Application of real time PCR for the quantitative detection of radiation-induced genomic DNA strand breaks

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The frequency of single strand breaks (SSBs) occurring on both strands of the pBR322 plasmid DNA region flanked by a pair of primers used for polymerase chain reaction (PCR) amplifications was determined after irradiation with ^{137}Cs γ rays. We verified that real time PCR is suitable for the detection and quantitative analysis of SSBs caused by γ ray irradiation. The utility of this approach was also supported by the comparison of the practical experimental data with the Monte Carlo simulation. The potential application of this PCR method for the detection of genomic DNA damage was also confirmed.

Key words: single strand break, radiation, scavenger, real-time PCR, plasmid DNA, Monte Carlo simulation, *Bombyx mori*

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INTRODUCTION

It is currently accepted that the biological effects of ionizing radiation are mainly a consequence of DNA damage. In an attempt to understand DNA damage associated with ionizing radiation, we examined strand break formation caused by irradiation of plasmid DNA in aqueous solution. Plasmids are a convenient model system in which to study DNA damage, since they have a well-defined size, are commercially obtainable in sufficiently high purity, and SSB detection is readily accomplished by gel electrophoresis (Milligan *et al.*, 1993; Murakami *et al.*, 2000). This method is based on the principle that single strand breaks (SSBs) occurring on plasmid DNA can be detected using differences in electrophoresis mobility between a closed circular DNA (CC) and an open circular (OC) DNA, with the latter being produced by nicking which is caused by irradiation (Tomita *et al.*, 1995; Shao *et al.*, 1999).

PCR is a useful and convenient tool for amplification of a given DNA sequence (Funatsuki *et al.*, 2001). PCR amplification ceases when SSBs occur on both strands within the DNA region flanked by the PCR primers. Therefore, this reaction could be applied to detect SSBs between the given primers. It is thus possible to detect *de novo* damage in the given region of irradiated DNA, regardless of whether the DNA is plasmid or genomic, by using PCR. An additional merit of the method is that only extremely small amounts of source DNA (at the pg order) are required for the assay. Furthermore, real time PCR has higher detection sensitivity and allows more robust quantitation than staining an electrophoresis gel to detect the PCR products followed by densitometry to quantitate the stained bands on the gel.

While plasmid DNA is uniform and for this, robust electrophoresis detection methods are available, genomic DNA includes both heterogeneous and repetitive sequences, so electrophoresis methods are not suitable for detection of SSBs in such DNA. Bulk damage of eukaryote genomic DNA is detectable by the COMET assay (Ostling and Johanson, 1984) but quantitative analysis is unreliable. By contrast, real time PCR method should be applicable to detect SSBs of genomic DNA.

In this study, quantitative analysis of SSBs in *Bam*HI digested plasmid pBR322 irradiated by γ rays was carried out by real time PCR in which different concentrations of a radical scavenger was included in the PCR reaction. The experimental results were compared with a Monte Carlo simulation based on the same conditions of irradiation dose, fragment size, and scavenger concentration. Monte Carlo methods are widely used for computational algorithms for simulating the behavior of various physical and

mathematical systems, and this is a suitable method for calculation using a computer, utilizing many techniques of computer simulation, for the repetition of algorithms and the large number of calculations. Experimental and Monte Carlo results were comparable, and therefore, a confirmation of the feasibility of this PCR approach for the detection of DNA damage by ionizing radiation in both plasmid DNA was obtained (Oikawa *et al.*, 2005).

Nucleolus organizer is constructed by a cluster containing a tandem array of about 240 copies of an rDNA gene in *Bombyx mori* (Gage, 1974). This cluster is opened at the stage of the first meiosis and rRNA is still synthesized regardless that the other chromosomes are sifted to the condensation stage (Tsuchida *et al.*, 1995). If this boundary region DNA could be also highly complex in aqueous solution as shown in testes at the first meiosis (Tsuchida *et al.*, 1995), it is expected to be detected as the difference on the radiation sensitivity depending on the complication degree. As the first approach, we confirmed that radiation damages to uniform DNA fragments of rDNA genes can be detected in aqueous solution by using this PCR method.

MATERIALS AND METHODS

Preparation of linear plasmid DNA

The plasmid DNA pBR322 (250 ng/μl stored in 10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 0.1 mM EDTA) was purchased from GIBCO. Linear plasmid DNA was prepared by the digestion of 1,250 ng stock DNA with 20 units *Bam*HI (New England Biolabs) for 30 min at 37°C, electrophoresis on 1% agarose gel (SeaKem GTG, BioProducts) in 1 x TAE buffer (40 mM Tris-HCl, pH 8.0, 40 mM acetic acid and 1 mM EDTA), staining of the gel with ethidium bromide to detect the DNA, and purification of the DNA with a QIAquick Gel Extraction Kit (QIAGEN).

PCR detection plasmid DNA digested with *BamHI*

Solutions including 0.5 units of *Bam*HI (NEB) and 1 μg pBluescript Phagemid Vector（Stratagene）DNA in 40 μl of total volume were incubated for 0, 30, 60, 90, 120, 150 and 180 min and then stopped by the addition of 1 μl of 500 mM EDTA. Electrophoresis was carried out on 1% agarose gel (TaKaRa LO3) and, after staining, amounts of DNA were determined by densitometry (Flour-S^{TM} MultiImager, BIO-RAD) after photographing the gel with a Polaroid MP4 Instant Camera System with UV (254 nm). Primers shown in Table 1 were used. The amplifications were performed using a

LightCycler™ Quick system 330, Rosh, with LightCycler™ FastStart DNA Master SYBRGreen kit under conditions shown in Table 2.

Irradiation by ^{137}Cs g rays and OH radical scavenger

A ^{137}Cs irradiation apparatus (gamma cell 40, AECL Canada)(Takada *et al.*, 2006) was used to irradiate plasmid DNA solutions that included Tris-HCl concentrations of 0, 0.5, 1.0, 1.5 or 2.0 mM as a radical scavenger. The DNA solution was prepared with a DNA concentration of 80 pg/ 20 μl (corresponding to about 1.8 x 10^7 copies as plasmid) in each micro-tube and stood on ice. For the diluted aqueous solutions with the above low OH radical scavenger concentrations, the main process of strand break formation (the reaction of OH radical with DNA) is thought to be an indirect effect. OH radical scavenging capacity was calculated as 1.62 x 10^6 s^{-1} for example 1 mM Tris-HCl and 0.1 mM EDTA using the reaction rate constant for OH radical of 1.5 x $10^9 M^{-1}s^{-1}$ for Tris-HCl and 1.2 x $10^9 M^{-1}s^{-1}$ for EDTA (Buxton *et al.*, 1988). Irradiation was done for 0, 30, 60, 100, 150, 200 and 250 min at a dose rate of 1.06 Gy/min. Samples prepared for all Tris-HCl concentrations were irradiated at the same time.

Estimation of DNA damage

Real time PCR was used for the quantitative analysis of plasmid DNAs damaged by ionizing radiation. The concentration of Mg^{2+} was varied at 2, 3, 4, and 5 mM. Use of real time PCR permits amplification of very low amounts of sample DNAs, maintaining uniform conditions for the amplification of all samples, possible verification of products by checking their T_m values, and calibration based on the same conditions. PCR amplification to four different lengths (1,003 bp, 749 bp, 505 bp, and 243 bp) was carried out to the DNA solutions irradiated from 0 to 265 Gy at each Tris-HCl concentration. One series of DNA solutions containing different Tris-HCl concentrations was prepared from the stock DNA solution in each experiment. Statistical analysis was performed with the Friedman' test (Friedman, 1937), which can be used to compare observations repeated on the same subjects. The null hypothesis for the comparison across repeated measures is that the distributions are the same across four different lengths of PCR amplification or across five different Tris-HCl concentrations in plasmid DNA solutions.

Detection of radiation induced damage to rDNA

The real-time PCR method was also applied to detect radiation-induced damage to genomic DNA, extracted from posterior silk glands of late fifth instar larvae of *Bombyx*

mori, strain N4 (Suzuki *et al.*, 1972; Maekawa *et al.*, 1988). A 4 μg aliquot of genomic DNA was digested with 50 units of *Eco*RI (NEB) for 1 h at 37°C and extracted with phenol-chloroform; 20 ng/μl the purified DNA in 1, 3 or 5 Tris-HCl, pH 8.0, was irradiated by ^{137}Cs for 0, 5, 10, 20, 30, 40, 60, 80, 100, 120, 150, 200, and 250 min at a dose of 1.06 Gy/min. The average value from two-time experiments for each dose was plotted. The amplification conditions used for the real-time PCR are shown in Table 2.

Monte Carlo simulation of DNA strand break induction

The Monte Carlo simulation of the induction process of DNA strand breaks by ^{137}Cs γ rays was carried out using the same conditions for the concentration of DNA and scavenger used in the experiment. In the simulation system, the OC pBR322 plasmid DNA was used instead of linear plasmid DNA, since the OC DNA can be dealt with more easily than linear DNA due to it being less flexible in the solution. The simulation was performed using the code system DBREAK, which allows the estimation of DNA strand break yields through the track structure simulation of electrons in liquid water, production and diffusion of water radicals, and the reaction of radicals with the pBR322 plasmid DNA model described in atomic representation. A strand break was assumed to be caused by the reaction of an OH radical with DNA. In the simulation model, the sequence of the DNA was not considered in the radical reaction process. That is, the reaction rate constant for reaction of DNA with OH radicals was assumed to be equal for all types of nucleotides. The details of the simulation model and method is described elsewhere (Tomita *et al.*, 1997, 1998; Watanabe and Saito, 2002). The validity of the model and simulation was shown by comparison with experimental data in several stages.

RESULTS

PCR detection of strand breaks of DNA digested with the restriction enzyme

Frequency of strand breaks was determined by both electrophoresis and PCR methods. Amount of CC plasmid DNA remaining after the digestion on an agarose gel (Fig. 1A) was quantitatively measured by densitometry (Fig. 1B). It is shown that CC plasmid DNA was decreased with increase of digestion time. Its decreased amount was also confirmed by the PCR reaction with appropriate primers (Fig. 1C). Both methods revealed comparable results and therefore, it is possible to detect strand breaks of DNA by using the PCR reaction.

Effect of DNA concentration in the solution for irradiation

Very low amounts of DNA (pg order) is sufficient to amplify by the PCR reaction while for the electrophoresis method, ng to μg of DNA is needed. Experiments were performed to determine whether these differences in DNA amounts of 40 pg/μl to 20 ng/μl affect the level of strand breakage by radicals. The results indicated no significant difference in strand breakage among the specimens in which radiation damage was given to different concentrations of DNA. Similar results were also obtained from PCR amplification for two different lengths of 505 bp (Fig. 2A) and 1,003 bp (Fig. 2B). Consequently, the PCR method was accepted as a comparable alternative to the electrophoresis method and the advantages of detecting strand breakage using the minimum amount of DNA should be emphasized.

Irradiation to linear shaped plasmid DNA

Plasmid DNAs were prepared in Tris-HCl at a concentration of 0, 0.5, 1.0, 1.5, or 2.0 mM, and irradiated with gamma rays at total dosages of 0, 31.8, 63.6, 106, 159, 212, or 265 Gy. Samples were amplified by real time PCR and plotted by the fit points program of LightCycler (Fig. 3, A, C, E and G). Strand breaks occurred significantly more frequently for longer length fragments (Friedman's test: $\chi^2 = 16.42–20.00$, d.f. = 3, $p < 0.001$). We thus confirmed that the frequency of strand breakage is higher as the region being amplified becomes longer. In addition, a higher concentration of the radical scavenger may also reduce significantly the amount of strand breaks on plasmid DNA (Friedman's test: $\chi^2 = 20.68–23.18$, d.f. = 4, $p < 0.001$).

Comparison with Monte Carlo simulation calculations

The calculation was carried out using break-induced probabilities (*p*) of 0.1 to 0.3 under the conditions specified in MATERIALS AND METHODS. As shown in Fig. 3 (B, D, F, and H), patterns calculated for the reasonable probability of 0.2 (Von Sontag, 1987) were very similar to the results obtained by real time PCR with the linear shaped plasmid. This finding indicates that the real time PCR method can be a useful procedure for the quantitative detection of radiation-induced damage to DNAs containing the specific sequence as a target.

Detection of radiation induced damage to genomic DNA

The real-time PCR method used for pBR322 was applied to genomic DNA irradiated with γ rays. The rDNA sequence was selected as a target for the PCR because it contains

a big cluster region with tandem repeats of about 240 copies of the 10.6 kb unit of the ribosomal RNA gene (Fig. 4A) and, therefore, more easily detected than a single copy gene. Genomic DNA was digested with *Eco*RI in order to produce target DNA molecules of a uniform size, since the originally extracted genomic DNA had a variety of sizes, which might deleteriously effect the subsequent PCR amplification. As shown in Fig. 4B, breaks in the presence of 1 mM Tris-HCl occurred more frequently on 981 bp fragments than on shorter fragments. These results suggest that for genomic DNA the frequency of strand breakage increases as the region being amplified becomes longer, as was the case with plasmid DNA. Similarly, increasing the concentration of the radical scavenger reduced the strand breaks on genomic DNA (Fig. 4B and C), as seen in plasmid DNA.

DISCUSSION

The amount of restriction products generated by a single-cut enzyme on the plasmid could be verified in proportion to the digestion rate as determined by the electrophoresis. The same result about the amount of restriction products was obtained with real time PCR. We confirmed that SSBs occurring on each strand of the DNA region flanked by both primers were detectable by the PCR method. Regardless of the concentration of the DNA in aqueous solution, the PCR detection efficiency was similar. The realtime PCR method was comparable with the electrophoresis method for detection of DNA damage. However, detection by realtime PCR is much easier and more convenient because of the very low amount of target DNA required, the ability to directly measure the amplified DNA without complicated procedures such as electrophoresis, gel staining densitometry etc., and the ability to verify the products with the T_m checking protocol. It was reported that PCR method for detection of DNA damages has higher sensitivity than the Southern hybridization method (Ayala-Torres *et al.*, 2000).

Quantitative analysis for detection of strand breaks by ionizing radiation of linear plasmid DNA was accomplished by the real time PCR methods using SYBR Green. The experimental results were compared with the results of Monte Carlo simulation. In this simulation, the shape of the pBR322 plasmid in the solution was assumed as OC. Since the data from both methods, experimental and simulation, were coincident with each other for all cases of several different radical scavenger concentrations and regardless of length of amplified products, the real time PCR method was verified as a feasible, alternative and potentially superior application method for the detection of SSBs caused by radiation. Since the DNA super-coiling suppresses real time PCR (Chen

et al., 2007), linear plasmid DNA is suitable for quantitative analysis. Slightly high value of real time PCR at 243 bp in Tris-HCl 2.0 mM might be deviation but not be caused by contaminated residues of CC plasmid DNA.

The application of this PCR method to irradiated genomic DNA was then attempted. On the rDNA cluster target region, damaged DNAs were reliably detected and the scavenger effect of the genomic DNA solution was also confirmed. SSBs occurring on a specific sequence or region of genomic DNA would thus be detectable by taking advantage of the PCR reaction, which only amplifies sequences flanked with both primers. This means that if the sensitivity to radiation-induced damage is sequence-dependent, this PCR method could be a very useful detection tool.

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Table 1. Primers sequences for real time PCR.

Primers	Sequences
pBluescript	
M13PrimerP7	CGCCAGGGTTTTCCCAGTCACGAC
M13PrimerP8	AGCGGATAACA ATTTCACACAGGAAAC
pBR322	
S1s	ATGCAGGAGTCGCATAAGGGAGAG
pBR2as	CCAAGGGTTGGTTTGCGCATTCAC
pBR3as	AGACTTTACGAAACACGGAAACCG
pBR4as	TCGAAGTTAGGCTGGTAAGAGCCG
pBR5as	GAGGGCGTGCAAGATTCCGAATAC
rDNA	
012as	CCTCAGAGCCAATCCTTATATTCC
017	ATGAAGCCGGAGATCTGATGACGG
016	AGGAGAGATCTTATGTCGATGCGG
07	TACTCTCTGCGGACCTTGAAAATTCAGGTG

Primers were constructed for the amplification of 243, 505, 749, and 1,003 bp fragments to pBR322 and also for detection of pBluescript digested with *Bam*HI. For rDNA, primers were constructed for the amplification of 177, 497, and 981 bp fragments.

Table 2. Amplification condition for real time PCR.

Primer	Length	Preheating	Denature	Annealing	Extention	Melting (speed)	Cooling
pBluescript							
P7+P8	275 bp	95℃, 10 min	95℃, 15 sec	60℃, 10 sec	72℃, 10 sec	65 95 (20 /sec)	40℃, 30 sec
pBR322							
S1s+pBR3as	1,003 bp	95℃, 10 min	95℃, 15 sec	60℃, 10 sec	72℃, 40 sec	65 95 (20 /sec)	40℃, 30 sec
S1s+pBR2as	749 bp	|	|	|	72℃, 30 sec	|	|
S1s+pBR4as	505 bp	|	|	|	72 , 20 sec	|	|
S1s+pBR5as	243 bp	|	|	|	72℃, 10 sec	|	|
rDNA							
012as+017	981 bp	|	|	|	72℃, 40 sec	|	|
012as+016	497 bp	|	|	|	72℃, 20 sec	|	|
012as+07	177 bp	|	|	|	72℃, 7 sec	|	|

Primer combinations for pBR322 and rDNA are indicated. PCR conditions for each primer combination are shown. Bar indicates the same conditions as those of the upper lane.

（ 図の説明）

Fig. 1. Detection of DNA damage by digesting with *Bam*HI. pBluescript DNAs were incubated for 0, 30, 60, 90, 120 and 150 min with *Bam*HI. Densitometry for the electrophoresis data was plotted with the value of non-irradiated DNA as 100%. The real time PCR data of the same sample as the electrophoresis was also plotted. A. Image of electrophoresis gel of pBluescript DNA incubated with *Bam*HI for the indicated time in the panel. B. Plotted densitometry data for the electrophoresis shown in panel A. C. Real time PCR for the same digestion samples as that of the electrophoresis.

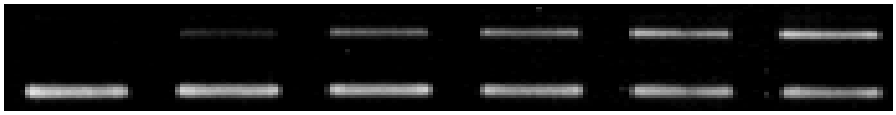
Fig. 2. Quantification of DNA damage after irradiation of ^{137}Cs for several DNA concentrations using real time PCR. DNA damage was determined as the amount of DNA without any breaks between each primer set. The solution contained Tris-HCl 1.0 mM (sc(OH)=1.5 x $10^6 s^{-1}$) and CC+OC pBR322 DNA of 40 pg/μl-20 ng/μl. Two amplification sizes (505 bp and 1,003 bp) were carried out by LightCycler (A and B, resptectively). The Y-axis is the amount of remaining DNA without any breaks between each primers set. Those amounts are based on the PCR value for non-irradiated DNA being 100%.

Fig. 3. Comparison between the experimental PCR data and the Monte Carlo simulation data calculated for the PCR amplification model. The left side panels (A, C, E, and G) are real time PCR results using irradiated linear plasmid DNA digested with *Bam*HI. Tris-HCl at a concentration shown in each panel was added to each DNA solution as a radical scavenger before irradiation. The right side panels (B, D, F, and H) are results of the simulation calculation. The data based on a *p* value of 0.2 for OC is only shown. OH radical scavenging capacity (sc(OH)) of solutions containing Tris-HCl at 0 mM, 0.5 mM, 1.0 mM, and 2.0 mM are estimated as 0 s^{-1}, 7.5 $10^5 s^{-1}$, 1.5 $10^6 s^{-1}$ and 3.0 $10^6 s^{-1}$, respectively, except using 1.2 $10^4 s^{-1}$ for 0.008mM Tris-HCl as simulation calculation. Statistical analysis was performed with the Friedman's test: χ^2 = 16.42, d.f. = 3, $p < 0.01$ (D); χ^2 = 19.42, d.f. = 3, $p < 0.01$ (F); χ^2 = 19.14, d.f. = 3, $p < 0.01$ (H). Names of those primers shown in Table 1 are indicated in this panel along with each predicted amplification size (243, 505, 749, and 1,003 bp). The X-axis is the irradiation dose (Gy) of γ ray by ^{137}Cs. The Y-axis is the same as that for Fig. 2. Precise break induction probability (*p* value) could be estimated by this comparison.

Fig. 4. Detection of SSB in rDNA region of genomic DNA irradiated by ^{137}Cs.

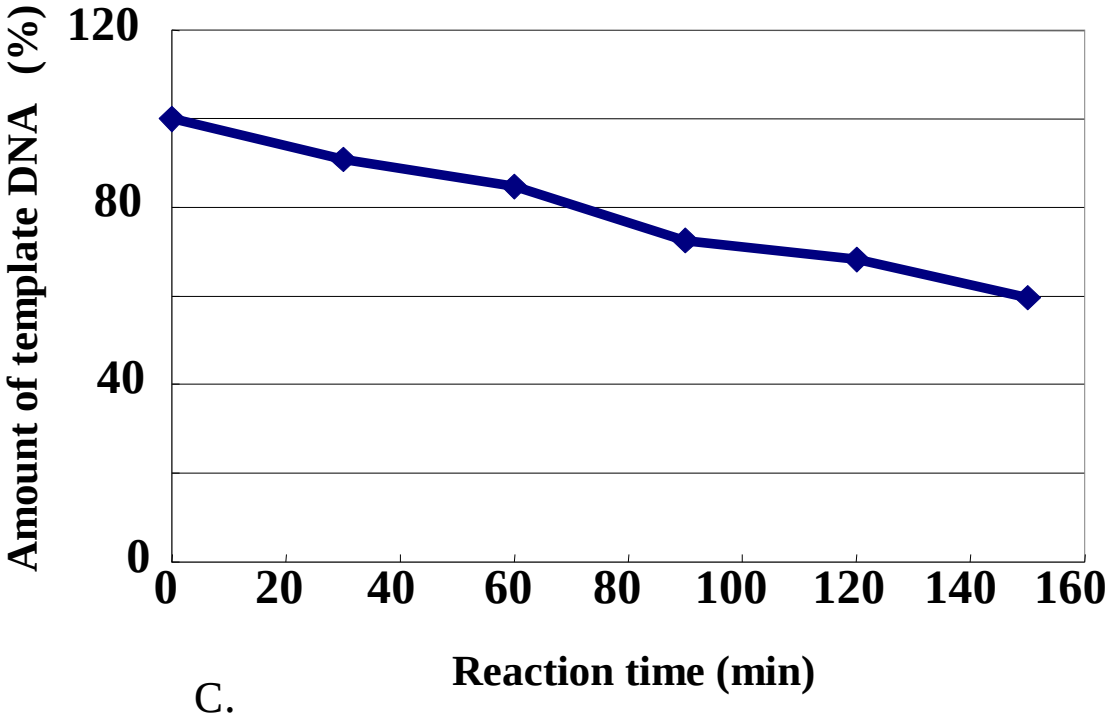
Irradiated genomic DNA was amplified by real-time PCR using primer combinations for the rDNA 10.6 kb unit (A). Sample values were calculated based on the amount for non-irradiated DNA as 100% and then plotted by the second derivative program. The X-axis is irradiation dose (Gy) of γ ray by ^{137}Cs. The Y-axis is same as that for Fig. 2. Scavenger concentrations of 1 mM (B), 3 mM (C), and 5 mM (D) were used.

A.



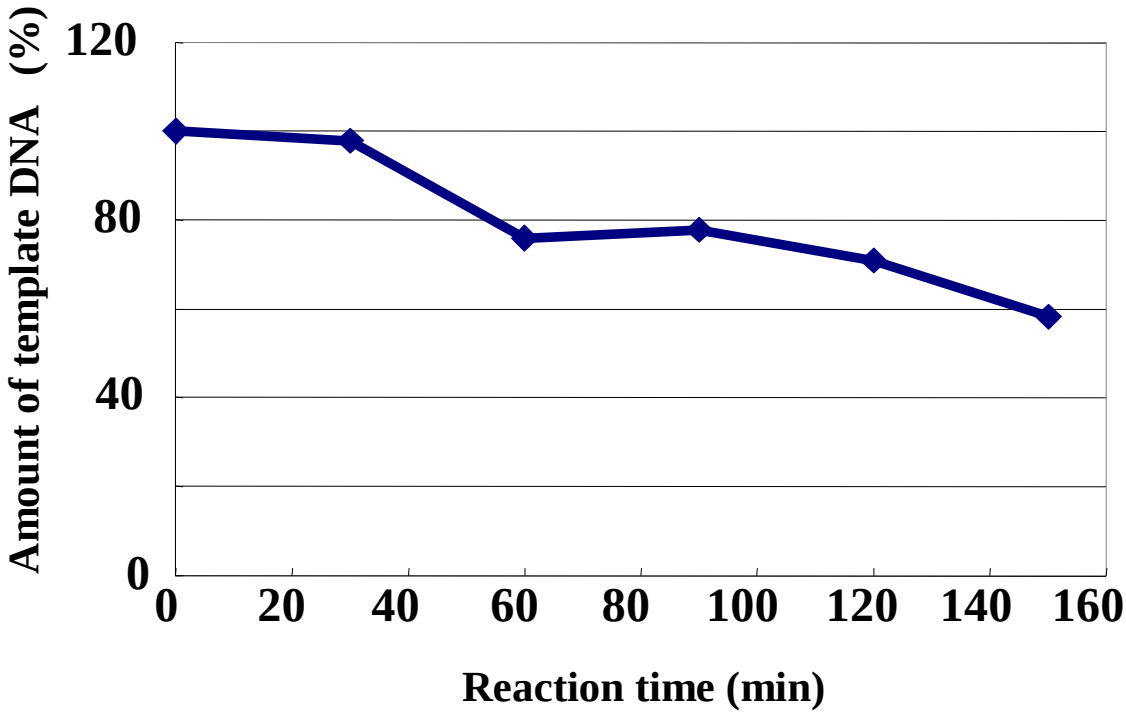
Reaction time (min)

B.



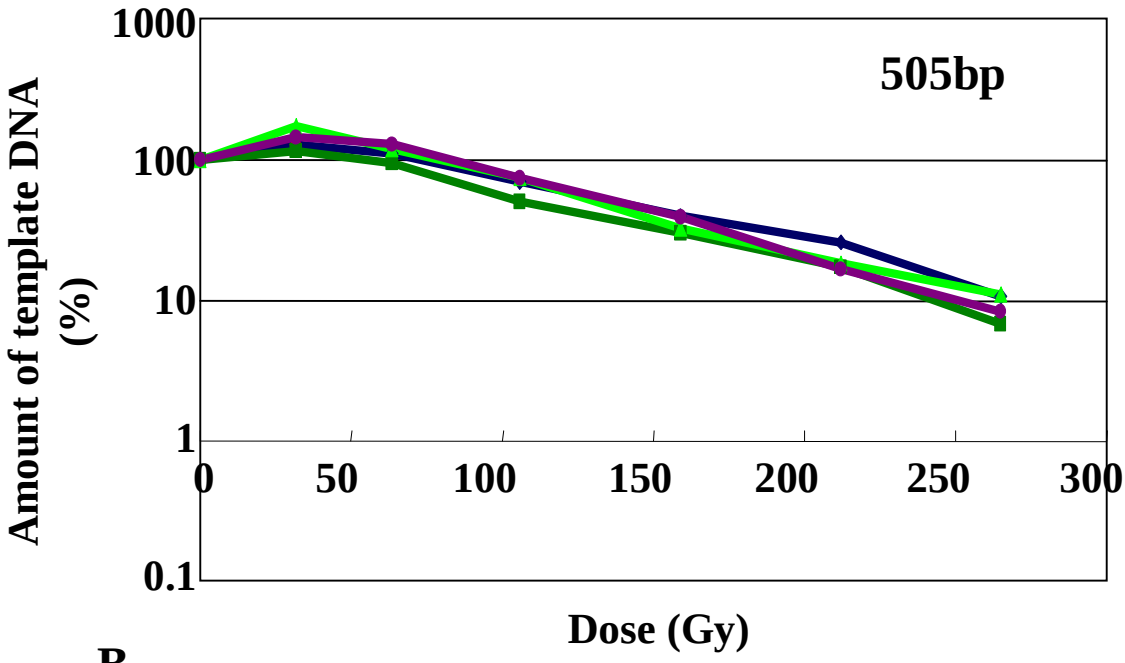
Reaction time (min)

C.

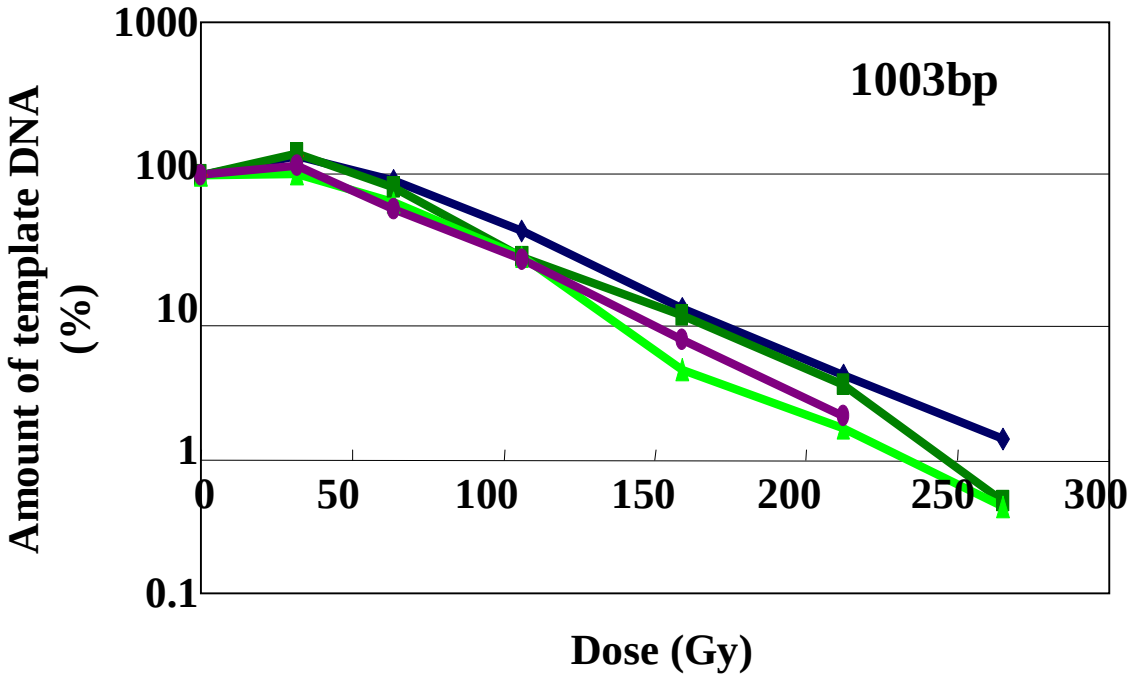


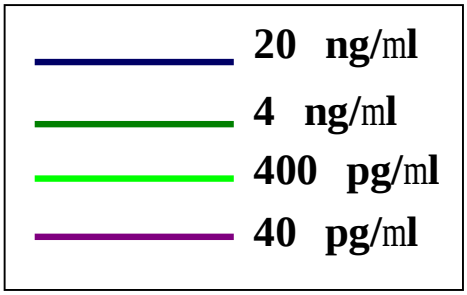
Reaction time (min)

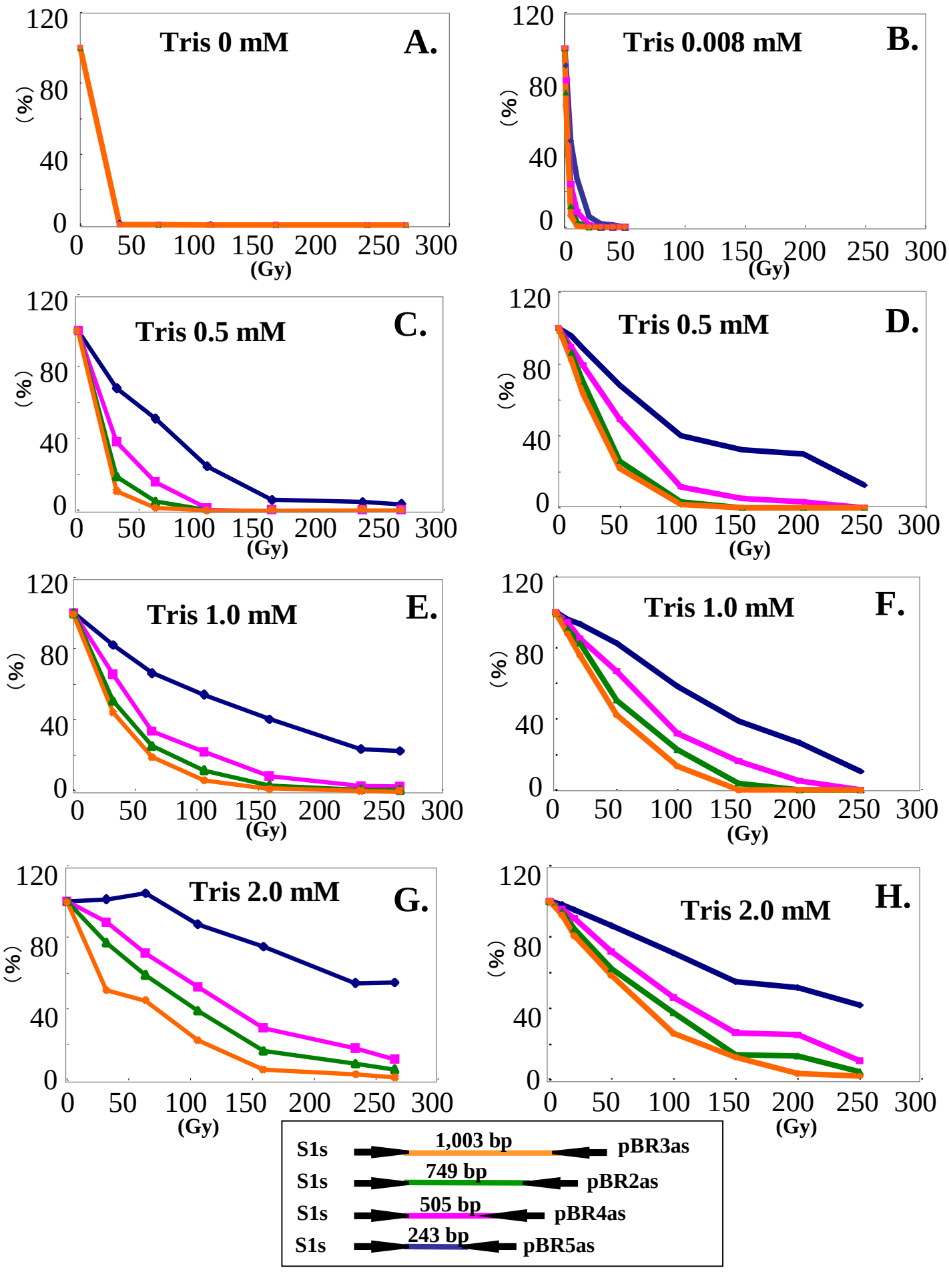
A.

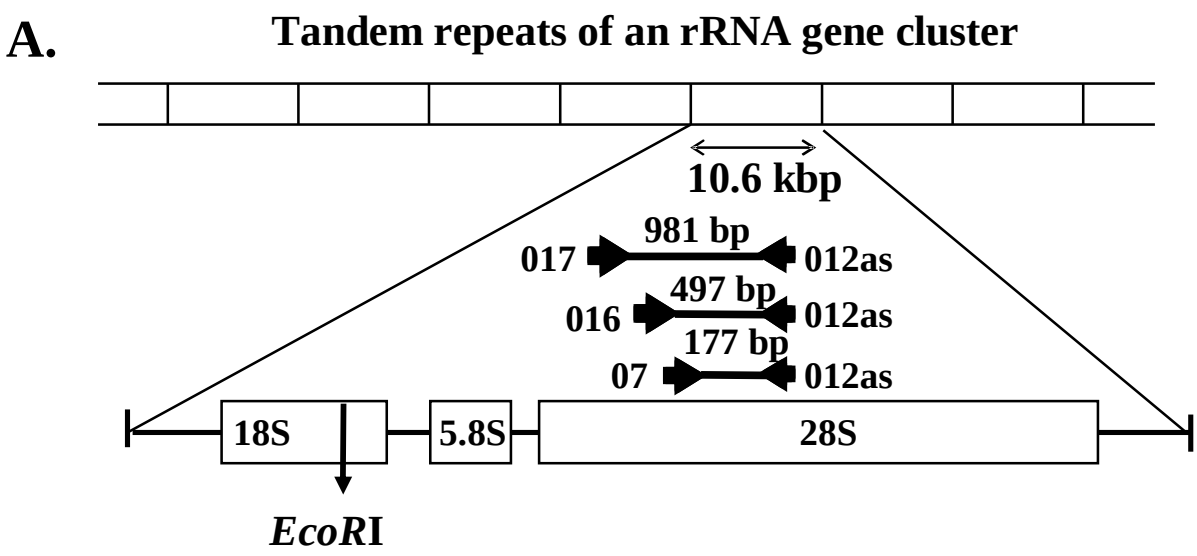

505bp
Amount of template DNA (%)
1000
100
10
1
0.1
0
50
100
150
200
250
300
Dose (Gy)

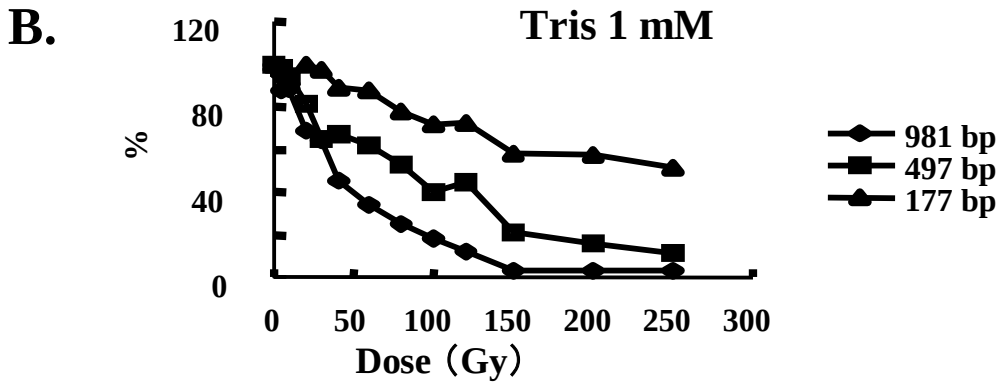
B.

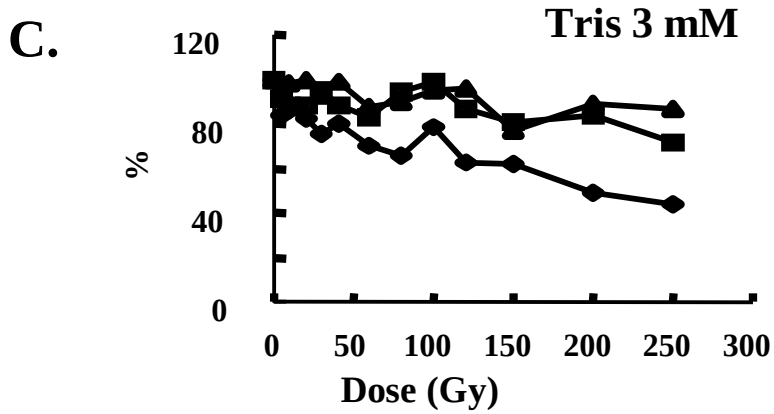

1003bp
Amount of template DNA (%)
1000
100
10
1
0.1
0
50
100
150
200
250
300
Dose (Gy)

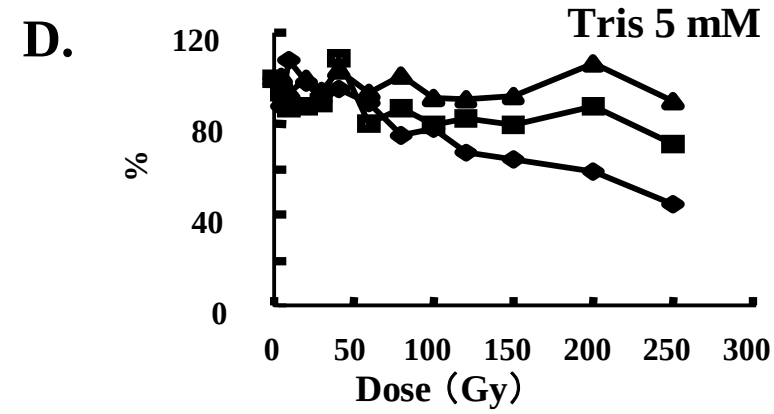

20 ng/ml
4 ng/ml
400 pg/ml
40 pg/ml


Tris 0 mM
A.
Tris 0.008 mM
B.
Tris 0.5 mM
C.
Tris 0.5 mM
D.
Tris 1.0 mM
E.
Tris 1.0 mM
F.
Tris 2.0 mM
G.
Tris 2.0 mM
H.
(%)
(Gy)
0
40
80
120
0 50 100 150 200 250 300
S1s
1,003 bp
pBR3as
749 bp
pBR2as
505 bp
pBR4as
243 bp
pBR5as


A.
Tandem repeats of an rRNA gene cluster
10.6 kbp
981 bp
017
012as
497 bp
016
012as
177 bp
07
012as
18S
5.8S
28S
EcoRI


B.
Tris 1 mM
120
80
40
0
%
0
50
100
150
200
250
300
Dose（Gy）
981 bp
497 bp
177 bp


C.
Tris 3 mM
120
80
40
0
%
0
50
100
150
200
250
300
Dose (Gy)


D.
Tris 5 mM
120
80
40
0
%
0
50
100
150
200
250
300
Dose（Gy）