Generally Recognized as Safe (GRAS) Notice

for

L-Threonine Fermentation Product as a Source of Threonine in Livestock and Poultry

Prepared for:

U.S. Food and Drug Administration

Center for Veterinary Medicine

Division of Animal Feeds

Prepared by:

CheilJedang Corporation

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1. PART 1 GRAS Notice

CJ CheilJedang Corporation (hereinafter referred to as "CJ") is submitting a GRAS notice for the substance L-Threonine Fermentation Product as a source of threonine in Livestock and poultry diets.

1.1 Name and Address of Organization

CJ CheilJedang Corporation
Ms. Stephanie LEE
330, Dongho-Ro,
Jung-Gu, SEOUL, 04560, KOREA
Tel : +82-2-6740-3367
E-mail : stephanie.lee@cj.net

CJ BIO America, Inc.
Keith Haydon, PhD
CJ BIO America, Inc.
3500 Lacey Road, Suite 230
Downers Grove, IL 60515
Tel: (630) 241-0112
E-mail: keith.haydon@cj.net

1.2 Name of the Notified Substance

The common or usual name of the subject substance of this notification is "L-Threonine Fermentation Product". It is a source of the essential nutrient threonine, and specifically the L-isomer of the compound. The level of threonine in the product a minimum of 75%. L-Threonine Fermentation Product also containing approximately 5-7% amino acid from biomass (dried *Corynebacterium glutamicum* cell).

1.3 Intended Conditions of Use

L-Threonine Fermentation Product is to be used as an ingredient in animal feed according to current good manufacturing and feeding practice as defined in 21 C.F.R § 582.1(b) ("Substances that are generally recognized as safe"). Threonine is an essential amino acid that is considered to be the second limiting amino acid in pig feed and probably as the third limiting amino acid in

poultry feed. Threonine will be incorporated into the diet at levels commensurate with the nutritional requirement. Therefore, the required level will be decided on a case-by-case basis by animal nutritionists, based on good feeding practice for the target species.

1.4 Statutory Basis for GRAS Determination

This GRAS conclusion is based on the scientific procedures as provided in 21 CFR 570.30(a) and (b).

1.5 Federal Food, Drug, and Cosmetic Act Premarket Approval Exemption

The submitter has determined that the use of L-Threonine Fermentation Product as produced by fermentation with *Corynebacterium glutamicum*, for use a nutrient (threonine) in livestock and poultry feed is Generally Recognized as Safe based on scientific procedure and is thus exempt from the premarket approval requirement of the Federal Food, Drug and Cosmetic Act (21 U.S.C § 301 et.seq.).

1.6 Availability of Information for FDA Review

CJ agrees to make the data and information pertaining to this submission available to FDA.

CJ agrees to both of the following procedures for making the data and information available to FDA:

(A) Upon FDA's request, CJ will allow FDA to review and copy the data and information during customary business hours at the address specified for where these data and information will be available to FDA; and

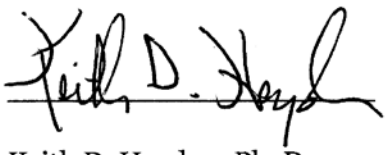
(B) Upon FDA's request, CJ will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for FDA evaluation or on paper.

1.7 Freedom of Information Act 5 U.S.C 552 Disclosure Exemption

CJ has placed proprietary and confidential information in three appendices: Appendix 1, "Composition and Impurity Reports (CONFIDENTIAL)"; Appendix 2, "Pre-Fermentation Information (CONFIDENTIAL)"; and Appendix 3, "L-Threonine Fermentation Product Manufacturing Process (CONFIDENTIAL)".

1.8 Certification of Complete, Representative Submission

To the best of our knowledge and belief, this GRAS notice is a complete, representative and balanced submission that includes unfavorable information, as well as favorable information, known to CJ and pertinent to the evaluation of the safety and GRAS status of the use of L-Threonine Fermentation Product produced by fermentation with genetically engineered *Corynebacterium glutamicum* as a source of threonine for livestock and poultry feed.



Keith D. Haydon, Ph. D.
CJ America - Bio
Director of Technical Services and Marketing

2. PART 2 GRAS Notice: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Scientific Data and Information that Identifies the Notified Substance

2.1.1 Name and Other Identities

Chemical name according to IUPAC nomenclature	L-2-Amino-3-hydroxybutanoic acid
Synonyms	(2S,3R)-2-Amino-3-hydroxybutyric acid
CAS No.	72-19-5
EC-No.	200-774-1
Appearance	Pale or dark brown powder
Molecular mass	119.12 g/mol
Molecular formula	$C_4H_9NO_3$
Structural formula	[redacted]

This GRAS notice covers L-Threonine Fermentation Product produced by fermentation with *Corynebacterium glutamicum*, with a minimum purity of 75% of L-Threonine. L-Threonine is the active substance in the L-Threonine Fermentation Product. L-Threonine belongs to the aspartate amino acid family. Due to its dedicated chemical properties, L-Threonine can only be found as free amino acid, which must not be transformed into a salt to be stable during production, storage and application.

2.1.2 Composition

The majority of the amino acid product is L-Threonine (≥75%). The product also contains other

free amino acids (< 2.0%), amino acid from biomass (<7%), sugar (<0.4%), organic acid (<0.2%), mineral (<5%) and moisture (<1%), and carrier (<7%). Refer to Appendix 1, Composition and Impurity Reports (Confidential) for additional information regarding the analytical assessment of the product composition. The carrier is used to assure a consistent threonine level in the final product from batch to batch.

Table 2-1: Chemical Composition Including Impurities

Test	Units	Method	Batch 01	Batch 02	Batch 03	Batch 04	Batch 05	Average
L-Threonine	%	AOAC 999.13					(b) (4)	77.94
Hydrolyzed amino acids (in insoluble Biomass part) (Total)								6.62
Aspartic acid		AOAC 994.12						0.62
Lysine								0.41
Serine								0.03
Glutamic acid								0.74
Glutamine								0.34
Glycine								0.37
Alanine								0.57
Valine								0.40
Cystine	%	AOAC 985.28						0.06
Isoleucine		AOAC 994.12						0.31
Leucine								0.51
Tyrosine								0.12
Phenylalanine								0.32
b-Alanine								0.02
Tryptophan		AOAC 988.15						0.06
Methionine		AOAC 985.28						0.25
Homoserine		AOAC 994.12						0.18
Threonine								0.56
Arginine								0.42
Proline								0.33

Free amino acids (Total, other than Threonine)	%	AOAC 999.13	(b) (4)	1.99
Lysine				1.07
Glutamic acid				0.20
Glycine				0.13
Alanine				0.03
Valine				0.05
Isoleucine				0.40
Leucine				0.01
Tyrosine				0.04
Phenylalanine				0.05
Homoserine				0.02
Moisture	%	AOAC 934.01		0.83
Ammonium		ASTM D4327 03		0.59
Sugars (Total)	%	AOAC 995.13		0.35
Glucose				0.07
Trehalose				0.28
Organic acids (Total)	%	Korean Feed Standards Codex, 1 of chapter 14		0.12
Malic Acid				0.01
Succinic Acid				0.04
Lactic Acid				0.07
Inorganic anions/cations	%	ASTM D4327-03 ASTM D 6919–03		3.93
Sodium				0.01
Potassium				0.48
Magnesium				0.04
Calcium				0.01

Chloride			(b) (4)	0.01
Phosphate				0.88
Sulfate				2.54
Ash[1]	%	AOAC 942.05		1.51
Carrier[1]	%			7.00
Total of quantified components[3]	%			99.42

[1] : Value of ash is included in inorganic anions/cations value.

[2]: Carrier: Amount of added.

ex. starch, dextrin, corn gluten meal, soybean mill run, corncob.

[3]: by calculation (%) = L-Threonine + Hydrolyzed amino acids + Free amino acids + Moisture Ammonium + Sugars + Organic acids + Inorganic anions/cations + Carrier, Ash is included in Inorganic anions/cations.

2.1.3 Fermentation Organism

The fermentation organism is a genetically modified strain of *Corynebacterium glutamicum*. The genetic modification and characterization of the production microorganism can be found in Appendix 2, "Pre-Fermentation Information (CONFIDENTIAL)." The safety of the production microorganism can found in Appendix 2, Section 6, and Appendix 9.

2.2 Manufacturing Process

L-Threonine Fermentation Product is produced by fermentation with *Corynebacterium glutamicum* as a production strain. After fermentation, the pH is lowered by adding H_2SO_4 and the temperature is increased for sterilization. The fermentation liquid is then concentrated and the concentrated liquid is transferred into the mixer granulator. After granulation, the wet granule is dried and separated by a mesh separator. The separated particle is packaged with the minimum content of 75% L-Threonine.

The applicant declares that no antimicrobial compounds (including antibiotics) were used in the production process.

The pre-fermentation process is provided in Appendix 2, "Pre-Fermentation Information (CONFIDENTIAL)," which includes the genetic engineering process, characterization and assessment of the production microorganism.

The full fermentation process and downstream manufacturing processes are provided in Appendix 3, "Manufacturing Process (CONFIDENTIAL)".

2.2.1 Ingredient Stability (Shelf Life)

Stability testing for L-Threonine Fermentation Product was performed using three typical batches. Stability results for zero-time to twelve months are presented in Table 2-2 (25°C, 60%RH) and Table 2-3 (40°C, 75% RH).

None of the tested samples showed a significant decrease in the level of the active substance L-threonine at the tested time points. The specified minimum 75% L-threonine content was maintained in all samples over the tested periods. The full report on product stability can be found in Appendix 4, "L-Threonine Fermentation Product Stability Study". The data supports product stability of at least 12 months.

Table 2-2: Shelf life of L-Threonine Fermentation Product in % (Target Value is a Minimum 75% L-Threonine) at 25°C, 60% RH During Storage of 12 Months
n.t.: Not tested.

Batch	Measurement	Zero-time		Time in months					
Lot		start value	unit	1	2	3	4	6	12
Gran.Threonine Lot T75-16-11A5-29	Threonine content	77.4	%	(b) (4)					
	moisture	1.30	%						
Gran.Threonine Lot T75-16-12A3-02	Threonine content	78.2	%						
	moisture	1.40	%						
Gran.Threonine Lot T75-16-11B2-30	Threonine content	77.7	%						
	moisture	1.20	%						

Table 2-3: Shelf life of L-Threonine Fermentation Product in % (Target Value is a Minimum 75% L-Threonine) at 40 °C, 75% RH During Storage of 6 Months

Batch	Measurement	Zero-time		Time in months				
Lot		start value	unit	1	2	3	4	6
Gran.Threonine Lot T75-16-11A5-29	Threonine content	77.4	%	(b) (4)				
	moisture	1.30	%					
Gran.Threonine Lot T75-16-12A3-02	Threonine content	78.0	%					
	moisture	1.40	%					
Gran.Threonine Lot T75-16-11B2-30	Threonine content	77.7	%					
	moisture	1.20	%					

The threonine levels were stable over the six months of testing, demonstrating product stability throughout the testing period at ambient temperatures or in accelerated conditions. This data supports product stability of at least one year.

2.2.1 Stability upon Addition to Animal Feed

A 12-week study in broiler mash feed (three batches) was conducted to demonstrate the stability of the product when mixed in a complete feed. The animal feed was assessed every four weeks. The full report can be found in Appendix 5, "Test Report No. 3.243-7 Granule Threonine -IFF Trial V-931-7 Stability Mash Feed".

Table 2-4: Stability of L-Threonine Fermentation Product in Mash Feed for Broilers

Added value 0.40 %			Time in months			
Nominal value 1.011 %		Blank	Zero	1	2	3

Sample number	Unit		S-0	S-1	S-2	S-3
Analysis method		DJ005[1]	DJ005[1]	DJ005	DJ005	DJ005
V-931-F-498	%	(b) (4)				
V-931-F-499	%					
V-931-F-500	%					

[1] Threonine (acid/oxidative hydrolysis); Method: EU 152/2009 (F), 03:2005, IC-UV

This study demonstrated that the L-Threonine Fermentation Product was a stable source of L-threonine when added to complete mixed feed over a three-month period, demonstrating by less than 10% variability over the time period.

2.3 Specifications

L-Threonine Fermentation Product specifications are based on the assay of five batches. The analytical data supporting the specifications is in reported Table 2-1 above and further discussed in Appendix 1, "Analytical Reports: Qualitative and Quantitative Composition of L-Threonine Fermentation Product (CONFIDENTIAL)". The product specifications are provided in Table 2-5 below.

Table 2-5: L-Threonine Fermentation Product Specifications

Component	Amount	Method
Threonine, minimum	75%	HPLC, AOAC 999.13
Moisture, maximum	5%	At 105℃ for 3hr, AOAC 934.01
Ash, maximum	5%	AOAC 942.05

2.4 Intended Use (Utility) of L-Threonine Fermentation Product

The L-Threonine Fermentation Product is to be used as a L-Threonine supplemental nutrient in animal feeds in accordance with good manufacturing or feeding practice as defined in 21 CFR 582.1(b) Substances that are generally recognized as safe. Threonine exists as a stereoisomer,

either as D-threonine or L-threonine. L-threonine is the physiologically relevant stereoisomer. L-threonine is an essential amino acid in all animal species (EFSA, 2015:13(9)). The level of supplementation varies between species and is dependent on the nutritional content of the diet (specifically the amino acids content). Therefore, the use of supplementation will be determined on a case-by-case basis by animal nutritionists, based on good feeding practice.

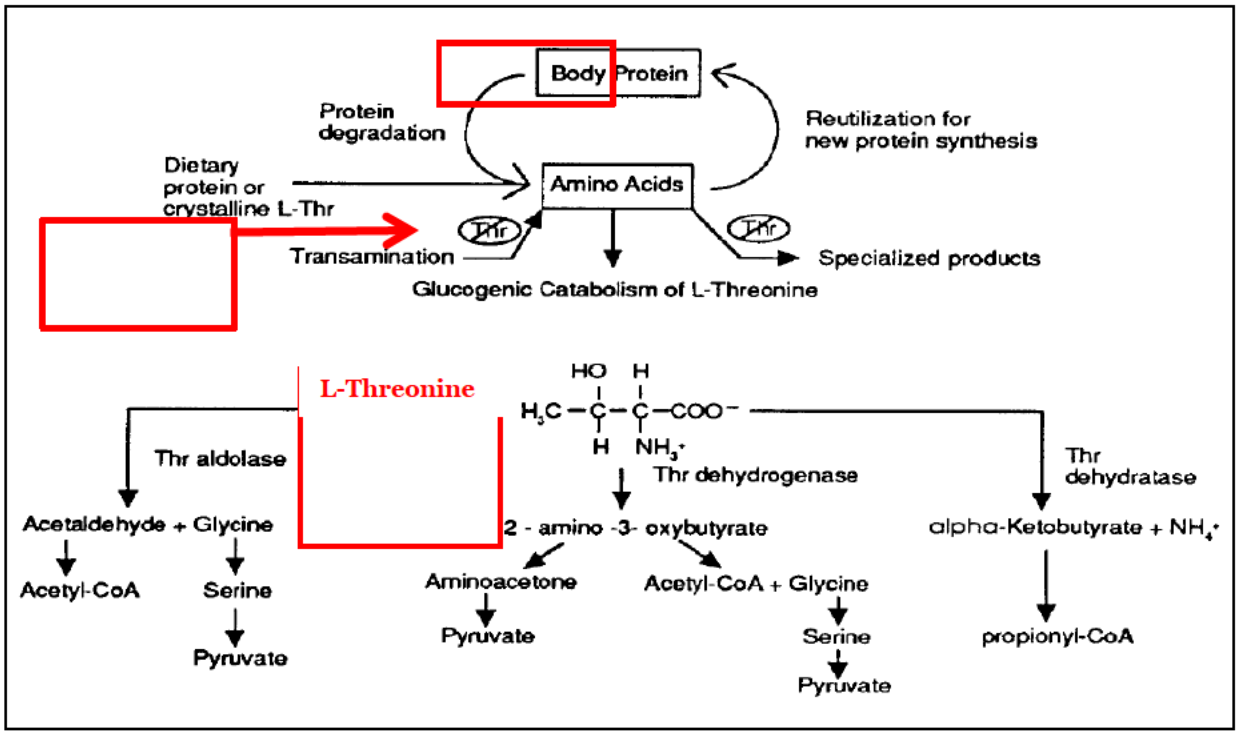


Fig. 2-1. Schematic Representation of L-threonine Catabolism (Kidd et al. 1996, J. Appl. Poult. Res.5(4):358-367)

Under normal USA feeding conditions, L-threonine is usually the second limiting amino acid, after L-lysine, in the diet of pigs and the third, after Sulphur amino acids and L-lysine, for poultry. L-threonine is proposed to be used in feeds in order to achieve the adequate amino acid profile and meet the requirements on L-threonine for livestock and poultry species.

It can be added directly to the feeding stuffs/complementary feeding stuffs or via premixture. No inclusion levels are proposed as the requirements in quantitative terms depend on the species, the physiological state of the animal, the performance level and the environmental conditions, as well as the amino acid composition of the non-supplemented diet. The formulator of the feed will determine the required level of amino acid supplementation.

The L-Threonine Fermentation Product is the subject of this GRAS noticed application. The active substance is L-threonine. Any component of L-Threonine Fermentation Product doesn't differ significantly from the constituents of the ordinary diet of the target animal.

The biomass portion of the L-Theronine Fermentation Product is dried, inactivated *Corynebacterium glutamicum*, which is the same biomass used in the Dried L-Lysine Fermentation product (AAFCO 36.16). According to the AAFCO Official Publication (AAFCO, 2018, AAFCO 2018 Official Publication CHAPTER SIX, 36.16, 387-388), Dried L-Lysine Fermentation product (AAFCO 36.16) may be effectively used as an alternative to L-lysine monohydrochloride (L-lysine without biomass product) as a supplemental lysine source in swine diets. The biomass has been demonstrated to not interfere with the lysine availability. This most recently has been confirmed in a publication comparing the bioavailability of L-lysine and Lysine Sulphate (Lysine Fermentation Product) in young swine (Htoo et al, 2016, J. Anim. Sci. 2016.94253–256) L-threonine and L-Threonine Fermentation Product may also be applied. Therefore, there is no expectation that the biomass will decrease the availability of threonine from the L-Threonine Fermentation Product. This was corroborated by a study in chicks (below).

The lack of effect of the *Corynebacterium glutamicum* biomass inclusion on bioavailability of the amino acids was corroborated by a 28-day chick utility as summarized below in Section 2.4.1. The full report and supporting date is provided in Appendix 6, "Utility Trial Report."

2.4.1 (b) (6) Utility Trial

A 28-day utility trial was conducted by (b) (6) to compare L-Threonine Fermentation Product to current commercially available L-Threonine (98%) (Appendix 6). The trial utilized 1320-day old Cobb 500 male chicks averaging 45.2 grams. Chicks were blocked on weight and assigned to one of 40 pens (33 chick/pen). Pens were randomly assigned to one of four dietary treatments. Dietary treatments were a: Positive Control (L- Threonine 98%); a Negative Control (same as Positive Control without L-Threonine 98% supplementation); Negative Control with L-Threonine Fermentation Product added at 100% of Positive Control threonine level; and Negative Control with L-Threonine Fermentation Product added at 150% of Positive Control threonine level. Pen weights and feed disappearance were recorded at day 14 (Starter Phase) and day 28 (Grower Phase). All feed was removed at day 14 and replaced with Grower Phase diets. Growth is a suitable measurement when determining the availability of an essential amino acid, when comparing to a negative control feed.

Table 2-6: Bioavailability Results of L-Threonine Fermentation Product Compared to Positive and Negative Control diets as Demonstrated by Growth[1,2]

Criteria	Positive Control (PC)	Negative Control (NC)	NC with L-Threonine	NC with L-Threonine	SEM	P-Value

			Fermentation Product 100%	Fermentation Product 150%		
Body Weights, grams						
Day 0	(b) (4)					
Day 14						
Day 28						
Feed Intake, grams/day						
Day 0 - 14						
Day 15 - 28						
Day 0 – 28						

[1]: Least square means

[2]: Means with differing superscript differ by listed p-value

The addition of L-Threonine regardless of source or level improved day 14 bird weight (b) (4). Birds fed 100% of the required threonine level (regardless of source) had increased (b) (4) on day 28 when compared to the negative control. Feed intake was not negatively impacted by the inclusion of biomass from day 0 to 14-day. Feed intake was actually significantly increased by L-Threonine Fermentation supplementation, regardless of level ((b) (4)). Threonine supplementation at 100% from either commercial 98% or L-Threonine Fermentation Product at 100% replacement rate increased ((b) (4)) day 28 bird weight as compared to the Negative Control. Birds fed threonine replacement rate to 150% of Positive Control result in statistically intermediate (b) (4) day 28 bird weight. Day 15 to 28-day feed intake was unaffected ((b) (4)) by threonine source or level. The data indicates that the L-Threonine Fermentation Product is a bioavailable source of the essential amino acid L-threonine in broiler chicks.

This study can be used as a corroborative sentinel study to demonstrate the L-threonine availability from Threonine Fermentation Product in animal feed. It also confirms, as previously demonstrated with the Lysine Fermentation product, the *Corynebacterium glutamicum* biomass does not impact bioavailability of the amino acid.

3. Part 3 GRAS Notice: Target Animal and Human Exposures

3.1 Target Animal Exposure

L-Threonine is an essential amino acid in all animal species (EFSA. 2015. EFSA Journal 2015,13(9)-4236, including livestock and poultry NRC, 1994(National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition) and NRC, 2012 (National Research Council. 2012. Nutrient Requirements of Swine). The level of supplementation varies between species and is dependent on the nutritional content of the diet (specifically the amino acids content). Therefore, the use of supplementation will be determined on a case-by-case basis by animal nutritionists, based on good feeding practice.

Based on the overall level of supplementation in the most fortified diets, (for example broilers, egg layers and swine), the maximum level of use threonine would in normal feeding practices be approximately from 0.05% to 0.10 % of the layers feed and approximately 0.075 - 0.14% % of the broilers feed (NRC, 1994 (National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition)). In swine feeds L-Threonine supplementation levels range from 0.075% to 0.20% depending on production phase and feed ingredients used in the diet (NRC, 1998 (National Research Council. 1998. Nutrient Requirements of Swine: Tenth Revised Edition)). Other species would be similar.

Therefore, although the level of use in the formulated feed will be based on the threonine content naturally occurring in the feed, a maximum would be considered 0.5% of the feed.

The impurities of L-Threonine Fermentation Product are all either essential nutrients or typical components of feed (amino acids, minerals and organic acids) and are consistent with normal components of feed, as such would not be a source of residues beyond that found in animal food products from traditionally fed animals.

3.2 Human Food Exposure

The threonine requirement is particularly nutritionally important in the human, since it has been suggested that, after the sulphur amino acids, it is the second rate-limiting amino acid in the maintenance requirement. (WHO. 2011. WHO Technical Report Series 935, Geneva Switzerland)

L-Threonine Fermentation Product is intended for use in animal feed only as a nutritional source of the essential amino acid, threonine. The other components of the ingredient are nutritional

and available for uptake, metabolism and growth. Therefore, the milk, meat, and eggs from animals fed L-Threonine Fermentation Product, should be no different than from animals fed a nutritionally complete diet. There is no expectation of a residue from the feeding of L-Threonine Fermentation Product.

Table 3-1 below demonstrates that availability of threonine in natural sources is quite limited, hence external supply is required to meet the optimal daily demand. Nutrition that comes from animal proteins can provide a more balanced amino acid profile, however, modern animal nutrition is more depended on vegetable protein. Hence the addition of supplemental Threonine is extremely important.

Table 3-1: Limiting Amino Acids in Foodstuffs (Kleemann et al. 1985. Amino acids. Vol.A2, pp. 57-97. Weinheim, Gemany: VCH Publishers)

Proteins	First limiting amino acid	Second limiting amino acid(s)
Peanut	Threonine	Lysine and Methionine
Fish	Methionine	Lysine
Casein	Methionine	Tryptophan
Torula yeast	Methionine	-
Sesame	Lysine	-
Skim milk	Methionine	-
Beans	Methionine	-
Sunflower seed	Lysine	Threonine
Soy protein	Methionine	Lysine
Wheat	Lysine	Threonine
Rice	Lysine	Threonine and Tryptophan
Rye	Lysine	Threonine and Tryptophan
Gelatine	Tryptophan	-
Maize	Lysine	Tryptophan and Threonine

The free amino acids produced by the degradation of proteins are absorbed by active transport through the small intestine mucosa and sodium. Absorbed free amino acids are used for continuous metabolism of intracellular proteins. Approximately 75% of the liberated amino acids are recycled by the animals.

4. Part 4 GRAS Notice: Self-Limiting Levels of Use

There is no self-limiting use information specific to this substance.

5. Part 5 GRAS Notice: Experience Based on Common Use in Food Before 1958

The GRAS determination is not based on common use in animal feed prior to 1958.

6. Part 6 GRAS Notice: Narrative

6.1 Safety of Corynebacterium glutamicum – Production Organism

Corynebacterium glutamicum is a gram positive bacteria belonging to the family of *Corynebacteriaceae*. These bacterial strains are scientifically recognized as safe and provide no negative impact to on human and the environment. Additionally, they have a long history of safe use in industrial production (Eggeling and Bott, 2005. Handbook of *Corynebacterium glutamicum*. CRC Press). Also, *Corynebacterium glutamicum* is a GRAS microorganism and has a "Qualified Presumption as Safe" (QPS) status (EFSA, 2011. EFSA Journal 2011, 9(12):2497). A description and summary of the QSP review of *Corynebacterium glutamicum* is provided in Appendix 9, Literature Review *Corynebacterium glutamicum*," Section 2.

Corynebacterium glutamicum is an authorized source for a number of feed ingredients. It is listed in the AAFCO OP (2018). It is the source organism for Condensed Extracted Glutamic Acid Fermentation Product (AAFCO Definition 36.1). It is also the source organism for Dried L-lysine Fermentation Product (AAFCO definition 36.16) as well as Liquid L-lysine Fermentation Product (AAFCO definition 36.17). As recent as 2014, the US Food and Drug Administration, Division of Animal Feeds (OS&C/FDA) had reviewed the safety assessment of this source organism for the use in animal feed. Based on that recent review, CJ was recommended to review the recent literature after 2003 to assure the assessment was complete. Appendix 9, Section 3 of this GRAS notice provides results of this extensive literature review. Overall, no studies were retrieved either in the electronic literature search (ELS) or follow-up selective searches that contained information indicating potential safety issues or hazards associated with *Corynebacterium glutamicum*. This is consistent with the previous safety assessment completed by the US FDA, Division of Animal Feeds.

6.2 Safety Considerations due to the Nature of Modification to Corynebacterium glutamicum

The production microorganism used to produce L-Threonine Fermentation Product is a genetically altered strain of *Corynebacterium glutamicum*. The full genetic modification process, safety assessment, and stability assessment is provided in Appendix 2, "Pre-Fermentation Information (CONFIDENTIAL)." The production strain is deposited in the Korean Centre of Microorganisms (KCCM). As shown in Appendix 2 of this notice, the assessment of the genetic engineering process demonstrates that there is no hazard imparted due to the engineering process. This data is summarized in the sections below.

6.2.1. Safety for humans and animals

The L-Threonine Fermentation Product is intended for use as a nutrient for animal consumption. Ordinarily, a GRAS notice will address the potential human dietary consumption of a component of animal feed due to consumption of animal products and tissues in which the component may be present. In this case, however, there is no need to determine the estimated daily intake (EDI) of the L-Threonine Fermentation Product for human consumption. The L-Threonine Fermentation Product and any of the described impurities (see above) will be metabolized when the animal consumes and digests its food (like all feed). The L-Threonine Fermentation Product derived from the genetically modified *Corynebacterium glutamicum* will be indistinguishable from other sources, as will be the potential impurities, which are all normal components of animal feed.

1) Information on any toxic, allergenic or other harmful effects on human or animal health

The genetic modifications made, resulting in strain *Corynebacterium glutamicum* KCCM80178, exclusively correspond to the over-expression of existing metabolic enzymes or the elimination of several enzymes. The initial parental strain *Corynebacterium glutamicum* is about the most used bacterium industrially. It has been used for the manufacturing of feed additives for many years and is generally accepted as safe. The assessment for the presence of open reading frames not associated with intended genetic changes and potential for spill-over effects were assessed and found not to provide any safety concern (Appendix 2).

2) Potential for DNA transfer or any capacity for enhanced gene transfer

To limit any potential transfer of genetic material to other organisms, the strategy of construction for *Corynebacterium glutamicum* KCCM80178 strain was based on procedures described below.

2-1) Any genetic material including plasmid to be autonomously replicable was not used.

2-2) All the genetic modifications were done on chromosome.

3) The resistance of antibiotics of Production strain

This study is to determine MIC of antibiotics of production strain of L-Threonine Fermentation Product. The broth tube dilution method is used to determine the susceptibility of a production strain *Corynebacterium glutamicum* KCCM80178. In regards to antibiotic resistance, to the knowledge of CJ, *Corynebacterium glutamicum* wild-type strains have not been reported to have any antibiotic resistance. This was confirmed by the test report of the “Determination of antibiotic minimum inhibitory concentration (MIC) of *Corynebacterium glutamicum* KCCM 80178. *Corynebacterium glutamicum* KCCM 80178 showed same antibiotic MIC with *Corynebacterium glutamicum* wild-type. These results indicated that there are no possible antibiotic resistance genes in the chromosome of the *Corynebacterium glutamicum* KCCM80178. The full test report is included in Appendix 2, Attachment 4.

4) The absence of viable cell in final product

CJ L-Threonine Fermentation Product is tested by viable production cell test. CJ L-Threonine Fermentation Product solution did not show any cell colony forming units during incubation. This result indicated that there is no viable production cell in the test sample. Even though the safety of production strain *Corynebacterium glutamicum* KCCM80178 has been confirmed as stated above, CJ did not allow any inclusion of the production strain in the final product. After fermentation, the pH is lowered by adding H_2SO_4 and the temperature increased for sterilization (100~120 ℃ for 5~20 minutes). The fermentation liquid is concentrated, and the concentrated liquid is transferred into the Mixer granulator. Any microorganism could not be viable in the final product after the cell inactivation step. Also during the L-Threonine Fermentation Product granulation process, the solution was heated to 60~100℃ to evaporate the water. This heating process also kills any remaining cells inside of the final product. Hence, it was confirmed there is no remaining viable production strain in the final product. The full test report is included in in Appendix 2, Attachment 5.

6.3 Safety Considerations for L-Threonine

L-Threonine Fermentation Product is a source of nutritional threonine that can be safely used in the in the production of proteins like all other sources of threonine.

Threonine is codified as a Generally Recognized as Safe amino acid for the use in animal feed (21 CFR 582.5881). In addition, it is an authorized feed ingredient as found in AAFCO OP (L-threonine definition 6.5). Threonine is an essential amino acid, as discussed in Section 2 of this notice and is formulated in diets that are deficient in naturally occurring threonine.

The European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed the safety and efficacy of various threonine compounds when used in animal diets (EFSA, 2015. EFSA Journal 2015, 13(9)-4236). The EFSA Panel noted that threonine additives in the feed of animals result in the incorporation of all absorbed threonine in tissue protein, and threonine that exceeds the threonine requirement of the animal is excreted. Consequently, no free threonine occurs or accumulates in target animal tissues. Only the L-stereoisomer form of threonine is used in animal feed and is digested, absorbed, and metabolized by the animal. This stereoisomer form of the amino acid is consistent with human nutrient needs. L-Threonine is an essential amino for humans. Free threonine is not a residue issue. Therefore, L-Threonine Fermentation Product presents no exposure risk to humans consuming tissues or products from the target animal.

6.4 Safety Considers of L-Threonine Fermentation Product

As seen in Table 2-1 in this dossier and in Appendix 1, "Analytical Reports: Qualitative and Quantitative Composition of L-Threonine Fermentation Product (CONFIDENTIAL)," there are no substances in the product that are not typical components of animal feed.

To corroborate the safety assessment, CJ conducted an acute toxicity study in rats as seen in Appendix 7, "Acute Toxicity". In this acute toxicity study, following a sighting test at a dose level of 300mg/kg and 2000 mg/kg, a further group of four fasted females were given a single oral dose of L-Threonine Fermentation Product as a solution in distilled water at a dose level of 2000 mg/kg body weight.

Clinical signs and body weight development were monitored during the study. The results were summarized as follows:

Mortality: No deaths were observed.
Clinical Observations: No signs of systemic toxicity.
Body Weight: All animals demonstrated expected gains in body weight.
Necropsy: No tissue abnormalities were noted at necropsy.

The acute oral median lethal dose (LD50) of L-Threonine Fermentation Product in the female Wistar strain rat was estimated to be greater than 2000 mg/kg body weight (Globally Harmonized Classification System Unclassified).

In the Bacterial Reverse Mutation Assay (OECD 471) that was performed on L-Threonine Fermentation Product, L-Threonine Fermentation Product was found to be non–mutagenic. The assay results can be found in Appendix 8, "Bacterial Reverse Mutation." These studies corroborate the safety assessment.

6.5 Safety Assessment of Known Impurities and/or Potential Contaminants

Based on the known composition of the product, there are no known impurities or contaminants introduced in the manufacture of the product that could raise safety concerns. The product is 75% L-Threonine and the specifications permit for 5% water and 5% inorganic compounds (generally sodium, sulphur and potassium). The use levels of threonine in the diet are small enough that these impurities cannot be considered nutritional source of minerals or free amino

acids as there are found at ppm levels (Table 6-1). Section 3 of this notice suggests the maximum level of use in the diet as 0.5% of feed.

Table 6-1: Feed Levels of L-threonine -Impurities

Substance	Average level in L-Threonine Fermentation Product, %	Feed Level when L-Threonine incorporated at 0.5%, expressed in ppm in the diet
Ammonium	0.59	(b) (4)
Sodium	0.01	
Potassium	0.48	
Magnesium	0.04	
Calcium	0.01	
Chloride	0.01	
Phosphate	0.88	
Sulfate	2.54	
Malic Acid	0.01	
Succinic Acid	0.04	
Lactic Acid	0.07	
Glucose	0.07	
Trehalose	0.28	
Lysine	1.07	
Glutamic acid	0.20	
Glycine	0.13	
Alanine	0.03	
Valine	0.05	
Isoleucine	0.40	
Leucine	0.01	
Tyrosine	0.04	
Phenylalanine	0.05	
Homoserine	0.02	

The levels of impurities are consistent with conventional feedstuffs, and none of the levels in the complete feed would be a concern.

6.6 Safety Assessment for Human Consumption

The L-Threonine Fermentation Product is intended for use as a nutrient for animal consumption. Ordinarily, a GRAS notice will address the potential human dietary consumption of a component of animal feed due to consumption of animal products and tissues in which the component may be present. In this case, however, there is no need to determine the estimated daily intake (EDI) of the L-Threonine Fermentation Product for human consumption. The L-Threonine Fermentation Product and any of the described impurities shown in Table 6-1 above will be metabolized when the animal consumes and digests animal feed containing L-Threonine Fermentation Product. The L-Threonine Fermentation Product derived from the genetically modified *Corynebacterium glutamicum* will be indistinguishable from other threonine sources, as will be the potential impurities, which are all normal components of animal feed. Non-threonine components of L-Threonine Fermentation Product are all typical feed components, mostly nutrients and will not be a concern for residues.

This same determination was made by the FDA in their support of the AAFCO definition 36.16 Dried L-Lysine Fermentation Product, AAFCO 36.17 Liquid L-Lysine Fermentation product and AAFCO 36.1 Condensed Extracted Glutamic Acid Fermentation Product.

In this regard, the European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed (EFSA, 2015. EFSA Journal 2015, 13(9)-4236) the safety and efficacy of threonine produced by *E.coli* K12 for use in the diets of all animal species. In the report, the EFSA Panel noted that threonine additives in animal feed results in the incorporation of all absorbed threonine in tissue protein. Doses exceeding the threonine requirement of the animal will be excreted. Consequently, no free threonine occurs or accumulates in target animal tissues and the only form of threonine that humans will be exposed to from its use in animal feed is in the form of protein that will be digested, absorbed, and metabolized consistent with human nutrient needs. The absence of residual threonine in the tissues of animals consuming any form of threonine in its diet will, therefore, not result in a subsequent human exposure or safety issue. As indicated by the analytical values displayed in Table 2-1, Appendix 1, and Table 3-1, residual components of L-Threonine Fermentation Product are at levels too low to present any risk of humans consuming the tissues of food animals fed the nutrient. All residual constituents are common metabolites or minerals and will be either excreted or metabolized. Therefore, they present no exposure risk to humans consuming tissues or products from the target animal. A review of the publicly available literature does not reveal information demonstrating that any of these residual constituents appears to present a risk of accumulation or harm to humans at the levels that would be consumed from animal tissue (IOM. 2006. Dietary Reference Intake, NAS/NAP). It should also be noted that L-threonine is an

essential amino acid for human nutrition is approved for direct addition to human food (21 CFR 582.1(b)).

In the Bacterial Reverse Mutation Assay (OECD 471), L-Threonine Fermentation Product was not mutagenic in this bacterial assay system (Appendix 8). The results indicate that the test article, L-Threonine Fermentation Product, was not mutagenic in this bacterial assay system.

6.7 Safety Conclusion

Based on the documentation provided in this GRAS Notification and as discussed above, CJ has concluded that L-Threonine Fermentation Product produced by fermentation with *Corynebacterium glutamicum* is generally recognized as safe via scientific procedures as a nutrient for animal consumption. The notifier has reviewed the available data and information and is not aware of any data and information that is, or may appear to be, inconsistent with your conclusion of GRAS status.

7. Part 7 GRAS Notice: List of Supporting Data and Information

7.1 Confidential Information

The only information that is considered confidential in this GRAS Notice is the information specific to the production of the genetically modified organism, the manufacturing process, and the documentation of the assays specific for the composition of the marketed product. None of the information to support the safety narrative, Section 6 of this notice, is considered to be confidential. All this information is provided in a summary basis in the body of the submission, as required by 21 CFR 570 Subpart E. Therefore, the summary of the manufacturing process, with the full disclosure of the safety assessment, are consistent with the general recognition standards.

7.2 Publically Available References

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EFSA. 2011. EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). EFSA Journal 2011, 9(12):2497.

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WHO. 2011. Joint WHO/FAO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition, WHO Technical Report Series No 935. WHO, Geneva.

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***See Appendix 9, "Literature Review Corynebacterium glutamicum" for Corynebacterium glutamicum* Literature Review References.**

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Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge. VA 22192-575.5
703 5907337 (F-a\ 703 580 8637)
Smedley@cfr-scrvicc.s.com

September 17, 2018

Dr. David Edwards
Director
Division of Animal Feeds (HFV-220),
Centerfor Veterinary Medicine,
Food and DrugAdministration,
7519 Standish PL,
Rockville, MD 20855

Subject: Filing of Animal GRAS Notificatbn
L-Threonine Fermentation Product

Notifier: CheilJedang Corporation (CJ)
330, Dongho-Ro,
Jung-Gu,SEOUL,04560,KOREA

Dear Dr. Edwards:

On behalf of CheilJedang Corporation, I am providing a copy of their animal General Recognized as safe notice for the use of L~Threonine Fermentation Product (75%) as produced by a genetically modified Cotynebacterium glutamicum for use as a source of L-threonine, a nutrient, in livestock and poultry diets. The submission is compliant with 21 CFR 570.210-255. The GRAS conclusion is based on scientific procedures.

Should you have any questions on the filing, please contact me direct\.

Sincerely,

KristiO.Smedley
Consultant to CheilJedang Corpiiration

Cc: Keith Hayden, CJ

ATTACHMENT:
CJ Letter of Representation—Smedley
GRAS Notice L-Threonine Fermentation Product

CJ AMERICA

May 31, 2018

David Edwards
Director
Division of Animal Feeds, HFV-220
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

Subject: CheilJedang Corporation
Authorization of Kristi Smedley as Regulatory Contact
AGRN L-Threonine Fermentation Product

Dear Dr. Edwards :

CheilJedang Corporation (CJ) is authorizing Dr. Kristi O. Smedley, Center for Regulatory Services, Inc. 5200 Wolf Run Shoals Road, Woodbridge, VA 22192 (Telephone 703 590 7337), to represent CheilJedang Corporation with respect to the Animal GRAS notice for L-Threonine Fermentation Product.

Should you have any questions on this matter, please contact the undersigned.

Sincerely,

Keith D. Haydon, Ph.D.
Director of Technical Services and Marketing

Cc: Kristi Smedley, CFR Services

REPORT

Chiral Purity Test of CJ L-Threonine Fermentation Product using HPLC

Original Final report date: August 21, 2018

CJ Research Institute of Biotechnology

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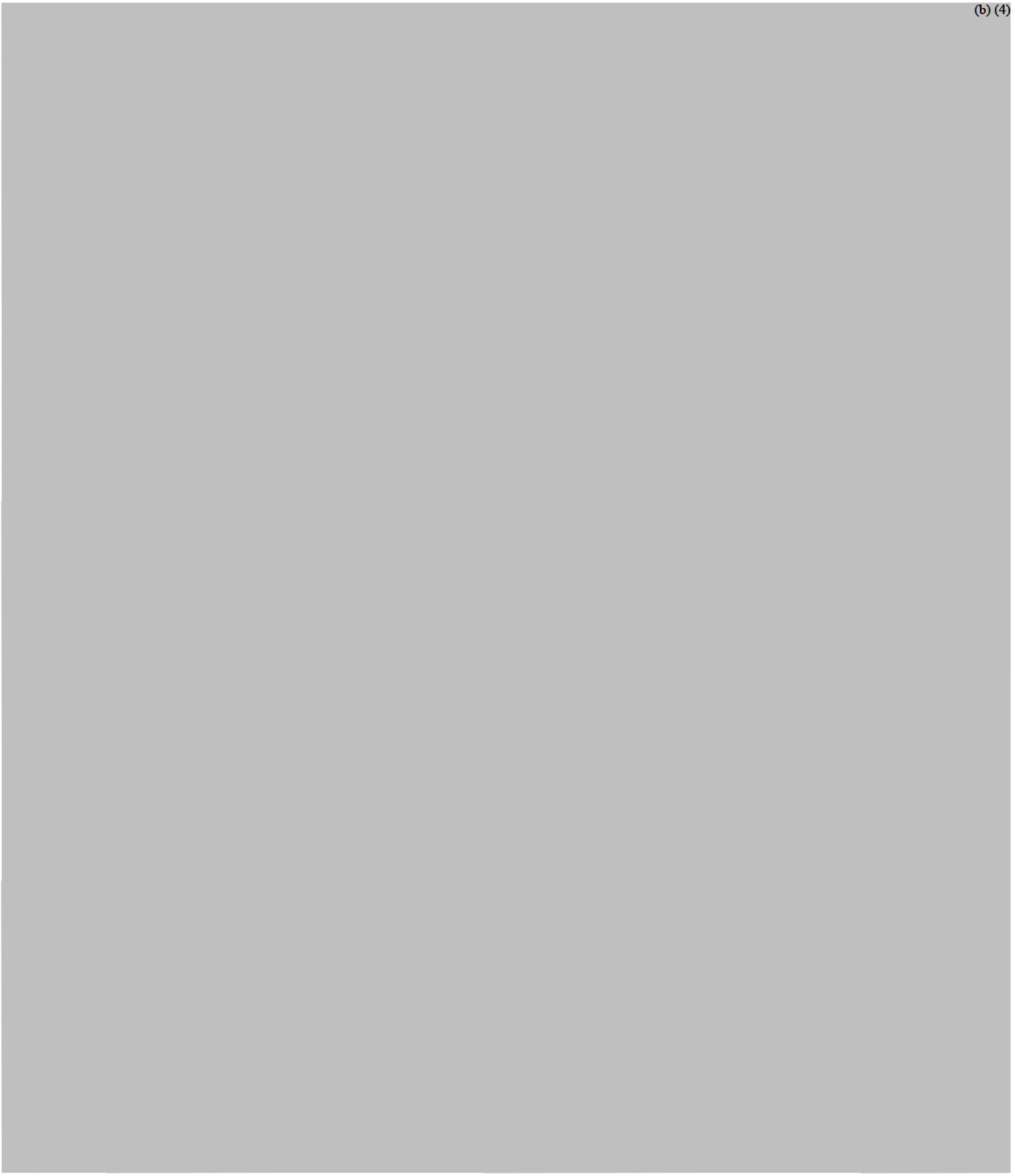

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APPENDIX 1: ANALYTICAL REPORTS (CONFIDENTIAL)

ANALYTICAL REPORT

Qualitative and Quantitative Composition of L-Threonine Fermentation Product (Document No.: CBM18007)

CJ Research Institute of Biotechnology

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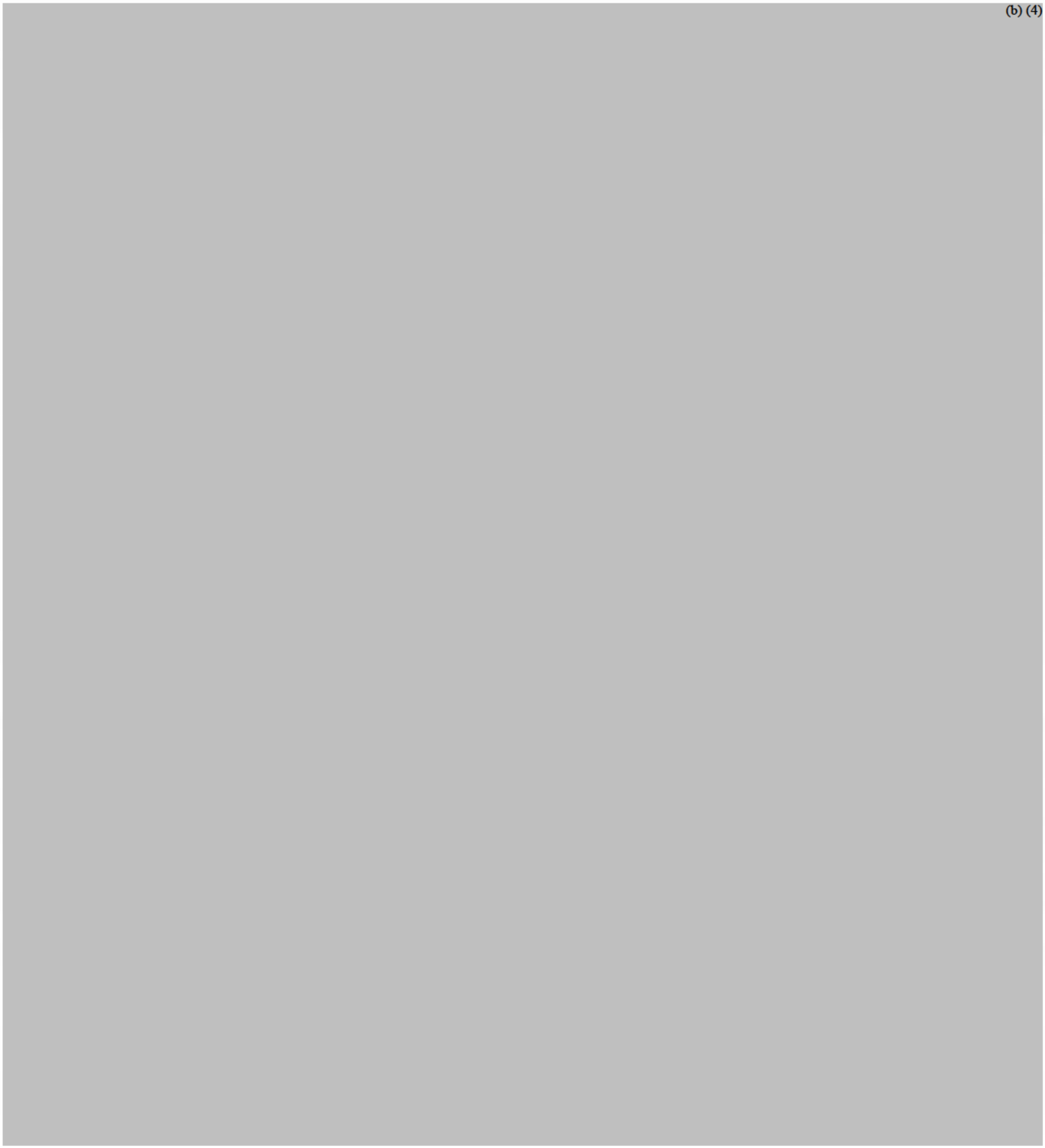

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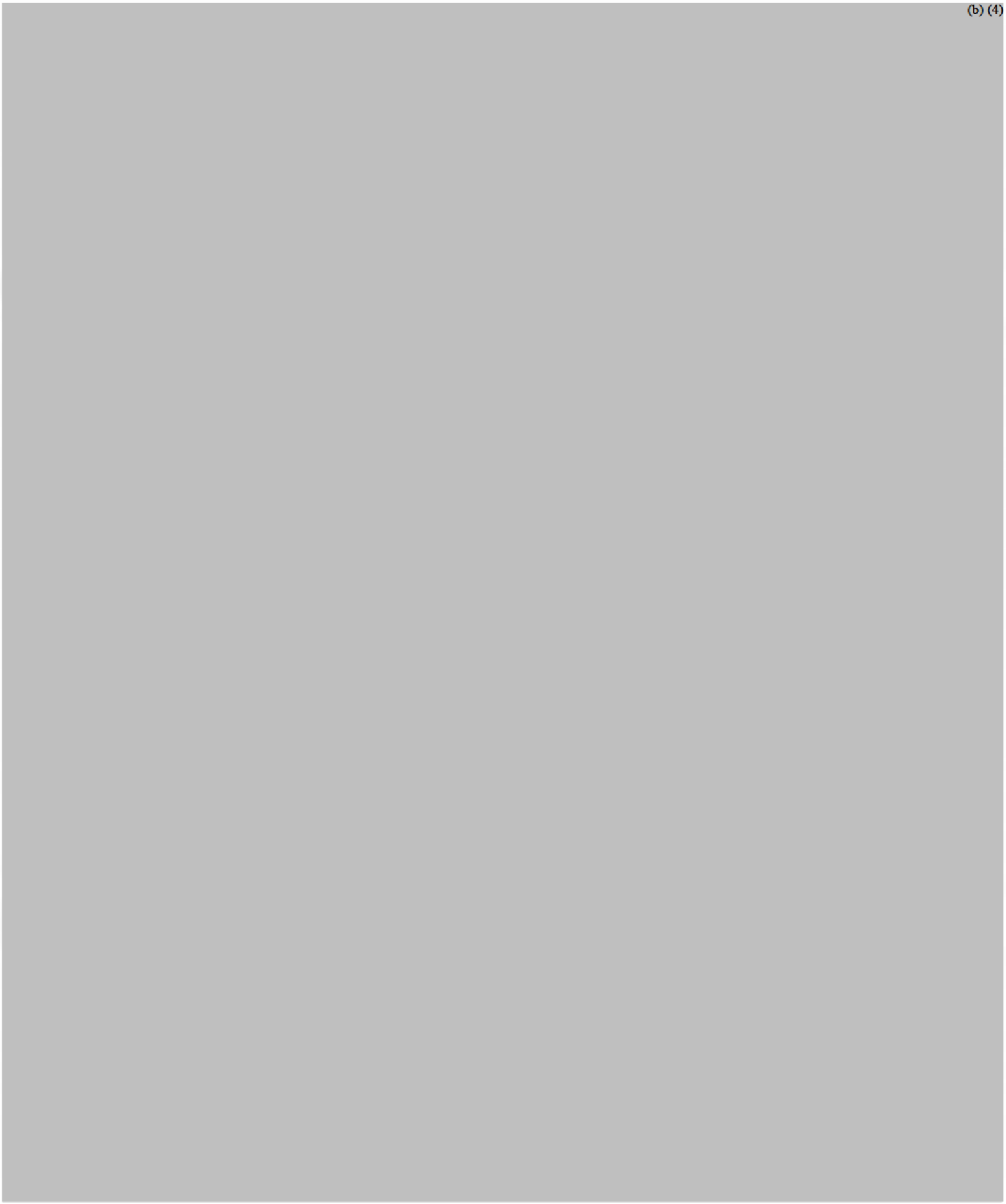

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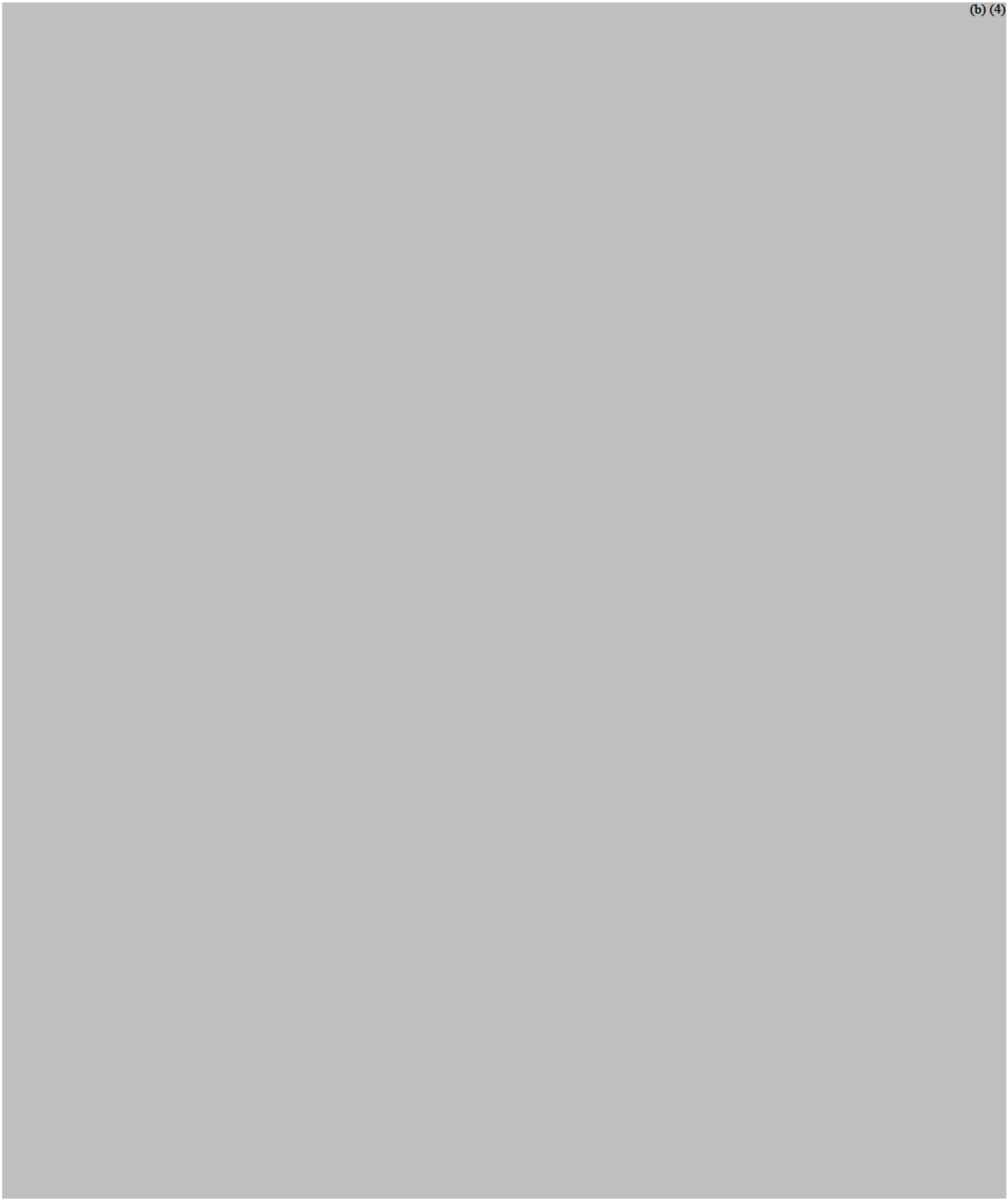

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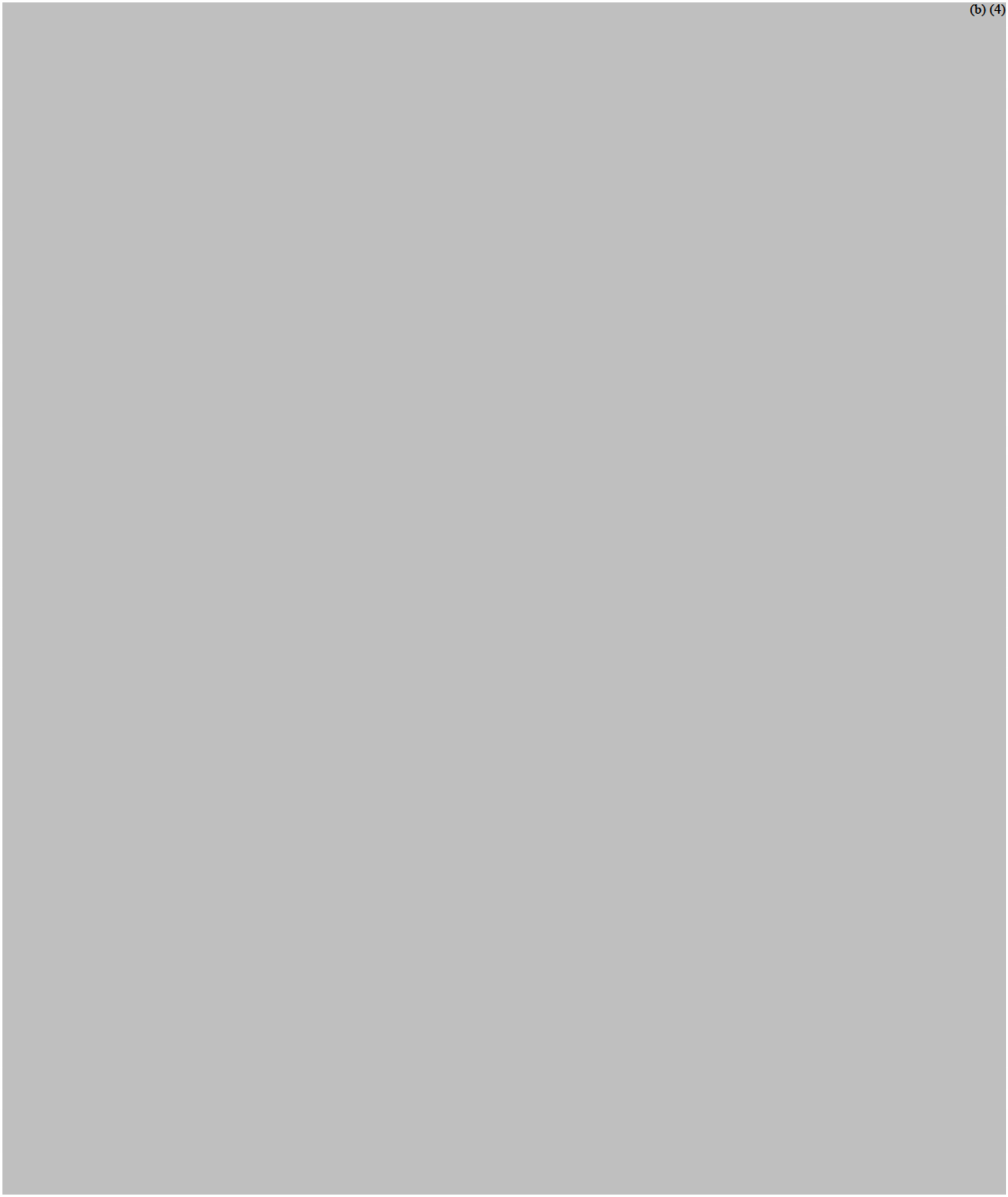

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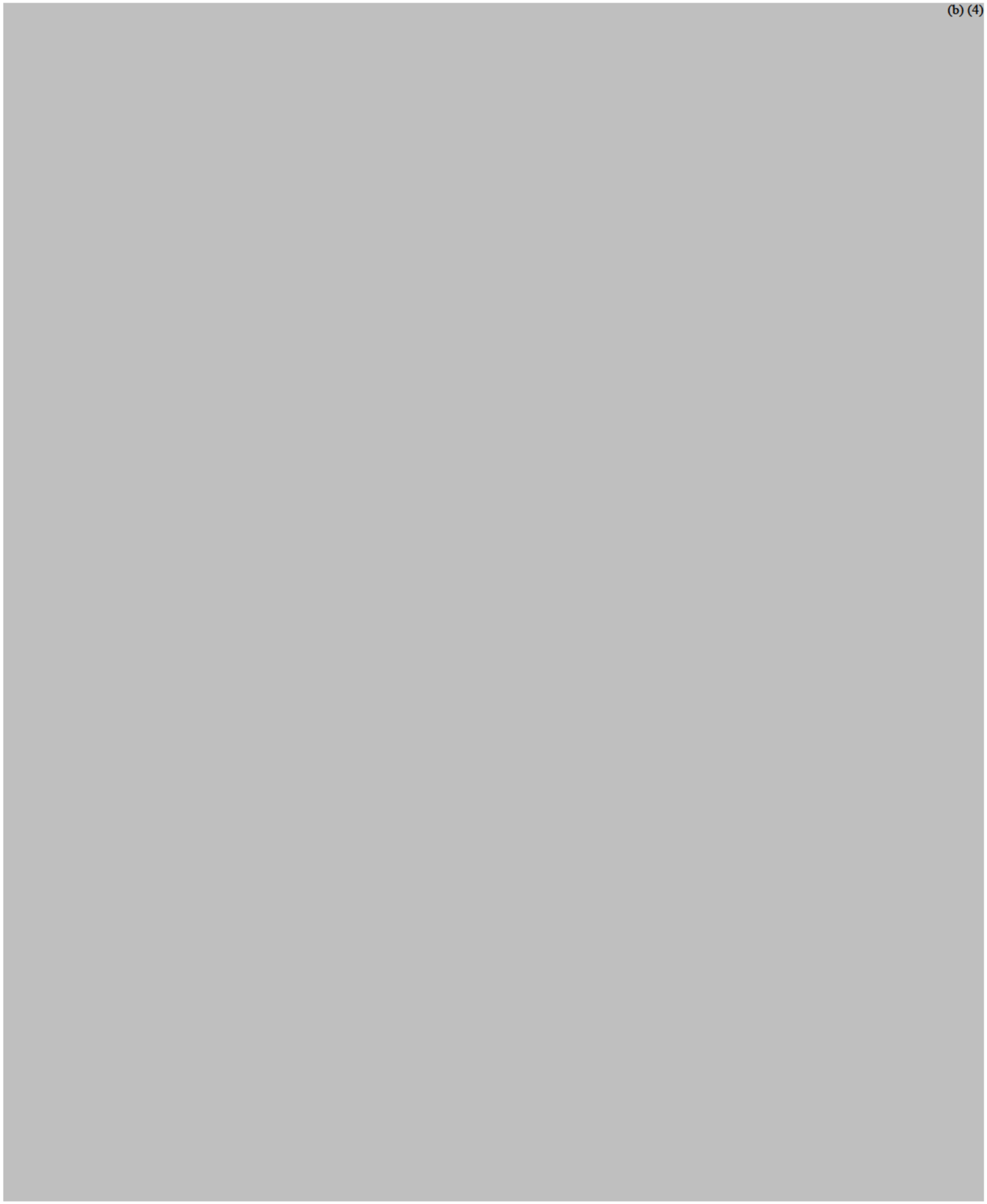

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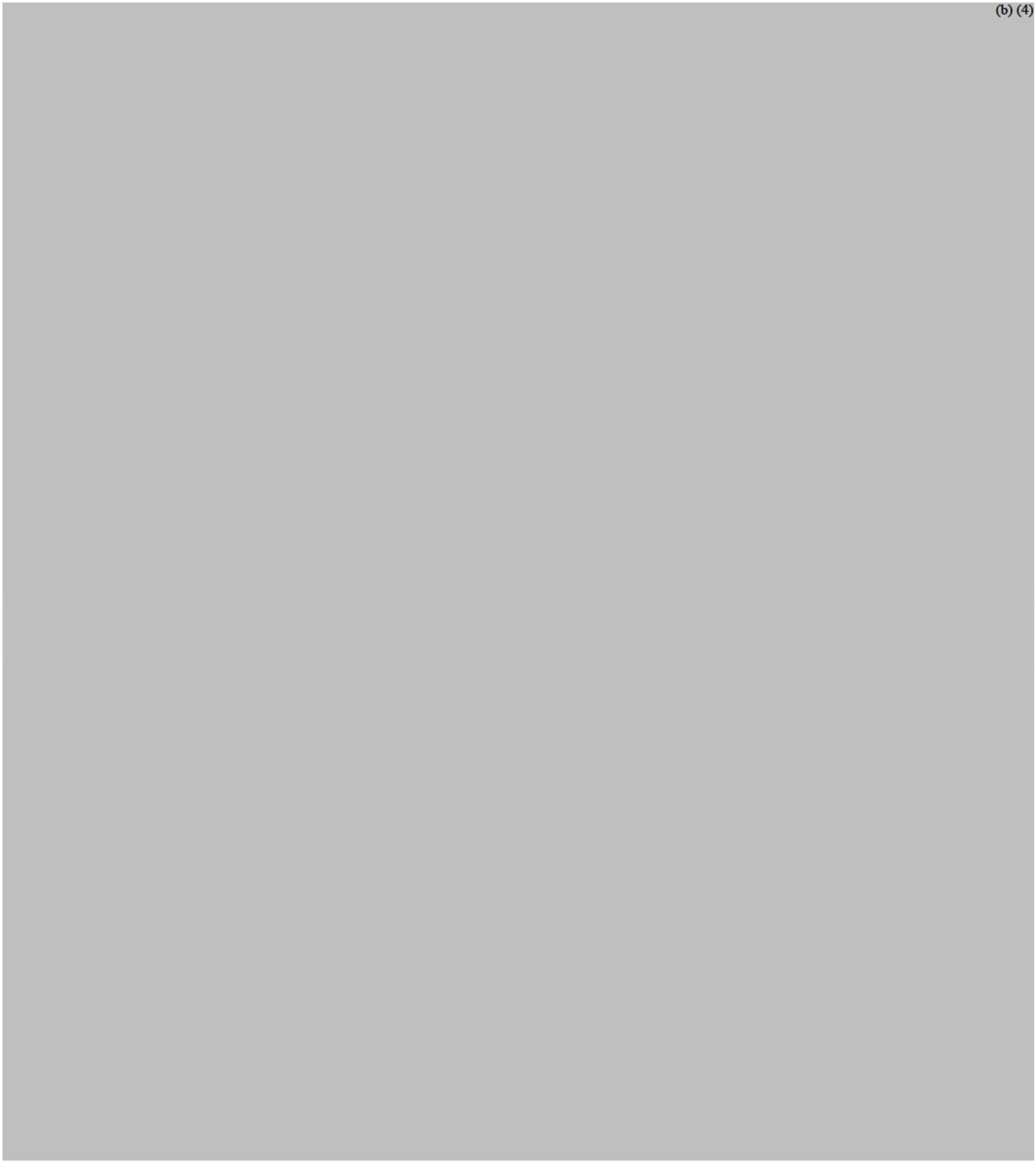

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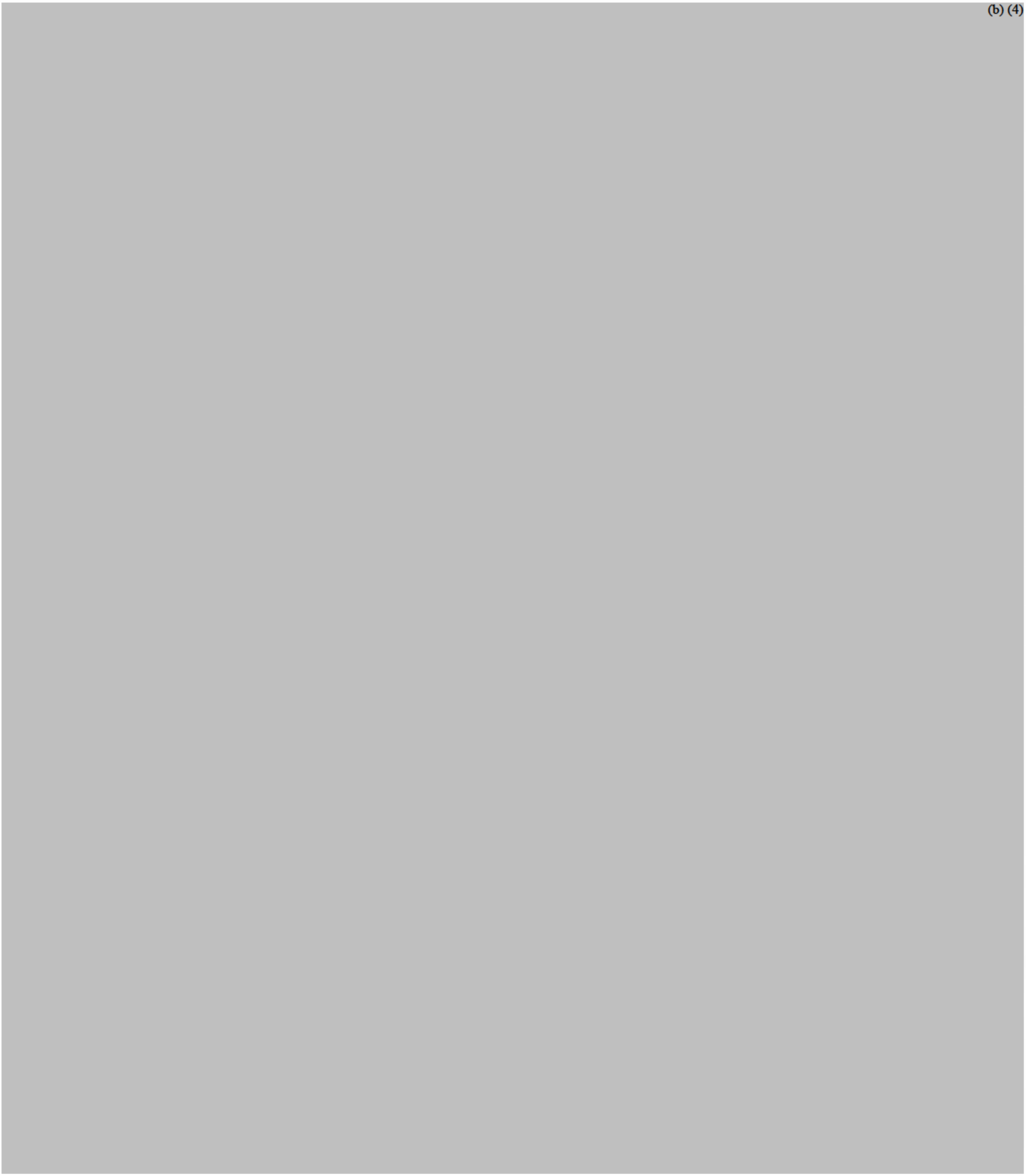

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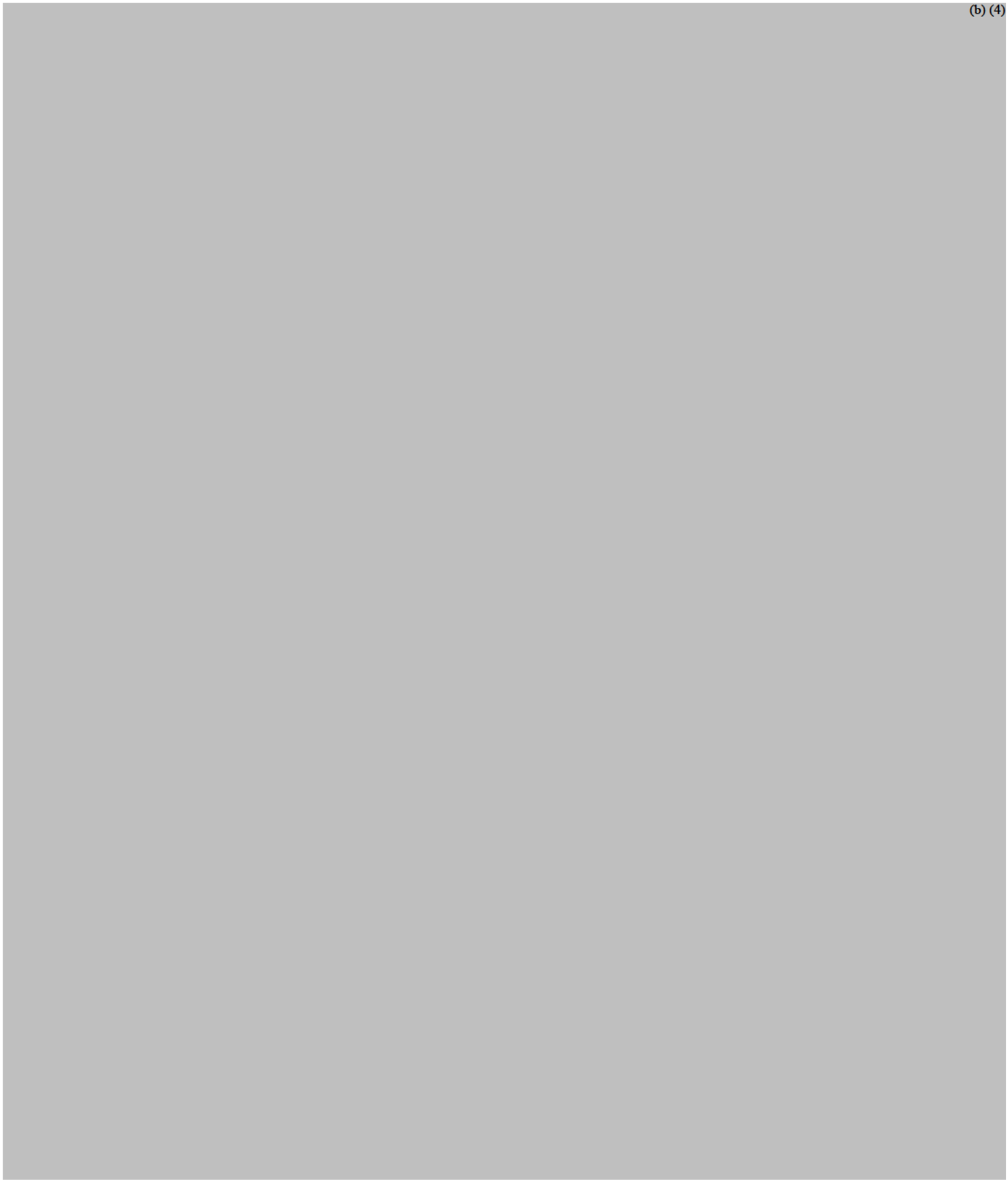

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Report I. Genetic Stability test of production strain

REPORT

The study about
Genetic stability of
L-threonine producing strain
< Confidential >

ORIGINAL FINAL REPORT DATE: May 08, 2018

CJ BlossomPark

TITLE: The study about Genetic stability of L-threonine producing strain

OBJECTIVE OF THE STUDY

This study was done to confirm the genetic stability of L-threonine production strain Corynebacterium glutamicum KCCM80178

SCHEDULE OF THE STUDY

Approval of protocol: Apr 08, 2018

Initiation of experiment: Apr 10, 2018

Termination of experiment: May 04, 2018

Submission of final report: May 08, 2018

TESTING PLACE

Name: CJ Blossom Park, BIO) Metabolic Engineering center
Address: 42, Gwanggyo-ro, Yeongtong, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, Seoul, Korea
Tel: +82-31-8099-2117

RESPONSIBLE STAFFS

Study Director	Suyon Kwon	(05/08/18)
Quality Assurance Manager	Kwang-woo Lee	(08/08/18)

1. Summary

Corynebacterium glutamicum KCCM80178, the producing strain of L-threonine, it was made by the deletion of the gene related to the side reaction and amplification of the gene related to the synthetic pathway. The method used for the amplification of the gene was known to very stable method which was not known to have any further mutation after the insertion.

Genetic stability of the producing strain KCCM80178 was confirmed by the stable production of the product from the different generations of stocks of the microorganism. Since all the amplified genes are related to the synthetic pathway of the product, genetic instability can induce the loss of production yield and change in culture time. Fermentation using KCCM80178 was repeated in the laboratories and all the data showed the similar production and culture profile.

The genetic stability of the production strain KCCM80178 was also confirmed by the PCR techniques. Genomic DNA was prepared from each step of the fermentation, and analysed by PCR analysis using the amplified genes as primer. The amplified genes were confirmed in the same location as the integration locus without any genetic location shift.

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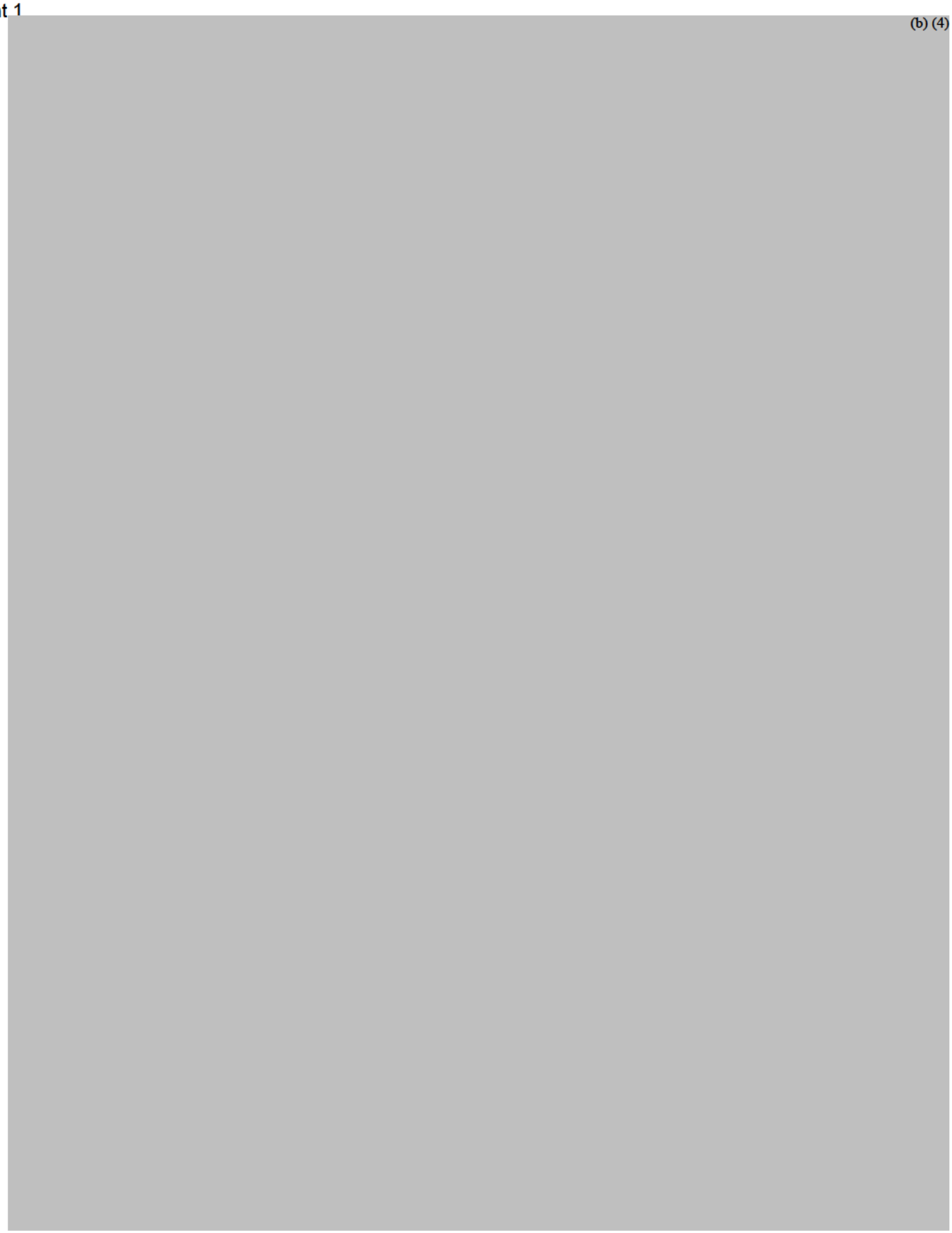

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Open Reading Frame Analysis of Genetically Modified Site

REPORT

The open reading frame analysis for the modified site on the *C. glutamicum* KCCM 80178

REPORT DATE: May 28, 2018
CJ BLOSSOM PARK

TITLE: The analysis of open reading frame for the modified site on the C.glutamicum KCCM 80178

OBJECTIVE OF THE STUDY

This study was done to analysis of open reading frame for the modified site on the C.glutamicum KCCM 80178

SCHEDULE OF THE STUDY

Data of Receipt: Apr 25, 2018

Data of Test: May 01, 2018

Data of Final report: May 28, 2018

TESTING FACILITY

Name: CJ Blossom Park, BIO) Metabolic Engineering center

Address: 42, Gwanggyo-ro, Yeongtong, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, Seoul, Korea

Tel: +82-31-8099-2117

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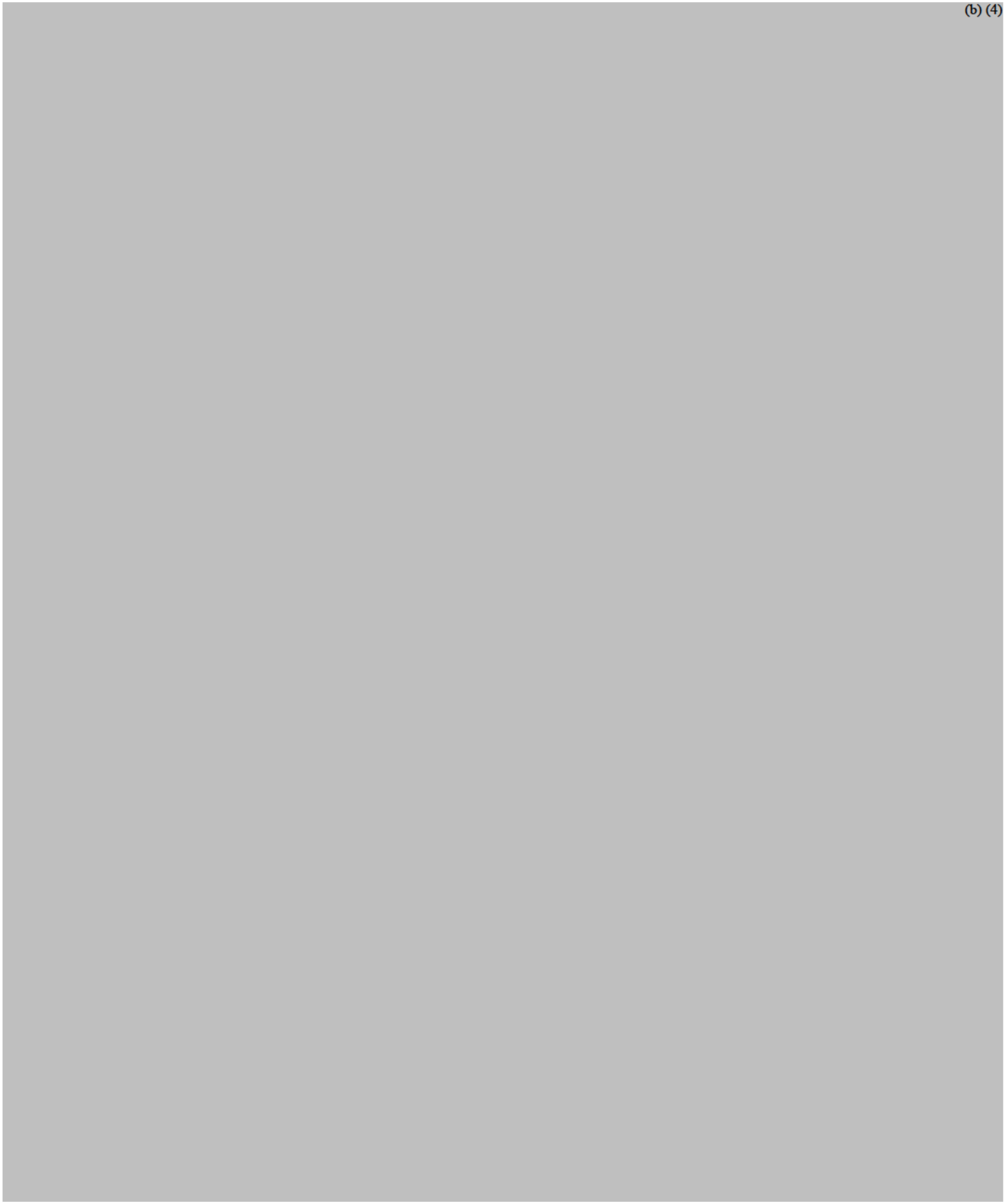

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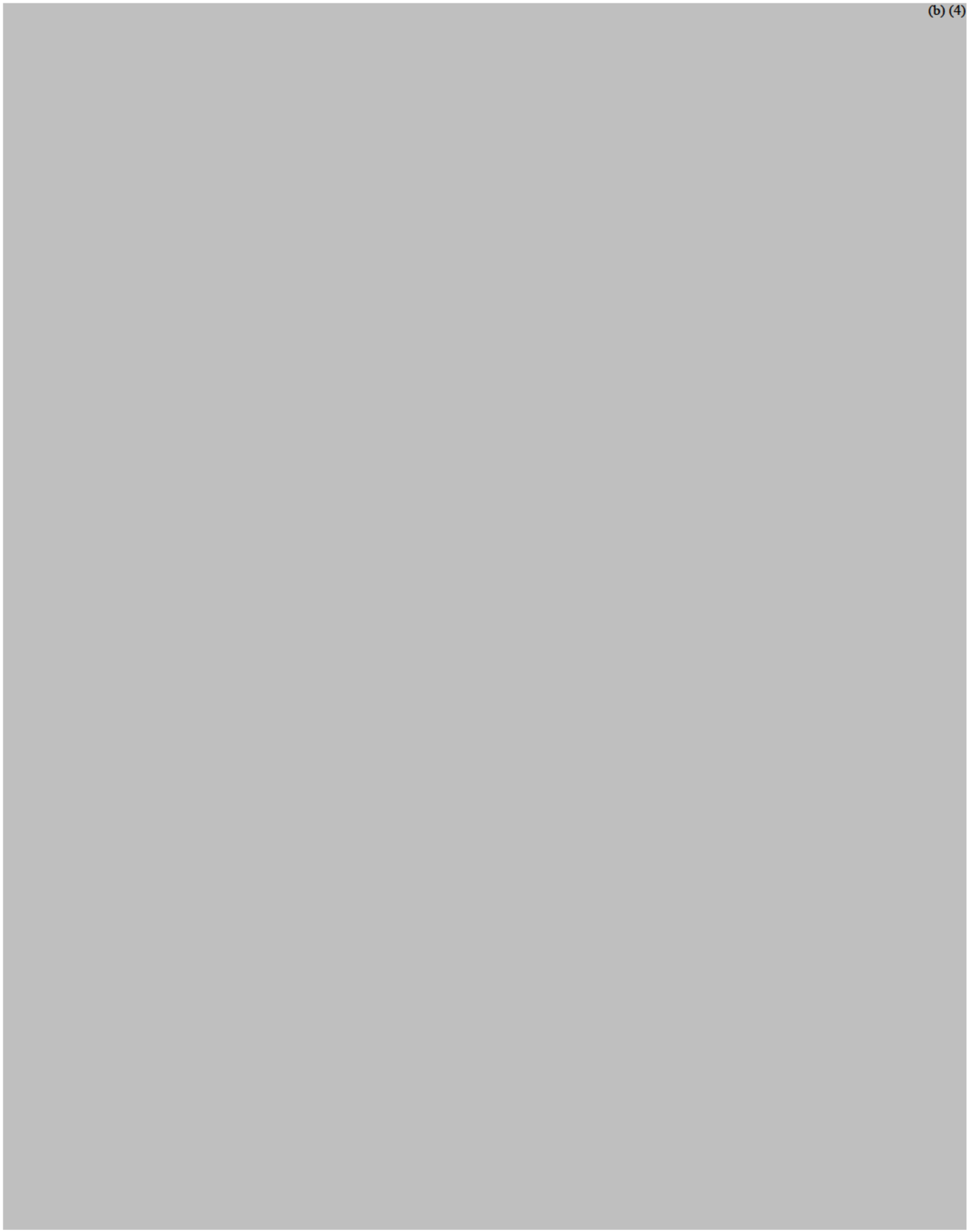

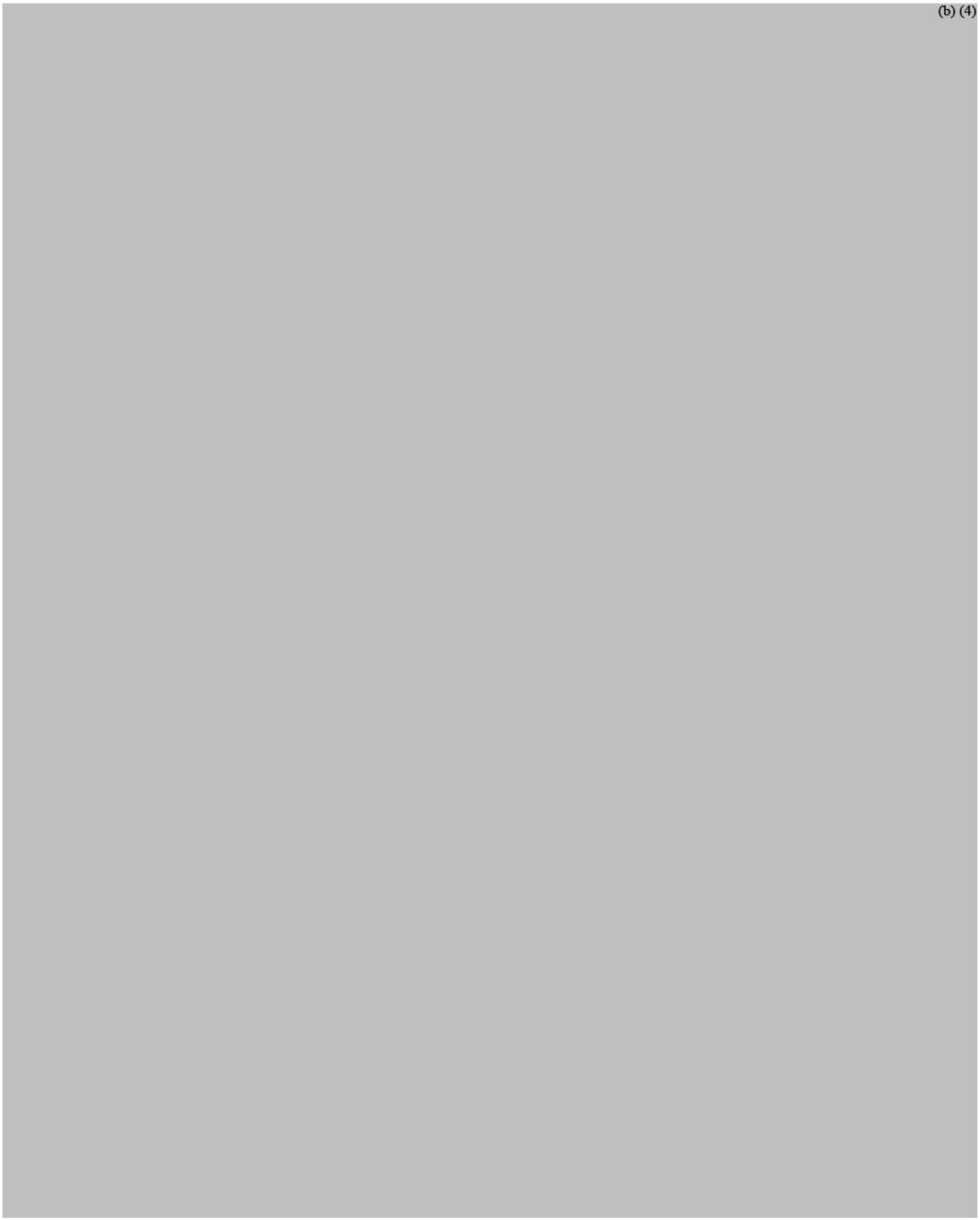

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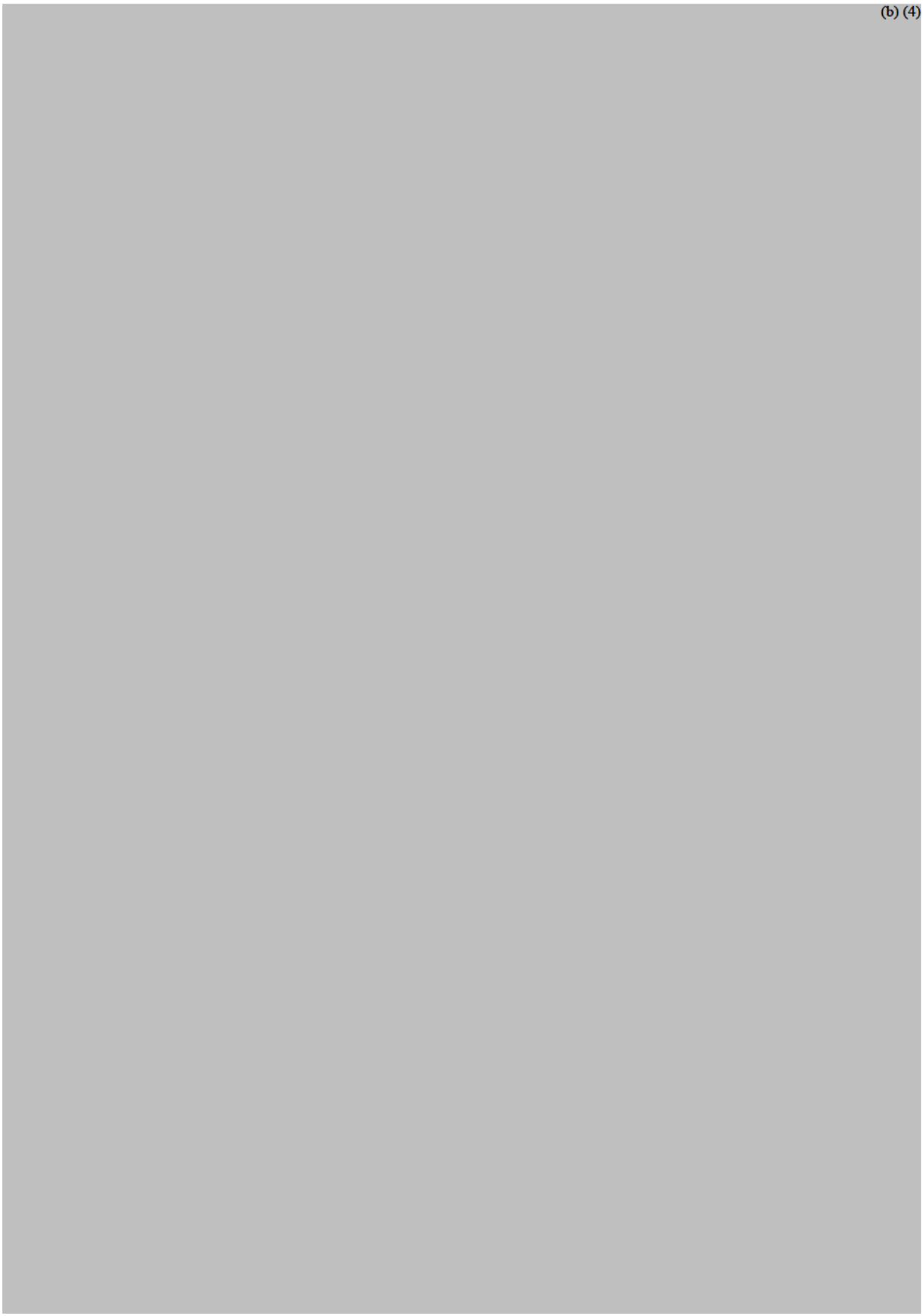

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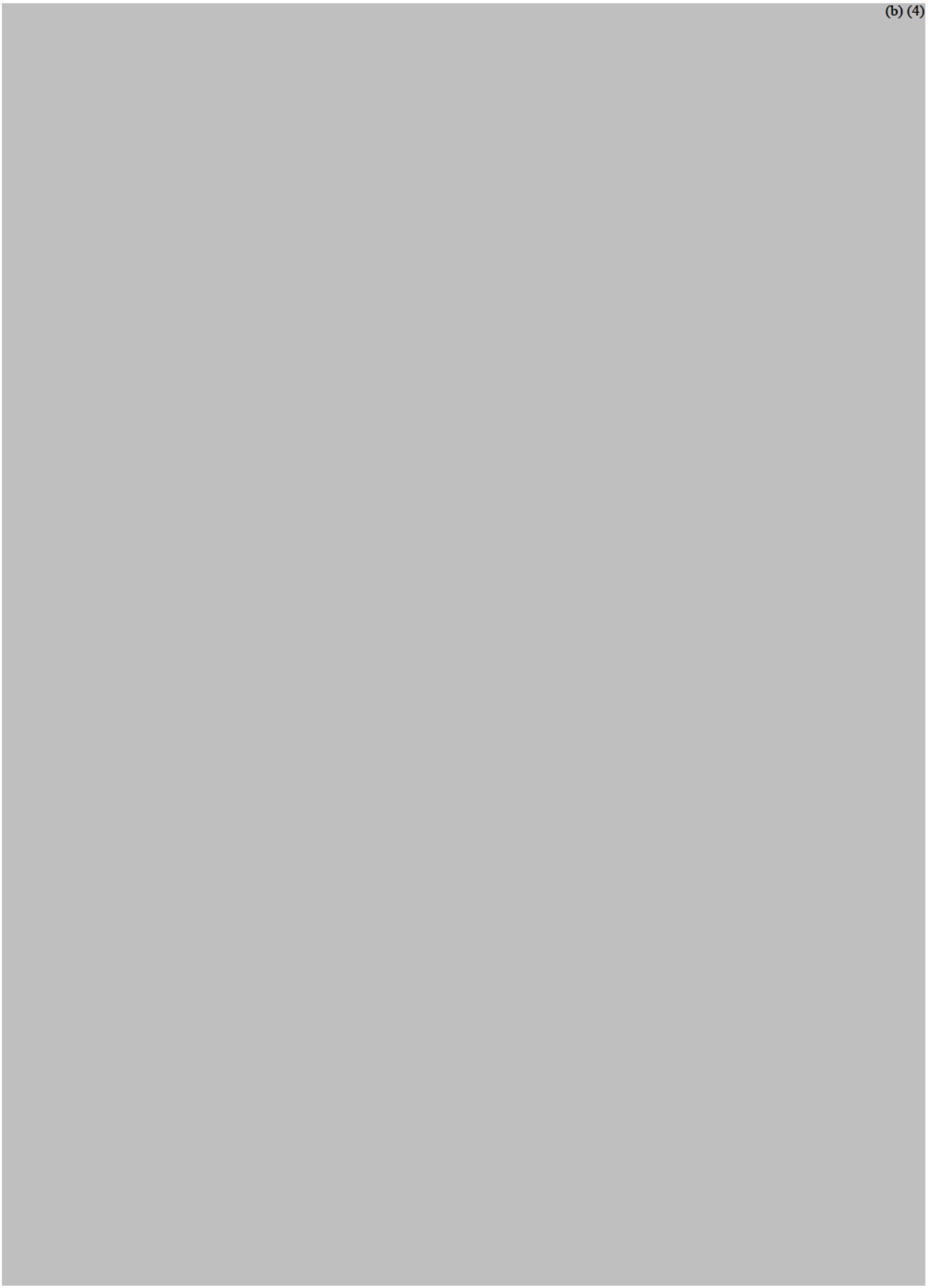

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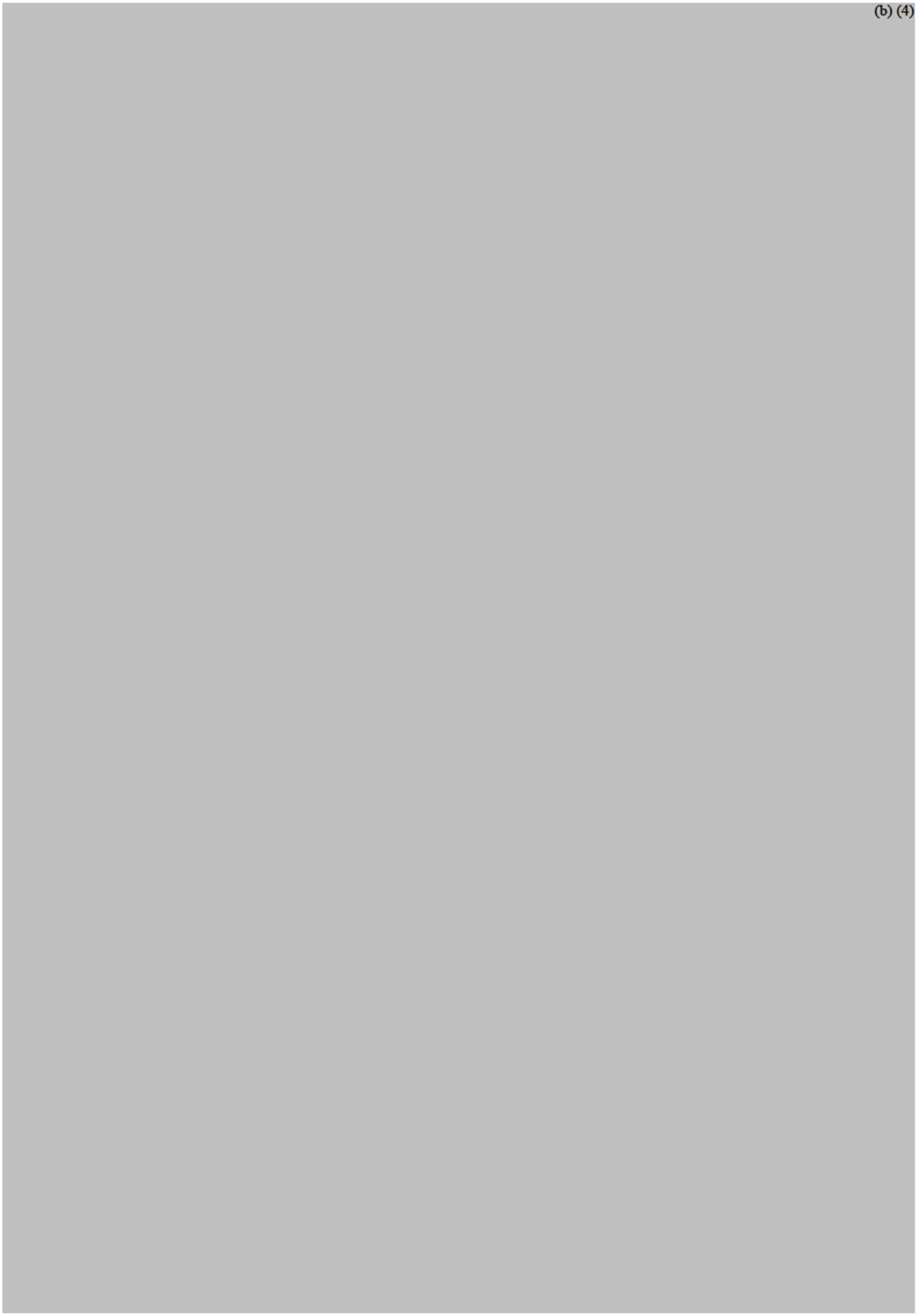

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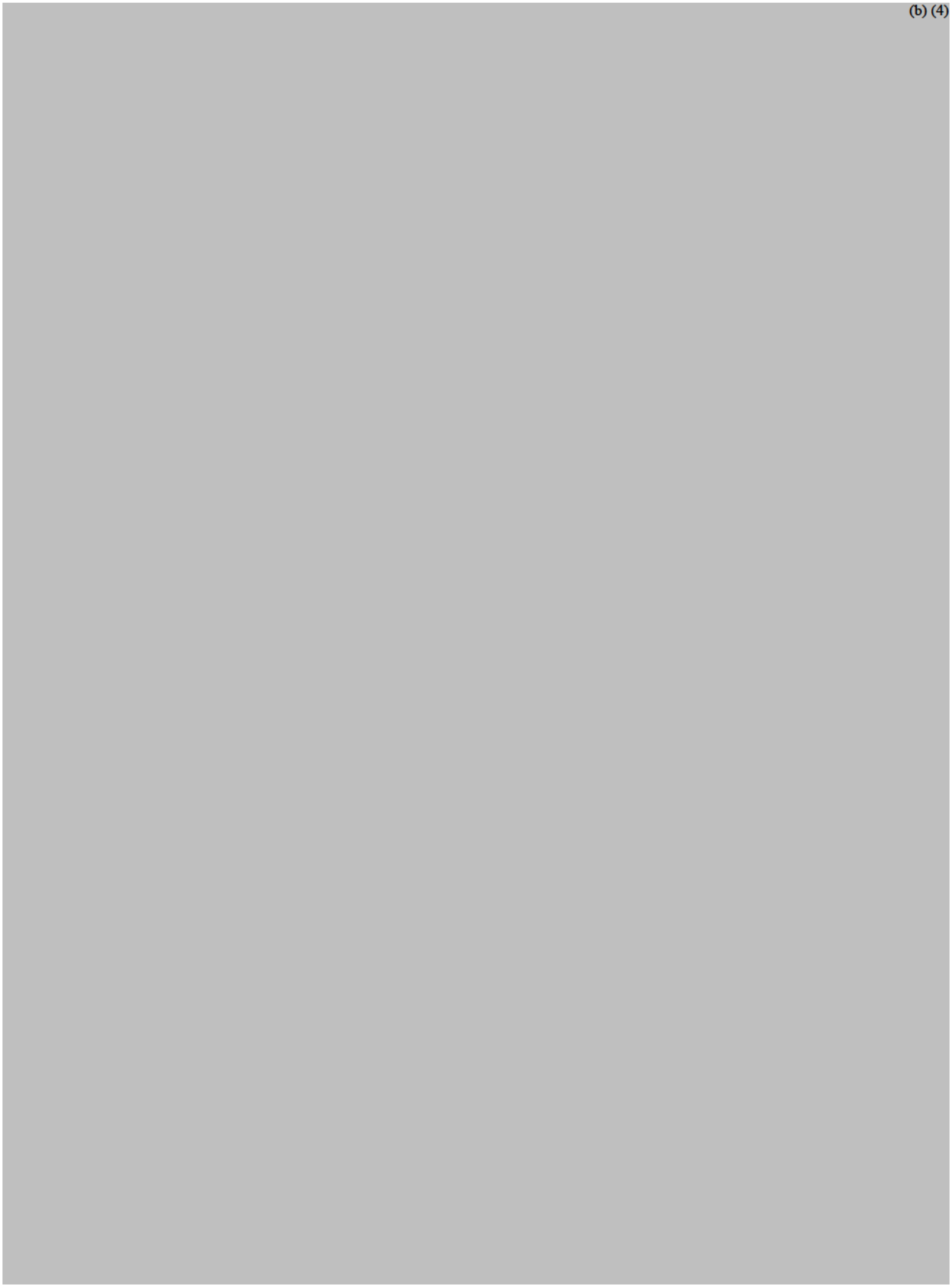

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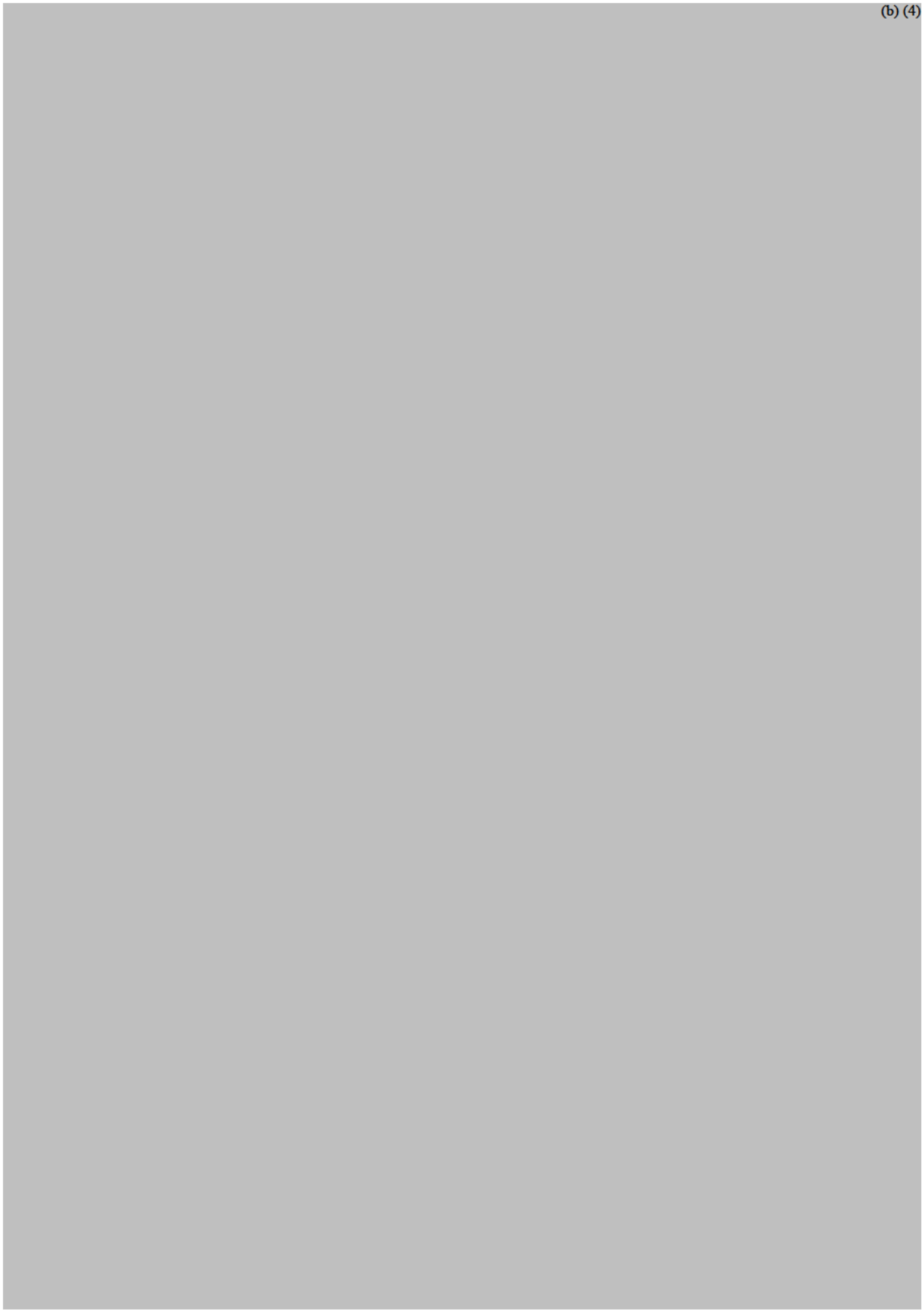

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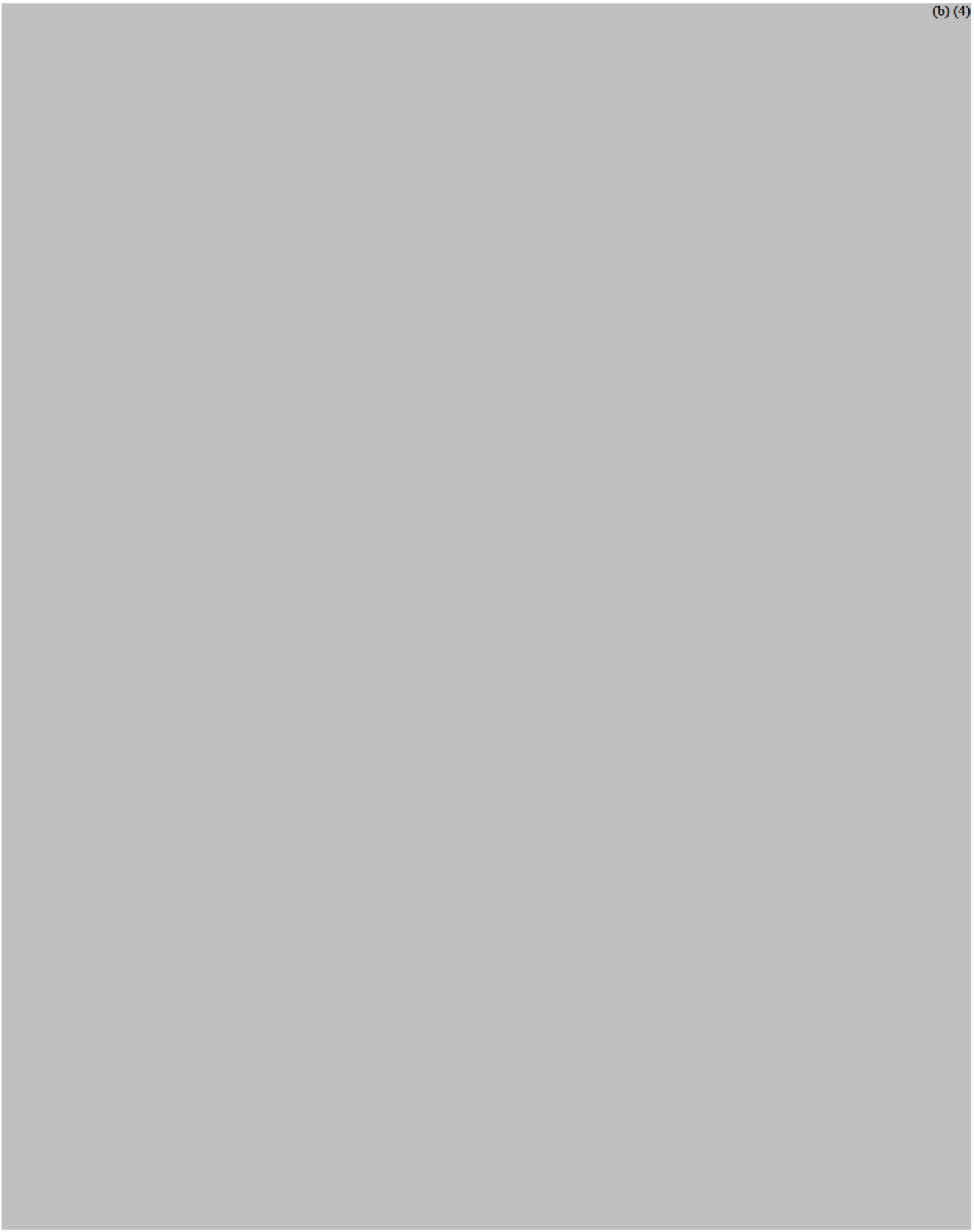

Appendix 2

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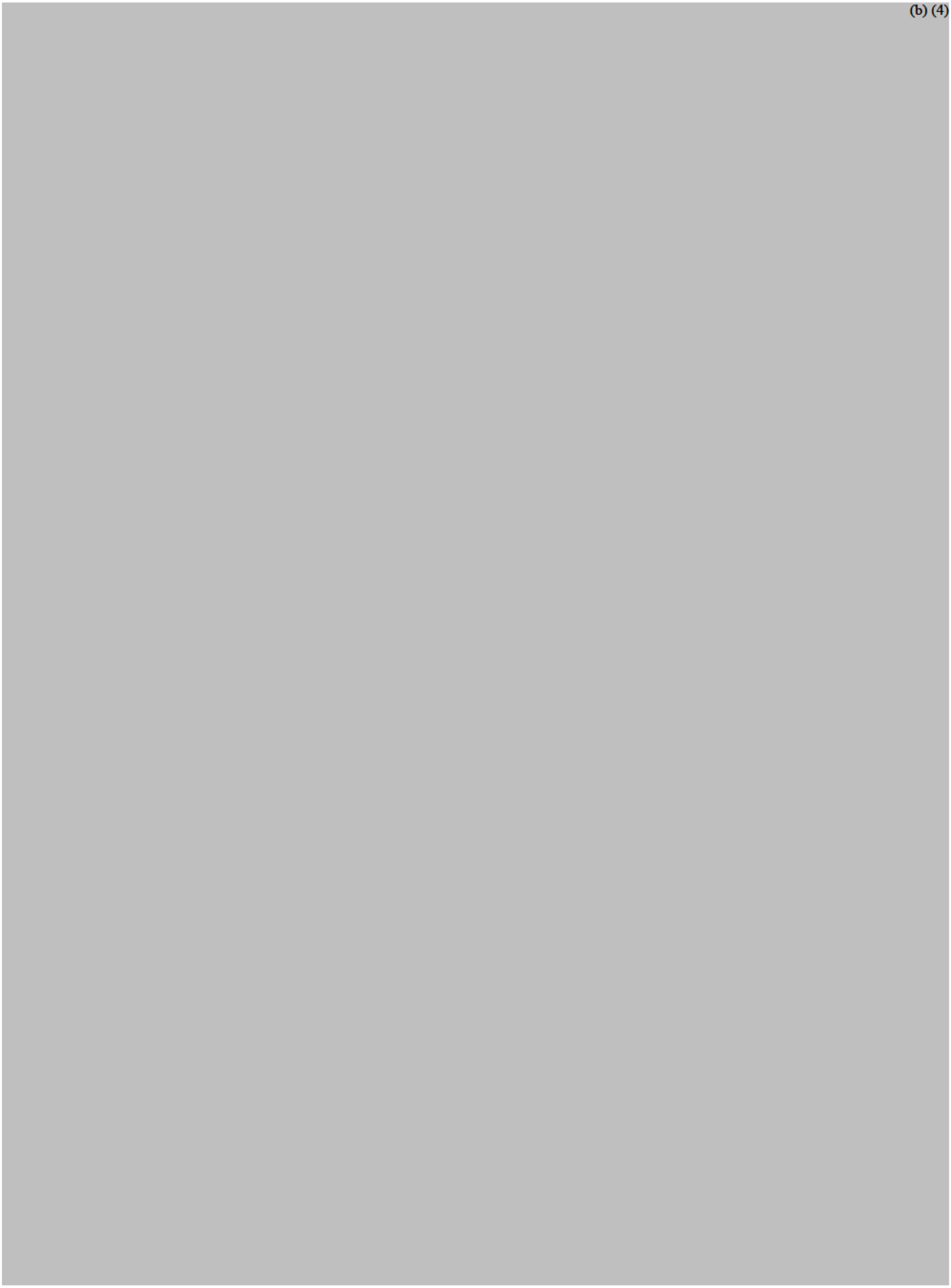

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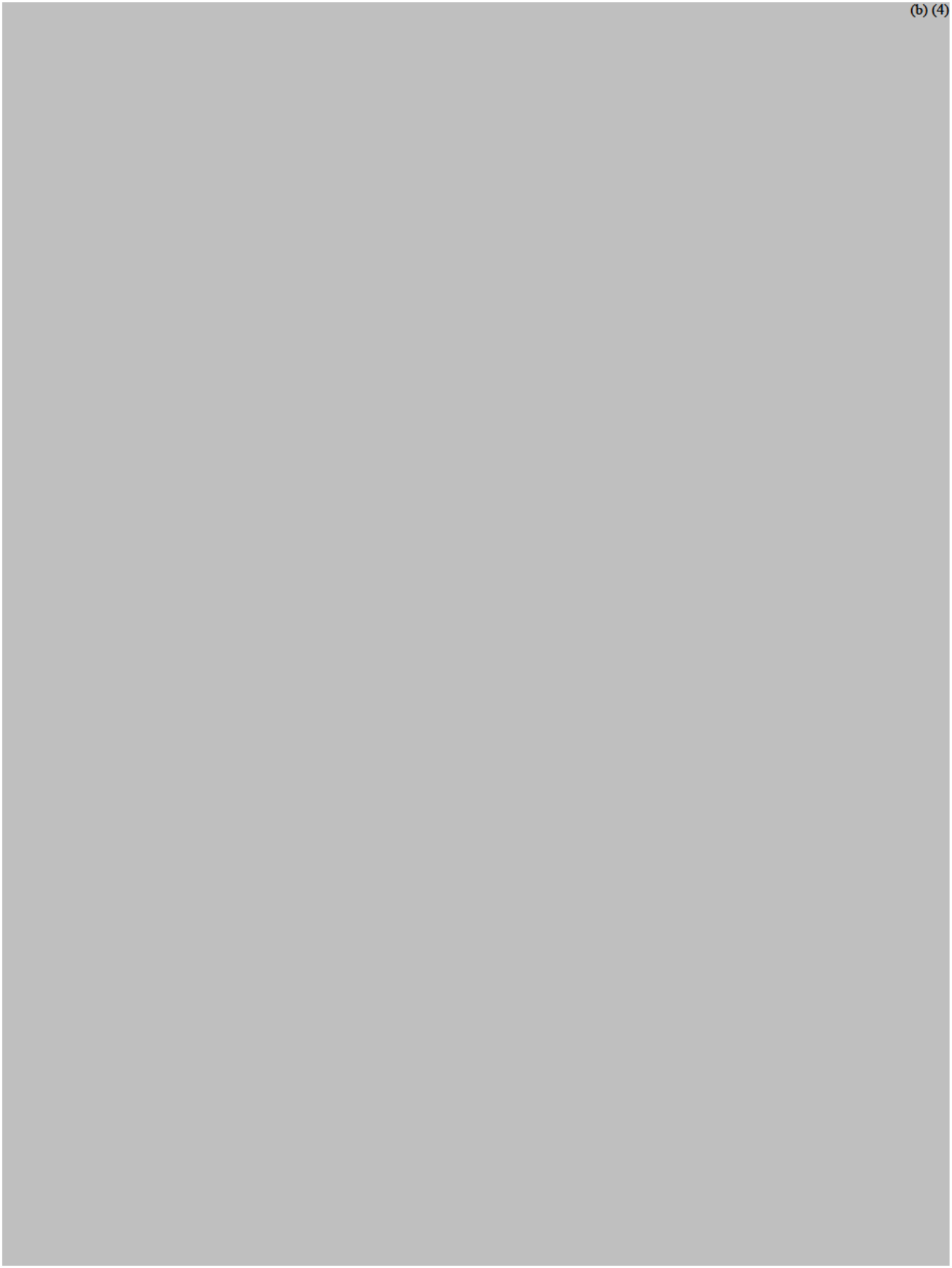

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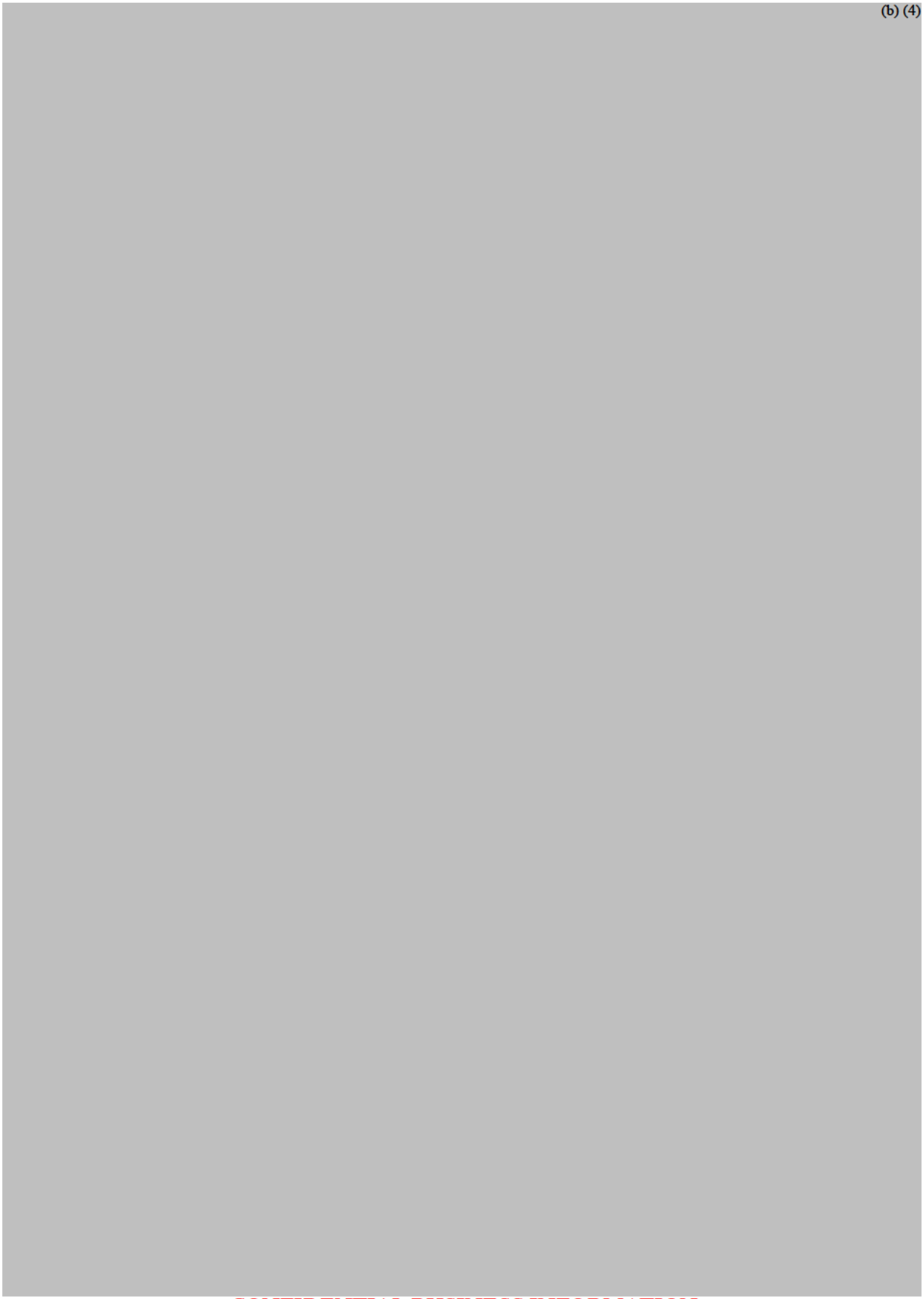

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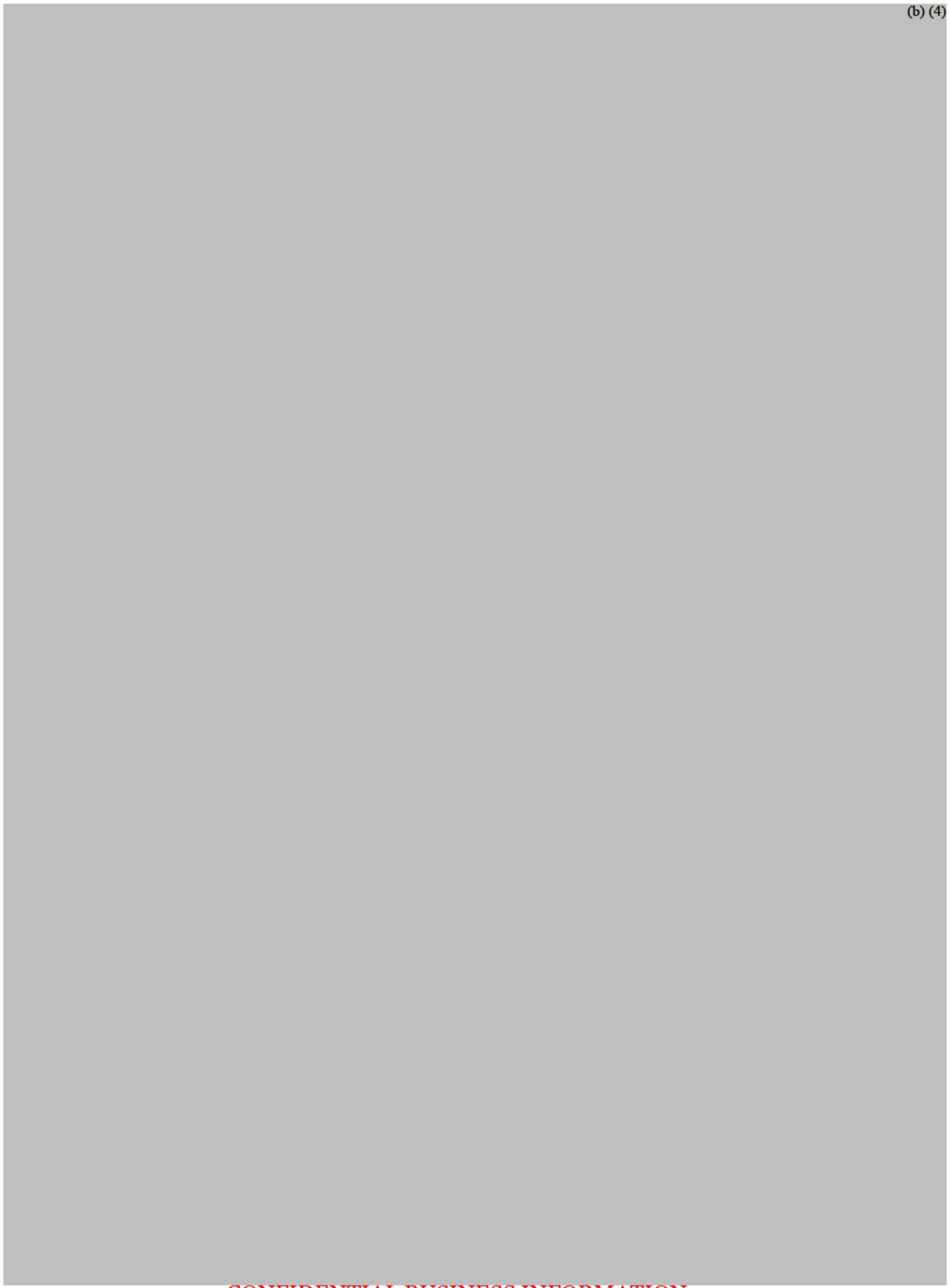

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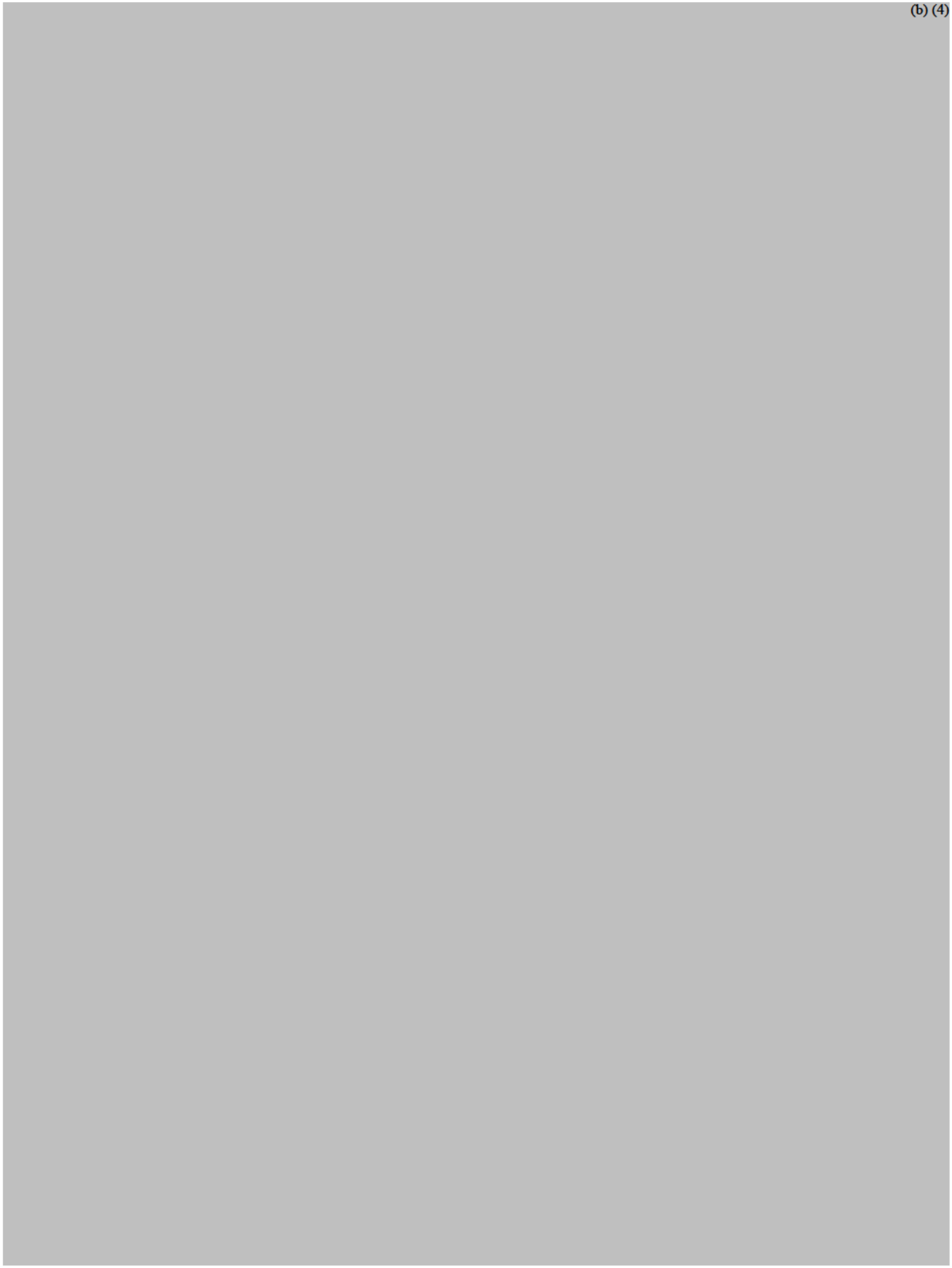

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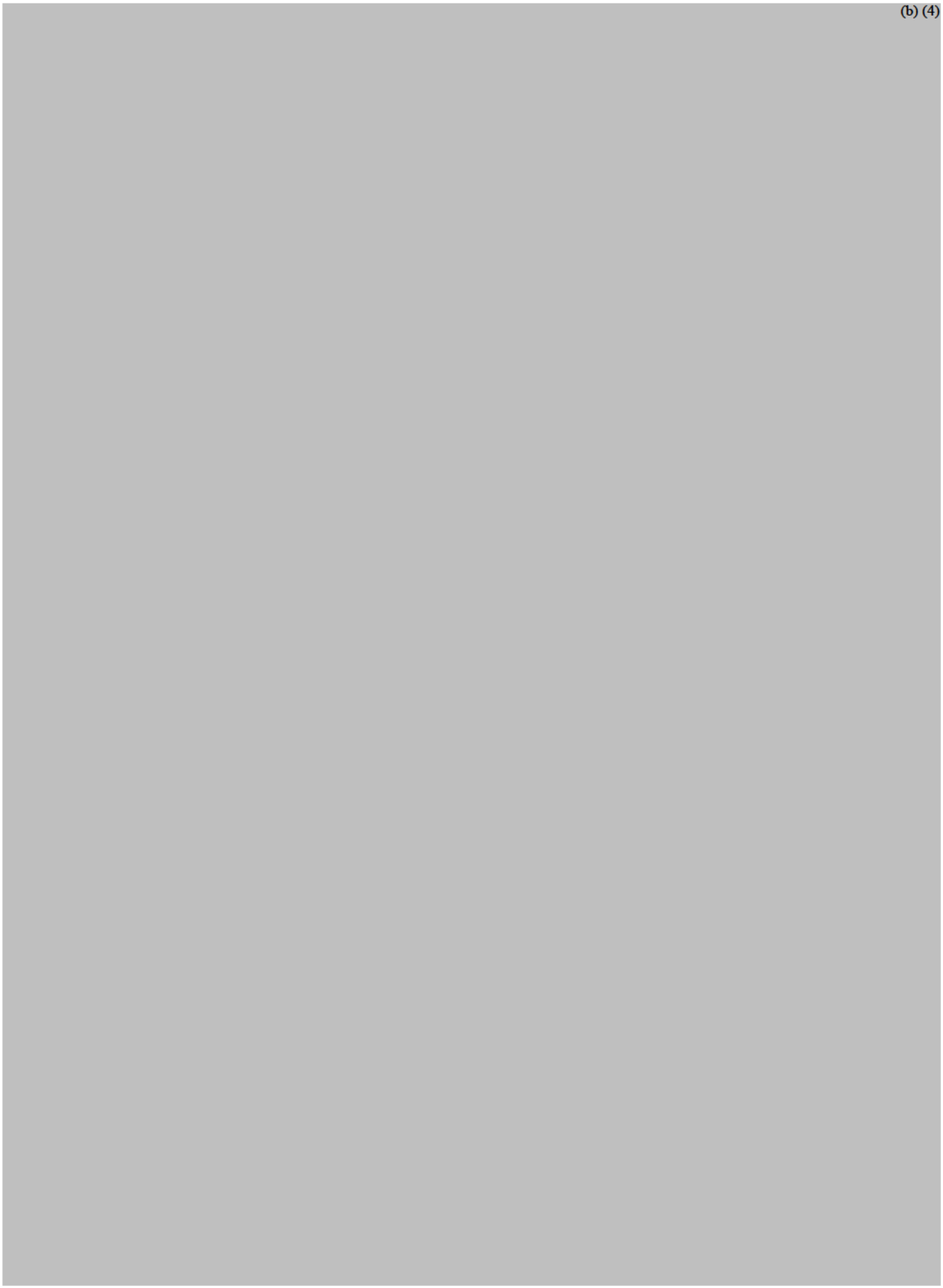

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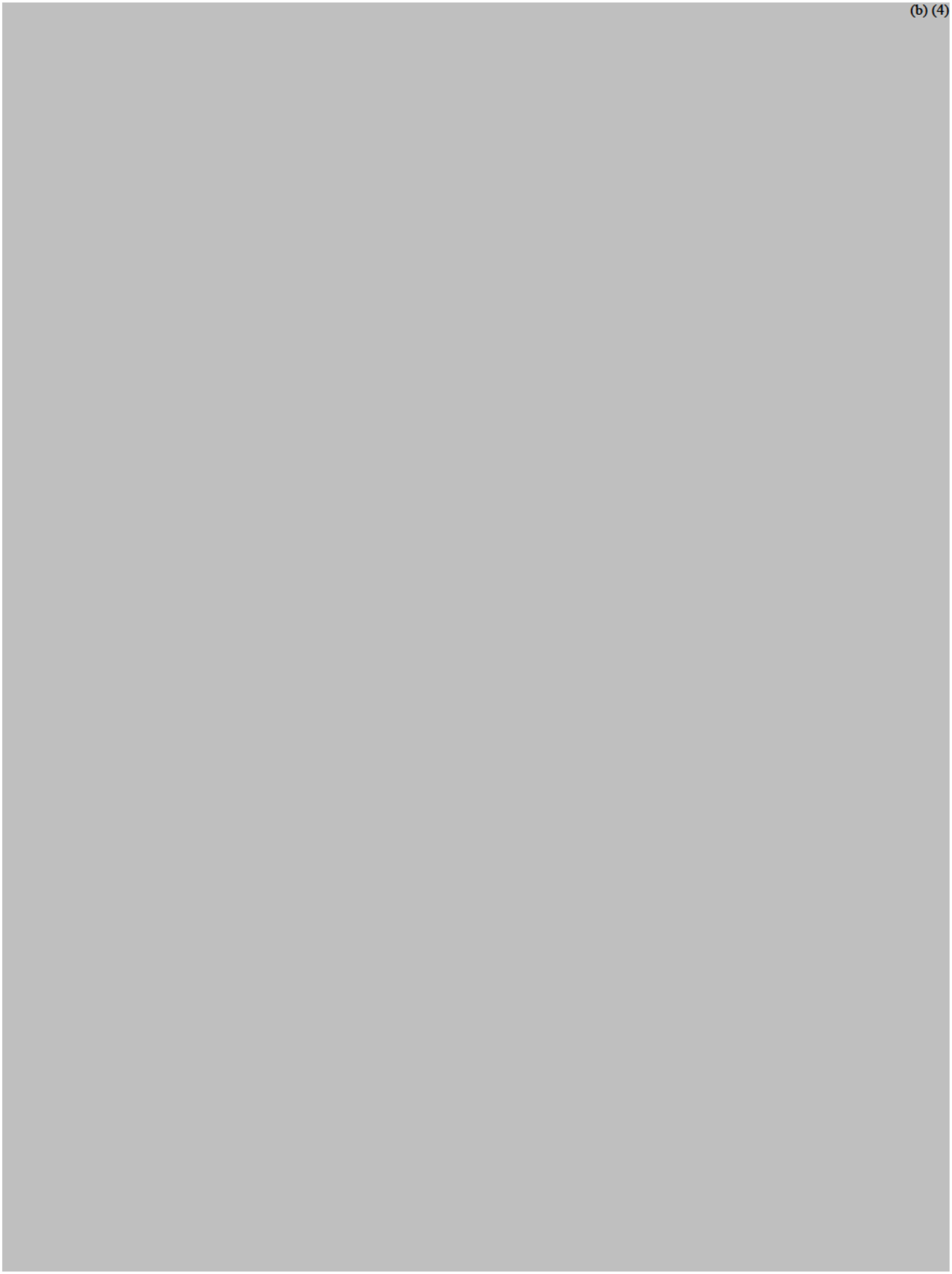

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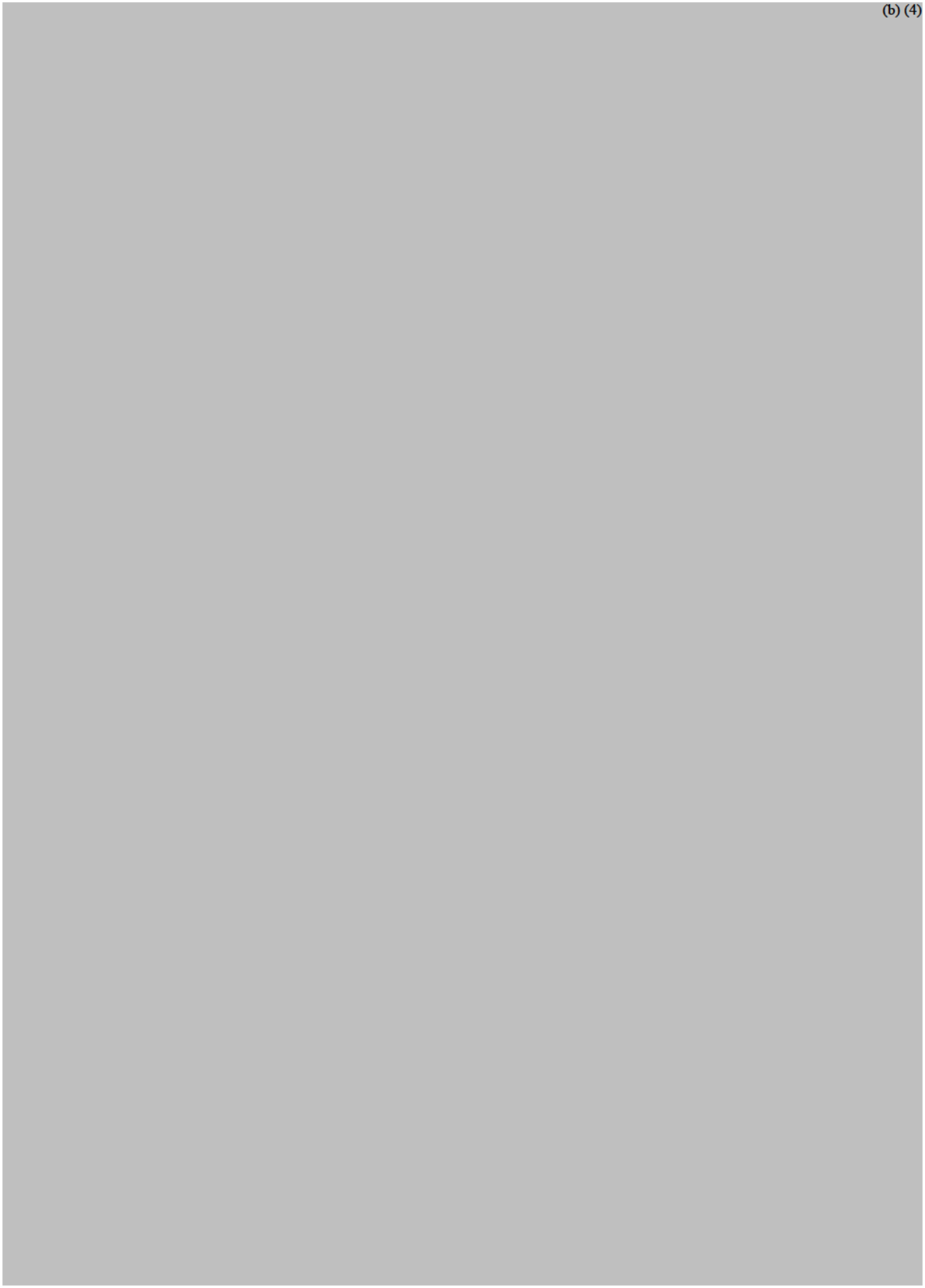

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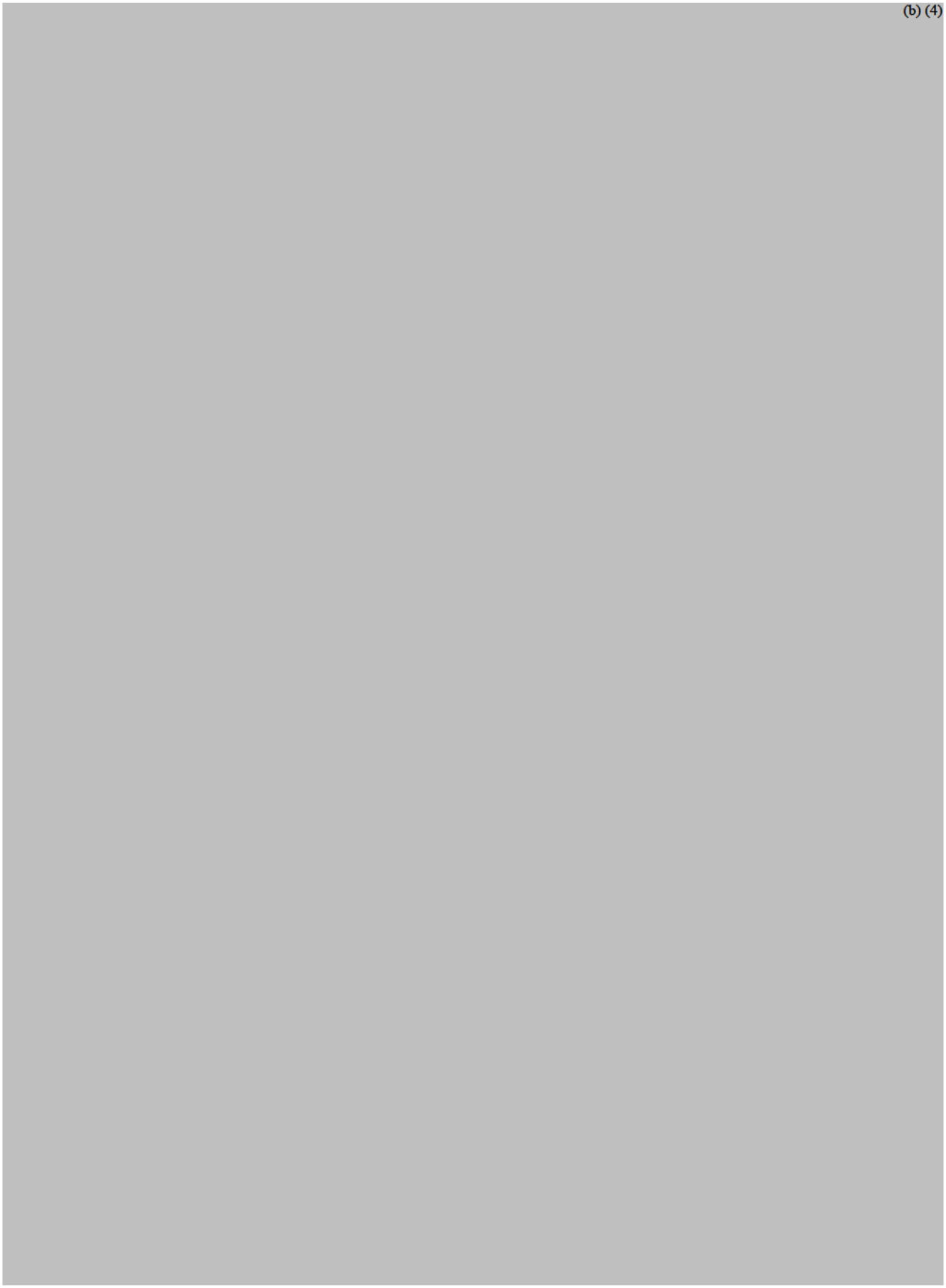

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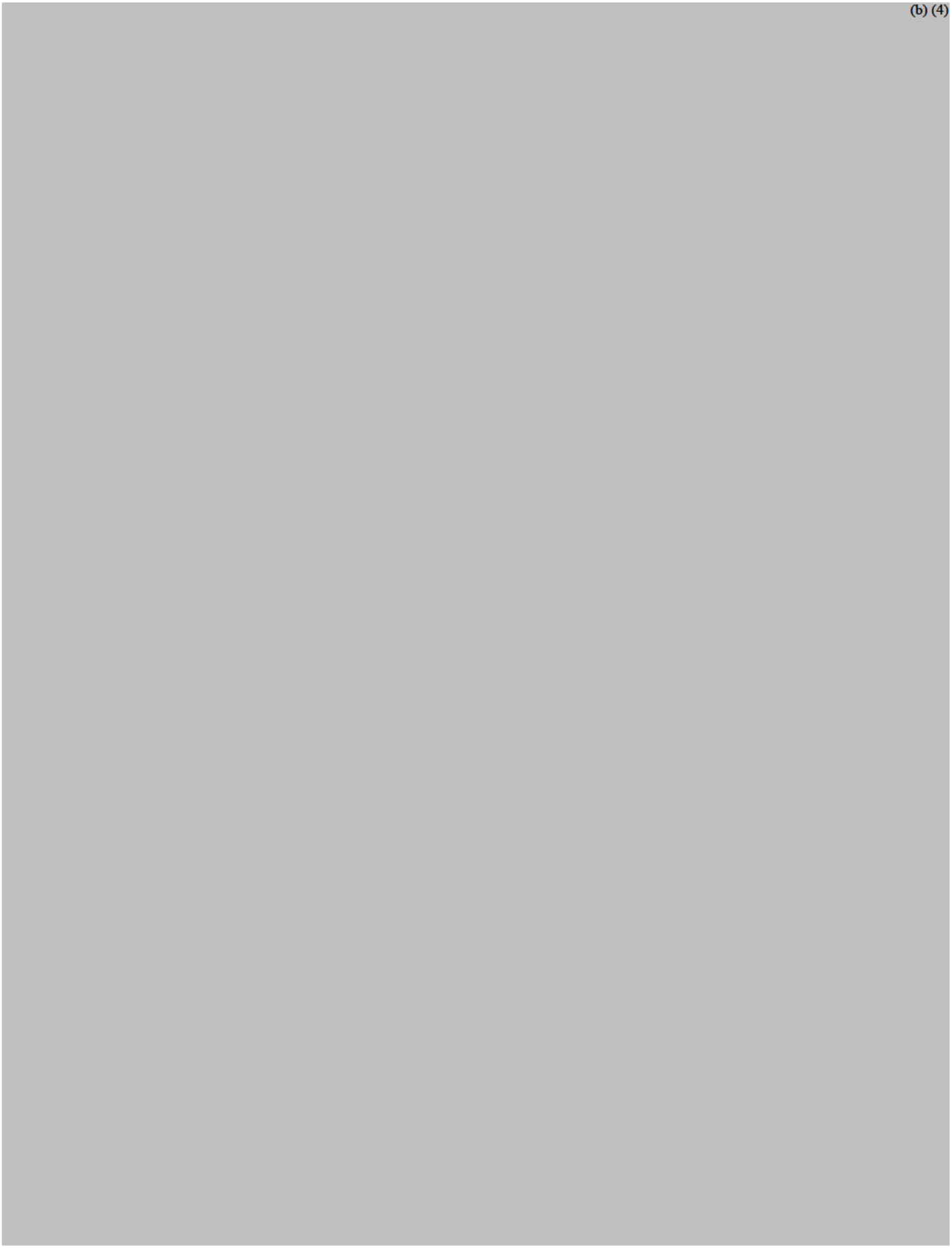

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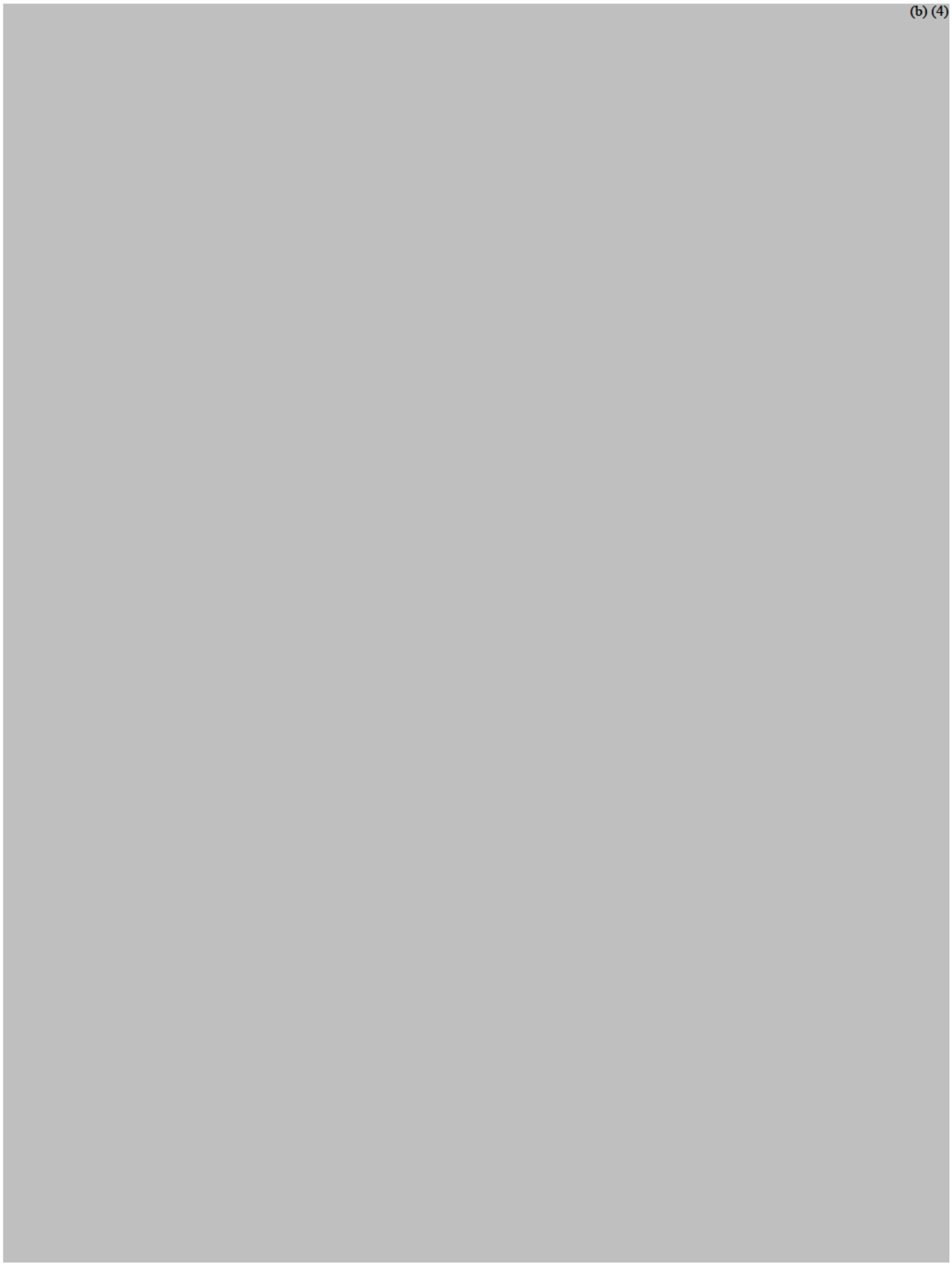

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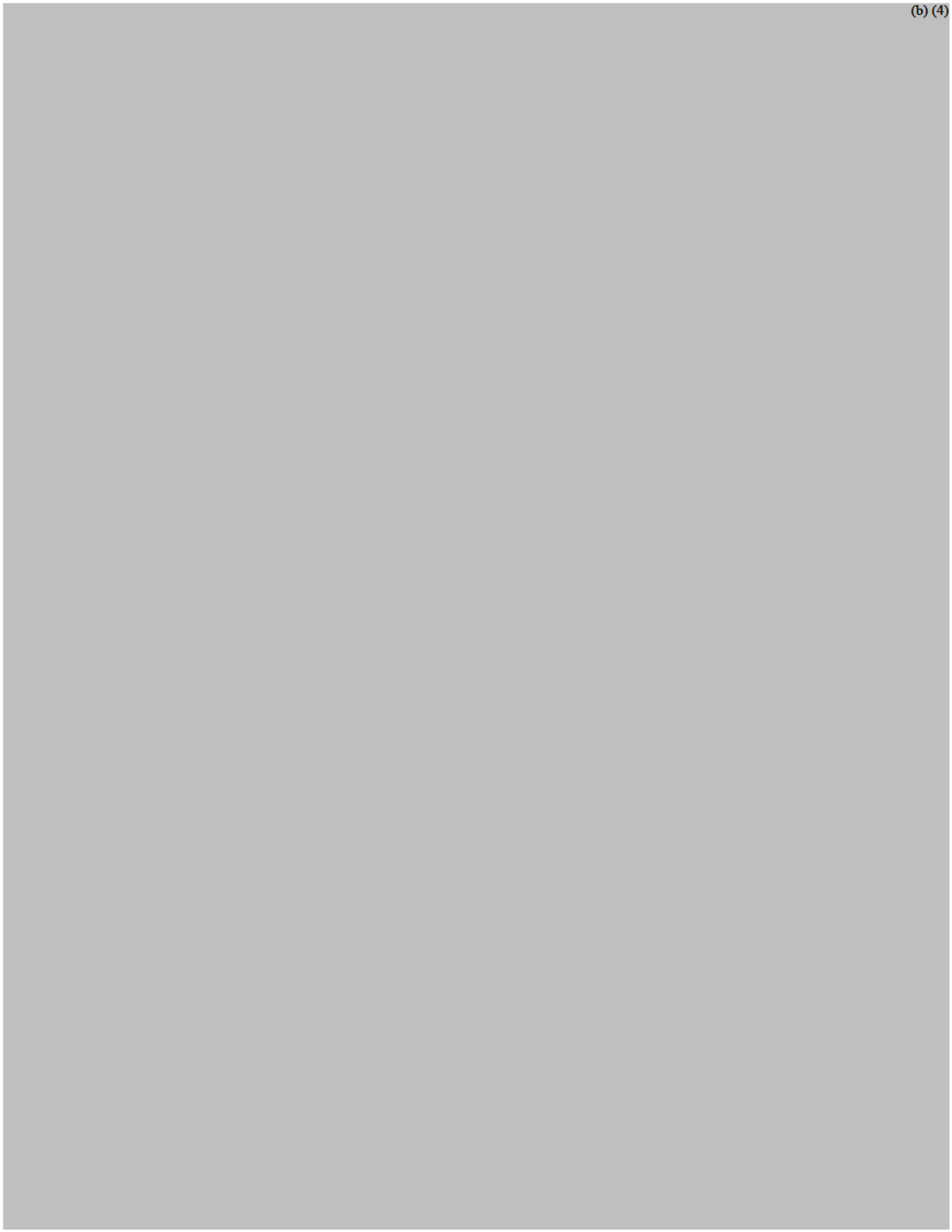

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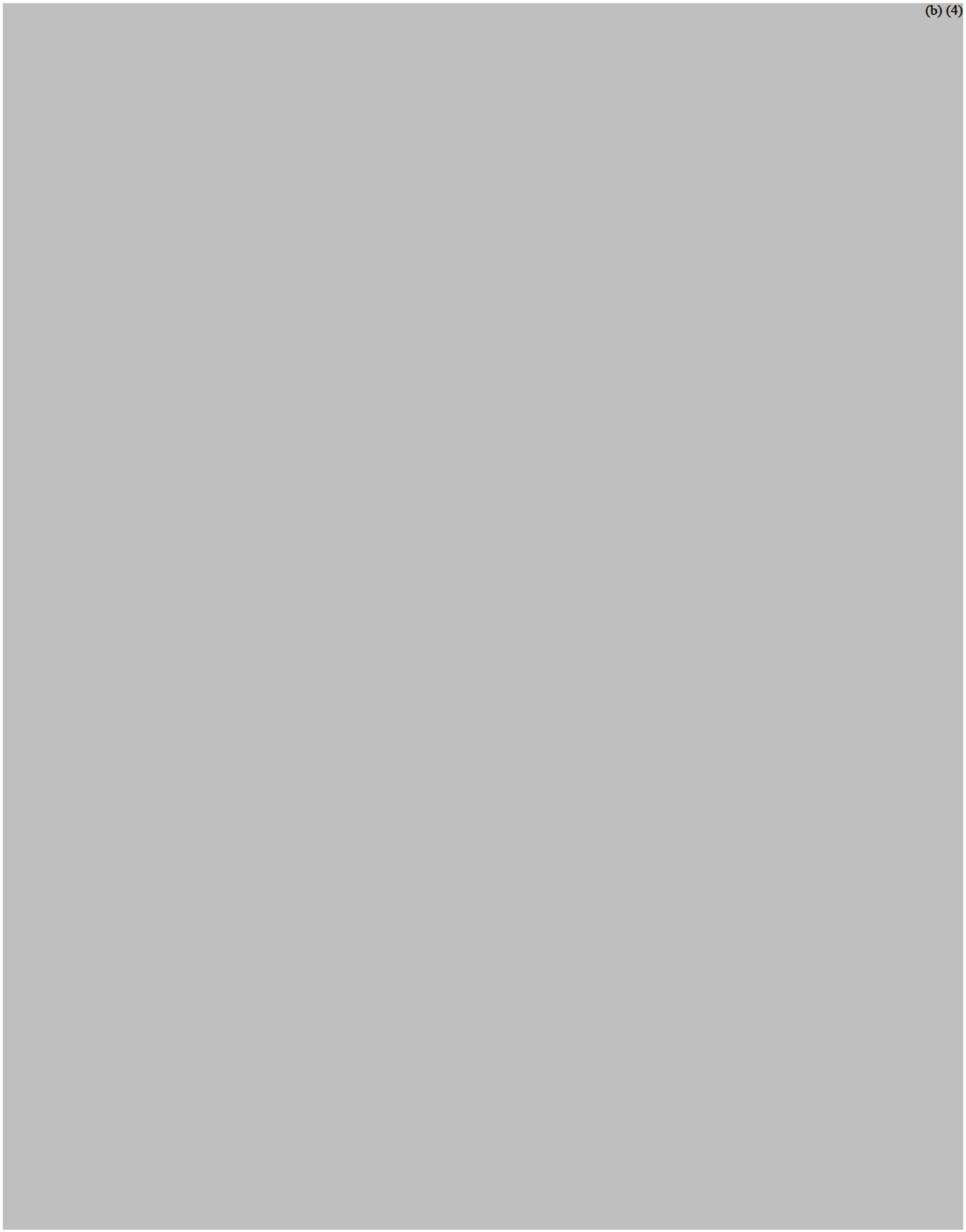

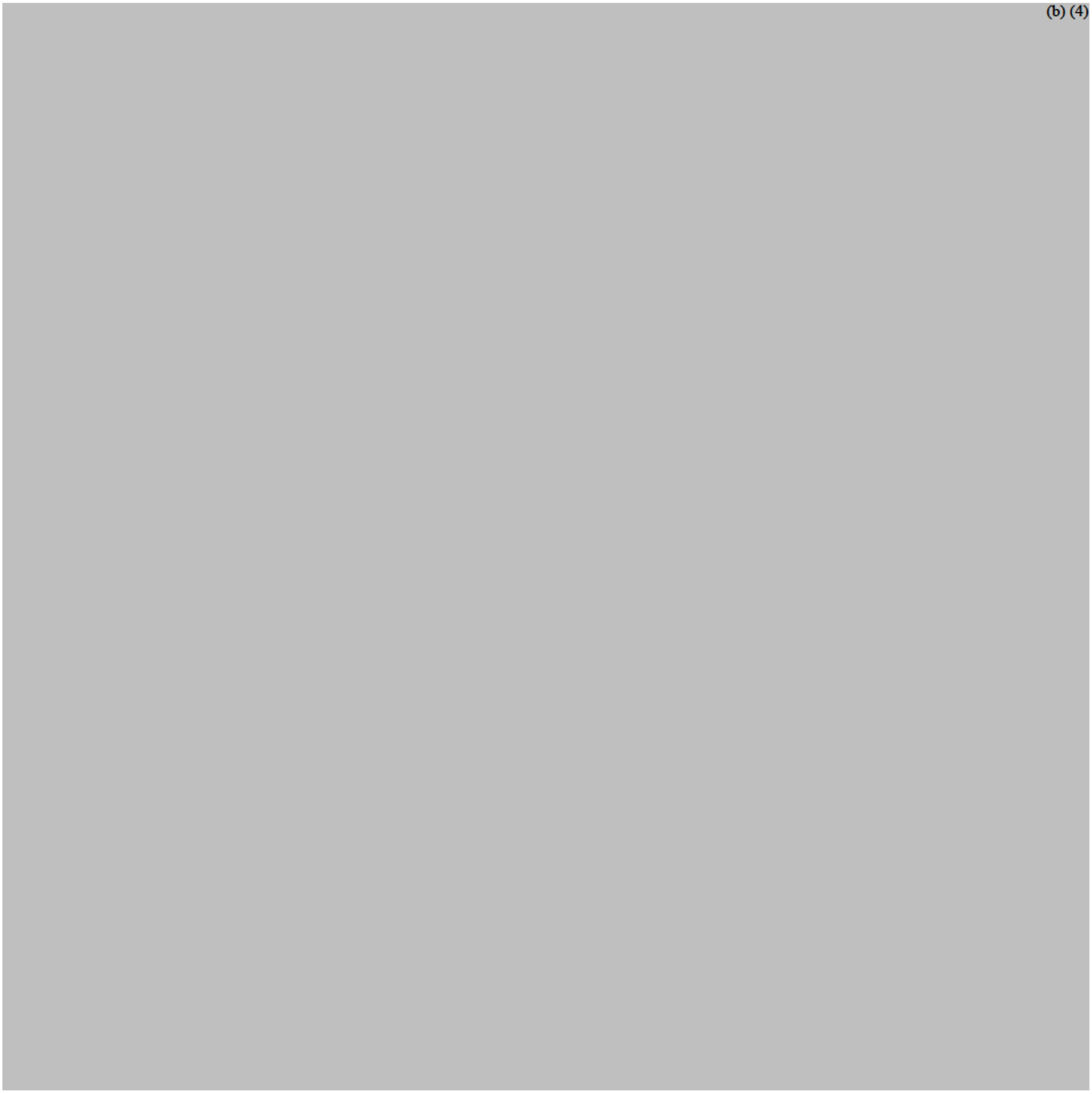
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(b) (4)

Appendix 2

Attachment 2

REPORT III. Open Reading Frame analysis of full genome sequence of production strain

REPORT

The open reading frame analysis for the Full Genome Sequence on the *C. glutamicum* KCCM 80178

REPORT DATE: August 17, 2018
CJ BLOSSOM PARK

TITLE: The analysis of open reading frame for full genome sequence on the *C. glutamicum* KCCM 80178

OBJECTIVE OF THE STUDY

This study was done to analysis of open reading frame for full genome sequence on the *C. glutamicum* KCCM 80178

SCHEDULE OF THE STUDY

Data of Receipt: July 13, 2018

Data of Test: July 16, 2018

Data of Final report: August 17, 2018

TESTING FACILITY

Name: CJ Blossom Park, BIO) Metabolic Engineering center

Address: 42, Gwanggyo-ro, Yeongtong, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, Seoul, Korea

Tel: +82-31-8099-2117

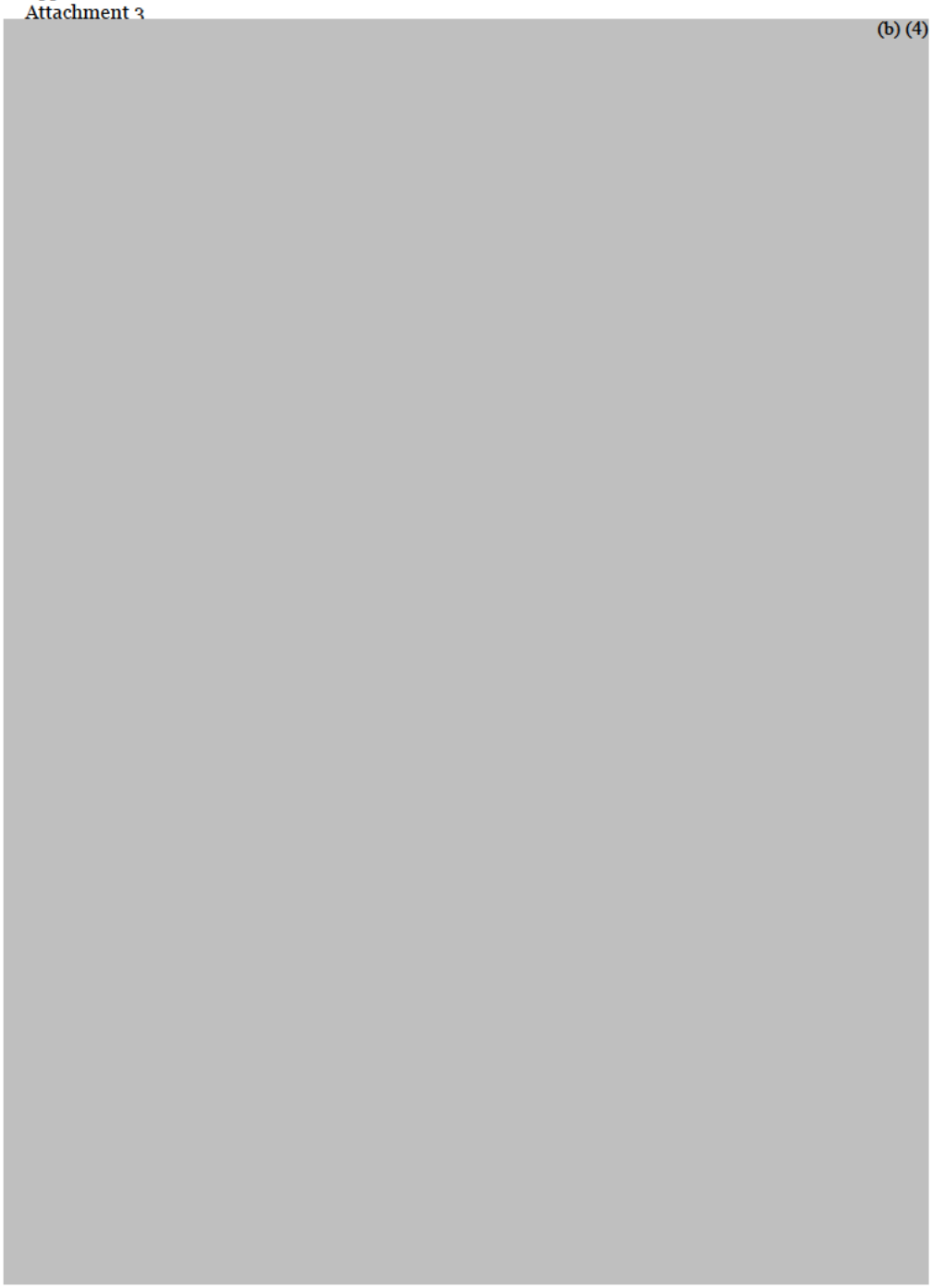
(b) (4)

Genes	Integrated locus	Location in Genome

(b) (6)

(b) (4)

(b) (4)



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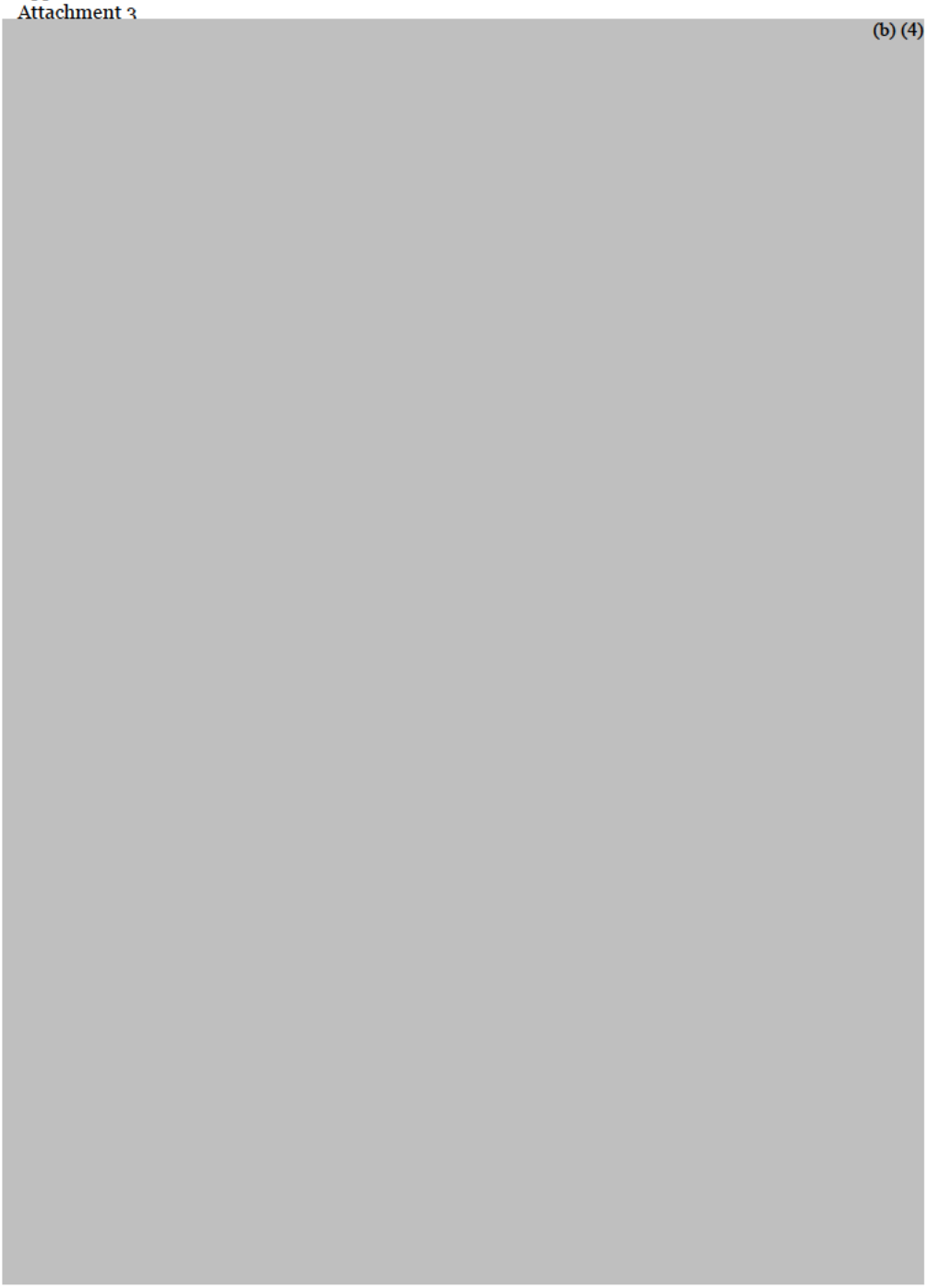

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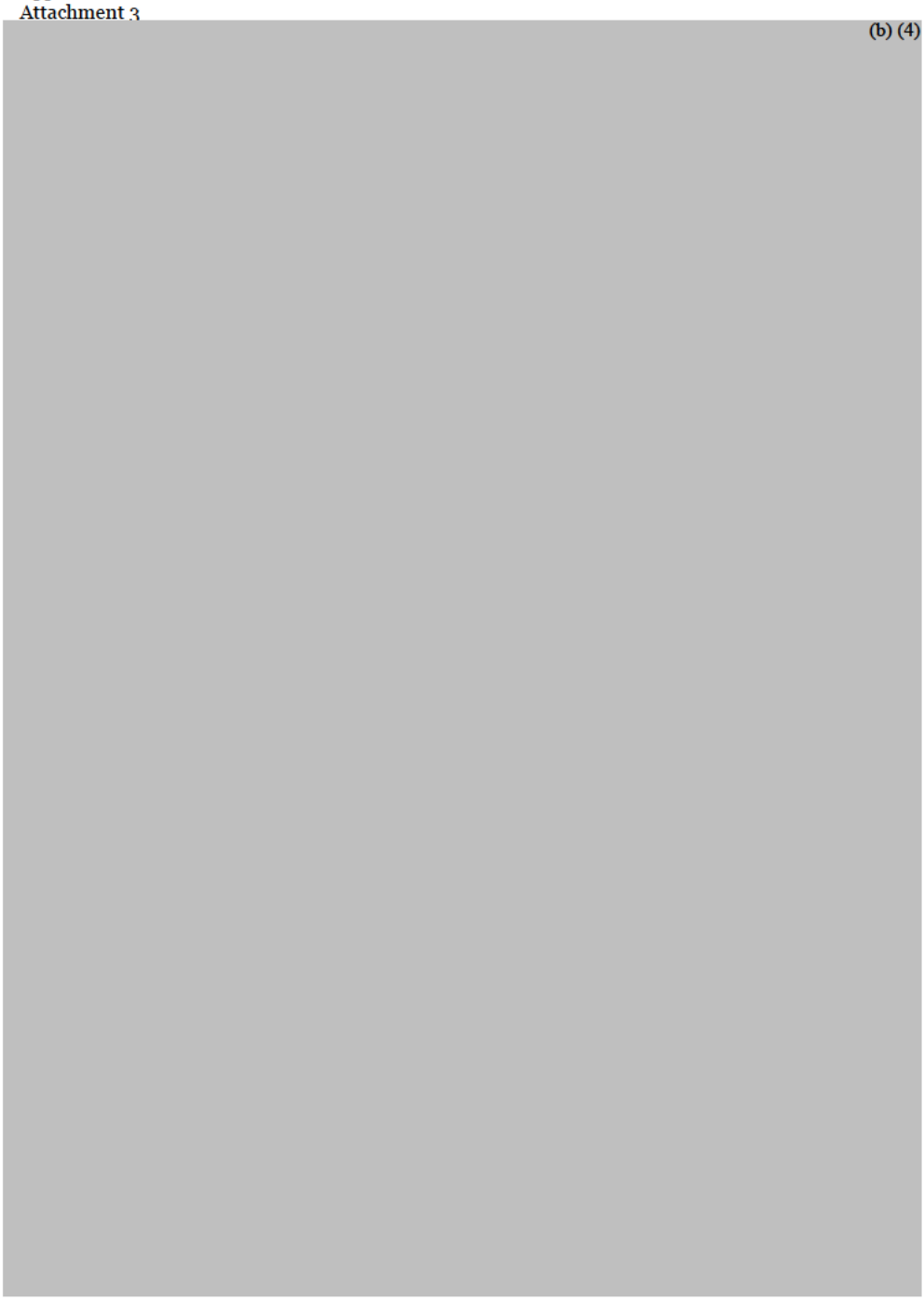

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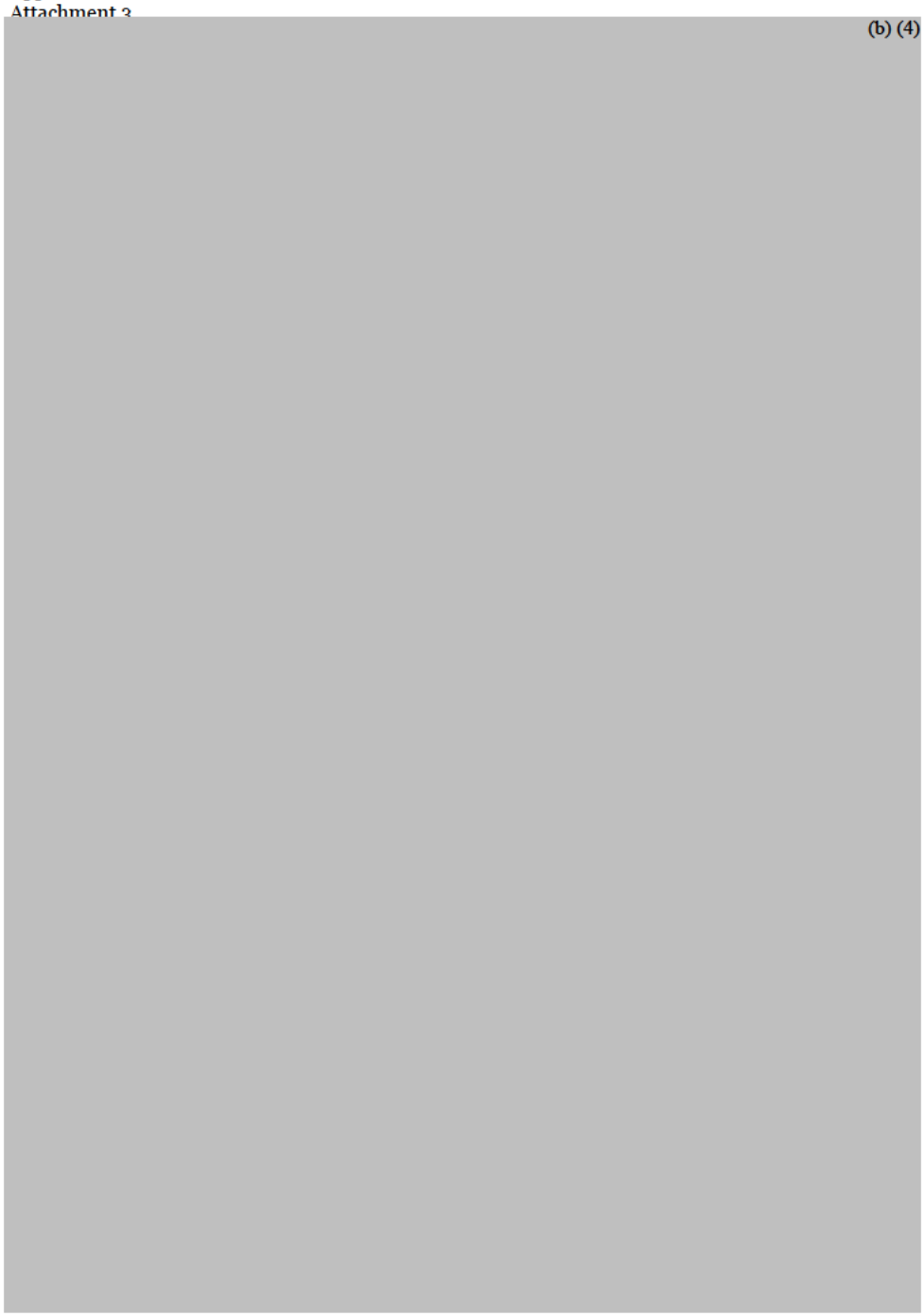

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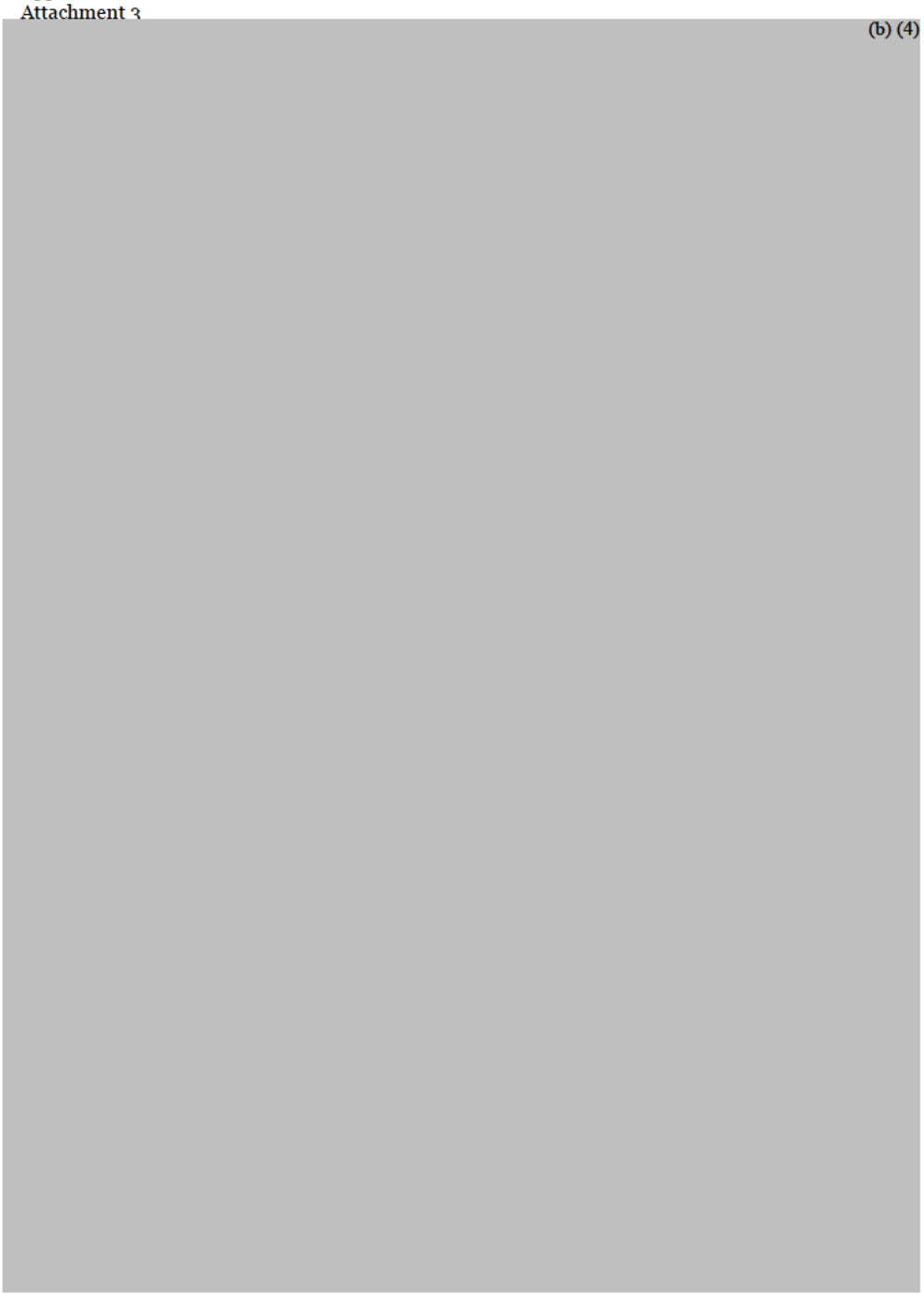

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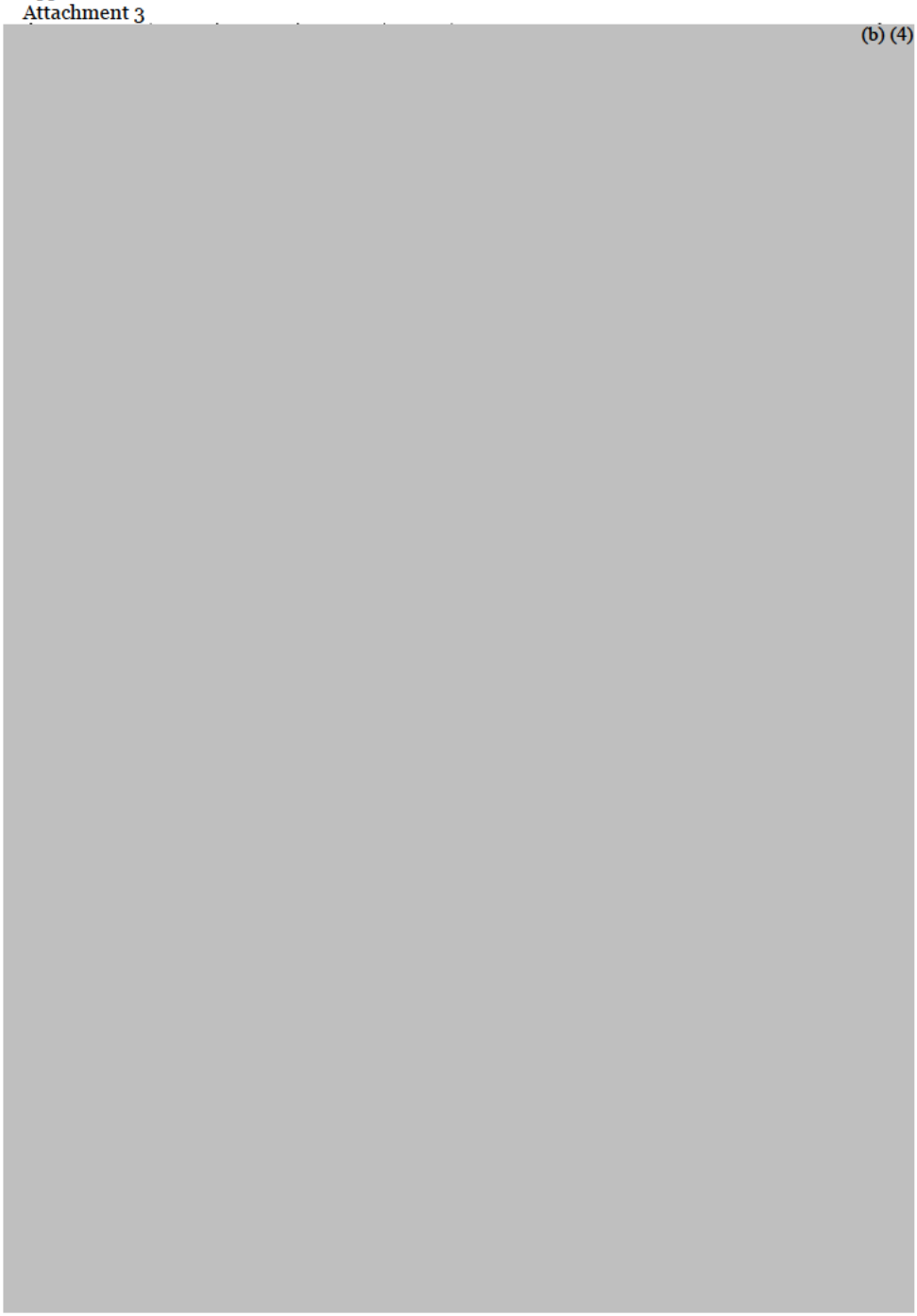

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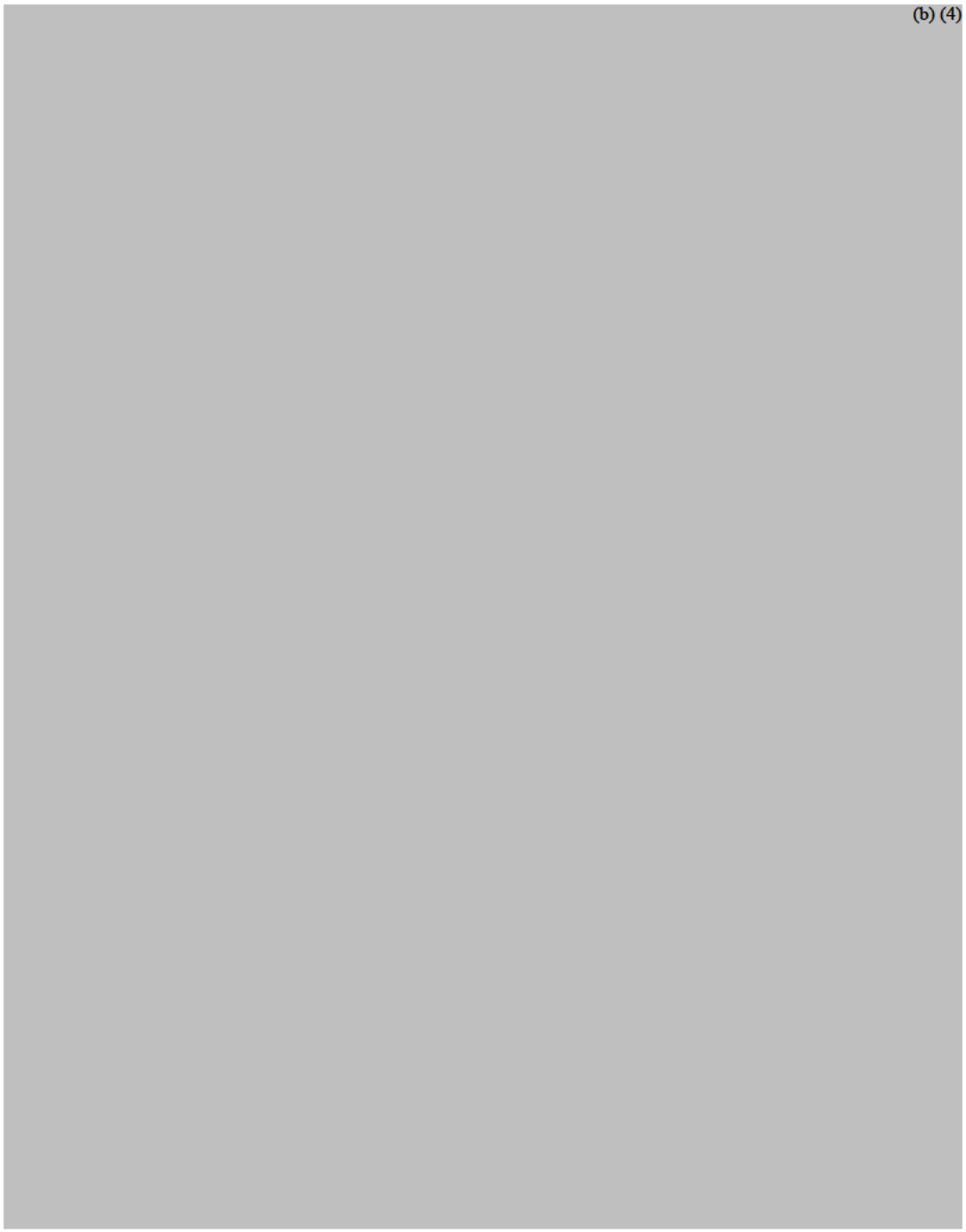

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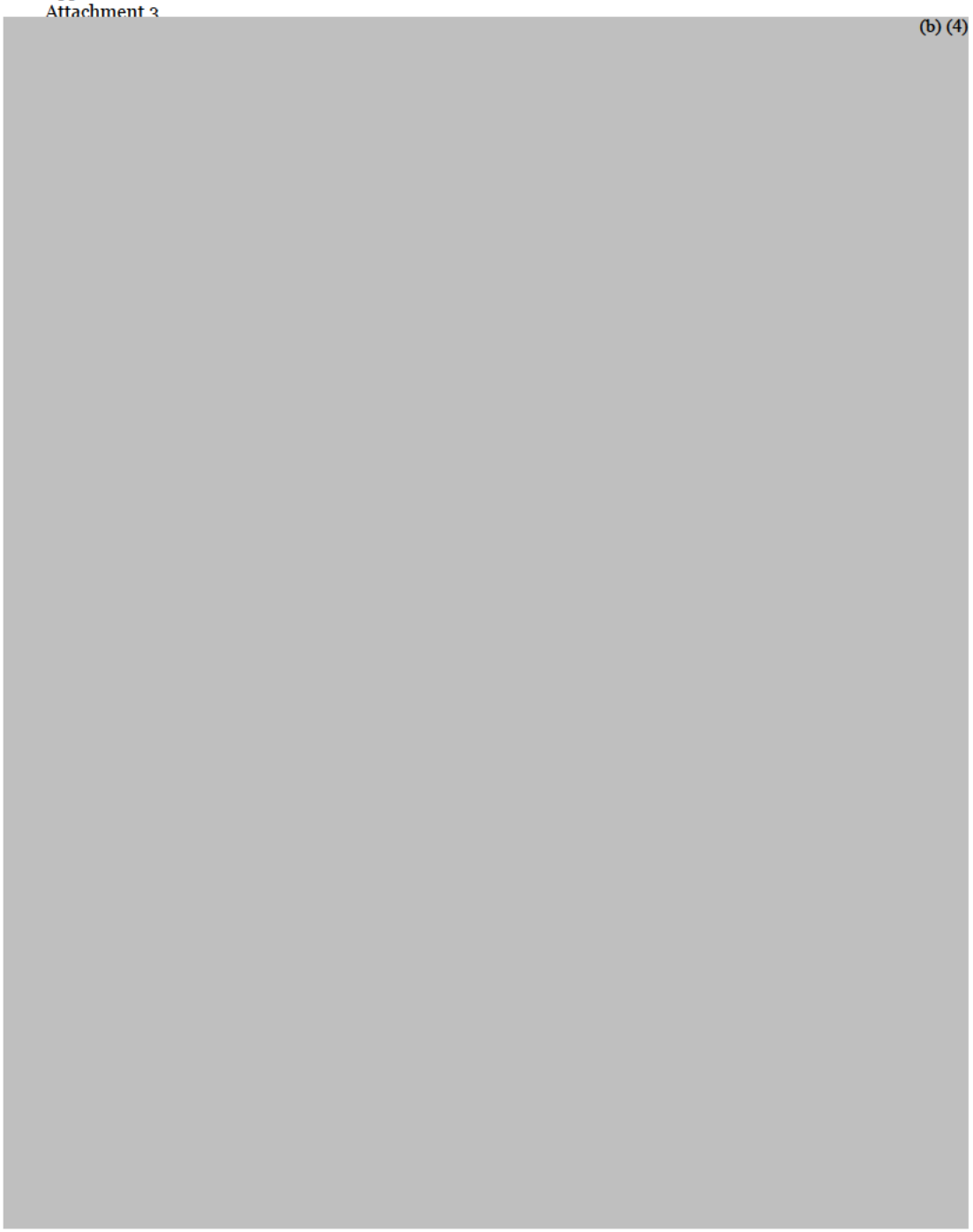

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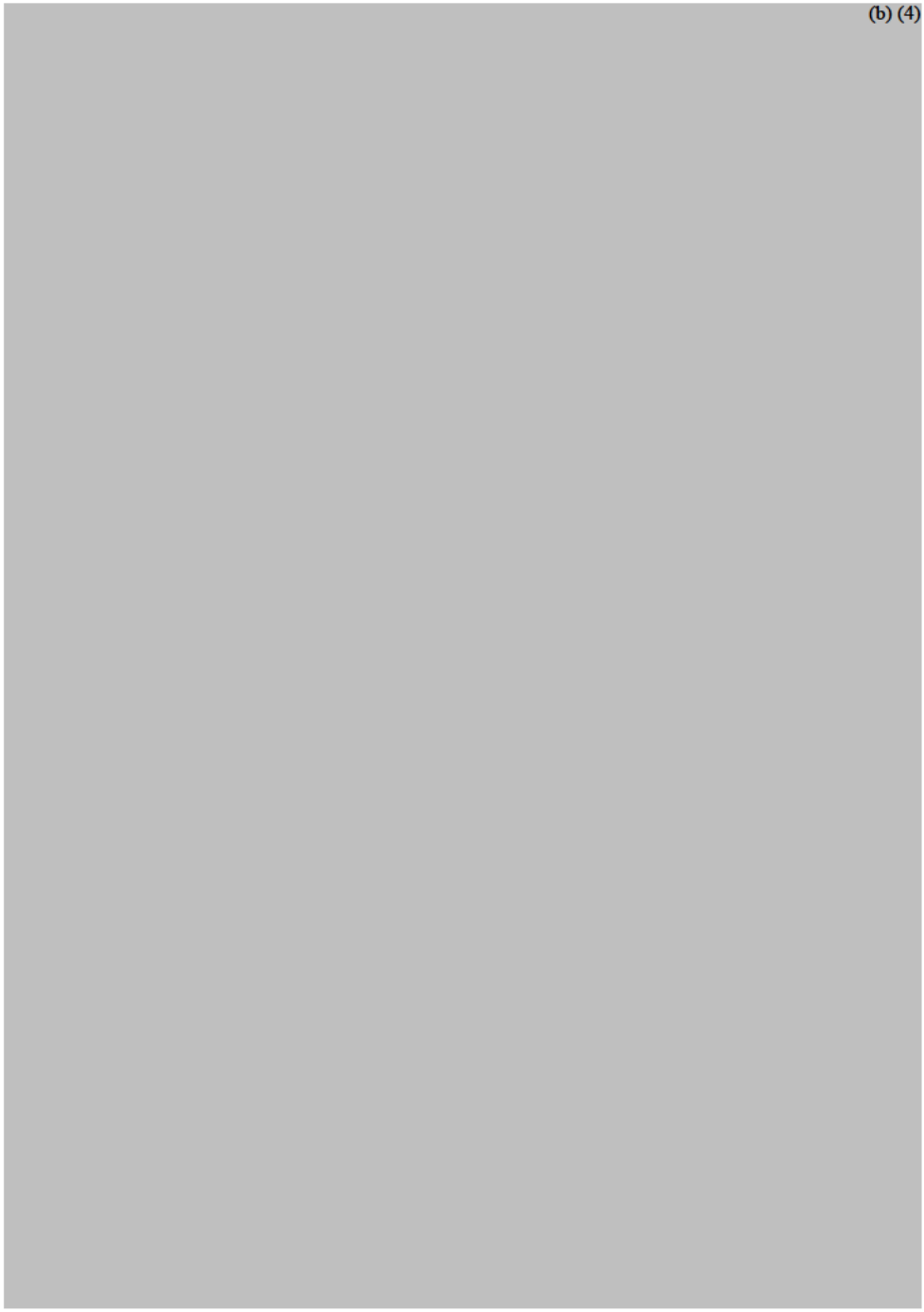

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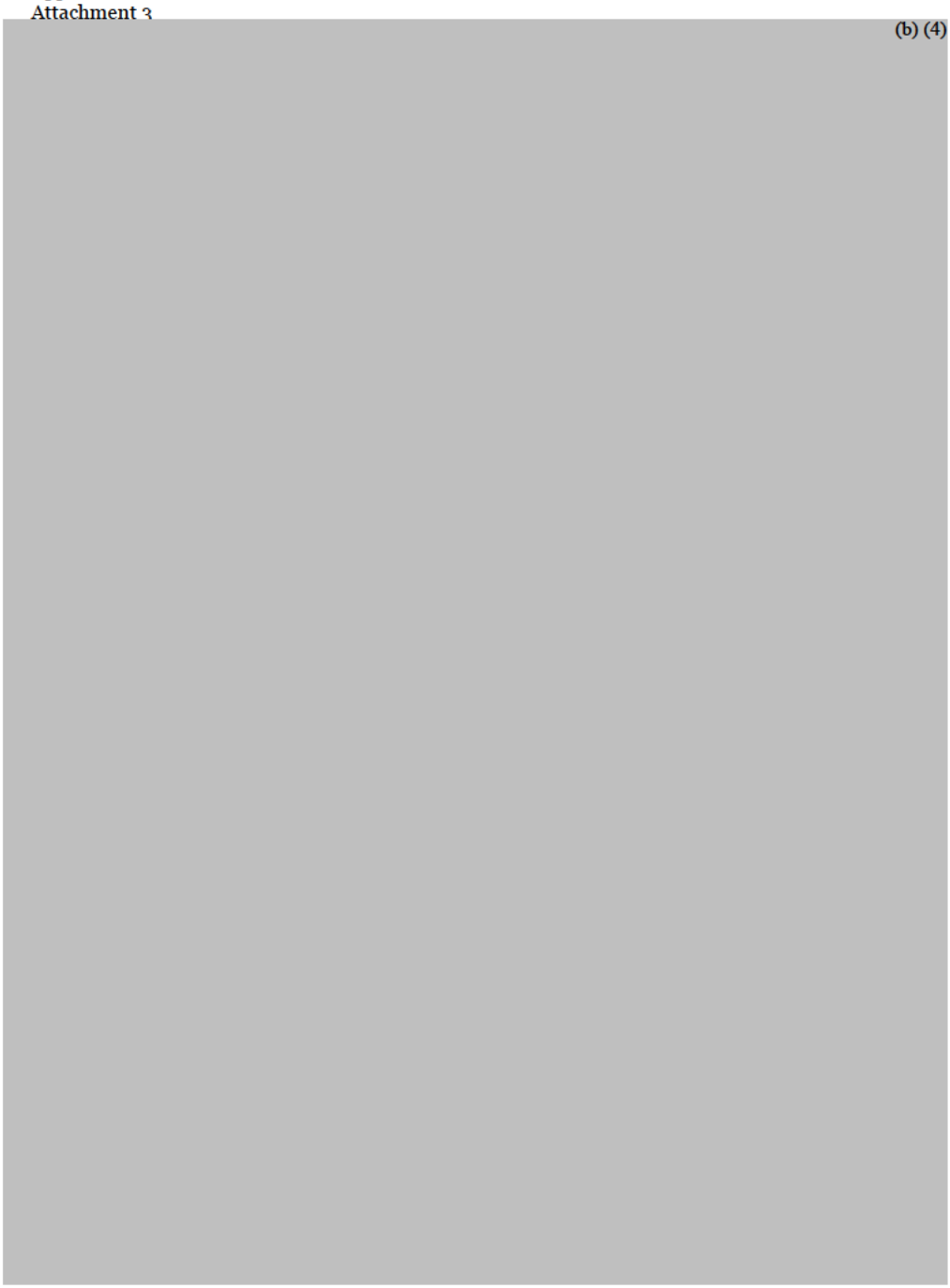

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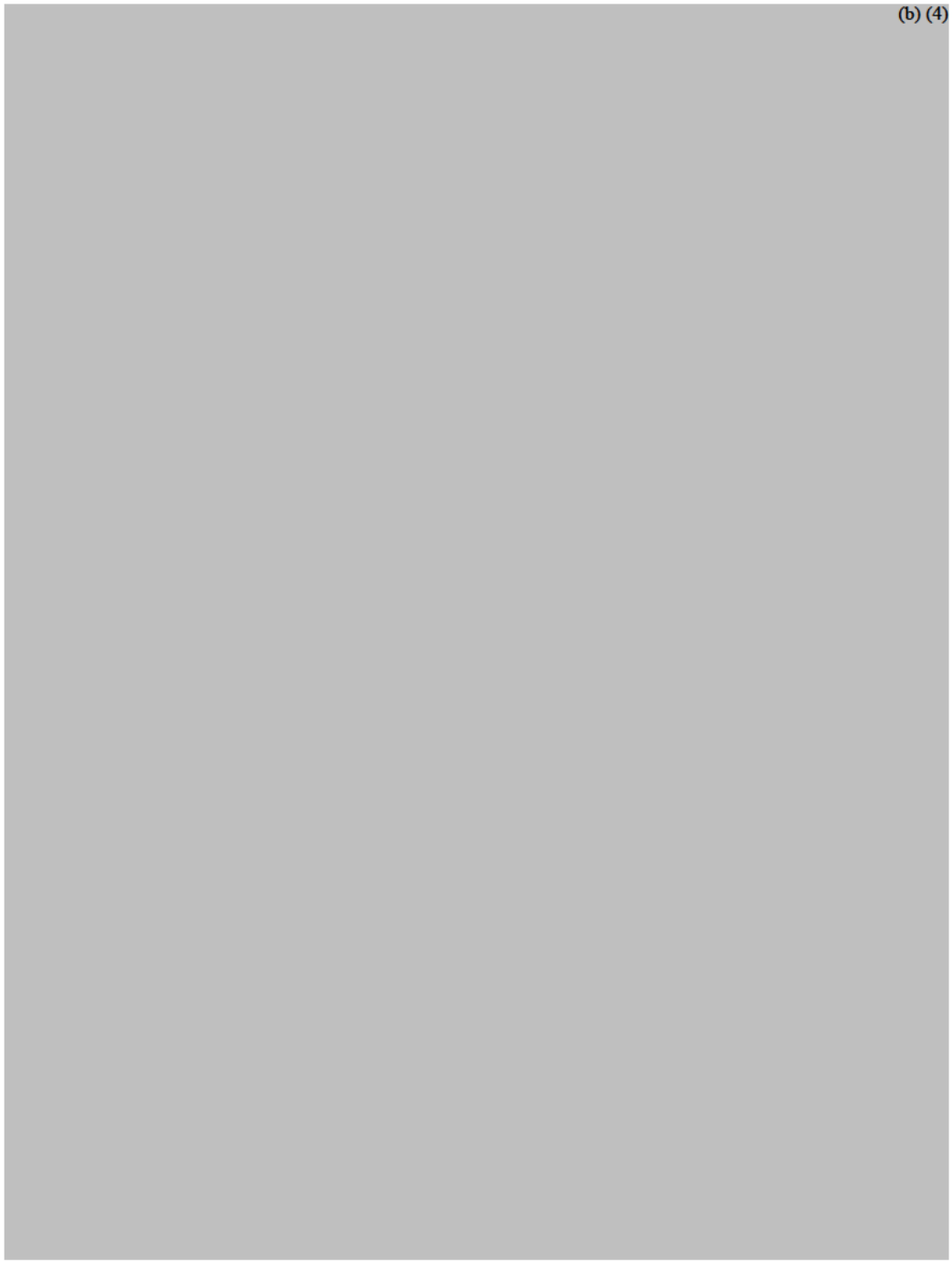

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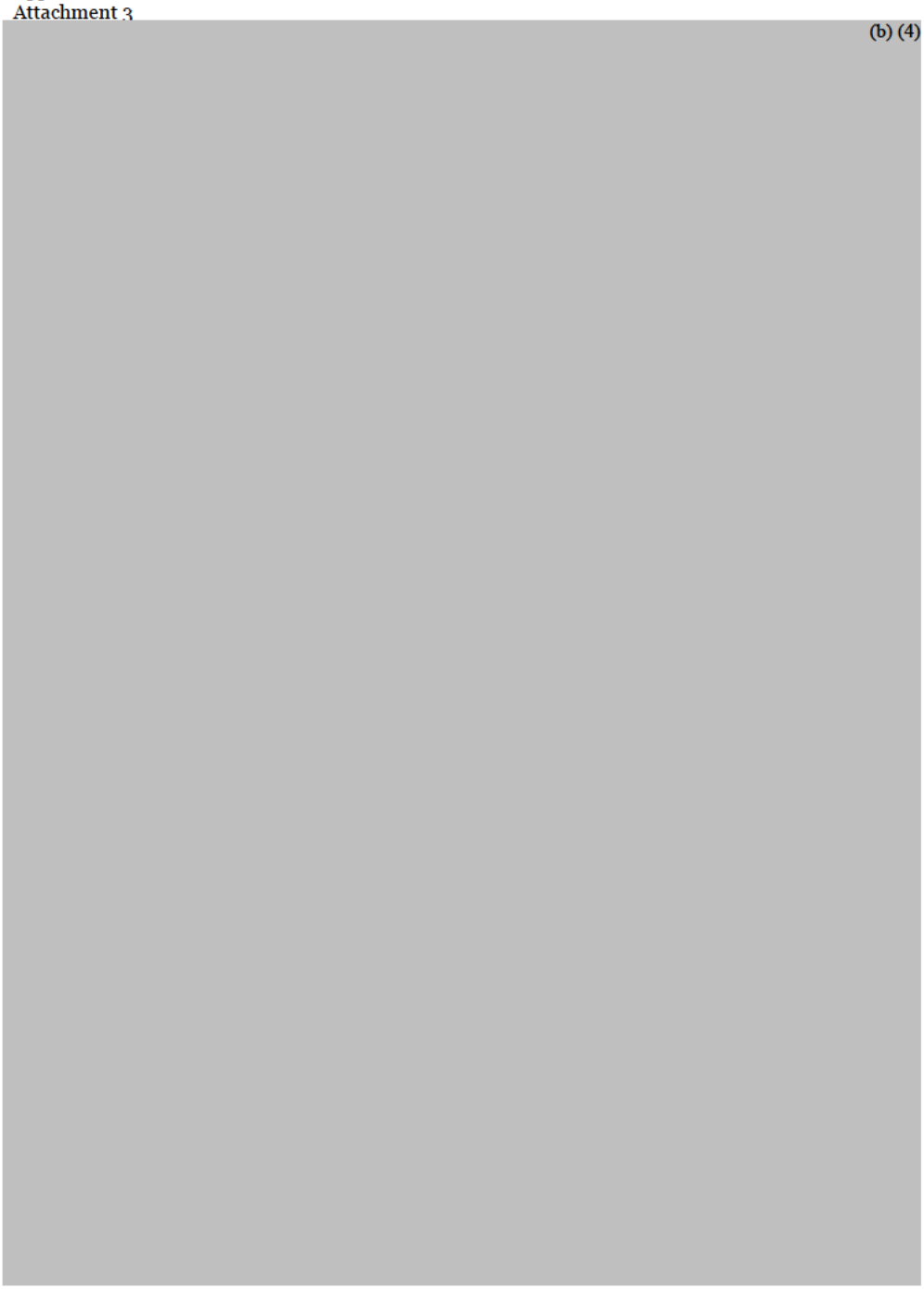

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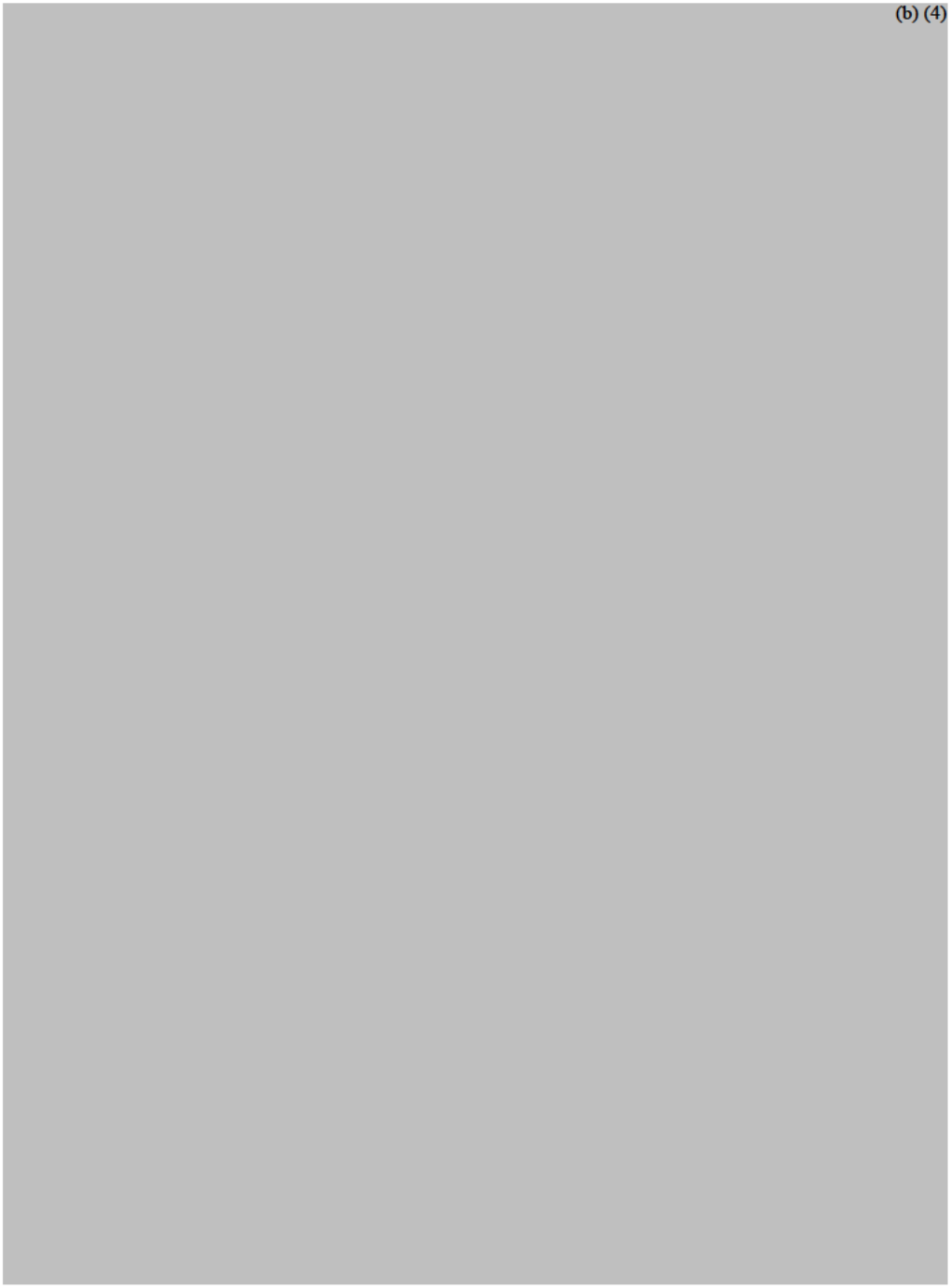

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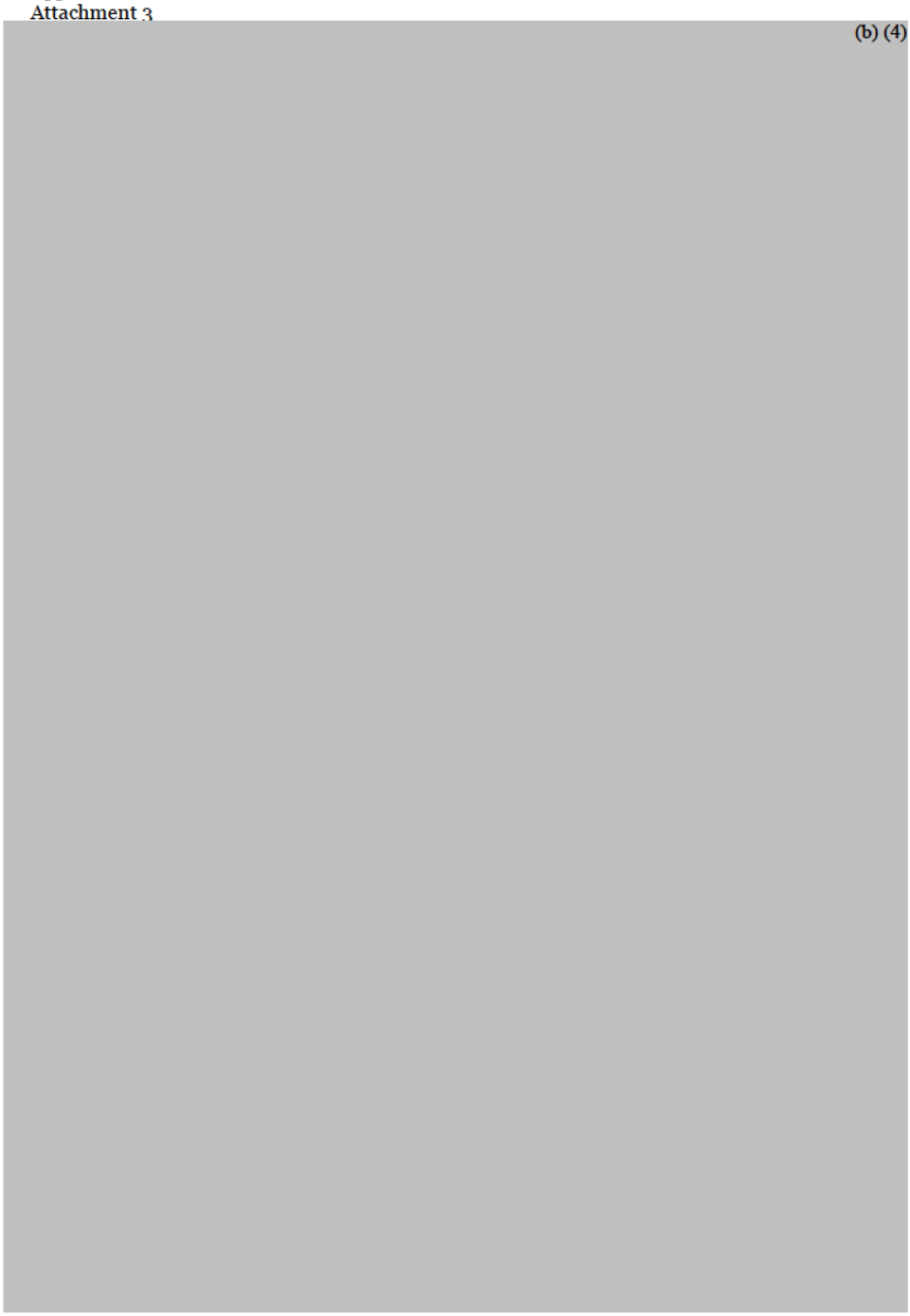

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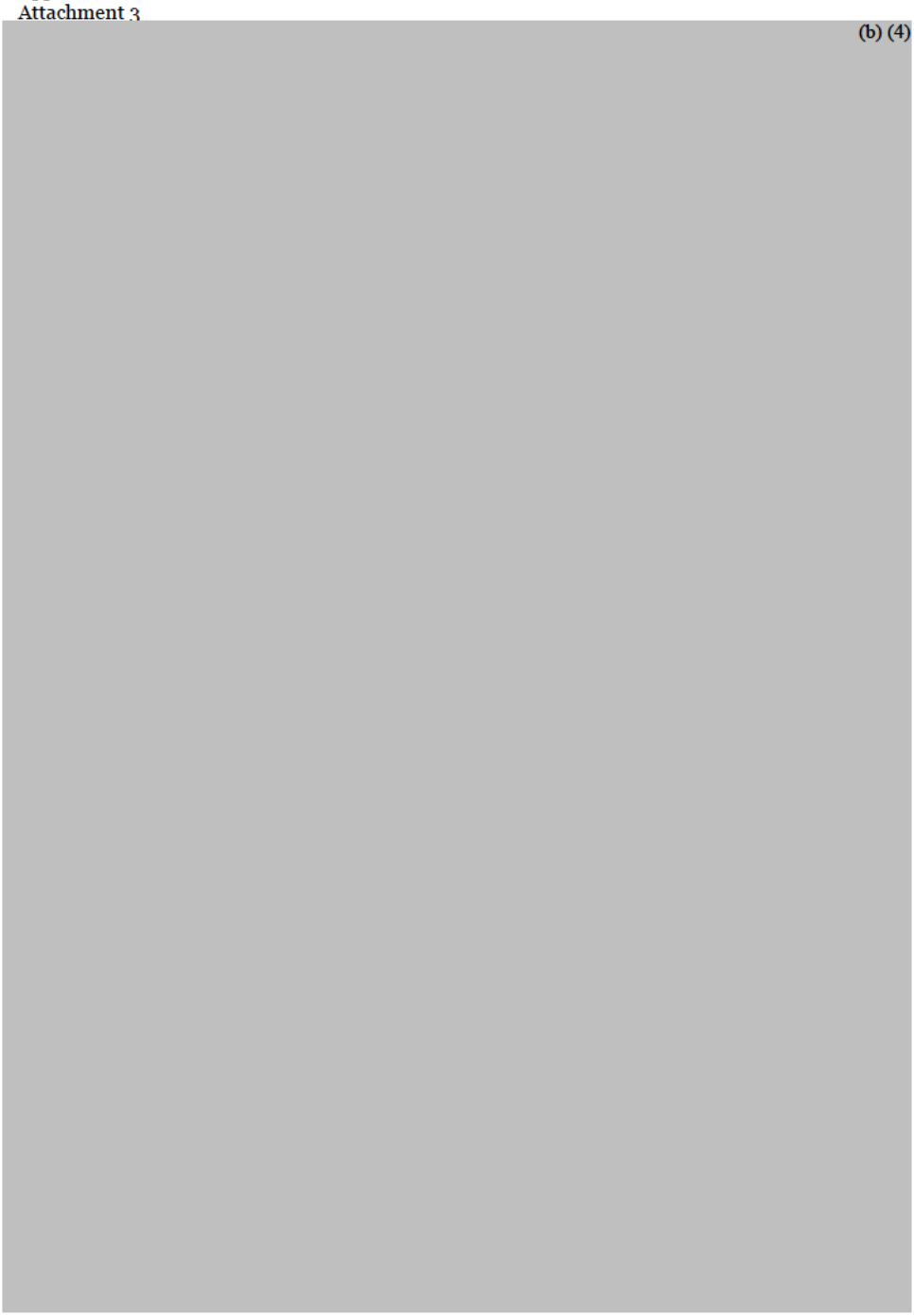

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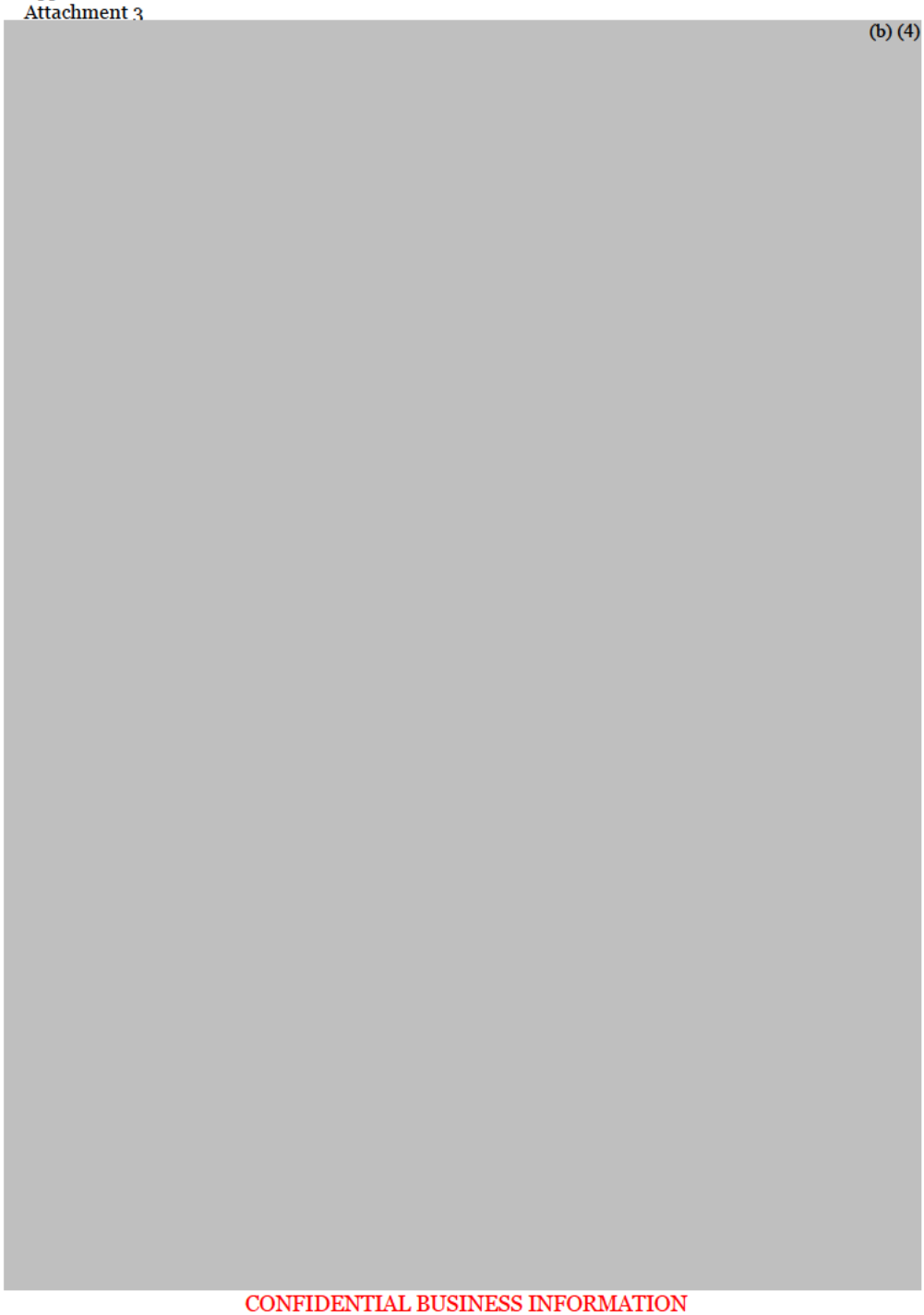

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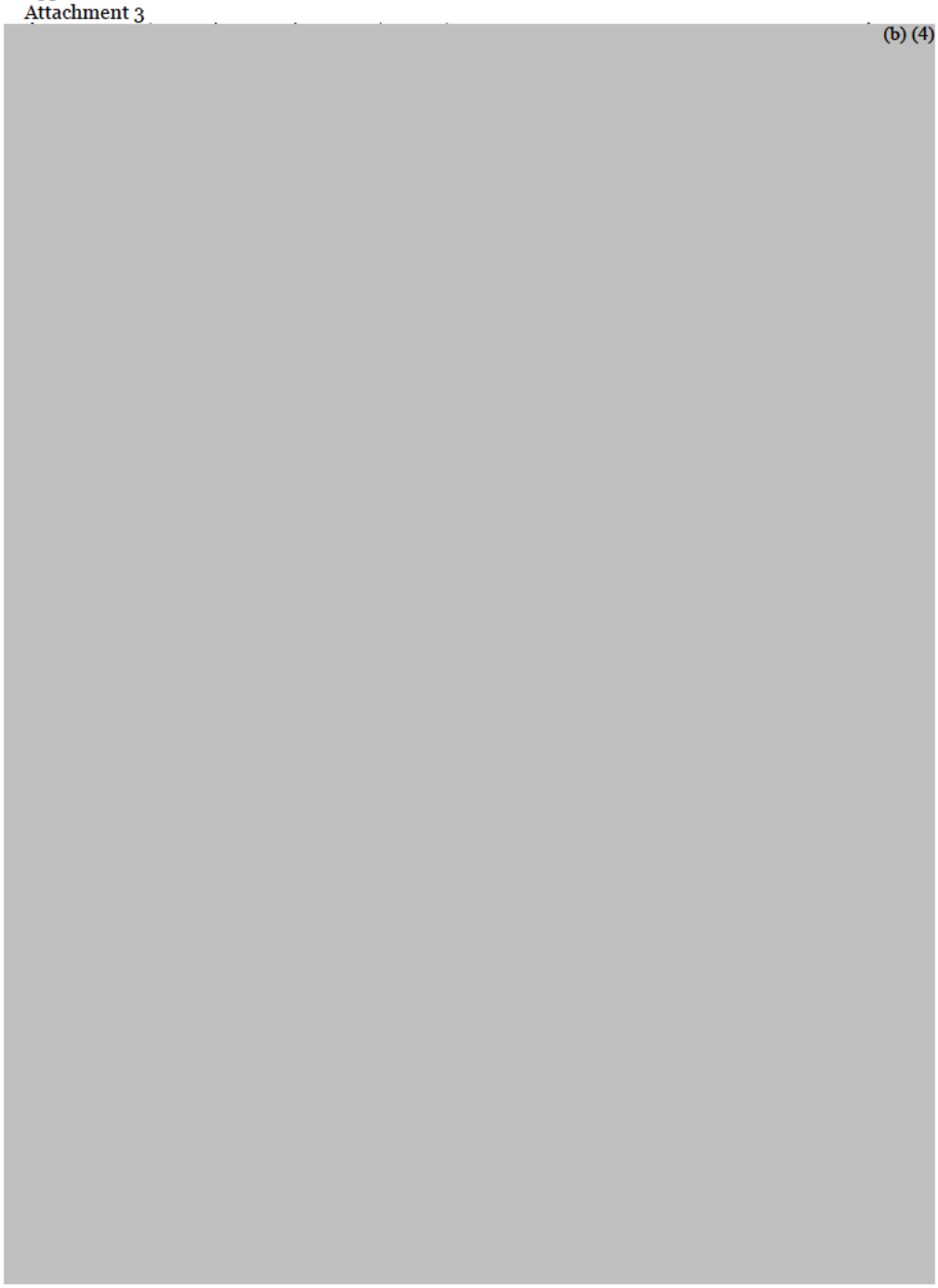

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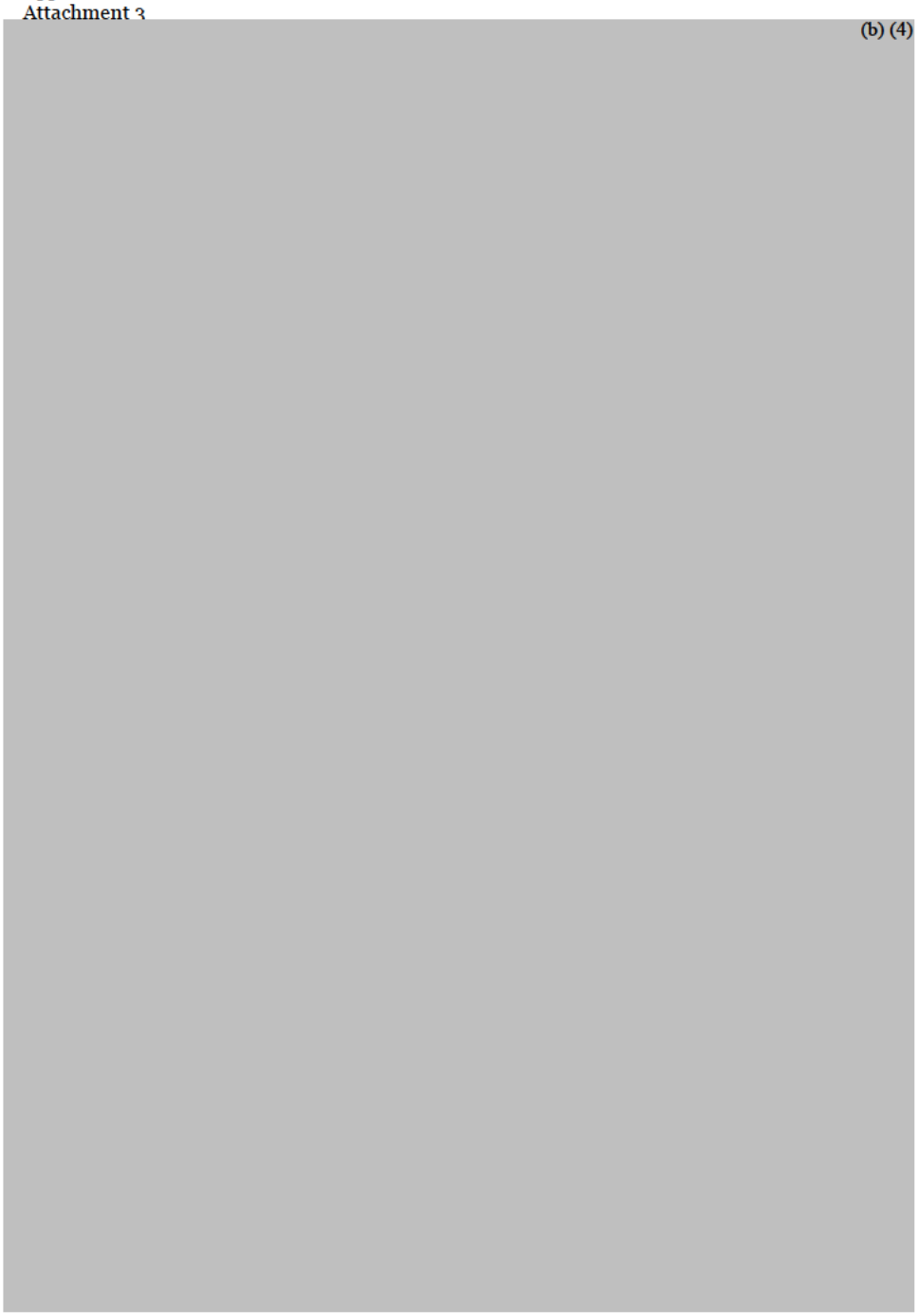

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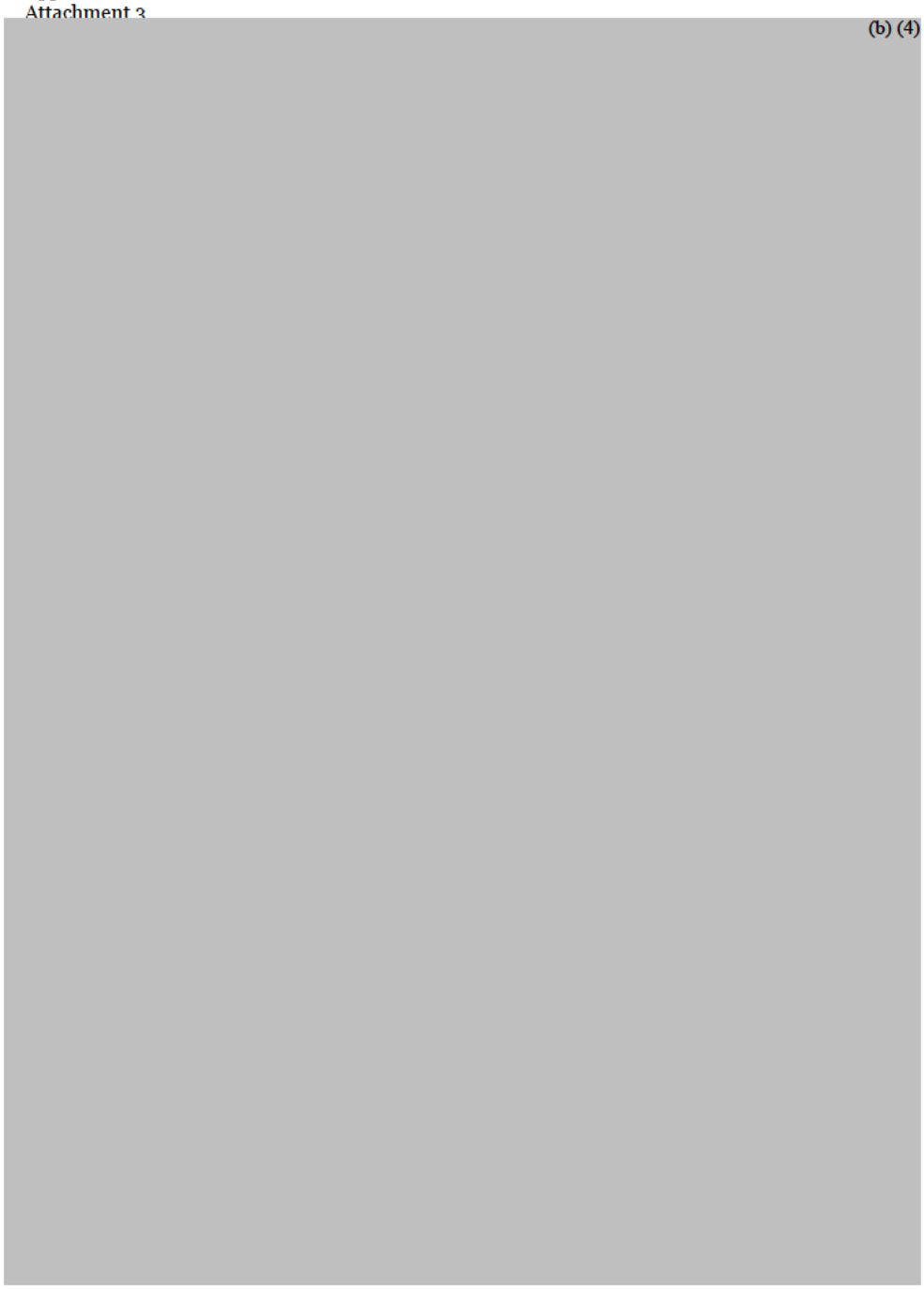

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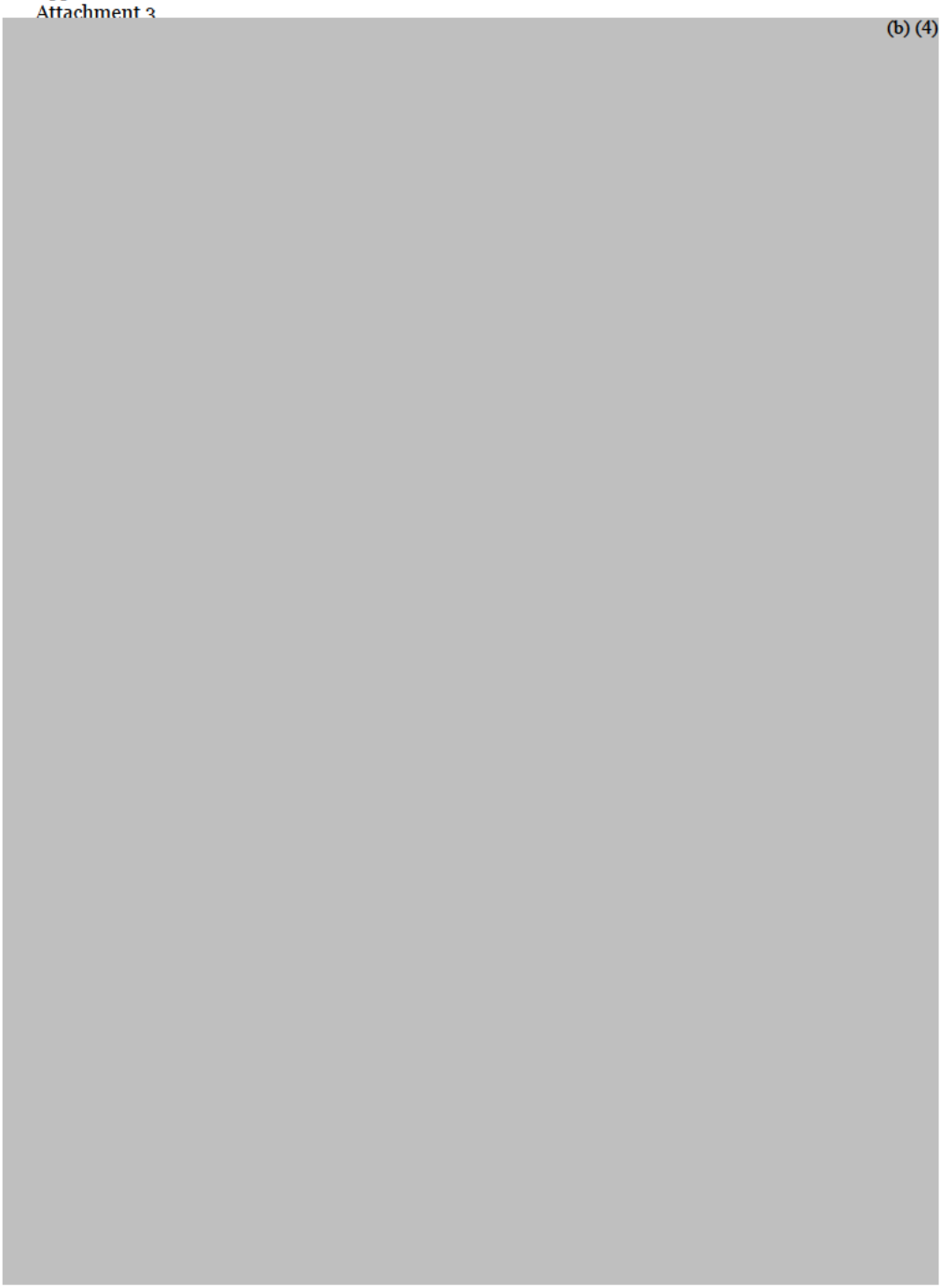

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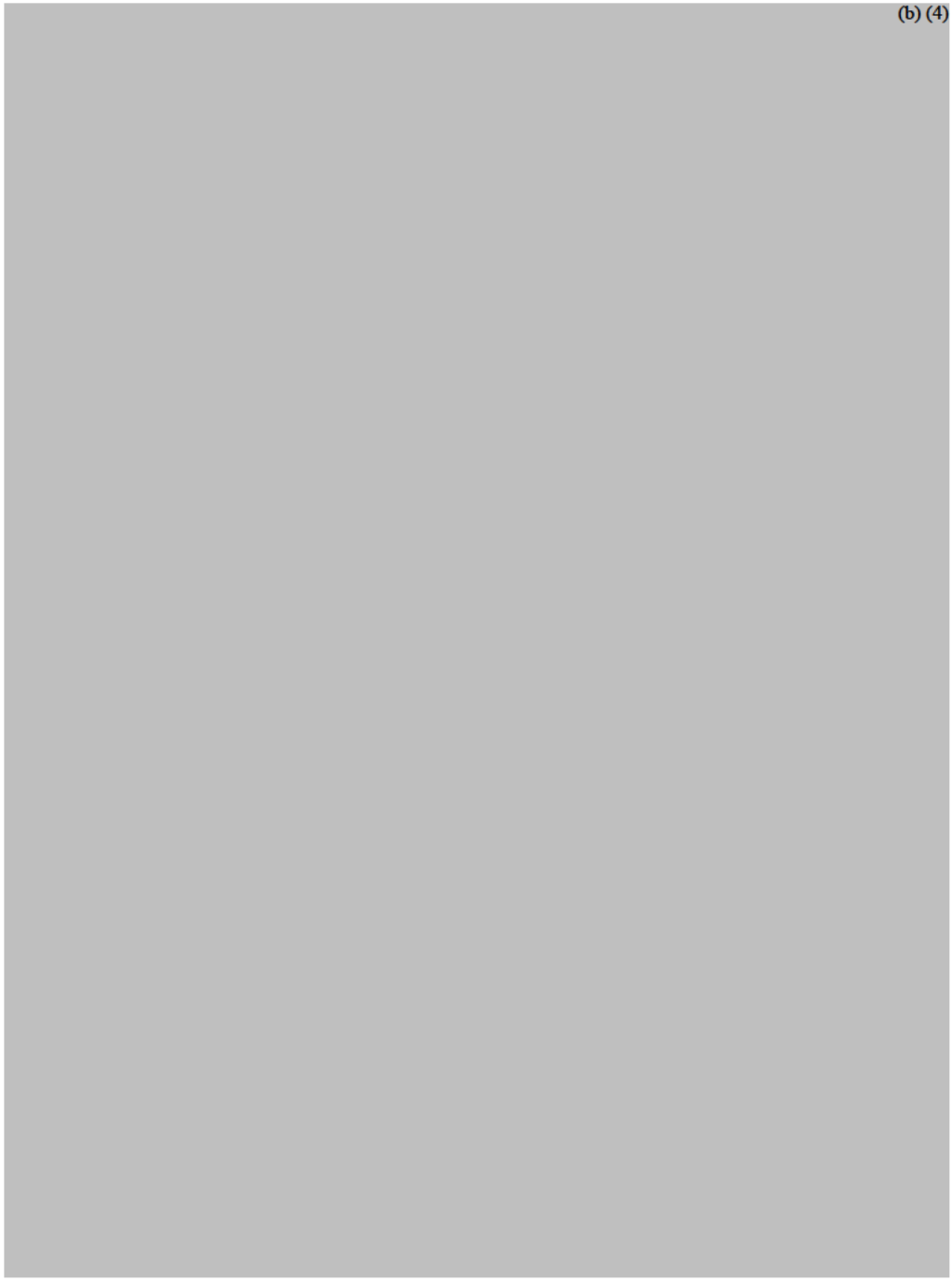

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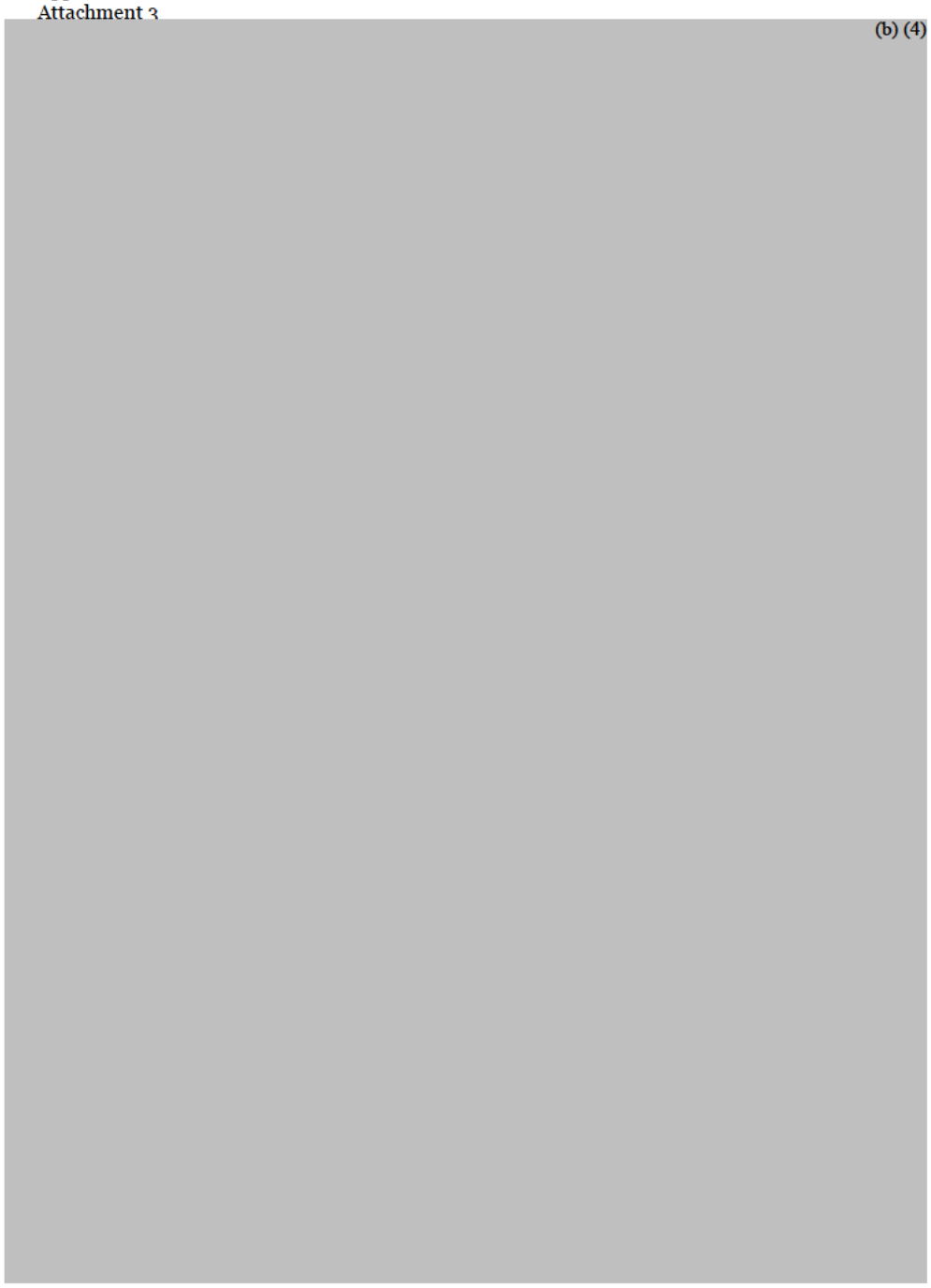

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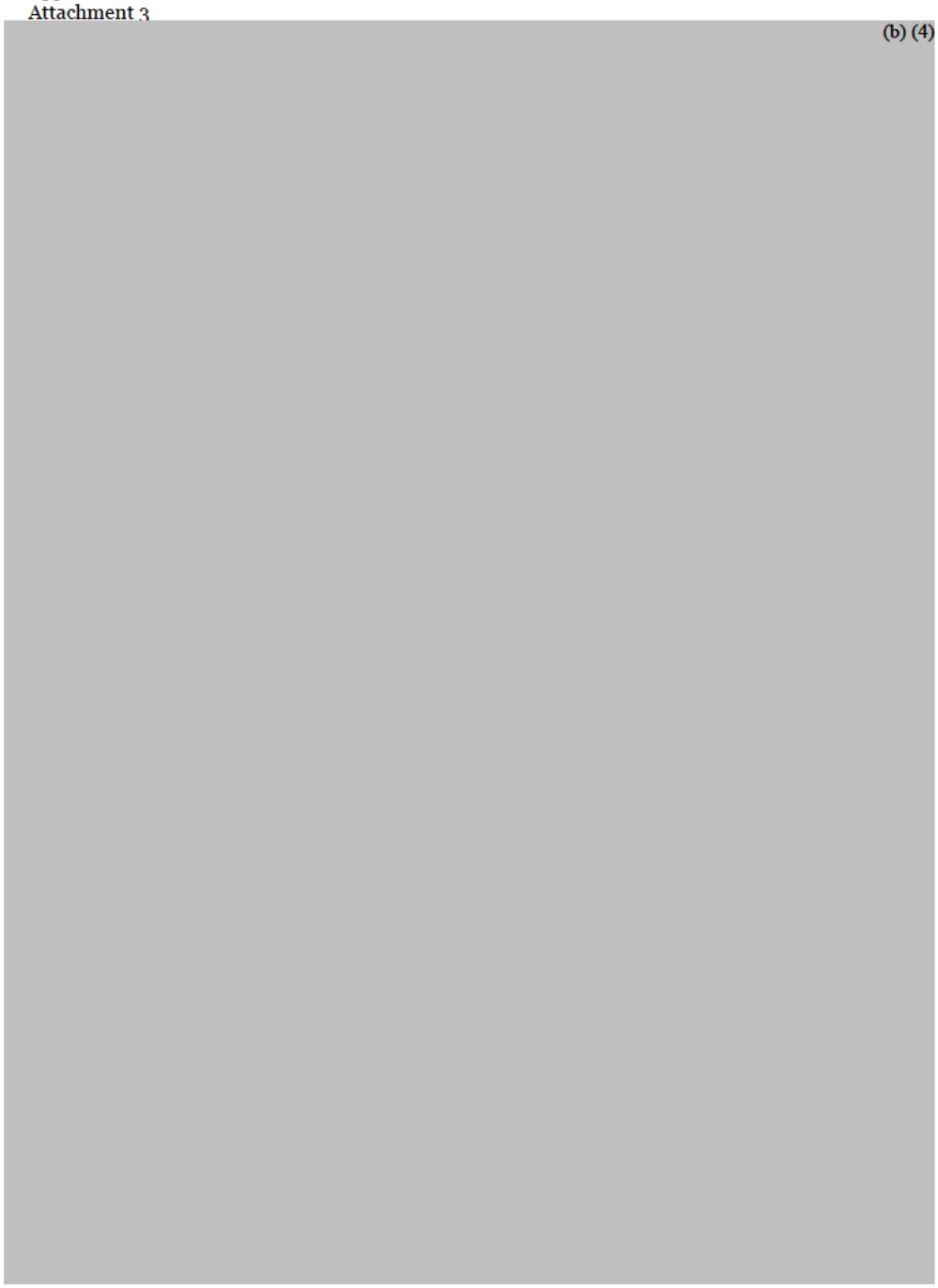

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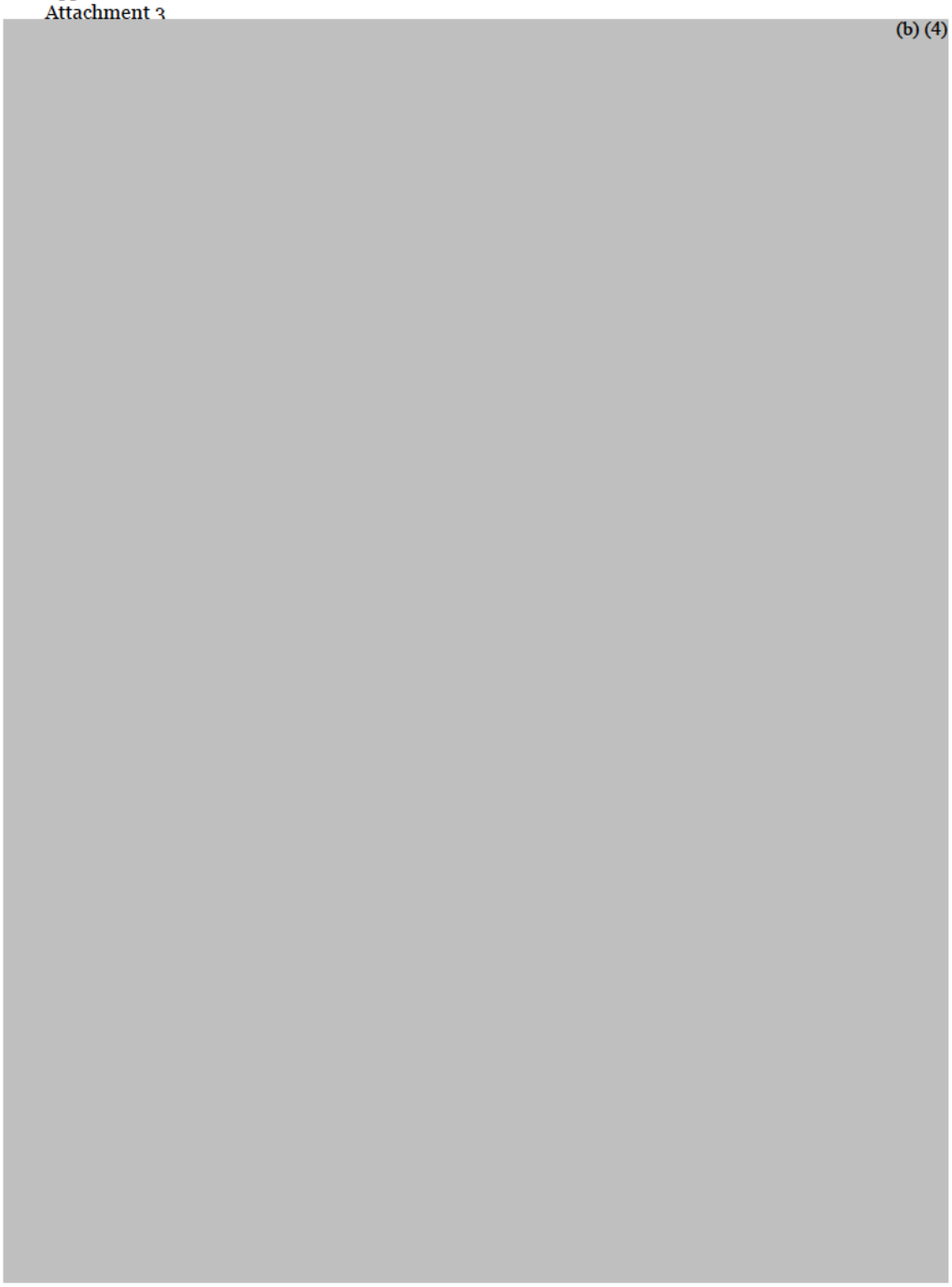

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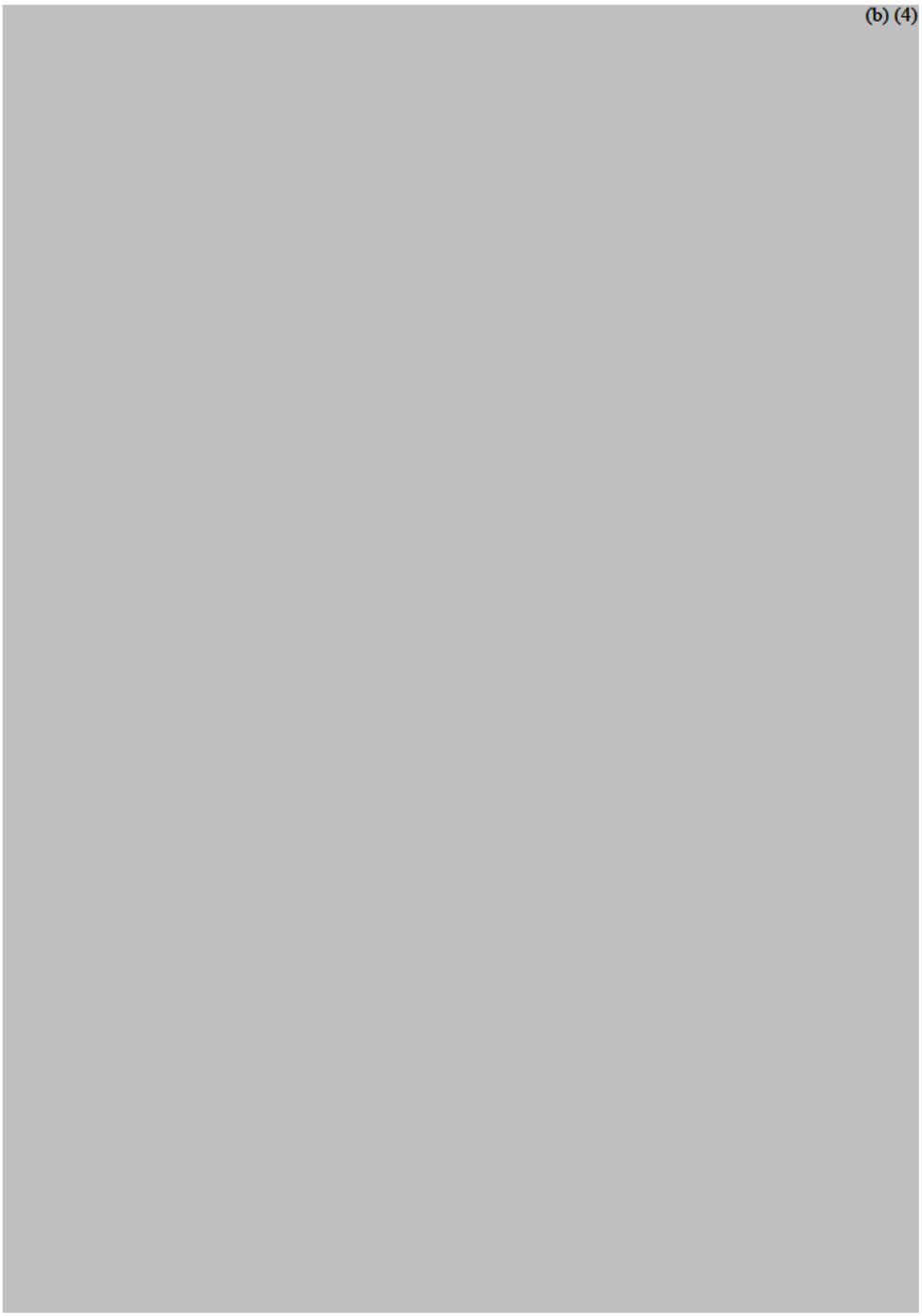

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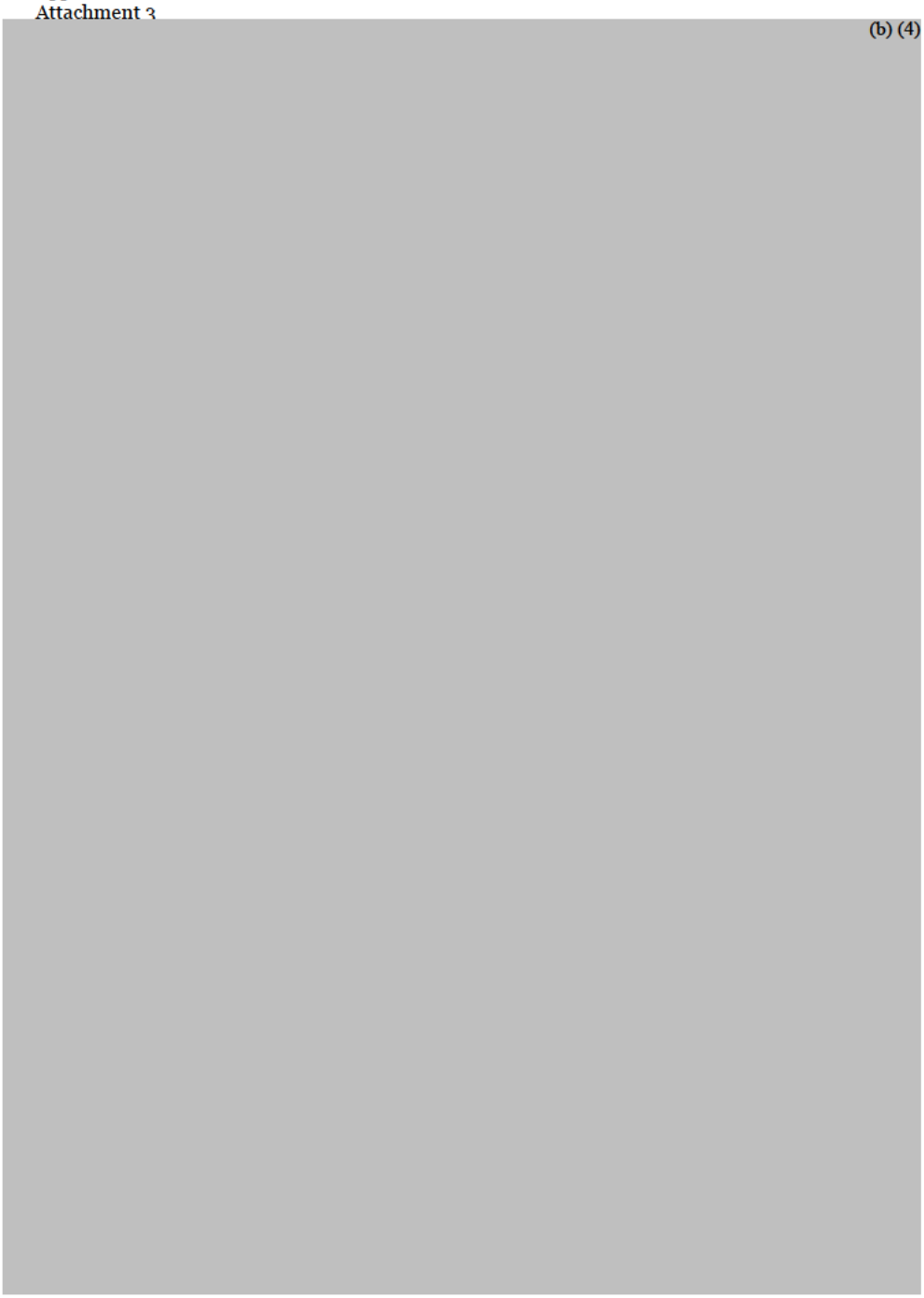

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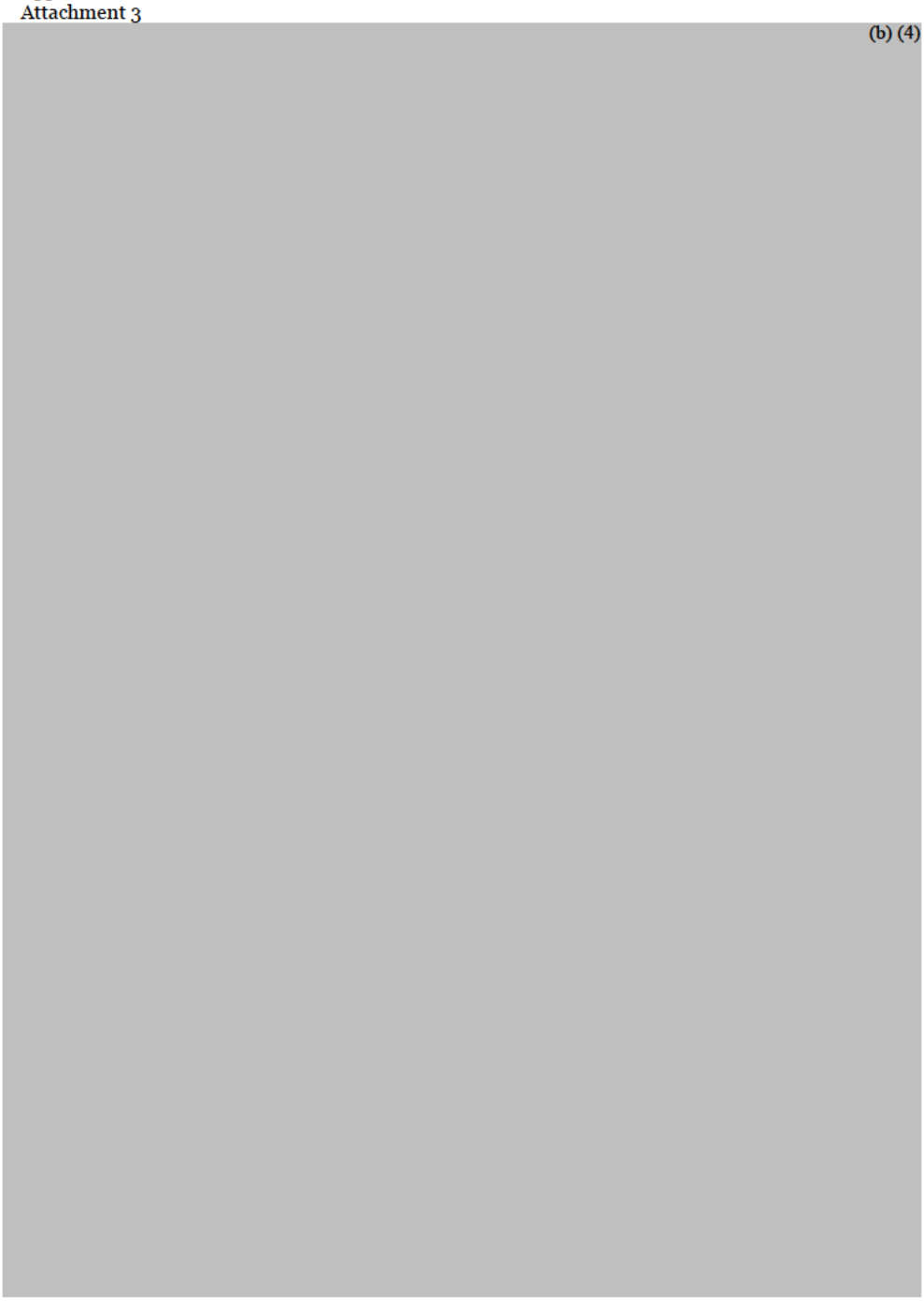

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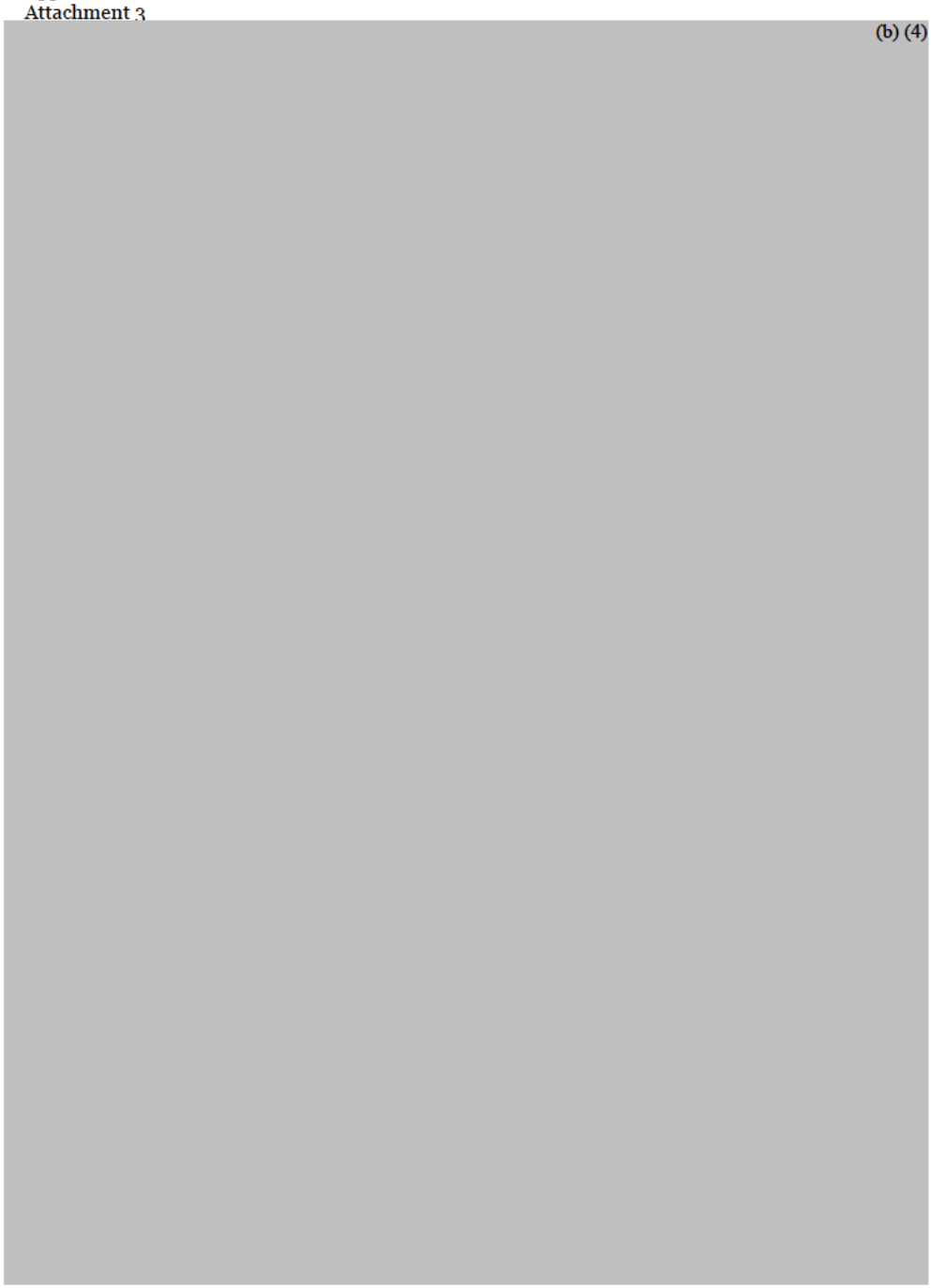

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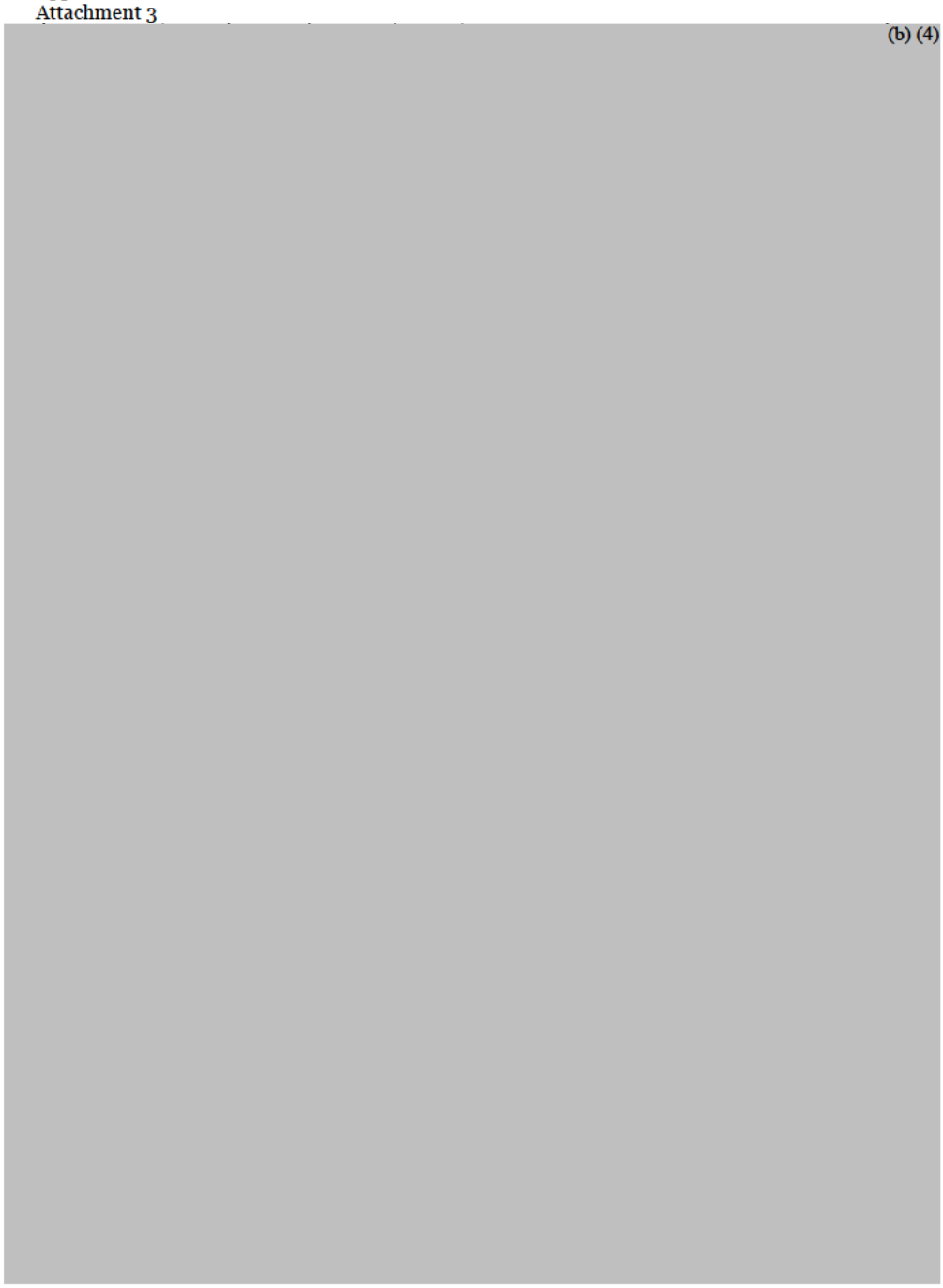

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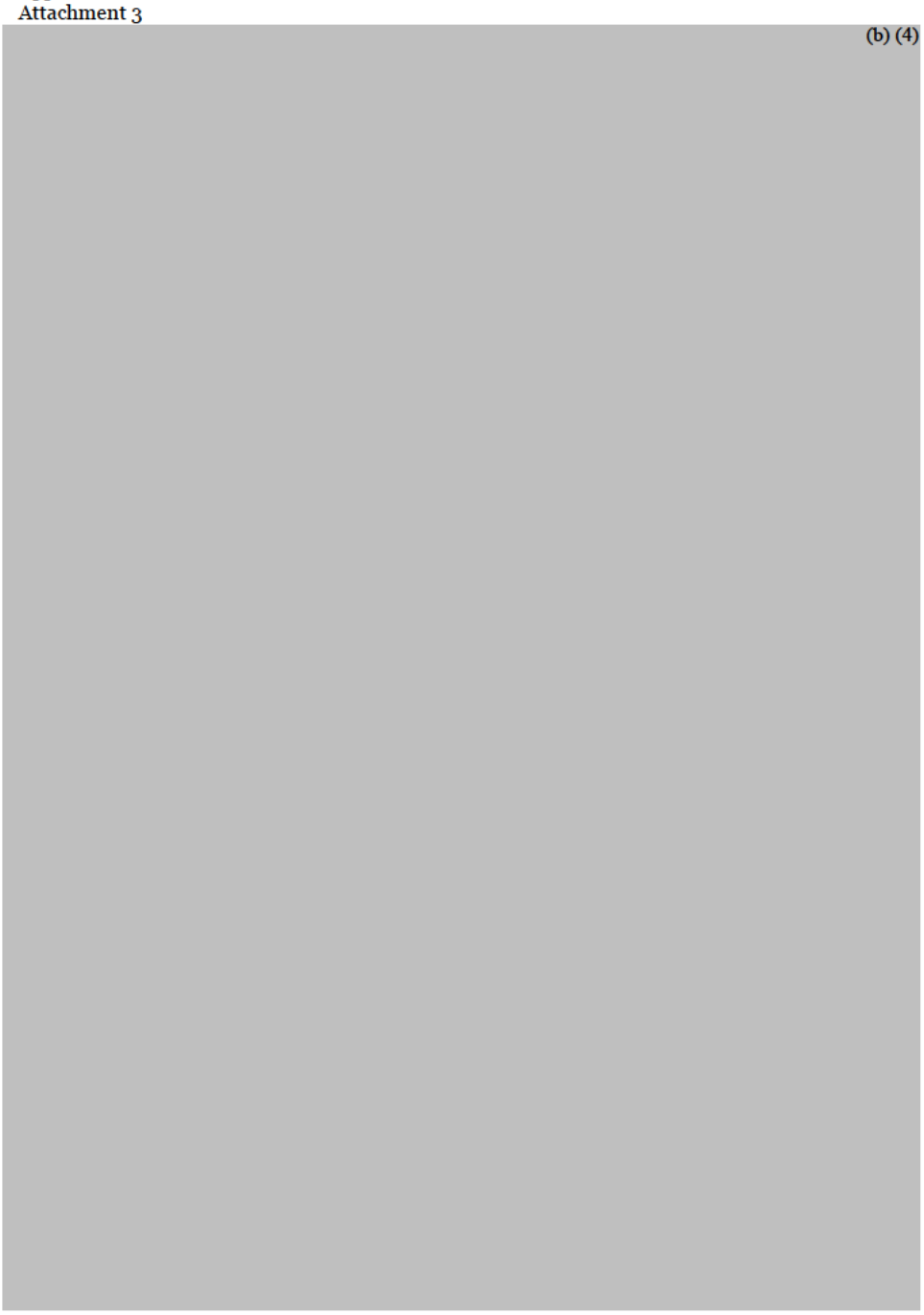

CONFIDENTIAL BUSINESS INFORMATION

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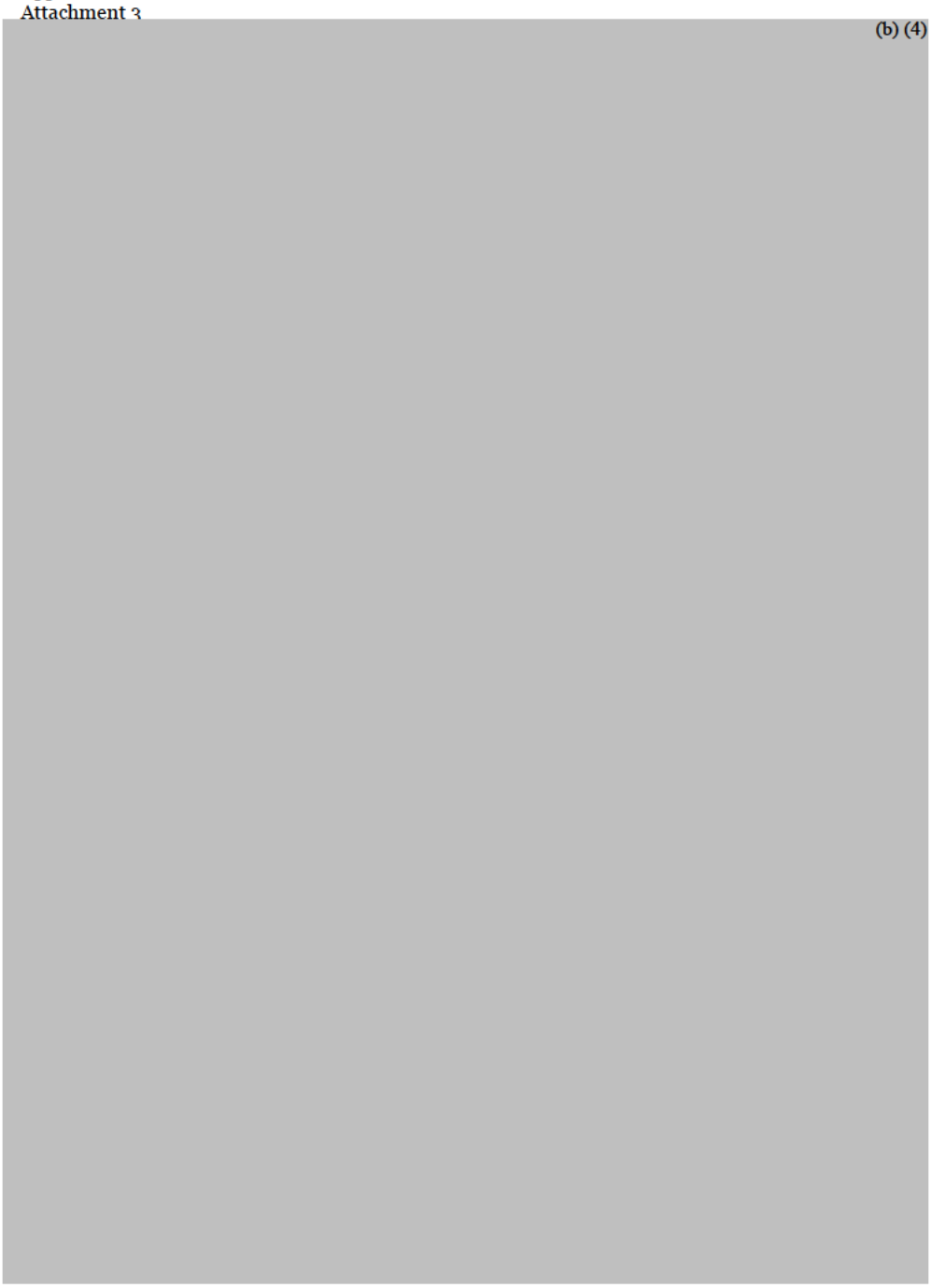

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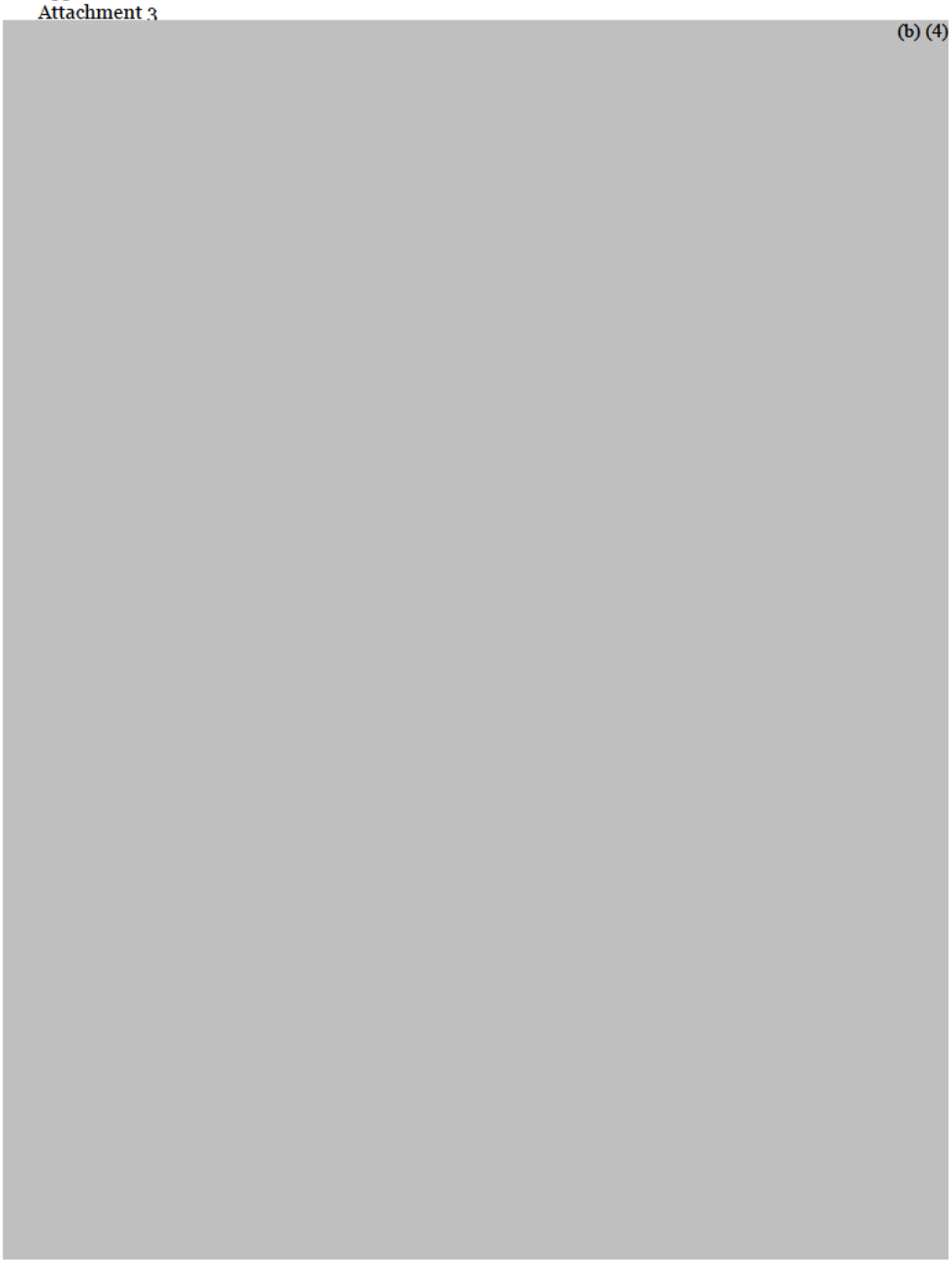

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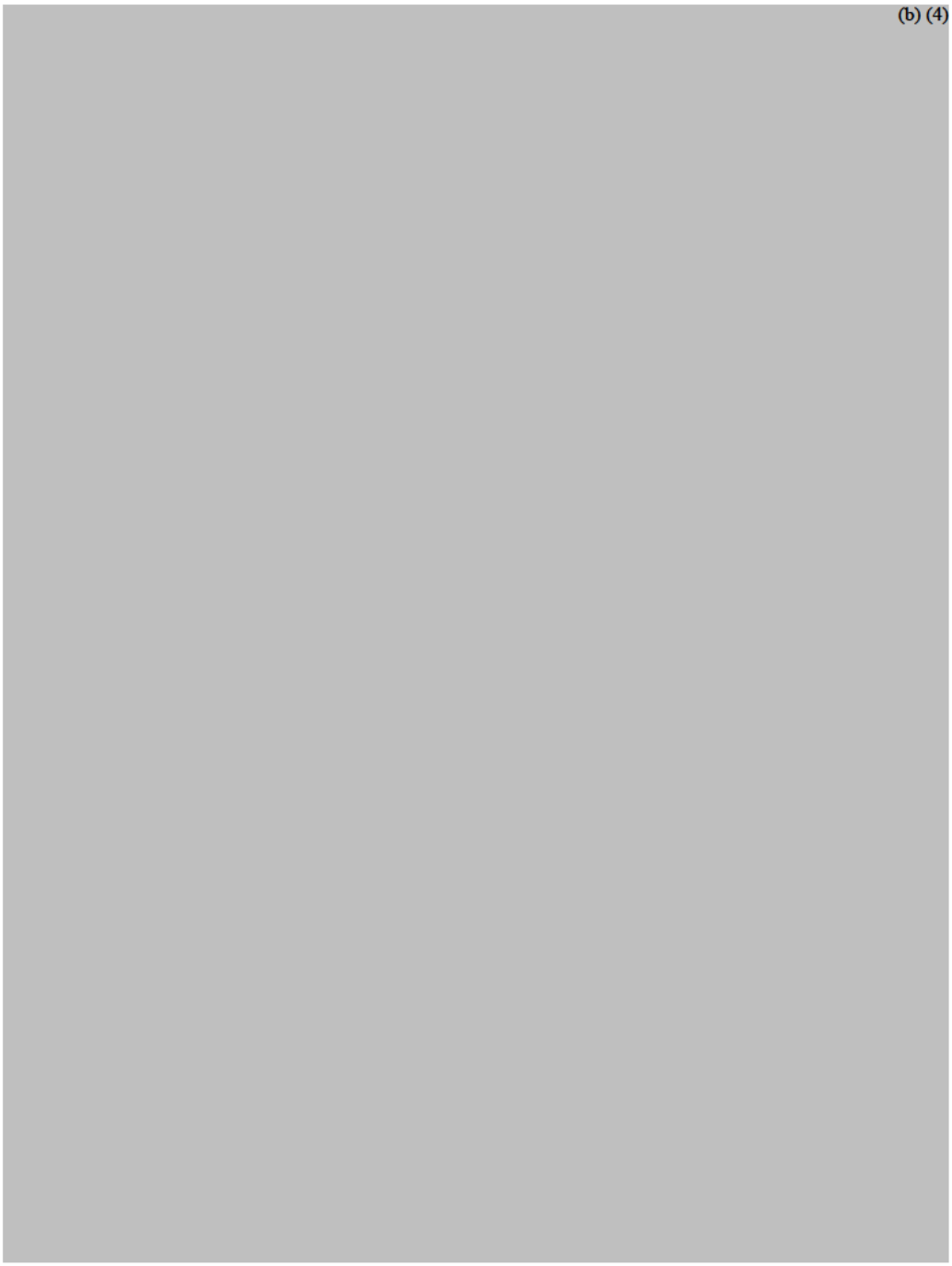

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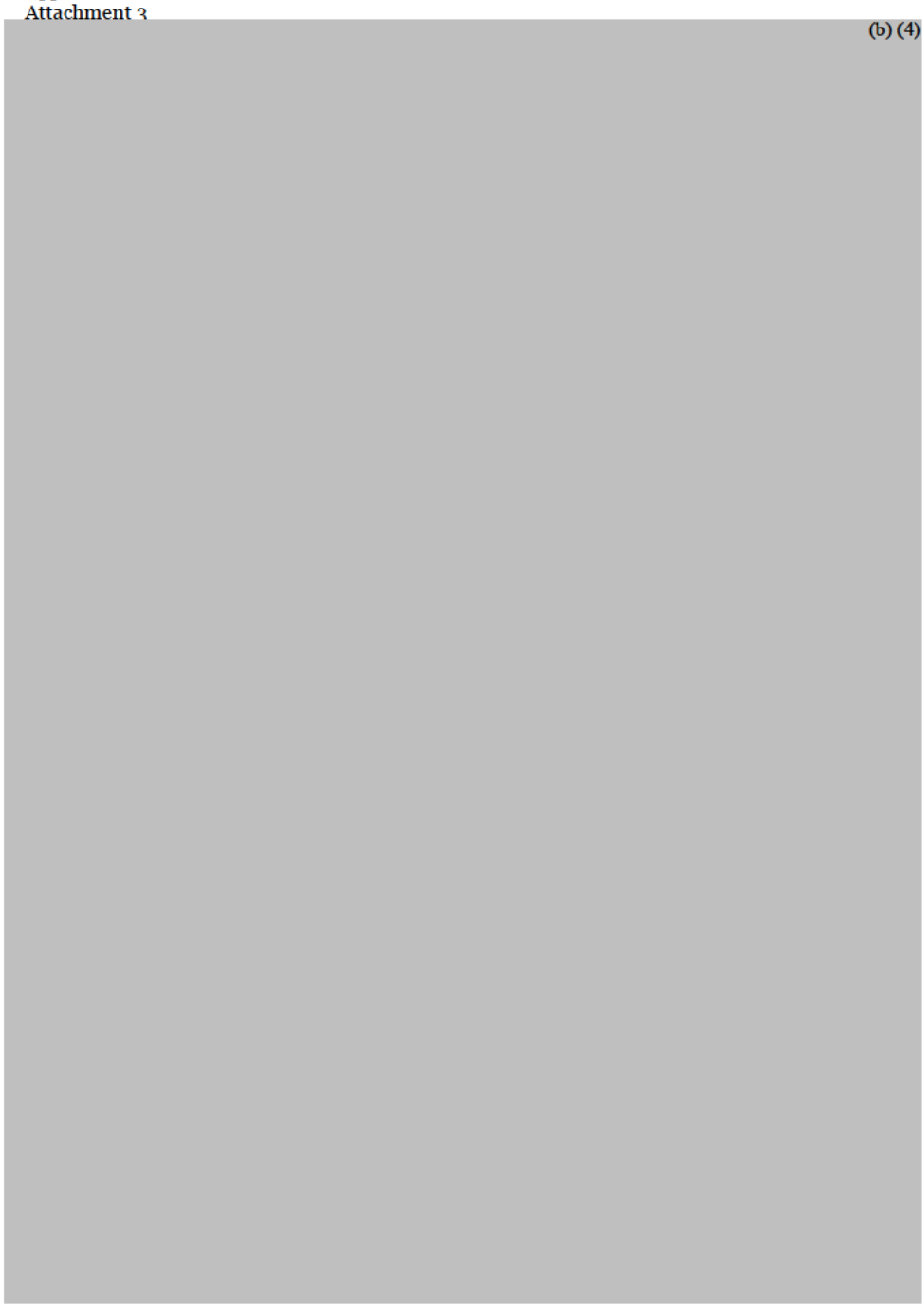

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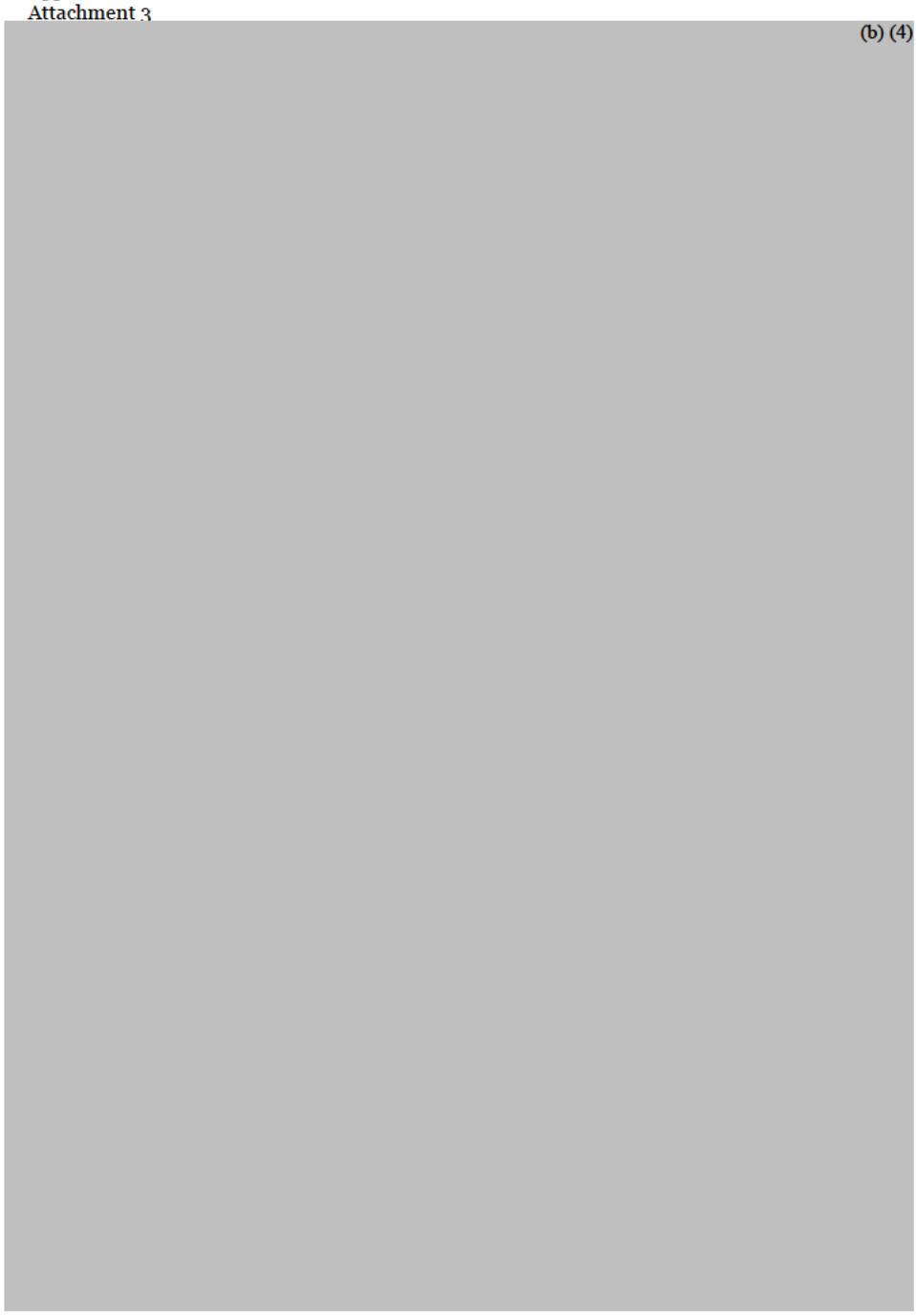

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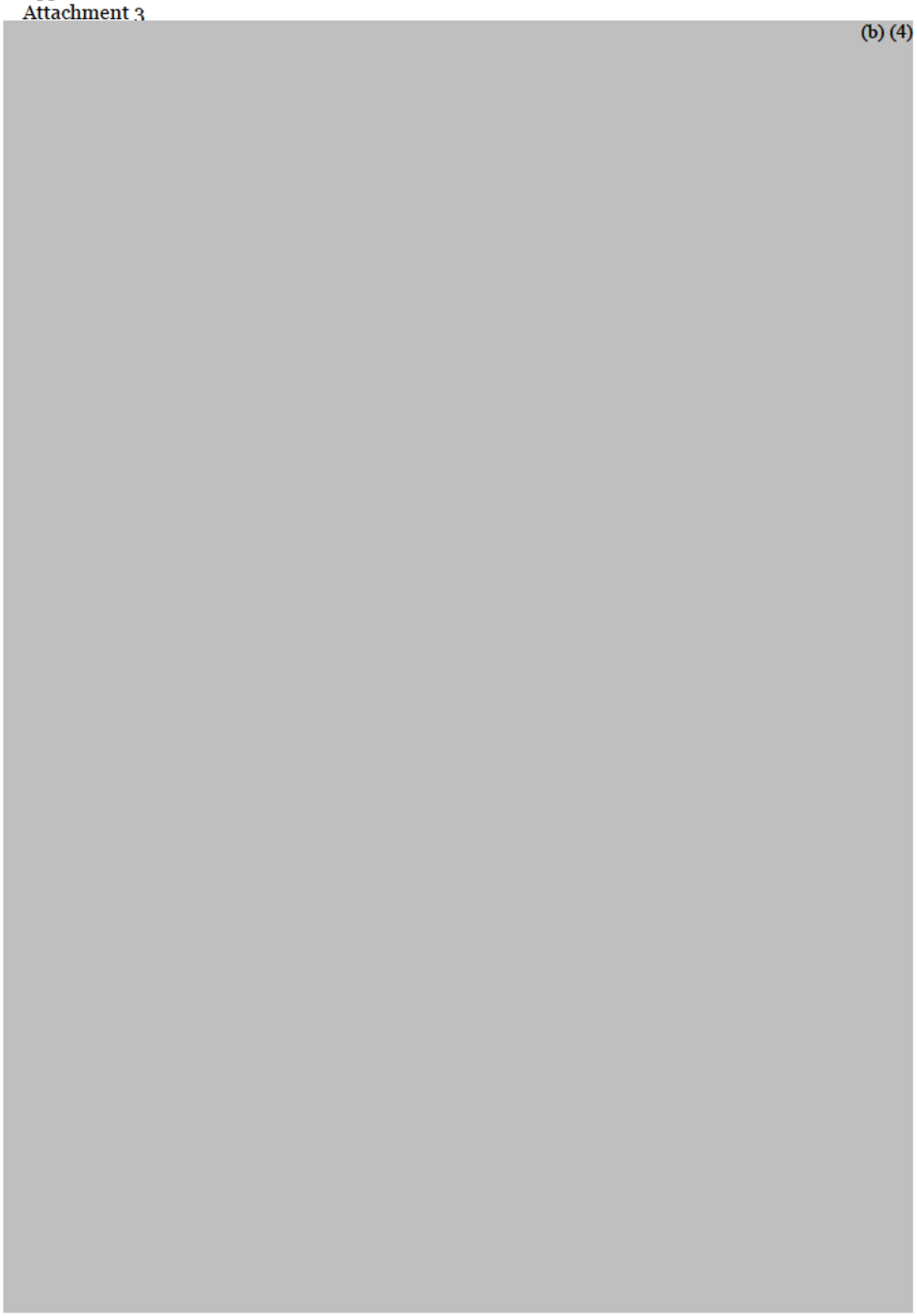

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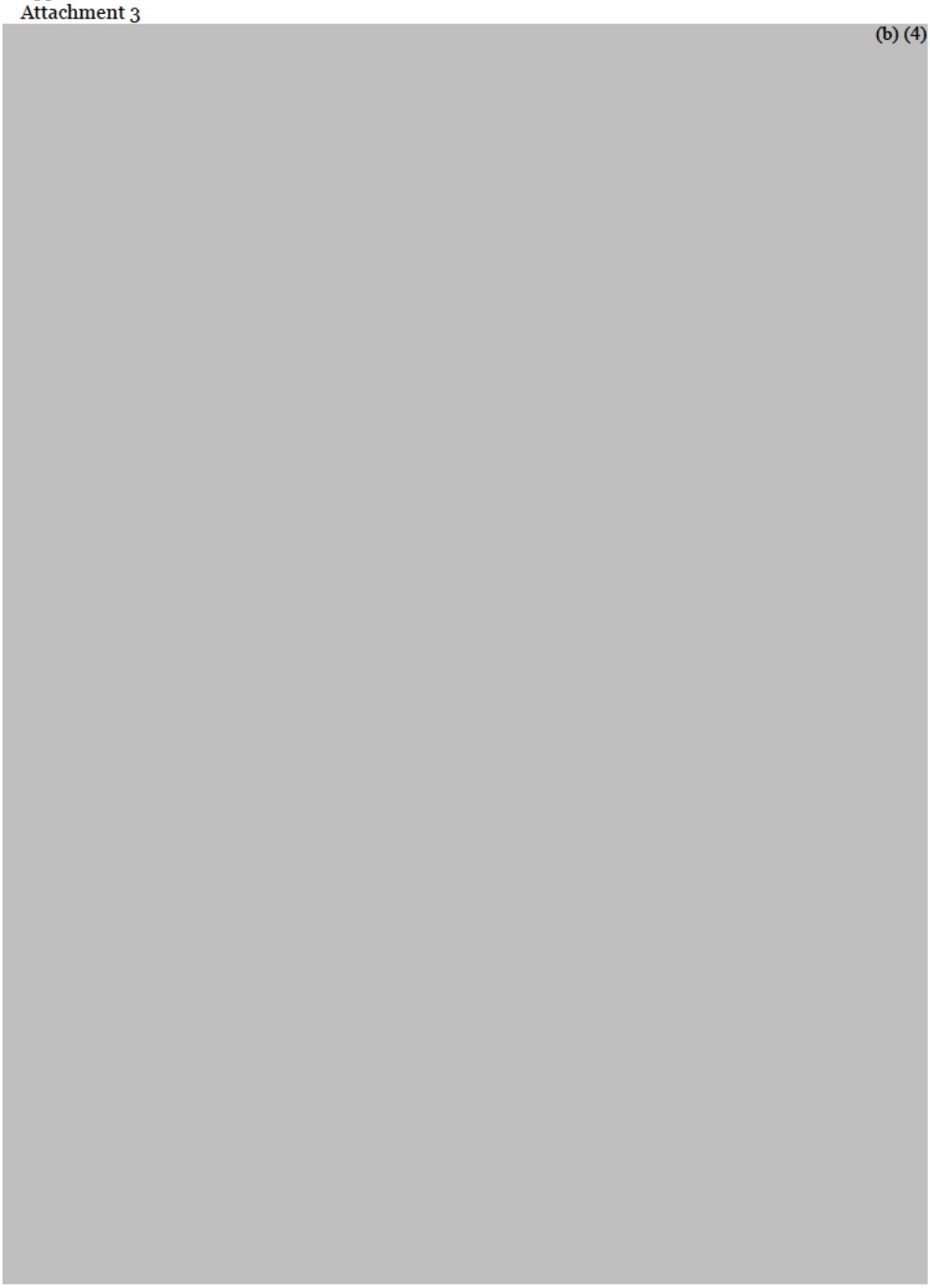

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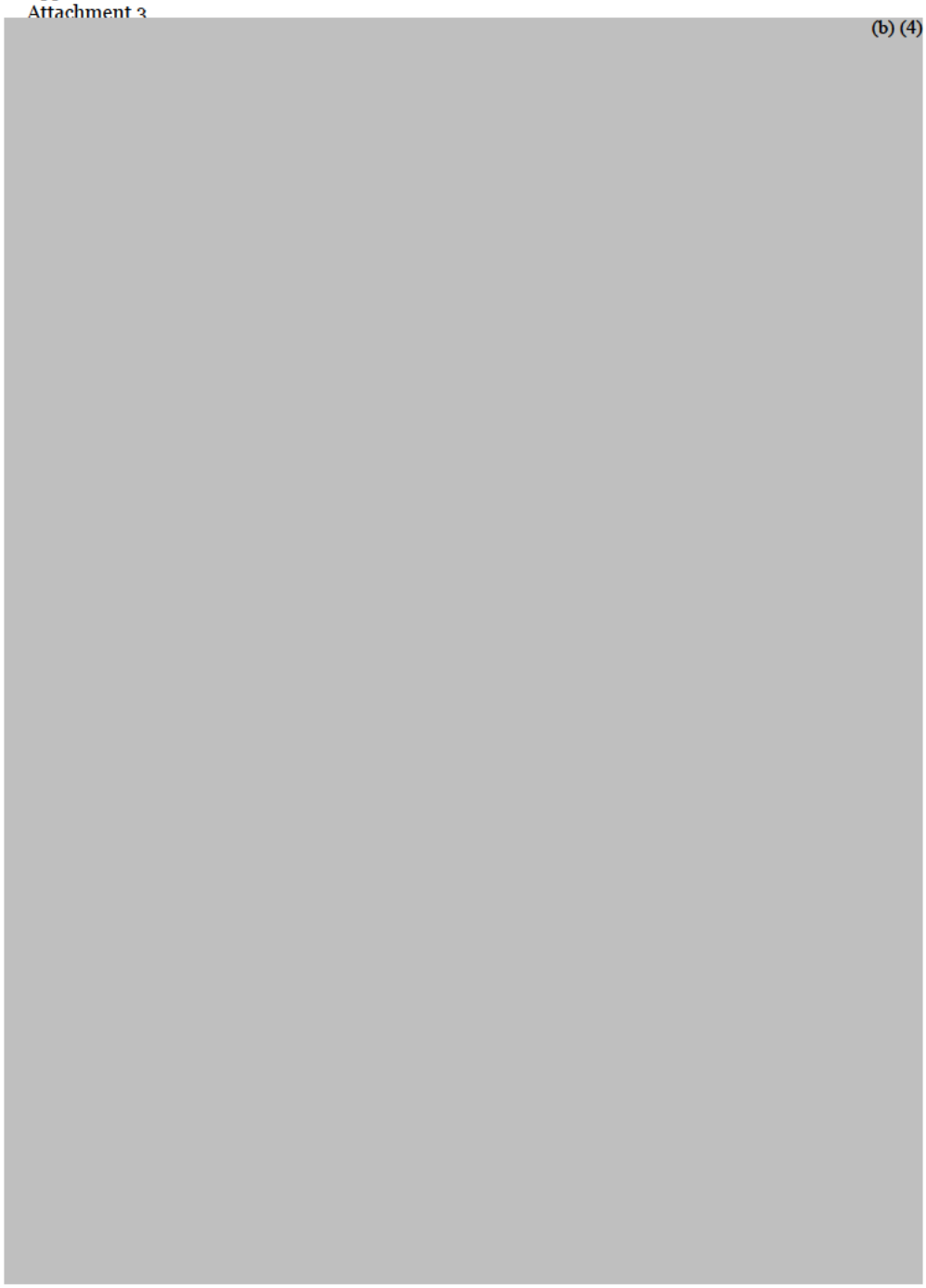

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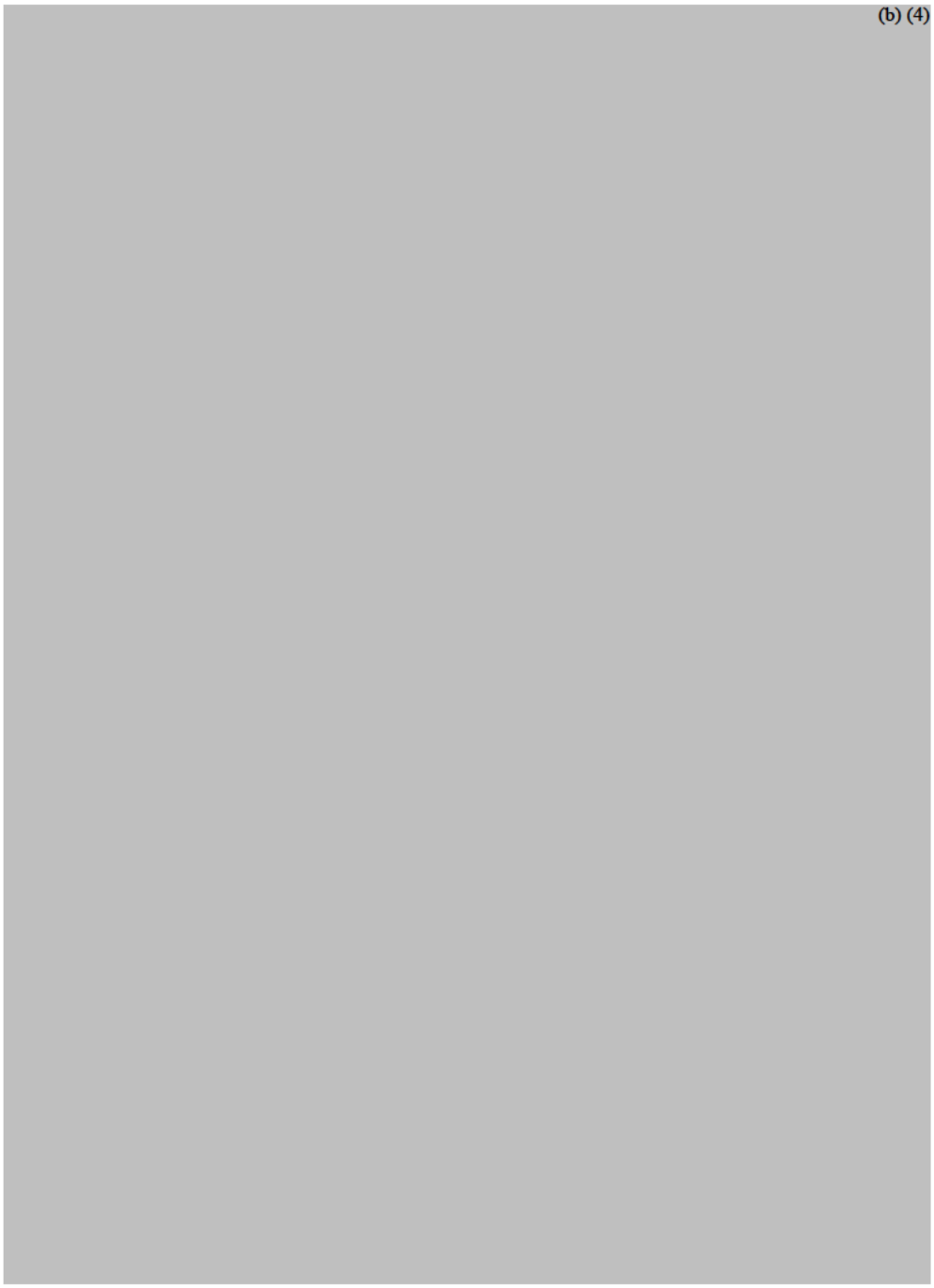

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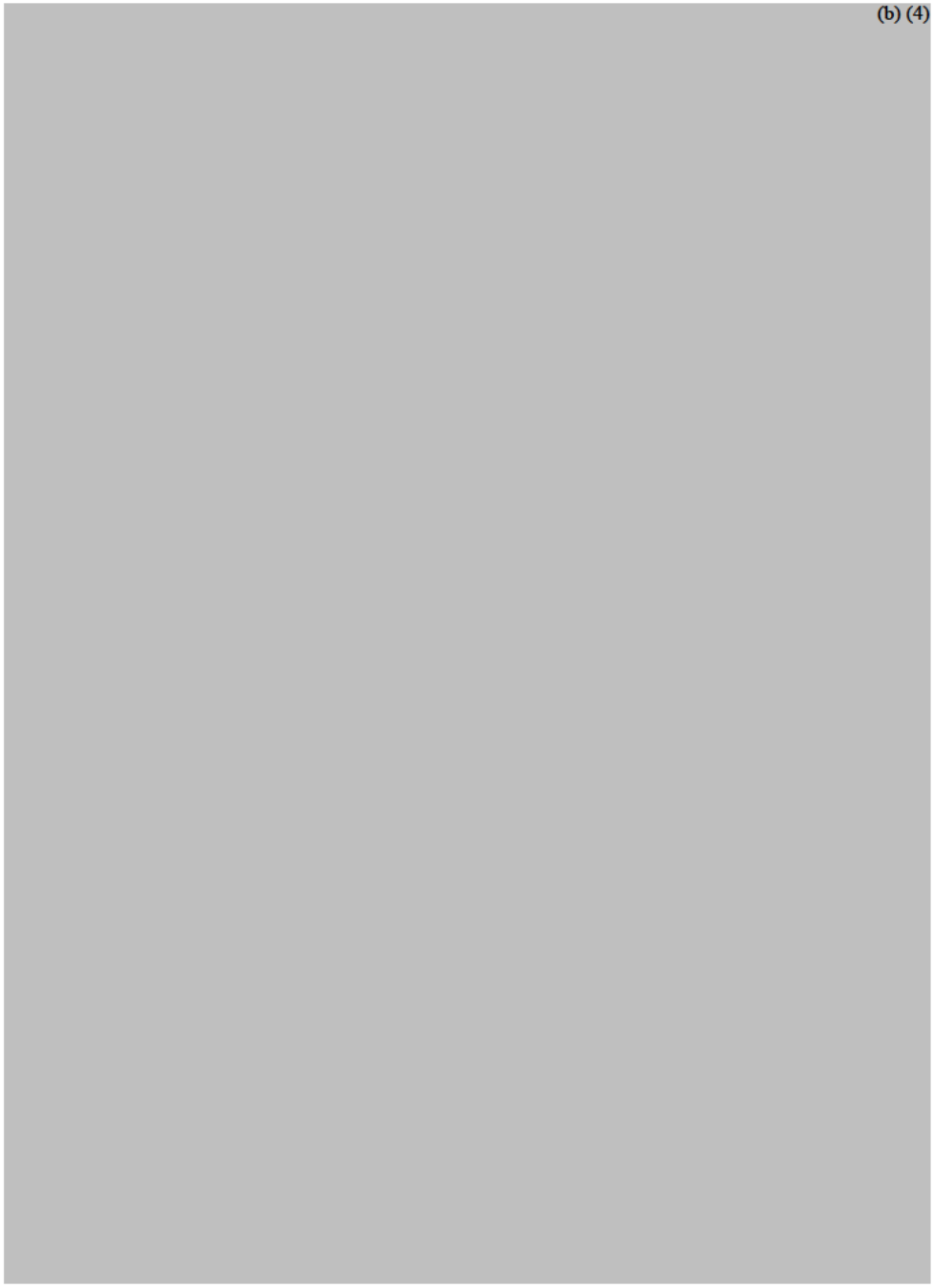

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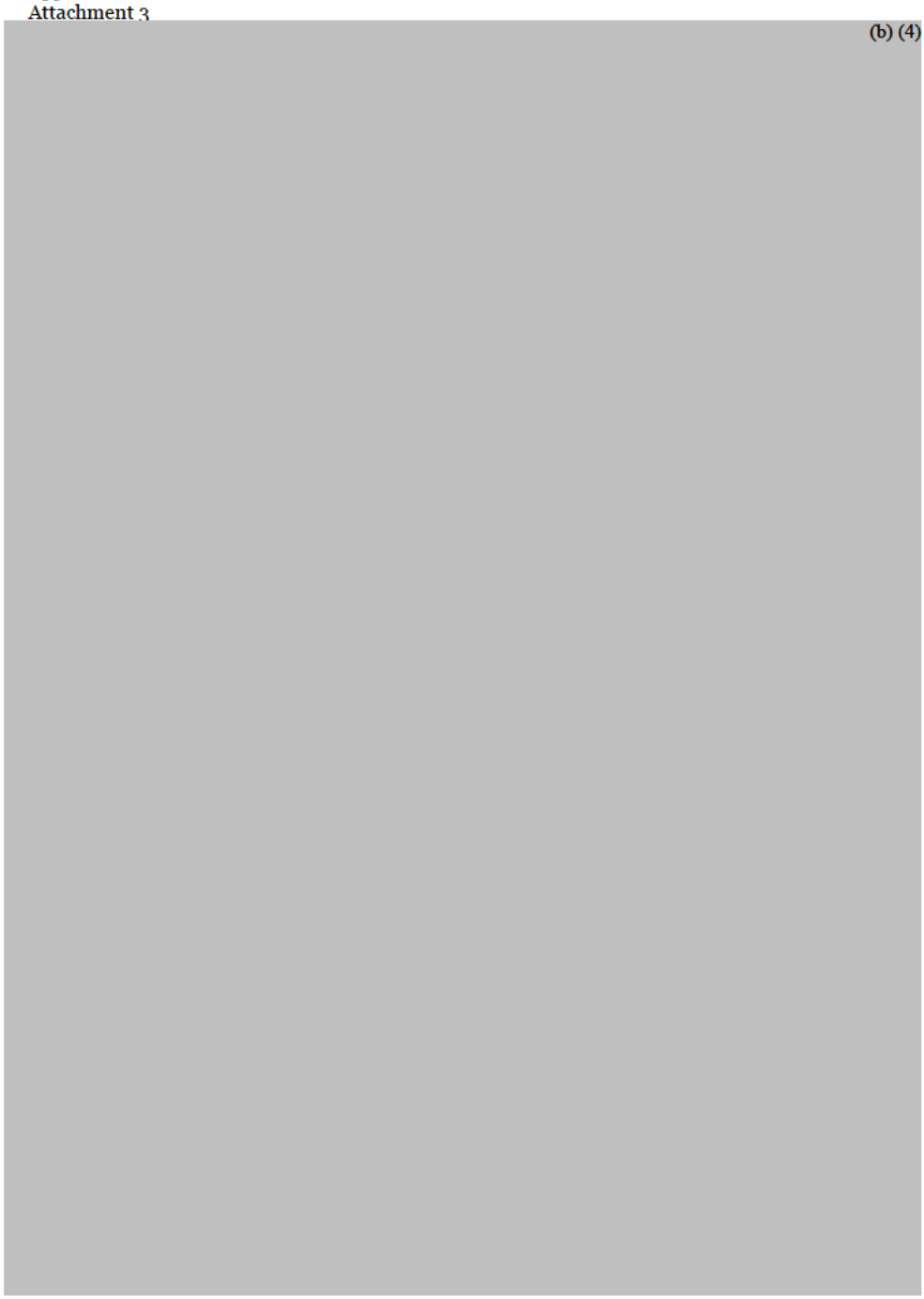

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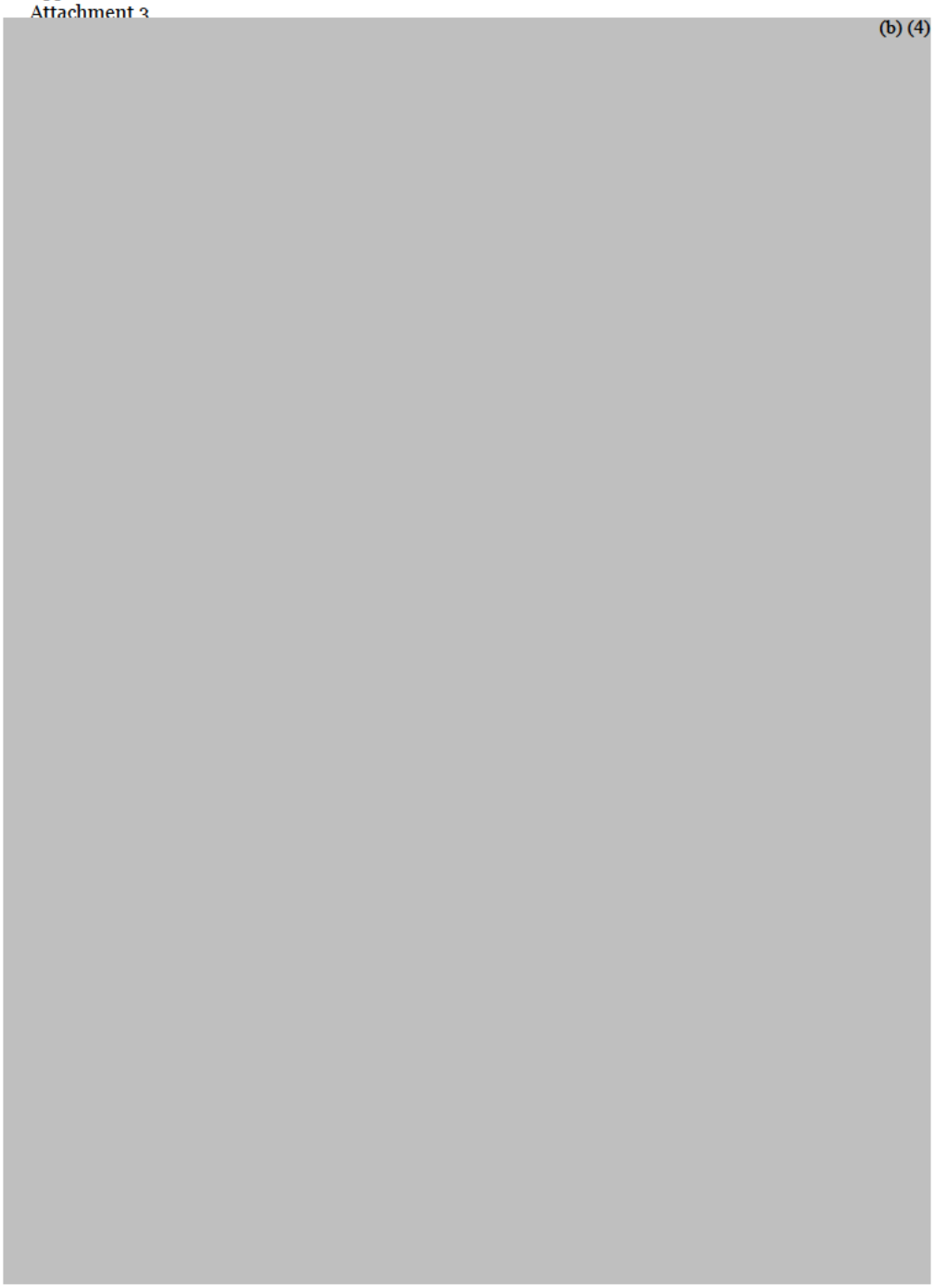

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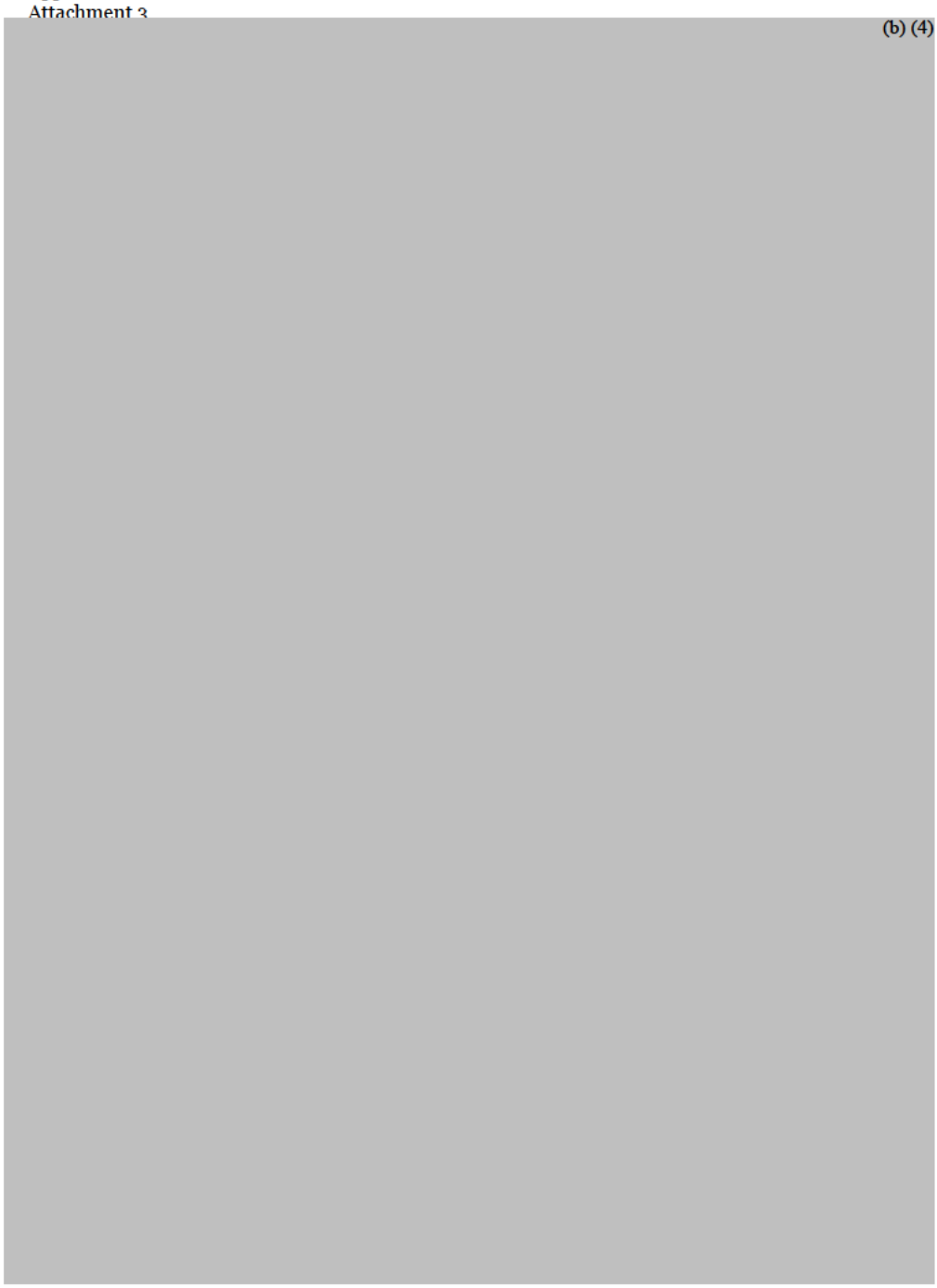

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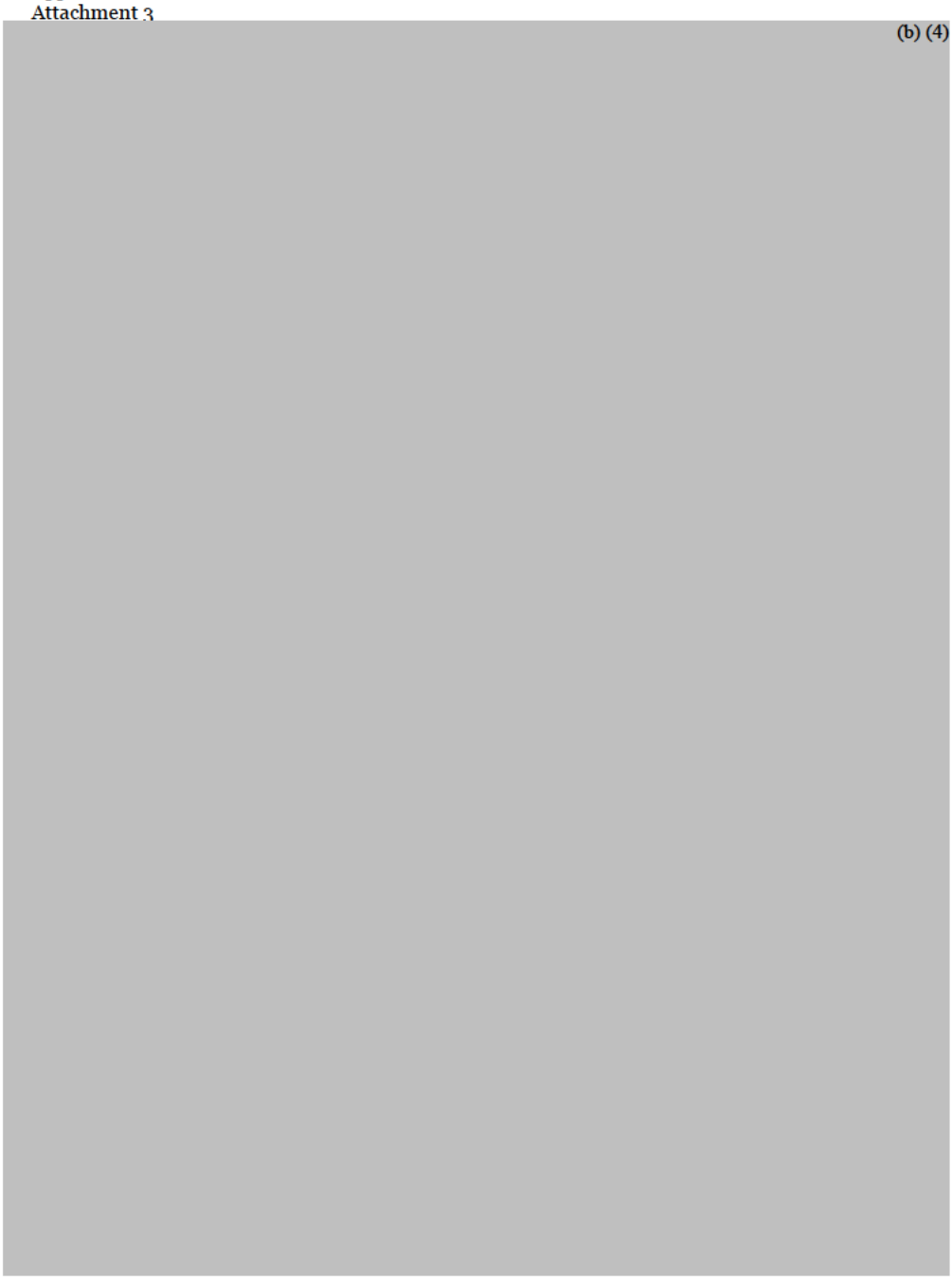

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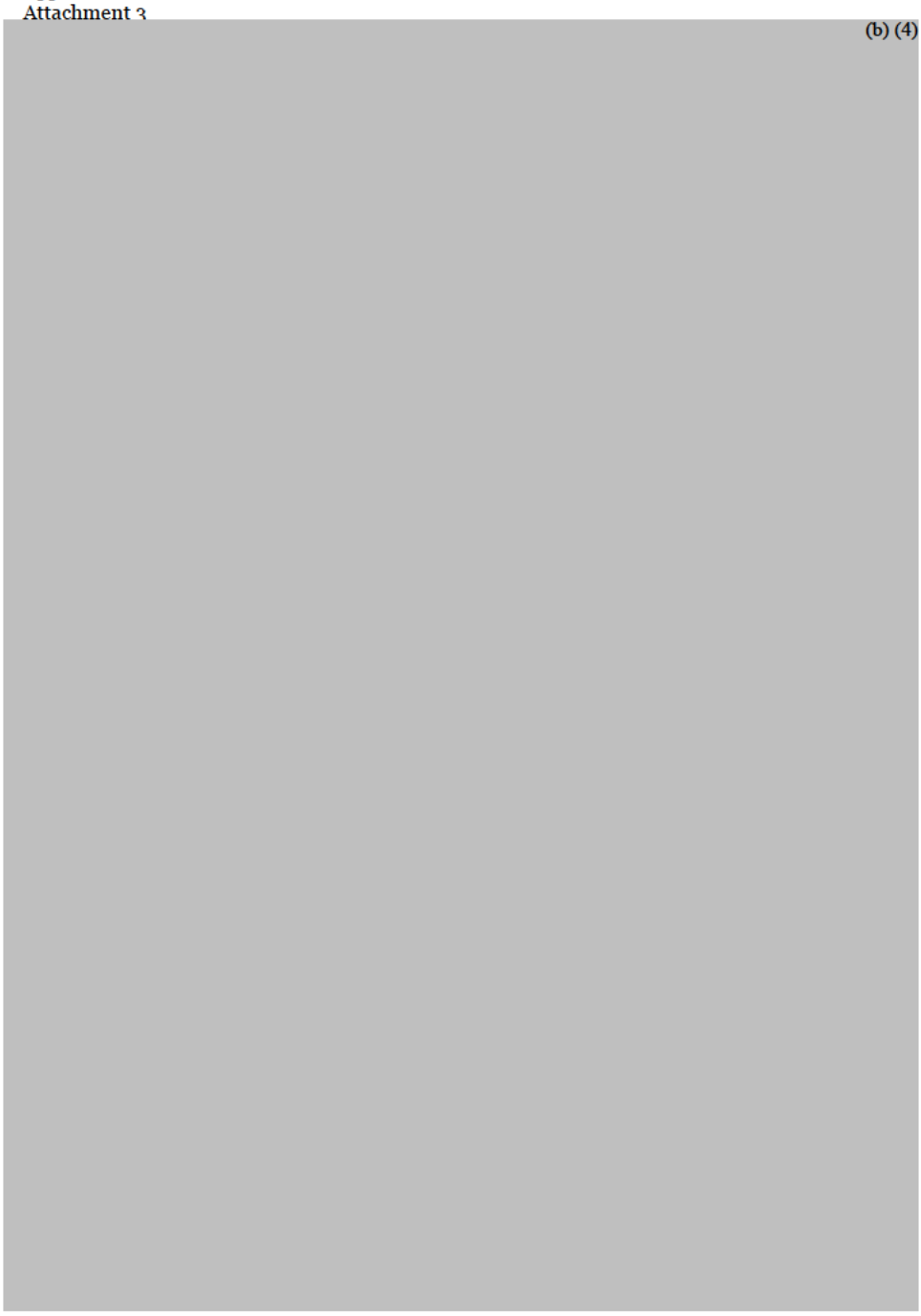

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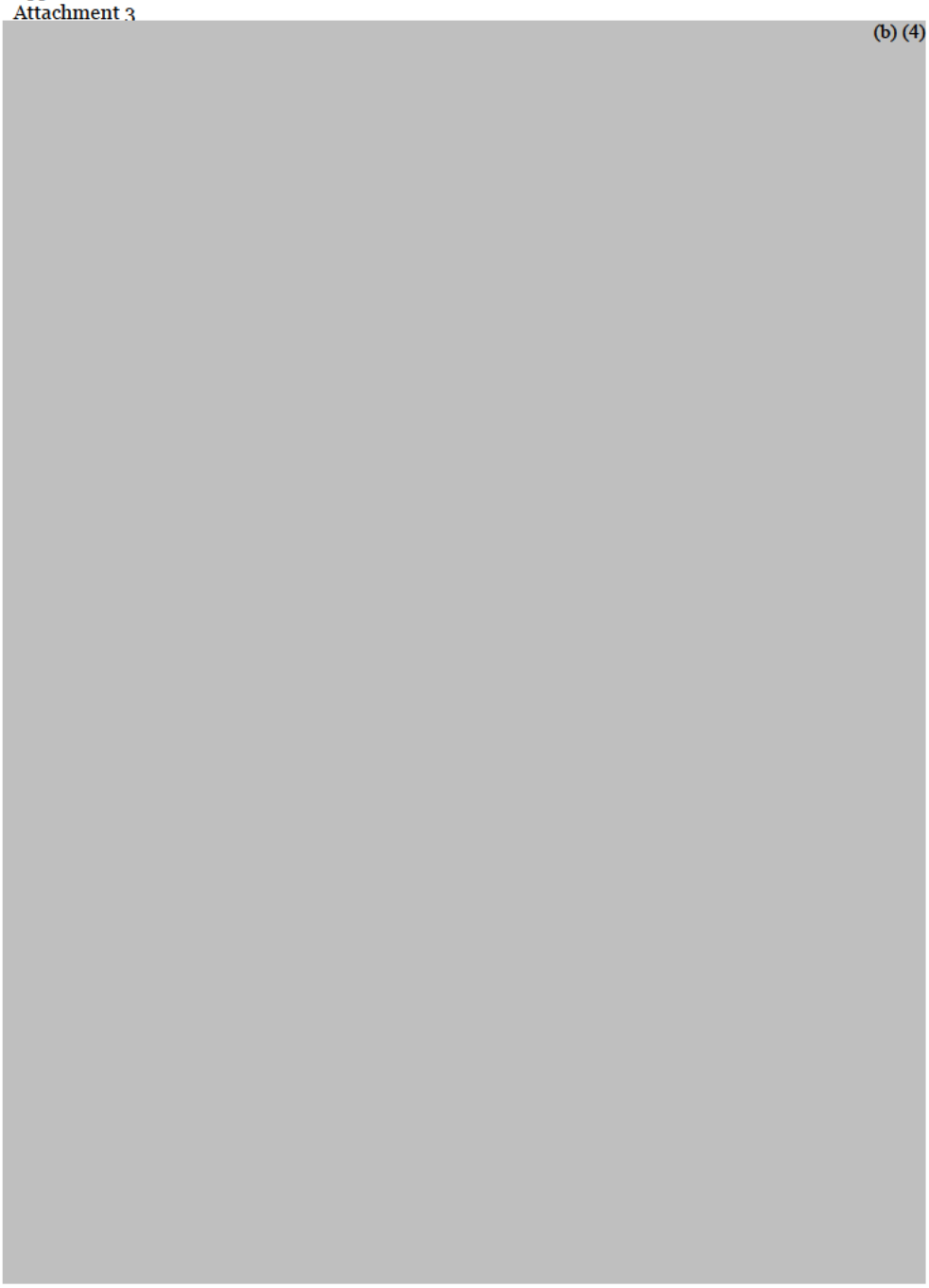

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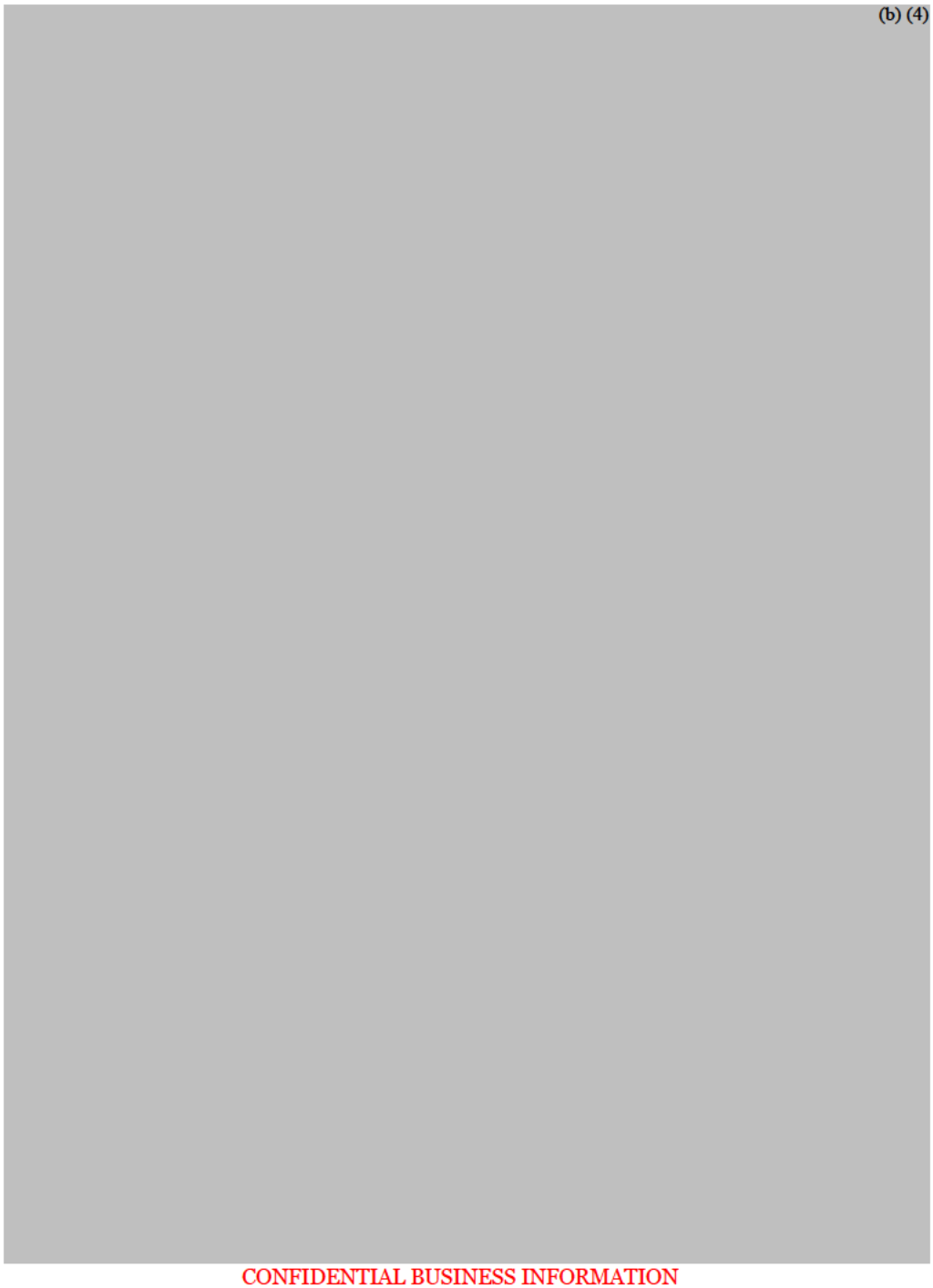

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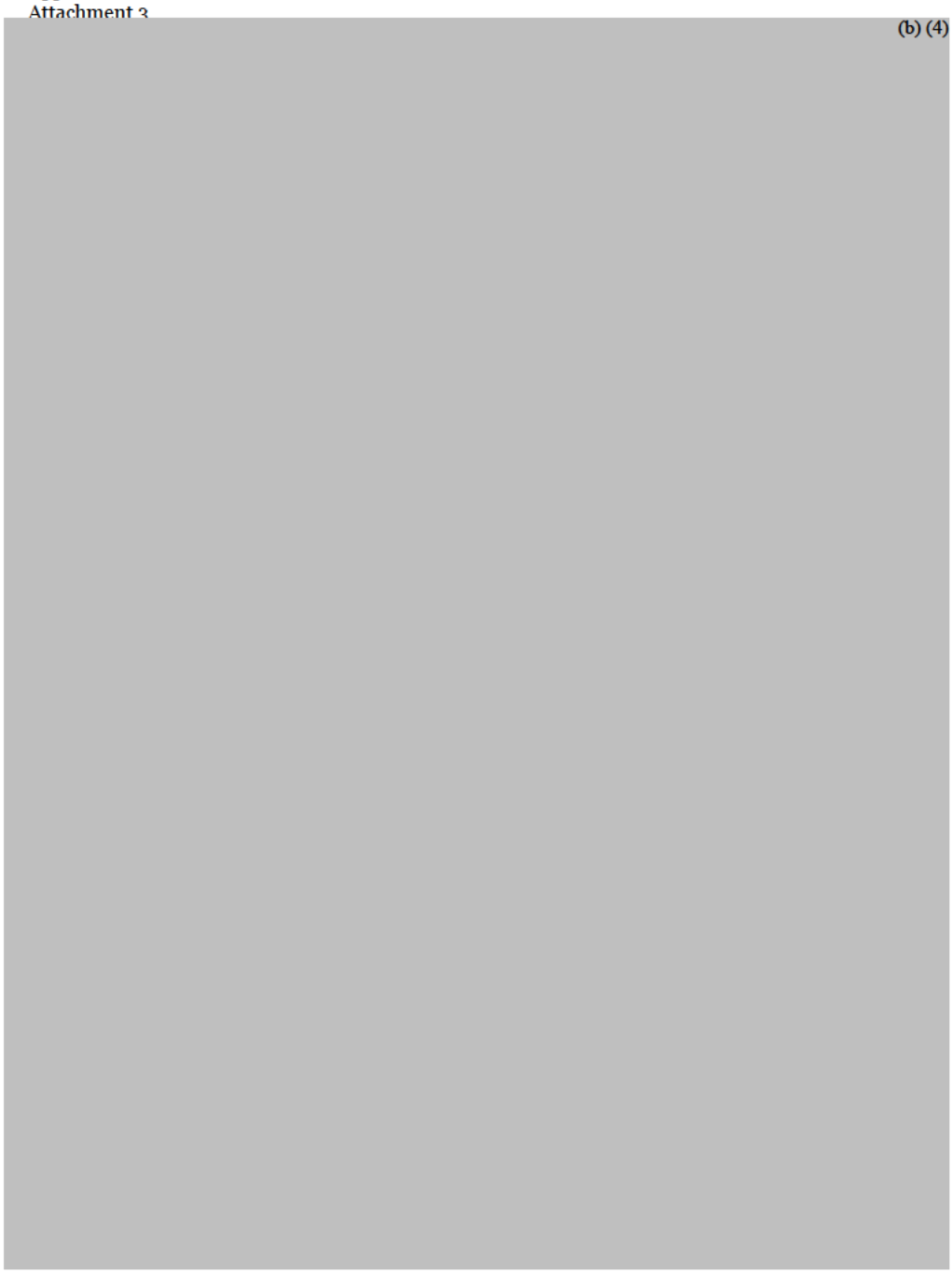

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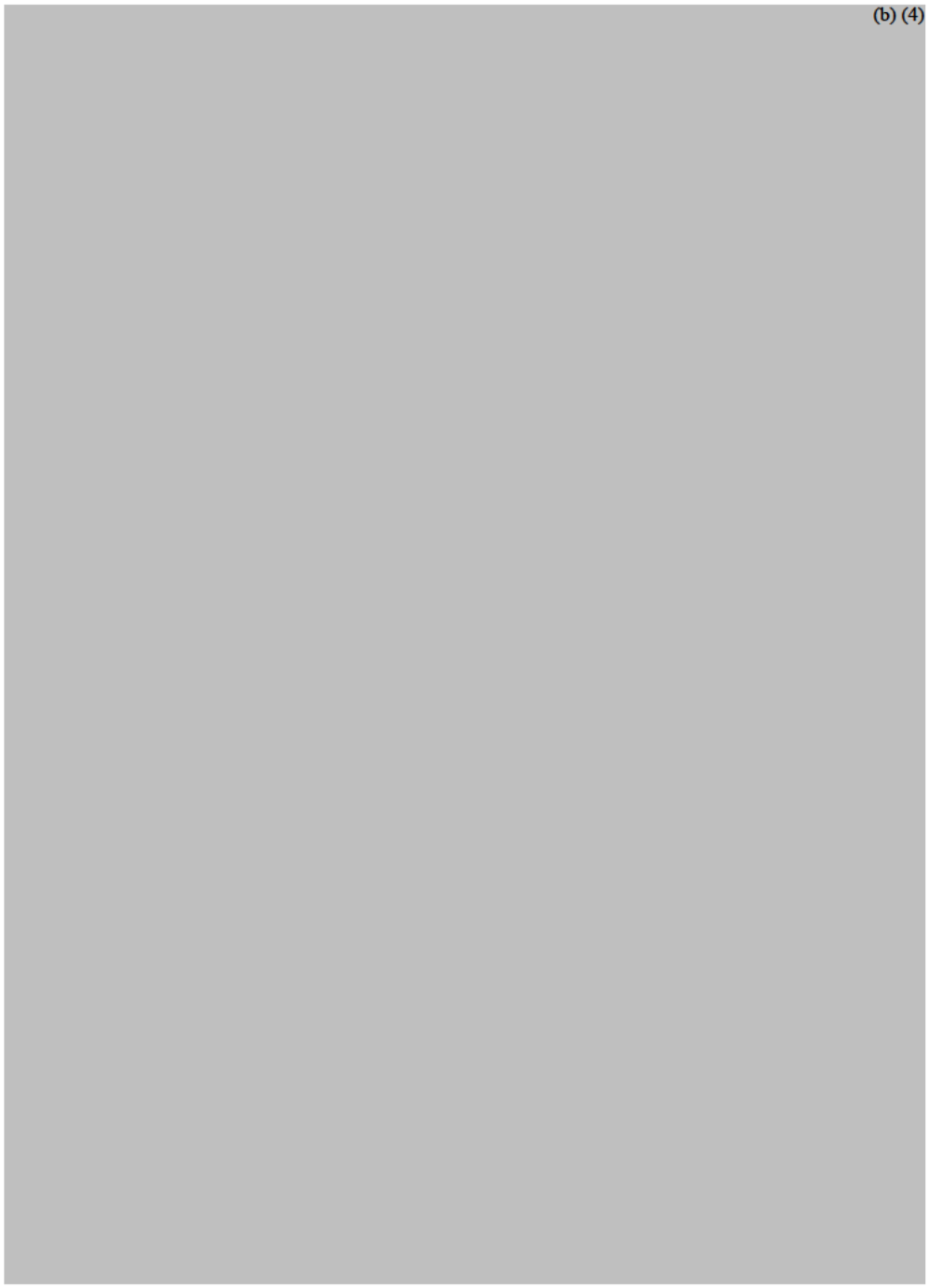

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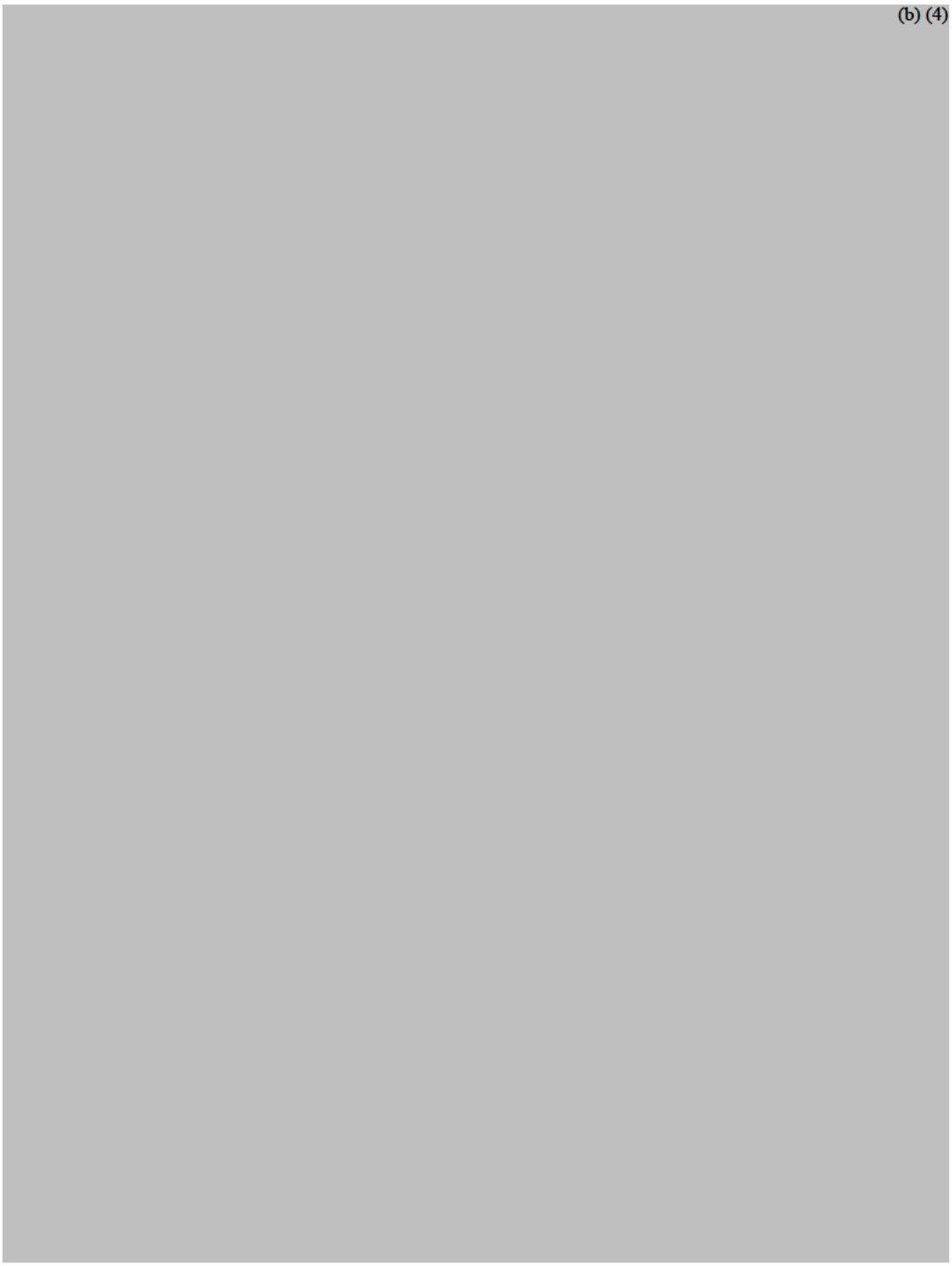

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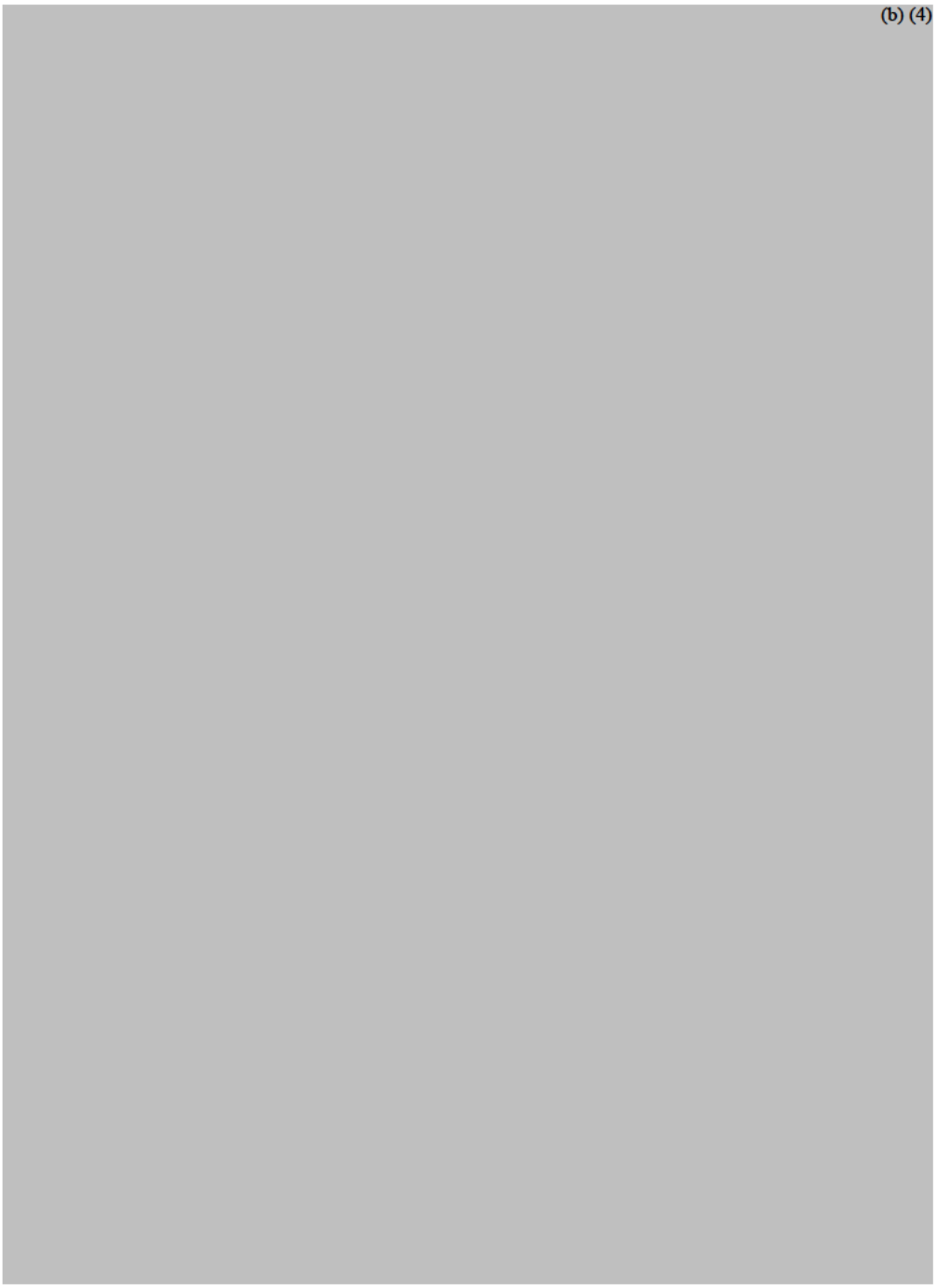

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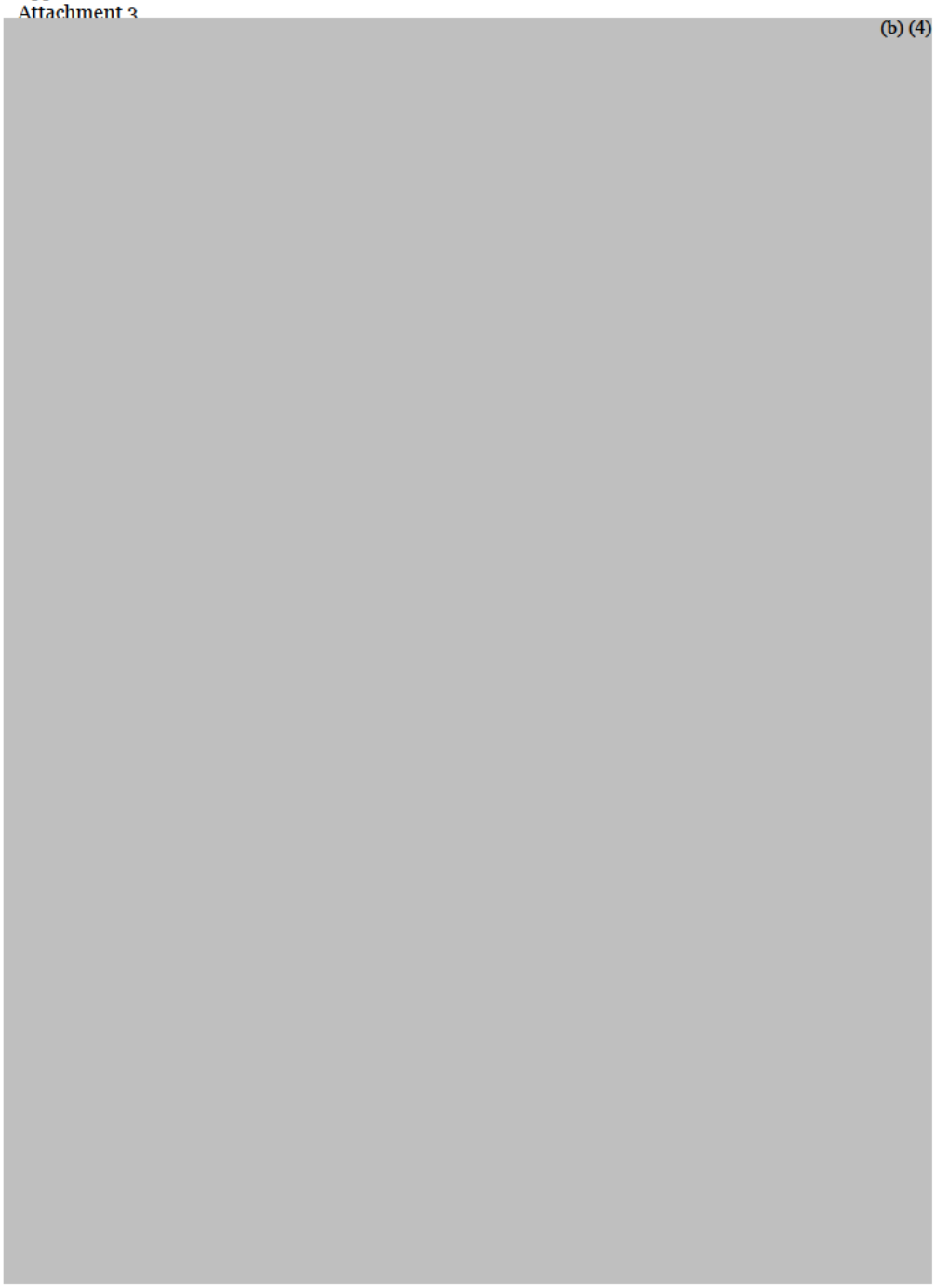

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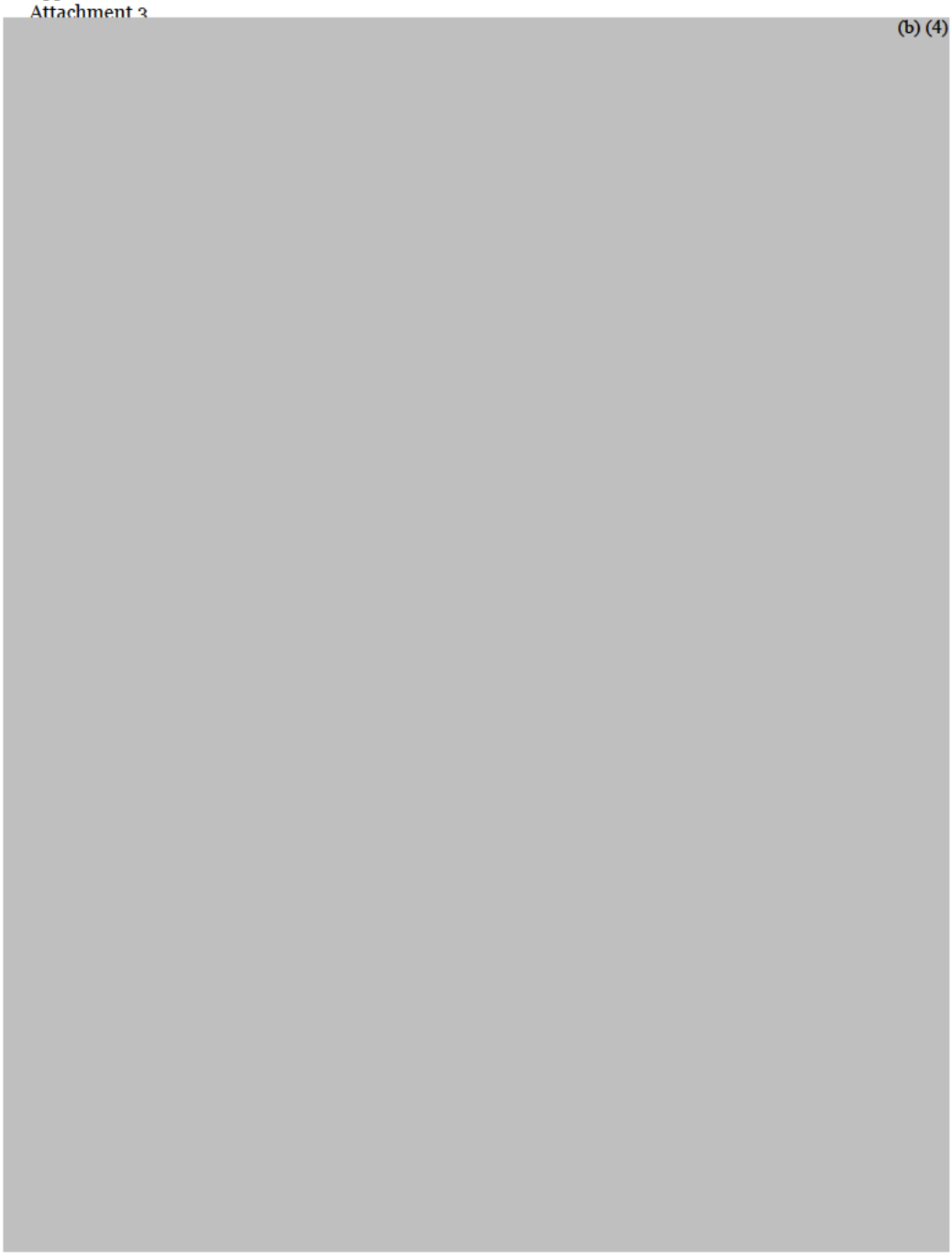

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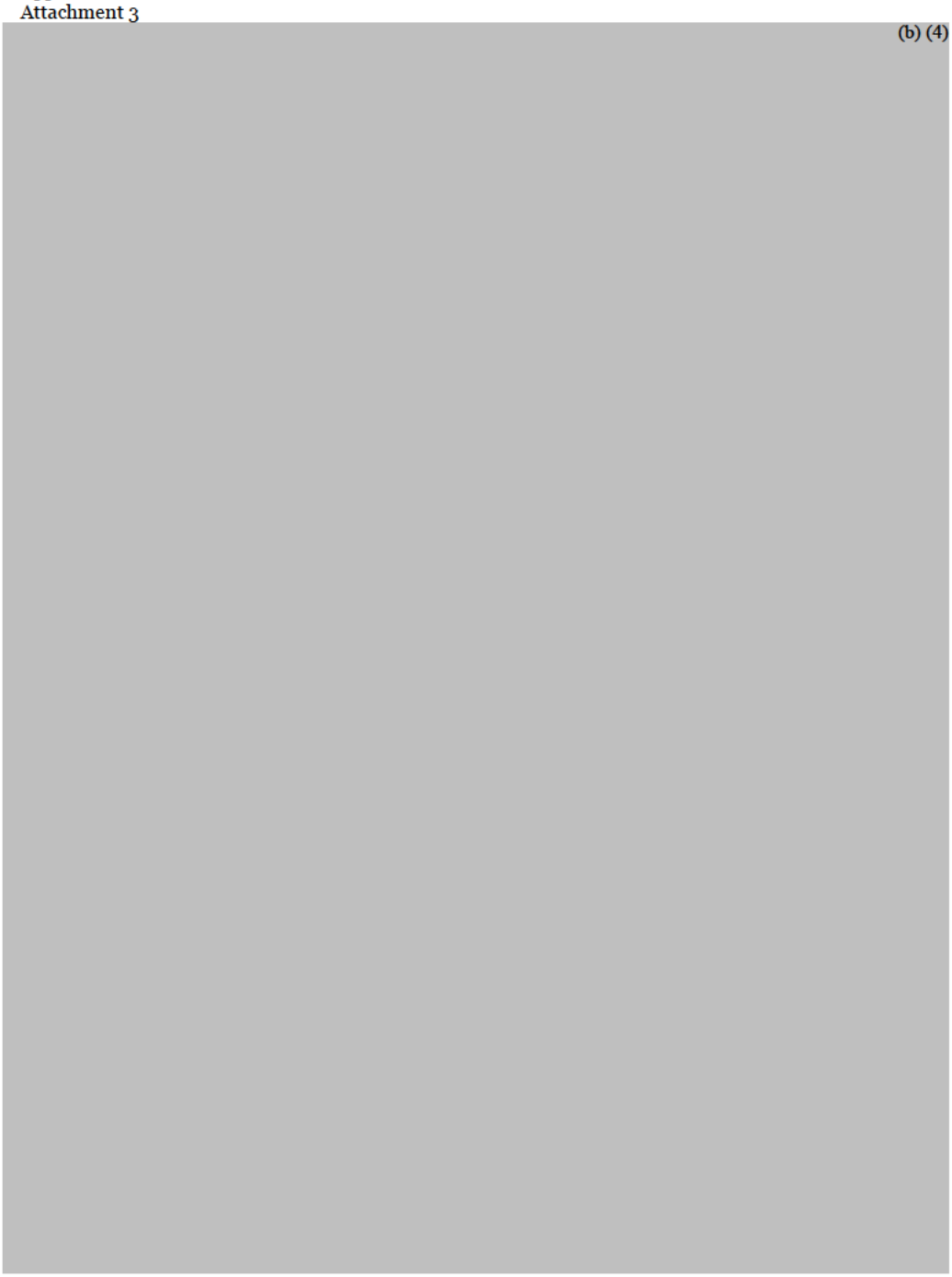

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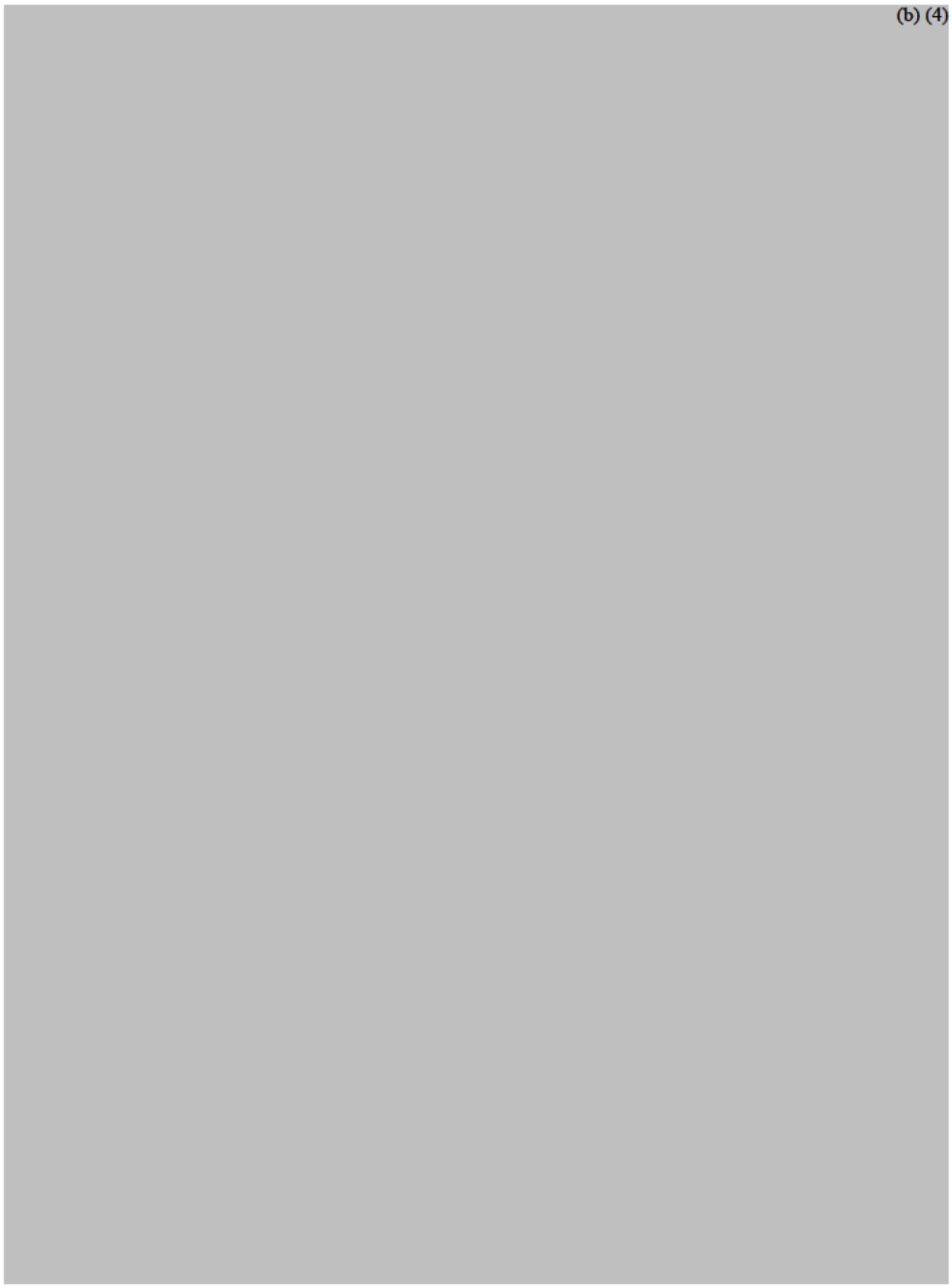

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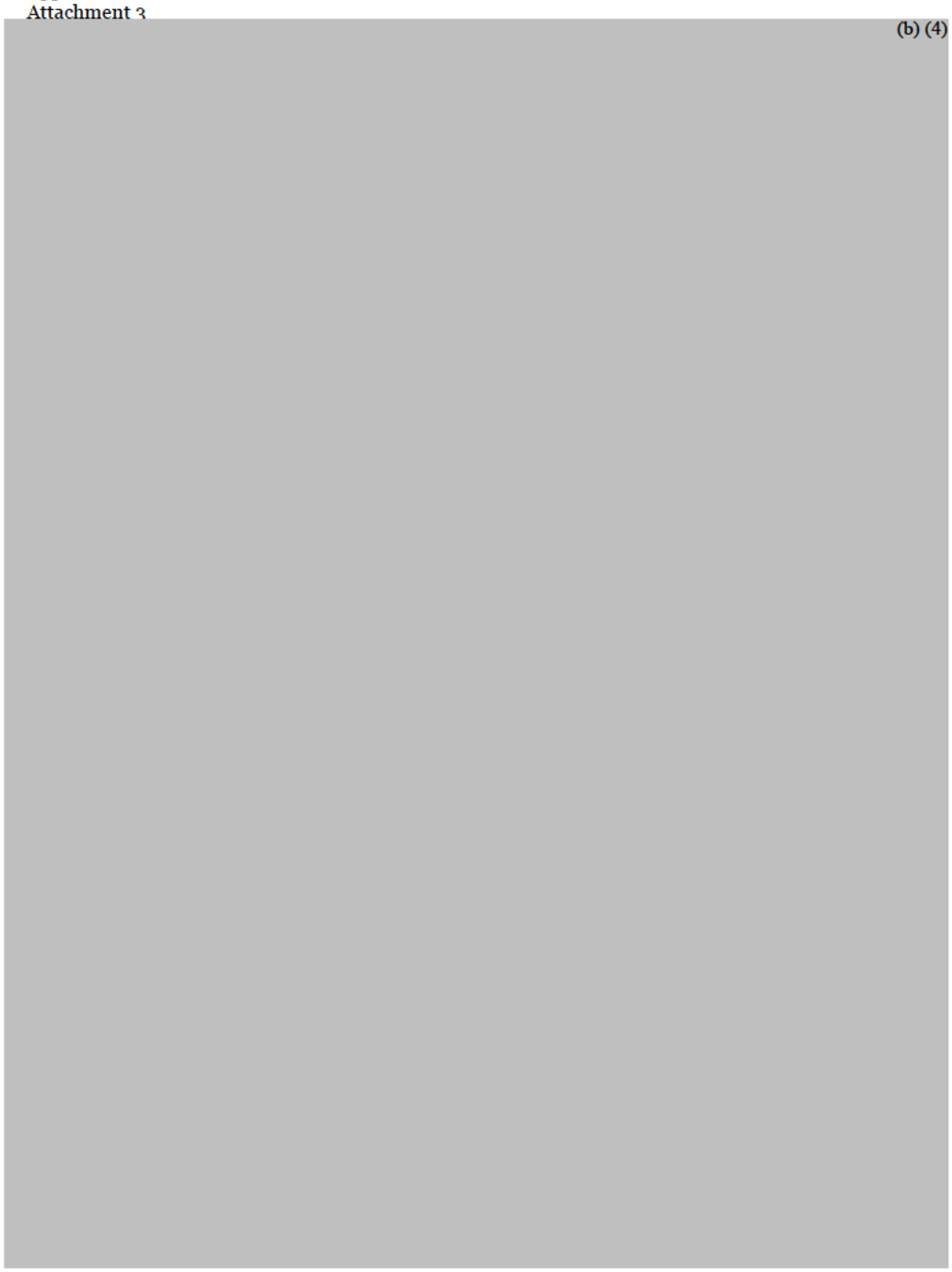

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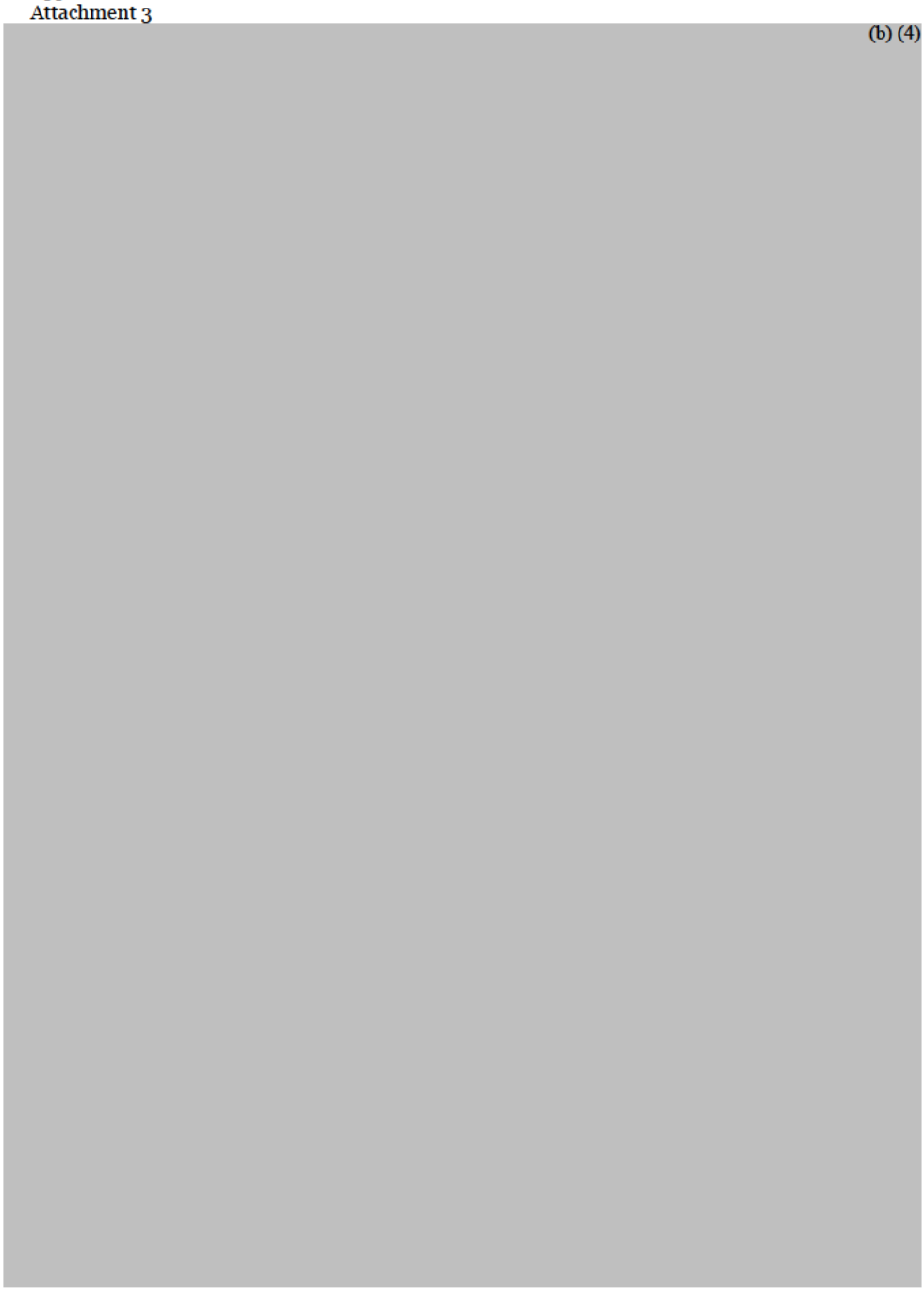

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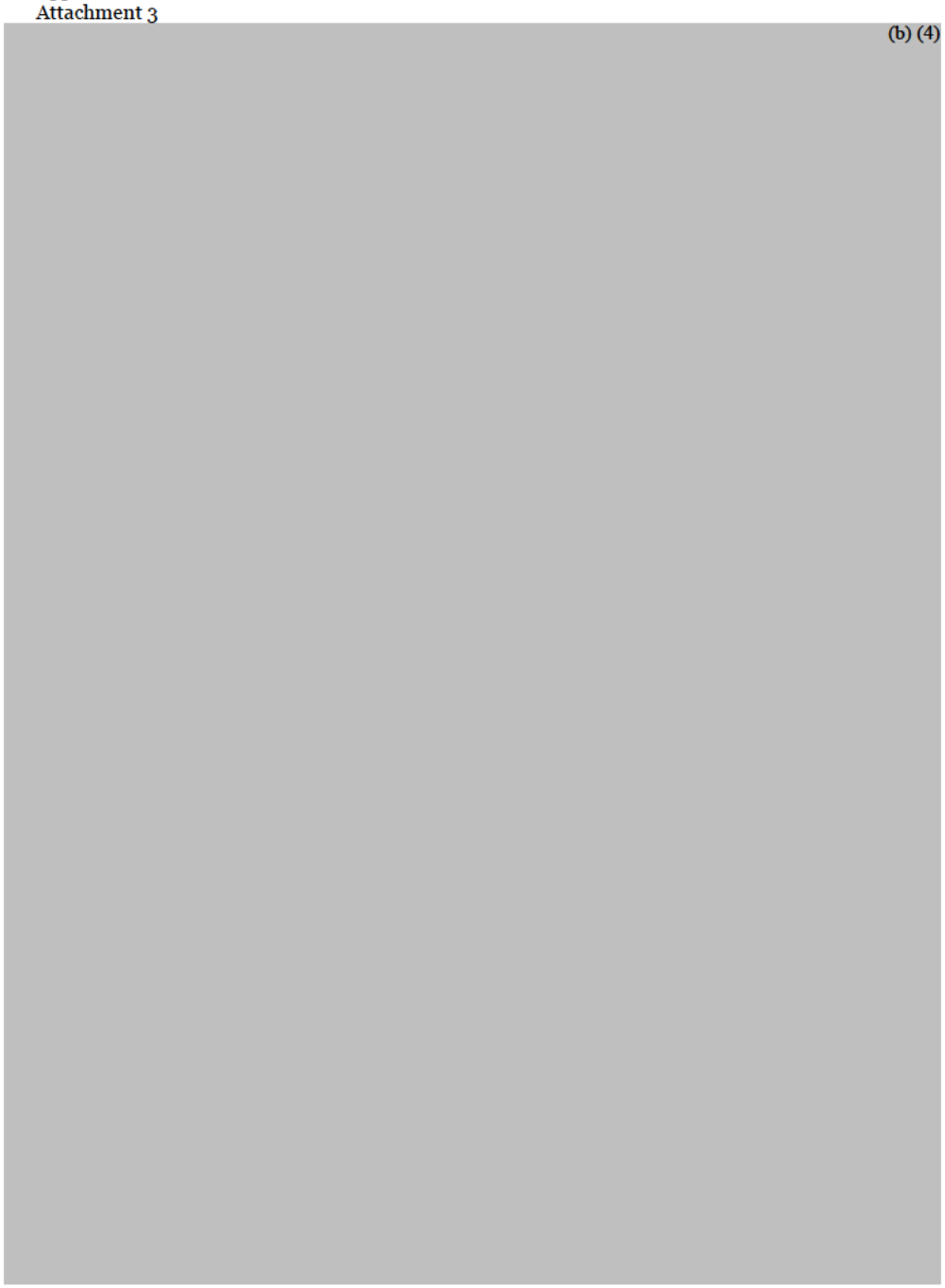

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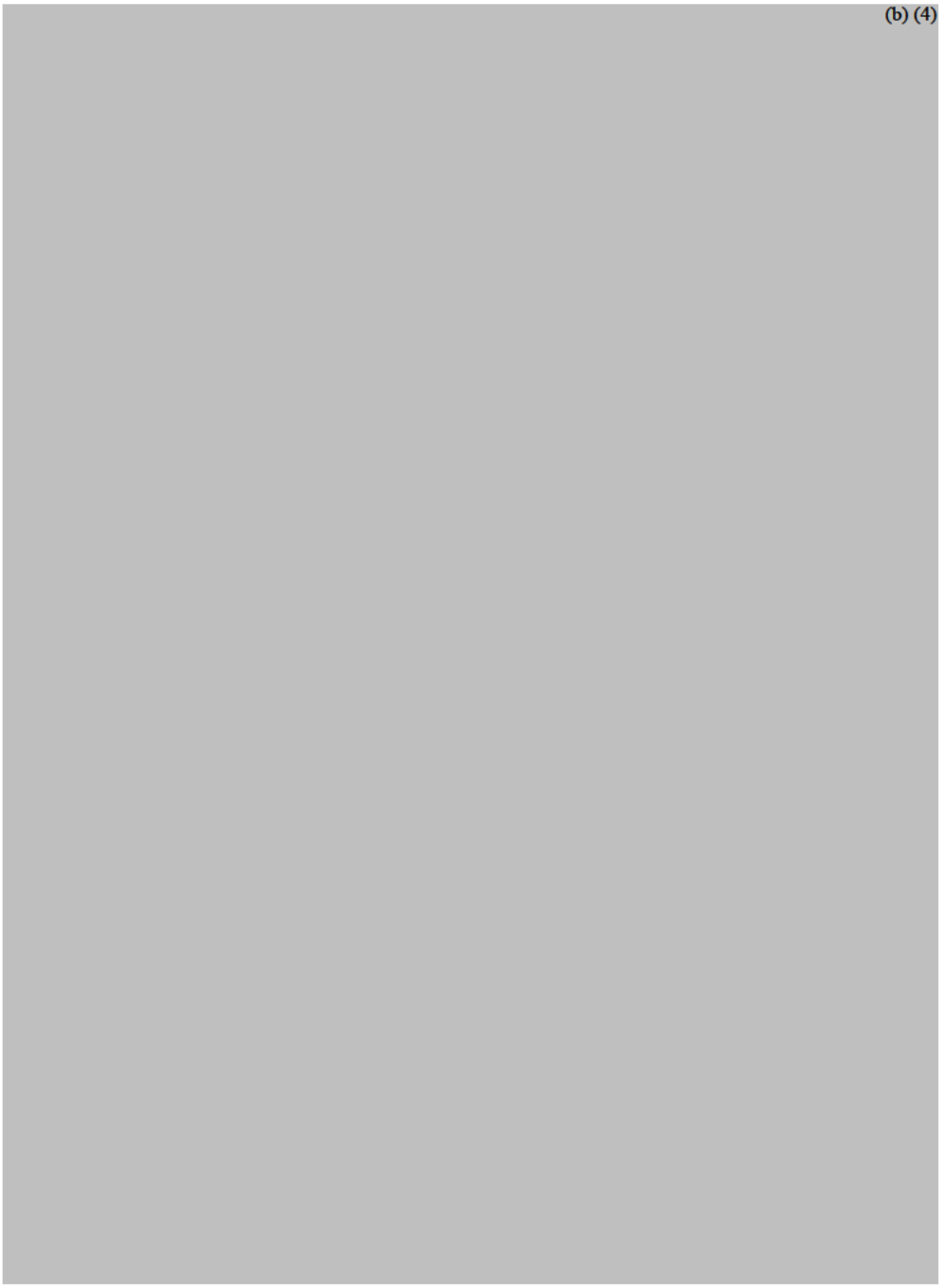

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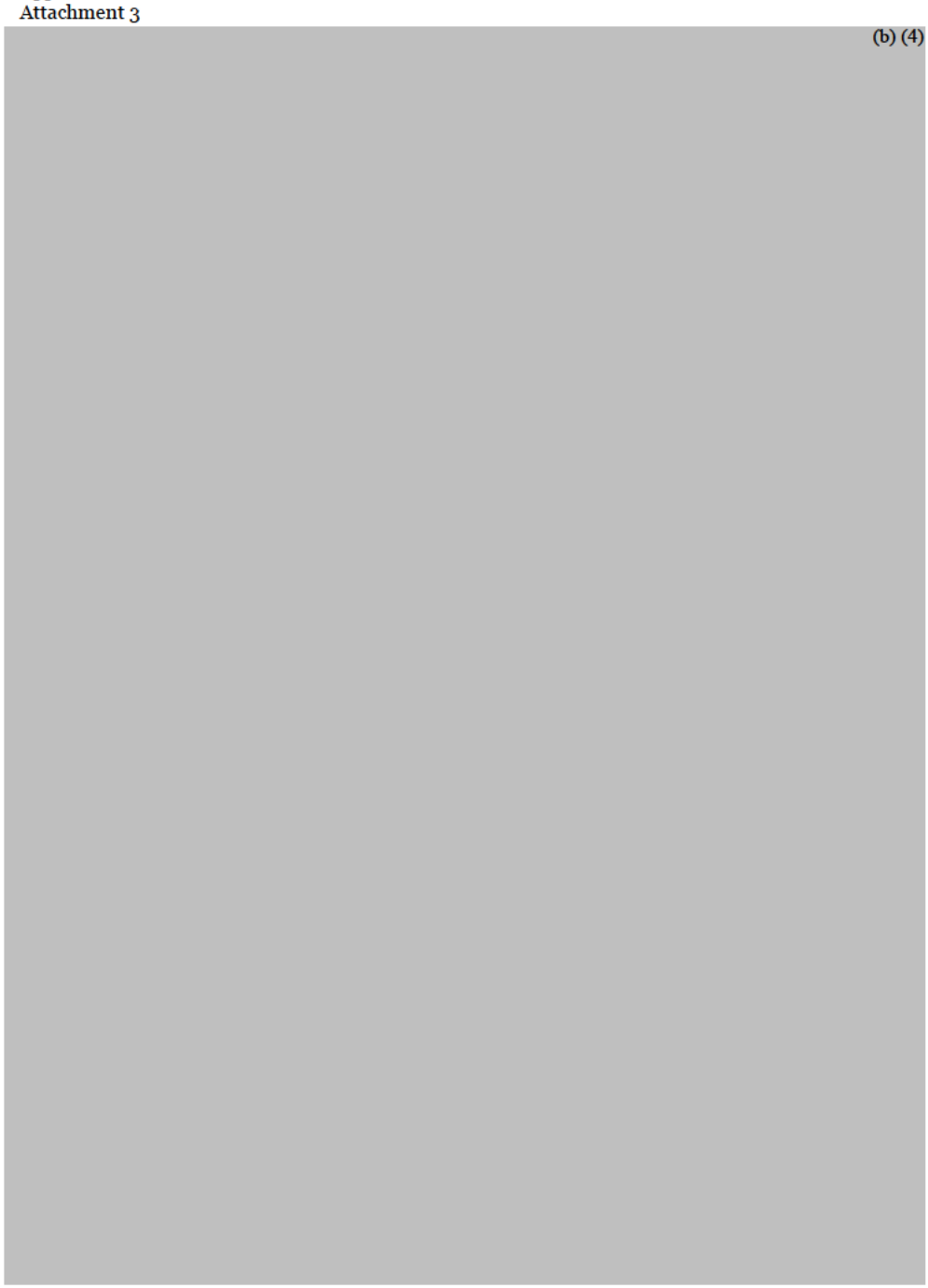

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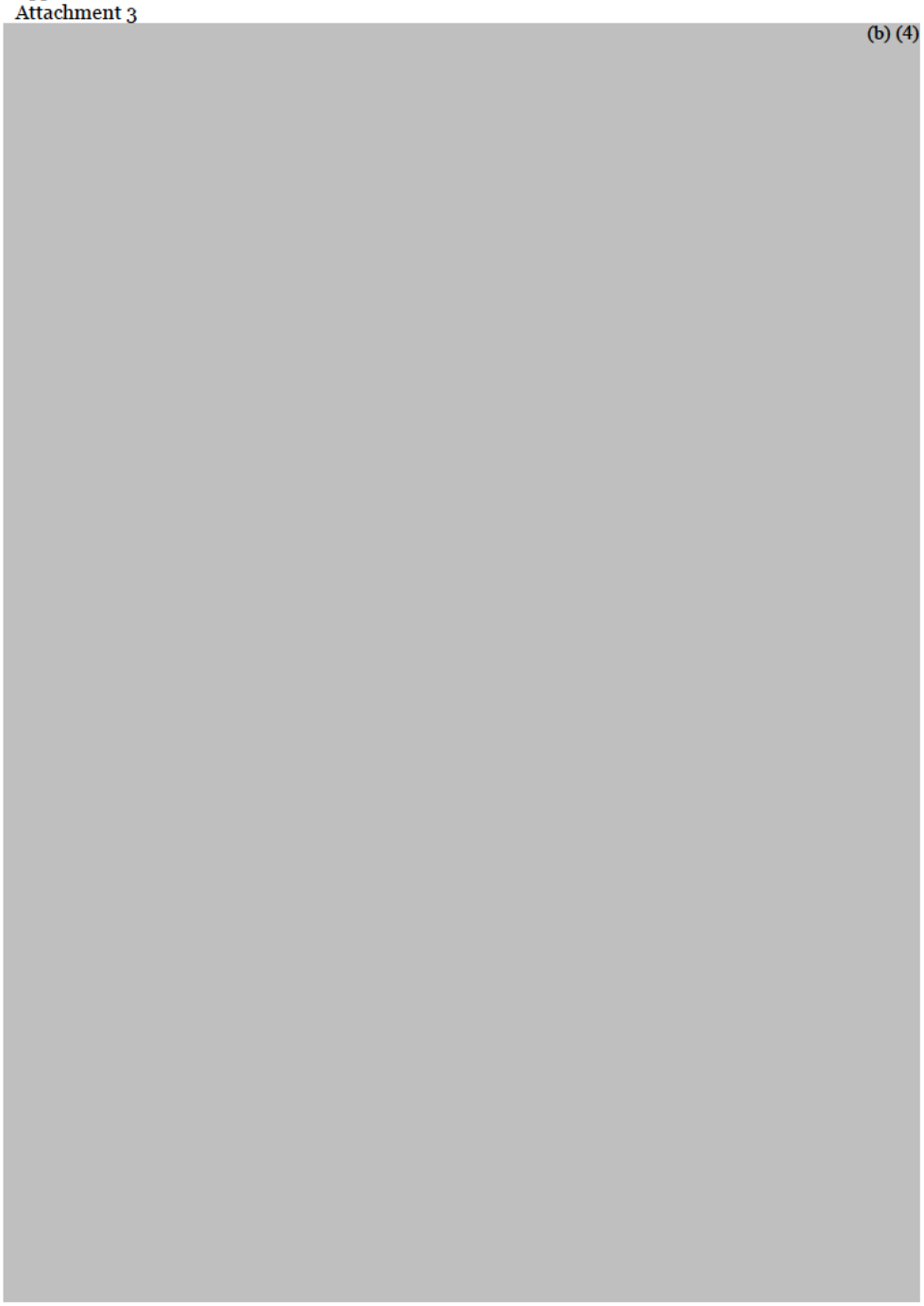

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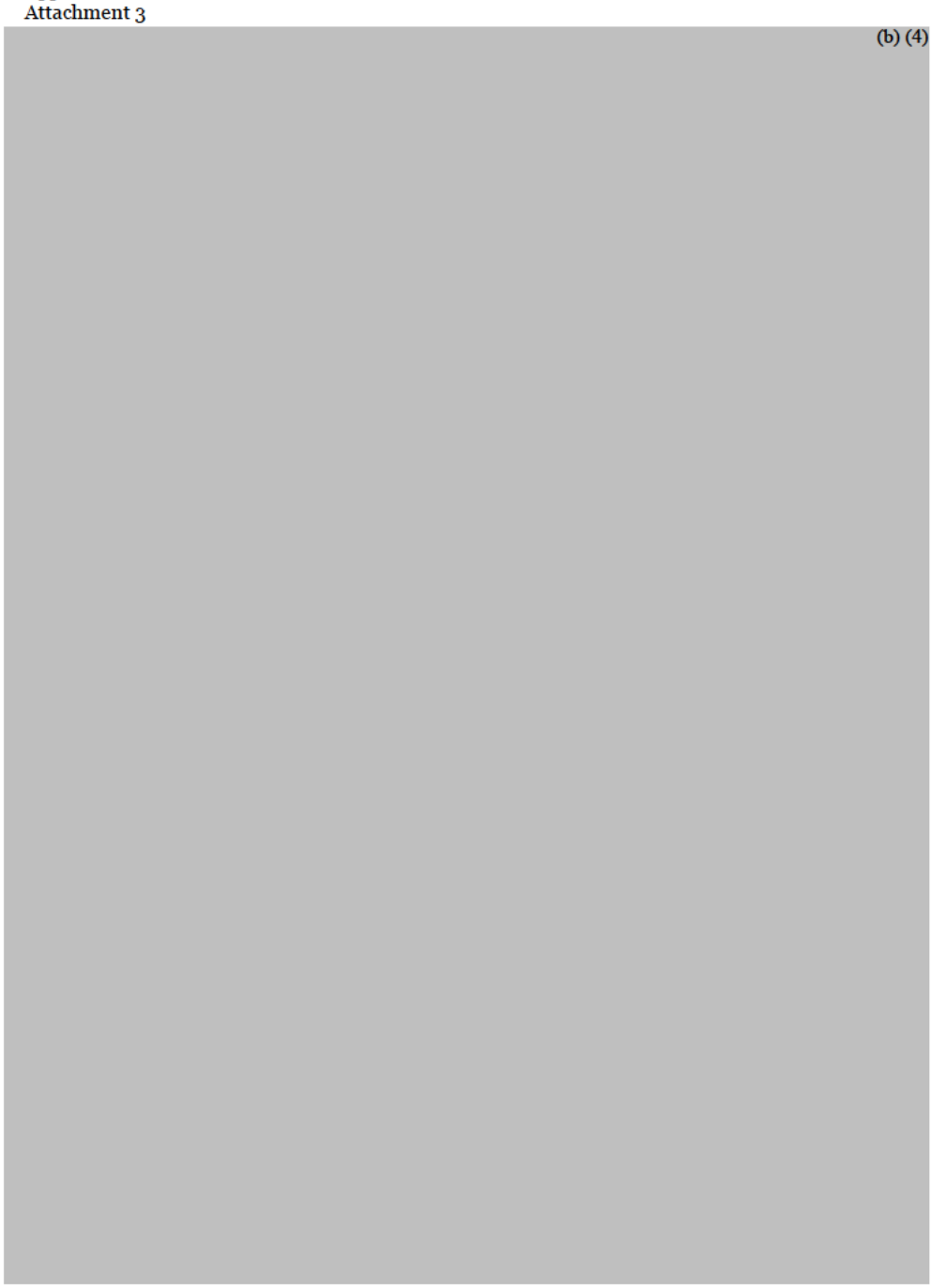

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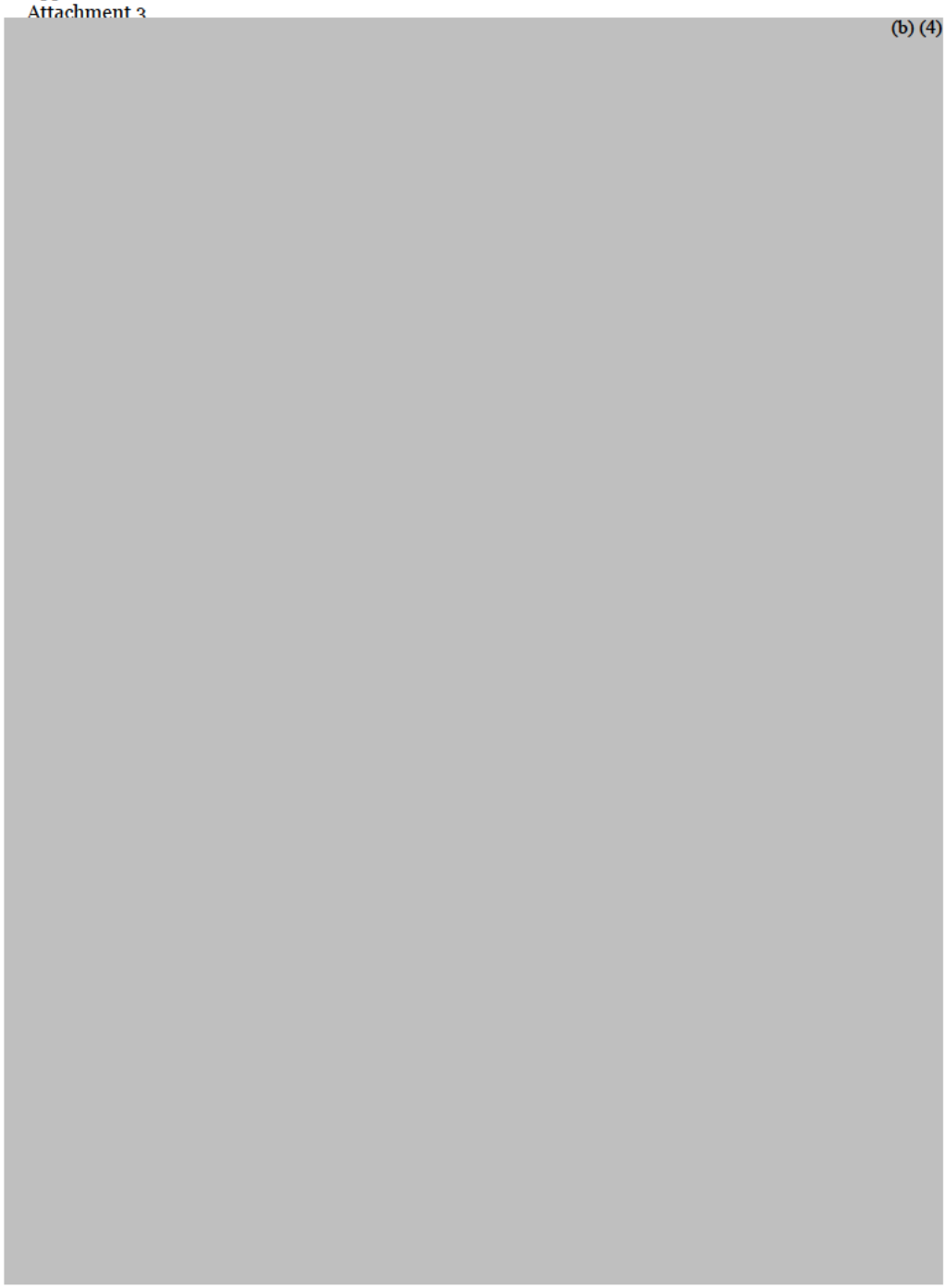

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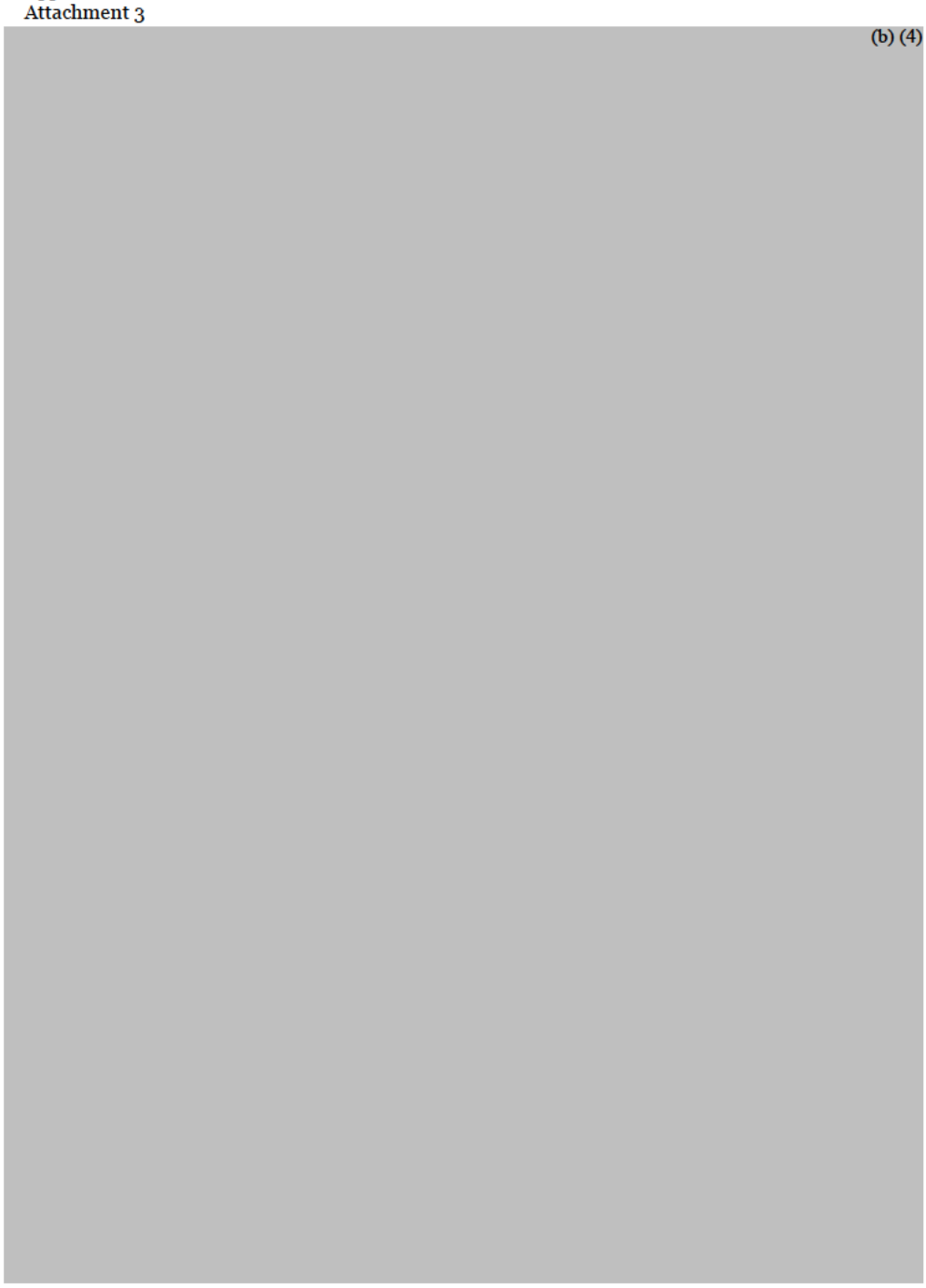

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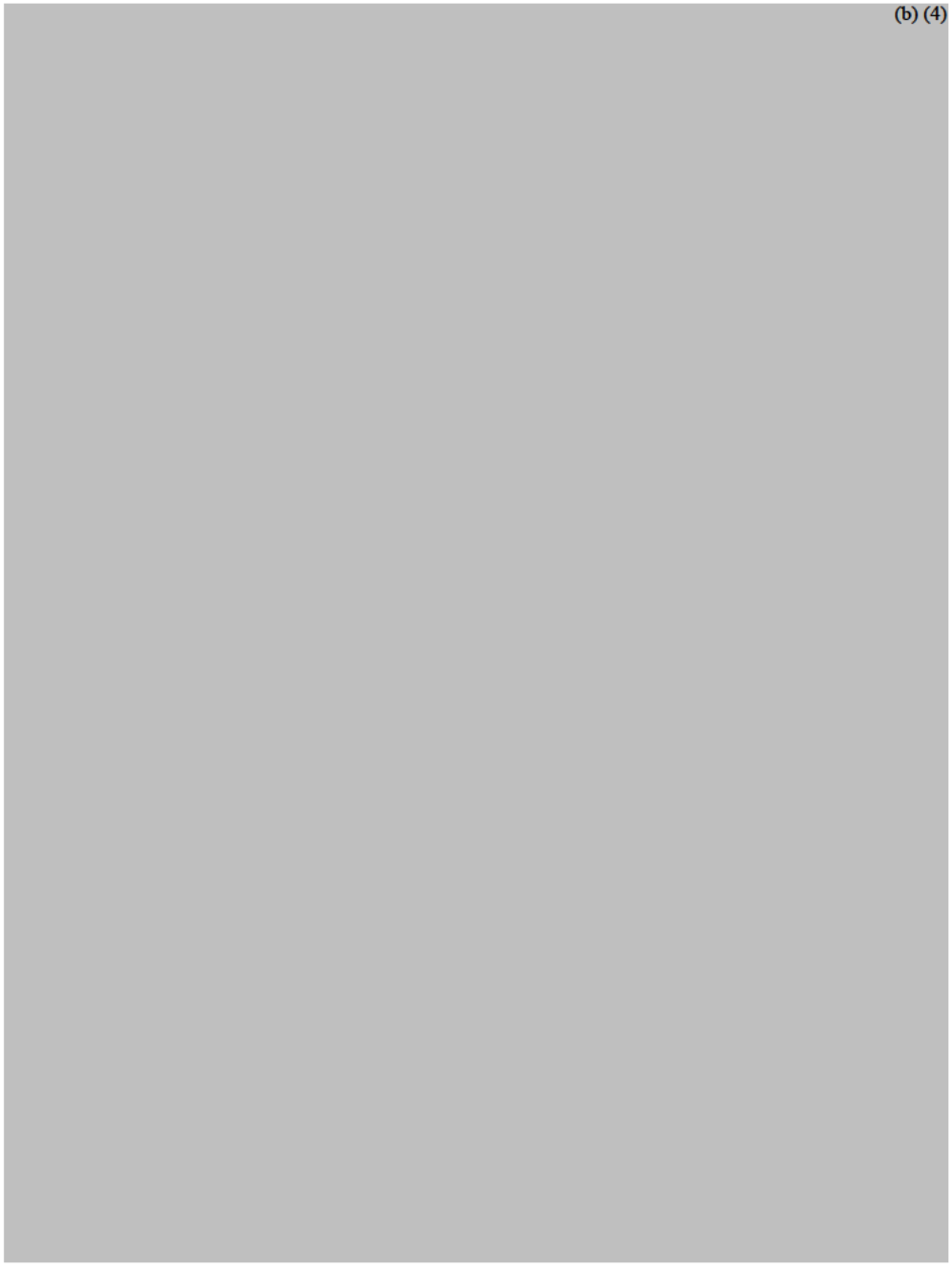

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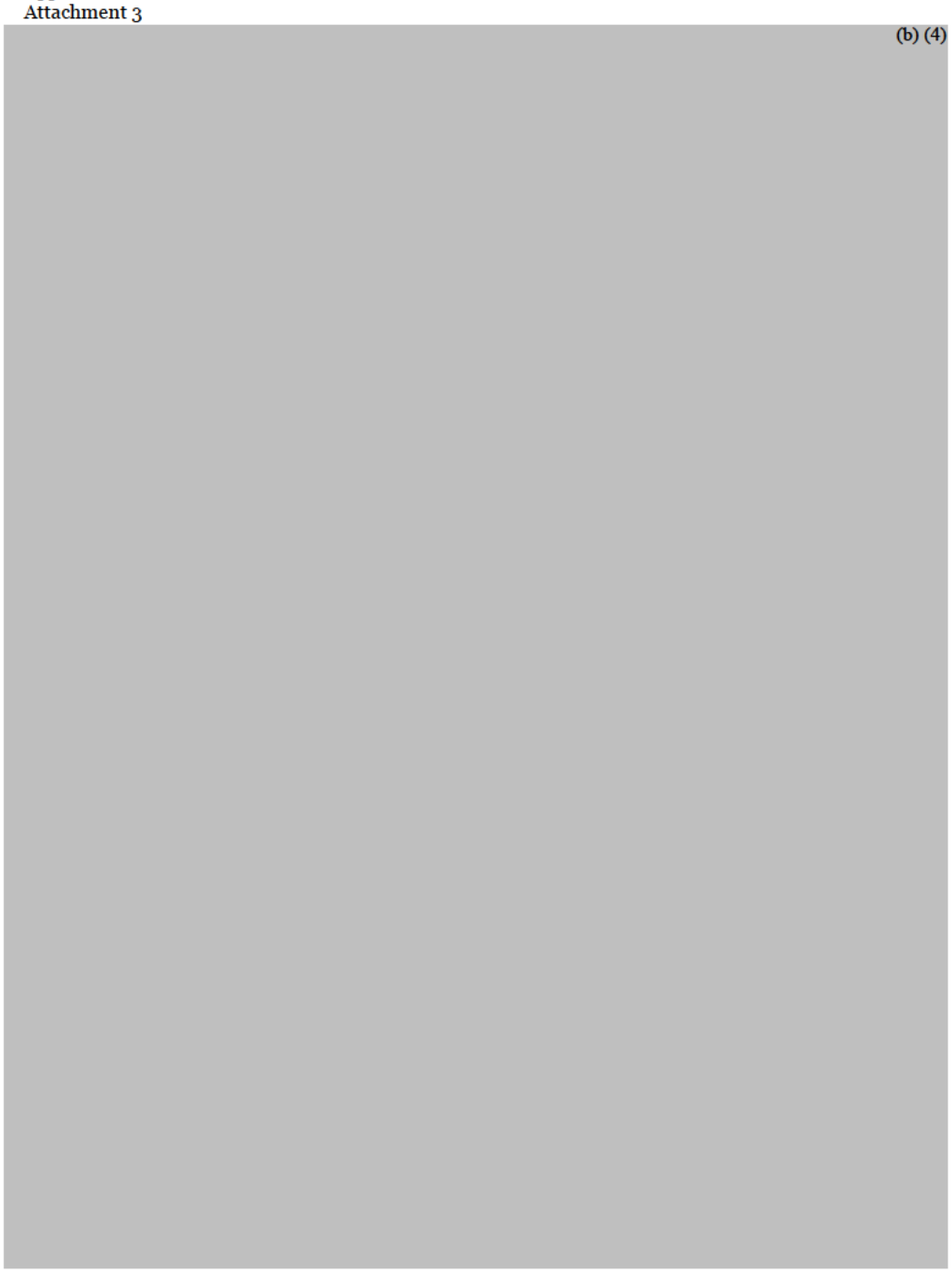

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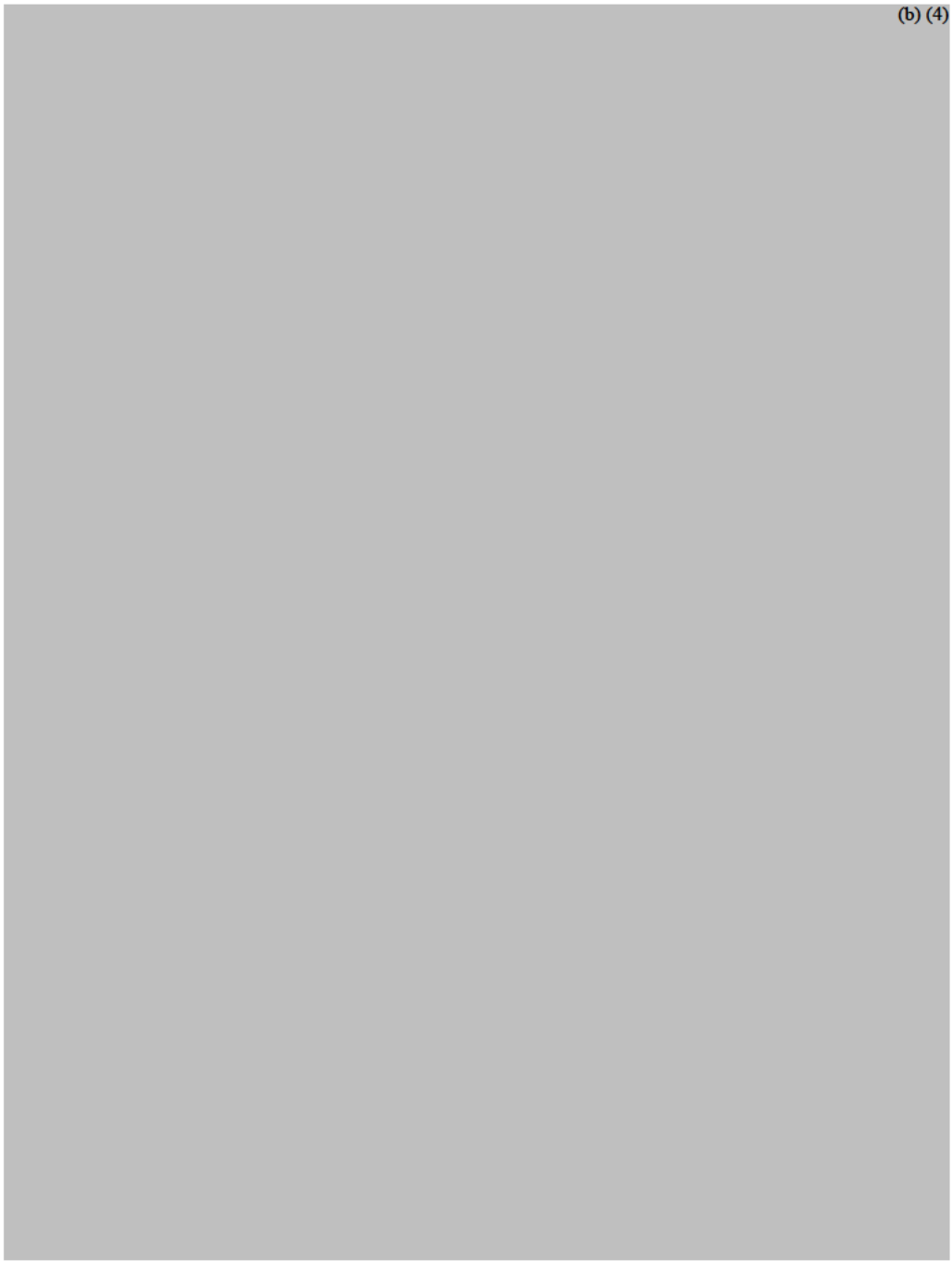

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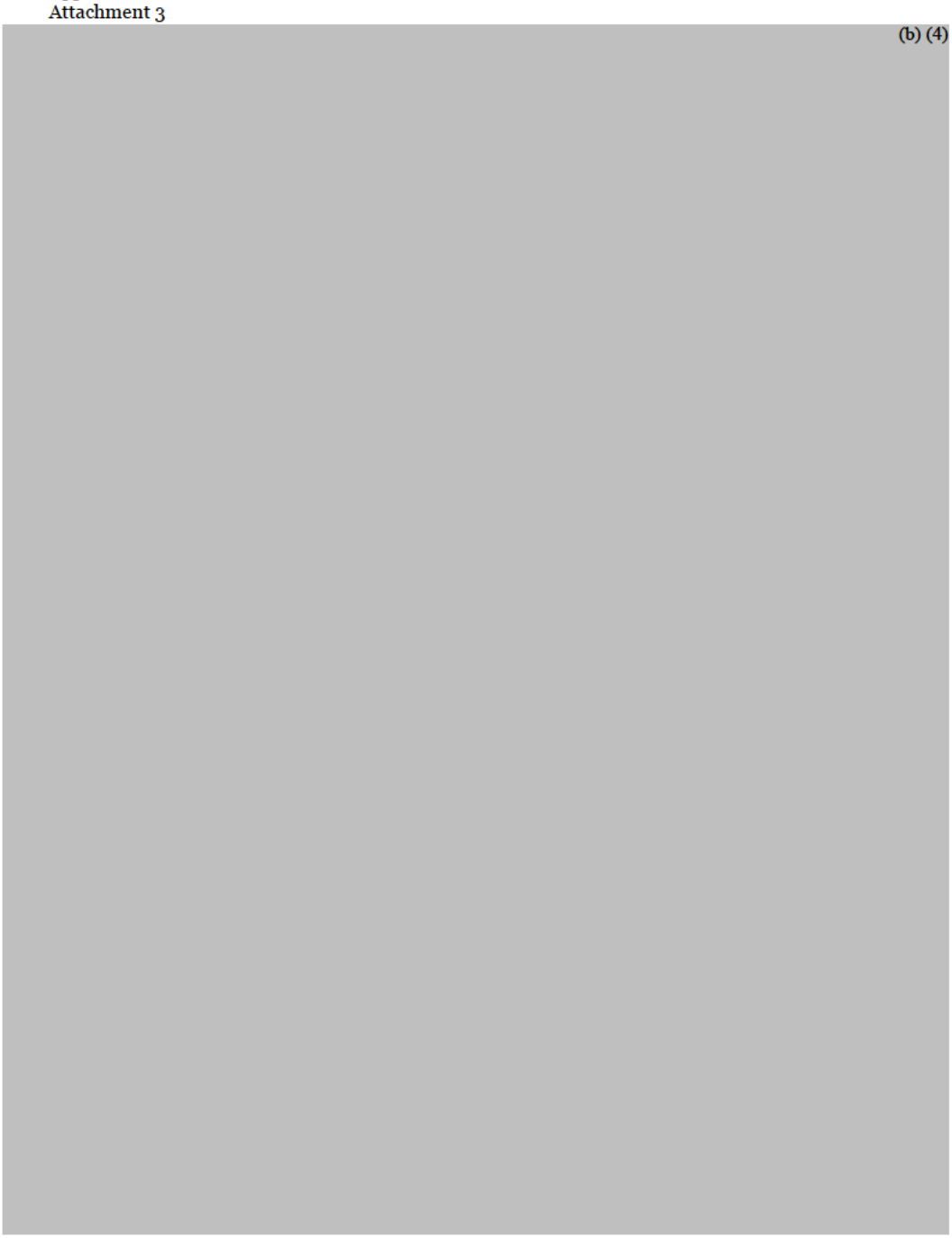

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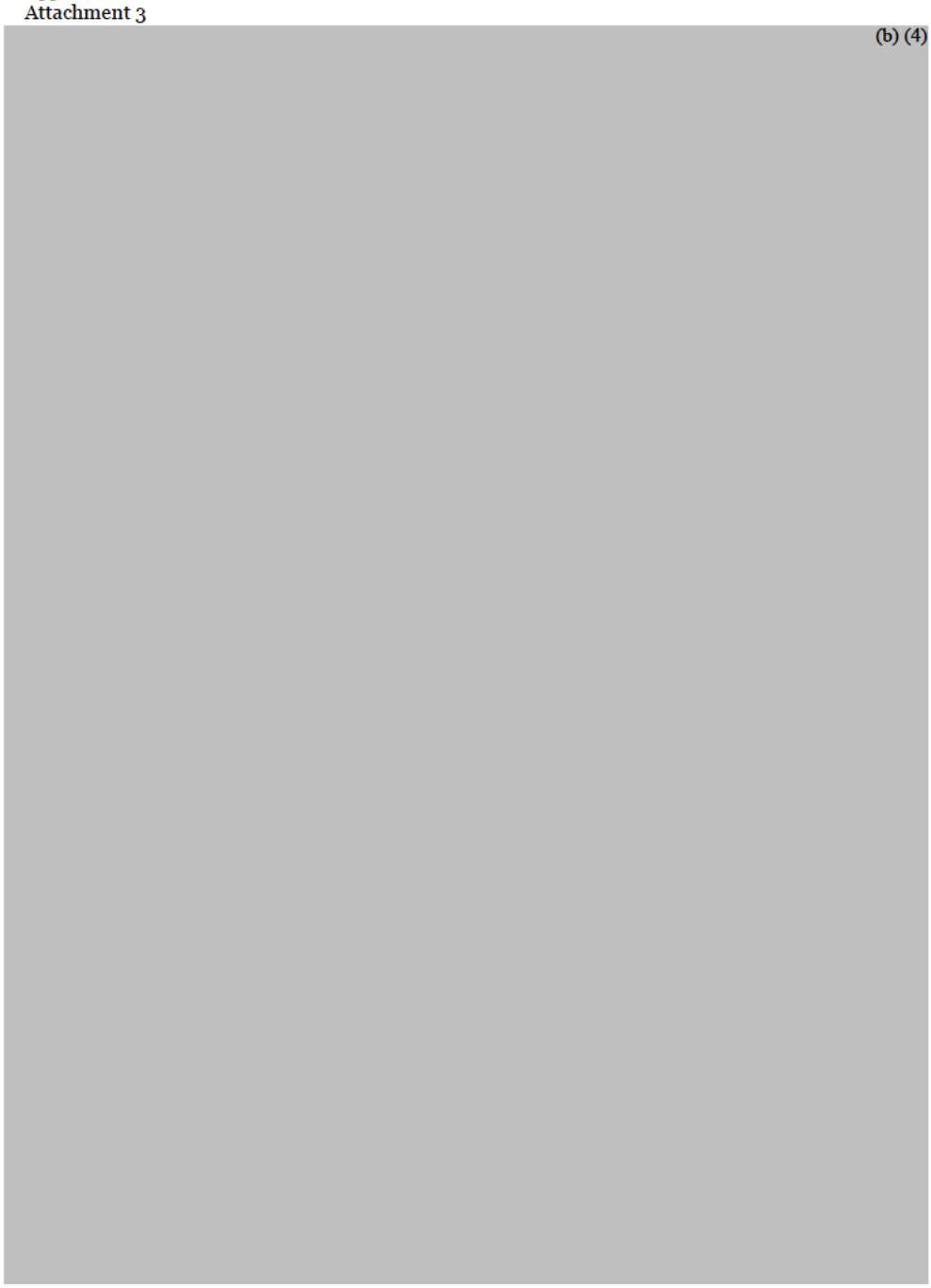

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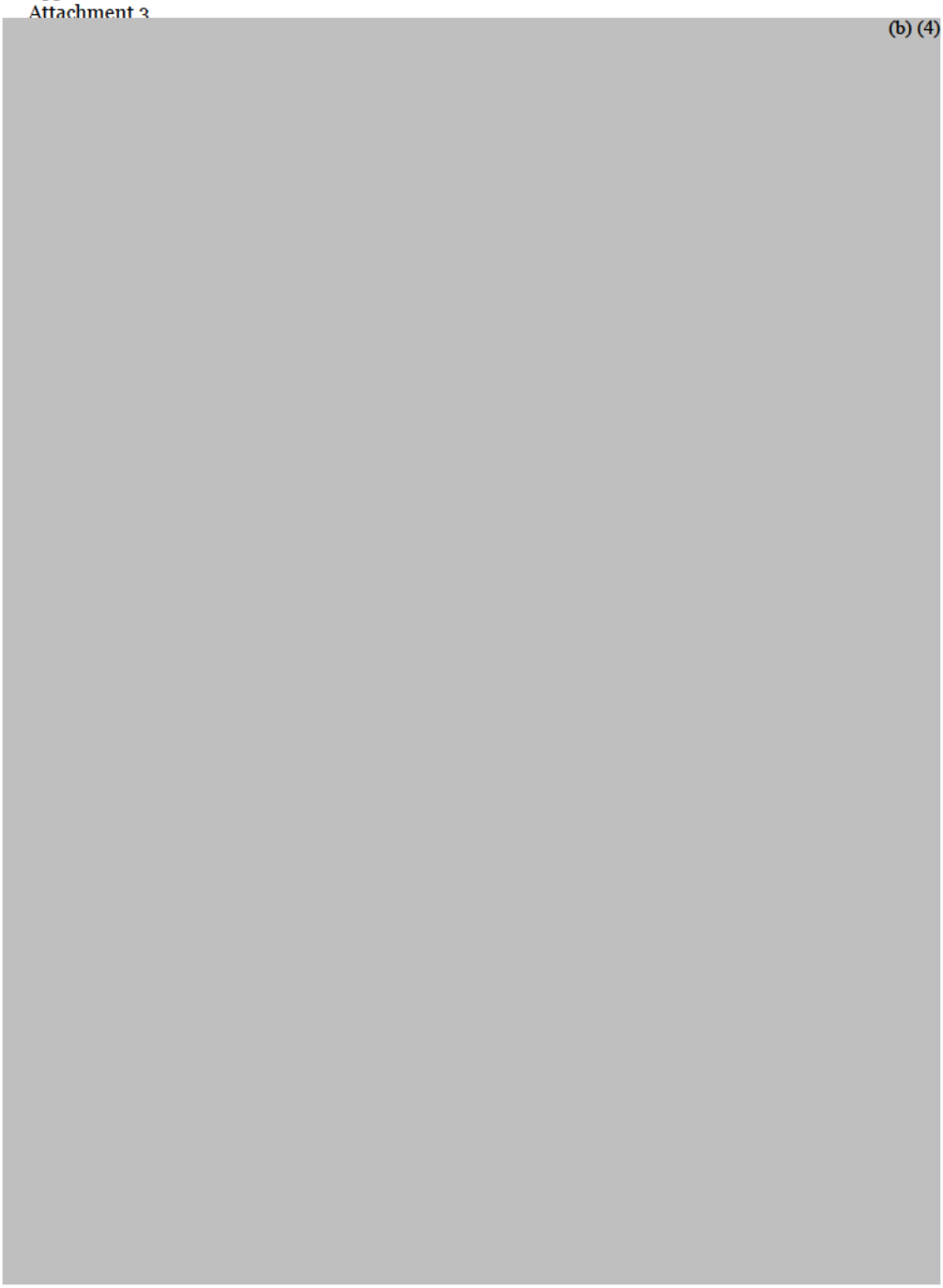

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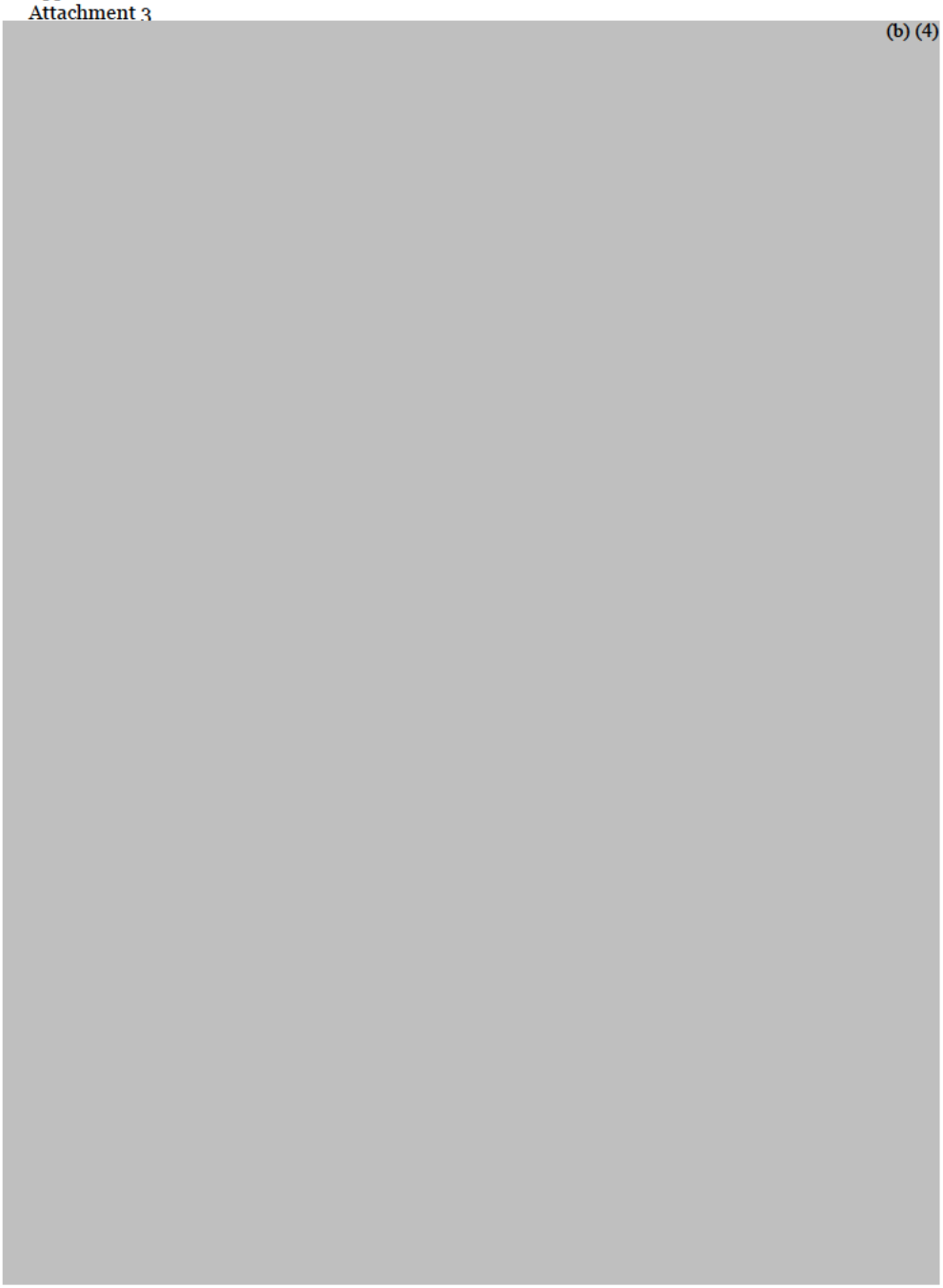

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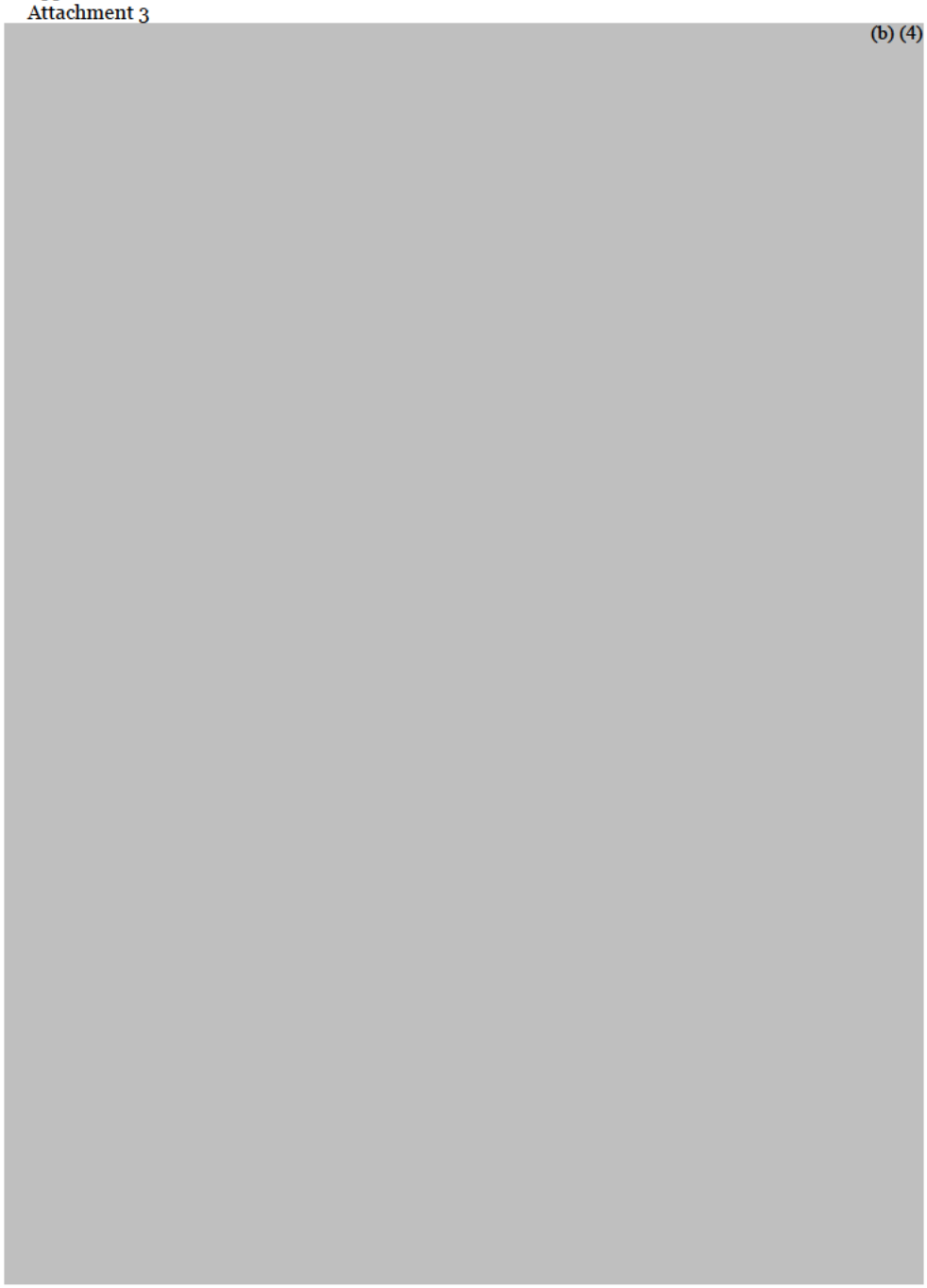

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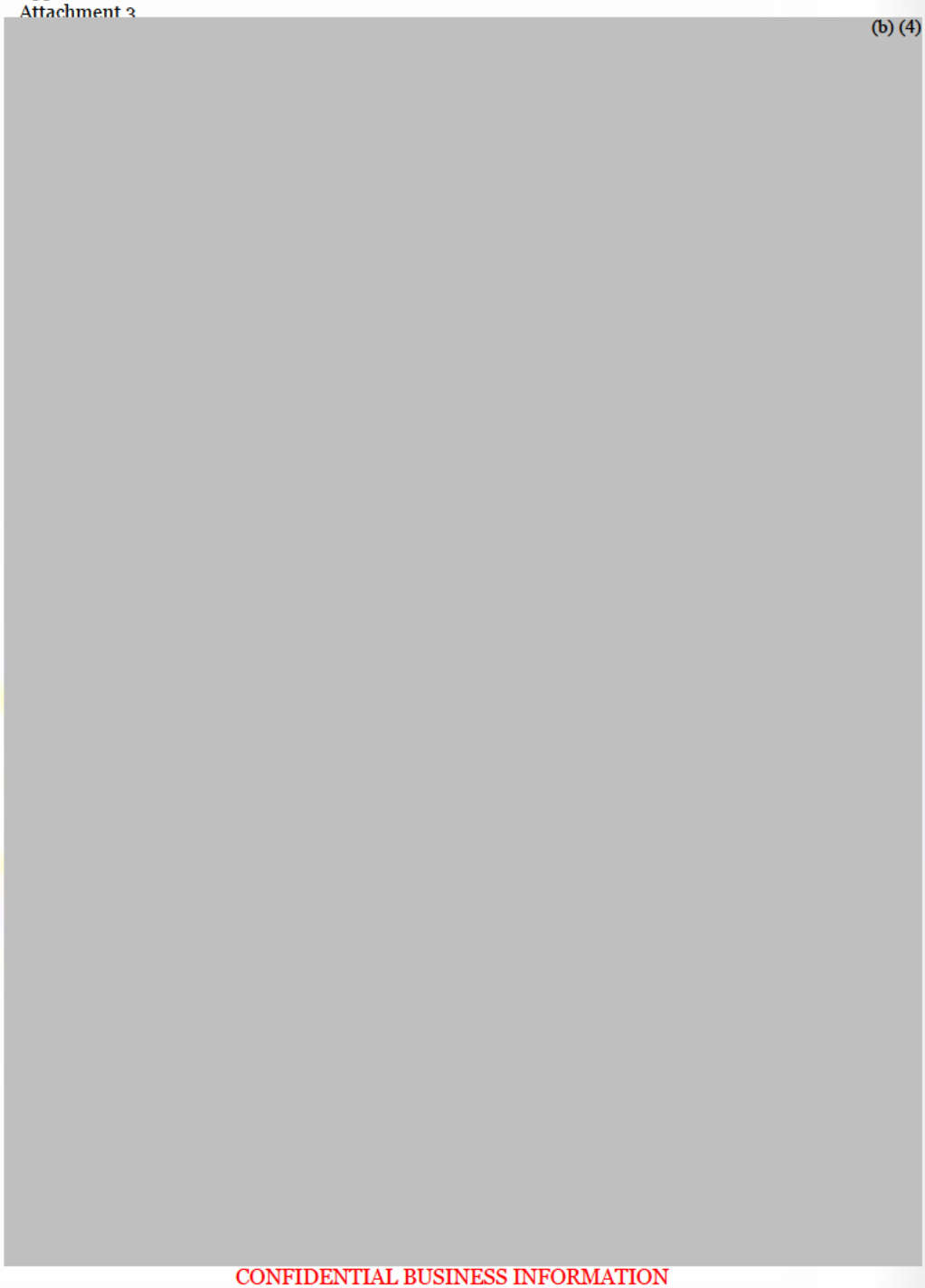

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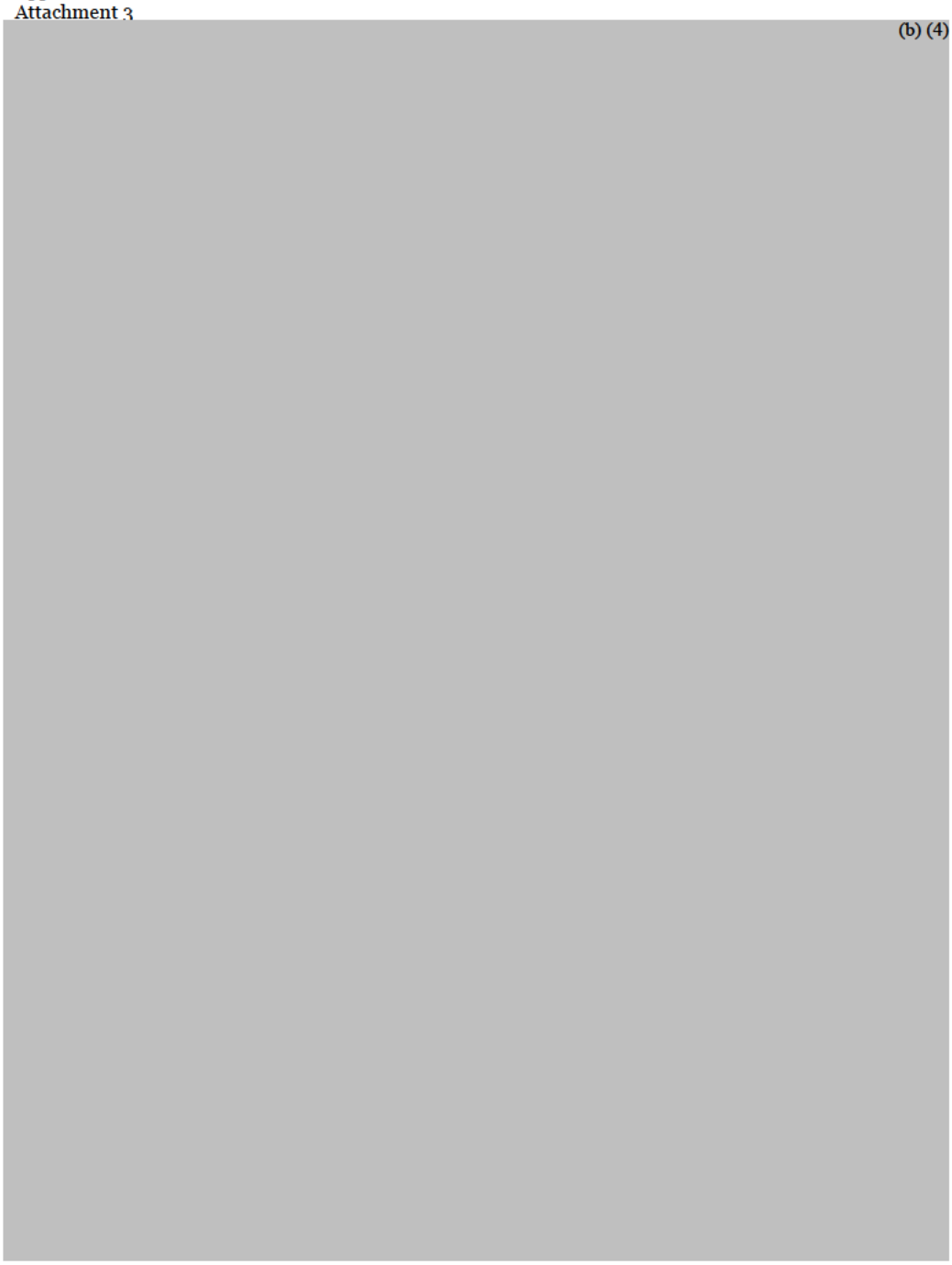

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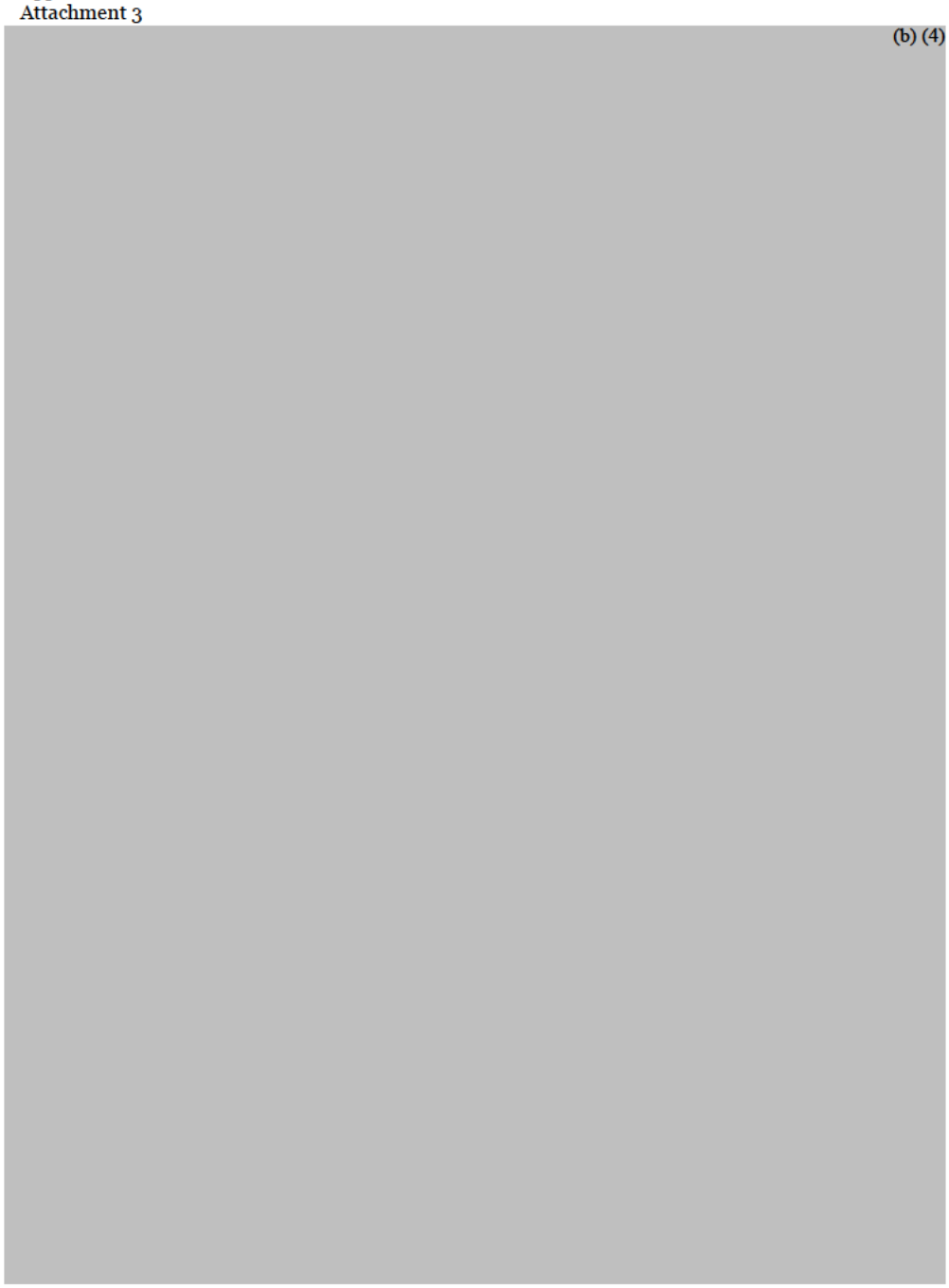

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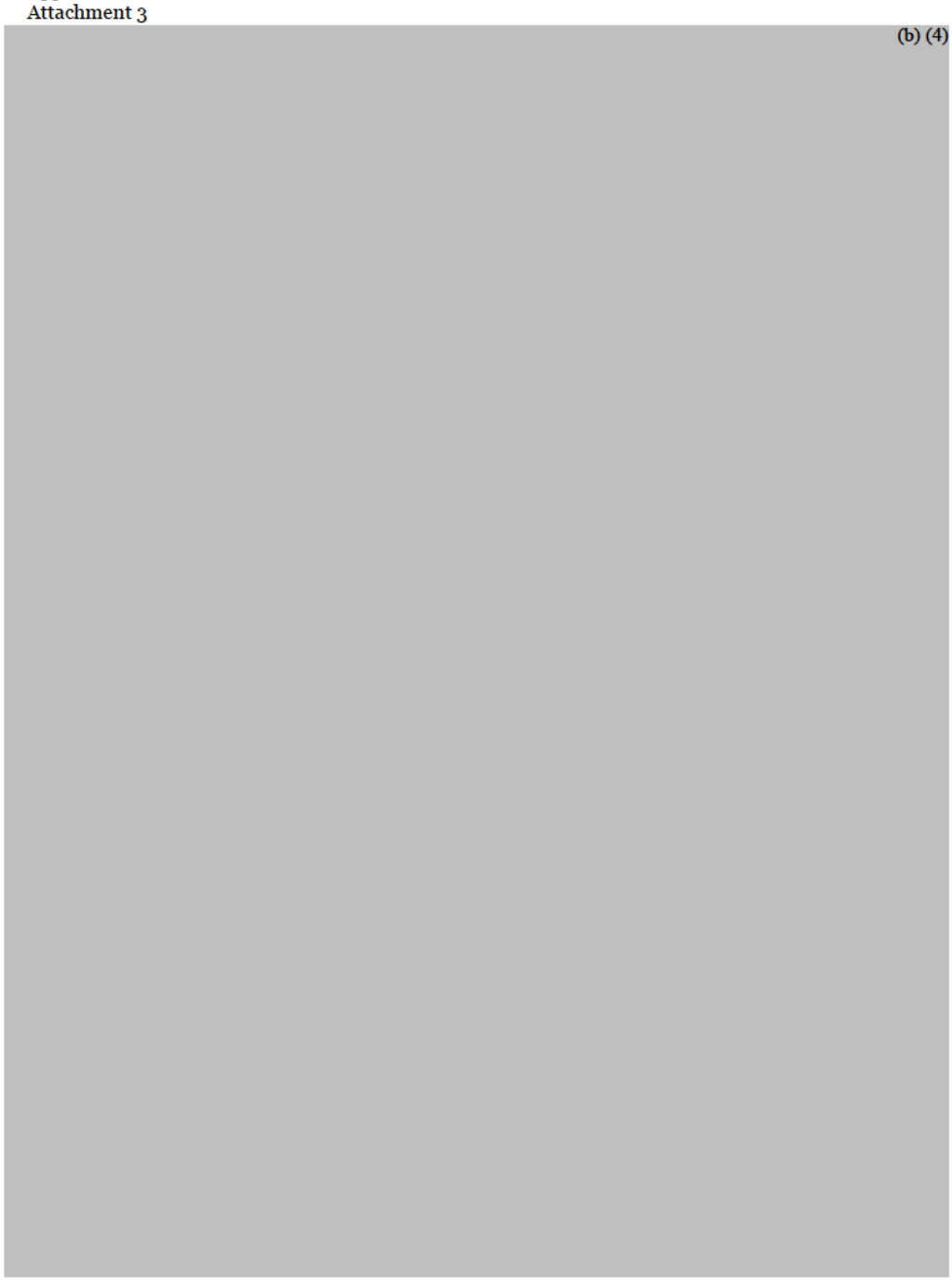

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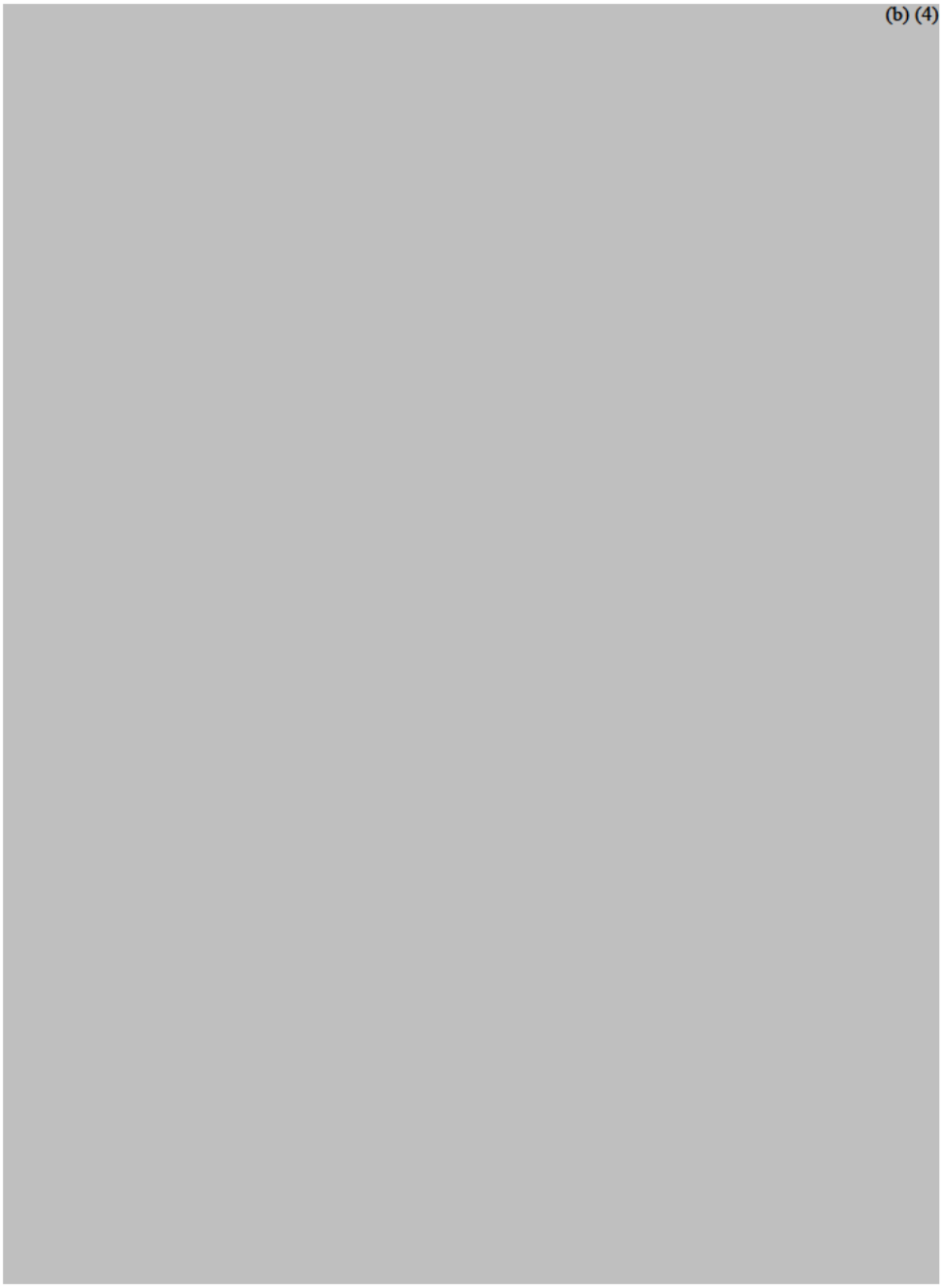

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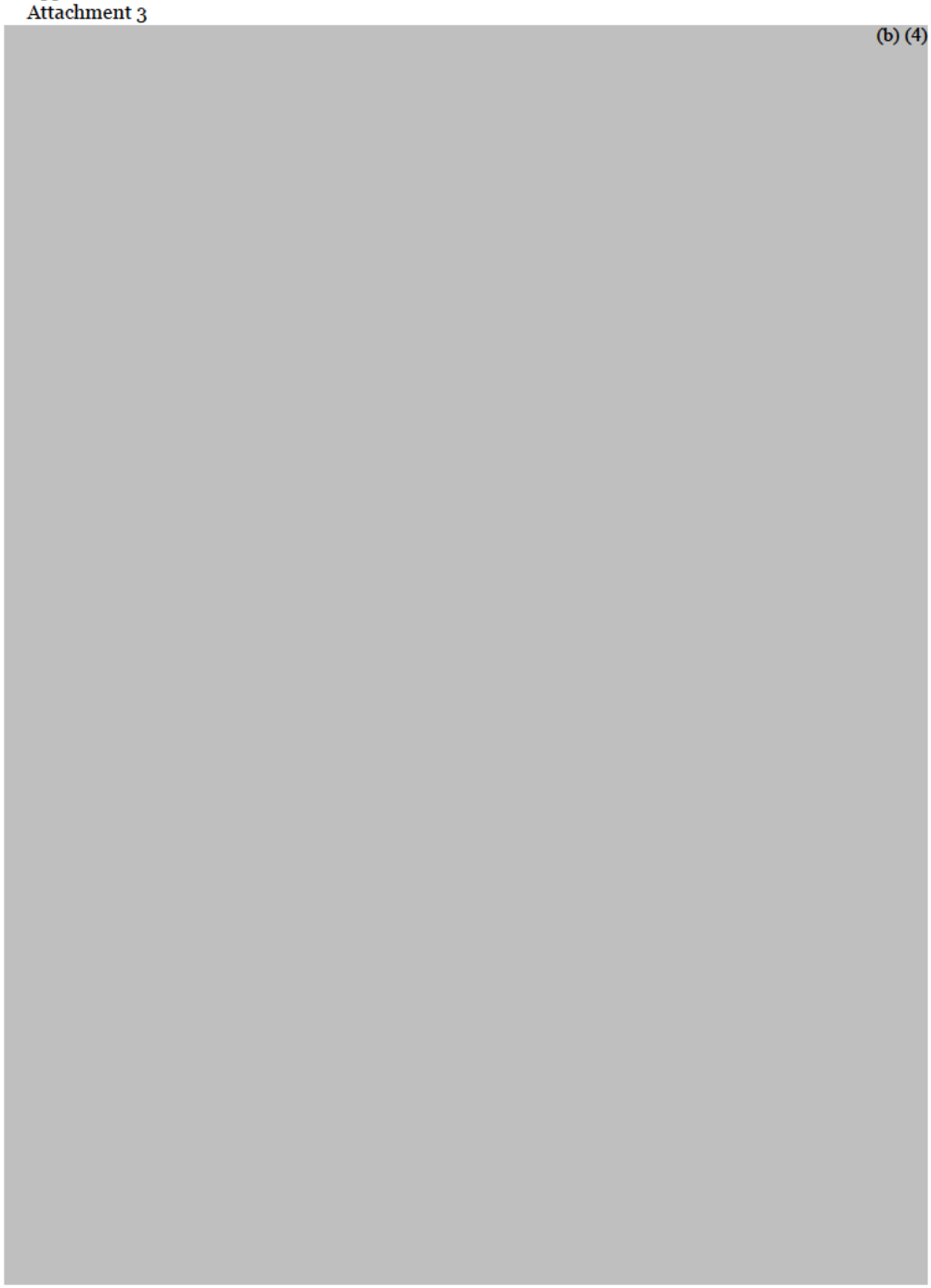

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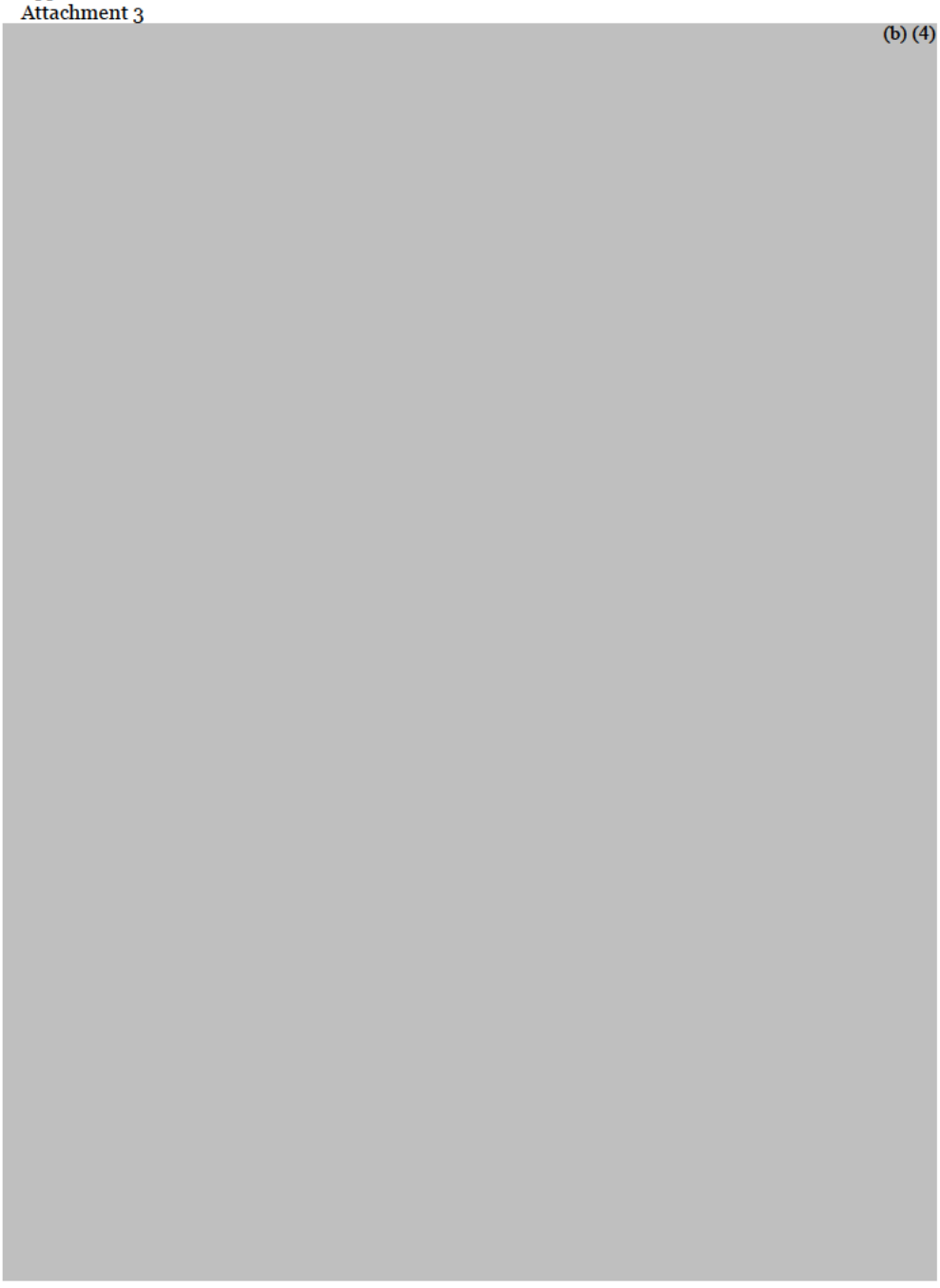

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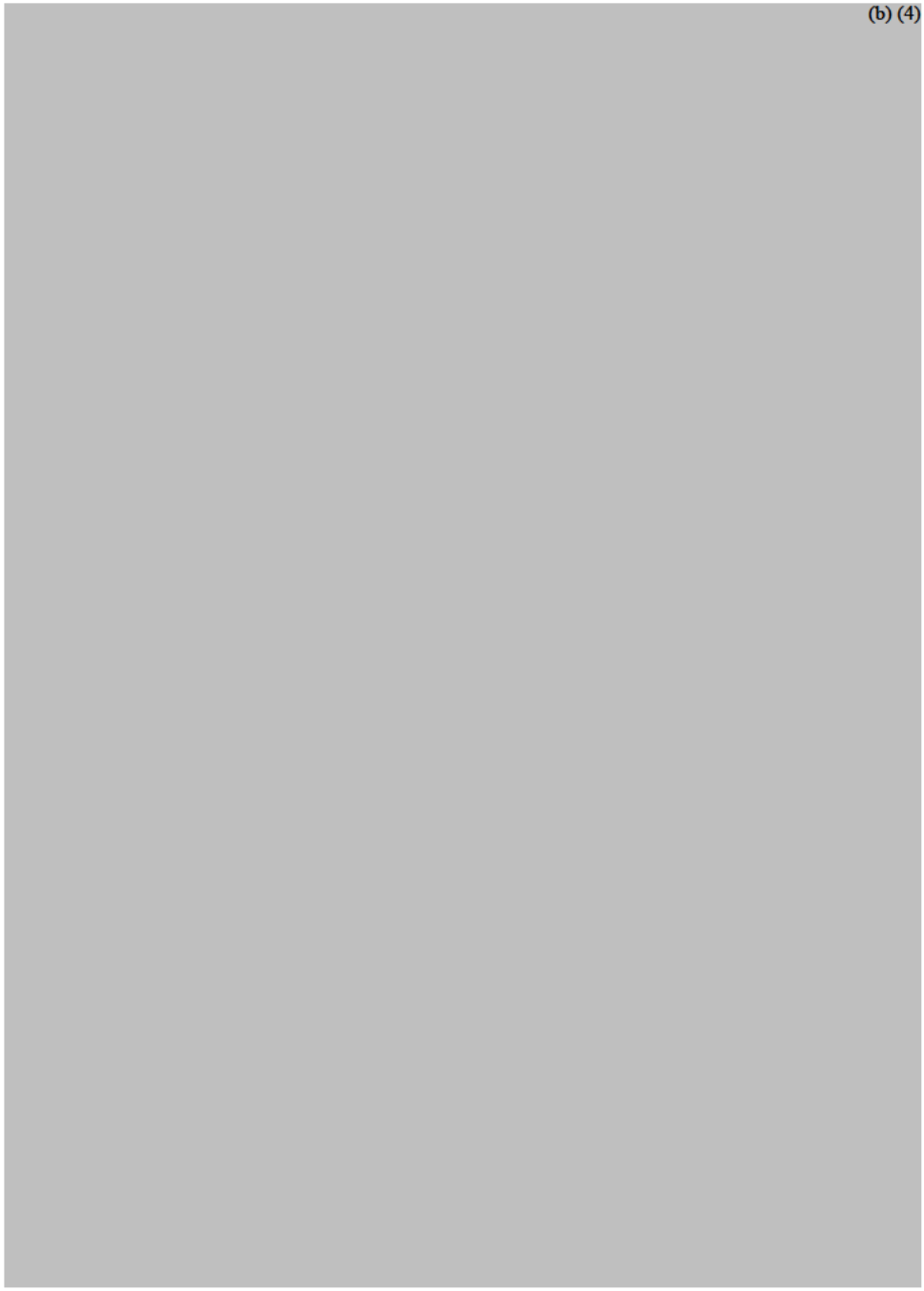

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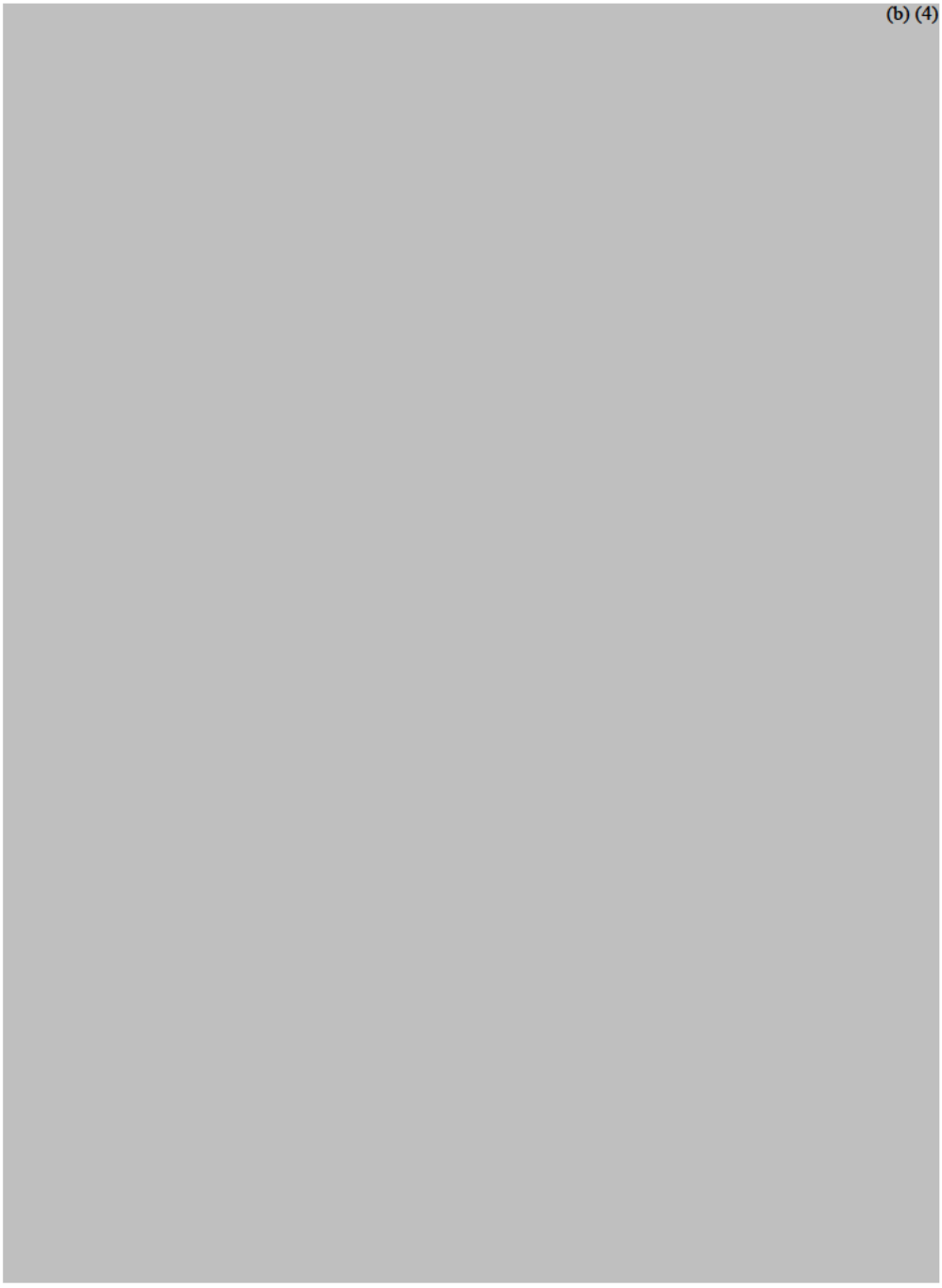

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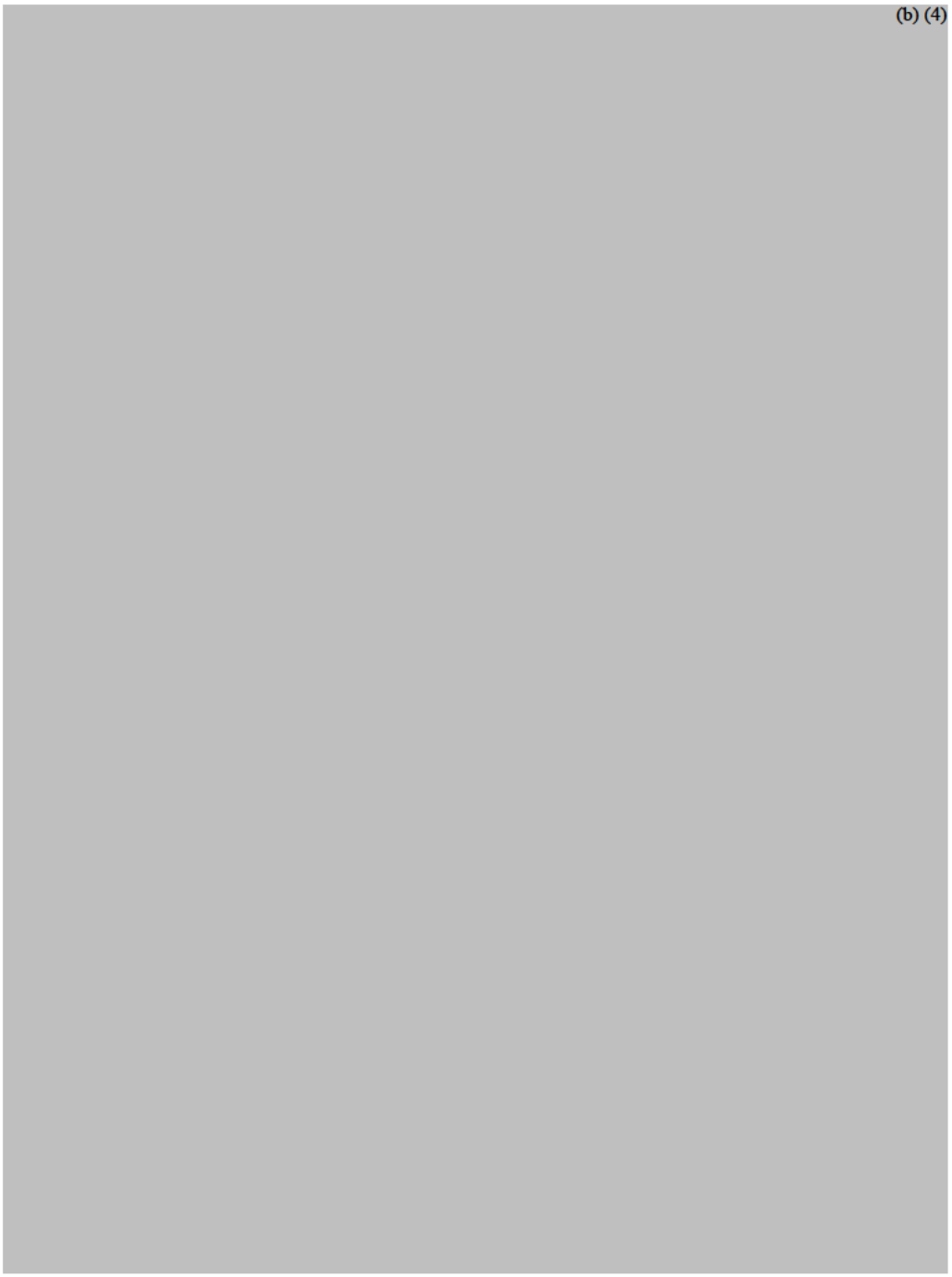

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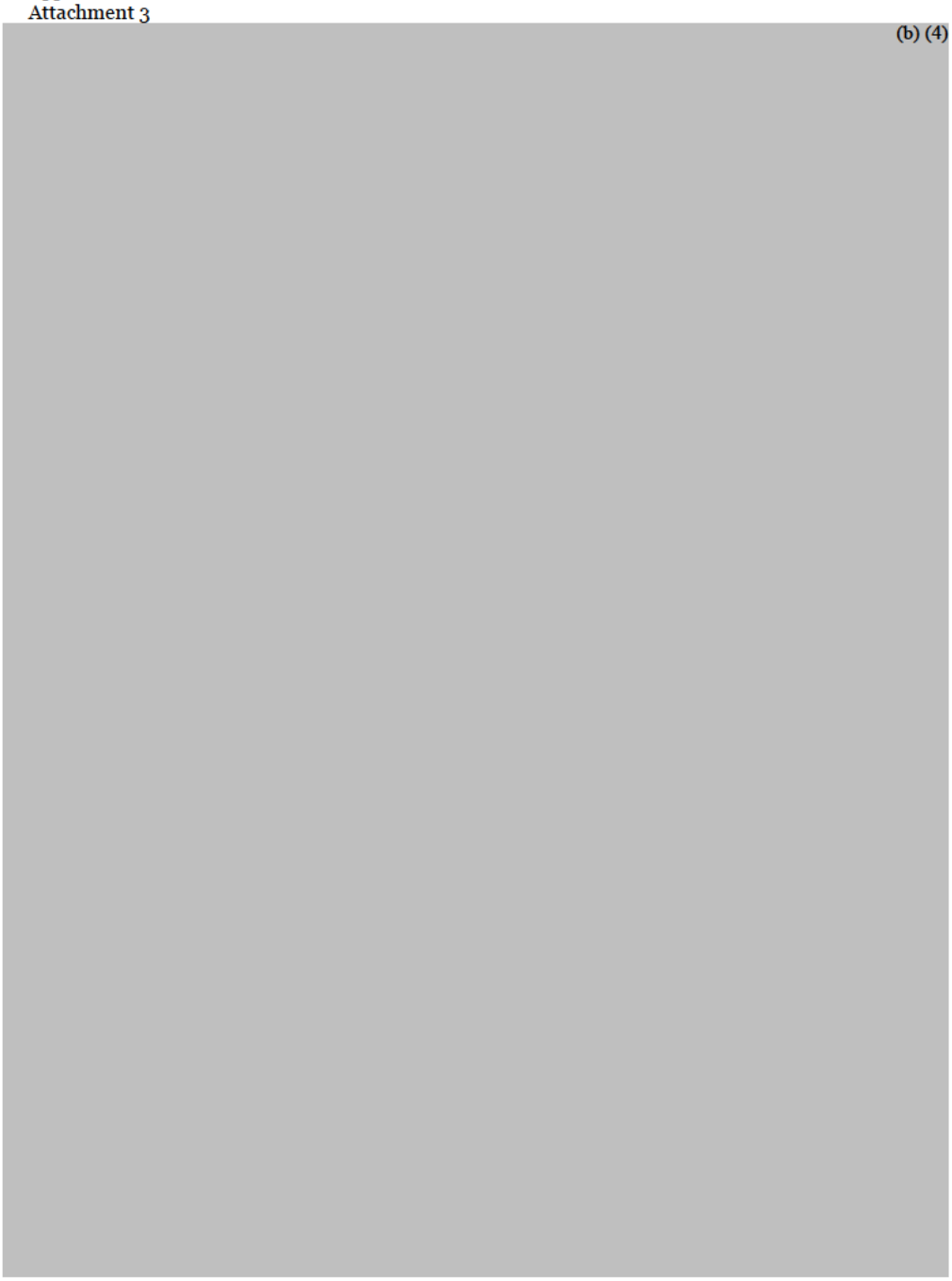

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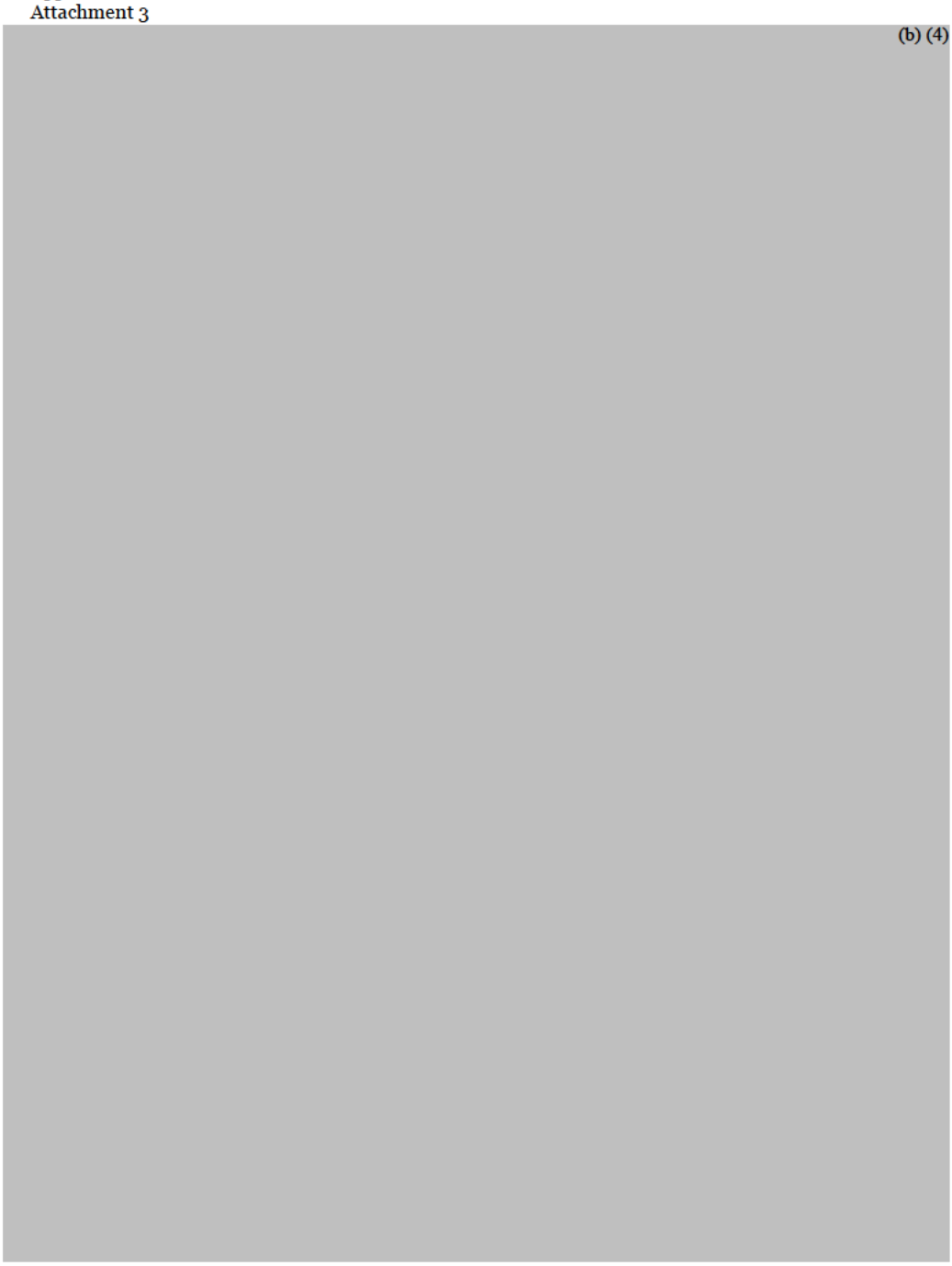

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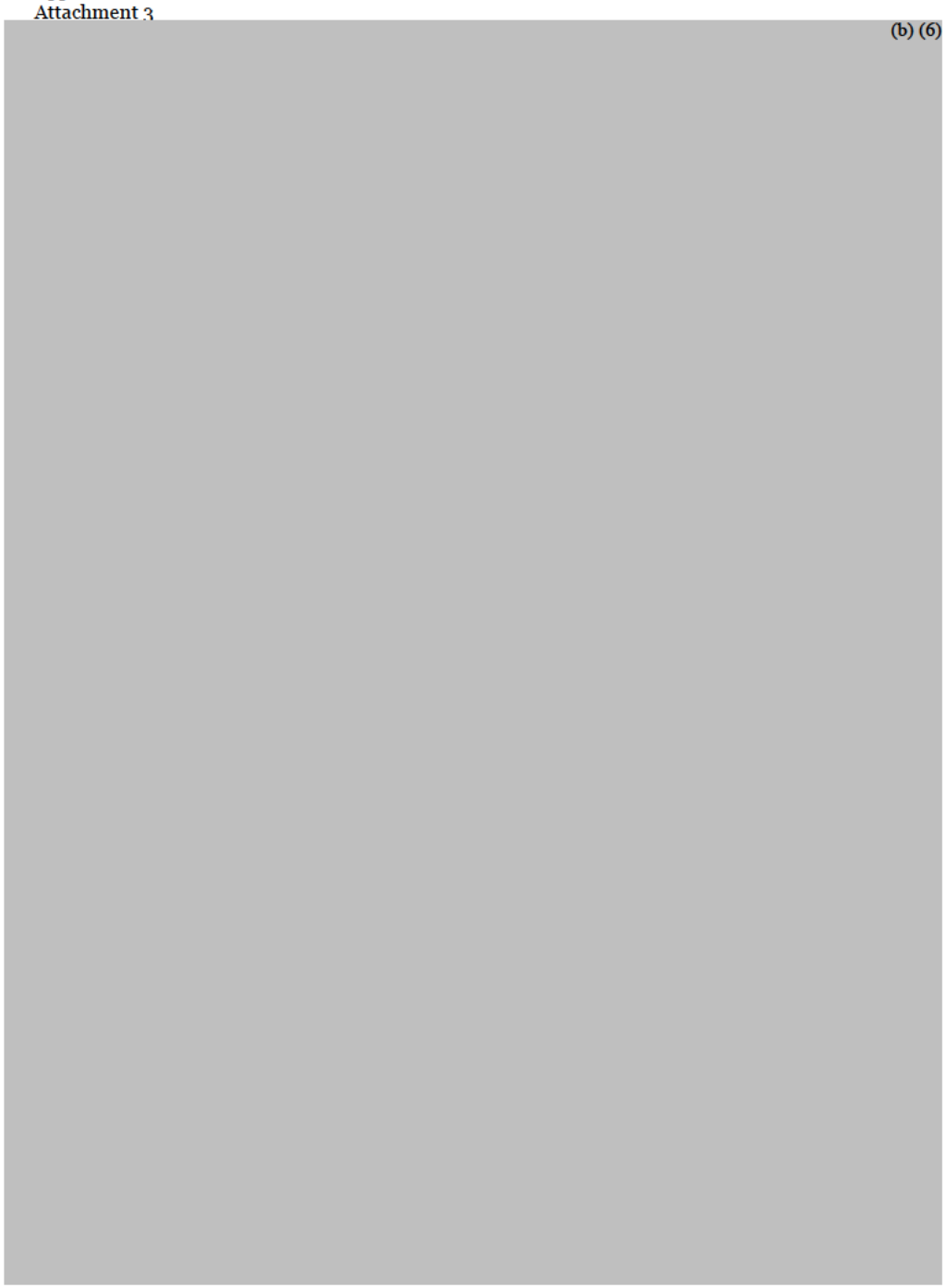

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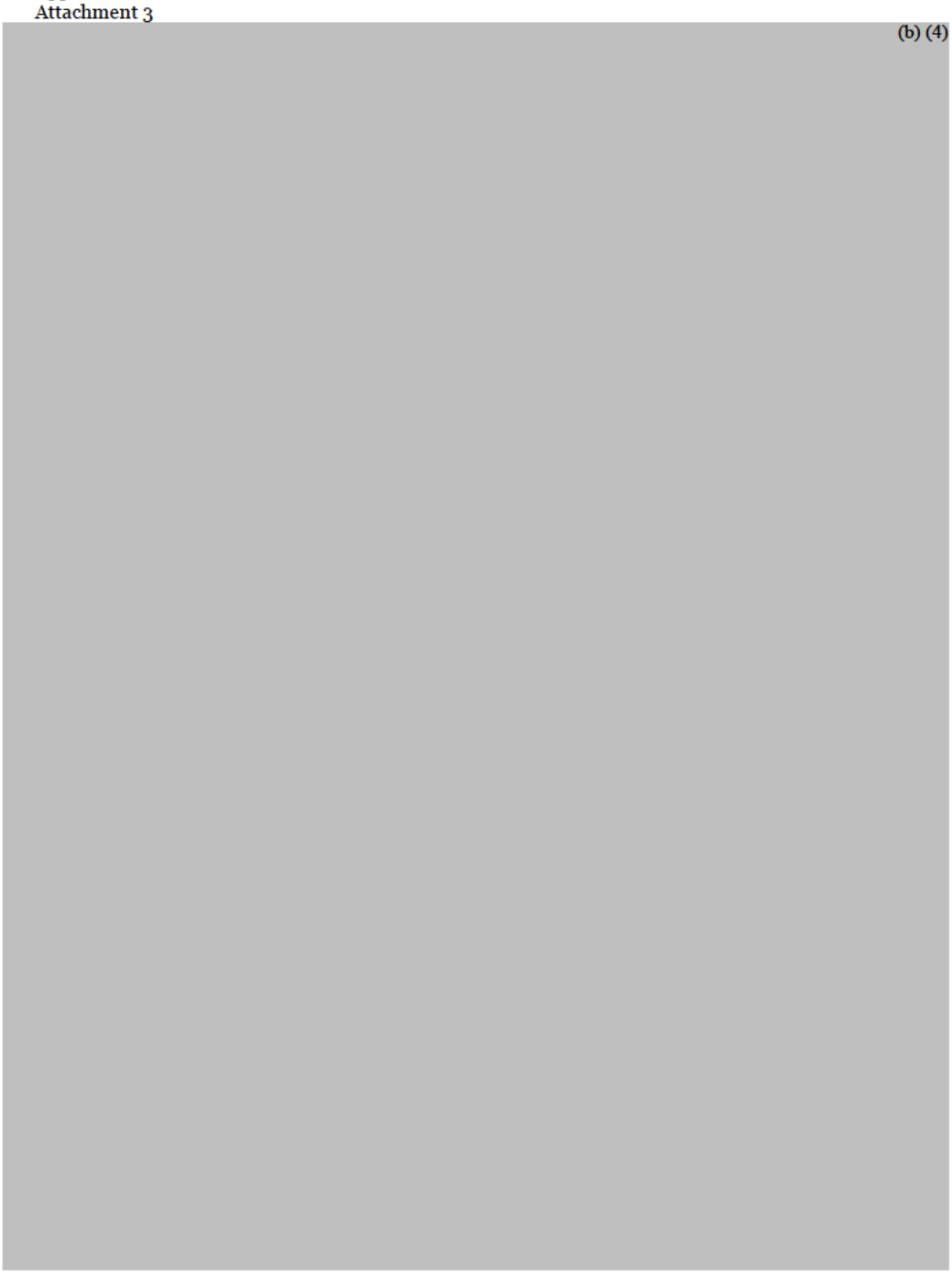

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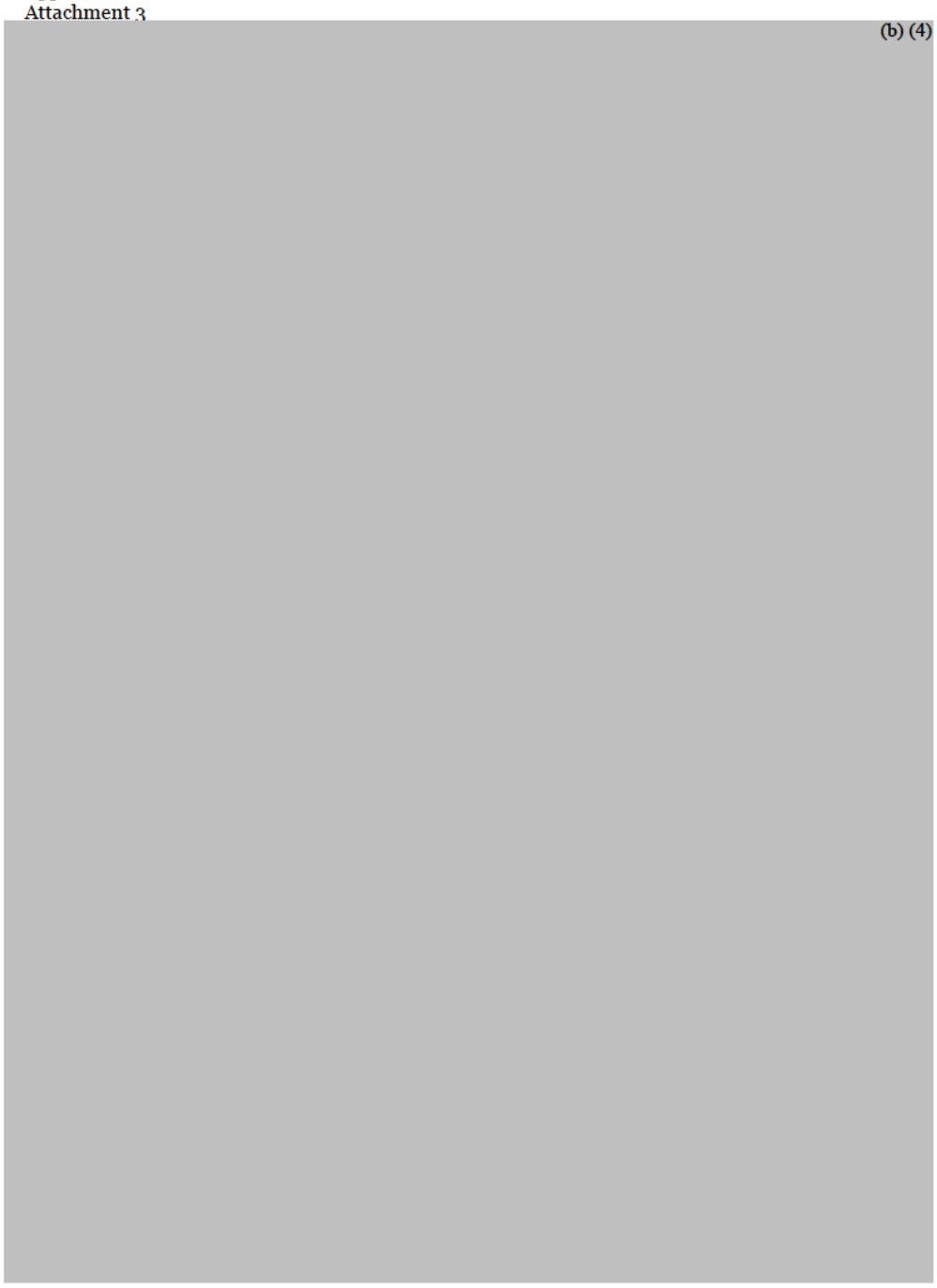

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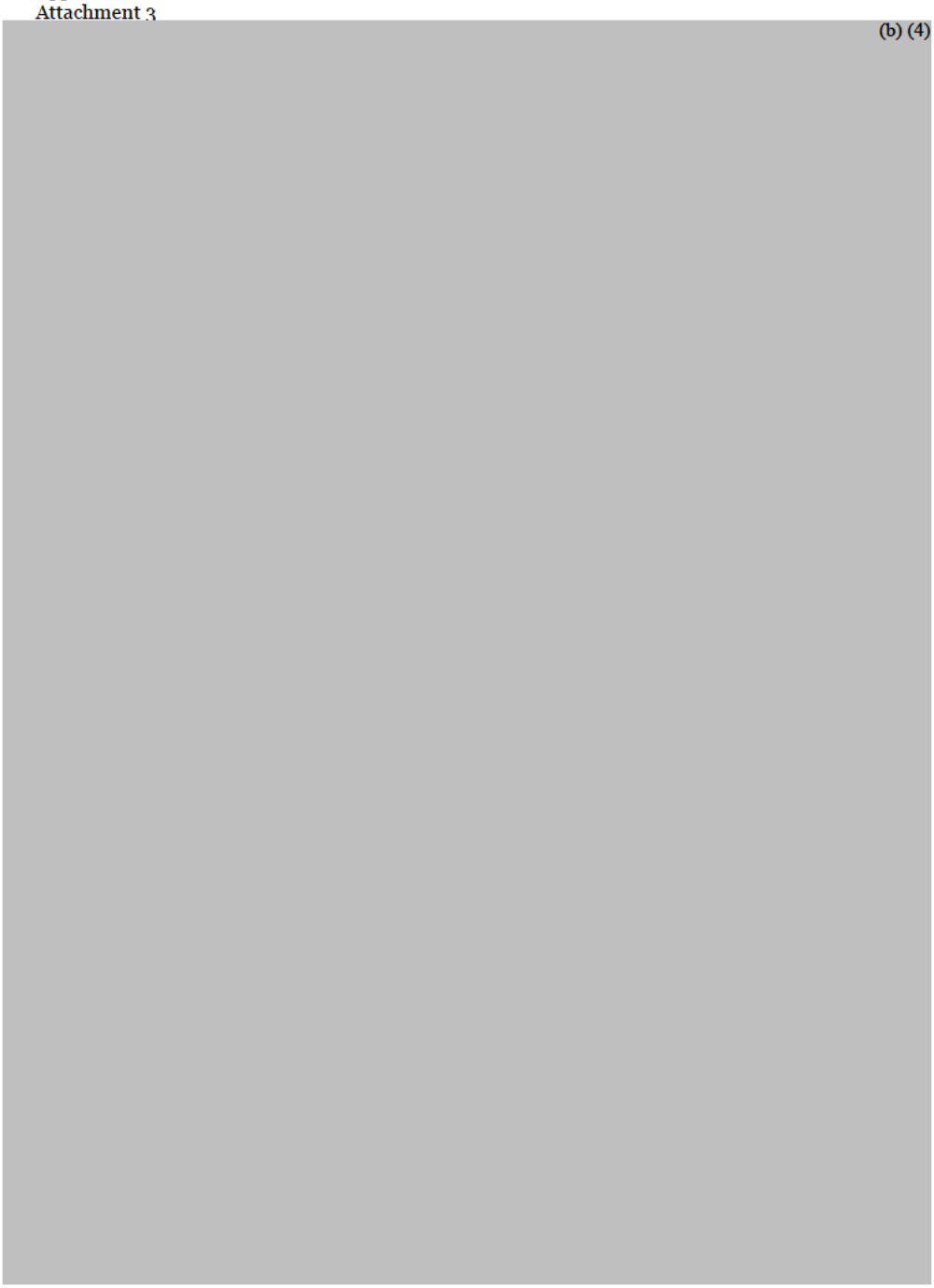

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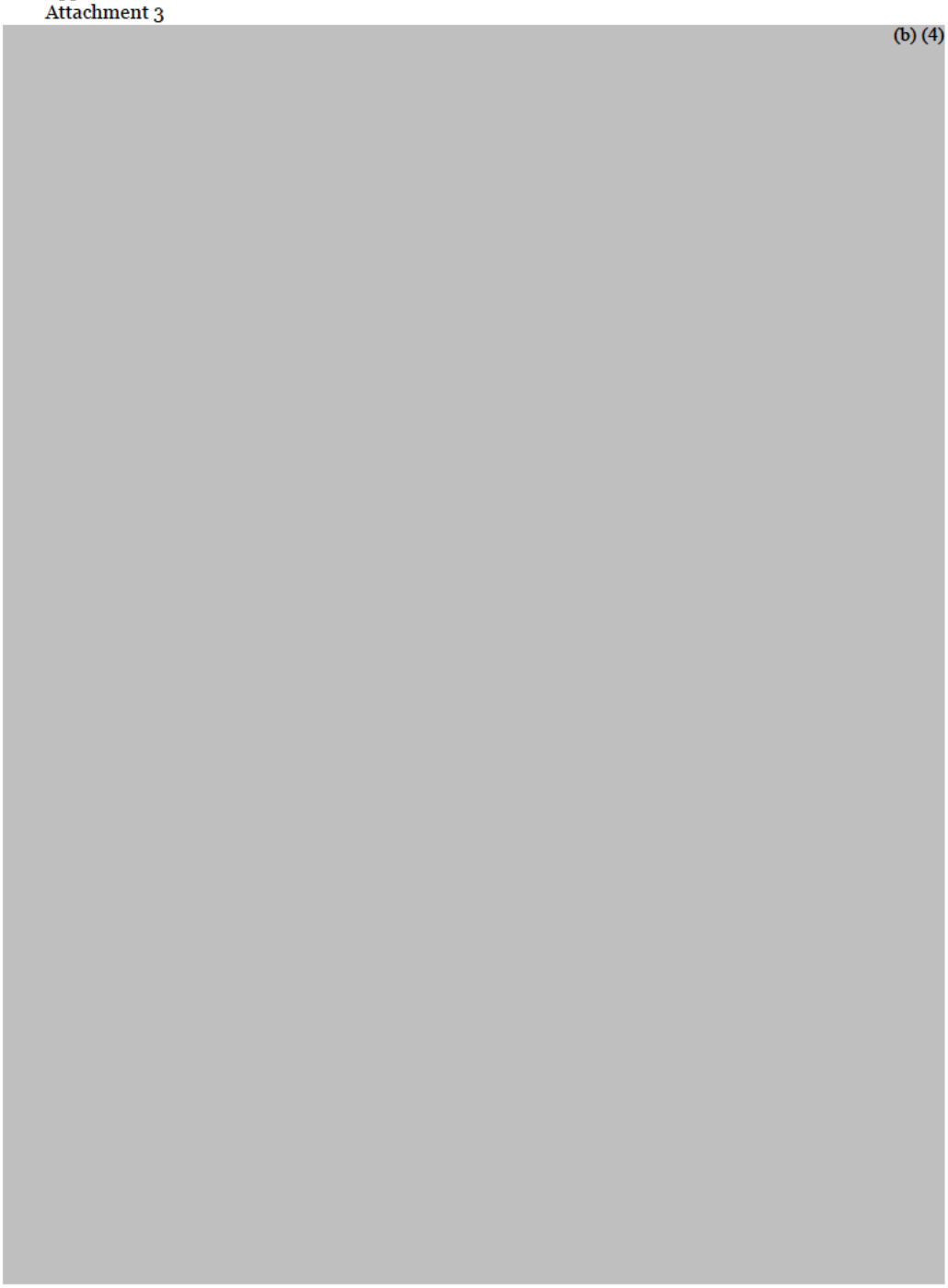

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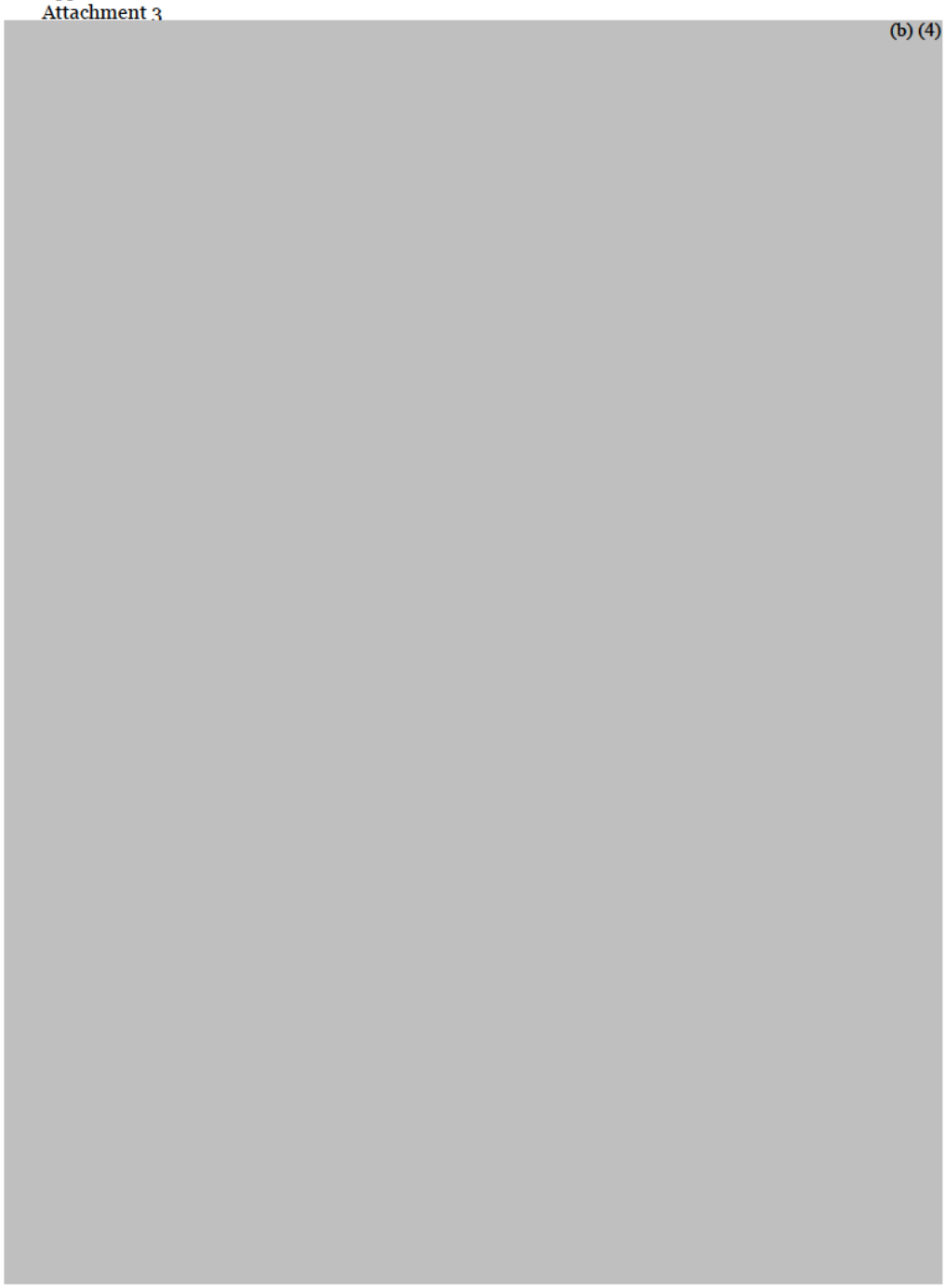

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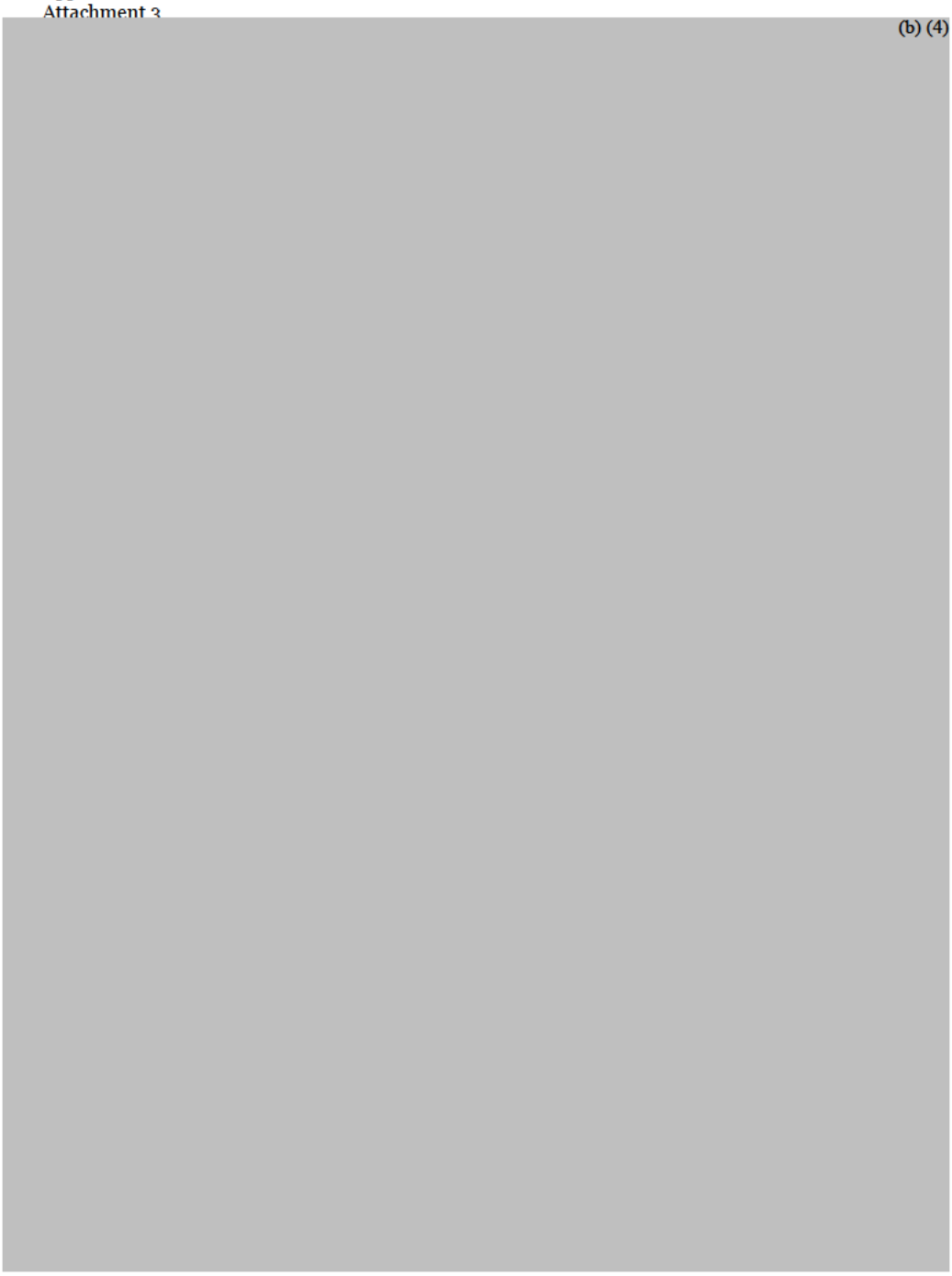

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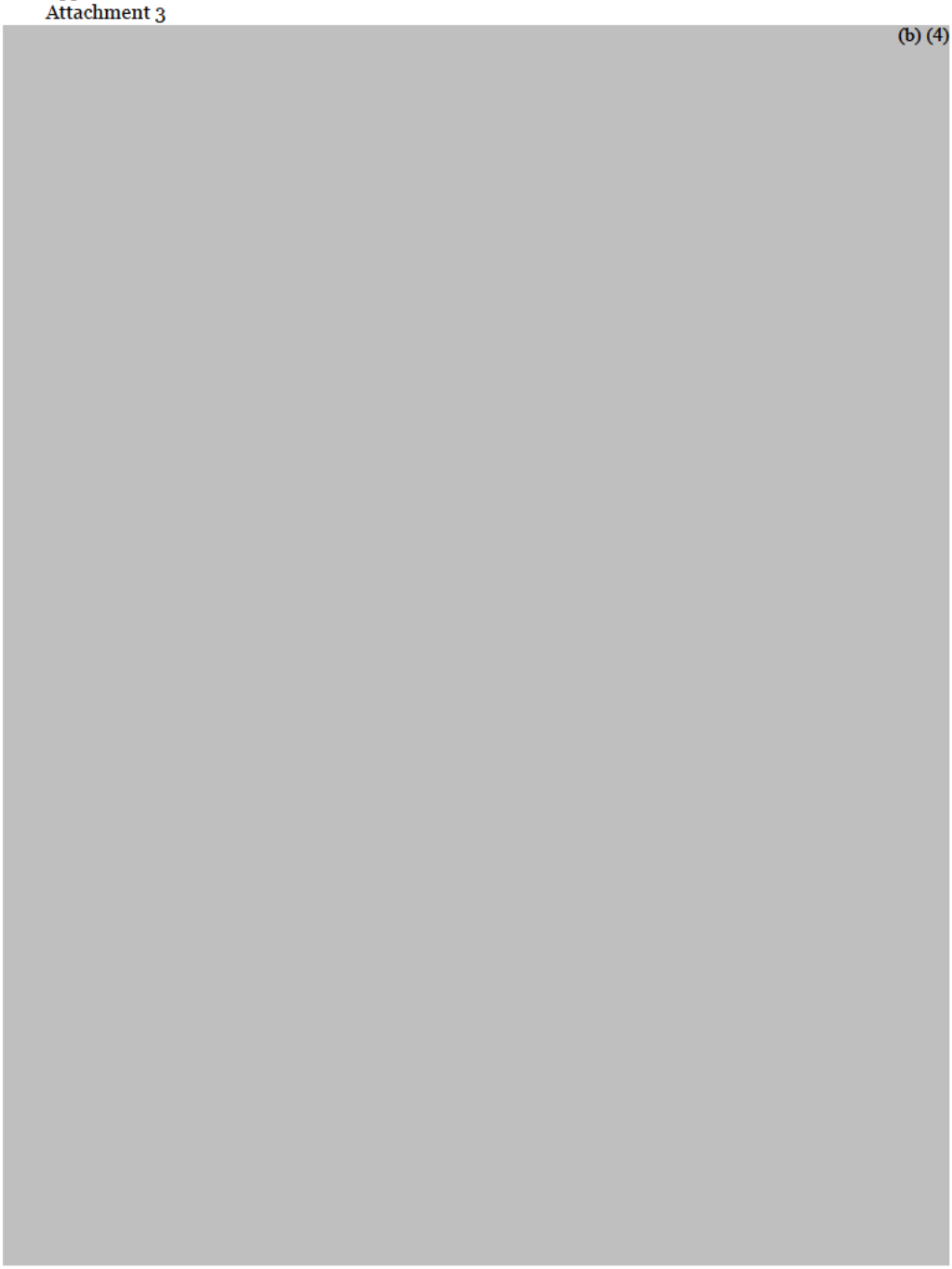

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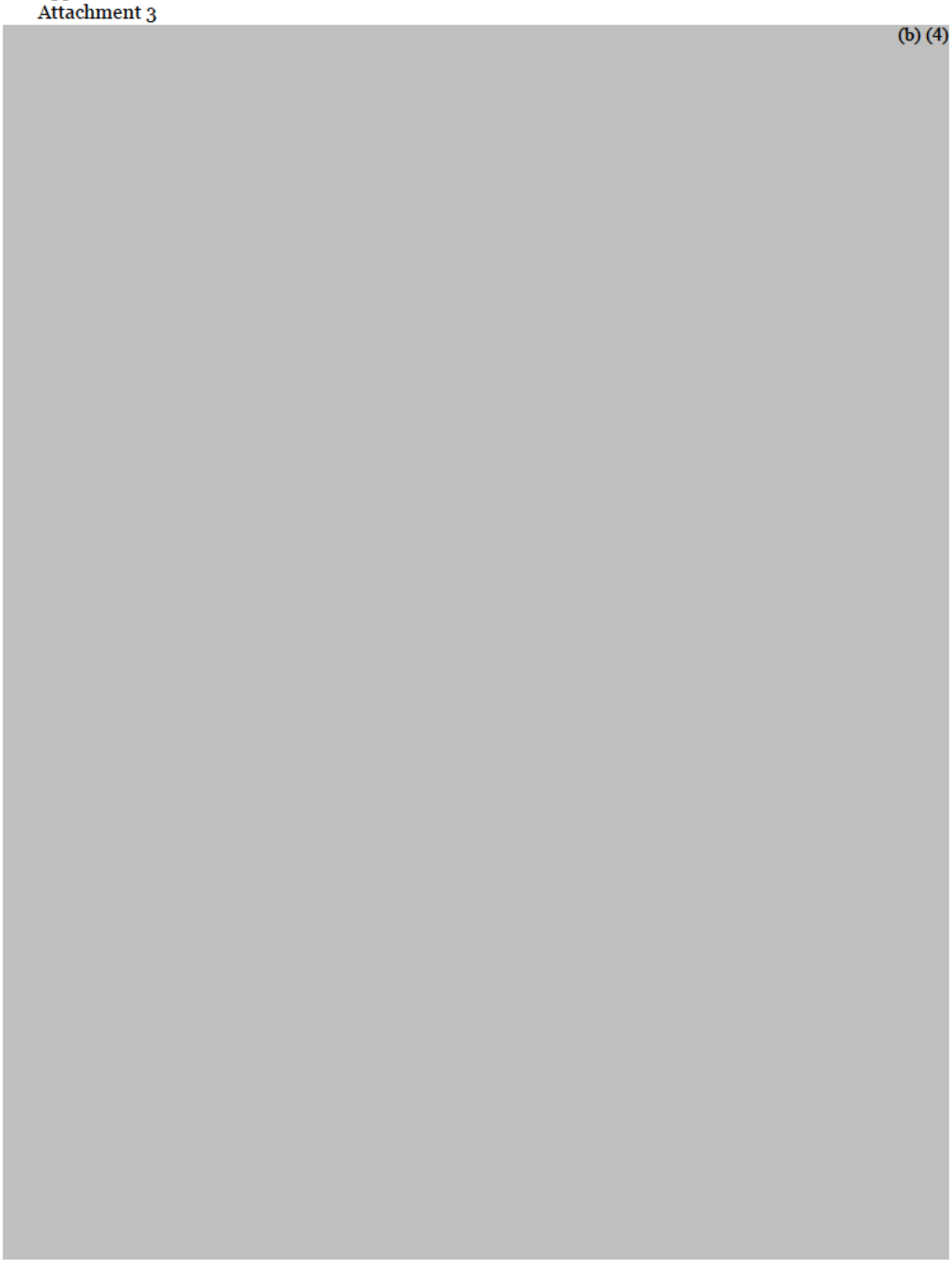

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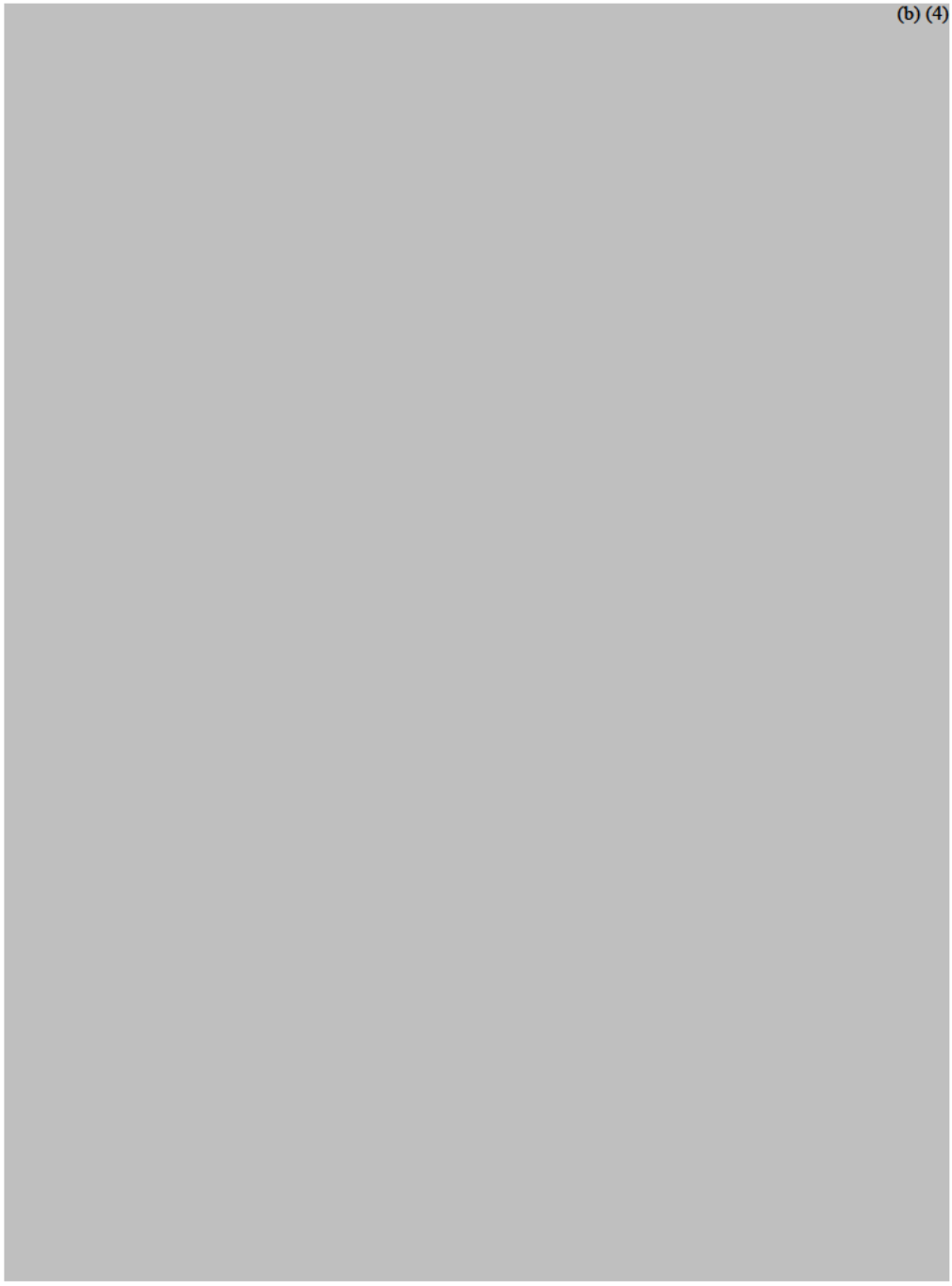

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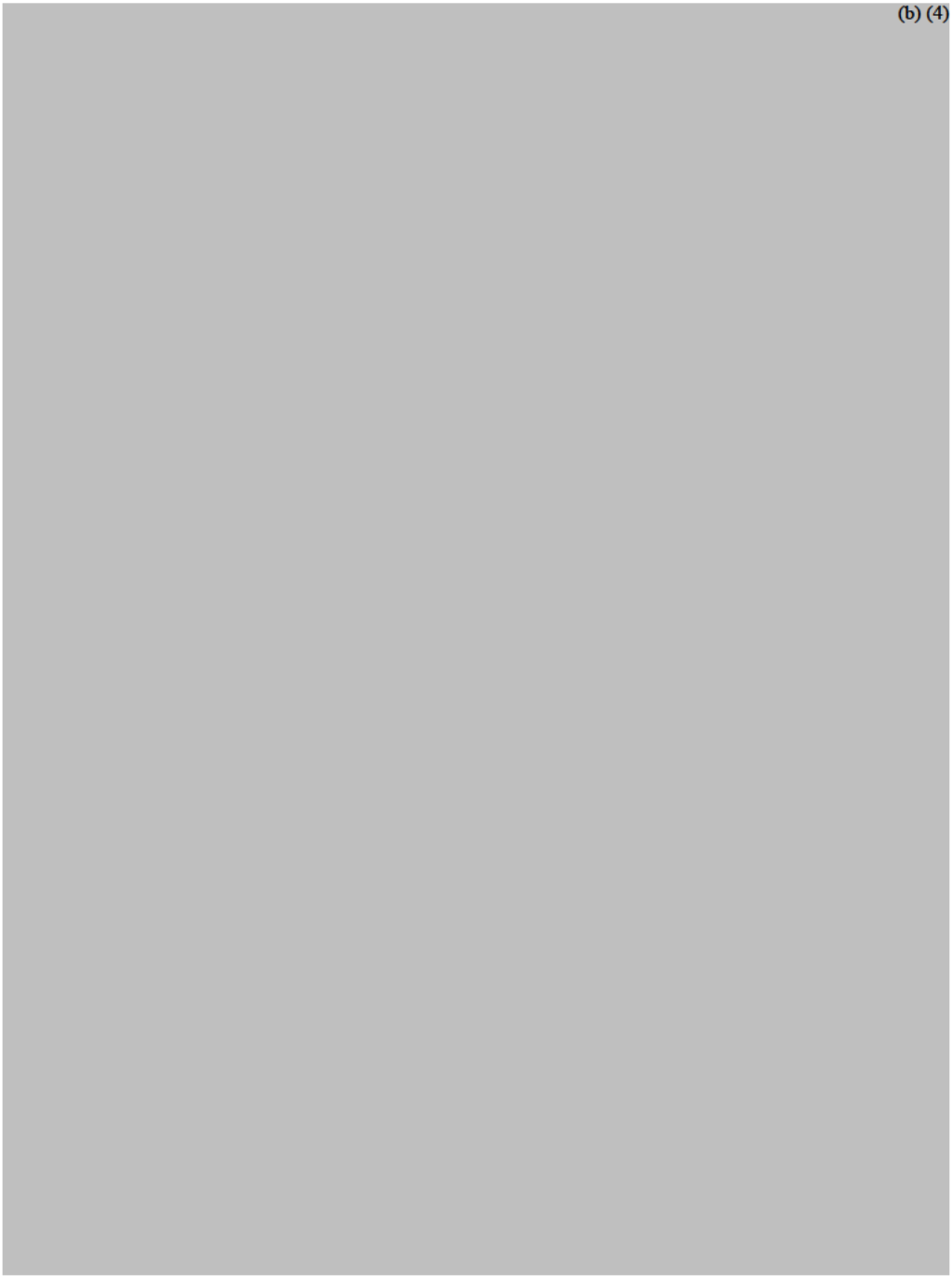

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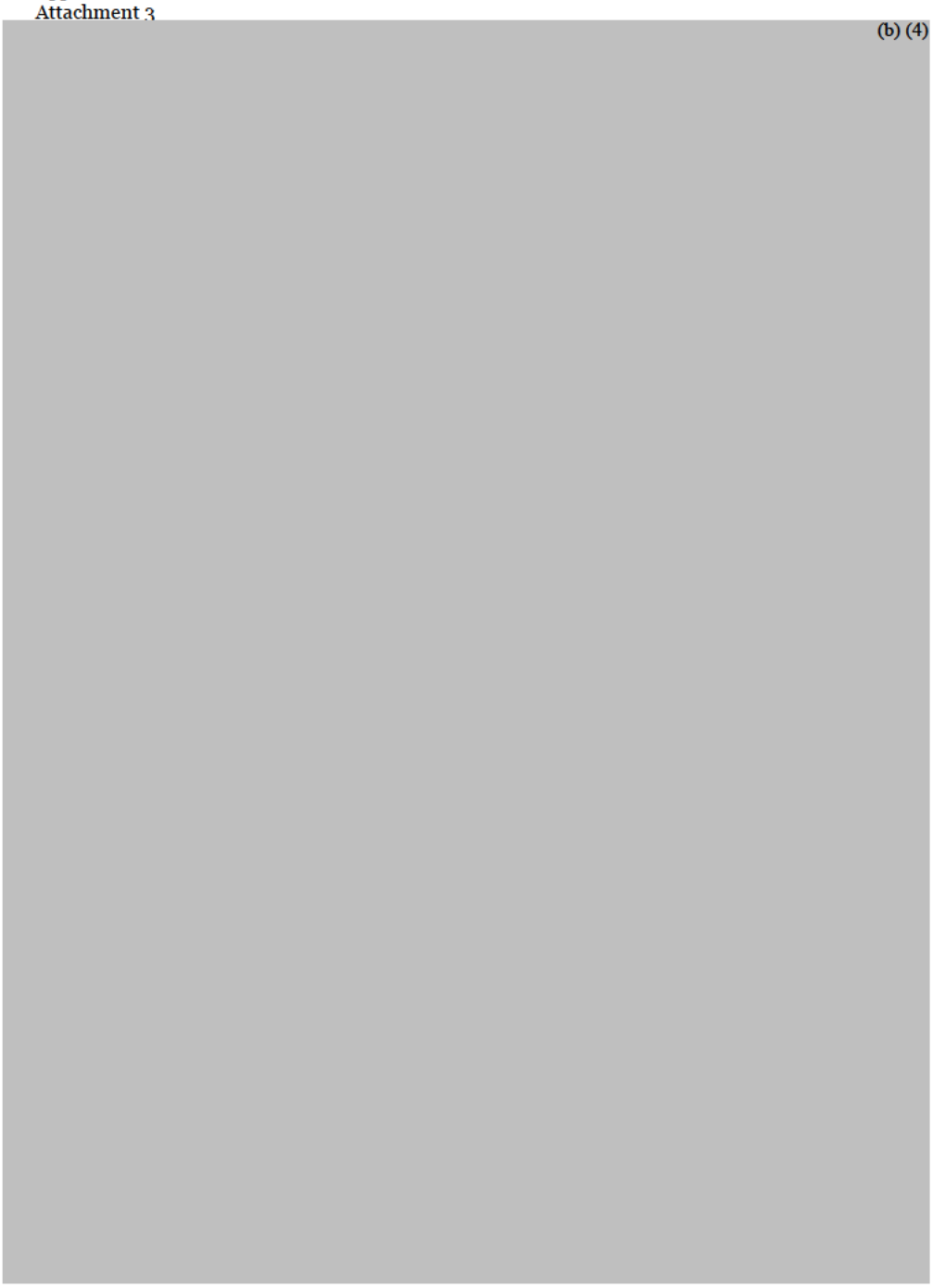

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Report IV. Antibiotics resistance of the Production strain

REPORT

Determination of antibiotic minimal inhibitory concentration (MIC) of production strain

< Confidential >

ORIGINAL FINAL REPORT DATE: May 08, 2018

CJ Blossom Park

TITLE: Determination of antibiotic minimal inhibitory concentration (MIC) of production strain

OBJECTIVE OF THE STUDY

This study was done to determine MIC of the L-threonine producing strain *Corynebacterium glutamicum* KCCM 80178.

SCHEDULE OF THE STUDY

Approval of protocol: April 09, 2018
Initiation of experiment: April 11, 2018
Termination of experiment: April 30, 2018
Submission of final report: May 08, 2018

TESTING FACILITY

Name: CJ Blossom Park, BIO) Metabolic Engineering center
Address: 42, Gwanggyo-ro, Yeongtong, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, Seoul, Korea
Tel: +82-31-8099-2117

RESPONSIBLE STAFFS

Study Director	Suyon Kwon	(05/08/18)
Quality Assurance Manager	Kwang-woo Lee	(05/08/18)


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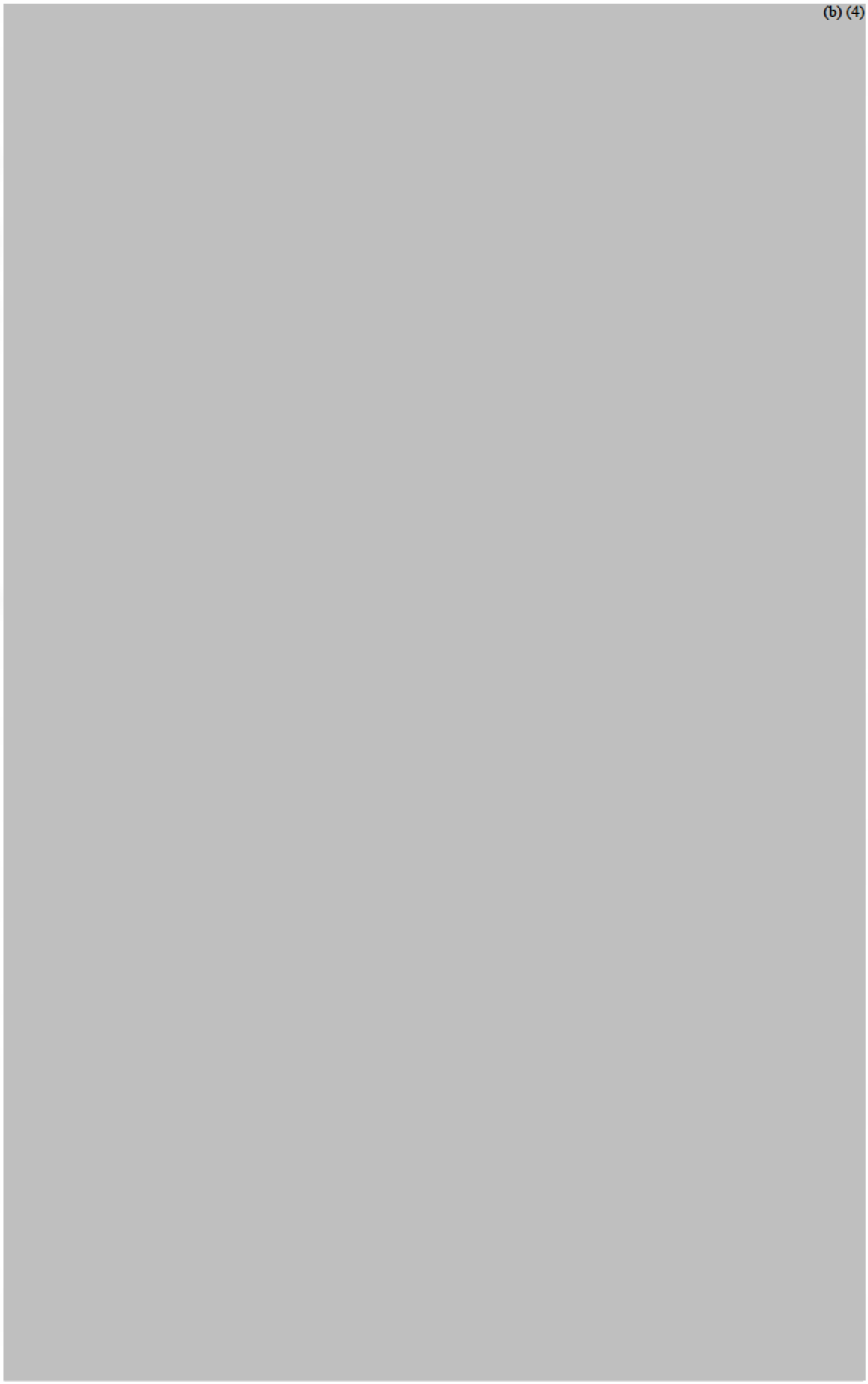
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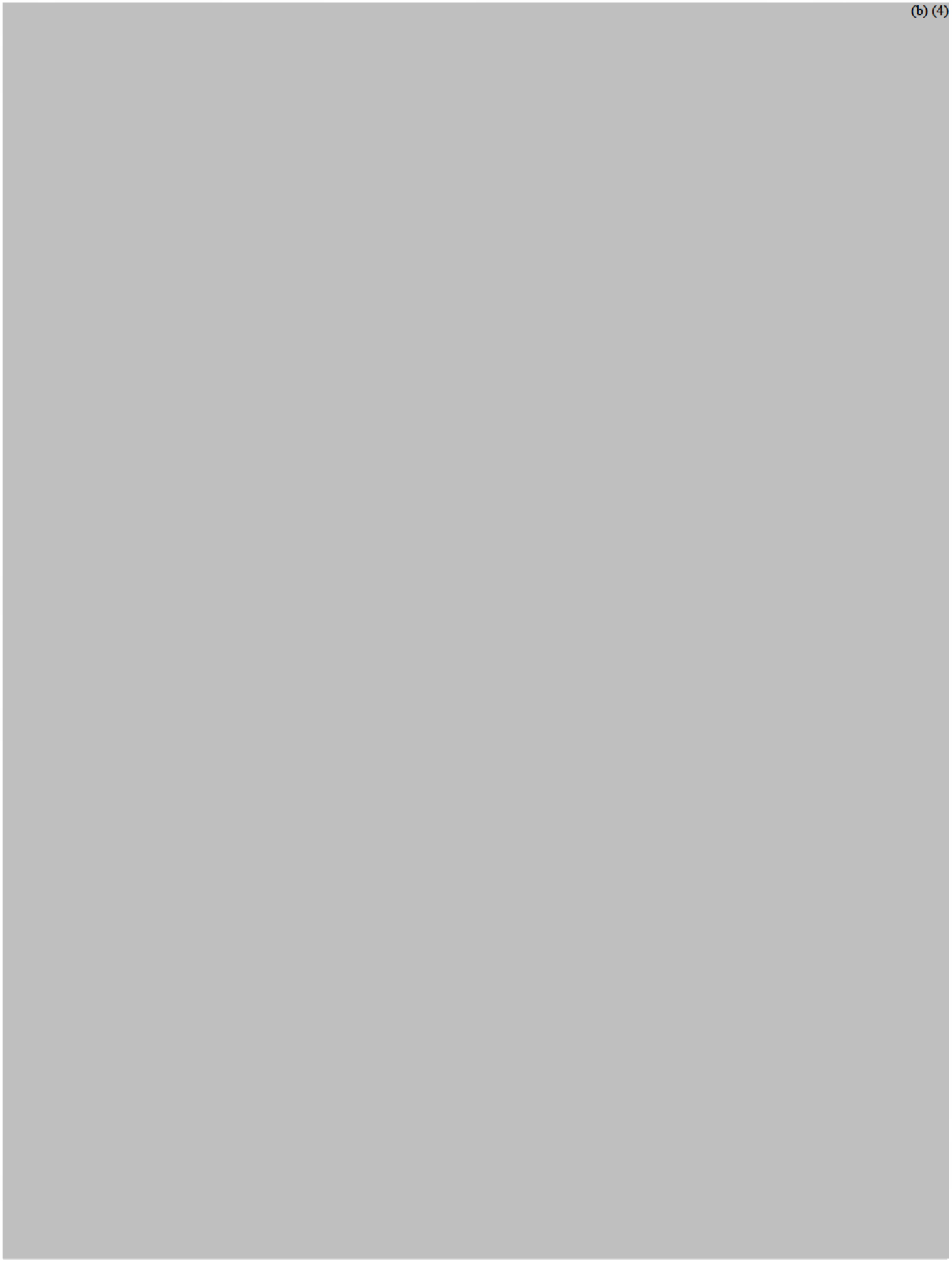

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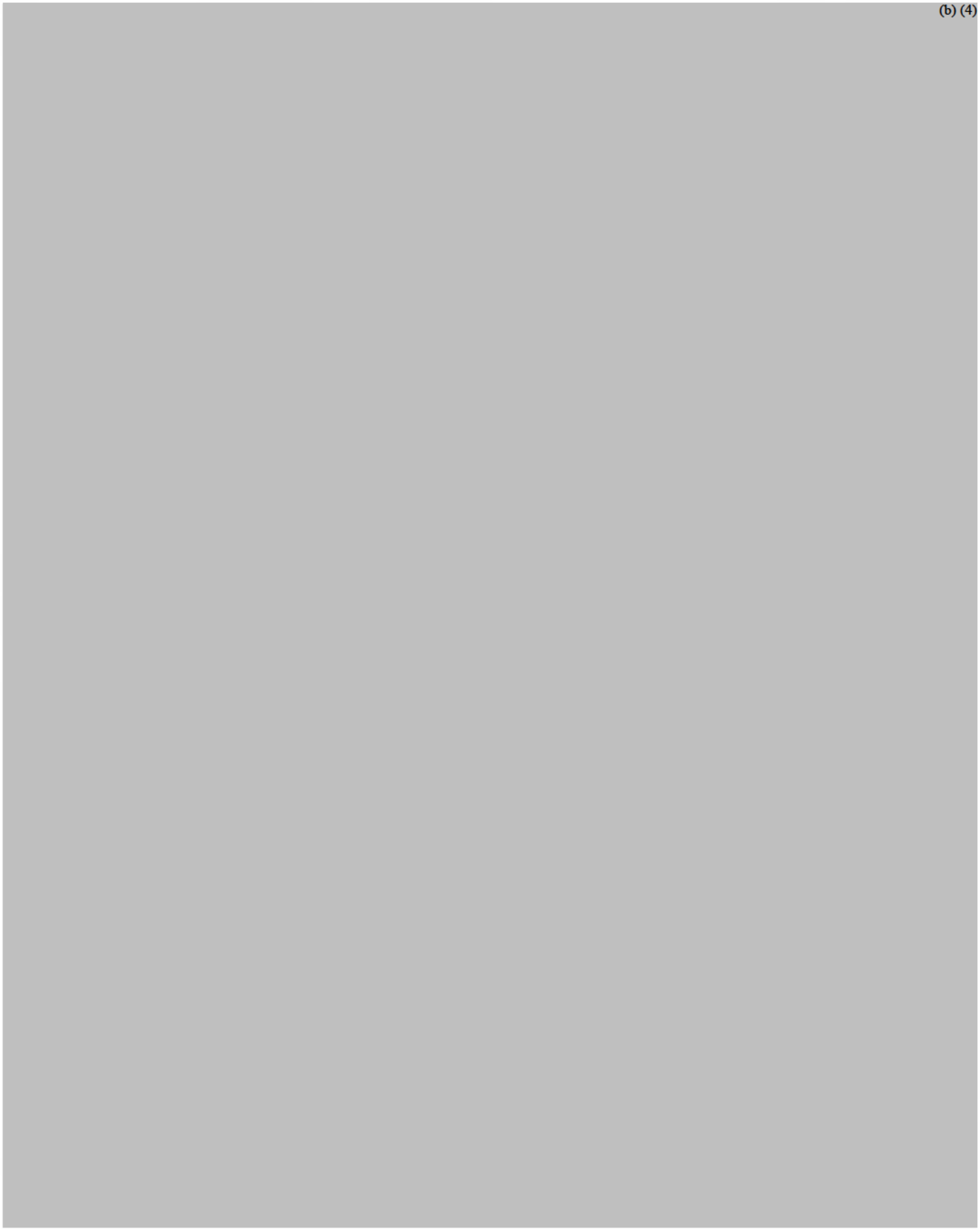

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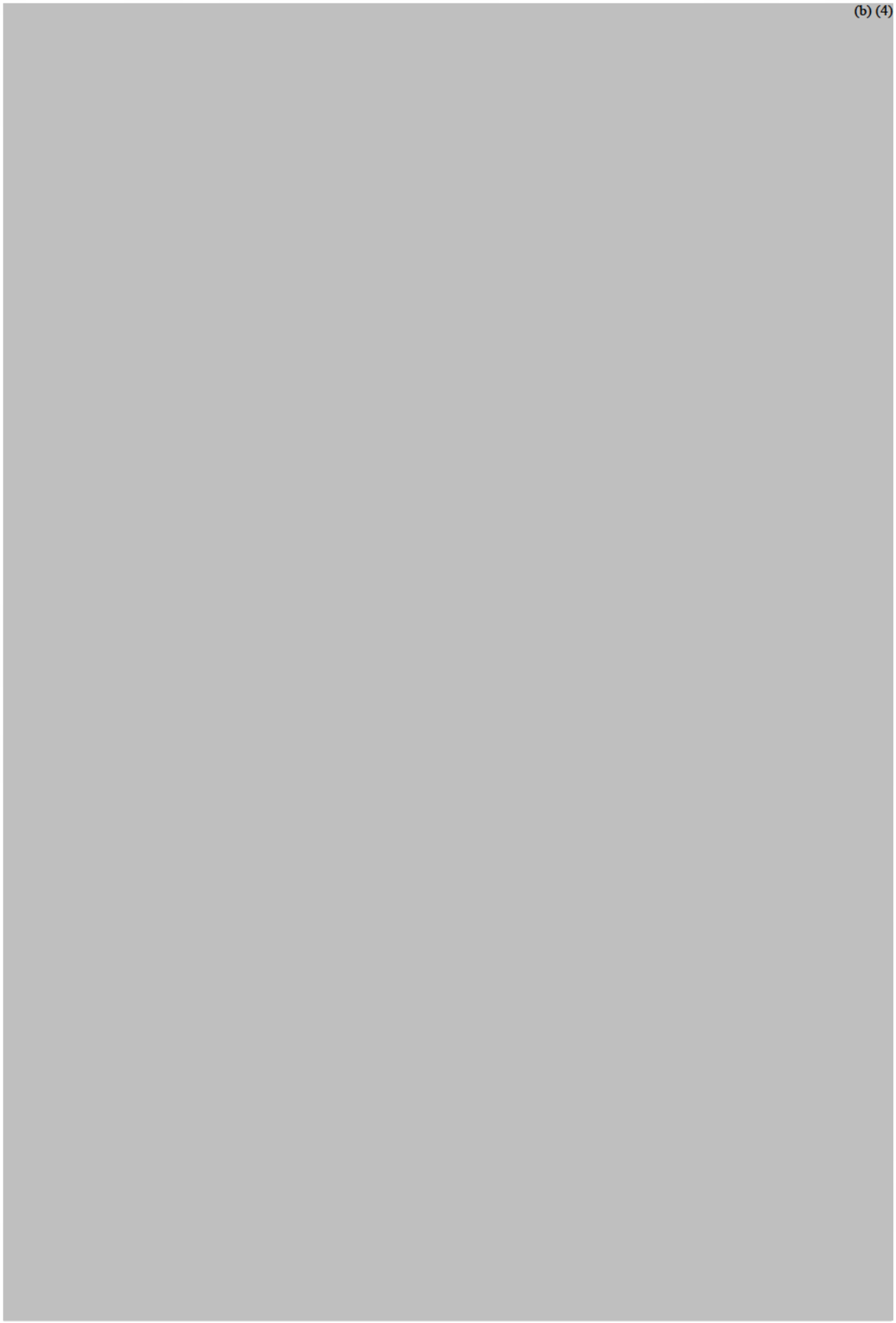


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Report V. viable cell in the final product

REPORT

Detection of residual production strain in L-threonine Fermentation Product

< Confidential >

ORIGINAL FINAL REPORT DATE: May 08, 2018

CJ Blossom Park

TITLE: Detection of residual production strain in L-threonine fermentation product

OBJECTIVE OF THE STUDY

This study was done to detect of residual production strain, *Corynebacterium glutamicum* KCCM 80178, in the final product

SCHEDULE OF THE STUDY

Data of Receipt: Apr 28, 2018

Data of Test: Apr 30, 2018

Data of Final report: May 08, 2018

TESTING FACILITY

Name: CJ Blossom Park, BIO) Metabolic Engineering center

Address: 42, Gwanggyo-ro, Yeongtong, Suwon-si, Gyeonggi-do, Korea CJ Blossom Park, Seoul, Korea

Tel: +82-31-8099-2117

RESPONSIBLE STAFFS

Study Director	Suyon Kwon	(05/08/18)
Quality Assurance Manager	Kwang-woo Lee	(05/08/18)

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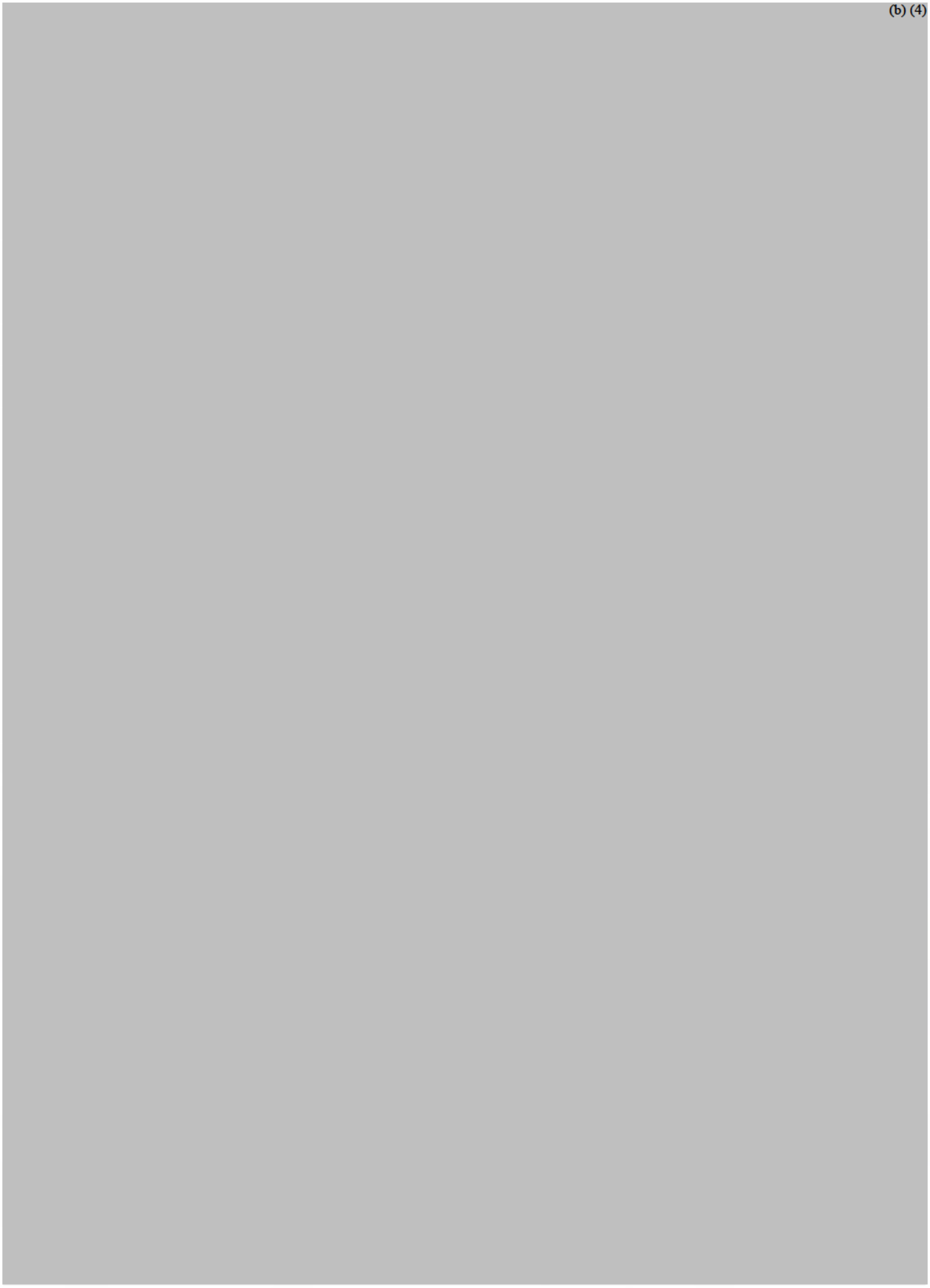

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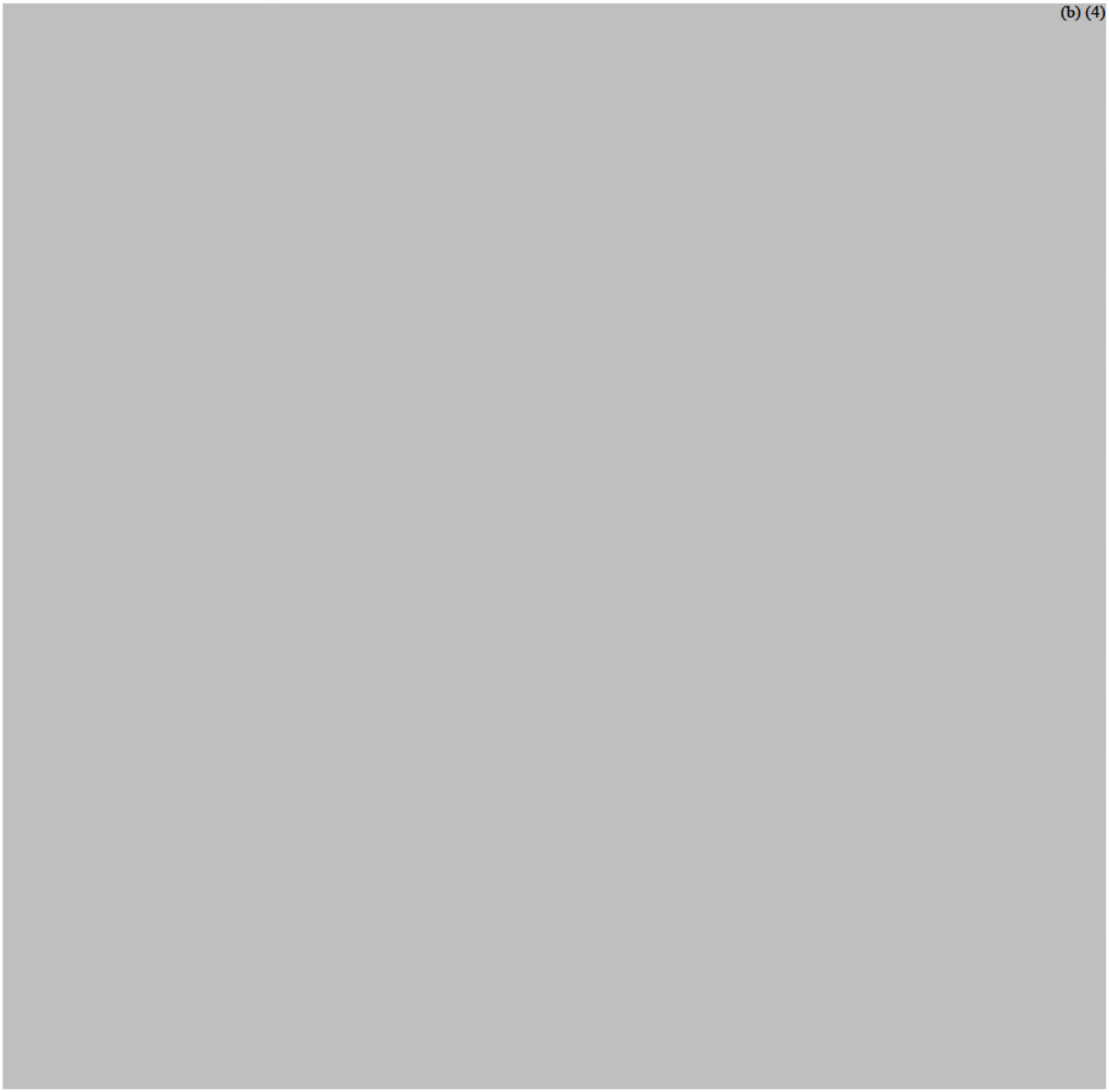

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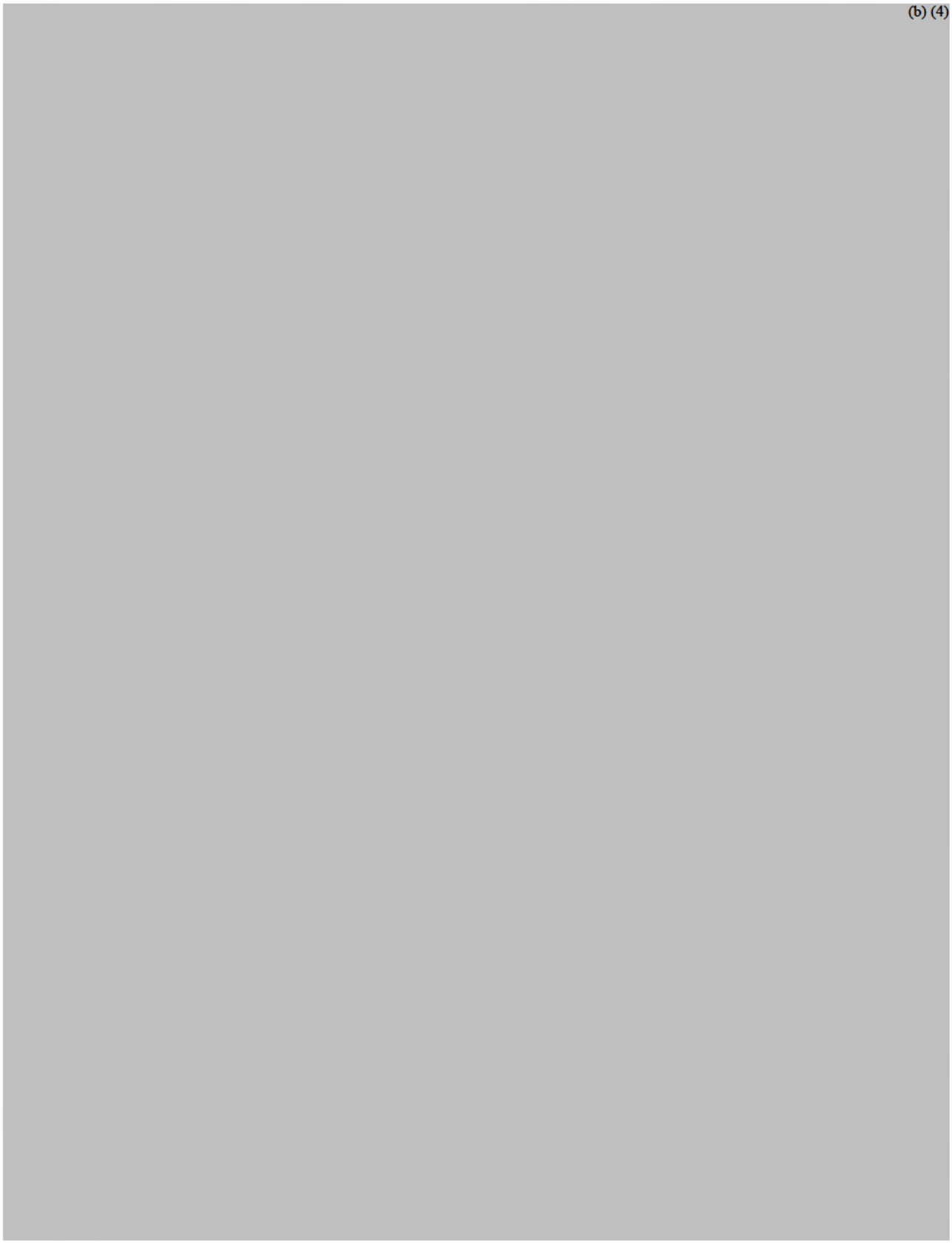

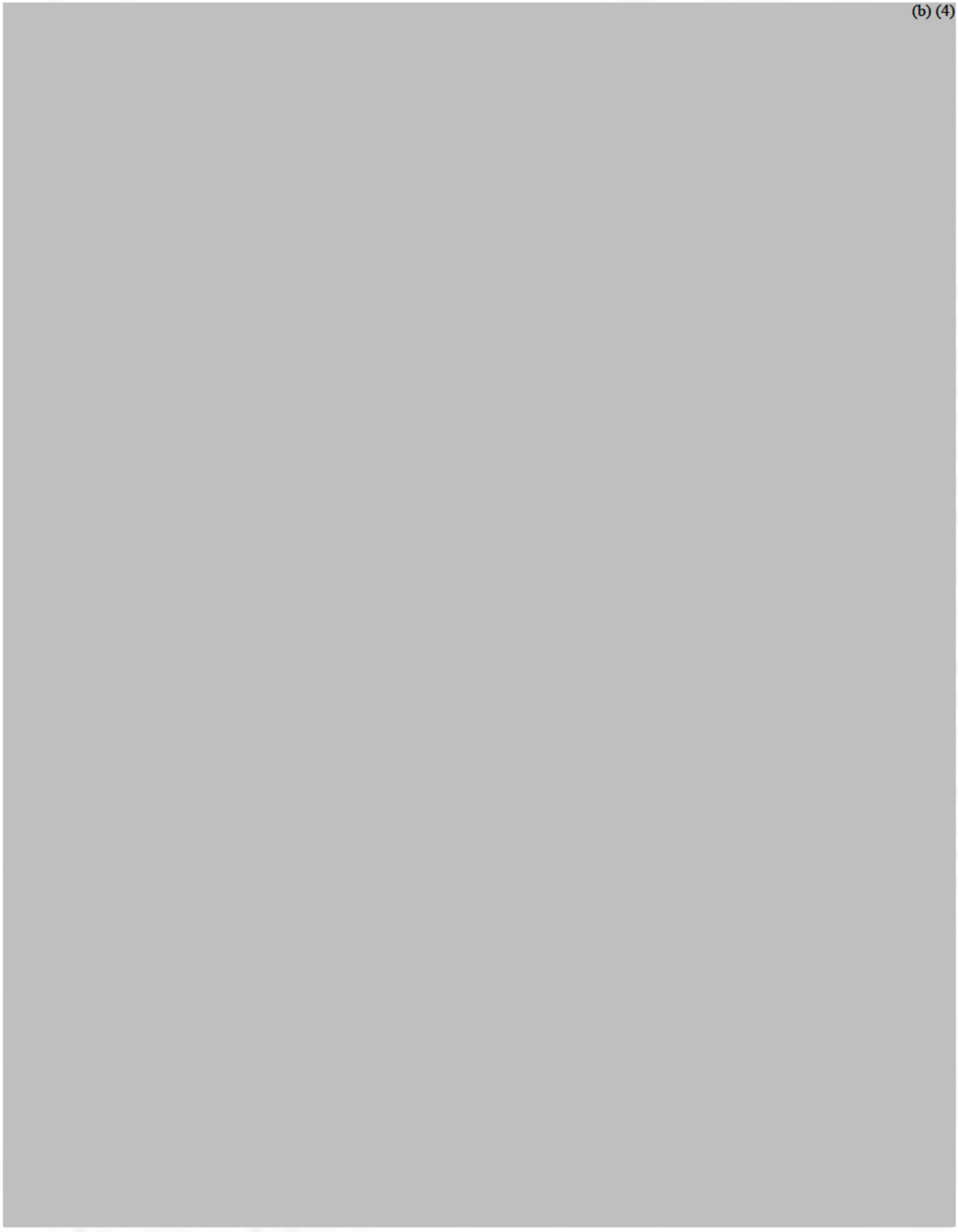
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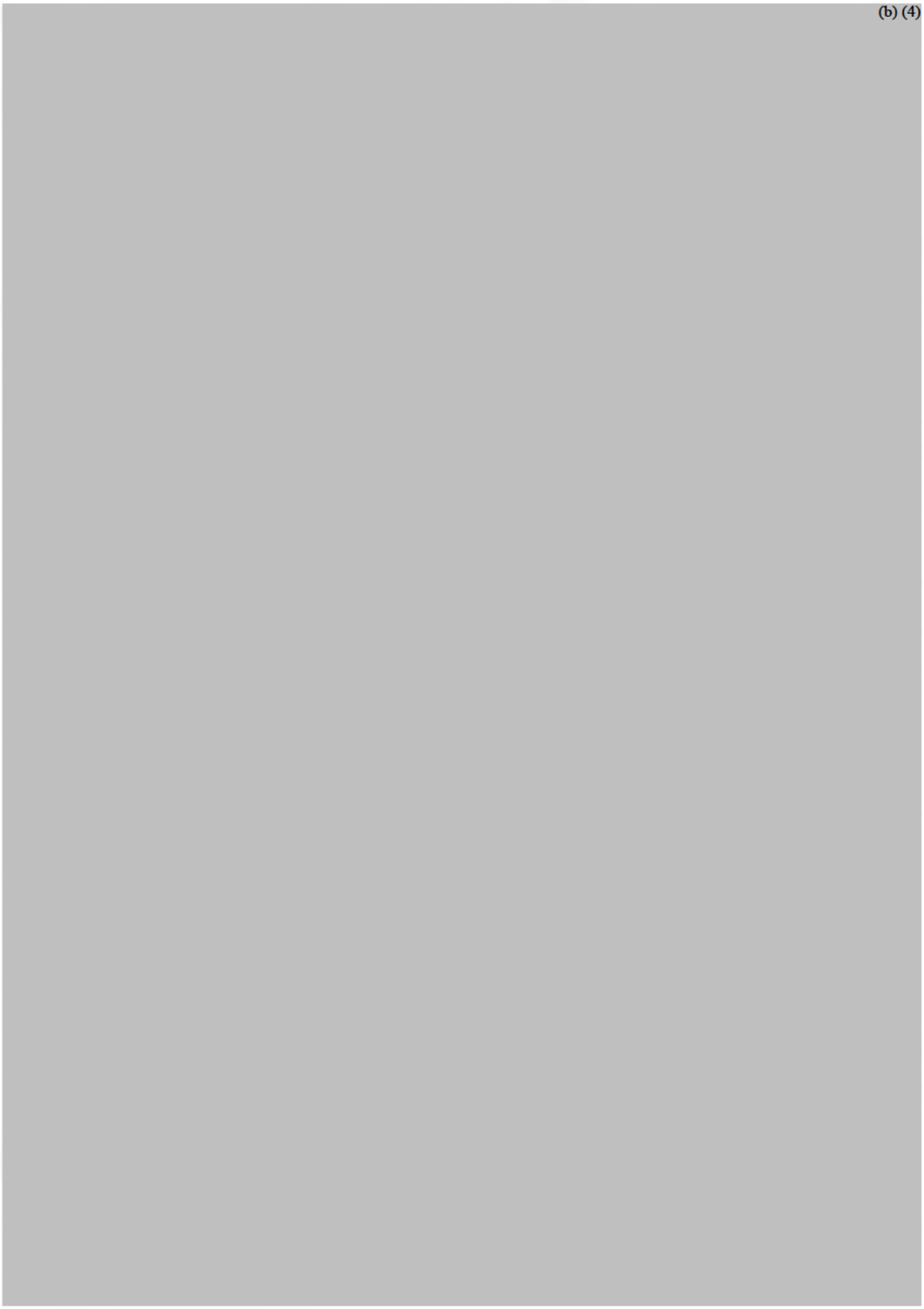
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APPENDIX 3
L-Threonine Fermentation Product Manufacturing Process
(CONFIDENTIAL)

Table of Contents

A. Manufacturing Process

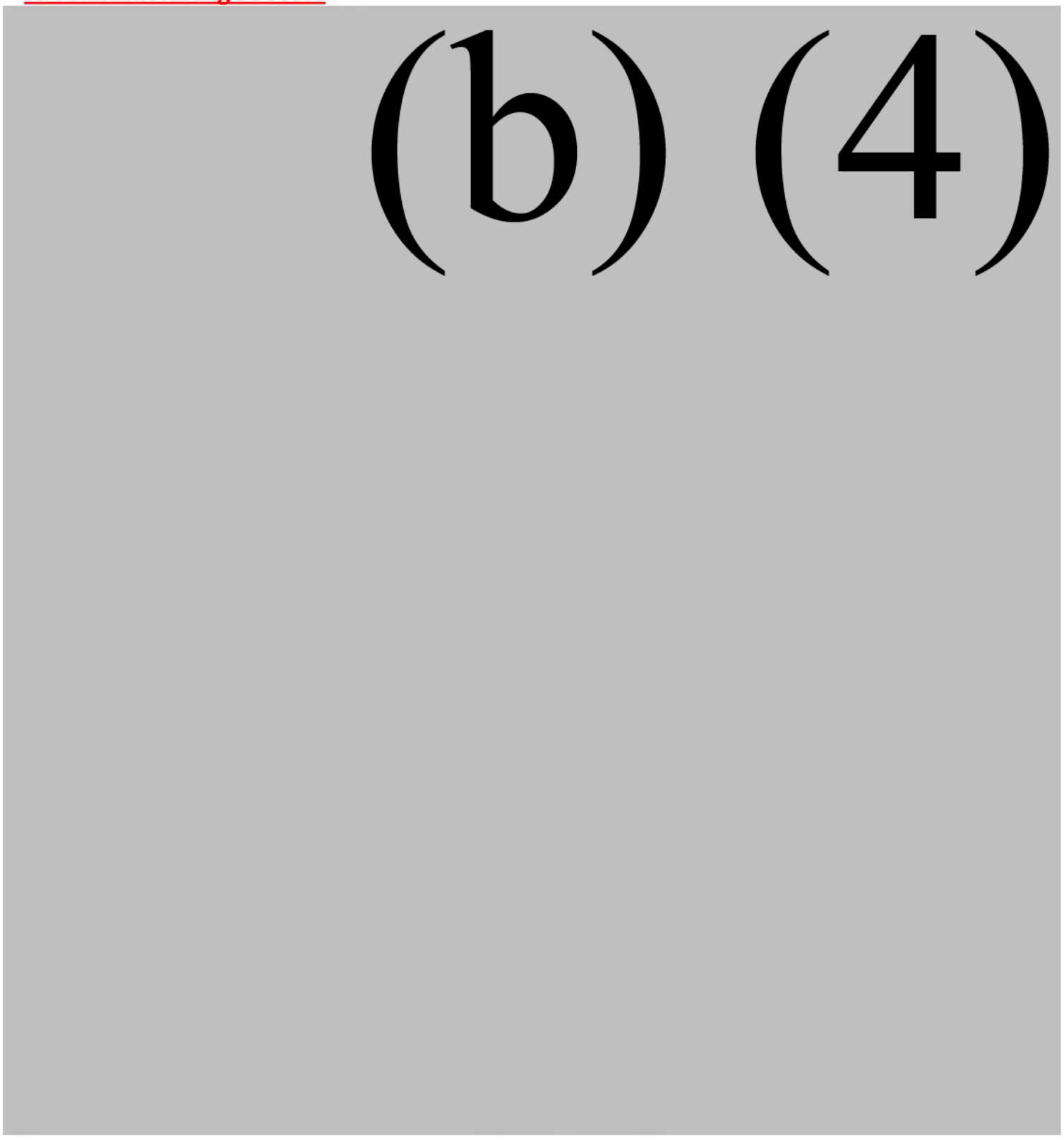
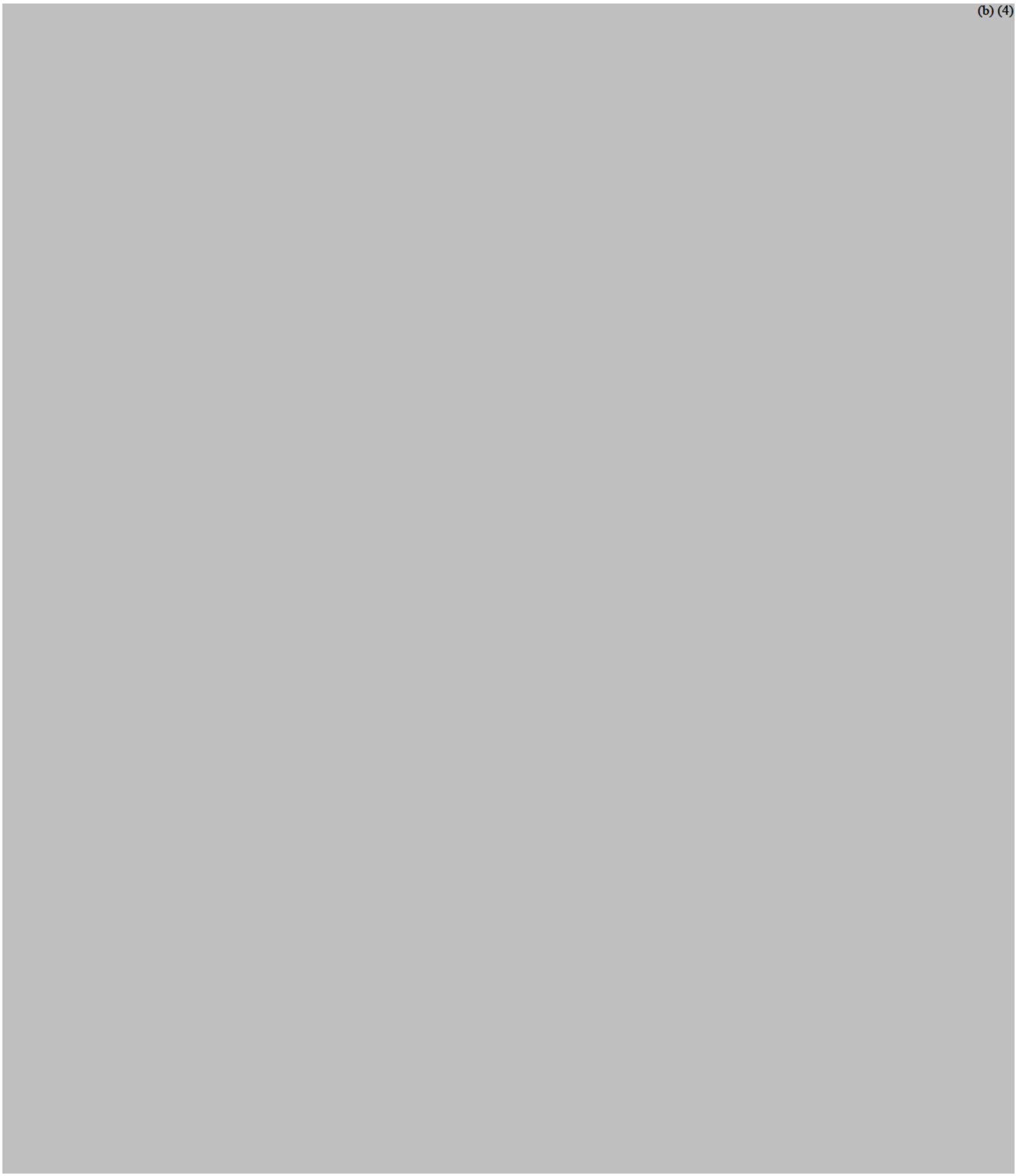


Figure Appendix 3, A-1, L-Threonine Fermentation Product Manufacturing Process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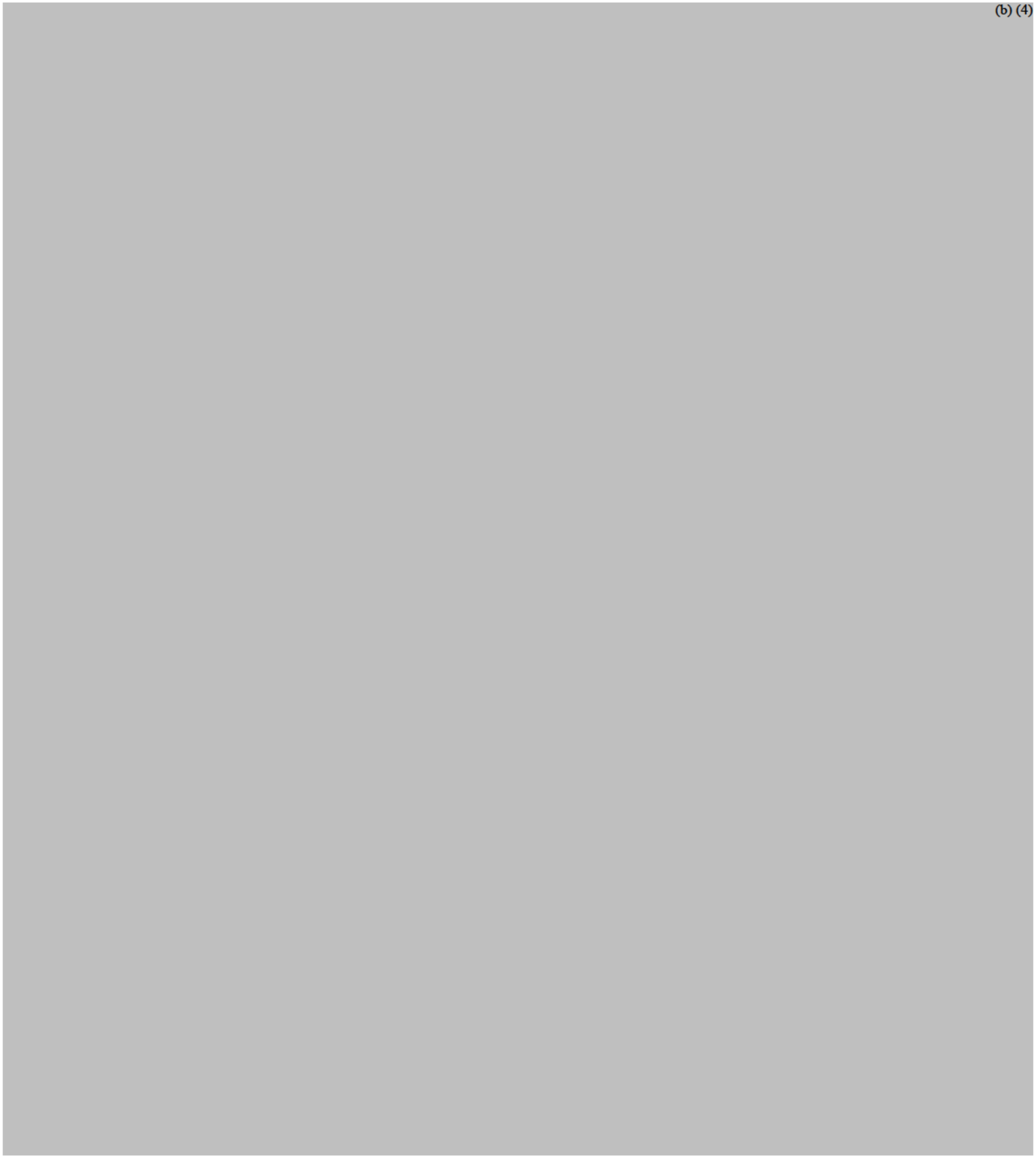
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APPENDIX 4

L-Threonine Fermentation Product Stability Study CBA

[redacted] (b) (6) - 12 month Stability

[redacted] (b) (6)

[redacted] (b) (6)

Report

laboratory: [redacted] (b) (6)

customer: CJ Cheiljedang Corporation
330, Dongho-RO;
Jung-gu, Seoul, 04560
South Korea

Mail: gemma.choi@cj.net

Registration: A05 1194fl1

date of delivery: 02.05.2017
sampling: which client admits
time of processing: 02.05.2017 – 21.11.2017
method: VDLUFA 4.11.6
date of the report: 24.11.2017

The results of analysis exclusively refer to the sample specified above.
Duplication of the test report is permitted only with previous agreement of the CBA GmbH Boehlen in part.

results

n.t. – not tested

Granule Threonine				Time Zero 10.05.2017		Samples tested at time (months)				
Lot	CBA-Number	Storage Conditions		start value	unit	1	2	3	4	6
Gran.Threonine Lot T75-16-11A5-29	A 17/05/1194	Standard (25°C/60%RH)	content	77,4	%	[redacted] (b) (4)				
			moisture	1,30	%					
Gran.Threonine Lot T75-16-12A3-02	A 17/05/1195	Standard (25°C/60%RH)	content	76,2	%					
			moisture	1,40	%					
Gran.Threonine Lot T75-16-11B2-30	A 17/05/1196	Standard (25°C/60%RH)	content	77,7	%					
			moisture	1,20	%					

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Report

Registration: **A05 1194fRoh**

Granule Threonine				Time Zero 10.05.2017		Samples tested at time (months)				
Lot	CBA-Number	Storage Conditions		start value	unit	12	18	24		
Gran.Threonine Lot T75-16-11A5-29	**A 17/05/1194**	Standard (25°C/60%RH)	content	77,4	%	(b) (4)				
			moisture	1,30	%					
Gran.Threonine Lot T75-16-12A3-02	**A 17/05/1195**	Standard (25°C/60%RH)	content	78,0	%					
			moisture	1,40	%					
Gran.Threonine Lot T75-16-11B2-30	**A 17/05/1196**	Standard (25°C/60%RH)	content	77,7	%					
			moisture	1,20	%					

Dr. (b) (6)
managing director

(b) (6)
head of laboratory

APPENDIX 5

Stability of L-Threonine Fermentation Product in Mash Feed- Test Report No. 3.243-7 granule Threonine -IFF Trial V-931-7 Stability mash feed

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Test Report No. 3.243-7 granule Threonine (Original)

IFF Trial V-931-7 Stability mash feed

Client:	CJ Europe GmbH Ober der Roeth 4 65824 Schwalbach am Taunus Germany
Subject matter:	Tests on stability of three batches granule Threonine in a broiler mash feed
Test material:	Broiler feed F-478 granule Threonine, batch T75-16-11A5-29 F-498 granule Threonine, batch T75-16-11B2-30 F-499 granule Threonine, batch T75-16-12A1-01 F-500
Order date:	21 April 2017
Study date:	Preparation of broiler feed mixtures in week 32 Analyses of the prepared samples during week 32 – 45, 2017
Contact person:	Gemma Eun-hui Choi

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Braunschweig-Thune, 8 January 2018
FO/Ke/Di

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(b) (4)

Table of Contents

Tables: 5

Figures: 1

Annexes: 1 Test report of the external laboratory

1. Responsibilities

Sponsor representative / monitor

CJ Europe GmbH
Gemma Eun-hui Choi
Ober der Roth 4
65824 Schwalbach am Taunus
Germany
phone: +49 (0) 6196 5901 68
fax: +49 (0) 6196 45 418
e-mail: gemma.choi@cj.net

Investigator

(b) (4)

Other persons involved in the study

1) (b) (4)

2) (b) (4)

3) (b) (4)

2. Objective

Referring to the order dated 21 April 2017, tests on the stability of three batches granule Threonine were performed in a broiler mash feed.

The feed mixtures were produced in the 32nd week 2017, the analyses of the stability samples took place during week 32 – 45, 2017.

3. Test material

Broiler feed	F-478
granule Threonine, batch T75-16-11A5-29	F-498
granule Threonine, batch T75-16-11B2-30	F-499
granule Threonine, batch T75-16-12A1-01	F-500

4. Material characterization

The broiler feed (F-478) was purchased from a local compound feed producer[1]. The granule Threonine batches (F-498 – F-500) were provided by the Client.

The broiler feed is characterised by its relevant ingredients and by its physical material properties. The formulation of the used broiler feed is listed in Table 1 of the annex according to the information of the supplier. The moisture content, the bulk and tap density of the broiler feed as well as information on its particle-size distribution are given in Table 2 of the annex.

5. Measuring methods

Moisture

The determination of the moisture content is carried out by measuring the mass difference after a drying time of 4 hours at a temperature of 103 °C.

Bulk density

The bulk density of the material is measured using the test unit according to Boehme as described in German standard DIN 1060.

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Tap density

Tap density is determined with the Becker-Rosenmueller equipment according to German standard DIN 53194.

Particle-size distribution

The determination of the particle-size distribution is carried out with a sieving machine according to the German standard DIN 66165 with sieves according to DIN ISO 3310. Sieves were used with mash sizes between 0.063 and 3.150 mm. The particle-size distribution of the broiler feed is shown as cumulative distribution function in Figure 1 of the annex.

6. Performance of the tests

6.1 Production of the broiler feed-mixtures with the three batches granule Threonine

The mixtures were prepared in a laboratory scale-batch mixer[2] with a mixing time of 3 min. Each batch of granule Threonine was mixed into the respective batch of broiler feed with an addition rate of 0.4 %. 4 collective samples of 250 g each were taken of each mixture. One of them was sent directly to the external laboratory[3] for analysis of the content of L-Threonine in the mixture. The remaining samples were stored in a climatic chamber at 25 °C and 60 % RH. Every four weeks samples were taken out of the climatic chamber and sent to the external laboratory for analysis. The composition of the batches is shown in Table 3 of the annex. Table 4 shows the sample encoding of the stability samples.

An additional retention sample of each batch was taken and kept at the Research Institute. The remaining material was disposed of.

7. Results of the analysis

The results of the analysis are compiled in Table 5 of the annex. The original test reports of the external laboratory are attached to this report.

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Annex

Table 1: Formulation and ingredients of the broiler feed "SoMi Thune Broiler 35" (F-478)

Composition according to the manufacturer:	
Maize	
Soy extraction meal with stock (steamer heated)[4]	
Wheat	
Fatty acids, vegetable	
Calcium carbonate	
Analytical components according to the manufacturer	**Percentage (%)**
Crude protein	15.00
Crude fat	7.30
Crude fibre	2.60
Crude ash	3.20
Calcium	0.50
Phosphorous	0.33
Sodium	0.03
Methionine	0.26
Lysine	0.74
Metabolisable energy	13.4 MJ ME/kg

Table 2: Physical material properties of the broiler feed "SoMi Thune Broiler 35" (F-478)

Physical properties	**Dimension**	**Broiler feed (F-478)**
Bulk density ρ_s	g/cm³	0.700
Tap density ρ_t	g/cm³	0.752
Moisture u	%	11.7
Particle size d_{10}	µm	150
Particle size d_{50}	µm	720
Particle size d_{90}	µm	1,750

[4] Made from genetic modified soybeans

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Test Report A 3 243-7 granule Threonine Stability broiler mash feed

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Table 3: Composition of broiler feed – amino acid mixtures

Batch No.	Ingredient	Amount
V-931-F-498	Broiler feed (F-478)	4,980 g
	granule Threonine (F-498), batch: T75-16-11A5-29	20 g
V-931-F-499	Broiler feed (F-478)	4,980 g
	granule Threonine (F-499), batch: T75-16-11B2-30	20 g
V-931-F-500	Broiler feed (F-478)	4,980 g
	granule Threonine (F-500), batch: T75-16-12A1-01	20 g

Table 4: Sample coding broiler feed – amino acid mixtures

Batch No.	Stability samples
V-931-F-498	V-931-F-498-S-0
	V-931-F-498-S-1
	V-931-F-498-S-2
	V-931-F-498-S-3
V-931-F-499	V-931-F-499-S-0
	V-931-F-499-S-1
	V-931-F-499-S-2
	V-931-F-499-S-3
V-931-F-500	V-931-F-500-S-0
	V-931-F-500-S-1
	V-931-F-500-S-2
	V-931-F-500-S-3

Table 5: Analysis results of the stability samples

Added value 0.40 %			Time in months			
Nominal value 1.011 %		Blank	Zero	1	2	3
Sample number	Unit		S-0	S-1	S-2	S-3
Analysis method		DJ005	DJ005[5]	DJ005	DJ005	DJ005
V-931-F-498	%	0.611	1.19	[redacted]		
V-931-F-499	%	0.611	1.05			
V-931-F-500	%	0.611	1.24			

(b) (4)

[5] Threonine (acid/oxidativ hydrolysis); Method: EU 152/2009 (F), ISO 13903:2005, IC-UV

(b) (4)

Test Report A.3.243-7 granule Threonine
Stability broiler mash feed

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(b) (4)

Figure 1: Cumulative frequency distribution Q_3 of broiler feed (F-478)

Annex

(b) (4)

(b) (4)

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(b) (4)

Person in charge ASM (b) (6)

Report date 11.12.2017

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(b) (4)

Analytical report AR-17-GF-046104-01

Sample Code 710-2017-26639012

Reference	Broilerfutter ohne Aminosäuresupplementierung
Sample sender	(b) (6)
Reception date time	30.11.2017
Transport by	Post
Client sample code	F-478
Number of containers	1
Reception temperature	room temperature
End analysis	11.12.2017

Test results

DJ007 Methionine (oxidative hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Methionine (Total)	0.269	g/100 g

DJ005 Threonine (acid / oxidativ hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	0.611	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge
ASM

(b) (6)

Report date 11.12.2017

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(b) (4)

Analytical report AR-17-GF-046101-01

Sample Code 710-2017-26639009

Reference	Multirobe Broilerfuttermehl mit L-Threonin
Sample sender	(b) (6)
Reception date time	30.11.2017
Transport by	Post
Client sample code	V-931-F-498-S-0
Number of containers	1
Reception temperature	room temperature
End analysis	11.12.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.19	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge (b) (6)
ASM

Report date 21.09.2017

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(b) (4)

Analytical report AR-17-GF-034434-01

Sample Code 710-2017-20385004

Reference	Futtermittel
Sample sender	(b) (6)
Reception date time	15.09.2017
Transport by	Post
Client sample code	V-831-F-496-S-1
Number of containers	1
Reception temperature	room temperature
End analysis	21.09.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)

Method EU 152/2009 (F), ISO 13903:2005, , IC-UV

Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.08	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge (b) (6)
ASM

Report date 16.10.2017

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(b) (4)

Analytical report AR-17-GF-037402-01

Sample Code 710-2017-22221004

Reference	Stabilitätsproben L-Threonin
Sample sender	(b) (6)
Reception date time	09.10.2017
Transport by	Post
Client sample code	V-931-F-496-S-2
Number of containers	1
Reception temperature	room temperature
End analysis	15.10.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.02	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge (b) (6)
ASM

Report date 07.11.2017

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(b) (4)

Analytical report AR-17-GF-040806-01

Sample Code 710-2017-24425004

Reference	Stabilitätsproben L-Threonin
Sample sender	(b) (6)
Reception date time	03.11.2017
Transport by	Post
Client sample code	V-931-F-496-S-3
Number of containers	1
Reception temperature	room temperature
End analysis	07.11.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.14	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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GfA Lab Service

(b) (4)

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(b) (4)

Person in charge ASM (b) (6)

Report date 11.12.2017

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(b) (4)

Analytical report AR-17-GF-046102-01

Sample Code 710-2017-26639010

Reference	Nullerobe Broilerfuttermehl mit L-Threonin
Sample sender	(b) (6)
Reception date time	30.11.2017
Transport by	Post
Client sample code	V-931-F-499-5-0
Number of containers	1
Reception temperature	room temperature
End analysis	11.12.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)

Method EU 152/2009 (F), ISO 13903:2005 , IC-UV

Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.05	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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(b) (4)

Person in charge
ASM

(b) (6)

Report date 21.09.2017

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(b) (4)

Analytical report AR-17-GF-034435-01

Sample Code 710-2017-20385005

Reference	Futtermittel
Sample sender	(b) (6)
Reception date time	15.09.2017
Transport by	Post
Client sample code	V-931-F-499-S-1
Number of containers	1
Reception temperature	room temperature
End analysis	21.09.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.08	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (6)

(b) (4)

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(b) (4)

Person in charge ASM (b) (6)

Report date 16.10.2017

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

Analytical report AR-17-GF-037403-01

Sample Code 710-2017-22221005

Reference	Stabilitätsproben L-Threonin
Sample sender	(b) (6)
Reception date time	09.10.2017
Transport by	Post
Client sample code	V-831-F-499-S-2
Number of containers	1
Reception temperature	room temperature
End analysis	15.10.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)

Method EU 152/2009 (F), ISO 13903:2005, , IC-UV

Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.07	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge (b) (6)
ASM

Report date 07.11.2017

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(b) (4)

Analytical report AR-17-GF-040807-01

Sample Code 710-2017-24425005

Reference	Stabilitätsproben L-Threonin
Sample sender	(b) (6)
Reception date time	03.11.2017
Transport by	Post
Client sample code	V-931-F-499-S-3
Number of containers	1
Reception temperature	room temperature
End analysis	07.11.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.12	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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(b) (4)

Person in charge ASM (b) (6)

Report date 11.12.2017

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(b) (4)

Analytical report AR-17-GF-046103-01

Sample Code 710-2017-26639011

Reference	Nullprobe Broilerfuttermehl mit L-Threonin
Sample sender	(b) (6)
Reception date time	30.11.2017
Transport by	Post
Client sample code	V-831-F-500-S-0
Number of containers	1
Reception temperature	room temperature
End analysis	11.12.2017

Test results

DJ006 Threonine (acid / oxidativ hydrolysis)

Method EU 152/2009 (F), ISO 13903:2005, , IC-UV

Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.24	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge (b) (6)
ASM

Report date 21.09.2017

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(b) (4)

Analytical report AR-17-GF-034436-01

Sample Code 710-2017-20385006

Reference	Futtermittel
Sample sender	(b) (6)
Reception date time	15.09.2017
Transport by	Post
Client sample code	V-931-F-500-S-1
Number of containers	1
Reception temperature	room temperature
End analysis	21.09.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)

Method EU 152/2009 (F), ISO 13903:2005, , IC-UV

Subcontracted to a (b) (6) accredited for this test.

Threonine (Total)	1.07	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge (b) (6)
ASM

Report date 16.10.2017

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(b) (4)

Analytical report AR-17-GF-037480-01

Sample Code 710-2017-22221006

Reference	Stabilitätsproben L-Threonin
Sample sender	(b) (6)
Reception date time	09.10.2017
Transport by	Post
Client sample code	V-931-F-500-S-2
Number of containers	1
Reception temperature	room temperature
End analysis	16.10.2017

Test results

DJ005 Threonine (acid / oxidativ hydrolysis)

Method EU 152/2009 (F), ISO 13903:2005, , IC-UV

Subcontracted to a (b) (4) accredited for this test.

Threonine (Total)	1.14	g/100 g

(b) (6)

Analytical Service Manager - Feed (b) (6)

(b) (4)

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Person in charge ASM (b) (6)

Report date 07.11.2017

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(b) (4)

Analytical report AR-17-GF-040808-01

Sample Code 710-2017-24425006

Reference	Stabilitätsproben L-Threonin
Sample sender	(b) (6)
Reception date time	03.11.2017
Transport by	Post
Client sample code	V-931-F-500-S-3
Number of containers	1
Reception temperature	room temperature
End analysis	07.11.2017

Test results

DJ005 **Threonine (acid / oxidativ hydrolysis)**
Method EU 152/2009 (F), ISO 13903:2005, , IC-UV
Subcontracted to (b) (6) accredited for this test.

Threonine (Total)	1.04	g/100 g

(b) (6)

(b) (6)

Analytical Service Manager - Feed

(b) (4)

APPENDIX 6

UTILITY TRIAL REPORT

Research Study

Evaluation of novel threonine source in a low threonine diet on broiler growth performance through 28 day of age

Protocol Title: Evaluation of a novel threonine source in a low threonine diet on broiler growth performance through 28 day of age.

Investigators:

Joe Lucas
Vice President and Managing Director
Joe.lucas@cj.net
CJ America – Bio
3500 Lacey Road Suite 230
Downers Grove, IL 60515

Keith Haydon, Ph.D.
Director of Technical Services and Marketing
Keith.haydon@cj.net
CJ America – Bio
3500 Lacey Road Suite 230
Downers Grove, IL 60515

Abstract

A 28-day growth assay was conducted using 1320 Cobb 500 male broilers in a small pen study (33 birds/pen) to determine the effectiveness of a novel L-threonine source (>75% L-threonine with biomass) fed during starter (0 – 14 days) and a grower phases (15 – 28 days). The assay evaluated four dietary treatments (10 reps/ treatment): 1. Positive Control (PC) diet using commercially available L-Threonine 98.5%, 2. Negative Control (NC, no L-threonine supplementation); 3. NC supplemented with 100% replacement of threonine level of the PC diet using the novel threonine source with biomass (NThr) and 4. NThr source fed at a 150% threonine replacement rate of the PC diet. In the starter phase (Day 0 – 14) birds fed the PC, and the 100 or 150% NThr replacement rates were heavier (P<.003) than the birds fed NC diet. Bird weights at 28 days were heavier (P<.04) for those fed the PC and 100% NThr diets than NC diet with the150% NThr fed birds being intermediate in weight. The only feed intake response was observed during the starter phase, with NThr 100 and 150% fed birds consuming more (P<.014) feed than the NC birds. No differences (P>.10) were observed in mortality. Mortality adjusted FCR (F/G) was lower for PC fed birds than NC or 150% NThr fed birds during the grower phase with 100% NThr bird being intermediate. Over the entire 28 day assay, the PC and 100% NThr fed birds had lower adjusted FCR than the NC or 150% NThr fed birds. The assay demonstrated the novel L-Threonine with biomass is an effective source of dietary threonine for broiler chicks.

I. Experimental Procedures:

Objective: Determine the effect of a novel threonine source containing greater than 75% L-threonine with fermentative biomass at 100 or 150% replacement rate on broiler performance compared to a positive control diet with commercial L-Threonine (>98.5%) or negative control diet without L-threonine supplementation.

Locations:

A. Live performance: Poultry Research Facility, (b) (6)

1. Pen size: 4 × 7 ft
2. Duration: 28 d
3. Group size: 33 birds/pen
4. Floor space: 0.85 ft^2/bird
5. Feeder type: Dry tube feeder (30-lb feed capacity)
6. Feeder space: 50 in. total; 1.4 in./bird
7. Water space: 4 nipple drinkers/pen (7 birds/nipple)
8. Lighting protocol:

B. Harvest: Poultry Research Facility, (b) (6)

Experimental Timelines:

Start date:	March 12, 2018
End date:	April 30, 2018
Preliminary report:	May 15, 2018
Final report issued:	May 31, 2018

Experimental Design:

Growth & carcass data

1. Design: Randomized complete-block
2. Replication factor: Live weight (by pen)
3. Replicates: 10

Animals

Genetics:	Cobb 500 (b) (6)
Number:	1,320
Gender:	Male
Age:	Hatch
Start weight:	~ 40 g

End weight:	~ 1.5 kg
Duration	28 days

II. Experimental Treatments: 4 treatments

A. Positive Control Diet with L-threonine
B. Negative Control Diet without L-threonine
C. NC + Novel Threonine (added to reach level of PC – 100%)
D. NC + Novel Threonine (added to reach above PC – 150%)

III. Experimental Procedures:

A. Animal care protocol: Care was provided following an approved Animal Use Protocol approved by the IACUC committee at (b) (4) Environmental conditions were monitored 3 times daily. Age appropriate temperature was provided and regulated. Heat was provided with multiple force draft heaters. House is cross-ventilated with adjustable vents on one end and 3 – 36 inch fans on the other end.

B. Allotment of animals to the experiment

1. Birds were assigned to pen based on day old chick weight. Initial pen weight of all replicate pens had a maximum of range of 30 grams.
2. Pens were then randomly allotted to dietary treatment from within replicate and immediately started on the study.
3. Pens remained on dietary treatments until the end of the experiment.
4. Minimum ventilation was run to supply necessary gas exchanges.
5. Birds were raised on used litter from 2 previous flocks.

C. Measurements:

1. Live performance:
   i. Total pen weights – start, d 14 and d 28
   ii. Feed disappearance – d 14 and d 28
   iii. Feed/gain ratio was adjusted for mortality by the following equation. Total feed consumed/ (pen weight gain + mortality weight).
   iv. Morbidity and mortality

D. **Experimental diet formulation (Table 1 and 2)**

1. Feedstuffs:
   i. Corn – yellow-dent:
   ii. Soybean meal:
   iii. Soy oil:

(b) (4)

2. Experimental test material:

a. Provided by CJ America

3. Experimental diet specifications:
    i. Two dietary phases – d 1-14 (starter) and d 15-28 (grower).
    ii. Diet components were mixed in a horizontal mixer.
    iii. Each diet was pelleted at 180F following 15 s of conditioning. The starter diet was crumbled following pelleting.
4. Diet sampling:
    i. Final experimental diets were sampled and analyzed. Sampling procedure included taking five 1 kg grab samples while the feed was exiting the mill. The grab samples were then combined, homogenized, and split into three equal samples. Amino acid analysis was conducted by a private third party laboratory.

IV. Statistical Procedures:

A. Prior to analysis, all data was checked for outliers. Any observation > 3 standard deviations in difference from the grand mean for that metric were removed from the dataset.

B. Cumulative body weight, body weight gain, feed intake, and mortality corrected feed conversion ratio were analyzed as a RCBD with four (4) treatments and 10 replicates.

C. Mortality was analyzed following an arc sine transformation.

V. Introduction

Threonine has long been recognized as the third limiting amino acids for the broiler. Although L-threonine from fermentation has been commercially available in feed grade form since the mid-1990's; wide scale adoption of threonine supplementation did not occur until 2000's.

Warnick and Anderson (1968) demonstrated in a 12% CP semi-purified soybean meal based diet that lysine, threonine and valine were next limiting essential amino acids after methionine. Schwartz and Bray (1975) using amino acid deletion technique with a 14% CP diet reported that deletion of threonine decreased gain by 31% from the control. Baker and Han (1994) proposed the first "ideal protein" concept for broilers with essential amino acids levels being expressed as a ratio to dietary lysine level. Their initial estimate for the threonine requirement was 67% for threonine. Kidd and Kerr (1996) ground-breaking work demonstrated that increasing dietary threonine levels improved breast yield, proved to be the catalysis for widespread adoption of threonine supplementation in the broiler industry as breast meat became the primary economic driver. Current estimate of global threonine usage range from 450,000 to 500,000 metrics tons, and demand is growing 40,000 to 50,000 metric tons per year (CJ personal communication).

The objective of this experiment was to evaluate a new threonine supplement from CJ, which contains a minimum of 75% L-threonine with the fermentative biomass as a replacement for commercially available L-threonine (98.5%) in broiler chicks.

VI. Results and Discussion

In the current experiment, body weight at day 14 and weight gain (Day 0 -14) were increased (P<.003) over the Negative Control (NC) with the addition of threonine either from commercial available 98.5% (Positive Control, PC) or novel L-threonine with biomass at both 100 (NThr100) and 150% (NThr150) replacement rates (Table 3.). In a recent study, Sigolo et. al, (2017) found dietary threonine need to be increased to 110% of requirement when reducing crude protein level 2.5% from 22 to 21.45% with ADG of 32 gm/day during the 21 day starter phase. Threonine levels in nur study were 58% of lysine in the NC and 65% of lysine in the PC with a crude protein of 21.1%, however, ADG were excellent (29 gm/day) during the starter period (0-14 days) as compared to ADG reported by Sigolo et. al. (2017) for (32 gms/day, day 0-21 days).

Body weights in the present trial at day 28 were lower (P<.04) for NC fed chicks as compared to the PC or NThr100 fed birds with those fed NThr150 being intermediate (P>.10). However, no significance (P>.20) in body weight gain was observed between treatments likely due to increased individual bird weight variation within replicate associated with age. Performance for the male birds from day 0 to 14 and 15 to 28 were excellent and were similar to target weight and ADG expectation as outline by Cobb 500 manual (2015).

No differences (P>.10) in mortality adjusted FCR (F/G) during the starter phase (Day 0 to 14, Table 4) was observed. Sigolo et. al. (2017) reported an improvement (P<.10) in FCR when feeding 110% of threonine requirement in birds fed 97.4% of CP requirement. However, it should be noted that Sigolo et. al. (2017) basal (100% of requirement) dietary total threonine level was .94% or 74% of total lysine, Whereas in the present study, PC diet digestible threonine level was set at 65% of digestible lysine. During the grower phase (Day 15 to 28) FCR was improved (P<.04) for PC fed birds as compared to NC or the NThr150 fed birds. FCR of the NThr100 fed birds was intermediate (P>.05). Sigolo et. al. (2017) also observed a numerical depression in in FCR with increasing threonine supplementation from 110% to 130% of requirement in both 97.5% and 100% CP requirement diets. FCR calculated over the entire 28 day growth assay was improved (P<.01) with threonine supplementation whether from commercially available source (PC) or with novel threonine source (NThr100 and NThr150) as compared to NC fed birds. FCR observed in the present study in NC fed broilers was 6% higher than expectations for Cobb 500 males (Cobb, 2015).

The lack of FCR response during the starter phase (day 1 to 14) could partially be attributed to the higher feed intake and growth rate observed for the NThr100 and NThr150 fed birds as compared to the NC fed birds (Table 5). Sigolo et. al. (2017) reported a numerical depression in feed intake with increasing threonine supplementation above 110% of requirement. PC fed birds were intermediate for day 1 to 14 feed intake. Feed intake during the grower phase (day 15 to 28) or measured over the 28 day trial period were not different (P>.10) among dietary treatments. Observed feed intakes were slightly higher than intakes suggest for Cobb (2015) for Cobb 500 males.

Mortality was unaffected (P>.10) by dietary treatment (Table 6). However, numerically higher mortality was observed for birds fed the NC treatment especially in the grower phase as compared to other treatments with supplemental dietary threonine.

VII. Conclusions

The current trial clearly demonstrated that the novel L-threonine supplement with biomass is an effective L-threonine source in broiler chicks. When replacing 100% of the L-threonine (98.5%), the novel L-threonine with biomass provided equal performance in both starter (day 0 to 14) and grower (day 15 to 28) phases to current commercially available feed-grade L-threonine.

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Table 1.Starter dietary formulations, calculated nutrient content, and analyzed nutrient content of treatment diets fed to male broilers (1 to 14 days-of-age)

Ingredient	Positive Control[1]	Negative Control	NC +100	NC +150
	%			
Corn	61.00	61.00	61.00	61.00
Soybean Meal	33.15	33.15	33.15	33.15
Soybean Oil	1.52	1.52	1.52	1.52
Limestone	1.33	1.33	1.33	1.33
Salt, NaCl	0.46	0.46	0.46	0.46
Monocalcium Phosphate	1.61	1.61	1.61	1.61
DL-Methionine, 99%	0.30	0.30	0.30	0.30
L-Lysine HCl, 78.8%	0.23	0.23	0.23	0.23
Vitamin Premix [1]	0.13	0.13	0.13	0.13
Trace Mineral Premix[2]	0.05	0.05	0.05	0.05
Salinomycin – SaCox[3]	0.05	0.05	0.05	0.05
L-Threonine, 98.0%[4]	0.088	--	--	--
L-Threonine Biomass, 75%[5]	--	--	0.117	0.175
Cellulose, Filler (wt: wt)[11]	0.087	0.175	0.058	--
Nutrient	Calculated Nutrient Content, %			
AME, kcal/kg	3036	3036	3036	3036
Protein[12]	21.10	21.04	21.10	21.13
dLys	1.18	1.18	1.18	1.18
dMet	0.58	0.58	0.58	0.58
dSAA	0.87	0.87	0.87	0.87
dThr	0.77	0.68	0.77	0.82
dArg	1.27	1.27	1.27	1.27
dVal	0.89	0.89	0.89	0.89
Calcium	0.90	0.90	0.90	0.90
Non-Phytate Phosphorus	0.45	0.45	0.45	0.45
Total Phosphorus	0.69	0.69	0.69	0.69
Sodium	0.19	0.19	0.19	0.19
Nutrient	Analyzed Nutrient Content, %			
Protein	21.69	20.23	21.64	21.41
Total Lysine	1.29	1.35	1.31	1.34
Total Threonine	0.84	0.79	0.84	0.92

[1] Vitamin premix added at this rate yields 7700 IU vitamin A, 5500 ICU vitamin D3, 55 IU vitamin E, 1.5 mg vitamin K-3, 0.01 mg B12, 6.6 mg riboflavin, 38.5 mg niacin, 9.9 mg d-pantothenic acid, 0.88 mg folic acid, 2.75 mg pyroxidine, 1.54 mg thiamine, 0.08 mg biotin per kg diet

[2] Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[3] Active drug ingredient salinomycin sodium, 60 g/lb (60 g/lton inclusion; (b) (4) (b) (4) For the prevention of coccidiosis caused by Eimeria tenella, Eimeria necatrix, Eimeria acervulina, Eimeria maxima, Eimeria brunetti and Eimeria mivati.

[4] (b) (4)

[5] CJ America, Downers Grove, IL

[6] The level of cellulose (wt: wt) was adjusted based on the amount of L-Threonine.

Table 2.Grower dietary formulations, calculated nutrient content, and analyzed nutrient content of treatment diets fed to male broilers (14 to 28 days-of-age)

Ingredient	Positive Control[1]	Negative Control	NC +100	NC +150
	%			
Corn	66.20	66.20	66.20	66.20
Soybean Meal	27.95	27.95	27.95	27.95
Soybean Oil	1.72	1.72	1.72	1.72
Limestone	1.27	1.27	1.27	1.27
Salt, NaCl	0.46	0.46	0.46	0.46
Monocalcium Phosphate	1.51	1.51	1.51	1.51
DL-Methionine, 99%	0.27	0.27	0.27	0.27
L-Lysine HCl, 78.8%	0.23	0.23	0.23	0.23
Vitamin Premix [1]	0.13	0.13	0.13	0.13
Trace Mineral Premix[2]	0.05	0.05	0.05	0.05
Salinomycin – SaCox[3]	0.05	0.05	0.05	0.05
L-Threonine, 98.0%[4]	0.085	--	--	--
L-Threonine Biomass, 75%[5]	--	--	0.113	0.170
Cellulose, Filler (wt: wt)[11]	0.085	0.170	0.057	--
Nutrient	Calculated Nutrient Content, %			
AME, kcal/kg	3102	3102	3102	3102
Protein[12]	18.99	18.93	18.99	18.99
dLys	1.05	1.05	1.05	1.05
dMet	0.53	0.53	0.53	0.53
dSAA	0.80	0.80	0.80	0.80
dThr	0.69	0.61	0.69	0.73
dArg	1.12	1.12	1.12	1.12
dVal	0.80	0.80	0.80	0.80
Calcium	0.84	0.84	0.84	0.84
Non-Phytate Phosphorus	0.42	0.42	0.42	0.42
Total Phosphorus	0.65	0.65	0.65	0.65
Sodium	0.19	0.19	0.19	0.19
Nutrient	Analyzed Nutrient Content, %			
Protein	19.06	18.52	18.89	18.57
Total Lysine	1.23	1.22	1.22	1.18
Total Threonine	0.79	0.72	0.79	0.80

[1] Vitamin premix added at this rate yields 7700 IU vitamin A, 5500 ICU vitamin D3, 55 IU vitamin E, 1.5 mg vitamin K-3, 0.01 mg B12, 6.6 mg riboflavin, 38.5 mg niacin, 9.9 mg d-pantothenic acid, 0.88 mg folic acid, 2.75 mg pyroxidine, 1.54 mg thiamine, 0.08 mg biotin per kg diet

[2] Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[3] Active drug ingredient salinomycin sodium, 60 g/lb (60 g/lton inclusion; (b) (4) (b) (4) For the prevention of coccidiosis caused by Eimeria tenella, Eimeria necatrix, Eimeria acervulina, Eimeria maxima, Eimeria brunetti and Eimeria mivati.

[4] (b) (4)

[5] CJ America, Downers Grove, IL

[6] The level of cellulose (wt: wt) was adjusted based on the amount of L-Threonine.

Table 3. Body weight and body weight gain of male broilers fed diets that vary in threonine level and source.

	Body Weight			Weight Gain	
	Day 0 (g)	Day 14 (g)	Day 28 (kg)	Day 1-14 (g)	Day 14-28 (kg)
Treatment					
Positive Control (PC)	45.1	[redacted]	[redacted]	(b) (4) [redacted]	1.103
Negative Control (NC)	45.2	[redacted]	[redacted]	[redacted]	1.077
NC + Novel Threonine (100%)	45.2	[redacted]	[redacted]	[redacted]	1.100
NC + Novel Threonine (150%)	45.2	[redacted]	[redacted]	[redacted]	1.085
PSEM	0.0	[redacted]	[redacted]	[redacted]	0.006
P-value	*0.683*	[redacted]	[redacted]	[redacted]	*0.232*

a,b Means in columns with different groupings differ significantly at $p \leq 0.05$

Table 4. Mortality corrected feed conversion ratio of male broilers fed diets that vary in threonine level and source.

	Starter	Grower	Day 1-28
Treatment			
Positive Control (PC)	1.254	1.530^{b}	1.460^{b}
Negative Control (NC)	1.264	1.570^{a}	1.486^{a}
NC + Novel Threonine (100%)	1.255	1.539^{ab}	1.460^{b}
NC + Novel Threonine (150%)	1.256	1.567^{a}	1.479^{a}
PSEM	0.003	0.006	0.003
P-value	*0.291*	*0.034*	*0.006*

a,b Means in columns with different groupings differ significantly at $p \leq 0.05$

Table 5. Feed intake (g/bird/day) corrected for mortality of male broilers fed diets that vary in threonine level and source.

	Starter	Grower	Day 1-28
Treatment			
Positive Control (PC)	36.6[ab]	130.3	81.3
Negative Control (NC)	36.1[b]	129.8	80.9
NC + Novel Threonioe (100%)	37.3[a]	129.8	81.6
NC + Novel Threonine (150%)	37.0[a]	130.7	82.0
PSEM	0.2	0.5	0.3
P-value	*0.014*	*0.894*	*0.486*

a,b Means in columns with different groupings differ significantly at $p \leq 0.05$

Table 6. Mortality (%) of male broilers fed diets that vary in threonine level and source.

	Starter	Grower	Day 1-28
Treatment			
Positive Control (PC)	1.80	0.00	1.82
Negative Control (NC)	1.50	0.61	2.12
NC + Novel Threonine (100%)	1.80	0.00	1.82
NC + Novel Threonine (150%)	0.30	0.30	0.61
PSEM	0.26	0.17	0.29
P-value	*0.144*	*0.552*	*0.277*

a,b Means in columns with different groupings differ significantly at $p \leq 0.05$

Observations

Removal of L-threonine negatively impacted average male BW of the NC fed broilers compared to the PC fed broilers. Additional of the novel threonine source to equivalent levels of the PC increased BW and reduced FCR.

APPENDIX 7— Acute Toxicity Report

(b) (4)

Report

L-Threonine: Acute Oral Toxicity in the Rat – Fixed Dose Method

(b) (4)	WW59WG
Sponsor Name:	CJ CheilJedang Corporation
Issue Date:	05 January 2017
Study Director:	(b) (4)
Testing Facility:	

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COMPLIANCE WITH GOOD LABORATORY PRACTICE

L-Threonine: Acute Oral Toxicity in the Rat – Fixed Dose Method

With the exception stated below the study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

- The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994)
- OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17
- EC Commission Directive 2004/10/EC of 11 February 2004

These principles of Good Laboratory Practice are accepted by the members of the OECD Mutual Acceptance of Data including the European Community/United States of America and Japan.

Due to the short-term nature of the study no analysis was carried out to determine the homogeneity, concentration or stability of the test item formulation. This exception is considered not to affect the purpose or integrity of the study.

(b) (6)

Study Director

(b) (4)

5/1/17

Date

Report (b) (4) WW59WG

QUALITY ASSURANCE STATEMENT

L-Threonine: Acute Oral Toxicity in the Rat – Fixed Dose Method

Study based activities at the Test Facility, (b) (4) were audited and inspected. The details of these audits and inspections are given below.

Type of Inspection	Date(s) of Inspection	Date Reporting to Study Director, Test Facility Management
Study Plan Verification	05 August 2016	05 August 2016
Process – based Test Item Preparation	03 August 2016	03 August 2016
Process – based Test System Preparation and Application	03 August 2016	03 August 2016
Process – based Assessment of Response	08 August 2016	08 August 2016
Process – based Necropsy	09 August 2016	09 August 2016
Report Audit	21 December 2016	21 December 2016

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

Quality Assurance

(b) (4)

QA Auditor

04 JAN 2017

Date

(b) (4)

1 SUMMARY

Introduction

The study was performed to assess the acute oral toxicity of the test item in the Wistar strain rat.

Methods

Following a sighting test at dose levels of 300 mg/kg and 2000 mg/kg, a further group of four fasted females was given a single oral dose of test item, as a solution in distilled water, at a dose level of 2000 mg/kg body weight. Clinical signs and body weight development were monitored during the study. All animals were subjected to gross necropsy.

Results

Mortality. There were no deaths.

Clinical Observations. There were no signs of systemic toxicity.

Body Weight. All animals showed expected gains in body weight.

Necropsy. No abnormalities were noted at necropsy.

Conclusion

The acute oral median lethal dose (LD_{50}) of the test item in the female Wistar strain rat was estimated to be greater than 2000 mg/kg body weight (Globally Harmonized Classification System – Unclassified).

The test item does not meet the criteria for classification according to Regulation (EC) No. 1272/2008, relating to the Classification, Labelling and Packaging of Substances and Mixtures.

2 INTRODUCTION AND PURPOSE

2.1 Purpose

The study was performed to assess the acute oral toxicity of the test item in the Wistar strain rat.

2.2 Justification

Rats are the preferred species of choice as historically used for safety evaluation studies and are specified in the appropriate test guidelines.

2.3 Study Details

Sponsor

CJ CheilJedang Corporation
CJ CheilJedang Building
292 Ssangnim-dong
Jung-gu
Seoul 100-400
KOREA

2.4 Study Schedule

Experimental start date	02 November 2016
Experimental completion date	05 December 2016

2.5 Animal Welfare

The study was designed and conducted to cause the minimum suffering or distress to the animals consistent with the scientific objectives and in accordance with the (b) (4) (b) (4) policy on animal welfare and the requirements of the United Kingdom's Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012. The conduct of the study may be reviewed, as part of the (b) (4) Ethical Review Process.

The study was conducted in accordance with the UK Home Office Guidance document on Regulatory Toxicology and Safety Evaluation Studies and the OECD guidance document on recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation.

2.6 Regulatory Testing Guidelines

The study was performed in compliance with the following regulations or guidelines:

- OECD Guideline for Testing of Chemicals No 420 "Acute Oral Toxicity - Fixed Dose Method" (2001)
- Method B1 bis Acute Toxicity (Oral) of Commission Regulation (EC) No. 440/2008

3 MATERIALS AND TEST METHODS

3.1 Test Item and Supporting Information

Information as provided by the Sponsor. A Certificate of Analysis supplied by the Sponsor is given in Annex 1.

Identification:	L-Threonine
Batch:	T75-16-02A5-29
Purity:	75.2%
Physical state/Appearance:	brown granular solid
Expiry Date:	29 May 2019
Storage Conditions:	room temperature in the dark

3.1.1 Test Item Preparation and Analysis

For the purpose of the study the test item was freshly prepared, as required, as a solution in distilled water.

The test item was formulated within 2 hours of being applied to the test system. It is assumed that the formulation was stable for this duration.

No analysis was conducted to determine the homogeneity, concentration or stability of the test item formulation. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

3.2 Test System

3.2.1 Animal Information

Female Wistar (RccHan™:WIST) strain rats were supplied by (b) (4) (b) (4) UK. On receipt the animals were randomly allocated to cages. The females were nulliparous and non-pregnant. After an acclimatization period of at least 5 days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were 8 to 12 weeks of age. The body weight variation did not exceed ±20% of the mean body weight at the start of treatment.

3.2.2 Animal Care and Husbandry

The animals were housed in groups of up to four in suspended solid-floor polypropylene cages furnished with woodflakes. With the exception of an overnight fast immediately before dosing and for approximately 3 to 4 hours after dosing, free access to mains drinking water and food ((b) (4) supplied by (b) (4) UK) was allowed throughout the study. The diet, drinking water and bedding were routinely

analyzed and were considered not to contain any contaminants that would reasonably be expected to affect the purpose or integrity of the study.

The temperature and relative humidity were set to achieve limits of 19 to 25 °C and 30 to 70% respectively. The rate of air exchange was at least fifteen changes per hour and the lighting was controlled by a time switch to give 12 hours continuous light and 12 hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.3 Study Design

In the absence of data regarding the toxicity of the test item, 300 mg/kg was chosen as the starting dose.

A single animal was treated as follows:

Dose Level (mg/kg)	Concentration (mg/mL)	Dose Volume (mL/kg)	Number of Rats
			Female
300	30	10	1

In the absence of toxicity at a dose level of 300 mg/kg, an additional animal was treated as follows:

Dose Level (mg/kg)	Concentration (mg/mL)	Dose Volume (mL/kg)	Number of Rats
			Female
2000	200	10	1

In the absence of toxicity at a dose level of 2000 mg/kg, an additional group of animals was treated as follows:

Dose Level (mg/kg)	Concentration (mg/mL)	Dose Volume (mL/kg)	Number of Rats
			Female
2000	200	10	4

A total of five animals were therefore treated at a dose level of 2000 mg/kg in the study.

All animals were dosed once only by gavage, using a metal cannula attached to a graduated syringe. The volume administered to each animal was calculated according to the fasted body weight at the time of dosing. Treatment of animals was sequential. Sufficient time was allowed between each dose group to confirm the survival of the previously dosed animals.

Clinical observations were made 30 minutes, 1, 2, and 4 hours after dosing and then daily for 14 days. Morbidity and mortality checks were made twice daily, early and late during normal working days, and once daily at weekends and public holidays.

Individual body weights were recorded on Day 0 (the day of dosing) and on Days 7 and 14.

At the end of the observation period the animals were killed by cervical dislocation. All animals were subjected to gross necropsy. This consisted of an external examination and opening of the abdominal and thoracic cavities. The appearance of any macroscopic abnormalities was recorded. No tissues were retained.

3.4 Data Evaluation

The test item was classified according to Annex 3 of the OECD Guidelines for Testing of Chemicals No. 420 "Acute Oral Toxicity - Fixed Dose Method" (adopted 17 December 2001) as shown in the Flow Chart in Annex 3.

Evaluation of data included identification of the number of animals that died during the study (or that were killed for humane reasons), and determination of the nature, severity, onset and duration of the toxic effects. If possible, the signs of evident toxicity were described. Evident toxicity refers to the toxic effects of sufficient severity that administration of the next higher dose level could result in development of severe signs of toxicity and probable mortality. Effects on body weights and abnormalities noted at necropsy were also identified.

Using the mortality data obtained, an estimate of the acute oral median lethal dose (LD_{50}) of the test item was made.

The results were also evaluated according to Regulation (EC) No. 1272/2008 on the Classification, Labelling and Packaging of Substances and Mixtures.

3.5 Major Computerized Systems

The following computerized system was used in the study:

Delta Controls – ORCAview

4 DEVIATIONS FROM STUDY PLAN

There were no deviations from the Study Plan.

5 ARCHIVING

Records and documentation relating to this study (including electronic records) will be maintained in the archives of (b) (4) for a period of 2 years from the date on which the Study Director signs the final report. This will include Study Plan, raw data and final report that support the reconstruction of the study. Specimens that no longer afford evaluation will be discarded in accordance with Standard Operating Procedures and without further notice.

At termination of the aforementioned period, the Sponsor will be contacted in order to determine the final disposition of these records and materials. After the specified period, the Sponsor is responsible for all costs associated with the retention, retrieval, onward transfer or destruction/disposal of these materials. If the Sponsor is unresponsive, the records will be destroyed in accordance with the (b) (4) Standard Operating Procedure.

In case records are transferred, the Sponsor should ensure that the materials and records in support of regulatory studies are retained and maintained under conditions that guarantee their integrity and continued access according to archiving requirements of the principles of GLP. The Sponsor should also ensure that such materials and records are retained for as long as required by relevant authorities.

(b) (4) will retain in its archive the Study Plan and final report, and any amendments, indefinitely.

6 RESULTS

6.1 Dose Level - 300 mg/kg

Individual clinical observations and mortality data are given in Appendix 1.

6.1.1 Mortality

There was no mortality.

6.1.2 Clinical Observations

No signs of systemic toxicity were noted during the observation period.

6.1.3 Body Weight

Individual body weights and body weight changes are given in Appendix 2.

The animal showed expected gains in body weight over the observation period.

6.1.4 Necropsy

Individual necropsy findings are given in Appendix 3.

No abnormalities were noted at necropsy.

6.2 Dose Level - 2000 mg/kg

Based on the results at a dose level of 300 mg/kg, a dose level of 2000 mg/kg body weight was investigated.

Individual clinical observations and mortality data are given in Appendix 4.

6.2.1 Mortality

There were no deaths.

6.2.2 Clinical Observations

No signs of systemic toxicity were noted during the observation period.

6.2.3 Body Weight

Individual body weights and body weight changes are given in Appendix 5.

All animals showed expected gains in body weight over the observation period.

6.2.4 Necropsy

Individual necropsy findings are given in Appendix 6.

No abnormalities were noted at necropsy.

7 CONCLUSION

The acute oral median lethal dose (LD_{50}) of the test item in the female Wistar strain rat was estimated to be greater than 2000 mg/kg body weight (Globally Harmonized Classification System – Unclassified).

The test item does not meet the criteria for classification according to Regulation (EC) No. 1272/2008, relating to the Classification, Labelling and Packaging of Substances and Mixtures.

8 REFERENCES

(b) (4)

The Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012.

UK HOME OFFICE (2005) Guidance on the Conduct of Regulatory Toxicology and Safety Evaluation Studies.

APPENDICES

Appendix 1 Individual Clinical Observations and Mortality Data - 300 mg/kg

Dose Level mg/kg	Animal Number and Sex	Effects Noted After Dosing (Hours)				Effects Noted During Period After Dosing (Days)												
		½	1	2	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
300	1-0 Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

Appendix 2 Individual Body Weights and Body Weight Changes - 300 mg/kg

Dose Level mg/kg	Animal Number and Sex	Body Weight (g) at Day			Body Weight Gain (g) During Week	
		0	7	14	1	2
300	1-0 Female	165	185	198	20	13

Appendix 3 Individual Necropsy Findings - 300 mg/kg

Dose Level mg/kg	Animal Number and Sex	Time of Death	Macroscopic Observations
300	1-0 Female	Killed Day 14	No abnormalities detected

Appendix 4 Individual Clinical Observations and Mortality Data - 2000 mg/kg

Dose Level mg/kg	Animal Number and Sex	Effects Noted After Dosing (Hours)				Effects Noted During Period After Dosing (Days)													
		½	1	2	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2000	2-0 Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3-0 Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3-1 Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3-2 Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3-3 Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

Appendix 5 Individual Body Weights and Body Weight Changes - 2000 mg/kg

Dose Level mg/kg	Animal Number and Sex	Body Weight (g) at Day			Body Weight Gain (g) During Week	
		0	7	14	1	2
2000	2-0 Female	154	170	189	16	19
	3-0 Female	177	190	210	13	20
	3-1 Female	167	189	200	22	11
	3-2 Female	174	188	198	14	10
	3-3 Female	185	190	214	5	24

Appendix 6 Individual Necropsy Findings - 2000 mg/kg

Dose Level mg/kg	Animal Number and Sex	Time of Death	Macroscopic Observations
2000	2-0 Female	Killed Day 14	No abnormalities detected
	3-0 Female	Killed Day 14	No abnormalities detected
	3-1 Female	Killed Day 14	No abnormalities detected
	3-2 Female	Killed Day 14	No abnormalities detected
	3-3 Female	Killed Day 14	No abnormalities detected

ANNEXES

Annex 1 Certificate of Analysis

CJ Research Institute of Biotechnology

42, Gwanggyo-ro, Yeongtong-gu, Suwon-si,
Gyeonggi-do,Korea
www.cj.co.kr
TEL : 031) 8099-2450 FAX : 031) 8099-2913

CJ CHEILJEDANG

Result of analysis

Certificate No.	2016-AN-033	Receipt No.	2016-AR-033
Client		Date of Receipt	2016-05-19
Client Name		Date of Test	2016-05-19
Client Tel		Use of Report	Reference test

Test Sample	L-Threonine
Manuf. Date	2016-05-29
Expiry Date	2019-05-29
Lot. No	T75-16-02A5-29
Quantity (kg)	

Test Item(s)	Test Result
L-Threonine	(b) (4)

• Information

• Temperature : (22~28) C, Relative Humidity : (30~50) %
• N.D : not detected (not quantifiable)
• The results shown in this test report refer only to the sample tested unless otherwise stated.
The Test Report cannot be reproduced, except in full

Tested by Taek Hee Nam

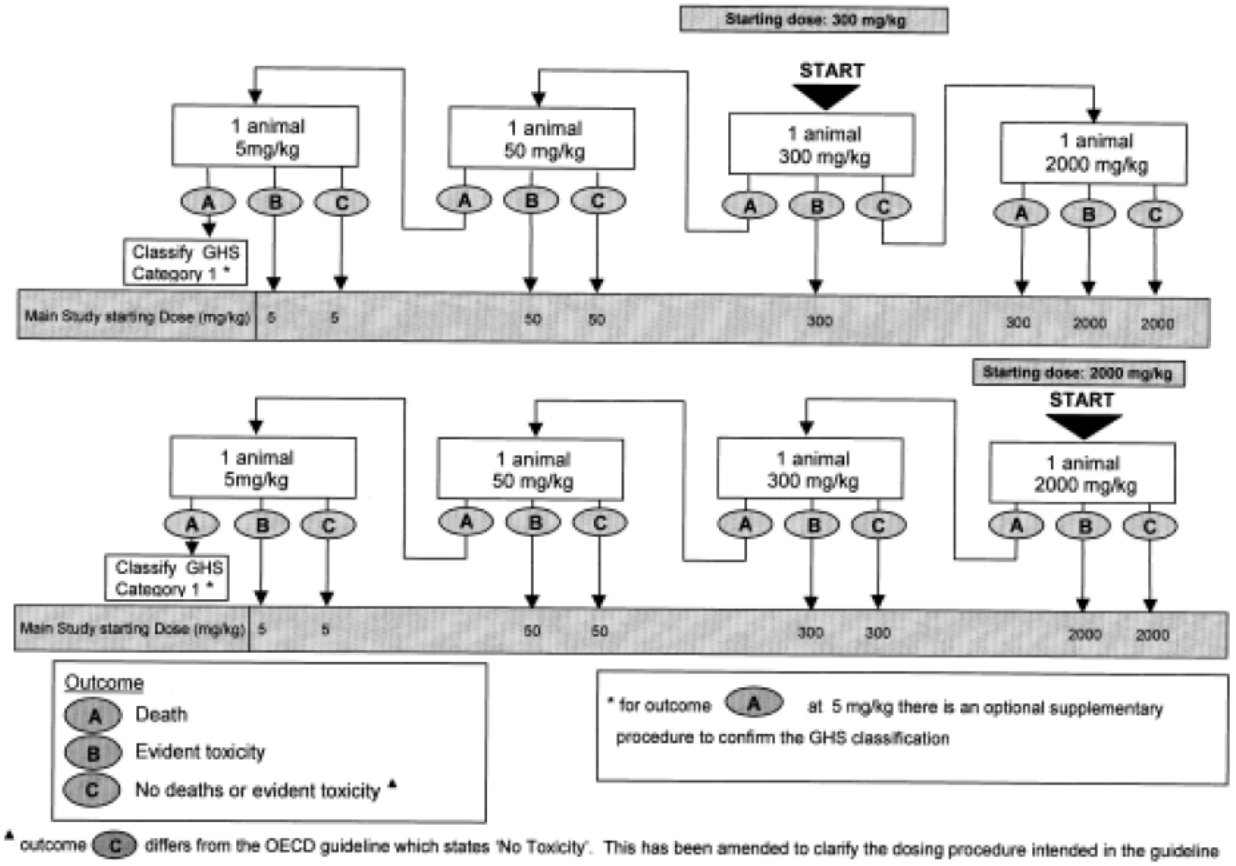
Approved by Technical Manager Seok Hun Yun

July 11 2016

CJ Research Institute of Biotechnology, BIO)Analysis Team

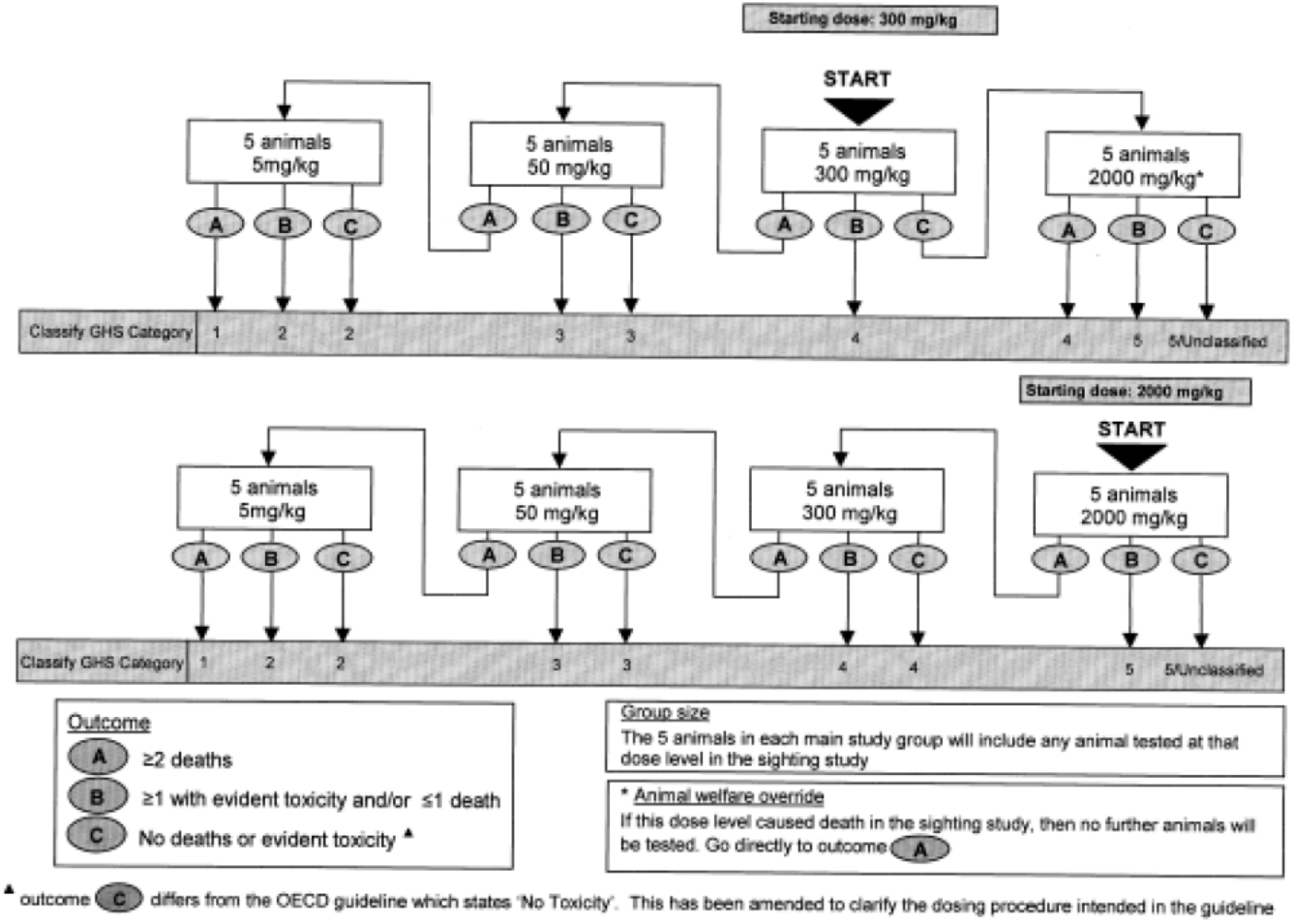
CJ BIO-AD form 100-01 REV.01

Annex 2 Flow Chart for the Sighting Test



▲ outcome C differs from the OECD guideline which states 'No Toxicity'. This has been amended to clarify the dosing procedure intended in the guideline

Annex 3 Flow Chart for the Main Test



▲ outcome C differs from the OECD guideline which states 'No Toxicity'. This has been amended to clarify the dosing procedure intended in the guideline

Annex 4 GLP Certificate

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

(b) (4)

TEST TYPE(S)

Analytical/Clinical Chemistry
Environmental Fate
Environmental Toxicity
Physical/Chemical Testing
Mutagenicity
Toxicology

DATE OF INSPECTION: 05/07/2016

DATE OF ISSUE: 28/11/2016

An Inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above named test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above named test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999).

Issued by
Dr Andrew J Gray
Head, UK GLP Monitoring Authority

Medicines & Healthcare products Regulatory Agency

MHRA

APPENDIX 8—Bacterial Reverse Mutation Assay

FINAL REPORT

Bacterial Reverse Mutation Assay with L-Threonine

Study Number: 18-VG-0143

Sponsor: CJ Cheiljedang BLOSSOM PARK,
BIO R&D Research Center

(b) (4)

(b) (4)

(b) (4) No. 18-VG-0143

GLP Compliance Statement

Bacterial Reverse Mutation Assay with L-Threonine

This study was conducted in accordance with OECD principles of Good Laboratory Practice (1997) ENV/MC/CHEM (98)17 and Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR, Part 58, US FDA, Revised as of April 1, 2017).

The study was performed following the approved protocol and SOPs in (b) (4) (b) (4) and the study objective defined in the protocol was achieved. There were no circumstances that may have affected the reliability of the data.

(b) (6)

May 08. 2018
Date

Study director

Address: (b) (4)

Contact: (b) (4)

E-mail: (b) (6)

Signature Page

(b) (6)

Study Director

(b) (4)

May 08. 2018
Date

(b) (6)

Management

(b) (4)

May 08, 2018
Date

Hyewon Um

Hyewon Um

Sponsor's representative

CJ Cheiljedang BLOSSOM PARK,
BIO R&D Research Center

May 04, 2018
Date

Quality Assurance Statement

Study Number: 18-VG-0143

Title: Bacterial Reverse Mutation Assay with L-Threonine

Study period: Apr 04, 2018 - May 08, 2018

Sponsor: CJ Cheiljedang BLOSSOM PARK, BIO R&D Research Center

Items	Inspected on	Inspection results confirmed to Study Director on	Inspection results reported to Management on
Protocol	Apr 03, 2018	Apr 04, 2018	Apr 05, 2018
Preparation of media and Inoculation of strains	Apr 10, 2018	Apr 10, 2018	Apr 11, 2018
Storage of test /reference article	Apr 11, 2018	Apr 11, 2018	Apr 12, 2018
Preparation of test /reference article	Apr 11, 2018	Apr 11, 2018	Apr 12, 2018
Status of bacterial strains	Apr 11, 2018	Apr 11, 2018	Apr 12, 2018
Identification	Apr 11, 2018	Apr 11, 2018	Apr 12, 2018
Chemical treatment	Apr 11, 2018	Apr 11, 2018	Apr 12, 2018
Scoring plates	Apr 13, 2018	Apr 13, 2018	Apr 19, 2018
Raw data	May 02, 2018	May 03, 2018	May 04, 2018
Final report (draft)	May 02, 2018	May 03, 2018	May 04, 2018
Final report	May 08, 2018	May 08, 2018	May 08, 2018

Hereby, I do certify that the detailed method in this final report was performed in accurately with OECD Guideline for Testing of Chemicals TG 471 (1997) 'Bacterial Reverse Mutation Test' and the raw data obtained in this study were reflected accurately in the final report and this study was performed in conformity with OECD Principles of Good Laboratory Practice (b) (4) and Good Laboratory Paractice for Nonclinical Laboratory Studies (21 CFR, Part 58, US FDA, Revised as of April 1, 2017)

Date: May 08, 2018

(b) (4)

Quality Assurance Person (b) (6)

Quality Assurance Manager

Study overview

Title	Bacterial Reverse Mutation Assay with L-Threonine
Objective	The objective of this study was to evaluate the test article, L-Threonine, for its ability to induce reverse mutation in the four histidine-requiring TA strains of *Salmonella typhimurium* and a tryptophan-requiring strain *Escherichia coli* WP2 *uvr*A.
Regulatory guideline	OECD Guideline for Testing of Chemicals TG 471 (1997) 'Bacterial Reverse Mutation Test'
Sponsor	CJ Cheiljedang BLOSSOM PARK, BIO R&D Research Center. CJ Blossom Park, 42, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16495, Republic of Korea +82-31-8099-2117 (TEL) , +82-31-8099-2901 (FAX) Sponsor's representative: Hyewon Um
Test Facility	(b) (4) Management: (b) (6)
Schedule	Apr 04, 2018: Approval of protocol (study initiation) Apr 10, 2018: Inoculation of test strains (experiment initiation) Apr 11, 2018: Chemical treatment Apr 13, 2018: Scoring plates (experiment completion) May 03, 2018: Submission of draft report May 08, 2018: Submission of final report
Contributing Scientists	Preparation/Storage of the Test article: Cell lines management: Main study person Archives: (b) (6)

Archives The protocol, final report, raw data, sample of the test article and other relevant evidential documents will be retained and stored in the Archives of (b) (4) (b) (4) for at least 5 years after the submission of final report for marketing authorization (US FDA basis).

Further storage of above materials shall be consulted with the sponsor.

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Summary

The test article, L-Threonine, was evaluated for its potential to induce reverse mutation in the four histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A in the presence and absence of exogeneous metabolic activation system.

The metabolic activation system consisting of the cofactor-supplemented post-mitochondrial fraction (S9) of liver homogenate from rats pretreated with Aroclor 1254 was used. The test strains were exposed to the test article using the direct plate incorporation method.

Test article for treatment was suspended in sterile distilled water for injection and serial dilutions were made. The dose ranges are presented in the table below. Concurrent negative and positive controls were also included, and triplicate plates were used for each dose.

Test strains	S9 mix	Dose (µg/plate)					
TA strains	+/-	12	37	111	333	1000	3000
WP2 *uvr*A	+/-	12	37	111	333	1000	3000

No substantial increases in numbers of revertants per plate of any of the test strains were observed following treatment with the test article at any dose level. There was no indication of cytotoxicity over the range of doses tested.

The mean revertant of the positive control for each test strain exhibited a clear increase over the mean revertant of the negative control for that strain.

The results indicate that the test article, L-Threonine, was not mutagenic in this bacterial assay system.

Materials and Methods

1. Test and reference articles

1) Test article (Appendix 5)

Name:	L-Threonine
Code No.:	C-2860
Lot No.:	T75-16-01A6-29
Date of receipt:	Feb 19, 2018
Amount:	10 g / tube × 1 tube
Appearance:	Pale brown granule
Purity:	L-Threonine 77.2%
Expiration date:	Jun 28, 2019
Storage conditions:	Room temperature
Supplier:	CJ Cheiljedang BLOSSOM PARK, BIO R&D Research Center

2) Vehicle (negative control article)

Name:	Sterile distilled water for injection
Lot No.:	48R7F95
Storage condition:	Room temperature (Refrigeration after opening)
Supplier:	(b) (4)
Justification of selection	The vehicle was selected according to the preliminary preparation.

3) Positive control articles

Positive control articles used in this study are listed in the following table. These positive control articles are among those recommended in the OECD guideline TG 471.

Metabolic activation	Positive controls (Abbr.)	CAS No.	Test Strains	Dose (μg/plate)
+	2-Aminoanthracene (2-AA)	613-13-8	TA100	1
			TA1535	2
			TA1537	1
			WP2 *uvr*A	6
	Benzo[a]pyrene (B[a]P)	50-32-8	TA98	1
-	Sodium azide (SA)	26628-22-8	TA100	0.5
			TA1535	0.5
	2-Nitrofluorene (2-NF)	607-57-8	TA98	2
	4-Nitroquinoline-1-oxide (4NQO)	56-57-5	WP2 *uvr*A	0.5
	Acridine Mutagen ICR 191(ICR-191)	17070-45-0	TA1537	0.5

Name	Supplier	Item No.	Lot No.	Date of Received	Storage Condition
2-AA	(b) (4)	A38800	(b) (4)	May 30, 2017	11 to 30 °C
B[a]P		48564		Jun 22, 2016	11 to 30 °C
SA		S8032		Oct 19, 2015	11 to 30 °C
2-NF		N16754		May 30, 2017	11 to 30 °C
4NQO		N8141		Mar 09, 2017	Below -15 °C
ICR-191		I3636		May 30, 2017	-1 to 10 °C

2. Preparation and analysis of dose formulation

1) Preparation of dose formulations

The test article was used without compensation for purity. The test article was weighed and mixed with vehicle by using a vortex mixer to make the highest dose. The highest dose was diluted with the same vehicle to make lower doses. The preparation was done just before treatment.

2) Preparation of positive control articles

Frozen stock solutions of SA which has been prepared with sterile distilled water for injection (DAIHAN PHARM Co., Ltd., Lot No. 48R7F95) was kept at below -15 °C. Stock solutions of 2-AA, B[a]P, 2-NF, 4NQO and ICR-191 prepared with DMSO (Sigma-Aldrich Co., #472301-500ML, Lot No. SHBH9867, ≥99.9 %) were kept frozen below -50 °C (B[a]P) and -15 °C (2-AA, 2-NF, 4NQO and ICR-191), respectively. The stock solutions were thawed just before the treatment.

3) Analysis of dose formulation

The dose formulation was not analyzed for concentration and stability.

3. Test system

1) Test system justification

The histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 (Maron and Ames, 1983) and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A (Green and Muriel, 1976) were used. These test strains are among those recommended by the test guideline of OECD TG 471. These strains have been shown to be sensitive to the mutagenic activity of a wide range of chemical classes. The specific genotypes of the test strains and detectable mutations are listed below.

Test strains	*his*/*trp* mutation	Additional mutation	Plasmid	Detection of mutation
TA100	*his*G46	*rfa* *uvr*B	pKM101	Base-pair substitution
TA1535	*his*G46	*rfa* *uvr*B	-	Base-pair substitution
TA98	*his*D3052	*rfa* *uvr*B	pKM101	Frame-shift
TA1537	*his*C3076	*rfa* *uvr*B	-	Frame-shift
WP2 *uvr*A	*trp*E	*uvr*A	-	Base-pair substitution

The *rfa* mutation in TA strains results in the partial loss of the lipopolysaccharide (LPS) barrier of cell wall and the mutation make the barrier more permeable to certain classes of large molecules. The *uvr*A or *uvr*B is essential for excision repair system of the test strain. Mutations of these genes result in a deficient DNA repair system and greatly enhance the sensitivity of these strains to some mutagens. The presence of plasmid pKM101 further increases the sensitivity of these strains to some mutagens.

2) Source of test strains and media

Source of test strains

Test strains were obtained from (b) (4) (b) (4) and subcultured in the (b) (4)

Culturing broth

The broth used to grow the test strains for mutagenicity assay was 2.5 % Oxoid Nutrient Broth No. 2 prepared in distilled water.

Minimal glucose agar (bottom agar) plates

The minimal glucose agar (25 mL per 15 x 90 mm petri dish) was Vogel-Bonner medium E supplemented with 1.5 % Bacto agar (Difco) and 2 % glucose. The minimal glucose agar for the WP2 *uvr*A strain was supplemented with additional 0.25 mL/L of 0.1 % L-tryptophan. Gamma ray-sterilized petri dishes were used.

Top agar

Top agar for selection of revertants was prepared with 0.6 % Bacto agar (Difco) and 0.5 % NaCl. The top agar for *Salmonella* strains was supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL.

3) Storage of test strains and phenotypic characterization

Frozen stocks of test strains

Frozen stock cultures for long-term storage were prepared from fresh overnight cultures. DMSO was added to the cultures (90 µL/mL) as a cryopreservative, and aliquots of cultures were stored at below -70 °C.

Master plates

The frozen stocks were thawed and cultured for 10 hours to prepare master plates of test strains. A part of each bacterial culture was used for the confirmation of genotypes. After confirming the genetic characteristics of the strains, then the master plates were used as the source of bacteria for mutagenicity assays.

Verification of genetic characteristics

The following genetic characteristics of the strains were verified according to the methods of Maron and Ames (1983).

Phenotypes	Test strains
histidine requirement	*Salmonella typhimurium* TA strains
presence of *uvr*B mutation	*Salmonella typhimurium* TA strains
presence of R-factor	*Salmonella typhimurium* TA strains
presence of *rfa* mutation	*Salmonella typhimurium* TA strains
number of spontaneous revertant	*Salmonella typhimurium* TA strains and *E. coli* WP2 *uvr*A
tryptophan requirement	*E. coli* WP2 *uvr*A
presence of *uvr*A mutation	*E. coli* WP2 *uvr*A

4. Metabolic activation system (S9 mix)

1) S9 and cofactor

S9

Origin of S9: Aroclor 1254- induced male Sprague-Dawley rat liver

Supplier: (b) (4)

Item No.: 11-01L

Lot No.: 3871

Protein content: 40.4 mg/mL

Storage condition: Kept in a freezer (below -15 °C)

Cofactor

Name: Cofactor-I

Supplier (b) (4)

Item No.: 309-50611

Lot No.: 999703

Storage condition: Refrigeration (-1 to 10 °C)

2) Preparation of S9 mix (per 1 mL, 5 % S9 v/v)

The S9 mix was prepared with S9 and cofactor solution just before use. The S9 mix contained: 8 µmol $MgCl_2 \cdot 6H_2O$, 33 µmol KCl, 5 µmol G-6-P, 4 µmol NADPH, 4 µmol NADH, 100 µmol sodium phosphate buffer (pH 7.4) and 50 µL S9. Prepared S9 mix was placed in crushed ice.

5. Experimental procedures

1) Selection of dose range

Dose ranges of this study were selected based on the results of a range-finding test conducted on the test article using the five test strains in both the presence and absence of metabolic activation system with two plates per dose (b) (4) number: 18-VG-0142P, a non-GLP study]. Six doses of test article ranging 8 to 5000 µg/plate were tested using the same methods of this study. The condition of the treatment mixtures and plates were checked for the formation of precipitation and cytotoxicity, if any. In the range-finding test, turbidity and precipitation were observed in the treatment mixtures of 3000 and 5000 µg/plate. At the time of colony counting, precipitation also observed in the plates of 1000, 3000 and 5000 µg/plate. Colony counting was possible up to 1000 µg/plate. Colony counting was not possible at 3000 and 5000 µg/plate. There was no significant increase or decrease in numbers of colony in all test strains at all doses.

Therefore, the high dose of this study was set at 3000 µg/plate for all test strains with additional 5 lower dose levels. The dose ranges are presented in the table below. Concurrent negative and positive controls were also included, and triplicate plates will be used for each dose.

Test strains	S9 mix	Dose (µg/plate)					
TA strains	+/-	12	37	111	333	1000	3000
WP2 *uvrA*	+/-	12	37	111	333	1000	3000

2) Plating procedures and scoring of plates

The test strains were exposed to the test article using the direct plate incorporation method.

A small amount of bacterial growth in each master plate was taken and transferred to a flask containing 20 mL of liquid medium (2.5 % Oxoid Nutrient Broth No. 2). Inoculated flasks were incubated for 10 hours in a shaker/incubator (37 ± 2 °C, 120 rpm). Overnight cultures were removed from incubation and the viable cell counts were determined by optical density (OD) at 600 nm, and the cultures were stored in a refrigerator until use.

For the plating assay, the followings were added to each sterile culture tube containing 2 mL of top agar held at 45 ± 2 °C in a dry bath: 0.5 mL of S9 mix (or sodium-phosphate buffer, pH 7.4 for the non-activating plates), 0.1 mL of bacterial culture and 0.1 mL of test article. The contents were vortexed for 2 - 3 second and overlaid onto the surface of the bottom agar.

Negative control plates were treated with 0.1 mL of vehicle instead of test article. The positive control plates were treated with positive control articles with the same method.

The sterility of the highest dose test article solution was checked by plating a 0.1 mL aliquot (mixed with 2 mL of top agar) on the minimal glucose agar. S9 mix was also checked for sterility by plating 0.5 mL with the same method.

After the top agar solidified, plates were inverted and incubated at 37 ± 2 °C for 50 ± 2 hours and then revertant colonies were counted with unaided eyes.

3) Identification of plates

Each plate was labeled with an oil-based pen to identify the study number, test strain, dose level and activation condition.

4) Observations

The turbidity and/or precipitation in the treatment mixture were checked with unaided eyes, and if settlement of fine particle was observed, it was considered as precipitation.

Revertant colonies were counted with unaided eyes. The condition of background lawn was scored relative to the negative control, and contamination and other abnormality of each plate were checked.

A dose level was considered to be cytotoxic if at least one of the following criteria was met:

(1) A clearing or diminution (reduction) of the background lawn that was accompanied by a substantial reduction in the number of revertants per plate.

(2) The presence of microcolonies (pinpoint colonies).

There is no common standard of 'decrease' for the number of revertants, so it was determined if the number of revertants per plate was less than 50 % of that of solvent control or when there is a reversal of an increasing trend of the number of colonies.

5) Presentation of the results

Mean revertant per plate and standard deviation were calculated from the triplicate plates per dose. The actual numbers of revertant were also presented. The 'increase factor' was calculated by dividing the value of treated plate by the value of negative control plate. The increase factors were rounded off to one decimal place.

6) Assay acceptance criteria

The assay was considered valid only if all of the following criteria were met.

(1) At least 0.5×10^8 CFU of bacteria/plate were plated.

(2) A minimum of three non-toxic dose levels were required to evaluate assay data.

(3) The mean number of spontaneous revertants per plate should be within the range presented in the following table.

Test strains	No. Revertant
TA100	75-200
TA1535	3-37
TA98	15-60
TA1537	4-31
WP2 *uvr*A	5-40

(4) The mean revertants per plate of a positive control for a respective test strain should be at least a 2-fold increase over the mean revertants per plate of the negative control for that test strain. The integrity of the S9 mix should be demonstrated by increases of revertants for the positive control plates treated with B[a]P and with 2-AA.

(5) There should be no microbial colonies due to the contamination in the plates for sterility check of test article and S9 mix.

6. Statistics and evaluation of the results

1) Statistical analysis

No statistical analysis was done.

2) Evaluation of results

The result was regarded as positive if there was a dose-related increase over the range tested and/or a reproducible increase at one or more doses in the number of revertant per plate in at least one strain with or without metabolic activation system. A positive result indicates that the test substance induces point mutation in the test strain.

The result was regarded as negative if the result did not meet the positivity criteria. The negative result indicates that the test article is not mutagenic in the test strains. Biological relevance of the results was also considered for the evaluation of the results.

Results

Dose formulations

The test article was suspended in the vehicle and turbidity or precipitation was observed at 3000 μg/plate.

Bacterial reverse mutation test (Table 1, Appendix 1 and Appendix 2)

Turbidity and precipitation was observed at 3000 μg/plate when the prepared test article was mixed with the top agar. At 1000 and 3000 μg/plate, precipitation was observed on the bottom agar at the time of plate scoring. Colony counting was not possible at 3000 μg/plate. There was no microbial colony due to contamination in any of the plates for sterility check of test article and S9 mix.

There were no reductions of revertants or cytotoxicity in TA100, TA1535, TA98 and TA1537 at any dose level of test article both in the presence and absence of metabolic activation system. Also, no increase in revertants was observed.

In WP2 *uvr*A, there were no reductions of revertants or cytotoxicity at any dose level of test article both in the presence and absence of metabolic activation system. Also, no increase in revertants was observed.

The mean revertant of the positive control for each test strain exhibited a clear increase over the mean revertant of the negative control for that strain.

The viable cell counts of test strains were $1.85 - 2.60 \times 10^9$ (TA strains) and 2.53×10^9 (*E. coli*) CFU/mL, and more than 0.5×10^8 CFU of bacteria/plate were plated.

Discussion and Conclusion

All criteria for a valid assay were met. For all of the test strains, in the presence and absence of S9 mix, there were no significant increases of the revertants per plate in all test strains, and the experimental results failed to meet the criteria for positivity.

Therefore, it was concluded that the test article, L-Threonine, did not induce reverse mutation in the test strains used in this study.

References

1) Basic Mutagenicity Tests: UKEMS Recommended Procedures, Edited by David J. Kirkland, Cambridge University Press, 1990. ISBN 0-521-39347-7.

2) Green, MHL and Muriel, WJ (1976): Mutagen testing using *trp+* reversion in *Escherichia coli*., Mutat. Res., 38:3-32.

3) GREEN, MHL (1984) Mutagen testing using *trp+* reversion in *Escherichia coli* in KILBEY, BJ, LEGATOR, M, NICHOLS, W and RAMEL, C (Eds.). *Handbook of Mutagenicity Test Procedures. Second edition*, p.161-187. Elsevier Science Publishers BV, Amsterdam.

4) Maron, DM and Ames, BN (1983): Revised methods for the *Salmonella* mutagenicity test, Mutat. Res., 113:173-215.

5) Vogel, HJ and Bonner, DM (1956): Acetylornithinase of *E. coli*: Partial purification and some properties, J. Biol. Chem., 218:97-106 (1956).

6) (b) (4) historical control data, 2006-2017, (b) (4)

Units and Abbreviations

Note: The following lists of codes, abbreviations and units are used by (b) (4)
Some, but not necessarily all, of this information may be needed for this report.

%	Percent
°	Degree
C	Celsius
L	Liter
mL	Milliliter
µL	Microliter
g	Gram
kg	Kilogram
mg	Milligram
µg	Microgram
ng	Nanogram
m	Meter
cm	Centimeter
mm	Millimeter
µm	Micrometer
nm	Nanometer
hr	Hour
min	Minute
sec	Second
rpm	Revolution per Minute
G-6-P	Glucose-6-phosphate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
GLP	Good Laboratory Practice Regulation
MFDS	Ministry of Food and Drug Safety
OECD	Organization for Economic Co-operation and Development
QAU	Quality Assurance Unit
SD	Standard Deviation
SOP	Standard Operating Procedures
SPSS	Statistical Package for the Social Sciences
DKBT	Diplomated Korean Board of Toxicology

(b) (4) No. 18-VG-0143

TABLE

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Table 1. Reverse mutagenicity assay results –summary

Test Strain	Chemical Treated	Dose (μg/plate)	Colonies/plate [factor][a] With S9 mix	Without S9 mix
TA100	Test article	0	119 ± 6	118 ± 9
		12	111 ± 9 [0.9]	141 ± 18 [1.2]
		37	105 ± 5 [0.9]	130 ± 16 [1.1]
		111	122 ± 12 [1.0]	111 ± 6 [0.9]
		333	112 ± 12 [0.9]	101 ± 6 [0.9]
		1000	118 ± 13 [1.0]	105 ± 8 [0.9]
		3000 TP	- ± - [-]	- ± - [-]
TA1535	Test article	0	10 ± 1	10 ± 2
		12	9 ± 2 [0.9]	9 ± 1 [0.9]
		37	8 ± 4 [0.8]	11 ± 1 [1.1]
		111	12 ± 2 [1.2]	11 ± 1 [1.1]
		333	13 ± 2 [1.3]	11 ± 2 [1.1]
		1000	12 ± 1 [1.2]	10 ± 3 [1.0]
		3000 TP	- ± - [-]	± [-]
TA98	Test article	0	30 ± 2	24 ± 4
		12	28 ± 3 [0.9]	28 ± 2 [1.2]
		37	30 ± 3 [1.0]	29 ± 4 [1.2]
		111	30 ± 2 [1.0]	27 ± 4 [1.1]
		333	31 ± 3 [1.0]	27 ± 5 [1.1]
		1000	34 ± 5 [1.1]	29 ± 4 [1.2]
		3000 TP	- ± - [-	- ± - [-]
TA1537	Test article	0	14 ± 1	11 ± 1
		12	17 ± 1 [1.2]	9 ± 3 [0.8]
		37	15 ± 4 [1.1]	8 ± 1 [0.8]
		111	17 ± 2 [1.2]	10 ± 1 [1.0]
		333	13 ± 4 [1.0]	10 ± 3 [0.9]
		1000	12 ± 3 [0.9]	8 ± 1 [0.8]
		3000 TP	- ± - [-]	- ± - [-]
E. coli WP2 *uvr*A	Test article	0	26 ± 4	20 ± 4
		12	23 ± 3 [0.9]	22 ± 4 [1.1]
		37	25 ± 3 [0.9]	21 ± 2 [1.1]
		111	29 ± 2 [1.1]	23 ± 4 [1.2]
		333	20 ± 3 [0.8]	26 ± 3 [1.3]
		1000	22 ± 1 [0.9]	24 ± 2 [1.2]
		3000 TP	- ± - [-]	- ± - [-]
Positive controls				
TA100	2-AA	1.0	1312 ± 153 [11.1]	
TA1535	2-AA	2.0	210 ± 5 [21.0]	
TA98	B[a]P	1.0	258 ± 51 [8.6]	
TA1537	2-AA	1.0	193 ± 8 [14.1]	
WP2 *uvr*A	2-AA	6.0	83 ± 1 [3.2]	
TA100	SA	0.5		400 ± 35 [3.4]
TA1535	SA	0.5		433 ± 52 [43.3]
TA98	2-NF	2.0		257 ± 24 [10.7]
TA1537	ICR-191	0.5		183 ± 9 [17.2]
WP2 *uvr*A	4NQO	0.5		182 ± 10 [9.2]

Test article: L-Theronine
T: Turbidity in the treatment mixture
P: Precipitation in the treatment mixture
a) Three plates/dose were used. No. of colonies of treated plate/No. of colonies of negative control plate
Abbreviations
2-AA, 2-aminoanthracene; SA, sodium azide; B[a]P, benzo[a]pyrene; ICR-191, acridine mutagen ICR 191; 4NQO, 4-nitroquinoline N-oxide; 2-NF, 2-Nitrofluorene.

APPENDICES

Appendix 1. Reverse mutagenicity assay results – individual plate counts

Test Strain	Chemical Treated	Dose (ug/plate)	Colonies/plate (Status of background lawn[a]) With S9 mix			Without S9 mix		
TA100	Test article	0	117 (N)	114 (N)	125 (N)	120 (N)	108 (N)	126 (N)
		12	108 (N)	122 (N)	104 (N)	130 (N)	161 (N)	131 (N)
		37	110 (N)	102 (N)	102 (N)	148 (N)	126 (N)	116 (N)
		111	134 (N)	111 (N)	120 (N)	114 (N)	104 (N)	114 (N)
		333	125 (N)	102 (N)	108 (N)	106 (N)	102 (N)	95 (N)
		1000	108 (P)	113 (P)	132 (P)	108 (P)	112 (P)	96 (P)
		3000 TP	- (O)	- (O)	- (O)	- (O)	- (O)	- (O)
TA1535	Test article	0	10 (N)	11 (N)	9 (N)	12 (N)	9 (N)	9 (N)
		12	11 (N)	8 (N)	7 (N)	9 (N)	8 (N)	10 (N)
		37	8 (N)	12 (N)	5 (N)	11 (N)	12 (N)	10 (N)
		111	14 (N)	12 (N)	11 (N)	10 (N)	12 (N)	10 (N)
		333	15 (N)	13 (N)	12 (N)	11 (N)	13 (N)	9 (N)
		1000	12 (P)	13 (P)	12 (P)	7 (P)	13 (P)	11 (P)
		3000 TP	- (O)	- (O)	- (O)	- (O)	- (O)	- (O)
TA98	Test article	0	30 (N)	32 (N)	28 (N)	20 (N)	25 (N)	27 (N)
		12	31 (N)	25 (N)	27 (N)	30 (N)	28 (N)	26 (N)
		37	32 (N)	26 (N)	32 (N)	25 (N)	31 (N)	32 (N)
		111	29 (N)	32 (N)	28 (N)	25 (N)	25 (N)	32 (N)
		333	33 (N)	32 (N)	28 (N)	21 (N)	29 (N)	31 (N)
		1000	39 (P)	34 (P)	30 (P)	24 (P)	31 (P)	32 (P)
		3000 TP	- (O)	- (O)	- (O)	- (O)	- (O)	- (O)
TA1537	Test article	0	15 (N)	13 (N)	13 (N)	11 (N)	10 (N)	11 (N)
		12	18 (N)	16 (N)	17 (N)	12 (N)	7 (N)	8 (N)
		37	18 (N)	11 (N)	16 (N)	9 (N)	8 (N)	8 (N)
		111	16 (N)	16 (N)	19 (N)	10 (N)	10 (N)	11 (N)
		333	17 (N)	12 (N)	10 (N)	7 (N)	13 (N)	9 (N)
		1000	9 (P)	15 (P)	13 (P)	8 (P)	7 (P)	9 (P)
		3000 TP	- (O)	- (O)	- (O)	- (O)	- (O)	- (O)
E. coli WP2 *uvrA*	Test article	0	24 (N)	23 (N)	31 (N)	23 (N)	20 (N)	16 (N)
		12	25 (N)	23 (N)	20 (N)	25 (N)	23 (N)	17 (N)
		37	28 (N)	23 (N)	23 (N)	19 (N)	22 (N)	22 (N)
		111	28 (N)	28 (N)	32 (N)	20 (N)	21 (N)	27 (N)
		333	22 (N)	22 (N)	17 (N)	23 (N)	28 (N)	26 (N)
		1000	23 (P)	23 (P)	21 (P)	26 (P)	24 (P)	22 (P)
		3000 TP	- (O)	- (O)	- (O)	- (O)	- (O)	- (O)
Positive controls								
TA100	2-AA	1.0	1446 (N)	1145 (N)	1346 (N)			
TA1535	2-AA	2.0	215 (N)	207 (N)	207 (N)			
TA98	B[a]P	1.0	228 (N)	228 (N)	317 (N)			
TA1537	2-AA	1.0	197 (N)	197 (N)	184 (N)			
WP2 uvrA	2-AA	6.0	82 (N)	83 (N)	84 (N)			
TA100	SA	0.5				389 (N)	440 (N)	372 (N)
TA1535	SA	0.5				423 (N)	387 (N)	489 (N)
TA98	2-NF	2.0				285 (N)	243 (N)	244 (N)
TA1537	ICR-191	0.5				173 (N)	185 (N)	191 (N)
WP2 uvrA	4NQO	0.5				171 (N)	190 (N)	184 (N)

Test article: L-Theronine

T: Turbidity in the treatment mixture

P: Precipitation in the treatment mixture

a) Status of background lawn (BL) and plate

N, normal BL; R, reduced BL; A, absent or almost absent BL; E, enhanced BL; O, obscured BL by precipitation; P, precipitation of test article in plate; M, presence of microcolonies; C, contaminated plate.

Abbreviations

2-AA, 2-aminoanthracene; SA, sodium azide; B[a]P, benzo[a]pyrene; ICR-191, acridine mutagen ICR 191; 4NQO, 4-nitroquinoline N-oxide; 2-NF, 2-Nitrofluorene

Appendix 2. Viable cell counts of test strains and results of sterility tests

Test strain	Viable cell counts (10^9 CFU/mL)	Sterility of test article Solution (highest dose)	Sterility of S9 mix
TA100	2.07	No colony due to contamination	No colony due to contamination
TA1535	2.10		
TA98	1.85		
TA1537	2.60		
WP2 *uvrA*	2.53		

Appendix 3. Historical control data

(Reverse mutation assays in the histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A)

All negative (vehicle) controls [Jan 2006 – Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	95	86	5	5	15	11	3	4	13	10
Max	210	213	29	33	52	51	35	25	44	42
Mean	140	137	13	13	30	24	13	10	24	21
SD	25	24	4	4	7	6	4	3	5	5
Confidence	91	91	4.7	5.7	17	12	5.4	4.1	14	11
Intervals (95 %)	181	183	20	21	43	36	20	17	35	31
No. of plates	795	795	771	771	783	786	780	777	789	783

Sterile distilled water for Injection controls [Jan 2006 – Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	95	86	5	7	15	13	5	4	13	10
Max	210	213	27	27	52	51	35	24	44	42
Mean	139	137	12	13	30	24	13	10	25	21
SD	25	24	3	3	7	6	4	3	5	5
Confidence	90	90	5.3	6.2	17	12	5.5	4.4	14	11
Intervals (95 %)	187	184	18	19	44	37	20	16	35	31
No. of plates	396	396	381	381	387	390	384	384	393	390

Dimethyl sulfoxide controls [Jan 2006 – Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	95	88	6	5	15	11	3	4	13	10
Max	198	207	29	33	51	44	28	25	39	39
Mean	139	135	13	13	29	23	13	10	24	20
SD	26	24	4	4	6	6	4	3	5	5
Confidence	89	89	5.1	5.4	17	11	5.2	3.8	14	11
Intervals (95 %)	190	181	20	21	42	35	21	17	34	30
No. of plates	321	321	312	312	318	318	315	315	318	315

Positive controls [a)] [Jan 2006 – Dec 2017]

Strain	TA100		TA1535		TA98		TA1537		WP2 *uvr*A	
S9 mix	+	-	+	-	+	-	+	-	+	-
Min	360	180	47	62	78	116	46	31	68	48
Max	2832	820	484	648	532	486	711	724	308	424
Mean	1106	465	160	296	212	290	158	175	142	164
SD	515	95	67	82	81	73	74	102	45	65
Confidence	95.5	278	28.22	134	53.4	146	12.3	-25	53.7	36.5
Intervals (95 %)	2116	651	93	457	371	435	304	374	229	291
No. of plates	567	768	744	744	606	498	753	651	558	756

a) See Table 1 for names of positive control articles and doses/plate

Appendix 4. Protocol

(b) (4)

PROTOCOL

Bacterial Reverse Mutation Assay with L-Threonine

Study Number: 18-VG-0143

Approval:

(b) (4)

Apr 04, 2018
Date

(b) (4)

Apr 05, 2018
Date

Hyewon Um

Apr 09 2018
Date

Hyewon Um
Sponsor's representative
CJ Cheiljedang BLOSSOM PARK,
BIO R&D Research Center

ORIGINAL
시험책임자

(b) (4) No. 18-VG-0143

Title	Bacterial Reverse Mutation Assay with L-Threonine
Objective	The objective of this study is to evaluate the test article, L-Threonine, for its ability to induce reverse mutation in the four histidine-requiring TA strains of *Salmonella typhimurium* and a tryptophan-requiring strain *Escherichia coli* WP2 *uvr*A.
Regulatory guideline	OECD Guideline for Testing of Chemicals TG 471 (1997) 'Bacterial Reverse Mutation Test'
Sponsor	CJ Cheiljedang BLOSSOM PARK, BIO R&D Research Center CJ Blossom Park, 42, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16495, Republic of Korea +82-31-8099-2117 (TEL) , +82-31-8099-2901 (FAX)
Test Facility	(b) (4)

Schedule		
	Apr 10, 2018	Inoculation of test strains (experimental initiation)
	Apr 11, 2018	Chemical treatment
	Apr 13, 2018	Scoring plates (experimental completion)
	May 08, 2018	Submission of draft report due date (expected data)

Contributing Scientists	Preparation/Storage of the test article: Cell lines management: Main study person: Archives:	(b) (6)

(b) (4) No. 18-VG-0143

Archives [SOP-AC-001~007]

The protocol (amendment and deviation, if any), raw data, sample of test article and other relevant evidential documents will be stored in the Archives of (b) (4) (b) (4) for at least 5 years after the submission of final report for marketing authorization (US FDA basis).

Further storage of above materials shall be consulted with the sponsor.

GLP compliance

OECD Principles of Good Laboratory Practice (1997) (b) (4)

Good Laboratory Paractice for Nonclinical Laboratory Studies (21 CFR, Part 58, US FDA, Revised as of April 1, 2017)

The amendments and deviation from the protocol (if any) will be documented, reviewed by Quality Assurance Unit (QAU), and approved by the study director, management and sponsor.

The QAU of (b) (4) inspects solely throughout the progression of study.

Final report [SOP-TO-007]

The final report will fully reflect the contents of the present protocol and consist of (but not limited to) cover page, statement of GLP compliance, quality assurance statement, synopsis, contents, summary, materials and methods, results, discussion and conclusion, references, tables and appendices.

1. Test and reference articles

1) Test article [SOP-TA-001]

Name:	L-Threonine
Code No.:	C-2860
Lot No.:	T75-16-01A6-29
Date of receipt:	Feb 19, 2018
Amount:	10 g / tube × 1 tube
Appearance:	Pale brown granule
Purity:	L-Threonine 77.2%
Expiration date:	Jun 28, 2019
Storage conditions:	Room temperature
Supplier:	CJ Cheiljedang BLOSSOM PARK, BIO R&D Research Center

2) Vehicle (Negative control)

Name:	Sterile distilled water for injection
Lot No.:	48R7F95
Supplier:	Room temperature (Refrigeration after opening)
Storage condition:	(b) (4)
Justification of selection:	The vehicle was selected according to the preliminary preparation.

3) Positive control article

Positive control articles for this study are listed in the following table. These positive control articles are among those recommended in the OECD guideline TG 471.

Metabolic activation	Positive controls (Abbr.)	CAS No.	Test Strains	Dose (μg/plate)
+	2-Aminoanthracene (2-AA)	613-13-8	TA100	1
			TA1535	2
			TA1537	1
			WP2 *uvr*A	6
	Benzo[a]pyrene (B[a]P)	50-32-8	TA98	1
-	Sodium azide (SA)	26628-22-8	TA100	0.5
			TA1535	0.5
	2-Nitrofluorene (2-NF)	607-57-8	TA98	2
	4-Nitroquinoline-1-oxide (4NQO)	56-57-5	WP2 *uvr*A	0.5
	Acridine Mutagen ICR 191 (ICR-191)	17070-45-0	TA1537	0.5

(b) (4) No. 18-VG-0143

Name	Supplier	Item No.	Lot No.	Date Received	Storage Condition
2-AA	(b) (4)	A38800	(b) (4)	May 30, 2017	11 to 30 °C
B[a]P		48564		Jun 22, 2016	11 to 30 °C
SA		S8032		Oct 19, 2015	11 to 30 °C
2-NF		N16754		May 30, 2017	11 to 30 °C
4NQO		N8141		Mar 09, 2017	Below -15 °C
ICR-191		I3636		May 30, 2017	-1 to 10 °C

2. Preparation and analysis of dose formulation

1) Preparation of dose formulations [SOP-TA-002]

The test article will be used without compensation for purity. The test article will be weighed and mixed with vehicle by using a vortex mixer to make the highest dose. The highest dose will be diluted with the same vehicle to make lower doses. The preparation will be done just before treatment.

2) Preparation of positive control articles

Frozen stock solutions of SA which has been prepared with sterile distilled water for injection (b) (4) was kept at below -15 °C. Stock solutions of 2-AA, B[a]P, 2-NF, 4NQO and ICR-191 prepared with DMSO (Sigma-Aldrich Co., #472301-500ML, Lot No. SHBH9867, ≥99.9 %) were kept frozen below -50 °C (B[a]P) and -15 °C (2-AA, 2-NF, 4NQO and ICR-191), respectively. The stock solutions will be thawed just before the treatment.

3) Analysis of dose formulation

The dose formulation will not be analyzed for concentration and stability.

3. Test system

1) Test system justification

The histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 (Maron and Ames, 1983) and a tryptophan auxotroph strain of *Escherichia coli* WP2 *uvr*A (Green and Muriel, 1976) will be used. These test strains are among those recommended by the test guideline of the OECD TG 471. These strains have been shown to be sensitive to the mutagenic activity of a wide range of chemical classes. The specific genotypes of the test strains and detectable mutations are listed below.

Test strains	*his*/*trp* mutation	Additional mutation	Plasmid	Detection of mutation
TA100	*his*G46	*rfa* *uvr*B	pKM101	Base-pair substitution
TA1535	*his*G46	*rfa* *uvr*B	-	Base-pair substitution
TA98	*his*D3052	*rfa* *uvr*B	pKM101	Frame-shift
TA1537	*his*C3076	*rfa* *uvr*B	-	Frame-shift
WP2 *uvr*A	*trp*E	*uvr*A	-	Base-pair substitution

2) **Source of test strains and media**

Source of test strains

Test strains, obtained from Molecular Toxicology Inc. ((b) (4)
USA) and subcultured in the (b) (4) will be used.

Culturing broth [SOP-MT-101]

The test strains for mutagenicity assay will be grown in 2.5 % Oxoid Nutrient Broth No. 2 prepared in distilled water.

Minimal glucose agar (bottom agar) plates [SOP-MT-101]

The minimal glucose agar (25 mL per 15 x 90 mm petri dish) will be Vogel-Bonner medium E supplemented with 1.5 % Bacto agar (Difco) and 2 % glucose. The minimal glucose agar for the WP2 *uvr*A strain will be supplemented with additional 0.25 mL/L of 0.1 % L-tryptophan. Gamma ray-sterilized petri dishes will be used.

Top agar [SOP-MT-101]

Top agar for selection of revertants will be prepared with 0.6 % Bacto agar (Difco) and 0.5 % NaCl. The top agar for *Salmonella* strains will be supplemented with 10 mL of 0.5 mM histidine/biotin solution per 100 mL.

3) **Storage of test strains and phenotypic characterization**

Frozen stocks of test strains [SOP-MT-107]

Frozen stock cultures for long-term storage were prepared from fresh overnight cultures. DMSO was added to the cultures (90 μL/mL) as a cryopreservative, and aliquots of cultures were stored at below -70 °C.

Master plates [SOP-MT-101/102]

The frozen stocks were thawed and cultured for 10 hours to prepare master plates of test strains. A part of each bacterial culture was used for the confirmation of genotypes. After confirming the genetic characteristics of the strains, then the stored master plates are used as the source of bacteria for mutagenicity assays.

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Verification of genetic characteristics [SOP-MT-106]

The following genetic characteristics of the strains were verified according to the methods of Maron and Ames (1983).

Phenotypes	Test strains
histidine requirement	*Salmonella typhimurium* TA strains
presence of *uvrB* mutation	*Salmonella typhimurium* TA strains
presence of R-factor	*Salmonella typhimurium* TA strains
presence of *rfa* mutation	*Salmonella typhimurium* TA strains
number of spontaneous revertant	*Salmonella typhimurium* TA strains and *E. coli* WP2 *uvrA*
tryptophan requirement	*E. coli* WP2 *uvrA*
presence of *uvrA* mutation	*E. coli* WP2 *uvrA*

4. Metabolic activation system (S9 mix)

1) S9 and cofactor

S9

Origin of S9: Aroclor 1254- induced male Sprague-Dawley rat liver

Supplier: (b) (4)

Item No.: 11-01L

Lot No.: to be specified in the final report

Protein content: to be specified in the final report

Storage condition: In a freezer (below -15 °C)

Cofactor

Name: Cofactor-I

Supplier: (b) (4)

Item No.: 309-50611

Lot No.: to be specified in the final report

Storage condition: Refrigeration (-1 to 10 °C)

2) Preparation of S9 mix (per 1 mL, 5 % S9 v/v) [SOP-MT-108]

The S9 mix will be prepared with S9 and cofactor solution just before use. The S9 mix will contain: 8 μmol $MgCl_2 \cdot 6H_2O$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADPH, 4 μmol NADH, 100 μmol sodium phosphate buffer (pH 7.4) and 50 μL S9. Prepared S9 mix will be placed in crushed ice.

5. Experimental procedures

1) Selection of dose range [SOP-MT-103]

Dose ranges of this study were selected based on the results of a range-finding test conducted on the test article using the five test strains in both the presence and absence of metabolic activation system with two plates per dose (b) (4) a non-GLP study]. Six doses of test article ranging 8 to 5000 μg/plate were tested using the same methods of this study. The condition of the treatment mixtures and plates were checked for the formation of precipitation and cytotoxicity, if any. In the range-finding test, turbidity and precipitation were observed in the treatment mixtures of 3000 and 5000 μg/plate. At the time of colony counting, precipitation also observed in the plates of 1000, 3000 and 5000 μg/plate. Colony counting was possible at 1000 μg/plate. At 3000 and 5000 μg/plate, colony counting was not possible. There was no significant increase or decrease in numbers of colony in all test strains at all doses.

Therefore, the high dose of this study was set at 3000 μg/plate for all test strains with additional 5 lower dose levels. The dose ranges are presented in the table below. Concurrent negative and positive controls were also included, and triplicate plates will be used for each dose.

Test strains	S9 mix	Dose (μg/plate)					
TA strains	+/-	12	37	111	333	1000	3000
WP2 *uvrA*	+/-	12	37	111	333	1000	3000

2) Plating procedures and scoring of plates [SOP-MT-102/103/104/105]

The test strains will be exposed to the test article using the direct plate incorporation method.

A small amount of bacterial growth in each master plate will be taken and transferred to a flask containing 20 mL of liquid medium (2.5 % Oxoid Nutrient Broth No. 2). Inoculated flasks will be incubated for 10 hours in a shaker/incubator (37 ± 2 °C, 120 rpm). Overnight cultures will be removed from incubation and the viable cell counts will be determined by optical density (OD) at 600 nm, and the cultures will be stored in a refrigerator until use.

For the plating assay, the followings will be added to each sterile culture tube containing 2 mL of top agar held at 45 ± 2 °C in a dry bath: 0.5 mL of S9 mix (or sodium-phosphate buffer, pH 7.4 for the non-activating plates), 0.1 mL of bacterial culture and 0.1 mL of test article. The contents will be vortexed for 2 - 3 second and overlaid onto the surface of the bottom agar.

Negative control plates will be treated with 0.1 mL of solvent instead of test article. The positive control plates will be treated with positive control articles with the same method.

The sterility of the most concentrated test article dilution will be checked by plating a 0.1 mL aliquot (mixed with 2 mL of top agar) on the minimal glucose agar. S9 mix will be also checked for sterility by plating 0.5 mL with the same method.

After the top agar solidified, plates will be inverted and incubated at 37 ± 2 °C for 50 ± 2 hours

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and then revertant colonies will be counted with unaided eyes.

3) **Identification of plates**

Each plate will be labeled with an oil-based pen to identify the study number, test strain, dose level and activation condition.

4) **Observations**

The turbidity and/or precipitation in the treatment mixture will be checked with unaided eyes, and if settlement of fine particle observed, it will be considered as precipitation.

Revertant colonies will be counted with unaided eyes. The condition of background lawn will be scored relative to the solvent control, and contamination and other abnormality of each plate will be checked.

A dose level will be considered to be cytotoxic if at least one of the following criteria is met:

(1) A clearing or diminution (reduction) of the background lawn that accompanied by a substantial reduction in the number of revertant per plate.

(2) The presence of microcolonies (pinpoint colonies).

There is no common standard of 'reduction', so it will be determined if the mean number of revertant per plate is less than 50 % of that of negative control or when there is a reversal of an increasing trend of the number of colonies.

5) **Presentation of the results**

Mean revertant per plate and standard deviation will be calculated from the triplicate plates per dose. The actual numbers of revertant will be also presented. The 'increase factor' will be calculated by dividing the value of treated plate by the value of negative control plate. The increase factors will be rounded off to one decimal place.

6) **Assay acceptance criteria**

The assay will be considered valid only if all of the following criteria are met.

(1) At least 0.5×10^8 CFU of bacteria/plate were plated.

(2) A minimum of three non-toxic dose levels were required to evaluate assay data.

(3) The mean number of spontaneous revertants per plate should be within the range presented in the following table.

Test strains	No. Revertant/plate
TA100	75-200
TA1535	3-37
TA98	15-60

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TA1537	4-31
WP2 *uvrA*	5-40

(4) The mean revertants per plate of a positive control for a respective test strain should be at least a 2-fold increase over the mean revertants per plate of the negative control for that test strain. The integrity of the S9 mix should be demonstrated by increases of revertants for the positive control plates treated with B[a]P and with 2-AA.

(5) There should be no microbial colonies due to the contamination in the plates for sterility check of test article and S9 mix.

6. Statistics and evaluation of the results

1) Statistical analysis

No statistical analysis will be done.

2) Evaluation of results

The result will be regarded as positive if there was a dose-related increase over the range tested and/or a reproducible increase at one or more doses in the number of revertant per plate in at least one strain with or without metabolic activation system. A positive result indicates that the test substance induces point mutation in the test strain.

The result will be regarded as negative if the result does not meet the positivity criteria. The negative result indicates that the test substance is not mutagenic in the test strains. A confirmatory test may be performed if it is not possible to make a definite judgement.

Biological relevance of the results will be also considered for the evaluation of the results.

7. References

1) Basic Mutagenicity Tests: UKEMS Recommended Procedures, Edited by David J. Kirkland, Cambridge University Press, 1990. ISBN 0-521-39347-7.

2) Green, MHL and Muriel, WJ (1976): Mutagen testing using *trp+* reversion in *Escherichia coli*., Mutat. Res., 38:3-32.

3) GREEN, MHL (1984) Mutagen testing using *trp+* reversion in *Escherichia coli* in KILBEY, BJ, LEGATOR, M, NICHOLS, W and RAMEL, C (Eds.). *Handbook of Mutagenicity Test Procedures. Second edition*, p.161-187. Elsevier Science Publishers BV, Amsterdam.

4) Maron, DM and Ames, BN (1983): Revised methods for the Salmonella mutagenicity test, Mutat. Res., 113:173-215.

5) Vogel, HJ and Bonner, DM (1956): Acetylornithinase of E. coli: Partial purification and some properties, J. Biol. Chem., 218:97-106 (1956).

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Units and Abbreviations

Note: The following lists of codes, abbreviations and units are used by (b) (4)
Some, but not necessarily all, of this information may be needed for this protocol.

%	Percent
°	Degree
C	Celsius
L	Liter
mL	Milliliter
µL	Microliter
g	Gram
kg	Kilogram
mg	Milligram
µg	Microgram
ng	Nanogram
m	Meter
cm	Centimeter
mm	Millimeter
µm	Micrometer
nm	Nanometer
hr	Hour
min	Minute
sec	Second
rpm	Revolution per Minute
G-6-P	Glucose-6-phosphate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
FDA	Food and Drug Administration
GLP	Good Laboratory Practice Regulation
MFDS	Ministry of Food and Drug Safety
OECD	Organization for Economic Co-operation and Development
QAU	Quality Assurance Unit
SD	Standard Deviation
SOP	Standard Operating Procedures
SPSS	Statistical Package for the Social Sciences
DKBT	Diplomated Korean Board of Toxicology

(b) (4) No. 18-VG-0143

Appendix 5. Certificate of analysis

CJ Research Institute of Biotechnology
42, Gwanggyo-ro, Yeongtong-gu, Suwon-si,
Gyeonggi-do,Korea
www.cj.co.kr
TEL : 031) 8099-2450 FAX : 031) 8099-2913

Result of analysis

Certificate No.	2016-AN-035	Receipt No.	2016-AR-035
Client		Date of Receipt	2016-07-19
Client Name		Date of Test	2016-07-19
Client Tel		Use of Report	Reference test

Test Sample	L-Threonine
Manuf. Date	2016-06-29
Expiry Date	2019-06-28
Lot. No	T75-16-01A6-29
Quantity (kg)	

Test Item(s)	Test Result
Appearance	Pale brown granule
L-Threonine	(b) (4)

- Information
- Temperature : (22-28) ℃, Relative Humidity : (30-55) %
- N.D : not detected (not quantifiable)
- The results shown in this test report refer only to the sample tested unless otherwise stated.

Tested by Taek Hee Nam

Approved by Technical Manager Seok Hun Yun

Aug 15, 2016

CJ Research Institute of Biotechnology, BIO)Analysis Team

CJ BIO-AD form 100-01 REV.01

Appendix 9 : Literature Review *Corynebacterium glutamicum* – with references

Review of the safety of *Corynebacterium glutamicum*

TABLE OF CONTENTS

1. INTRODUCTION

This document addresses the safety of the microorganism *Corynebacterium glutamicum*. It presents scientific data and information gathered from in-depth literature reviews which demonstrate that *C. glutamicum* can be used as a microorganism for the industrial production of amino acids and other substances which in turn can be safely added to feed for food-producing animals and poses no risk or health hazards to humans consuming products from food-producing animals consuming the substance. This review, as prescribed by the Division of Animal Feed staff, is intended to refresh the detailed safety review assessment completed in 2003 by the Division with the addition of *Corynebacterium glutamicum* and *Corynebacterium glutamicum* derived ingredients as an authorized feed ingredient.

2. EVALUATION BY EFSA

1.1 Qualified presumption of safety (QPS)

A wide variety of microorganisms are intentionally added at different stages into the food chain, either directly or as a source of food and feed additives, enzymes or plant protection products. The qualified presumption of safety (QPS) approach was developed by the EFSA Scientific Committee to provide a generic concept to prioritize and to harmonize risk assessment within EFSA of microorganisms intentionally introduced into the food chain (EFSA, 2005, 2007).

The list of QPS microorganisms has been continuously revised and updated since it was established in 2007. The publication of the overall assessment of the taxonomic units (TU) previously recommended for the QPS list is carried out every three years (EFSA, 2007, 2012). The recommendations provided concerning that list of microorganisms are maintained and re-evaluated based on extensive literature reviews and expert knowledge. (EFSA, 2007, 2018).

1.2 Re-evaluation using literature review

The bi-annual re-evaluation of microorganisms begins with a literature review for each TU that is notified to EFSA. QPS recommended TU and those which represent new TU notifications are annually reviewed (EFSA, 2007). The literature review for a new TU is broader to cover the history of use, the potential safety concerns and the ecology. Relevant databases such as Web of Science Core Collection, CAB Abstracts, BIOSIS Citation Index, MEDLINE and Food Science Technology Abstracts are searched using the TU in combination with common keywords (e.g. toxin, disease, antibiotic/antimycotic resistance, safety, syndrome) and respective animal categories. The search terms are broad and cover synonyms or former names of taxonomic units (EFSA, 2012, 2013, 2017). Findings from the literature review are then evaluated, taking into consideration recommendations given in the previous QPS Opinion. A detailed description of the methodology used in carrying out the literature review can be found in EFSA (2013, 2017). A summary of the literature search strategy for the most recent QPS update for *C. glutamicum* is given in Table 1.

Table 1.	***Corynebacterium glutamicum***
String for species	
"*Corynebacterium glutamicum*" OR "*C glutamicum*" OR "*Brevibacterium lactofermentum*" OR "*B lactofermentum*"	
Outcome	**String**
1) Antimicrobial/Antibiotic/Antimycotic	"antimicrobial resistan*" OR "antibiotic resistan*" OR "antimicrobial susceptibil*"
2) Infection/Bacteremia/Fungemia/Sepsis	infection* OR abscess* OR sepsis* or septic* OR bacteremia OR bacteraemia OR toxin* OR "pathogen*"
3) Type of disease	Not applied
4) Mortality/Morbidity	clinical* OR death* OR morbidit* OR mortalit* OR disease* OR illness*
5) Disease Risk	opportunistic OR virulen*
Flow records by search strategy resulted in 78 papers being identified using title screening, of which 8 papers were identified using title/abstract screening, of which 1 was identified using article appraisal and was considered relevant for QPS. Following the review of that paper (Yang and Yang, 2017), it was concluded that there were no safety concerns identified in the only article considered relevant for QPS exercise (EFSA, 2018).	
A literature review did not reveal new information about adverse health effects or on safety concerns since the last update (EFSA, 2013). The QPS recommendation has been confirmed.	
Source: EFSA (2018).	

1.3 QPS Classification of Corynebacterium glutamicum

The QPS approach is currently used for microorganisms in the three broad categories within which most of the species notified to EFSA fall: bacteria, yeasts and viruses (EFSA, 2005, 2007). Here only information as it relates to the QPS assessment of the bacterium *C. glutamicum* is presented.

As noted, each updated QPS Opinion is based on a review of newly available scientific literature and recommendations given in the previous years' opinions. Scientific opinions on the update of the list of QPS-recommended biological agents intentionally added to food or feed that include *C. glutamicum* are reported for the years 2007, 2008, 2010, 2011, 2012, 2013, 2016 and 2017. The recommendations given in each QPS Opinion for these respective years are summarized in Appendix 1. The recommendations unanimously confirm that *C. glutamicum* meets the QPS criteria for humans and animals and there are no adverse health effects or on safety concerns.

3. LITERATURE SEARCH (2003-2018)

1.4 Method Used

An electronic literature search (ELS) was conducted by (b) (4) to collect scientific studies, articles, reports and other documents deemed to be relevant for a review of the safety/risk assessment of *C. glutamicum*. The ELS was carried out from February 18 to 23,

2018 using the Google Scholar database and included information published from 2003 onwards. A detailed description of the ELS strategy employed and a listing of the search "strings" used and "hits" obtained is detailed in Appendix 2. The ELS was based on the search terms or "strings" used by EFSA in the 2017 QPS re-evaluations for *C. glutamicum* (Section 2.2.1), but adapted to the Google Scholar and its specific structure. The information collected from the ELS was reviewed and follow-up selective searches were made using the Web of Science Core Collection, CAB Abstracts and Global Health, BIOSIS Citation Index and Current Contents.

1.5 Relevant Records Retrieved

The "hits" or records retrieved in the ELS search were compiled and each publication was reviewed and judged whether it contained information relevant to the safety of C. glutamicum (Appendix 2, Table 2). Some examples of the topics addressing *C. glutamicum* in the records retrieved include the role of pathogenic and non-pathogenic *Corynebacterium spp*., particularly in human clinical trials (Camello et al., 2003; Roux et al., 2004; Bernard, 2005; Eguchi et al., 2008; Olender, 2012; Oliveira et al., 2017), genetic and biochemical characterization of *C. glutamicum* and site directed mutagenesis (Zhang et al., 2012), gene identification and sequencing (Ikeda and Nagakawa, 2003; Khamis et al., 2004; Ordonez et al., 2005; Yukawa et al., 2007), gene deletion and the effect on cell morphology and antibiotic resistance (Möker et al., 2004; Oritz-Pérez et al., 2010; Bernard, 2012) and carcass degradation (Kim et al., 2017).

Overall, no studies were retrieved either in the ELS or follow-up selective searches that contained information indicating potential safety issues or hazards associated with *C. glutamicum*. Those records retrieved from the searches that support the accepted safe use of different strains of C. glutamicum for amino acid production are reviewed in the following narrative.

4. NARRATIVE - CORYNEBACTERIUM GLUTAMICUM

The scientific data and information presented in the following sections demonstrate that *C. glutamicum* can be safely used as a microorganism for the industrial production of amino acids under the conditions of intended use for the target animals and humans consuming food derived from food-producing animals consuming the substance.

1.6 Taxonomy and Characteristics

The genus *Corynebacterium* belongs to the taxonomic class *Actinobacteria* that represents gram-positive bacteria with a high guanine and cytosine content in their DNA (Stackebrandt et al., 1997; Ventura et al., 2007). The genus Corynebacterium which currently has 110 validated species, is highly diversified and includes species that are of medical, veterinary, or biotechnological relevance (Pascual et al., 1995; Khamis et al., 2004; Bernard, 2012; Soares et al., 2013; Oliveira et al., 2017; Dalen et al., 2018).

One of the most prominent members among the genus *Corynebacterium* is *C. glutamicum*, a bacterium isolated in 1956 from an avian-feces-contaminated soil sample collected from Ueno Zoo in Tokyo (Japan) with a natural capacity to accumulate L-glutamate extracellularly in a biotin-limited medium (Kinoshita et al., 1957; Udaka, 1960; Shiio et al., 1962). *C. glutamicum* belongs to a broad, diverse group of mycolic acid-containing bacteria that share

the property of having an unusual cell envelope composition and architecture, differing from those of other gram-positive bacteria (Peuch et al., 2001).

C. glutamicum is a nonmotile, facultative anaerobic, Gram-positive biotin-auxotrophic soil bacterium, which forms rod-shaped, straight, or slightly curved cells (Becker and Whittman, 2017). The genome of the wild-type strain *C. glutamicum* ATCC 13032 possesses a circular chromosome of 3.3 Mb and a plasmid of 0.5 Mb (Becker et al., 2016) and contains about 3000 genes (Bathe et al., 1996; Becker and Whittman, 2017). Further typical characteristics comprise a cell wall with arabinogalactan and mycolic acids with 26 to 36 carbon atoms; and a murein sacculus with peptidoglycan cross-linked via meso-diaminopimelic acid (Whittman and Becker, 2007). *C. glutamicum* can use a variety of carbon sources as growth and energy substrates, including sugars, sugar alcohols, organic acids and aromatic compounds (Becker et al., 2016). For information on taxonomical studies see Abe et al (1967) and Liebl (2005).

Although some *Corynebacterium spp*. have been detected as components of the bacterial community of cheese surface (Monnet et al., 2006), only *C. glutamicum* is considered of relevance for industry feed and food production sectors.

1.7 Amino Acid Production

The global amino acid market is more than $US 7 billion and is forecast to reach $US 11.6 billion by the year 2015 and $US 35 billion by 2022 (Radiant Insights, Inc., 2015). Global volume consumption of feed grade amino acids, estimated at 4.5 million metric tons in 2017, is projected to reach 6.2 million metric tons by 2022. Poultry feed constitutes the largest consumer of feed amino acids globally with 2017 market share of 43.4% (Business Wire, 2017).

C. glutamicum has many fundamental physiological properties that make it an important industrial workhorse. These properties are listed by Lee et al (2016) as follows: (i) not pathogenic and generally recognized as a safe strain (GRAS); (ii) fast growth to high cell densities; (iii) genetically stable owing to the lack of a recombination repair system; (iv) limited restriction-modification system; (v) no autolysis and maintenance of metabolic activity under growth arrested conditions; (vi) low protease activity favoring recombinant protein production; (vii) plasticity of metabolism and strong secondary metabolism properties; and (viii) broad spectrum of carbon utilization (pentoses, hexoses, and alternative carbon sources); stress tolerance to carbon sources.

C. glutamicum's inability to form spores, relatively few growth requirements and natural capability to produce and secrete glutamate in high amounts makes it one of the most important platform microorganisms used for industrial production of amino acids. The practice of developing amino acid overproducing strains by mutagenesis and selection is a very well-established technique (Rowlands, 1984). Different strains have been utilized for decades by the industry to produce glutamate, lysine, tryptophan, threonine, isoleucine, valine and leucine as described in the "Handbook of *Corynebacterium glutamicum*" (Eggeling and Bott, 2005).

Amino acids have a wide variety of characteristics in terms of nutritional value, taste, medicinal action, and chemical properties, and thus have many potential uses, e.g., in food additives, feed supplements, pharmaceuticals, cosmetics, polymer materials, and agricultural chemicals (Ikeda and Takeno, 2013). Industrial amino acids produced by microorganisms are identical to those naturally found in vegetables and animals (Bercovici and Fuller, 1995).

Over the past decades, global competition among leading companies in the field steadily demanded innovation to improve key performance indicators: yield, titer, and productivity

(Becker et al., 2016). For this reason, *C. glutamicum* has become one of the best characterized microorganisms worldwide with regard to substrate spectrum and nutrient requirement (Buschke et al., 2013), catabolic and anabolic pathways and their regulation (Kalinowski et al., 2003; Schroder and Tauch, 2010) underlying biochemistry (Blombach and Seibold, 2010) and response to environmental conditions (Ehira et al., 2009).

1.7.1 Production methods

The two microbiological (biotechnology) methods for the industrial production of amino acids are the use of microbial enzymes or immobilized cells (enzymatic method) and fermentation (semi or direct) (Ivanov et al., 2013). The fermentation process is briefly addressed here to illustrate that the purification step within the fermentation process ensures a safe product.

Fermentation processes typically comprise three steps: fermentation, crude isolation and purification (Kusumoto, 2011; Ikeda and Takeno, 2013; Ivanov et al., 2013). In the fermentation process, the desired amino acid is specifically produced by the fermentation microorganism (e.g. *C. glutamicum* in the production of L-glutamine, L-lysine, L-valine). During the crude isolation process, most impurities contained in the fermentation broth are removed by combining various technologies. Final purification is performed to ensure the required quality for the intended use. The final product is obtained as a crystalline powder. The product is released only after quality tests have verified that the product meets specific requirements, and the normal functioning of each process step has been verified. All manufacturing processes to produce amino acids must comply with current good manufacturing practice requirements.

1.8 Other Uses

C. glutamicum is also employed in the production of L-phenylalanine (Shu and Liao, 2002), L-serine (Stolz et al. 2007) and for secreted protein production (Kikuchi et al., 2003; Umakoshi et al., 2011). The bacterium can be engineered for production of isobutanol (Blombach et al., 2011) and succinate (Litsanov et al., 2013).

Products for health and nutrition have the longest history in industrial biotechnology, with *C. glutamicum* being one of the major producers Meanwhile, processes for other products including non-proteinogenic amino acids, vitamins, flavors and fragrances and other nutrients and health care products are also on the rise (Burnett et al., 2013; Becker et al., 2016).

1.9 Genetic engineering

The past quarter century has seen rapid developments in strain development technology. Metabolic engineering has repeatedly led to successful yield improvements, especially in the field of amino acid production by *C. glutamicum* (Kirchner and Tauch, 2003; Eggeling and Bott, 2005; Wendisch, 2006; Becker and Whittmann, 2012; Zahoor et al., 2012; Burkovski, 2013; Buschke et al, 2013; Heider and Wendisch, 2015).

1.10 Safety Concerns

The species *C. glutamicum*, which serves as recipient and donor strain is generally considered to be non-pathogenic and no safety concerns are reported for this bacterial species for humans and animals. It is not known to produce toxins or present any other

hazards (Nelson et al., 2000; Kalinowski et al., 2003; Bernard, 2005; Olender, 2012; Oliviera et al., 2017).

As discussed in Section 2, *C. glutamicum* meets the EFSA premarket qualified presumption of safety (QPS) assessment criteria when used for fermentation of amino acids.

C. glutamicum is listed as a fermentation organism in several AAFCO feed ingredient definitions (e.g. 36.1, 36.16 and 36.17 (AAFCO 2016). Moreover, amino acids produced by an aerobic fermentation process using C. glutamicum are generally recognized as a safe (GRAS) for humans and food producing animals.

The publication of the C. glutamicum ATCC 13032 genome sequence by two independent groups (Ikeda and Nagakawa, 2003; Kalinowski et al., 2003) provided a platform that allowed for a better understanding and easier engineering of the bacterium. Due to its importance as an amino acid producer, *C. glutamicum* is one of the most-investigated and documented microorganisms (Jetten and Sinskey, 1995; Sahm et al., 1995, 2000; Krömer et al., 2004; Leuchtenberger et al. 2005; Dong et al., 2011; Schneider et al., 2011; Ikeda and Takeno, 2013; Lv et al., 2015; Hirasawa and Shimizu, 2016; Wendisch et al., 2016). Lee et al (2016) reviewed the literature and found that as of 2015 over 2,700 papers and 1,700 patents have been reported relating to *C. glutamicum*. The breadth and depth of research carried out on *C. glutamicum* substantiates the accepted safety of using this bacterium by the industry.

In addition to being used for the industrial production of amino acids, *Corynebacterium spp*. have a long history of safe use in food production, including preparation of fermented maize, sorghum, millet, African oil bean seed, rice, soybean and cassava (Caplice and Fitzgerald, 1999; Tateno et al., 2007; Osungbaro, 2009).

1.10.1 Nonpathogenicity

Many of the genes present in the completely sequenced genome of *C. glutamicum* are highly conserved in sequence and gene order within the other members of the *genus Corynebacterium* (Ikeda and Nakagawa, 2003; Kalinowski *et al* 2003). As a non-pathogenic member of the genus, *C. glutamicum* is of increasing interest as a model organism for other members of the suborder including important pathogens such as *C. diphtheriae*, *Mycobacterium tuberculosis* and *M. leprae* (Camello et al., 2003; Gibson et al., 2003; Moeker et al., 2004; Olender et al., 2012; Tauch and Burkovski, 2015; Cashmore et al., 2017).

5. SUMMARY AND CONCLUSIONS

The data and scientific information presented in this document demonstrate that there are no known safety issues regarding the use of *C. glutamicum* in the production of compounds for use in food for humans and for food-producing animals. *C. glutamicum* is generally considered to be non-pathogenic and no safety concerns are envisaged. The ELS and follow-up selected literature reviews carried out did not reveal any hazards associated with C. glutamicum when added to food or feed. These findings agree with the EFSA QPS Opinions issued from 2005 onwards.

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7. APPENDIX 1

Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA

Scientific opinions for *C. glutamicum* for each year are extracted from the respective reference cited.

Year 2007

EFSA. 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007, 587:1-16.

Corynebacterium glutamicum

C. glutamicum is a soil bacterium widely used for the biotechnological production of amino acids. Amino acid producing strains have been selected and improved by mutagenesis as well as by using recombinant DNA technology. *C. glutamicum* belongs to a genus which also includes significant human pathogenic bacteria. Although some *Corynebacterium* species have been detected as components of the bacterial community of cheese surface, only *C. glutamicum* is considered of relevance for feed and food sectors. Only this species has been considered for the QPS assessment because of its significant role in the industrial production of amino acids.

Taxonomic unit defined

The genus *Corynebacterium* belongs to a branch of the *Actinomycetales* that also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus*. Bacterial species belonging to this branch of the Gram-positive bacteria share particular characteristics, such as high G+C content (47– 74%) and a specific cell envelope organisation, mainly characterized by the presence of peptidoglycan, arabinogalactan and mycolic acids. The genus currently contains 63 species, which colonize different environments.

Is the body of knowledge sufficient?

The characteristics, the physiology and the genetics of *C. glutamicum* are well known. The genome sequence of this industrial bacterium has been determined (Kalinowski et al., 2003), reflecting the considerable biotechnological importance of these organisms.

Are there safety concerns?

C. glutamicum plays an important role in the amino acid fermentation industry. No safety concerns are reported for this bacterial species for humans and animals, and no information on the presence of acquired antibiotic resistances in this bacterial species is available. However, it should be kept in mind that the direct exposure of consumers to this bacterial species is expected to be very low.

Can the safety concerns be excluded?

C. glutamicum has generally been considered to be non-pathogenic and no safety concerns are envisaged. However, its history of use is as a source of amino acids and has not, to date, involved the direct and deliberate exposure of humans or livestock.

Units proposed for QPS status

There is a long history of safe use of *C. glutamicum* as an amino acid producer; consequently, *C. glutamicum* is proposed for QPS status with the qualification that this status applies only when the species is used for production purposes only.

Year 2008

EFSA. 2008. Scientific Opinion of the Panel on Biological Hazards on a request from EFSA on the maintenance of the QPS list of microorganisms intentionally added to food or feed. EFSA Journal 2008, 923, 1-48.

Corynebacterium glutamicum

QPS status applies only when the species is used for production purposes.

Year 2010

EFSA. 2010. EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA Journal 2010;8(12):1944. 56 pp.

Corynebacterium glutamicum

QPS recommendation only when the species is used for amino acid production.

Year 2011

EFSA. 2011. EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). EFSA Journal 2011;9(12):2497. 82 pp.

Corynebacteria

A literature review did not reveal new information about adverse health effects or on safety concerns since the last update (EFSA, 2010). The QPS recommendation has been confirmed.

Antimicrobial resistance aspects regarding the qualification

While no actual antibiotic MIC determinations for *C. glutamicum* appear to have been done, the antibiotic sensitivity of a strain used for amino acid production, has been tested using a disc method (Costa-Riu et al., 2003). The strain was sensitive to ampicillin, kanamycin, streptomycin, tetracycline, susceptible to gentamicin and resistant to norfloxacin, and chloramphenicol. However, the susceptibility test was not performed according to the methodology recommended by the CLSI guideline (Anonymous, 2007). There is no new information that would require a modification in the qualification of the antimicrobial resistance.

Year 2012

EFSA. 2012. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Panel on Biological Hazards. EFSA Journal 2012, 10(12):3020. 84 pp.

Corynebacteria

A literature review did not reveal new information about adverse health effects or safety concerns with regards to the last update (EFSA, 2011). The QPS recommendation has been confirmed.

Antimicrobial resistance aspects regarding the qualification

While no actual antibiotic MIC determinations for *C. glutamicum* appear to have been done, the antibiotic sensitivity of a strain used for amino acid production, has been tested using a disc method (Costa-Riu et al., 2003). The strain was sensitive to ampicillin, kanamycin, streptomycin, tetracycline, gentamicin and resistant to norfloxacin, and chloramphenicol. The susceptibility test was not performed according to the methodology recommended by the CLSI guideline (CLSI, 2007). There is no new information that would require a modification in the qualification of the antimicrobial resistance.

Year 2013

EFSA. 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA Panel on Biological Hazards. EFSA Journal 2013;11(11):3449, 107 pp.

Corynebacterium glutamicum

A literature review did not reveal new information about adverse health effects or safety concerns with regards to the last update (EFSA, 2012). The QPS recommendation has been confirmed.

Antimicrobial resistance aspects regarding the qualification

No new relevant information in the last year was published on the antimicrobial susceptibility or resistance of *C. glutamicum,* therefore no modifications in the qualification of the antimicrobial resistance are proposed.

Year 2016

EFSA. 2017. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA Journal 2017, 15(3):4664, 178 pp.

Corynebacterium glutamicum

Taxonomy

Since the last update on the QPS status (EFSA, 2013), no new information on the taxonomy of the *C. glutamicum* has been published.

Update of the body of knowledge on safety concerns

The total number of references found through the ELS was 188; after screening at title/abstract level, 33 passed to the full text phase; of those, two were considered relevant for the QPS assessment. A literature review did not reveal any new information about adverse health effects or safety concerns since the last update (EFSA, 2013).

Revision of antimicrobial resistance aspects

The involvement of class 1 integrons in the AMR towards streptomycin/spectinomycin and tetracycline in *C. glutamicum* isolates has been confirmed and reviewed by Deng et al. (2015). No additional relevant information was published in the last year on the antimicrobial susceptibility or resistance of *C. glutamicum*.

Update on other qualifications

This TU has the following qualification 'QPS only applies when the species is used for amino acid production'. Due to a lack of knowledge in relation to history of use of the viable organisms and because other members of the same genus are pathogenic, the qualification is confirmed.

Other relevant information

No new relevant information was identified.

Conclusion regarding a QPS recommendation

The QPS recommendation is confirmed for C. glutamicum as well as the qualification.

Year 2017

EFSA. 2018. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. EFSA Journal 2018, 16(1):5131, 43 pp.

Corynebacterium glutamicum

No safety concerns identified in the only article considered relevant for QPS exercise.

8. APPENDIX 2

Electronic Literature Search for safety / risk assessment of *Corynebacterium glutamicum*

Project: Electronic Literature Search for safety / risk assessment of *Corynebacterium glutamicum*

An electronic literature search (ELS) on *Corynebacterium glutamicum* was conducted to collect studies, articles, reports and reviews that are deemed likely to be relevant for further safety / risk assessment of *Corynebacterium glutamicum*.

The search was conducted with the following information:

1. Name of the database searched: Google Scholar (https://scholar.google.co.in).
2. Dates on which the database searched: Between 18th Feb. 2018 and 23rd Feb. 2018.
3. Time period between which the database searched: Publications between 2003 and till date.
4. Other restrictions applied: Search terms present in 'allintitle' and 'anywhere' excluding patents and citations.
5. Languages searched: For pages written in any language.
6. Publications searched: Articles published in any peer reviewed journal; book or book chapters; theses; published reviews; etc.
7. Search strategy applied, and records retrieved: Recorded in Table 1.

Selection of articles: A stepwise exercise was performed to select articles that are deemed likely to be relevant for further safety / risk assessment of *Corynebacterium glutamicum* and the shortlisted articles were made available for the 'full review' at the end of ELS.

1. Step 1: Check if the word "Corynebacterium" is mentioned in title, keywords and/or abstract
2. Step 2: Check if the term "Corynebacterium glutamicum" is described in abstract
3. Step 3: Read the abstract
4. Step 4: Select articles for the 'full review' if abstract describes "Corynebacterium glutamicum" or "Corynebacterium spp" and at least some indicative information that the article covers either safety aspects; hazards / disease events in plant, animals and humans; toxin production; or carry genes for antimicrobial resistance. Further detailed evaluation on deemed likely to be included or excluded for the 'full review' was recorded in Table 2.

Table 1: Electronic Literature Search (ELS) Strategy and Retrieved Hits:

Strategy number	Terms	Hits	Notes
#1	allintitle: "Corynebacterium glutamicum"	1970	First 50 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#2	allintitle: "Corynebacterium"	4020	First 50 hits were checked following 'selection of articles' as mentioned above and recorded in table

Strategy number	Terms	Hits	Notes
			2.
#3	#2 resistance	39	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#4	#2 resistant	41	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#5	#2 antibiotic resistance	2	Both hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#6	#2 antibiotic resistant	4	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#7	#2 antimicrobial susceptibility OR susceptibilities	8	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#8	#2 infection OR infections	221	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#9	#2 abscess OR abscesses	29	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#10	#2 sepsis OR septic	21	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#11	#2 bacteremia OR bacteraemia	27	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#12	#2 toxic OR toxin OR toxins	34	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#13	#2 pathogen OR pathogenic OR pathogenicity	74	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#14	#2 opportunistic OR	38	All hits were checked following 'selection of articles' as mentioned above and recorded in table

Strategy number	Terms	Hits	Notes
	virulence OR virulent		2.
#15	#2 safety OR risk	7	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#16	#2 mutagenic OR mutagenicity	00	
#17	#2 toxicity OR toxicology	8	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#18	#2 clinical OR clinically	79	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#19	#2 death OR deaths	2	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#20	#2 morbidity OR morbidities	00	
#21	#2 mortality OR mortalities	2	Both hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#22	#2 disease OR diseases	23	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#23	#2 illness OR illnesses	6	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#24	anywhere: "Corynebacterium glutamicum"	254	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#25	#24 resistance	193	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#26	#24 resistant	208	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.

Strategy number	Terms	Hits	Notes
#27	#24 antibiotic resistance	140	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#28	#24 antibiotic resistant	163	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#29	#24 antimicrobial susceptibility OR susceptibilities	103	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#30	#24 infection OR infections	107	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#31	#24 abscess OR abscesses	8	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#32	#24 sepsis OR septic	15	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#33	#24 bacteremia OR bacteraemia	9	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#34	#24 toxic OR toxin OR toxins	137	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#35	#24 pathogen OR pathogenic OR pathogenicity	124	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#36	#24 opportunistic OR virulence OR virulent	95	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#37	#24 safety OR risk	100	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#38	#24 mutagenic OR mutagenicity	21	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.

Strategy number	Terms	Hits	Notes
#39	#24 toxicity OR toxicology	86	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#40	#24 clinical OR clinically	89	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#41	#24 death OR deaths	92	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#42	#24 morbidity OR morbidities	12	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#43	#24 mortality OR mortalities	99	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#44	#24 disease OR diseases	141	First 20 hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#45	#24 illness OR illnesses	19	All hits were checked following 'selection of articles' as mentioned above and recorded in table 2.
#46			

Table 2: Relevant References / Articles:

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
#1 / 1970	allintitle: "Corynebacterium glutamicum"	**Handbook of Corynebacterium glutamicum** Eggeling L, Bott M. CRC Press, 2005. ISBN: 9781420039696	Review / Exclude Not relevant to safety of C. glutamicum
		The Corynebacterium glutamicum genome: features and impacts on biotechnological processes Ikeda M, Nakagawa S. Applied Microbiology and Biotechnology, 2003. Vol. 62(2 – 3), pp 99 – 109.	Review / Exclude Not relevant to safety of C. glutamicum
		Comparative analysis of the Corynebacterium glutamicum group and complete genome sequence of strain R Yukawa H, et al. Microbiology, 2007. Vol. 153, pp. 1042 – 1058. doi: 10.1099/mic.0.2006/003657-0	Review / Exclude Not relevant to safety of C. glutamicum
		Deletion of the genes encoding the MtrA–MtrB two-component system of Corynebacterium glutamicum has a strong influence on cell morphology, antibiotics susceptibility and expression of genes involved in osmoprotection Möker N, et al. Molecular Microbiology, 2004. Vol. 54 (2), pp. 420 – 438.	Review / Exclude Not relevant to safety of C. glutamicum
		Analysis of Genes Involved in Arsenic Resistance in Corynebacterium glutamicum ATCC 13032 Ordóñez E, et al. Appl. Environ. Microbiol., 2005. Vol. 71 (10), pp. 6206 – 6215.	Review / Exclude Not relevant to safety of C. glutamicum
#2 / 4020	allintitle: "Corynebacterium"	**rpoB Gene Sequencing for Identification of Corynebacterium Species**	Review / Exclude Not relevant to safety

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		La Scola B, et al. J. Clin. Microbiol., 2004. Vol. 42 (9), pp. 3925 – 3931.	of C. glutamicum
		Several results repeated	
#3 / 39	allintitle: Corynebacterium resistance	**The CGL2612 Protein from Corynebacterium glutamicum is a Drug Resistance-Related Transcriptional Repressor Structural and Functional Analysis of a newly identified transcription factor from genomic DNA Analysis** Itou H, et al. The Journal of Biological Chemistry, 2005. Vol. 280, pp. 38711 – 38719.	Review / Exclude Not relevant to safety of C. glutamicum
		High Frequency of Macrolide Resistance Mechanisms in Clinical Isolates of Corynebacterium Species Ortiz-Pérez A, et al. Microbial Drug Resistance 2010. Vol. 16(4), pp. 273 – 277.	Review / Exclude Not relevant to C. glutamicum
		Antibiotic Resistance and Detection of the Most Common Mechanism of Resistance (MLSB) of Opportunistic Corynebacterium Olender A. Chemotherapy, 2013. Vol. 59, pp. 294 – 306. https://doi.org/10.1159/000357467	Review / Exclude Not relevant to safety of C. glutamicum
		Mechanisms of Antibiotic Resistance in Corynebacterium spp. Causing Infections in People Olender A. 2012 https://www.intechopen.com/ https://cdn.intechopen.com/pdfs-wm/34699.pdf	Review / Exclude Not relevant to safety of C. glutamicum
		The identification and resistance analysis to 66 strains of corynebacterium clinical isolates Zhang LWZ. Chinese Journal of Laboratory Diagnosis, 2007. Vol. 7.	Exclude (based on abstract; no translation of full paper)) Not relevant to safety

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		http://en.cnki.com.cn/Article_en/CJFDTOTAL-ZSZD200707029.htm	of C. glutamicum
		Antimicrobial Resistance in Corynebacterium spp., Arcanobacterium spp., and Trueperella pyogenes. Feßler AT, Schwarz S. Microbiology Spectrum, 2017. Vol. 5(6). DOI: 10.1128/microbiolspec.ARBA-0021-2017	Review / Exclude Not relevant to safety of C. glutamicum
		Extracytoplasmic function sigma factor σD confers resistance to environmental stress by enhancing mycolate synthesis and modifying peptidoglycan structures in Corynebacterium glutamicum Koichi Toyoda, Toyoda K, Masayuki I. Molecular Microbiology, 2018. Vol. 107 (3), pp. 312 – 329.	Review / Exclude Not relevant to safety of C. glutamicum
		Phenotypic and genotypic characterization of high-level macrolide and lincosamide resistance in Corynebacterium species in Canada and the distribution of the ermX resistance determinant among Corynebacterium species Singh, Cathleen. Theses, 2010.	Review / Exclude Not relevant to safety of C. glutamicum
		A National Survey of Multi-Drug Resistance in Ophthalmic Clinical Isolates of Corynebacterium in Japan Eguchi H, et al., Investigative Ophthalmology and Visual Science, 2008. Vol.49, pp. 5530	Review / Exclude Not relevant to safety of C. glutamicum
		Several results repeated	
#4 / 41	allintitle: Corynebacterium resistant	**Adaptive evolution of Corynebacterium glutamicum resistant to oxidative stress and its global gene expression profiling**	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Lee JY, et al. Biotechnology Letters, 2013. Vol. 35(5), pp 709 – 717.	
		Genetic and biochemical characterization of Corynebacterium glutamicum ATP phosphoribosyltransferase and its three mutants resistant to feedback inhibition by histidine Zhang Y. et al. Biochinie, 2012. Vol. 94 (3), pp. 829 – 838.	Review / Exclude Not relevant to safety of C. glutamicum
		Infectious keratitis caused by fluoroquinolone-resistant Corynebacterium Fukumoto A, et al. Japanese Journal of Ophthalmology, 2011. Vol. 55 (5), pp 579 – 580.	Review / Exclude Not relevant to C. glutamicum
		Generation of branched-chain amino acids resistant Corynebacterium glutamicum acetohydroxy acid synthase by site-directed mutagenesis Guo Y, et al. Biotechnology and Bioprocess Engineering, 2014. Vol. 19 (3), pp 456 – 467.	Review / Exclude Not relevant to safety of C. glutamicum
		Characteristics of Multidrug-Resistant Corynebacterium spp. Isolated from Blood Cultures of Hospitalized Patients in Japan Qin L, et al. Japanese Journal of Infectious, 2017. Vol. 70 (2).	Review / Exclude Not relevant to safety of C. glutamicum
		Methodology of identification of Corynebacterium and the drug resistant mechanisms to tetracycline and macrolides antibiotics Cao J, et al. Chinese Journal of Nosocomiology, 2013. Vol. 2.	Review / Exclude Not relevant to safety of C. glutamicum
		Effect of surface active agents, chelating agents and antibiotics on l-methionine fermentation by a multiple analogue resistant mutant	Review / Include Article discusses antibiotic resistance.

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Corynebacterium glutamicum x300 Ganguly S, Satapathy KB. European Chemical Bulletin, 2014. Vol. 3(4), pp. 346 – 351.	
		Few results repeated	
#5 / 2	allintitle: Corynebacterium antibiotic resistance	Results repeated	
#6 / 4	allintitle: Corynebacterium antibiotic resistant	none	
#7 / 8	allintitle: Corynebacterium antimicrobial susceptibility OR susceptibilities	**Antimicrobial Susceptibility and Species Identification of Corynebacterium spp. Strains Collected in Europe and USA Medical Centers (2006-2010)** Sader HS, et al. Sentry Antimicrobial Surveillance, 2012. P1092 ECCMID 2012 JMI Laboratories North Liberty, IA, USA	Review / Exclude Not relevant to safety of C. glutamicum
		Few results repeated	
#8 / 221	allintitle: Corynebacterium infection OR infections	**Inflammatory pseudotumor of the liver revealing gynecological Corynebacterium infection** Marie I, et al. Scandinavian Journal of Gastroenterology, 2005. Vol. 40 (7), pp. 875 – 877.	Review / Exclude Not relevant to safety of C. glutamicum
		Corynebacterium-associated skin infections Blaise G, et al. International Journal of Dermatology, 2008. Vol. 47 (9), pp. 884 – 890.	Review / Exclude Not relevant to safety of C. glutamicum
		Corynebacterium Species Isolated from Bone and Joint Infections Identified by 16S rRNA Gene Sequence Analysis Raoult D, et al. J. Clin. Microbiol., 2004. Vol. 42 (5), pp. 2231 – 2233.	Review / Exclude Not relevant to safety of C. glutamicum
		Case of erythema nodosum associated with	Review / Exclude

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		granulomatous mastitis probably due to Corynebacterium infection Kubo Y, et al. The Journal of Dermatology, 2014. Vol. 41(9), pp. 821 – 823.	Not relevant to safety of C. glutamicum
		[Wound infections due to opportunistic corynebacterium species] Olender A, Łetowska I. Medycyna Doswiadczalna i Mikrobiologia, 2010. Vol. 62 (2), pp. 135 – 140.	Review / Exclude (based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum
		Identification of Corynebacterium spp. isolated from bovine intramammary infections by matrix-assisted laser desorption ionization-time of flight mass spectrometry dos Santos MV, et al. Veterinary Microbiology, 2014. Vol. 173 (1 – 2), pp. 147 – 151.	Review / Exclude Not relevant to safety of C. glutamicum
		Ocular Infections Caused by Corynebacterium Species Eguchi H. Infection Control, 2013. Dr. Silpi Basak (Ed.), In Tech, DOI: 10.5772/56214.	Review / Exclude Not relevant to safety of C. glutamicum
		Hardware Infection with Corynebacterium spp.: a Case Report and Review of the Literature Clarridge III JE, et al. Clinical Microbiology Newsletter, 2014. Vol. 36(2), pp. 9 – 13.	Review / Exclude Not relevant to safety of C. glutamicum
		Cerebrospinal fluid shunt infection caused by Corynebacterium sp: Case report and review Randi BA, et al. Brain Injury, 2014. Vol. 28(9), pp. 1223 – 1225.	Review / Exclude Not relevant to safety of C. glutamicum
		Transmission dynamics of intramammary infections caused by Corynebacterium species	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	**Search Strategy**	**Selected Publications**	**Include / Exclude** **Justification**
		Delen G, et al. Journal of Dairy Science, 2018. Vol. 101 (1), pp. 472 – 479.	
		Modelling and dynamics of intramammary infections caused by Corynebacterium species Rachah A, et al. 7th International Conference on Modeling, Simulation, and Applied Optimization (ICMSAO), 2017. Conference proceedings.	Review / Exclude Not relevant to safety of C. glutamicum
		Few results repeated	
#9 / 29	allintitle: Corynebacterium abscess OR abscesses	none	
#10 / 21	allintitle: Corynebacterium sepsis OR septic	none	
#11 / 27	allintitle: Corynebacterium bacteremia OR bacteraemia	none	
#12 / 34	allintitle: Corynebacterium toxic OR toxin OR toxins	none	
#13 / 74	allintitle: Corynebacterium pathogen OR pathogenic OR pathogenicity	**Corynebacterium - occurrence and pathogenicity for humans and animals. [Corynebacterium - występowanie i chorobotwórczość dla ludzi i zwierząt.]** Banaszkiewicz T, Krukowski H. Medycyna Weterynaryjna, 2011. Vol.67 No.4 pp.229-232	Exclude (based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum
		Insight of Genus Corynebacterium: Ascertaining the Role of Pathogenic and Non-pathogenic Species Oliveira A, et al. Front. Microbiol., 2017.	Review / Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		https://doi.org/10.3389/fmicb.2017.01937	
		Few results repeated	
#14 / 38	allintitle: Corynebacterium opportunistic OR virulence OR virulent	**Molecular armory or niche factors: virulence determinants of Corynebacterium species** Tauch A, Burkovski A. FEMS Microbiology Letters, 2015. Vol. 362(23), fnv185, https://doi.org/10.1093/femsle/fnv185	Review / Exclude Not relevant to safety of C. glutamicum
		Few results repeated	
#15 / 7	allintitle: Corynebacterium safety OR risk	**Safety and efficacy of l-arginine produced by Corynebacterium glutamicum KCCM 80099 for all animal species** EFSA. EFSA Journal, 2017. DOI: 10.2903/j.efsa.2017.4858	Review / Include Assessment reviews safety, efficacy and toxicity
#17 / 8	allintitle: Corynebacterium toxicity OR toxicology	**Transcriptomic analysis of Corynebacterium glutamicum in the response to the toxicity of furfural present in lignocellulosic hydrolysates** Park HS, et al. Process Biochemistry, 2015. Vol. 50(3), pp. 347 – 356.	Review / Exclude Not relevant to safety of C. glutamicum
#18 / 79	allintitle: Corynebacterium clinical OR clinically	**The clinical course of peritoneal dialysis-related peritonitis caused by Corynebacterium species** Szeto CC, et al. Nephrology Dialysis Transplantation, 2005. Vol. 20 (12), pp. 2793 – 2796. https://doi.org/10.1093/ndt/gfi123	Review / Exclude Not relevant to safety of C. glutamicum
		Nondiphtherial Corynebacterium species isolated from clinical specimens of patients in a university hospital, Rio de Janeiro, Brazil Camello TCF, et al. Braz. J. Microbiol., 2003. Vol. 34 (1).	Review / Exclude Not relevant to safety of C. glutamicum
		Antibiotic susceptibility of	Review / Exclude

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		Corynebacterium isolated from clinical specimens Chen D, et al. Chinese Journal of Clinical Laboratory Science, 2011. Vol. 3	Not relevant to safety of C. glutamicum
		Relationship Between Susceptibility to Quinolones in Corynebacterium Ophthalmic Clinical Isolates and the GyrA Gene Mutations Katome T, et al. Investigative Ophthalmology & Visual Science, 2008. Vol. 49 (13).	Review / Exclude Not relevant to safety of C. glutamicum
		Relationship Between Mutations in the gyrA Gene and Quinolone Resistance in Ophthalmic Clinical Isolates of Corynebacterium Species Eguchi H, et al., Investigative Ophthalmology & Visual Science, 2006. Vol. 47 (13), pp. 3566.	Review / Exclude Not relevant to safety of C. glutamicum
		Endophthalmitis Caused by Corynebacterium Species: Clinical Features, Antibiotic Susceptibility, and Treatment Outcomes Kuriyan AE, et al. Ophthalmology retina, 2017. Vol. 1 (3), pp. 200 – 205.	Review / Exclude Not relevant to safety of C. glutamicum
#19 / 2	allintitle: Corynebacterium death OR deaths	none	
#21 / 2	allintitle: Corynebacterium mortality OR mortalities	**Biodegradation of Contaminated Environments Using Corynebacterium glutamicum and Its Application to Livestock Mortalities Burials** [rest of the details are in Chinese]	Exclude (based on abstract; no translation of full paper)) Not relevant to safety of C. glutamicum
#22 / 23	allintitle: Corynebacterium disease OR diseases	**Corynebacterium species and coryneforms: An update on taxonomy and diseases attributed to these taxa** Bernard K. Clinical Microbiology Newsletter, 2005. Vol. 27(2), pp 9	Exclude Not relevant to safety of C. glutamicum

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
		– 18. DOI: https://doi.org/10.1016/j.clinmicnews.2005.01.002.	
#23 / 6	allintitle: Corynebacterium illness OR illnesses	none	
#24 / 254	anywhere: "Corynebacterium glutamicum"	Few results repeated	
#25 / 193	anywhere: "Corynebacterium glutamicum" resistance	none	
#26 / 208	anywhere: "Corynebacterium glutamicum" resistant	none	
#27 / 140	anywhere: "Corynebacterium glutamicum" antibiotic resistance	none	
#28 / 163	anywhere: "Corynebacterium glutamicum" antibiotic resistant	**Drivers of bacterial genomes plasticity and roles they play in pathogen virulence, persistence and drug resistance** Patel S. Infection, Genetics and Evolution, 2016. Vol. 45, pp. 151 – 164.	Exclude Not relevant to safety of C. glutamicum
#29 / 103	anywhere: "Corynebacterium glutamicum" antimicrobial susceptibility OR susceptibilities	none	
#30 / 107	anywhere: "Corynebacterium glutamicum" infection OR infections	none	
#31 / 8	anywhere: "Corynebacterium glutamicum"	Corynebacterium ulcerans, an emerging human pathogen Hacker E, et al. Future	Exclude Not relevant to C.

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
	abscess OR abscesses	Microbiology, 2016. Vol. 11 (9). https://doi.org/10.2217/fmb-2016-0085	glutamicum
#32 / 15	anywhere: "Corynebacterium glutamicum" sepsis OR septic	none	
#33 / 9	anywhere: "Corynebacterium glutamicum" bacteremia OR bacteraemia	none	
#34 / 137	anywhere: "Corynebacterium glutamicum" toxic OR toxin OR toxins	none	
#35 / 124	anywhere: "Corynebacterium glutamicum" pathogen OR pathogenic OR pathogenicity	none	
#36 / 95	anywhere: "Corynebacterium glutamicum" opportunistic OR virulence OR virulent	none	
#37 / 100	anywhere: "Corynebacterium glutamicum" safety OR risk	none	
#38 / 21	anywhere: "Corynebacterium glutamicum" mutagenic OR mutagenicity	none	
#39 / 86	anywhere: "Corynebacterium glutamicum" toxicity OR toxicology	none	
#40 / 89	anywhere: "Corynebacteriu	none	

Search Strategy No. / hits	Search Strategy	Selected Publications	Include / Exclude Justification
	m glutamicum" clinical OR clinically		
#41 / 92	anywhere: "Corynebacterium glutamicum" death OR deaths	none	
#42 / 12	anywhere: "Corynebacterium glutamicum" morbidity OR morbidities	none	
#43 / 99	anywhere: "Corynebacterium glutamicum" mortality OR mortalities	none	
#44 /141	anywhere: "Corynebacterium glutamicum" disease OR diseases	none	
#45 / 19	anywhere: "Corynebacterium glutamicum"	none	