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Development and characterization of a novel host cell DNA assay using ultra-sensitive fluorescent nucleic acid stain "PicoGreen"

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ABSTRACT

Development of a novel host cell DNA assay using PicoGreen is described, which is capable of detecting short double stranded DNAs (ds-DNAs) in cell culture supernatants and process intermediates. Examination of this PicoGreen DNA assay was carried out by determination of the DNA length detection limit, observation of short ds-DNAs in cell culture supernatants and process intermediates, evaluation of dose dependency and a supersensitizing protocol, and comparison of the novel assay with conventional assays for measuring host cell DNA concentration in real samples. The PicoGreen DNA assay was capable of detecting ds-DNAs as short as 20 bp, and the sensitivity of the PicoGreen DNA assay was comparable to that of the Threshold system with application of additional SDS/Proteinase K digesting and DNA concentrating steps. Also, the amount of DNA identified in both cell culture supernatant and process intermediates was clearly underestimated by the Threshold system results when compared with the PicoGreen DNA assay results. The PicoGreen DNA assay clearly provides better accuracy and is a simpler procedure for measuring host cell DNA levels in cell culture supernatant and process intermediates than the conventional method with the Threshold system. This newly developed DNA assay will be prominent among host cell DNA assays for measuring host cell DNA levels in bio-pharmaceuticals.

1. Introduction

The number of recombinant proteins produced using mammalian cells and microbe cultivation systems has increased dramatically in recent years. Among mammalian cells, Chinese hamster ovary (CHO) cells have been the first choice as a host because they are well characterized and many recombinant proteins produced from CHO cells have obtained regulatory approval [1,2].

It is important to evaluate the clearance of impurities in the manufacturing of recombinant proteins, and host cell DNA is one of the impurities relevant to this process. Although host cell DNA is not taken into consideration as a risk-factor, it must be reduced to very low levels. The acceptable residual amount of host cell DNA specified in Food and Drug Administration (FDA) guidelines is 100 pg/dose, and the acceptable limit of host cell DNA allowed by the World Health Organization (WHO) and the European Union (EU) is up to 10 ng/dose [3–5].

The Threshold system is generally used instead of dot blot hybridization for verifying DNA clearance [6], and quantitative real-time PCR has recently been used for monitoring host cell DNA [7]. However, the Threshold system and quantitative real-time PCR are unable to detect short stranded DNA [8], although short stranded DNA probably exists in cell culture supernatant during recombinant protein production. Moreover, the Threshold system lacks cost-performance and throughput efficiency. The Quant-iT™ PicoGreen® ds-DNA Assay Kit specifically detects ds-DNA [9]. This kit is broadly used for measuring the amount of DNA templates during DNA sequencing or polymerase chain reaction (PCR), for quantitation of amplified PCR products [10], for determination of enzymatic activities [11,12], for quantitation of adenovirus DNA or total viral DNA [13,14], and for DNA quantitation in capillary electrophoresis [15]. It enables us to measure DNA amounts with high throughput. However, to date, there are no reports of monitoring host cell DNA using this kit.

The aims of this study are: determining the presence of short ds-DNA in cell culture supernatants and process intermediates of pharmaceutical recombinant protein production systems, developing a novel host cell DNA assay which is able to detect short ds-DNA in cell culture supernatant, process intermediates, and drug products using the PicoGreen DNA assay, and improving the sensitivity of the PicoGreen DNA assay system to the level of the Threshold system.

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2. Experimental

2.1. Materials

Quant-iT™ PicoGreen® ds-DNA Assay Kit (Invitrogen), DNA marker (20 bp DNA Ladder, TaKaRa), 4.5% agarose gel (Lonza), DNA Extractor Kit (Wako), 2-propanol (Wako), Sodium dodecyl sulfate (Bio-Rad), and Proteinase K (Roche Diagnostics), were purchased for carrying out these studies. The Threshold system (molecular devices) was employed according to the manufacturer's instructions. Cell culture supernatants, process intermediates, and drug substances of recombinant humanized monoclonal IgG products were used as samples. Cell culture supernatant was obtained as filtrate of the CHO cells culture, and process intermediates were obtained by purifying the cell culture supernatant through several steps, such as an affinity chromatography step, an ion exchange chromatography step, and other processes. These samples were stored at −80 °C until analysis.

2.2. DNA quantification

DNA quantification using the Quant-iT™ PicoGreen® ds-DNA Assay Kit was carried out according to the manufacturer's instructions. Lambda DNA standard attached to the kit was used as the standard DNA. Various concentrations of standard DNA solution were prepared by diluting standard DNA with TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Samples and standard DNA solutions were mixed with an equal volume of diluted PicoGreen reagent (1:200 in TE buffer), and were incubated for 5–30 min at room temperature in microplate wells. Three hundred and eighty-four well microplates (Corning) were used for the microplate assay. Sample volume in the microplate assays was 50 μL. Fluorescent intensity was measured with an EnVision 2102 Multilabel Reader (PerkinElmer). Samples and standard DNAs were excited at 480 nm and the fluorescence was measured at 520 nm. DNA concentration of standard DNA solutions and fluorescent intensity were plotted, and a standard curve was calculated. DNA amounts of samples were calculated using the standard curve.

The methodology of the Threshold system is based on denaturing DNA into single stranded DNA (ss-DNA), labeling protein complexes, binding immobilized labeled complexes to a nitrocellulose membrane, and detecting a change in pH using a light-addressable potentiometric sensor. DNA amounts of samples were analyzed and compared with a standard curve generated with known amounts of ss-DNA.

2.3. DNA extraction

DNA extraction using a DNA Extractor Kit was carried out according to the manufacturer's instructions. Twenty microliters of *N*-lauroyl sarcosinate solution and 500 μL of NaI solution containing glycogen were added to 400 or 500 μL of samples. The mixtures were incubated at 55 °C for 10 min. The mixtures were added to 900 μL isopropanol and vortexed, and then let stand at room temperature for 10 min. After a brief centrifugation (10,000 rpm × 10 min), white pellets became visible. The supernatants were discarded and residual droplets on the tube wall were removed by placing the tube upside down on filter paper. Four hundred microliters of washing solution (A) was added to the tube and vortexed vigorously as the pellets were detached from the tube wall. After centrifugation (10,000 rpm × 5 min), the supernatants were discarded. This washing protocol with washing solution (A) was repeated once more. One thousand five hundred microliters of washing solution (B) containing glycogen was added to the tube and vortexed. After centrifugation (10,000 rpm × 5 min), the supernatants were discarded, and the resulting pellets were vacuum-dried. The pellets contained DNA and carrier glycogen, and the pellets were reconstituted with TE buffer.

2.4. Gel electrophoresis and DNA extraction from gel

Sample was electrophoresed on 4.5% agarose gel, and a portion of the gels containing short ds-DNAs was cut off. DNA in the cut-off gel was extracted using a 0.22 μm membrane filter (TaKaRa) and the DNA Extractor Kit.

2.5. Improvement of the PicoGreen DNA assay

2.5.1. Evaluation of extracting process in the PicoGreen DNA assay

The DNA Extractor Kit was applied to check whether DNA extraction from sample solutions and enrichment of DNA concentration were possible. DNA standards in TE buffer were extracted using the DNA Extractor Kit, and TE buffer was added to the extracted DNAs to result in either a 1/5 of initial volume ("five times concentrated") or the same initial volume ("not concentrated"). DNA amounts in the "five times concentrated" and the "not concentrated" samples were determined using the PicoGreen DNA assay, and percent recovery for each was calculated.

2.5.2. Evaluation of protein digestion by SDS/Proteinase K in the PicoGreen DNA assay

DNA standards were added to drug substances of product A, diluted by TE buffer. SDS/Proteinase K solution (SDS concentration, 5.0% [w/v]; Proteinase K concentration, 2 mg/mL) was added to samples, and the sample mixtures were incubated for 10, 20, or 60 min at 55 °C. After incubation, DNA in the sample mixtures was extracted and concentrated five-times using the DNA Extractor Kit. The amount of DNA in the samples was then determined using the PicoGreen DNA assay, and the percent recovery of DNA contents was calculated.

2.6. Quantification of DNA in cell culture supernatants and process intermediates using the PicoGreen DNA assay and the Threshold system

Cell culture supernatants and process intermediates of product A and B were used as samples, and diluted with TE buffer. Protein digestion, DNA extraction and five-times enrichment of DNA concentration were performed as described. The amount of DNA in the samples were then determined using the PicoGreen DNA assay and the Threshold system.

3. Results

3.1. Reactivity of short double stranded DNA

Reactivity of short ds-DNA was examined as follows. Electrophoresis and DNA extraction from gels of DNA marker (20 bp DNA Ladder) were carried out as described in Section 2. The short ds-DNAs (20–100 bp) were obtained, and DNA quantification of these samples was carried out by the PicoGreen DNA assay and the Threshold system. Results of agarose gel electrophoresis and reactivities of various lengths of ds-DNA (20–100 bp) in the Threshold system and the PicoGreen DNA assay are shown in Fig. 1. The results suggest that the PicoGreen DNA assay can detect ds-DNAs as short as 20 bp, which are undetectable by the Threshold system. Although the true value is unknown, the measured value in the Threshold system fell as the size of the DNA fragment decreased. However, the measured value in the PicoGreen DNA assay did not.

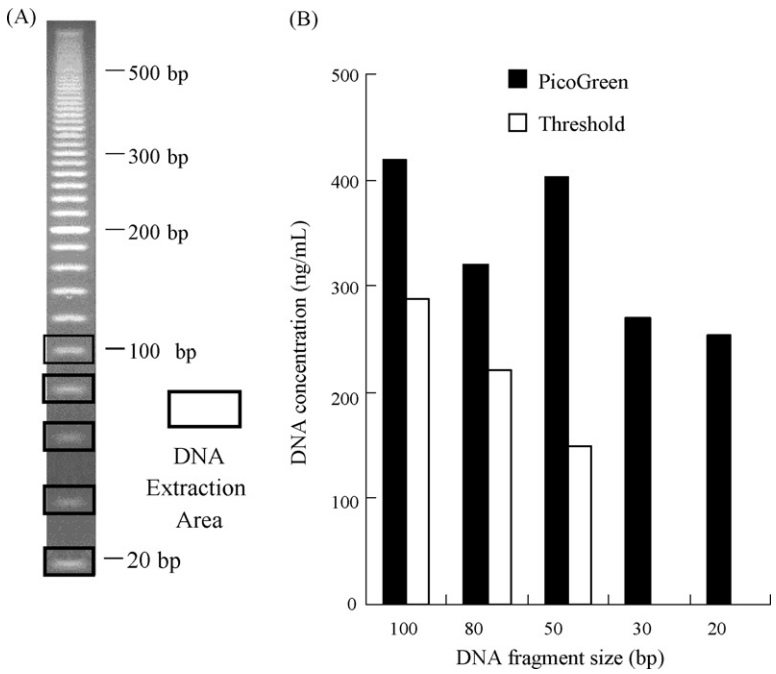

Fig. 1. Agarose gel electrophoresis of DNA marker (A) and reactivity of short ds-DNAs using the Threshold system and the PicoGreen method (B). The areas enclosed by the black line show the extracted ds-short DNAs.

3.2. Identification of DNA in culture supernatants and process intermediates

3.2.1. Determination of short ds-DNA in culture supernatants and process intermediates

The presence of short ds-DNAs in cell culture supernatants and process intermediates was determined as follows. DNA was extracted from cell culture supernatants and process intermediates using the DNA Extractor Kit. Electrophoresis of the extracted DNA was carried out, and gels of short ds-DNAs (approximately 20 bp) were cut off. DNA in the cut-off gel was extracted using a 0.22 μm membrane filter and the DNA Extractor Kit. The amounts of total ds-DNA and short ds-DNA were then determined using the PicoGreen DNA assay, and the percent ratio of short ds-DNA to total ds-DNA was calculated. The results of agarose gel electrophoresis and DNA quantification using the PicoGreen DNA assay are shown in Fig. 2, which revealed the presence of short ds-DNA in cell culture supernatants and process intermediates. The ratio of short ds-DNA to total ds-DNA is shown in Table 1. Here, it was assumed that the DNA extraction efficiency of total ds-DNA and short ds-DNA from the gel was the same. This result indicates that the percent ratio of short ds-DNA to total ds-DNA was 32% in process intermediates (an eluate of affinity chromatography) in spite of being 0.3% in cell culture supernatants.

3.2.2. Determination of ss-DNA in cell culture supernatant

Determination of ss-DNA in cell culture supernatant was carried out as follows. The amount of DNA in cell culture supernatants with or without a denaturation process was determined using the Threshold system. If DNA was detected without the denaturation process, the presence of ss-DNA in cell culture supernatant was verified. The results of ss-DNA quantitation in cell culture supernatants with or without denaturation using the Threshold system is shown in Fig. 3. The results suggest that ss-DNA was below the detection limit, and it was therefore thought that the ss-DNA in cell culture supernatants would not affect a DNA assay using the PicoGreen system.

3.3. Improvement of the PicoGreen DNA assay

3.3.1. Determination of dose dependency and LOD/LOQ of the PicoGreen DNA assay

DNA standards of various concentrations were prepared with TE buffer, excited at 480 nm, and the fluorescence was measured at 520 nm. DNA concentration and fluorescent intensity were plotted, and based on the plotted result, R^2, limit of detection (LOD), and limit of quantitation (LOQ) were calculated. Dose dependency and LOD/LOQ of the PicoGreen DNA assay are shown in Fig. 4 and Table 2, respectively. The results indicate that the calculated R^2 value was 0.9960 and the calculated LOD and LOQ were 102 and 310 pg/mL, respectively.

3.3.2. Evaluation of the extracting process in the PicoGreen DNA assay

Evaluation of the extracting process in the PicoGreen DNA assay is shown in Fig. 5. The results suggest that the effect of the extracting process using the DNA Extractor Kit was negligible, and a five-times enrichment of DNA concentration was achieved by this protocol.

3.3.3. Evaluation of protein digestion by SDS/Proteinase K in the PicoGreen DNA assay

A fluorescence intensity increase of 19% represents 0.1% IgG in samples, according to the instructions of the Quant-iT™ PicoGreen® ds-DNA Assay Kit. Therefore, DNA extraction by the DNA Extractor Kit was needed for samples containing proteins such as IgG. However, it is predicted that all proteins in samples are not removed by the DNA Extractor Kit alone. So, it is assumed that the protein digestion by SDS/Proteinase K before DNA extraction increased the efficacy of the protein removal from the sample mixture. The results are shown in Fig. 6, and suggest that protein digestion was important for DNA recovery, and that the duration required for digestion was 10 min.

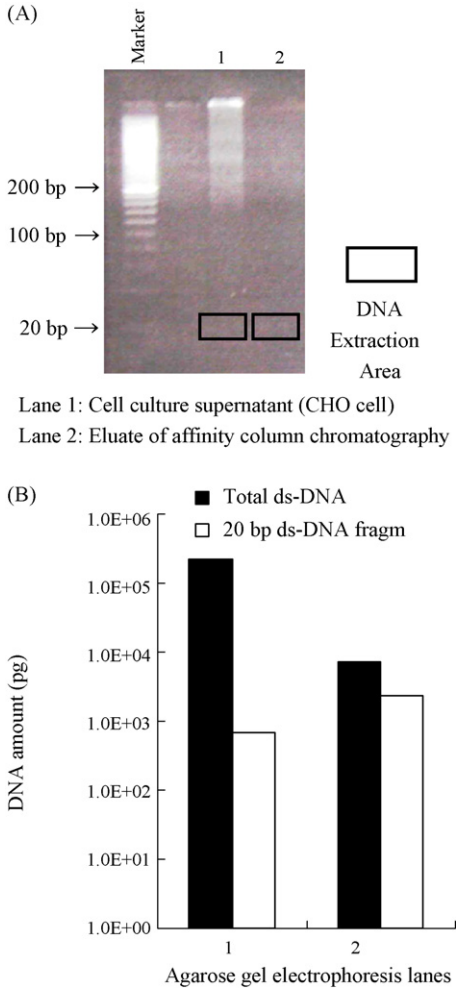

Fig. 2. Agarose gel electrophoresis of DNAs from samples (A) and the amounts of total DNA and short ds-DNA (B). The areas enclosed by the black line show the extracted ds-short DNAs.

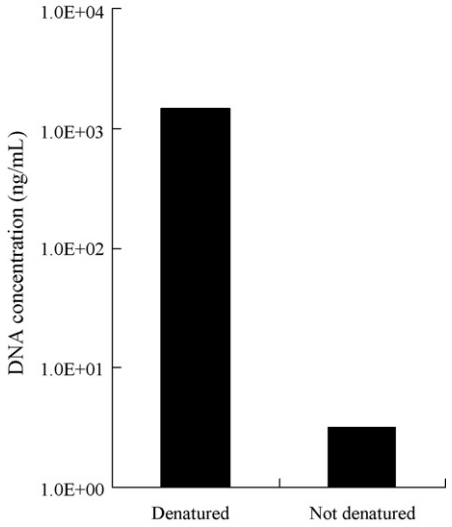

Fig. 3. Amount of ss-DNA in cell culture supernatants. "Not denatured" shows that ss-DNA was below the detection limit in the Threshold system.

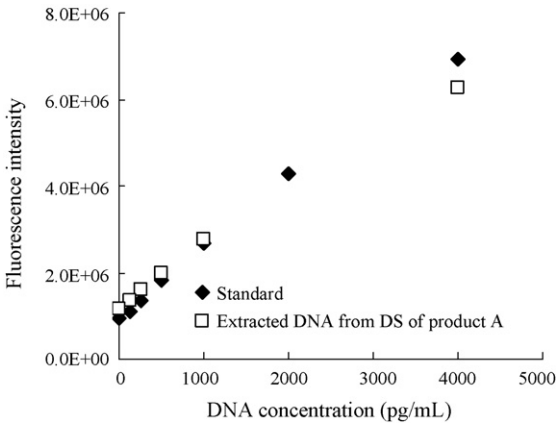

Fig. 4. Dose dependency of standard DNA and extracted DNA from drug substance of product A.

3.3.4. Comparison of dose dependent curves with or without addition of drug substance

DNA standards of various concentrations were added to drug substance of product A, and diluted five times with TE buffer. Protein digestion, DNA extraction and five-times enrichment of DNA concentration were performed. Extracted amounts of DNA were then determined using the PicoGreen DNA assay. DNA concentrations and fluorescent intensity were plotted. Based on the potted result, R^2, LOD, and LOQ were calculated. Also, dose dependent curves with or without addition of drug substance were compared. Comparison of dose dependent curves with or without addition of drug substance is shown in Fig. 4 and Table 2. The results indicate that linearity and LOD/LOQ of DNA standards in drug substance were almost comparable to that in TE buffer.

Table 2
Linear regression parameters of standard and extracted DNA from drug substance of product A.

	Standard	Product A
Slope	1489	1260
Y-intercept	1032649	1291913
R^2	0.9960	0.9955
Average of S.D.	46103	74317
LOD (pg/mL)	102	195
LOQ (pg/mL)	310	590

Limit of detection (LOD) = 3.3 × average of S.D./slope; limit of quantitation (LOQ) = 10 × average of S.D./slope.

Table 1
Comparison of the amounts of total ds-DNA and short ds-DNA.

Sample		DNA amount (pg)	Rate (%) (20 bp/total)
Cell culture supernatant	Total	2.3×10^5	0.30
	20 bp DNA fragment	6.8×10^2	
Eluate of affinity column chromatography	Total	7.4×10^3	32
	20 bp DNA fragment	2.3×10^3	

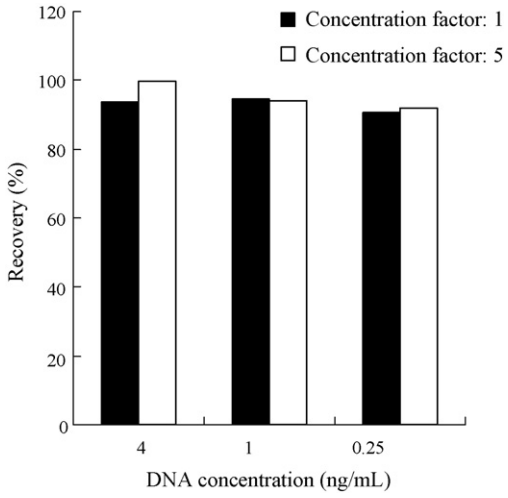

Fig. 5. DNA recovery from DNA extraction and concentration.

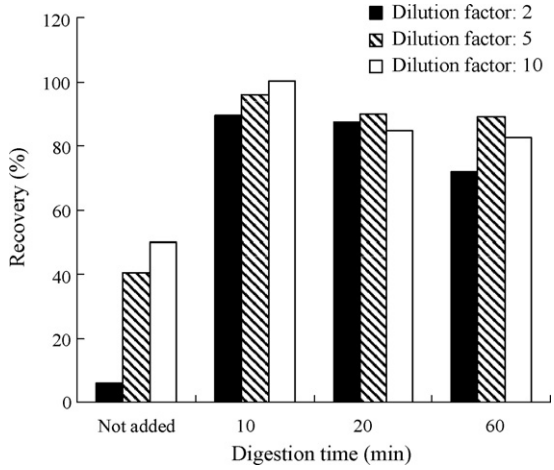

Fig. 6. DNA recovery after protein digestion. PicoGreen standard DNA added to DS of product A was used as the sample, diluted 2, 5 and 10 times by TE buffer.

3.4. Quantification of DNA in cell culture supernatants and process intermediates using the PicoGreen DNA assay and the Threshold system

Quantification of DNA in cell culture supernatant and process intermediates using the PicoGreen DNA assay and Threshold system are shown in Table 3. The results suggest that both the PicoGreen DNA assay and the Threshold system were able to determine the DNA contents in cell culture supernatants and process intermediates, and the sensitivity of the novel PicoGreen DNA assay system was comparable to that of the Threshold system. However, as expected, DNA contents were clearly underestimated in the Threshold system compared to the novel PicoGreen DNA assay system (Table 3). As shown in Table 3, host cell DNA contents in cell culture supernatants of product A and B were underestimated 27 and 40% by the Threshold system. Host cell DNA contents in process intermediates (step 1) of product A and B were also underestimated by 51 and 61%, respectively, by the Threshold system.

Table 4
Summary of types of DNAs detectable by the Threshold system and the PicoGreen DNA assay.

	ds-DNA		ss-DNA	
	Long	Short	Long	Short
In Samples	Present	Present	Absent	Unknown
PicoGreen	Detected	Detected	Not detected	Not detected
Threshold	Detected	Not detected	Detected	Not detected

4. Discussion

This study was undertaken to assess whether the DNA assay using the Quant-iT™ PicoGreen® ds-DNA Assay Kit was able to determine the quantity of ds-DNA as short as 20 bp, which is almost undetectable by the Threshold system. Also, short ds-DNA in both cell culture supernatants and process intermediates were observed, with much higher concentrations being present in process intermediates than in cell culture supernatant. Furthermore, ss-DNA, which is not measured with the PicoGreen DNA assay, was below the detection limit in cell culture supernatants, and it was therefore thought to be unlikely to affect the PicoGreen DNA assay. Various DNA types existed in cell culture supernatants/process intermediates, and those detectable by the Threshold system and the PicoGreen DNA assay are summarized in Table 4. As shown in Table 4, the PicoGreen DNA assay is able to detect both long and short ds-DNA in cell culture supernatants/process intermediates. However, short ds-DNA is not detectable by the Threshold system. Because of this lack of short ds-DNA detection capability, there is risk of underestimation of host cell DNA contents in cell culture supernatants/process intermediates. Long ss-DNA, which is below detectable levels in cell culture supernatants, is detectable by the Threshold system. Short ss-DNA is undetectable by both the PicoGreen DNA assay and the Threshold system. It is therefore thought that the PicoGreen DNA assay is more suitable than the Threshold system to determine host cell DNA contents in cell culture supernatants/process intermediates.

Table 3
DNA amounts in cell culture supernatants and process intermediates.

Product	Process step	DNA amount in diluted sample (pg/mL)		DNA amount (pg/mg product)		Reactivity (%) Threshold/PicoGreen
		Threshold	PicoGreen	Threshold	PicoGreen	
Product A	Cell culture supernatant	3.0×10^2	4.1×10^2	1.5×10^7	2.0×10^7	73
	Step 1	63	2.6×10^2	4.2×10^2	8.6×10^2	49
	Step 2	82	57	19	21	87
	Step 3	<30	<50	<6.8	<19	–
	Step 4	<30	<50	<17	<49	–
	Drug substance	<30	<50	<6.0	<5.9	–
Product B	Cell culture supernatant	1.2×10^2	4.1×10^2	4.1×10^6	6.8×10^6	60
	Step 1	65	1.7×10^2	1.5×10^2	3.9×10^2	39
	Step 2	<30	<50	<21	<59	–
	Step 3	<30	<50	<7.1	<59	–
	Step 4	<30	<50	<43	$<1.2 \times 10^2$	–
	Drug substance	<30	<50	<6.0	<4.6	–

Table 5
Comparison of various DNA determination methods.

Method	PicoGreen[a]	Threshold[a]	Q-PCR	Hybridization
Limit of detection[b]	50 pg/mL	30 pg/mL	5 pg/mL	10 ng/mL
Dynamic range[b]	50–800 pg/mL	30–480 pg/mL	5 pg/mL to 1 μg/mL	10–2500 ng/mL
Type of DNA[c]	ds-DNA	ss-DNA	Specific for target sequence	Random sequence
Specificity[c]	Non-specific	Non-specific	Specific	Specific
Limit of DNA fragment size (bp)[d]	20	100	150	50
Assay time (h)[d]	3	6	2	48
Sample throughput[d]	High	Medium	High	Low

[a] This study.
[b] Dr. Marc Bailey, detection and quantification of process-related impurities, WCBP2006, 25th January 2006.
[c] Ref. [8].
[d] Ref. [16].

The detection limit of the Quant-iT™ PicoGreen® ds-DNA Assay Kit is specified as 250 pg/mL in the product instructions, and the actual LOD and LOQ results obtained from this study were 102 and 310 pg/mL, respectively. Although it is possible to achieve the acceptable levels (10 ng/dose) of a WHO guideline with this sensitivity, it is clearly inferior to the sensitivity (approximately 30 pg/mL) of the Threshold system. Therefore, a DNA extraction/enrichment process following protein digestion by SDS/Proteinase K was applied to increase the detection sensitivity of the PicoGreen DNA assay. As a result, detection sensitivity comparable to the Threshold system was achieved.

The host cell DNA contents in cell culture supernatants, process intermediates, and drug substance of two different products were determined using the novel PicoGreen DNA assay system, including a DNA extraction/enrichment process following protein digestion by SDS/Proteinase K. It was confirmed that the host cell DNA content determined by the Threshold system was clearly underestimated compared to the DNA content determined by the novel PicoGreen DNA assay system. Furthermore, every DNA contents only exception of the Product B's drug substance might be underestimated by Threshold system compared to the novel PicoGreen DNA assay system although most of those values were under detection limits for both DNA assay systems.

In addition to the PicoGreen DNA assay and the Threshold system, a comparison of various other DNA determination methods, including dot blot hybridization and quantitative real-time PCR is shown in Table 5 [8,16]. Dot blot hybridization possesses a relatively wide dynamic range (10–2500 ng/mL) and an ability to detect DNA fragments as short as 50 bp. However, the detection limit of dot blot hybridization is 10 ng/mL, which does not fulfill the WHO guideline. Among these DNA determination methods, quantitative real-time PCR possesses the widest dynamic range (5 pg/mL to 1 μg/mL) and the highest detection sensitivity (5 pg/mL), and the protocol for DNA quantitation is also the simplest. However, the possibility of underestimating the DNA content must be considered because its detection limit of DNA length is 150 bp, which is even longer than the Threshold system limit (100 bp), and DNA fragments without a DNA primer sequence are not detectable by quantitative real-time PCR. Therefore, based on the characteristic features of these DNA determination methods, it is thought that the PicoGreen DNA assay is the most suitable DNA determination method for clearance evaluation of host cell DNA in cell culture supernatants and process intermediates among the four determination methods.

5. Conclusions

The PicoGreen DNA assay has been developed, which has a higher detection capability of short ds-DNA than the conventional method with the Threshold system, and overall detection sensitivity comparable to the Threshold system. Using this method, the measurement of host cell DNA levels in cell culture supernatants and process intermediates is available with better accuracy, simpler procedure and higher throughput. This novel host cell DNA assay using PicoGreen will be most suitable in various host cell DNA assays for measuring host cell DNA levels in bio-pharmaceuticals.

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References

[1] D.C. Andersen, L. Krummen, Curr. Opin. Biotechnol. 13 (2002) 117–123.
[2] L. Chu, D.K. Robinson, Curr. Opin. Biotechnol. 12 (2001) 180–187.
[3] WHO, WHO Weekly Epidemiological Record, 72 (1997) 141–145.
[4] Food and Drug Administration (FDA), Center for Biologics Evaluation and Research, February 28, 1997.
[5] EU, The European Agency for the Evaluation of Medical Products: Evaluation of medicinal products for human use, CPMP/BWP/1143/00, 2001.
[6] V.T. Kung, P.R. Panfili, E.L. Sheldon, R.S. King, P.A. Nagainis, B. Gomez Jr., D.A. Ross, J. Briggs, R.F. Zuk, Anal. Biochem. 187 (1990) 220–227.
[7] P.M. Nissom, Biologicals 35 (2007) 211–215.
[8] T. Wolter, A. Richter, Bioprocess Int. 3 (2005) 40–46.
[9] V.L. Singer, L.J. Jones, S.T. Yue, R.P. Haugland, Anal. Biochem. 249 (1997) 228–238.
[10] E.L. Romppanen, K. Salvolainen, I. Mononen, Anal. Biochem. 279 (2000) 111–114.
[11] S.J. Choi, F.C. Szoka, Anal. Biochem. 281 (2000) 95–97.
[12] G. Tolun, R.S. Myers, Nucleic Acids Res. 31 (2003) e111, 1-6.
[13] P. Murakami, M.T. McCaman, Anal. Biochem. 274 (1999) 283–288.
[14] K. Otawa, H. Satoh, Y. Kanai, M. Onuki, T. Mino, Lett. Appl. Microbiol. 46 (2008) 434–438.
[15] C. Guillo, J.P. Ferrance, J.P. Landers, J. Chromatogr. A 1113 (2006) 239–243.
[16] J.J. Wolf, L. Wang, F. Wang, Expert Rev. Vaccines 6 (2007) 547–558.