L-Ornithine-L- Aspartate
Microbiological Examination Suitability
Validation Report

Jing Jing Pharmaceutical Co.,Ltd.

1. Abstract

Microbiological suitability of counting method has been verified to make sure this examination is suitable for microbiological enumeration in L-Ornithine-L-Aspartate.

This method is suitable for Total aerobic microbial count (TAMC),Total yeasts and moulds count (TYMC) and e.coli in L-Ornithine-L-Aspartate.

2. Team member

Li Binghua,Wu Cunjing,Wang Zheng,Li Jianfei

3. Responsibilities

Leader:Review this method validation protocol and report,supervise and coordinate the validation work, handle any deviation in this validation,

QC:Draft the validation protocol, carry out method validation and prepare the validation report.

QA：Review this method validation protocol and report,approval method validation protocol and report.

4. References

Good Manufacturing Practices for Pharmaceutical Products (2010 Revision) chapter10:quality control and quality assurance;

Good Manufacturing Practices for Pharmaceutical Products:Quality control laboratory and material system;

Chinese Pharmacopeia 1105 “Microbiological Examination of Nonsterile Products”：Microbial Enumeration Tests”

Chinese Pharmacopeia 1106 “Microbiological Examination of Nonsterile Products”：Tests for specified Microorganisms”

5. Testing method

Table 2 Specification

Product Name	Testing Items	Specification
L-Ornithine-L-Aspartate	Total aerobic microbial count	≤1000cfu/g
	Total yeasts and moulds count	≤100cfu/g
	e.coli	Absence /g

6. Preparation

6.1. Instruments

Table 1 Instruments

Instrument	Vendor	Model	Instrument No.	Calibration date	Calibration certificate
electric balance	METTLER 702EDO	PL203	EM-ZV-005	2022.04.26-2023.04.25	LXZ722-01472
biochemical incubator	Shanghai yiheng scientific Instruments Co., Ltd	2RH-250	TD-Z4-012	2022.04.25-2023.04.24	RGG422-JZ02517
incubator	Jinjing pharmaceutical Co.,Ltd	/	TD-Z4-030	2022.04.25-2023.04.24	RGG422-JZ02531
vertical sterilization cabinet	Shandong xinhua medical equipment Co., Ltd	2MQ-C	10-HY-002	2022.05.06-2023.05.05	/
pulse vacuum sterilization cabinet	Shandong xinhua medical equipment Co., Ltd	XG1-D	10-HY-001	2022.05.06-2023.05.05	/

6.2. Stains and suspensions

6.2.1. Test strains

Manufacturer has qualified the following test strains information including name,source,variation or not,and passage.The viable microorganisms used for inoculation are not more than 5 passages.

Table 3 Test Strains

Strain name	Source	No.	Passages	Variation
Staphylococcus aureus	NMPA	26003-9α5-2-31003-03	3	No
Pseudomonas aeruginosa	NMPA	10104-2α31-1-31002-03	3	No
Bacillus subtilis	NMPA	64501-2α29-2-31002-03	3	No
E.coli	NMPA	44102-2A1-2-31002-03	3	No
Candida albicans	NMPA	98001-2α23-1-31002-03	3	No
Aspergillus niger	NMPA	98003-0A1005-31002-03	3	No

6.2.2. Preparation of microbial suspension

Prepare the test strains including e.coli, Staphylococcus aureus, Candida albicans, Pseudomonas aeruginosa , Aspergillus niger and Bacillus subtilis according to “Test strains preparing standard operation procedure”SOP-012-JYF033.

6.3. L-Ornithine-L-Aspartate Product

Batch No.:C552210001,C552210002,C552211001

6.4. Mediums

The suitability test of the medium used should meet the requirements.

Table 4 Medium information

Medium	Batch No.	Supplier
Soybean-casein digest agar	2112112	Beijing zhiyao technology development company
Sabouraud dextrose agar	22010052	Beijing zhiyao technology development company
Soybean casein digest broth	210113	Beijing zhiyao technology development company
Sabouraud dextrose broth	2103232	Beijing zhiyao technology development company
MacConkey agar	2004092	Beijing zhiyao technology development company
MacConkey broh	210714	Beijing zhiyao technology development company
pH7.0 sterile buffered sodium chloride-pettone solution	210428	Beijing zhiyao technology development company
Sodium chloride	2020010114	Tianjin city zhiyuan chemical reagents Co.,Ltd

7. Suitability testing

7.1. Preparation

Take one batch of L-Ornithine-L-Aspartate to have test in the same condition of incubation and enumeration.

Table 5 Preparation and use of test microorganism for TAMC ,TYMC

Medium	Test strain		Blank	Acceptable criteria	Conclusion
Sabouraud dextrose agar	Candida albicans	0.9	No bacteria growth	No bacteria growth in blank solution.The recovery rate should be in 50-200%	conforms
	Aspergillus niger	0.9			conforms
Soybean-casein digest agar	Pseudomonas aeruginosa	1.0	No bacteria growth		conforms
	Staphylococcus aureus	0.9			conforms
	Bacillus subtilis	0.9			conforms

	Candida albicans	0.9			conforms
	Aspergillus niger	0.8			conforms

Table 6 Preparation and use of test microorganism for e.coli

Culture	Growth-promotion capacity	Inhibitory capability	Indicating capacity	Acceptable criteria	Conclusion
MacConkey agar	Bacterial growth well on the E.coli medium	No bacterial growth on the Staphylococcus aureus medium .		Growth-promotion capacity:Bacterial growth well on the E.coli medium; Inhibitory capability:No bacterial growth on the Staphylococcus aureus medium .	conforms
MacConkey broth	Bacterial growth well on the E.coli medium		The bacteria growth and indicative reaction in the e.coli culture medium is consistent with in the reference medium.	Growth-promotion capacity:Bacterial growth well on the E.coli medium; Indicating capacity:The bacteria growth and indicative reaction in the e.coli culture medium is consistent with in the reference medium.	conforms

7.2. Test Results

Table 7 Test results of TAMC (Batch No.2201052)

Medium name:Sabouraud Dextrose Agar medium	batch No.： 2201052	Preparing No.:20220707
Reference medium name: Sabouraud Dextrose Agar reference medium	batch No.：135013-2021004	Preparing No.:20220707
☐incubate in 30-35℃ thermostatic room(room No:/) for 3days; ☑incubate in 20-25℃ biochemical incubator(Type:LRH250,No:TD-Z4-012) for 5days;		
Starting date:2022.07.13 Time:15：42	Ending date:2022.07.18 Time：15：50	

Strain name	Strain No.	Microbial	Microbial counting for reference group (cfu/ml)	Incubation days										Average(cfu)	Ration with the reference group(should be in 0.5-2)	Comparing the colony shape
				1		2		3		4		5				
				Dish 1	Dish 2	Dish 1	Dish 2	Dish 1	Dish 2	Dish 1	Dish 2	Dish 1	Dish 2			
Candida albicans	98001-2α 23-1-210 03-03	Control group	83	0	2	49	53	76	80	80	81	80	81	81	0.9	Sharp and size is consistent
		Product group		1	4	54	61	88	90	91	93	91	93	92		
Aspergillus niger	98003-0 A1005-2	Control group	32	0	0	0	2	19	22	31	35	31	35	33	0.9	Sharp and

	1003-03	Product group		0	0	3	4	21	24	34	39	34	39	37		size is consistent
		Control group Product group Control group Product group														
negative control group				0	/	0	/	0	/	0	/	0	/	/		
Reviewer				Li Jiangbing		Li Jiangbing		Li Jiangbing		Li Jiangbing		Li Jiangbing		Li Jiangbing	Li Jiangbing
Date				2022.07.14		2022.07.15		2022.07.16		2022.07.17		2022.07.18		2022.07.18	2022.07.18
Note	/														

Test date:2022.07.13

Review date:2022.07.18

Table 8 Test results of TAMC (Batch No.Batch 2112112)

Medium name:Soybean-casein digest agar	batch No.： 2112112	Preparing No.:20220114
Reference medium name: Soybean-casein digest agar	batch No.：135025-201804	Preparing No.:20220114

☑incubate in 30-35℃ thermostatic room(room No:TD-Z4-030) for 3days;

☐incubate in 20-25℃ biochemical incubator(Type:LRH250,No:/) for 5days;

Starting date:2022.01.18 Time:16:07	Ending date:2022.01.23 Time：16:10

Strain name	Strain No.		Microbial counting for reference group (cfu/ml)	Incubation days										Average(cfu)	Ration with the reference group(0.5-2)	Comparing the colony shape
				1		2		3		4		5				
				Dish 1	Dish 2	Dish 1	Dish 2	Dish 1	Dish 2	Dish 1	Dish 2	Dish 1	Dish 2			
Staphylococcus	26003-9α4-1-21003-03	Control group	61	30	33	53	59	54	59					57	1.0	Sharp and size is consistent
		Product group		32	41	56	64	56	64					60		
Pseudomonas aeruginosa	10104-2α29-2-21003-03	Control group	63	29	34	56	63	56	63					60	0.9	Sharp and size is consistent
		Product group		35	38	59	68	60	68					64		
Bacillus subtilis	63501-2α28-2-210	Control group	68	19	23	52	50	45	54					50	0.9	Sharp and size

	02-03	Product group		22	27	49	59	50	62					56		is consistent
Candida albicans	98001-2α 21-2-410 002-03	Control group	61	9	14	22	30	48	52	48	54	48	54	51	0.9	Sharp and size is consistent
		Product group		19	26	42	50	56	59	56	62	56	62	59		
Aspergillus niger	98003-0 A1004-3 1002-03	Control group	26	0	0	1	3	10	12	14	17	14	17	16	0.8	Sharp and size is consistent
		Product group		0	0	3	4	14	21	18	22	18	24	21		
negative control group				0	/	0	/	0	/	0	/	0	/	/		
Reviewer				Li Jiangbing		Li Jiangbing		Li Jiangbing		Li Jiangbing		Li Jiangbing		Li Jiangbing	Li Jiangbing	
Date				2022.01.19		2022.01.20		2022.01.21		2022.01.22		2022.01.23		2022.01.23	2022.01.23	
Note			/													

Test date:2022.01.18

Review date:2022.01.23

Table 9 Growth promotion testing (Batch No.210714)

<table>
<tr><td colspan="3">Medium name:MacConkey broth</td><td colspan="3">batch No.：210714</td><td colspan="3">Preparing No.:20210914</td></tr>
<tr><td colspan="3">Reference medium name: MacConkey reference broth</td><td colspan="3">batch No.：1905282</td><td colspan="3">Preparing No.:20210914</td></tr>
<tr><td colspan="9">incubate in 30-35℃ thermostatic room(room No:TD-ZK-030) for 18-24hours;</td></tr>
<tr><td colspan="3">Starting date:2021.09.16</td><td colspan="6">Ending date:2021.09.17</td></tr>
<tr><td colspan="3" rowspan="2">Test strains name and code / Number of colony</td><td rowspan="2">The number of testing colony (cfu/ml)</td><td colspan="2"></td><td rowspan="2">Results</td><td rowspan="2">Negative</td><td rowspan="2">Conclusion</td></tr>
<tr><td>Dish 1</td><td>Dish 2</td><td>Average</td></tr>
<tr><td>Growth promotion capacity</td><td>E.coli</td><td>44102-3α33-1-41003-03</td><td>32</td><td>41</td><td>37</td><td>Bacteria growth well in the control group</td><td rowspan="3">No bacteria growth</td><td rowspan="3">Conforms</td></tr>
<tr><td>Inhibiting microbial growth capacity</td><td>Staphylococcus aureus</td><td>26003-9α2-2-41003-03</td><td>48</td><td>60</td><td>54</td><td>No bacteria growth</td></tr>
<tr><td>Indicating capacity</td><td colspan="6"></td></tr>
<tr><td>Note</td><td colspan="8"></td></tr>
</table>

Test date:2021.09.16

Review date:2021.09.17

Approval date:2021.09.17

Table 10 Growth promotion testing (Batch No.Batch 2004092)

<table>
<tr><td colspan="3">Medium name:MacConkey agar</td><td colspan="3">batch No.：2004092</td><td colspan="3">Preparing No.:20210308</td></tr>
<tr><td colspan="3">Reference medium name: MacConkey reference agar</td><td colspan="3">batch No.：135009-202503</td><td colspan="3">Preparing No.:20210308</td></tr>
<tr><td colspan="9">incubate in 30-35℃ thermostatic room(room No:TD-ZK-030) for 18-24hours;</td></tr>
<tr><td colspan="3">Starting date:2021.02.11</td><td colspan="6">Ending date:2021.03.12</td></tr>
<tr><td colspan="3" rowspan="2">Test strains name and code / Number of colony</td><td colspan="3">The number of testing colony (cfu/ml)</td><td rowspan="2">Results</td><td rowspan="2">Negative</td><td rowspan="2">Conclusion</td></tr>
<tr><td>Dish 1</td><td>Dish 2</td><td>Average</td></tr>
<tr><td>Growth promotion capacity</td><td>E.coli</td><td>44102-3α31-2-41002-03</td><td>32</td><td>41</td><td>37</td><td>Colony size and shape characteristic in testing medium is consistent with in the reference medium</td><td rowspan="3">No bacteria growth</td><td rowspan="3">Conforms</td></tr>
<tr><td>Inhibiting microbial growth capacity</td><td colspan="6"></td></tr>
<tr><td>Indicating capacity</td><td>E.coli</td><td>44102-3α31-2-41002-03</td><td>32</td><td>41</td><td>37</td><td>Indicator reaction in testing medium is consistent with in the reference medium</td></tr>
<tr><td>Note</td><td colspan="8"></td></tr>
</table>

Test date:2021.03.11

Review date:2021.03.12

Approval date:2021.03.12

Table 11-e.coli method suitability result

Testing	Group	2101001	2102002	2102003
e.coli	Testing group	(+)	(+)	(+)
	Negative control group	(-)	(-)	(-)
Note：(+)Indicates that the corresponding test bacteria are detected;				

7.3. Conclusion:

The test results conform with the requirements.These mediums are suitable for the testing.

8. Microbial counting

6.1 Test strains:

Staphylococcus aureus,Bacillus subtilis, Pseudomonas aeruginosa,Candida albicans and Aspergillus niger

6.2 Solution Preparing:

1)Product solution:Take 10g of LOLA, add sterile buffered sodium chloride-pettone solution pH7.0 to 100mL, mix well as 1:10 product solution.

2)Testing group:Take 10ml of 1:10 product solution ,filter,rinse the filter membrane by 100ml of sterile buffered sodium chloride-pettone solution pH7.0 for 3times.Add 1ml of the testing strains in the last rinseing solution(not more than 100cfu).Filter.Take the filter membrane.incubate on the plate of Soybean-casein digest agar or Sabouraud dextrose agar.Prepare 2 filter membrane each medium.Calculate the average of microbial counting($A_{average}$).

3)Reference group:Take 10ml of 1:10 product solution ,filter,rinse the filter membrane by 100ml of sterile buffered sodium chloride-pettone solution pH7.0 for 3times.Filter.Take the filter membrane.incubate on the plate of Soybean-casein digest agar or Sabouraud dextrose agar.Prepare 2 filter membrane each medium.Calculate the average of microbial counting($B_{average}$).

4)Inoculum group:Take 1ml of testing strains solution(not more than 100cfu/ml) into plate.Prepare 2plates each bacteria in parallel.Calculate the average of microbial counting($C_{average}$).

6.3 Test procedure

Incubate staphylococcus aureus,Bacillus subtilis and pseudomonas aeruginosa on the Soybean-casein digest agar in 30-35℃for not more than 3days.;

Incubate Candida albicans and Aspergillus niger on the Soybean-casein digest agar in 30-35℃for not more than 5days.;Iincubate Candida albicans and Aspergillus niger on the Sabouraud dextrose agar in 30-35℃for not more than 5days.;

Test for 3times in parallel.

6.4 Acceptable criteria

Ratio = (Inoculum group-Product group)/Control group should be in the range of 0.5-2;

6.5 Test results

Table 12 TAMC,TYMC counting method(Batch No.C552210001)

Group	Stains name	Product batch No.	C552210001	Test No.	No.1
		Number of colony A_1(cfu)	Number of colony A_2(cfu)	Number of colony $A_{avergae}$(cfu)	The recovery rate of testing group
Testing group	Pseudomonas aeruginosa	74	84	79	0.65
	Staphylococcus aureus	58	66	62	0.65
	Bacillus subtilis	48	53	51	0.52
	Candida albicans(aerobic)	61	84	73	0.66
	Aspergillus niger(aerobic)	62	72	67	0.73
	Candida albicans	36	51	44	0.90
	Aspergillus niger	30	34	32	0.86

Reference group	TAMC B(cfu)		$B_{average}$(cfu)	TYMC B(cfu)		$B_{average}$(cfu)
	38	42	40	6	8	7

Inoculum group	Stains name	Number of colony C_1(cfu)	Number of colony C_2(cfu)	Number of colony $C_{avergae}$(cfu)
	Pseudomonas aeruginosa	52	68	60
	Staphylococcus aureus	33	34	34
	Bacillus subtilis	20	22	21
	Candida albicans(aerobic)	42	57	50
	Aspergillus	35	38	37

	niger(aerobic)			
	Candida albicans	36	45	41
	Aspergillus niger	28	29	29
Recovery rate	Recovery rate=(A-B)/C			
Conclusion	Ratio = (Inoculum group-Product group)/Control group is in the range of 0.5-2.It confirms with the requirements.			
Note	/			
Analyst	Li Bingjia	Date	2022.11.05	
Reviewer	Wu Cunjing	Date	2022.11.10	

Table 13 TAMC,TYMC counting method(Batch No.C552210002)

Group	Stains name	Product batch No.	C552210002	Test No.	No.2
		Number of colony A_1(cfu)	Number of colony A_2(cfu)	Number of colony $A_{avergae}$(cfu)	The recovery rate of testing group
Testing group	Pseudomonas aeruginosa	62	78	70	1.02
	Staphylococcus aureus	38	44	41	0.91
	Bacillus subtilis	22	22	22	0.52
	Candida albicans(aerobic)	39	42	41	0.77
	Aspergillus niger(aerobic)	37	40	39	0.82
	Candida albicans	28	30	29	0.78
	Aspergillus niger	27	29	28	0.93

Reference group	TAMC B(cfu)		$B_{average}$(cfu)	TYMC B(cfu)		$B_{average}$(cfu)
	10	12	11	0	1	1

Inoculum group	Stains name	Number of colony C_1(cfu)	Number of colony C_2(cfu)	Number of colony $C_{avergae}$(cfu)
	Pseudomonas aeruginosa	54	62	58

	Staphylococcus aureus	30	36	33
	Bacillus subtilis	19	22	21
	Candida albicans(aerobic)	35	43	39
	Aspergillus niger(aerobic)	33	35	34
	Candida albicans	33	38	36
	Aspergillus niger	26	31	29
Recovery rate	Recovery rate=(A-B)/C			
Conclusion	Ratio = (Inoculum group-Product group)/Control group is in the range of 0.5-2.It confirms with the requirements.			
Note	/			

Analyst	Li Bingjia	Date	2022.11.05
Reviewer	Wu Cunjing	Date	2022.11.10

Table 14 TAMC,TYMC counting method(Batch No.C552211001)

Group	Stains name	Product batch No.	C552211001	Test No.	No.3
		Number of colony A_1(cfu)	Number of colony A_2(cfu)	Number of colony $A_{avergae}$(cfu)	The recovery rate of testing group
Testing group	Pseudomonas aeruginosa	48	52	50	0.82
	Staphylococcus aureus	27	32	30	0.77
	Bacillus subtilis	26	28	27	0.81
	Candida albicans(aerobic)	36	36	36	0.70
	Aspergillus niger(aerobic)	27	30	29	0.56
	Candida albicans	29	30	30	0.71
	Aspergillus niger	27	33	30	1.08

Reference	TAMC B(cfu)	$B_{average}$(cfu)	TYMC B(cfu)	$B_{average}$(cfu)

group	9	10	10	2	3	3
Inoculum group	Stains name	Number of colony C_1(cfu)	Number of colony C_2(cfu)	Number of colony $C_{avergae}$(cfu)		
	Pseudomonas aeruginosa	48	50	49		
	Staphylococcus aureus	25	26	26		
	Bacillus subtilis	21	21	21		
	Candida albicans(aerobic)	35	38	37		
	Aspergillus niger(aerobic)	28	39	34		
	Candida albicans	36	39	38		
	Aspergillus niger	23	26	25		
Recovery rate	Recovery rate=(A-B)/C					
Conclusion	Ratio = (Inoculum group-Product group)/Control group is in the range of 0.5-2.It confirms with the requirements.					
Note	/					

Analyst	Li Bingjia	Date	2022.11.05
Reviewer	Wu Cunjing	Date	2022.11.10

9. Control bacteria testing

9.1. Solution preparation:

Product solution:Take 10g of LOLA, add sterile buffered sodium chloride-pettone solution pH7.0 to 100mL, mix well as 1:10 product solution.

Take 10ml of 1:10product solution.rinse 100ml for 3times.Filter.Prepare 2filter membrane.Take 3 bottles of Soybean-casein digest agar,100ml each bottle.Separately put one filter membrane into 2bottles

Positive reference group:Put 1ml of reference bacteria into one of bottle as positive reference group.The number of colony should not more than 100cfu.

Negative reference group:Take the filter membrane which filter by 10ml of sterile buffered sodium chloride-pettone solution pH7.0 into the third bottle.

incubate in 30-35℃for 18--24hours.There is no bacteria growth in the negative reference group.

9.2. Test procedure:

Transfer 1ml of above solution.Inoculate into 100ml of MacConkey broth.Incubate in 42-44℃for 24-48hours.Take MacConkey broth.Streak on the plate of MacConkey agar.incubate in 30-35℃ for 18-72hours.Test for 3times in parallel.

9.3. Acceptable criteria:

There should be bacteria growth in the positive reference group.

The characteristic of colony on the MacConkey agar should be consistent with the characteristic of E.coli,which should be bright peach or slightly red, colony center dark peach, round, flattened, neat edge, smooth surface, moist.

9.4. Test results:

Table 15 Control bacteria testing method(Batch No.C552210001)

Product batch No.	C552210001	Test No.	No.1
Group name	Soybean-casein digest agar	MacConkey broth	Colony morphology
Negative reference group	Negative	Negative	/
Product group	Negative	Negative	/
Positive group	Positive	Positive	Bright peach or slightly red, colony center dark peach, round, flattened, neat edge, smooth surface, moist
Conclusion	There is bacteria growth in the positive reference group. The characteristic of colony on the MacConkey agar is consistent with the characteristic of E.coli. It confirms with the requirements.		
Note	/		
Analyst	Li Bing jia	Date	2022.11.05
Reviewer	Wu Cunjing	Date	2022.11.08

Table 16 Control bacteria testing method(Batch No.C552210002)

Product batch No.	C552210002	Test No.	No.2
Group name	Soybean-casein digest	MacConkey broth	Colony morphology

	agar		
Negative reference group	Negative	Negative	/
Product group	Negative	Negative	/
Positive group	Positive	Positive	Bright peach or slightly red, colony center dark peach, round, flattened, neat edge, smooth surface, moist
Conclusion	There is bacteria growth in the positive reference group. The characteristic of colony on the MacConkey agar is consistent with the characteristic of E.coli. It confirms with the requirements.		
Note	/		
Analyst	Li Bing jia	Date	2022.11.05
Reviewer	Wu Cunjing	Date	2022.11.08

Table 17 Control bacteria testing method(Batch No.C552211001)

Product batch No.	C552211001	Test No.	No.3
Group name	Soybean-casein digest agar	MacConkey broth	Colony morphology
Negative reference group	Negative	Negative	/
Product group	Negative	Negative	/
Positive group	Positive	Positive	Bright peach or slightly red, colony center dark peach, round, flattened, neat edge, smooth surface, moist
Conclusion	There is bacteria growth in the positive reference group. The characteristic of colony on the MacConkey agar is consistent with the characteristic of E.coli. It confirms with the requirements.		

Note	/		
Analyst	Li Bing jia	Date	2022.11.05
Reviewer	Wu Cunjing	Date	2022.11.08