

## Video Article

# Colorimetric Analysis of Alkaline Phosphatase Activity in *S. Aureus* Biofilm

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## Abstract

Alkaline phosphatase (ALP) is a common enzyme expressed in both prokaryotic and eukaryotic cells. It catalyzes the hydrolysis of phosphate monoesters from many molecules at basic pH and plays an indispensable role in phosphate metabolism. In humans, eukaryotic ALP is one of the most frequently used enzymatic signals in diagnosing various diseases, such as cholestasis and rickets. In *S. aureus*, ALP is detected exclusively on the cell membrane; it is also expressed as a secretory form as well. Yet, little is known about its function in biofilm formation.

The purpose of this manuscript is to develop a quick and reliable assay to measure ALP activity in *S. aureus* biofilm that does not require protein isolation. Using p-nitrophenyl phosphate (pNPP) as a substrate, we measured ALP activity in *S. aureus* biofilm formed in 96-well tissue culture plates. Activity was based on the formation of the soluble reaction product measured by 405 nm absorbance. The high throughput nature of the 96 well tissue culture plate method provides a sensitive and reproducible method for ALP activity assays. The same experimental set up can also be extended to measure other extracellular molecular markers related to biofilm formation.

## Introduction

Alkaline phosphatase (ALP) is ubiquitously expressed in both prokaryotic and eukaryotic cells<sup>1</sup>. It can catalyze the hydrolysis of monophosphate from different molecules such as nucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid. In humans, eukaryotic ALP is present in many tissues including liver, bone, intestine and placenta<sup>2</sup>. It plays important roles in protein phosphorylation, cell growth, apoptosis, stem cell processes as well as normal skeletal mineralization. Eukaryotic ALP is also a key serum indicator for the presence of diseases in bone, liver, and other tissues/organs when elevated<sup>3,4</sup>.

Prokaryotic ALP has been detected in a variety of bacterial cells, including *E. coli*<sup>5</sup>, *S. aureus*<sup>6,7</sup> and some common rumen bacteria in the soils<sup>8</sup>. Bacterial ALP activity has been used as a biosensor in the detection of pesticides, heavy metals<sup>9</sup> and bacterial contamination<sup>10</sup>. The constitutive expression of ALP has been used to identify *Staphylococci*<sup>11</sup> and to differentiate *Serratia* from *Enterobacter*<sup>12</sup>. It is further suggested that constitutive ALP production is correlated with pathogenicity in *staphylococci*<sup>13</sup>. Although ALP has been studied in different settings<sup>3,4</sup>, yet little is reported for its activity and function in biofilms cultures.

A biofilm has been documented to have a differently functioning bacterial life as compared with its free-living bacterial cell counterpart<sup>14</sup>. In *S. aureus*, the formation of biofilm has been identified in a variety of clinical conditions and accounts for the antibiotic resistance and chronic inflammation<sup>15,16</sup>. Many molecules had been reported to be found in a biofilm matrix such as polysaccharides, proteins, nucleic acids, and lipids, but the mechanism of biofilm formation is not fully understood<sup>14</sup>. To understand the role of ALP in biofilm formation, we cultured *S. aureus* biofilms in 96 well tissue culture plates and measured the ALP activity using para-nitrophenylphosphate (pNPP).

The molecule pNPP is a ready-to-use substrate for ALP and has been widely used to measure ALP activity<sup>6,17,18</sup>. This colorimetric assay is based on the conversion of para-nitrophenyl phosphate (pNPP) to para-nitrophenol resulting in a colored product at 405 nm. Compared to other conventional ALP assay, such as agarose gel electrophoresis<sup>19</sup>, wheat germ agglutinin (WGA) precipitation, and WGA-HPLC<sup>20</sup>, this assay is highly specific, sensitive, easy to reproduce, and most importantly, allows for high throughput.

## Protocol

### 1. Medium preparation

1. Prepare 1 L of Tryptic Soy Broth (TSB): Into 1 L of distilled water, add 15 g of pancreatic digest of casein, 5 g of papaic digest of soybean meal, 3 g of sodium chloride, 2.5 g of dextrose, and 2.5 g of dipotassium phosphate. Sterilize before use.
2. For Tryptic Soy Agar (TSA), add 15 g of agar to 1 L of TSB, autoclave. Then let it cool down to room temperature. Next, pour at a ratio of 20 mL per Petri dish (100 mm x 15 mm).
3. Biofilm culture medium: Add 10 g of glucose to 1 L of autoclaved TSB. Sterilize by filtration using a 0.2 µm filter.

## 2. *S. aureus* biofilm formation in 96 well tissue culture plate

NOTE: The *S. aureus* biofilm is cultured as previously described<sup>6,21</sup>.

1. Inoculate one individual colony of *S. aureus* from TSA in 10 mL of TSB and grow overnight at 37 °C.
2. Dilute the overnight culture into 10 mL of TSB/glucose (10 g/L) at a 1:100 (v/v) ratio. Vortex gently to mix for a consistent concentration.
3. Transfer 200  $\mu$ L of diluted culture into a total of 18 wells (more for experimental groups, such a non-biofilm control to suggest a relationship to ALP activity<sup>6</sup>) from one 96 well plate. Use triplets for each time point: 0 min, 15 min, 30 min, 45 min, 60 min, and 75 min. See step 3.2.
4. Incubate the 96 well plate at 37 °C for 24 h for biofilm formation<sup>6,21</sup>.
5. Decant the supernatant by aspiration.
6. Wash each well with 1x phosphate buffered solution (PBS, pH 7.4), centrifuge for 5 min at 15,000 x g and decant the supernatant by aspiration.

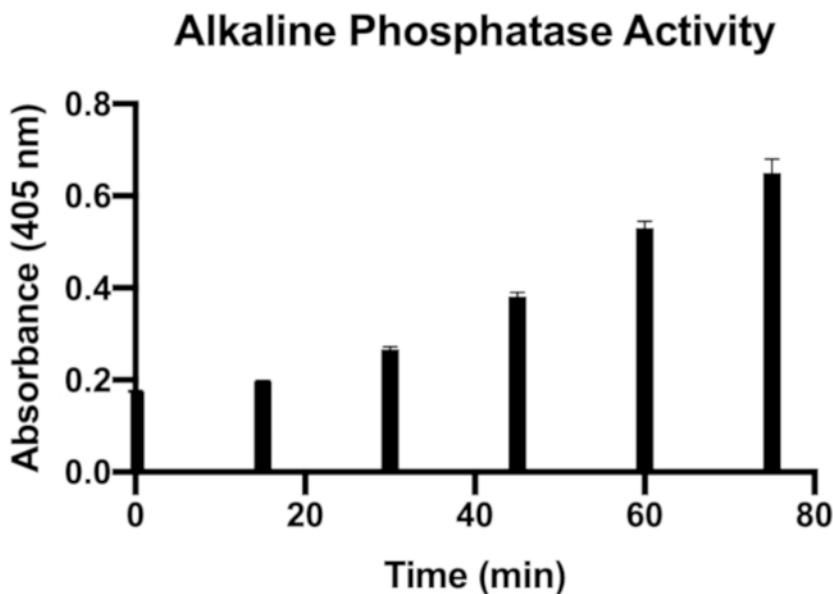
## 3. Measurement of ALP activity in *S. aureus* biofilm

NOTE: ALP activity was measured as described<sup>6,18</sup>.

1. Add 75  $\mu$ L of buffered ALP substrate consisting of commercially available pNPP to each well from step 2.6 and begin recording the time. Record each time point in triplicate.
2. After incubation for a varied time: 0 min (control), 15 min, 30 min, 45 min, 60 min, and 75 min respectively, add 75  $\mu$ L of 5 M NaOH to stop the reaction.
3. Centrifuge the plate for 5 min at 15,000 x g.
4. Transfer 100  $\mu$ L of supernatant into a new 96 well plate for colorimetric measurement.
5. Measure the absorbance of each well at 405 nm using a 96 well plate reader. Use the 0 min time point wells to control for 405 nm background noise.
6. Repeat the entire experiment in three individual 96 well plates.

### Representative Results

**Figure 1** shows a representative result of ALP activity from biofilm cultures of *S. aureus* in 96 well tissue culture plates. 75  $\mu$ L of commercially available pNPP solution was added to each well and incubated at room temperature. After incubation for different times (0 min, 15 min, 30 min, 45 min, 60 min, and 75 min, respectively) 75  $\mu$ L of 5 M NaOH was added to stop the reaction. The product was then measured in a 96 well plate reader at 405 nm. Each time point was repeated in triplets from a single 96 well plate, and the entire experiment was repeated in 3 individual 96 well plates.



**Figure 1:** *S. aureus* biofilm cultures were incubated with pNPP substrate for different time (0 min, 15 min, 30 min, 45 min, 60 min and 75 min) and their ALP activity was measured at 405 nm. Error bars represent standard deviation. [Please click here to view a larger version of this figure.](#)

### Discussion

In our assay, we used pNPP as the ALP substrate. This is a working solution designed for ELISA and no dilution is needed. After hydrolysis by ALP, a yellow product develops and can be measured at 405 nm. At the end of the enzymatic reaction, we briefly centrifuged the 96 well plate

and transferred the supernatant to a fresh 96 well plate to measure absorbance using the plate reader. We found that this extra centrifugation step is critical, since it increases the consistency of absorbance at 405 nm, probably by eliminating any possible suspended cells incurred during enzymatic reaction.

For biofilm formation, we use 200  $\mu$ L of a 1:100 (v/v) dilution for overnight culture as reported<sup>6,21</sup>. This is the optimum dilution for *S. aureus* to form biofilm compared to other dilutions (data not shown). Since the purpose of this paper is to measure ALP activity in biofilm, it was crucial that we maintained optimum biofilm culture conditions. This was further emphasized by the choice in using glucose at 10 g/L for optimum biofilm growth<sup>6</sup>. The dilution factor and glucose concentration need to be optimized if different bacterial cells are used for biofilm formation.

During incubation of the biofilm culture with pNPP substrate, it will take time for the colored product to develop. We measured ALP activity after 15- and 30-min time points which is when the visible yellow color is observed. With the current concentration, we noticed an increased ALP activity for up to 75 min as indicated in **Figure 1**. After 75 min, no increase of the ALP activity was observed.

Finally, since ALP in *S. aureus* is an exclusively membrane bound protein<sup>7</sup>, its activity can be tested without cell lysis. There are benefits to an enzyme activity assay that does not require whole cell destruction. Notably, the protein isolation process can sometimes yield a lower active enzyme quantity<sup>22</sup>. However, this protocol cannot be used if ALP is not in its membrane bound form.

As previously reported<sup>6</sup>, ALP activity is elevated in biofilm culture as compared to its platonic counterpart. The experimental settings used in the current study can be extended to investigate factors/compounds that affect ALP activity in *S. aureus* biofilm. The findings from these studies will provide additional insight into how to control bacterial biofilm formation.

## Disclosures

The authors have nothing to disclose.

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