| | FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM<br>*Standard Operating Procedure* | |
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| Title: Determining bacterial ODs using the Spectronic 20D+ | | | |
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# Determining bacterial ODs using the Spectronic 20D+

## FILE NAME: 5.3.2-Using the Spectronic 20D+.docx

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# SECTION 1 INTRODUCTION

## 1.1 Purpose

The purpose of this document is to provide instructions for using the Spec 20D to determining bacterial ODs and to explain the basic principles behind these measurements.

## 1.2 Scope

This SOP applies to the Food Safety and MQIP Labs.

## 1.3 Definitions

OD: Optical Density

## 1.4 Safety

Bacterial cultures are splash hazards. Lab PPE is required when working in the lab.

## SECTION 2 MATERIALS

- Spectronic 20D+ (Manufactured by Thermo Spectronic)

- Bacterial Culture (can be grown in side arm, or various tubes, or on plates).
- Appropriate tubes; 13 mm tubes (green caps), 16 mm tubes (red caps) and 18 mm tubes (blue caps).

**If dilutions are required**

- Dilute sample in the same growth medium in which your culture was cultivated.
  Example: if your sample was grown in BHI then it should be diluted in BHI.

- Tubes (13 mm, Green caps) for dilutions

- 1000 µl pipette, 200 µl pipette

# SECTION 3 PROCEDURES

## 1.1 Turn on the machine

To turn on the machine, rotate the left knob clockwise. The machine needs to warm up 15 ***minutes before use.*** Each time you use the Spec20D+ please print your name on the sign-up sheet and indicate the timeframe for which you will be using the equipment.

Since many people rely on this machine, signing in to use it does not make you the exclusive user but serves as notification to others to keep the machine on or turn off when they are finished.

Also, if you are keeping the machine on for an indefinite amount of time, place an in "use ign" on the machine so that someone does not inadvertently shut the machine off.

**3.2 Adjust the wavelength on the machine to 600 nm (For an explanation as to why 600 nm is used see pages 9-11.**

**3.3 Adjusting the transmittance on the machine.**

- The amount of light transmitted should be zero.
- Press the mode button until the red light is displayed beside the transmittance function.
- Turn the left knob in small increments until the transmittance = 0.0

**3.4 Place a blank tube containing the media you will be taking measurements on*.**

- If you are using BHI for monitoring growth use BHI for dilutions.
- If you are using another solution to re-suspend, dilute etc. use this solution as a blank.

*Please also note that the minimum amount of liquid needed for obtaining optical densities is 3 ml for 13 mm tubes, 4 ml for 16 mm tubes, and 5 ml for 18 mm tubes.

- Remove fingerprints, liquid etc, by wiping down sides of the tube with Kimwipe and visually inspecting the tube for fingerprints, smudges etc.
- Make sure the tube is inserted all the way down in the tube holder (see page 11 (2)).

**3.5 Adjust to absorbance mode for the blank by pressing the mode button once.(Aredlight should be displayed besides absorbance.)**

**3.5.1** Blank machine by turning the right knob in small increments. Digital readout should be 0.0.*

*Close the lid on the sample tube holder or place something over the tube, sidearm, etc, to block the light. The spectrophotometer measure absorbed light, so light coming in from another source will give false OD readings. (See pages 9-11 for concepts).

If you are having a hard time getting the machine to reach zero, the transmittance is

likely not adjusted correctly. Do not force the knob or you will break it.

**3.6 You can now place your sample to be read inside of the machine.**

- Remember to wipe off fingerprints
- Make sure tube is all the way down in the tube holder.
- Make sure your sample is mixed homogenously by either vortexing or by pipetting up and down.

**The reading that you obtain should be between 0.10 and 0.62 to be inside the linear range of this machine for this operation.** If the OD reading is <0.10 or >0.62, then the reading is not in the linear range and the readings are not correct (see pages 9-11 for an explanation). If you sample is above 0.62 dilute it (See calculations below) using appropriate solution (eg BHI if you are growing your sample in BHI). If the reading for your sample is <0.10 you have either diluted your sample too much or the bacterial density in your sample is not yet sufficient for an accurate measurement.

Record your optical density measurements in your lab book.Even if OD values are outside the linear range, you should still record these values in your lab book! Make sure you record readings for diluted an undiluted samples (if taken) and **also record how much sample volume and diluent volume was used to prepare dilutions.** These records will allow corrections to be made if dilutions were not calculated or performed correctly

Once the OD for your sample is inside the linear range, record your value. Multiply the OD value measured by your dilution factor to get the actual optical density reading for your sample (see pages 8-9 for trouble shooting and details). For example if the OD600 reading for a 4 fold dilution of a sample is 0.500, the actual OD600 of the sample is 2.0 (0.500 x 4 = 2.0)

**3.7 Turn off the instrument when you are finished.**

# SECTION 4 TROUBLESHOOTING

Certain problems have arisen with the consistency of data points within experiments or the consistency of data from similar experiments between different individuals. These problems have mostly been attributed to:

- Allowing light in from the top of the tube holder.
- Obtaining measurements using the incorrect wavelength.
- Improper adjustment of transmittance of the spec.
- Improper zeroing of the blank.
- Improper re-suspension of the sample.
- Not removing fingerprints from the tube.
- Incorrect performance or calculation of dilution factors.
- Obtaining measurements outside the linear range for optical density readings.

**Following the protocol detailed above should avoid any of these problems. Helpful specific additional troubleshooting hints are outlined below**

**Dilution factors**

Any dilution factor can be used, so long as the diluted sample meets the volume minimum requirement (3ml in 13mm tube, etc page 5) and is in the linear range. If your pipetting is accurate and you are in the linear range the dilution factor shouldn't matter. Only experience will tell how much you need to dilute but keep in mind that *L. monocytogenes* grown shaking (210 rpm) at 37˚C in a side arm doubling time is about 45 min (OD doubles every 45 min) and that the $OD_{600}$ of *L. monocytogenes* does not reach above approximately 2.4. While dilution schemes are fairly straightforward, here are some standard dilution volumes:

## SECTION 4 TROUBLESHOOTING (CONT'D)

**Standard Dilution Factor Table**

| Dilution factor | Sample Volume (ml) | Volume of Diluent (ml) | Total Volume in tube (ml) |
|---|---|---|---|
| 10 fold | 0.5 | 4.5 | 5.0 |
| 5 fold | 1.0 | 4.0 | 5.0 |
| 4 fold | 1.0 | 3.0 | 4.0 |
| 3 fold | 1.0 | 2.0 | 3.0 |
| 2 fold | 1.5 | 1.5 | 3.0 |

If you need to conserve sample, you can minimize the sample volume needed by using the minimum total volume (3ml in a 13 mm tube) required for taking OD measurements. However, this requires calculating the sample volume needed and using a given dilution factor constant.

Where:
SV = sample volume
DV= amount of diluent needed
TV= total volume
DF= dilution factor desired

The equation takes into consideration that amount of sample plus the amount of diluent will add up to the target total volume.

**SV + DV = TV** Example: 0.75 ml +2.25 ml =3ml

In the example below, the target total volume is 3.0 ml (the minimum total volume needed to get a reading using the smallest tube [13 mm]) and the dilution factor desired is 4 fold.

The sample volume (SV) needed to make a certain total volume is the total volume (TV) divided by the dilution factor (DF).

## SECTION 4 TROUBLESHOOTING (CONT'D)

SV=TV/DF Example: SV=3ml/4 = 0.75 ml

The diluent volume (DV) needed is the total volume (TV) minus the sample volume (SV).

DV=TV-SV Example: DV=3 ml-0.75 ml = 2.25 ml

Overall:

SV + DV=TV Again 0.75 ml +2.25 ml =3 ml
SV=TV/DF
DV=TV-SV

## The principle behind $OD_{600}$ measurements

First of all, an $OD_{600}$ reading is not an absorbance reading. It actually relies on the reflection and dissipation of light photons and therefore is **linear for a very small range (0.1-0.62).**

True absorbance readings are performed in non-turbid (clear solutions, not always colorless) which rely on the absorbance of light at a certain wavelength by aromatic rings (example: 260nm for DNA, or various colorimetric assays).

As you'll recall, the electromagnetic spectrum is composed of many varying wavelengths.

## SECTION 4 TROUBLESHOOTING (CONT'D)

The shorter the wavelength is, the more energy dense it is (gamma rays, UV light, x-rays). The longer wavelengths are used for other purposes like FM or AM radio. The spectronic 600 only uses light in the visible range (medium wavelength), see table 2.

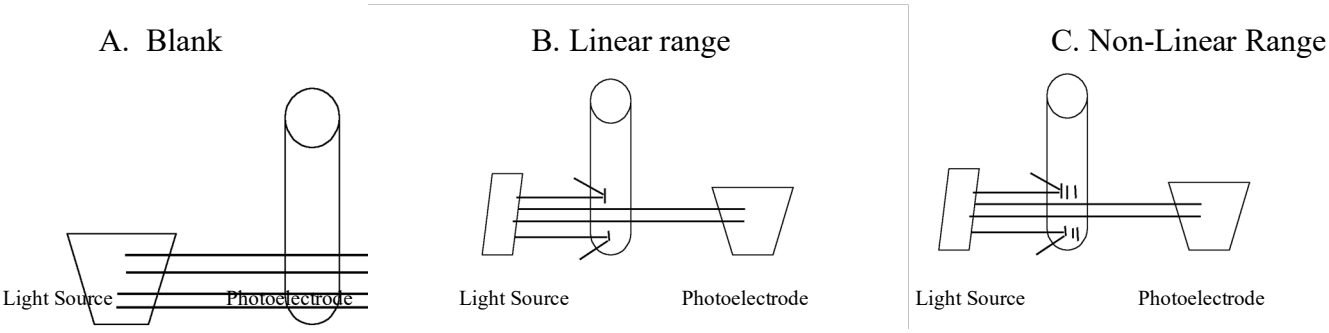
| Approximate Wavelengths of Colors (in nm) | | | | | |
|---|---|---|---|---|---|
| 780-622 | 622-597 | 597-577 | 577-492 | 492-455 | 455-390 |
| Red | Orange | Yellow | Green | Blue | Violet |

The wavelength used for $OD_{600}$ readings is 600 nm (yellow/orange), which provides the best range for optical observations.

The principle behind an $OD_{600}$ is to measure the turbidity of a solution by measuring the transmitted light. This employs a light emitting source (light bulb) and light capturing or measuring device (photoelectrode). When you blank a solution you are setting the machine to measure 100% of the light transmitted (0% absorbed) in that solution. When a sample is added, the machine measures the amount of light that is transmitted (if only 50% of the light is transmitted, then 50% was absorbed.)

As you can see in figure below, all of the light that passes through the test tube is reaching the spectrophotometer (This is represented above the blank tube). In figure B you can see that 50% of the light transmitted is reaching the photoelectrode, while the other 50% is diffracted and does not reach the photoelectrode. In figure C, there are more bacteria than in figure B, however, 50% of the light is still reaching the photoelectrode. As you can see in figure C, the amount of light that is diffracted is not linearly related to the amount of bacteria that are in the test tube.

SECTION 4 TROUBLESHOOTING (CONT'D)




In the linear range, the amount of light transmitted to the photoelectrode is directly proportional to the amount of bacteria in the tube. However, if measurements are made outside the linear range, the relationship is no longer proportional and is inaccurate. This is due to the concept shown in figure c which demonstrates that the more bacteria that are in the tube, the more likely it is that a significant portion of the bacteria are not diffracting light.

## SECTION 4 TROUBLESHOOTING (CONT'D)

**Problems with machine itself.**

(1) I cannot fit my tube in the machine.

There are several sizes of tube adaptors that fit into the machine. These adaptors require a small allen key to remove and replace them. Take the small allen key, turn the allen screw counterclockwise remove the tube adaptor. Place the adaptor inside the adaptor hole and lightly screw the allen screw into position (clockwise).

(2) I put my tube in but nothing happens.

One likely explanation is that if you are using a 13 mm tube and the cap will have to be removed to take a reading. The machine does not transmit light until a lever is depressed inside of the tube adaptor hole. Make sure you tube is all the way down in the machine.

(3) My blank keeps giving me different readings.

- There are fingerprints on your tube. Wipe them off with a Kimwipe and try again.
- The machine was not warmed up before you zeroed the blank. Re-zero and try again.
- Someone else used the machine and was using a different blank.
- You are using a different solution to blank.

(4) The machine is not giving the proper readings that I am used to, or the machine is not giving correct readings.

- Check that you have performed the transmittance tare and blank zero correctly.
- The lightbulb is burning or burnt out. Replace the light bulb (operators manual pages 3-3 through 3-5). If you are uncomfortable performing this procedure, ask Tom.
- If the light bulb is okay. The photoelectrode is broken. Order a new one (thermospectronic.com) and put it in the machine.

## SECTION 5 REFERENCES

http://www.glenbrook.k12.il.us/gbssci/phys/Class/light/u12l1a.html Lesson 1 and Lesson 2

Spectronic 20D+ Operation manual

## SECTION 6 METHOD VERSION & CHANGES

| VERSION | DATE | EDITOR | COMMENTS |
|---|---|---|---|
| Version 1 | 03/07/2005 | | Original SOP |
| Version 2 | 04/29/2020 | Ser15 | Formatted to new template. |