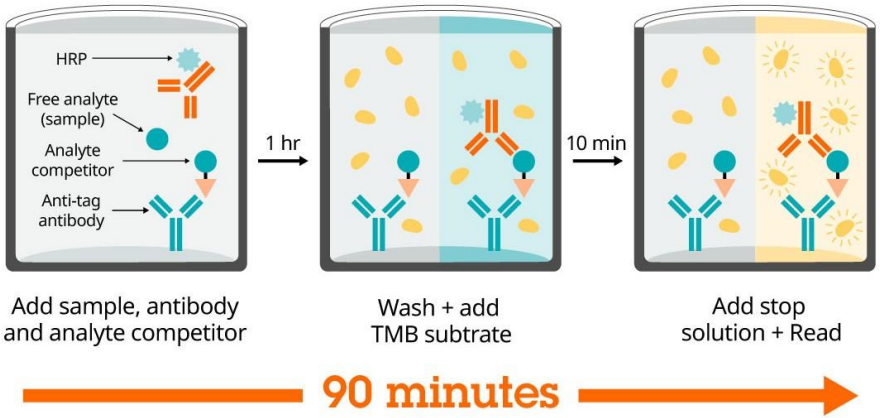
ab324665 – Cotinine SimpleStep ELISA® Kit – Extracellular

For the quantitative measurement of Cotinine in serum, plasma (citrate), plasma (EDTA), plasma (heparin), urine, saliva, and cell culture supernatant.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab324665



Storage and Stability: Store kit at 2-8°C immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

Materials Supplied

Item	Quantity 1 x 96 tests	Quantity 10 x 96 tests	Storage Condition
Cotinine Lyophilized CaptSure™ Conjugate	1 vial	10 x 1 vial	+4°C
Cotinine HRP Conjugate 50X	80 µL	10 x 80 µL	+4°C
Cotinine Lyophilized Standard	2 Vials	10 x 2 Vials	+4°C
Antibody Diluent 5BI	8 mL	10 x 8 mL	+4°C
Sample Diluent NS	12 mL	2 x 50 mL	+4°C
Wash Buffer PT 10X	20 mL	200 mL	+4°C
TMB Development Solution	12 mL	120 mL	+4°C
Stop Solution	12 mL	120 mL	+4°C
SimpleStep Pre-Coated 96-Well Microplate	96 wells	10 x 96 wells	+4°C
Plate Seal	1	10	+4°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.

Prepare only as much reagent as is needed on the day of the experiment. The CaptSure and HRP Conjugates have only been tested for stability in the provided 50X formulation.

1X Wash Buffer PT: Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

CaptSure Conjugate: To reconstitute the lyophilized CaptSure conjugate, centrifuge at 10,000 g for 2 minutes. Add 100 µL of Sample Diluent NS, let sit at room temperature for 10 minutes and resuspend well by inverting the tube by hand and gently pipetting. Unused reconstituted conjugate can be stored frozen at -20°C. Avoid repeated freeze-thaw cycles.

CaptSure Conjugate Solution: Prepare CaptSure Conjugate Solution by diluting the 50X CaptSure Conjugate in Antibody Diluent 5BI. To make 2 mL of the Capture Conjugate Solution combine 40 µL 50X CaptSure Conjugate with 1.96 mL Antibody Diluent 5BI. Mix thoroughly and gently.

HRP Conjugate Solution: Prepare HRP Conjugate Solution by diluting the 50X HRP Conjugate in Antibody Diluent 5BI. To make 2 mL of the HRP Conjugate Solution combine 40 µL 50X HRP Conjugate with 1.96 mL Antibody Diluent 5BI. Mix thoroughly and gently.

Standard Preparation

Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Reconstitute the Cotinine standard sample by adding the volume of Sample Diluent NS indicated on the standard vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 15,000 pg/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 288 µL of Sample Diluent NS into tube number 1 and 180 µL of Sample Diluent NS into numbers 2-8.
4. Use the **Stock Standard** to prepare the following dilution series. Standard #8 contains no standard and is the Blank control:

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	**Stock Standard**	72	288	15,000	3,000
2	Standard#1	180	180	3,000	1,500
3	Standard#2	180	180	1,500	750
4	Standard#3	180	180	750	375
5	Standard#4	180	180	375	187.5
6	Standard#5	180	180	187.5	93.8
7	Standard#6	180	180	93.8	46.9
8	Blank Control	0	180	N/A	N/A

Sample Preparation

Typical Sample Dynamic Range	
Sample Type	**Range**
Serum	3.13 – 25%
Human Plasma – Citrate	0.13 – 1%
Human Plasma – EDTA	0.13 – 1%
Human Plasma – Heparin	0.13 – 1%
Mouse/Rat Plasma – Citrate	3.13 – 25%
Mouse/Rat Plasma – EDTA	3.13 – 25%
Mouse/Rat Plasma – Heparin	3.13 – 25%
Urine	3.13 – 50%
Saliva	3.13 – 50%
Cell Culture Supernatant	3.13 – 50%

Serum Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:4 into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.
Plasma Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:100 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
Urine Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted urine samples at -20°C or below. Avoid repeated freeze-thaw cycles.
Saliva Centrifuge saliva at 800 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.
Cell Culture Supernatants Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

Assay Procedure

Equilibrate all materials and prepared reagents to room temperature prior to use.
We recommend that you assay all standards, controls and samples in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
3. Add 60 µL of all sample or standard to appropriate wells.
4. Add 30 µL of the CaptSure Conjugate Solution to each well.
    a. Optional – for non-specific binding wells, add 30 µL of Antibody Diluent 5BI in lieu of CaptSure Conjugate Solution.
5. Add 30 µL of the HRP Conjugate Solution to each well.
6. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
7. Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 30 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
8. Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
   Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
9. Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
10. Alternative to 8 – 9: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin

recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:
www.abcam.com/protocols/the-complete-elisa-guide

For technical support contact information, visit: www.abcam.com/contactus

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Additional information

ASSAY SPECIFICITY

This kit is designed for the quantification of Cotinine.

The standard in this kit is free Cotinine.

Cotinine signal was detected in pooled Human serum, plasma (heparin), plasma (EDTA), plasma (citrate), saliva and urine.

Spiked experiments were used to validate Human serum, saliva and urine from low concentration or non-smoking donors. Spiked experiments were also used to validate cell culture supernatant and Mouse and Rat serum, plasma (heparin), plasma (EDTA), and plasma (citrate).

Milk, CSF, cell extracts, and tissue extracts have not been tested with this kit.

For the measurement of Cotinine in cell extracts and tissue extracts, use Cotinine ELISA Kit - Intracellular ab324664.

CROSS REACTIVITY

Cross reactivity was determined for related compounds at 3,000 and 30,000 pg/mL. Cross reactivity is reported as interpolated concentration relative to Cotinine.

Compound	Cross Reactivity – 3,000 pg/mL (%)	Cross Reactivity – 30,000 pg/mL (%)
trans-3'Hydroxycotinine (3HC)	96	-
Nicotine	0	2

INTERFERENCE

3,000 and 30,000 pg/mL of Nicotine were tested for interference with 750 pg/mL of Cotinine. No interference was observed.

SPECIES REACTIVITY

Validated in Human, Mouse and Rat samples, reactivity is species independent.

CALCULATION

- Optional: Non-specific binding (NSB) well subtracted values can be calculated by averaging the absorbance values for the NSB wells and subtracting the average NSB absorbance value from all other absorbance values.
- Create a standard curve by plotting the average absorbance value for each standard concentration (y-axis) against the target concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
- Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- Determine the concentration of the target analyte in the sample by interpolating absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target analyte in the sample.
- Samples generating absorbance values lower than that of the highest concentration standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance value greater than that of the lowest concentration standard should be retested in a less dilute form.
- Optional: The binding percentage, B/B_0, can be calculated by dividing the average absorbance value for each standard or sample by the average absorbance of the zero standard (B_0).

TYPICAL DATA

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Standard Curve Measurements				
Concentration (pg/mL)	O.D. 450 nm		Mean O.D.	B/B_0 (%)
	1	2		
NSB	0.038	0.041	0.040	-
0 (B_0)	1.626	1.650	1.638	100%
46.88	1.397	1.427	1.412	86%
93.75	1.211	1.203	1.207	74%
187.5	0.822	0.848	0.835	51%
375	0.507	0.522	0.515	31%
750	0.284	0.277	0.280	17%
1,500	0.155	0.148	0.151	9%
3,000	0.087	0.088	0.088	5%

Table 1. Example of Cotinine standard curve in Sample Diluent NS. The Cotinine standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

TYPICAL SAMPLE VALUES

Sensitivity:

The calculated minimal detectable dose (MDD) is 22.8 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=24) and subtracting 2 standard deviations then extrapolating the corresponding concentration.

Recovery

Three concentrations of Cotinine were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
25% Human Serum	108	104 - 114
1:200 Human Plasma – Citrate	109	104 - 118
1:200 Human Plasma – EDTA	104	98 - 107
1:200 Human Plasma – Heparin	108	104 - 114
25% Mouse Serum	98	97 - 99
25% Mouse Plasma – Citrate	99	99 - 100
25% Mouse Plasma – EDTA	104	102 - 107
25% Mouse Plasma - Heparin	104	103 - 106
25% Rat Serum	101	96 - 110
25% Rat Plasma – Citrate	101	98 - 107
25% Rat Plasma – EDTA	104	95 - 115
25% Rat Plasma - Heparin	106	99 - 111
50% Human Saliva	95	88 - 100
50% Human Urine	87	84 - 90
50% Cell Culture Media*	109	101 - 117

*Media is DMEM containing 10% fetal calf serum.

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Cotinine was measured in the following biological samples in a 2-fold dilution series. Linearity assessment was performed on pooled Human samples with variable Cotinine concentrations. Linearity may be attainable at a higher percentage for samples with low cotinine levels. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	1% Human Serum	1% Human Plasma (Citrate)	1% Human Plasma (EDTA)	1% Human Plasma (Heparin)	1% Human Saliva	1:400 Human Urine
Undiluted	pg/mL	2,001	1,263	1,758	520	1,401	1,775
	% Expected value	100	100	100	100	100	100
2	pg/mL	991	647	928	253	744	901
	% Expected value	99	102	106	97	106	101
4	pg/mL	471	313	457	119	364	455
	% Expected value	94	99	104	92	104	103
8	pg/mL	210	156	230	73	181	232
	% Expected value	84	99	105	113	103	105

Free Cotinine was spiked in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	25% Human Serum*	50% Human Saliva*	50% Human Urine*	50% Cell Culture Media
Undiluted	pg/mL	919	1,990	2,096	1,482
	% Expected value	100	100	100	100
2	pg/mL	393	1,044	973	787
	% Expected value	85	105	93	106
4	pg/mL	186	522	559	388
	% Expected value	81	105	107	105
8	pg/mL	107	241	240	192
	% Expected value	93	97	92	104
16	pg/mL	53	107	118	100
	% Expected value	92	86	90	108

*Human serum samples are pooled from individual donors with low cotinine levels. Saliva and Urine samples are from non-smoking donors.

Dilution Factor	Interpolated value	25% Mouse Serum	25% Mouse Plasma (Citrate)	25% Mouse Plasma (EDTA)	25% Mouse Plasma (Heparin)
Undiluted	pg/mL	829	798	736	842
	% Expected value	100	100	100	100
2	pg/mL	370	368	344	424
	% Expected value	89	92	94	101
4	pg/mL	183	161	154	179
	% Expected value	88	81	84	85
8	pg/mL	84	90	79	89
	% Expected value	81	90	86	85

Dilution Factor	Interpolated value	25% Rat Serum	25% Rat Plasma (Citrate)	25% Rat Plasma (EDTA)	25% Rat Plasma (Heparin)
Undiluted	pg/mL	930	913	911	923
	% Expected value	100	100	100	100
2	pg/mL	396	399	383	458
	% Expected value	85	87	84	99
4	pg/mL	201	196	194	198
	% Expected value	87	86	85	86
8	pg/mL	104	96	100	113
	% Expected value	90	84	88	98

Precision

Mean coefficient of variations of interpolated values of Cotinine from one concentration of human serum and human urine within the working range of the assay.

	Intra-assay	Inter-assay
N=	8	3
CV (%)	2.8	6.7

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:
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