Antimicrobial susceptibility testing
Agar dilution method

Rakuno Gakuen University
School of Veterinary Medicine

WOAH Collaborating Centre
for Food Safety

World Organisation
for Animal Health
Founded as OIE

2025. Jul.

Rakuno Gakuen University
Veterinary AMR Center, NVAL, MAFF

Exercise Flow

July

8th AM

Prepare a dilution series of antimicrobials.

Mix 2mL antimicrobial and 18 mL MHA (Mueller–Hinton Agar) in petri dishes and solidify and dry.

8th PM

Inoculate strains to the plates prepared in AM .

9th AM

Assessment

First day ①

Dilution scheme of antimicrobials

(1) Antimicrobial drugs

Purchase antimicrobial standards
(For example Sigma-Aldrich Co.)

(2) Storage method

・Antimicrobial powders should be stored at or below -20℃
In a container containing a silica gel desiccant to prevent absorption of moisture.

silica gel

【Calculating required volume of diluent 】

$$\text{Diluent amount (ml)} = \frac{\text{Potency (µg/mg)} \times \text{Weight (µg)}}{\text{Stock concentration (µg/ml)}}$$

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Tetracycline hydrochloride, meets USP testing specifications
Product Number	T4062
Product Brand	Sigma-Aldrich
CAS Number	64-75-5
Molecular Formula	$C_{22}H_{24}N_2O_8 \cdot HCl$
Molecular Weight	480.90
Storage Temp	−20°C

TEST	SPECIFICATION	LOT 095K13182 RESULTS
IDENTITY	PASS	PASS
CRYSTALLINITY	PASS	PASS
PH TEST	1.8 TO 2.8 (1% SOLUTION)	2.38
LOSS ON DRYING	NMT 2.0%	0.24%
LIMIT OF 4-EPIANHYDRO-TETRACYCLINE	NMT 2.0%	0.05%
SPECIFIC ROTATION	-240 DEG TO -255 DEG	-245 DEG
HEAVY METALS	NMT 0.005%	0.004%
ASSAY	NLT 900 UG/MG	981 UG/MG
EXPIRATION DATE		
QC ACCEPTANCE DATE		

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

For example: Tetracycline

highest concentration to be tested : 512 µg/ml

Stock concentration=512 × 10 = 5120 µg/ml

Potency =981µg/mg

Diluent amount=>9mL ・・10mL (1test)

10test × 10mL =100mL

$$\text{Diluent amount (ml)} = \frac{\text{Potency 981 (µg/mg)} \times \text{Weight (µg)}}{\text{Stock concentration (µg/ml) 5120}}$$

100

$$\text{Weight} = \frac{100 \times 5120}{981} = 521.9 \text{ mg}$$

target value

But Actual weight=533mg (For example)

$$\text{Diluent amount (ml)} = \frac{\text{Potency 981 (µg/mg)} \times 533 \text{(µg)}}{\text{Stock concentration (µg/ml) 5120}}$$

=102.124mL

Final:533mg Tetracycline is soluble in 102.124mL DW

①

(3) Preparation of a standard solution

① Weigh the required amount of the drug to be used.

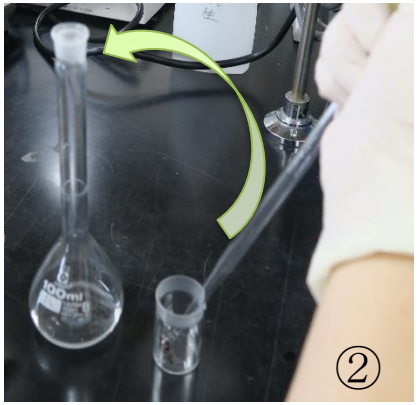
② Dissolve in a solvent (Determine the amount of solvent needed for a standard solution in advance).

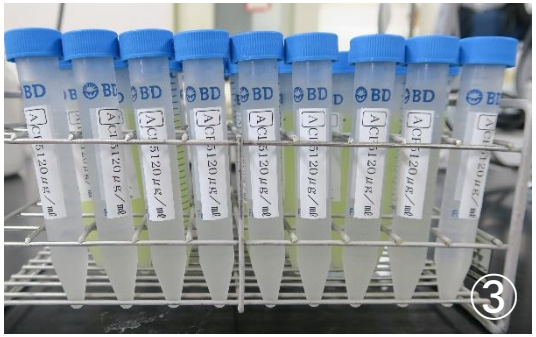
Water-soluble drugs: Dissolve in sterile purified water.

Drugs with limited solubility in water: The drug stock solution should be prepared by dissolving in a small amount of a solvent and then adding sterile purified water.

③Dispense the solution to each volume

④Most drug stock solutions can be used until about 6 months after storage in a sterile polyethylene or polypropylene bottle at or below -70℃. However, once dissolved, use them entirely on the same day.





Tricks and Traps

hygrometer

- Weigh after the drug has returned to ambient temperature before using

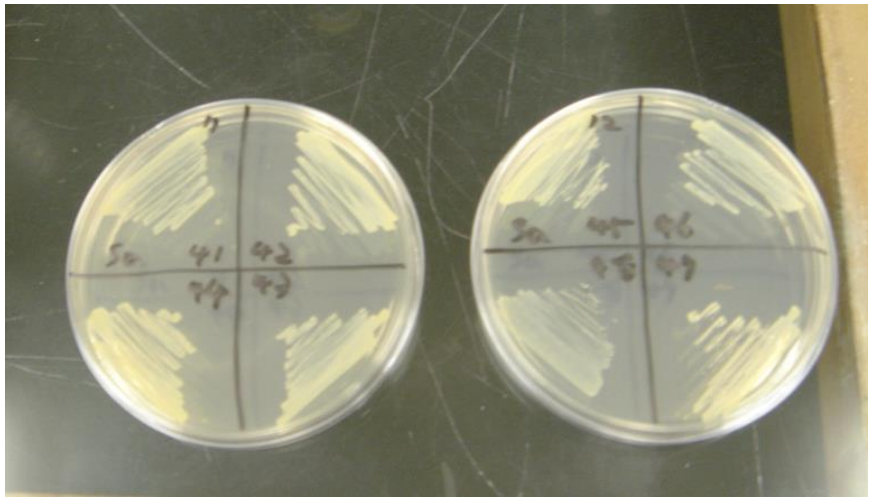
- Weigh at or below 45% relative humidity since some antimicrobials are hygroscopic.

- Water-soluble drugs: In general, these drugs should be dissolved in sterile purified water.

- Insoluble drugs or limited solubility in water: Drug stock solutions are prepared by dissolving in a minimum volume of a solvent, such as ethanol, methanol, a buffer, or an aqueous solution of sodium hydroxide, and then diluting in purified water.

First day ②

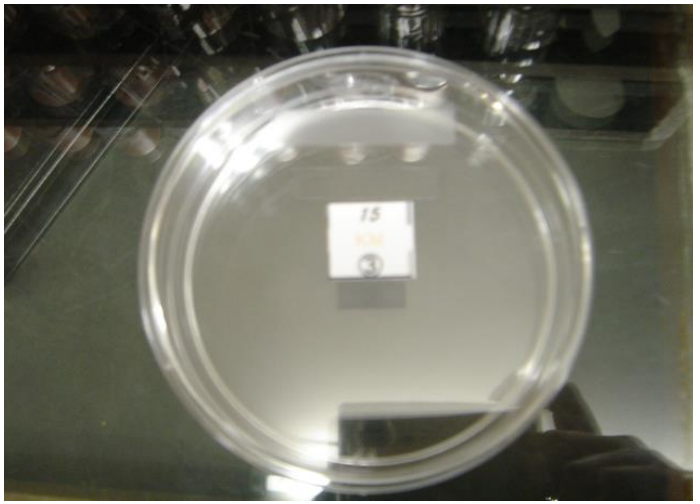
Grow isolated wild strains and 4 quality control strains for MIC measurements on nutrient agar, HI nutrient agar, etc.



Isolation, Identification and Preservation (−70 ～ −80° C)

First day ③

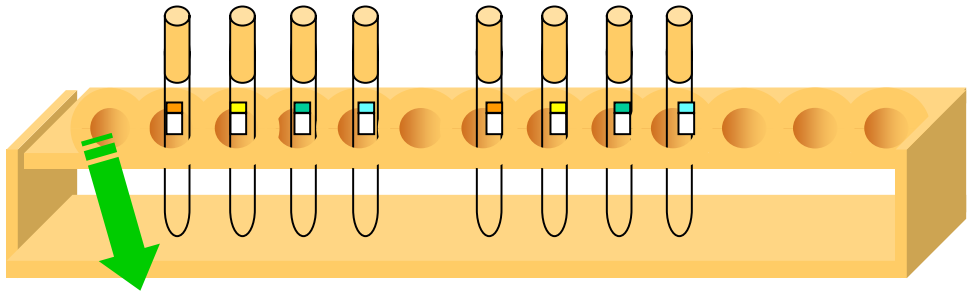
Number the master tubes, dilution tubes and petri dishes.



Numbering of the Master and Dilution Tubes

(1) Master tubes

B	C	D	E
CP	CP	CP	CP
1280	160	20	2.5
2 : 6	1 :7	1 : 7	1 : 7

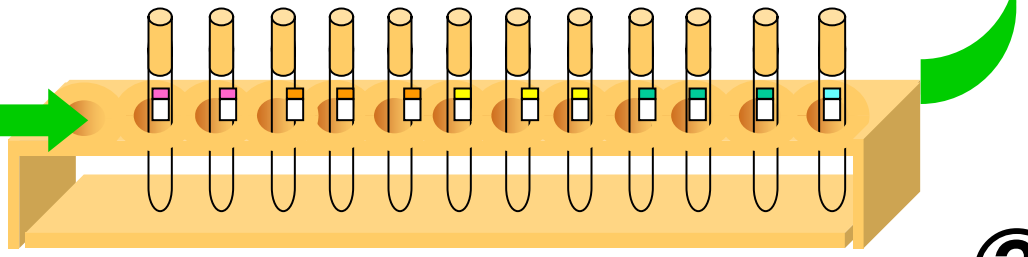


Undiluted solution goes into Tube A

(2) Dilution tubes

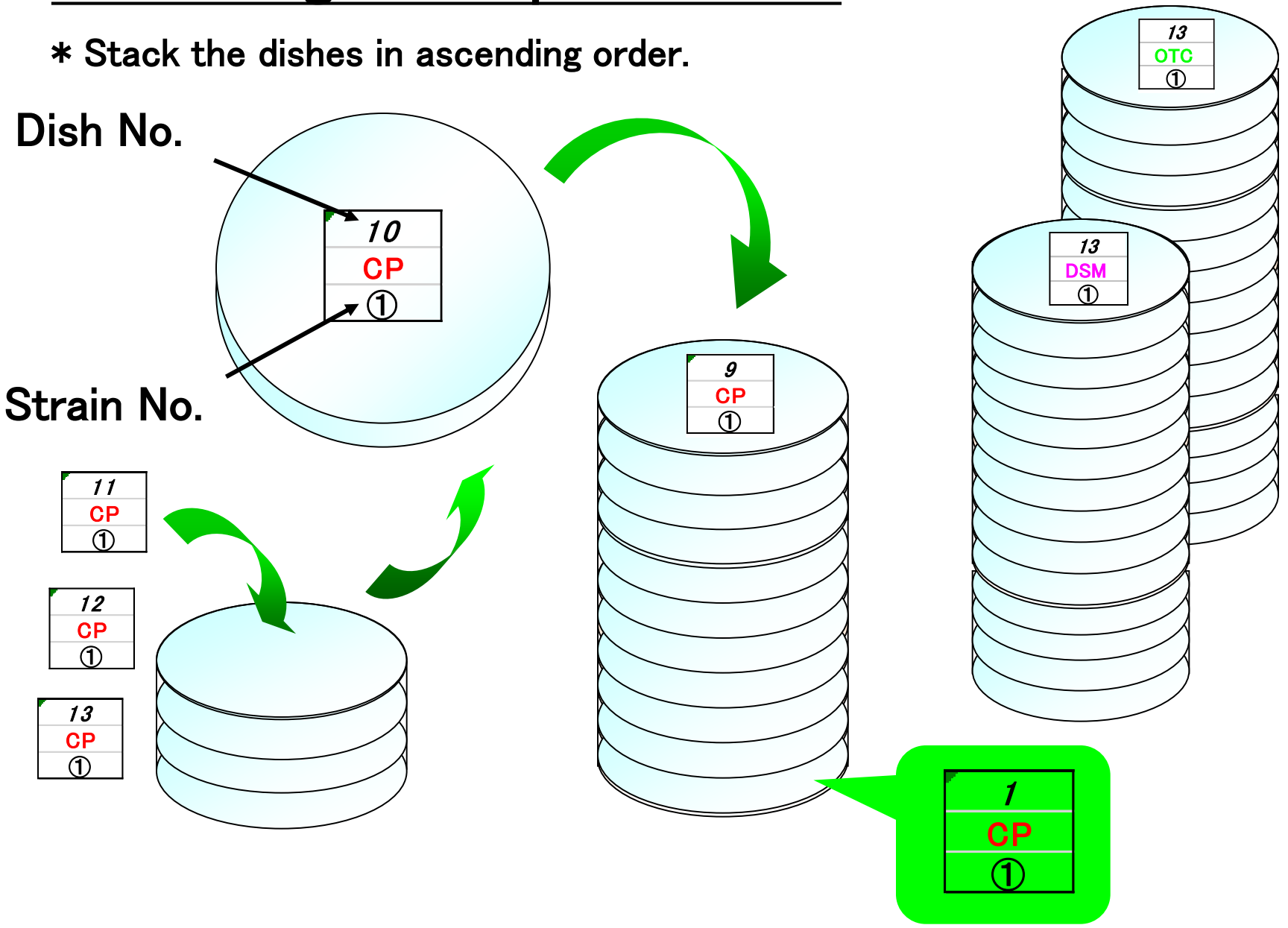
A	A	B	B	B	C	C	C	D	D	D	E
2	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
CP	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP
2	1	2	1	1	2	1	1	2	1	1	2
2	3	2	3	7	2	3	7	2	3	7	2

Undiluted solution goes into Tube “A–1”



Numbering of the petri dishes

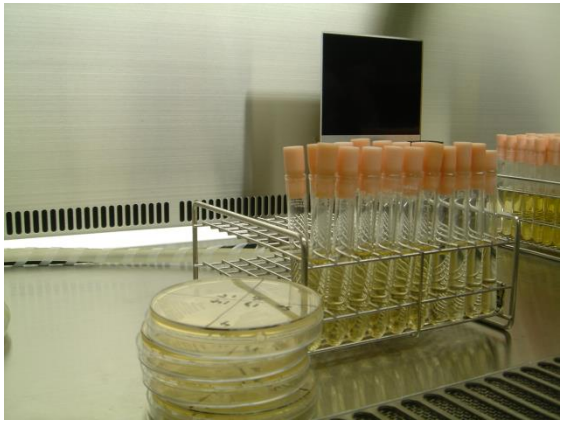
* Stack the dishes in ascending order.

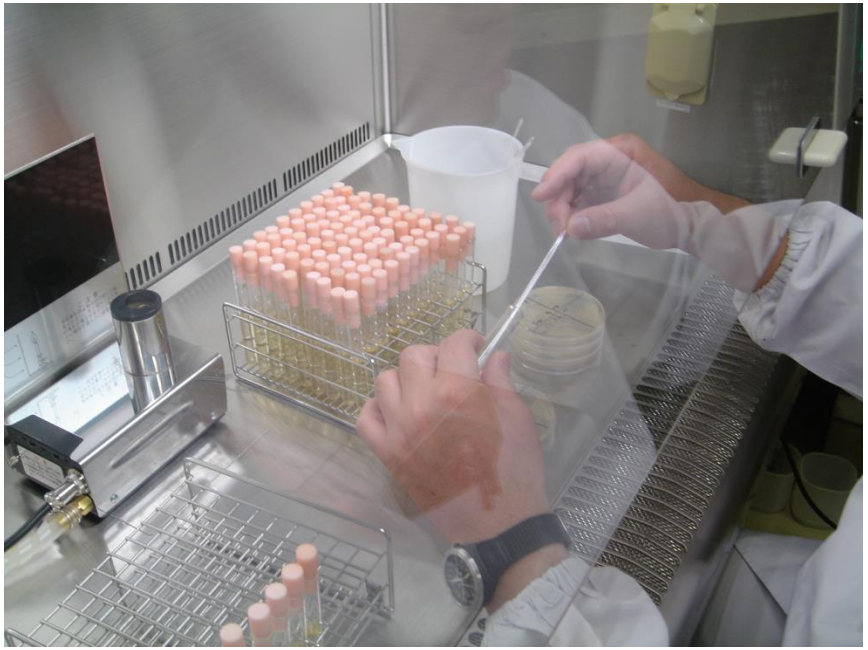


Second day ④

- Inoculation of Test Bacteria -

On the evening before MIC measurement, inoculate strains grown on plates into 4 mL TSB, and incubate at 35℃ to a concentration of McFarland 0.5 (2–6h)





Second day ⑤
– Dissolution of Drug –

Freeze a stock solution of drug until use, or prepare a stock solution the previous day or the day of use.

Thaw the drug on the previous day or the morning of preparing the MIC plates. (4℃ is better.)

Second day ⑥

Melt the required amount of Mueller–Hinton agar (MHA) medium and keep at 50℃ in a constant–temperature bath.

Second day ⑦

Dispense a dilution solution (water, buffer, etc.) into the master tubes and dilution tubes.

Dispensing of dilution solution into the master and dilution tubes

(1) Master tubes A～E

A	B	C	D	E
CP	CP	CP	CP	CP
5120	1280	160	20	2.5
–	2 : 6	1 : 7	1 : 7	1 : 7

Diluted reagent conc. (μg/mL)

The volume ratio to achieve the label conc. (Master tube : sterilized DW)

(2) Dilution tubes

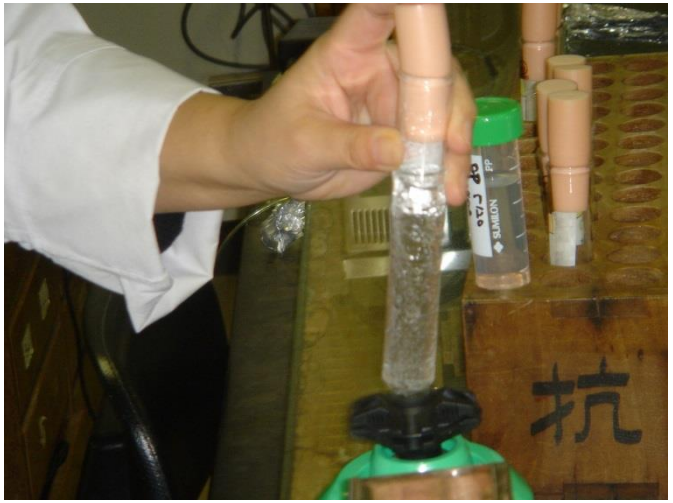
	A	A	B	B	B	C	C	C	D	D	D	E
	2	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP
Master soln (mL)	2	1	2	1	1	2	1	1	2	1	1	2
Sterilized DW (mL)	2	3	2	3	7	2	3	7	2	3	7	2
	2560	1280	640	320	160	80	40	20	10	5	2.5	1.25

Dispense each of MHA

Final conc. (μg/mL)	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125

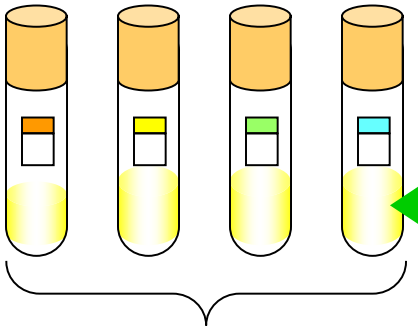
Preparation of Master Dilution Series

Prepare master dilution series B, C or later from the thawed drug stock solution (either master A or B).

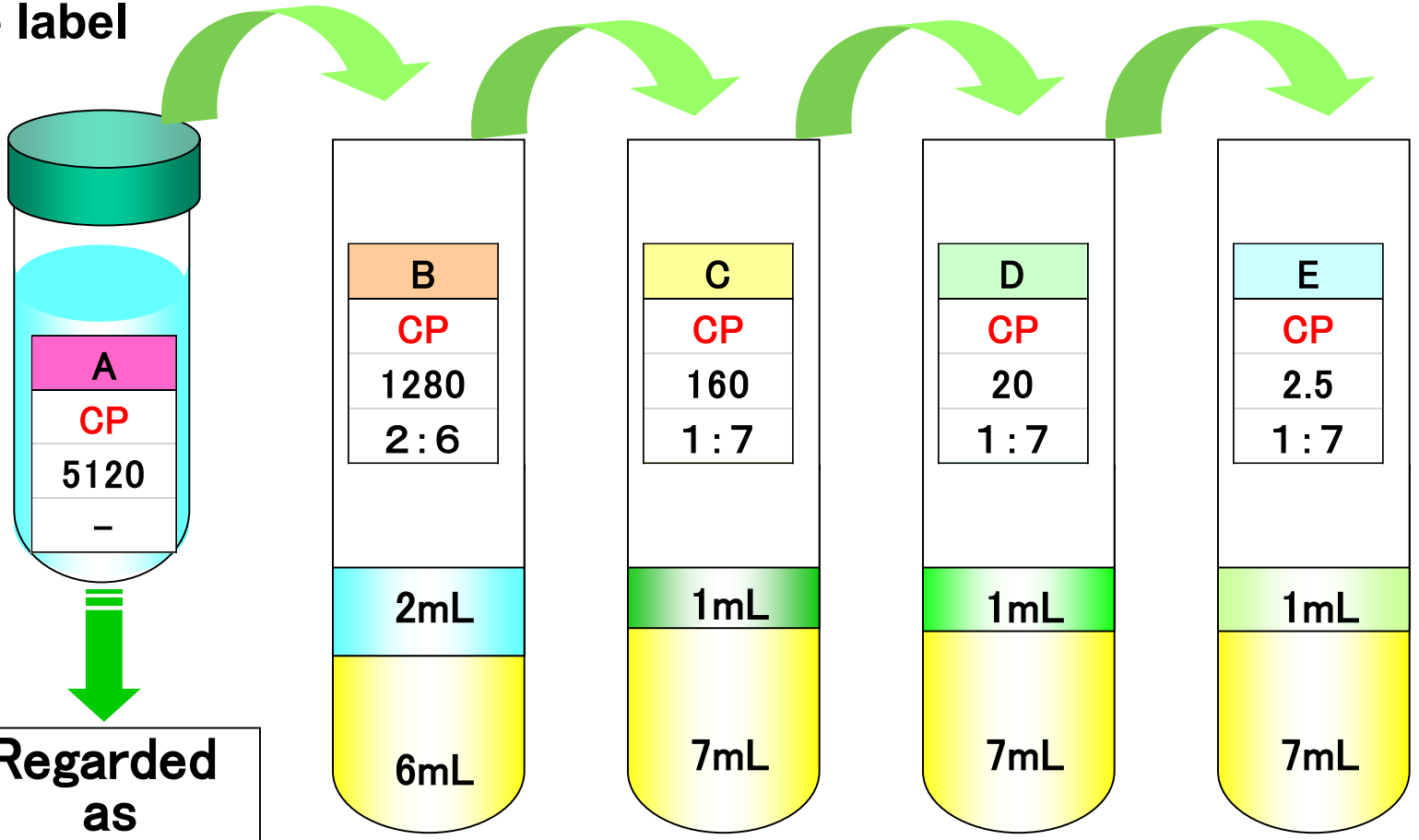


(1) Master tubes

Transfer the solution of the preceding tube according to the label



Diluted solution

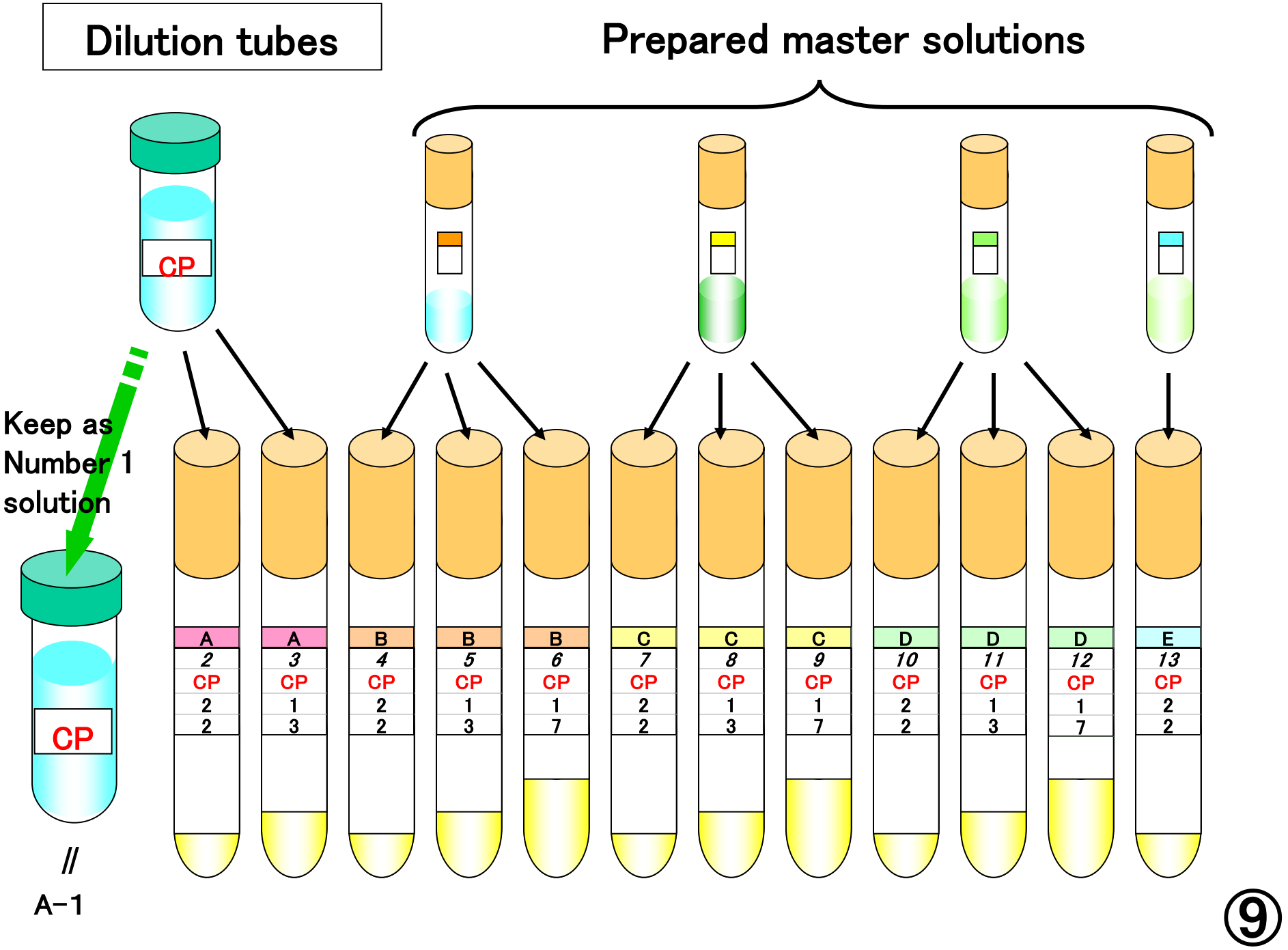


Regarded as Master A

Preparation of Drug Dilution Series

From master dilution series, prepare dilution series to be dispensed into petri dishes.

If solutions with lower concentrations are dispensed first, one pipette will suffice.


Dilution tubes
Prepared master solutions
CP
Keep as
Number 1
solution
CP
//
A-1
A
2
CP
2
2
A
3
CP
1
3
B
4
CP
2
2
B
5
CP
1
3
B
6
CP
1
7
C
7
CP
2
2
C
8
CP
1
3
C
9
CP
1
7
D
10
CP
2
2
D
11
CP
1
3
D
12
CP
1
7
E
13
CP
2
2

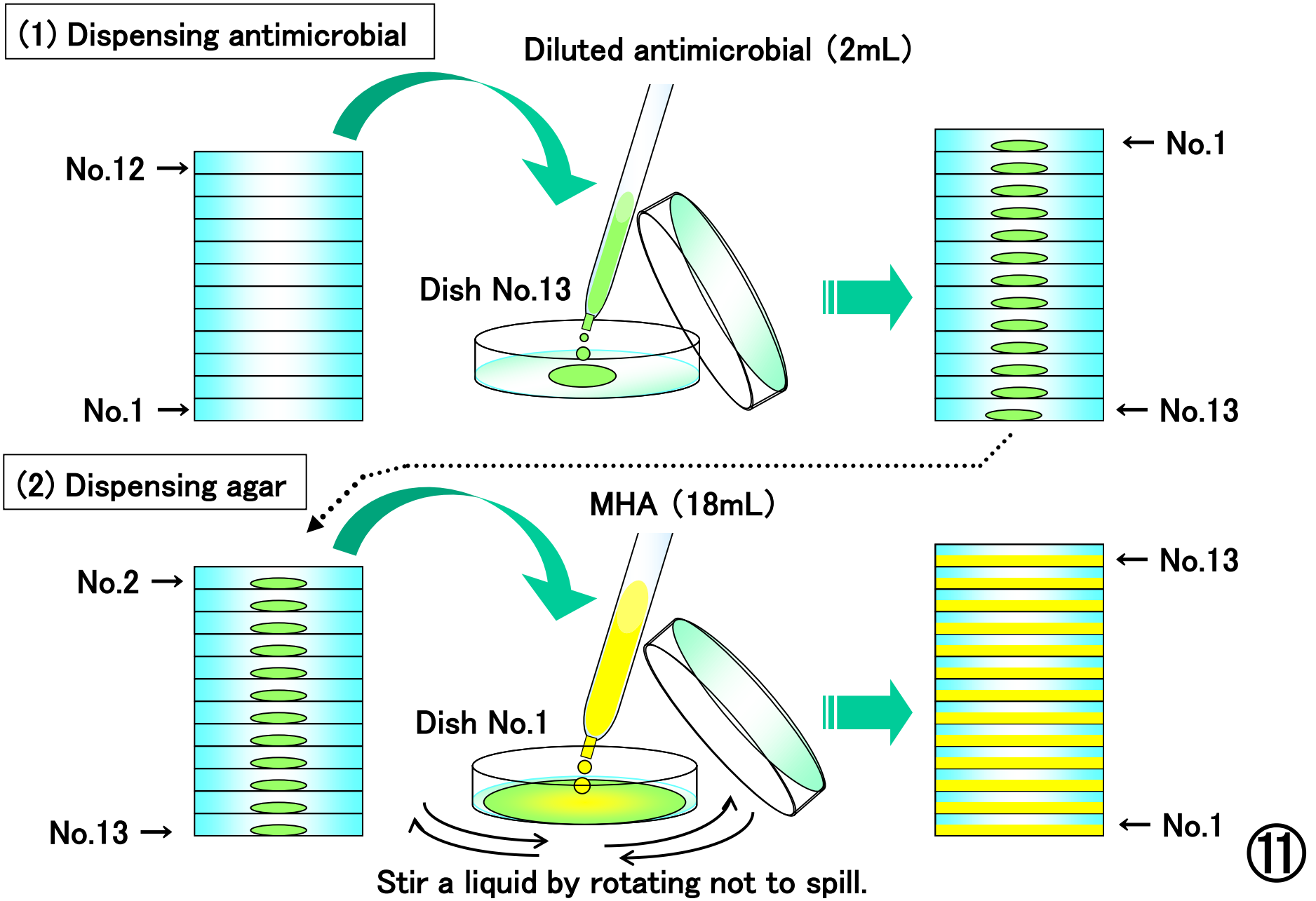
Dispensing of diluted solutions into petri dishes

Dispense 2 mL each of the diluted solutions into the numbered dishes according to the numbers.

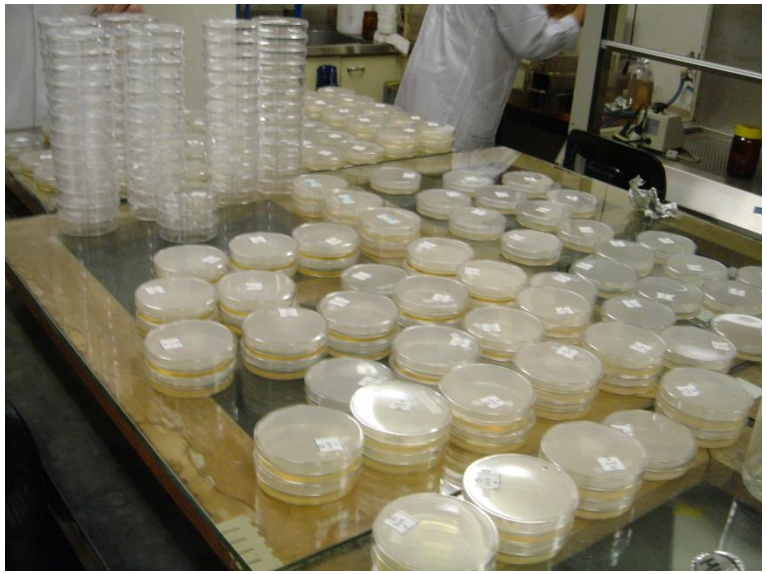
Dispensing of MHA into dishes

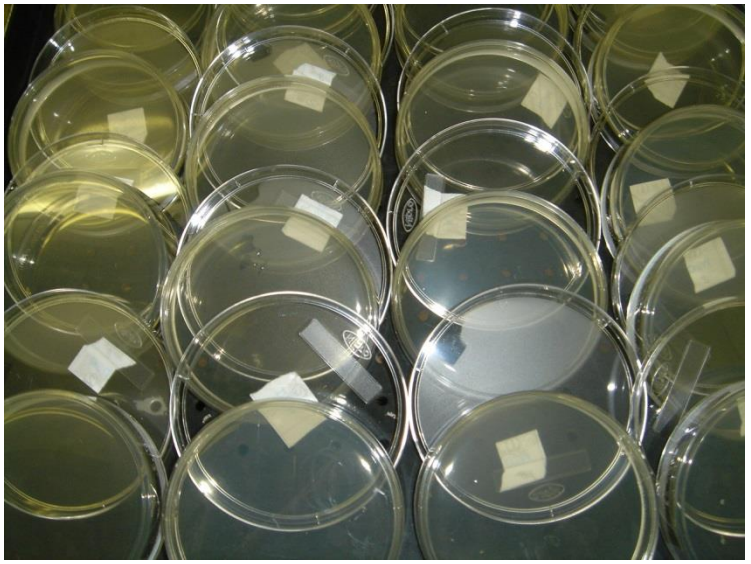
Dispense 18 mL each of MHA kept at 50℃ into the dishes, mix well with the drug, and after solidifying, let it dry.

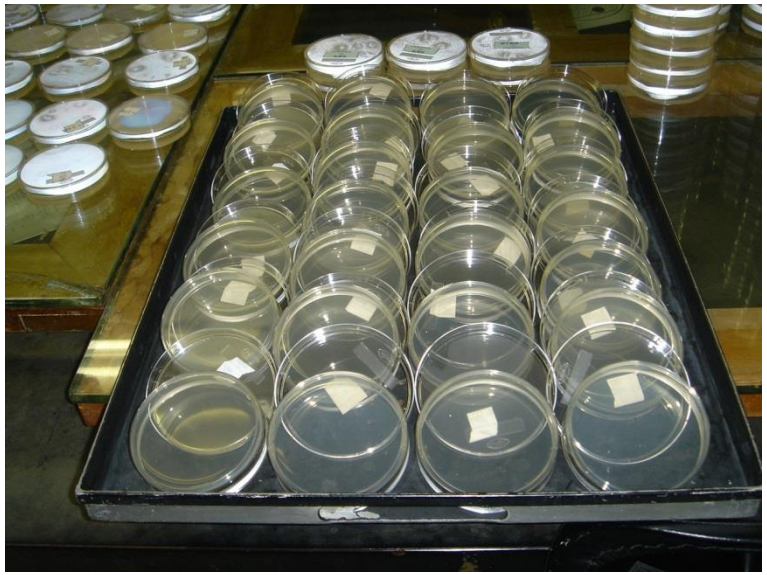
Dispensing of drug and agar



Drying of dishes

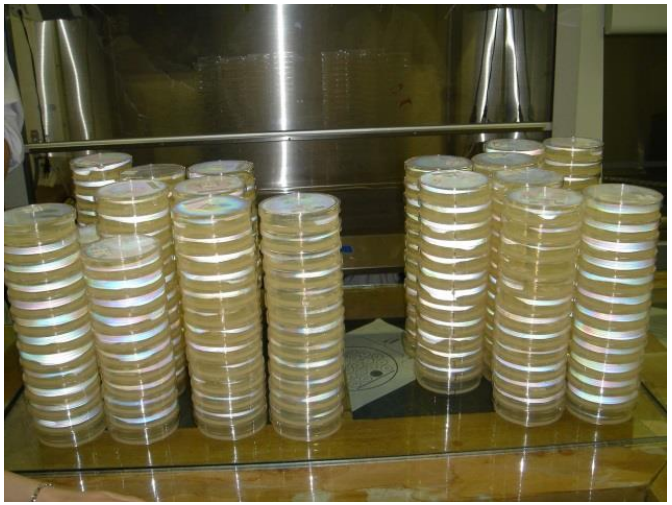


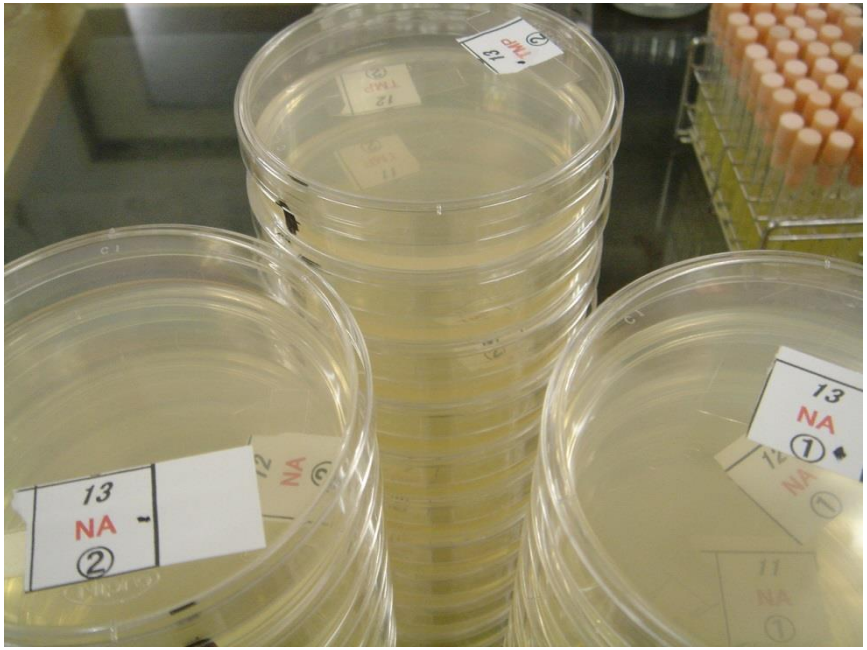




Alignment of plates

Align the dried plates
in order of inoculation.
- The largest number is
at the top (“13”).
- Note the group number !



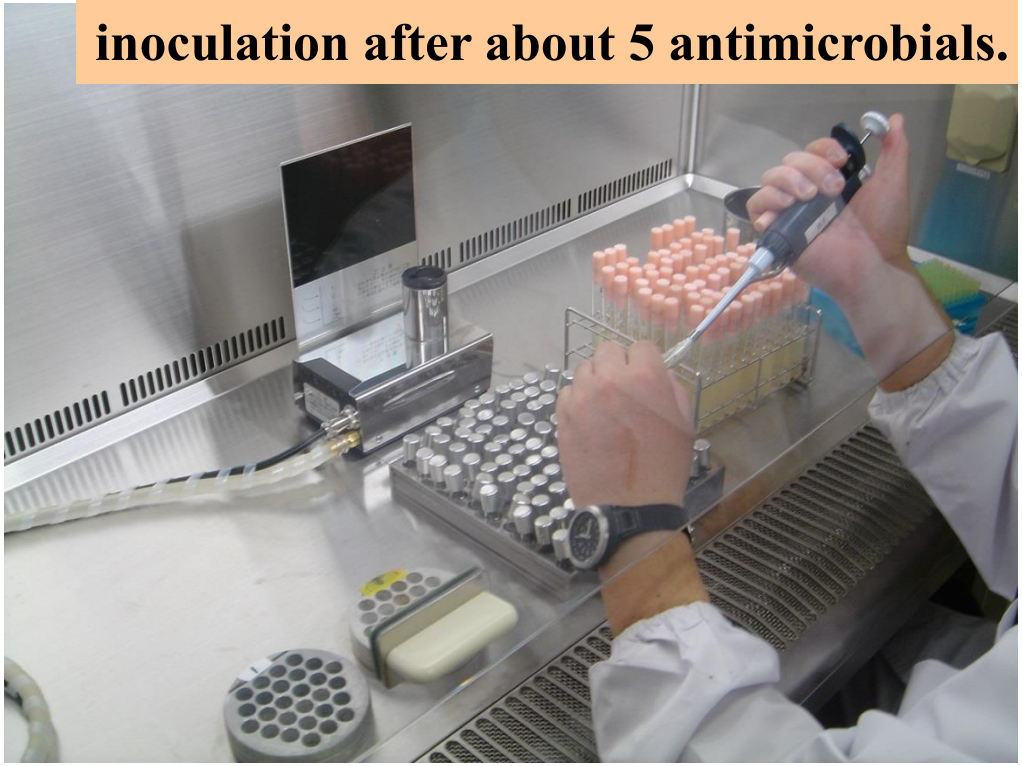


Marks indicating the direction of inoculation should be placed when the plates are aligned immediately before inoculation.

Dilution/Preparation of Bacterial Solutions

- Dilute/prepare bacterial solutions

Prepare several solutions when examining multiple antimicrobials with the same strain group.
Use new bacterial solutions for inoculation after about 5 antimicrobials.



Inoculation of bacterial solutions

- Inoculate the prepared bacterial solutions to the plates containing the drug

DO NOT let the multipoint inoculator to touch other tubes. If you do so, make sure to sterilize them before using again.

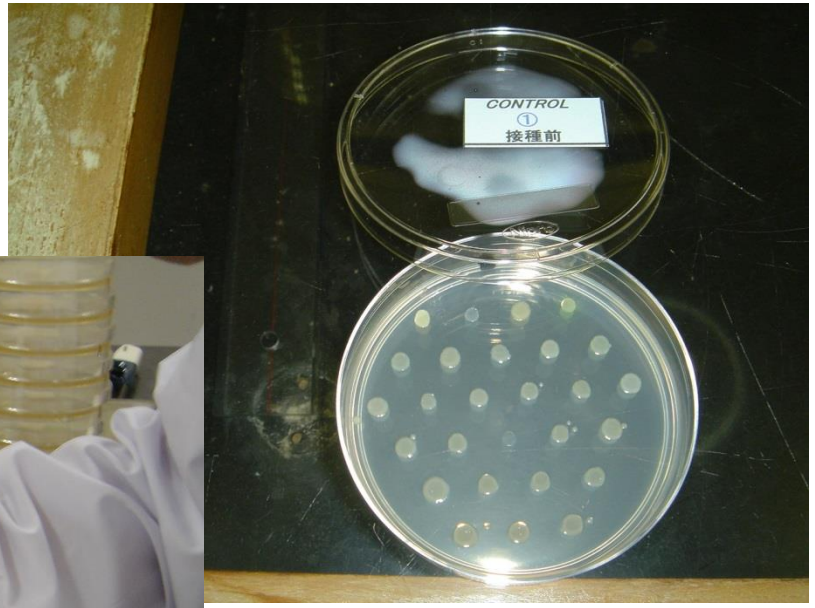
Up to 2 plates can be inoculated with the bacterial solution using the same multipoint inoculator.

Leave them at room temperature until the start of incubation.

Invert the plates, and then start incubation (at 35 ℃ for 16–20 hours).

Third day

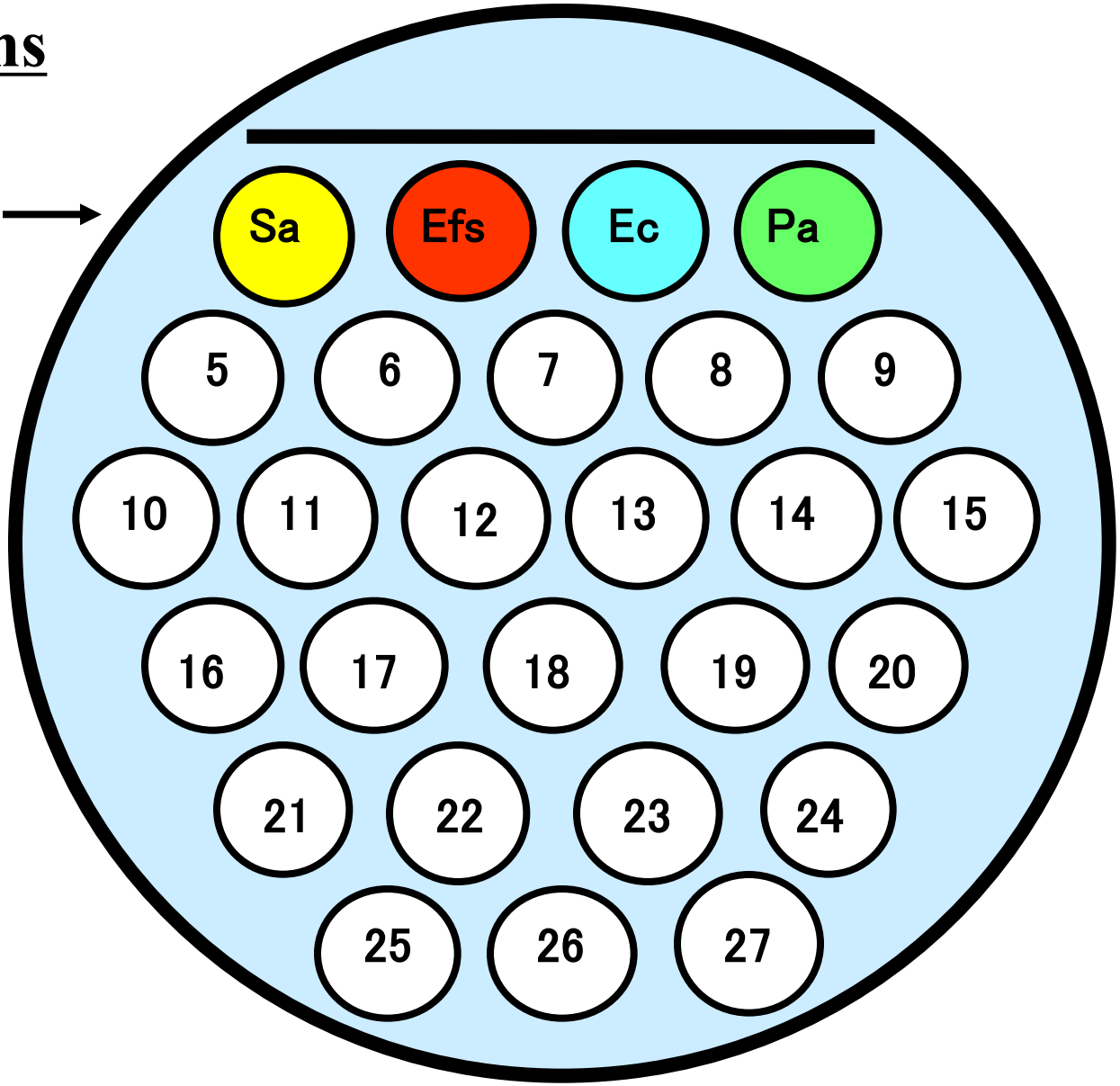
Assessment



Arrangement of the bacterial strains

Quality control strains
(4 strains)

Subject strains
(~23 strains)

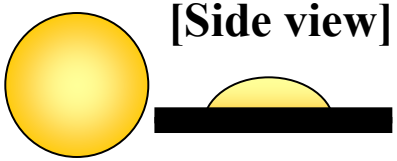


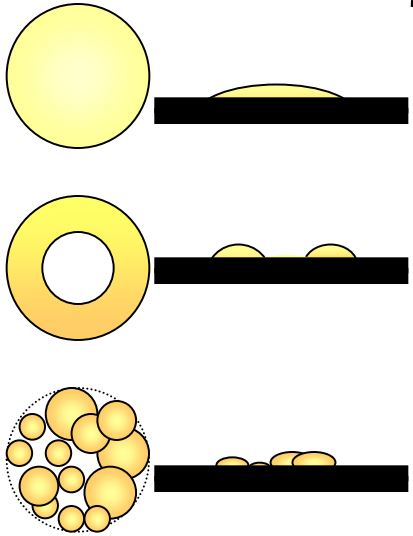
Assessment : +

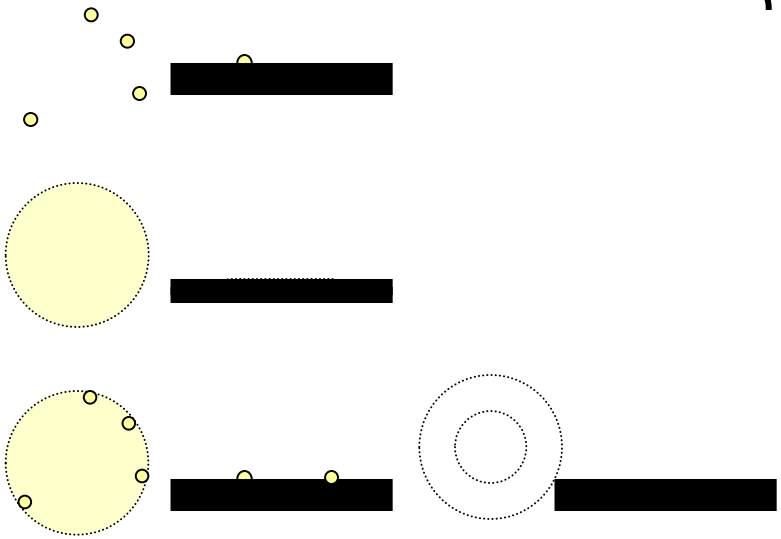
Assessment : −

[Overhead view]

[Side view]







Similar growth as the DW control plate.

Examples

+

(1) Slightly thinner than those on the DW control plate.

(2) Equally thick as those on the DW control plate, but with an empty center.

(3) Equally thick as those on the DW control plate, but are large clumps.

+ w

(1) Minute and thin colonies spread sporadically.

(2) Resembling drops of water stuck to a dusty glass panel.

(3) Minute colonies spread sporadically over ones resembling drops of water stuck to a dusty glass panel.

− w

Only the inoculation mark of the multipoint inoculator is left.

−

Assessment and Record

Strain: E. coli/Enterococcus

Drug name＋Plate No.: ABPC ＋ ①

Date of inoculation: 2003/6/25, Date of assessment: 2003/6/26.

	Strain name	Antimicrobial concentration (μg／ml)													MIC
			13	12	11	10	9	8	7	6	5	4	3	2	1	
		C	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	(μg/ml)
1	*S.aureus* ATCC29213	＋	＋	＋	＋	－	－	－	－	－	－	－	－	－	－	1
2	*E.faecalis* ATCC29212	＋	＋	＋	＋	＋	－	－	－	－	－	－	－	－	－	2
3	*E.coli* ATCC25922	＋	＋	＋	＋	＋	＋	＋	－	－	－	－	－	－	－	8
4	*P.aeruginosa* ATCC27853	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	－	－	－	－	64
5	B-1	＋	＋	＋	＋	＋	＋	＋	＋	－	－	－	－	－	－	16
6	B-2	＋	＋	＋	＋	＋	＋	＋	－	－	－	－	－	－	－	8
7	B-3	＋	－	－	－	－	－	－	－	－	－	－	－	－	－	≦0.125
8	B-4	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	512<
9	B-5	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	＋	－	512
10	P-1	＋	＋	＋	－	－	－	－	＋	＋	＋	－	－	－	－	0.5 ?
11	P-2	＋	＋	＋	＋	＋	－	＋	－	－	－	－	－	－	－	Retest ?
12	P-3	－	＋	＋	＋	＋	＋	－	－	－	－	－	－	－	－	Retest
13	P-4	－	－	－	－	－	－	－	－	－	－	－	－	－	－	Retest

Important points

Make sure that the MICs for QC strains are within the quality control range.

Errors of reagent dilution

Contamination of inoculated bacteria

To handle multiple samples, stickers are used to number master dilutions, dilution tubes and petri dishes.
For those requiring sticker labels, an Excel file will be sent.