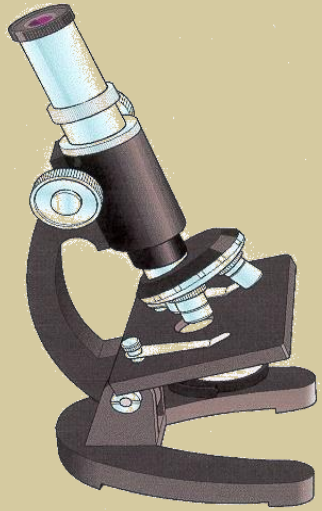
Suitability of Microbial Limits Test Methods



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MicroWorks, Inc.

- cGMP compliant Microbiological test laboratory located in Crown Point, IN
- Testing includes sterility, endotoxin analysis, raw material testing, Microbial Limits testing, Container closure integrity testing
- DEA licensed, Schedule 2-5
- Microbiological consulting services including qualification of cleanrooms (EMPQ), Set up of EM Programs, Micro method validation

Course Objectives

- High level understanding of the Microbial limits test
- References
- Setting up the test
- Transfer Steps
- Reading the Results
- Suitability testing
- Release of Media

Microbial Limits

- Testing performed on non-sterile products to demonstrate they are suitable for their intended use.
- Quantitative testing- Result in a number indicating the amount of bioburden detected in the product.
- Qualitative-Results in a positive or negative result.

References

- **USP 61**
- Quantitative testing
- Enumeration of mesophilic bacteria and fungi that grow under aerobic conditions.
- Can be performed by pour plating, spread plating or membrane filtration.
- Most probable number procedures may be used where levels are very low.

- **USP 62**
- Qualitative testing
- Also known as testing for specified organisms.
- Test included in the chapter include: *Salmonella, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, Bile-Tolerant Gram Negative (BTGN), *Clostridium*, and *Candida albicans.*

USP <1111>

- Informational chapter that goes with USP <61> and <62>
- Provides guidance regarding acceptance criteria
- The route of administration plays a key role in determining what is an acceptable limit.
- The health of the intended user plays a role as well.

Setting up the <61> Test

- The amount of product to be tested is weighed or pipetted into a diluent (may be phosphate buffer, TSB, letheen broth, DE broth, or other suitable diluent) to make a 1:10 dilution.
- Typically 10g is tested.
- The pH of the diluent is adjusted so that it is in the 6-8 range.
- The product is dissolved by shaking or vortexing until it goes into solution.

Setting up the <61> Test

- The sample is then pipetted into plates and media is added.
- For TAMC (Total aerobic microbial count) the media is typically TSA or TSA with lecithin and tween. Incubation is 30-35C for 3-5 days.
- For TYMC (Total yeast and mold count) the media is typically SDA or SDA with lecithin and tween. Incubation is 20-25C for 5-7 days.

Plating samples for USP <61>

- TSA or TSA with lecithin and tween is added to two of the plates. These plates are incubated at 30-35C for 3-5 days.
- SDA or SDA with lecithin and tween is added to two of the plates. These plates are incubated at 20-15C for 5-7 days.
- Different media may be needed depending on method development/validation.

Setting up USP <61>

- 10g of product is dissolved in diluent
- may be TSB, Lactose broth, phosphate buffer, D/E Neutralizer or other diluent that is shown to be suitable.
- The pH of the solution is adjusted to be between 6-8.
- 1 mL aliquots are aseptically plated.

Reading USP <61> Plates

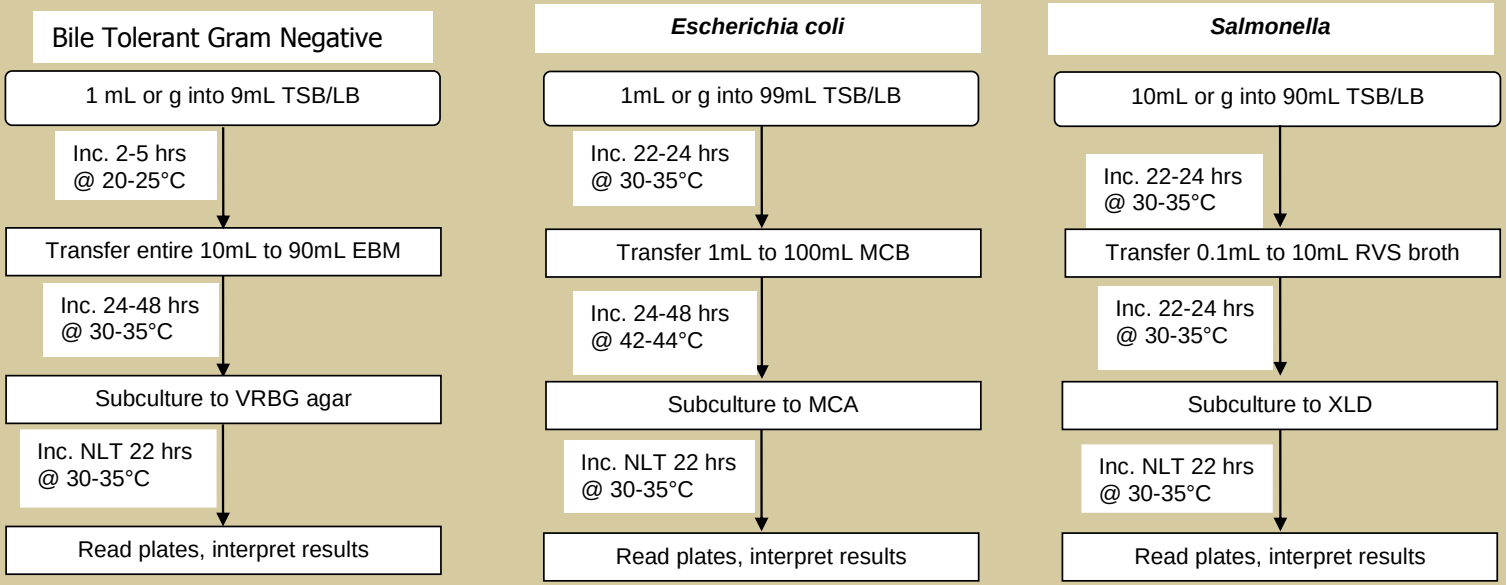
- Once plate incubation is completed the plates are examined for colonies.
- The number of colonies are counted and the results are calculated, taking into consideration what dilution was plated.
- For example: If the colony counts are 6 on one plate and 10 on the other. The total is 16/2 for an average count of 8. If the test was a 1/10 dilution the result is multiplied by 10 so the result is 80 cfu/g.

Setting up the <62> Testing

- Depending on the organisms that are specified the method will vary.
- Organisms for testing based on specification of the product, compendial specification, route of administration, country where it will be sold, origin of sample, etc.
- The amount of sample to be tested is typically 1-10 g. Testing for *E.coli* and *Salmonella* are typically 10g.

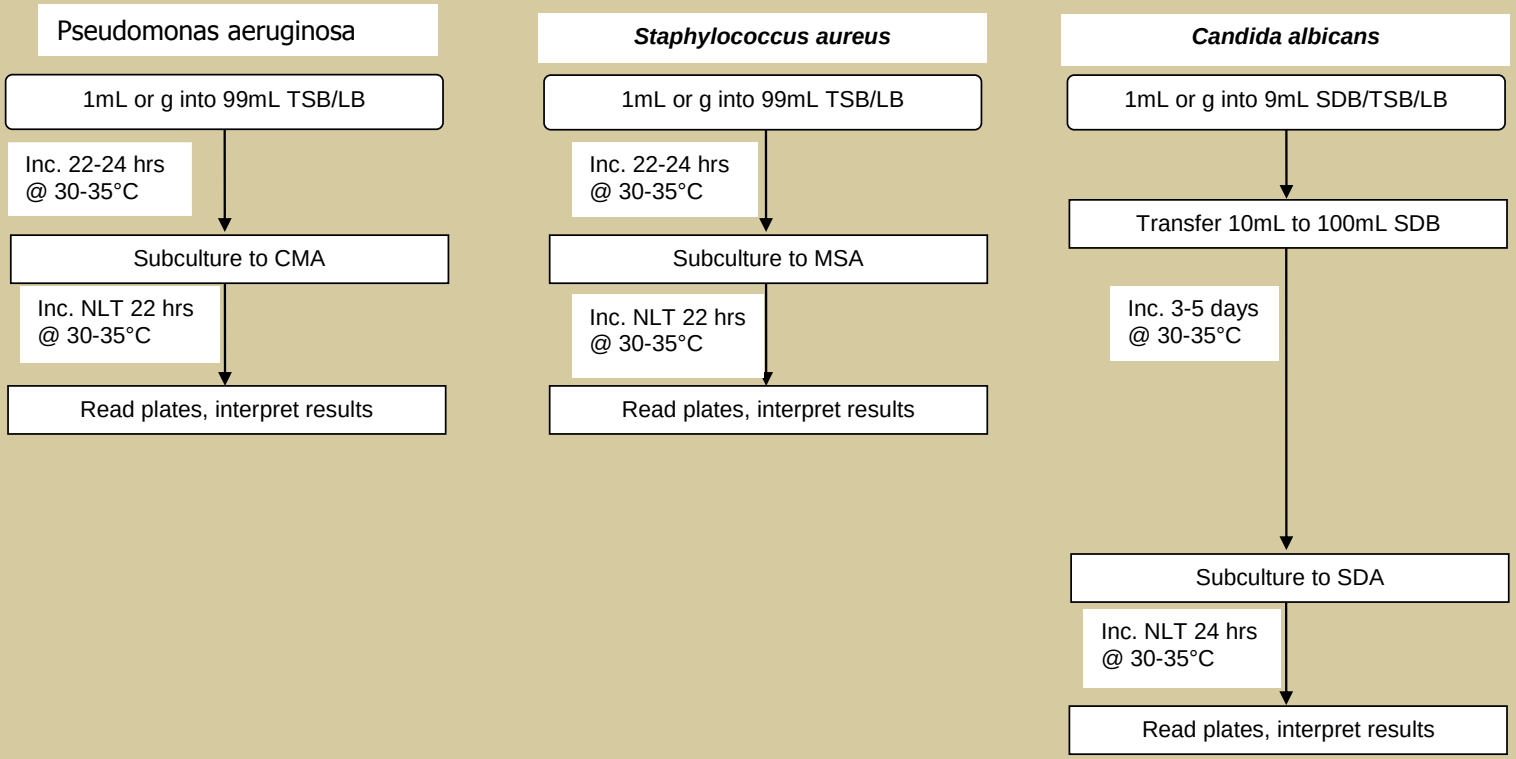
USP <62> Testing

Attachment 2. USP <62> Testing Flowcharts
BTGN, *E. coli*, *Salmonella*



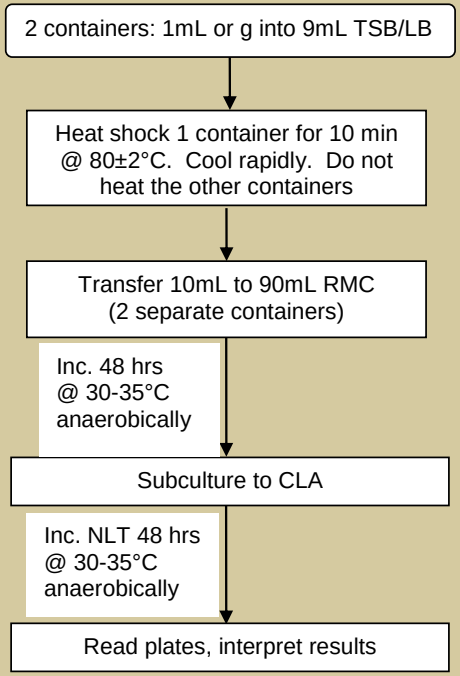
USP <62> Testing

Attachment 3. USP <62> Testing Flowcharts
Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans



USP <62> Testing

Attachment 4. USP <62> Testing Flowchart
Clostridia



Reading Results for USP <62>

- Since this is a qualitative test record whether or not there is growth on the plate.
- If there is growth on the plate determine if the growth is the organism being tested for.
- Gram stain the organism and observe it microscopically.
- If the organism is not the same morphology and gram reaction as the organism being tested the test is negative for the target organism.

Reading Results for USP <62>

- If the organism appears to be the same morphology and gram reaction as the organism being tested for perform an ID of the organism for confirmation.
- If the organism is the same as the organism being tested for the results is positive.
- If the organism is not the same as the organism being tested for it is reported as negative.
- Need to investigate the impact of the organism.

Method Suitability

- Method suitability is performed for each product that is to be tested by the Microbial Limits method.
- This is done to ensure that any inhibition that the product may have is overcome by the test method to ensure if contamination is present in the sample it will be detected.

Method Suitability for <61>

- To demonstrate that a variety of organisms can be detected in the presence of the sample.
- Sample is prepared according to the proposed method.
- pH is adjusted to between 6-8.
- Aliquots of the sample are spiked with representative organisms.
- Organisms plated without the presence of the product. These plates are the positive controls.

Method Suitability for USP <61>

- Representative Organisms include:
- *Staphylococcus aureus* (SA)-gram positive cocci
- *Pseudomonas aeruginosa* (PA)-gram negative rods
- *Bacillus subtilis* (BS)-gram positive rods with spores
- *Aspergillus brasiliensis* (AB)-mold
- *Candida albicans* (CA)-yeast

Method Suitability for USP <61>

- Bacteria are plated with TSA or TSA with lecithin and tween and incubated at 30-35C for ≤3 days.
- Yeast and mold are plated on TSA or TSA with lecithin and tween and incubated at 30-35C for ≤5 days and on SDA or SDA with lecithin and tween and incubated at 20-25C for ≤5 days.
- Other media may need to be substituted based on method development.

Method Suitability for USP <61>

- After incubation the plates are counted and the plates with the product (test samples) are compared to the positive control plates to see if the product caused inhibition.
- The percent recovery is calculated:

$$\text{Percent recovery} = \frac{\text{Test Sample}}{\text{Positive Control}} \times 100$$

Method Suitability for USP <61>

- Percent recovery must be between 50 and 200% for all organisms for the test to be valid.
- If any of the organisms are inhibited modifications of the method are needed to overcome the inhibition.
- This might include dilution, using a different primary enrichment, using a different media to plate, extending the incubation, filtering the product….

Example of Suitable TAMC Method

Total Aerobic Microbial Count, USP <61>			
Organism Challenge	Inoculum Count (cfu/plate)	% Recovery	Meets Acceptance Criteria Yes/No
Staphylococcus aureus	34.5	113%	Yes
Pseudomonas aeruginosa	36.5	99%	Yes
Bacillus subtilis	42.5	94%	Yes
Aspergillus brasiliensis	33.5	78%	Yes
Candida albicans	32.5	105%	Yes

Summary of sample preparation and testing:	
Method Suitability	Routine Test Method
Weighed 10g product into a sterile container. Prepared a 1:10 dilution by adding enough Phosphate Buffer to equal 100g. Adjusted pH by adding 0.4 mL 2M NaOH.	Weigh a minimum of 10g product into a sterile container. Add Phosphate Buffer to prepare a 1:10 dilution. Adjust pH by adding 0.4 mL 2M NaOH per 100 mL prepared sample solution.
Pour-plated 1mL in duplicate with TSA.	Pour-plate 1mL in duplicate with TSA.
Incubated for 44 hours and 20 minutes at 30-35˚ C.	Incubate for 3–5 days at 30-35˚ C.

Example of Suitable TYMC Method

Total Yeast and Mold Count, USP <61>			
Organism Challenge	Inoculum Count	% Recovery	Meets Acceptance Criteria Yes/No
Aspergillus brasiliensis	31.5	92%	Yes
Candida albicans	43.5	123%	Yes

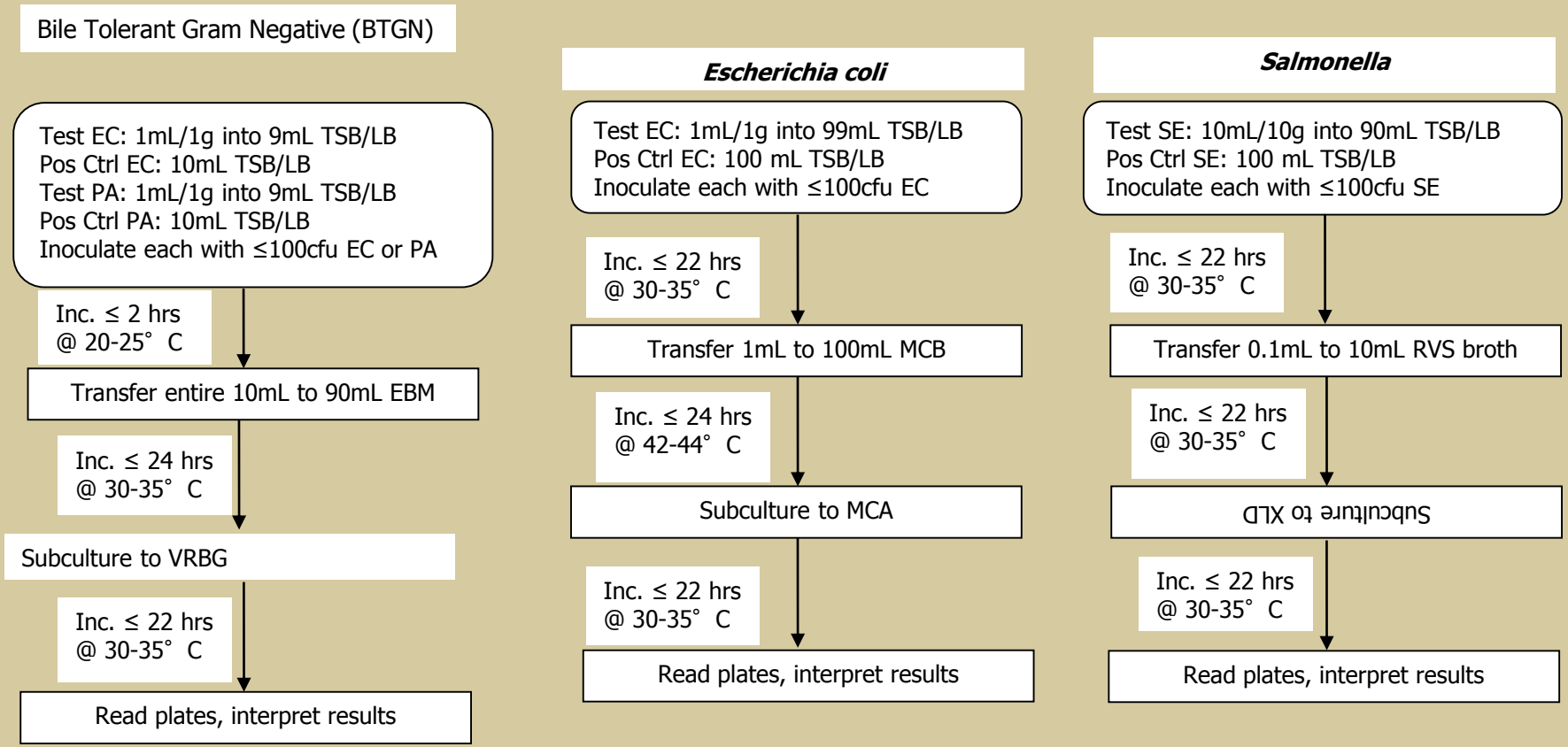
Summary of sample preparation and testing:	
Method Suitability	Routine Test Method
Weighed 10g product into a sterile container. Prepared a 1:10 dilution by adding enough Phosphate Buffer to equal 100g. Adjusted pH by adding 0.4 mL 2M NaOH.	Weigh a minimum of 10g product into a sterile container. Add Phosphate Buffer to prepare a 1:10 dilution. Adjust pH by adding 0.4 mL 2M NaOH per 100 mL prepared sample solution.
Pour-plated 1mL in duplicate with SDA.	Pour-plate 1mL in duplicate with SDA.
Incubated for 49 hours and 20 minutes at 20-25˚ C.	Incubate for 5–7 days at 20-25˚ C.

Suitability for USP 62

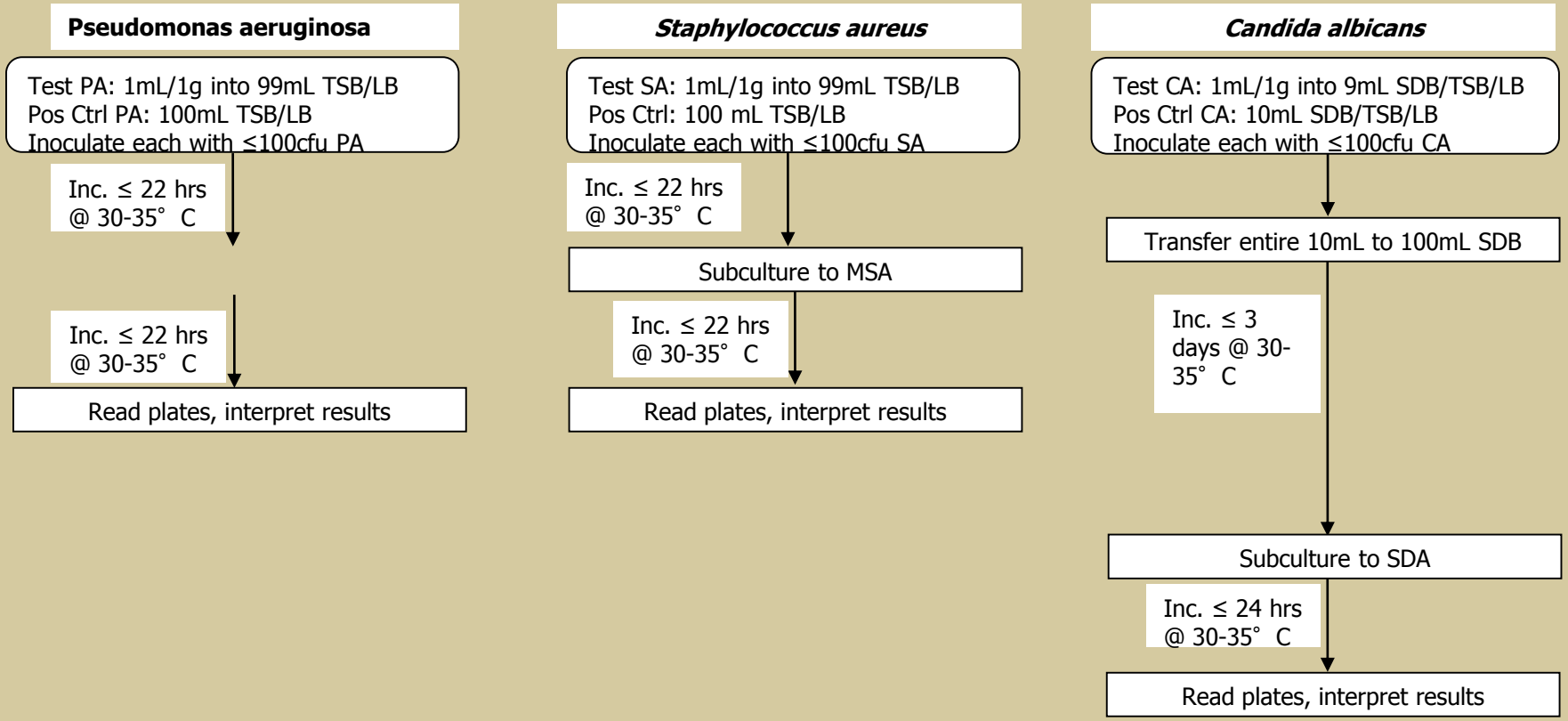
- Each specified organism requires a separate suitability test.
- Incubation steps are all performed at <time that the step will be performed routinely.
- For example, if method states, incubate for 18-24 hours, the suitability testing needs to be performed at <18 hours.

Organism							
Day	BTGN	EC	SE	PA	SA	CS	CA
Day 1	1g/1mL Product into 9mL TSB/LB	1g/1mL Product into 99mL TSB/LB	10g/10mL Product into 90mL TSB/LB	1g/1mL Product into 99mL TSB/LB	1g/1mL Product into 99mL TSB/LB	1g/1mL Product into 9mL TSB/LB tubes in duplicate	1g/1mL Product into 9mL SDB/TSB/ LB
	Inc. ≤2 h at 20-25˚ C					Heat one tube for 10 min. at 80˚ C	
	Transfer each 10mL TSB/LB to 90mL EBM broth					Transfer contents of each to 100mL RMC	Transfer 10mL to 100mL SDB
	Inc. ≤24 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C	Inc. ≤48 h at 30-35˚ C anaerobic	Inc. ≤3 days at 30-35˚ C
Day 2	Subculture to VRBG agar	Transfer 1mL to 100mL MCB	Transfer 0.1mL to 10mL RVS broth	Subculture to CMA	Subculture to MSA	N/A	N/A
	Inc. ≤22 h at 30-35˚ C	Inc. ≤24 h at 42-44˚ C	Inc. ≤22 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C		
Day 3	Read plates	Subculture to MCA	Subculture to XLD	Read plates	Read plates	Subculture to CLA	N/A
		Inc. ≤22 h at 30-35˚ C	Inc. ≤22 h at 30-35˚ C			Inc. ≤48 h at 30-35˚ C, anaerobic	
Day 4		Read plates	Read plates			N/A	Subculture to SDA
							Inc. ≤24 h at 30-35˚ C
Day 5						Read plates	Read plates

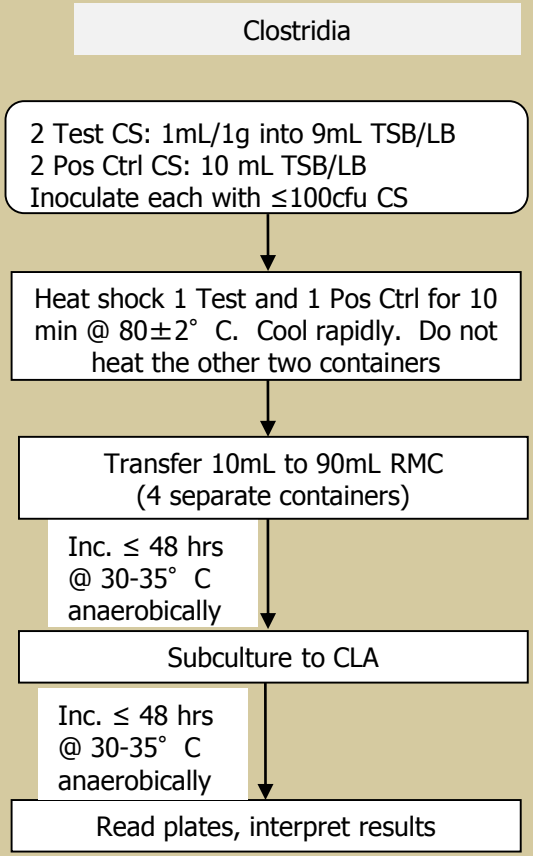
Example USP <62> Suitability Flowcharts



Example USP <62> Suitability Flowcharts



Example USP <62>Suitability Flowcharts



Acceptance Criteria for USP <62> Suitability

- Inoculum of challenge organism must be <100 cfu.
- Negative control must be negative.
- Growth obtained from challenge test must be comparable to growth of positive control. If not, additional method development work is needed.

Media for Microbial Limits Testing

- Media for USP <62> testing is specifically to select for the target organism and to inhibit other organisms that might complete with the target organism.
- Releasing selective media: Inoculate with <100 cfu of the target organism. Incubate at less than the shortest time it will be incubated.
- Also inoculate with >100 cfu of organisms that should be inhibited.

Setting up Media Release

Growth Promotion Testing for Non-Selective Media

Media	Organism	ATCC No.	Inoculum CFU	Incubation Temp	Incubation Time	Appearance
TSA	S. aureus	6538	≤100	30-35° C	≤ 3 days	Round yellow colonies
	P. aeruginosa	9027	≤100	30-35° C	≤ 3 days	Yellow-green to blue-green undulate round colonies
	B. subtilis	6633	≤100	30-35° C	≤ 3 days	Large flat colonies
	A. brasiliensis	16404	≤100	30-35° C	≤ 5 days	Fuzzy black colonies
	C. albicans	10231	≤100	30-35° C	≤ 5 days	Large milky colonies
(RMC) Reinforced Medium for Clostridia	*C. sporogenes*	11437 or 19404	≤100	30-35° C anaerobic	≤ 48 hrs	Growth
(LB) Letheen Broth	*P. aeruginosa*	9027	≤100	30-35° C	≤ 48 hrs	Growth
	B. subtilis	6633	≤100	30-35° C	≤ 48 hrs	Growth
	S. aureus	6538	≤100	30-35° C	≤ 48 hrs	Growth

Growth Promotion Testing for Selective Media

Media	Organism	ATCC No., or equivalent	Inoculum CFU	Incubation Temp	Incubation Time	Expected Results	Appearance
(CMA) Cetrimide Agar	*P. aeruginosa*	9027	≤100	30-35° C	≤ 22 hrs	Growth promoting	Yellow green to blue colonies
	E. coli	8739	≥100	30-35° C	≥ 72 hrs	Inhibitory	Inhibition of growth
(M-Endo) M-Endo Agar	*P. aeruginosa*	9027	≤100	30-35° C	≤ 48 hrs	Atypical	Clear to opaque colonies; no red color
	E. coli	8739	≤100	30-35° C	≤ 48 hrs	Typical	Red colonies with greenish metallic sheen
	S. aureus	6538	≥100	30-35° C	≥ 48 hrs	-	Marked to complete inhibition
(MSA) Mannitol Salt Agar	*S. aureus*	6538	≤ 100	30-35° C	≤ 22 hrs	Growth promoting + indicative	Small to large colonies with yellow zones
	E. coli	8739	≥ 100	30-35° C	≥ 72 hrs	Inhibitory	Marked to complete inhibition

Summary

- Microbial Limits testing is one of the most common test methods in the lab.
- All methods are shown to be suitable before tests are performed.
- The method that is used for suitability testing details how the sample is prepared and how long it is incubated.
- The set up of the test needs to follow the same method every time it is performed.
- All media needs specifications for release testing.
- Specifications vary from client to client and product to product so it is important to verify the requirements for each test.