THC Metabolite ELISA Kit

Item No. 701570

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TABLE OF CONTENTS

GENERAL INFORMATION
3 Materials Supplied
4 Safety Data
4 Precautions
4 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION
6 Background
7 About This Assay
8 Principle of Assay
10 Definition of Key Terms

PRE-ASSAY PREPARATION
12 Buffer Preparation
13 Testing for Interference
14 Sample Preparation
15 Sample Matrix Properties

ASSAY PROTOCOL
18 Preparation of Assay-Specific Reagents
20 Plate Set Up
22 Performing the Assay

ANALYSIS
24 Calculations
26 Performance Characteristics

RESOURCES
30 Assay Summary
31 Plate Template
32 Troubleshooting
33 References
34 Notes
35 Warranty and Limitation of Remedy

GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
701571	THC Metabolite Antibody	1 vial/100 dtn
701572	THC Metabolite-AP Tracer	1 vial/100 dtn
701573	THC Metabolite ELISA Standard	1 vial
400080	Tris Buffer Concentrate (10X)	1 vials/10 ml
411007	AP Wash Buffer Concentrate (150X)	1 vial/5 ml
400009/400008	Goat Anti-Mouse IgG-Coated Plate	1 plate
400012	96-Well Cover Sheet	1 cover
400089	*p*NPP Substrate Solution	2 vials/12 ml
400040	ELISA Tracer Dye	1 vial
400042	ELISA Antiserum Dye	1 vial

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's THC Metabolite ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 405 nm
2. Adjustable pipettes and a repeating pipettor
3. An orbital microplate shaker
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Materials used for **Sample Preparation** (see page 14)

INTRODUCTION

Background

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive chemical in *Cannabis*.[1] Upon consumption, this cannabinoid (CB) acts as a partial agonist at both CB receptor 1 (CB_1) and CB_2.[2,3] Its actions are analogous to the endogenous neurotransmitter anandamide when it binds to receptors and alters mental and physical functions in the body.[4] Once in the liver, Δ^9-THC is metabolized by the cytochrome P450 (CYP) isoforms CYP2C9, CYP2C19, and CYP3A4, to the psychoactive metabolite 11-hydroxy-Δ^9-THC.[5,6] 11-hydroxy-Δ^9-THC is then further oxidized to the non-psychoactive metabolite 11-nor-9-carboxy-Δ^9-THC. This carboxy form is the most abundant metabolite of Δ^9-THC. For this reason, it is often used as the main urinary marker for *Cannabis* consumption.[6]

About This Assay

Cayman's THC Metabolite ELISA Kit is a competitive assay that can be used for quantification of the predominant THC metabolite, 11-nor-9-carboxy-Δ^9-THC, in plasma, serum, and urine. The assay has a range from 0.017-5 ng/ml with a midpoint of approximately 0.295 ng/ml (50% B/B_0) and a sensitivity of approximately 0.072 ng/ml (80% B/B_0).

Principle of the Assay

This assay is based on the competition between free THC metabolite (11-nor-9-carboxy-Δ^9-THC) and a THC metabolite-alkaline phosphatase conjugate (THC Metabolite-AP Tracer) for a limited amount of THC Metabolite Monoclonal Antibody. Because the concentration of the THC Metabolite AP-Tracer is held constant while the concentration of free THC metabolite varies, the amount of THC Metabolite-AP Tracer that is able to bind to the THC Metabolite Monoclonal Antibody will be inversely proportional to the concentration of free THC metabolite in the well. This antibody-THC metabolite complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then *p*NPP Substrate Solution (which contains the substrate to AP) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of THC Metabolite-AP Tracer bound to the well, which is inversely proportional to the amount of free THC metabolite present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{Bound THC Metabolite-AP Tracer}] \propto 1/[\text{THC metabolite}]$$

A schematic of this process is shown in Figure 1, on page 9.

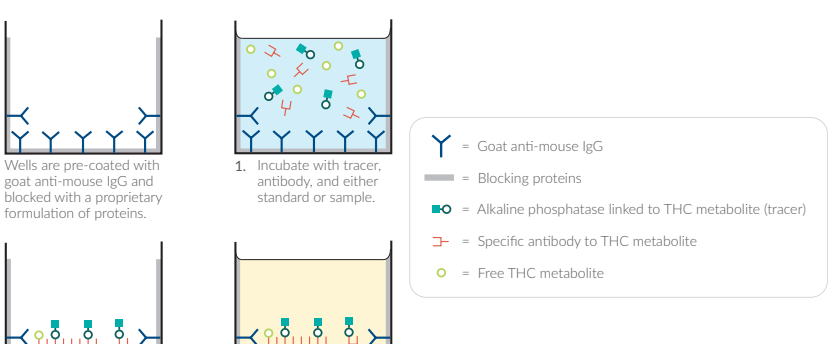


Figure 1. Schematic of the THC Metabolite ELISA

Definition of Key Terms

Blank (Blk): background absorbance caused by *p*NPP Substrate Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

Total Activity (TA): total enzymatic activity of the THC metabolite AP-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a sample or standard well to the average absorbance of the maximum binding (B_0) wells.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months.

1. **Tris Assay Buffer (1X) Preparation**

   Dilute the contents of one vial of Tris Buffer Concentrate (10X) (Item No. 400080) with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water.*

2. **AP Wash Buffer (1X) Preparation**

   Dilute 5 ml of AP Wash Buffer Concentrate (150X) (Item No. 411007) to a total volume of 750 ml with ultrapure water. Smaller volumes of AP Wash Buffer (1X) can be prepared by diluting the AP Wash Buffer Concentrate 1:150 in ultrapure water.

Testing for Interference

This assay has been validated in human plasma, serum, and urine. Other samples types should be tested for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 1.3 ng/ml and 0.070 ng/ml (*i.e.*, between 20-80% B/B_0, which is the linear portion of the standard curve). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated THC metabolite concentration, sample purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the end user and tested for compatibility in the assay.

Sample Preparation

Plasma and Serum

Plasma and serum purification is advised for this kit. Sample purification should be performed using a method similar to the following protocol.

1. To 0.4 ml sample, add 1 ml of methanol. Mix and incubate at room temperature for 5 minutes.
2. Centrifuge at 5,000 x g for 5 minutes.
3. Carefully transfer supernatant to a clean tube and repeat methanol purification on the sample twice more.
4. Evaporate supernatant to dryness under inert gas.
5. Reconstitute with 0.4 ml (original volume of sample) of the assay buffer and assay immediately. *NOTE: If samples cannot be assayed immediately, store at -80°C.*

Urine

Urine samples should be diluted into Tris Assay Buffer (1X) prior to testing in the assay.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.

Sample Matrix Properties

Linearity

Human plasma, serum, and urine were spiked with 11-nor-9-carboxy-Δ^9-THC and prepared as described in the Sample Preparation section. Samples were serially diluted with Tris Assay Buffer (1X), and evaluated for linearity using the THC Metabolite ELISA Kit. The results are shown in the table below.

Dilution	Concentration (ng/ml)	Dilutional Linearity (%)
Plasma		
8	4.95	100
16	4.81	97.2
32	4.52	91.3
64	4.65	93.9
Serum		
8	5.77	100
16	6.09	105.5
32	5.91	102.4
64	5.84	101.2
Urine		
10	6.98	100
20	6.58	94.3
40	6.24	89.4
80	5.65	80.9

Table 1. Dilutional linearity of human plasma, serum, and urine

Spike and Recovery

Human plasma, serum and urine samples were spiked with different amounts of 11-nor-9-carboxy-Δ^9-THC, purified as described in the Sample Preparation section (see page 14), then serially diluted and evaluated using the THC Metabolite ELISA kit.

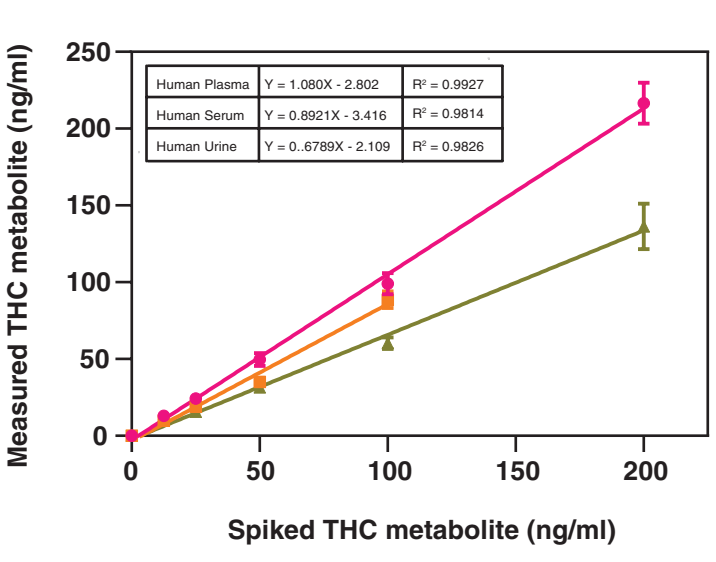


Figure 2. Spike and recovery in human plasma, serum, and urine

Parallelism

To assess parallelism, THC-positive human plasma, serum, and urine samples were purified and assayed at multiple dilutions with the THC Metabolite ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below.

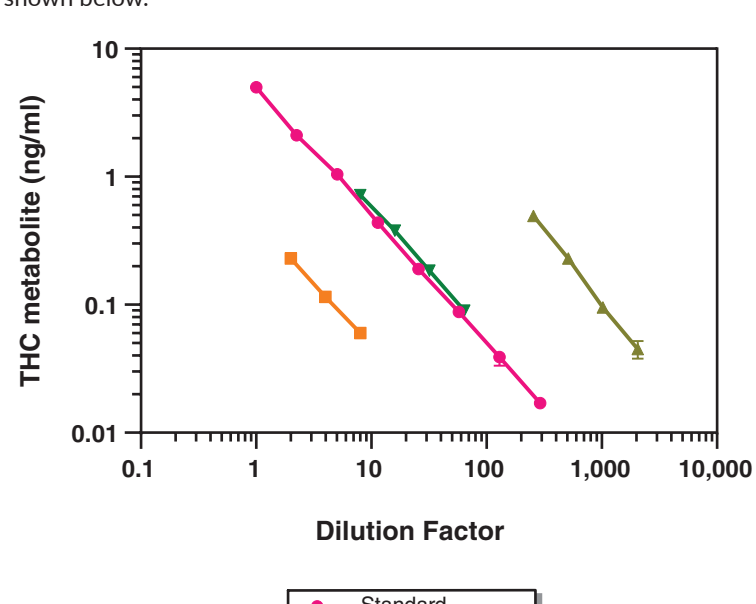


Figure 3. Parallelism of plasma, serum, and urine in the THC Metabolite ELISA Kit

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

THC Metabolite ELISA Standard

Equilibrate a pipette tip by repeatedly filling and expelling the tip with THC Metabolite ELISA Standard (Item No. 701573) several times. Using the equilibrated pipette tip, transfer 100 µl of the standard into a clean test tube, then dilute with 900 µl ultrapure water. The concentration of this solution (the bulk standard) will be 50 ng/ml. *NOTE: Store this solution at 4°C. It will be stable for at least one week.*

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1 through #8. Aliquot 900 µl Tris Assay Buffer (1X) to tube #1 and 500 µl Tris Assay Buffer (1X) to tubes #2-8. Transfer 100 µl of the bulk standard (50 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than two hours.

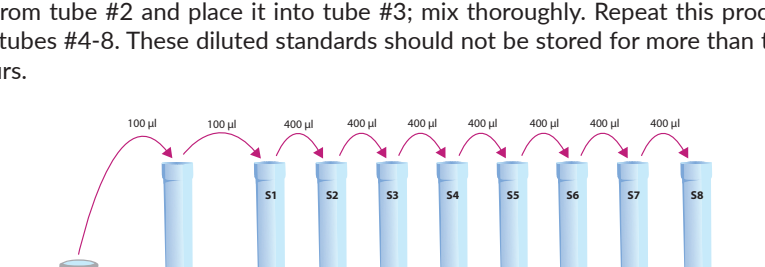


Figure 4. Preparation of the THC metabolite standards

THC Metabolite-AP Tracer

Reconstitute THC Metabolite-AP Tracer (Item No. 701572) with 6 ml of Tris Assay Buffer (1X). Store the reconstituted THC Metabolite-AP Tracer at 4°C (*do not freeze!*) and use within six week(s). A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer). *NOTE: Do not store tracer with dye for more than 24 hours.*

THC Metabolite Antibody

Reconstitute the THC Metabolite Antibody (Item No. 701571) with 6 ml of Tris Assay Buffer (1X). Store the reconstituted THC Metabolite Antibody at 4°C (*do not freeze!*) and use within six week(s). A 20% surplus of antibody has been included to account for any incidental losses.

Antibody Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). *NOTE: Do not store antibody with dye for more than 24 hours.*

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two blank (Blk), two NSB, and three maximum binding wells (B_0), and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results. Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.*

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 24 for more details). We suggest you record the contents of each well on the template sheet provided (see page 31).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 5. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **Tris Assay Buffer (1X)**

   Add 100 µl Tris Assay Buffer (1X) to NSB wells. Add 50 µl Tris Assay Buffer (1X) to B_0 wells.

2. **THC Metabolite ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **THC Metabolite-AP Tracer**

   Add 50 µl to each well *except* the TA and Blk wells.

5. **THC Metabolite Antibody**

   Add 50 µl to each well *except* the TA, NSB, and Blk wells within 15 minutes of the addition of the tracer.

Incubation of the Plate

Cover each plate with a 96-Well Plate Cover (Item No. 400012) and incubate 2 hours at room temperature with shaking.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
2. Add 200 µl of *p*NPP Substrate Solution (Item No. 400089) to each well.
3. Add 5 µl of the reconstituted tracer to the TA wells.
4. Cover the plate with the 96-Well Plate Cover. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.6 A.U. (blank subtracted)) in 60 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep *p*NPP Substrate Solution from splashing on the cover. *NOTE: Any loss of pNPP Substrate Solution will affect the absorbance readings.*
3. Read the plate at a wavelength of 405 nm.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively, a spreadsheet program can be used. The data should be plotted as either %B/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysistools/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA vaues are not used in the standard curve calculations. Rather, they are used as a diagnostic tool. Erratic absorbance values could indicate the presence of organic solvents in the buffer or other technical problems (see page 32 for Troubleshooting). Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* THC metabolite concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample by identifying the %B/B_0 on the standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any dilution of the original sample prior to its addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference, which could be eliminated by purification.

NOTE: If there is an error in the B_0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off standard curve. Only the linear part of this standard curve should be used in the calculations.

Performance Characteristics

Representative Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the value of your samples.

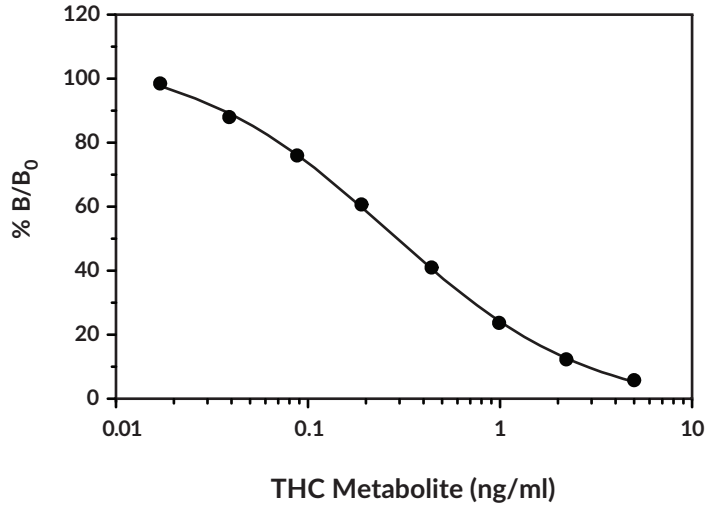
Absorbance at 405 nm (60 minutes)

THC Metabolite Standards (ng/ml)	Blank-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0	%CV* Intra-assay Precision	%CV* Inter-assay Precision
NSB	0.003	--	--	--	--
B_0	0.734	0.731	--	--	--
TA	0.802	0.799	--	--	--
5.0	0.046	0.043	5.8	8.6	6.6
2.2	0.094	0.091	12.4	6.1	4.5
0.98	0.177	0.174	23.7	5.3	5.6
0.44	0.303	0.300	41.0	8.0	4.4
0.19	0.447	0.444	60.7	9.8	7.3
0.087	0.560	0.557	76.1	11.7	7.7
0.039	0.647	0.644	88.1	17.9	24.2**
0.017	0.724	0.721	98.5	43.3**	41.8**

Table 2. Typical results

**%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve*

***Evaluate data in this range with caution*



Assay Range = 0.017-5.0 ng/ml
Sensitivity (defined as 80% B/B_0) = 0.072 ng/ml
Mid-point (defined as 50% B/B_0) = 0.30 ng/ml
Lower Limit of Detection (LLOD) = 0.037 ng/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with Tris Assay Buffer (1X).

Figure 6. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (human urine) in a single assay.

Matrix Control (ng/ml)	%CV
0.719	6.5
0.193	9.4
0.088	10.0

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing eight replicates of three matrix controls (human urine) in separate assays on different days.

Matrix Control (ng/ml)	%CV
0.753	10.3
0.224	12.0
0.098	15.4

Table 4. Inter-assay precision

Cross Reactivity:

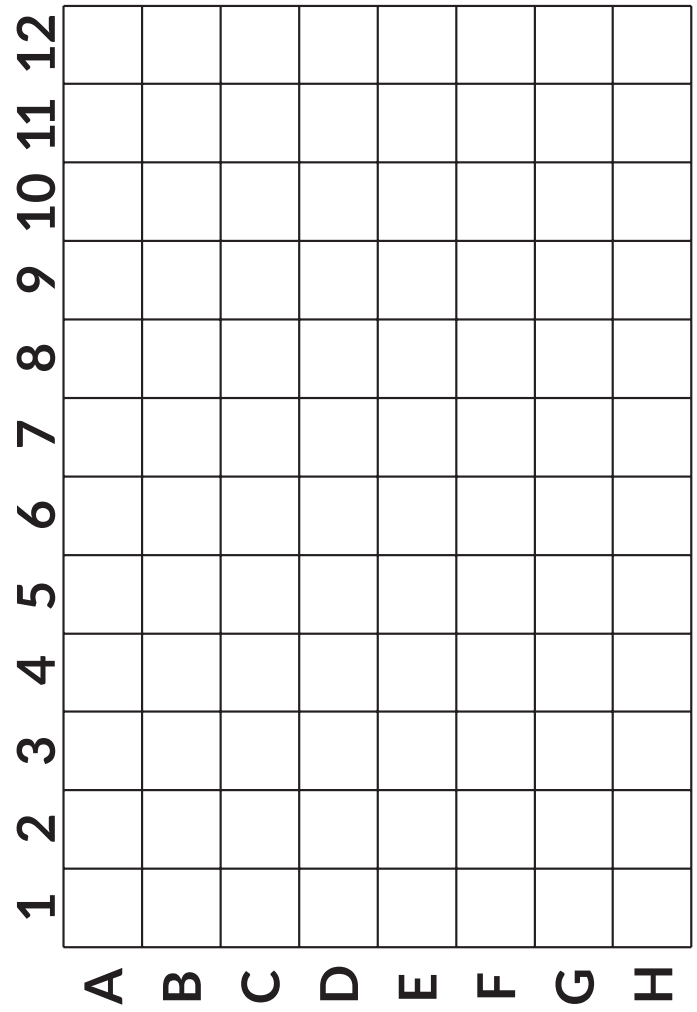
Compound	Cross Reactivity
(−)-11-nor-9-carboxy-Δ^8-THC	100%
(−)-11-nor-9-carboxy-Δ^9-THC	100%
(±)-11-nor-hydroxy-Δ^9-THC	28.3%
Δ^8-THC	18.04%
(±)-11-hydroxy-Δ^9-THC	14.67%
Δ^9-THC	12.34%
(+)-11-nor-9-carboxy-Δ^9-THC Glucuronide	0.35%
Cannabinol	0.09%

Table 5. Cross reactivity of the THC Metabolite ELISA

RESOURCES

THC Metabolite Assay Summary					
Procedure	**Blk**	**TA**	**NSB**	**B_0**	**Standards/ Samples**
Reconstitute and mix	Mix all reagents gently				
Tris Assay Buffer (1X)	-	-	100 µl	50 µl	-
Standards/Samples	-	-	-	-	50 µl
THC Metabolite-AP Tracer	-	-	50 µl	50 µl	50 µl
THC Metabolite Antibody	-	-	-	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate for 2 hours at room temperature on orbital shaker				
Aspirate	Aspirate wells and wash 5 x ~300 µl with AP Wash Buffer (1X)				
*p*NPP Substrate	200 µl	200 µl	200 µl	200 µl	200 µl
TA - Apply Tracer	-	5 µl	-	-	-
Seal	Seal plate and incubate for 60 minutes at room temperature on orbital shaker, protected from light				
Read	Read absorbance at 405 nm				

Table 6. Assay Summary



Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>0.100 O.D.)	A. Poor washing; ensure proper washing is used B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose-response curve)	Standard is degraded or contaminated
Analysis of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present; consider sample purification prior to analysis.

References

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NOTES

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