Test cycle #

The antibacterial synergetic effect of titanium dioxide and blue light - experiment

All included operations need to be performed according to the protocol, all exceptions must be written down.
Each operation section on the cover page should be signed by the performing team member.

Resuspension of the freeze-dried bacteria

30 min

Date: Signature:

Pipetting of the suspension onto the test tiles

60 min

Signature:

Photon disinfection

2 h | 4 h | 6 h

Signature:

Dissolving of the bacteria into buffer solution

30 min

Signature:

Preparation of the dilution series

15 min

Signature:

Plating and incubation

15 min

Signature:

Counting of the CFUs

30 min

Date: Signature:

Introduction

The purpose of this experimentation is to observe the gained benefit of the use of TiO_2-coating during photon disinfection. This is achieved by comparing the viability of the model bacteria spread onto coated and non-coated test tiles. The inactivation is monitored in four time points, taking place every two hours. To control for the cell death not caused by aBL, identical control samples are created and kept in dark during the photon disinfection process.

One test cycle contains 48 samples in total, half of which are controls. All samples including controls are evenly divided between TiO_2-coated and non-coated test tiles. Following photon disinfection, tiles are furtherly processed in four batches of twelve according to their time point.

The duration of a test cycle is one full workday of nine hours and – on the following day – a one hour counting period of the formed bacterial colonies. After each cycle, all generated waste must be disposed of according to the common biolaboratory practices.

Used strain

The selected bacteria for this experiment is gram-negative model bacteria *Escherichia coli*.

First batch was freeze-dried in 10th of January and it contains about 108 million CFUs per ampule according to the viability test performed in 11th of January.

Second batch was freeze-dried in 28th of February with a higher concentration of 1.78 billion CFUs per ampule; viability tested in 5th of March.

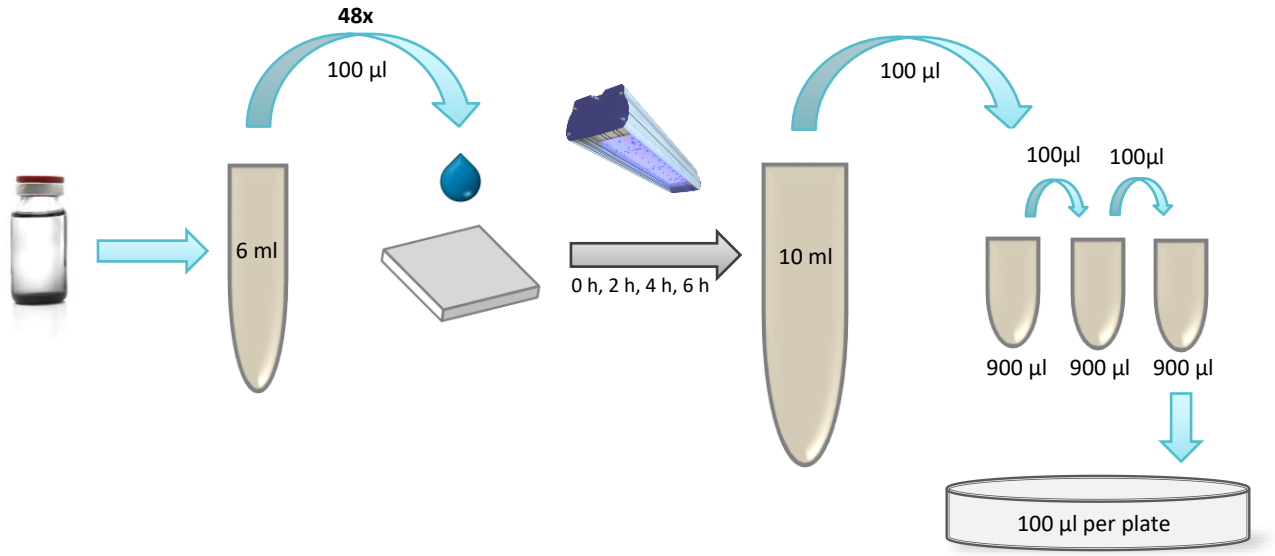
Figure 1. Ampule

Term definitions

Suspending – In this context refers to the preparation of cell suspension. Suspension refers to heterogenic mixture where solid particles have been diluted into liquid, in this case cells.

Vortexing – Refers to mechanic stirring of the solution in test or sample tubes with a vortex mixer. Stirring intensity is described in the form of rounds per minute (rpm).

Figure 2. Process diagram of the test cycle



Preparations

Before the start of each test cycle, required supplies need to be gathered and checked. These include the needed amount of growth medium, buffer solution, petri-dishes and sterile laboratory material. All necessary equipment can be found from the bio laboratory of *Turku University of Applied Sciences*.

Table 1. Required supplies for one test cycle

Supply	Pcs per round	Remarks
Auto pipette, 10 - 100 µl	1 pcs	Calibration
Auto pipette, 100 - 1 000 µl	1 pcs	Calibration
Auto pipette, 1 000 - 5 000 µl	1 pcs	Calibration
Pipette tip box, 50 - 200 µl	1 pcs	Autoclaving
Pipette tip box, 100 - 1 000 µl	1 pcs	Autoclaving
Pipette tip box, 1 000 - 5 000 µl	1 pcs	Autoclaving
Eppendorf-tubes, 1.5 ml	144 pcs	Autoclaving
Falcon-tubes, 15 ml	1 pcs	-
Falcon-tubes, 50 ml	48 pcs	-
Eppendorf-tube rack, 3 x 6 spots	2 pcs	-
Falcon-tube rack, 12 spots	2 pcs	-
Drigalski-spatula	48 pcs	Disposable
TSA-plates	48 pcs	-
TSB-medium	5 ml	Autoclaving
PBS-solution	~610 ml	Autoclaving

For each round, falcon-tubes and petri-dishes must be carefully labeled to avoid any intermingling of the samples. Markings should not smear when ethanol disinfection is applied as a part of aseptic operating in laboratory. Due to the high number of samples, following markings system is implemented.

Samples: T#S#P#

Controls: T#C#P#

Table 2. Marking system

Marking	Explanation	Example
T#	Number of time point	**A4**N#R#
S#	Number of sample	A#**N2**R#
C#	Number of control	A#**K2**R#
P#	Number of parallel	A#N#**R3**

TSB-medium and PBS-solution are prepared according to the following instructions. Before reaching the desired volume, pH needs to be adjusted to 7.4 ± 0.2.

Table 3. TSB-medium reagents, 1L

Reagent	Tryptone	Soytone	Sodium chloride
Description / Formula	Assortment of peptides	Enzymatic digest of soy	NaCl
Amount in mass	15 grams	5 grams	15 grams

Table 4. PBS-solution reagents, 1L

Reagent	Sodium chloride	Soytone	Disodium phosphate	Monopotassium phosphate
Description / Formula	NaCl	KCl	Na_2HPO_4	KH_2PO_4
Amount in mass	8 grams	0.2 grams	1.44 grams	0.24 grams

Resuspension of the freeze-dried bacteria

Freeze-dried ampules are stored in -80 °C and should be retrieved only just before the start of the cycle. Ampules should only be handled with cut resistant gloves, this is due to the extreme stress ampules are exposed during the freeze-drying process. Inside the ampules there is a mild vacuum of nitrogen to improve shelf life. The number of needed ampules is dependent on the used batch and on the desired cell concentration – translating into the amount of countable CFUs.

I. Supplies are **gathered** into a class II microbiological safety cabinet (MSC II), all materials and the cabinet itself is **disinfected** with ethanol

- Auto pipette
  - 100 - 1 000 microliters (µl)
- Pipette tip box
  - 100 - 1 000 µl
- Falcon-tube
  - 15 milliliters (ml)
- Falcon-tube rack
- Beaker

II. Ampules containing the freeze-dried bacteria, are **disinfected** and **transferred** into MSC II

- Needed number of ampules must be calculated beforehand

  *Number of samples include 6 + 6 spare ones (1.2 ml) to ensure that suspension suffices to all test tiles

  **Dilution factor

$$PMYs = \frac{viable\ cells}{samples^{*}} \cdot DF^{**} = \frac{n_{vc}}{(30+30)} \cdot 10^{-5}$$

$$viable\ cells = PMYs \cdot samples^{*} \cdot DF^{**} = n_{PMY} \cdot (30+30) \cdot 10^{-5}$$

$$ampules = \frac{viable\ cells}{viable\ cells\ per\ ampule} = \frac{n_{vc}}{n_{vc}/ampule}$$

III. Rubber caps of the ampules are **opened**

IV. TSB-medium is **pipetted** to each opened ampule

- Tip needs to be changed, in the case of accidental contact with the ampule
- 1.0 ml per ampule

V. Ampules are **closed** with the rubber caps they came with

VI. Ampules are severally **vortexed**

- Inside MSC II
- 20 seconds (s)
- 2 000 rpm

VII. The content of ampules is equally **divided** between two 15 ml falcon-tubes

- If necessary, suspension is diluted by using TSB-medium
- 3.0 ml per falcon-tube

VIII. Filled falcon-tubes are **stored** in a cold room, in the case of necessary wait time

- +4 °C

Pipetting of the suspension onto the test tiles

Test tiles are made out of stainless steel and are 2.0 x 2.0 square centimetres (cm^2) in size. Half of them are coated with titanium dioxide (TiO_2) nanoparticles, which increases the hydrophobicity of the tile-surface. Pipetting onto the test ties should be performed inside the same MSC II where the photon disinfection takes place. Tiles should be placed thusly, that once the light is switched on all the tiles are exposed to the same level of intensity. Control samples are pipetted onto test tiles in another safety cabinet, one without a light source. In order to minimize the drying time, suspension is spread onto tiles as widely as possible to increase its surface area. In both cabinets, ventilation is switched on and the work lights stay turned off, to prevent photocatalytic coating from activating.

I. Supplies are **gathered** into MSC II, all materials and the cabinet are **disinfected** with ethanol
- Auto pipette
  - 100 - 1 000 microliters (µl)
- Pipette tip box
  - 100 - 1 000 µl
- Beaker

II. 48 test tiles are **disinfected**, **dried** and **placed** into two selected safety cabinets
- All tiles should be easy to access while pipetting
- Coated and non-coated tiles should not mix
- The level of intensity should be near equal
- 12+12 tiles in each cabinet

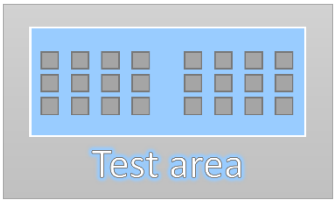


Figure 3. Test tile placement

III. Falcon-tubes which contain the suspension, are **vortexed**
- 20 s
- 2 000 rpm

IV. Falcon-tubes are **disinfected** and **transferred** into two selected safety cabinets
- Photon disinfection - cabinet
- Control storage - cabinet

V. Suspension is **pipetted** and spread onto test tiles
- Simultaneously in both cabinets
- 100 µl per test tile
  - 24 samples
  - 24 controls


Figure 4. Suspension spread onto tiles

VI. Suspension is **allowed to dry**
- ~45 min
- Until all tiles are dry

Table 5. Test tiles per cycle

	Samples		Controls	
	TiO_2-coated	Non-coated	TiO_2-coated	Non-coated
Time points	4	4	4	4
Replicates	3	3	3	3
In total	12 pcs	12 pcs	12 pcs	12 pcs

Photon disinfection

During photon disinfection test tiles are kept in two separate light isolated MSC IIs. Beforehand, outsides of the cabinets are covered with black plastic to prevent excess light from entering. Ventilation is switched off at the start of photon disinfection. In every time point, twelve (3+3+3+3) test tiles are transferred to the next operation; dissolving of the bacteria into buffer solution. During the transfer, aBL is switched off and timer is on pause. To ensure that results from all cycles are comparable, the light source and the placement area must stay still during or between test cycles.

Photon disinfection phase must start before 9:30 am, so that the samples from the last time point are ready for the next phase at 3:30 pm at the latest. Laboratory must be vacated before 4:00 pm.

I. Test tiles are **checked**, by all present team members
- Suspension has not crossed over tiles edges
- Suspension is completely dried onto the tiles surface
- Deposits are equal in size

II. Time point **zero** (0 h), twelve first test tiles are transferred into the next phase
- See the next section of the protocol

III. Light source is **switched on**
- **Start time:** _____

IV. Time point **2 h**, next twelve test tiles are transferred into the next phase
- Light source is switched off during transfer
- Timer is paused during transfer
- **Transfer time:** _____

V. Time point **4 h**, next twelve test tiles are transferred into the next phase
- Light source is switched off during transfer
- Timer is paused during transfer
- **Transfer time:** _____

VI. Time point **6 h**, test tiles are transferred into the next phase
- Light source is switched off
- Timer is stopped
- **Transfer time:** _____

Dissolving of the bacteria into buffer solution

Next three phases are performed in parts, one time point at a time. Dried bacterial deposits are dissolved from the tiles into individual 50 ml falcon-tubes by vortexing. Before the transfer, falcon-tubes are filled with 10 ml of PBS-solution inside a safety cabinet. At this point, it is important to check the labels and that the tubes are in the correct order. Transfer takes place inside the MSC IIs after which caps are closed, and tubes are collected back into the rack. In every time point, twelve test tiles are handled in identical fashion.

I. Supplies are **gathered** into MSC II, all materials and the cabinet are **disinfected** with ethanol
- Auto pipette
  - 1 000 - 5 000 µl
- Pipette tip box
  - 1 000 - 5 000 µl
- 12x Falcon-tubes
- 2x Falcon-tube rack
  - 12 spots
- Scissors
- Beaker

II. Falcon-tubes are **arranged** on the rack and the caps are **opened**
- Storage bag is opened with scissors

III. PBS-solution is **pipetted** into each falcon-tube and the caps are **closed**
- 10 ml per tube

IV. The rack is **disinfected** and **transferred** into MSC II, where photon disinfection takes place

V. Samples of the time point are **transferred** into falcon-tubes one by one according to the labels
- 6 samples per time point

VI. The rack is disinfected and transferred into MSC II, where controls are stored

VII. Controls of the time point are **transferred** into falcon-tubes one by one according to the labels
- 6 controls per time point

VIII. The rack is **removed** from the MSC II and **placed** next to vortex mixer

IX. Falcon-tubes are **vortexed** one by one
- 2 min
- 2 000 rpm

X. Processed falcon-tubes are **collected** into another rack and **checked**

Preparation of the dilution series

Three-part dilution series are prepared form the buffer solutions, containing the samples. Between preparation of every dilution, short vortexing is applied. In addition to supplies, ~120 ml of PBS-solution is needed. Sample solutions are diluted into concentration of $1{:}10^{-3}$, to achieve the desired pmy count. Prepared dilutions are transferred into the next phase.

I. Supplies are **gathered** into MSC II, all materials and the cabinet are **disinfected** with ethanol
- Auto pipette
  - 10 - 100 µl
- Auto pipette box
  - 50 - 200 µl
- 48x Eppendorf-tubes
  - 1.5 ml
- Eppendorf-tube rack
  - Sample order marked down
  - 3 x 12

II. Eppendorf-tubes are **arranged** on the rack and their caps are **opened**
- 3 in a row
- 12 in a line

III. PBS-solution is **pipetted** into each eppendorf-tube
- 900 µl per tube

IV. One row of eppendorf-tubes is **left opened** at a time, others are **closed**

V. The rack containing the falcon-tubes is **disinfected** and **transferred** into MSC II
- Samples can be processed after the completion of the previous phase

Next operations are repeated to all samples and controls

VI. Falcon-tube is **vortexed**
- 10 s
- 2 000 rpm

VII. Suspension is **pipetted** into the first opened eppendorf-tube
- 100 µl

VIII. Eppendorf-tube is **vortexed**
- 5 s
- 2 000 rpm

IX. From the vortexed eppendorf-tube, dilution is **pipetted** into the next tube
- Kärki vaihdetaan jokaisen pipetoinnin välissä
- 100 µl

X. Previous two operations are **repeated** (VIII.) until all three dilutions are ready

Plating and incubation

To beginning incubation, prepared 1:10^{-3} dilutions are pipetted onto TSA-plates. At this point MSC II should be cleared from all needles supplies, so that there is enough room for the plates. Eppendorf-tube rack should be placed next to the vortex mixer and plate labels should be checked before the start of pipetting. Samples are processed one at a time and ready plates are stacked to save workspace. Spreading of the pipetted dilution is done by disposable drigalski spatulas to speed up this operation and to maximise repeatability.

I. Supplies are **gathered** into MSC II, all materials and the cabinet are **disinfected** with ethanol
- Auto pipette
  - 100 - 1 000 µl
- Auto pipette box
  - 100 - 1 000 µl
- 12x TSA-plates
- 12x Drigalski spatulas
- Beaker

II. Eppendorf-tube, which contains the 1:10^{-3} dilution is **vortexed**
- 2 s
- 2 000 rpm

III. Vortexed eppendorf-tube is **disinfected** and **transferred** into MSC II

IV. Matching TSA-plate is **opened**, and the cap is **placed** upside down next to the plate

V. Dilution is **pipetted** onto the TSA-plate
- 100 µl

VI. Pipetted dilution is evenly **spread** onto the plates surface

VII. TSA-plates cap is **closed** and **stacked** on top of each other

VIII. Previous six operations are **repeated** (II.) until suspension is spread onto all plates

IX. Pipetted dilution is **allowed to dry**
- 5 min

X. TSA-plates are carefully **taped** together into two stacks and **incubation is initiated**
- Samples and controls into separate stacks
- Bottoms pointing up
- 16-20 h
- +37 °C
- **Aloitusaika:** _____
  - When all plates are ready

Counting of the CFUs

Each test cycle includes 48 TSA-plates, which are all counted twice. Marker used during the counting process must be erasable with ethanol. Counting is performed by two team members after which average is calculated from the two gained results. Count is repeated if samples the standard deviation (s) is 2.0 or over. Gained results are gathered into previously prepared Microsoft Excel data table.

I. TSA-plate is **placed** on top of the calculation grid and the light is **switched on**
- Bottom pointing up

II. CFUs are **counted** and **marked** down (first time)

III. Result is **inserted** into Excel table and markings are **wiped** with ethanol

IV. CFUs are **counted** and **marked** down (second time)

V. Twice counted TSA-plates are **taped** back into stacks and **stored** in a cold room
- +4 °C