.
8. If using NSB wells, pipette 120 µL assay diluent into those two wells.
9. Make final 1:300 dilution of conjugate (50 µL into 15 mL assay diluent), mix, and immediately pipette 100 µL into each well.

10. Pipette 100 µL antiserum into all wells, except the NSB wells (if used).
11. Place adhesive cover over plate. Incubate plate on a microplate incubator/shaker for 1.5 hours at 37°C, with constant mixing at 500-600 rpm.
12. Wash plate 4 times with 1X wash buffer. Blot.
13. Add 200 µL TMB solution to each well.
14. Mix plate for 5 minutes at 500 rpm. Incubate in dark at room temperature for an additional 25 minutes.
15. Add 50 µL stop solution to each well. Mix for 3 minutes at 500 rpm.
16. Wipe plate bottom clean and read within 10 minutes of adding stop.

Calculations

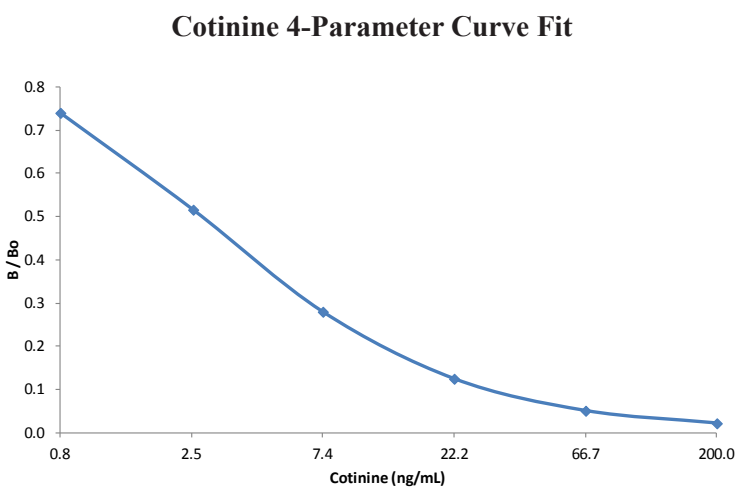
1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the average OD of the zero, standards, controls, and unknowns.
3. Calculate the percent bound (B/Bo) for each standard, control, and unknown by dividing the average OD (B) 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 unknowns by interpolation using software capable of logistics. We recommend a non-linear regression 4-parameter curve fit.
5. Samples with cotinine values greater than 200 pg/mL should be diluted with assay diluent and rerun for accurate results. If a dilution of the sample is used, multiply results by the dilution factor.

When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.

Typical Results

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

Well	Sample	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	NSB	0.008	NA	NA	NA



Quality Control

The Salimetrics' high and low salivary 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.

Material Safety Information*

Hazardous Ingredients

Liquid stop solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. MSDS sheets are available from Salimetrics upon request.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard 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, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

HS Salivary Cotinine Quantitative EIA Kit Performance Characteristics

Precision

The intra-assay precision was determined from 10 samples each of four levels of cotinine.

Sample	N	Mean (ng/mL)	Std Dev (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.

Sample	N	Mean (ng/mL)	Std Dev (ng/mL)	Coefficient of Variation (%)
Low	8	6.07	0.55	9.04
High	8	102.23	4.30	4.21

Recovery

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

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

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 zero is 0.15 ng/mL.

Sample Dilution Recovery

Two saliva samples were diluted with assay diluent and assayed.

Sample	Dilution Factor	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
Sample 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
Sample 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.

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

Group	N	Mean (ng/mL)	Std Dev (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.

Salimetrics quantitative enzyme immunoassay for salivary cotinine discriminates smokers from non-smokers, and differentiates primary from secondary smoke exposure. (8)

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**	**Std Dev**
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**	**Std Dev**
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 breastmilk 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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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."