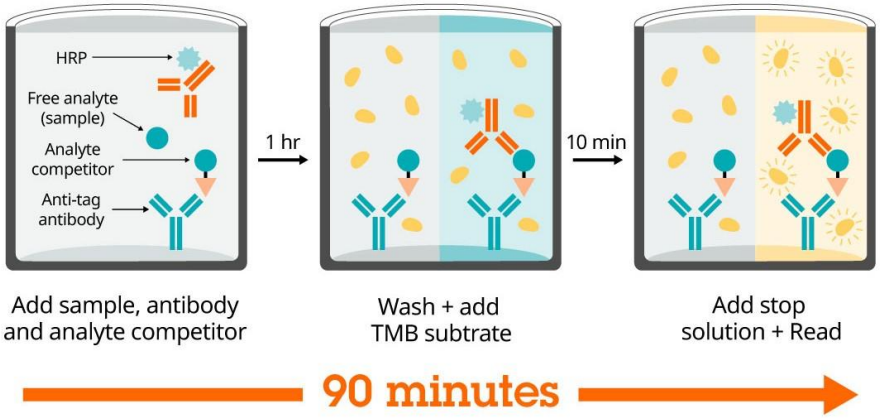
ab324664 – Cotinine SimpleStep ELISA® Kit – Intracellular

For the quantitative measurement of Cotinine in cell and tissue extracts.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab324664



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
Cell Extraction Buffer PTR 5X	10 mL	2 x 50 mL	+4°C
Sample Diluent NS	12 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

Sample Diluent NS is provided but not necessary for this product.

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.
- Method for determining total protein concentration in sample (BCA assay recommended).
- 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 lyophilized and 50X formulation.

1X Cell Extraction Buffer PTR (For cell and tissue extracts only): Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 8 mL deionized water and 2 mL Cell Extraction Buffer PTR 5X. Mix thoroughly and gently. If required protease inhibitors can be added.

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 1X Cell Extraction Buffer PTR, let sit at room temperature for 10 minutes and resuspend well by inverting the tube by hand and gently pipetting. Unused conjugate can be stored frozen at -20°C. Avoid repeated freeze-thaw cycles.

CaptSure Conjugate Solution: Prepare CaptSure Conjugate Solution by diluting the reconstituted CaptSure Conjugate in Antibody Diluent 5BI. To make 2 mL of the Capture Conjugate Solution combine 40 µL reconstituted 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 1X Cell Extraction Buffer PTR 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 1X Cell Extraction Buffer into tube number 1 and 180 µL of 1X Cell Extraction Buffer 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**
A549 Cell Extract	62.5 - 500 µg/mL
HeLa Cell Extract	62.5 - 500 µg/mL
Jurkat Cell Extract	62.5 - 500 µg/mL
Human Lung Tissue Extract	62.5 - 500 µg/mL

Preparation of extracts from cell pellets Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C. Rinse cells twice with PBS. Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer PTR. Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Preparation of extracts from adherent cells by direct lysis (alternative protocol) Remove growth media and rinse adherent cells 2 times in PBS. Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR per confluent 15 cm diameter plate). Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Preparation of extracts from tissue homogenates Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (Dounce homogenizer recommended). Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly. Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

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.

Spiked experiments were used to validate cell and Human lung extracts.

Serum, plasma (heparin), plasma (EDTA), plasma (citrate), cell culture supernatant, saliva, urine, milk, and CSF samples have not been tested with this kit.

For the measurement of Cotinine in serum, plasma, cell culture supernatant, saliva, and urine, use Cotinine ELISA Kit - Extracellular ab324665.

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	0.037	0.041	0.039	-
0 (B_0)	1.411	1.420	1.416	100%
46.88	1.358	1.314	1.336	94%
93.75	1.281	1.190	1.235	87%
187.5	1.086	1.039	1.062	75%
375	0.863	0.828	0.845	60%
750	0.523	0.505	0.514	36%
1,500	0.284	0.271	0.278	20%
3,000	0.151	0.144	0.148	10%

Table 1. Example of Cotinine standard curve in 1X Cell Extraction Buffer PTR. 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 18.8 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

3 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 (%)
500 µg/mL A549 Cell Extract	98	95 – 101
500 µg/mL HeLa Cell Extract	98	92 – 104
500 µg/mL Jurkat Cell Extract	102	101 – 106
500 µg/mL Human Lung Tissue Extract	100	95 – 106

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.

Free Cotinine was spiked in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTR

Dilution Factor	Interpolated value	500 µg/mL A549 Cell Extract	500 µg/mL HeLa Cell Extract	500 µg/mL Jurkat Cell Extract	500 µg/mL Human Lung Extract
Undiluted	pg/mL	1,556	1,550	1,577	1,480
	% Expected value	100	100	100	100
2	pg/mL	754	757	780	742
	% Expected value	97	98	99	100
4	pg/mL	355	374	372	361
	% Expected value	91	96	94	98
8	pg/mL	184	181	182	174
	% Expected value	95	93	92	94

Precision

Mean coefficient of variations of interpolated values of Cotinine from Human Lung Extract spiked with two concentrations of free Cotinine within the working range of the assay.

	Intra-assay	Inter-assay
N=	8	3
CV (%)	2.8	4.5

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