# 21120.10562 SARS CoV 2 Whole Genome Sequencing Verification report

## Copy of version 1.0 (approved and current)

**Last Approval or Periodic Review Completed** 19-Mar-2021

Periodic review not required

**Effective Date** 19-Mar-2021

**Uncontrolled Copy** printed on 19-Mar-2021 13:07

**Printed By** (b) (6)

**Organization** Eurofins - Viracor

**Author**
(b) (6)

**Comments for version 1.0**
Initial version



**Approval and Periodic Review Signatures**

| Type | Description | Date | Version | Performed By | Notes |
|---|---|---|---|---|---|
| Approval | (b) (6) | 19-Mar-2021 12:22 | 1.0 | (b) (6) (b) (6) | |
| Approval | (b) (6) Approval | 19-Mar-2021 1:29 | 1.0 | (b) (6) (b) (6) | |
| Approval | (b) (6) Approver - | 17-Mar-2021 21:32 | 1.0 | (b) (6) (b) (6) | |

**Version History**

| Version | Status | Type | Date Added | Date Effective | Date Retired |
|---|---|---|---|---|---|
| 1.0 | Approved and Current | Initial version | 17-Mar-2021 | 19-Mar-2021 | Indefinite |

# Verification report to establish performance of the SARS Coronavirus 2 (SARS-CoV-2) Whole Genome Sequencing (WGS) assay

## A. Introduction / Objective

An outbreak of coronavirus disease 2019 (COVID-19) caused by the 2019 novel coronavirus (SARS-CoV-2) began in Wuhan, Hubei Province, China in December 2019, and has spread throughout China as well as numerous other countries, including the United States. The outbreak was declared a Public Health Emergency of International Concern on 30 January 2020 by the World Health Organization. Signs and symptoms of COVID-19 include fever, cough, and shortness of breath. Person-to-person spread of SARS-CoV-2 appears to occur mainly by respiratory transmission. Based on the incubation period of illness for Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) coronaviruses, as well as observational data from reports of travel-related COVID-19, CDC estimates that symptoms of COVID-19 occur within 2–14 days after exposure. Preliminary data suggest that older adults and persons with underlying health conditions or compromised immune systems might be at greater risk for severe illness from this virus.

In order to evaluate epidemiological trends and patterns regarding newly identified circulating genetic variants of SARS-CoV2 public health agencies are performing genome-wide sequencing on a proportion of positive samples collected from routine testing. The emerging variants of concern (B.1.1.7 – the UK variant; B.1.351 – variant from South Africa; and P.1 – Brazilian variant) are currently being investigated due to concerns that these novel strains may be associated with increased transmission rates, increased mortality rates, and / or a decrease in the efficaciousness of available vaccines. Eurofins Genomics (Ebersberg, Germany) has developed a whole genome sequencing assay using Illumina next-generation sequencing chemistries / platforms. Eurofins-Viracor verified the performance of this method with the goal of assisting various US-based health authorities in tracking these variants as they emerge.

The primary objective of this study was to evaluate the performance of the Eurofins SARS-CoV-2 virus (SARS-CoV-2) specific Whole Genome Sequencing Assay to detect and sequence SARS-CoV-2 RNA in upper respiratory (nasal/nasopharyngeal swab) specimens. This assay is intended to sequence genomic RNA extracted from the SARS-CoV-2 virus. This assay is intended for use with specimens collected from individuals meeting SARS-CoV-2 virus clinical criteria (e.g., clinical signs and symptoms).

This verification report is intended to provide documented evidence of equivalent limit of detection (LoD), (b) (4) between Eurofins Genomics Laboratories and Eurofins Viracor.



## B. Scope

This verification report includes the extraction kit/reagent methods/automated extraction platforms, assessment of limit of detection (LoD), (b) (4) and acceptance criteria for each of these approaches, for the SARS-CoV-2 WGS assay. This verification report is intended to document the equivalent perfromance of the Eurofins Genomics SARS-CoV2 WGS assay after transfer to Viracor-Eurofins. **Table 1** is a summary of the report including acceptance criteria.

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**Table 1.** Summary of verification criteria for SARS-COV-2 WGS assay

| Performance Characteristic | Action | Results | Pass/Fail |
|---|---|---|---|
| Limit of Detection (LoD) | The SARS-CoV-2 WGS LoD was previously established by Eurofins Genomics at 100 copies per assay. The LoD was re-confirmed using twist SARS-CoV2 RNA control material (b) (4) (b) (4) (b) (4) (b) (4) were evaluated and prepared with the Twist SARS-CoV2 synthetic RNA positive control material. | The LoD for SARS-CoV2 WGS was previously determined by Eurofins Genomics to be 100 copies/ARTIC PCR Assay reaction. This LoD was re-confirmed by Viracor. (b) (4) (b) (4) (b) (4) (b) (4) (b) (4) (b) (4) (b) (4) | **PASS** |

| | |
|---|---|
| (b) (4) | **PASS** |
| | **PASS** |

Data located in Run Packet #1 SARS WGS tab in Binder B-2020-027-E

## C. Materials

The following materials (or suitable equivalents) were used:

1. GSD NovaPrime® RNA Extraction (b) (4) Kit, catalog # (b) (4)
2. ThermoFisher KingFisher Flex instrument with associated disposables
3. (b) (4)
4. (b) (4) Internal RNA Control (MS2). Stock (part # (b) (4) ), a (b) (4) (b) (4)
5. (b) (4) Instrument with disposables
6. (b) (4) ((b) (4) catalog # (b) (4)
7. (b) (4) SARS-COV-2 part (b) (4)
8. ARTIC PCR primer pools
9. SARS-CoV-2-negative human upper respiratory NP/NS
10. (b) (4) thermal cycler
11. (b) (4) catalog # (b) (4)
12. (b) (4) ((b) (4) catalog # (b) (4) )
13. NEBNext ULTRA II FS DNA (NEB E7805L)
14. Purification Beads (Beckman Coulter catalog # (b) (4) )
15. (b) (4) ((b) (4) catalog # (b) (4) )
16. Qubit FLEX fluorometer (ThermoFisher catalog #Q33327)
17. (b) (4) (b) (4) catalog # (b) (4)
18. NextSeq 500/550 Mid output (b) (4) (Illumina catalog # (b) (4)
19. Agilent BRAVO liquid handler
20. (b) (4) pipette tips (b) (4)
21. (b) (4) deep-well plates (b) (4)
22. (b) (4) PCR plates (b) (4)
23. (b) (4) BioAnalyzer
24. (b) (4) ((b) (4) catalog # (b) (4) )
25. Agilent TapeStation 4200
26. TapeStation (b) (4) tapes (Agilent catalog # (b) (4) )
27. TapeStation (b) (4) reagents (Agilent catalog # (b) (4)
28. RNase-, DNase-free water, (b) (4) , catalog # (b) (4)
29. Pipette tips with aerosol barrier: 10µL, 200µL, and 1000µL sizes
30. Pipettes to accommodate tip sizes listed above
31. (b) (4) statistical software (b) (4)
32. (b) (4) statistical software (b) (4)
33. Twist RNA: Synthetic RNA control for variant of the SARS-CoV-2 virus: (b) (4) (Twist Bioscience, Cat. No. (b) (4) )

## D. Methods

**Sample preparation**

All samples were (b) (4)

(b) (4)

**Nucleic acid extraction**

Nucleic acid extraction for respiratory specimens was performed for each sample following instructions in SOP 21120.9718 *KingFisher NovaPrime Nucleic Acid Isolation* with the following modifications:

KingFisher with GSD NovaPrime® RNA Extraction (AE1) Kit
GSD NovaPrime® RNA Extraction reagents and protocol were performed following manufacturer recommendations with the following exceptions: KingFisher protocol followed a (b) (4) (b) (4) that was provided by the kit manufacturer. The protocol used (b) (4) (b) (4) . Once reagents and samples were added to the respective deep-well plates the KingFisher executed binding, wash and elution protocols.

A Twist SARS-CoV2 RNA positive control was processed in parallel with each verification run for positive control of the reverse transcription, ARTIC PCR amplification, and library preparation.

**Nucleic acid amplification**

**cDNA Synthesis**
RNA extracted and purified by the KingFisher was converted into cDNA using (b) (4) (b) (4) according to the following table:

(b) (4)

Reverse transcription was performed using the (b) (4) thermalcycler running the following protocol:

(b) (4)

**ARTIC PCR**
Following reverse transcription, each cDNA sample was subjected to amplification using ARTIC SARS-CoV2 Primer Pools (b) (4) These primer pools were designed to amplify approximately 90 amplicons each with each amplicon averaging ~400bp. Mapping these amplicons to a reference sequence illustrates the 'tiled' approach used for primer design resulting in coverage of the entire SARS-CoV2 genome.

ARTIC PCR reactions were amplified using primer pools supplied by Eurofins Genomics Laboratories (EGL) and (b) (4) reagents according to the following tables:

(b) (4)

ARTIC PCR was performed using the (b) (4) thermalcycler running the following protocol:

(b) (4)

(b) (4)

**Magnetic Bead Cleanup**(b) (4)

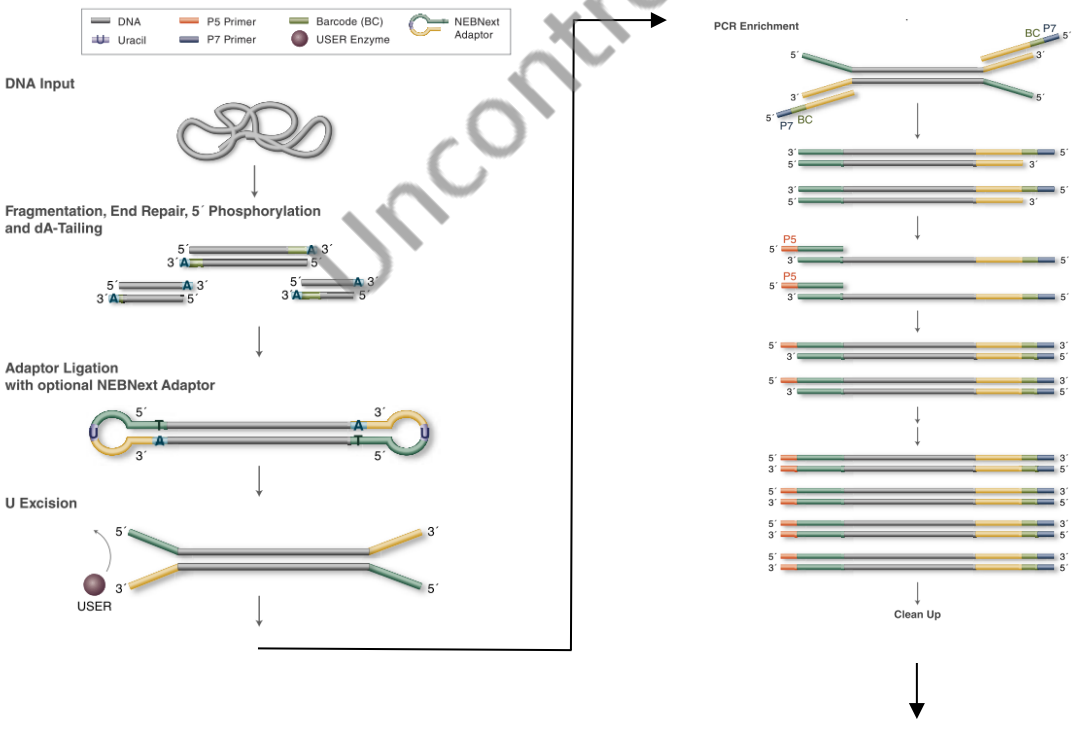
Following pooling and dilution, purification of the ARTIC PCR reactions was performed manually with Beckman-Coulter SPRIselect magnetic beads. (b) (4)
(b) (4)
(b) (4)

**ARTIC PCR Product Quantification and Normalization**

The concentration of amplified amplicons in each sample was quantified using the Qubit FLEX fluorometer and (b) (4) reagents. Values collected were used to (b) (4)
(b) (4)

**NGS Library Preparation**

Preparation of libraries was performed using the NEBNext Ultra II FS library prep kit in conjunction with the BRAVO liquid handling platform. This protocol followed the NEB ULTRA II FS DNA Library Kit for Illumina v2.2.




Sequencing (NextSEQ 500/550)

**End Repair / Fragmentation**

Following normalization, amplicons underwent enzymatic fragmentation and end repair using the NEBNext Ultra II FS library prep kit. A total of (b) (4) (b) (4) according to the table below:

(b) (4)

End Repair / Fragmentation was performed using the (b) (4) thermalcycler running the following protocol:

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4) **PCR**

(b) (4)

(b) (4)

(b) (4)

**Library Quantification and Normalization**

The mass of each purified indexed library was quantified using the Qubit FLEX fluorometer and (b) (4) (b) (4) reagents. (b) (4) (b) (4) The average fragment size of each library was assumed to be approximately 400bp for these calculations.

**Library Pooling**

Once libraries were (b) (4) (b) (4)

**Library QC**

The fragment size distribution of the final pooled library was confirmed using the Agilent TapeStation 4200 or ThermoFisher BioAnalyzer 2100 DNA fragment analyzer prior to preparation for sequencing.

**Library Sequencing on the Illumina NextSEQ 500/550**

Pooled (b) (4) libraries were denatured, (b) (4) (b) (4) and sequenced on the NextSEQ 500 or 550 instrument using a NextSEQ Mid Output 500/550 flow cell and reagents running a 2x150 cycle paired-end sequencing protocol.

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## Performance Characteristics Evaluation

**Limit of detection (LoD)**

SARS-CoV-2 WGS limit of detection was performed using a pre-characterized Twist SARS-CoV2 synthetic RNA control stock. The LoD for SARS-CoV2 WGS was previously determined to be 100 copies/ARTIC PCR Assay. This LoD was re-confirmed with (b) (4) ARTIC PCR Assay, (b) (4) /ARTIC PCR Assay and (b) (4) /ARTIC PCR Assay using this material.

- **Acceptance criteria**

The LoD will be confirmed if the previous baseline LoD reported and Viracor method generate a

(b) (4)

(b) (4)

- **Acceptance criteria**

(b) (4)

(b) (4)

- **Acceptance criteria**

(b) (4)

## General Acceptance Criteria

**Acceptance criteria for controls**

(b) (4)

(b) (4)

**ARTIC PCR Acceptance Criteria**

- ARTIC PCR must produce a (b) (4) to continue through sequencing. This acceptance criterion was successfully met. (b) (4) are included in the run packet.

**NextSeq Acceptance Criteria**

Sequencing data was assessed using the (b) (4)
(b) (4)

**SARS CoV-2 WGS Acceptance Criteria**

- Acceptance criteria for WGS require (b) (4)
(b) (4)

## E. Analysis

**Eluate Check: Qubit quantification**

The Qubit fluorometer was used to determine dilution factors for library input dilutions. A .csv file was generated, saved as an excel file, and included in the run packet.

**Library Quality Check: Qubit Flex and TapeStation or BioAnalyzer**

The Qubit Flex fluorometer and Agilent 4200 TapeStation in conjunction with (b) (4)
or BioAanlyzer in conjunction with BioAanlyzer kits and reagents were used to determine the (b) (4)
(b) (4) The values reported
were used to determine (b) (4)
was performed to verify that the pooled library (b) (4) (b) (4)

(b) (4)

A Qubit .csv file was generated, saved as an excel file, and included in the run packet. A TapeStation or BioAnalyzer portable digital file (.pdf), along with a Sample Table .csv, was also be generated for each run performed. The Sample Table was printed along with the .pdf report for inclusion in the run packet.

**Sequencing Analysis**

Upon completion of NextSeq runs, cluster density and Q≥30 scores were evaluated to verify that the data are of sufficient quality to be run through the (b) (4) . Q≥30 scores must be ≥80%,

(b) (4)

**SARS CoV-2 Analysis**

SARS CoV-2 WGS sequencing results were analyzed with (b) (4)
Instructions for the (b) (4) are summarized below.

(b) (4)

## F. Results and Conclusions

Results were summarized graphically and/or in tables.

A verification report was generated with all the results obtained from this verification protocol.

Footnotes were included in the verification report with the run packet numbers from which the data originated.

**Controls and negative samples**

All (b) (4) were negative within all SARS-CoV2 ARTIC PCR runs performed during this verification. Since there was no detected amplification for these negative controls during QC steps (Qubit quantification after ARTIC PCR), these samples were removed from library preparation and thus not sequenced.

A Twist SARS-CoV2 RNA positive control was used during the verification for positive control of the reverse transcription, ARTIC PCR amplification, and library preparation and performed as expected.

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**Limit of Detection (LoD):**

The LoD for SARS-CoV2 WGS was previously determined by Eurofins Genomics to be 100 copies/ARTIC PCR assay reaction. This LoD was re-confirmed by Viracor, using commercially available twist SARS-CoV2 synthetic RNA control material. (b) (4) (b) (4) were evaluated and prepared with the Twist SARS-CoV2 synthetic RNA positive control material. An (b) (4) sample was also included as part of the cDNA and ARTIC PCR assay reactions. (b) (4) sample passed QC steps for ARTIC PCR, and so it was not included in library preparation or sequencing.

LoD results are summarized in **Table 2**. Samples for (b) (4) (b) (4) (b) (4) All operators generated the same results with respect lineage of samples tested (**Table 3**).

LoD results for (b) (4) copies/assay reaction, the (b) (4) tested, generated genome coverage at the (b) (4) The data is shown in **Table 2.** Samples at this (b) (4) failed the acceptance criteria and the pangoLEARN SARS-CoV2 lineage classifier could not determine a probable strain designation. Therefore, a lineage could not be determined due to an (b) (4) (b) (4) to the SARS-CoV2 reference genome for (b) (4) reactions (**Table 3**).

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**Table 2:** LoD Summary Table.

(b) (4)

Data located in Run Packet #1 SARS WGS tab in Binder B-2020-027-E

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**Table 3:** LoD Lineage

(b) (4)

Data located in Run Packet #1 SARS WGS tab in Binder B-2020-027-E

(b) (4)

Previously characterized positive samples were used for (b) (4) (b) (4) testing.

(b) (4)

the final lineage results. Results are listed in **Appendix A** and a summarized version of these results is shown in **Table 4**.

**Table 4:** (b) (4)

(b) (4)

Data located in Run Packet #1 SARS WGS tab in Binder B-2020-027-E

(b) (4)

(b) (4)

(b) (4) Results are listed in **Appendix A** and a summarized version of these results is shown in **Table 4**.

(b) (4)

**Clinical Evaluation** (b) (4)

(b) (4)

Results for all samples are listed in **Appendix B** and **Table 5**.

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**Table 5:** (b) (4) **Lineage Table**

(b) (4)



Data located in Run Packet #1 SARS WGS tab in Binder B-2020-027-E

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**Conclusions:**

The performance characteristics of the SARS CoV-2 RT Whole Genome Sequencing assay met the acceptance criteria specified in *21120.10413 Verification protocol to establish performance of the SARS Coronavirus 2 (SARS-CoV-2) Whole Genome Sequencing (WGS) assay*. I have reviewed the verification and the performance of the method is considered acceptable for patient testing.

## G. Exceptions

Any deviations or exceptions to this protocol were documented on the appropriate laboratory records and data packets and addressed in the verification report. Instances in which the pre-specified acceptance criteria were not met were identified and evaluated in the verification report. Verification approval/rejection was not meant to be exclusively determined based on pass/fail outcome. Rather, criteria failures were investigated and evaluated based on the nature of the violation and its assessed impact in the context of clinical testing after further discussion between the design review committee members and Eurofins Viracor technical team.

Magnetic Bead Cleanup (b) (4) was performed manually. The BRAVO protocol for this step was not implemeted before verification began.

NGS Library Preparation was run with the full volume protocol as described by NEB for the NEBNext UltraII FS Library Prep kit. The (b) (4) protocol was not implemented before verification began.

Ideal sequencing metrics are typically collected when $C_T$ values for samples are (b) (4) using the in-house developed and validated SARS-CoV2 (b) (4) assay. SARS-Cov2 samples from this verification that did not meet whole genome sequencing quality metrics were assessed using (b) (4)

Two Twist SARS-CoV2 RNA positive controls prepared for verification runs (b) (4) did not pass sequencing acceptance criteria. These eluates were evaluated by (b) (4) to determine $C_T$ values. The reported $C_T$ value for these preparations was (b) (4) This value approximates the (b) (4) $C_T$ cutoff and explains the failure. It should be noted that the positive controls used for runs (b) (4) were from a different preparation than the positive control(s) used for runs (b) (4)

(b) (4) samples (b) (4) did not pass sequencing acceptance criteria. These eluates were assessed by (b) (4) to determine $C_T$ values. (b) (4) reported a $C_T$ value of (b) (4) which is near the LoD of the assay; sample (b) (4) (b) (4) generated a $C_T$ value of (b) (4) This is within acceptable assay C range and was identified as an assay failure of unknown origin. (b) (4) (b) (4)



## H. References

Policy for Diagnostics Testing in Laboratories Certified to Perform High Complexity Testing under CLIA prior to Emergency Use Authorization for Coronavirus Disease-2019 during the Public Health Emergency Immediately in Effect Guidance for Clinical Laboratories and Food and Drug Administration Staff. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Devices and Radiological Health. February 29, 2020.

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EP17-A Vol 24 No 34, replaces EP17-P. Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline.Clinical and Laboratory Standards Institute.

(b) (4)

(b) (4)

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR 3rd, Smith TF. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev. 2006 Jan;19(1):165-256. Review. Erratum in: Clin Microbiol Rev.2006Jul;19(3):595

Guidance for Industry; Bioanalytical Method Validation. U.S. Department of Health and Human Services, FDA Food and Drug Administration, Center for Drug Evaluation and Research Center for Veterinary Medicine, May 2001 BP.

MM3-A2, Vol. 26 No.8. Molecular Diagnostics Methods for Infectious Diseases; Approved Guideline, Second Edition, Clinical and Laboratory Standards Institute. Wayne, PA. 2006.

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TaqPath™ COVID-19 Combo Kit, Instructions For Use; Publication Number MAN0019181, Revision C.0. ThermoFisher Scientific; Pleasonton, CA. 19 April 2020.

Molecular Microbiology: Diagnostic Principles and Practice, Second Edition. David H. Persing. ASM Press. 2011. Washington, D.C.

(b) (4)

Submission guidelines for nucleic acid amplification tests for infectious agents, State of New York Department of Health. February, 2011.

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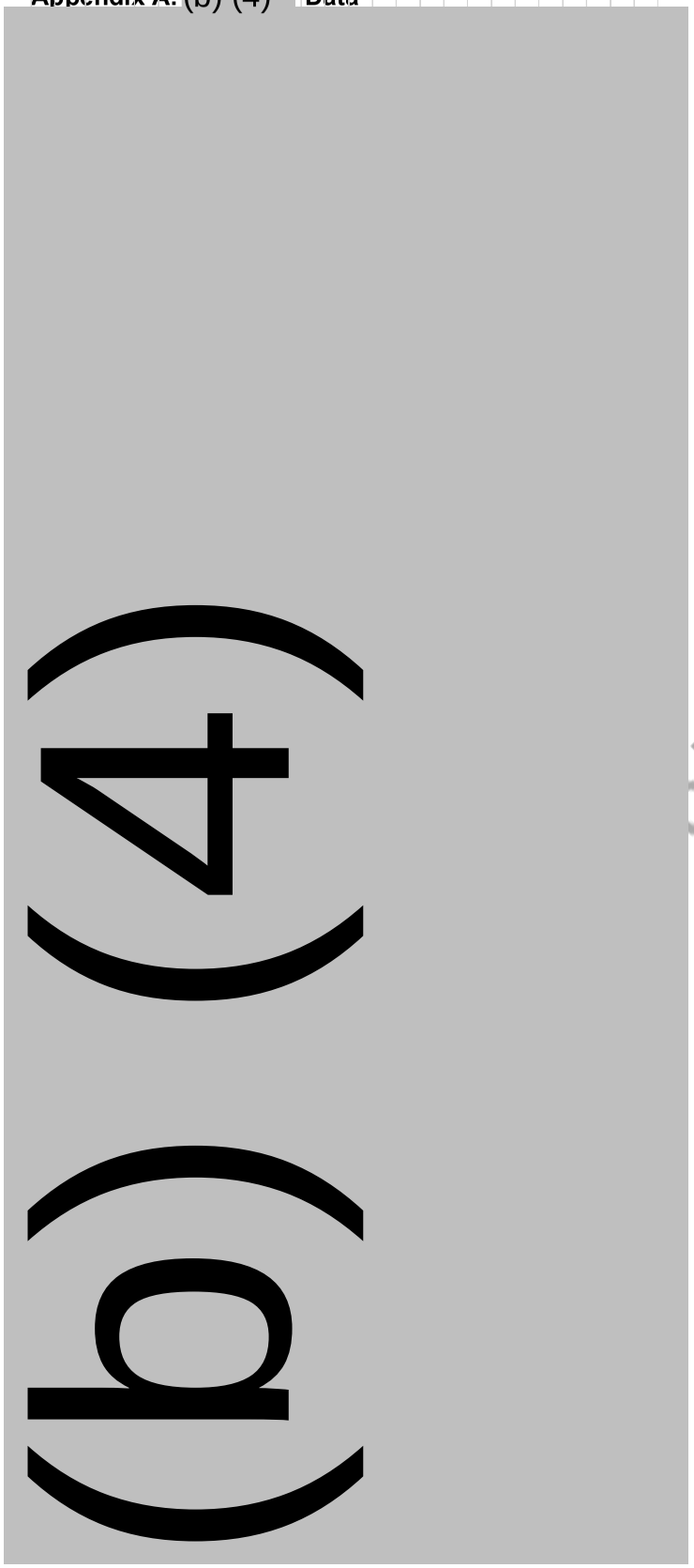
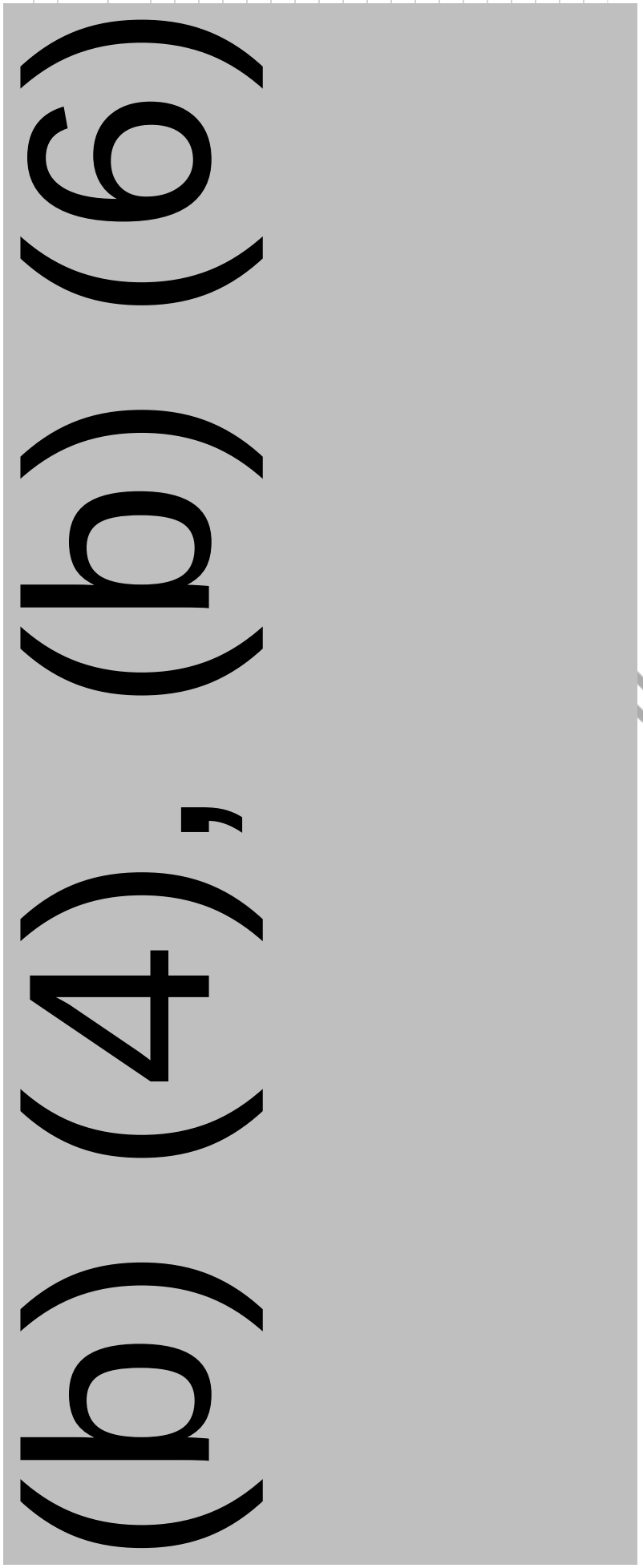
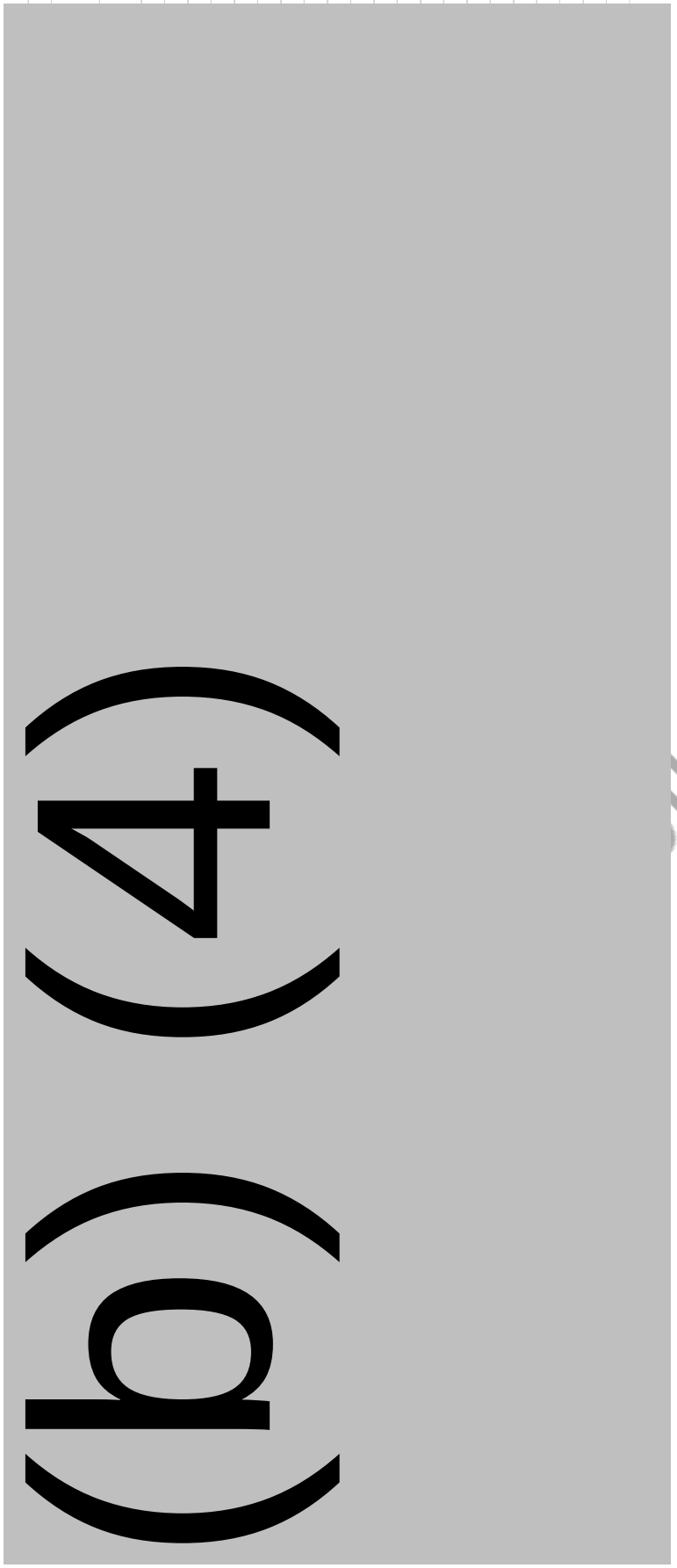
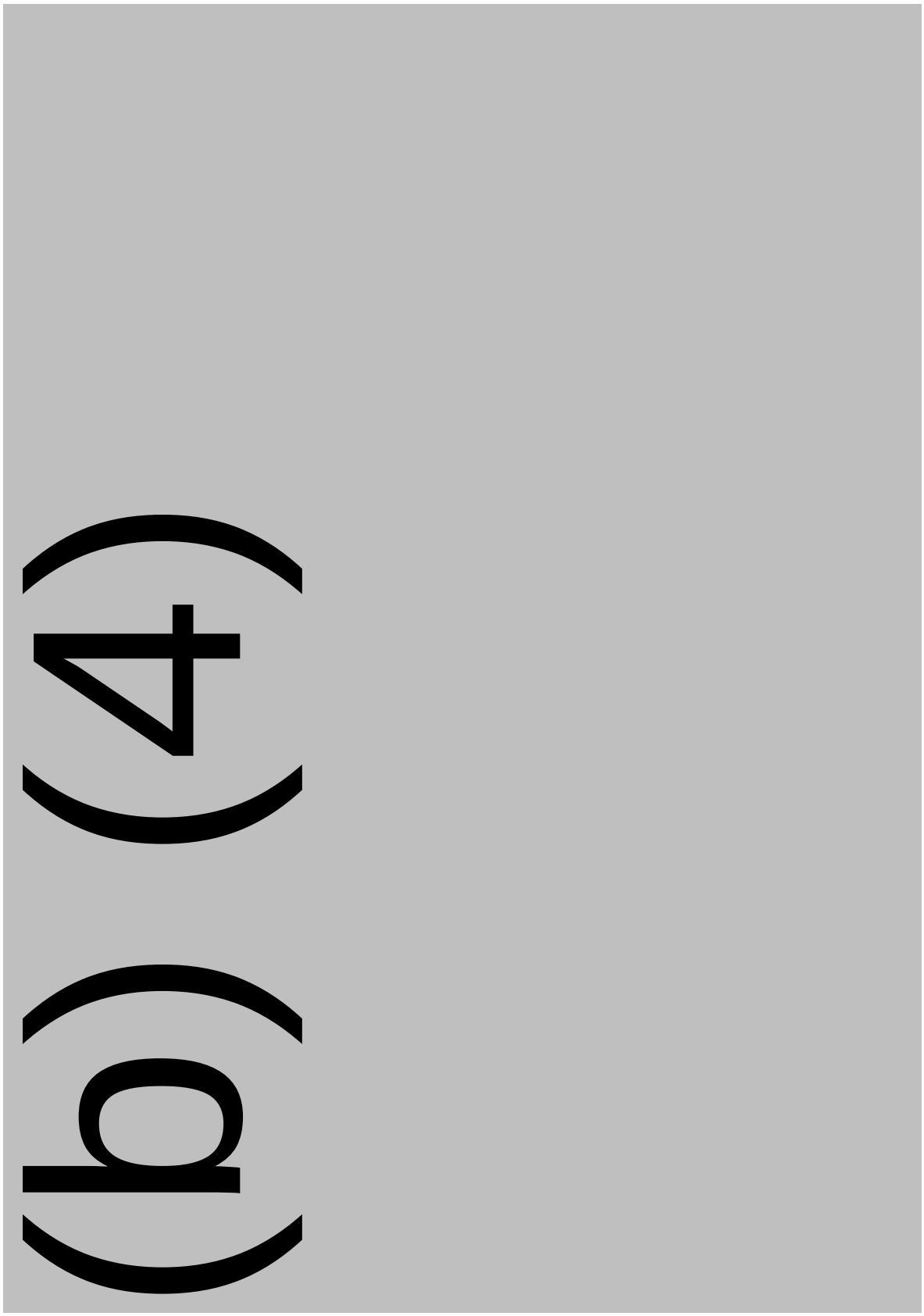
**Appendix A:** (b) (4) **Data**

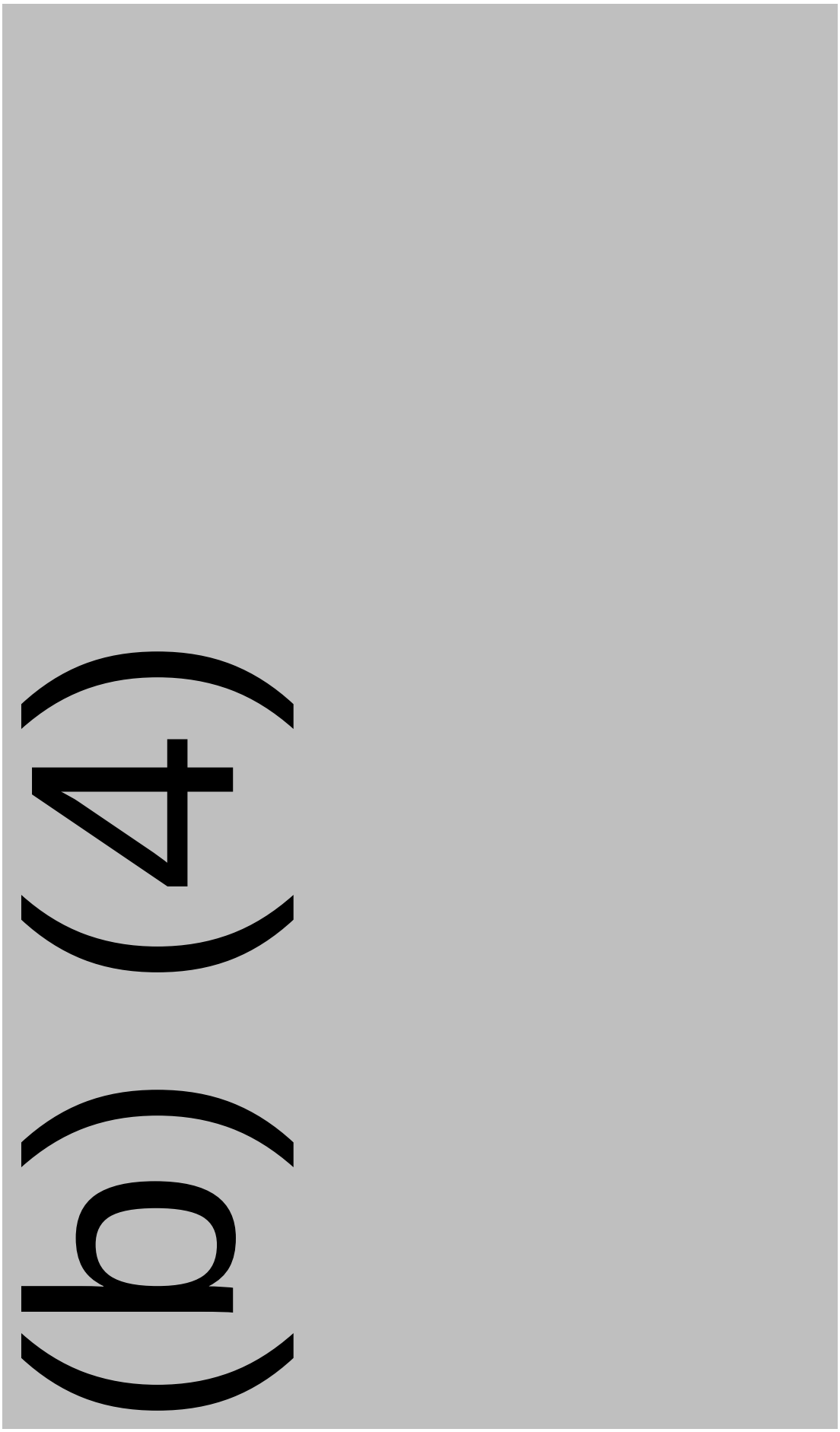


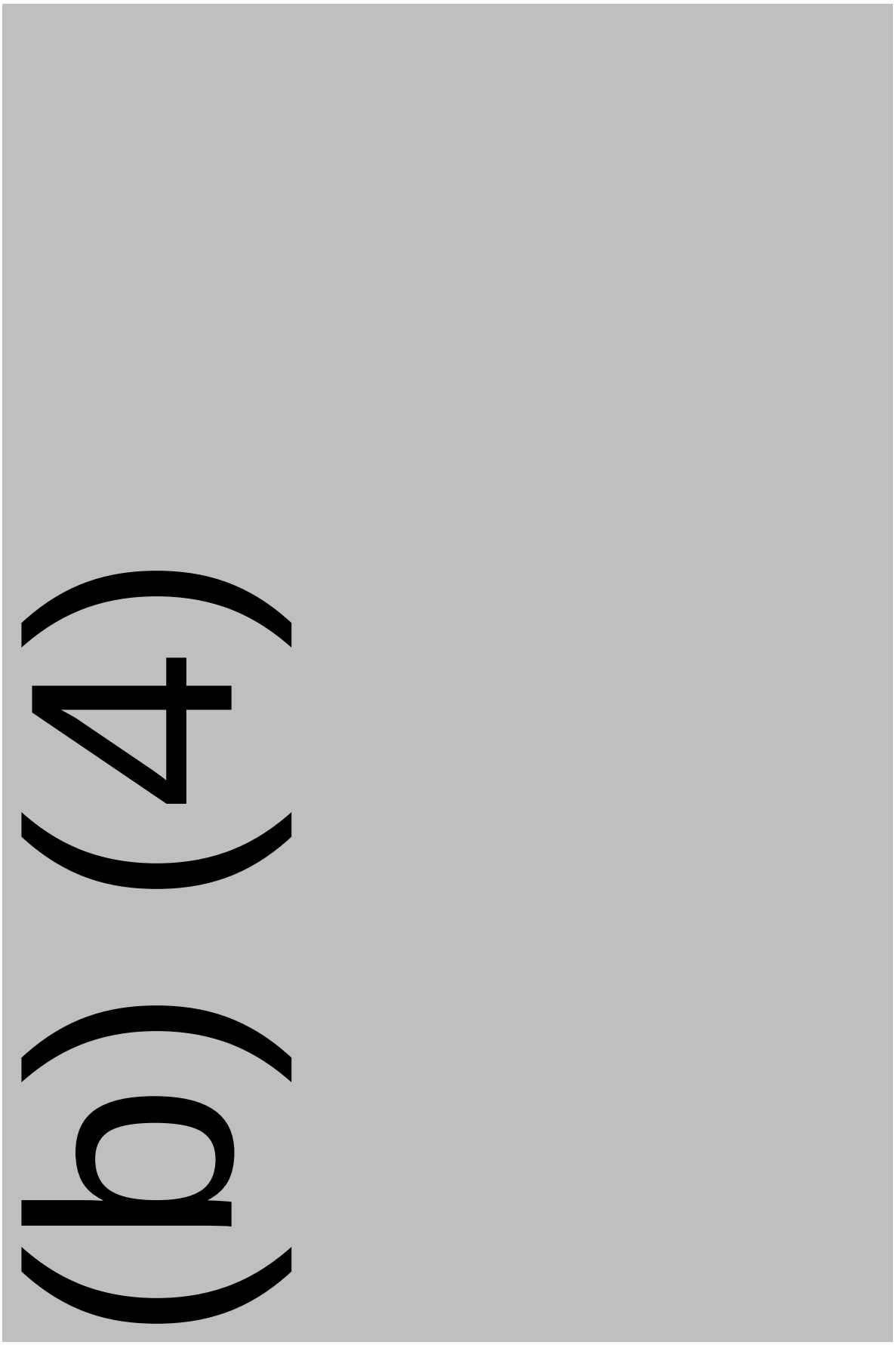




(b) (4)







**Appendix B:** (b) (4) **Sample** (b) (4)