



AMP'D[®] ELISA Signal Amplification Kit

Catalog #: ENZ-KIT-100

Sizes

ENZ-KIT-100-0001: 1 assay

ENZ-KIT-100-0005: 5 assays



Product Manual

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Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Please contact Enzo Life Sciences Technical Support if necessary.

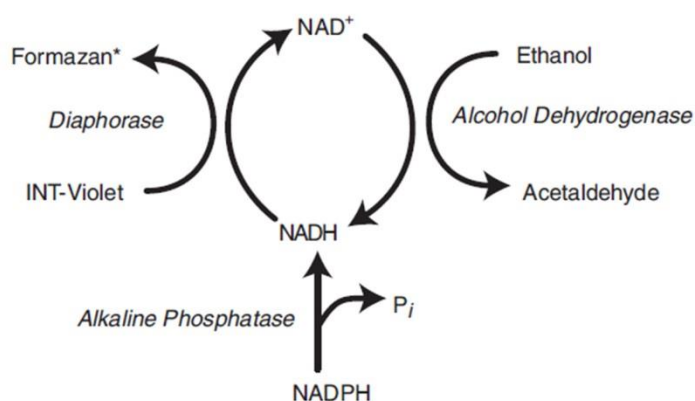
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DESCRIPTION

The AMP'D® ELISA Signal Amplification kit is designed to replace traditional alkaline phosphatase (AP) substrates, such as pNPP (p-Nitrophenyl phosphate), with a combination substrate and amplifier system that results in greater sensitivity when compared to a classic (sandwich) substrate enzyme linked-immunosorbent assay (ELISA). In a conventional detection system, enzyme bound to the microtiter plate interacts directly with the substrate producing a color change where the resulting absorbance is directly proportional to the amount of captured analyte. In the AMP'D ELISA system, bound AP converts a substrate that is utilized in a second enzyme reaction system which is initiated by addition of the amplifier reagent. It is this amplification step that allows for greater (amplified) color production at lower analyte concentrations resulting in an increase in assay sensitivity.

INTRODUCTION

The critical substrate component, NADPH, when added to wells containing AP, is reduced to NADH via release of a phosphate group. This reaction is allowed to proceed for an amount of time with the accumulated NADH being proportional to the amount of analyte/bound AP-conjugate. Upon the addition of the reconstituted amplifier reagent, this first reaction is quenched and the NADH feeds a second redox enzyme system. Here diaphorase utilizes NADH to reduce the idonitrotetrazolium salt into formazan (purple color) producing NAD⁺. A counter enzymatic reaction then occurs where the NAD⁺ is reduced to NADH while ethanol is oxidized to acetaldehyde via alcohol dehydrogenase. This set of enzymatic reactions is also allowed to proceed for a period of time, recycling the NADH thus amplifying the original AP/substrate reaction. The resulting color intensity is ultimately proportional to the amount of bound analyte. The reaction schematic is shown below.



*Colored end product

Alkaline phosphatase signal amplification.



Handle
with care



Avoid
freeze /
thaw cycles

SAFETY WARNINGS & PRECAUTIONS

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- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

MATERIALS SUPPLIED

1. **AMP'D Substrate,**
Lyophilized, Catalog No. 80-2596
ENZ-KIT-100-0001 (1 assay): 1 vial
ENZ-KIT-100-0005 (5 assays): 5 vials
Lyophilized signal amplification substrate containing NADPH.
2. **AMP'D Amplifier**
Lyophilized, Catalog No. 80-2598
ENZ-KIT-100-0001 (1 assay) 1 vial
ENZ-KIT-100-0005 (5 assays) 5 vials
Lyophilized signal amplification amplifier containing alcohol dehydrogenase and diaphorase.
3. **AMP'D Substrate diluent**
ENZ-KIT-100-0001 (1 assay): Catalog No. 80-2597, 6 mL
ENZ-KIT-100-0005 (5 assays): Catalog No. 80-2630, 30 mL
A 1x diluent, containing preservatives, used to reconstitute lyophilized substrate.
4. **AMP'D Amplifier diluent**
ENZ-KIT-100-0001 (1 assay): Catalog No. 80-2599, 6 mL
ENZ-KIT-100-0005 (5 assays): Catalog No. 80-2631, 30 mL
A 1x diluent, containing preservatives, used to reconstitute lyophilized amplifier.



Reagents require separate storage conditions.

STORAGE

Upon receipt, the kit should be stored at 4°C. The substrate and amplifier are provided lyophilized. Once reconstituted, the substrate is stable at 4°C for 1 week and the amplifier is stable at -20°C for 1 week. A slight pink color may develop in the amplifier over time but this will not negatively affect the assay results.

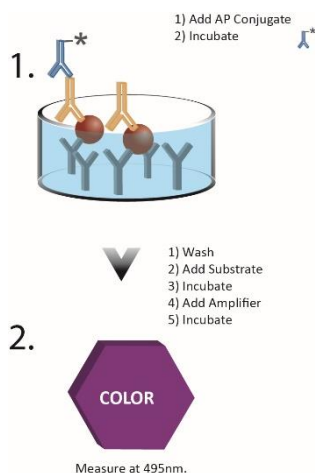
OTHER MATERIALS NEEDED

1. **0.3M H₂SO₄ Stop Solution:** To 59 mL of deionized water, slowly add 1 mL of concentrated sulfuric acid (MW 98, specific gravity 1.84, purity 96-98%). **Note:** When using sulfuric acid, exercise caution and follow manufacturer's safety recommendations. When making dilutions **always add acid to water.**
2. **Wash buffer:** Tris buffered saline (TBS): 0.05M Tris-HCl, 0.1M NaCl, pH 7.5.
3. Deionized or distilled water.
4. Precision pipets for volumes between 50 µL and 1,000 µL.
5. Repeater pipet for dispensing 50 µL.
6. Graduated cylinders.
7. A microplate shaker.
8. Microplate reader capable of reading at 495 nm.

PROCEDURAL NOTES

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Reconstitute the substrate 10 minutes before use by adding 5.5ml of 1x substrate diluent directly to the substrate vial. Gently mix until dissolved. The reconstituted substrate is stable at 4°C for 1 week.
4. Reconstitute the amplifier 10 minutes before use by adding 5.5ml of 1x amplifier diluent directly to the amplifier vial. Gently mix until dissolved. The reconstituted substrate is stable at -20°C for 1 week.
5. Avoid using phosphate in assay wash buffers as it may potentially inhibit the substrate reaction.
6. The substrate and amplifier reactions develop rather quickly and require very precise timing. To ensure identical incubation times in each well, add substrate, amplifier and stop solution in the same well-to-well order.

RECOMMENDED SIGNAL AMPLIFICATION SYSTEM PROTOCOL



1. Complete a traditional ELISA that includes alkaline phosphatase as the enzyme for substrate conversion.
2. Remove excess alkaline phosphatase by washing the wells thoroughly (4x400 μ L) with TBS buffer.
3. Pipet 50 μ l of the reconstituted Substrate Solution into each well.
4. Incubate for 15 minutes at room temperature on a plate shaker at ~500 rpm.
5. Pipet 50 μ L of the reconstituted Amplifier Solution into each well. **It is important to add the substrate and amplifier to the wells in the same sequence.**
6. Incubate for 15 minutes at room temperature on a plate shaker at ~500 rpm.
7. To stop the reaction, pipet 50 μ L of 0.3M H₂SO₄ to each well and read the absorbance at 495 nm.

FACTORS THAT MAY AFFECT RESULTS

1. Substrate and Amplifier Incubation Steps:

Both steps of the two-step amplification process have linear reaction kinetics. In the substrate incubation, NADPH is dephosphorylated to NADH. This reaction is stopped by the addition of the amplifier which contains phosphate to inhibit the AP enzymatic reaction, in addition to the enzymes required for amplification. In the amplifier incubation, the rate of color development is proportional to the amount of NADH produced during the substrate incubation. For a given amount of bound AP-conjugate, the optimal sensitivity versus background signal is obtained when the substrate and amplifier incubation times are identical. Increasing both incubation times equally will result in a higher signal than increasing either incubation alone.

2. Length of Incubation:

Incubation times for the amplification reagents can vary depending on the sensitivity/signal desired or required. Our recommendation is an overall incubation of 30 minutes – 15 minutes for the substrate and 15 minutes for the amplifier. This would give an approximate 10-fold increase in sensitivity. If more or less sensitivity is required, incubation time should be adjusted appropriately. For example, if the

overall incubation time is increased from 30 to 60 minutes, the individual substrate and amplifier incubation times would be 30 minutes each. This should be done to achieve optimal signal/sensitivity versus background signal.

Signal amplification in sandwich ELISA.

Total incubation time of Amplification Reagents (min)	Approximate Fold Increase in Sensitivity
30 (15 for substrate, 15 for amplifier)	8
60 (30 for substrate, 30 for amplifier)	20
90 (45 for substrate, 45 for amplifier)	50

It should be noted that increasing the overall time for incubation will increase the signal in wells containing the target antigen but may also increase the signal in background wells. It is important to determine the incubation time(s) that will maximize both signal and sensitivity while keeping background acceptable.

3. Temperature:

Since the amplification reactions are driven by enzymes, the temperature at which these reactions take place is an important factor. We recommend that the amplification reagents be used at a constant temperature between 20°C and 25°C.

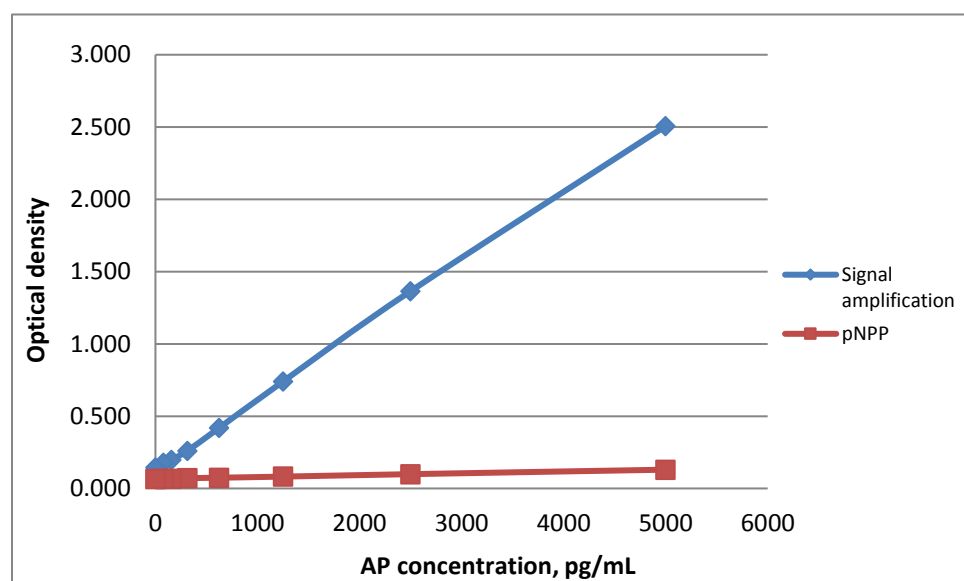
4. Reagent Volumes and Concentrations:

Even higher signal may be achieved by increasing the final assay volumes and perhaps by using a more concentrated amplifier. However, the reagents provided in this kit have been paired and we only provide stability and performance specifications for reaction volumes and concentrations as described in this kit.

TYPICAL ALKALINE PHOSPHATASE TITER

Signal from a titer of AP was measured using either a traditional pNPP substrate (405 nm) or reconstituted signal amplification reagents (495 nm). Up to a 10-fold increase in sensitivity was observed with the new Signal Amplification system. The results shown below are for illustration only and should not be used to calculate results.

Sample	Signal amplification reagents	pNPP	AP conc (pg/mL)
S1	2.51	0.130	5,000
S2	1.36	0.099	2,500
S3	0.74	0.082	1,250
S4	0.42	0.075	625
S5	0.26	0.071	312.5
S6	0.20	0.069	156.3
S7	0.18	0.068	78.1
S8	0.14	0.065	0





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NOTES



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