8681 Robert Fulton Drive
Columbia, MD 21046
T 877-ITX-PHAGE
F 410-625-2506
E info@intralytix.com
W intralytix.com

A-0000

intralytix
SAFETY BY NATURE™

4 December 2024

Attn: Dr. David Edwards
Director
U.S. Food and Drug Administration
MPN 2, Room E436
12225 Wilkins Avenue
Rockville, MD 20852

Re: Intralytix GRAS Notification for SalmoFresh™ to control *Salmonella* contamination of pet food and animal feed.

Dear Dr. Edwards,

In accordance with the Federal Register [81 Fed. Reg. 159 (17 August 2016)] issuance on GRAS notifications (21 CFR Part 570), Intralytix is pleased to submit a notice that we have concluded, through scientific procedures, the bacteriophage cocktail, SalmoFresh™, is generally recognized as safe and is not subject to the pre-market approval requirements for the use in pet foods and animal feeds, generally, as a processing aid to control *Salmonella enterica*.

SalmoFresh is currently GRAS affirmed (GRN 435) for various human foods including ready-to-eat and raw poultry products; ready-to-eat and raw red meat carcasses, subprimals, and trimmings; fish; shellfish; fresh and processed fruits and vegetables; and grain and grain products at up to 1×10^8 PFU/g of food. We believe that the intended use of SalmoFresh is also safe at the proposed use level of up to 1×10^8 PFU/g of pet food and animal feed. The method of manufacture and identity of SalmoFresh™ are the same as listed in the original GRAS notification for human foods (GRN 435). SalmoFresh application to pet food and animal feed products will result in insignificant dietary exposure of various ingredients (including endotoxins) and is not a safety concern. A review of literature, last conducted on 7th November 2024, continues to strongly support the safety of lytic phages and the absence of adverse effects in animals and humans. Included as supporting data for this submission, are efficacy studies (see Appendix 7.1.20.1 through 7.1.20.5) that show that SalmoFresh significantly reduces *Salmonella* contamination in various pet foods. In addition, efficacy studies submitted on 17th April 2023, as part of GRN 435

(also included with this submission), demonstrate that SalmoFresh significantly reduces *Salmonella* contamination on grain and grain products, that are major components of animal feed. Altogether, the available evidence indicates that (i) application of SalmoFresh can effectively reduce the levels of *Salmonella* in various human and animal/pet foods and animal feeds, (2) lytic phages – including component phages of SalmoFresh - are safe when administered to humans and other animals orally (including through their food), and (3) application of SalmoFresh at up to the maximum application rate of 1×10^8 PFU/g of pet food and animal feed will result in negligible increases in the dietary intake of phages, endotoxins, sodium, and potassium and is, therefore, of no safety concern.

Thank you for your time and consideration in reviewing this request. Should you have any questions regarding the submission, please feel free to contact me.

Sincerely,

(b) (6)

Alexander Sulakvelidze
President and CEO

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By

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1 SIGNED STATEMENTS AND CERTIFICATION

1.1 STATEMENT OF INTENT

In accordance with 21 CFR 570.220 regulations for GRAS notifications, Intralytix is pleased to submit a notice that we have concluded through scientific procedures the bacteriophage preparation, SalmoFresh™, is generally recognized as safe and is not subject to the premarket approval requirements for the use in pet food and animal feed as a processing aid to control *Salmonella* under the intended use conditions described within this notification.

1.2 NAME & ADDRESS OF NOTIFIER

Intralytix, Inc.
8681 Robert Fulton Dr.
Columbia, MD 21046
Tel: 877-489-7424
Fax: 410-625-2506

1.3 COMMON OR USUAL NAME

Intralytix produces a lytic bacteriophage preparation with potent lytic activity against the Gram-negative bacterium *Salmonella enterica* under the trade name SalmoFresh™.

1.4 CONDITIONS OF USE

SalmoFresh™ is intended for use as an antimicrobial to control *Salmonella enterica* contamination on companion pet food and animal feed when applied to food surfaces up to $1x10^8$ PFU/gram of food.

1.5 BASIS FOR THE GRAS CONCLUSION

Pursuant to the GRAS rule, Intralytix has concluded that SalmoFresh™ is GRAS for the above defined "Conditions of Use" (Section 1.4) through scientific procedures, in accordance with 21 CFR 570.30 (a) and (b).

1.6 SALMOFRESH™ IS NOT SUBJECT TO PREMARKET APPROVAL

Because Intralytix has concluded that SalmoFresh™ is GRAS for its intended use on pet food and animal feed, it is not subject to the premarket approval requirements for the use in pet food and animal feed, generally, as a processing aid to control *Salmonella* under the intended use conditions described within this notification.

1.7 AVAILABILITY OF INFORMATION

The data and information that are the basis for Intralytix's conclusion that SalmoFresh™ is GRAS are available for review and copying by FDA during customary business hours, at the location below, or will be sent to FDA upon request, made to:

Intralytix
Attn: Amit Vikram
8681 Robert Fulton Dr.
Columbia, MD 21046
avikram@intralytix.com

1.8 FREEDOM OF INFORMATION ACT

It is our view that the information contained in this notification is not exempt from disclosure under the Freedom of Information Act.

1.9 CERTIFICATION

To the best of our knowledge, this GRAS notification is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of SalmoFresh™.

1.10 SIGNATURE

(b) (6)

11/27/2024

Alexander Sulakvelidze
President and CEO
asulakvelidze@intralytix.com

Date

2 IDENTITY AND SPECIFICATIONS OF SALMOFRESH™

2.1 IDENTITY

SalmoFresh consists of a mixture of equal proportions of up to six individually purified lytic bacteriophages (hereinafter referred to as "Component Phage(s)" or "Component Monophage(s)"). Each of these monophages is specifically effective against *Salmonella enterica* serotypes. The component phages in SalmoFresh were isolated from water of the (b) (6) and a mix of water samples collected from the (b) (6) area.

The current SalmoFresh article of commerce is a liquid made up of approximately equal parts of up to six monophages that combined have a minimum lytic titer of 10.0 ± 0.33 $\log_{10}$ plaque-forming units (PFU) per mL. This article of commerce is a concentrate that is diluted with water at the application site to form the SalmoFresh working solution which is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food.

The specific purpose of including up to six monophages in the article of commerce is to enable quick response to real-life situations when uncommon / new *Salmonella* strains may be emerging and contaminating food products. The ability to utilize various blends of up to six lytic phages allows our phage biocontrol technology to ensure continued optimal efficacy which, in turn, is critical for ensuring the safety of foods. Namely, it helps in (i) warranting the broadest possible lytic activity of the cocktail for effective control of various *Salmonella* strains, and (ii) reducing/mitigating the risk of development of bacterial resistance against SalmoFresh and/or natural emergence of *Salmonella* clones against which the current version of SalmoFresh is not optimally effective.

The product may be safely used as an antimicrobial in accordance with the following conditions[1]:

1. The phages are produced on host *Salmonella* strains grown in media free of animal products.
2. The potency of the cocktail is ≥ 9.7 $\log_{10}$ PFU/mL.
3. The phages do not contain functional sequences of any toxin-encoding genes described in 40 CFR 725.421(d).
4. The phages do not contain sequences derived from genes encoding bacterial 16S ribosomal RNA.

[1] The inclusion criteria for SalmoFresh are essentially the same ten criteria for inclusion set forth in the EcoShield PX (GRN 834) and CampyShield (GRN 966) that granted flexibility for both preparations.

5. The cocktail consists of a mixture of approximately equal proportions of up to six different individually purified bacteriophages lytic against *Salmonella*.
6. The *Salmonella* host strains used for production do not encode any functional toxin genes, or if such genes are present, their expression byproducts are not present in the final phage cocktail preparation or are present at negligible levels that do not pose any safety risk.
7. The cocktail achieves positive lytic results by a spot titer assay against one or more *Salmonella* strains available in reference collections (e.g. ATCC).
8. The cocktail contains ≤25,000 EU/mL of endotoxin at SalmoFresh concentration of 9.0 $\log_{10}$ PFU/mL.
9. The cocktail is determined to be free from bacterial contamination.
10. The phage cocktail is used in accordance with the conditions of use outlined in Section 1.4.

SalmoFresh is a concentrate that is normally diluted with water at the application site to form the SalmoFresh working solution, typically with a lytic titer of ~9.0 $\log_{10}$ PFU/mL. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food.

2.1.1 PHAGE CLASSIFICATION

The component phages in SalmoFresh were isolated by Intralytix's scientists from the environment. They have not been genetically modified. Each monophage was fully characterized by a variety of methods, including pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), electron microscopy (EM), full-genome sequence analysis, lytic aetivity against *Salmonella enterica* strains, and lytic activity against non-*Salmonella enterica* strains.

The component bacteriophages currently included in SalmoFresh are lytic double-stranded DNA phages from the class *Caudoviricetes*. Their phylogeny is listed below:

Name	Family	Subfamily	Genus
SBA-1781	Unclassified	*Ounavirinae*	*Felixounavirus*
SKML-39	*Ackermannviridae*	*Aglimvirinae*	*Agtrevirus*
SPT-1	Unclassified	*Ounavirinae*	*Felixounavirus*
SSE-121	Unclassified	*Vequintavirinae*	*Seunavirus*
STML-13-1	*Ackermannviridae*	*Cvivirinae*	*Kuttervirus*
STML-198	*Straboviridae*	*Tevenvirinae*	*Gelderlandvirus*

2.1.2 POTENTIAL TOXICANTS

The *Salmonella enterica* host strains are Gram-negative bacteria. As with all Gram-negative bacteria, they produce bacterial endotoxin or lipopolysaccharide (LPS). Intralytix tests every lot of SalmoFresh for LPS to ensure it meets the release criteria. Endotoxins are further discussed below, in Sections 6.2.3, 6.3.1.3, and 6.3.2.2.

2.2 METHOD OF MANUFACTURE

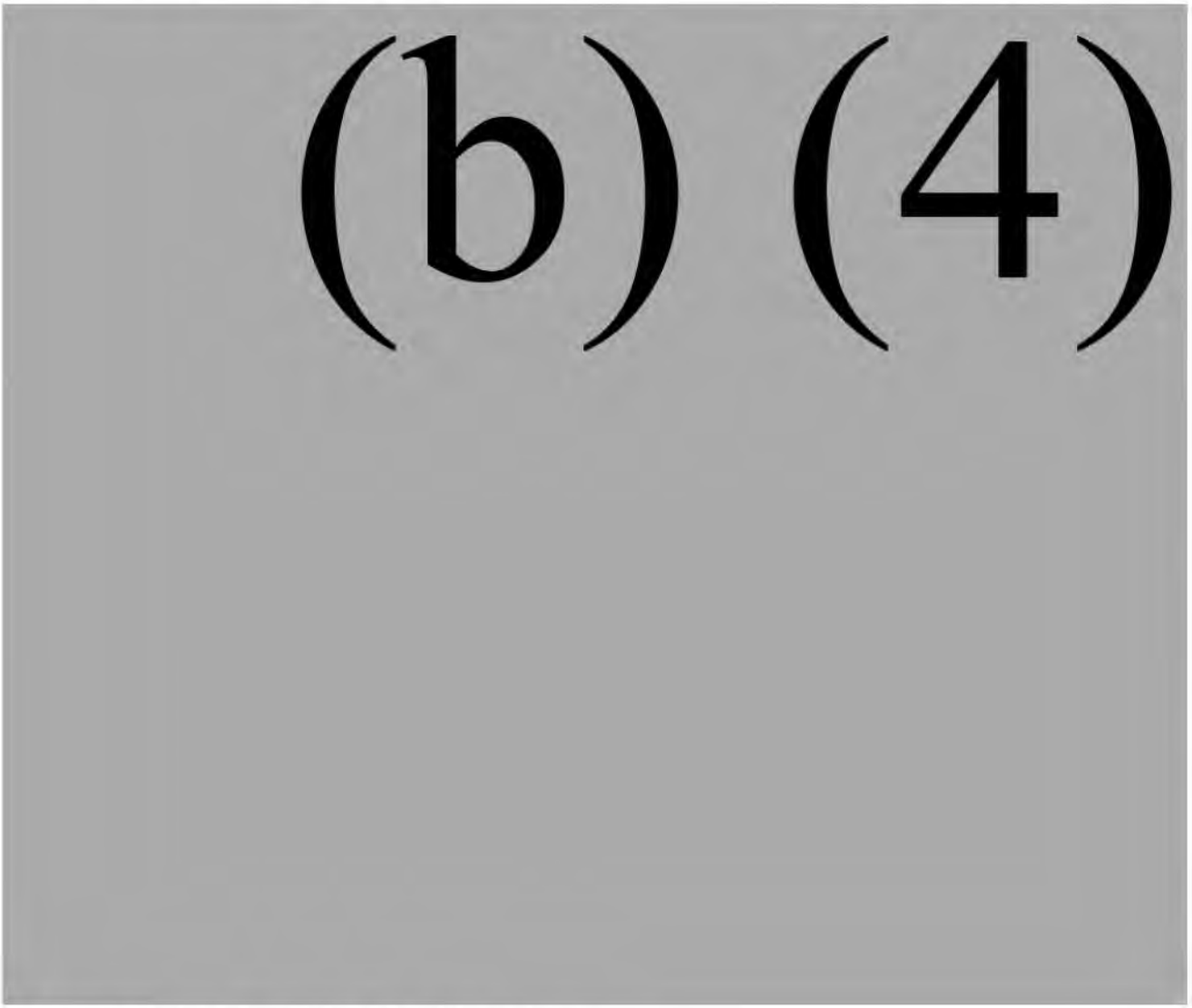


Figure 2 provides an overall schematic of the process.

2.3 SPECIFICATIONS

2.3.1 MONOPHAGE SPECIFICATIONS

Due to the two-step manufacturing process, there are two levels of quality control. (b) (4) (listed in Table 1) before it can be used to prepare a lot of SalmoFresh. The specification sheets for each component monophage are included in Appendices 7.1.1-7.1.6 and the listed methods are provided in Appendices 7.1.7-7.1.9.

Table 1 Product specifications for individual monophage lots

Test	Specification	Acceptance criteria	Method
Potency (Lytic titer)	Minimum titer necessary to produce a final product	NLT 9.8 $\log_{10}$ PFU/mL	Plaque assay (Intralytix method ITXM-E9)
Purity (Microbial)	Negative for total aerobic microbial count	No growth	Growth promotion (Intralytix method ITXM-E1)
Identity	Identification of each monophage is confirmed	Positive identification	RFLP (Intralytix method ITXM-E3)

2.3.2 COMPLIANCE WITH MONOPHAGE SPECIFICATIONS

Results from three representative batches of each of the SalmoFresh component monophages are presented in Table 2. All batches meet the specifications. Certificates of analysis are provided in Appendices 7.1.7-7.1.14. The analytical batch data demonstrates that the monophages can each be reproducibly manufactured in compliance with the product specifications and each exhibit acceptable batch to batch variation.

Table 2 QC results for three representative batches of each component SalmoFresh™ monophage

Phage SBA-1781		(b) (4)		
Potency (Lytic titer)	NLT 9.8 $\log_{10}$ PFU/mL	(b) (4)		
Purity (Microbial)	No growth			
Identity (RFLP)	Positive identification			
Specifications met:		03/18/20	08/24/22	03/27/24

Test	Acceptance criteria	Batch #		
	Phage SKML-39			
Potency (Lytic titer)	NLT 9.8 log_{10} PFU/mL	(b) (4)		
Purity (Microbial)	No growth			
Identity (RFLP)	Positive identification			
	Specifications met:	01/29/19	03/04/20	12/20/23
	Phage SPT-1			
Potency (Lytic titer)	NLT 9.8 log_{10} PFU/mL	(b) (4)		
Purity (Microbial)	No growth			
Identity (RFLP)	Positive identification			
	Specifications met:	12/15/20	03/30/22	01/31/24
	Phage SSE-121			
Potency (Lytic titer)	NLT 9.8 log_{10} PFU/mL	(b) (4)		
Purity (Microbial)	No growth			
Identity (RFLP)	Positive identification			
	Specifications met:	05/14/20	02/24/21	07/07/22
	Phage STML-13-1			
Potency (Lytic titer)	NLT 9.8 log_{10} PFU/mL	(b) (4)		
Purity (Microbial)	No growth			
Identity (RFLP)	Positive identification			
	Specifications met:	09/01/21	09/07/23	01/19/24
	Phage STML-198			
Potency (Lytic titer)	NLT 9.8 log_{10} PFU/mL	(b) (4)		
Purity (Microbial)	No growth			
Identity (RFLP)	Positive identification			
	Specifications met:	05/14/20	11/25/20	12/23/20

2.3.3 PRODUCT SPECIFICATIONS

Only after all component monophages have met the release specifications can a lot of SalmoFresh be blended/produced. Each lot of SalmoFresh is analyzed to ensure it meets the following release specifications listed in Table 3. The specification sheet for SalmoFresh is

included in Appendix 7.1.15 and the listed methods are provided in Appendices 7.1.7 and 7.1.16-7.1.18.

Table 3 Product specifications for *Salmonella* phage preparation

Test	Specification	Acceptance criteria	Method
Potency (Lytic titer)	Within 0.33 log_{10} PFU/mL of intended concentration	For 30X: 10.48 ± 0.33 log_{10} PFU/mL For 100X: 11.00 ± 0.33 log_{10} PFU/mL For 500X: 11.70 ± 0.33 log_{10} PFU/mL	Plaque assay (b) (4)
Purity (Microbial)	Negative for total aerobic microbial count	No growth	Growth promotion (b) (4)
Purity (Endotoxin)	Below specified limits	≤25,000 EU/mL (at ~9.0 ± 0.33 log_{10} PFU/mL)	LAL (Limulus amoebocyte lysate) test (b) (4))
Identity	All component monophages present	All component monophages added during blend	Identity by Input (b) (4)

2.3.4 COMPLIANCE WITH PRODUCT SPECIFICATIONS

Results from three non-consecutive representative batches of the SalmoFresh are presented in Table 4. All batches meet the specifications. Certificates of analysis are provided in Appendix 7.1.16. The analytical batch data demonstrates that SalmoFresh can be manufactured in compliance with the product specifications and exhibits acceptable batch to batch variation.

Table 4 QC results for three batches of SalmoFresh™ (100X)

Test	Acceptance criteria	(b) (4)		
Potency (Lytic titer)	11.00±0.33 log10 PFU/mL			
Purity (Microbial)	No growth			
Purity (Endotoxin)	≤ 25,000 EU/mL (at ~9.0 log10 PFU/mL)			
Identity	All component monophages added			
Specifications met:		10/23/20	09/16/22	06/16/23

2.4 CHARACTERISTIC PROPERTIES

SalmoFresh is a clear to opalescent odorless liquid. The phage component of SalmoFresh (typical working concentration of ~1 x 10^9 PFU/mL) is roughly estimated to be 0.0000342% by weight and the remainder is 0.1M sodium chloride. Typical chemical analysis of SalmoFresh (at the standard working concentration of ~1 x 10^9 PFU/mL) is shown below. The values shown are derived (averages) from the chemical analysis of three separate SalmoFresh lots.

Table 5 Typical chemical analysis of SalmoFresh™

Property / analysis / composition	SalmoFresh	SalmoFresh	SalmoFresh (b) (4)	SalmoFresh typical
Total nitrogen (mg/L)	(b) (4)			2.9
pH				7.59
Specific gravity (at 25°C)				1.008
Arsenic (mg/L)				ND
Barium (mg/L)				ND
Cadmium (mg/L)				0.011
Calcium (mg/L)				0.256
Chromium (mg/L)				0.023
Cobalt (mg/L)				ND
Copper (mg/L))				0.076
Iron (mg/L)				0.080
Lead (mg/L)				0.022
Magnesium (mg/L)				ND
Manganese (mg/L)				ND
Nickel (mg/L)				ND
Phosphorus (mg/L)				10.1
Potassium (mg/L)				5.5
Silicon (mg/L)				ND
Sodium (mg/L)				225
Tin (mg/L)				ND
Zinc (mg/L)				0.049
Chloride (mg/L)				359
Nitrate (as N) (mg/L)				0.16
Nitrite (as N) (mg/L)				ND
Total Organic Carbon (mg/L)				23
Total Kjeldahl Nitrogen (mg/L)				2.7
Total Dissolved Solids (mg/L)				603
Total Suspended Solids (mg/L)				ND
Total Phosphorous (mg/L)				11.4
Silica (mg/L)				2.4
Endotoxin content (EU/mL)				3788

ND = none detected

2.5 STABILITY

2.5.1 MONOPHAGE STABILITY

The shelf life of SalmoFresh article of commerce is 12 months when stored at 5 ± 3°C in a dark, UV-protected area in an unopened original container.

Potency (lytic titer) is monitored during the stability studies. The stability of three representative lots of each of the component monophages are shown below. The specification for the monophage potency is ≥9.8 log10 PFU/mL.

SBA-1781

Data for the long-term stability of component monophage SBA-1781 at 5 ± 3°C (the recommended storage condition) are presented in Table 6. The stability testing results for the SBA-1781 lots have met potency specifications through 24 months of storage.

Table 6 Stability Data for SBA-1781

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
03/12/20	0	12.1	08/22/22	0	12.6	03/25/24	0	12.1
06/30/20	110	11.6	11/16/22	86	12.6	10/23/24	212	11.9
09/15/20	187	11.6	02/23/23	185	12.4			
12/08/20	271	11.5	05/18/23	269	12.7			
03/18/21	371	11.6	08/02/23	345	12.7			
09/14/21	551	11.8	02/14/24	541	12.3			
03/10/22	728	11.4	08/14/24	723	12.4			

SKML-39

Data for the long-term stability of component monophage SKML-39 at 5 ± 3°C (the recommended storage condition) are presented in Table 7. The stability testing results for the SKML-39 lots have met potency specifications through 36 months of storage.

Table 7 Stability Data for SKML-39

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
01/23/19	0	12.2	04/03/20	0	11.9	12/18/23	0	12.5
08/02/19	191	11.6	07/01/20	89	11.7	07/24/24	219	12.2
01/29/20	371	11.7	09/10/20	160	11.7			
			12/02/20	243	11.7			
			02/25/21	328	12.0			
			08/26/21	510	11.8			
			02/16/22	684	12.1			
			03/20/23	1081	11.8			

SPT-1

Data for the long-term stability of component monophage SPT-1 at 5 ± 3°C (the recommended storage condition) are presented in Table 8. The stability testing results for the SPT-1 lots have met potency specifications through 24 months of storage.

Table 8 Stability Data for SPT-1

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
12/11/20	0	12.8	03/23/22	0	12.7	01/24/24	0	12.9
02/16/22	432	12.8	06/23/22	92	12.3	07/24/24	182	12.8
			09/23/22	184	12.8			
			12/20/22	272	12.9			
			03/20/23	362	12.8			
			09/20/23	546	12.5			
			03/01/24	709	12.4			

SSE-121

Data for the long-term stability of component monophage SSE-1 at 5 ± 3°C (the recommended storage condition) are presented in Table 9. The stability testing results for the SSE-1 lots have met potency specifications through 24 months of storage.

Table 9 Stability Data for SSE-121

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
05/06/20	0	11.8	02/22/21	0	12.0	07/05/22	0	11.7
08/10/20	96	11.7	05/18/21	85	12.1	10/19/23	471	11.4
11/02/20	180	11.0	08/16/21	175	11.8			
02/12/21	282	11.5	11/18/21	269	11.8			
05/05/21	364	11.6	02/16/22	359	11.9			
11/03/21	546	11.5	08/17/22	541	11.9			
05/03/22	727	11.5	02/23/23	731	11.9			

STML-13-1

Data for the long-term stability of component monophage STML-13-1 at 5 ± 3°C (the recommended storage condition) are presented in Table 10. The stability testing results for the STML-13-1 lots have met potency specifications through 24 months of storage.

Table 10 Stability Data for STML-13-1

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
08/25/21	0	12.0	09/05/23	0	12.4	01/15/24	0	12.4
05/23/22	271	11.9	11/28/23	84	12.0	07/24/24	191	12.3
08/24/22	364	11.8	02/22/24	170	12.4			
02/24/23	548	11.9	05/29/24	267	12.6			
08/02/23	707	11.9	08/28/24	358	12.6			

STML-198

Data for the long-term stability of component monophage STML-198 at 5 ± 3°C (the recommended storage condition) are presented in Table 11. The stability testing results for the STML-198 lots have met potency specifications through 24 months of storage.

Table 11 Stability Data for STML-198

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
04/29/20	0	12.2	11/24/20	0	11.6	12/21/20	0	11.7
07/22/20	84	11.9	02/26/21	94	11.8	03/22/21	91	11.8
10/29/20	183	11.9	05/18/21	175	11.7	06/17/21	178	12.0
01/26/21	272	12.1	08/19/21	268	11.9	09/17/21	270	11.8
05/05/21	371	11.9	11/15/21	356	11.8	12/09/21	353	11.8
10/29/21	548	12.1	05/17/22	539	12.1	06/15/22	541	11.8
04/27/22	728	12.0	11/15/22	721	11.9	12/13/22	722	12.0

2.5.2 PRODUCT STABILITY

As stated above, the shelf life of SalmoFresh article of commerce is 12 months when stored at 5 ± 3°C in a dark, UV-protected area in the unopened original container.

Potency (lytic titer) is monitored during the stability studies. The specification for SalmoFresh (100X) potency is 11.00±0.33 $\log_{10}$ PFU/mL.

Data for the long-term stability of three representative lots of SalmoFresh (100X) at 5 ± 3°C (the recommended storage condition) are presented in Table 12. The stability testing results for the SalmoFresh (100X) lots have met potency specifications through the shelf life of 12 months of storage.

Table 12 Stability Data for SalmoFresh™ (100X)

(b) (4)

Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result	Analysis Date	Days	Titer Result
8/30/2022	0	10.78	9/21/2020	0	10.97	9/22/2020	0	11.00
12/5/2022	97	10.75	12/22/2020	92	11.13	12/22/2020	91	11.22
2/27/2023	181	10.67	3/26/2021	186	11.25	3/26/2021	185	11.28
5/31/2023	274	10.84	5/7/2021	228	11.18	6/23/2021	274	11.16
8/29/2023	364	10.71	9/22/2021	366	11.25	9/22/2021	365	11.25
2/22/2024	541	10.79	3/16/2022	541	11.29	3/23/2022	547	11.39
8/28/2024	729	10.75	9/20/2022	729	11.18	9/20/2022	728	11.38

2.5.3 STABILITY SUMMARY

The stability date presented above for both component monophages and the SalmoFresh product support the 12-month shelf-life.

2.6 CONDITIONS OF INTENDED USE

SalmoFresh is intended for use as an antimicrobial to control *Salmonella enterica* contamination on companion pet food and animal feed when applied to food surfaces up to $1x10^8$ PFU/gram of food.

2.7 REGULATORY STATUS

Several phage products, including SalmoFresh, have been GRAS affirmed for human food use (see Section 6.4.1).

2.8 INFORMATION TO ESTABLISH UTILITY FOR THE TARGET ANIMAL

Companion animal food is generally comprised of meat, meat byproducts, poultry, and poultry byproducts. In addition, fresh produce, such as peas, potato, beets etc., may be added to pet food formulations. The primary components of livestock feed are grains, such as wheat, corn, soybean, hay, silage (typically produced by fermenting raw plants), and other plant parts.

Salmonella is a major foodborne pathogen in poultry and meat products and a significant concern in vegetables. According to CDC, there were 4 outbreaks due to fresh produce and 2 associated with grains within a short duration of 21 months from Jan 2023 to Aug 2024. *Salmonella* in pet food and animal feed components serves as a source of contamination to companion animals and livestock. SalmoFresh, as well as several other phage products, has been shown to significantly reduce *Salmonella* contamination of various ingredients used in pet food and animal feed such as meat, poultry, fresh produce, and grain products (Table 13 and Table 14). In addition to the studies presented in Table 13, studies submitted as part of this package (Appendix 7.1.20) demonstrate that SalmoFresh significantly reduces *Salmonella* on different pet foods.

Altogether, these studies establish that SalmoFresh is efficacious in reducing *Salmonella* contamination of various food products that are used as components of pet food and animal feed. Furthermore, as described in Section 6.2.1 and Section 6.5, phages are components of the natural microbiota of animals and phage administration in live animals is safe.

Table 13 Efficacy of SalmoFresh™ on various food products that are used as components of pet food and animal feed

Target	Study description	Reference
***Salmonella* spp.**	A phage cocktail* was sprayed onto experimentally contaminated raw pet food ingredients, including chicken, tuna, turkey, cantaloupe, and lettuce, and reduced the levels of the targeted bacteria by 0.4-1.1 logs.	(Soffer et al. 2016)
S. Enteritidis, S. Montevideo, S. Senftenberg, S. Typhimurium	Spray application of phage cocktail* demonstrated a dose dependent reduction in *Salmonella* population on dry kibbles that were inoculated with a mixture of 4 *Salmonella* serotypes. Phage treatment resulted in up to ~2 log MPN/g reduction in *Salmonella* population.	(Heyse et al. 2015)
***Salmonella* spp.**	Treatment of chicken breast fillets with a combination of phage and chemical antimicrobials did not produce a synergistic effect on the reduction of *Salmonella*; however, application of chlorine or PAA followed by spraying with phage significantly reduced *Salmonella* from chicken skin by up to 2.5 logs, compared to use of chlorine, low levels of PAA, or phage alone (0.5-1.5 logs).	(Sukumaran et al. 2015)
***Salmonella* spp.**	Treatment of chicken breast fillets by dipping or surface application of a *Salmonella*-specific bacteriophage preparation and storage at 4°C significantly reduced *Salmonella* contamination by up to 0.9 log; further, storing the meat in modified atmospheric packaging after surface application produced a higher reduction in bacterial counts (up to 1.2 logs).	(Sukumaran et al. 2016)
***Salmonella* spp.**	Phage biocontrol reduced *Salmonella* by 2-3 log on lettuce and sprouts. Pairing of phage cocktail with chlorinated water resulted in 2.7-3.8 log reduction in viable *Salmonella* counts.	(Zhang et al. 2019)
Salmonella Heidelberg	Cantaloupe slices, apple slices and romaine hearts were inoculated with ca. 4.0 lug *Salmonella*. A sequential application of PAA or chlorine followed by $1x10^8$ PFU/g SalmoFresh resulted in up to 3.34 log reduction in *Salmonella*.	(Moye et al. 2020)
Salmonella Newport	Mini whole cucumbers were inoculated with 5 log *Salmonella* and were either sliced or used whole for experiment. SalmoFresh application at 4.76×10^7 PFU/cm^2 resulted in 1.83 log reduction on whole cucumbers, and up to 1.48 log reduction on sliced cucumber at 10°C and 2.37 log reduction on whole cucumbers at 22°C.	(Sharma et al. 2017)
***Salmonella* spp.**	Counts of *Salmonella* were decreased by ~3.5 logs at 5 and 10°C and ~2.5 logs at 20°C on melon slices after application of a four-phage cocktail§; treatment of apple slices with phage showed no reduction of bacteria.	(Leverentz et al. 2001)

* Studies performed with SalmoLyse®, an alternate tradename for SalmoFresh™

§ Studies performed with SCPLX-1, an early version of SalmoFresh™

Table 14 Efficacy of *Salmonella* targeting phages on various food matrices

Target	Product	Study description	Reference
***Salmonella* spp.**	SJ2	*Salmonella* levels were reduced by 1-2 logs in raw and pasteurized cheeses created using milk that was treated with phage, while cheese made from milk without phage saw *Salmonella* counts rise ~ 1 log.	(Modi et al. 2001)
***Salmonella* spp.**	Felix-O1	*Salmonella* counts were decreased by 1.8-2.1 logs after phage application to chicken frankfurters.	(Whichard et al. 2003)
***Salmonella* spp.**	PHL4	The levels of *Salmonella* recovered from experimentally contaminated broiler and naturally contaminated turkey carcasses were reduced by as high as 100% or 60% respectively after phage administration.	(Higgins et al. 2005)
***Salmonella* spp.**	Six phages	Levels of *Salmonella* were decreased ~3 logs after application of a phage cocktail to sprouts; addition of an antagonistic bacteria to the phage cocktail increased this reduction to ~6 logs.	(Ye et al. 2010)
***Salmonella* spp.**	FO1-E2	In chocolate milk and mixed seafood, *Salmonella* levels were reduced to undetectable after phage treatment and storage for 24 h at 8°C and remained below the limit of detection. When foods were treated with phage and stored at 15°C, *Salmonella* counts were reduced to undetectable levels within 24-48 h for hot dogs, sliced turkey breast, and chocolate milk, but regrowth occurred after 5 days. *Salmonella* levels were initially inhibited ~0.5-2 logs and ~1-3 logs in egg yolk and mixed seafood respectively after phage addition; but bacterial recovery matched controls in egg yolks after two days, while the log reduction was maintained in seafood.	(Guenther et al. 2012)
***Salmonella* spp.**	UAB_Phi 20, UAB_Phi78, UAB_Phi87	*Salmonella* counts were decreased by ~1 log on the shells of fresh eggs and 2-4 logs on lettuce 60 mins after application of the phage cocktail. After an initial reduction of 1-2 logs when chicken breasts were dipped in the phage cocktail, no further decrease in the bacterial counts was observed over the next seven days at 4°C. The levels of *Salmonella* were reduced 2-4 logs on pig skin after phage application and storage for 6 h at 33°C.	(Spricigo et al. 2013)
***Salmonella* spp.**	wksl3	*Salmonella* counts were decreased by ~3 logs on chicken skin after application of a single phage, and no further decrease in bacterial levels was observed over the next seven days at 8°C. Phage cocktail was also administered to mice to test for safety. Mice received a single dose of phage orally and displayed no adverse effects.	(Kang et al. 2013)
***Salmonella* spp.**	Five phages	The levels of *Salmonella* were decreased by ~1 log on chicken skin after application of a five-phage cocktail comprised of closely related phages. The reduction was comparable to treatment with 200 ppm	(Hungaro et al. 2013)

Target	Product	Study description	Reference
		dichloroisocyanurate, 10 ppm peroxyacetic acid, and 2% lactic acid.	
***Salmonella* spp.**	P22	After the administration of a single temperate phage and storage at 4°C, levels of *Salmonella* decreased by 0.5-2 logs on chicken samples; below the limits of detection in whole and skimmed milk; by ~3 logs in apple juice; by ~2 logs in liquid egg; and by ~2 logs in an energy drink.	(Zinno et al. 2014)
***S.* Enteritidis**	Five phages	The levels of S. Enteritidis were reduced by as much as 3.2 log and 2.8 log after 10 days storage at 18°C and 4°C, respectively in raw salmon fillets. The phage treatment of smoked salmon fillets resulted in 1.9 log and 1.2 log reduction in *Salmonella* after 10 days storage at 18°C and 4°C, respectively.	(Galarce et al. 2014)
***Salmonella* spp.**	SJ2	Application of the phage SJ2 significantly reduced *Salmonella* in experimentally contaminated ground pork and eggs with a larger reduction observed at room temperature compared to 4°C. After treatment, *Salmonella* colonies were screened for phage resistance, and more phage-resistant *Salmonella* isolates were recovered from eggs compared with ground pork.	(Hong et al. 2016)
***Salmonella* spp.**	PhageGuard S™ (formerly Salmonelex™)	Boneless chicken thighs and legs were experimentally contaminated with *Salmonella* and treated with phage solution. A larger reduction of *Salmonella* was achieved when the bacteriophage preparation was diluted in tap water compared to filtered water prior to application, and the phage cocktail was more effective against *Salmonella* isolated from other sources compared to those from ground chicken.	(Grant et al. 2017)
***Salmonella* spp.**	PhageGuard S™	Treatment with a bacteriophage cocktail or irradiation significantly reduced (~1 log) the level of *Salmonella* on experimentally contaminated ground beef trim, and a combination of these methods was even more effective and decreased bacterial contamination by ~2 logs.	(Yeh et al. 2018)
***Salmonella* spp.**	PhageGuard S™	Bacteriophage application reduced the *Salmonella* levels >1 log on skinless and skin-on poultry products.	(Hagens et al. 2018)
***Salmonella* spp.**	BSPM4, BSP101, BSP22A	The phage cocktail treatment achieved a reduction of 4.7-5.8 log *Salmonella* on lettuce and cucumber.	(Bai et al. 2019)
***Salmonella* spp.**	LPSTLL, LPST94, LPST153	Application of phage cocktail reduced 3.0 log *Salmonella* inoculum to below detectable limits on chicken breast and in milk. Phage cocktail was effective against *Salmonella* biofilm grown for 72 h on microtiter plates and steel chips, resulting in >5.23 log reduction in *Salmonella* viable cells.	(Islam et al. 2019)

3 TARGET ANIMAL AND HUMAN EXPOSURES

3.1 ESTIMATED EXPOSURE FOR ANIMALS

3.1.1 APPLICATION RATES

The current SalmoFresh article of commerce is a concentrate that is typically diluted with water at the application site to form the SalmoFresh working solution. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below $1x10^8$ PFU/g of food. Future preparations may be sold in a more concentrated form, but the accompanying instructions for dilution and application rate will be appropriately adjusted to ensure the final concentration of phage on the food articles is always at or below $1x10^8$ PFU/g of pet food / animal feed.

3.1.2 DIETARY INTAKES

SalmoFresh is anticipated to be used on all types of pet foods and animal feed, including, but not limited to, food for dogs, cats, and livestock. Regardless of the type of animal being fed, the pet food and animal feed is treated at or below $1x10^8$ PFU/g of pet food / animal feed. Therefore, the initial calculations for dietary exposure below are calculated based on the amount present in the food, not the individual types of animals.

a. Dietary intake for SalmoFresh non-phage components

The following calculation assumes the worst-case scenario, where all food consumed by an animal is treated at the highest rate of SalmoFresh application ($1x10^8$ PFU/g):

Amount of SalmoFresh applied per gram of pet food and animal feed:

$$\frac{1x10^8 \text{ PFU}}{\text{g pet food \& animal feed}} \times \frac{1 \text{ mL SalmoFresh}}{1x10^{10} \text{ PFU}} = \frac{0.01 \text{ mL SalmoFresh}}{\text{g pet food \& animal feed}}$$

Therefore, every 1g of pet food and animal feed eaten would have 0.01mL of SalmoFresh added. In the worst-case scenario, assuming all the food eaten by the companion animal or livestock is treated with SalmoFresh, the SalmoFresh would represent 1% of the animal's food. SalmoFresh is >99% water,[2] so further calculations are present below to represent the number of phages that would be added to the diet.

[2] See FDA GRAS notice inventory GRN000435 (accessed 20Mar24)

b. Dietary intake for SalmoFresh phages

The following calculation determines the approximate weight of phages added to pet food and animal feed, again assuming the worst-case scenario of $1x10^8$ PFU/g of SalmoFresh application:

Weight of total phages consumed (in micrograms) per gram of pet food and animal feed:

$$\frac{1x10^8 \text{ PFU}}{\text{g pet food \& animal feed}} \times \frac{3.45x10^{-16} \text{ g}}{\text{phage}} \times \frac{1x10^6 \text{ µg}}{\text{g}} = \frac{0.035 \text{ µg}}{\text{g pet food \& animal feed}}$$

Where:

$3.45x10^{-16}$ g = mass of one phage, calculated for the largest monophage

Therefore, the dietary concentration of phages in pet food and animal feed is just 35 ppb:

$$\frac{0.035 \text{ µg}}{\text{g pet food \& animal feed}} = 35 \text{ ppb}$$

The exposure of animals to SalmoFresh phages was estimated based on representative body weight and feed intake data for major categories of animal (Rychen et al. 2017). These intake estimates are summarized Table 15, which presents the total phage consumption by various animals in a worst-case scenario, i.e., where all the food/feed consumed by the animals is treated with SalmoFresh.

Table 15 Estimated daily intake of SalmoFresh™ phages (at maximum use level of $1x10^8$ PFU/g) on a body weight basis

Animal category	Bodyweight (kg)	Feed intake (kg DM/day)	Daily intake of SalmoFresh (PFU / kg body weight)	Daily intake of SalmoFresh (ug phage / kg body weight)
Chicken for fattening	2	0.158	7.9E+09	2.7
Laying hen	2	0.106	5.3E+09	1.8
Turkey for fattening	3	0.176	5.9E+09	2.0
Piglet	20	0.88	4.4E+09	1.5
Pig for fattening	60	2.20	3.7E+09	1.3
Sow lactating	175	5.28	3.0E+09	1.0
Veal calf (milk replacer)	100	1.89	1.9E+09	0.7
Cattle for fattening	400	8.0	2.0E+09	0.7
Dairy cow	650	20.0	3.1E+09	1.1
Sheep/goat	60	1.2	2.0E+09	0.7
Horse	400	8.0	2.0E+09	0.7

Animal category	Bodyweight (kg)	Feed intake (kg DM/day)	Daily intake of SalmoFresh (PFU / kg body weight)	Daily intake of SalmoFresh (ug phage / kg body weight)
Rabbit	2	0.1	5.0E+09	1.7
Salmon	0.12	0.0021	1.8E+09	0.6
Dog	15	0.250	1.7E+09	0.6
Cat	3	0.060	2.0E+09	0.7
Ornamental fish	0.012	0.000054	4.5E+08	0.2

The daily intake is scaled to body weight (g dry matter (DM)/kg bodyweight) for the main animal species/categories (Rychen et al. 2017)

c. Dietary intake for endotoxins

As mentioned earlier, the only toxin of possible concern is endotoxin. The following calculation determines the approximate added endotoxin per g of pet food and animal feed, assuming worst case seenarios of both the highest rate ($1x10^8$ PFU/g) of SalmoFresh application and a maximum allowed endotoxin content (25,000 EU in $1x10^9$ PFU) being present in SalmoFresh:

Total endotoxin (EU) added per g of pet food and animal feed:

$$\frac{2.5x10^4 \text{ EU}}{1x10^9 \text{ PFU}} \times \frac{1x10^8 \text{ PFU}}{\text{g pet food \& animal feed}} = \frac{2.5x10^3 \text{ EU}}{\text{g pet food \& animal feed}}$$

Using an activity of 10 EU/ng pure endotoxin, based on estimated values of 12–25 IU/ng (Luchi and Morrison 2000), 10 IU/ng (Liebers et al. 2006), 10 EU/ng (Rapala et al. 2002), the amount of endotoxin per gram of pet food and animal feed in parts per million can be calculated as follows:

$$\frac{2.5x10^3 \text{ EU}}{\text{g pet food \& animal feed}} \times \frac{1x10^{-9} \text{ g endotoxin}}{10 \text{ EU}} = \frac{2.5x10^{-7} \text{ g endotoxin}}{\text{g pet food \& animal feed}} = 0.25\text{ppm}$$

Therefore, the maximum possible dietary concentration of endotoxin in pet food and animal feed would be just 0.25 ppm. To further put this into perspective, the typical concentration of endotoxin in human saliva is 1mg of endotoxin per mL of saliva (data was not available for the typical levels in pets).

$$\frac{2.5x10^{-7} \text{ g endotoxin}}{\text{g pet food \& animal feed}} \div \frac{0.001 \text{ g endotoxin}}{\text{mL human saliva}} = 0.025\%$$

The added endotoxin per gram of pet food and animal feed would be just 0.025% of what is typically already present in a milliliter of human saliva.

While data was not available for the typical levels of endotoxin in animal saliva, the levels of endotoxin naturally found in animal food can be as high as $2x10^6$ EU/g, as shown in Table 16.

Table 16 Levels of endotoxin found in animal feed (adapted from (Wallace et al. 2016)) and pet food (Intralytix)

Feed material	Average/max concentration (units quoted)	Average/max concentration (EU/g feed, calculated)*
Pig feed	13/60 mg/kg	1.3E+05 / 6.0E+05
Hay	0.05 mg/kg	5.0E+02
Pelleted goat feed	10 mg/kg	1.0E+05
Corn silage	1/200 IU/mg	1.0E+03, 2.0E+05
Duck feed	50, 93 IU/mg	5.0E+04, 9.3E+04
Horse feed oats	>50 ng/mg	5.0E+05
Pig feed	7.5-259, mean 64.7, ng/mg	7.5E+04-2.6E+06, mean 6.5E+05
Raw dog food (beef based) §	n/a	3E+05
Wet dog food (chicken based) §	n/a	1E+02
Cat food (rabbit based) §	n/a	1E+04

* Using an activity of 10 EU/ng pure endotoxin, based on estimated values of 12 25 IU/ng (Luchi et al. 2000), 10 IU/ng (Liebers et al. 2006), 10 EU/ng (Rapala et al. 2002) (EU (Endotoxin Unit) IU (International endotoxin Unit))

§ Pet food was tested internally as described by Dutkiewicz et al. (1989) using the LAL method described in Section 7.1.17

With a median concentration of $1x10^5$ EU/g in pet food and animal feed, the worst-case scenario of endotoxin per gram of food would be 2.5% of what is already present.

$$\frac{2.5x10^3 \text{ EU}}{\text{g pet food and animal feed}} : \frac{1x10^5 \text{ EU}}{\text{g pet food and animal feed}} = 2.5\%$$

Based on the above dietary intake calculations, it is evident that the exposure to SalmoFresh resulting from its use in pet food and animal feed under specified conditions of use will not result in a significant increase in exposure to both phages and other components of SalmoFresh.

3.1.3 RESIDUES

As SalmoFresh is not a chemical product, no reaction or hydrolytic products are formed after application in either pet food or animal feed (including in food for food-producing animals).

3.2 HUMAN EXPOSURE BY CONSUMPTION OF TARGET ANIMALS

The use of SalmoFresh in animal feed does not pose any significant risk of harm to humans who consume edible products from animals that consume SalmoFresh treated feed.

SalmoFresh has been previously GRAS affirmed for direct application on human foods (GRN 435) including ready-to-eat and raw poultry products, ready-to-eat and raw red meat products, fish, shellfish, fresh and processed fruits and vegetables, and grain and grain products.

Bacteriophages are already natural components of the animal gut microbiota (Shi et al. 2021; Wang et al. 2023; Li et al. 2021; Xie et al. 2016; Callaway et al. 2006; Firlieyanti et al. 2016) and commonly consumed by livestock in their feed and water (Bergh et al. 1989; Calei et al. 1998; Furuse et al. 1983; Xie et al. 2016). As is often noted as a hurdle with the use of phages in animals, the low pH and enzymes typically present in the stomach can inactivate phages (Bianchessi et al. 2024; Jończyk et al. 2011). SalmoFresh consumed with animal feed will likely be inactivated by the low pH of the stomach. As demonstrated by Sukumaran et al. (2015), SalmoFresh titer was reduced by 2.5-3 log after exposure to an acidic solution.

Given bacteriophage sensitivity to exposure to high temperatures, any presence of bacteriophage found in edible tissue or other edible products would be reduced far below the levels currently approved for SalmoFresh when applied to human food after commonly used bacterial contamination controls in the production and preparation of the food. Examples of these types of contamination controls include, but are not limited to, the pasteurization of milk or eggs, HACCP based production and preparation of animal products for human food and Ready-to-Eat (RTE) food items in accordance with regulations outlined in the Food Safety Modernization Act and USDA regulations, and the act of cooking meat and other edible tissue of food producing animals.

Therefore, it can be reasonably expected that consumption of SalmoFresh treated animal feed by food producing animals will not significantly increase exposure of humans to SalmoFresh phages.

4 SELF-LIMITING LEVELS OF USE

This part is not applicable. There are no self-limiting levels of use associated with SalmoFresh that would result in the animal food being unpalatable or technologically impractical.

5 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

This section is not applicable to this notification.

6 NARRATIVE

6.1 INTRODUCTION

SalmoFresh is a GRAS affirmed bacteriophage preparation for reduction of *Salmonella enterica* in various human food products (including ready-to-eat foods) when applied at a rate of up to 1×10^8 PFU/g of food (GRN No. 435). In the following sections, the data and information providing the basis for our conclusion that SalmoFresh is also GRAS when applied onto pet food and animal feed under the intended use conditions, is presented. The information provided below, and elsewhere in this document, that is generally available has been properly cited. The list of references is presented in Part 7.

6.2 COMPONENTS OF SALMOFRESH™

SalmoFresh is a mixture of component bacteriophages together with added 0.1 M sodium chloride; due to the method of production, there may also be small amounts of residual production by-products. The primary active ingredient is not a single chemical substance but a mixture of naturally occurring lytic bacteriophages. In the appropriate sections below, we consider separately the safety of the:

- Phages (active component)
- Added salts
- Manufacturing by-products

6.2.1 PHAGES

The safety and ubiquity of bacteriophages have been well established. The pertinent safety data on bacteriophages is reviewed below. The published literature on phages and other information developed by Intralytix are discussed below.

Bacteriophages are the most ubiquitous organisms on earth. This abundance of phages in the environment, and the continuous exposure of animals to them, explains the excellent tolerance of animals to phages.

- Bacteriophage population densities are estimated to be in the range of 10^7 to 10^9 plaque forming units (PFU) per gm or per mL depending upon the matrix under consideration (Aziz et al. 2015; Bergh et al. 1989) and the total number of phages in the biosphere has been estimated to be in the range of 10^{30} to $\sim10^{32}$ (Brüssow and Hendrix 2002).

- The biology of phages has been exhaustively studied. The studies have clearly shown that lytic bacteriophages are obligate intracellular parasites of bacteria and are not infectious in animals and humans (Sulakvelidze et al. 2001; Hesse and Adhya 2019).

Bacteriophages are components of the natural microbiota of animals and humans and are routinely isolated from animals and associated environments such as water, farm soil, etc.

- Phages have been isolated from canine and feline feces, indicating phages are part of natural microflora in both species. Bacteriophages of the families *Microviridae*, *Siphoviridaae*, *Ackermannviridae*, *Podoviridae*, *Myoviridae* and unclassified phages are reported in dog gastrointestinal tracts (Shi et al. 2021; Wang et al. 2023). CrAssphage, a phage abundantly present in the human gastrointestinal tract, was identified in cat feces, suggesting that phages are naturally present in the GI of cats (Li et al. 2021).
- Bacteriophages are naturally present in cattle (Xie et al. 2016; Callaway et al. 2006). In the study by Xie et al. (2016), all samples of feces, feed, soil, and water from a Texas beef cattle feedlot contained at least one *Salmonella* phage, indicating that bacteriophages are widespread in feedlots and already present in the feed and water of beef cattle.
- Bovine ruminal contents were reported to have at least 5×10^7 PFU/mL phage particles (Paynter et al. 1969). Given that the total ruminal fluid in an adult cow ranges from 25-50 gallons (or ~95–190 L) (Evans and Hooser 2010), a conservative estimate (using the lower rumen fluid volume) would be that there are 4.7×10^{12} phage particles present in bovine rumen. Another study estimated between 3×10^9 and 1.6×10^{10} phage particles per ml of ruminal fluid of sheep (Klieve and Swain 1993). Assuming a 4L total ruminal fluid content of an adult sheep (Warner and Stacy 1968; Ulyatt 1964), a conservative estimate (using the lower phage content estimate) indicates there are $1.2\text{x}10^{13}$ phage particles present in the ovine rumen. Altogether these studies indicate that phages are naturally present in ruminal fluid in high numbers.
- A review by Letarov and Kulikov (2009) of studies regarding the abundance of phages in mammals and birds found that a diverse range of phages are generally present in high numbers in all mammals including cattle, sheep, dogs and cats and birds (such as chicken) ranging from 10^5 to 10^{10} PFU/g of fecal content or PFU/mL of ruminal fluid.
- *Campylobacter* specific bacteriophages have been isolated from chicken livers indicating phages are naturally present in chickens (Firlieyanti et al. 2016).

- Several *Salmonella* specific phages were isolated from chicken feces and farm sewage water (Cao et al. 2022), indicating phages are a natural part of the GI tract of chickens.
- Several bacteriophages targeting EPEC and EHEC were isolated from chicken skin, chicken intestine, beef lung, and beef intestine (Lukman et al. 2020).

Bacteriophages are natural components of all fresh, unprocessed foods and are commonly consumed by animals via various foods.

- Animals are constantly exposed to phages through drinking water and foods (Armon and Kott 1993; Armon et al. 1997; Furuse et al. 1983; Bergh et al. 1989; Calci et al. 1998).
- In a study from Texas A&M University male-specific and somatic coliphages were detected in all animal feeds, feed ingredients, and poultry diets examined, even after the samples were stored at -20°C for 14 months (Maciorowski et al. 2001).
- Bacteriophages have been readily isolated from a wide range of food products, including ground beef, pork sausage, chicken, farmed freshwater fish, common carp and marine fish, oil sardine, raw skim milk, and cheese (Atterbury et al. 2003; Greer 1983; Greer et al. 2007; Pujato et al. 2014; Whitman and Marshall 1971; Kennedy et al. 1984; Gautier et al. 1995; Hsu et al. 2002; Suarez et al. 2002; Eller et al. 2012; Hoai and Yoshida 2016; Hoai et al. 2018; Tyutikov et al. 1983; He and Yang 2015).
- Several studies have suggested that 100% of the ground beef and chicken meat sold at retail contain different levels of various bacteriophages. For instance, bacteriophages were recovered from 100% of examined fresh chicken and pork sausage samples and from 33% of delicatessen meat samples (Kennedy et al. 1984). The levels ranged from 3.3 to 4.4 $\log_{10}$ PFU/100 g of fresh chicken, up to 3.5 $\log_{10}$ PFU/100 g of fresh pork, and up to 2.7 $\log_{10}$ PFU/100 g of roast turkey breast samples.
- *E. coli*- and *Shigella*-specific bacteriophages were isolated from 100% of beef and 68% of mixed salad purchased in a variety of markets (Imamovic and Muniesa 2011).

Because of (i) their highly specific nature and (ii) the everyday exposure of humans and animals to them (including daily consumption of bacteriophages with various foods and drinking water), bacteriophages do not adversely affect humans and animals, nor do they deleteriously affect their GI microflora.

- In a trial to determine the safety of bacteriophage application, oral administration of *E. coli*-specific phage T4 to 15 healthy adult volunteers did not elicit any adverse effects and was well-tolerated (Bruttin and Brüssow 2005). Additionally, no substantial phage replication was detected. It did not cause a decrease in total fecal *E. coli* counts upon oral administration. No adverse events related to phage application were observed in any of the volunteers (Bruttin et al. 2005).
- A pharmacokinetic and toxicological study using mice and guinea pigs did not show any signs of acute toxicity or histological changes, even when the dose administered was 3,500-fold higher than the human dose projected in the course of the study (Sulakvelidze and Kutter 2005b).
- High doses of *Listeria* phage preparations (i.e. ListShield and P100) were administered to laboratory animals (mice and rats) without any adverse effects (Mai et al. 2010; Carlton et al. 2005).
- A long-term toxicity study with a *Shigella* phage preparation (under the tradename ShigActive™) in mice, showed no significant effect on any health or toxicity markers in the mice. Additionally, and in contrast to an antibiotic, the phage preparation did not significantly affect the microbiota of the treated mice (Mai et al. 2015).
- Pet food treated with the *Salmonella* phage preparation that is the subject of this GRAS notification significantly reduced the *Salmonella* while showing no adverse effect on dogs and cats consuming the treated food (Soffer et al. 2016). Furthermore, phage coated pet food did not impact their normal gut microbiota. This study is further discussed in Section 6.5.1 and 6.5.2.

Bacteriophages are also commensals of the human gastrointestinal tract (GIT), and play an important role in regulating the diversity and community structure of the GIT microbiome (Barr 2017).

- The number of bacteriophages in the human gastrointestinal tract could be as high as one quadrillion (Dalmasso et al. 2014; Shkoporov and Hill 2019).
- A study suggests that humans routinely absorb up to 30 billion phages every day (Nguyen et al. 2017) without any adverse effect.
- Phages specific to *E. coli, Bacteroides fragilis*, and various *Salmonella* serotypes have been isolated from human fecal specimens in concentrations as high as 10^5 PFU/100 g of feces (Calci et al. 1998; Barr 2017; Furuse et al. 1983; Havelaar et al. 1990). The recent data based on metagenomic analyses (using partial shotgun sequencing) of an uncultured viral community from human feces suggested that bacteriophages are the second most abundant category after bacteria in the

uncultured fecal library (Reyes et al. 2010; Breitbart et al. 2003) with an estimated 10^{12}-10^{15} phages typically present in the human gastrointestinal tract (Dalmasso et al. 2014; Shkoporov et al. 2019).

- Recent evidence suggests that GIT phages may offer human health benefits. Specifically, the abundance and diversity of GIT phages is associated with improved human health (Moreno-Gallego et al. 2019; Gogokhia et al. 2019).

In recent years, investigators around the globe have studied the effect of bacteriophage administration on livestock animals. While these studies were focused on reducing colonization, morbidity, and mortality from foodborne pathogens, they do provide some insight into the safety of phages and ability to improve animal health.

- Bacteriophage administration as a supplement to *Salmonella* challenged broilers using feed has shown a significant reduction in *Salmonella* in cecum and liver and resulted in reduced GI tract inflammation and improved health of the birds (Sarrami et al. 2023). Multiple studies including long term studies have shown beneficial effect of bacteriophage administration to poultry (Sklar and Joerger 2001; Atterbury et al. 2007; Lim et al. 2011; Fiorentin et al. 2005; Andreatti Filho et al. 2007) and reviewed by Wernicki et al. (2017).
- Prophylactic and post-infection administration of *Salmonella* phage PSE showed a positive influence on the ileal microbiome of quail by increasing *Lactobacillus* spp. and presumably reducing the *Salmonella* (Ahmadi et al. 2016).
- Administration of bacteriophage cocktail, INT-401, significantly reduced *Clostridium perfringens* induced necrotic enteritis and mortality in broiler chickens. Administration of INT-401 via oral gavage, feed, and drinking water significantly improved weight gain, and the feed conversion ratio in 48 day old birds (Miller et al. 2010).

Phages have been used therapeutically in humans for more than 100 years, without any serious side effects (Sulakvelidze et al. 2001; Sulakvelidze and Barrow 2005a). During the long history of therapeutic use in Eastern Europe and the former Soviet Union (and, before the antibiotic era, in the United States, France, Australia, and other countries), phages have been administered to humans in various forms, including orally, rectally, locally (skin, eye, ear, nasal mucosa, etc.); in tampons, rinses, and creams, as aerosols or intrapleural injections, and intravenously.

- A randomized, double-blind, placebo controlled clinical trial evaluated safety and tolerability of ShigActive, a phage preparation targeting *Shigella* spp. via oral administration. ShigActive or placebo was administered three times daily for 7 days. No adverse events were recorded for trial evaluation period of up to 90 days. Approximately 50% of subjects experienced mild gas, bloating and/or abdominal

pain either during 7 days of treatment or immediately after treatment completion on 8th day. All events were deemed not clinically relevant and were observed at the same frequency as placebo treatment. Additionally, ShigActive treatment did not elicit any significant inflammatory response in the GI-tract or alter the fecal microbiome (Chen et al. 2024).

- Critical analysis of in-vitro and in-vivo studies did not reveal any harmful effects or serious complications because of bacteriophage use in humans, including children (Gorski et al. 2018). On the contrary, the available data indicate phages may exert anti-inflammatory and downregulate hyperactive immune system effects and may help protect the overall health (Gorski et al. 2018; Guglielmi 2017). Recent reviews summarize the results of some of the human therapy studies involving bacteriophages (Chan et al. 2013; Abedon et al. 2011; Golkar et al. 2014; Vandamme 2014; Gordillo Altamirano and Barr 2019; Kutateladze and Adamia 2008; Międzybrodzki et al. 2012; Gorski et al. 2018).
- Phages have also been administered to humans for non-therapeutic purposes without any adverse effects. For example, phage preparations have been used extensively to monitor humoral immune functions in humans including patients with Down's syndrome, the Wiskott-Aldrich syndrome, and immunodeficient patients in the United States in the 1970s-1990s (Lopez et al. 1975; Ochs et al. 1992). In some of the studies (including several studies performed by the FDA), the purified phages were injected intravenously into HIV-infected patients or other immunodeficient individuals without any apparent side effects (Smith et al. 2014; Rubinstein et al. 2000; Ochs et al. 1971).
- Phages have also been administered to humans via various sera and FDA-approved vaccines commercially available in the United States (Ochs et al. 1971; Milch and Fornosi 1975; Moody et al. 1975; Merril et al. 1972).
- No serious adverse immunologic or allergic sequelae have ever been reported because of human or animal exposure to phages (Sulakvelidze et al. 2001; Abedon et al. 2011).

In summary, extensive literature suggests that (i) bacteriophages are natural inhabitants of humans, animals, and birds, (ii) bacteriophages are naturally present in food, feed, and water commonly consumed by animals including companion animals, poultry, and livestock, and (iii) bacteriophage administration to companion animals, poultry, and livestock is safe.

6.2.1.1 LYTIC PHAGES ARE GRAS

All lytic phages are, by nature, GRAS. There are two major types of phages: "virulent" (also called "lytic") and "temperate" (often mistakenly called "lysogenic"). Lytic phages lyse host

bacteria without integrating into the host genome. In contrast, temperate phages may integrate into the host genome and a small subset of these may transduce undesirable bacterial genes, such as those encoding toxins or antibiotic resistance. Both lytic and temperate phages are ubiquitous in the environment, including the human and animal gut, the human oral cavity, foods sold at retail, sewage, and many other places that we encounter daily. Humans shed large numbers of both lytic and temperate phages into the environment every day – estimated to be on the order of $4x10^9$ phages daily per person (Sulakvelidze et al. 2005a). Temperate phages are found in almost all bacterial genera, including *Staphylococcus*, *Vibrio*, *Pseudomonas*, *Salmonella*, *Shigella*, *Bacillus*, *Corynebacterium*, *Listeria*, and *Streptococcus* (Jacob and Wollman 1959; Langley et al. 2003; Schicklmaier and Schmieger 1995; Eggers et al. 2001). Indeed, some strains can release as many as five different types of temperate phages. Although the possibility of added gene transfer events is highly unlikely to bring danger to any individual consuming temperate phages, the use of such phages on an industrial scale could increase the overall risk of potentially harmful genes being acquired by new bacterial strains. Therefore, Intralytix identifies and uses only lytic phages in its phage preparations (including SalmoFresh).

6.2.1.2 SALMOFRESH™ MONOPHAGES ARE GRAS

The component phages in SalmoFresh were each characterized by various approaches, including electron microscopy, genotypic fingerprinting, and full genome sequence analysis. The component phages in SalmoFresh are members of the class *Caudoviricetes*, containing linear double-stranded DNA phages, as defined by the International Committee on the Taxonomy of Viruses (ICTV) (Turner et al. 2023; Zhu et al. 2022).

Intralytix has fully sequenced all component monophages included in SalmoFresh. This approach is used to exclude bacteriophages carrying sequences encoding any undesirable genes, including genes encoding bacterial toxins (including genes listed in 40 CFR § 725.421), other known toxin genes, genes associated with drug resistance, and bacterial 16S rRNA genes (which would indicate prior evidence of transduction).

Table 17 summarizes the current phage genomes' properties; the full phage sequencing report is included in Appendix 7.1.21.1. Analysis of the sequences yielded the following results:

- No toxin genes (including, but not limited to, genes listed in 40 CFR § 725.421) have been identified in any of the component monophages.
- No 16S ribosomal RNA genes have been identified in any of the component monophages.
- No antibiotic resistance genes have been identified in any of the component monophages.

Summary: All six monophages (SBA-1781, SKML-39, SPT-1, SSE-121, STML-198, STML-13-1) included in the current version of SalmoFresh are linear double stranded DNA phages: SBA-1781 and SPT-1 are closely related to *Salmonella* Phage Mushroom, a member of the *Felixounavirus*. SKML-39, SSE-121, STML-198, and STML-13-1 are T4-like phages in the genus *Agtrevirus*, *Seunavirus*, *Gelderlandvirus*, and *Kuttervirus*, respectively. All six phages are classified as lytic and do not contain any toxin genes listed in the US Code of Federal Regulations (40 CFR § 725.421) nor any other undesirable genes. Therefore, all six monophages are suitable for use in animals.

Table 17 Genome size and composition of phages contained in SalmoFresh™

Phage	Size (bp)	GC%	# CDS	# tRNA	Undesirable genes		
					CFR genes	Others	16S rRNA
SBA-1781	(b) (4)				0	0	0
SKML-39					0	0	0
SPT-1					0	0	0
SSE-121					0	0	0
STML-13-1					0	0	0
STML-198					0	0	0

6.2.1.3 SalmoFresh™ is Specific to *Salmonella*

Lytic activity of the SalmoFresh monophages is targeted against *Salmonella* strains. SalmoFresh has been screened for its lytic activity against 1,397 *Salmonella* isolates in the Intralytix collection, representing more than 60 serotypes, including those belonging to the most common/highly pathogenic serotypes Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Kentucky, Thompson, Georgia, Agona, Grampian, Senftenberg, Alachua, Infantis, Reading, and Schwarzengrund. The serotypes and their relative proportion in the Intralytix collection is presented in Figure 1. As shown in Table 18, SalmoFresh is effective against the collection.

Table 18 *Salmonella enterica* in Intralytix's collection and the percent susceptible to SalmoFresh™

Species	# isolates in Intralytix collection	Percent kill by SalmoFresh™ $1x10^9$ PFU/mL
Salmonella enterica	1397	99.57%

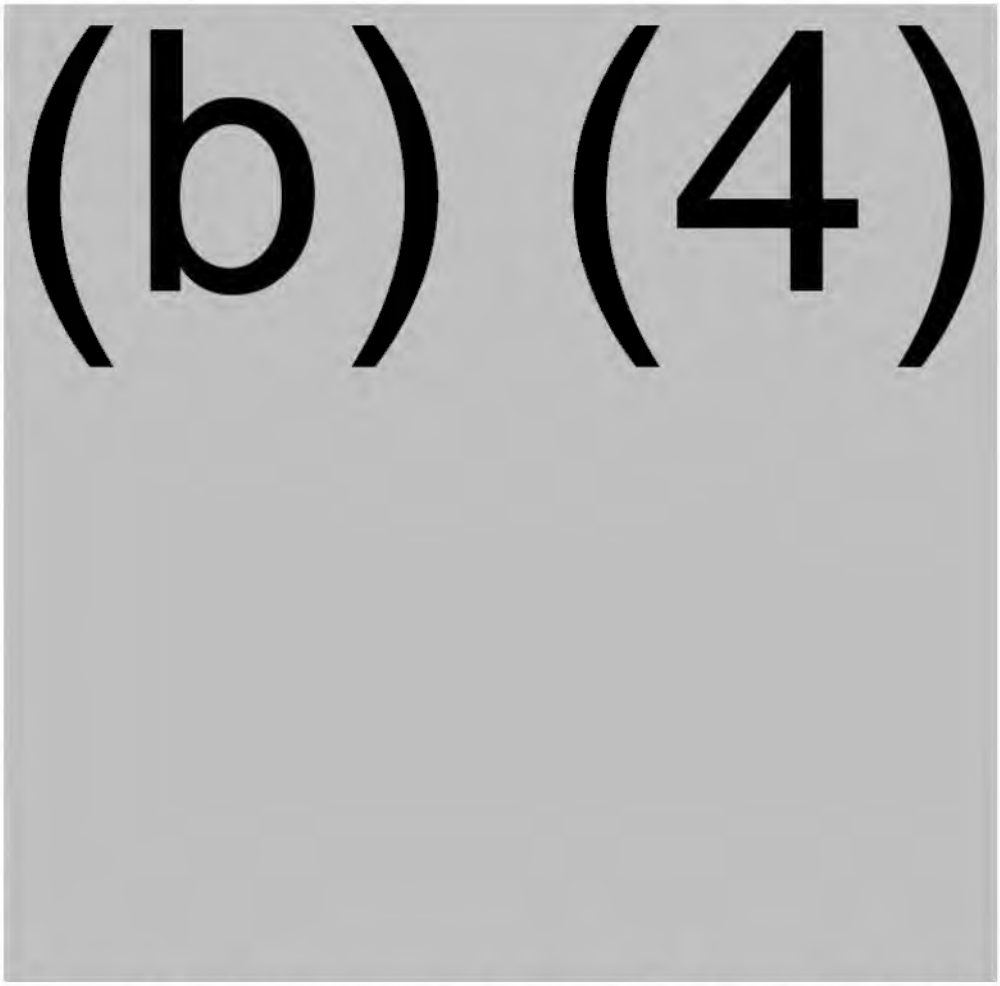


Figure 1 Salmonella serotypes and their proportion in Intralytix collection

SalmoFresh is also highly specific. As shown in Table 19, SalmoFresh does not lyse any of the non-targeted Gram-positive isolates examined. These isolates include 5 strains each of *Staphylococcus aureus*, *Listeria* spp., and *Enterococcus* spp. SalmoFresh also does not lyse several non-*Salmonella* Gram-negative strains, including 5 strains each of *Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Shigella* spp. Of the 5 strains of *E. coli* tested, 2 *E. coli* O157:H7 were susceptible to SalmoFresh. This is not unexpected as the SalmoFresh monophages are genetically similar to other known *E. coli* phages and *Salmonella* and *E. coli* are closely related members of the *Enterobacteriaceae* family.

Table 19 Lytic activity of SalmoFresh™ against non-Salmonella enterica strains of bacteria

Non-Salmonella enterica strains		Species	Susceptibility to SalmoFresh™ ($1x10^9$ PFU/mL)
Intralytix ID	Original ID		
(b) (4)	ATCC25923	*Staphylococcus aureus*	-
	ATCC29213	*Staphylococcus aureus*	-
	ATCC700699	*Staphylococcus aureus*	-
	ATCC49775	*Staphylococcus aureus*	-
	ATCC14458	*Staphylococcus aureus*	-
	ATCC19117	*Listeria monocytogenes*	-
	ATCC19118	*Listeria monocytogenes*	-
	ATCC51724	*Listeria innocua*	-
	ATCC19116	*Listeria monocytogenes*	-
	ATCC33090	*Listeria innocua*	-
	WCC188	*Enterococcus spp.*	-
	ATCC11823	*Enterococcus faecalis*	-
	ATCC19433	*Enterococcus faecalis*	-
	1133455	*Enterococcus avium*	-
	1126611	*Enterococcus faecalis*	-
	ATCC19606	*Acinetobacter baumannii*	-
	HER1401	*Acinetobacter baumannii*	-
	4308-2	*Acinetobacter baumannii*	-
	3247-1	*Acinetobacter baumannii*	-
	1673-2	*Acinetobacter baumannii*	-
	ATCC10145	*Pseudomonas aeruginosa*	-
	ATCC15692	*Pseudomonas aeruginosa*	-
	ATCC51674	*Pseudomonas aeruginosa*	-
	ATCC43390	*Pseudomonas aeruginosa*	-
	ATCC39324	*Pseudomonas aeruginosa*	-
	514	*Shigella dysenteriae*	-
	045-311082	*Shigella flexneri*	-
	300	*Shigella flexneri*	-
	90	*Shigella sonnei*	-
	ATCC9290	*Shigella sonnei*	-
	ATCC43895	*Escherichia coli* O157:H7	+
	ATCC35401	*Escherichia coli* O78:H11	-
	ATCC700728	*Escherichia coli* O157:H7	+
	ATCC11303	*Escherichia coli*	-
	ATCC12435	*Escherichia coli*	-

6.2.2 SODIUM CHLORIDE

Sodium chloride (salt) is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

6.2.3 BY-PRODUCTS

The *Salmonella enterica* host strains are Gram-negative bacteria. As with all Gram-negative bacteria, they produce bacterial endotoxins or lipopolysaccharides (LPS). Intralytix tests every lot of SalmoFresh to ensure its LPS levels fall below the established release criteria. Endotoxins are further discussed in Sections 6.3.1.3 and 6.3.2.2.

6.3 MANUFACTURING OF SALMOFRESH™

SalmoFresh is manufactured using Intralytix's standard procedures. These procedures have been reviewed by the FDA for the manufacturing of several of Intralytix's bacteriophage food safety products, SalmoFresh™ (GRN 435), ListShield™ (21 CFR §172.785, GRN 528), EcoShield™ (FCN No. 1018), EcoShield™ PX (GRN 834), ShigaShield™ (GRN 672), and CampyShield™ (GRN 966) and are currently used to manufacture commercial lots of these products.

(b) (4)

6.3.1 STARTING MATERIALS

There are four starting materials for manufacture of SalmoFresh component monophages:

- Animal-product free media
- Host strains
- Monophages
- Antifoam (as needed)

The safety of each is considered separately below.

6.3.1.1 ANIMAL-PRODUCT FREE MEDIA

Vegan media is used for bacterial growth and phage propagation. The main components are described here and have an existing regulatory status as GRAS or affirmed as food or for food application(s).

Phytone Peptone and Soytone: Peptones are GRAS affirmed at 21 CFR § 184.1553 for use as processing aids, among other uses, at levels not to exceed good manufacturing practice. Peptones are protein hydrolysates consisting of free amino acids and short peptides in an aqueous salt solution.

Yeast Extract: Yeast extract is a commonly used food ingredient. For example, baker's yeast extract is GRAS affirmed as a flavoring agent or adjuvant at up to 5% in foods generally. 21 CFR § 184.1983.

Sodium Chloride: Sodium chloride "salt" is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

Magnesium Sulfate: Magnesium sulfate salt is GRAS affirmed at 21 CFR § 184.1443 for use as a processing aid, among other uses, at levels not to exceed good manufacturing practice. Magnesium sulfate is a component of the animal-product free growth media used in SalmoFresh production.

6.3.1.2 ANTIFOAMING AGENT

(b) (4) is polypropylene glycol-based, Kosher-certified product, approved for a variety of food additive uses, both direct and indirect (b) (4) . (b) (4)

6.3.1.3 HOST STRAINS

The component monophages are produced on several *Salmonella enterica* strains. These host strains were characterized at Intralytix through sequencing. The full host strain sequencing report is included in Appendix 7.1.21.2.

As described in the sequencing report, the *Salmonella enterica* host strains are not known to produce any enterotoxins that could compromise the safety of the final product. Therefore, the only production host strain-related toxin that is relevant for SalmoFresh safety is endotoxin or LPS. As with all Gram-negative bacteria, *Salmonella* outer membrane contains lipopolysaccharides or LPS. If sufficiently high amounts of LPS enter the bloodstream, it can trigger the signaling cascade for macrophage/endothelial cells to secrete pro-inflammatory cytokines and nitric oxide that may lead to "endotoxic shock." Because SalmoFresh phages are propagated in *Salmonella* host strains, host cells lyse during the process (as the result of phage lytic cycle) and *Salmonella* LPS is present in the resulting phage lysates. Most of the endotoxin is removed during phage purification process. However, as a standard quality control protocol, Intralytix analyzes every SalmoFresh batch for the presence and levels of the LPS endotoxin in the final product. All product lots must be at or below 25,000 endotoxin unit (EU)/mL at $1x10^9$

PFU/mL level to pass the release criteria for LPS. This level is safe, as explained in Section 3.1.2.

6.3.1.4 MONOPHAGES

The safety of monophages is discussed in Section 6.2.1.

6.3.2 QUALITY CONTROL

6.3.2.1 MONOPHAGES

The following tests are performed upon each monophage lot:

Lytic titer

The lytic titer test measures the lytic titer of each monophage lot, by determining the number of plaque forming units per milliliter (PFU/mL). The specification for each monophage lot is the titer is $\geq$9.8 $\log_{10}$ PFU/mL which is the minimum titer necessary to be able to produce a final product. The method has been qualified by Intralytix. Intralytix has established the error associated with this method of titration as $\pm$0.33 $\log_{10}$ PFU/mL (Anderson et al. 2011).

Microbial purity

The microbial purity test confirms that the monophage solution does not contain viable microbes. Briefly, samples of each monophage solution are tested by a) direct plating onto non-selective agar and b) after enrichment. The specification is that each monophage lot must be free from bacterial contamination.

Identity

Genomic fingerprinting is used to confirm the identity of each monophage lot. The acceptance criteria for the specification is the genomic fingerprint matches the reference phage genome as determined during the development of the master phage bank. The method to determine restriction fragment length polymorphism (RFLP) is well established in the scientific literature; please see Santos et al. (2010) for a reference specific to *Salmonella*-targeting bacteriophages. With regards to our specific bacteriophages, the method has been confirmed to be valid by analyzing different lots of bacteriophages and demonstrating the reproducibility.

6.3.2.2 SALMOFRESH™

The following tests are performed upon each batch of SalmoFresh:

Lytic titer test

The lytic titer test method confirms the titer (PFU/mL) of the SalmoFresh preparation. The specification for this test is dependent upon the concentration of the finished good. For example, SalmoFresh 100X has a lytic titer of 11.0±0.33 $\log_{10}$ PFU/mL. The method has been qualified by Intralytix. Intralytix has established the error associated with this method of titration as ±0.33 $\log_{10}$ PFU/mL (Anderson et al. 2011).

Microbial purity

The microbial purity test is a determination of the viable microbial contamination in a phage solution. Briefly, a representative sample of each lot of SalmoFresh is tested by combining with a concentrated growth media and incubating for 14 days. Growth is monitored visually and by plating. Both positive and negative controls are included. The specification for this test is that SalmoFresh must be free from bacterial contamination.

Endotoxin content test

Endotoxins are toxins associated with host bacteria, of which a residual amount could be present in the phage preparations. A commercially available quantitative Limulus Amebocyte Lysate (LAL)-based test specifically for measurement of endotoxin is currently used by Intralytix. The specification for this test is each lot of SalmoFresh must contain ≤25,000 EU/mL (at 9.0 $\log_{10}$ PFU/mL).

Identity test

The identity of the finished good is confirmed by a review of the Batch Production Record information to confirm that each lot of the component monophage was added at the appropriate concentration by an Intralytix production employee and the addition of the component monophage was confirmed by a second production employee. This review is conducted initially by Intralytix Quality Control personnel and then confirmed by Intralytix Quality Assurance prior to the release of the finished product.

6.4 SUBSTANTIAL EQUIVALENCE TO APPROVED PRODUCTS

6.4.1 PREVIOUSLY APPROVED BACTERIOPHAGE PREPARATIONS

Although bacteriophage products have not been GRAS affirmed or approved as a food additive for pet food and animal feed, several lytic bacteriophage products targeting various foodborne bacterial pathogens have already been designated GRAS and/or cleared for human food safety usage and other applications by several regulatory agencies:

- SalmoFresh™, a phage preparation containing six *Salmonella*-specific lytic phages is GRAS (GRN 435) for human food safety applications.
- SalmoFresh is also listed by the FSIS for use on various poultry products (FSIS Directive 7120.1).
- ListShield™ (formerly known as LMP-102,) a phage preparation containing six lytic *Listeria monocytogenes*-specific phages, is FDA-cleared as a food additive (21 CFR §172.785).
- ListShield is also GRAS (GRN 528).
- ListShield is also listed by the FSIS for use on various RTE meats and poultry products (FSIS Directive 7120.1).
- ListShield is also EPA-registered for use on non-food contact surfaces in food processing plants to prevent or significantly reduce contamination of *Listeria monocytogenes* (EPA registration #74234-1).
- Listex™[3], a phage preparation containing a single *Listeria monocytogenes* lytic phage, P100, is GRAS (GRN 218).
- Listex is also listed by the FSIS for use as processing aid when applied at a level of $1x10^7$ to $1x10^9$ PFU/g food product (FSIS Directive 7120.1).
- Salmonelex™[4], a phage preparation containing two *Salmonella*-specific phages is GRAS (GRN 468).
- SalmPro® a phage preparation containing two *Salmonella*-specific phages is GRAS (GRN 752).
- EcoShield™ (formerly ECP-100) a phage preparation containing three lytic *E. coli* O157:H7-specific phages, is FDA-cleared for use as a food contact substance (FCN No. 1018).
- EcoShield is also listed by the FSIS for use as processing aid on red meat parts and trim prior to grinding (FSIS Directive 7120.1).
- EcoShield PX, a phage preparation containing three to eight lytic phages specific to shiga-toxin producing *E. coli*, is affirmed as GRAS (GRN 834).
- PhageGuard E™, a phage preparation containing two *E. coli*-specific lytic phages is GRAS (GRN 757).

[3] Currently marketed as PhageGuard L.

[4] Currently marketed as PhageGuard S.

- An *E. coli* specific phage preparation containing six of 12 *E. coli* specific bacteriophages is GRAS (GRN 724)
- ShigaShield™, a phage preparation containing five lytic phages to *Shigella* spp. is affirmed as GRAS (GRN 672).
- AgriPhage, a phage preparation targeting *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*, is EPA-registered for use on tomatoes and peppers (EPA Reg. No. 67986-1).
- Two bacteriophage preparations – one *Salmonella*-targeting and one *E. coli* O157:H7-targeting – are listed by the FSIS for use as processing aids on the hides and feathers of live animals before slaughter (FSIS Directive 7120.1).
- *Salmonella* Enteritidis phage SP8 preparation is GRAS affirmed (GRN 1134) at the levels of up to $2x10^8$ PFU/g of food.
- Applied Phage Meat S2, a phage preparation that contains any combination of six phages out of a set of eleven qualified phages is GRAS affirmed (GRAS 1038).
- Applied Phage Vegetable S2, a phage preparation that contains any combination of six phages out of a set of eleven qualified phages is GRAS affirmed (GRAS 1070).

The regulatory agencies represented in the preceding list each separately concluded that different bacteriophage preparations were safe and effective. The variety of these previously cleared or registered bacteriophage preparations attests to the general safety of bacteriophages and therefore supports their natural GRAS status. SalmoFresh is one of the phage preparations already granted GRAS status by the CFSAN FDA for human foods; based on this and on additional information presented in this document, we believe it is also GRAS for the intended use on pet food and animal feed when applied to food at up to $1x10^8$ PFU / gram of food.

6.5 STUDIES IN TARGET ANIMALS

Although the safety of the SalmoFresh lytic phages is well documented and they have been designated as GRAS for human uses, short-term feeding studies in both dogs and cats were conducted to provide confirmation that no unanticipated health effects were seen when the animals were fed SalmoFresh-treated pet food products (Soffer et al. 2016). Note: The studies were performed with the SalmoFresh phage cocktail under the alternate tradename of SalmoLyse. These studies are summarized in the following sections – and as anticipated based on the available safety data and safe use in humans, no adverse findings were noted during these studies.

6.5.1 DOG FEEDING STUDY

The feeding study publication can be found in Section 7.1.20.5.

6.5.1.1 Study Design

Twelve dogs were fed twice daily, for 14 days, the PHNC house diet (modified mini chunks; (b) (4)) that was enrobed with the 6 phage SalmoFresh mixture ($2 \times 10^7 \pm 0.3$ log PFU/g). Gastrointestinal (GI) health was evaluated through quantification of intake, fecal scoring, measurements of body weight (BW), and body condition scoring (BCS).

6.5.1.2 Results

There were no significant adverse events reported and no medical reason to remove any dog during the 14-day feeding period. One dog exhibited one event of a fecal score of 1 on the afternoon of day three of the study. This event was isolated and consistent with historical fecal scores for that individual (9 events of fecal score 1 in the last 7 months). One dog had diet orts for two consecutive days (38 and 43 g out of 182 g/d offered).

Both events, the fecal score of 1 and the orts, could be attributed to heat stress because these events occurred during days of extremely high ambient temperature that reached 94°F and a heat index over 100°F (data obtained from the National Climatic Data Center of the National Oceanic and Atmospheric Administration).

During the course of the study, there was no incidence of emesis (productive or non-productive) or diarrhea, which are the main signs of GI discomfort. The only event of liquid feces was isolated and consistent with the history of that particular dog and was potentially caused by heat stress. Furthermore, fecal scores were distributed as it has been observed historically (two-year data) for this panel of dogs. Additionally, parameters such as intake, BW, and BCS were not affected, suggesting no impact on nutrient intake, digestion, and absorption. These data suggest that intake of a SalmoFresh-coated diet does not have an impact on the gastrointestinal health of dogs.

6.5.2 CAT FEEDING STUDY

The feeding study publication can be found in Section 7.1.20.5.

6.5.2.1 Study Design

Twelve cats were fed once daily, for 14 days, the PHNC house diet (b) (4)) that was enrobed with a 6 phage SalmoFresh mixture ($2 \times 10^7 \pm 0.3$ log PFU/g). Gastrointestinal health was evaluated through quantification of intake, fecal scoring, measurements of body weight (BW), and body condition scoring (BCS).

6.5.2.2 Results

There were no significant adverse events reported and no medical reason to remove any cat during the 14-day feeding period.

During the course of the study, there was no incidence of emesis (productive or non-productive) or diarrhea, which are the main signs of GI discomfort. Additionally, parameters such as intake and BW were not affected suggesting no impact on nutrient intake, digestion, and absorption. Because of the subjective nature of the BCS system, the slight change observed in BCS can be attributed to the normal variation of these values.

These data suggest that intake of a SalmoFresh-coated diet does not have an impact on the gastrointestinal health of cats.

6.5.3 LITERATURE REVIEW

Numerous studies were identified in the published literature in which animal diets were supplemented with phages to evaluate the effect of the phages on the animals. Some of those studies are summarized in the table below.

Table 20 Selected studies using oral application of bacteriophage to animals

Animal	Target bacteria	Phage(s)	Study description	Ref.
Chicken	*S.* Gallinarum	CJΦ01	Six-week-old chicks consumed feed supplemented with bacteriophage at 1E6 PFU/kg for ~28 days, with no adverse events	(Lim et al. 2011)
Chicken	*S.* Enteritidis	CJ07	Layer chickens were fed feed supplemented with bacteriophage at E5, E7, or E9 PFU/g for 21 days; neither clinical signs nor mortality was observed in any group throughout the experiment	(Lim et al. 2012)
Chicken	*S.* Typhimurium	Three-phage cocktails	Phage given orally, alone and with a competitive exclusion product, showed no adverse effects on challenged chicks	(Toro et al. 2005)
Chicken	*S.* Enteritidis	Three-phage cocktail	Chicks received bacteriophage at E8 PFU/ml in their drinking water for 1 day prior to challenge; no adverse effects were observed in any group throughout the experiment	(Borie et al. 2008)
Chicken	*C. jejuni*	Phage NCTC 12673, vB_CcM-LmqsCPL1/1	Phage in drinking water (E7 PFU/mL, ad libitum) for 3 days, alone and with a competitive exclusion product, showed no adverse effects on broilers	(Peh et al. 2024)
Chicken	*S.* Typhimurium, *S.* Enteritidis, *S. gallinarum*, *E. coli*, and	Cocktail of phages targeting *Salmonella*, *E. coli* and *C. perfringens*	Combination of phages added to feed (E8 PFU/g) for 35 days showed no adverse events	(Upadhaya et al. 2021)

Animal	Target bacteria	Phage(s)	Study description	Ref.
	Clostridium perfringens			
Chicken	*S.* Typhimurium	SPFM10, SPFM14	Phage delivered in feed (E5-E7 PFU/day) to chickens for 42 days, no adverse effects were reported.	(Thanki et al. 2023)
Chicken	*S.* Enteritidis	Phage cocktail	Oral application of phages (E11 PFU) to challenged birds showed no adverse effects on the animals	(Fiorentin et al. 2005)
Swine	*Salmonella* spp., *E. coli*, *Staphylococcus aureus*, and *Clostridium perfringens*	Phage cocktail	Phage in feed (E9/kg), alone and with a probiotic product, for 35 days showed no adverse effects on grower pigs	(Kim et al. 2014)
Swine	*S.* Typhimurium	Phage with probiotics	Phage in feed (E9/kg) for 14 days prior to challenge showed no adverse effects compared to control feed.	(Gebru et al. 2010)
Swine	*S.* Typhimurium	Felix 01	Single oral (and intramuscular) application of phages (E10 PFU) to challenged pigs showed no adverse effects at 6 hrs post treatment	(Lee and Harris 2001)
Swine	*S.* Typhimurium	Phage cocktail	Oral application of phages (E9 PFU) every two hours for six hours to challenged pigs showed no adverse effects at 6 hrs post treatment	(Wall et al. 2010)
Swine	*E. coli* (ETEC)	Phage cocktail	Piglets challenged with *E. coli* and fed phage-supplemented feed (E9/kg) for 7 days showed no adverse effects due to phage consumption	(Han et al. 2016)
Swine	*E. coli* (ETEC)	Single phage	Challenged piglets fed phage-supplemented feed (E7/kg) for 21 days showed no adverse effects	(Lee et al. 2017)
Swine	*E. coli*	A221	Oral application of phages (E9 PFU) for 7 days to challenged piglets showed no adverse effects	(Mao et al. 2023)
Swine	*E. coli* (MDR)	vB_EcoM-RPN170, vB_EcoM-RPN171, vB_EcoM-RPN187, vB_EcoM-RPN226, vB_EcoM-RPN242, vB_EcoP-RPN243	Oral application of low (E9) and high (E10) doses of phages to pigs once a day for three days showed no adverse effects, and the microbiota in animals that received phage was similar to the control animals	(Imklin et al. 2022)

Additional studies have examined bacteriophage application in animals through non-oral routes. These studies also demonstrate that there are no adverse effects on the animals and are summarized in the table that follows.

Table 21 Selected studies on bacteriophage administration in livestock via non-oral routes

Animal	Target bacteria	Safety summary	Ref.
Dogs	*Pseudomonas aeruginosa*	Topical administration of phages to the ear canal of dogs did not elicit any adverse effects, inflammation or toxicity	(Hawkins et al. 2010).
Dog	*P. aeruginosa*	A clinical case report showed that phages applied to the interdigital region of a dog were well tolerated	(Grecu et al. 2023)
Cat	*P. aeruginosa*	Throughout the period of topical application of phages to a leg infection, no side effects were observed.	(Braunstein et al. 2024)
Chickens	*E. coli*	Aerosol spray of phage (E8 - E10 PFU) onto chickens showed no adverse effects on the animals.	(Huff et al. 2002)
Mice	*S. aureus*	Intramammary application of E7 PFU did not induce any inflammatory response including neutrophilic and lymphocytic inflammatory responses.	(Breyne et al. 2017)
Mice	*S. aureus* (MRSA)	Intravenous and intraperitoneal administration of E5 or E7 PFU showed no adverse effects on mice.	(Iwano et al. 2018)
Mice	*S. aureus* (MRSA)	Phage application (E9 PFU) to abdominal cavity was considered safe in mice and cytokine experiments showed no bias.	(Wang et al. 2016)
Cows	*E. coli* (MDR)	Intramammary application of E8 PFU for 3 days showed no adverse effects in dairy cows with induced mastitis.	(Guo et al. 2021)
Cows	*E. coli* (ETEC, EPEC)	Suppository application of phage/probiotic for 5 days showed no adverse effect on the animals.	(Alomari et al. 2021)

In addition to the selected studies presented in Table 21, the safety of phages has been evaluated in a number of other species including sheep, horse, rats, mice, rabbits, and fish; insects such as greater wax moths and Drosophila; and nematode (*Caenorhabditis elegans*) etc. (Penziner et al. 2021; Yang et al. 2024). The extensive testing in animals, including at least 11 mammal species, a fish, 2 insects, and a nematode species has demonstrated that phage application across all of them is safe and does not elicit any adverse effects (Penziner et al. 2021).

6.6 SUMMARY AND BASIS FOR GRAS

SalmoFresh is an all-natural product made of up to six *Salmonella*-specific lytic bacteriophages. All phages included in SalmoFresh are lytic phages and they have been rigorously characterized (including full genome sequencing) prior to inclusion in the cocktail.

Bacteriophages are the oldest, most ubiquitous organisms on earth, with their numbers estimated to be between 10^{30} and 10^{32}. Phages are present everywhere – including in mouths, on skin, and within gastrointestinal tracts. They are also common and natural ingredients of all fresh,

unprocessed foods. The omnipresence of phages (including in foods) and their daily consumption by humans and animals makes them naturally GRAS.

In further recognition of their safety, several lytic bacteriophage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage and other applications by several regulatory agencies.

Although all lytic bacteriophages are, by nature, GRAS, the phages in SalmoFresh must be verified to be lytic and to not contain any undesirable genes, including those listed in 40 CFR § 725.421. The genomes of the component bacteriophages in SalmoFresh have been sequenced. Bioinformatic analysis of the component phages' sequences shows none contain any undesirable genes, including antibiotic resistance genes, 16S rRNA sequences, or known toxin genes.

SalmoFresh is manufactured using Intralytix's well-established standard procedures. SalmoFresh is produced (b) (4). The final SalmoFresh product contains no preservatives or additives. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, SalmoFresh™ (GRN 435), ListShield™ (21 CFR §172.785; GRN 528), EcoShield™ (FCN No. 1018), ShigaShield™ (GRN 672), EcoShield™ PX (GRN 834), and CampyShield™ (GRN 966) and are currently used to manufacture commercial lots of these products. Intralytix's phage manufacturing procedures and quality systems are also SQF-certified by the Safe Quality Food Institute, ensuring rigorous quality standards of all our commercial phage preparations for food safety applications. SalmoFresh is OMRI-listed, Kosher, and Halal.

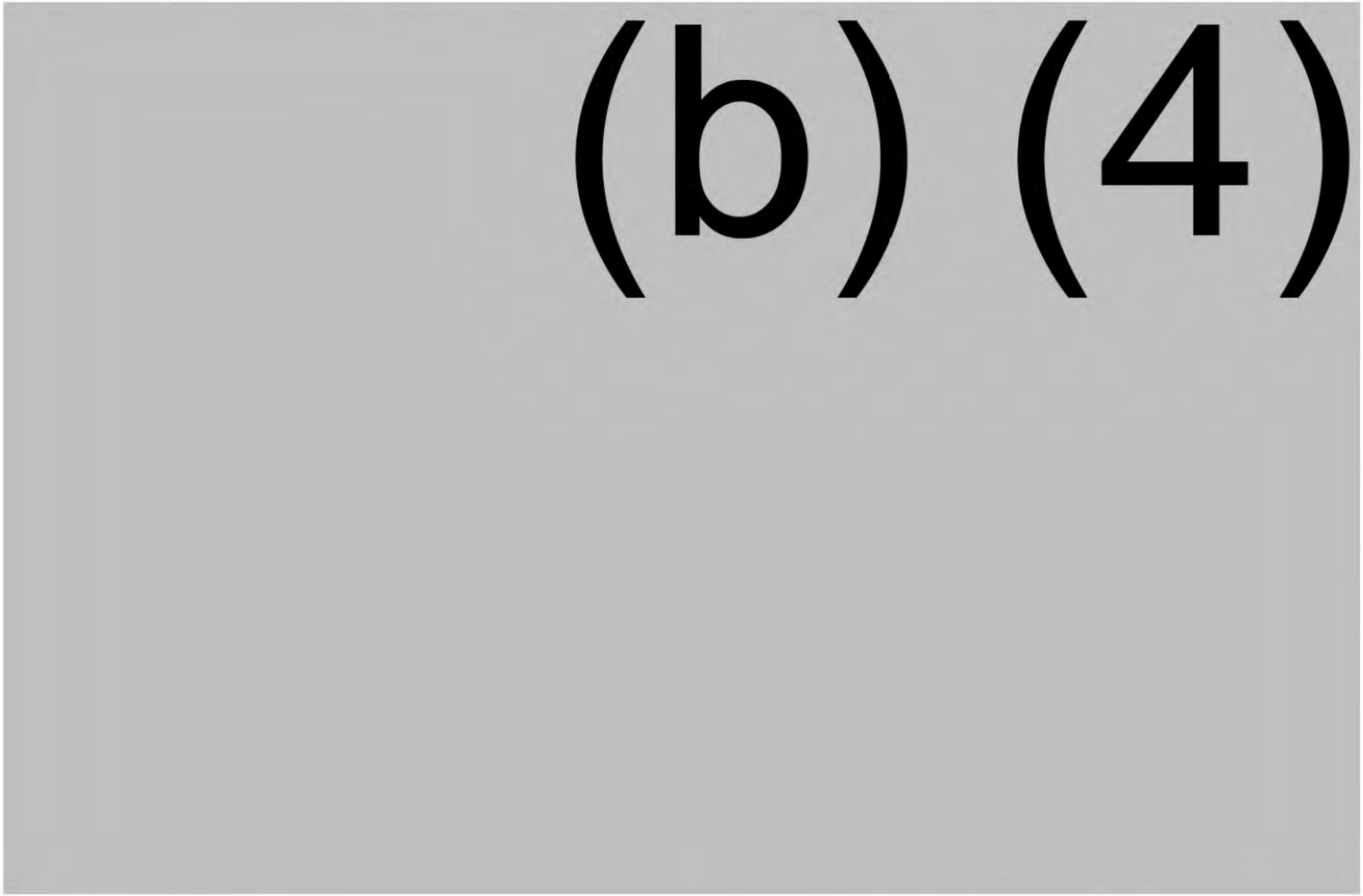
The only manufacturing byproduct of potential concern during SalmoFresh manufacturing is LPS. Intralytix tests every lot of SalmoFresh for LPS to ensure it meets the release criteria. The LPS levels of the SalmoFresh lot (at the typical working concentration of ~$1x10^9$ PFU/mL) must be below 25,000 EU/mL for the lot to be released. This standard is the same as the maximum LPS level previously cleared by the FDA for SalmoFresh (GRN 435), EcoShield (FCN 1018), EcoShield PX (GRN 834), ShigaShield (GRN 672), and CampyShield (GRN966).

The proposed application rate for SalmoFresh is up to $1x10^8$ PFU per gram of food article. Assuming the maximum application rate of $1x10^8$ PFU/g to all foods within the target food groups, the average daily consumption of these foods would contain a mere 0.035 µg phage particles per g of pet food or animal feed. The added phage is negligible.

SalmoFresh for pet food and animal feed is identical to SalmoFresh (GRN 435) for human food applications and technically equivalent to other lytic bacteriophage preparations that have been previously designated GRAS and/or cleared by the FDA and other regulatory agencies.

In summary, the data presented in this document fully supports our designation of SalmoFresh as GRAS for pet food and animal feed applications. The basis for our conclusion is four-fold. First,

scientific literature extensively documents that lytic bacteriophages pose no safety concerns to animals and humans. Second, all bacteriophages in SalmoFresh are lytic, non-genetically modified, and free of undesirable genes. Third, Intralytix's manufacturing process ensures the safety and quality of the final SalmoFresh product. And fourth, the estimated daily intake of the SalmoFresh phage preparation is negligihle when compared to the natural levels found in animals and on animal foods and feed.


(b) (4)

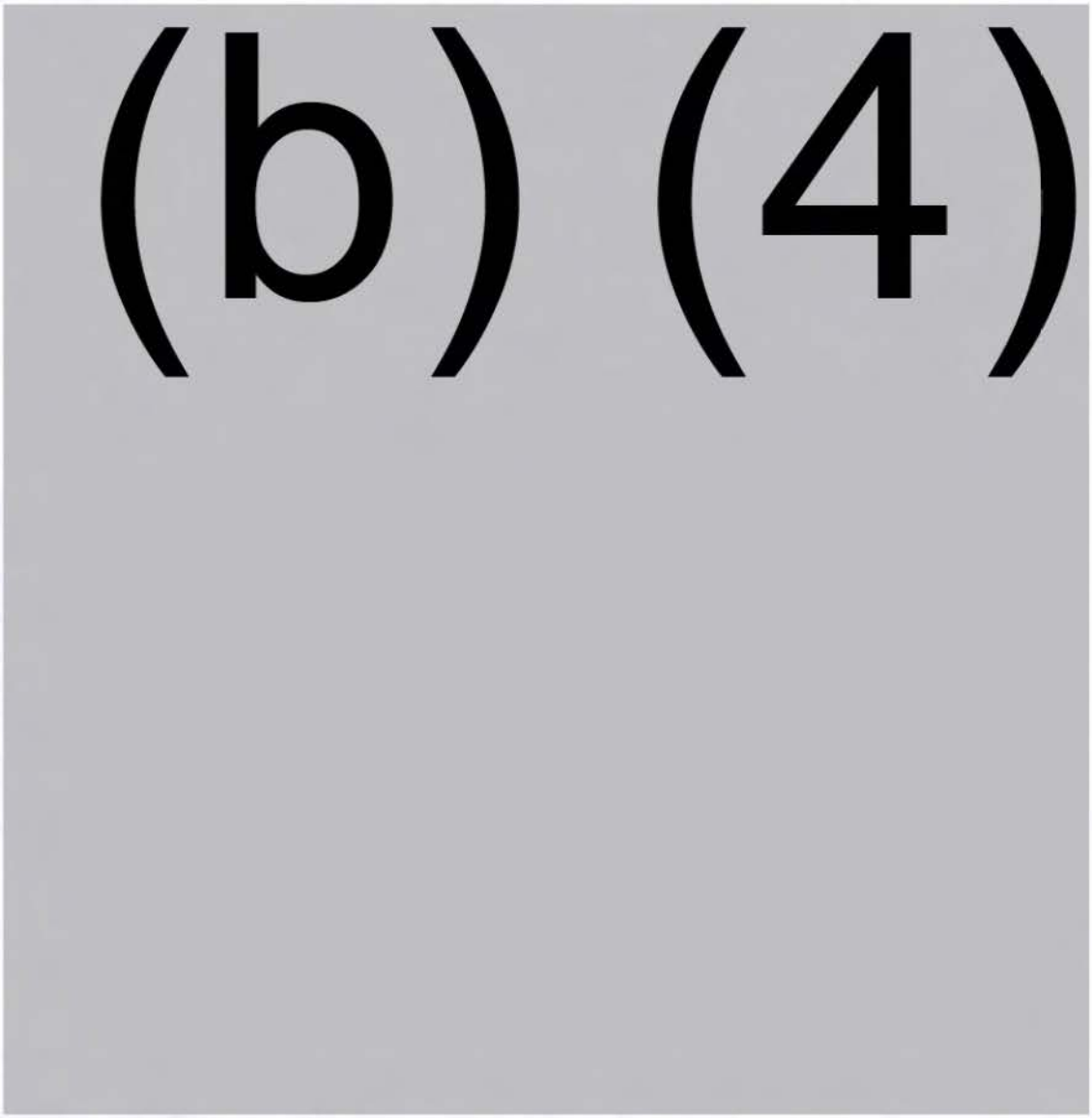


Figure 2 Overview of SalmoFresh™ manufacturing process

7 LIST OF SUPPORTING DATA AND INFORMATION

7.1 APPENDICES (INCLUDES NOT GENERALLY AVAILABLE DATA)

This section includes not generally available data, with the exception of Appendices 7.1.20.4 and 7.1.20.5.

7.1.1 SPECIFICATION SHEET: SBA-1781

intralytix

PRODUCT SPECIFICATION SHEET

(b) (4), (b) (6)

(b) (4)

7.1.2 SPECIFICATION SHEET: SKML-39

PRODUCT SPECIFICATION SHEET



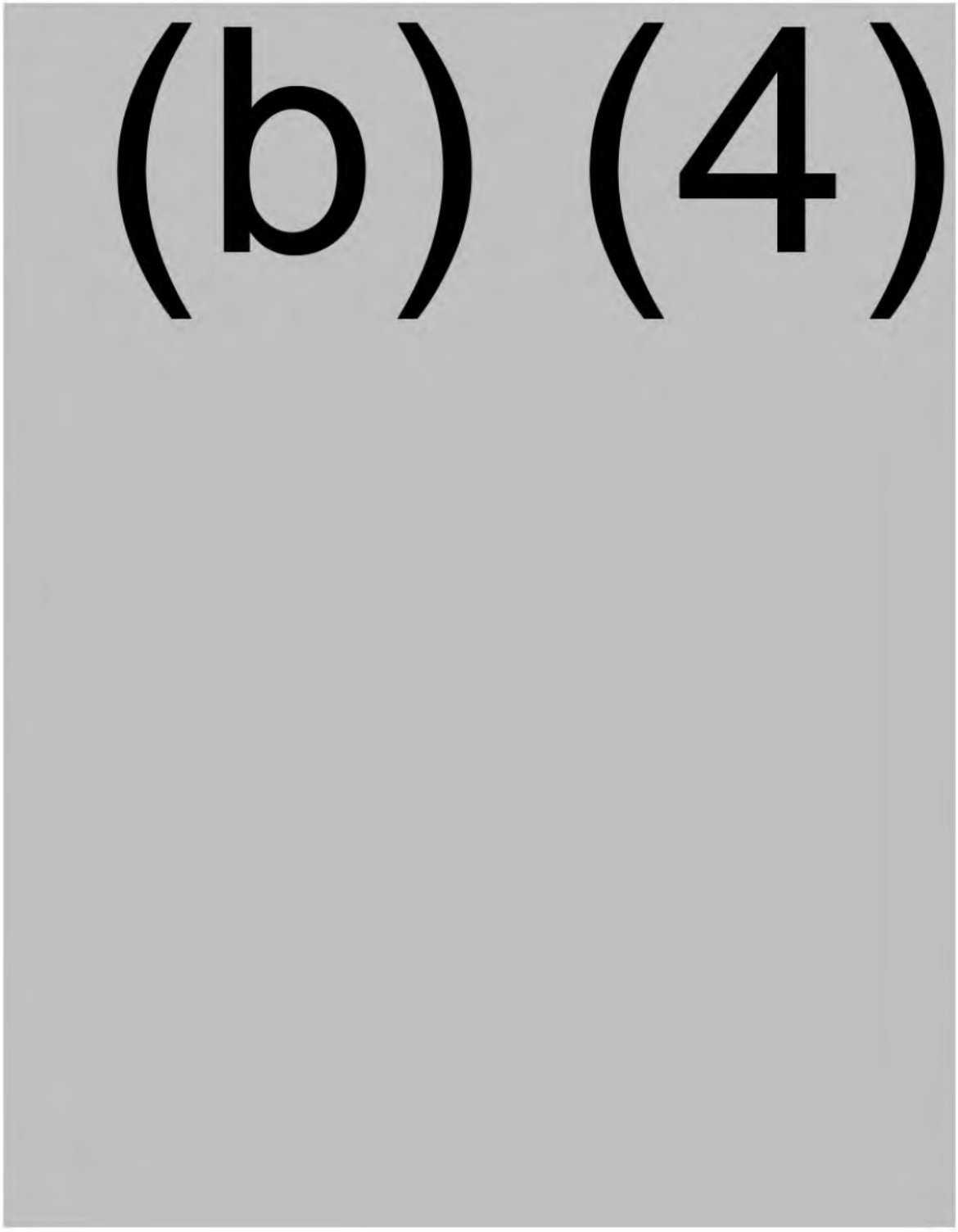
7.1.3 SPECIFICATION SHEET: SPT-1

intralytix

PRODUCT SPECIFICATION SHEET

(b) (4), (b) (6)

7.1.4 SPECIFICATION SHEET: SSE-121



7.1.5 SPECIFICATION SHEET: STML-13-1

intralytix

PRODUCT SPECIFICATION SHEET

(b) (4), (b) (6)

(b) (4)

7.1.6 SPECIFICATION SHEET: STML-198

intralytix

PRODUCT SPECIFICATION SHEET

(b) (4), (b) (6)

(b) (4)

7.1.7 (b) (4)

intralytix
SAFETY BY NATURE

STANDARD OPERATING PROCEDURE

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AUTHORED BY:
APPROVED BY:

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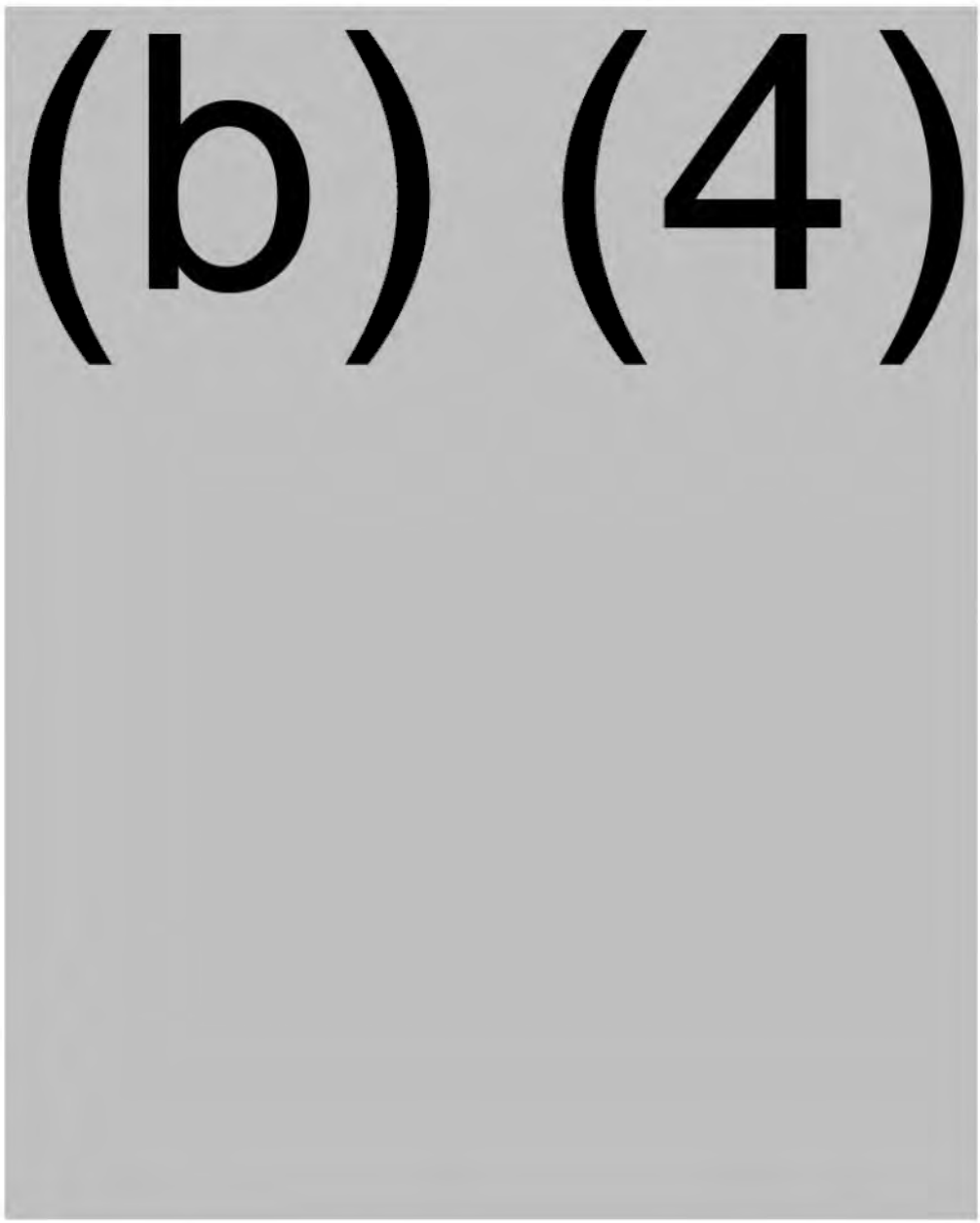
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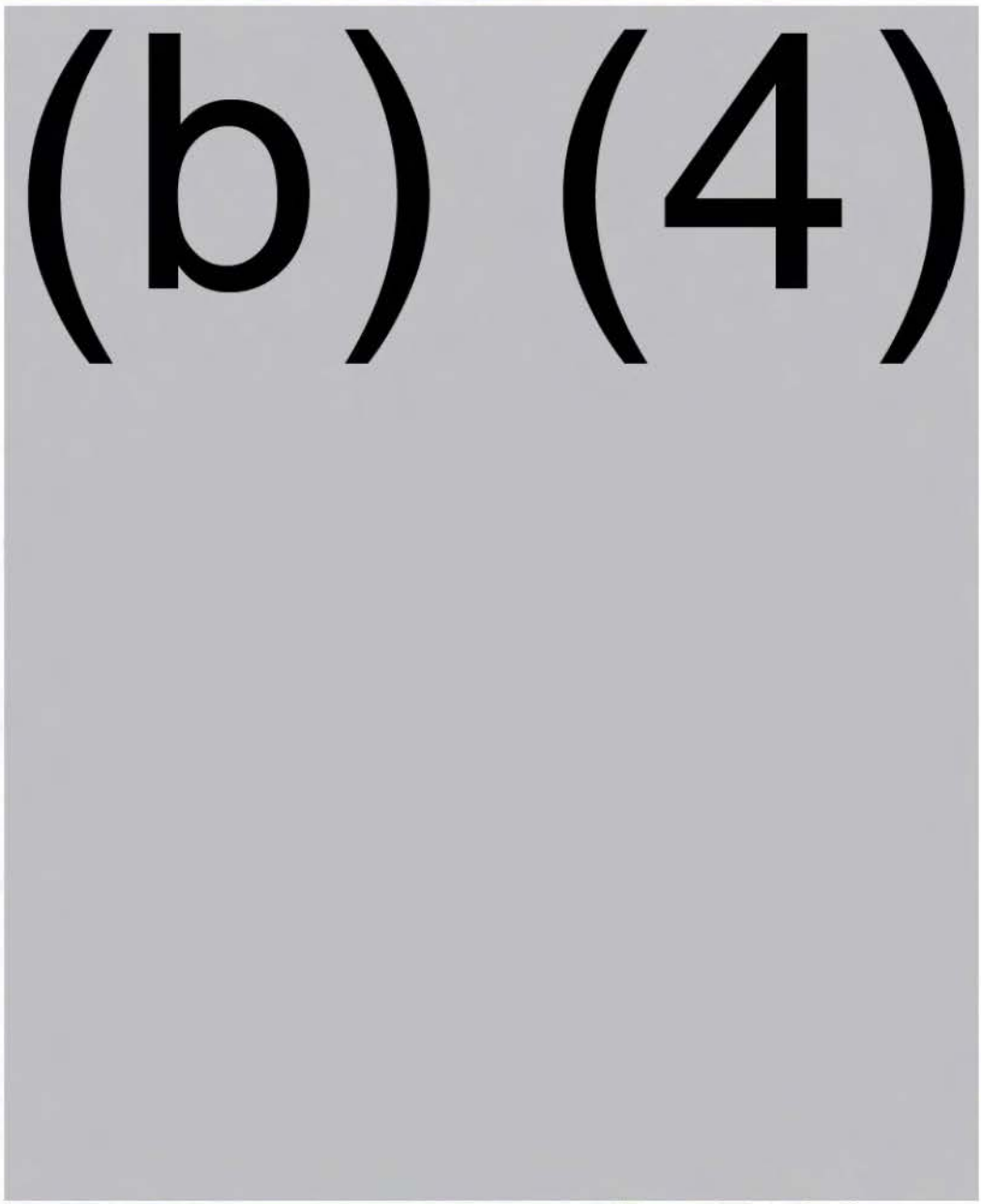
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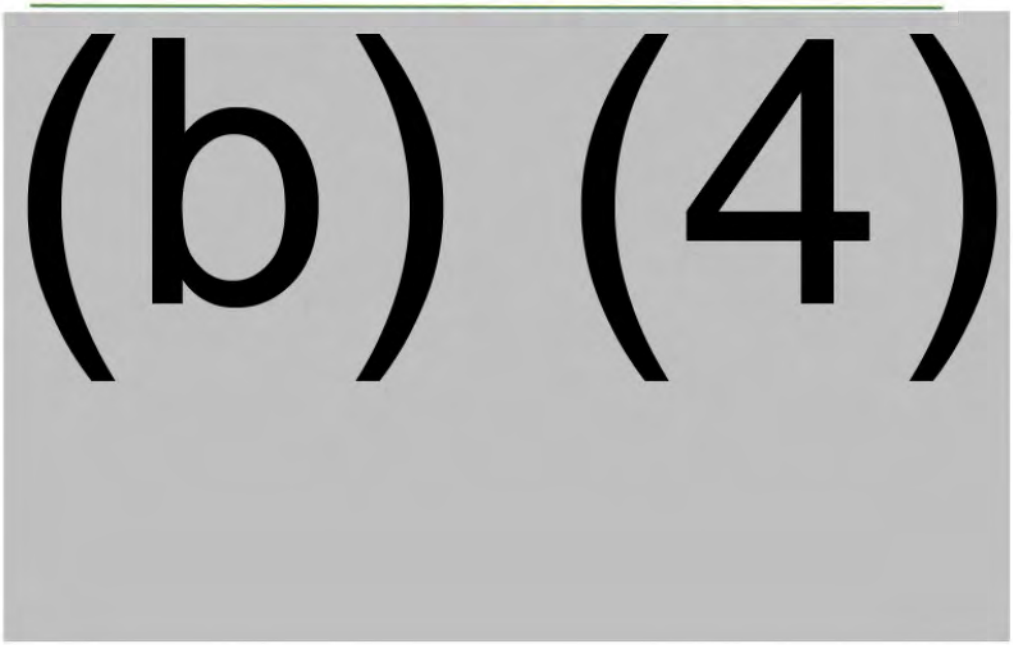
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8.0 REVISION HISTORY

(b) (4)

7.1.8 (b) (4)

intralytix
SAFETY BY NATURE

LABORATORY METHODS

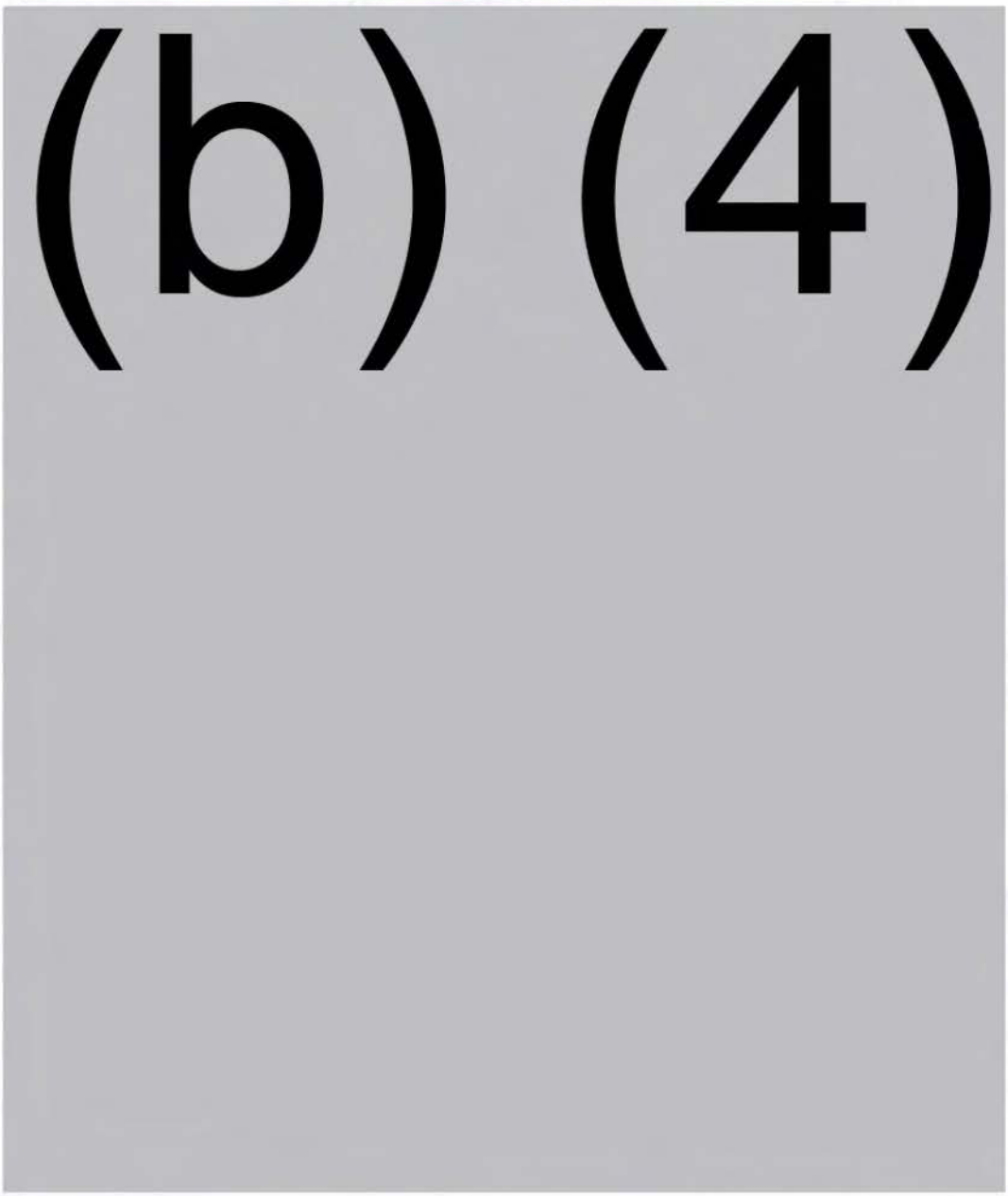
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TITLE:		REVISION NUMBER:	1
AUTHORED BY		DATE:	May 26, 2023
APPROVED BY:		DATE:	May 26, 2023

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intralytix

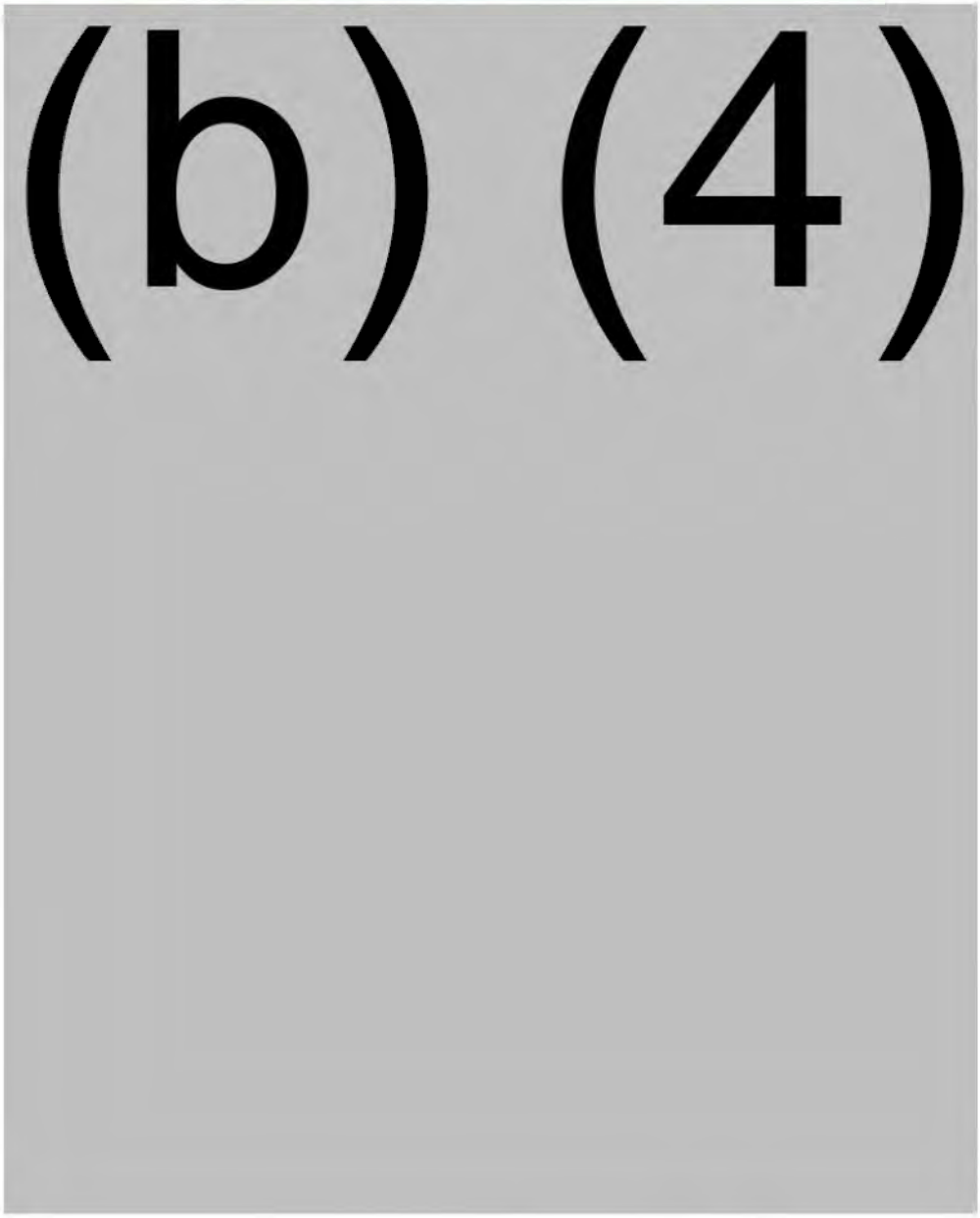
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intralytix

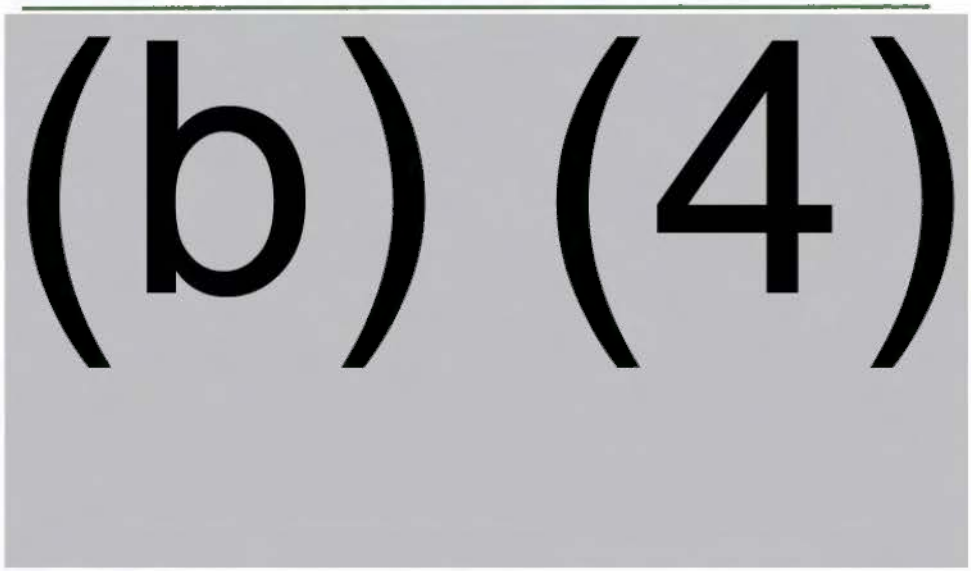
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8.0 REVISION HISTORY

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7.1.9 (b) (4)

intralytix
SAFETY BY NATURE

LABORATORY METHODS

PROCEDURE #: (b) (4), (b) (6)

TITLE:

AUTHORED BY:

APPROVED BY:

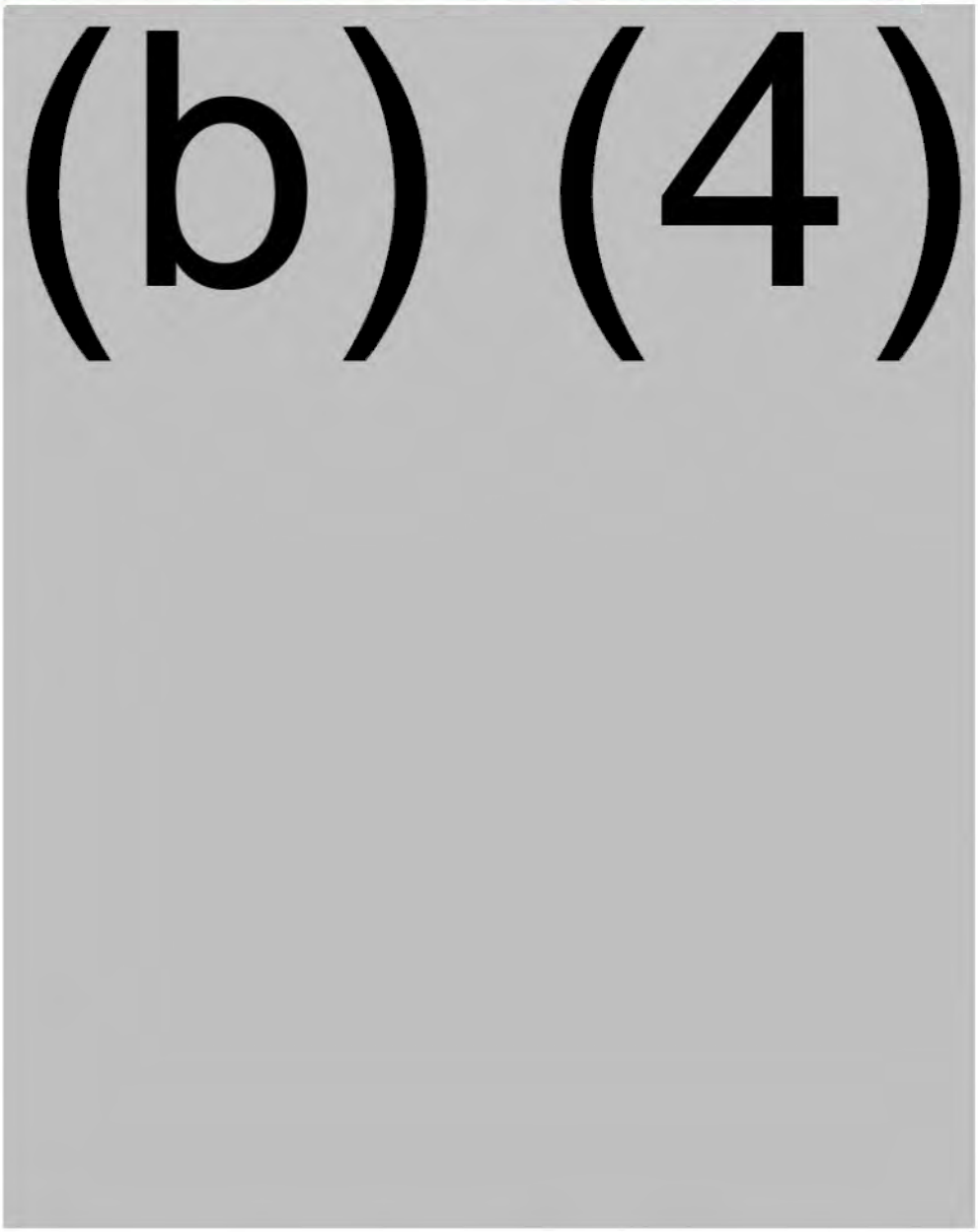
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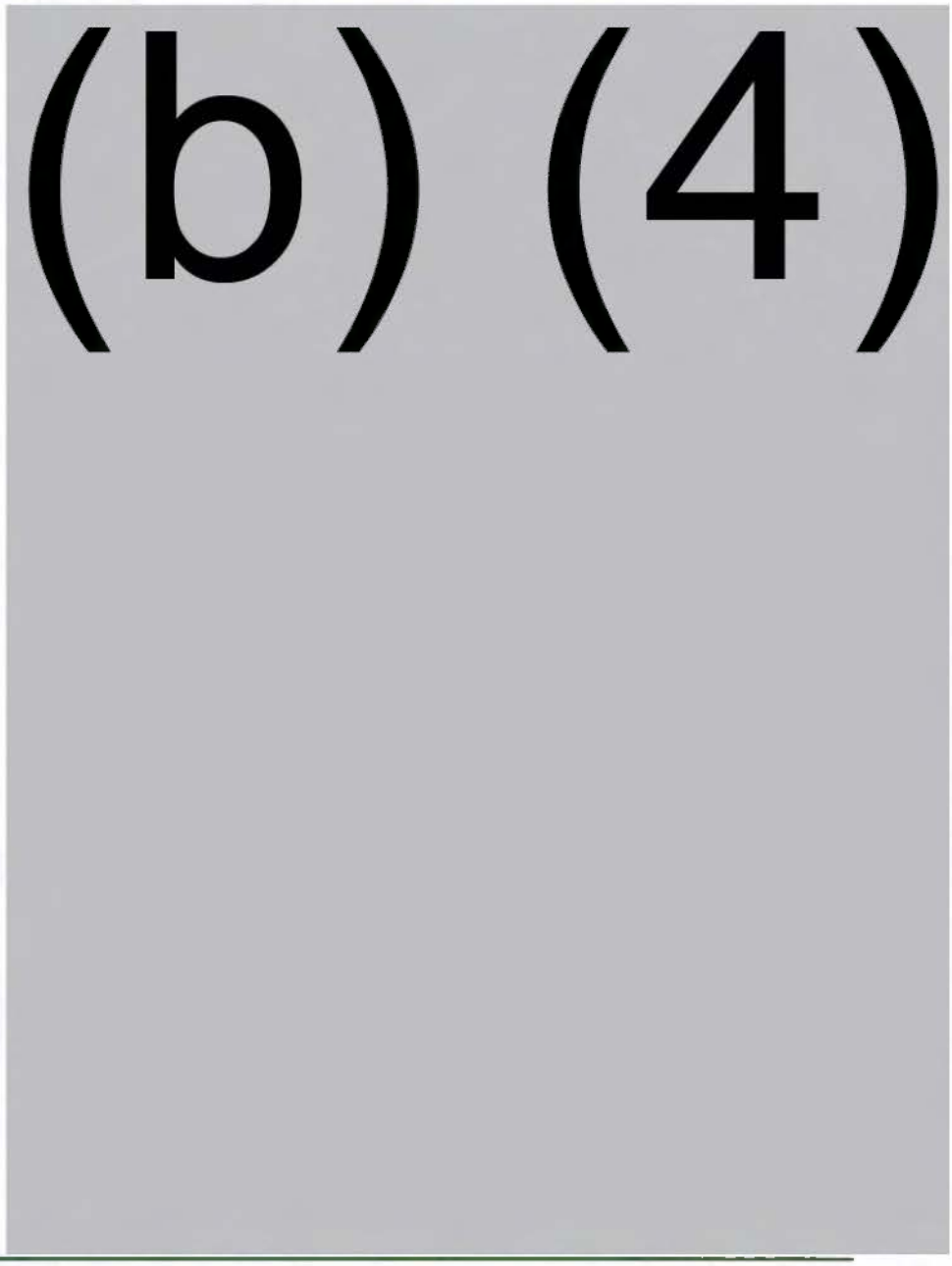
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(b) (4)

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CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SBA-1781 Monophage	October 08, 2024	SBA-1781

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	March 12, 2020	March 12, 2020	March 12, 2021

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	March 18, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	March 18, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	March 18, 2020	
Purity (Microbial)	No growth	March 18, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SBA-1781 Monophage	October 08, 2024	SBA-1781

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	August 18, 2022	August 22, 2022	August 18, 2023

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	August 24, 2022	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^7 PFU/mL	August 23, 2022	
Purity (Microbial)	No growth	August 24, 2022	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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Columbia, MD 21046
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Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SBA-1781 Monophage	October 08, 2024	SBA-1781

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	March 20, 2024	March 25, 2024	March 20, 2025

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	March 27, 2024	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^9 PFU/mL	March 26, 2024	
Purity (Microbial)	No growth	March 27, 2024	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

7.1.10 COAS: SKML-39

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CERTIFICATE OF ANALYSIS
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Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SKML-39 Monophage	October 08, 2024	SKML-39

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	January 23, 2019	January 23, 2019	January 23, 2020

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	January 29, 2019	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^8 PFU/mL	January 29, 2020	
Purity (Microbial)	No growth	January 29, 2019	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SKML-39 Monophage	October 08, 2024	SKML-39

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	February 27, 2020	February 27, 2020	February 27, 2021

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	March 05, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^9 PFU/mL	March 03, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	March 04, 2020	
Purity (Microbial)	No growth	March 04, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SKML-39 Monophage	October 08, 2024	SKML-39

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	December 13, 2023	December 18, 2023	December 13, 2024

Test	Specification	Test Date	Result:
Identity (RFLP)	Matches reference pattern	December 20, 2023	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^9 PFU/mL	December 19, 2023	
Purity (Microbial)	No growth	December 20, 2023	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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7.1.11 COAS: SPT-1

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Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SPT-1 Monophage	August 23, 2021	SPT-1

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	December 08, 2020	December 11, 2020	December 08, 2021

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	December 11, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	December 15, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	December 11, 2020	
Purity (Microbial)	No growth	December 11, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

1 / 1

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Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SPT-1 Monophage	October 08, 2024	SPT-1

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	March 23, 2022	March 23, 2022	March 23, 2023

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	March 29, 2022	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^8 PFU/mL	March 29, 2022	
Purity (Microbial)	No growth	March 30, 2022	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SPT-1 Monophage	October 08, 2024	SPT-1

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	January 24, 2024	January 24, 2024	January 24, 2025

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	January 30, 2024	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	January 31, 2024	
Purity (Microbial)	No growth	January 31, 2024	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

1 / 1

7.1.12 COAS: SSE-121

intralytix SAFETY BY NATURE

CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SSE-121 Monophage	October 08, 2024	SSE-121

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	May 06, 2020	May 06, 2020	May 06, 2021

Test	Specification	Test Date	Result:
Identity (RFLP)	Matches reference pattern	May 14, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	May 13, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	May 19, 2020	
Purity (Microbial)	No growth	May 14, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported result: accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

1 / 1

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Columbia, MD 21046
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Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SSE-121 Monophage	October 08, 2024	SSE-121

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	February 17, 2021	February 22, 2021	February 17, 2022

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	February 23, 2021	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	February 23, 2021	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	February 24, 2021	
Purity (Microbial)	No growth	February 24, 2021	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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Columbia, MD 21046
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Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
SSE-121 Monophage	October 06, 2024	SSE-121

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	June 29, 2022	July 05, 2022	June 29, 2023

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	July 08, 2022	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^9 PFU/mL	July 06, 2022	
Purity (Microbial)	No growth	July 07, 2022	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

7.1.13 COAS: STML-13-1

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Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
STML-13-1 Monophage	November 26, 2024	STML-13-1

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	August 25, 2021	August 25, 2021	August 25, 2022

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	September 01, 2021	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^9 PFU/mL	September 01, 2021	
Purity (Microbial)	No growth	September 01, 2021	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

1 / 1

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E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
STML-13-1 Monophage	October 08, 2024	STML-13-1

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	August 30, 2023	September 05, 2023	August 30, 2024

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	September 08, 2023	(b) (4)
Potency (Lytic Titer)	NLT 6.3 x 10^9 PFU/mL	September 06, 2023	
Purity (Microbial)	No growth	September 07, 2023	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

CERTIFICATE OF ANALYSIS
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Intralytix, Inc.
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Columbia, MD 21046
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Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
STML-13-1 Monophage	October 08, 2024	STML-13-1

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	January 11, 2024	January 15, 2024	January 11, 2025

Test	Specification	Test Date	Result
Identity (RFLP)	Matches reference pattern	January 18, 2024	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	January 17, 2024	
Purity (Microbial)	No growth	January 19, 2024	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

7.1.14 COAS: STML-198

intralytix SAFETY BY NATURE

CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
STML-198 Monophage	October 08, 2024	STML-198

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	April 29, 2020	April 29, 2020	April 29, 2021

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	May 21, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^{9} PFU/mL	May 01, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL @ 9.0 ± 0.33 log10 PFU/mL	May 19, 2020	
Purity (Microbial)	No growth	May 14, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
STML-198 Monophage	October 08, 2024	STML-198

Lot Number	Manufacture Date	Pull Date	Expiration Date
(b) (4)	November 18, 2020	November 24, 2020	November 18, 2021

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	November 25, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	November 24, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	November 25, 2020	
Purity (Microbial)	No growth	November 25, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

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CERTIFICATE OF ANALYSIS
STABILITY TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Product Number
STML-198 Monophage	October 08, 2024	STML-198

Lot Number	Manufacture Date	Full Date	Expiration Date
(b) (4)	December 16, 2020	December 21, 2020	June 21, 2021

Test	Specification	Test Date	Results
Identity (RFLP)	Matches reference pattern	December 22, 2020	(b) (4)
Potency (Lytic Titer)	NLT 6.3×10^9 PFU/mL	December 22, 2020	
Purity (Endotoxin)	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	January 05, 2021	
Purity (Microbial)	No growth	December 23, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project.

(b) (6)

Responsible Quality Official

(b) (4)

1 / 1

7.1.15 SPECIFICATION SHEET: SALMOFRESH™

intralytix

PRODUCT SPECIFICATION SHEET

Description: SalmoFresh™
Spec Level: Rev 1
Supersedes: N/A

Item Number: 02SP
Effective Date: 11/06/2024

1. **Physical Description**: Colorless, clear to slightly opalescent liquid
2. **3rd Party Certifications:** Kosher Certified, Halal Certified, OMRI Listed
3. **Ingredient Listing:**
   a. Saline, A mixture of approximately equal proportions of six lytic bacteriophages with lytic activity against Salmonella spp.
   b. Lytic bacteriophages – SPT-1, SSE-121, STML-198, STML-13-1, SBA-1781, and SKML-39
4. **Analytical Requirements**

	Method	Acceptance Criteria
Identity Tests		
Appearance	Visual	Colorless, clear to slightly opalescent liquid
Identity by Input	(b) (4)	All component monophages are present
Potency Test – 30X		
Potency by Titer		10.48 ± 0.33 log10 PFU/mL
Potency Test – 100X		
Potency by Titer		11.00 ± 0.33 log10 PFU/mL
Potency Test – 500X		
Potency by Titer		11.70 ± 0.33 log10 PFU/mL
Contamination Limits		
Sterility		No Growth
Endotoxin		NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL

5. **Finished Product Packaging Requirements:** The finished product is filled into polyethylene terephthalate copolyester (PETG) square media bottles capped with a natural HDPE screw cap closure. A tamper-evident shrink sleeve neckband is added to the cap to prevent tampering with the finished goods.

ITXPSS-5 Rev 1 1 of 3

Approved by: (b) (6) Date: 11/06/2024

intralytix

PRODUCT SPECIFICATION SHEET

Description: SalmoFresh™
Spec Level: Rev 1
Supersedes: N/A

Item Number: 02SP
Effective Date: 11/06/2024

6. **Label Information:**

a. **Sample Label**

(b) (4)

b. **Label Masters**

i. (b) (4)

7. **Product Size and Order Information:**

Intralytix Part #	Volume (mL)	Container Size
30X		

(b) (4)

Approved by: (b) (6) Date: 11/06/2024

intralytix

PRODUCT SPECIFICATION SHEET

Description: SalmoFresh™ Item Number: 02SP
Spec Level: Rev 1 Effective Date: 11/06/2024
Supersedes: N/A

02SP-2000-500	2000	2000 mL

8. **Shelf Life and Storage Conditions:**
   a. **Storage Conditions:** Store at 2°-8°C (36°-46°F) protected from light. Do not freeze.
   b. **Shelf Life:** Shelf life is 12 months from date of manufacture in original sealed containers when stored at the recommended storge conditions. Recommended to be used within 2 weeks of opening.
9. **Shipping Requirements:**

Container type:	Shipper is taped properly and securely.
How to ship:	Ship in an insulated shipping container with ice packs via overnight shipment with a commercial shipping company.
Expiring dating:	Expiration date is listed on all product labels
Cert. of Analysis:	One (1) COA is sent with the shipment

10. **Special Instructions:** None
11. **Known Allergens:** None
12. **Revision History**

Date: 11/6/2024 Revised By: (b) (6) Reason: See CR134
Date: _____ Revised By: _____ Reason: _____
Date: _____ Revised By: _____ Reason: _____
Date: _____ Revised By: _____ Reason: _____
Date: _____ Revised By: _____ Reason: _____
Date: _____ Revised By: _____ Reason: _____
Date: _____ Revised By: _____ Reason: _____

Approved by: (b) (6) Date: 11/06/2024

7.1.16 (b) (4)

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LABORATORY METHODS

PROCEDURE # (b) (4)

EFFECTIVE DATE: 04/05/2021

(b) (6)

REVISION NUMBER: ORIGINAL

DATE: Mar 31, 2021

APPROVED BY:

DATE: Mar 31, 2021

(b) (4)

(b) (4)

(b) (4)

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intralytix

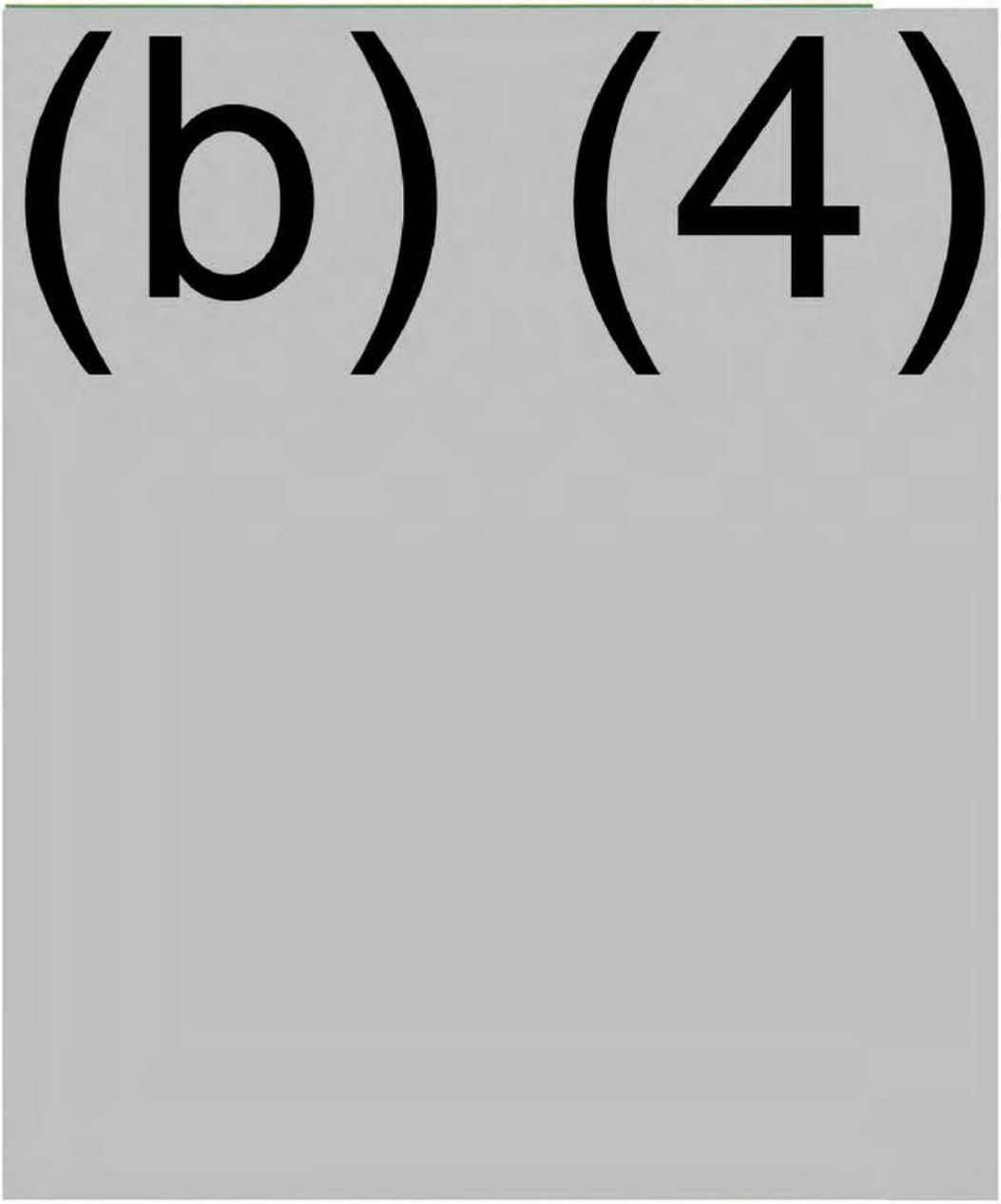
(b) (4)

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(b) (4)

intralytix

(b) (4)



(b) (4)

intralytix

(b) (4)

(b) (4)

(b) (4)

8.0 REVISION HISTORY

DATE	Rev	Author	DESCRIPTION OF CHANGE
(b) (4)			

7.1.17 (b) (4)

intralytix
SAFETY BY NATURE

LABORATORY METHODS

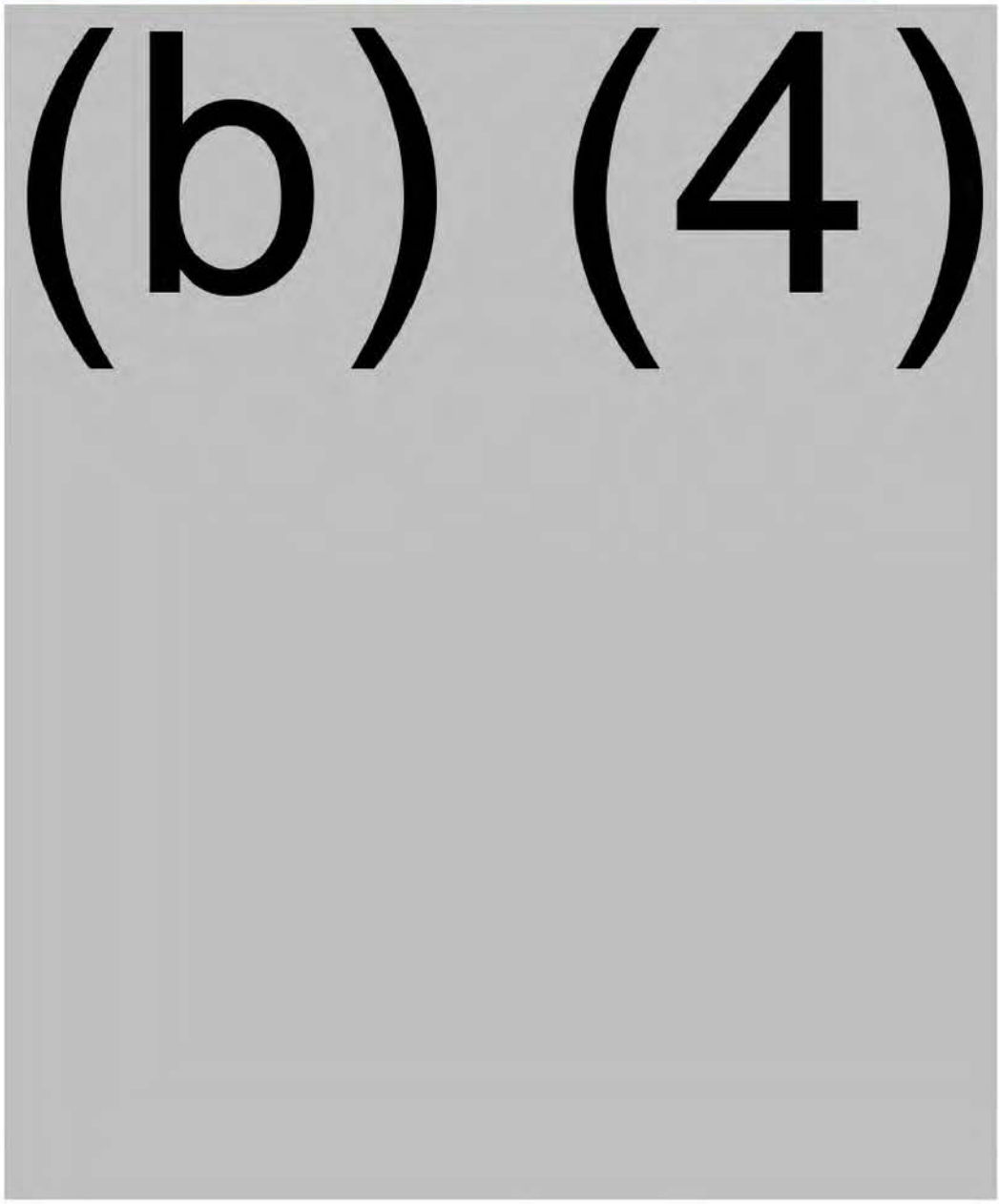
PROCEDURE # (b) (4)
EFFECTIVE DATE: 07/21/2021

TITLE: (b) (4)
REVISION NUMBER: ORIGINAL

AUTHORED BY: (b) (6)
DATE: Jul 14, 2021

APPROVED BY:
DATE: Jul 14, 2021

(b) (4)


(b) (4)

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8.0 REVISION HISTORY

(b) (4)

7.1.18 (b) (4)

(b) (4)

intralytix

Identity by Input

LABORATORY METHODS

PROCEDURE # (b) (4) EFFECTIVE DATE: 09/10/2024

TITLE: (b) (4) REVISION NUMBER: ORIGINAL

AUTHORED BY (b) (6) DATE: 09/05/24

APPROVED BY DATE: 09/05/24

(b) (4)

(b) (4)

intralytix
(b) (4)

(b) (4)

8.0 REVISION HISTORY

(b) (4)

7.1.19 COAS: SALMOFRESH™

CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Catalog Number	Safety Data Sheet
SalmoFresh™	April 30, 2021	02SP	(b) (4)
Lot Number	**Manufacture Date**	**Pull Date**	**Expiration Date**
(b) (4)	September 22, 2020	September 22, 2020	September 22, 2021

Test	Specification	Test Date	Results
Endotoxin Content	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	September 25, 2020	(b) (4)
Identity Test	All component phages are present	October 09, 2020	
Microbial purity	No growth	October 23, 2020	
Potency	11 ± 0.33 log10 PFU/mL	September 23, 2020	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project. This product is Star-K Kosher-certified, IFANCA Halal-certified, OMRI listed, and was produced in a SQF-certified facility.

(b) (6)

April 30, 2021

Responsible Quality Official

(b) (4)

CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Catalog Number	Safety Data Sheet
SalmoFresh™	October 08, 2024	02SP	(b) (4)
Lot Number	**Manufacture Date**	**Pull Date**	**Expiration Date**
(b) (4)	August 30, 2022	August 30, 2022	August 30, 2023

Test	Specification	Test Date	Results
Endotoxin Content	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	September 09, 2022	(b) (4)
Identity Test	All component phages are present	September 02, 2022	
Microbial purity	No growth	September 16, 2022	
Potency	11 ± 0.33 log10 PFU/mL	September 01, 2022	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project. This product is Star-K Kosher-certified, IFANCA Halal-certified, OMRI listed, and was produced in a SQF-certified facility.

(b) (6)

October 08, 2024

Responsible Quality Official

(b) (4)

CERTIFICATE OF ANALYSIS
RELEASE TESTING

Intralytix, Inc.
8681 Robert Fulton Drive
Columbia, MD 21046
USA

Phone: 1-877-ITXPHAGE
E-mail: info@intralytix.com

Product Name	Date of Certification	Catalog Number	Safety Data Sheet
SalmoFresh™	October 08, 2024	02SP	(b) (4)
Lot Number	**Manufacture Date**	**Pull Date**	**Expiration Date**
(b) (4)	May 30, 2023	May 31, 2023	May 30, 2024

Test	Specification	Test Date	Results
Endotoxin Content	NMT 25,000 EU/mL at 9.0 ± 0.33 log10 PFU/mL	June 14, 2023	(b) (4)
Identity Test	All component phages are present	June 14, 2023	
Microbial purity	No growth	June 16, 2023	
Potency	11 ± 0.33 log10 PFU/mL	June 01, 2023	

This product meets Intralytix, Inc. stringent quality control standards. The reported results accurately reflect the raw data of the project. This product is Star-K Kosher-certified, IFANCA Halal-certified, OMRI listed, and was produced in a SQF-certified facility.

(b) (6)

October 08, 2024

Responsible Quality Official

(b) (4)

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7.1.20 EFFICACY

Efficacy of SalmoFresh on pet food and animal feed.

7.1.20.1

7.1.20.2

7.1.20.3

(b) (4)

7.1.20.4 **Heyse S, Hanna LF, Woolston J, Sulakvelidze A, Charbonneau D. 2015. Bacteriophage cocktail for biocontrol of Salmonella in dried pet food. J Food Prot 78:97-103**

7.1.20.5 **Soffer N, Abuladze T, Woolston J, Li M, Hanna LF, Heyse S, Charbonneau D, Sulakvelidze A. 2016. Bacteriophages safely reduce Salmonella contamination in pet food and raw pet food ingredients. Bacteriophage 6:e1220347.**

7.1.21 SEQUENCING REPORTS

7.1.21.1

7.1.21.2

(b) (4)

7.2 LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BCS	Body condition scoring
BW	Body weight
CBER	Center for Biologics Evaluation and Research
CDC	Centers for Disease Control and Prevention
CDS	Coding DNA sequence
CEO	Chief Executive Officer
CFR	Code of Federal Regulations
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony forming units
DM	Dry matter
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EM	Electron microscopy
EPA	U.S. Environmental Protection Agency
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EU	Endotoxin unit
FCN	Food contact notification
FDA	U.S. Food and Drug Administration
FSIS	Food Safety and Inspection Service
GI	Gastrointestinal
GIT	Gastrointestinal tract
GRAS	Generally Recognized as Safe

HACCP	Hazard Analysis and Critical Control Point
HIV	Human immunodeficiency virus
ICTV	International Committee on the Taxonomy of Viruses
ID	Identification
ITX	Intralytix
IU	International endotoxin unit
LAL	Limulus Amebocyte Lysate
LPS	Lipopolysaccharide
MDR	Multidrug resistant
MOI	Multiplicity of infection
MPN	Most probable number
MRSA	Methicillin resistant Staphylococcus aureus
ND	None detected
NLT	Not less than
OMRI	Organic materials research institute
PAA	Peracetic acid
PFGE	Pulse-field electrophoresis
PFU	Plaque forming units
PHNC	Pet Health & Nutrition Center
QC	Quality control
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RTE	Ready-to-eat
SQF	Safe quality foods
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
UV	Ultra-violet

7.3 REFERENCES (FOR GENERALLY AVAILABLE DATA)

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Bacteriophage Cocktail for Biocontrol of *Salmonella* in Dried Pet Food

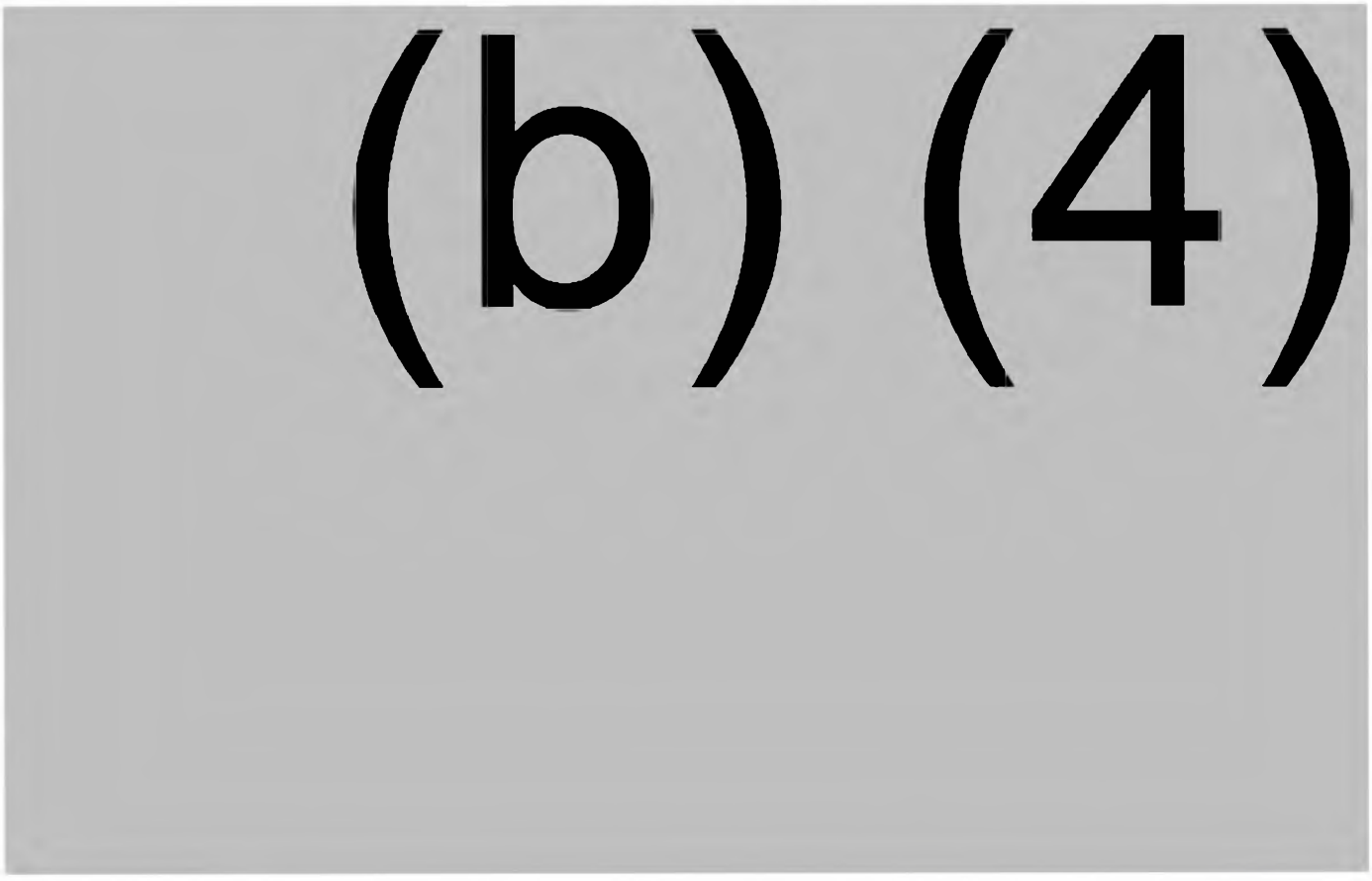
SERENA HEYSE,[1] LEIGH FARRIS HANNA,[1] JOELLE WOOLSTON,[2] ALEXANDER SULAKVELIDZE,[2] AND DUANE CHARBONNEAU[1]*

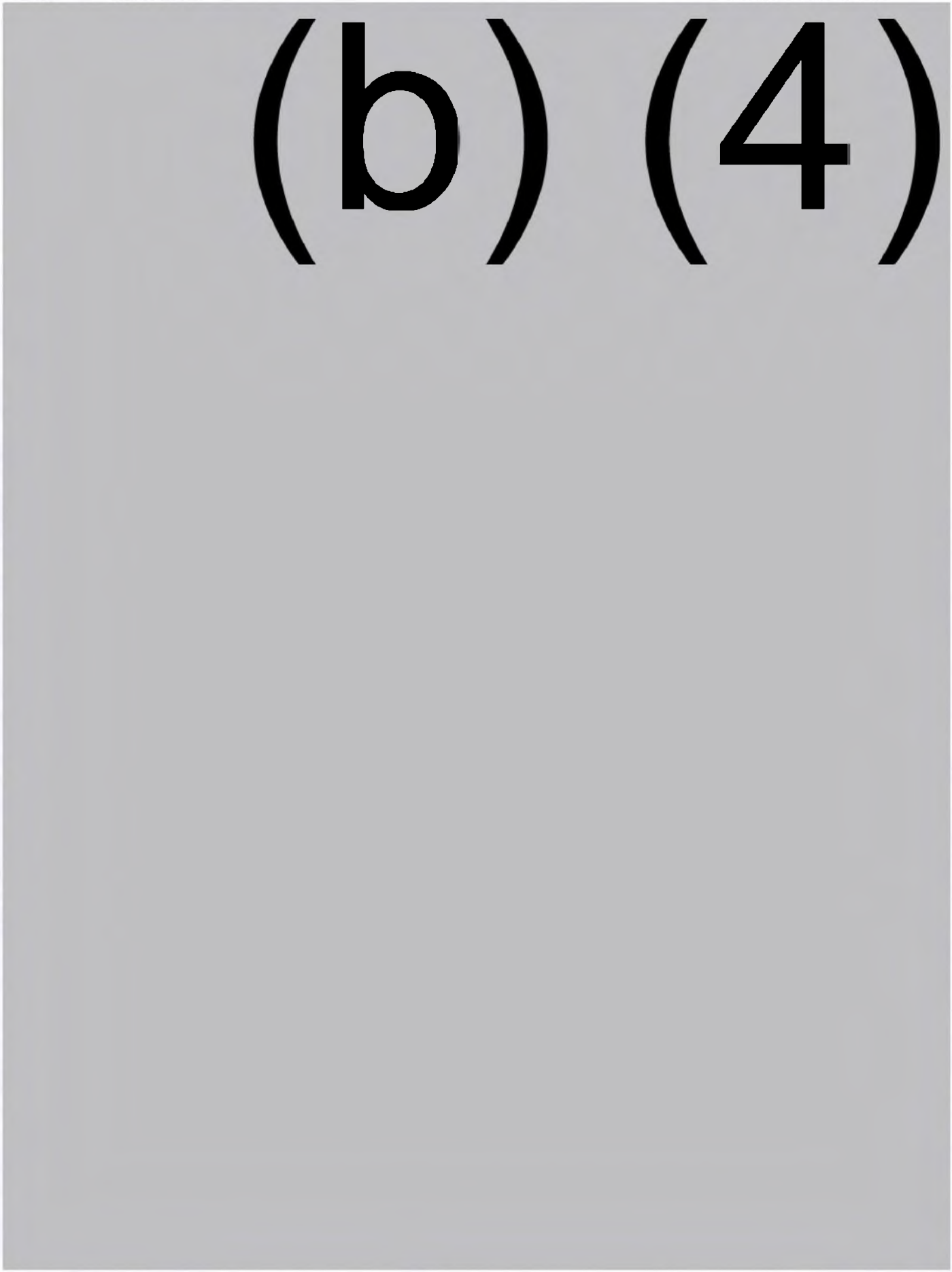
[1]*The Procter & Gamble Company, 8700 Mason-Montgomery Road, Mason, Ohio 45040; and* [2]*Intralytix, Inc., 701 East Pratt Street, Baltimore, Maryland 21202, USA*

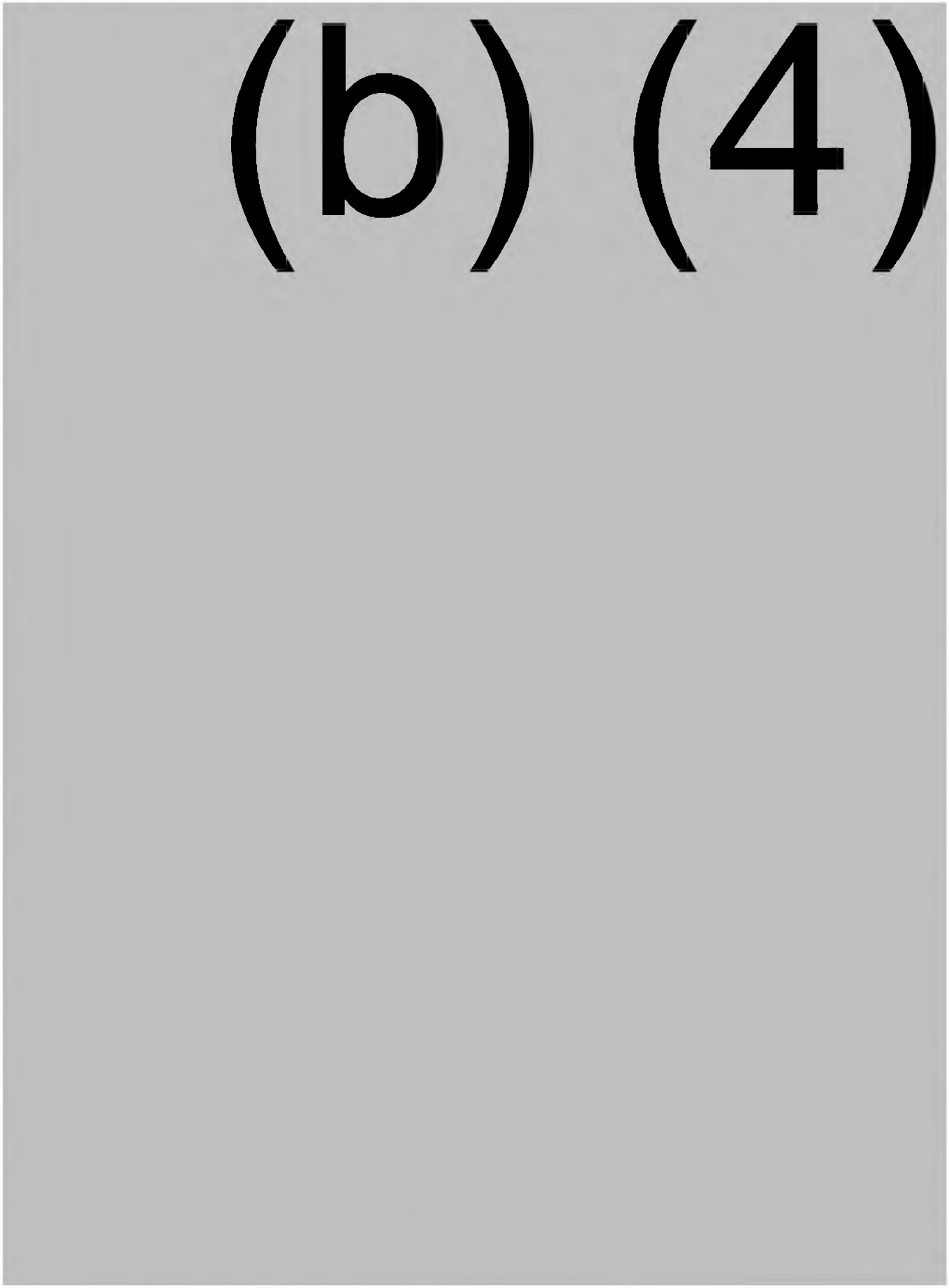
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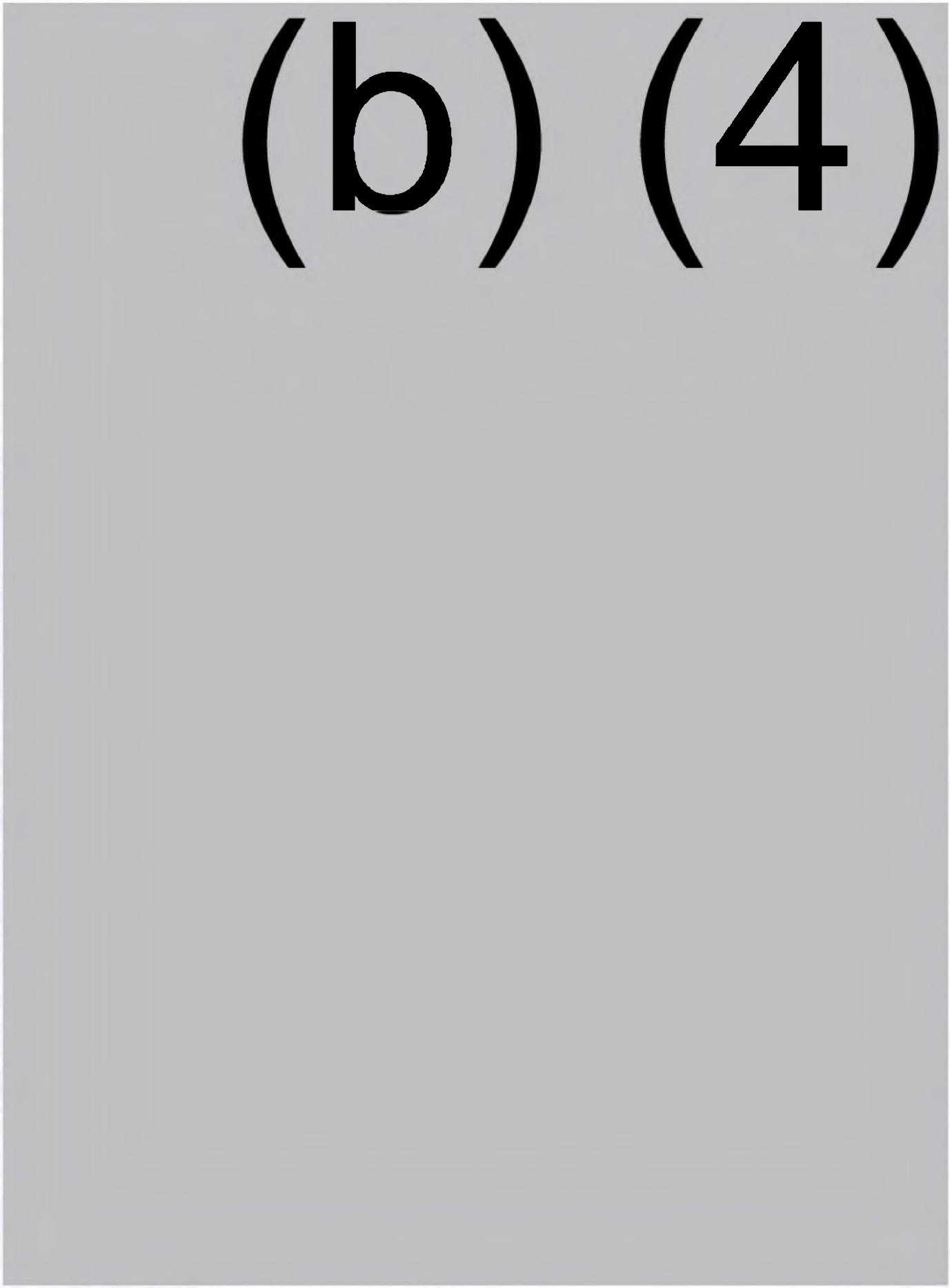
ABSTRACT

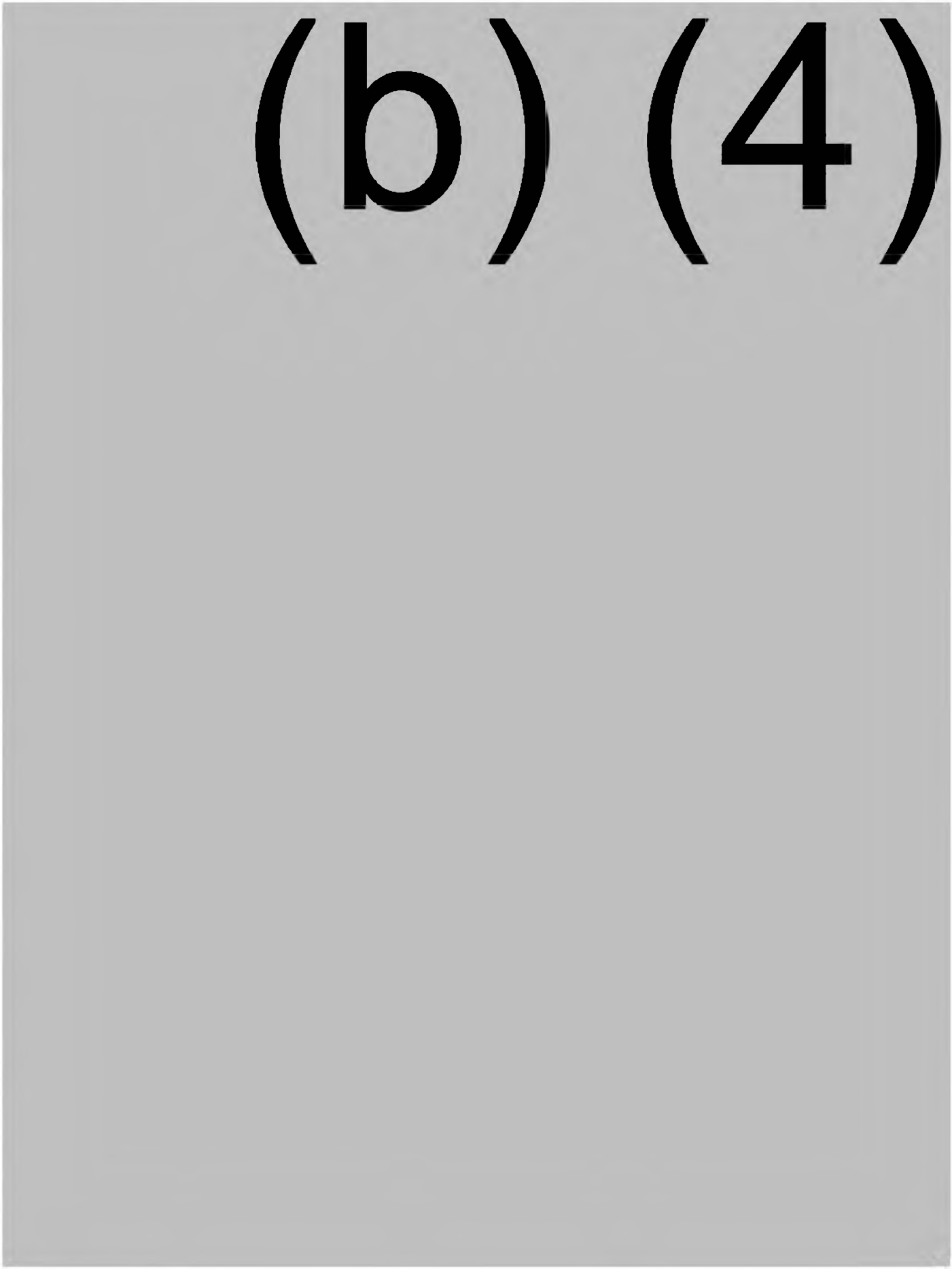
Human salmonellosis has been associated with contaminated pet foods and treats. Therefore, there is interest in identifying novel approaches for reducing the risk of *Salmonella* contamination within pet food manufacturing environments. The use of lytic bacteriophages shows promise as a safe and effective way to mitigate *Salmonella* contamination in various food products. Bacteriophages are safe, natural, highly targeted antibacterial agents that specifically kill bacteria and can be targeted to kill food pathogens without affecting other microbiota. In this study, we show that a cocktail containing six bacteriophages had a broad-spectrum activity in vitro against a library of 930 *Salmonella enterica* strains representing 44 known serovars. The cocktail was effective against 95% of the strains in this tested library. In liquid culture dose-ranging experiments, bacteriophage cocktail concentrations of $\geq 10^8$ PFU/ml inactivated more than 90% of the *Salmonella* population (10^1 to 10^3 CFU/ml). Dried pet food inoculated with a mixture containing equal proportions of *Salmonella* serovars Enteritidis (ATCC 4931), Montevideo (ATCC 8387), Senftenberg (ATCC 8400), and Typhimurium (ATCC 13311) and then surface treated with the six-bacteriophage cocktail ($\geq 2.5 \pm 1.5 \times 10^6$ PFU/g) achieved a greater than 1-log ($P < 0.001$) reduction compared with the phosphate-buffered saline–treated control in measured viable *Salmonella* within 60 min. Moreover, this bacteriophage cocktail reduced natural contamination in samples taken from an undistributed lot of commercial dried dog food that tested positive for *Salmonella*. Our results indicate that bacteriophage biocontrol of *S. enterica* in dried pet food is technically feasible.

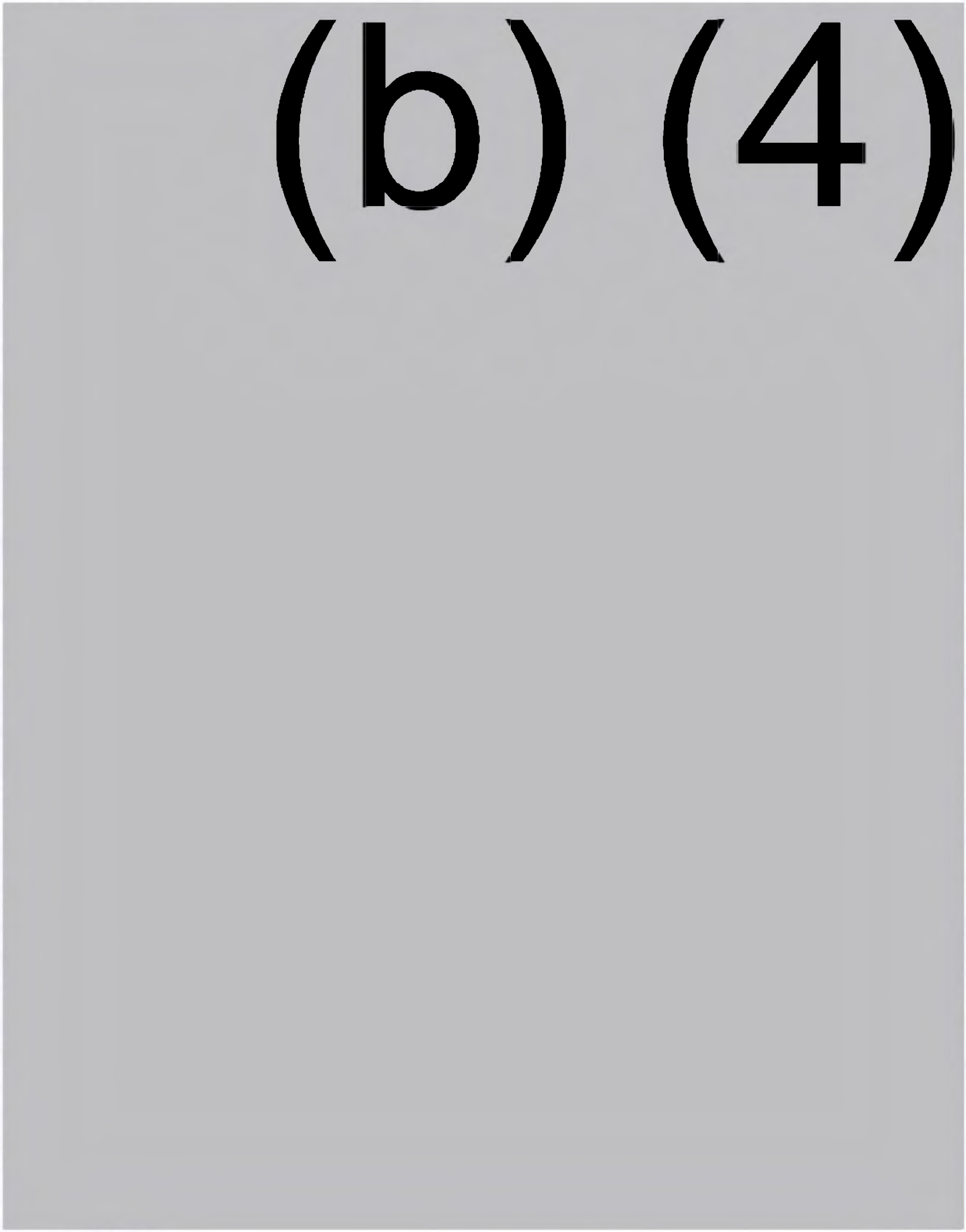


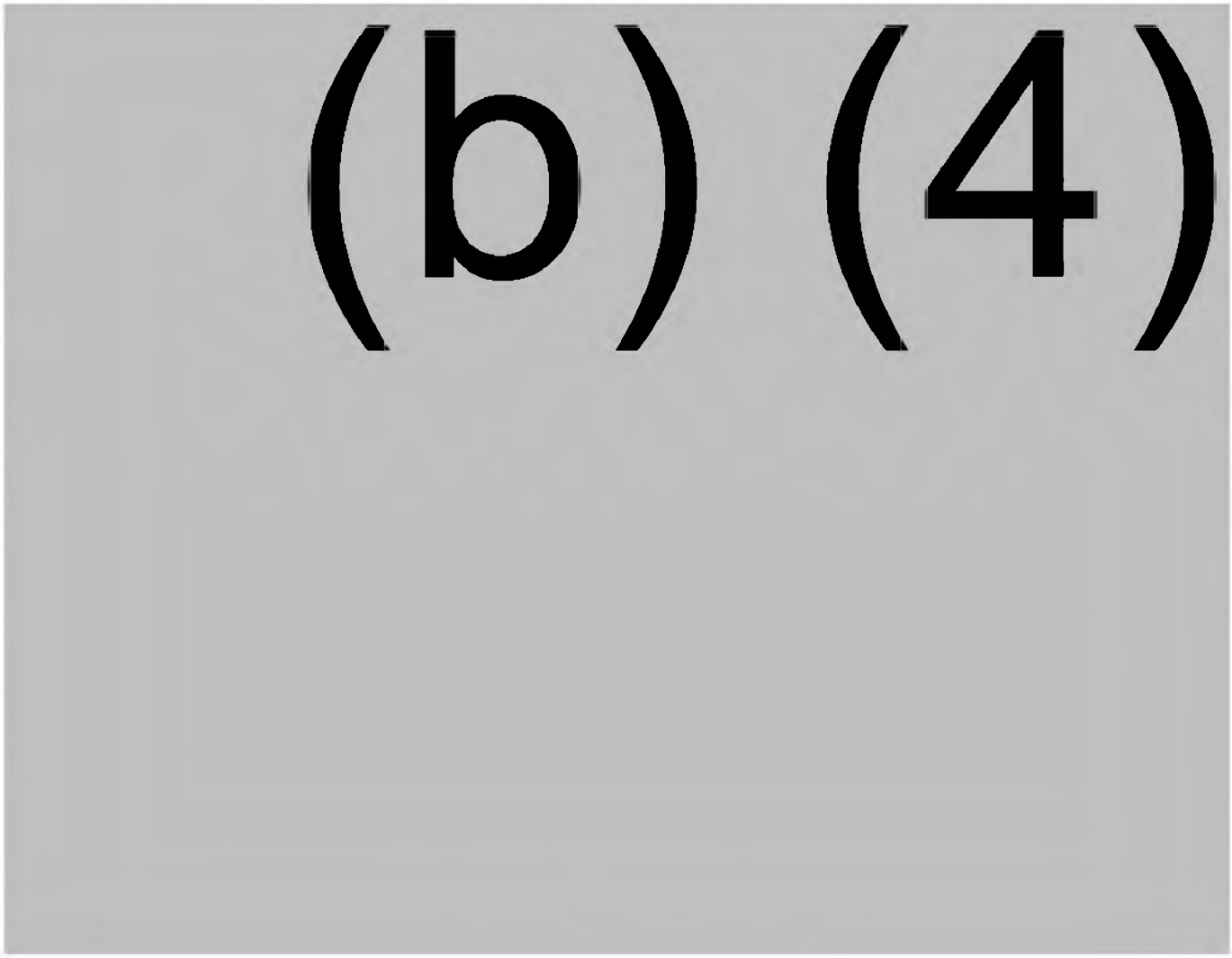

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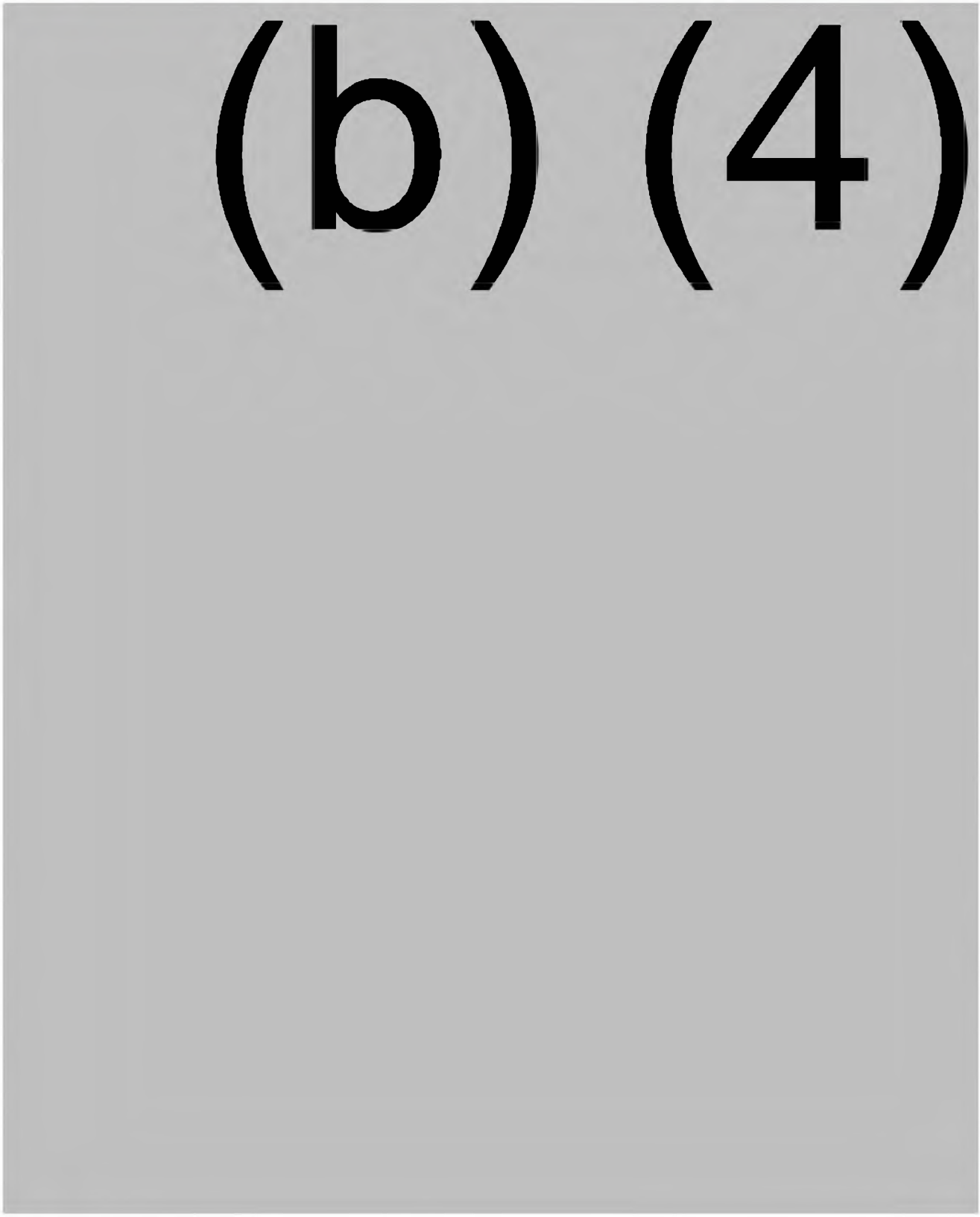

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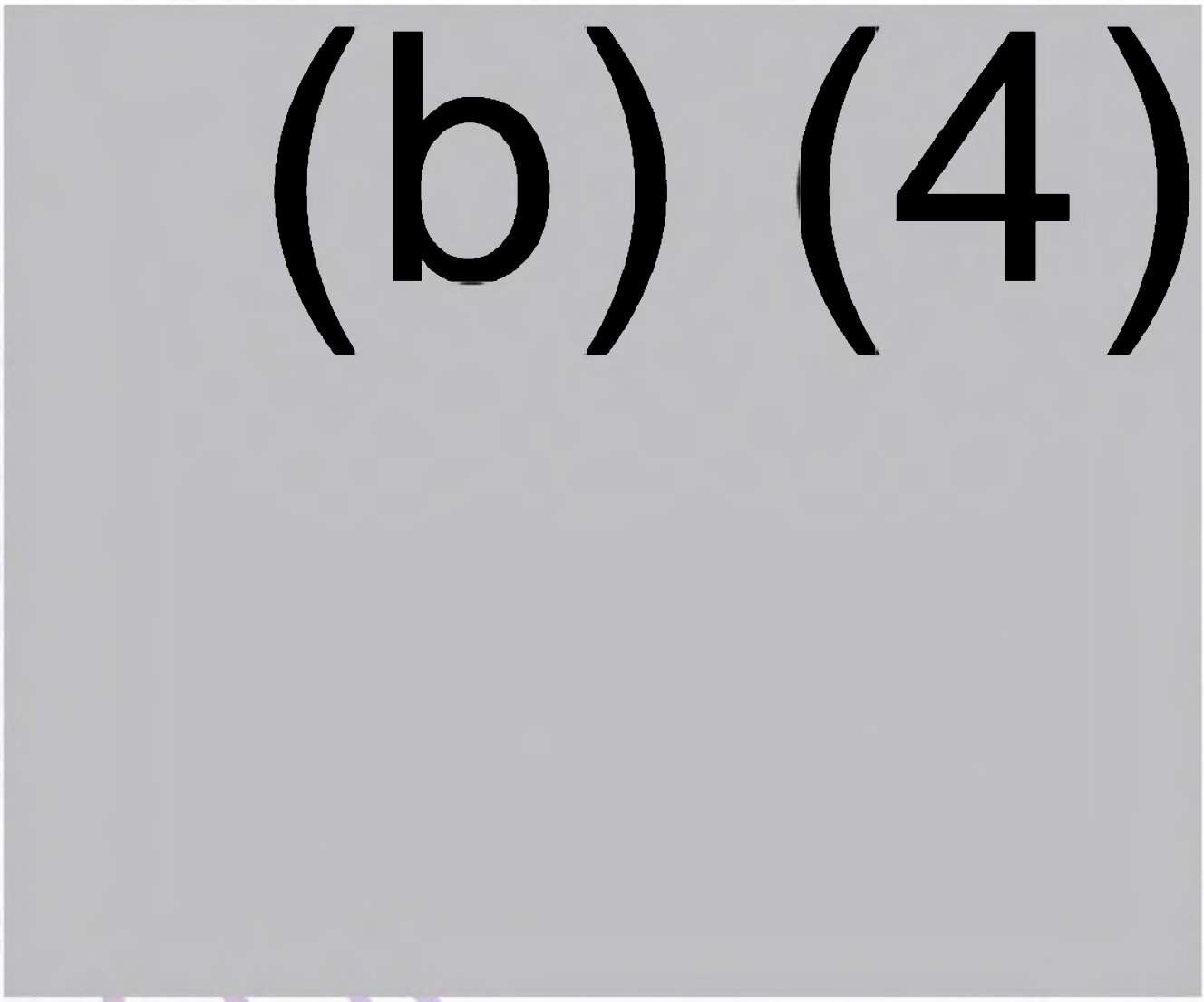

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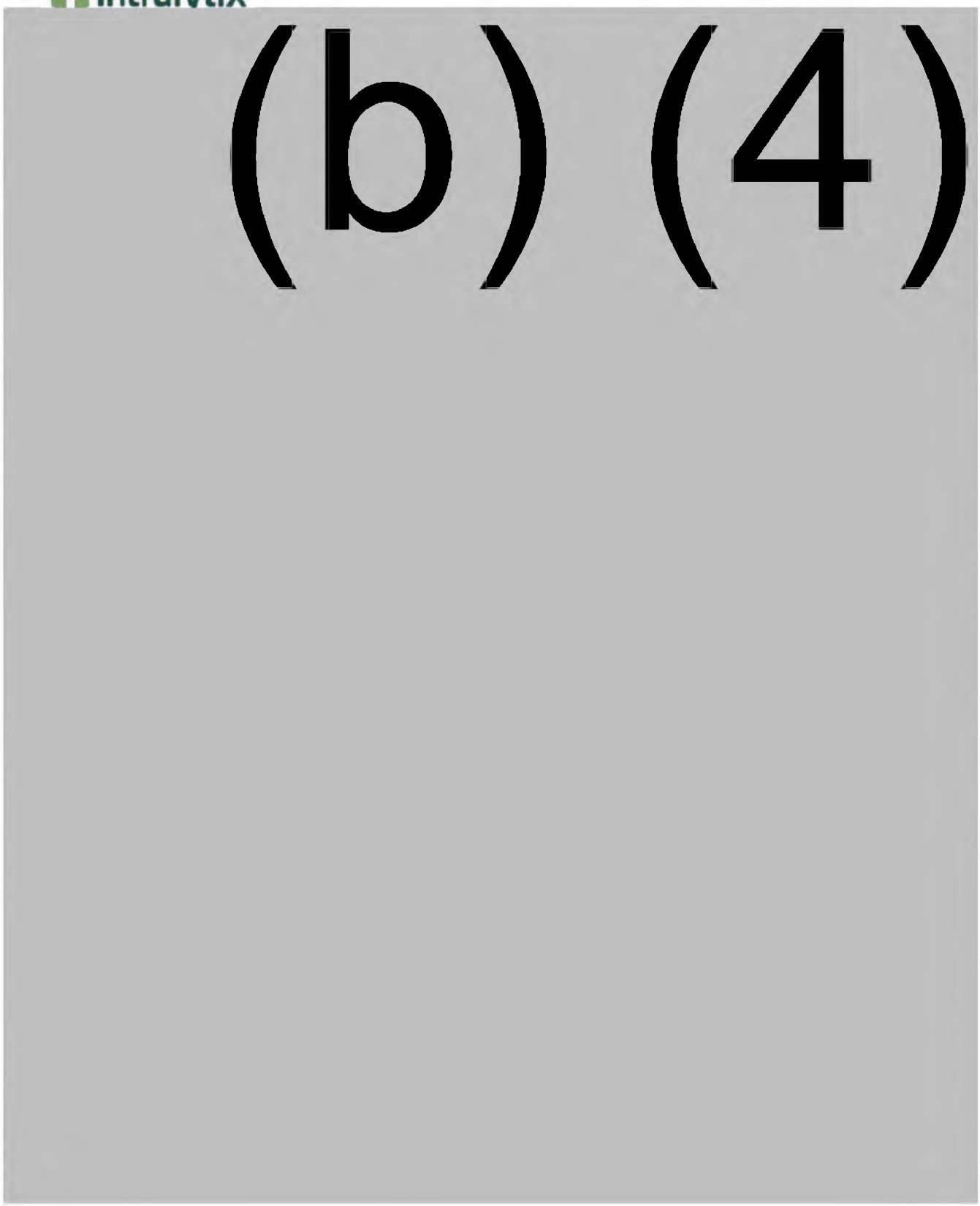
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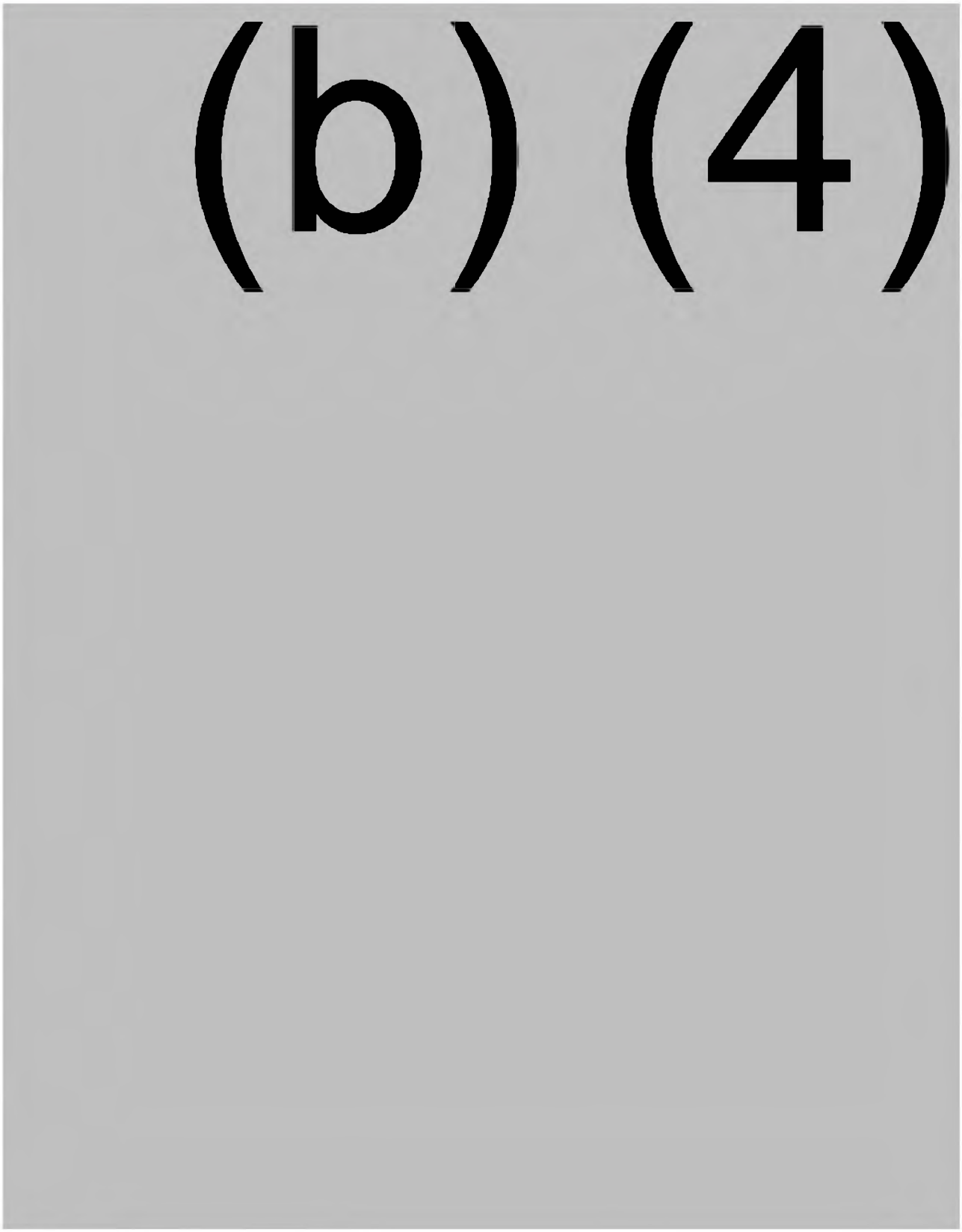
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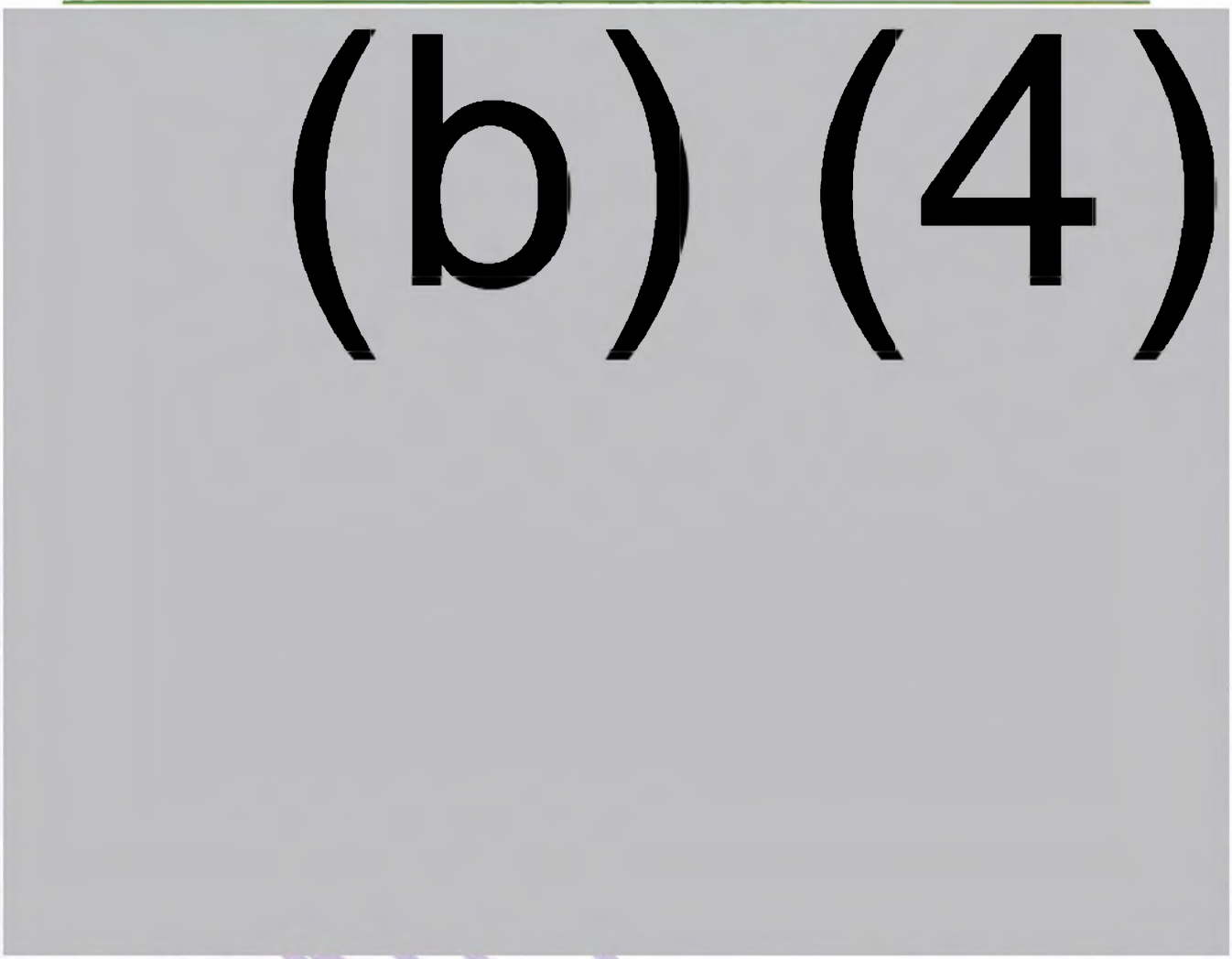
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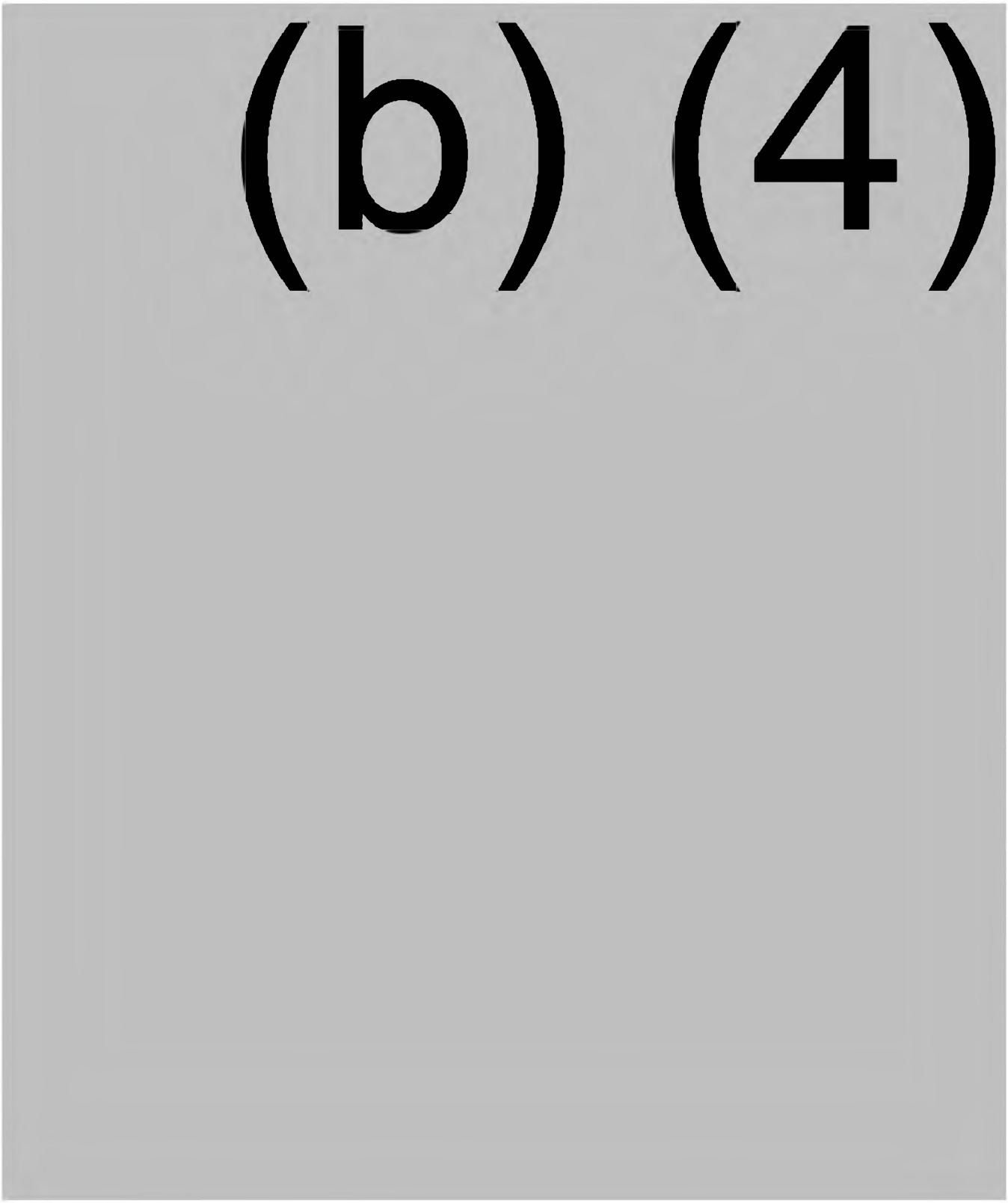
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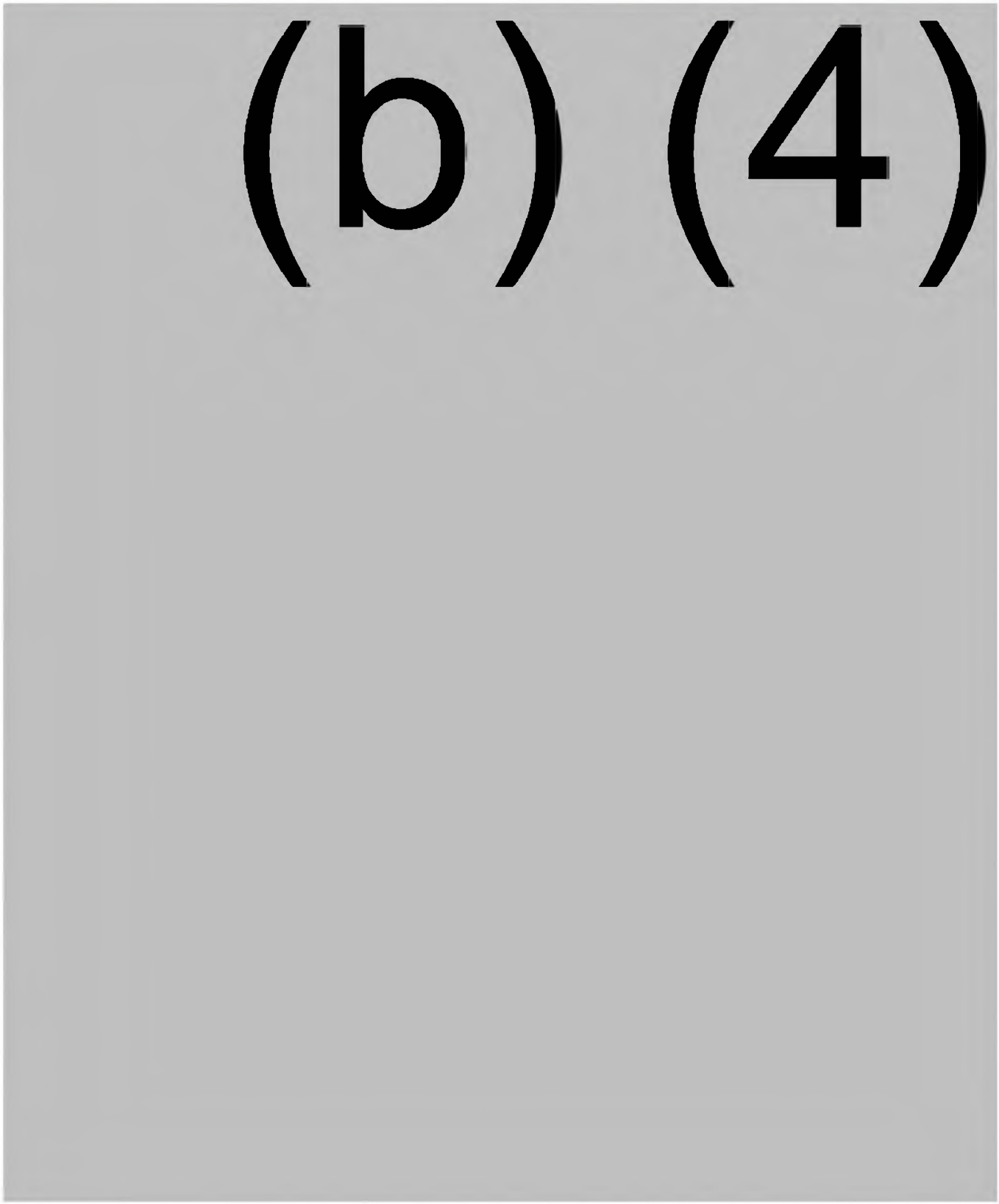

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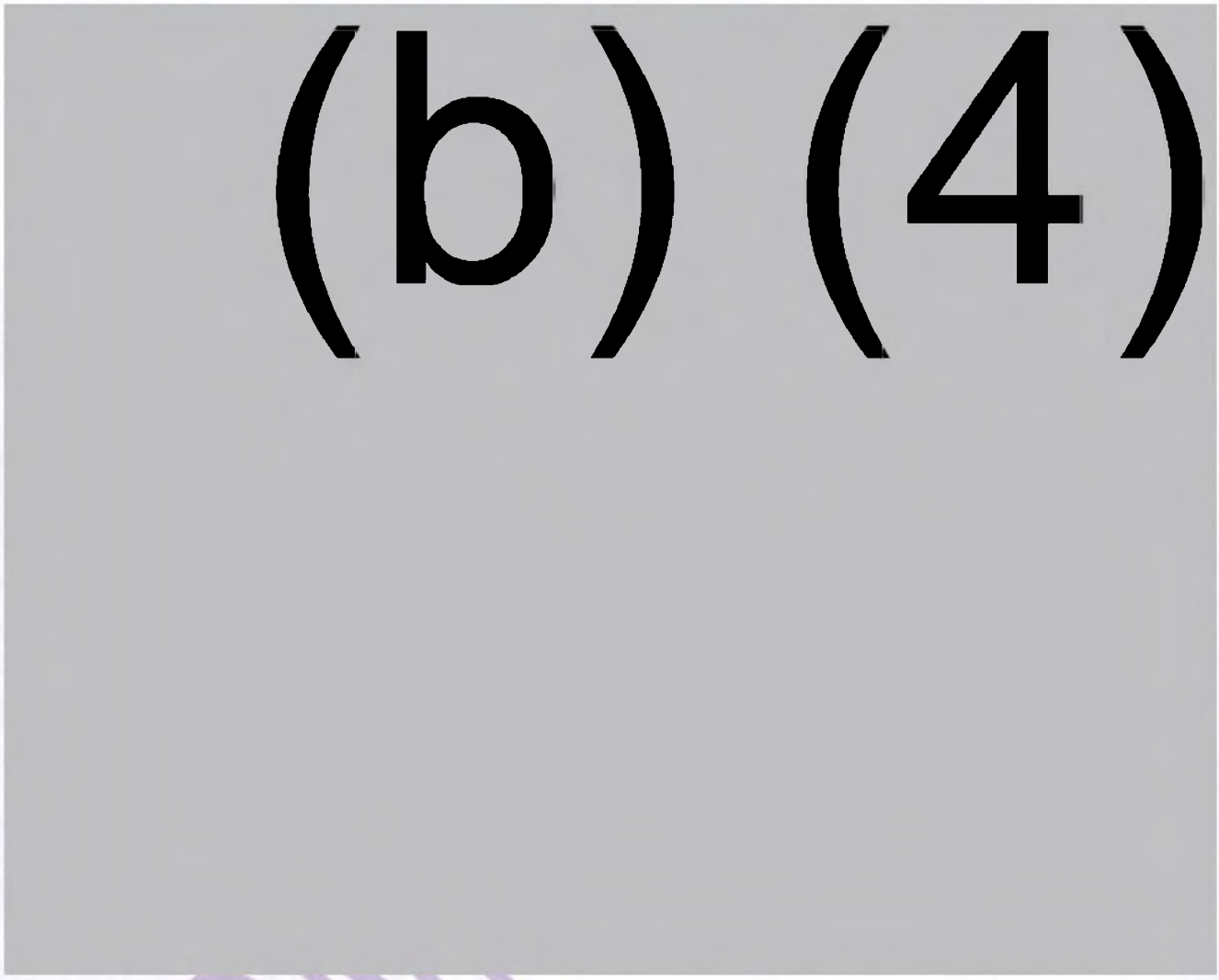
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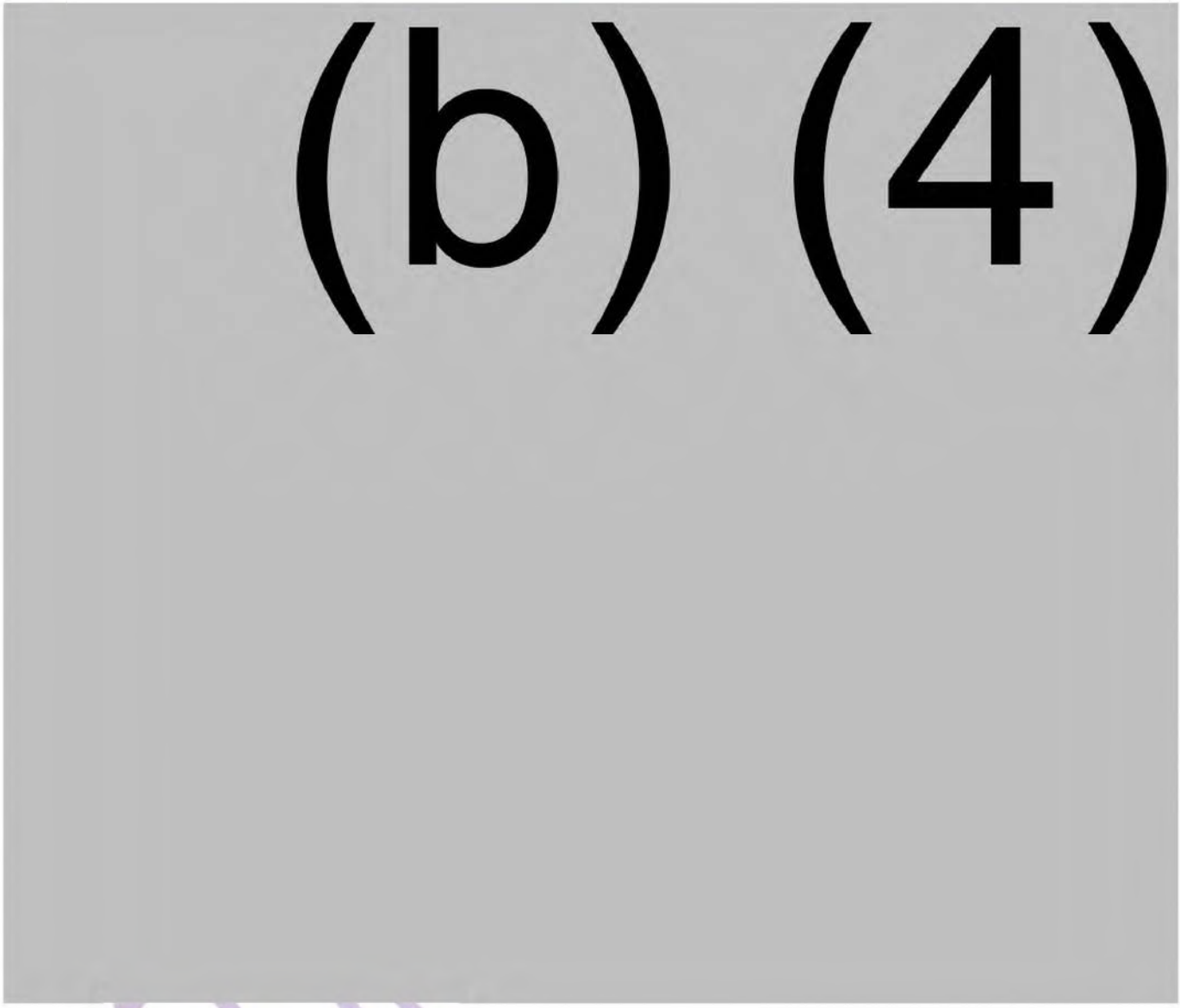
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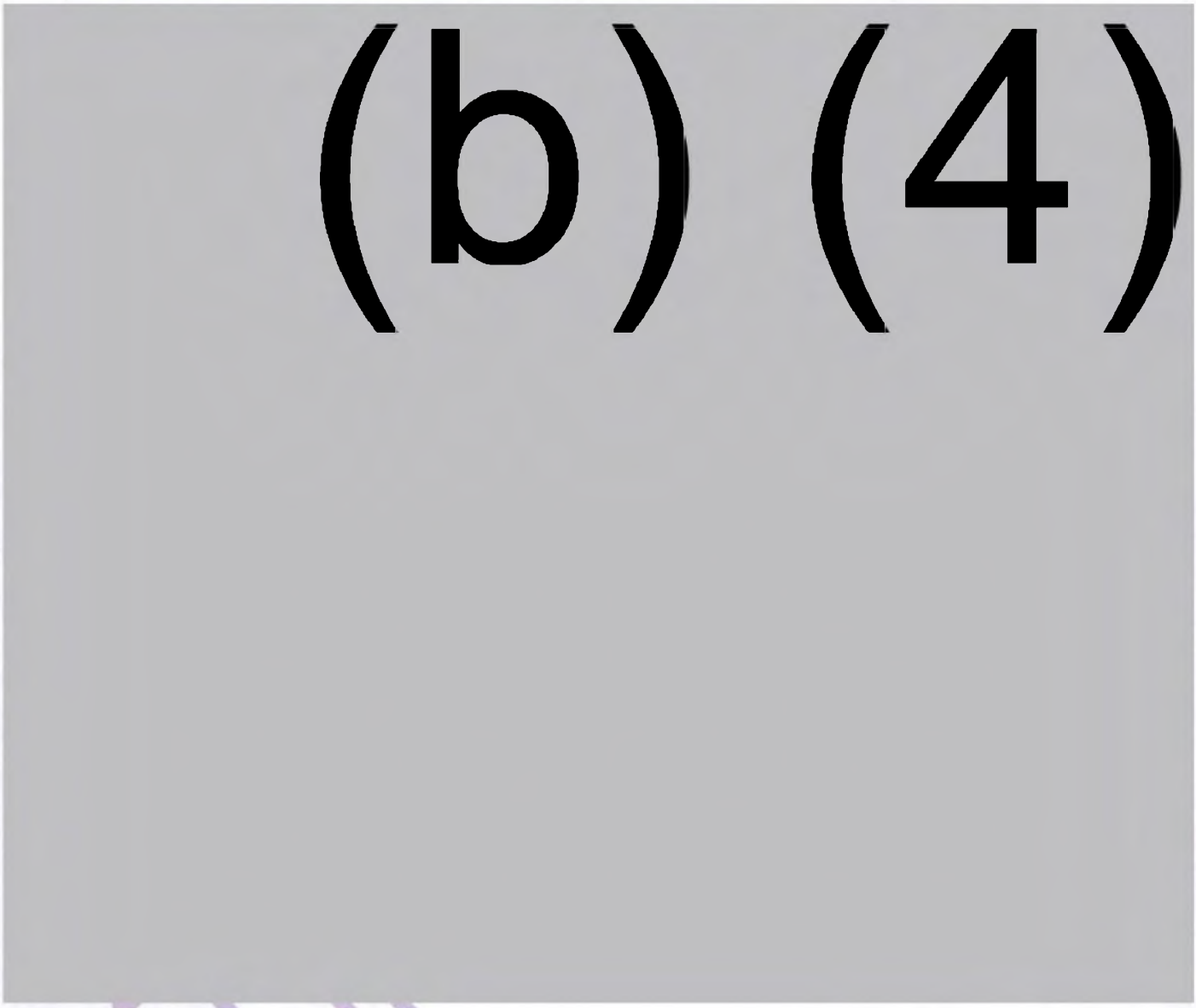
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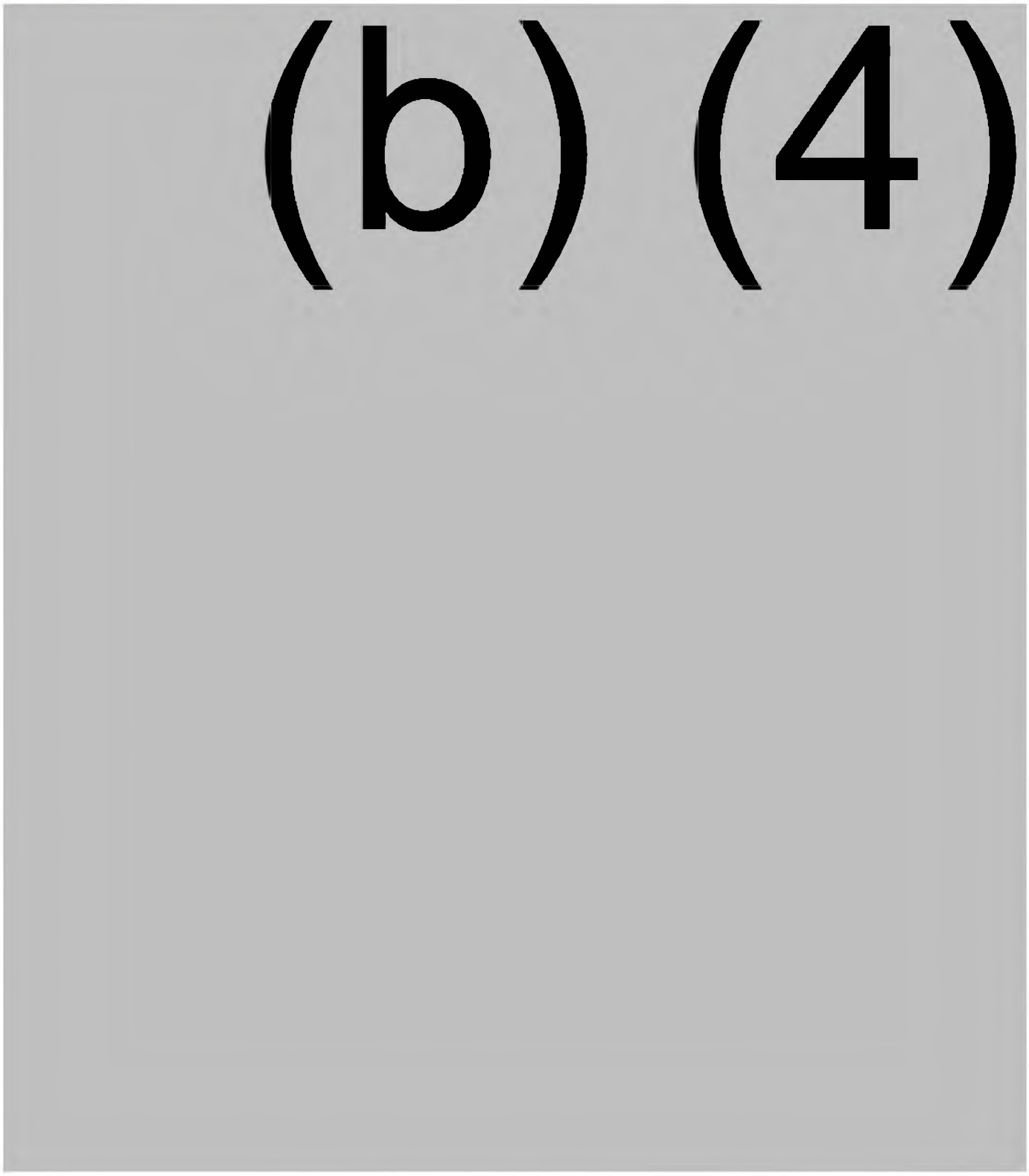

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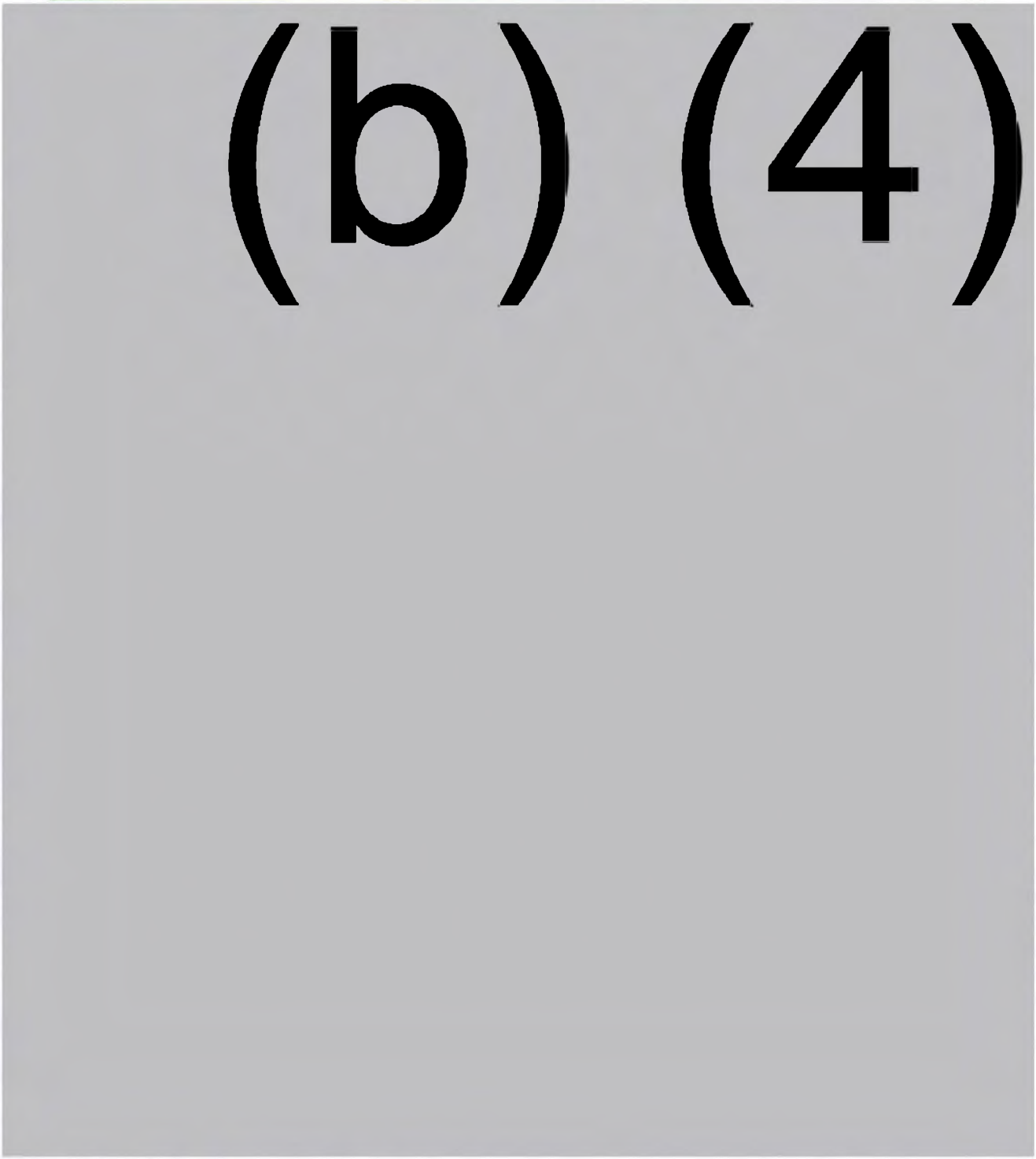


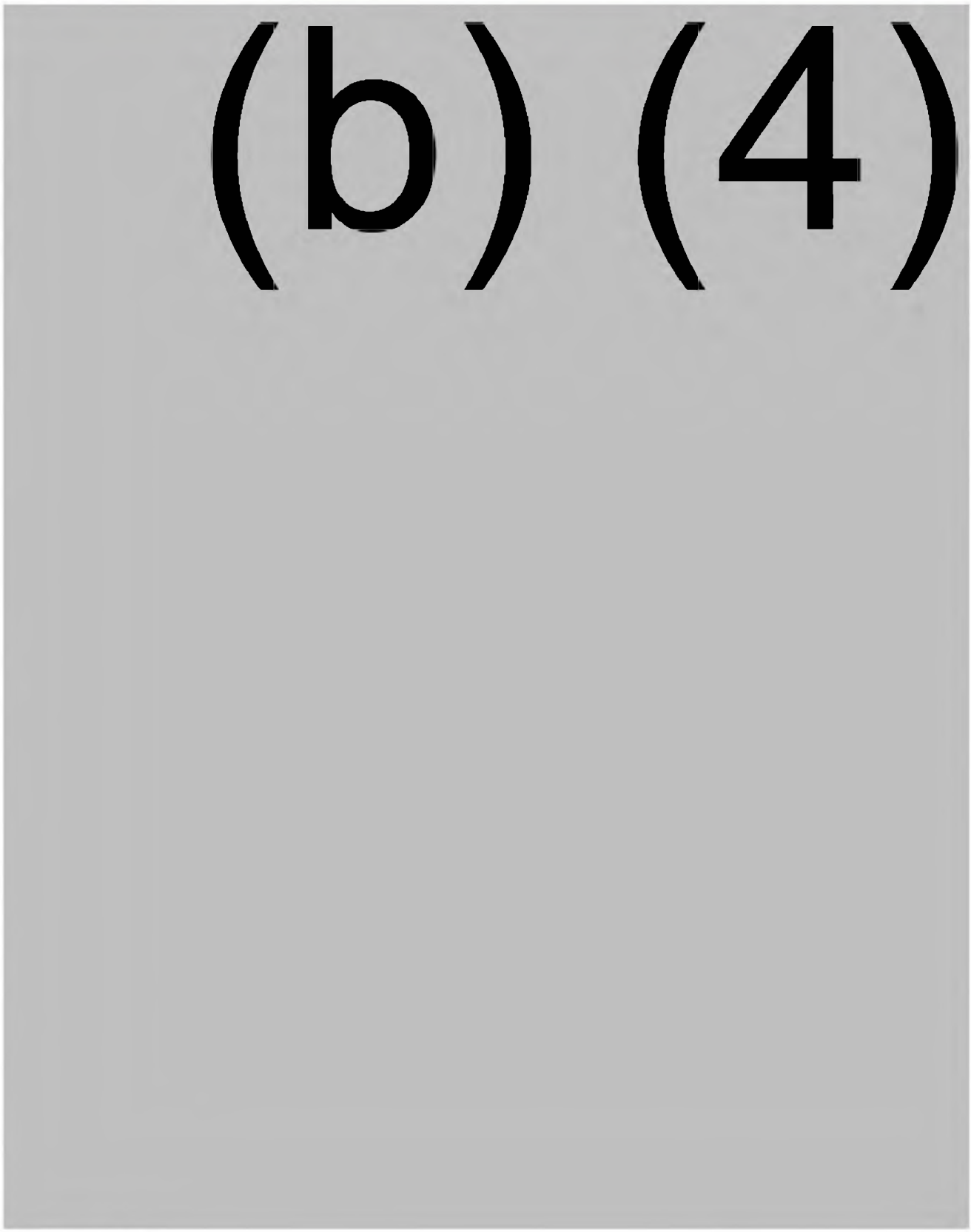
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Prepared by:
Intralytix
8681 Robert Fulton Drive
Columbia, MD 21046
www.intralytix.com

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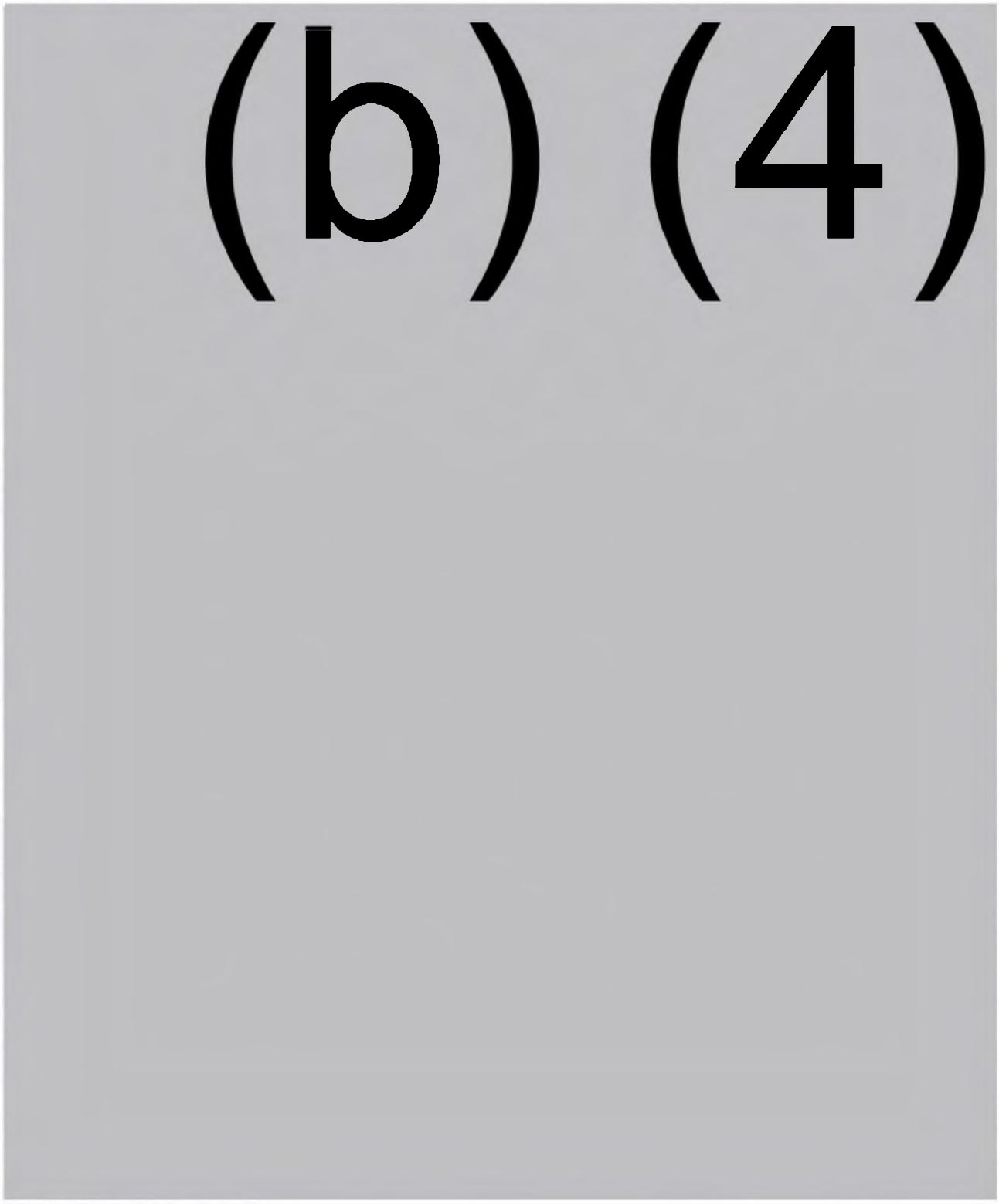
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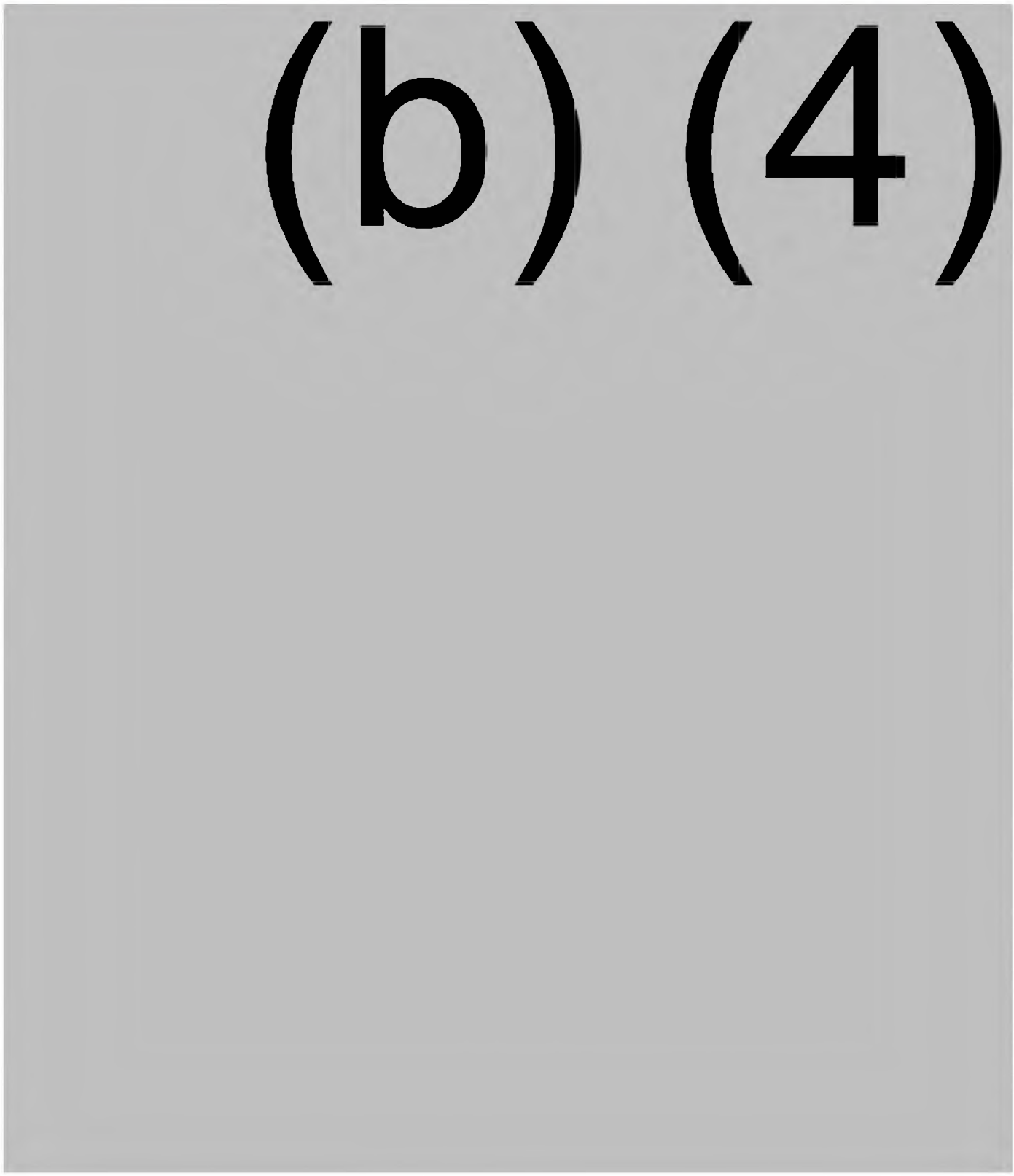
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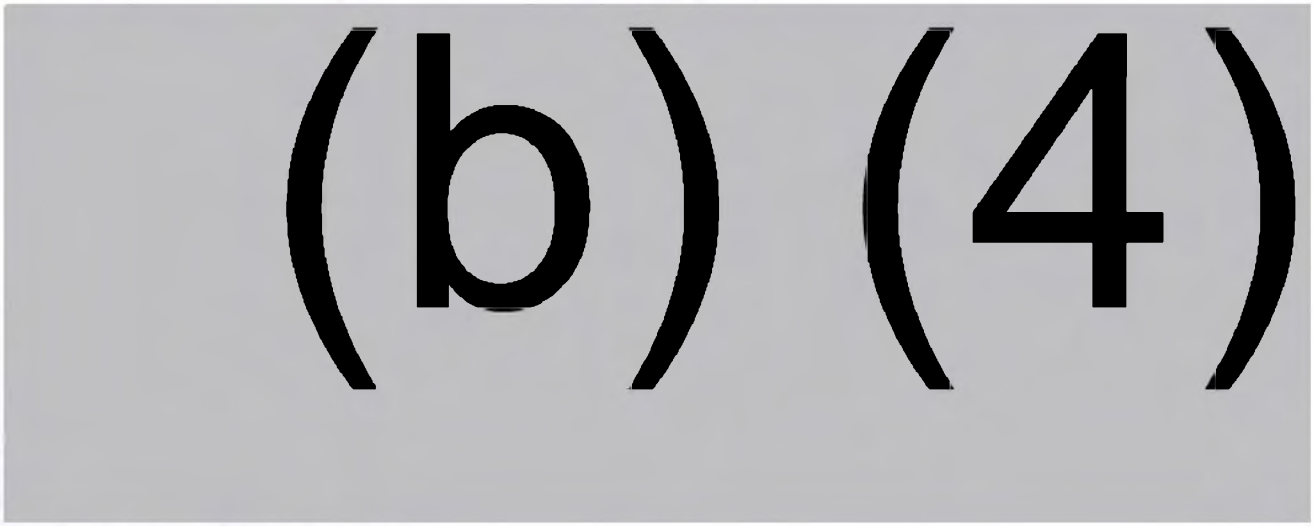
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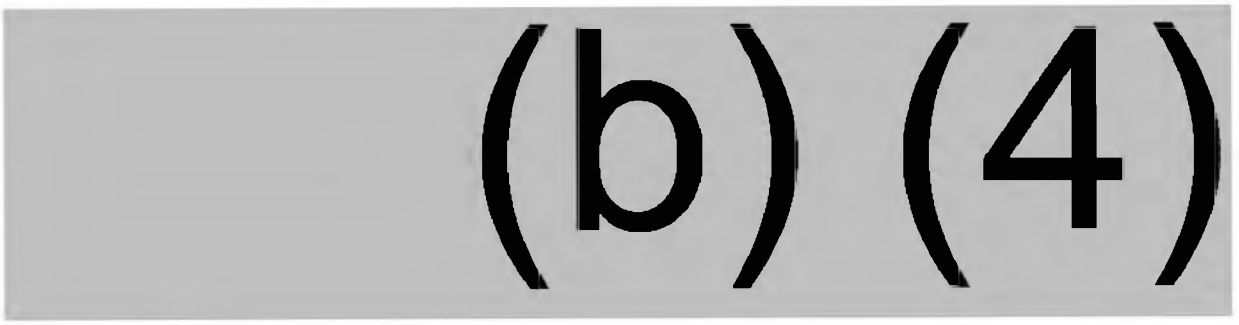


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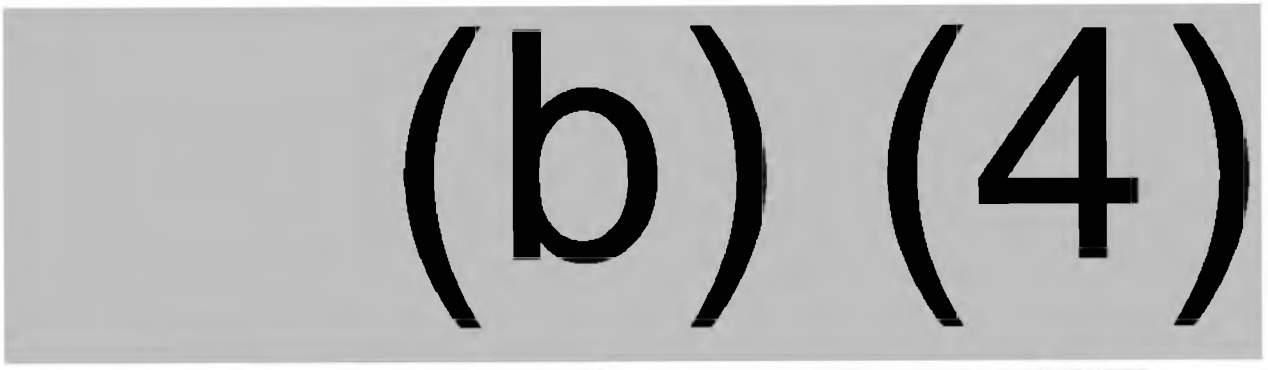


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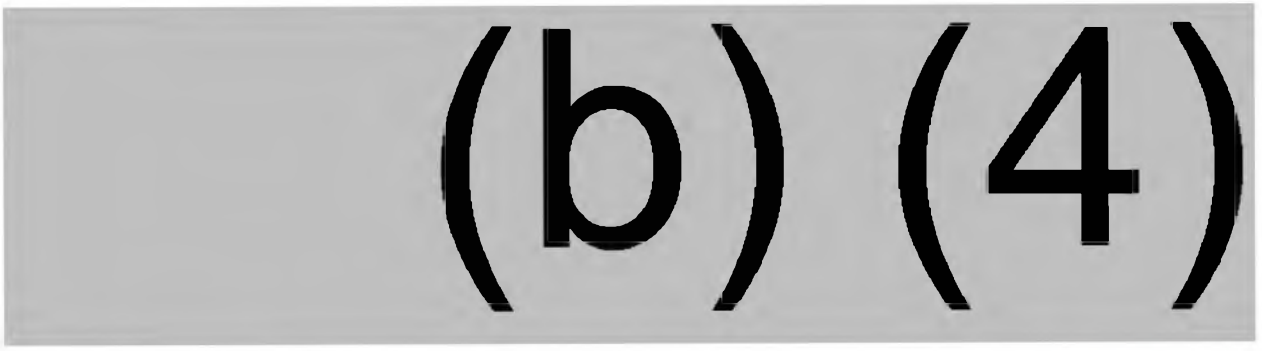


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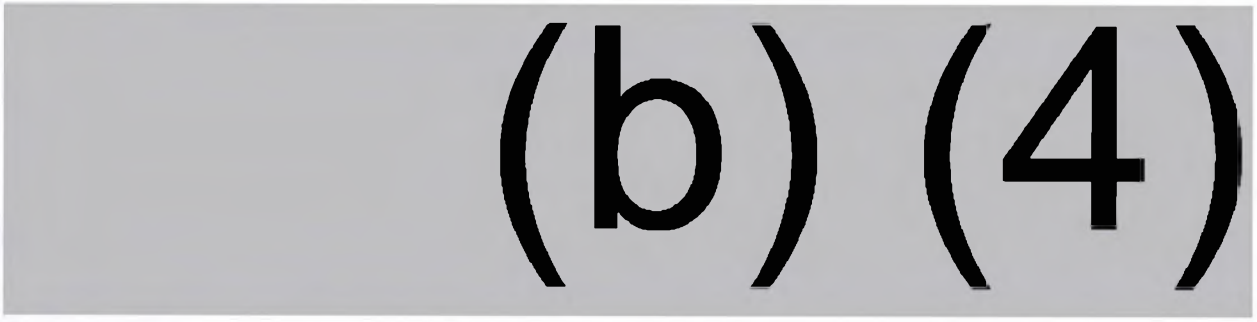


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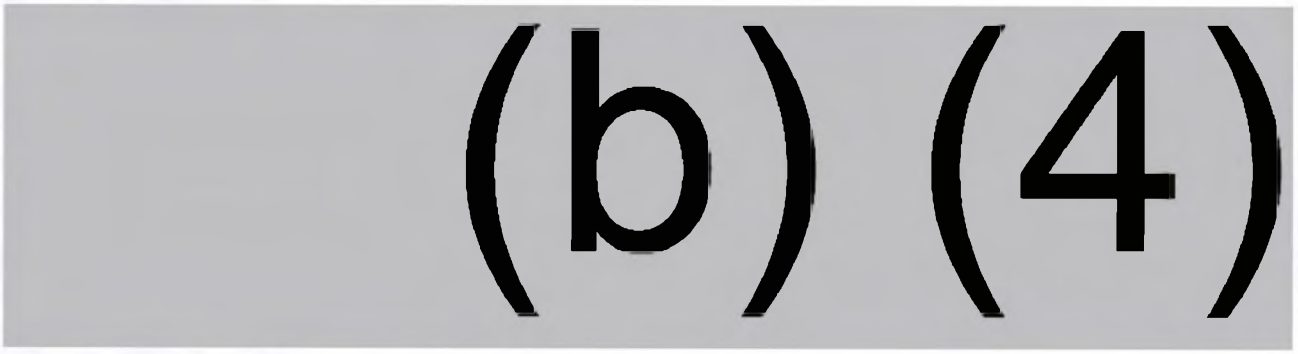


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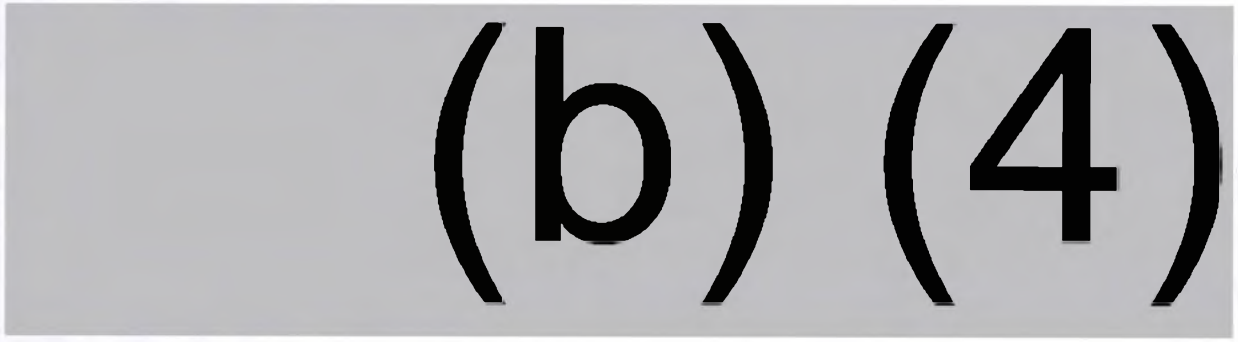


Figure 6 ViPTree (b) (4)

4 Signatures

Report authored by:

(b) (6) Date

Report reviewed by:

(b) (6) Date

5 Revision History

Date	Rev	Author	Description of change
10/07/2024	0	(b) (6)	Original
11/27/2024	1		Changed the order of the phage genus on page 5 and 8.

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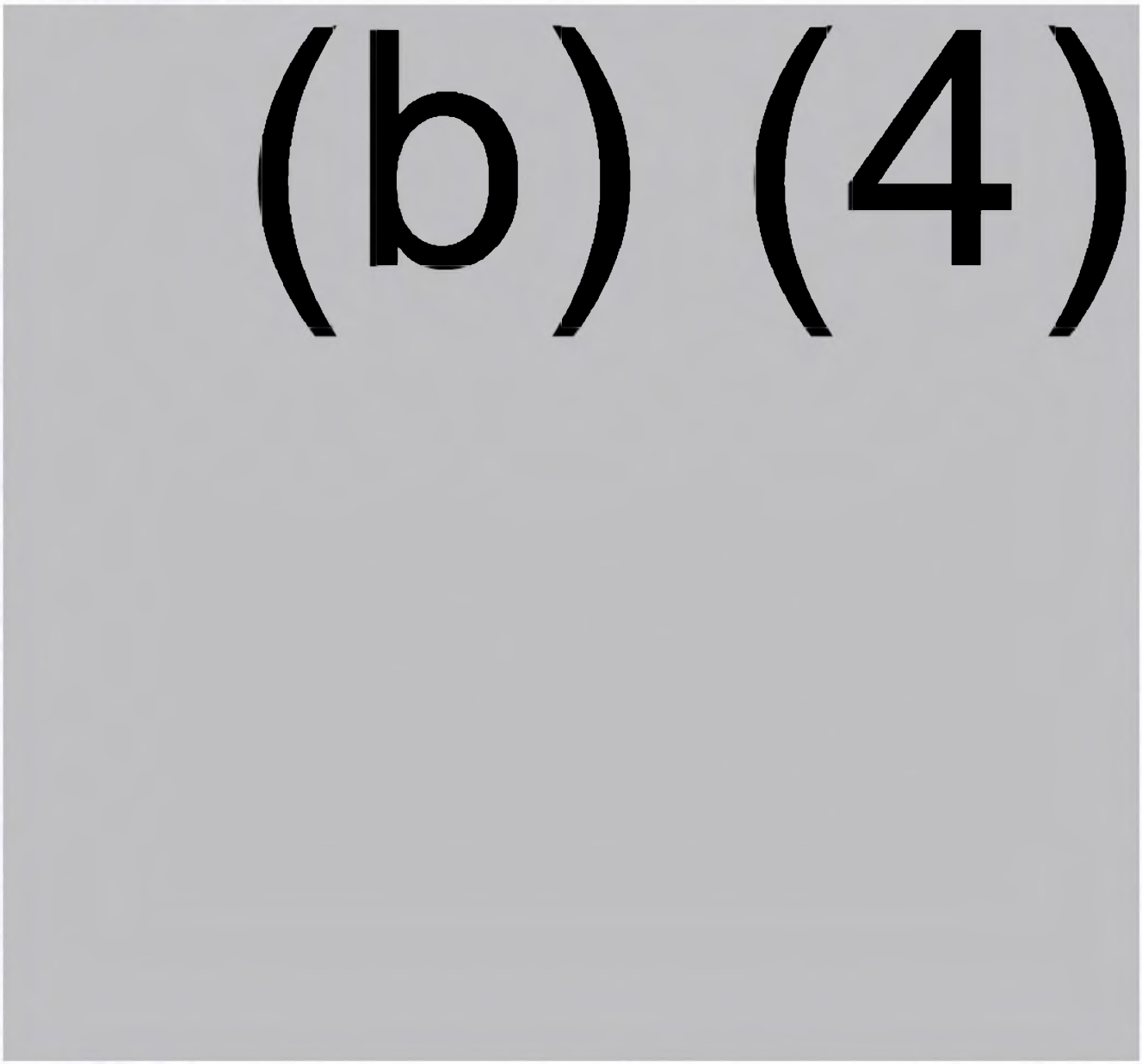
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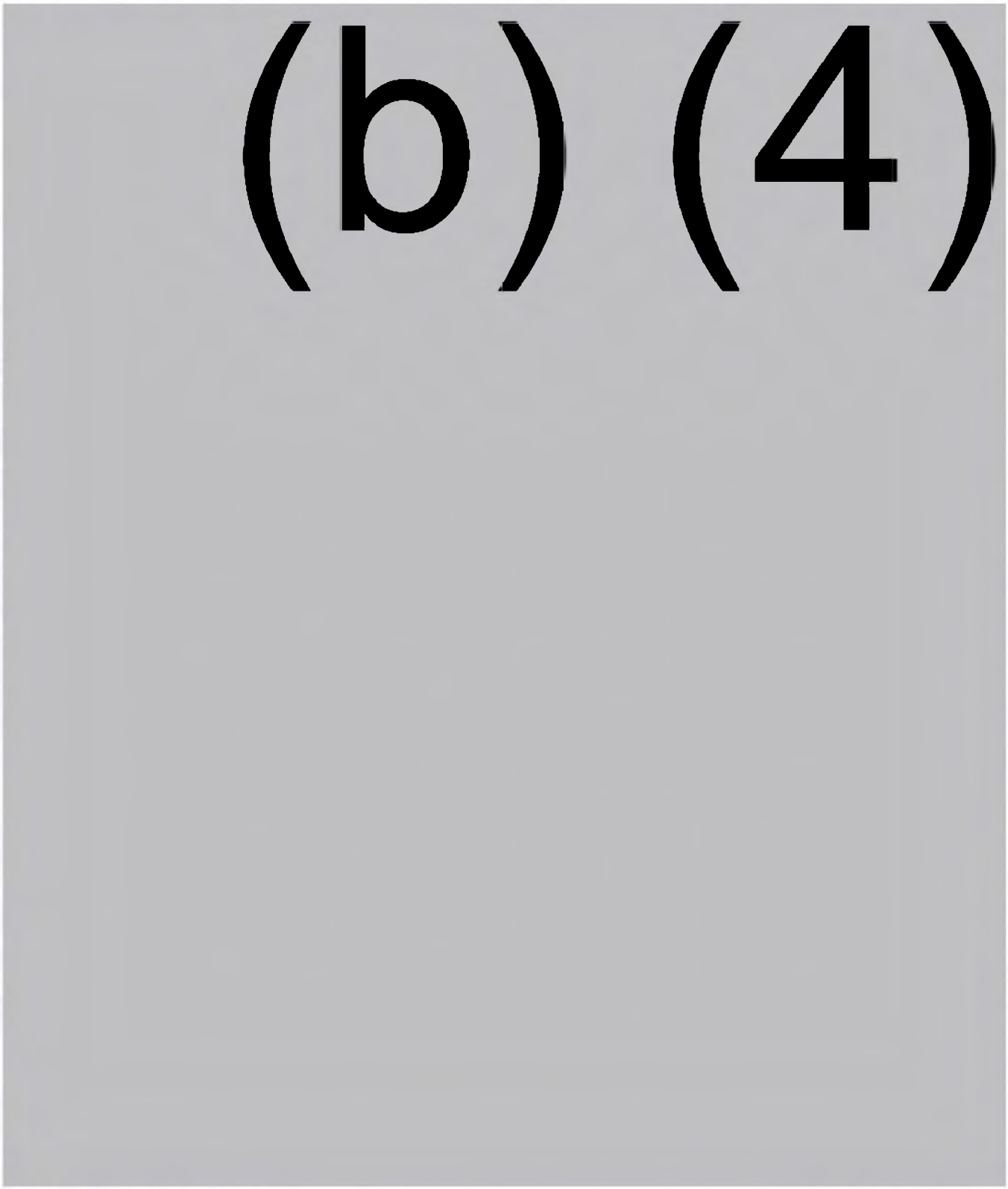
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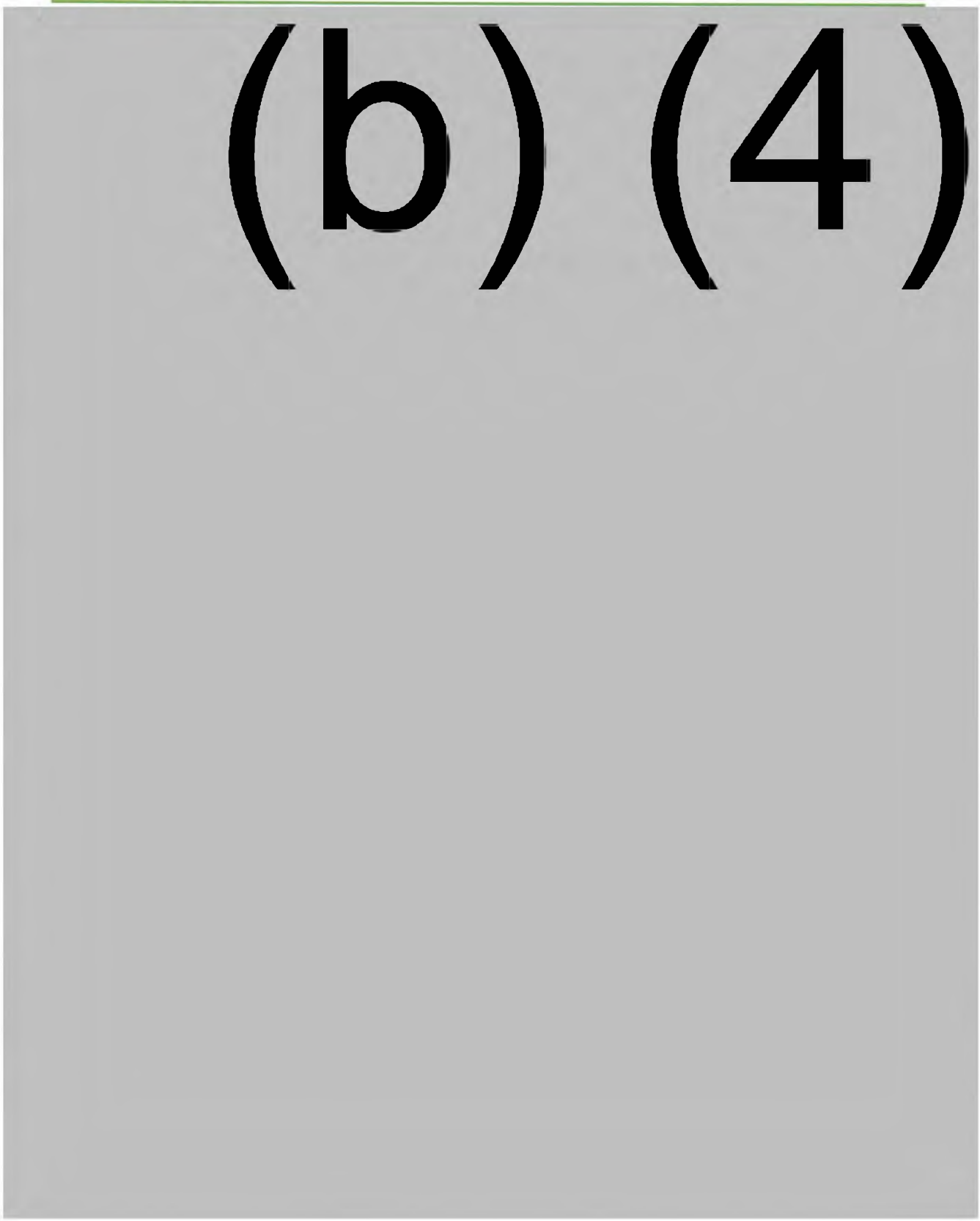
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Intralytix
8681 Robert Fulton Drive
Columbia, MD 21046
www.intralytix.com

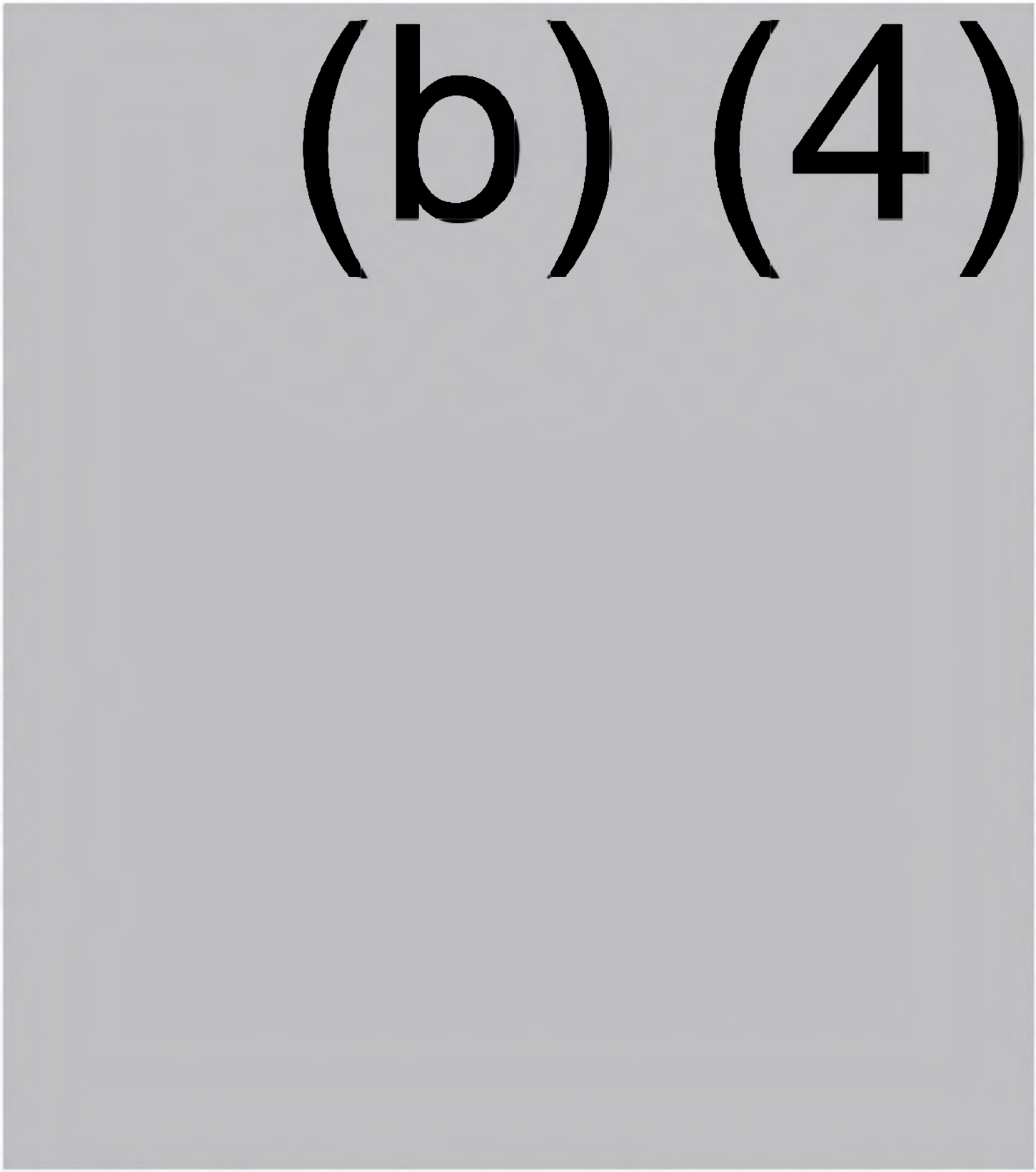
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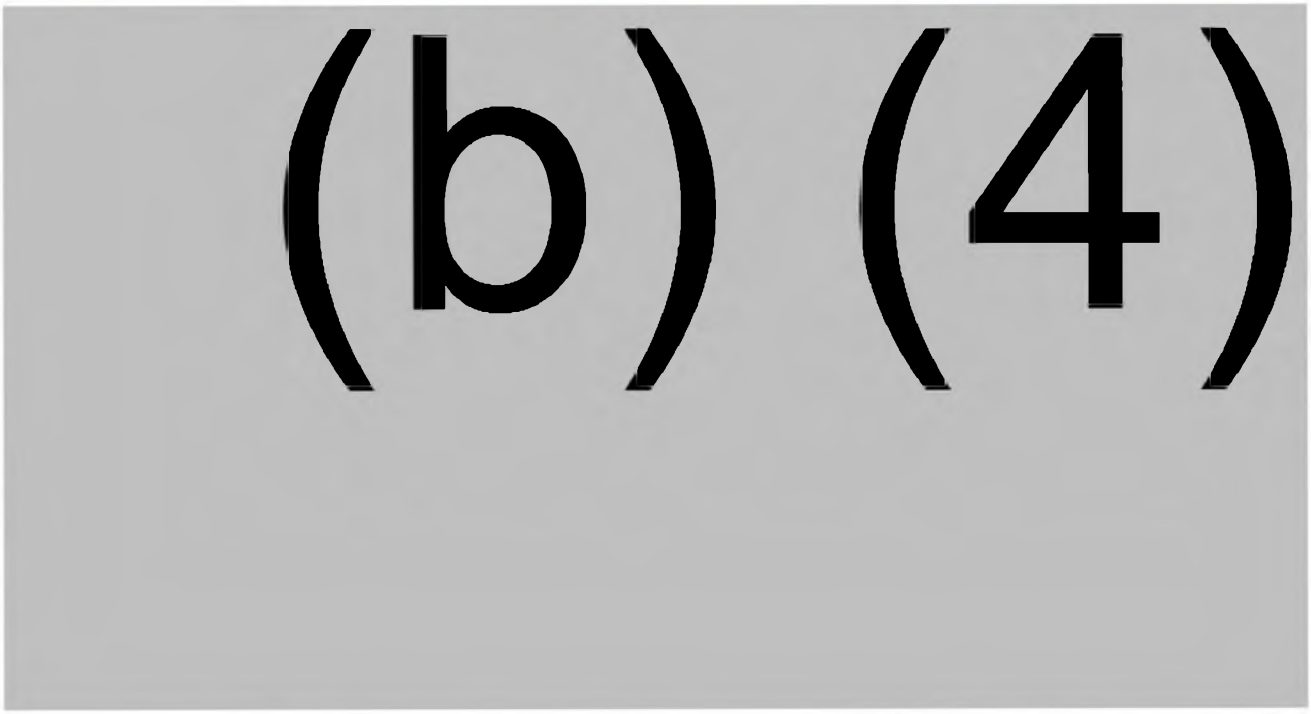


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4 Additional figures

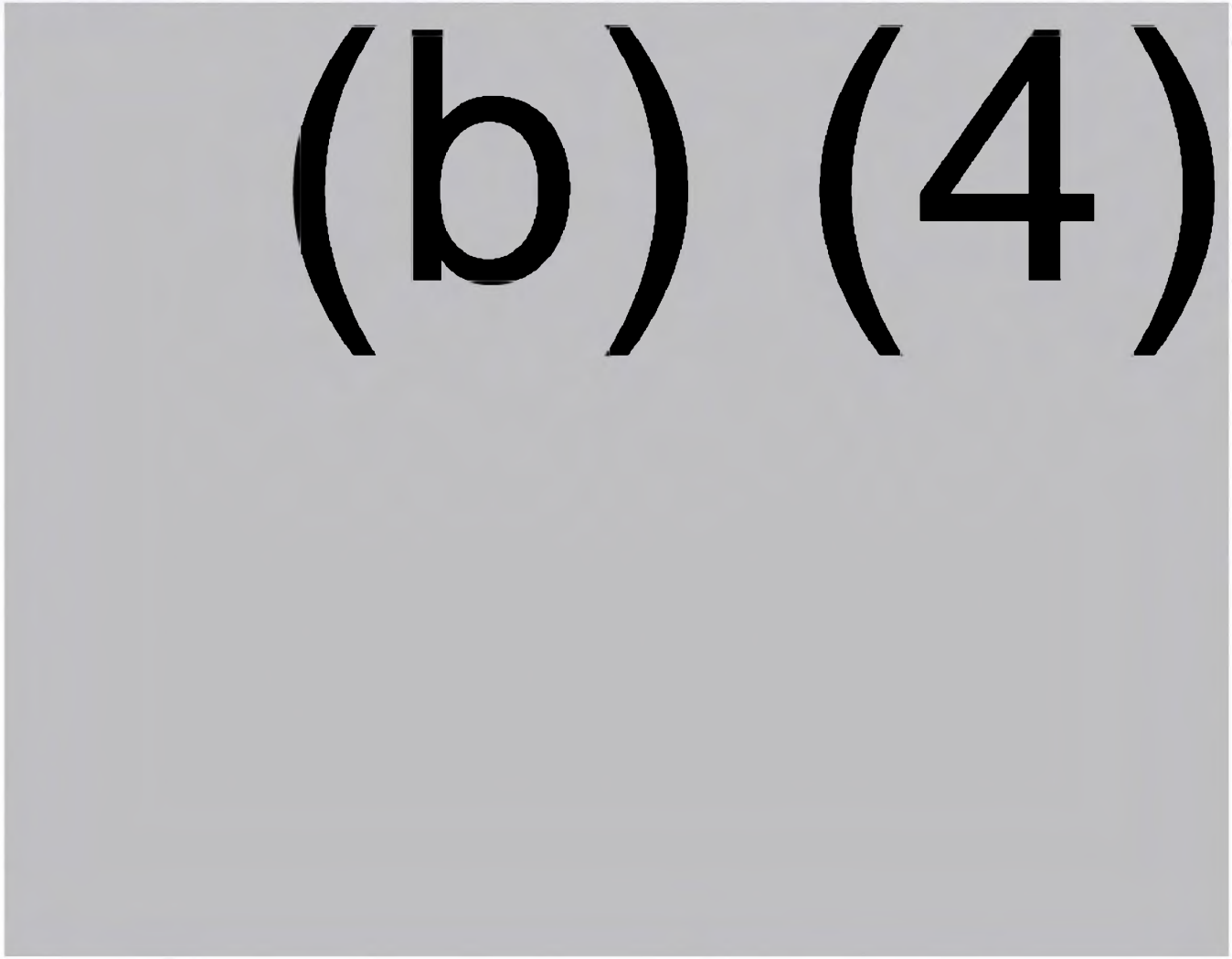


Figure 1 **Circular map of host SA121 genome with** (b) (4)

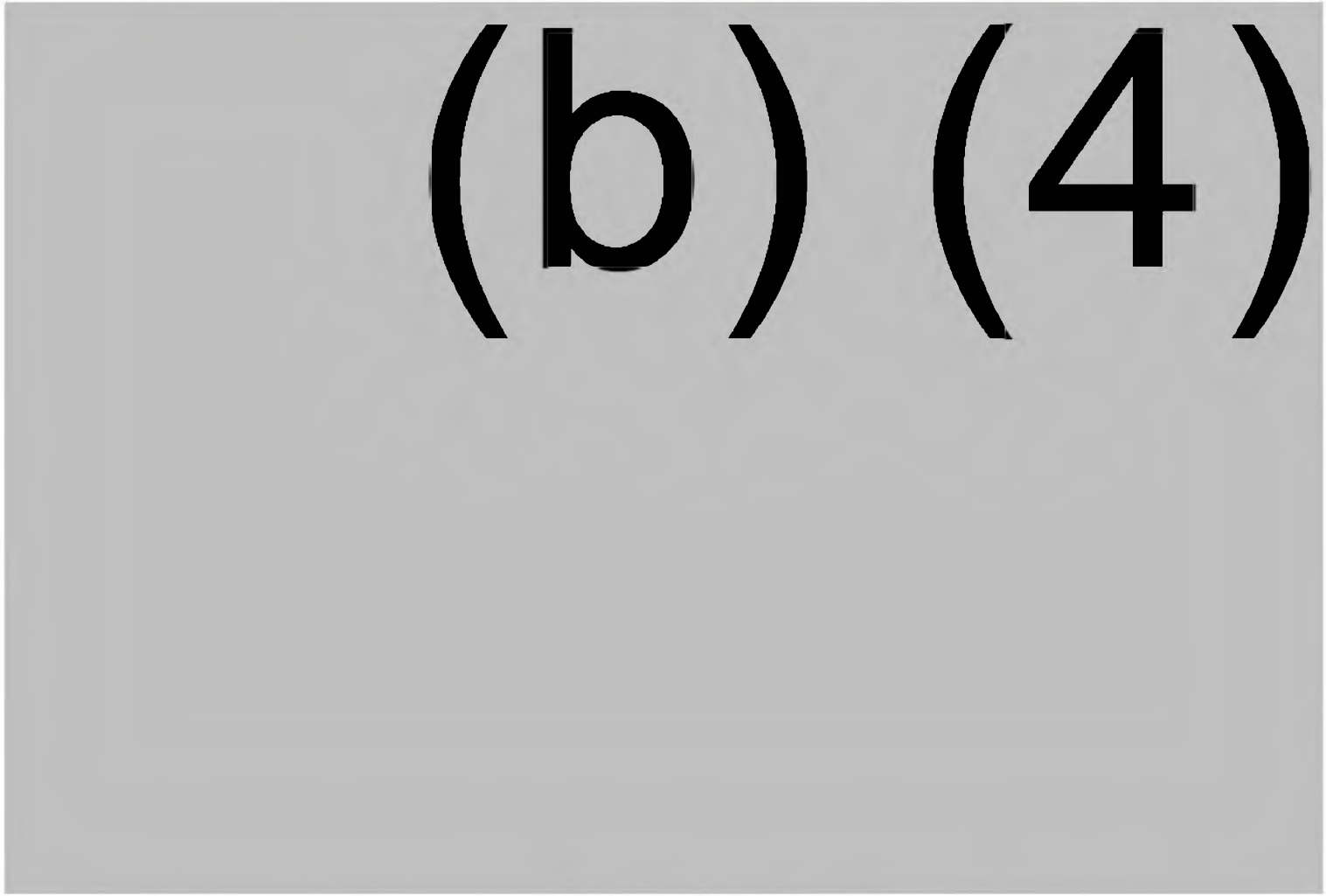


Figure 2 **Circular map of host SE378 genome with** (b) (4)

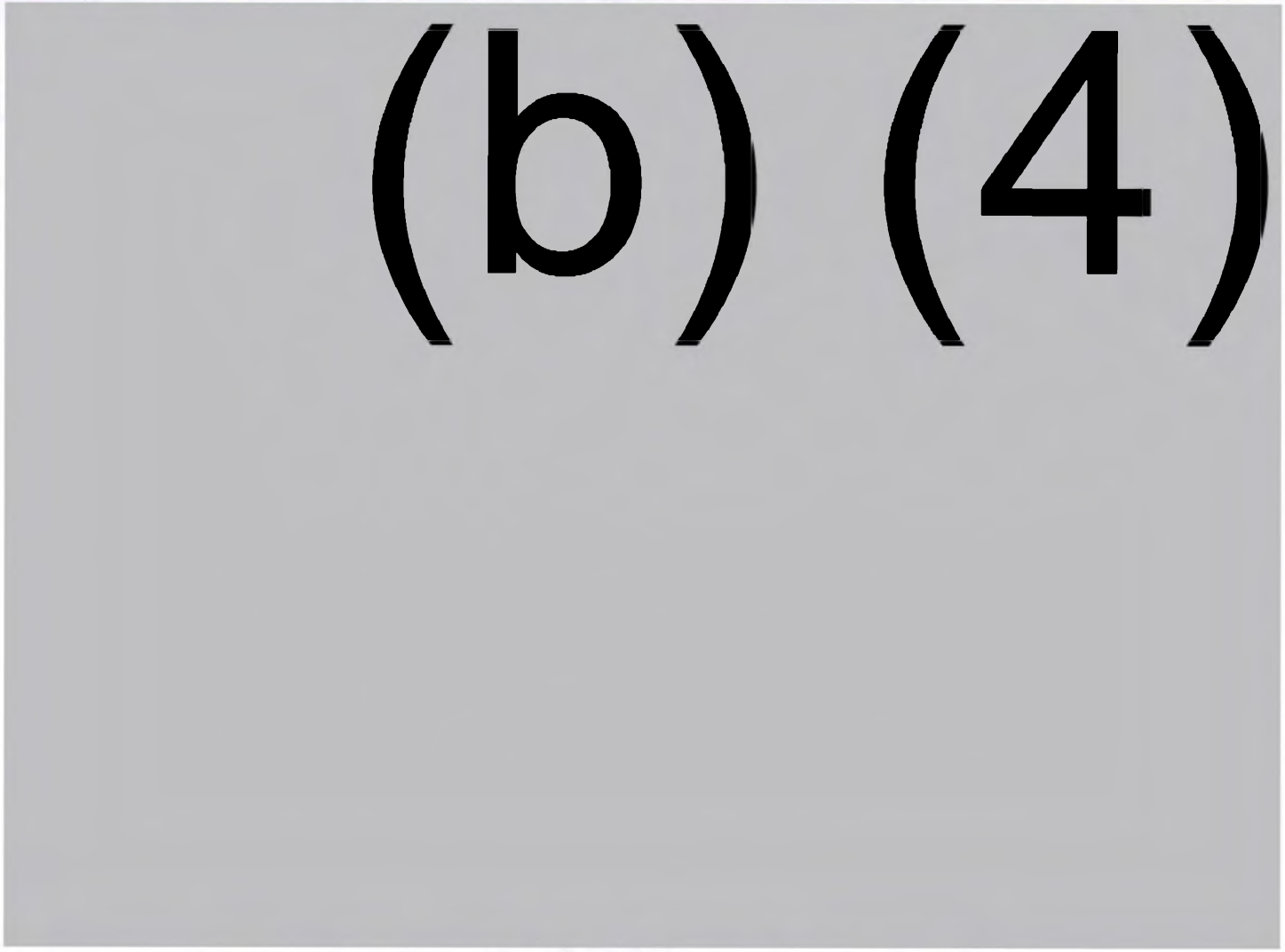


Figure 3 **Circular map of host SH178 genome with** (b) (4)

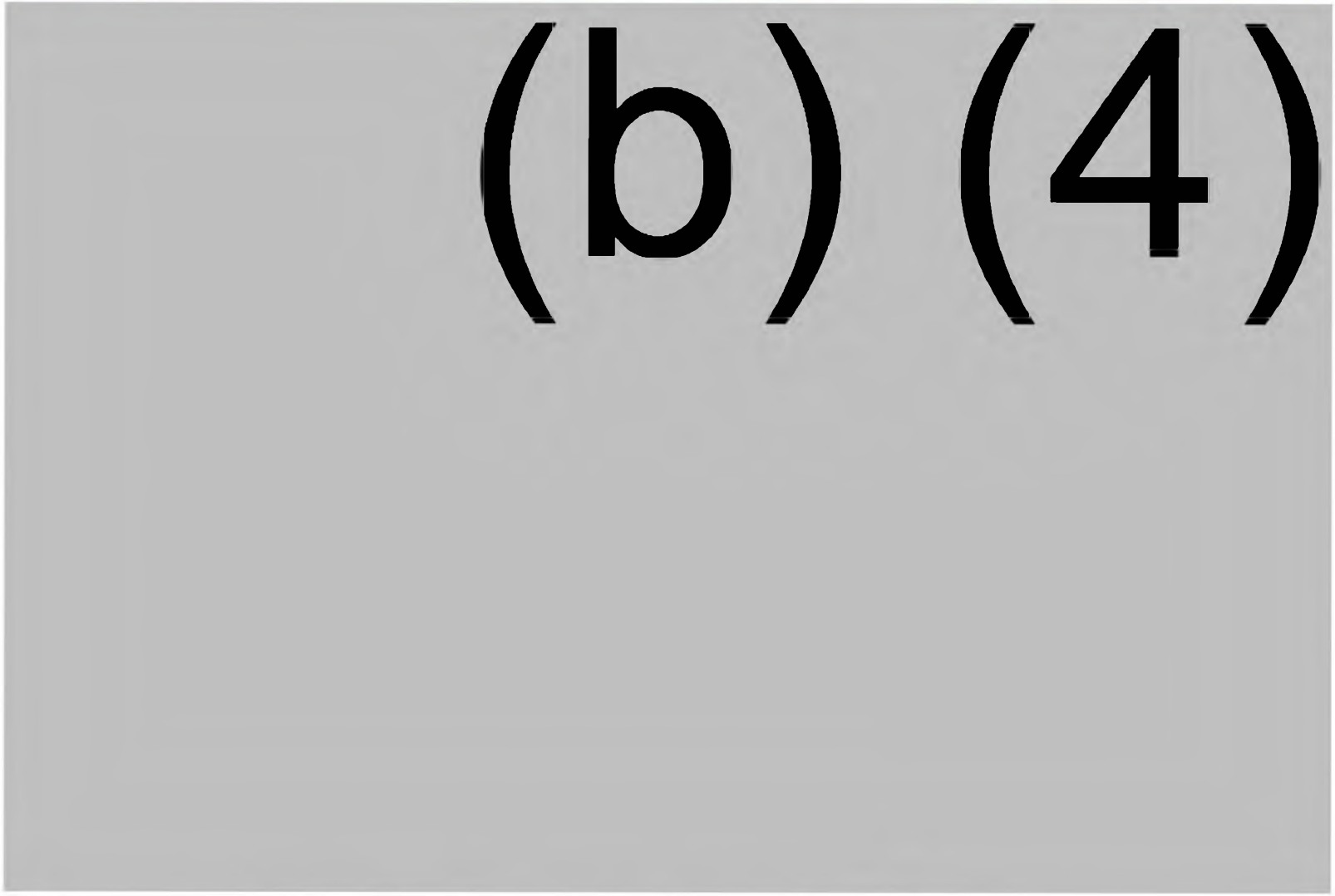


Figure 4 Circular map of host SK39 genome with (b) (4)

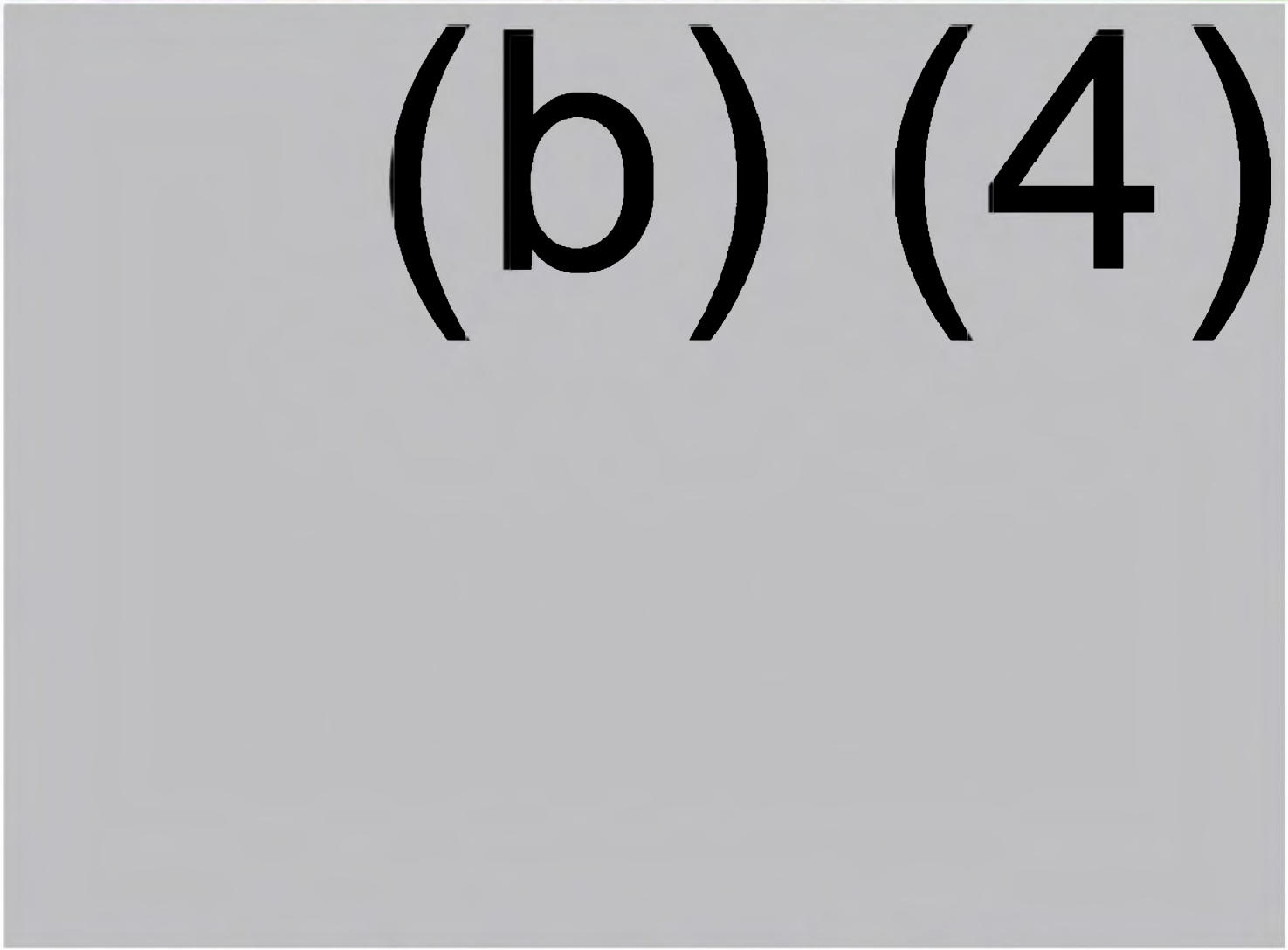


Figure 5 Circular map of host ST13 genome with (b) (4)

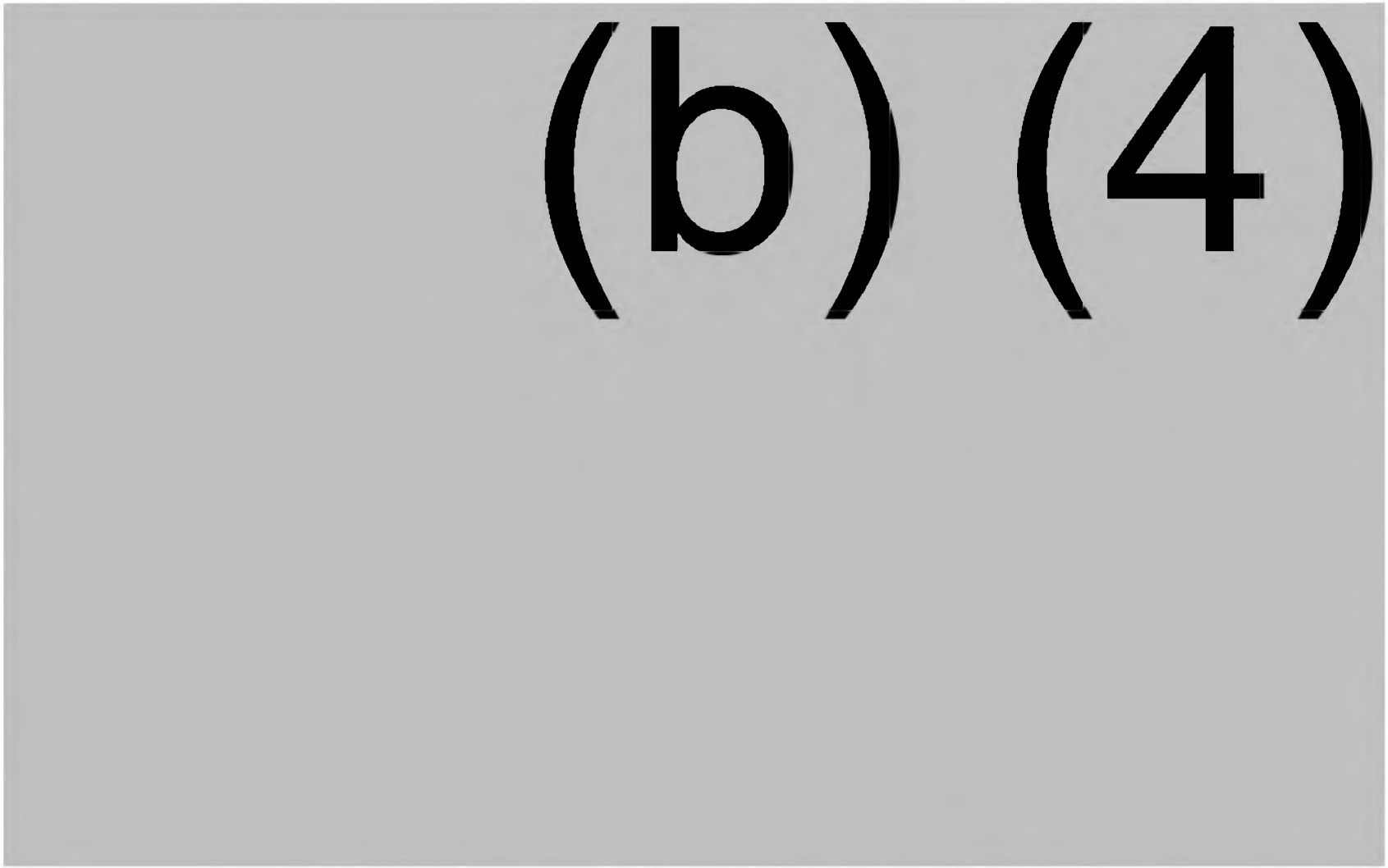


Figure 6 **Circular map of host ST198 genome with** (b) (4).

5 Signatures

Report authored by:

(b) (6) Date

Report reviewed by:

(b) (6) Date

6 Revision History

Date	Rev	Author	Description of change
10/30/2024	0	(b) (6)	Original

7 References

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2016, VOL. 6, NO. 3, e1220347 (8 pages)
http://dx.doi.org/10.1080/21597081.2016.1220347

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RESEARCH PAPER

Bacteriophages safely reduce *Salmonella* contamination in pet food and raw pet food ingredients

Nitzan Soffer[a], Tamar Abuladze[a], Joelle Woolston[a], Manrong Li[a], Leigh Farris Hanna[b], Serena Heyse[b], Duane Charbonneau[b], and Alexander Sulakvelidze[a]

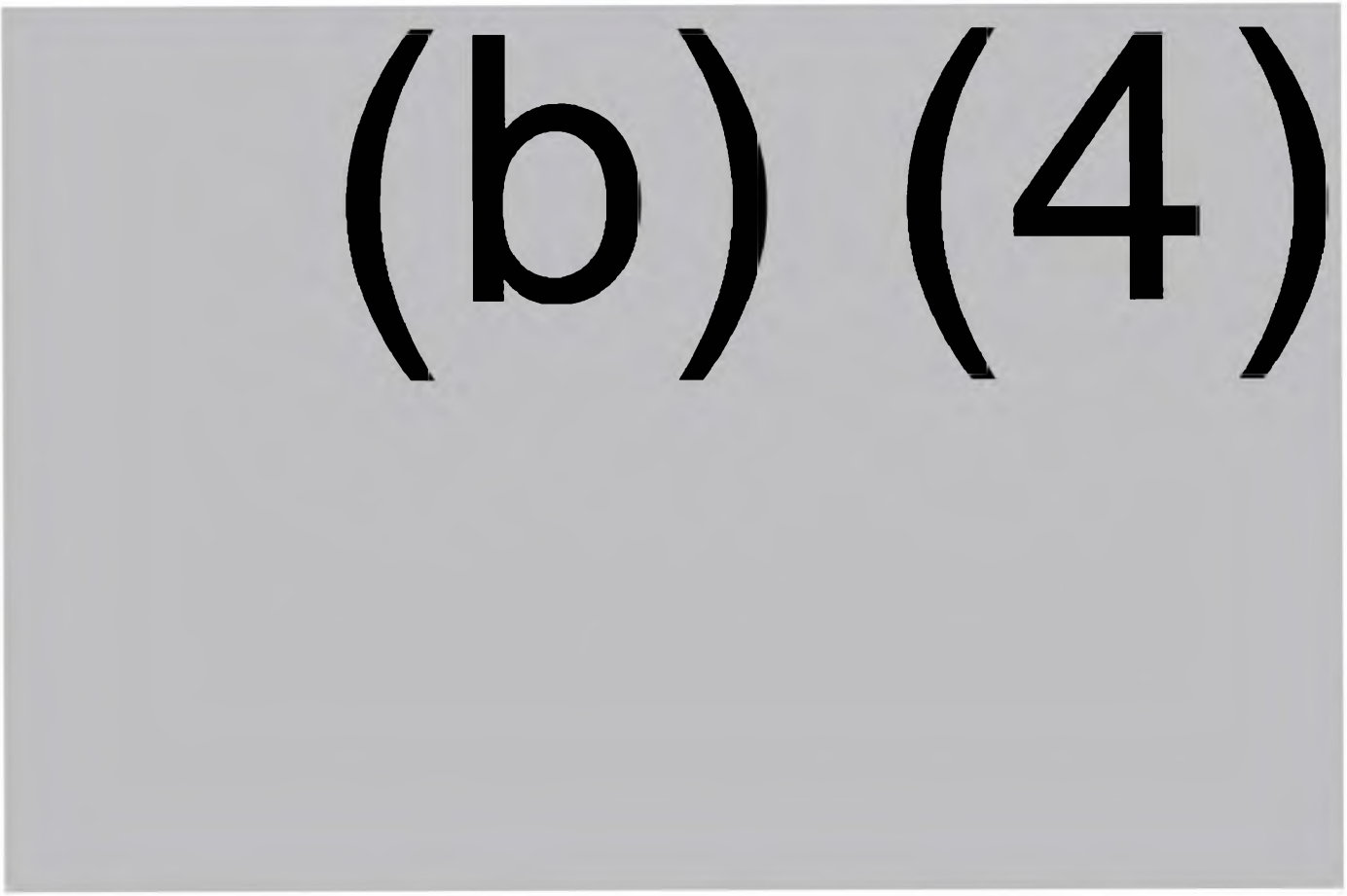
[a]Intralytix, Inc., Baltimore, MD, USA; [b]The Procter and Gamble Company, Mason, OH, USA

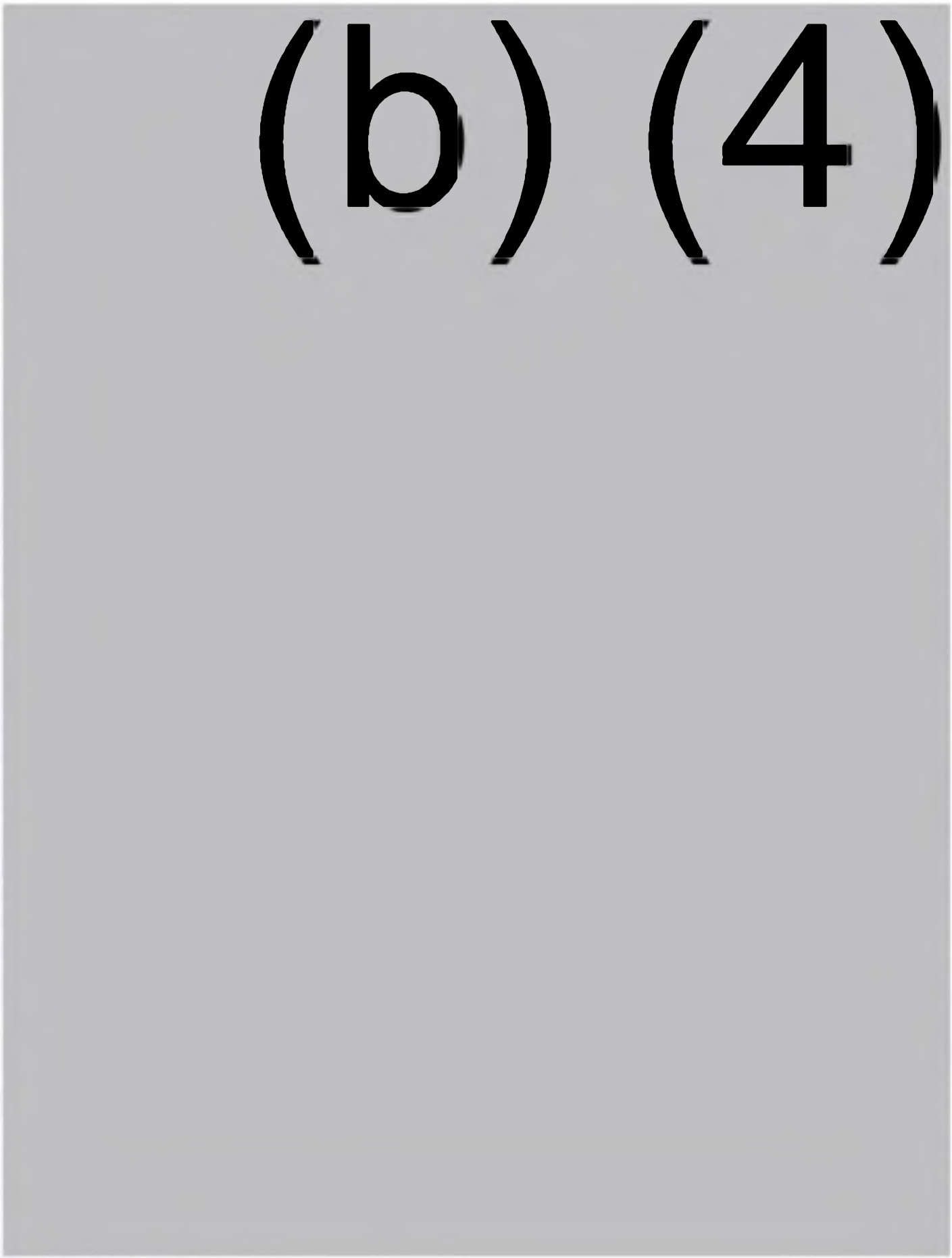
ABSTRACT

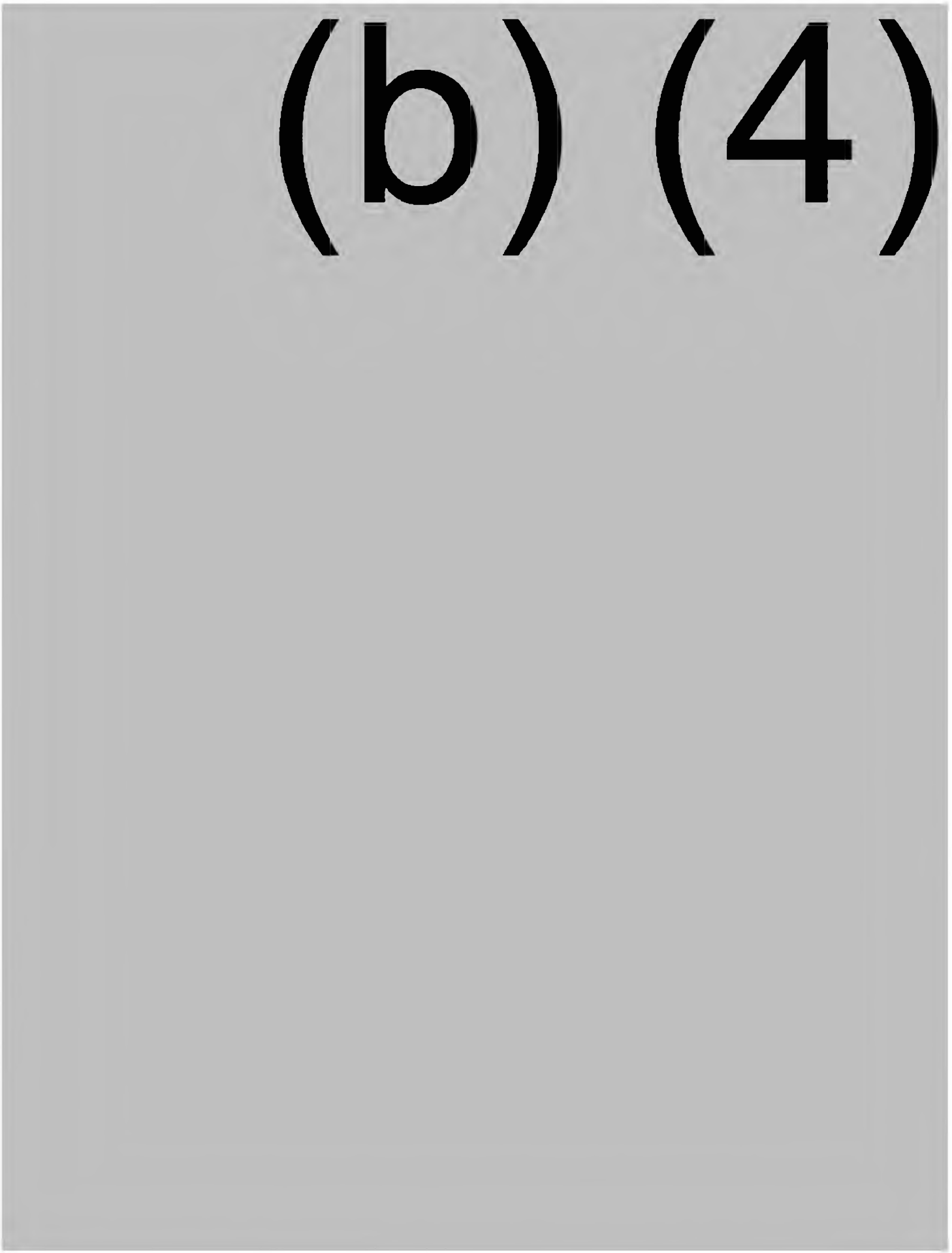
Contamination of pet food with *Salmonella* is a serious public health concern, and several disease outbreaks have recently occurred due to human exposure to *Salmonella* tainted pet food. The problem is especially challenging for raw pet foods (which include raw meats, seafood, fruits, and vegetables). These foods are becoming increasingly popular because of their nutritional qualities, but they are also more difficult to maintain *Salmonella*-free because they lack heat-treatment. Among various methods examined to improve the safety of pet foods (including raw pet food), one intriguing approach is to use bacteriophages to specifically kill *Salmonella* serotypes. At least 2 phage preparations (SalmoFresh™ and Salmonelex™) targeting *Salmonella* are already FDA cleared for commercial applications to improve the safety of human foods. However, similar preparations are not yet available for pet food applications. Here, we report the results of evaluating one such preparation (SalmoLyse®) in reducing *Salmonella* levels in various raw pet food ingredients (chicken, tuna, turkey, cantaloupe, and lettuce). Application of SalmoLyse® in low (ca. $2–4\times10^6$ PFU/g) and standard (ca. 9×10^6 PFU/g) concentrations significantly ($P < 0.01$) reduced (by 60–92%) *Salmonella* contamination in all raw foods examined compared to control treatments. When SalmoLyse®-treated (ca. 2×10^7 PFU/g) dry pet food was fed to cats and dogs, it did not trigger any deleterious side effects in the pets. Our data suggest that the bacteriophage cocktail lytic for *Salmonella* can significantly and safely reduce *Salmonella* contamination in various raw pet food ingredients.

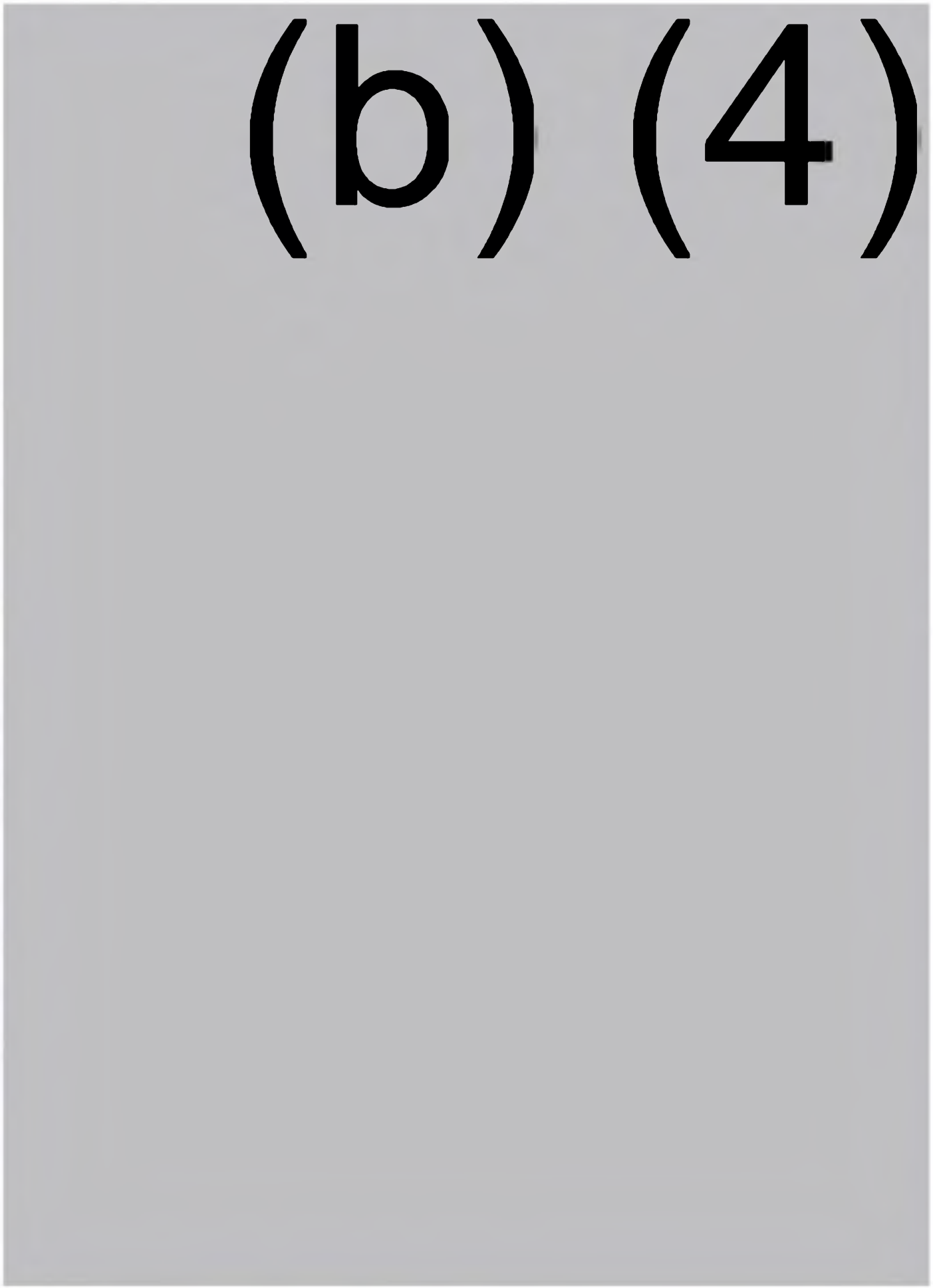
ARTICLE HISTORY
Received 16 March 2016
Revised 27 July 2016
Accepted 28 July 2016

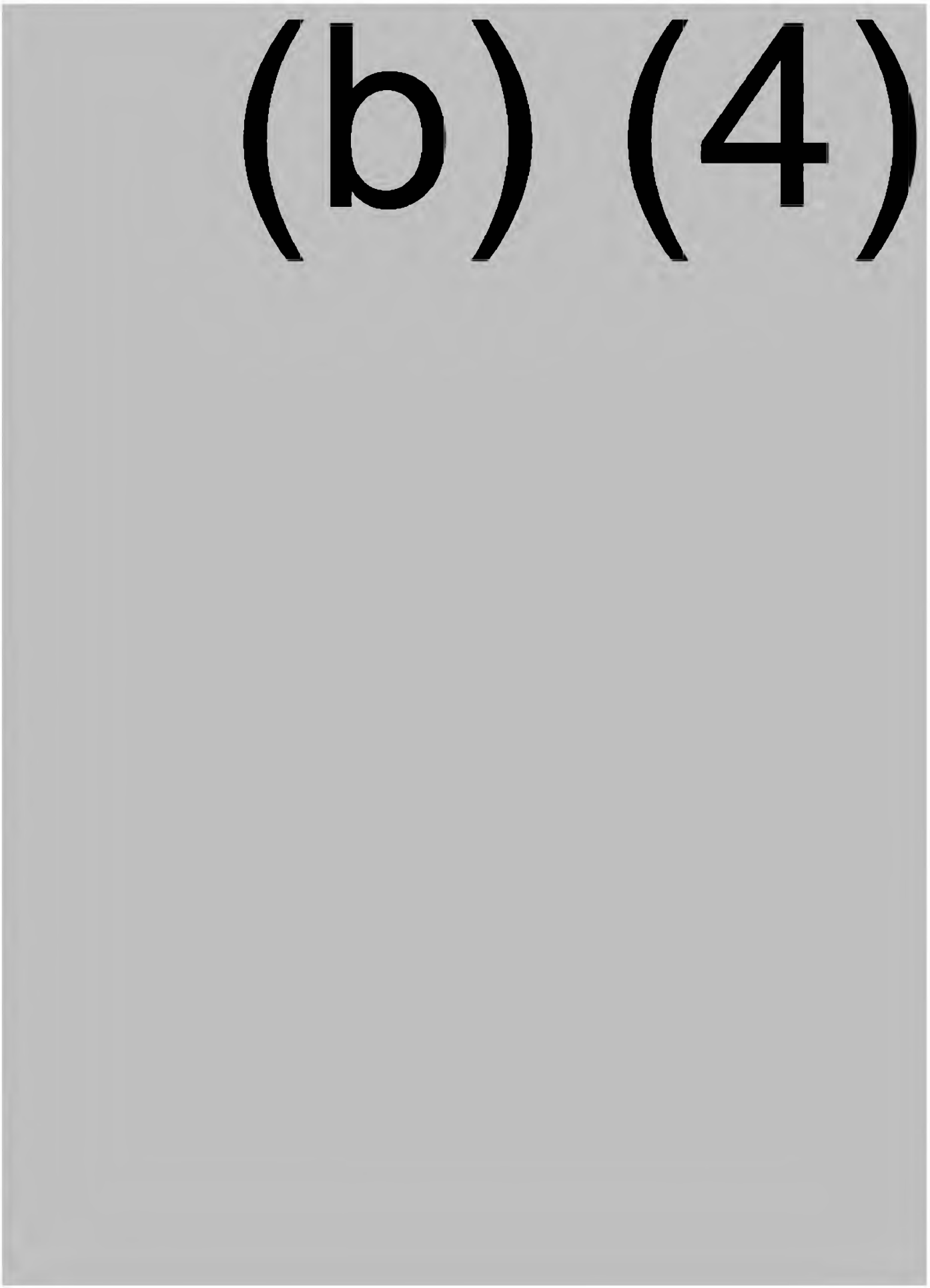
KEYWORDS
bacteriophage; food safety; phage; pet food; raw pet food; *Salmonella*; SalmoLyse®

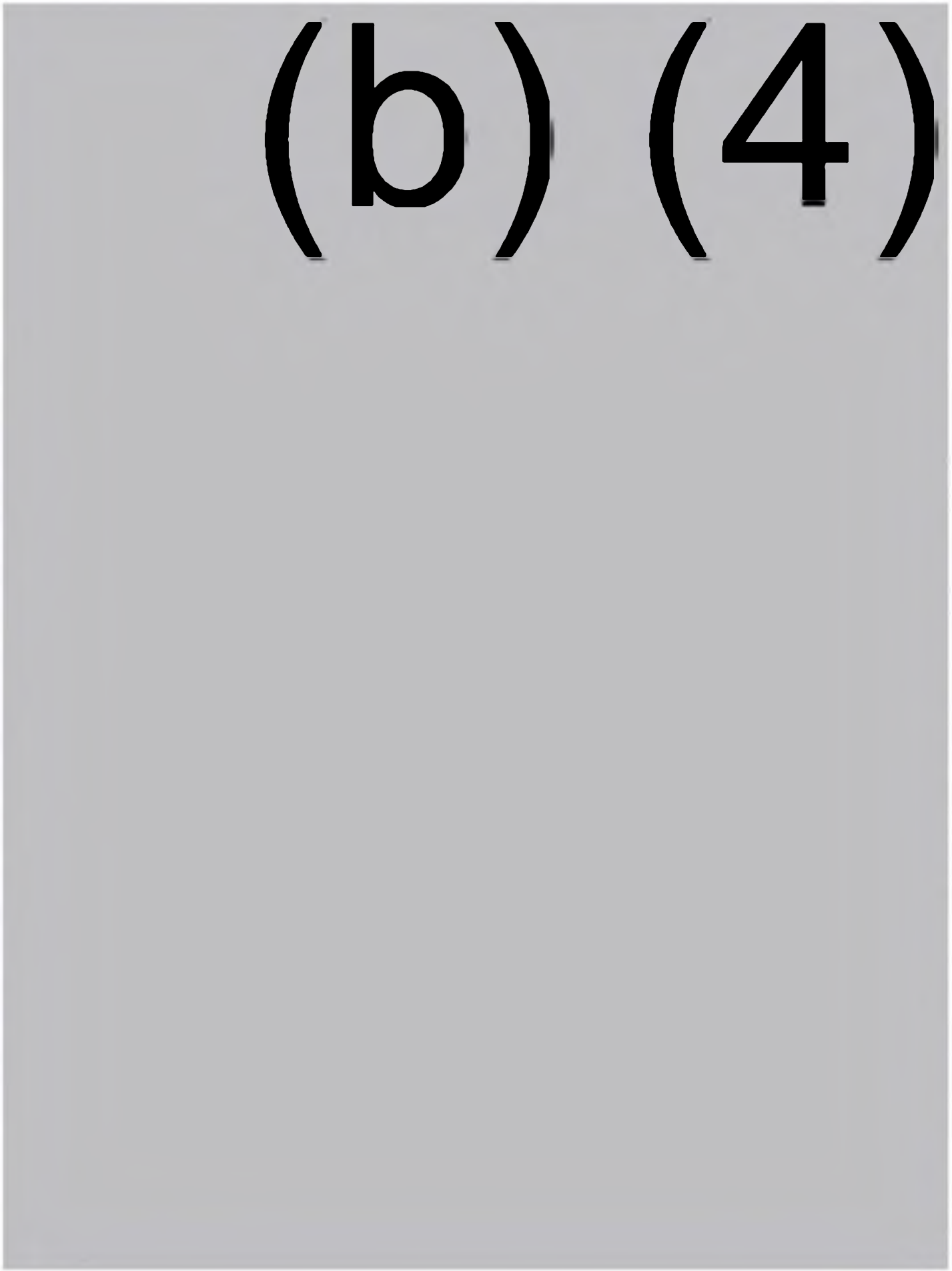


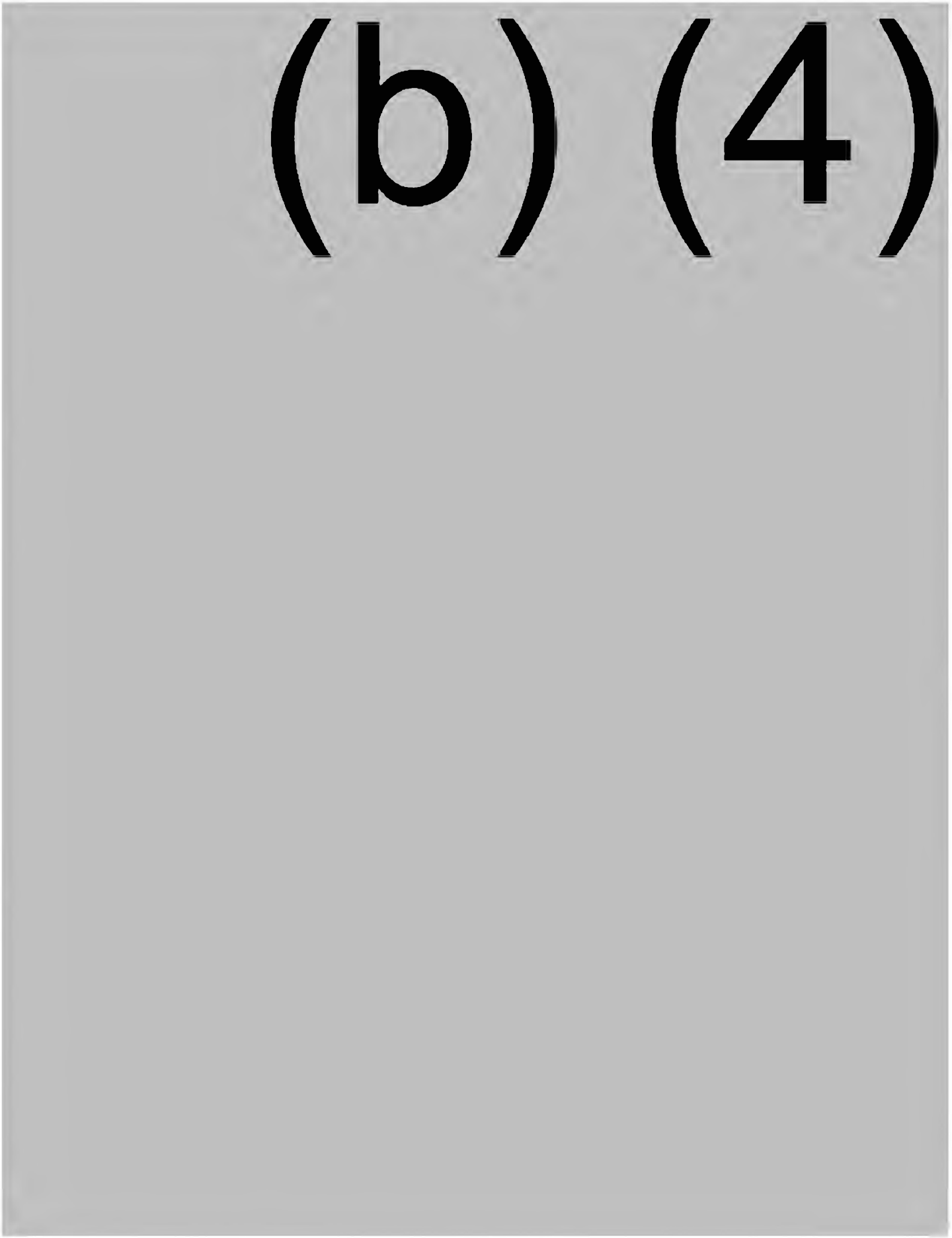

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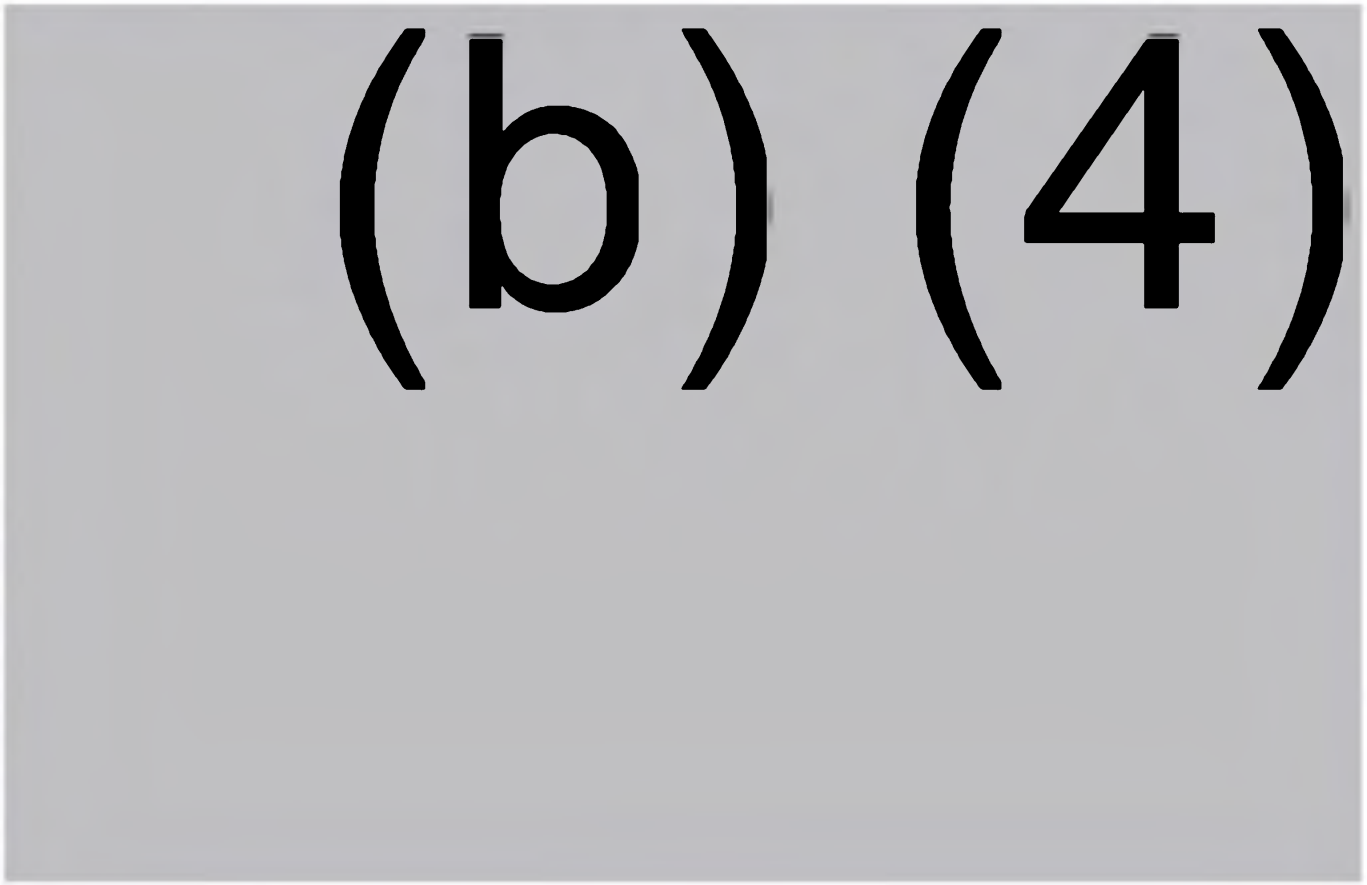

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Response to CVM query

Query - The literature review in section 6.5.3, including Table 20, does not include a comparison of the notified substance to the phage products used in the referenced studies. For example, Table 13 indicates the similarity of the substance in the listed references to the notified substance with the use of an asterisk (*) or section sign (§).

Response - Table 20 in the original submission summarizes studies demonstrating efficacy of phage administration to different animal species with no adverse effects. These studies show that administration of lytic phages, in general, is safe across several different animal species (no known adverse effects have been recorded in any of the studies). These studies were not conducted with SalmoFresh or its component monophages, although the phages listed in Table 20 are technically equivalent to phages present in SalmoFresh. Therefore, it is fully expected that SalmoFresh phages will be also as safe and effective as the phages listed in Table 20.

As per CVM FDA request of January 7, 2025, appropriately amended Section 6.5.3 is presented below.

6.5.3. LITERATURE REVIEW

Numerous studies are available in published peer reviewed literature in which animal diets were supplemented with phages. Some of those studies are summarized in the table below.

Table 1 Selected studies using oral application of bacteriophage to animals

Animal	Target bacteria	Phage(s)	Study description	Ref.
Chicken	*S.* Gallinarum	CJΦ01	Six-week-old chicks consumed feed supplemented with bacteriophage at 1E6 PFU/kg for ~28 days, with no adverse events	(Lim et al. 2011)
Chicken	*S.* Enteritidis	CJ07	Layer chickens were fed feed supplemented with bacteriophage at E5, E7, or E9 PFU/g for 21 days; neither clinical signs nor mortality was observed in any group throughout the experiment	(Lim et al. 2012)
Chicken	*S.* Typhimurium	Three-phage cocktails	Phage given orally, alone and with a competitive exclusion product, showed no adverse effects on challenged chicks	(Toro et al. 2005)
Chicken	*S.* Enteritidis	Three-phage cocktail	Chicks received bacteriophage at E8 PFU/ml in their drinking water for 1 day prior to challenge; no adverse effects were observed in any group	(Borie et al. 2008)
Chicken	*C. jejuni*	Phage NCTC 12673, vB_CcM-LmqsCPL1/1	Phage in drinking water (E7 PFU/mL, ad libitum) for 3 days, alone and with a competitive	(Peh et al. 2024)

Animal	Target bacteria	Phage(s)	Study description	Ref.
			exclusion product, showed no adverse effects on broilers	
Chicken	*S.* Typhimurium, *S.* Enteritidis, *S. gallinarum, E. coli,* and *Clostridium perfringens*	Cocktail of phages targeting *Salmonella*, *E. coli* and *C. perfringens*	Combination of phages added to feed (E8 PFU/g) for 35 days showed no adverse events	(Upadhaya et al. 2021)
Chicken	*S.* Typhimurium	SPFM10, SPFM14	Phage delivered in feed (E5-E7 PFU/day) to chickens for 42 days, no adverse effects were reported.	(Thanki et al. 2023)
Chicken	*S.* Enteritidis	Phage cocktail	Oral application of phages (E11 PFU) to challenged birds showed no adverse effects on the animals	(Fiorentin et al. 2005)
Swine	*Salmonella* spp., *E. coli*, *Staphylococcus aureus*, and *Clostridium perfringens*	Phage cocktail	Phage in feed (E9/kg), alone and with a probiotic product, for 35 days showed no adverse effects on grower pigs	(Kim et al. 2014)
Swine	*S.* Typhimurium	Phage with probiotics	Phage in feed (E9/kg) for 14 days prior to challenge showed no adverse effects compared to control feed.	(Gebru et al. 2010)
Swine	*S.* Typhimurium	Felix 01	Single oral (and intramuscular) application of phages (E10 PFU) to challenged pigs showed no adverse effects at 6 hrs post treatment	(Lee and Harris 2001)
Swine	*S.* Typhimurium	Phage cocktail	Oral application of phages (E9 PFU) every two hours for six hours to challenged pigs showed no adverse effects at 6 hrs post treatment	(Wall et al. 2010)
Swine	*E. coli* (ETEC)	Phage cocktail	Piglets challenged with *E. coli* and fed phage-supplemented feed (E9/kg) for 7 days showed no adverse effects due to phage consumption	(Han et al. 2016)
Swine	*E. coli* (ETEC)	Single phage	Challenged piglets fed phage-supplemented feed (E7/kg) for 21 days showed no adverse effects	(Lee et al. 2017)
Swine	*E. coli*	A221	Oral application of phages (E9 PFU) for 7 days to challenged piglets showed no adverse effects	(Mao et al. 2023)
Swine	*E. coli* (MDR)	vB_EcoM-RPN170, vB_EcoM-RPN171, vB_EcoM-RPN187, vB_EcoM-RPN226, vB_EcoM-RPN242, vB_EcoP-RPN243	Oral application of low (E9) and high (E10) doses of phages to pigs once a day for three days showed no adverse effects, and the microbiota in animals that received phage was similar to the control animals	(Imklin et al. 2022)

Noteworthy, the phages used in the studies presented in Table 20 are technically equivalent to the phages present in SalmoFresh. Therefore, the safety assessment can be extended to SalmoFresh

and its component monophages. Additional studies have examined bacteriophage application in animals through non-oral routes. These studies also demonstrate that there are no adverse effects on the animals and are summarized in the table below. Similarly, the phages used in the studies presented in Table 21 are technically equivalent to the phages present in SalmoFresh.

Table 2 Selected studies on bacteriophage administration in livestock via non-oral routes

Animal	Target bacteria	Safety summary	Ref.
Dogs	*Pseudomonas aeruginosa*	Topical administration of phages to the ear canal of dogs did not elicit any adverse effects, inflammation or toxicity	(Hawkins et al. 2010).
Dog	*P. aeruginosa*	A clinical case report showed that phages applied to the interdigital region of a dog were well tolerated	(Grecu et al. 2023)
Cat	*P. aeruginosa*	Throughout the period of topical application of phages to a leg infection, no side effects were observed.	(Braunstein et al. 2024)
Chickens	*E. coli*	Aerosol spray of phage (E8 - E10 PFU) onto chickens showed no adverse effects on the animals.	(Huff et al. 2002)
Mice	*S. aureus*	Intramammary application of E7 PFU did not induce any inflammatory response including neutrophilic and lymphocytic inflammatory responses.	(Breyne et al. 2017)
Mice	*S. aureus* (MRSA)	Intravenous and intraperitoneal administration of E5 or E7 PFU showed no adverse effects on mice.	(Iwano et al. 2018)
Mice	*S. aureus* (MRSA)	Phage application (E9 PFU) to abdominal cavity was considered safe in mice and cytokine experiments showed no bias.	(Wang et al. 2016)
Cows	*E. coli* (MDR)	Intramammary application of E8 PFU for 3 days showed no adverse effects in dairy cows with induced mastitis.	(Guo et al. 2021)
Cows	*E. coli* (ETEC, EPEC)	Suppository application of phage/probiotic for 5 days showed no adverse effect on the animals.	(Alomari et al. 2021)

In addition to the studies presented in Table 2, the safety of phages has been evaluated in a number of other animal species including sheep, horse, rats, mice, rabbits, and fish; insects such as greater wax moths and Drosophila; and nematode (*Caenorhabditis elegans*) (Penziner et al. 2021; Yang et al. 2024). The extensive testing in animals, including at least 11 mammal species, a fish, 2 insects, and a nematode species has demonstrated that phage application across all of them is safe and does not elicit any adverse effects (Penziner et al. 2021). Thus, it is reasonable to conclude that lytic phages, including those present in SalmoFresh, are safe for animal consumption and are not expected to cause any negative side effects.

Howell, Lauren

From: Alexander Sulakvelidze <asulakvelidze@intralytix.com>
Sent: Wednesday, May 28, 2025 10:34 AM
To: Animalfood-premarket
Subject: [EXTERNAL] RE: [EXT] Status Update for GRAS Notice AGRN #74 Lytic Bacteriophage Preparation
Attachments: Response to CVM Questions May 28 2025.pdf

Categories: Yellow Category

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Lauren,

Please find attached our responses to the questions in your questionnaire sent on May 14, 2025.

Let me know if anything requires further clarification or if you have any additional questions or comments.

Thank you!

Sandro

RECEIVED DATE
MAY 28, 2025

Alexander "Sandro" Sulakvelidze, Ph.D.
President and CEO
Intralytix, Inc.
8681 Robert Fulton Dr.
Columbia, MD 21046

Phone: 410-625-2533
Fax: 410-625-2506
E-mail: asulakvelidze@intralytix.com
www.intralytix.com

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Wednesday, May 14, 2025 5:01 PM
To: Alexander Sulakvelidze <asulakvelidze@intralytix.com>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: [EXT] Status Update for GRAS Notice AGRN #74 Lytic Bacteriophage Preparation

Dear Dr. Sulakvelidze,

CVM is currently evaluating Animal GRAS Notice No. 74, Lytic Bacteriophage Preparation, which was submitted on behalf of Intralytix, Inc. At this time, CVM has identified some questions regarding the chemistry, manufacturing, and controls; utility; and microbial safety sections that could potentially be addressed in a minor, clarifying amendment. Please see the questions below:

Chemistry, Manufacturing, and Controls

A. Specifications, Batch Analysis, and Potential Contaminants/Impurities

1. The established specification limit for the endotoxin content in the marketed product (<25,000 EU/mL (at ~9.0 $\log_{10}$ PFU/mL)) is significantly higher than the analytical results from the batch analysis in Table 4 and lot analysis data provided in Table 5 (the highest analyzed endotoxin content was <5,000 EU/mL (at ~9.0 $\log_{10}$ PFU/mL)). The notifier should establish the specification limit for the endotoxin content based on the provided batch analysis data.

2. The chemical analysis data provided in Table 5 of the current submission are the same as those included in GRN 435, submitted in 2012 for human food use. Also, the chemical analysis data provided in Table 5 does not contain data for mercury. The notifier should provide chemical analysis data including the analysis of mercury content using recently manufactured batches of the marketed product, SalmoFresh™. The notifier should also provide the analytical methods that are used for this chemical analysis.

B. Stability

3. The specification sheets provided for monophages indicate that real time stability studies were designed to measure phage identity, titer, endotoxin testing, and microbial purity. However, stability data were provided only for phage titers. The notifier should provide stability data for the microbial purity and endotoxin testing for the duration of the claimed shelf-life of two years for the component monophages.

C. Homogeneity

4. Inconsistent application methods of the product may lead to suboptimal surface coverage and inconsistent reduction in *Salmonella*. Therefore, the notifier should clearly describe the recommended application method procedure of SalmoFresh™ onto the animal feed for target animal species. The notifier should also provide data or information to demonstrate that the marketed product, SalmoFresh™, can be distributed uniformly onto targeted animal feed.

Utility

1. The data provided by the notifier in the utility studies are based on a bacteriophage preparation of six bacteriophages (SBA-1781, SKML-39, SPT-1, SSE-121, STML-13-1, and STML-198). Thus, the notified substance should be identified as the bacteriophage preparation consisting of the six individually purified lytic, double-stranded DNA bacteriophages (SBA-1781, SKML-39, SPT-1, SSE-121, STML-13-1, and STML-198). For example, the statement on page 9 that "SalmoFresh consists of a mixture of equal proportions of up to six individually purified lytic bacteriophages...." should be revised.

2. Based on the utility data, CVM recommends that the intended use of the phage cocktail be updated to reflect the notified substance's demonstrated utility to reduce but not eliminate *Salmonella* in animal food: "the notified substance helps control *Salmonella* contamination in animal food and animal food ingredients."

3. The notifier has provided data for reducing *Salmonella* in different animal food matrices with different treatment conditions, e.g. one hour at room temperature for the *Salmonella*-inoculated kibble, two minutes at room temperature for the undistributed *Salmonella*-contaminated commercial kibble, five minutes at room temperature for turkey trims, raw chicken, tuna, cantaloupe, and lettuce, and one hour or twenty-four hours at 4 °C for raw chicken and beef pet food, raw beef dog food, raw pork dog food,

and raw chicken dog food. The notifier should clarify the conditions of use to ensure the intended effect can be achieved.

4. Lytic specificity: When used in animal food, the phage cocktail should have demonstrated utility for those *Salmonella* serotypes that are pathogenic for animal species listed in FDA CVM's Compliance Policy Guide (CPG) 690.800, *Salmonella* in Foods for Animals. If the notifier intends the notified substance to be used in feeds for specific animal species, information should be provided with multiple disease-associating/representative isolates/strains in those pathogenic serotypes for the target animal species:

    a. Poultry: *Salmonella* Pullorum and *Salmonella* Gallinarum
    b. Swine: *Salmonella* Choleraesuis
    c. Sheep: *Salmonella* Abortusovis
    d. Horse: *Salmonella* Abortusequi
    e. Dairy and beef cattle: *Salmonella* Dublin

    Alternatively, if this information is not available at this time, the notifier may revise the intended use of the notified substance to narrow the types of animal food to which the phage will be applied.

5. The notifier provides several names for the bacteriophage cocktail, such as SalmoFresh, SalmoLyse, SalmoFresh 27SP. Please clarify if they all refer to the notified substance.

Microbial Safety

1. We note that the notifier provides a "inclusion criteria" on page 7 of this GRAS notice, which states that "[t]he product may be safely used as an antimicrobial in accordance with the following conditions". Please clarify whether the notifier intends to substitute new *Salmonella* host strains or phage isolates for the existing ones in the final marketed formulation. Any new strains would not be covered by this GRAS notice.

CVM requests that the information be sent no later than Wednesday, May 28, 2025 by 5:00 PM ET in order to complete the evaluation of this GRAS notice.

Sincerely,
Lauren

Lauren R. Howell, MPH
Consumer Safety Officer

U.S. Food and Drug Administration
Department of Health and Human Services
Tel: 240-402-8012



Response to CVM Questions

Chemistry, Manufacturing, and Controls

A. Specifications, Batch Analysis, and Potential Contaminants/Impurities

1. The established specification limit for the endotoxin content in the marketed product (<25,000 EU/mL (at ~9.0 $\log_{10}$ PFU/mL)) is significantly higher than the analytical results from the batch analysis in Table 4 and lot analysis data provided in Table 5 (the highest analyzed endotoxin content was <5,000 EU/mL (at ~9.0 $\log_{10}$ PFU/mL)). The notifier should establish the specification limit for the endotoxin content based on the provided batch analysis data.

Response: To the best of our knowledge, there are currently no regulatory limits on endotoxin levels in either animal or human foods in the United States. However, GRAS Notice GRN 435 specifies an endotoxin threshold of ≤25,000 EU/mL (approximately 9.0 Log_{10} PFU/mL) for SalmoFresh in human food applications, a level deemed acceptable by the FDA CFSAN. Intralytix has voluntarily established this limit as part of its Good Manufacturing Practice (GMP) program, ensuring that SalmoFresh is produced to the highest standards of quality and safety for both animal and human food applications. As discussed in the original Animal GRAS Notice No. 74 in Section 3.1.2, even at the highest endotoxin concentration of 25,000 EU/mL (at ~9.0 Log_{10} PFU/mL), the worst-case scenario of endotoxin contributed to animal food would be only 2.5% of what is typically present in those foods, and therefore safe for oral consumption.

As outlined in Animal GRAS Notice No. 74, SalmoFresh is produced by growing individual monophages on their specific host bacterial strains, followed by a series of filtration steps to remove bacterial remnants, including endotoxins. While Intralytix follows strict quality control procedures, because of the nature of phage manufacturing, there may be instances where endotoxin levels exceed 5,000 EU/mL. However, as stated in Animal GRAS Notice No. 74, if endotoxin concentrations surpass 25,000 EU/mL, the affected SalmoFresh lot will not be released for commercial use. By setting the endotoxin limit at ≤25,000 EU/mL (at approximately 9.0 Log_{10} PFU/mL), Intralytix is able to ensure the product's quality and safety without imposing excessive production constraints. Therefore, in order to (i) provide a degree of reasonable flexibility in the production process, (ii) maintain consistency in our internal quality controls and established Methods and Standard Operating Procedures, and (iii) align with GRAS Notice GRN 435 for SalmoFresh, we request retaining the endotoxin specification of ≤25,000 EU/mL (at ~9.0 Log_{10} PFU/mL) in Animal GRAS Notice No. 74.

2. The chemical analysis data provided in Table 5 of the current submission are the same as those included in GRN 435, submitted in 2012 for human food use. Also, the chemical analysis data provided in Table 5 does not contain data for mercury. The notifier should provide chemical analysis data including the analysis of mercury content using recently manufactured batches of the marketed product, SalmoFresh™. The notifier should also provide the analytical methods that are used for this chemical analysis.

Response: The manufacturing process and ingredients for SalmoFresh have remained unchanged since the submission of GRN 435, so we have provided the same data previously submitted. However, in response to the request from the FDA CVM, we have sent three recently produced batches of SalmoFresh for chemical analysis, including testing for mercury. We will update the FDA CVM with the results and the analytical methods that were used for this analysis as soon as they are available.

B. Stability

3. The specification sheets provided for monophages indicate that real time stability studies were designed to measure phage identity, titer, endotoxin testing, and microbial purity. However, stability data were provided only for phage titers. The notifier should provide stability data for the microbial purity and endotoxin testing for the duration of the claimed shelf-life of two years for the component monophages.

Response:
Historically, for other food-related submissions, only potency (phage titer) stability data for the monophages has been required. To address the FDA CVM's request, Intralytix evaluated the microbial stability of the component monophages in SalmoFresh™ using available retention samples. Most monophage batches listed in Section 2.5.1 of the original application had retention samples available, except SKML-39. Therefore, one SKML-39 sample from a previous manufacturing run (lot # (b) (4)) was included in the testing. As shown below, the microbial purity of these lots remained stable over time - maintaining stability for at least 2 years in the available SKML-39 lot # (b) (4), and over 5 years in the oldest tested retention sample, SBA1781 lot (b) (4).

Monophage	Batch / Lot #	Days	Microbial purity results
SBA1781	(b) (4)	1895	No growth
SBA1781		1007	No growth
SPT1		1624	No growth
SPT1		1154	No growth
SSE121		1840	No growth
SSE121		1553	No growth
STML131		1364	No growth
STML131		629	No growth
STML198		1847	No growth
STML198		1616	No growth
SKML39		748	No growth

"Toxin Testing" (e.g., endotoxin testing) is not included in the analytical requirements on the specification sheets for the monophages; instead, our standard procedure for SalmoFresh production lots is to test endotoxin levels at the time of manufacturing to ensure they meet our established specifications. We do not perform endotoxin testing during SalmoFresh storage, as endotoxin levels are not expected to increase in microbiologically pure phage preparations. However, we do conduct such testing for two other technically equivalent phage products currently in clinical trials in the USA, which target other Gram-negative bacteria: *Shigella* (product name: ShigActive; clinicaltrials.gov #NCT05182749) and adherent-invasive *E. coli* (AIEC) (product name: EcoActive; clinicaltrials.gov #NCT03808103). Testing of these products indicates that endotoxin levels remain stable during long-term storage - up to 60 months for EcoActive and 48 months for ShigActive, based on current data.

C. Homogeneity

4. Inconsistent application methods of the product may lead to suboptimal surface coverage and inconsistent reduction in *Salmonella*. Therefore, the notifier should clearly describe the recommended application method procedure of SalmoFresh™ onto the animal feed for target animal species. The notifier should also provide data or information to demonstrate that the marketed product, SalmoFresh™, can be distributed uniformly onto targeted animal feed.

Response: Our team actively collaborates with customers to optimize the phage application based on the customer's product type and processing workflow. In general, our recommendation is to evenly apply SalmoFresh™ over the food matrix to maximize the coverage of the product surface. For example, when treating foods as they move along a conveyor belt, this may involve installing spray systems with multiple strategically positioned nozzles and incorporating mechanisms to flip the product. This ensures

complete and uniform coverage, including the side that was in contact with the belt. We have also made significant progress in designing and optimizing spray systems to deliver consistent and reliable applications of our phage products, including SalmoFresh, onto the surface of various food items, as detailed below.

Intralytix has collaborated with a leading US-based spray system manufacturer to identify and implement optimal methods for applying phages onto various food surfaces. This work has focused on ensuring optimal: (1) spray droplet size that maximizes efficiency of phage delivery - minimizing losses due to parabolic rebound from the food surface when droplets are too large and preventing unintended drift by air currents in the facility when droplets are too small, and (2) pressure range and application distance and pattern to reduce harmful shear stress and maximize surface coverage. As a result of these efforts, phage particles sprayed onto food surfaces reach their target with minimal losses, and in a more viable state. We provide all our customers with this computer-controlled, phage-optimized spray system or work with them to retrofit their existing systems. This latter includes ensuring the use of appropriate nozzle sizes and operation within our recommended ranges for spray pressure and distance, to ensure effective phage delivery across diverse food matrices. Of note, our atomized spray application method is currently patent-pending (U.S. Patent Application No. 19/049966), and its development was supported by an SBIR grant from the United States Department of Agriculture (award #2023-70439-39191), specifically designed to deliver optimal SalmoFresh™ coverage across a variety of food products.

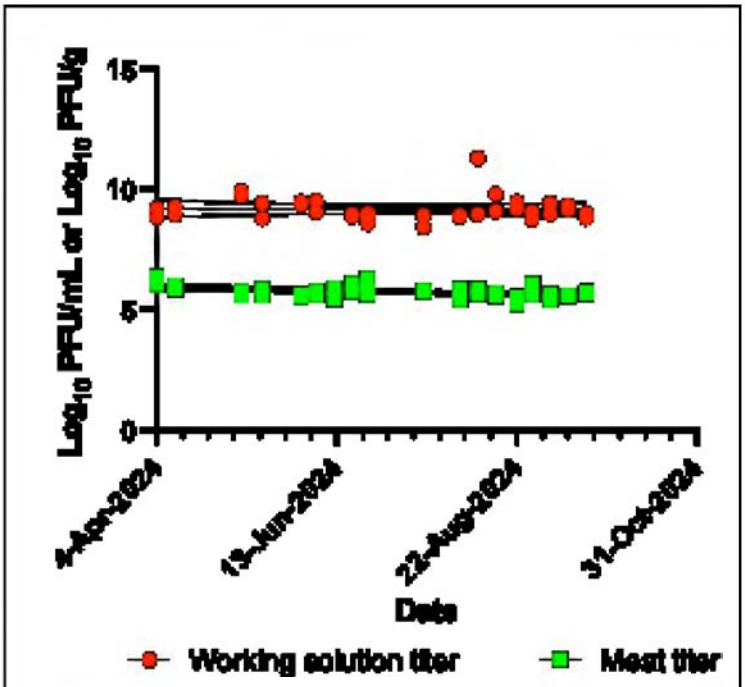


Figure 1. SalmoFresh™ phage recoveries from solution prior to application onto ground poultry (red dots) and from treated ground poultry samples post-application (green dots), measured over a 7-month period.

As shown in Figure 1, data collected over approximately seven months demonstrate that the atomized spray method provides highly consistent application of SalmoFresh on ground poultry. In this study, conducted at a large commercial facility in the southeastern United States, phage was applied to 8,000-pound batches of ground

poultry. Periodic 1-pound representative samples were collected and analyzed to assess phage presence and concentration. The results showed a very consistent phage recovery throughout the study period, indicating a reliable and uniform application of SalmoFresh.

Utility

1. The data provided by the notifier in the utility studies are based on a bacteriophage preparation of six bacteriophages (SBA-1781, SKML-39, SPT-1, SSE-121, STML-13-1, and STML-198). Thus, the notified substance should be identified as the bacteriophage preparation consisting of the six individually purified lytic, double-stranded DNA bacteriophages (SBA-1781, SKML-39, SPT-1, SSE-121, STML-13-1, and STML-198). For example, the statement on page 9 that "SalmoFresh consists of a mixture of equal proportions of up to six individually purified lytic bacteriophages...." should be revised.

Response: As described in our response to Utility question #5 below, the data provided includes studies performed with SalmoFresh™, SalmoLyse™, and SalmoFresh™ 27SP. SalmoFresh™ and SalmoLyse™ are different trade names referring to the same substance - a six-bacteriophage cocktail specifically designed to target *Salmonella*. The name SalmoFresh™ 27SP is an internal catalog number which refers to a blend of SalmoFresh that includes three of the six bacteriophages (e.g., STML-198, SPT-1, and SSE-121). This version of the cocktail was included to demonstrate the technical equivalency of adjusting the cocktail composition. Please see the response to the Microbial Safety question below for further explanation.

2. Based on the utility data, CVM recommends that the intended use of the phage cocktail be updated to reflect the notified substance's demonstrated utility to reduce but not eliminate *Salmonella* in animal food: "the notified substance helps control *Salmonella* contamination in animal food and animal food ingredients."

Response: We concur with the CVM's recommendation to revise the language in Section 2.6 to state: "SalmoFresh is intended for use as an antimicrobial to help control *Salmonella* contamination in animal food and animal food ingredients when applied at up to 1×10^{8} PFU per gram of food or food ingredient."

3. The notifier has provided data for reducing *Salmonella* in different animal food matrices with different treatment conditions, e.g. one hour at room temperature for the *Salmonella*-inoculated kibble, two minutes at room temperature for the undistributed *Salmonella*-contaminated commercial kibble, five minutes at room

temperature for turkey trims, raw chicken, tuna, cantaloupe, and lettuce, and one hour or twenty-four hours at 4°C for raw chicken and beef pet food, raw beef dog food, raw pork dog food, and raw chicken dog food. The notifier should clarify the conditions of use to ensure the intended effect can be achieved.

Response: Data submitted in Animal GRAS Notice 74 demonstrate that SalmoFresh™ can effectively reduce *Salmonella* levels within 2 to 5 minutes at room temperature. Additionally, data from Human Foods GRAS Notice (GRN 435) show that SalmoFresh™ significantly reduces *Salmonella* on various food matrices, including raw chicken and beef products, within 15 minutes at 4°C. Based on these findings, we recommend a minimum contact time of 5 minutes for SalmoFresh™ application. We generally advise that food processors apply SalmoFresh™ during the final stages of processing, immediately prior to packaging, which ensures minimum contact time. Our team collaborates closely with each customer or processor to tailor the application method and timing to their specific operational workflows, ensuring maximum effectiveness.

4. Lytic specificity: When used in animal food, the phage cocktail should have demonstrated utility for those *Salmonella* serotypes that are pathogenic for animal species listed in FDA CVM's Compliance Policy Guide (CPG) 690.800, *Salmonella* in Foods for Animals. If the notifier intends the notified substance to be used in feeds for specific animal species, information should be provided with multiple disease-associating/representative isolates/strains in those pathogenic serotypes for the target animal species:
    a. Poultry: *Salmonella* Pullorum and *Salmonella* Gallinarum
    b. Swine: *Salmonella* Choleraesuis
    c. Sheep: *Salmonella* Abortusovis
    d. Horse: *Salmonella* Abortusequi
    e. Dairy and beef cattle: *Salmonella* Dublin

Alternatively, if this information is not available at this time, the notifier may revise the intended use of the notified substance to narrow the types of animal food to which the phage will be applied.

Response: SalmoFresh™ has been shown to have lytic activity against ~99.6% of 1,397 *Salmonella* isolates from the Intralytix collection, representing more than 60 distinct serotypes. Its intended use on animal food is to reduce the presence of *human*-pathogenic *Salmonella*, thereby helping to protect both pet owners and the eventual human

consumers of animals raised on such feed. According to CDC's BEAM Dashboard [1], the serotypes most commonly associated with foodborne illness of humans include Enteritidis, Heidelberg, Newport, I 4,[5],12:i:-, and Typhimurium. SalmoFresh™ targets 100% of these serotypes in our collection, which includes over 200 strains each of Enteritidis and Typhimurium, over 40 strains each of Heidelberg and Newport, and 8 strains of *I 4,[5],12:i:-*.

SalmoFresh™ was not specifically developed to target *Salmonella* serotypes associated with animal disease, and we currently do not maintain a large collection of such strains for testing. However, based on our limited internal collection of strains belonging to such serotypes, SalmoFresh™ has demonstrated effectiveness against 100% of the animal-pathogenic strains available, including 4 strains of Dublin, 2 of Gallinarum, and 1 of Choleraesuis. The collection does not currently include the additional animal-pathogenic *Salmonella* serotypes mentioned. However, if and when Intralytix decides to develop a *Salmonella*-targeting phage preparation for specific animal health applications (e.g., *Salmonella* Choleraesuis for swine), we will acquire the relevant strains and update the cocktail formulation with appropriate lytic phages to effectively target those serotypes.

5. The notifier provides several names for the bacteriophage cocktail, such as SalmoFresh, SalmoLyse, SalmoFresh 27SP. Please clarify if they all refer to the notified substance.

Response: SalmoFresh™ and SalmoLyse™ are different trade names referring to the same substance - a six-bacteriophage cocktail specifically designed to target *Salmonella*. The name SalmoFresh™ 27SP is an internal catalog number which refers to a blend of SalmoFresh that includes three of the six bacteriophages (e.g., STML-198, SPT-1, and SSE-121). This version of the cocktail was included to demonstrate the technical equivalency of adjusting the cocktail composition. Please see the response to the Microbial Safety question for further explanation.

Microbial Safety

1. We note that the notifier provides a "inclusion criteria" on page 7 of this GRAS notice, which states that "[t]he product may be safely used as an antimicrobial in accordance with the following conditions". Please clarify whether the notifier intends to substitute new *Salmonella* host strains or phage isolates for the existing

[1] Centers for Disease Control and Prevention (CDC). BEAM (Bacteria, Enterics, Amoeba, and Mycotics) Dashboard. Atlanta, Georgia: U.S. Department of Health and Human Services. www.cdc.gov/ncezid/dfwed/BEAM-dashboard.html. Accessed 05/21/2025.

ones in the final marketed formulation. Any new strains would not be covered by this GRAS notice.

Response: The intention is to maintain flexibility in modifying the finished product within the manufacturing and process controls outlined in the GRAS notification. This approach mirrors the flexibility granted to EcoShield PX (GRN 834) and CampyShield (GRN 966) by FDA CFSAN. The inclusion criteria for SalmoFresh™ align with the same criteria set forth in those notifications. As described in Section 2.1, paragraph 3, the inclusion of up to six monophages in the article of commerce is specifically designed to enable rapid response to real-world situations – for example, when a single serotype becomes overwhelmingly predominant in a particular food processing facility and requires a more targeted strategy. The ability to deploy various combinations of up to six lytic phages, all covered under the GRAS notice, ensures continued optimal efficacy of the phage biocontrol product, which is essential for maintaining food safety.

Any new lytic phages not listed within this current submission will be fully characterized using the criteria described in Section 2.1 of Animal GRAS Notice 74 before inclusion in the product. Furthermore, we will notify CVM of any updates that include new monophages not listed within this current submission.

Howell, Lauren

From: Alexander Sulakvelidze <asulakvelidze@intralytix.com>
Sent: Tuesday, June 10, 2025 11:03 AM
To: Animalfood-premarket
Cc: Joelle Woolston
Subject: [EXTERNAL] RE: [EXT] Status Update for GRAS Notice AGRN #74 Lytic Bacteriophage Preparation
Attachments: Response to CVM Questions of June 4 2025.pdf

Categories: Yellow Category

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Lauren,

Please find attached our responses to the questions / comments presented by CVM during our TEAMS call on June 4, 2025.

We have not yet received the results of the chemical analysis. The samples have been submitted to (b) (4) for testing, and we expect to receive their report by June 20, 2025. We will forward the report to CVM as soon as it becomes available. (b) (6) , I have asked Joelle Woolston, our VP for Lab Operations, to send the (b) (4) report to you directly upon receipt, (b) (6). Please be on the lookout for an email from Joelle instead of me. She is copied on this email for your reference.

Let me know if anything requires further clarification or if you have any additional questions or comments.

Thank you!

RECEIVED DATE
JUN 10, 2025

Sandro

Alexander "Sandro" Sulakvelidze, Ph.D.
President and CEO
Intralytix, Inc.
8681 Robert Fulton Dr.
Columbia, MD 21046

Phone: 410-625-2533
Fax: 410-625-2506
E-mail: asulakvelidze@intralytix.com
www.intralytix.com

Response to CVM Comments from Teams meeting held on June 4, 2025

Comment 1. The Dublin strain used in the study by Heyse *et al*. was noted as resistant to SalmoFresh, yet in Intralytix's response dated 05/28/25, the strain was listed as susceptible. CVM requested (1) an updated list of serotypes examined for susceptibility to SalmoFresh, and (2) further data to support that SalmoFresh is active against the Dublin strain/serotype.

Response. An updated list of serotypes tested for susceptibility to SalmoFresh is presented in Table 1 below. Some of the strains/serotypes and their susceptibility data have been revised from those reported in the Heyse *et al*. paper, as explained below.

The Intralytix *Salmonella* collection is continuously expanding and currently comprises 1,431 *Salmonella* isolates. Intralytix regularly reviews and updates its strain libraries to incorporate newly acquired data and improve strain characterization. As part of these efforts, the serotype assignments of certain strains have been revised over time, either through internal research or updates from external culture collections. For example, one of the three *Salmonella* Gallinarum strains (ATCC 9184) referenced in the 2015 publication by Heyse *et al*. was originally classified as *S.* Gallinarum by the American Type Culture Collection (ATCC) but has since been reclassified by ATCC as *Salmonella* Hillingdon. Our internal records have been updated accordingly. Another example is *Salmonella* Grampian. In the above-referenced study by Heyse *et al*., eight Grampian strains were reported. However, based on our internal validation, one of those strains has since been reclassified as *Salmonella* Oranienburg. As a result, our current catalog reflects seven Grampian strains.

The internal reviews of the collection also include performing additional or repeat testing of the susceptibility to the relevant cocktails. Initially, screening for lytic activity was performed by spot test method at 37°C. Since the 2015 paper by Heyse *et al*., the spot test method has been updated, and the screening occurs at 30°C. This lower temperature enhances visualization, enabling clearer interpretation and improved reproducibility of lysis zones on the *Salmonella* bacterial lawns. Several of the isolates have been screened again at a lower temperature and their susceptibility patterns have been updated based on the new testing method. This included the Dublin strain (ATCC 15480) mentioned in our conversation with CVM on 06/04/25, which was found to be susceptible to the phage cocktail when re-tested at the lower temperature.

As requested by CVM, below is a summary list of *Salmonella* serotypes (including the number of strains per serotype) currently in the Intralytix collection, along with their most recent susceptibility to SalmoFresh (Table 1).

Table 1 Summary list of *Salmonella* strains / serotypes in Intralytix collection and their susceptibility to SalmoFresh

Serotype	# of strains	# strains susceptible to SalmoFresh
4,[5],12:i:-	8	8
Agona	39	39
Alachua	6	6
Albany	1	1
Anatum	10	10
Berta	3	3
Braenderup	5	5
Brandenburg	2	2
Bredeney	2	2
Choleraesuis	1	1
Derby	6	6
Dublin	4	4
Enteritidis	212	212
Gallinarum	2	2
Georgia	17	17
Grampian	7	7
Hadar	174	174
Havana	3	3
Heidelberg	60	60
Hilingdon	1	1
Indiana	1	1
Infantis	63	63
Javiana	6	6
Kedougou	1	1
Kentucky	42	42
Larochelle	2	2
Litchfield	2	2
Liverpool	2	2
Livingstone	5	5
London	1	1
Mbandaka	82	78
Meleagridis	2	2
Minnesota	1	1
Montevideo	21	21
Muenchen	6	6
Muenster	5	5
Newport	41	41

Serotype	# of strains	# strains susceptible to SalmoFresh
Oakum	1	1
Ohio	20	20
Oranienburg	6	6
Orion	2	2
Othmarschen	1	1
Ouakam	2	2
Paratyphi B	4	4
Poona	1	1
Putten	1	1
Reading	8	8
Rissen	1	1
Saint Paul	4	4
Schwarzengrund	35	34
Senftenberg	31	31
Soerenga	1	1
Stanley	13	13
Tennessee	8	8
Thompson	104	104
Tilburg	1	1
Typhi	2	2
Typhimurium	264	264
Uganda	1	1
Urbana	2	2
Virchow	2	2
Westhampton	15	15
Worthington	3	3
Unknown	52	52
Totals	1,431	1,426

Comment 2. CVM provided feedback concerning the uniform distribution of SalmoFresh on animal feed, specifically referencing Figure 1 included in our response dated 05/28/25. CVM requested that Intralytix present uniformity data from a minimum of 10 samples and suggested that Intralytix either (1) supplies additional information related to the data shown in Figure 1, including clarifying whether the dataset included at least 10 randomly selected samples, or (2) performs a new mixability study utilizing 10 randomly selected samples from a batch treated with SalmoFresh to further validate uniform application.

Response. In our response dated 05/28/25, we presented a figure demonstrating the uniform distribution of phage on ground poultry. For convenience, that figure is also included below. The data presented was collected over a span of 7 months as SalmoFresh was used daily by a large-scale commercial poultry processor. During these operations, multiple batches of 8,000 lbs per batch of ground poultry were processed daily and SalmoFresh was applied to the ground poultry batches in a blender using an atomized sprayer system.

Each week, two 1-lb samples of the SalmoFresh treated meat in their final packaging were randomly collected from two different production batches. Additionally, samples of the phage working solution corresponding to each ground poultry batch were collected (e.g., the SalmoFresh solution prior to spraying onto the ground poultry). All samples were collected by facility personnel trained in sample collection procedures in accordance with guidelines provided by the Food Safety and Inspection Service (FSIS), USDA.

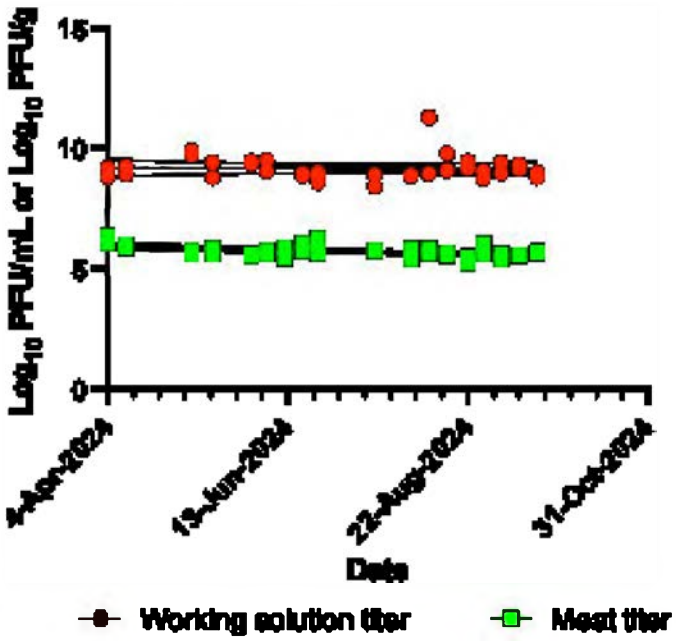


Figure 1. SalmoFresh™ phage recoveries from solution prior to application onto ground poultry (red dots) and from treated ground poultry samples post-application (green dots), measured over a 7-month period.

A total of 56 ground poultry samples and 56 working solution samples were collected over a 7-month period and analyzed for phage recovery and application uniformity. As noted above, SalmoFresh was applied to multiple production batches each day, with two random batches sampled weekly. **As a result, the data in Figure 1 reflect the consistency of SalmoFresh application based on 56 sampling events - representing a dataset roughly five times larger than what CVM has requested**. These samples collectively represent treatment across more than 12 million pounds of ground poultry, providing a robust assessment of application uniformity under real-world commercial conditions.

Generally, we observed a consistent ~2-log difference between the phage application dose (i.e., phage working solution) and the recovered levels. This lower recovery was

fully expected and is likely due to three main factors: (1) not 100% of phage particles actually reach the food on which they are being sprayed, (2) the phages become trapped within the ground poultry matrix, making recovery more difficult, and (3) a time lapse of approximately 24 hours between phage application at the facility and phage recovery testing at Intralytix. However, and directly relevant to the FDA's question / comment about application uniformity, phage recovery results were highly consistent - demonstrating uniform application of SalmoFresh across large batches of ground poultry over an extended period. Additional data supporting the uniformity of phage application, based on analysis of multiple samples resembling mash food, is provided below in our response to CVM Comment #3.

Comment 3. Along the same lines as Comment 2, CVM provided comments regarding the overall assessment of the uniformity of phage distribution within various food matrices, with the use of SalmoFresh on mash food products as an example.

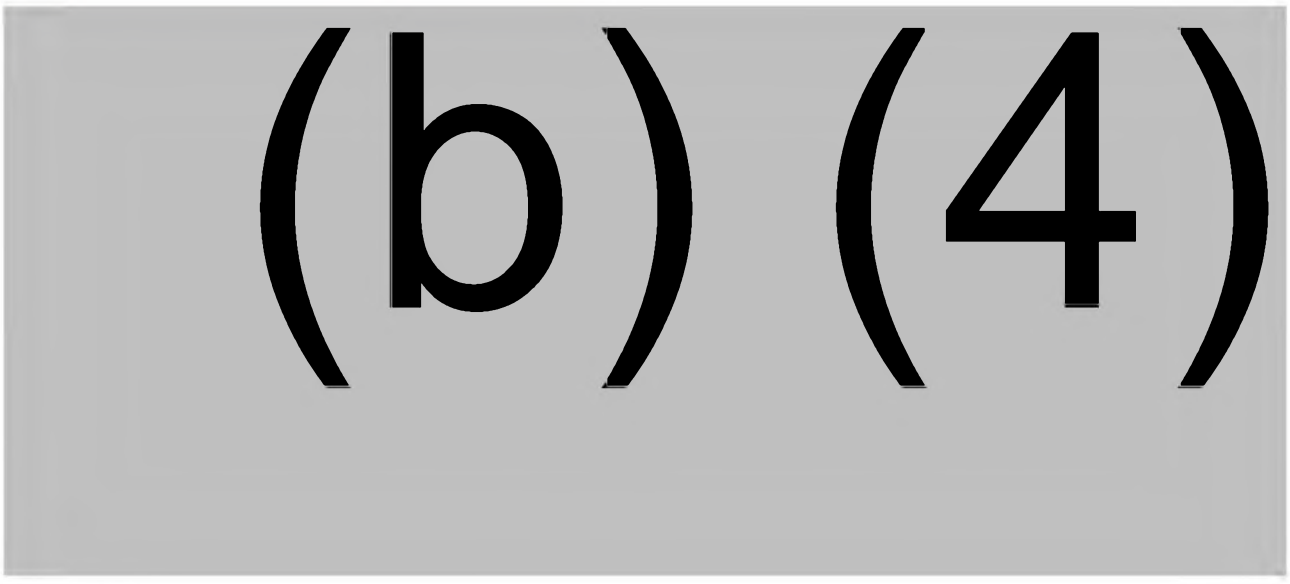
Response. Preliminary studies performed in preparation for the Heyse *et al*. paper included examining the content uniformity of the phage cocktail on dry pet kibble across a production batch (data not published). This study examined how uniformly phage was distributed across a high-volume dog formula produced on equipment 1/16th the size of full-scale production equipment. During production, phage was applied during the enrobing process via two technology coater systems to a concentration of approximately 2×10^7 PFU/g kibble. Kibble samples were collected at the beginning, middle, and end of the run to examine the uniformity of phage application throughout the run. Bacteriophage was recovered from the collected kibble samples by adding 25 g of kibble to 225 mL saline, stomaching for 2 minutes, centrifuging to pellet large debris, filtered through a 0.22 filter, and serially diluted. Appropriate dilutions were titered on the titer host following standard titration methods.

As shown in Table 2, this study demonstrated that phage applied to dry kibble in the pilot plant via spray application with two different coating systems was uniformly applied as the samples collected at the beginning, middle, and end of the production run demonstrated a low level of variance (0.15 log PFU/g kibble). As seen in the ground poultry example provided in the response to Comment 2, the recovered bacteriophage titers were less than the application concentration of 2×10^7 PFU/g kibble, due to the low extraction efficacy of recovering phage from the kibble matrix. However, these results demonstrate that phage is uniformly applied to kibble using a spray application independent of enrobing system.

Table 2 Summary of titers recovered from kibble samples taken throughout kibble production

	Coater A		Coater B	
	Average (Log PFU/g)	Standard Error (Log PFU/g)	Average (Log PFU/g)	Standard Error (Log PFU/g)
Beginning	5.07	0.0404	4.43	0.0404
Middle	4.92	0.0404	4.54	0.0404
End	4.95	0.0404	4.58	0.0404

With respect to the uniformity of phage distribution in other types of food matrices - particularly mash food products used by the CVM as an example - our data (summarized below) demonstrate a similarly uniform distribution to that observed with dry kibbles. Mash foods - coarse-milled grains blended with vitamins and minerals - undergo processing similar to that of wheat milling. We have conducted both laboratory and field trials to evaluate phage application uniformity and effectiveness on this type of food matrix, using EcoShield™ PX, a technically equivalent to SalmoFresh phage preparation targeting Shiga toxin-producing *Escherichia coli* (STEC). The lab trial utilized two *E. coli* O157:H7 strains while the field trial was conducted in a commercial milling facility with one non-pathogenic surrogate *E. coli* strain to assess application consistency and effectiveness under real-world conditions. The results demonstrated that EcoShield PX application effectively reduced the *E. coli* levels on flour and other milled fractions. This work has now been accepted for publication in *Journal of Food Protection* (Vikram, A., McCarty, K., Callahan, M.T., Sullivan, J., Sulakvelidze, A., (2025). Biocontrol of two *Escherichia coli* O157:H7 strains and a non-pathogenic surrogate *E. coli* in wheat grains and wheat milling operations using a lytic bacteriophage cocktail. Journal of Food Protection, 100557. https://doi.org/10.1016/j.jfp.2025.100557.). The milled flour and other fractions (e.g. bran and mill feed) produced during the trial did not enter commerce.



(b) (4)

(b) (4)

Comment 4. CVM requested chemical analysis including mercury analysis data from 3 SalmoFresh lots.

Response. The samples have been submitted for chemical analysis with (b) (4) . The expected return date of the QA approved reports from (b) (4) is June 20, 2025. We will provide these reports to CVM as soon as we receive them.

Howell, Lauren

From: Joelle Woolston <jwoolston@intralytix.com>
Sent: Thursday, June 19, 2025 2:39 PM
To: Animalfood-premarket; Alexander Sulakvelidze
Subject: RE: [EXTERNAL] RE: [EXT] Status Update for GRAS Notice AGRN #74 Lytic Bacteriophage Preparation
Attachments: (b) (4)MB_Level_1 06 19 2025 1424.pdf

Categories: Yellow Category

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Lauren,

As promised, please find the (b) (4) testing report attached. The results of the chemical analysis data, including the analysis of mercury content, using recently manufactured batches of SalmoFresh are within. The report includes the analytical methods that were used for this chemical analysis.

Please let me know if there are any questions.

Best regards,

Joelle

RECEIVED DATE
JUN 23, 2025

(b) (4), (b) (6)

CERTIFICATE OF ANALYSIS

(b) (4)

Project Description

Liquid Samples

For:

Joelle Woolston

INTRALYTIX INC

8681 Robert Fulton DR

Columbia, MD 21046

(b) (6)

Thursday, June 19, 2025

Please find enclosed the analytical results for the samples you submitted to (b) (4). Review and compilation of your report was completed by (b) (4), (b) (6). If you have any questions, comments, or require further assistance regarding this report, please contact your service representative listed above.

I certify that all test results meet all of the requirements of the accrediting authority listed within this report. Analytical results are reported on a 'as received' basis unless specified otherwise. Analytical results for solids with units ending in (dry) are reported on a dry weight basis. A statement of uncertainty for each analysis is available upon request. This laboratory report shall not be reproduced, except in full, without the written approval of (b) (4) ries. The reported results are related only to the samples analyzed as received.

(b) (4), (b) (6)

(b) (4), (b) (6)

CERTIFICATE OF ANALYSIS

(b) (4)

INTRALYTIX INC

Joelle Woolston
8681 Robert Fulton DR
Columbia, MD 21046

Project Name: Liquid Samples

Project / PO Number: N/A
Received: 06/04/2025
Reported: 06/19/2025

Case Narrative

No time on COC, logged per labels.

Sample Summary Report

Sample Name	Laboratory ID	Client Matrix	Sample Type	Sample Begin	Sample Taken	Lab Received
0223E30100A64-A12	(b) (4)	Aqueous			06/03/25 09:50	06/04/25 11:00
0224B23100A42-H27		Aqueous			06/03/25 10:25	06/04/25 11:00
0224G26100A08		Aqueous			06/03/25 10:10	06/04/25 11:00

(b) (4), (b) (6)

CERTIFICATE OF ANALYSIS

(b) (4)

Analytical Testing Parameters

Client Sample ID: 0223E30100A64-A12
Sample Matrix: Aqueous
Lab Sample ID: (b) (4) **Collection Date:** 06/03/2025 9:50

Inorganics Total	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Calculation							
Nitrogen, Total as N	(b) (4)	0.200	mg/L		06/10/25 0919	06/10/25 1649	(b) (6)
EPA 351.2, Rv. 2 (1993)							
Total Kjeldahl Nitrogen (TKN)		0.200	mg/L		06/10/25 0858	06/10/25 1208	
EPA 353.2, Rv. 2 (1993)							
Nitrate & Nitrite as N		0.0500	mg/L		06/10/25 0919	06/10/25 1649	
SM 2540 D-2015							
Total Suspended Solids (TSS)		5.00	mg/L		06/04/25 1312	06/04/25 1422	
Wet Chem - DW/EPA 365.4							
Phosphorus as P		0.200	mg/L		06/10/25 0906	06/10/25 1003	
Wet Chem - DW/SM 2540 C-2015							
Total Dissolved Solids (TDS)		10.0	mg/L		06/06/25 1434	06/06/25 1505	
Wet Chem - DW/SM 5310 C-2014							
Total Organic Carbon (TOC)		1.00	mg/L		06/04/25 0843	06/05/25 0028	

Metals Total by AA	Result	RL	Units	Note	Prepared	Analyzed	Analyst
EPA 7470A							
Mercury	(b) (4)	0.000200	mg/L		06/17/25 0730	06/18/25 1114	(b) (6)

Metals Total by ICP	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Calculation							
Silica as SiO2 (Calc)	(b) (4)	1.71	mg/L		06/10/25 0943	06/11/25 1953	(b) (6)
EPA 200.7/EPA 200.7, Rv. 4.4 (1994)							
Arsenic		0.0100	mg/L		06/10/25 0943	06/11/25 1953	
Barium		0.00800	mg/L		06/10/25 0943	06/11/25 1953	
Cadmium		0.00160	mg/L	Q2	06/10/25 0943	06/11/25 1953	
Calcium		0.400	mg/L		06/10/25 0943	06/11/25 1953	
Chromium		0.00400	mg/L		06/10/25 0943	06/11/25 1953	
Cobalt		0.00400	mg/L		06/10/25 0943	06/11/25 1953	
Copper		0.00800	mg/L	Q2	06/10/25 0943	06/11/25 1953	
Iron		0.0800	mg/L		06/10/25 0943	06/19/25 1156	
Lead		0.0160	mg/L	Q2	06/10/25 0943	06/11/25 1953	
Magnesium		0.400	mg/L		06/10/25 0943	06/11/25 1953	
Manganese		0.00800	mg/L		06/10/25 0943	06/11/25 1953	
Molybdenum		0.0160	mg/L		06/10/25 0943	06/11/25 1953	
Nickel		0.0160	mg/L	Q2	06/10/25 0943	06/11/25 1953	
Phosphorus as P		0.800	mg/L		06/10/25 0943	06/11/25 1953	
Potassium		0.800	mg/L		06/10/25 0943	06/11/25 1953	
Silicon		0.800	mg/L		06/10/25 0943	06/11/25 1953	
Sodium		0.400	mg/L		06/10/25 0943	06/11/25 1953	
Tin		0.400	mg/L	Q2	06/10/25 0943	06/11/25 1953	

(b) (4), (b) (6)

(b) (4)

CERTIFICATE OF ANALYSIS

(b) (4)

Client Sample ID:	0223E30100A64-A12		
Sample Matrix:	Aqueous		
Lab Sample ID:	(b) (4)	**Collection Date:**	06/03/2025 9:50

Metals Total by ICP	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Zinc	(b) (4)	0.0200	mg/L		06/10/25 0943	06/11/25 1953	(b) (6)

Anions by IC	Result	RL	Units	Note	Prepared	Analyzed	Analyst
EPA 300.0, Rv. 2.1 (1993)							
Chloride	(b) (4)	4.00	mg/L	**D3**		06/12/25 1723	(b) (6)
Nitrate as N		0.600	mg/L	**H2**		06/05/25 1110	
Nitrite as N		0.200	mg/L	**H2**		06/05/25 1110	

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

(b) (4)

Client Sample ID:	0224B23100A42-H27
Sample Matrix:	Aqueous
Lab Sample ID:	(b) (4)
Collection Date:	06/03/2025 10:25

Inorganics Total	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Calculation							
Nitrogen, Total as N	(b) (4)	0.200	mg/L		06/10/25 0919	06/10/25 1651	(b) (6)
EPA 351.2, Rv. 2 (1993)							
Total Kjeldahl Nitrogen (TKN)		0.200	mg/L		06/10/25 0858	06/10/25 1210	
EPA 353.2, Rv. 2 (1993)							
Nitrate & Nitrite as N		0.0500	mg/L		06/10/25 0919	06/10/25 1651	
SM 2540 D-2015							
Total Suspended Solids (TSS)		5.00	mg/L		06/04/25 1312	06/04/25 1422	
Wet Chem - DW/EPA 365.4							
Phosphorus as P		0.200	mg/L		06/10/25 0906	06/10/25 1003	
Wet Chem - DW/SM 2540 C-2015							
Total Dissolved Solids (TDS)		10.0	mg/L		06/06/25 1434	06/06/25 1505	
Wet Chem - DW/SM 5310 C-2014							
Total Organic Carbon (TOC)		1.00	mg/L		06/04/25 0843	06/05/25 0051	

Metals Total by AA	Result	RL	Units	Note	Prepared	Analyzed	Analyst
EPA 7470A							
Mercury	(b) (4)	0.000200	mg/L		06/17/25 0730	06/18/25 1119	(b) (6)

Metals Total by ICP	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Calculation							
Silica as SiO2 (Calc)	(b) (4)	1.71	mg/L		06/10/25 0943	06/11/25 2006	(b) (6)
EPA 200.7/EPA 200.7, Rv. 4.4 (1994)							
Arsenic		0.0100	mg/L		06/10/25 0943	06/11/25 2006	
Barium		0.00800	mg/L		06/10/25 0943	06/11/25 2006	
Cadmium		0.00160	mg/L	Q2	06/10/25 0943	06/11/25 2006	
Calcium		0.400	mg/L		06/10/25 0943	06/11/25 2006	
Chromium		0.00400	mg/L		06/10/25 0943	06/11/25 2006	
Cobalt		0.00400	mg/L		06/10/25 0943	06/11/25 2006	
Copper		0.00800	mg/L	Q2	06/10/25 0943	06/11/25 2006	
Iron		0.0800	mg/L		06/10/25 0943	06/19/25 1200	
Lead		0.0160	mg/L	Q2	06/10/25 0943	06/11/25 2006	
Magnesium		0.400	mg/L		06/10/25 0943	06/11/25 2006	
Manganese		0.00800	mg/L		06/10/25 0943	06/11/25 2006	
Molybdenum		0.0160	mg/L		06/10/25 0943	06/11/25 2006	
Nickel		0.0160	mg/L	Q2	06/10/25 0943	06/11/25 2006	
Phosphorus as P		0.800	mg/L		06/10/25 0943	06/11/25 2006	
Potassium		0.800	mg/L		06/10/25 0943	06/11/25 2006	
Silicon		0.800	mg/L		06/10/25 0943	06/11/25 2006	
Sodium		0.400	mg/L		06/10/25 0943	06/11/25 2006	
Tin		0.400	mg/L	Q2	06/10/25 0943	06/11/25 2006	
Zinc		0.0200	mg/L		06/10/25 0943	06/11/25 2006	

(b) (4), (b) (6)

(b) (4)

CERTIFICATE OF ANALYSIS

(b) (4)

Client Sample ID:	0224B23100A42-H27
Sample Matrix:	Aqueous
Lab Sample ID:	(b) (4)
Collection Date:	06/03/2025 10:25

Anions by IC	Result	RL	Units	Note	Prepared	Analyzed	Analyst
EPA 300.0, Rv. 2.1 (1993)	(b) (4)						(b) (6)
Chloride		4.00	mg/L	D3		06/12/25 1740	
Nitrate as N		0.600	mg/L	H2		06/05/25 1126	
Nitrite as N		0.200	mg/L	H2		06/05/25 1126	

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

(b) (4)

Client Sample ID:	0224G26100A08
Sample Matrix:	Aqueous
Lab Sample ID:	(b) (4)
Collection Date:	06/03/2025 10:10

Inorganics Total	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Calculation							
Nitrogen, Total as N	(b) (4)	0.400	mg/L		06/10/25 0919	06/10/25 1653	(b) (6)
EPA 351.2, Rv. 2 (1993)							
Total Kjeldahl Nitrogen (TKN)		0.400	mg/L		06/10/25 0858	06/10/25 1246	
EPA 353.2, Rv. 2 (1993)							
Nitrate & Nitrite as N		0.0500	mg/L		06/10/25 0919	06/10/25 1653	
SM 2540 D-2015							
Total Suspended Solids (TSS)		5.00	mg/L		06/04/25 1312	06/04/25 1422	
Wet Chem - DW/EPA 365.4							
Phosphorus as P		0.200	mg/L		06/10/25 0906	06/10/25 1004	
Wet Chem - DW/SM 2540 C-2015							
Total Dissolved Solids (TDS)		10.0	mg/L		06/06/25 1434	06/06/25 1505	
Wet Chem - DW/SM 5310 C-2014							
Total Organic Carbon (TOC)		1.00	mg/L		06/04/25 0843	06/05/25 0115	

Metals Total by AA	Result	RL	Units	Note	Prepared	Analyzed	Analyst
EPA 7470A							
Mercury	(b) (4)	0.000200	mg/L		06/17/25 0730	06/18/25 1122	(b) (6)

Metals Total by ICP	Result	RL	Units	Note	Prepared	Analyzed	Analyst
Calculation							
Silica as SiO2 (Calc)	(b) (4)	1.71	mg/L		06/10/25 0943	06/11/25 2020	(b) (6)
EPA 200.7/EPA 200.7, Rv. 4.4 (1994)							
Arsenic		0.0100	mg/L		06/10/25 0943	06/11/25 2020	
Barium		0.00800	mg/L		06/10/25 0943	06/11/25 2020	
Cadmium		0.00160	mg/L	Q2	06/10/25 0943	06/11/25 2020	
Calcium		0.400	mg/L		06/10/25 0943	06/11/25 2020	
Chromium		0.00400	mg/L		06/10/25 0943	06/11/25 2020	
Cobalt		0.00400	mg/L		06/10/25 0943	06/11/25 2020	
Copper		0.00800	mg/L	Q2	06/10/25 0943	06/11/25 2020	
Iron		0.0800	mg/L		06/10/25 0943	06/19/25 1205	
Lead		0.0160	mg/L	Q2	06/10/25 0943	06/11/25 2020	
Magnesium		0.400	mg/L		06/10/25 0943	06/11/25 2020	
Manganese		0.00800	mg/L		06/10/25 0943	06/11/25 2020	
Molybdenum		0.0160	mg/L		06/10/25 0943	06/11/25 2020	
Nickel		0.0160	mg/L	Q2	06/10/25 0943	06/11/25 2020	
Phosphorus as P		0.800	mg/L		06/10/25 0943	06/11/25 2020	
Potassium		0.800	mg/L		06/10/25 0943	06/11/25 2020	
Silicon		0.800	mg/L		06/10/25 0943	06/11/25 2020	
Sodium		0.400	mg/L		06/10/25 0943	06/11/25 2020	
Tin		0.400	mg/L	Q2	06/10/25 0943	06/11/25 2020	
Zinc		0.0200	mg/L		06/10/25 0943	06/11/25 2020	

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

(b) (4)

Client Sample ID:	0224G26100A08
Sample Matrix:	Aqueous
Lab Sample ID:	(b) (4)
Collection Date:	06/03/2025 10:10

Anions by IC	Result	RL	Units	Note	Prepared	Analyzed	Analyst
EPA 300.0, Rv. 2.1 (1993)							
Chloride	(b) (4)	4.00	mg/L	D3		06/12/25 1757	(b) (6)
Nitrate as N		0.600	mg/L	H2		06/05/25 1142	
Nitrite as N		0.200	mg/L	H2		06/05/25 1142	

Definitions

D3:	Dilution was performed due to high target analyte concentration.
H2:	Initial analysis was within holding time. Reanalysis was done past holding time.
mg/L:	Milligrams per Liter
Q2:	LCS recovery is above acceptance limits.
RL:	Reporting Limit

Cooler Receipt Log

Cooler ID: Default Cooler Temp: 5.6°C

Cooler Inspection Checklist

Ice Present or not required?	Yes	Shipping containers sealed or not required?	Yes
Custody seals intact or not required?	Yes	Chain of Custody (COC) Present?	Yes
COC includes customer information?	Yes	Relinquished and received signature on COC?	Yes
Sample collector identified on COC?	Yes	Sample type identified on COC?	Yes
Correct type of Containers Received	Yes	Correct number of containers listed on COC?	Yes
Containers Intact?	Yes	COC includes requested analyses?	Yes
Enough sample volume for indicated tests received?	Yes	Sample labels match COC (Name, Date & Time?)	No
Samples arrived within hold time?	Yes	Correct preservatives on COC or not required?	Yes
Chemical preservations checked or not required?	Yes	Preservation checks meet method requirements?	Yes
VOA vials have zero headspace, or not recd.?	Yes		

Report Comments

Reviewed and Approved By:

(b) (6)

Project Manager

Reported: 06/19/2025 14:24

The data and information on this, and other accompanying documents, represents only the sample(s) analyzed. This report is incomplete unless all pages indicated in the footnote are present and an authorized signature is included. ***The services were provided under and subject to (b) (4)'s standard terms and conditions which can be located and reviewed at (b) (4).***

(b) (4), (b) (6)

CHAIN OF CUSTODY RECORD

Number
Instructions on back

TO BE COMPLETED BY MICROBAC

Temperature Upon Receipt (°C) 5.6
Therm ID N
Holding Time

Samples Received on Ice? ☐ Yes ☐ No ☒ N/A
Custody Seals Intact? ☐ Yes ☒ No ☐ N/A

Lab Report Address
Client Name: Intralytix
Address: 8681 Robert Fulton Drive
City, State, Zip: Columbia, MD 21046
Contact: Joelle Woolston, jwoolston@intralytix.com
Telephone No.: (443) 275-9685
Send Report via: ☐ Mail ☒ Fax ☒ e-mail (address)

Invoice Address
Client Name: Intralytix
Address: 8681 Robert Fulton Drive
City, State, Zip: Columbia, MD 21046
Contact: (b) (6)
Telephone No.: (b) (6)
Send Invoice via: ☐ Mail ☐ Fax ☒ e-mail (address) (b) (6)

Turnaround Time
☒ Routine (5 to 7 business days)
☐ RUSH* (notify lab)
(needed by)
Report Type
☒ Results Only ☐ Level 1 ☐ Level 2 ☐ Level 3 ☐ Level 4 ☐ EDD

Project: SalmoFresh
Location: Intralytix
PO No.:
Compliance Monitoring? ☐ Yes ☒ No
☐ Agency/Program

Sampled by (PRINT): (b) (4)
Sampler Signature:
Sampler Phone No.: (443) 275-9712

* Matrix Types: Soil/Solid (S), Sludge, Oil, Wipe, Drinking Water (DW), Groundwater (GW), Surface Water (SW), Waste Water (WW), Other (specify)
** Preservative Types: (1) HNO3, (2) H2SO4, (3) HCl, (4) NaOH, (5) Zinc Acetate, (6) Methanol, (7) Sodium Bisulfate, (8) Sodium Thiosulfate, (9) Hexane, (0) Unpreserved

REQUESTED ANALYSIS

Lab ID	Client Sample ID	Date Collected	Time Collected	No. of Containers	Matrix	Grab / Comp	Preservative Types **	Additional Notes
	0223E30100A64-A12	06/03/25		4	DW		1, 2, U	Phosphorus, TDS, TKN, TOC, TSS, Hg, Chloride/Nitrite/Nitrate
	0224B23100A42-H27	06/03/25		4	DW		1, 2, U	Heavy metals: As, Ba, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg,
	0224G26100A08	06/03/25		4	DW		1, 2, U	Mn, Mo, Ni, P, K, Si, Na, Sn, Zn

M S F 0 1 8 9

Intralytix
Rec'd 06/04/2025 11:06
By: Stephanie Murphy
Temp 5.6 (Signature)

Possible Hazard Identification ☐ Hazardous ☒ Non-Hazardous ☐ Radioactive
Sample Disposition ☒ Dispose as appropriate ☐ Return ☐ Archive

Comments
All tests described in the service proposals attached, with the exception of pH and specific gravity, which are not needed.

Relinquished By (signature)	Date/Time	Received By (signature)	Date/Time
Michele Cop	6-3-25 11:00 AM		
Relinquished By (signature)	Date/Time	Received By (signature)	Date/Time
Relinquished By (signature)	Date/Time	(b) (6)	Date/Time 6/4/25 1120

rev. 7/18/18

Page of

MICROBAC®

(b) (4) r #

COOLER TEMP >6° C LOG

SAMPLE ID	Bottle 1 °C	Bottle 2 °C	Bottle 3 °C	Bottle 4 °C	Bottle 5 °C	Bottle 6 °C

6/4/25 SM

pH Lot # (b) (4) pH Exceptions

SAMPLE ID	Bottle 1	Bottle 2	Bottle 3	Bottle 4	Bottle 5	Bottle 6

6/4/25 SM

PRESERVATIVE EXCEPTIONS

✓ NONE

AS NOTED

6/4/25 SM

Document Control # 1957
Last 04-10-2019

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