Chemiluminescent Labeling kit

Catalog No. ADI-907-001

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Description

The Chemiluminescent Labeling kit is designed to allow the rapid attachment of a chemiluminescent Acridinium Ester to lysine groups on most antibodies, proteins, nucleic acids and some peptides. The kit contains enough materials to perform up to 5 IgG labeling experiments, each of 1 mg of IgG. The following materials are included: Acridinium Ester with a N-hydroxysuccinimide Ester group, dry solvent for dissolving the Acridinium Ester, buffer for diluting the antibody, protein or nucleic acid, a solution of lysine for stopping the labeling reaction, a gel filtration column for separation of the labeled product from excess Acridinium Ester, column running buffer, a set of Trigger Solutions for detection of the labeled antibody, protein or nucleic acid, and a labeling worksheet.

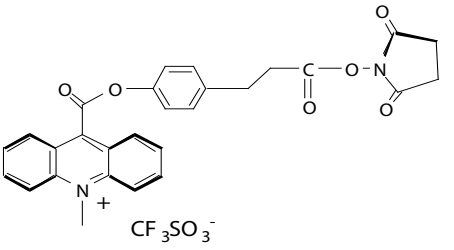
Uses of Acridinium Ester Labeled Proteins, Peptides and Nucleic Acids

The Acridinium Ester labeled samples can be used for a number of different purposes. They can be added to solutions of the unlabeled materials as an alternative to the use of ^{125}I labeled tracer molecules. They can be used as labeled antigens for competitive immunoassays, and as labeled signal antibodies for immunometric assays. Chemiluminescent labeled nucleic acids may be used as reporter molecules in DNA probe based assay systems.

The uses of 4-(2-succinimidyl-oxycarbonylethyl) phenyl-10-acridinium-9-carboxylate trifluoromethyl sulfonate, or more simply the $A^+ C_2$ NHS Ester, as a means for labeling antibodies, antigens and DNA has been described in a number of publications[1-9]. These publications describe the use of these labeled materials to generate super sensitive, rapid assays for a variety of molecules.

The structure of the labeling compound is shown in Figure 1.

Figure 1. Acridinium C_2 NHS Ester



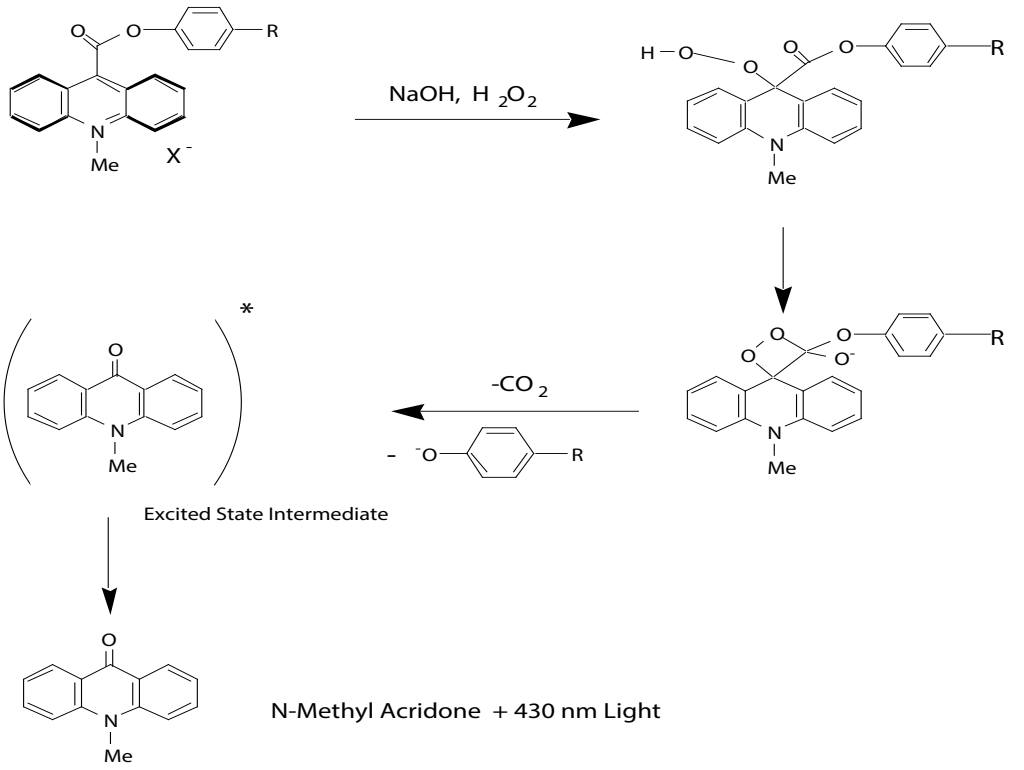
Acridinium C_2 NHS Ester

4-(2-succinimidyl-oxycarbonylethyl)-phenyl-10-acridinium-9-carboxylat
trifluoromethyl sulfonate

$C_{29}H_{23}F_3N_2O_9S$ Formula Weight= 632.55

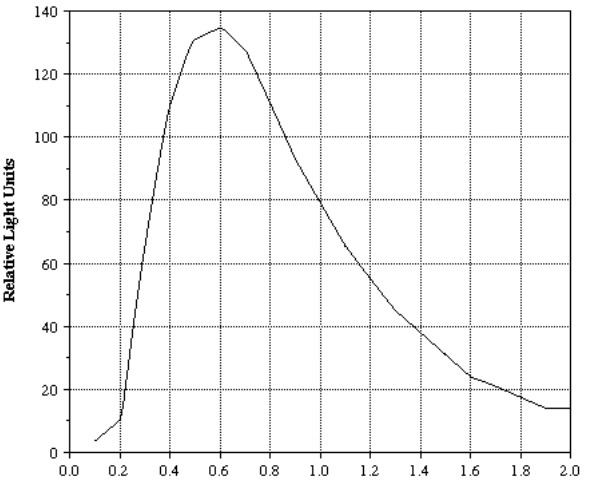
The mechanism of the chemiluminescent reaction is provided in Figure 2:

Figure 2. Mechanism of Chemiluminescent Reaction



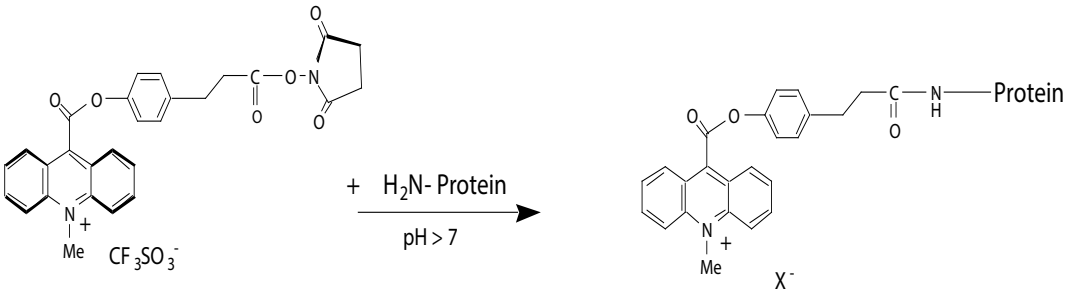
The injection of the Trigger Solutions causes the rapid chemiluminescent reaction to take place with the emission of blue light. The emission is centered on 430 nm and is ideally suited for maximum sensitivity by the bialkalia photomultiplier tubes found in most commercial luminometers. Typical emission kinetics are shown in Figure 3.

Figure 3. Emission Kinetics of CL Reaction



Description of Labeling Reaction

The Acridinium Ester supplied in this kit has a N-hydroxysuccinimidyl (NHS) ester labeling group attached to a 2 carbon spacer arm. The NHS ester labeling group will covalently attach to any primary amino group on the protein, peptide or nucleic acid to be labeled. At pH's above neutral the NHS ester labeling group is subject to nucleophilic attack by the amine group on the protein, peptide or nucleic acid. The NHS group is displaced from the Acridinium Ester to form a stable amide bond between the Acridinium Ester and the protein, peptide or nucleic acid. The labeling reaction is shown below for a protein amine group.



Acridinium Ester - Protein Conjugate

Precautions

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1. Some kit components contain azide, which may react with lead and copper plumbing, or with acids. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. The Dimethyl Formamide (DMF), Catalog No. 80-0087, is both flammable and a lachrymator. Care should be exercised in handling this solvent.
3. The Acridinium Ester, Catalog No. 80-0086, and the end product of the chemiluminescent reaction, N-Methyl acridone, have been shown to be either very weakly genotoxic or non-genotoxic. Care should be taken in the use of this reagent. The Acridinium Ester solution should be handled carefully as DMF has the property of allowing rapid transdermal penetration of some organic compounds.
4. The Trigger Solutions, Catalog Nos. 80-0092 and 80-0093, contain dilute solutions (<0.2M) of nitric acid and sodium hydroxide. Both solutions are CAUSTIC and care should be used in handling them.

Materials Supplied

1. **Acridinium Ester with N-Hydroxysuccinimide Ester Group, 100 µg, Catalog No. 80-0086**
   Protect from moisture.
2. **Dry Dimethyl Formamide, 0.5 mL, Catalog No. 80-0087**
   Protect from moisture. Contains drying agent.
   Caution: **LACHRYMATOR, CORROSIVE, FLAMMABLE**.
3. **Bicarbonate Labeling Buffer, 5 mL, Catalog No. 80-0088**
   0.1M bicarbonate buffer, pH 8.5. Contains no preservative.
4. **10% Lysine Solution, 0.5 mL, Catalog No. 80-0089**
   Solution of lysine in bicarbonate buffer, pH 8.5 and sodium azide as preservative.
5. **Gel Filtration Column, Catalog No. 80-0090**
   Prepackaged gel filtration column containing 10 mL of desalting media. Complete with end cap and plug.
6. **Column Buffer 10x Concentrate, 100 mL, Catalog No. 80-0091**
   Citrate buffered saline, containing detergent at pH 5.0 and gentamicin as preservative.
7. **Set of Trigger Solutions, 30 mL each. Catalog Nos. 80-0092 & 80-0093**
   Solutions for detecting Acridinium Ester labeled materials in a suitable luminometer. Trigger 1 should be injected immediately prior to Trigger 2 inside a suitable luminometer. The injection of Trigger 2 must be made directly in front of the luminometer PMT. Light emission takes place immediately and lasts approximately 2 seconds.
   Caution: **Caustic.**
8. **Labeling Worksheet, 1 sheet, Catalog No. 30-0019**

Storage

All components of this kit are stable at 4 °C until the kit's expiration date.

Materials Needed but Not Supplied

1. 12 x 75 mm **borosilicate glass** test tubes and suitable test tube racks.
   NOTE: Sodalime tubes phosphoresce and may give high backgrounds.
2. Precision pipets for volumes between 1 µL and 1,000 µL.
3. A micro magnetic stir bar (~ 3 x 10 mm) and magnetic stirrer.
4. A ring stand.
5. Disposable beakers and graduated cylinders for diluting buffer concentrates.
6. A luminometer capable of injecting 2 trigger solutions and measuring flash chemiluminescence. Please refer to the list of luminometer manufacturers on Page 10.

Procedural Notes

1. Do not mix reagents from different lot numbers or use reagents beyond the expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. **Use only disposable containers, pipets and other laboratory ware when working with Acridinium Esters to avoid chemiluminescence contamination of containers, buffers and other reagents.**
4. White polysytrene plates or clear plastic tubes may be used in place of the glass test tubes for testing fractions.

Reagent Preparation

1. Column Buffer

Dilute the Column Buffer Concentrate 1:10 by measuring 10 mL of Concentrate and adding 90 mL of deionized water for every 100 mL of Column Buffer needed. Typically 40-60 mL of buffer are needed for each labeling experiment.

2. Column Preparation

Remove the upper cap and clamp the column vertically in a ring stand. Pour off the shipping buffer which contains a preservative. Cut off the tip and allow the column to drain. Add 20 mL of the Column Buffer to the top of the column and allow the column to completely drain. Flow will stop when the buffer level reaches the top frit.The column is now ready to use.

3. Sample Preparation

The sample to be labeled must be free of all aliphatic primary amine and free of all preservatives. Samples should not contain Tris based buffers, sodium azide and other nucleophilic materials. Salt free lyophilized preparations or concentrated solutions are ideal. These can be diluted in the Bicarbonate Labeling Buffer to achieve optimum pH for labeling.

4. Acridinium Ester Preparation

Prepare the Acridinium Ester solution just prior to labeling the sample by adding 50 µL of the dry DMF solvent and vortexing. Use solution immediately or store desiccated at -80 °C.

Labeling Worksheet and Calculations

There is sufficient Acridinium Ester to label a total of 5 mg of an IgG antibody with a 5-fold molar excess of the Acridinium Ester. The examples and calculations given here represent a typical protein labeling protocol and will have to be modified for alternative starting materials. Use the supplied Labeling Worksheet, Catalog No. 30-0019, to calculate the ratios of Acridinium Ester to peptide, protein or nucleic acid for optimal labeling. The Labeling Worksheet also contains a flowsheet for the labeling reaction.

Labeling Procedure

Refer to the Labeling Worksheet prior to starting the labeling reaction.

1. Allow all the components of the kit to come to room temperature prior to opening.

2. Allow the peptide, protein or nucleic acid to come to room temperature. If the material to be labeled is lyophilized, add sufficient Labeling Buffer to make a 5 mg/mL solution. If the material to be labeled is in solution, dilute with Labeling Buffer to bring the concentration to 5 mg/mL.

3. Place the calculated amount of the peptide, protein or nucleic acid to be labeled in a test tube and add a micro stir bar. Add the calculated volume of the Acridinium Ester solution in DMF to the peptide, protein or nucleic acid solution and vortex. Stir at room temperature for 30 minutes.

4. After 30 minutes add 10 µL of the 10% Lysine Solution to stop any further reaction and vortex. Stir at room temperature for 15 minutes.

5. Place fourteen 12 x 75 mm numbered test tubes in a rack for fraction collection. Apply the labeled sample to the top of the washed, drained column. Let the sample enter the column bed and collect the eluant in tube 1.

   With a clean pipet tip add 800 µL of Column Buffer to the reaction tube and vortex to mix. Apply this to the column and collect the eluant in tube 1.

   With a clean pipet tip follow with the 1 mL aliquots of the Column Buffer, to collect 1 mL fractions. The column will stop draining automatically as each 1 mL aliquot is added.

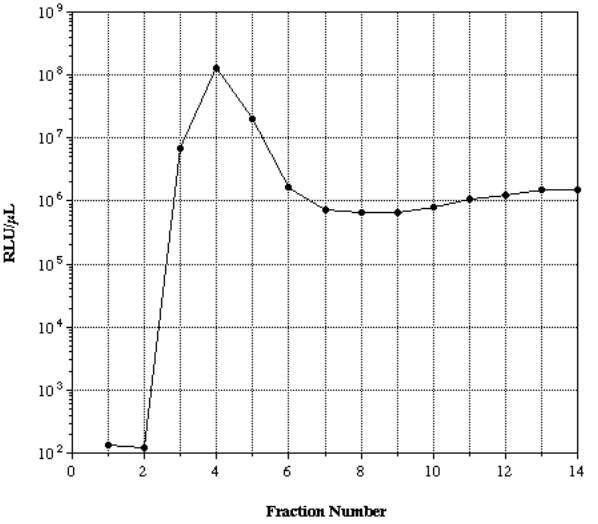
6. Pipet 1 µL of each fraction into numbered duplicate 12 x 75 mm test tubes or microtiter wells. Read each tube in a luminometer using Triggers 1 & 2. Any fractions that display more than 1 x 10^6 RLU/µL should be diluted into Column Buffer and retested. Plot column fraction number versus RLU/µL. Refer to the graph on Page 8 as an example. Pool the first fraction(s) that contain light activity.

   The pooled sample should be aliquoted and frozen at -20 °C for long term (>1 week) stability.

7. The column should be washed with 20 mL of Column Buffer, tightly capped and stored at room temperature for future use.

Typical Results

The following data is for the Acridinium Ester labeling of mouse IgG. The graph below shows the Relative Light Units (RLU) for 1 µL of each fraction. The RLU's for the peak fractions have been calculated from the light activity of the diluted fractions. In this example fraction # 4 was aliquoted and stored at -20 °C.



Determination Of Acridinium Incorporation into Proteins, Antibodies, and DNA

CAUTION:

This method requires that the labeled protein, antibody, or DNA is acidified to a pH of < 1.5. This will cause denaturation of most proteins, which may be irreversible. We suggest using a small aliquot (~50-100 µL) of the labeled protein for this determination.

The Acridinium Ester attached to your protein, antibody, or DNA exists in two forms at neutral pH's. The salt form is favored by pH's below about 4; above pH 4 water adds across the central Acridinium ring to add an OH group at the 9 position. This form is termed the pseudo-base form. The pseudo-base form loses all of the typical yellow color associated with the salt form. To measure the amount of Acridinium Ester attached, the pseudo-base form must be converted into the salt form by addition of acid.

Note: This procedure assumes you are working with a "normal" protein that has a reasonable optical density at 280 nm, and a protein spectra that does not change dramatically when measured at pH <1.5.

1. Dilute the labeled protein solution with water so that the resulting solution will have an optical density at 280 nm of between 0.1 and 1.5. Add 5 µL of concentrated hydrochloric acid for each mL of diluted protein solution. DNA samples should be handled in the same fashion.

2 . Measure the optical densities at 367 and either 280 nm for proteins or 260 nm for DNA. The molar extinction coefficient for the Acridinium Ester at 367 nm is 14,650. Take your measured OD at 367 and divide by 14,650 to obtain the concentration of the Acridinium Ester in Moles/liter.

3 . Calculate the correction factor at 280 nm due to the Acridinium Ester by multiplying the OD at 367 nm by 0.17. This correction is for the OD at 280 nm of the Acridinium Ester salt form. This correction factor must be subtracted from the measured OD at 280 nm. After subtracting this correction factor, the remaining OD at 280 nm is due to your protein. The correction factor for the OD at 260 nm is 4.95. Follow the same calculation procedures as outlined for proteins.

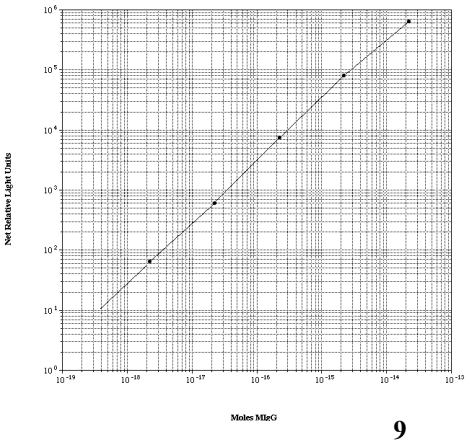
4. Calculate the protein or DNA concentration by dividing the corrected OD at 280 or 260 nm by the OD for a 1 mg/mL solution of the protein, antibody, or DNA . Convert the concentration in mg/mL into Moles/Liter by dividing by the molecular weight.

5 . Divide the Acridinium Ester concentration in Moles/Liter calculated in Step 2. above by the protein concentration in Moles/Liter from Step 4. above to obtain the molar ratio of Acridinium Ester attached to your protein or DNA molecules.

Sensitivity Determination

The fraction containing the Acridinium Ester labeled mouse IgG was used for the sensitivity determination. The labeled protein was serially diluted from 1:100 to 1:10^6 in Column Buffer and duplicate measurements were made in a Berthold tube luminometer. Acridinium Ester chemiluminescence takes 2 seconds using the Trigger Solutions provided. The data is shown in Figure 4.

Figure 4. Sensitivity Determination



Sensitivity was calculated by determining the RLU's emitted from the buffer blank and comparing to the RLU's emitted by the lowest concentration of labeled MIgG. Here, the difference in RLU's was compared to two (2) standard deviations from the zero.

In this case the lowest amount of MIgG that could be detected was 0.6 x 10^{-18} Moles.

Luminometer Set Up Suggestions

The luminometer to be used for measuring the labeled fractions and the assays developed using the labeled materials **must** be able to be compatible with "Flash" type chemiluminescent systems. The Trigger Solutions provided must be injected with the sample in the light collection position of the luminometer. A list of luminometer manufacturers is provided below. If you are in doubt about the suitability of your instrument to measure these labeled molecules, please contact your luminometer manufacturer.

The luminometer should inject 50-300 µL of Trigger Solution 1, 0.5 to 1.2 seconds prior to injection of the same volume of Trigger Solution 2. Light emission will start immediately when Trigger 2 is injected and last for about 2 seconds. Integration of the luminescence signal should be initiated prior to, or at the time of injection of Trigger 2 and last for 2 seconds. If in doubt about your luminometer set up please contact your luminometer manufacturer.

We have provided a list of Luminometer Manufacturers and their U.S. phone numbers. Please be aware that this list is neither a complete list of all the available manufacturers, nor an endorsement of any manufacturer's instrumentation for a specific purpose.

Luminometer Manufacturers

1. Anthos Labtec
Plate Luminometers
Phone Numbers (800) 336-3233
(301) 695-1461
Fax Number (301) 695-1066

2. BioOrbit (Man-Tech Associates)
Tube Luminometers
Phone Number (716) 743-1320
Fax Number (519) 763-9995

3. Dynatech Laboratories
Plate Luminometers
Phone Numbers (800) 336-4543
(703) 631-7800
Fax Number (703) 631-7816

4. EG&G Berthold (Wallac)
Tube & Plate Luminometers
Phone Numbers (800) 638-6692
(301) 963-3200
Fax Number (301) 963-7780

5. Laboratory Technologies
Tube Luminometers
Phone Numbers (800) 542-1123
(708) 529-3112
Fax Number (708) 529-3141

6. Labsystems
Plate Luminometers
Phone Numbers (800) 522-7763
(617) 449-8060
Fax Number (617) 455-9799

7. MGM Instruments
Tube Luminometers
Phone Numbers (800) 551-1415
(203) 248-4008
Fax Number (203) 288-8378

8. Source Scientific
Tube Luminometers
Phone Numbers (800) 888-9285
(714) 898-9001
Fax Number (714) 891-1229

References

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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