Acetylcholine/Choline Fluorometric/Colorimetric Assay Kit

Item No. 702530

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400794	Assay Buffer A (5X)	1 vial/10 ml	4°C
400795	Choline Standard	1 vial/100 µl	-20°C
401075	Acetylcholine Standard	1 vial/100 µl	-20°C
400796	Acetylcholine Converter Enzyme	1 vial	-20°C
400797	Choline Detection Enzyme	1 vial	-20°C
400798	HRP Assay Reagent	1 vial	-20°C
400610	MaxiProbe	2 vials/250 µl	-20°C
400777	96-Well Black Wall, Clear Bottom, Assay Plate	1 plate	RT
400012	96-Well Cover Sheet	1 ea	RT

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 Acetylcholine/Choline Fluorometric/Colorimetric Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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 in the **Materials Supplied** section (see page 3) 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 fluorescence with excitation and emission wavelengths of 530 nm (530-540 nm) and 590 nm (580-600 nm), respectively, or a plate reader capable of measuring absorbance at 570 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of pure water; glass-distilled water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 11)
5. A 37°C incubator

INTRODUCTION

Background

Choline is an essential micronutrient and a precursor of the neurotransmitter acetylcholine and the methyl donor betaine, which has roles in epigenetic regulation.[1,2] Alongside its pleiotropic role in cellular homeostasis, choline influences many cellular processes, including lipid metabolism, membrane fluidity, mitochondrial bioenergetics, and epigenetic regulation of gene expression. It is primarily metabolized in the liver and dysregulation of choline-related genes and choline-restricted diets are associated with the development of non-alcoholic fatty liver disease (NASH).[1] Levels of choline in cerebrospinal fluid (CSF) and putamen are decreased in patients with Huntington's disease.[3]

Acetylcholine is a neurotransmitter that binds to nicotinic and muscarinic acetylcholine receptors (AChRs) in the central and peripheral nervous systems.[4] It mediates motor function at the neuromuscular junction and functions in the parasympathetic and sympathetic nervous systems. The actions of acetylcholine are terminated primarily via the action of acetylcholinesterase (AChE), which breaks it down into acetate and choline. Acetylcholine has roles in multiple cognitive and behavioral processes, including learning and memory, reward, appetite, mood regulation, and motivation.[4,5] Dysregulation of acetylcholine homeostasis is associated with various diseases, such as multiple sclerosis, Alzheimer's disease, and myasthenia gravis.[6-7]

About This Assay

Cayman's Acetylcholine/Choline Fluorometric/Colorimetric Assay Kit provides methods for measuring acetylcholine, choline, and acetylcholine + choline (total) levels in biological samples, such as tissue homogenates, cell culture medium, serum, and plasma. In the assay, the Acetylcholine Converter Enzyme catalyzes the conversion of acetylcholine to choline, which is further converted to betaine aldehyde and hydrogen peroxide (H_2O_2) by the Choline Detection Enzyme. In the presence of horseradish peroxidase (HRP), H_2O_2 reacts stoichiometrically with MaxiProbe to produce a fluorescent compound (Figure 1). Background from H_2O_2 in the sample is determined by omitting the converter and detection enzymes in sample background reactions. To determine choline levels alone, the Acetylcholine Converter Enzyme is excluded from the reaction mixture. The concentration of acetylcholine can be calculated by subtracting choline levels from the total measurement.

This assay offers the option to quantify by either fluorescence or absorbance. For fluorescence detection, measurements can be taken using excitation and emission wavelengths of 530 and 590 nm, respectively. Alternatively, the absorbance can be measured at 570 nm. It is at the user's discretion to choose the mode of measurement (and corresponding standard curve preparation) that best fits their needs. When read fluorometrically, this assay has a range of 0-10 µM and a lower limit of detection (LLOD) of 0.005 µM and 0.04 µM for the choline and total acetylcholine+choline assays, respectively. When read colorimetrically, the range is 0-100 µM with an LLOD of 0.04 µM and 0.07 µM for the choline and total acetylcholine+choline assays, respectively.

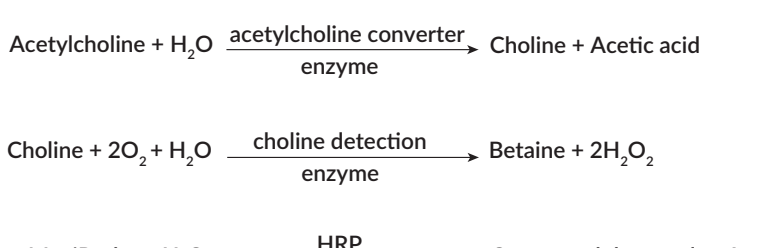


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer A (5X) - (Item No. 400794)**

   This vial contains 10 ml of Assay Buffer A (5X). Thaw at room temperature and dilute the contents with 40 ml of pure water. Assay Buffer A (1X) will be stable for two months when stored at 4°C.

   NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with pure water.

2. **Acetylcholine Standard - (Item No. 401075)**

   This vial contains 100 µl of 10 mM acetylcholine in Assay Buffer A (1X). If not using all at once, prepare aliquots and store at -20°C. Limit freeze-thaw cycles to three.

   Prior to each assay, dilute 10 µl of the 10 mM Acetylcholine Standard with 990 µl of Assay Buffer A (1X) to yield a 100 µM bulk acetylcholine standard. The bulk acetylcholine standard will be stable for two hours at room temperature.

3. **Choline Standard - (Item No. 400795)**

   This vial contains 100 µl of 10 mM choline in Assay Buffer A (1X). If not using all at once, prepare aliquots and store at -20°C. Limit freeze-thaw cycles to three.

   Prior to each assay, dilute 10 µl of the 10 mM Choline Standard with 990 µl of Assay Buffer A (1X) to yield a 100 µM bulk choline standard. The bulk choline standard will be stable for two hours at room temperature.

NOTE: Use the bulk standards in the preparation of the standard curves as shown on pages 20 and 21.

4. **Acetylcholine Converter Enzyme - (Item No. 400796)**

   This vial contains lyophilized Acetylcholine Converter Enzyme. Reconstitute the contents of the vial with 300 µl of pure water and place on ice. *Do not vortex*. If not using all at once, prepare aliquots and store at -20°C, where it will be stable for two months. Limit freeze-thaw cycles to three.

5. **Choline Detection Enzyme - (Item No. 400797)**

   This vial contains lyophilized Choline Detection Enzyme. Reconstitute the contents of the vial with 300 µl of pure water and place on ice. *Do not vortex*. If not using all at once, prepare aliquots and store at -20°C, where it will be stable for two months. Limit freeze-thaw cycles to three.

6. **HRP Assay Reagent - (Item No. 400798)**

   This vial contains lyophilized HRP. Reconstitute the contents of the vial with 450 µl of pure water and place on ice. *Do not vortex*. If not using all at once, prepare aliquots and store at -20°C, where it will be stable for at least two months when stored at -20°C. Limit freeze-thaw cycles to three.

7. **MaxiProbe - (Item No. 400610)**

   Each vial contains 250 µl of MaxiProbe in DMSO. It is ready to use as supplied. The unopened vial should be thawed at room temperature protected from light. If not using all at once, prepare aliquots and store at -20°C. Limit freeze-thaw cycles to three.

Sample Preparation

Samples should be processed and assayed immediately after collection; samples that cannot be assayed immediately should be flash frozen and stored at -80°C. To fall within the range of the standard curve, it may be necessary to dilute samples with Assay Buffer A (1X) prior to the assay. See below for dilution recommendations.

Tissue Homogenate

1. Collect tissue and mince into small pieces.
2. Prepare a homogenization buffer by combining 2 ml of Assay Buffer A (1X) with 2 ml of pure water and 0.5 ml of 0.6 M HCl. This is a sufficient volume to process 100 mg of tissue. The addition of protease inhibitors is highly recommended (see **Interferences**, on page 30).
3. Per 20 mg of minced tissue, add 900 µl of ice-cold homogenization buffer and homogenize using a tissue homogenizer/tissue tearor/Dounce homogenizer or similar instrument for 20-30 seconds.
4. Neutralize with 100 µl of 0.6 M Tris, pH 8.5, then centrifuge at 10,000 x g for 10 minutes at 4°C.
5. Transfer the supernatant to a new tube and keep on ice.
6. The amount of acetylcholine and choline varies between tissue types. It is recommended that samples be assayed at several dilutions for measured values to fall within the range of the standard curve.
7. Normalize the values obtained from tissue samples to protein concentration using Cayman's Protein Determination (BCA) Kit (Item No. 701780), Protein Determination Kit (Item No. 704002), or a similar protein quantification assay. Alternatively, values can be normalized to the initial mass (mg) of tissue used.

Cell Lysate

1. Pellet cells (5-8 x 10^6 cells) by centrifugation (*i.e.* 500-1,000 x g for 10 minutes at 4°C). For adherent cells, harvest using a cell scraper instead of proteolytic enzymes.
2. Prepare a lysis buffer by combining 8 ml of pure water with 8 ml of Assay Buffer A (1X) and 2 ml of 0.6 M HCl. This is a sufficient volume to process 21 samples.
3. Add 500 µl of ice-cold lysis buffer and 100-200 mg of glass beads (500-750 µm) to the cell pellet.
4. Vortex for 30 seconds and leave on ice for 30 seconds. Repeat this step four times.
5. Neutralize by adding 100 µl of 0.6 M Tris-HCl, pH 8.5, and centrifuge at 10,000 x g for 10 minutes at 4°C.
6. Transfer the supernatant to a new tube and keep on ice.
7. The amount of acetylcholine and choline varies between cell types. It is recommended that the fluorometric format be used and that samples be assayed at several dilutions for measured values to fall within the range of the standard curve.
8. Normalize the values obtained from cell lysates to protein concentration using Cayman's Protein Determination (BCA) Kit (Item No. 701780), Protein Determination Kit (Item No. 704002), or a comparable protein quantification assay. Alternatively, results can be normalized to the number of cells used during lysis.

Cell Culture Supernatant

1. Centrifuge cells at 1,200 x g for 10 minutes at 4°C and collect the supernatant.
2. Dilute the supernatant with Assay Buffer A (1X). Dilutions starting from 1:10 are recommended for the fluorometric and colorimetric formats.

NOTE: Typically, phenol red does not interfere with the assay if samples are diluted as recommended.

Cerebrospinal Fluid (CSF)

Typically, it is not necessary to dilute CSF prior to running the assay. It is recommended that the fluorometric format be used.

Plasma

1. Collect blood in vacutainers containing heparin or EDTA.
2. Centrifuge at 1,000 x g for 15 minutes at 4°C.
3. Transfer the top plasma layer into a clean test tube without disturbing the white buffy layer.
4. Deproteinate through a 10 kDa cut-off spin filter following the manufacturer's protocol. Use the filtrate in the assay.
5. Typically, it is not necessary to dilute plasma prior to running the assay. It is recommended that the fluorometric format be used.

Serum

1. Collect blood in vacutainers without an anticoagulant.
2. Allow samples to clot undisturbed for 30-60 minutes at room temperature.
3. Centrifuge at 1,000-2,000 x g for 15-30 minutes at 4°C.
4. Transfer the top serum layer into a clean test tube.
5. Deproteinate through a 10 kDa cut-off spin filter following the manufacturer's protocol. Use the filtrate in the assay.
6. Typically, it is not necessary to dilute serum prior to running the assay. It is recommended that the fluorometric format be used.

Sample Matrix Properties

Spike and Recovery

HeLa cell lysate was spiked with acetylcholine, processed as described in the Sample Preparation section, serially diluted with Assay Buffer A (1X), and evaluated using the Acetylcholine/Choline Fluorometric/Colorimetric Assay Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

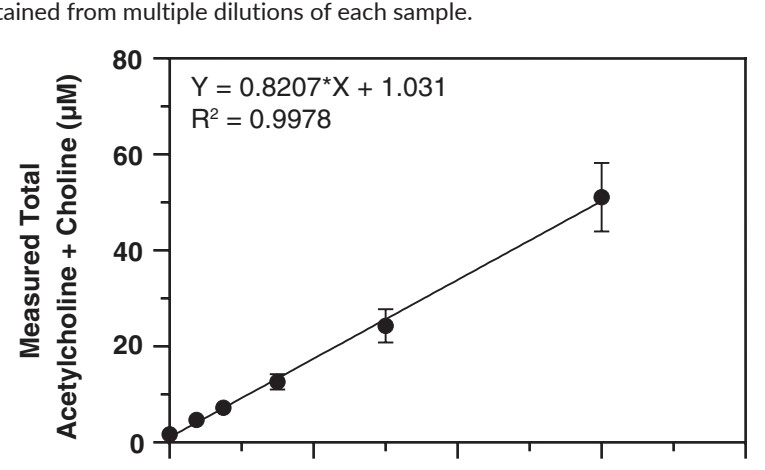


Figure 2. Spike and recovery of acetylcholine in HeLa cell lysate in the fluorometric assay

Parallelism

To assess parallelism, samples were processed as described in the Sample Preparation section, serially diluted with Assay Buffer A (1X), and evaluated using the Acetylcholine/Choline Fluorometric/Colorimetric Assay Kit. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

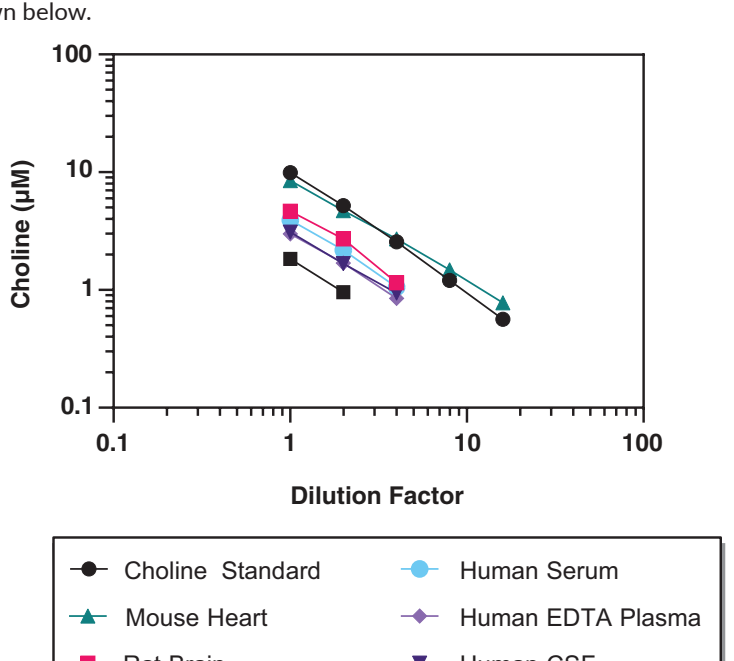


Figure 3. Parallelism of choline in various matrices in the fluorometric assay

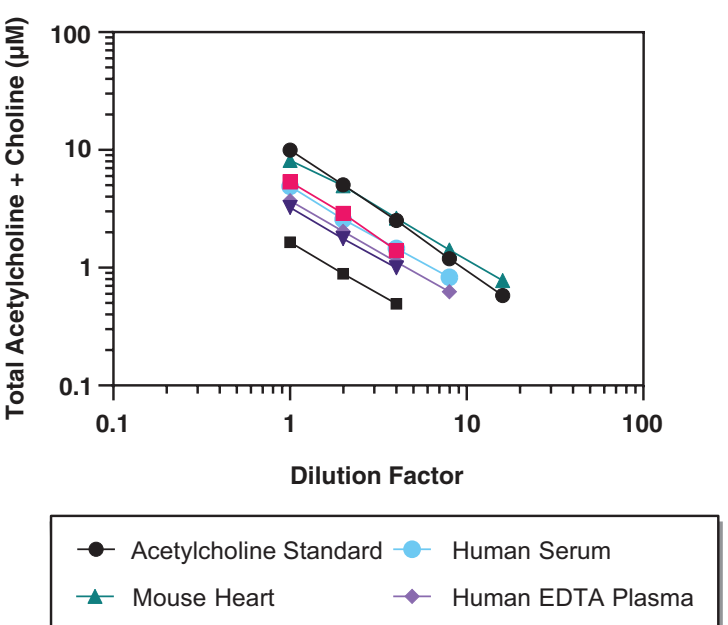


Figure 4. Parallelism of total acetylcholine+choline in various matrices in the fluorometric assay

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each standard, sample, and sample background be assayed at least in duplicate. A typical layout in duplicate is shown in Figure 5. It is suggested that the contents of each well are recorded on the template sheet provided (see page 37).

	1	2	3	4	5	6	7	8	9	10	11	12
A	AA	AA	T1	T1	T7	T7	CA	CA	C1	C1	C7	C7
B	AB	AB	T2	T2	T8	T8	CB	CB	C2	C2	C8	C8
C	AC	AC	T3	T3	T9	T9	CC	CC	C3	C3	C9	C9
D	AD	AD	T4	T4	T10	T10	CD	CD	C4	C4	C10	C10
E	AE	AE	T5	T5	T11	T11	CE	CE	C5	C5	C11	C11
F	AF	AF	T6	T6	T12	T12	CF	CF	C6	C6	C12	C12
G	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
H	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12

AA-AF = Acetylcholine Standard Wells
T1-T12 = Total Acetylcholine + Choline Sample Wells
CA-CF = Choline Standard Wells
C1-C12 = Choline Sample Wells
B1-B12 = Sample Background Wells

Figure 5. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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.

General Information

- The final volume in the assay is 200 µl in all of the wells.
- All reagents should be prepared as described above. The enzymes should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate, but it is at the user's discretion to do so.
- The assay is performed at 37°C.
- Monitor the fluorescence with excitation and emission wavelengths of 530 and 590 nm, respectively, or monitor the absorbance at 570 nm.
- Total and individual acetylcholine and choline levels can be measured on the same plate by using the appropriate reaction mixes.

Standard Curve Preparation

NOTES:

1. This assay can be read using fluorescence or absorbance. Choose the standard curve preparation that matches the assay format selected.

2. To measure total acetylcholine + choline levels, use the acetylcholine standard curve. To measure choline levels alone, use the choline standard curve. To measure acetylcholine levels alone, prepare both standard curves and run both acetylcholine and choline assays (see Table 1, below).

3. Prior to preparing the standards, ensure that the Acetylcholine or Choline Standard has been diluted as described in the Reagent Preparation section (see page 9).

Target(s) to Measure	Acetylcholine Standard Curve	Choline Standard Curve
Total	✓	--
Acetylcholine	✓	✓
Choline	--	✓

Table 1. Standard curve selection

Fluorometric Standard Curve Preparation

Use the 100 µM bulk standard (either acetylcholine or choline) diluted as described in the **Reagent Preparation** section (see page 9) to prepare the fluorometric assay standards.

Label six clean test tubes A-F. Pipette 360 µl Assay Buffer A (1X) to tube A. Pipette 200 µl of Assay Buffer A (1X) to tubes B-F. Transfer 40 µl of the bulk standard (100 µM) to tube A. Mix gently. Serially dilute the standard by transferring 200 µl from tube A to tube B. Mix gently. Next, transfer 200 µl from tube B to tube C. Mix gently. Repeat the process for tubes D-E. Do not add any standard to tube F. The diluted standards will be stable for two hours at room temperature.

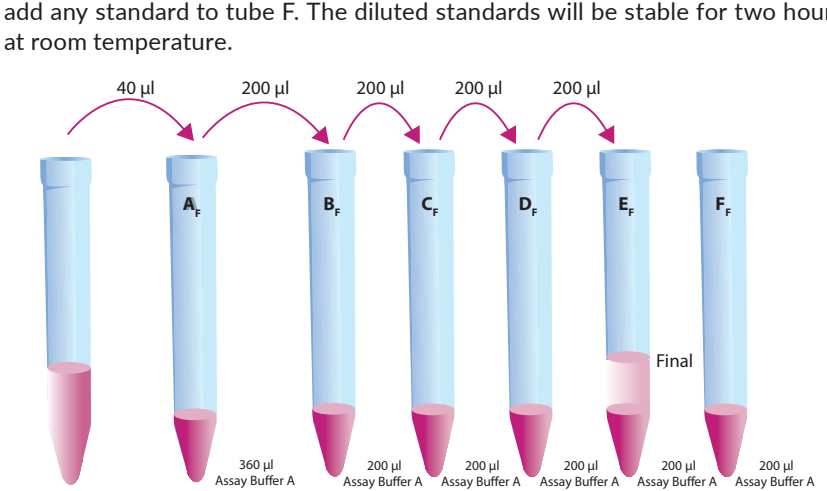


Figure 6. Preparation of the acetylcholine/choline standards for the fluorometric assay format

Colorimetric Standard Curve Preparation

Use the 100 µM bulk standard (either acetylcholine or choline) diluted as described in the Reagent Preparation section (see page 9) to prepare the colorimetric assay standards.

Label six clean test tubes A-F. Pipette 200 µl of Assay Buffer A (1X) to tubes B-H. Transfer 400 µl of the bulk standard (100 µM) to tube A. Serially dilute the standard by transferring 200 µl from tube A to tube B. Mix gently. Next, transfer 200 µl from tube B to tube C. Mix gently. Repeat the process for tubes D-E. Do not add any standard to tube F. The diluted standards will be stable for two hours at room temperature.

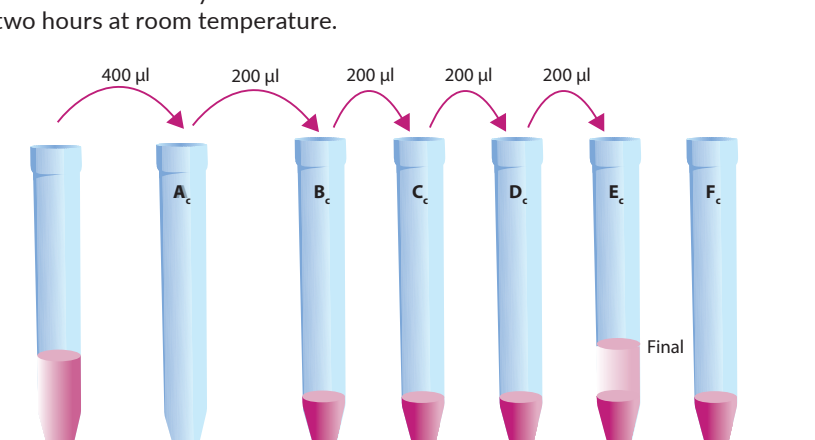


Figure 7. Preparation of the acetylcholine/choline standards for the colorimetric assay format

Performing the Assay

1. The reaction mixes should be selected based on the purpose of the experiment as outlined in Table 2, below. To measure the total acetylcholine+choline levels, prepare the total reaction mix. To measure choline, prepare the choline reaction mix. To measure acetylcholine, prepare both reaction mixes. The same sample background mix can be used for both choline and total acetylcholine + choline measurement if it is included on the same plate.

Target(s) to Measure	Total Reaction Mix	Choline Reaction Mix	Background Mix
Total	✓	--	✓
Acetylcholine	✓	✓	✓
Choline	--	✓	✓

Table 2. Reaction mix selection

2. Prepare the reaction and background mixes according to Table 3, below. Store the reaction mixes on ice, where they will be stable for 20 minutes. These are sufficient volumes to assay 12 samples in duplicate for total, acetylcholine, and choline levels including sample background (see Figure 5, page 17). The same sample background wells can be used for both choline and total acetylcholine + choline measurement if it is included on the same plate. Scale accordingly.

Component	Total Reaction Mix	Choline Reaction Mix	Background Mix
Assay Buffer A (1X)	7,600 µl	7,700 µl	7,800 µl
Acetylcholine Converter Enzyme	100 µl	--	--
Choline Detection Enzyme	100 µl	100 µl	--
HRP Assay Reagent	100 µl	100 µl	100 µl
MaxiProbe	100 µl	100 µl	100 µl
Total volume	8,000 µl	8,000 µl	8,000 µl

Table 3. Preparation of reaction and background mixes

3. Add the appropriate amount of reagents to the designated wells following the table below.

Reagent	Acetylcholine Determination				Sample Background Wells
	Total Acetylcholine + Choline		Choline Determination		
	Acetylcholine Standard Wells	Total Sample Wells	Choline Standard Wells	Choline Sample Wells	
Total Reaction Mix	160 µl	160 µl	--	--	--
Choline Reaction Mix	--	--	160 µl	160 µl	--
Background Mix	--	--	--	--	160 µl
Acetylcholine Standard	40 µl	--	--	--	--
Choline Standard	--	--	40 µl	--	--
Sample	--	40 µl	--	40 µl	40 µl

Table 4. Pipetting summary

4. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate for 30 minutes at 37°C.
5. Remove the plate cover and read fluorescence using excitation and emission wavelengths of 530 and 590 nm, respectively. If the colorimetric method is used, read absorbance at 570 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence or absorbance of each standard, sample, and sample background wells.
2. Subtract the average fluorescence or absorbance value of standard F (standard 0) from itself and all other standards to obtain corrected signal values.
3. Plot the corrected signal values of each standard as a function of the final concentration of choline and/or acetylcholine from Figures 6 and 7, on pages 20 and 21. See Figures 8 and 9, on pages 28 and 29, for typical standard curves.
4. Subtract the average signal of the sample background from the average signal of the corresponding sample to yield the corrected sample measurement (CSM).

5. Calculate the total acetylcholine + choline concentration of the samples using the equation obtained from the linear regression of the acetylcholine standard curve substituting the CSM for each sample.

$$\text{Total Acetylcholine + Choline (µM)} = \left[\frac{\text{CSM - (y-intercept)}}{\text{slope}_{\text{acetylcholine}}}\right] \text{x sample dilution}$$

6. Calculate the choline concentration of the samples using the equation obtained from the linear regression of the choline standard curve substituting the CSM for each sample.

$$\text{Choline (µM)} = \left[\frac{\text{CSM - (y-intercept)}}{\text{slope}_{\text{choline}}}\right] \text{x sample dilution}$$

7. Calculate acetylcholine concentration for the samples using the equation below:

$$\text{Acetylcholine (µM)} = \text{[total acetylcholine + choline] - [choline]}$$

Performance Characteristics

The assay range of either acetylcholine or choline assays is 0-10 µM when read fluorometrically and 0-100 µM when read colorimetrically.

The LLOD is defined as a concentration two standard deviations higher than the mean zero value. The LLOD for choline and total acetylcholine + choline in the fluorometric assay are 0.005 µM and 0.04 µM, respectively, and the LLOD for choline and total acetylcholine + choline in the colorimetric assay are 0.04 µM and 0.07 µM, respectively.

The lower limit of quantification (LLOQ) is the lowest standard concentration in which the mean signal value - 1.64 X S.D is higher than the signal + 1.64 x S.D. of the zero standard. The LLOQ for both choline and total acetylcholine + choline in the fluorometric assay is 0.63 µM, and the LLOQ for both choline and total acetylcholine + choline in the colorimetric assay is 6.3 µM.

Precision

When a series of 24 acetylcholine-spiked rat brain and HepG2 lysate measurements were performed on the same day in the total acetylcholine + choline assay, the intra-assay coefficients of variation were 3% and 1% in the colorimetric assay, and 2% and 1% in the fluorometric assay, respectively. When a series of six mouse heart homogenate and five acetylcholine-spiked HepG2 lysate measurements were performed on different days under the same experimental conditions in the total acetylcholine + choline assay, the inter-assay coefficients of variation were 4% and 6% for the colorimetric assay and 5% and 8% for the fluorometric assay, respectively.

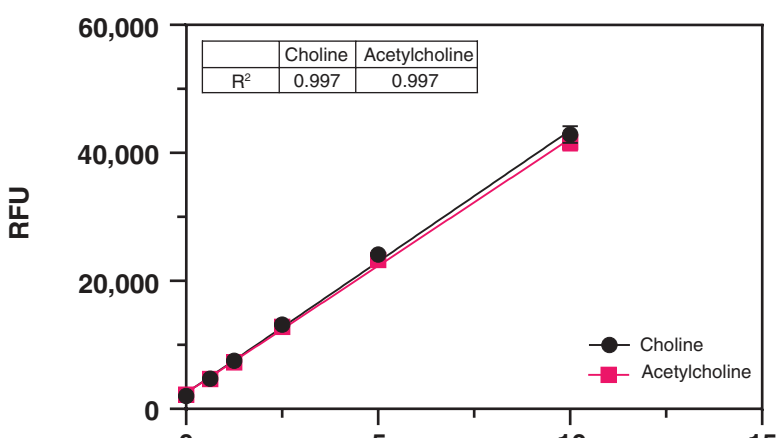


Figure 8. Choline and acetylcholine standard curves for the fluorometric format

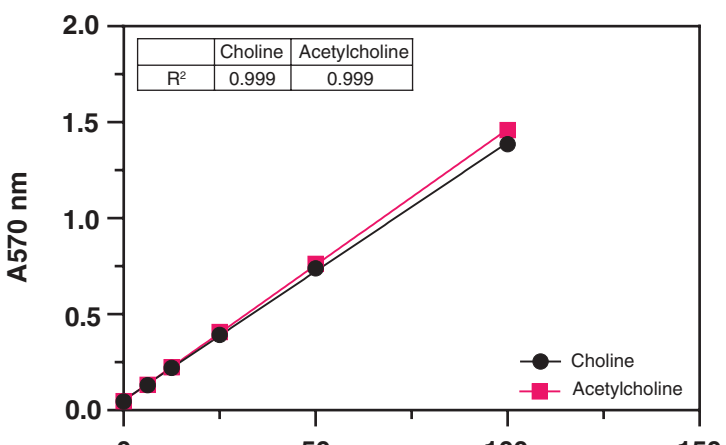


Figure 9. Choline and acetylcholine standard curves for the colorimetric format

Interferences

The standards were prepared in the solutions listed below and tested for potential interference in the assay. A solution is considered to interfere if it causes the measured result to deviate by more than ±5% compared to standards prepared in Assay Buffer A (1X).

Reagent		Will Interfere (Yes or No)
Buffers	M-PER	No
	T-PER	No
	RIPA	No
Detergents	SDS (0.1%)	No
	Triton X-100 (0.1%)	No
	Tergitol (1%)	No
	Polysorbate 20 (1%)	Yes
Chelators	EDTA (2 mM)	No
Protease Inhibitors	Aprotinin (0.06 µg/ml)	No
	Bestatin (40 µg/ml)	No
	E-64 (0.5 µg/ml)	No
	Leupeptin, Microbial (10 µM)	No
	Pepstatin A (0.7 µg/ml)	No
	AEBSF (hydrochloride) (100 µM)	No
	Pefabloc SC (1 mg/ml)	No
	Aprotinin (2 µg/ml)	No
Solvents	DMSO (20%)	No
Others	BSA (0.1%)	No
	Glycerol (20%)	No
	Sucrose (2%)	No
	Triglyceride (Intralipid, 20 mg/dl)	Yes
	Hemoglobin (500 mg/dl)	Yes
	Hemoglobin (50 mg/dl)	No
	Bilirubin (30 mg/ml)	Yes
	Bilirubin (3 mg/ml)	No
	Bilirubin conjugate (12.5 mg/ml)	Yes
	Bilirubin conjugate (1.5 mg/ml)	No

Table 5. Interferences

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/technique B. Bubble in the well	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No signal above the zero standard's in sample wells	Target is below detection limit or absent	Concentrate to enrich sample and verify presence of target
Signal is higher than the top standard in sample wells	Sample is too concentrated	Dilute the sample and assay again
Low signal in all wells, including standards	A. Incorrect detection wavelength(s) used B. Missing component (*e.g.*, detection reagent, enzyme mix, substrate, *etc.* not added) C. Reagent degradation	A. Confirm correct instrument settings (ex/em wavelengths or absorbance) B. Make sure to add all components to the wells C. Check reagent integrity and storage/handling conditions
The fluorometer exhibited 'MAX' value for the wells	The *gain* setting is too high	Reduce the *gain* or utilize the instrument's optimal *gain* feature and re-read

Assay Summary

Item No.	Reagent	Procedure
400794	Assay Buffer (5X)	Dilute to 1X with pure water
400795	Choline Standard	Dilute 1:100 with Assay Buffer (1X) prior to preparing the standards (see Figures 6 and 7 on pages 20 and 21, respectively).
401075	Acetylcholine Standard	Dilute 1:100 with Assay Buffer (1X) prior to preparing the standards (see Figures 8 and 9 on pages 28 and 29, respectively).
400796	Acetylcholine Converter Enzyme	Reconstitute with 300 µl of pure water
400797	Choline Detection Enzyme	Reconstitute with 300 µl of pure water
400798	HRP Assay Reagent	Reconstitute with 450 µl of pure water
400610	MaxiProbe	Ready to use as supplied

Table 6. Reagent preparation summary

Component	Total Reaction Mix	Choline Reaction Mix	Background Mix
Assay Buffer A (1X)	7,600 µl	7,700 µl	7,800 µl
Acetylcholine Converter Enzyme	100 µl	--	--
Choline Detection Enzyme	100 µl	100 µl	--
HRP Assay Reagent	100 µl	100 µl	100 µl
MaxiProbe	100 µl	100 µl	100 µl
Total volume	8,000 µl	8,000 µl	8,000 µl

Table 7. Preparation of reaction and background mixes

	Standard Wells	Sample Wells	Sample Background Wells*
Prepare the Total Reaction and Background Mixes. Use within 20 minutes.			
Add Total Reaction Mix	160 µl	160 µl	--
Add Background Mix	--	--	160 µl
Add Acetylcholine Standards	40 µl	--	--
Add Sample	--	40 µl	40 µl
Cover and incubate for 30 minutes at 37°C			
Read: ex 530 nm/em 590 nm; or A570			

Table 8. Assay summary for measuring total acetylcholine + choline**

**Only one set of background reactions is needed for each sample if choline levels are also being measured on the same plate.*

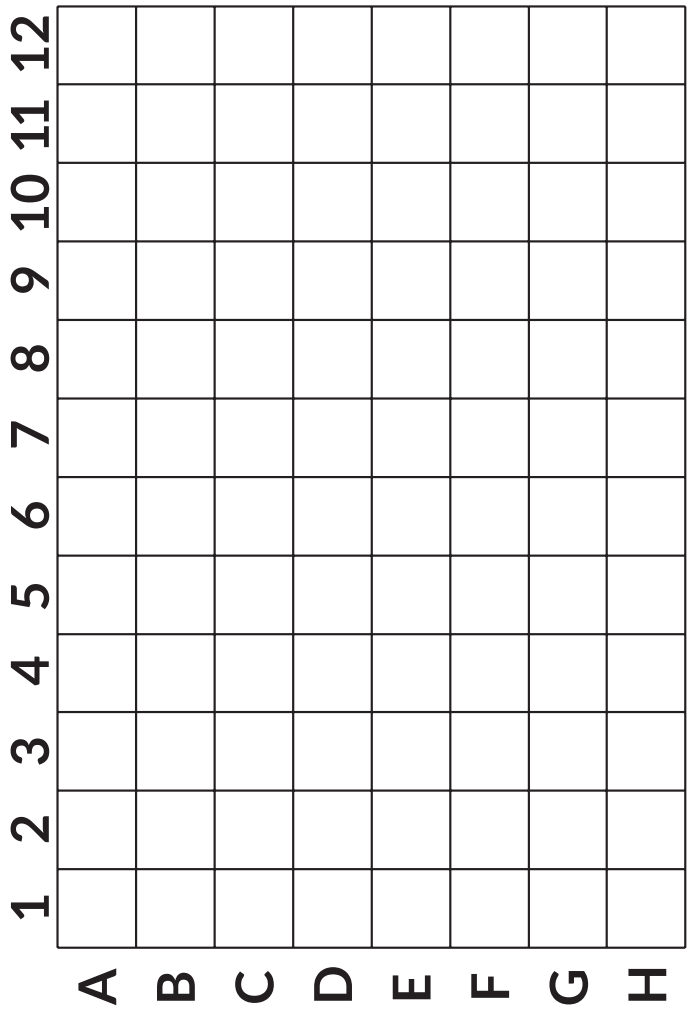
***To measure acetylcholine, perform both total acetylcholine + choline and choline reactions.*

	Standard Wells	Sample Wells	Sample Background Wells*
Prepare the Choline Reaction and Background Mixes. Use within 20 minutes.			
Add Choline Reaction Mix	160 µl	160 µl	--
Add Background Mix	--	--	160 µl
Add Choline Standards	40 µl	--	--
Add Sample	--	40 µl	40 µl
Cover and incubate for 30 minutes at 37°C			
Read: ex 530 nm/em 590 nm; or A570			

Table 9. Assay summary for measuring choline**

**Only one set of background reactions is needed for each sample if total acetylcholine+choline levels are also being measured on the same plate.*

***To measure acetylcholine, perform both total acetylcholine + choline and choline reactions.*



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NOTES

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