USER MANUAL

Acetylcholine Kit

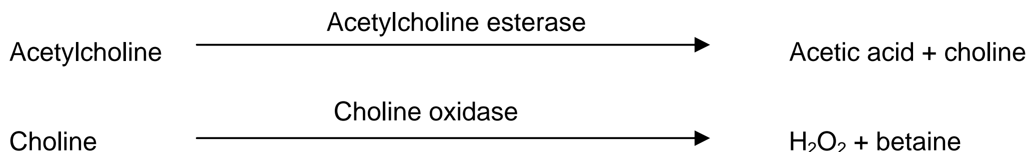
Warning

The Acetylcholine Kit is developed specially for sensitive determination of acetylcholine and/or choline (with a concentration below the nanomol range) in biological samples. Introduction of organic solvents into the system except as described below may irreversibly deactivate the IMmobilized Enzyme Reactor. Consequently, you should thoroughly flush the lines of your LC with isopropanol followed by deionized water to remove any organic solvents prior to installation of the system. The Choline analytical column has been packed with a derivatized silica material. Introduction of basic solvents (pH > 8.0) or acidic solvents (pH < 2.5) into the column may dissolve the silica material and damage the column. You should thoroughly familiarize yourself with the contents of this manual before using your system. Improper use will invalidate the warranty.

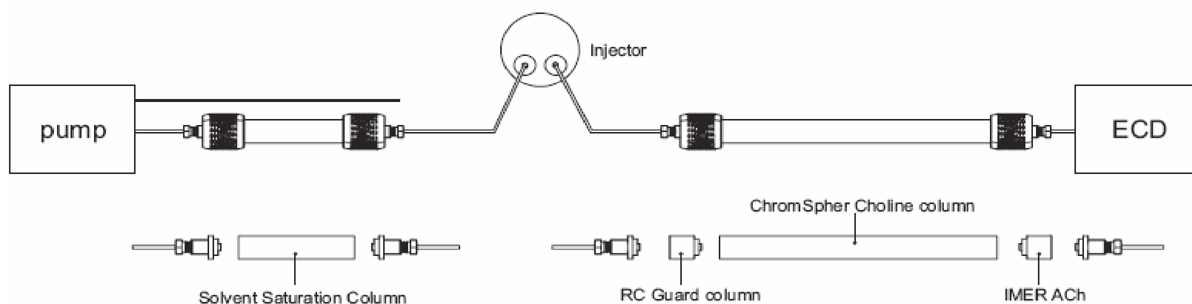
Introduction

The Acetylcholine Kit contains:

- One Choline cartridge column, packed with chemically derivatized silica material. It is designed specifically for the separation of acetylcholine and choline using a phosphate buffer as eluent only.
- Three ACh IMERs (IMmobilized Enzyme Reactors) containing silica base material, loaded with chemically bonded acetylcholine esterase and choline oxidase. Base material properties, column dimensions and enzyme amounts are optimized for the post-column stoichiometric conversion of acetylcholine to betaine and choline, and choline to acetic acid and hydrogen peroxide in the femtomole to nanomole range, with minimal influence on peak broadening.



- One dummy IMER; an empty IMER to put in place of the IMER when the analytical column and the guard column are flushed with organic solvents.



- Five ChromSep guard columns, packed with material similar to the analytical packing material, in order to protect the Choline column from pollutants in the sample.
- One stainless steel Solvent Saturation column, filled with reversed phase material to protect the analytical column from dissolving and to prevent stripping of the functional groups. The solvent saturation column is placed between the pump and the injector.
- ChromSep hardware for the analytical system consisting of a ChromSep stainless steel housing, two end heads and Valco type end fittings. The hardware is designed to contain the guard column, the analytical column and the IMER in one single housing.
- ChromSep hardware for the Solvent Saturation column consisting of a ChromSep stainless steel housing, two end heads and Valco type end fittings.

Eluent

The eluent recommended for this system is 0.2 M potassium phosphate buffer, with a pH of 7.5, to which 2 mM tetramethylammonium chloride (TMACl) and 5 mM KCl are added. The pH of 7.5 is chosen as a compromise between enzyme activity and column stability. Lower pH values will result in a higher column stability but a lower enzyme activity, and thus a lower detection sensitivity. Prevent the use of eluents with a pH lower than 2.5 or higher than 8.0 for they will decrease column life time. Avoid the use of eluents containing organic modifiers like acetonitrile, for this will deactivate the the immobilized enzymes. Hydrogen bridge forming modifiers like ethanol do not have such a devastating effect on enzyme stability, but their use is not recommended, since the analytical circumstances and resolution have already been optimized with the stationary phase choice and the use of the phosphate buffer as eluent. To make up the buffer different salts can be used, but one has to take into consideration the influence of the salts and salt concentrations on enzyme activity. To influence retention of both acetylcholine and choline, change of TMACl concentration can be used. A higher TMACl concentration results in a higher solvent strength and faster elution. The TMACl concentration should always be kept below 5 mM to prevent enzyme deactivation. Eluents must be degassed prior to use to prevent detection and pumping problems, and filtered through a 0.5 µm filter. Always check your eluents for microbial growth before starting up the system, otherwise the column may get clogged and back pressure will rise up to unacceptable levels.

Flow and Pressure

The optimal flowrate is 0.6 ml/min. Do not exceed 1.5 ml/min or a pressure of 200 bar (20 MPa, 2800 psi). Furthermore a high flowrate might result in incomplete conversion by the IMER. Increasing or decreasing the flowrate must always be done in small steps, to prevent packing bed disturbances. High column pressures nearly always result from improper use of the column. Use of a guard column (see below) will usually prevent contaminants from accumulating on the analytical column.

Temperature

The Acetylcholine system can be operated at ambient temperature. The use of a column thermostat may result in a higher enzyme activity or a better retention time reproducibility, but is not strictly necessary.

Guard Columns

A guard column must always be used because sample and eluent contamination can result in excessive column pressures and altering selectivity. Replacement of the guard column is advised on a regular base, before or when increased column pressure and/or loss of performance is observed. Sets of 5 replacement guard columns are available (contact supplier for ordering information).

Storage

The analytical and guard columns can best be stored after rinsing with first 10 ml K_3PO_4 pH 7.5 than 10 ml deionized water followed by rinsing with 10 ml methanol. All with a flow rate of 1 ml/min.

Make sure that the reactor is taken out of the holder after rinsing with 10 ml K_3PO_4 pH 7.5 and replaced by the dummy before the columns are rinsed with methanol.

No specific precautions have to be taken to prevent the analytical or guard column from drying out. The IMERs must be stored in 0.2 M K_3PO_4 pH 7.7 and are not allowed to dry out, for this will result in a decrease of activity. Storage of the IMERs is preferably done in the refrigerator at 4 °C but storage at ambient temperature is very well possible. The IMERs are loaded with more enzyme than is strictly necessary for operation. Therefore a small decrease in activity will not be noticed. For overnight storage it is best to continuously rinse the system with the eluent at a low flow rate (like 0.2 ml/min).

Pump Cleaning

We noticed that it can be important to clean the HPLC pump thoroughly when starting up the system. It is something which should be done before column conditioning and no columns should be connected to the pump! Please check if it is possible to use the strong acids with your pump. If the pump cannot withstand the solvents used for the pump cleaning it should not be done.

Make sure that all parts that are not acid-resistant such as: nylon inlet filters, column and flow cell are not connected during this step.

The cleaning procedure is:

1. Flush with 50 ml 20% nitric acid
2. Flush with 50 ml 30% acetic acid
3. Flush with 100 ml of HPLC water to make sure that all acid is removed from the pump.
4. Flush with 100 ml of the eluent, which you are going to use

Column Conditioning

Before starting up the analysis the columns must be conditioned properly. A not properly conditioned column may give rise to problems like bad performance, changing separation etc. First connect the pre-saturation column between pump and injector and rinse with 40 column volumes of eluent. Please take care to do this without connecting the guard column, analytical column, IMER and electrochemical detector. Then connect the guard and analytical column but leave out the IMER and use the dummy IMER. Rinse the guard column and the analytical column with eluent for 40 column volumes. Finally put the IMER in the right position and rinse with eluent for 20 column volumes. After this procedure the electrochemical detector can be connected and the system can be equilibrated.

Sample Treatment

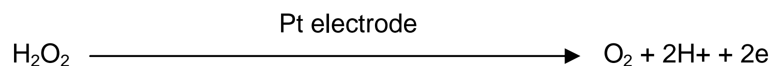
The key to long column life is proper treatment of samples prior to injection. Avoid the introduction of particulate matter and oils or fats into the column by either mobile phases or samples. In particular, you should avoid introduction of particulate matter. These will ultimately cause an increase in operating pressure and may be difficult or impossible to remove.

Sample Volume

Sample volume merely depends on the sample concentration of the analytes. Injection of low sample volumes usually creates no problems, but injection of high sample volumes might result in broad peaks. It is not advised to inject higher volumes than 100 µl. Considering the high sensitivity of the analysis system, in most cases this is largely sufficient.

Detection Sensitivity

Detection of the hydrogen peroxide takes place with an electrochemical detector (ECD).



$$H_2O_2 \xrightarrow{\text{Pt electrode}} O_2 + 2H+ + 2e$$

The detection sensitivity mainly depends on the type of ECD used. With a proper ECD it is possible to reach a detection limit of 50 fmol. But for this purpose a thorough detection optimization is necessary. The detection of hydrogen peroxide requires the use of a platinum electrode.

When the reference electrode (Ag/AgCl) uses the eluent as reference solution, addition of for instance 5 mM KCl to the eluent has no influence on retention or enzyme stability. As a compromise between detection response and detection selectivity a working electrode potential of +400 mV is advised.

Possible Causes of Performance Loss

1. **Extra column band broadening.** Make sure the tubing length and tubing ID are kept to a minimum.
2. **Insufficient equilibration time with eluent.**
3. **Improper pH or ionic strength of eluent.**
4. **Improper eluent cation present.** Prepare fresh eluent.
5. **Stationary phase contamination.**

High column pressure accompanies resolution loss

- Particulate accumulation on frit or packing bed:
  - Sample origin: filter or centrifuge samples
  - Eluent origin: use mobile phase filter (0.2 µm pores), close eluent reservoirs
  - System origin: flush all lines and pumps; install in-line system filter.

- **Accumulation of proteinaceous material:**
  - Microbial growth in samples
  - Microbial growth in eluent

- **Normal column pressure accompanies performance loss**
  Organic contamination
  - Fats, oils, lipids in sample: stationary phase surface becomes coated
  - Non-specific organics from sample or improperly prepared eluent
  - Non-specific organics introduced into eluent after preparation (e.g. from atmosphere, during transfer etc.)

Possible Operations to Correct Contamination

1. **Prepare fresh eluent** In many cases, performance loss is traced to eluent contamination. Therefore, prepare fresh eluent and flush all liquid lines before using column; eluent should be filtered through 0.2 to 0.4 µm membranes (in-line solvent filter).

2. **Column regeneration**

To regenerate the column:

- first **take out the IMER** and replace it with the dummy IMER
- then invert the system (guard, anal. column and dummy)
- rinse with 30 ml 1 M ammonium nitrate at 0.4 ml/min
- rinse with 30 ml methanol/water (40:60 v/v) at 0.4 ml/min
- rinse with 30 ml iso-propanol at 0.4 ml/min
- rinse with 30 ml water at 0.4 ml/min
- rinse with 30 ml of eluent at 0.4 ml/min
- invert the system to the original position
- take out the dummy put back the IMER
- equilibrate with the eluent