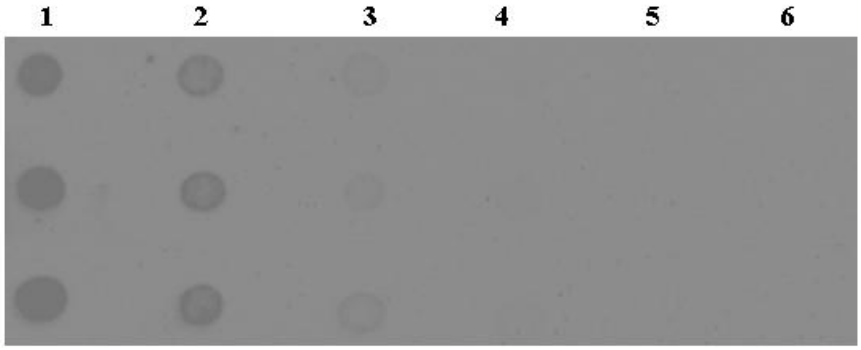
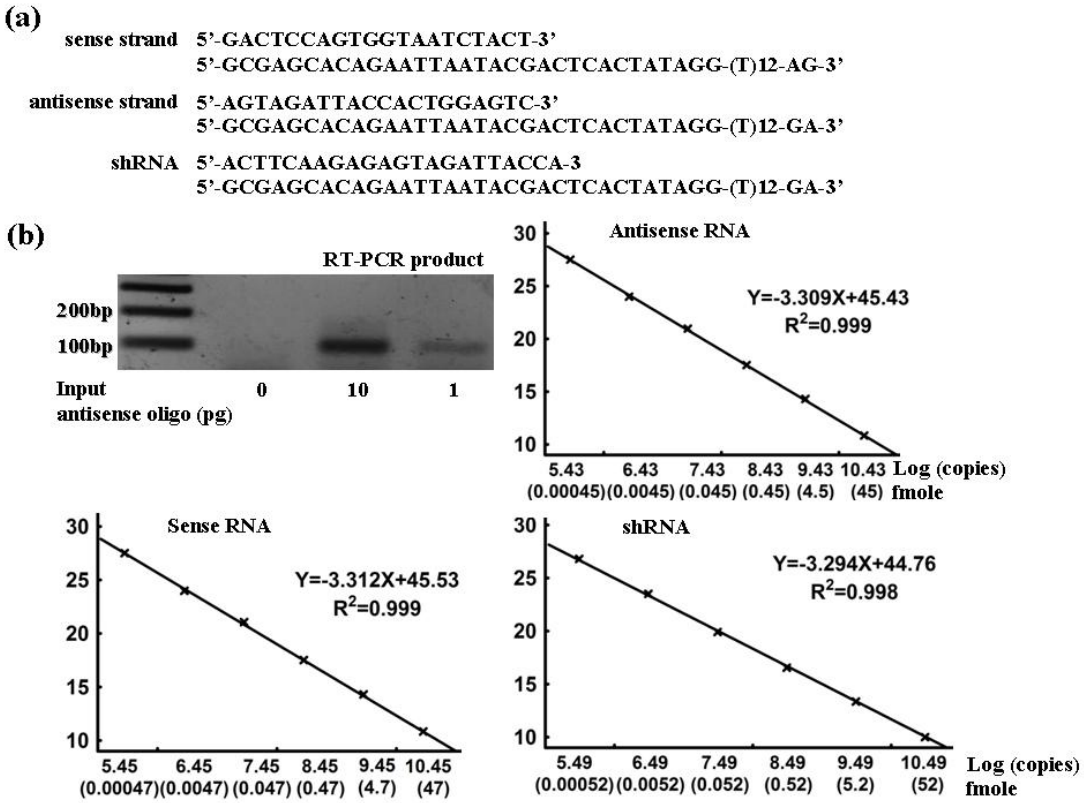
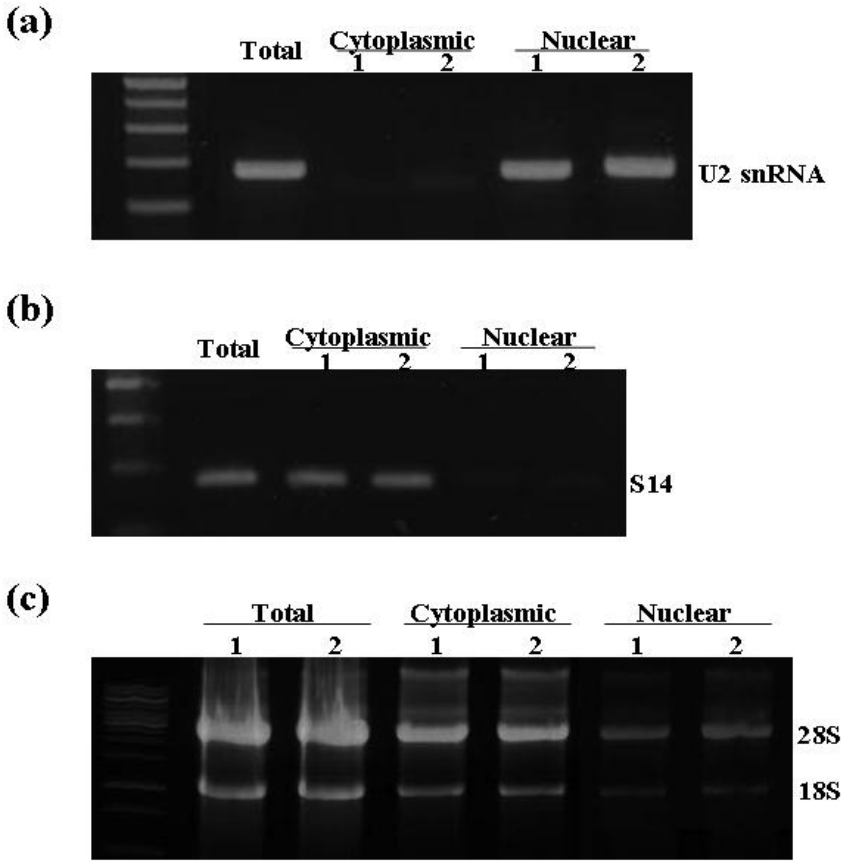


ng	**1.0**	**0.2**	**0.04**	**0.008**	**0.0016**	**0.00032**
fmol	**166**	**33.2**	**6.64**	**1.33**	**0.26**	**0.052**
copy	**$1.0*10^{11}$**	**$2.0*10^{10}$**	**$4.0*10^{9}$**	**$8.0*10^{8}$**	**$1.6*10^{8}$**	**$3.2*10^{7}$**

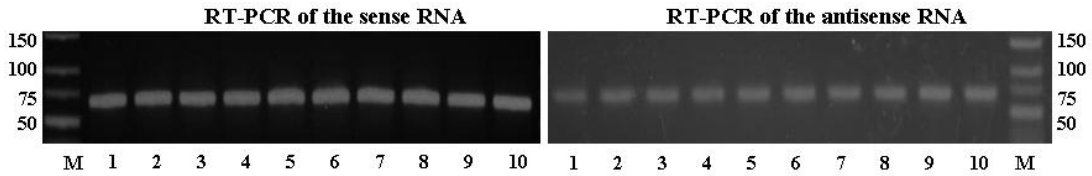
Analysis of the minimal detection level via Northern blotting for quantitation of dsRNA duplexes. The following amounts of the sense oligomer, 5-fold serially diluted, were added to 2 μg of total tRNA, and then spotted into a nylon membrane (left to right): 1.0, 0.2, 0.04, 0.008, 0.0016 and 0.00032 ng. A 2 nM solution of the DIG-labeled antisense RNA was used as probe for hybridization. Clearly, lower than 0.04 ng of the sense RNA, equal to 6.64 fmol or 4.0×10^{9} copies, would not be detected by this method. In this manuscript, the amount of dsRNA duplexes generated from a well of a 6-well plate, $7x10^{7}$ copies, is almost 50-fold lower than the minimal detection level by Northern blotting. Therefore, it is unfeasible to quantitation of the tiny amounts of dsRNA duplexes generated in the transfected and transduced cells by Northern bloting using the DIG-labeled probe.



Development of RT-PCR for quantitation of dsRNA duplexes and shRNA generated in cells. (a) Sequences of the respective forward and reverse primers utilized for analysis by RT-PCR of sense RNA, antisense RNA and shRNA. (b) Amplification of the antisense RNA by RT-PCR using 0, 1 and 10 pg input oligomer as template (30 cycles). Standard curves for plotting Ct *versus* the corresponding amount of input RNA oligomer for quantitation of the sense RNA, antisense RNA and shRNA.



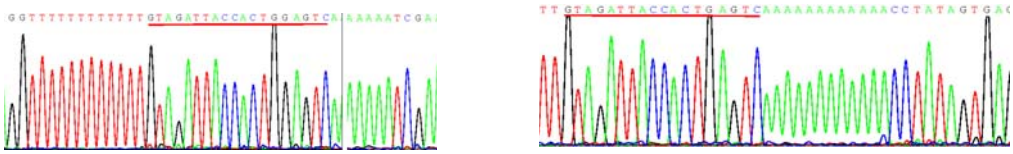
Confirmation of the integrity and quality of the isolated cytoplasmic and nuclear RNA. (a) Analysis of U2 snRNA by RT-PCR. An intense product was observed when the nuclear fraction rather than the cytoplasmic fraction was used as the template. (b) Analysis of S14 by RT-PCR. A major PCR band was obtained when used the cytoplasmic fraction rather than the nuclear as the template. (c) Analysis of ribosomal RNA location. The major 28S and 18S ribosomal RNA were detected in cytoplasm rather than in nuclear. Data shown are derived from an experiment that was conducted in duplicate for each sample.



1: *dsRNA* 2: *dsRNA(O)* 3: *dsRNA(O'* 4: *dsRNA-sense* 5: *dsRNA-antisense* 6: *hU6/U6-dsRNA-mU6/U6*

7: *hU6/U6/O-dsRNA-mU6/U6* 8: *hU6-dsRNA-mU6/U6* 9: *hU6/O-dsRNA-mU6/U6* 10: *hU6-dsRNA/O/mU6/U6*

1



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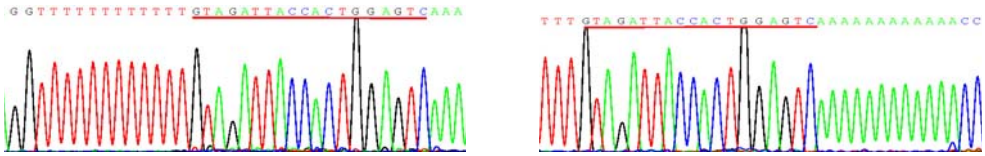

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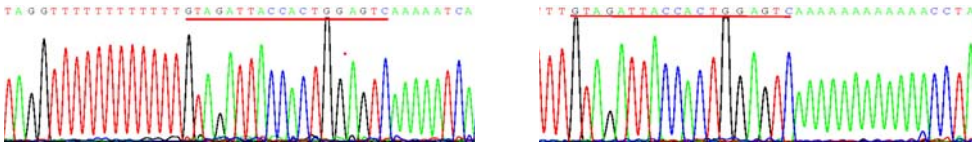

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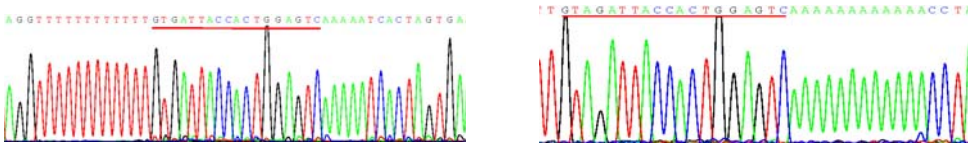

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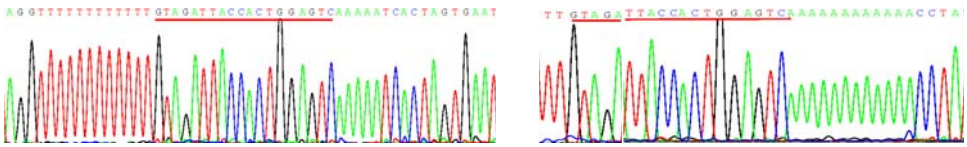

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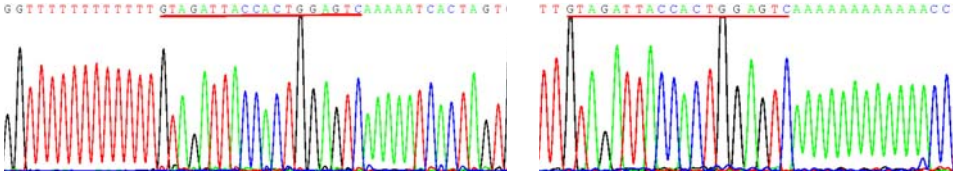

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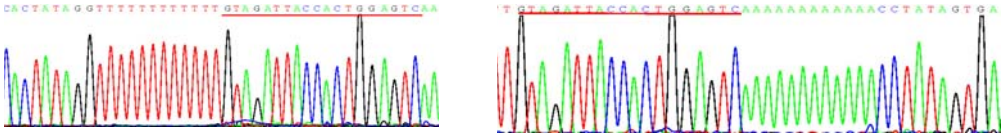

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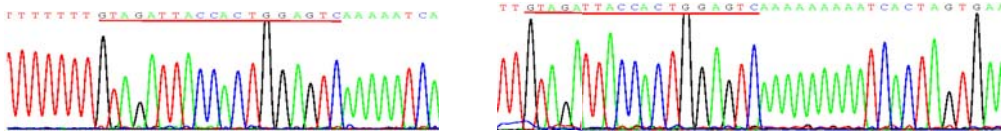

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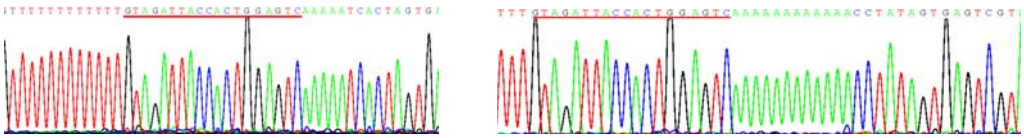
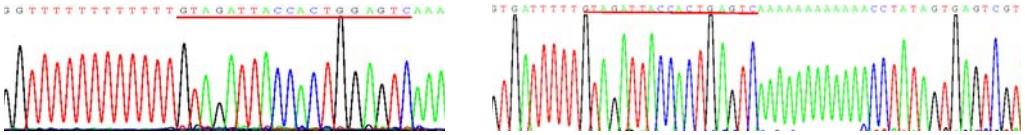
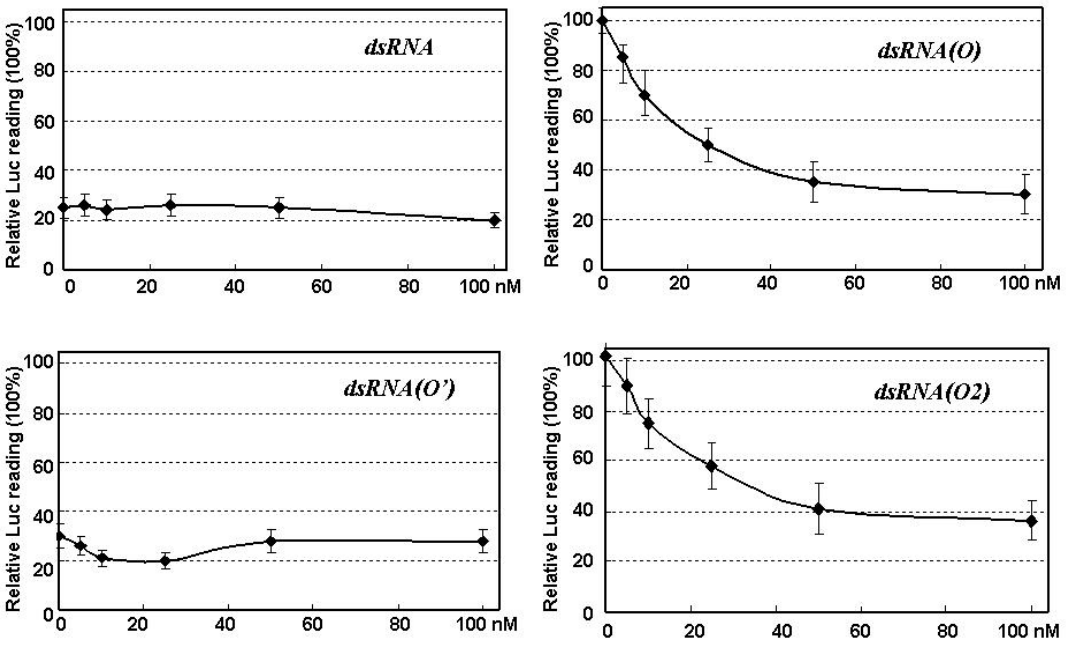
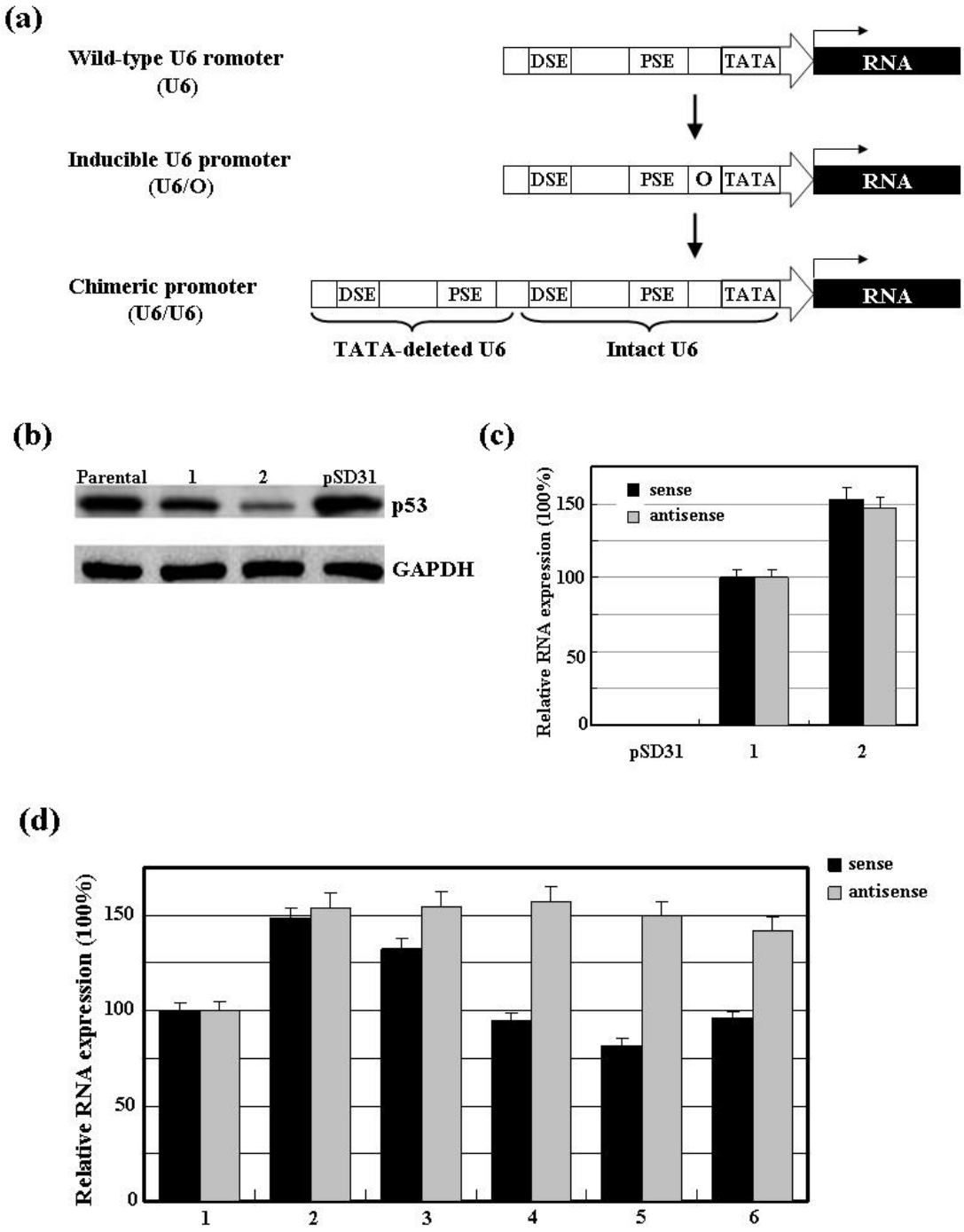


10 

Mapping of dsRNA duplexes generated from various expression cassettes. The RNA transcripts were captured and amplified by RT-PCR. The PCR products were cloned into TA vector and then harvested from TOP10 cells for sequencing. 1, 2, 3, …, 10 represented corresponding RT-PCR and sequencing data for *dsRNA*, *dsRNA(O)*, *dsRNA(O')*, *dsRNA-sense*, *dsRNA-antisense*, *hU6/U6-dsRNA-mU6/U6*, *hU6/U6/O-dsRNA-mU6/U6*, *hU6-dsRNA-mU6/U6*, *hU6/O-dsRNA-mU6/U6*, *hU6-dsRNA/O/mU6/U6*.



Confirmation of siRNA strand antagonism using the firefly luciferase reporter and dox titration system. pDual-Luci was co-transfected with *pSD31-siRNA*, *-siRNA(O)*, *-siRNA(O')* or *–siRNA(O2)* into 293FT cells cultured in the presence of 0, 5, 10, 25, 50 and 100 nM doxcycline. The luciferase reading decreased following dox titration from low concentration to high concentration for *pSD31-siRNA(O)* and *–siRNA(O2)*. By contrast, *pSD31-siRNA(O')* behaved differently with firefly luciferase reading decreased following initial dox titration (5, 10, 25 nM). But such trend reversed following continuous dox titration (50, 100 nM). Renilla luciferase reporter was used as an internal control and the ratio of firefly luciferase reading to renilla luciferase reading without siRNA cassette was normalized to 100%. Data shown are derived from an experiment that was conducted in triplicate for each sample.



(a) Schematic representation of the wild-type U6 promoter, which consists of DSE, PSE and a TATA box; an inducible U6 promoter, which contained a tetracycline operator (O) between PSE and the TATA box; and a chimeric promoter, which consisted of a TATA box-deleted U6 promoter and an intact U6 promoter. Deletion of the TATA box from the upstream region of the U6 promoter was designed to prevent transcription of RNAs other than dsRNA duplexes. (b) Western blotting analysis of knockdown of expression of p53 in transfected cells, as indicated. (c) Levels of

expression of the various dsRNA duplexes in transfected 293T cells with the *mU6-dsRNA-hU6* (1) and the *mU6/U6-dsRNA-hU6/U6* cassette (2) detected by real-time RT-PCR. (d) Levels of expression of the dsRNA duplexes in 293FT cells that harbored a variety of dsRNA-expression cassettes by real-time RT-PCR. The data are shown relative to levels of dsRNA duplexes in 293FT cells that harbored pSD31-*dsRNA*. Data shown are derived from results of an experiment that was conducted in triplicate for each sample.