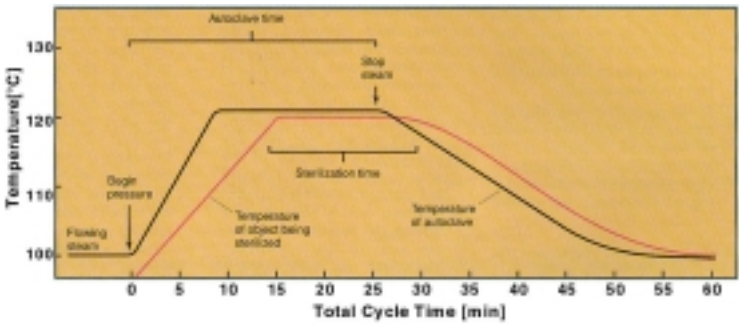
Technical Procedures and Handling

1 Autoclave HiClave (Wolf) HA-300MII

Purpose: Sterilization of culture media with autoclave.

Procedure: Hiclave Wolf HA-300MII:

- Before it is attached to mains and switched on, exchange the demineralized water from the main tank.
- Drain the water of previous sterilization cycle from the sterilization compartment.
- Mark any items to be autoclaved with the appropriate **indicator tape** (thermopaper sensitive to 120°C), this is to verify the temperature within the chamber will reach the preset value.
- Charge baskets with items and place in autoclaver; place the interior temperature sensor amongst the items to be sterilized.
  Note: If bottles with contents have to be autoclaved, make sure that the cap is not firmly closed to allow pressure equalization within the bottle.
- Close and lock main door of autoclaver, lower safety lid, verify autoclaver program settings (121°C for 15mins) and run the preset sterilization cycle.
- Upon termination of sterilization cycle (after approx. 1h 50mins), use gloves to unload the autoclaver firmly close any sealable bottles.

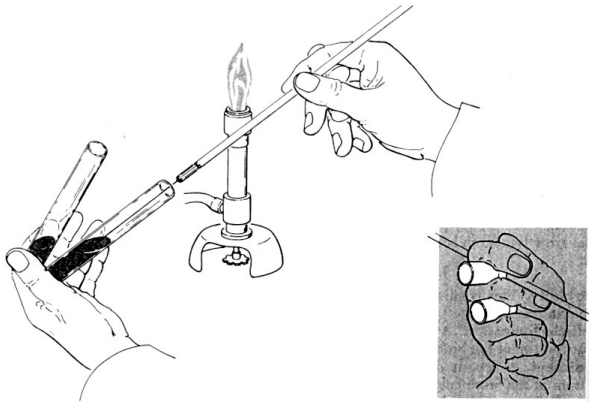


2 Aseptic Technique

Purpose: Culture media are a perfect breeding ground for a amply large selection of bacterial strains. Thus, it is important procedure in order to ensure appropriate results without the influence of contaminants interfering with the inoculum.

Procedure: Transfer of bacteria from one culture medium to the next

- Prior to sterilization, a tube containing medium is usually loosely capped with a metal or plastic closure. This prevents the entry of more contaminants but permits free interchange of air or other gases.
- To start a bacterial culture, a number of cells (the inoculum) are transferred (inoculated) into a sterilized medium.
- In the inoculation procedure the needle or loop that is used to transfer microorganisms should be heated to redness by flaming immediately before and after the transfer. Flaming destroys living forms on the surface of the needle or loop.
- The mouth of the tubes from which cultures are taken and into which they are transferred to, should also be passed through the burner flame immediately before and after the needle is introduced and removed (except for plastic petri dishes). In addition to destroying organisms on the lips of the tubes, flaming creates outward convection currents, which decrease the chance of airborne contamination.
  Note: Flame the inoculating loop before and after inoculation.

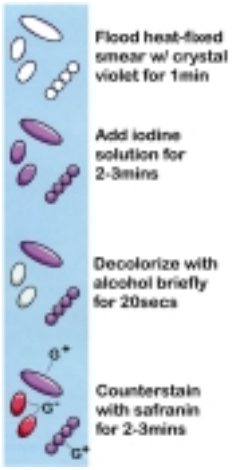


3. Gram Staining Reaction

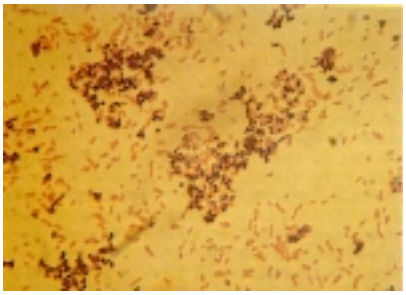
Purpose: An important differential staining procedure widely used in bacteriology.
The gram differentiation is based upon the color reaction exhibited by bacteria when they are treated with crystal violet dye followed by an iodine-potassium-iodide solution. Certain organisms lose the violet color rapidly when ethyl alcohol is applied, while others lose their color more slowly. After the decolorization step, a counterstain (safranin) is used. The **Gram-positive** (G^{Pos}) will retain a **blue** or **purple** color and will not take the counterstain safranin. The **Gram-negative** (G^{Neg}) microorganisms that are unable to retain the crystal violet stain will take the counterstain and will exhibit a **pink** or **red** color. It is important to note that the basis for this differentiation is one of rate, rather than an absolute characteristic of bacteria. For this reason, the procedure must be performed with great care.

Procedure:

- A slide containing dried suspension of microorganisms is flooded on a vertical rack suspended in a flat pan for a minute or two with a dilute solution of dye (*crystal violet*).
- Rinse with an iodone containing solution (*Lugol*) and drip enough iodine solution onto the slide to cover the dried suspension - everything turns purple (in case of GRAM-Pos strains, iodine will react with the thick peptidoglycan layer to form a protective coating).
- Flush briefly with ethanol to decolorize unprotected spots (until no dye will come off).
- Rinse quickly with deionized water. Counterstain by covering the dried suspension for 2 to 3 minutes with safranin solution. (G^{Pos} cells stain purple, G^{Neg} cells pink to red).
- Finally rinse with deionized water. Let slide dry and observe under microscope.
  Note: Use latex gloves and protective clothing for the entire staining procedure.



Photomicrograph of bacteria that are gram-positive (blue-purple) and gram-negative (pink-red)

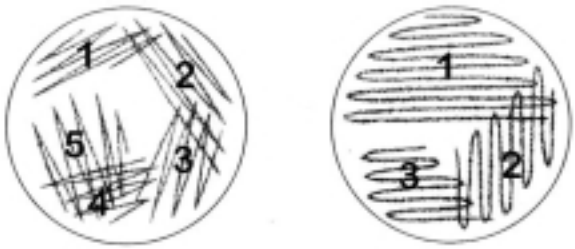


4 Streak plate Method

Purpose: The streak plate technique is another example of a dilution technique. It involves the spreading of a single loopful of material containing microorganisms over the surface on an agar medium which has been allowed to solidify.

Procedure: Description of the 2 most common methods out of a set of representative streaking techniques.

- Pipet or apply a tiny amount from an inoculation loop onto the edge of the agar.
  Motion of loop in the two methods of streaking plates are shown on the right.
- Spread the first dilution streak in a zick-zack manner; according to the method used, flame and cool the loop between strokes 1 and 2; 2 and 3; (3 and 4; 4 and 5).
- Incubate at appropriate temperature for the desired time.

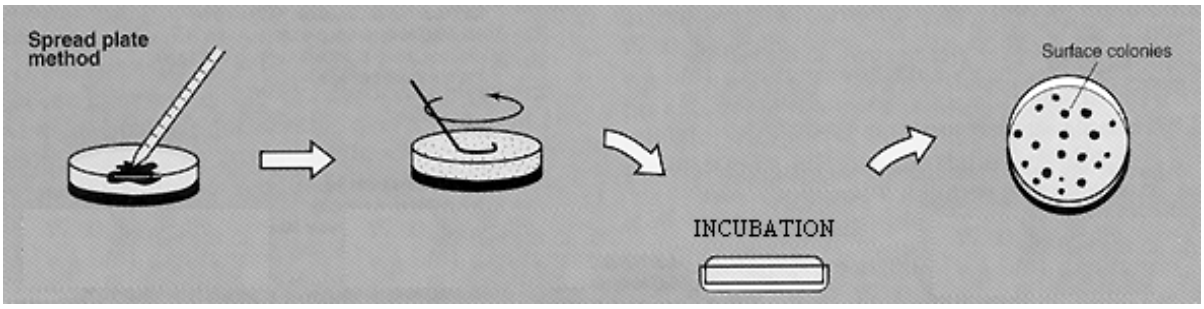


5 Spread plate Method

Purpose: This procedure is a simple method to dilute a sample (usually from a serial dilution row) onto a medium dish to obtain a statistical result regarding the original suspension.

Procedure: Use of this technique requires solidified agar medium.

- Extract a certain amount containing the broth microorganisms and dip it onto the center of the dish.
- Dip the glass spreader (hockey stick) into ethanol and ignite it with the burners flame.
  Note: Keep burning spreader away from the beaker containing the EtOH; in case burning drops of fluid do ignite the EtOH reservoir, cover the beaker immediately to suffocate the flame.
- Place stick onto the rim of dish and agar to cool it off - try not to touch the inoculum with the hot instrument.
- Spread inoculum evenly and homogeneously across the agar plate.
- Incubate at appropriate temperature for the desired time.



Medium used for Agar Plates

1. **NA and Endo-agar** (used in experiment 1.1 and 7.1)

NA: Nutrient agar (predominantly used for drinking water analysis and verification of sterilization requirements)
Endo-agar: MERCK (Selective medium for the verification water-borne contaminants of the genera *Escherichia* and other coliformes which rely on lactose as the primary C-source); Lactose utilizers will turn red; i.e. positive result;

Procedure: 2 bottles of 400mL were used in total to supply the entire course; thus execute procedure twice!

- Weigh the listed amounts, except for the agar (table below - according to the volume desired) into a 500mL beaker (use digital precision flat pan balance to weigh tiny amounts).
- Add approx. 350mL demineralized water, add magnetic rod, place mixture on magnetic stirrer, and allow complete homogenization of solution.
- Slowly add the agar to the solution and keep running for another 5mins.
- Reconvert solution into measuring cylinder and fill up the missing amount of water to the 400mL mark.
- Pour content back into the 500mL beaker.
- Use pH meter to monitor the pH of NA-agar: Remove electrode from electrolyte container, rinse with demineralized water, insert pH-meter into beaker, and add a few drops of 1M HCl until the desired pH is reached (act slowly).
- Once finished, rinse the pH-electrode again with demineralized water and place back into storage compartment.
- Pour content into sealable container and autoclave at 121°C for 15mins.
- Store bottles with medium in incubator at 50°C.

NA-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
Meat extract	1g	0.4g	1g
Yeast extract	2g	0,8g	1g
Peptone	5g	2g	2.5g
NaCl	5g	2g	2.5g
Agar	15g	6g	7.5g
desired pH	7.4	7.4	7.4

Endo-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
Peptone	10g	4g	5g
K_2HPO_4	3.5	1.4g	1.75g
Lactose	10g	4g	5g
$NaSO_3$	2.5g	1g	1.25g
Fuchsin	0.4g	0.16g	0.2g
Agar	12.5g	5g	6.25g
desired pH	n.a.	n.a.	n.a.

2. **BCP and YTA agar** (used in experiment 4 and 5)

BCP: Bromcresol purple glucose agar
YTA: Yeast extract tryptone agar

Procedure: 400mL were used in total to supply the entire course.

- Weigh the listed amounts, except for the agar (table below - according to the volume desired) into a 500mL beaker (use digital precision flat pan balance to weigh tiny amounts).
- Add approx. 350mL demineralized water, add magnetic rod, place mixture on magnetic stirrer, and allow complete homogenization of solution.
- Slowly add the agar to the solution and keep running for another 5mins.
- Reconvert solution into measuring cylinder and fill up the missing amount of water to the 400mL mark.
- Use pH meter to monitor the pH of BCP agar: Remove electrode from electrolyte container, rinse with demineralized water, insert pH-meter into beaker, and add a few drops of 1M HCl until the desired pH is reached (act slowly).
- Once finished, rinse the pH-electrode again with demineralized water and place back into storage compartment.
- Pour content into sealable container and autoclave at 121°C for 15mins.
- Store bottles with medium in incubator at 50°C.

BCP-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
Bactotrypton	10g	4g	5g
Yeast extract	5g	2g	2.5g
K_2HPO_4	2g	0.8g	1g
BCP indicator solution	2mL	0.8mL	1mL
Glucose	10g	4g	5g
Agar	15g	6g	7.5g
desired pH	7.1 - 7.2	7.1 - 7.2	7.1 - 7.2

YTA-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
Bacto-Trypton	10g	4g	5g
Yeast extract	5g	2g	2.5g
K_2HPO_4	5g	0.8g	2.5g
Glucose	1g	0.4g	0.5g
Agar	15g	6g	7.5g
desired pH	n.a.	n.a.	n.a.

3. **M2 agar** (used in experiment 6 and 7)

M2: Casein Hydrolysate (Hycase) agar

Procedure: 2 bottles of 400mL were used in total to supply the entire course; thus execute procedure twice!

- Weigh the listed amounts, except for the agar (table below - according to the volume desired) into a 500mL beaker (use digital precision flat pan balance to weigh tiny amounts).
- Add approx. 350mL demineralized water, add magnetic rod, place mixture on magnetic stirrer, and allow complete homogenization of solution.
- Slowly add the agar to the solution and keep running for another 5mins.
- Reconvert solution into measuring cylinder and fill up the missing amount of water to the 400mL mark.
- Pour content back into the 500mL beaker.
- Use pH meter to monitor the pH: Remove electrode from electrolyte container, rinse with demineralized water, insert pH-meter into beaker, and add a few drops of 1M HCl until the desired pH is reached (act slowly).
- Once finished, rinse the pH-electrode again with demineralized water and place back into storage compartment.
- Pour content into sealable container and autoclave at 121°C for 15mins.
- Store bottles with medium in incubator at 50°C.

M2-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL*
HyCase	5g	2g	2.5g
Yeast extract	5g	2g	2.5g
NaCl	200g	80g	100g
Tris	12.1g	4.84g	6.05g
$MgCl_2 \cdot 6H_2O$	20g	8g	10g
KCl	2g	0.8g	1g
$CaCl_2 \cdot 2H_2O$	0.2g	0.08g	0.1g
Agar	20g	8g	10g
desired pH	7.4	7.4	7.4

Ingredients per 400mL - experiment 6.2

M2-agar	0.5% NaCl	5% NaCL	10% NaCl	15% NaCl	20% NaCl
HyCase			2g		
Yeast extract			2g		
NaCl	**2**	**20**	**40g**	**60g**	**80g**
Tris			4.84g		
$MgCl_2 \cdot 6H_2O$			8g		
KCl			0.8g		
$CaCl_2 \cdot 2H_2O$			0.08g		
Agar			8g		
desired pH			7.4		

(*) it turned out that 400mL were not sufficient to supply the entire group of 19 students with sufficient agar; an average student used 20mL of agar per petri dish; some gets spilled, others do not use the pipette to limit the poured amounts, etc. Thus, future courses should prepare 500mL of agar.

4. **Ascidianus medium** (used in experiment 6)

Enriched minimal medium for *Ascidianus brierleyi*;

Procedure: 2 bottle of 400mL was each were used in total to supply the entire course; thus, execute procedure twice.

- Weigh the listed amounts, (table below - according to the volume desired) into a 500mL beaker (use digital precision flat pan balance to weigh tiny amounts).
- Add 400mL demineralized water, add magnetic rod, place mixture on magnetic stirrer, and allow complete homogenization of solution.
- Pour content into sealable container and autoclave at 121°C for 15mins.
- Once **cooled down**, add yeast extract and sulfur.
- Store bottles with medium in incubator at 50°C.

NB-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
$(NH_4)_2SO_4$	3g	1.2g	1.5g
$K_2HPO_4 \cdot 3H_2O$	0.5g	0.2g	0.25g
$MgSO_4 \cdot 3H_2O$	0.5g	0.2g	0.25g
KCl	0.1	40mg	50mg
$Ca(NO_3)_2$	0.01	4mg	5mg
Yeast extract	2g	0.8g	1g
Sulfur	10g	4g	5g
desired pH	6	6	6

5. **LB agar** (used in experiment 7)

LB: Luria Broth (Complex medium for fast growing *Escherichia* and other Entero-bacteria species)

Procedure: 2 bottles of 400mL were used in total to supply the entire course; thus execute procedure twice!

- Weigh the listed amounts, except for the agar (table below - according to the volume desired) into a 500mL beaker (use digital precision flat pan balance to weigh tiny amounts).
- Add approx. 350mL demineralized water, add magnetic rod, place mixture on magnetic stirrer, and allow complete homogenization of solution.
- Slowly add the agar to the solution and keep running for another 5mins.
- Reconvert solution into measuring cylinder and fill up the missing amount of water to the 400mL mark.
- Pour content into sealable container and autoclave at 121°C for 15mins.
- Store bottles with medium in incubator at 50°C.

LB-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
Bacto-Trypton	10g	4g	5g
Yeast extract	5g	2g	2.5g
NaCl	10g	4g	5g
Agar	15g	6g	7.5g

6. **NB broth** (used in experiment 8)

NB: Nutrient broth (predominantly for *Bacillus* and *Pseudomonas* species)

Procedure: 1 bottle of 400mL was used in total to supply the entire course.

- Weigh the listed amounts (table below - according to the volume desired) into a 500mL beaker (use digital precision flat pan balance to weigh tiny amounts).
- Fill up with demineralized water till the 400mL mark, add magnetic rod, place mixture on magnetic stirrer, and allow complete homogenization of solution.
- Use pH meter to monitor the pH: Remove electrode from electrolyte container, rinse with demineralized water, insert pH-meter into beaker, and add a few drops of 1M HCl until the desired pH is reached (act slowly).
- Once finished, rinse the pH-electrode again with demineralized water and place back into storage compartment.
- Pour content into sealable container and autoclave at 121°C for 15mins.

NB-agar	Ingredients per liter	Ingredients per 400mL	Ingredients per 500mL
Meat extract	3g	0.4g	1.5g
Peptone	5g	2g	2.5g
desired pH	7	7	7

References

Logan, Niall A.; (1994); *Bacterial Systematics*; Blackwell Scientific Pub.; Oxford, UK.

Madigan, Martinko & Parker (1997); *Brock Biology of Microorganisms*; 8th ed. Prentice Hall, Inc.; New Jersey - USA.

Seeley, H.W., VanDemark P.H. and Lee, J.L. (1991); *Microbes in Action*; A Laboratory Manual of Microbiology; 4th ed. W.H. Freeman Co.; New York – USA

Skoog, West, Holler (1996); *Fundamentals of Analytical Chemsitry* 7th ed; Saunders College Publ.; Orlando FL - USA

Stan-Lotter H. (1999); *Mikrobiologische Übungen WS 99/00*; Universität Salzburg, SBG - AT

Web related sites:

- Growth pattern: http://www.cellsalive.com/gallery.htm
- German Collection of Microorganisms and Cell Cultures: http://www.dsmz.de/species/strains.htm
- American Society for Microbiology: http://intl-aem.asm.org/search.shtml
- American Type Culture Collection: http://www.atcc.org/

PRGM Sheet: HP-41CX software solution for experiment 8;

allocate memory for n>30:
XEQ SIZE 70 for n ≤ 50
XEQ SIZE 120 for n ≤ 100
XEQ SIZE 270 for n ≤ 250

1	LBL Gauss	standard deviation	
2	SF 00	activate flag “program running”	
3	N = ?		
4	PROMPT		
5	STO 10	enter number total number of single events and store in memory 10	memory 10 = *n*
6	0		
7	STO 11	reset summation memory (later on used as arithmetic mean $\bar{x}$)	memory 11 = 0
8	STO 12	reset loop counter memory 12 = 0	
9	STO 13	reset auxiliary memory for 1st term in parenthesis $\Sigma(x_n \quad \bar{x})^2$	memory 13 = 0
10	14		
1	STO 14	set counter for individual events (x) to starting position	memory 14 = 14
2	LBL 01	start of sub-routine #1	
3	1		
4	ST+ 12	increment counter 12 = appendix for alpha display	
5	ST+ 14	increment counter 14	
6	FIX 0	eliminate comma display (scientific notation)	
7	X		
8	ARCL 12		
9	append=?	display n w/ n^{th} index according to value of counter	
20	ENG 4	reestablish scientific notation	
1	PROMPT	enter value of n^{th} element	
2	STO IND 14	store individual value under indirectly addressed memory 14	
3	ST+ 11	add individual event to summation memory 11	
4	RCL 12		
5	RCL 10		
6	X≠Y?		
7	GTO 01	repeat sub-routine #1 as long as counter 12 is lower than *n*	
8	RCL 11		
9	RCL 10		
30	/		
1	STO 11	memory 11 now used for arithmetic mean $\bar{x}$	memory 11 = $\bar{x}$
2	MEAN=		
3	ARCL X		
4	PROMPT	display arithmetic mean	
5	LBL 02	start of sub-routine #2	
6	RCL IND 14	retrieve individual event from indirectly addressed memory....	
7	RCL 11	....and deduct arithmetic mean	
8	-		
9	X^2	raise to the power of 2 and....	
40	ST+ 13	....add preliminary result to memory 13	memory 13 = $\Sigma(x_n \quad \bar{x})^2$
1	1		
2	ST- 12	decrement loop counter 12	
3	ST- 14	decrement indirect addressee 14	
4	RCL 12		
5	X≠0?		
6	GTO 02	repeat sub-routine #2 as long as counter 12 is greater than 1	
7	RCL 13		
8	RCL 10	$\Sigma(x_n \quad \bar{x})^2$ include denominator of equation of standard deviation	
9	1		
50	-		
1	/	standard deviation = memory 13*/(n-1)*....	stack X = $\Sigma(x_n \quad \bar{x})^2$ / *(n-1)*
2	SQRT	....and take square root	
3	SD=		
4	ARCL X		
5	AVIEW	display standard deviation = stack X	
6	PROMPT	halt program to enable readout of display	
7	RCL 11	recall *n*	
8	/		
9	100	standard deviation [%] = *SD·100/n*	stack X = *SD·100 / n*
60	*		
1	SD-% =		
2	ARCL X		
3	AVIEW	display standard deviation in percent	
4	CF 00	clear flag “program running”	
65	END	end of program	