	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ** **PRODUCTON**
Autoclave, LB media, Agar plates **Module Hours: 3.5**	**Effective Date: 03/11/2024** **PRQs: Buffers and Stock Solutions**	**Revision 2.0** **Author: Z. Hazlett-Klein** **Checked by** **Editor: M. Stowell**

Background

Sterile laboratory techniques are essential to prevent microbial contamination and the introduction of unknown, uncontrollable variables into one's experiments. The **autoclave** (also called the steam sterilizer) is a machine that provides a physical method of sterilization by killing bacteria, viruses, and even spores present in or on a material.[1] Autoclaves sterilize material by heating the contents of the chamber to a defined temperature, producing steam from the liquid within the chamber, and raising and maintaining the pressure for enough time to kill all microbial contaminants on the material within. As these machines use high heat and pressure, proper knowledge and use of the autoclave is imperative to prevent injury and to properly sterilize equipment.

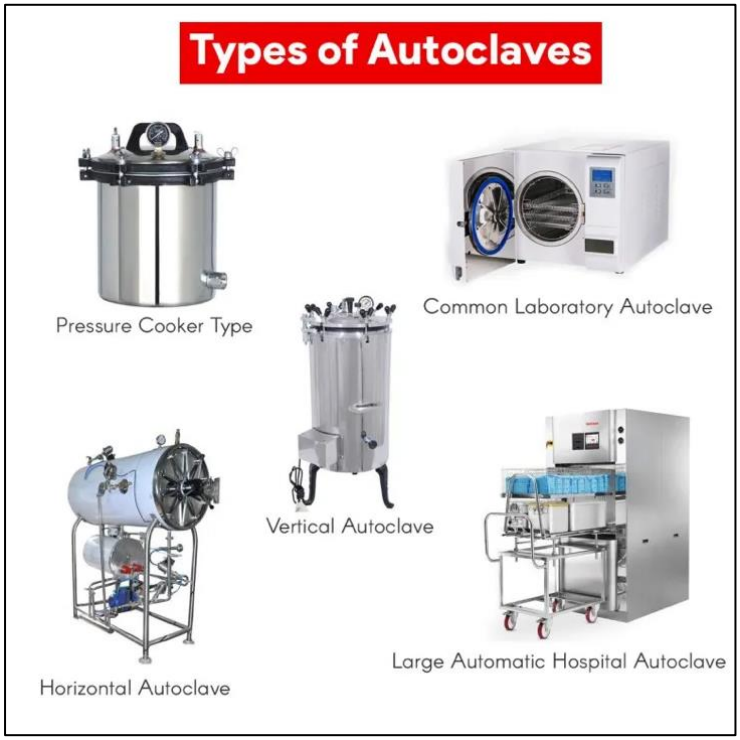

Figure 1. Types of Autoclaves. Image Source: microbeonline.com

There are many different types of autoclaves (Figure 1), but the principles of steam sterilization for each are the same. Prior to use of whichever autoclave is available, make sure to familiarize yourself with the model and any operating instructions specific to that model.

In the MCDB Skills Center, we use a Steris Amsco Century V120 Sterilizer (Figure 2). As autoclaves can be used to sterilize materials from liquid media in covered flasks to biohazardous waste, the proper cycle and cycle settings must be chosen to achieve

Figure 2. AMSCO Century V120 Sterilizer.

optimum results. The Century V120 comes with three primary cycles: Gravity, prevacuum, and liquid. The gravity cycle is the most basic cycle where the air is displaced with steam by gravity through the drain port. This sterilization cycle is typically used to sterilize glassware, unwrapped goods, waste, utensils, and red biohazard bags. The prevacuum cycle is similar to the gravity cycle only the air is mechanically removed from the chamber and load through a series of vacuum and pressure pulses. This cycle is typically used to sterilize more porous material such as wrapped goods, packs, animal cage bedding, cages, red biohazard bags. The liquid cycle is a cycle with slightly lower temperature and longer sterilizatation time used to sterilize liquid media in vented glass or autoclavable plastic containers.[2] In the following protocol, we provide directions for sterilizing liquid media in vented containers.

1. Purpose

The purpose of this SOP is to instruct students on the proper use and safety precautions of the laboratory autoclave.

2. Scope

This procedure applies to qualified skills center users.

3. Responsibility

3.1. It is the responsibility of the user to understand and perform the procedure described in this document.
3.2. It is the responsibility of the user performing the procedure to fully document any deviations from the written procedure.
3.3. It is the responsibility of the user to become trained on the procedure.

4. Definitions

4.1 Autoclave: A pressurized, heated chamber meant to sterilize contents via steam sterilization
4.2 Sterilization: The process of killing any bacteria, viruses, or spores for the purpose of creating a material free of contaminants.
4.3 Autoclave indicator tape: Tape applied to material being autoclaved that changes color once the material is appropriately autoclaved.

5. Materials/Equipment

Liquid Media Preparation/Sterilization

5.1 Heat resistant gloves
5.2 Autoclave tape
5.3 Lab equipment to be autoclaved
  5.3.1 2 x 500 ml glass or polycarbonate Erlenmeyer flasks (with aluminum foil or vented cap to cover)
5.4 Lysogeny Broth (LB) media (500 ml)
5.5 Shallow autoclavable pan or tub (optional)
5.6 An autoclave

LB Agar Plate Preparation/Sterilization

5.7 37 g pre-mixed powder (We have pre-weighed 37g capsules) consisting of:
  5.7.1 5.0 g yeast extract

5.7.2 10.0 g peptone from casein
5.7.3 10.0 g sodium chloride
5.8 Dry agar-agar
5.9 1 L sterile H20
5.10 20-30, 60 mm x 15 mm sterile plates which can hold 5-10 mL of agar and on which you can individually distinguish a maximum of ~100 bacterial colonies
5.11 Autoclavable flasks
5.12 Sterile pipettes + pipettman
5.13 Ice bucket
5.14 Antibiotic
5.14.1 Check with proctors to see which antibiotic to use for LB Agar plates.
5.14.2 If no dissolved antibiotic is available, you can dissolve antibiotic at 1000X concentration in the appropriate solvent. See table on THIS PAGE[2] for antibiotic stock and working concentrations.
5.15 Autoclave tape
5.16 Shallow autoclavable pan or tub (optional)
5.17 Heat resistant gloves
5.18 An autoclave
5.19 60ºC water bath
5.20 Bacterial strains for testing proper LB agar plating
5.20.1 One with antibiotic resistance to the antibiotic used in protocol and
5.20.2 One without that antibiotic resistance
5.20.3 Ask proctor if you need help locating these

6. **Procedure**

(Adapted from Sapkota A., 2021[1] and ThermoFisher – Pouring Agar Plates[2])

Watch video on how to prepare LB agar plates prior to running procedure: https://www.youtube.com/watch?v=ey19jM6y7-c

Liquid Media Preparation/Sterilization

6.1 Prepare equipment for autoclave sterilization

6.1.1 Fill glass or polycarbonate Erlenmeyer flasks with desired volume of media. Follow recipe instructions on LB media powder container if starting from dry reagent.

6.1.2 Cover glass flasks with aluminum foil or polycarbonate flasks with their vented screw caps. If using polycarbonate flasks with non-vented screw-on caps, screw cap on halfway (not tight or it might explode in the autoclave).

6.1.3 Label all equipment to be sterilized with a small amount of autoclave tape.

6.1.4 If autoclaving multiple pieces of equipment, place all pieces in a shallow autoclavable tray or tub.

6.1.5 Consider pausing here if desiring to sterilize liquid media here with media for LB agar plates (protocol shown below).

6.2 Before using the autoclave, check for any items left from the previous cycle.

6.3 Select “Open Door” on autoclave touchscreen and place the materials to be sterilized inside the chamber.

6.1 Select “Close Door” to close chamber.

6.2 Choose the appropriate liquid sterilization cycle

6.2.1 Select “Cycle Select” and “Liquid Cycle”

6.2.2 Select sterilization time based on information in Table 1.

6.2.3 Run Cycle.

6.3 When the run is complete, the heater will switch off. Allow the autoclave to cool until the pressure gauge indicates the pressure inside has lowered down to that of the atmospheric pressure. DO NOT OPEN prior to the chamber reaching atmospheric pressure.

6.4 Select “Open Door” and, using heat resistant gloves, remove the sterilized materials from the chamber.

Volume of Liquid in One Container	Minimum Recommended Sterilize Time* at 121°C (250°F) minutes
75 mL	25
250 mL	30
500 mL	40
1000 mL	45
1500 mL	50
2000 mL	55
> 2000 mL	55 + 10 min/L

* *Minimum sterilize times are based on obtaining a 10^{-6} Sterility Assurance Level (SAL) with standard test loads. Your specific loads may require different sterilize times to achieve this level of sterility, or you may require a different SAL.*

Table 1. Liquid Cycle Parameters[3]

LB Agar Plate Preparation/Sterilization

6.5 Use the LB capsules (1 capsule makes 40 mL of LB broth) add dry agar to be able to reach a final concentration of 1.5% w/v. The precise mass you measure out will be based on the number of plates you'd like to pour (I.e. total desired volume of LB-agar you wish to prepare).

6.5.1 Ex. Because we'd like to make 10 plates, and our plates can hold a maximum of 10 mL, we'll want 100 mL of media total. If using 47mm plates, you can make the same volume of LB agar, but fill with approximately half as much and discard the rest in the trash after it solidifies.

6.5.2 Importantly, we'll actually make a bit more than 100 mL (~120 mL) just in case we spill anything or have small errors in measurement. You should also always make a bit more gel-agar mix than you think you'll need.

6.5.3 So for 120 mL of LB-agar, we'll need:
3 LB capsules

6.5.4 Determine how much dry agar reagent to add to reach a final concentration of 1.5% w/v in 120 mL total volume.

6.6 Transfer the LB-agar powder you've measured out into an appropriately sized bottle for autoclaving. We make 400 mL of agar in 1 L Flasks and 120 mL of agar in 250 mL flasks. The extra empty volume is necessary to prevent your molten agar from boiling over in the autoclave.

6.7 Transfer the sterile water (in our case 120 mL) to the same bottle and swirl to form a medium/agar colloid.

6.8 Cover the opening of the bottle with its cap or aluminum foil (but do not make an air-tight seal!) and tape the bottle with autoclave tape. The autoclave tape will darken during the autoclave process if your sample has spent at least 10 min at 121 ℃. Use lab tape to label the bottle with your initials, the date, and the bottle contents. This will clear up any confusion later if you forget your bottle in the autoclave.

6.9 Place the gel mix in a shallow autoclavable pan or tub in the autoclave and run on a setting that gets the sample to at least 121 ℃ under 20 psi for at least 30 min. The high pressure will prevent your gel mix from boiling over at high temperature.

6.9.1 See Table 1 for proper sterilization conditions

6.10 While your samples are sterilizing in the autoclave, you should prepare your plate pouring station:

6.10.1 Find an empty section of lab bench with a working flame.

6.10.2 Spray down the bench with a 70% ethanol solution and wipe down with a paper towel.

6.10.3 Count out the appropriate number of plates and stack them on your lab bench.

6.10.4 Label the plates with the date and the medium they will contain including the identity of the antibiotic. You can always label the bag that you will store the plates in once they have solidified. Alternatively, ask the proctors if there are any batch color code labels for specific plate types.

6.11 Prepare a water bath at 60 ℃ with sufficient water to submerge ~75% of the bottle containing your molten gel mixture.

6.11.1 You will cool the molten agar in the water bath prior to adding the antibiotic. 60 ℃ is a good temperature because the molten agar will remain liquid at this temperature but most antibiotics will not break down at this temperature. Check with your antibiotic's manufacturer to make sure this is true in your case.

6.12 Retrieve your molten agar mix from the autoclave.

6.12.1 Allow the autoclave to cool until the pressure gauge indicates the pressure inside has lowered down to that of the atmospheric pressure. DO NOT OPEN prior to the chamber reaching atmospheric pressure.

6.12.2 Open the door slowly and keep your face and unprotected hands away from the door has hot steam will pour out when the door opens.

6.13 Partially submerge your molten gel-mix in the 60 ℃ water bath.

6.13.1 Leave the molten gel-mix in the water bath for at least 5 min. Do not let any of the water bath water touch the neck or top of the bottle as this water is not likely to be sterile. Cooled agar should be warm to the touch; as a rule of thumb, if you cannot take the molten agar out of the water bath wearing only lab gloves, it's not likely cool enough to add antibiotic to. To be certain your agar is at the right temperature, we recommend using a laser thermometer.

6.14 Light the flame at the plate pouring station and dilute your antibiotic into your ~60 ℃ molten gel mix using sterile technique. The following table provides typical stock and final concentration targets for Kan and Amp.

	Final Concentration	Stock Concentration
Kanamycin	50 micrograms/mL	50 mg/mL
Ampicillin	100 micrograms/mL	100mg/mL

6.15 Swirl the agar bottle to ensure even distribution of the antibiotic throughout the agar.

6.16 Open one plate at a time next to the flame and begin pouring. Measure your desired amount of agar with a pipete for the first plate to get a good idea of what that volume looks like in your particular plate.

6.17 For the remainder of the plates, pour directly from the bottle.

6.17.1 Be sure to swirl your plates after pouring to remove bubbles and ensure an even distribution of agar over the bottom of the plate. Cap each plate after pouring and stack as you pour.

6.17.2 If your agar partially solidifies in the bottle while you're pouring, you should stop pouring and re-make the gel-mix. If you're making plates without any antibiotic you can alternatively re-liquefy the agar by running it through the autoclave again or by microwaving (if you microwave, beware of over-boiling!).

6.18 Leave your plates out on the bench to solidify.

6.18.1 Leave plates out at room temperature overnight to allow them to dry. After overnight drying, place the plates in a plastic bag with an absorbent material to reduce condensation. The plates are then stored at 4 ℃ until use.

6.19 Once your plates have solidified and dried, you should test them to make sure the antibiotic functions properly:

6.19.1 Take out two plates.

6.19.2 On the first plate, use a sterile toothpick or pipette tip to streak out a strain that you know to be resistant to the antibiotic.

6.19.3 On the second plate, streak out a strain that's not resistant to the antibiotic.

6.19.4 Incubate both plates overnight at the appropriate growth temperature and check for growth. See our sample data section below for positive and negative test results.

6.20 Clean up lab space. Make sure all plates are labeled properly.

7. Troubleshooting

Reference operation manual (https://cdn.vanderbilt.edu/vu-web/lab-wpcontent/sites/20/2019/03/22193200/steris-manual.pdf) or contact proctor if you encounter any problems.

8. References

1. Sapkota A. (November 2021) *Autoclave- Definition, Parts, Principle, Procedure, Types, Uses*. Microbe Notes. Retrieved from: https://microbenotes.com/autoclave/
2. Pouring LB Agar Plates. ThermoFisher. Retrieved from: https://www.addgene.org/protocols/pouring-lb-agar-plates/
3. *Amsco Century Medium Steam Sterilizer Operation Manual*. (December 2005). Retrieved from https://cdn.vanderbilt.edu/vu-web/lab-wpcontent/sites/20/2019/03/22193200/steris-manual.pdf

9. Module Mastery Tasks

A) This task will test your ability to properly steam sterilize **liquid media** using an autoclave.

1. Prepare two 250 ml Erlenmeyer flasks containing 120 ml LB media as described above.
2. Prepare and label each: one to be autoclaved and one to remain unsterilized.
3. Take a picture of both flasks before autoclave sterilization.
4. Autoclave one of the two flasks, making sure to follow the protocol and proper safety precautions.
5. Afterward, set both flasks in a safe place at room temperature for one week.
6. Take a picture of both flasks after one week.
7. Observe differences in each and explain why they might look differently.
8. Submit pictures and short summary of results and conclusion to proctor.

B) This task will test your ability to prepare **LB agar plates** using autoclave sterilization procedures.

1. Make a batch of LB agar plates (10 total) using the sterile plate making protocol described above.
2. Take a picture of the positive (with Kan) and negative (without Kan) test controls that show your plates were prepared properly.
3. Describe how the results show that the plates were prepared properly.

4. Describe how the autoclave is essential for this process and why this is valuable for future experiments.