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## An In Vitro Model to Study the Effect of 5-Aminolevulinic Acid-Mediated Photodynamic Therapy on Staphylococcus aureus Biofilm --Manuscript Draft--

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**TITLE:**

An *In Vitro* Model to Study the Effect of 5-Aminolevulinic Acid-mediated Photodynamic Therapy on *Staphylococcus Aureus* Biofilm

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**KEYWORDS:**

Photodynamic therapy, 5-aminolevulinic acid, biofilm, *Staphylococcus aureus*, protocol, model, *in vitro*

**SUMMARY:**

This manuscript describes a protocol to study the antimicrobial effect of 5-aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) on a *Staphylococcus aureus* biofilm. This protocol can be used to develop an *in vitro* model to study the treatment of bacterial biofilms with PDT in the future.

**ABSTRACT:**

*Staphylococcus aureus* (*S. aureus*) is a common human pathogen, which causes pyogenic and systemic infections. *S. aureus* infections are difficult to eradicate not only due to the emergence

of antibiotic-resistant strains but also its ability to form biofilms. Recently, photodynamic therapy (PDT) has been indicated as one of the potential treatments for controlling biofilm infections. However, further studies are required to improve our knowledge of its effect on bacterial biofilms, as well as the underlying mechanisms. This manuscript describes an *in vitro* model of PDT with 5-aminolevulinic acid (5-ALA), a precursor of the actual photosensitizer, protoporphyrin IX (PpIX). Briefly, mature *S. aureus* biofilms were incubated with ALA and then exposed to light. Subsequently, the antibacterial effect of ALA-PDT on *S. aureus* biofilm was quantified by calculating the colony forming units (CFUs) and visualized by viability fluorescent staining via confocal laser scanning microscopy (CLSM). Representative results demonstrated a strong antibacterial effect of ALA-PDT on *S. aureus* biofilms. This protocol is simple and can be used to develop an *in vitro* model to study the treatment of *S. aureus* biofilms with ALA-PDT. In the future, it could also be referenced in PDT studies utilizing other photosensitizers for different bacterial strains with minimal adjustments.

## INTRODUCTION:

*S. aureus* is an important Gram-positive pathogen that colonizes the skin and mucosa of human hosts. Its ability to form biofilms is considered an important aspect of its pathogenesis<sup>1</sup>. Bacterial biofilms are a community of bacteria embedded in a self-produced matrix, which is composed of extracellular polymeric substances, including polysaccharide, DNA, and protein. This matrix plays a significant role in the persistence of bacterial infections, contributing to a high degree of resistance to the human immune system and current anti-microbial therapies<sup>2</sup>. Antibiotics are still the major treatment for biofilm infections, although the effects of antibiotics on biofilms are limited. It has been shown previously that cells in biofilms are 10–1,000 times more resistant to antibiotics compared to their planktonic counterparts<sup>3</sup>. Thus, alternative strategies are needed to conquer this issue.

PDT, an alternative treatment for bacterial infections, uses the light of an appropriate wavelength to activate photosensitizers. This leads to the production of reactive oxygen species (ROS), which are lethal to target cells by disrupting the cell wall, inactivating enzymes, and damaging DNA<sup>4</sup>. This multi-target characteristic makes it difficult for bacteria to develop resistance to the PDT treatment.

The antimicrobial effect of PDT on bacterial and fungal biofilms, with multiple photosensitizers, such as toluidine blue, malachite green, methylene blue, chlorine e6, and porphyrins, has been studied in previous reports<sup>5-13</sup>. 5-ALA, a prodrug of the actual photosensitizer, PpIX, is characterized by its small molecular weight and rapid clearance<sup>12,14</sup>. These advantages give ALA-PDT major potential as a therapeutic application. Although the effect of ALA-PDT on planktonic bacteria has been studied by many groups<sup>12</sup>, the antimicrobial effect of ALA-PDT on bacterial biofilms has not yet been elucidated. Meanwhile, it is difficult to compare the results between previous studies. One of the reasons is that the different protocols are used by diverse groups. Thus, this protocol describes an *in vitro* model of an ALA-PDT system based on our previous work<sup>15</sup>. The effect of this model was confirmed by CFU calculation and viability staining with CLSM.

**PROTOCOL:**

**1. Biofilm Formation**

**1.1. Biofilm formation in 96-well microplates**

1.1.1. Retrieve the *S. aureus* strain USA300 and 3 biofilm-forming clinical strains (C1–C3) stored at -80 °C.

Note: The ability of the clinical strains to form biofilms was determined by the microtiter plate assay described previously<sup>15</sup>.

1.1.2. Inoculate the bacterium in 5 mL tryptone soya broth (TSB) medium, and cultivate in an incubator with shaking at 37 °C overnight to the stationary phase.

1.1.3. Centrifuge the overnight bacterial culture at 4,000 x g for 10 min at room temperature and then discard the supernatant. Resuspend the pellets in phosphate buffered saline (PBS) to a final concentration of  $2.0 \times 10^9$  CFU/mL.

Note: The concentration of the bacteria was estimated by measuring optical density and further determined by plate count<sup>16</sup>, revealing that 1 OD<sub>600</sub> of suspension contained  $1.5 \times 10^8$  CFU/mL.

1.1.4. Dilute the bacterial suspension to 1:200 ( $1.0 \times 10^7$  CFU/mL) in TSB medium containing 0.5% glucose. Inoculate 200 µL of bacterial suspension into each well of a cell-culture-treated polystyrene 96-well microplate.

1.1.5. Incubate the microplate statically at 37 °C for 24 h under a well oxygenated environment.

Note: The incubation time for mature biofilm formation may vary for different bacterial strains; this should be determined before the PDT experiment<sup>15</sup>.

1.1.6. Discard the media in the wells and wash the microplate wells gently with PBS three times and then discard the supernatant.

Note: The step should be carried out very gently to avoid disturbing the formed biofilm.

**1.2. Biofilm formation in dishes**

1.2.1. Inoculate the *S. aureus* strain USA300 into 5 mL of TSB medium, and cultivate in an incubator with shaking at 37 °C overnight to the stationary phase.

1.2.2. Centrifuge the overnight bacterial culture at 4,000 x g for 10 min at room temperature and then discard the supernatant. Resuspend the pellets in PBS to a final concentration of  $2.0 \times 10^9$  CFU/mL. Then, dilute the bacterial suspension to 1:200 ( $1.0 \times 10^7$  CFU/mL) in TSB medium containing 0.5% glucose. Inoculate 2 mL of bacterial suspension into a 35-mm optical quality glass

bottom cell culture dish, and incubate statically at 37 °C for 24 h.

Note: The concentration of the bacteria was estimated by measuring the optical density.

1.2.3. Aspirate the media with a pipette, and then rinse the biofilms in the dish gently with PBS three times and then, discard the supernatant carefully.

Note: Avoid touching the pipette tip to the bottom of the dish. The step 2.2 should be performed immediately after this step to prevent drying of the formed biofilm.

## **2. Light Irradiation**

2.1. Store 5-ALA in a 4 °C refrigerator. Before the experiment, dilute 5-ALA with PBS to 10 mM.

Note: 5-ALA solution should be freshly prepared before the experiment.

2.2. In the experimental group, add 200 µL of 10 mM ALA to each well of the microplate or 2 mL to the culture dish. Cover the plate/dish with aluminum foil, and incubate for 1 h. Then, irradiate the plate/dish with a light-emitting diode (LED) with a light intensity of 100 mW/cm<sup>2</sup> for 1 h to achieve a light dose of 360 J/cm<sup>2</sup> at a major wavelength of 633 ± 10 nm<sup>17</sup>.

Note: In order to let the light energy be effectively and equally delivered to the biofilm in all of the wells/dishes, fix the distance from the peak of the light source to the well/dish at 6.0 cm, and limit the experimental region to the central irradiation area (10 cm x 8 cm). To ensure that the results are reproducible, the experiments should be performed at the same room temperature. In the LED irradiation step, to avoid direct exposure of the plate to other light sources, such as sunlight, room lighting or lamplight, the LED was turned on before moving the plate/dish to the irradiation area, and the light was bright enough to finish the operation.

2.3. Set up the control groups (three control groups were set up in our experiment).

2.3.1. For the first control group (ALA-LED-), add 200 µL of PBS to each well of the microplate or 2 mL to the culture dish. Cover the plate/dish with aluminum foil, and incubate it for 2 h.

2.3.2. For the second control group (ALA+LED-), add 200 µL of 10 mM ALA to each well of the microplate or 2 mL to the culture dish. Cover the plate/dish with aluminum foil, and incubate it for 2 h.

2.3.3. For the third control group (ALA-LED+), add 200 µL of PBS to each well of the microplate or 2 mL to the culture dish. Cover the plate/dish with aluminum foil, and incubate it for 1 h. Then expose the plate to the LED with 360 J/cm<sup>2</sup> light irradiation at a major wavelength of 633 ± 10 nm<sup>17</sup>.

Note: In the LED irradiation step, avoid direct exposure of the plate to other light sources, such as sunlight, room lighting, or lamplight.

### **3. Determination of the Effectiveness of PDT Treatment**

Note: To confirm the effect of ALA-PDT on the *S. aureus* biofilms, the viability of the cells with or without ALA-PDT was evaluated by CFU counting as well as by viability staining.

#### **3.1. Determination of the remaining viable bacterial cells**

3.1.1. After ALA-PDT treatment, discard the media in the wells and wash the wells with PBS three times to remove all non-adherent cells for both experimental and control groups.

Note: This step should be carried out very gently.

3.1.2. Scrape the adherent bacteria cells thoroughly from the wells with the pipette tip and collect the cells in conical tubes.

3.1.3. Centrifuge the bacterial suspension at 4,000 x g for 10 min at 4 °C, then discard the supernatant.

3.1.4. Resuspend the bacteria in 1 mL of 0.25% pancreatin enzyme in PBS and incubate at 37 °C for 1.5 h.

3.1.5. Centrifuge at 4,000 x g for 10 min; discard the supernatant, then resuspend the pellet in 200 µL of PBS.

3.1.6. Make 1:10 serial dilutions of the cell solution with PBS; then, add 5 µL of each serial dilution sample onto the tryptone soya agar (TSA) plate. Incubate the TSA plate at 37 °C for 16 h; then, count (by naked eye) the number of bacterial colonies (CFU/mL).

#### **3.2. Observation of *S. aureus* biofilms by CLSM**

3.2.1. After light irradiation, wash the biofilms in the culture dish with PBS three times.

Note: This step should be carried out very gently.

3.2.2. Add 1 mL of 1 µM green-fluorescent nuclear and chromosome stain that is permeable to the prokaryotic cell membranes (*e.g.*, SYTO9) and 1 mL of 1 µM propidium iodide (PI) for 20 min to stain the biofilm as well as dead cells.

3.2.3. Observe viable cells (green fluorescence, Ex/Em 485 nm/530 nm) and dead cells (red fluorescence, Ex/Em 485 nm/630nm) under a CLSM with a 63X 1.4-NA oil-immersion objective lens.

3.2.4. Generate images using microscopy software.

## REPRESENTATIVE RESULTS:

The viability of the bacteria in the biofilms was decreased after ALA-PDT treatment when compared to the controls (ALA-LED-, ALA+LED-, and ALA-LED+) in both USA300 and the three clinical strains (**Figure 1**).

To confirm the results from the CFU assay and observe the antibacterial effect of ALA-PDT on the *S. aureus* biofilm *in situ*, the USA300 biofilms were visualized by CLSM with viability staining. The viable and dead cells were stained with green and red fluorescence, respectively. The image showed that most of the bacteria in the biofilms were killed by ALA-PDT, which was consistent with the results of the CFU assay (**Figure 2**).

## FIGURE AND TABLE LEGENDS:

**Figure 1: The effect of ALA-PDT on biofilms.** CFU/mL was log-transformed and shown as the mean  $\pm$  standard deviation in USA300 and three clinical strains (C1–C3) treated with ALA-PDT (ALA+LED+) or under the control conditions (ALA-LED-, ALA+LED-, ALA-LED+).

**Figure 2: Representative CLSM images of *S. aureus* biofilms with LIVE/DEAD staining.** Biofilms formed by USA300 bacteria were treated with or without ALA-PDT (panel A: ALA-LED-, panel B: ALA+LED-, panel C: ALA-LED+, panel D: ALA+LED+), and then stained with SYTO9 (green fluorescence) and PI (red fluorescence) to represent the live and dead bacteria independently. A 63X 1.4-NA oil immersion objective was used.

## DISCUSSION:

PDT has been a well-studied therapy for the treatment of cancer since it was invented more than 100 years ago<sup>18</sup>. Over the last decade, PDT has been applied as an antimicrobial strategy and has shown effectiveness against some antibiotic-resistant pathogenic bacteria<sup>12</sup>. Compared to the planktonic state, bacterial biofilms appear to be more resistant to antibiotic treatment<sup>3</sup>, while the effect of ALA-PDT on biofilms has not been fully investigated yet.

In this article, an *in vitro* ALA-PDT system was described, and the antibacterial effect of this model on *S. aureus* biofilms was demonstrated. Two methods were used to test the effect of ALA-PDT on *S. aureus* biofilms in this protocol. While the CFU test demonstrated the antimicrobial effect by calculating the viable cells after treatment, fluorescent viability staining with CLSM not only confirmed the results of the CFU test but also detected the morphological character of the live and dead bacteria *in situ*. Using both analytical techniques together is an ideal approach for determining the effect of ALA-PDT on biofilms. Based on the CLSM results, the dead bacterial cells were predominantly distributed in the upper layer, while some of the bacteria in the bottom layer remained alive<sup>15</sup>. The latter may be the source of bacterial colonies in the CFU assay. A similar result has been observed in a study conducted by O'Neill *et al.*, which was explained by the low accumulation of photosensitizers in the inner layer or the inability of the light to penetrate these regions<sup>19</sup>.

In this protocol, the mature biofilm was incubated with 10 mM of ALA for 1 h before exposure to

PDT. These parameters were chosen based on the results of two pre-experiments. First, the antimicrobial effect of ALA was tested against biofilms without light irradiation using different concentrations of ALA and different amounts of time for incubation with *S. aureus* biofilms. The groups without any bactericidal effect were chosen as the candidates. Second, the PDT effect was detected in these candidate groups, and the group with the most potent bactericidal effect was finally chosen in this protocol. Thus, the parameters used in this protocol ensured that there was no bactericidal effect of ALA alone. A 1-h incubation time is significantly shorter than those used in previous studies<sup>17,20</sup>, making it convenient for *in vitro* studies and for potential application in future clinical treatments.

There are several critical points for successful use of this model. First, the whole process involving the manipulation of ALA should be performed in darkness. Second, the manipulation of the mature biofilms should be gentle to avoid disturbing the formed biofilm. Third, in the CFU test, scraping the bacteria from the bottom of the plates should be thorough. Finally, the freshly prepared ALA should be used; therefore, it is better to prepare ALA right before the experiment.

Although this protocol can be used to test the effect of ALA-PDT on *S. aureus* biofilms *in vitro*, it is still different from the clinical situation *in vivo*. For example, biofilms in the human body are usually formed by multiple bacterial strains<sup>21,22</sup>, and the environment *in vivo* is more complex than that *in vitro*, which might influence the effect of PDT. Therefore, future *in vivo* experiments are needed for a full evaluation of the antibacterial effects of ALA-PDT on *S. aureus* biofilms. However, because of its advantages of convenience and the ethical issues of conducting *in vivo* studies, this *in vitro* platform will be useful and practical to improve the study of the effects of ALA-PDT on *S. aureus* biofilms. It should also be noted that although ALA, a precursor of the photosensitizer PpIX, has favorable characteristics, including rapid clearance, less and shorter-lasting cutaneous photosensitivity, limited light penetration restricted to superficial lesions and especially its non-cumulative toxicity<sup>14</sup>, the light dose and photosensitizer concentration required to achieve bacteria killing may still have a bystander effect on host cell viability. Thus, studying the modification of ALA<sup>23</sup> for targeted therapy of selected pathogenic bacterial strains like *S. aureus* is valuable for future antimicrobial therapy.

This protocol can not only be used for the study of the effect of ALA-PDT on *S. aureus* strains in the future but can also be referenced to study its effect on biofilms formed by other bacteria. The parameters, such as the concentration of ALA and the duration of the bacteria incubation with ALA, may vary among different bacterial strains, but the principles discussed above are commonly shared.

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**DISCLOSURES:**

The authors have nothing to disclose

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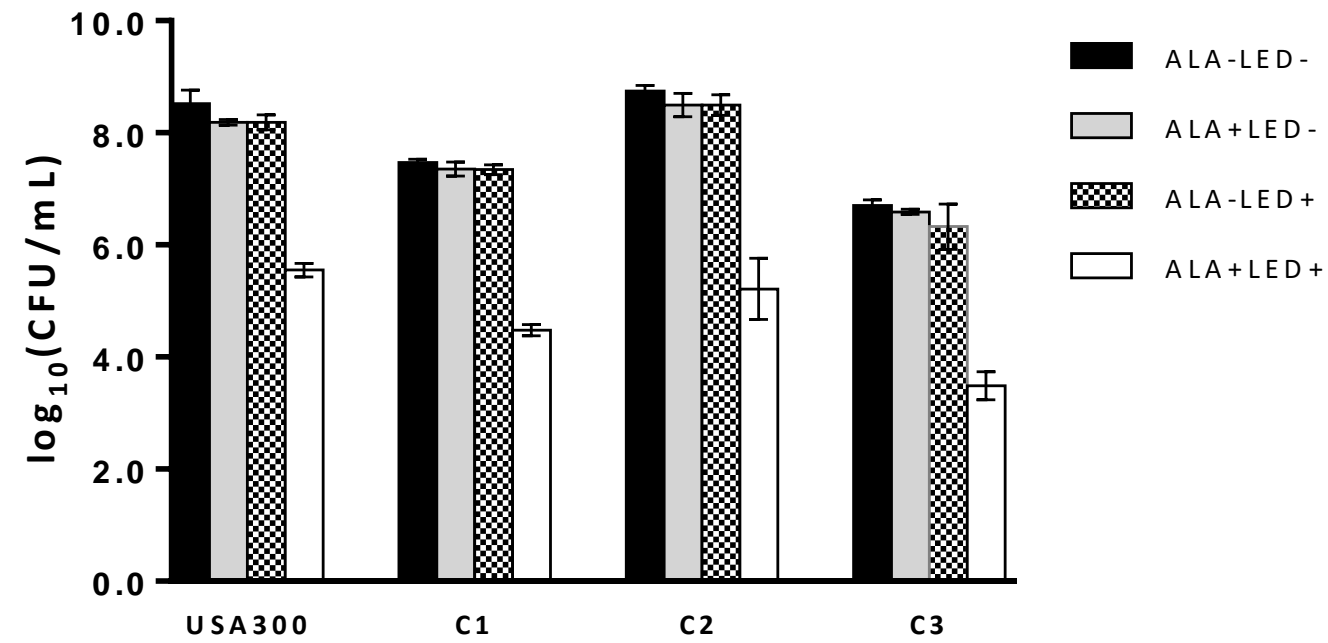
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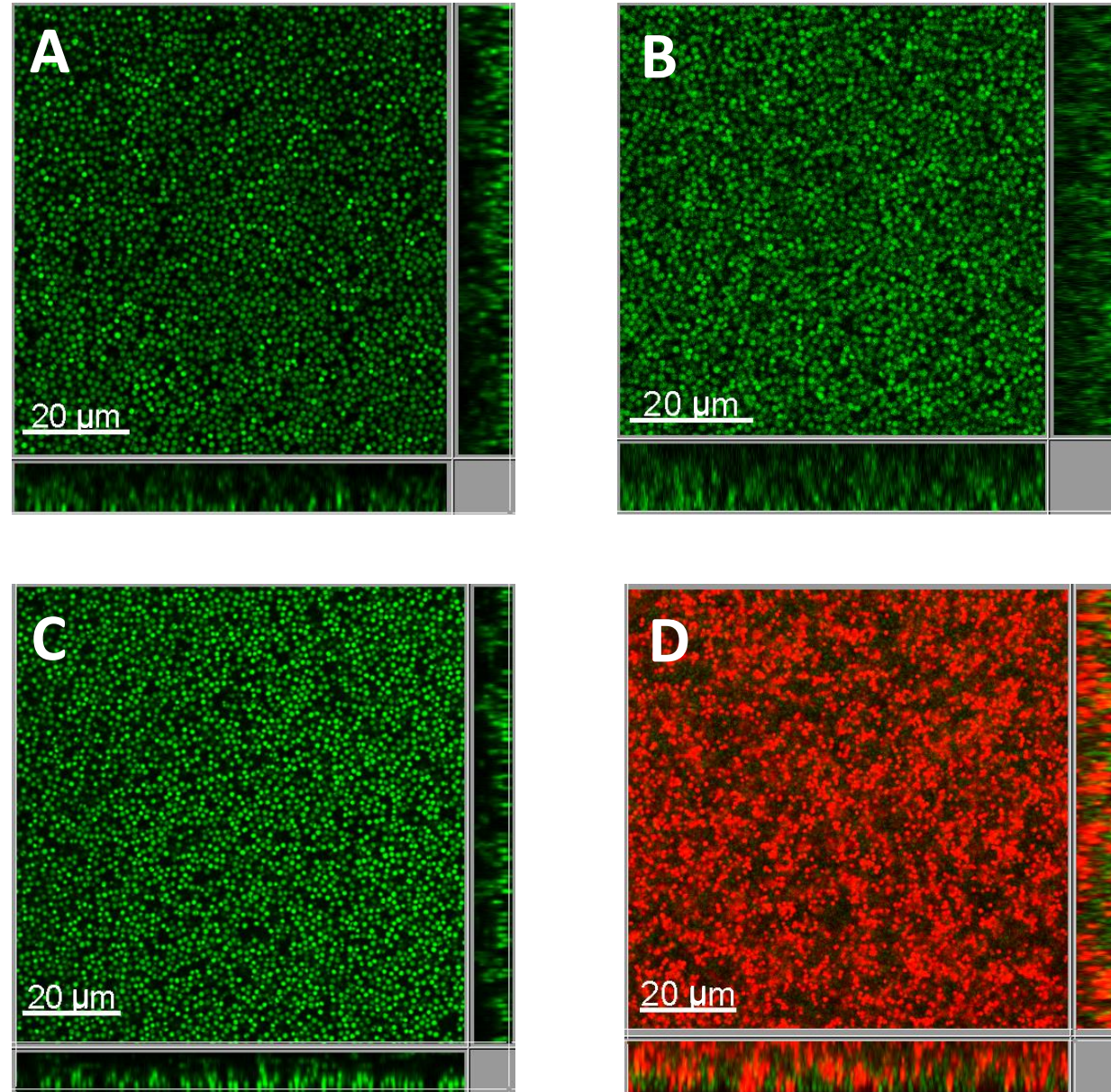
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**Fig 1**

**Fig 2**

Name of Material/ Equipment	Company	Catalog Number
Tryptone Soya Broth (TSB)	OXOID	CM0129B
Tryptone Soya Agar (TSA)	OXOID	CM0131
SYTO9	Thermo Fisher Scientific	L7012
Propidium iodide (PI)	Thermo Fisher Scientific	L7012
Pancreatin	Sigma-Aldrich	P3292
5-aminolevulinic acid （ALA）	Fudan Zhangjiang Bio-Pharm	3.1
<i>Staphylococcus aureus</i> strain USA300	/	/
<i>Staphylococcus aureus</i> clinical strains (C1-C3)	/	/
96-well microplate	Corning Inc	3599

Fluorodish	NEST Biotechnology	801001
Eppendorf Safe-Lock Tubes, 1.5 mL	Eppendorf	0030120086
Eppendorf microcentrifuge 5417	Eppendorf	Z365998   SIGMA
Incubator	Thermo Fisher Scientific	SHKE4000
Light emitting diode (LED)	Wuhan Yage Optic and Electronic Technique CO	LED-IB
Leica TCS SP8 confocal laser-scanning microscope	Leica Microsystems	
Leica LAS AF software	Leica Microsystems	
IMARIS software	Bitplane	

Comments/Description
The LIVE/DEAD BacLight Bacterial Viability Kits
The LIVE/DEAD BacLight Bacterial Viability Kits
The source of USA 300 references “Tenover FC, Goering RV. J Antimicrob Chemother. 2009 Sep; 64(3):441-6”.
All clinical strains were isolated from patients with chronic rhinosinusitis in the Department of Otorhinolaryngology-Head and Neck Surgery, Eye and ENT Hospital of Fudan University [Zhang QZ, Zhao KQ, Wu Y, et al. PLoS One. 2017 Mar; 12(3): e0174627].
Clear Flat Bottom Polystyrene TC-Treated Microplates, Individually Wrapped, with Lid, Sterile

Glass bottom, Non-pyrogenic
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Article Title:

An in vitro Model to Study the Effect of 5-aminolevulinic acid-mediated Photodynamic Therapy on Staphylococcus aureus Biofilm

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Date:

Nov. 15th, 2017

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We are submitting a revised version of our manuscript “An *In Vitro* Model to Study the Effect of 5-Aminolevulinic Acid-Mediated Photodynamic Therapy on *Staphylococcus aureus* Biofilms” (JoVE57604\_R1). We would like to thanks for the reviewer’s insightful comments and have addressed the comments in the revised manuscript. All changes are highlighted with green text. Below are our point-by-point responses to the comments and questions.

Editorial comments:

1. Need a reference.

R: Following the editor’s suggestion, we have added a citation [Harris F, et al. *Med Res Rev.* 2012, 32 (6): 1292-1327] as reference 12. (Line 72)

2. Mention centrifuge speed (in g), and duration. Is the supernatant discarded?

R: Following the editor’s suggestion and question, we have revised the manuscript to read as follows: “Centrifuge the overnight bacterial culture at 4000 x g for 10 min at room temperature and then discard the supernatant.” (Lines 94-95)

3. When and how is the CFU/ml measured?

R: Thanks for your question, we have added the following description in the step 1.1.3: “The concentration of the bacteria was estimated by optical density and further determined by plate count<sup>16</sup>, revealing that 1 OD<sub>600</sub> of suspension contained 1.5 x10<sup>8</sup> CFU/ml.” (Lines 95-97)



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4. How did you determine the formation time? If you have described this in more detail, please cite a reference.

R: Thanks for your question, the detail of biofilm assay has been reported in our previous work [Zhang QZ, et al. PLoS One. 2017, 12(3): e0174627]. We have cited this work as reference 15 in the revised manuscript. (Line 107)

5. Is the media in the dish first aspirated?

R: Thanks for your question, we have revised the statement to now read “Discard the media in the wells and wash the microplate wells gently with PBS three times and then discard the supernatant.” (Lines 109-110)

6. If there are any care instructions e.g. avoid touching the pipette tip to the bottom of the well, please add it as a note.

R: The step should be carried out very gently to avoid disturbing the formed biofilm as the description in the Note of 1.1.6. (Line 112)

7. Mention centrifuge speed (in g), and duration. Is the supernatant discarded?

R: Following the editor's suggestion and question, we have revised the manuscript to read as follows: “Centrifuge the overnight bacterial culture at 4000 x g for 10 min at room temperature and then discard the supernatant.” (Lines 119-120)

8. When and how is the CFU/ml measured?

R: Thanks for your question, we have added the following description in the step 1.2.2: “The concentration of the bacteria was estimated by optical density.” (Lines 120-121)

9. Is the media in the dish first aspirated?

R: Thanks for your question, we have revised the statement to now read “Aspirate the media with pipette, and then rinse the biofilms in the dish gently with PBS three times; then, discard the supernatant carefully.” (Lines 126-127)



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10. It looks like it will be important to also state that 2.2 should be performed immediately after this to prevent drying of the films.

R: Following the editor's suggestion, we have added the following description in the Note of 1.2.3: "The step 2.2 should be performed immediately after this step to prevent drying of the formed biofilm." (Lines 129-130)

11. If there are any care instructions e.g. avoid touching the pipette tip to the bottom of the well, please add it as a note.

R: Following the editor's suggestion, we have added the following description in the Note of 1.2.3: "Avoid touching the pipette tip to the bottom of the dish." (Line 129)

12. This should be performed with ambient light turned off. How is this achieved?

R: Thanks for the editor's question. Before we move the plate/dish to the irradiation area, the LED has been turned on, and the light is enough for us to finish the operation to avoid exposure of the plate/dish to the ambient light. We have added the following description in the Note of 2.2: "In the LED irradiation step, to avoid direct exposure of the plate to other light sources, such as sunlight, room lighting or lamplight, LED was turned on before moving the plate/dish to the irradiation area, and the light was bright enough to finish the operation." (Lines 148-150)

13. Why not just say 2 hours here and delete the next sentence?

R: Thanks for your suggestion, we have revised the statement to now read "For the first control group (ALA-LED-), add 200  $\mu$ L of PBS to each well of the microplate or 2 mL to the culture dish. Cover the plate/dish with aluminum foil, and incubate it for 2 h" (Lines 154-155)

14. Still in the dark?

R: Yes, the plate/dish were covered with aluminum foil in the process.



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15. Please note and verify the edits.

R: Following the editor's suggestion, we have revised the edits to now read "Then expose the plate to the LED with 360 J/cm<sup>2</sup> light irradiation at a major wavelength of 633 ± 10nm<sup>17</sup>. (Lines 163-164)

16. Also room lighting?

R: We have added the following description in the Note of 2.3.3: "In the LED irradiation step, avoid direct exposure of the plate to other light sources, such as sunlight, room lighting or lamplight." (Lines 166-167)

17. Is the PBS/ALA aspirated before washing?

R: We have revised the description to now read "After ALA-PDT treatment, discard the media in the wells and wash the wells with PBS three times to remove all non-adherent cells for both experimental and control groups." (Lines 176-177)

18. What kind of scraping tool do you use?

R: Thanks for your question. We used pipette tip as the scraping tool in our experiment. This information has been added in the step 3.1.2: "Scrape the adherent bacteria cells thoroughly from the wells with the pipette tip and collect in conical tubes." (Lines 181-182)

19. 0.25 % in PBS?

R: We have revised the description of 3.1.4 to now read "Resuspend the bacteria in 1 ml of 0.25% pancreatin enzyme in PBS and incubate at 37 °C for 1.5 h." (Lines 187-188)

20. How are the colonies counted/calculated? Under a microscope? Mention magnification.

R: Thanks for your question. The bacterial colonies were counted by naked eye.





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21. 1 mL 1  $\mu$ M?

R: Thanks for your question. We have revised the description of 3.2.2 to now read “1 mL 1 $\mu$ M propidium iodide (PI) for 20 min to stain the biofilm as well as dead cells.” (Lines 206-207)

22. Reference?

R: Following the editor’s suggestion, we have added a citation [St Denis TG, et al. Virulence. 2011, 2(6): 509-520] as reference 18. (Line 240)

23. Reference?

R: Following the editor’s suggestion, we have added a citation [Mah TF, et al. Trends Microbiol. 2001, 9(1): 34-39] as reference 3. (Line 242)

24. How did you test for this? This should likely be added to the results section along with a figure showing this. OR cite a reference where you have already published this.

R: Thanks for your question. This phenomenon has been published in our previous work [Zhang QZ, et al. PLoS One. 2017, 12(3): e0174627]. We have cited this work as reference 15 in the revised manuscript. (Line 253)

25. Needs references.

R: Following the editor’s suggestion, we have added a citation [Shi H, et al. Photodiagnosis Photodyn Ther. 2016, 15: 40-45] as reference 14. (Line 286)

26. Reference

R: Following the editor’s suggestion, we have added a citation [Wu J, et al. ACS Appl Mater Interfaces. 2017, 9(17): 14596-14605] as reference 23. (Line 288)



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