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Patent Pending

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**Introduction**

The Acetyltransferase activity kit is a complete kit for the screening of candidate compounds that may alter normal acetyltransferase activity. Please read the complete kit insert before performing this assay.

Acetylation is an important covalent molecular modification. Originally identified as the method by which certain bacteria were able to deactivate anti-microbial compounds, acetylation is now also known as an important partitioning and signaling modification. Acetyltransferases are enzymes that covalently transfer an acetyl group from a donor molecule (Acetyl CoA) to an acceptor. Acetyl CoA serves as a universal donor while the acceptor varies with the acetyltransferase. Acceptors include histones, kinases, transcription factors, receptors, neurotransmitter precursors like choline and serotonin, and anti-microbial agents like chloramphenicol and fluoroquinones. Acetylation can signal an increase or decrease in activity based on the context of the message. Frequently located at critical junctions in metabolic pathways, Acetyltransferases and their regulation have become attractive therapeutic targets to treat everything from insomnia to cancer.

**Principle**

1. Enzyme and reaction mix spiked with acceptor substrate are added to wells of a black 96-well plate. The plate is then incubated.
2. Ice cold isopropyl alcohol is added to stop the reaction. The detection solution is then added and the plate is again incubated.
3. The plate is transferred to a plate reader and fluorescence is measured at 380ex/520em.
Materials Supplied

1. **Black Microtiter Plate**
   One plate of 96 wells, Catalog No. 80-1675
   The plate is ready to use.

2. **Transferase Assay Buffer Concentrate**
   15 mL, Catalog No. 80-1648
   A 10X concentrated buffer containing detergent and preservative.

3. **Acetyltransferase Reaction Buffer Concentrate**
   0.1 mL, Catalog No. 80-1651
   A 50X concentrated buffer containing Acetyl CoA.

4. **Transferase Detection Solution Concentrate**
   0.15 mL, Catalog No. 80-1650
   A 100X concentrated solution of fluorescent substrate in DMSO.

5. **Acetyltransferase Positive Control**
   0.2 mL, Catalog No. 80-1647
   A Stock Solution of buffer and reaction end product.

6. **Foil Plate Sealer**
   3 each, Catalog No. 10-3126

7. **Acetyltransferase Assay Layout Sheet**
   1 each, Catalog No. 30-0240
Storage

Acetyltransferase Reaction Buffer Concentrate and Transferase Detection Solution concentrate must be stored at -20°C, and Acetyltransferase Positive Control must be stored at -70°C. Transferase Assay buffer Concentrate can be stored at -20°C or 4°C. All kit components are stable at their recommended storage temperatures until the kit expiration date. Recommended storage temperatures do not necessarily reflect shipping conditions.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Acetyl CoA dependent acetyltransferase.
3. Appropriate acceptor substrate.
4. Inhibitor/activator compounds to be screened.
5. Precision pipets for volumes between 5 μL and 1,000 μL.
6. Disposable beakers for diluting buffer concentrates.
7. Graduated cylinders.
8. A microplate shaker.
9. Disposable microtubes, 0.5 and 1.5 mL.
10. Microplate reader capable of measuring fluorescence at 380ex/520em.
11. Crushed ice and container.
12. Isopropyl alcohol (ice cold).
Reagent Preparation

1 Transferase Assay Buffer

Prepare the assay buffer by diluting 15mL of the supplied Transferase Assay Buffer Concentrate with 135mL of deionized water. This solution can be stored at 4°C for 3 months, or the kit’s expiration, whichever is earlier. The 1X assay buffer is used to prepare dilutions of Acetyltransferase Reaction Buffer, Transferase Detection Solution, enzymes, substrates, and compounds to be screened.

2 Transferase Detection Solution

Count the total number of wells needed for compound screening and add 6 (for the zero, positive control, and blank wells in duplicate). Use the following formula to calculate the volume of 1X Detection Solution required.

A. Total volume required
   \[ \text{Total number of wells needed} + 6 \times 100 \, \mu\text{L} = \underline{_______} \, \mu\text{L} \]

B. Volume of Transferase Detection Solution Concentrate required
   \[ \text{Total volume required (from A. above)} \times 0.01 = \underline{_______} \, \mu\text{L} \]

C. Volume of 1X Transferase Assay Buffer required
   \[ \text{Total volume required (from A. above)} \times 0.99 = \underline{_______} \, \mu\text{L} \]

Prepare 1X Detection Solution by combining the appropriate reagent volumes calculated in B and C above. For example, to prepare 2 mL of 1X Detection Solution, combine the following volumes: 20 \( \mu\text{L} \) of the supplied Transferase Detection Solution Concentrate and 1980 \( \mu\text{L} \) 1X Transferase Assay Buffer. Diluted Detection Solution should be kept on ice and used within 4 hours of preparation. Any unused 1X Detection Solution should be discarded.

3 Acetyltransferase Reaction Mix

Count the total number of wells needed for compound screening and add 4 (for the zero and blank wells, in duplicate). Use the following formula to calculate the volume of 1X Acetyltransferase Reaction Mix required.

A. Total volume required
   \[ \text{Total number of wells needed} + 4 \times 25 \, \mu\text{L} = \underline{_______} \, \mu\text{L} \]

B. Volume of Acetyltransferase Reaction Buffer Concentrate required
   \[ \text{Total volume required (from A. above)} \times 0.02 = \underline{_______} \, \mu\text{L} \]

C. Volume of acceptor substrate required
   \[ \text{Determined empirically based on enzyme used} = \underline{_______} \, \mu\text{L} \]
D. Volume of 1X Transferase Assay Buffer required

\[ \text{[Total volume required (from A. above)] \times 0.98 - [volume of substrate required]} = \underline{\text{__________ \( \mu \)L}} \]

Prepare 1X Reaction Mix on ice by combining the appropriate reagent volumes calculated in B, C and D above. For example, to prepare 2 mL of Reaction Mix spiked with 22 \( \mu \)L of acceptor substrate, combine the following volumes: 40 \( \mu \)L Acetyltransferase Reaction Buffer Concentrate, 22 \( \mu \)L substrate and 1938 \( \mu \)L 1X Transferase Assay Buffer. Diluted Reaction Mix should be kept on ice and used within 8 hours of preparation. Any unused 1X Reaction Mix should be discarded.

4 Positive Control

A positive control is included in the kit to verify the activity of the kit components. It should not be used to calculate the concentration of acetyltransferase activity in samples. Store the Positive Control at -70°C and avoid freeze-thaws.

Prepare enough Positive Control to test in duplicate. Use the following formula to calculate the volume of Positive Control required. A minimum of 2 wells of the positive control are recommended per assay.

A. Total volume required

\[ \text{[Total number of wells needed]} \times 50 \mu L = \underline{\text{__________ \( \mu \)L}} \]

B. Dilute Acetyltransferase Positive Control Stock 1:100 by adding 5\( \mu \)L of Positive Control to 495\( \mu \)L of 1X Transferase Assay Buffer.

C. Volume of 1:100 Positive Control required

\[ \text{[Total volume required (from A. above)] \times 0.025 = \underline{\text{__________ \( \mu \)L}} \]

D. Volume of 1X Transferase Assay Buffer required

\[ \text{[Total volume required (from A. above)] \times 0.975 = \underline{\text{__________ \( \mu \)L}} \]

Prepare Positive Control on ice by combining the appropriate reagent volumes calculated in C and D above. For example, to prepare 200\( \mu \)L of Positive Control, combine the following volumes: 5\( \mu \)L of (1:100) Positive Control and 195\( \mu \)L of 1X Transferase Assay Buffer. Diluted Positive Control should be kept on ice and used within 2 hours of preparation. Any unused 1X Positive Control should be discarded.
**Sample Handling**

This assay is suitable for use with all Acetyl CoA-dependent acetyltransferases. It is necessary to titrate each enzyme / substrate system in the assay to determine optimal conditions.

This assay should only be used to screen purified *in vitro* samples in buffer systems without reductants.

It is recommended that an end-point assay be performed to determine the optimal concentration of enzyme/substrate to use prior to screening candidate compounds. Make serial dilutions of the acetyltransferase of interest in the assay buffer. Initial concentrations of 100 nM are recommended. A kinetic assay format is also an available option.

The positive control provided may also be used to test colored compounds for interference in the assay. A suitable protocol follows.

**End point / Kinetic Assay Procedure**

Refer to the Assay Layout Sheet to determine the number of wells to be used. Cover unused wells tightly with a plate sealer. **DO NOT REUSE WELLS!**

1. Pipet 25 µL of 1X Transferase Assay Buffer into the blank wells.
2. Pipet 25 µL of acetyltransferase dilutions into the bottom of the appropriate wells.
3. Pipet 25 µL of 1X Reaction Mix into each well.
4. Cover plate with foil plate sealer. Incubate for 30 min (for end point format) shaking* at room temperature.

   For **Kinetic** measurements, incubate identical reaction wells for the desired periods of time. The duplicate wells for each time point can be stopped as in step #6 below.

5. Pipet 50 µL of 1X Positive Control into the bottom of the appropriate wells.
6. Pipet 50 µL of ice cold isopropyl alcohol into each well.
7. Pipet 100 µL 1X Detection Solution into each well.
8. Cover plate with foil plate sealer. Incubate for 10 min at room temperature, without shaking.
9. Read fluorescence at 380ex/520em.

* Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.
Example of Endpoint Assay Results

1. Plot the mean of the duplicate relative fluorescence units (RFU) at 380ex/520em versus Enzyme concentration. (Figure 1)

2. Calculate the signal-to-noise ratio: $\frac{\text{Mean RFU for enzyme dilution}}{\text{Mean RFU blank}}$.

![Figure 1: Chloramphenicol Acetyltransferase Titration in Acetyltransferase Assay](image)

Chloramphenicol acetyltransferase (CAT) was titered in the assay using 100µM of the Substrate chloramphenicol. Serial dilutions of the enzyme were prepared in Transferase Assay Buffer. Mean relative fluorescence was plotted against CAT concentration to generate the following graph. This is for illustration purposes only. The investigator must titrate their enzyme / substrate system in the assay.

Based on this titration data, the acetyltransferase concentration of 100nM produces a maximum signal within the detection range of the plate reader, with a signal to noise ratio sufficient for easy detection of altered enzyme activity.

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.
**Example Kinetic Assay Results**

100nM CAT was tested with 100μM Chloramphenicol substrate in the kinetic assay format. Mean relative fluorescence was plotted against the stop time interval to generate this graph. This graph is for illustration purposes only. The kinetic assay must be optimized by the investigator, with their enzyme / substrate system.
Typical Inhibition Assay Procedure

Refer to the Assay Layout Sheet to determine the number of wells to be used. Cover unused wells tightly with a plate sealer. **DO NOT REUSE WELLS!**

1. Pipet 25μL of 1X Transferase Assay Buffer into the blank wells.
2. Pipet 10μL of 1X Transferase Assay Buffer into the zero wells.
3. Pipet 10μL of inhibitor dilution into the bottom of the appropriate wells.
4. Pipet 15μL of acetyltransferase at chosen working concentration into appropriate wells.
5. Cover plate with foil plate sealer. Incubate for 10 min at room temperature without shaking.
6. Pipet 25μL of 1X Reaction Mix into each well.
7. Cover plate with foil plate sealer. Incubate for 30 min shaking* at room temperature.
8. Pipet 50μL of ice cold isopropyl alcohol into each well.
9. Pipet 100μL of 1X Detection Solution into each well.
10. Cover plate with foil plate sealer. Incubate for 10 min at room temperature without shaking.
11. Read fluorescence at 380ex/520em.

* Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.

**Calculation of Results**

Several options are available for the calculation of the inhibition of acetyltransferase. We recommend that the data be handled by a software package utilizing a suitable curve fitting program to determine the percent inhibition. If data reduction software is not readily available, the data can be calculated as follows:

1. Calculate the mean net RFU for each sample by subtracting the mean blank RFU from the mean RFU for the samples:
   
   Mean Net RFU = Mean Sample RFU - Mean Blank RFU

2. Percent inhibition should be calculated using the following formula for each inhibitor dilution:

   Percent Inhibition = \[
   \frac{(\text{Mean Net Zero (non-inhibited enzyme) RFU} - \text{Mean Net Inhibited enzyme RFU}) \times 100}{\text{Mean Net Zero (non-inhibited enzyme) RFU}}
   \]
**Typical Inhibition Assay Results**

Using Chloramphenicol Acetyltransferase (CAT) and Chloramphenicol as an enzyme substrate system, percent inhibition for dilutions of Iodoacetamide was tested. The results shown below are for illustration only and **should not** be used to calculate results from another assay.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Inhib. Conc. (mM)</th>
<th>Mean RFU</th>
<th>Mean Net RFU</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100mM</td>
<td>8,707</td>
<td>2,161</td>
<td>93.6%</td>
</tr>
<tr>
<td>2</td>
<td>50mM</td>
<td>15,575</td>
<td>9,029</td>
<td>73.1%</td>
</tr>
<tr>
<td>3</td>
<td>25mM</td>
<td>24,951</td>
<td>18,405</td>
<td>45.2%</td>
</tr>
<tr>
<td>4</td>
<td>12.5mM</td>
<td>30,110</td>
<td>23,564</td>
<td>29.8%</td>
</tr>
<tr>
<td>0</td>
<td>0mM</td>
<td>40,127</td>
<td>33,581</td>
<td>0%</td>
</tr>
<tr>
<td>blank</td>
<td>------</td>
<td>6,889</td>
<td>0</td>
<td>------</td>
</tr>
</tbody>
</table>

**Typical Inhibition Curve**

![Typical Inhibition Curve](image)
**Typical Enzyme Interference Assay Procedure**

*Prepare dilutions of the colored compound to be screened in 1X Transferase Assay Buffer.

1. Pipet 50μL of 1X Transferase Assay Buffer into the blank wells.
2. Pipet 25μL of 1X Transferase Assay Buffer into the zero wells.
3. Pipet 25μL of colored compound dilution into appropriate wells.
4. Pipet 25μL of 1X Positive Control into the zero wells, and wells containing colored compound dilutions.
5. Pipet 50μL of ice cold isopropyl alcohol into each well.
6. Pipet 100μL 1X Detection Solution into each well.
7. Cover plate with foil plate sealer, incubate for 10 min at room temperature without shaking.
8. Read fluorescence at 380ex/520em.

To determine whether or not colored compounds will interfere with the assay, calculate and compare the signal to noise ratio of the colored compound dilutions to the signal to noise ratio of the zero wells.

**Interfering Substances**

The following solvents were tested for interference with the fluorescent signal generated in the assay. The table lists the percentage of signal in the presence of interferant relative to the zero for each solvent.

<table>
<thead>
<tr>
<th>% Interferant</th>
<th>DMSO</th>
<th>DMF</th>
<th>Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>108</td>
<td>68</td>
<td>49</td>
</tr>
<tr>
<td>6.25</td>
<td>107</td>
<td>82</td>
<td>71</td>
</tr>
<tr>
<td>3.12</td>
<td>107</td>
<td>88</td>
<td>86</td>
</tr>
<tr>
<td>1.56</td>
<td>112</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>0.78</td>
<td>114</td>
<td>104</td>
<td>95</td>
</tr>
<tr>
<td>0.39</td>
<td>115</td>
<td>109</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Percent interferant is relative to a 50 μL total reaction volume.

Diluents containing bovine serum albumin (BSA), or other Thiol containing reagents, should be treated with N-Ethylmaleimide (NEM) prior to use in the assay. We recommend reacting 10% BSA with 1 mM NEM for 1 hour at room temperature, however this procedure should be optimized for each reagent.
References


Notes
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