Product Number(s): **KF17101**

NEUROMICS

Cholinesterase Detection Kit

Instruction Manual

Background

Originally isolated from the Calabar bean vine, Physostigma venenosum, physostigmine (a carbamate ester alkaloid) mimics the natural substrate, acetylcholine, thus allowing it to act as a cholinesterase inhibitor. Cholinesterase inhibition is facilitated by the ability of the physostigmine inhibitor to target the cholinesterase active site and subsequently carbamylate the reactive serine residue within the active site region of the enzyme. The carbamylated enzyme intermediate is much more stable than the acylated-enzyme intermediate which results from its reaction with the natural substrate, acetylcholine. This feature allows us to utilize physostigmine as a cell-permeant cholinesterase-targeting probe when labeled with a green fluorescent tag (fluorescein). In this assay, fluorescein has been conjugated to a physostigmine analog, eseroline, through a 5-carbon spacer linked to the carbamoyl carbonyl group of the physostigmine side chain. The resulting conjugate (Physostigmine – Fluorescein, Ph-Fl) can then be used to detect the activity of cholinesterase enzymes.

Overview

This unique kit is not an ELISA and does not use antibodies - instead it is based on physostigmine (Ph), a known cholinesterase inhibitor, linked to a green fluorescent label, fluorescein (Fl). The Ph-Fl reagent is cell permeant, so you don't have to lyse the cells or permeabilize the membranes. Because this kit does not use antibodies, there is no cross-reaction with inactive or pro forms of the enzyme. Only cells with the active cholinesterase enzyme will fluoresce. The bound-cholinesterase form of this complex, following a brief wash step, can be quantitated using 3 different fluorescence detection methods:

- **96-well microtiter plate fluorometry for quantitation**
  Using a fluorescence plate reader (with **black** microtiter plates), cholinesterase activity can be quantitated as the amount of green fluorescence emitted from bound Ph-Fl probes. Cell populations with increasing cholinesterase activity will have a higher RFU intensity than cell populations with less activity.

- **Fluorescence microscopy for qualitative analysis**
  Viewing cells through a fluorescence microscope, cholinesterase-positive cells will fluoresce green, while negative cells will appear mostly unstained.

- **Flow cytometry for quantitation**
  Using a flow cytometer, analysis is done using a 15 mW argon ion laser at 488 nm. Fluorescein is measured on the FL1 channel, and a log FL1 (X-axis) versus number of cells (Y-axis) histogram may be generated. On this histogram, there will appear two cell populations represented by two peaks. The majority of the negative cells will occur within the first log decade of the FL1 (X) axis (first peak), whereas the cholinesterase-positive cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity.

The Ph-Fl reagent has an optimal excitation range from 488 - 492 nm, and emission range from 515 - 535 nm (the excitation / emission pairs which best approximate this optimal range should be used). Cells labeled with the Ph-Fl reagent may be read immediately or preserved for 24 hours using the fixative.

Following the suggested protocols listed here, each 500 µL sample of your cell culture (grown up to 1 x 10^6 cells/mL) requires 10 µL of the 51X Ph-Fl working solution (equal to 2 µL of the 255X Ph-Fl stock concentrate. Your cells may require more or less of this reagent, and the length of the incubation time may vary based on your protocol.

Cholinesterase Kit Contents

KF17101-25

- 1 amber vial of lyophilized Cholinesterase reagent, Ph-Fluorescein (Ph-Fl), 25 tests per vial,
- 1 bottle of 15 mL 10X Cellular Wash Buffer
- 1 bottle of 6 mL Fixative

KF17101-100

- 4 amber vials of lyophilized Cholinesterase reagent, Ph-Fluorescein (Ph-Fl), 25 tests per vial
- 1 bottle of 60 mL 10X Cellular Wash Buffer
- 1 bottle of 6 mL Fixative

General Cholinesterase Protocol

Labeling cells with the Cholinesterase Detection Kit can be completed within a few hours. However, the Cholinesterase Detection Kit is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for your experimental manipulation of the cells. Therefore, as the 51X Ph-Fl working solution must be used immediately, the Ph-Fl reagents should be prepared at the end of your experimental process. The following is a quick overview of the Cholinesterase Detection Kit protocol:

1. Culture cells to a density optimal for your specific experiment, but not to exceed 10^6 cells/mL.
2. Expose your cells to your experimental conditions.
3. At the same time, culture a non-treated negative control cell population at the same density as the experimental population for every labeling condition.
4. Dilute the cellular wash buffer in dH2O to 1X.
5. Reconstitute the Ph-Fl reagent with 50 uL DMSO to create the 255X Ph-Fl stock concentrate.
6. Add 200 uL PBS to the reconstituted reagent to created the 51X Ph-Fl working solution.
7. Add 10 uL of the 51X Ph-Fl working solution to 500 uL of your cells to label them, yielding a final concentration of 20 uM Ph-Fl at 1X in the cells.
8. Incubate cells for about 1 hour.
9. Remove the Ph-Fl-containing media and wash cells.
10. Add fresh 1X cellular wash buffer, media, or PBS.
11. If desired, fix cells.
12. Analyze data via microtiter plate fluorometry, fluorescence microscopy, or flow cytometry.

Recommended Materials/Equipment (not all are required):

- Cultured cells with media
- Reagents to do your experiment
- 15 mL polystyrene centrifuge tube (1 per sample)
- Amber vials or polypropylene tubes for storage of the 255X stock concentrate at –20°C, if aliquoted
- 150 mL or 600 mL graduated cylinder
- Slides
- Hemocytometer
- Slides
- Hemocytometer
- Centrifuge at <300 X g
- 37°C CO2 incubator
- Vortexer
- Pipette(s) capable of dispensing at 10uL, 50uL, 200uL, 300uL, 1mL
- dH2O, 135 mL or 540 mL needed
- Phosphate Buffered Saline (PBS) pH 7.4, up to 100 mL needed
- Dimethyl Sulfoxide (DMSO), 50uL or 200uL needed
- Ice or 4°C refrigerator to store cells

Instrumentation (not all are required):

- 96-well fluorescence plate reader with excitation at 488 nm, emission 520 nm filter pairings, and black round or flat bottom 96-well microtiter plates.
- Fluorescence microscope with appropriate filters (excitation 490 nm, emission >520 nm) and slides.
- Flow cytometer equipped with a 15 mW, 488 nm argon excitation laser, with appropriate filters (excitation 490 nm, emission >520 nm).

Storage and Shelf-Life

- Store the unopened kit (and each unopened component) at 2°C to 8°C until the expiration date.
- Protect the cholinesterase reagent Ph-Fl from light at all times.
- Once reconstituted, the 255X stock concentrate should be stored at -20°C protected from light. This reagent is stable for 6 months and may be thawed twice during that time.
- Once diluted, store the 1X cellular wash buffer at 2 - 8°C for 30 days.

Safety Information

- Use gloves while handling the Ph-Fl reagent, cellular wash buffer, and fixative.
- Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be tossed in standard trash bins.

Preparation of 1X Cellular Wash Buffer

The cellular wash buffer is supplied as a 10X concentrate which must be diluted to 1X with dH20 prior to use.

1. If necessary, gently warm the 10X concentrate to completely dissolve any salt crystals that may have come out of solution. Do not let it boil.
2. Dilute 1:10 in dH2O
    - For example, add 10 mL 10X wash buffer to 90 mL dH2O to make 100 mL
    - For the 25-test kit, add the entire bottle of 10X Cellular Wash Buffer (15 mL) to 135 mL of dH2O to make 150 mL.
    - For the 100-test kit, add the entire bottle of 10X Cellular Wash Buffer (60 mL) to 540 mL of dH2O to make 600 mL.
3. Let the solution stir for 5 minutes or until all crystals have dissolved.
4. If not using the 1X cellular wash buffer the same day it was prepared, store it covered at 2° - 8°C for 30 days.

The cellular wash buffer contains a small amount of sodium azide which should not affect the cells during the short amount of time they are in the wash buffer. However, if you do not want to use the Cellular Wash Buffer, just use your own fresh cell culture media instead to wash the cells. We do not recommend using PBS to wash the cells.

Warning: The wash buffer contains sodium azide, which is harmful if swallowed or absorbed through the skin. Sodium azide can react with lead and copper sink drains forming explosive compounds. When disposing of excess wash buffer, flush sink with copious amounts of water.

Fixative

If the stained cell populations cannot be evaluated immediately upon completion of the staining protocol, cells may be fixed and analyzed up to 24 hours later on a microscope or flow cytometer. The fixative is a formaldehyde solution designed to cross-link cell components and will not interfere with the carboxyfluorescein labeling once the labeling reaction has taken place.

After labeling, add the fixative into the cell solution at a 1:10 ratio. For example, add 100 uL fixative to 900 uL cells. Fixed cells may be stored on ice or at 2-8°C up to 24 hours.

- Do not use ethanol-based or methanol-based fixatives - they will inactivate the Ph-Fl label.
- Never add the fixative until the staining and final wash steps have been completed.

Reconstitution of the Ph-Fl Vial

The Ph-Fl reagent is supplied as a highly concentrated lyophilized powder (the amber vial may appear empty as the reagent is lyophilized onto the insides of the vial). It must first be reconstituted in DMSO, forming a 255X Ph-Fl stock concentrate, and then diluted 1:5 in PBS to form a final 51X Ph-Fl working solution. For best results, the 51X Ph-Fl working solution should be prepared immediately prior to use; however, the reconstituted 255X Ph-Fl stock concentrate can be stored at –20°C for future use.

The newly reconstituted 255X Ph-Fl stock concentrate must be used or frozen immediately after it is prepared and protected from light during handling.

1. Reconstitute each vial of lyophilized Ph-Fl with 50 uL DMSO. This yields a 255X stock concentrate. (The 25-test kit contains 1 vial; the 100-test kit contains 4 vials.)
2. Mix by swirling or tilting the vial, allowing the DMSO to travel around the base of the amber vial until completely dissolved. At room temperature (RT), this reagent should be dissolved within a few minutes.
3. Or, if using later, aliquot and store it at –20°C.
4. If immediately using this solution, dilute it to 51X.

Storage of the 255X Ph-Fl Stock for Future Use

If not all of the 255X Ph-Fl stock concentrate will be used the same time it is reconstituted, the unused portion may be stored at -20°C for 6 months. During that time, the 255X Ph-Fl stock concentrate may be thawed and used twice. After the second thaw, discard any remaining 255X Ph-Fl stock concentrate. If you anticipate using it more than twice, make small aliquots in amber vials or polypropylene tubes and store at -20°C protected from light. Following this protocol, each 500 uL cell sample uses 2 uL of 255X stock concentrate, yielding a 20 uM solution of Ph-Fl at 1X. When ready to use, follow Section 13 below.

Preparation of 51X Ph-Fl Working Solution for Immediate Use

Using the reconstituted 255X Ph-Fl stock, prepare the 51X working-strength Ph-Fl solution by diluting the stock 1:5 in PBS at pH 7.4. Following the suggested protocols here, each 500 uL sample to be tested requires only 10 uL of 51X Ph-Fl solution (or 2 uL of the 255X Ph-Fl stock).

1. If you are using the entire vial, simply add 200 uL PBS pH 7.4 to each reconstituted vial (each vial contains 50 uL of the 255X Ph-Fl stock; this yields 250 uL of a 51X Ph-Fl working solution).
2. If not using the entire vial, dilute the 255X Ph-Fl stock 1:5 in PBS, pH 7.4. For example, add 10 uL of the 255X Ph-Fl stock to 40 uL PBS (this yields 50 uL of a 51X Ph-Fl working solution). Store the unused 255X Ph-Fl stock at –20°C.
3. Mix by inverting or vortexing the vial at RT. The 51X Ph-Fl working solution must be used the same day that it is prepared.

General Procedure

Staining cells with the Cholinesterase Detection Kit can be completed within a few hours. However, the kit is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental process. Therefore, as the 51X Ph-Fl working solution must be used immediately, it should be prepared at the end of the experimental process. Cells may be manipulated in test tubes, or in plates. The following is a quick overview of the labeling protocol:

1. Expose your cell population to your experimental system (such as induction of apoptosis). Also prepare non-treated negative controls for reference (such as a non-induced cell population).

Cell density in the cell culture flasks should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used and your experimental conditions.

2. Reconstitute each vial of Ph-Fl lyophilized reagent with 50 uL DMSO and mix thoroughly to solubilize all of the Ph-Fl probe contained in the vial. Allow the DMSO to travel around the base of the amber vial until completely dissolved. At room temperature (RT), this reagent should be dissolved within a few minutes. This yields a 255X stock concentrate that may be stored at -20°C for future use, or further diluted to stain the cells.
3. Add 200 uL PBS to each reconstituted vial and mix. This yields 250 uL of a 51X Ph-Fl working solution that will be used to label the cells. Each 500 uL cell sample will use 10 uL of the working solution.

4. Label each cell sample using a 1:51 v/v ratio of the working solution. For example, add 10 uL of the 51X Ph-Fl working solution to 500 uL of your cell suspension. This ratio of Ph-Fl probe/sample yields a 20 uM Ph-Fl probe concentration in the labeled sample. You may use a different volume of cells, and your particular cell line may require a different concentration of the Ph-Fl probe.

When using the assay for the first time, optimize the incubation period for your particular cell line and experimental conditions by setting up several samples to incubate for different lengths of time.

5. Incubate the labeled cells for 60 minutes at 37ºC in a CO2 incubator protected from light. Resuspend the cells at least once during this incubation period to facilitate even equilibration of the probe into the cells. Active cholinesterase will begin to react with the Ph-Fl probe within 15 minutes of addition to the sample. Your particular cell line and experimental conditions may require a longer incubation period (samples typically incubate for 1-4 hours).

When using this assay for the first time, set up several samples to incubate for different lengths of time to optimize the incubation period for your particular cell line and experimental conditions.

6. Dilute the 10X cellular wash buffer 1:10 in DI H2O. For example, add 10 mL 10X wash buffer to 90 mL DI H2O to make 100 mL.
7. After staining the cells and incubating them with the Ph-Fl reagent, the cells need to be washed to remove any unbound Ph-Fl reagent. The Ph-Fl reagent is always fluorescent, so any unbound reagent left with the cells may lead to high backgrounds or false-positives. Wash the cells usingthe 1X Cellular Wash Buffer, or your cell culture media. Cells are washed by removing the labeled media, rinsing the cells in fresh buffer and centrifuging them, or letting them incubate further in fresh buffer.

Several different wash techniques may be used to wash your cells (See Below)

8. After washing, resuspend the cells in fresh buffer.
9. If not analyzing the cells immediately, cells may be fixed for viewing up to 24 hours later. Add the fixative at a 1:10 ratio into the volume of cell suspension to be fixed. For example, if the cells were resuspended in 500 uL, add 50 uL fixative.
10. Evaluate the cells. The Ph-Fl reagent has an optimal excitation range from 488 - 492 nm, and emission range from 515 - 535 nm (the excitation / emission pairs which best approximate this optimal range should be used).
    a. Viewing cells through a fluorescence microscope, cholinesterase-positive cells will fluoresce green, while negative cells will appear mostly unstained.
    b. When analyzing on a fluorescence plate reader (with black microtiter plates), use 488 nm excitation coupled with 530 nm emission pairing along with a 515 nm lower wavelength cut-off filter. Cholinesterase activity can be quantitated as the amount of green fluorescence emitted from bound Ph-Fl probes. Cell populations with more cholinesterase activity will have a higher RFU intensity than cell populations with less activity. When using this assay for the first time, optimize the incubation period for your particular cell line and experimental conditions by setting up several samples to incubate for different lengths of time.
    c. Using a flow cytometer, analysis is done using a 15 mW argon ion laser at 488 nm. Fluorescein is measured on the FL1 channel, and a histogram may be generates using the log FL1 (X-axis) versus the number of cells (Y-axis). On this histogram, there will appear two cell populations represented by two peaks. The majority of the negative cells will occur within the first log decade of the FL1 (X) axis (first peak), whereas the cholinesterase-positive cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity.

Washing Non-Adherent Cell Populations

Non-adherent cells may easily be lost in the wash process, so care is needed when aspirating the media. If growing suspension cells in a plate, the entire plate may be gently spun in the wash process (however this may be harmful to some cell lines so you may want to try several wash techniques). Here is one method of washing the cells:

1. After staining and incubating the cells in Section 14, Step 5, add an extra 1-2 mL of the wash buffer (or other buffer as mentioned above) to each tube.
2. Mix the cell suspension thoroughly.
3. Pellet down the cells by centrifuging them at <300 x g for 5-6 minutes at RT.
4. Carefully remove and discard the supernatant.
5. Resuspend the cell pellets in about 1 mL of wash buffer.
6. Pellet the cells down again by centrifuging them at <300 x g, but reduce the centrifugation time down to 3-4 minutes.
7. Repeat steps 9-11.
8. Carefully remove and discard the supernatant.
9. Resuspend the cells in 500 uL of the wash buffer or just PBS or cell media.
10. Fix the cells, if desired
11. Evaluate the cells.

Washing Adherent Cell Populations

Adherent cells need to be carefully washed to avoid the loss of any cells which round up and come off the plate surface. Loose cells may be harvested from the plate or slide surface and treated as suspension cells, while those remaining adherent to the surface should be washed as adherent cells. The washed non-adherent cells can then be recombined with the adherent portion when the analysis is performed. If growing adherent cells in a plate, the entire plate may be gently spun as part of the wash process (however this may be harmful to some cell lines so you may want to try several wash techniques). Here is one method of washing the cells:
After staining and incubating the cells, pour off or aspirate the Ph-Fl-labeled media.
Add 1-2 mL of the wash buffer.

1. Let the wash buffer incubate with the cells for several minutes at RT protected from light (15 minutes to 1 hour, cells may be placed in the incubator).
2. Harvest the loose cells and wash as suspension cells.
3. Carefully aspirate the supernatant and discard.
4. Wash the cells up to 3 times by repeating Steps 7-9. Some cells may need to be washed more than others depending on the type of instrument used. Cells evaluated by flow cytometry may not need to be washed as much as cells evaluated in a plate reader or microscope, as the sheathing fluid acts as a wash buffer. Cells analyzed in a plate reader often have to be washed the most, as the fluorometer measures total fluorescence within the well and any excess reagent will lead to higher RFUs.
5. Cells to be analyzed in a flow cytometer can be trypsinized off the surface of the flask or slide and subsequently resuspended in buffer.
6. Recombine loose cells with adherent cells.
7. Fix the cells if desired
8. Evaluate the cells