

Constant extraction of DNA from paraffin-embedded gastric surgical and biopsy specimens for polymerase chain reaction (PCR) analysis

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Summary

In order to see how large paraffin-embedded tissue is enough for extraction of DNA, of which the solution is employed as a template DNA solution of polymerase chain reaction (PCR), this study compare in the PCR for human β -globin (HBG) gene the DNA solutions gotten from paraffin-embedded tissue sections of various largeness by means of TaKaRa DEXPAT™ and the DNA solutions about 10 times concentrated by means of Amicon Microcon Microconcentrator. The about 10 times concentrated DNA solution from a section of 2 mm x 2 mm x 100 μ m largeness could be the template DNA for the PCR, whereas the DNA solution extracted from a section of 2 cm x 1 cm x 20 μ m largeness by means of DEXPAT™ was enough for the DNA template solution for the PCR. The DNA solution gotten by means of TaKaRa DEXPAT™ included a large amount of protein so that the phenol extracted and ethanol sedimentation are suggested to be good procedure to concentrate DNA and diminish the back ground in agar-gel electrophoresis of PCR product. The DNA extracted from thin sections included larger amount of short stranded DNA so that the positive control PCR was important to see whether the extracted DNA is enough for PCR analysis. The DNA in the paraffin-embedded tissue stored long time included an amount of the shorter than 250 bp and longer than 110 bp long stranded DNA.

Introduction

Since polymerase chain reaction (PCR) analysis was introduced in surgical pathology, it became easy to detect specific DNA sequences of micro-organisms, viruses and several kind of human genes by means of PCR employing DNA extracted from paraffin sections of biopsy or surgical material. Method of extracting DNA from paraffin-embedded tissue section has been developing. TaKaRa DEXPAT™ that is a fluid product to extract DNA from paraffin-embedded tissue section gives an easy performance of DNA extraction in comparison with the routine method that comprises dewax in xylene, proteinase K digestion, phenol extraction and ethanol sedimentation. On the other hand, Amicon Microcon microconcentrator gives an easy way to concentrate DNA solution or to change its buffer, although the routine DNA extraction method can concentrate DNA by adding an adequate volume of buffer after ethanol sedimentation of DNA.

But it is difficult sometimes to see the amount of paraffin-embedded tissue section enough for a template DNA solution of the PCR analysis, especially when the paraffin-embedded tissue is small such as that of endoscopic mucosal biopsy material. Then, this study aimed to see how large the paraffin-embedded tissue section is necessary to extract constantly an enough DNA amount for the PCR analysis.

Figure 1. The largeness of the specimens, from which DNA was extracted

SP01) One section of gastric wall with moderate to poorly differentiated adenocarcinoma.

SP03) One section of gastric wall without carcinoma.

SP02) One tiny piece of the gastric mucosa, which is of the same largeness as an usual endoscopic biopsy specimen. The mucosa includes adenocarcinoma.

SP04) One tiny piece of gastric mucosa, which is of the same largeness as an usual endoscopic gastric mucosa. Carcinoma is not recognized in this specimen.

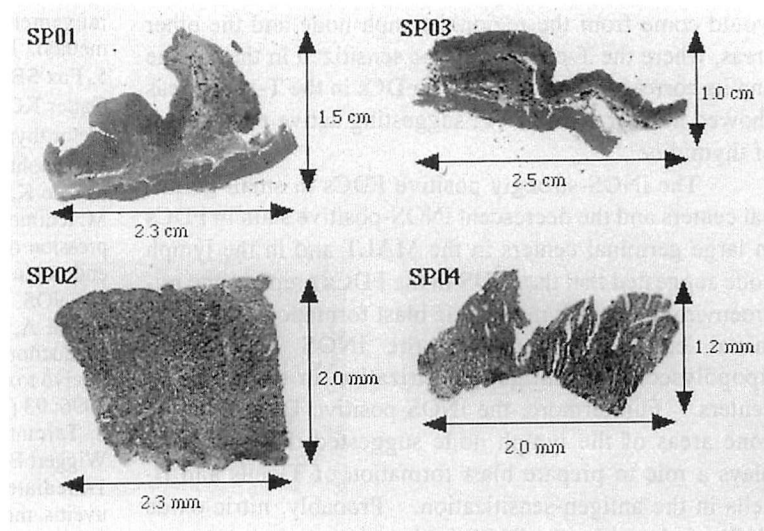


Figure 2. The nature of DNA extracted from paraffin-embedded tissue by means of DEXPAT™

The DNA was extracted from one 20 μm thick section of SP01 and SP03 by means of TaKaRa DEXPAT. A trace band of amplified DNA is seen in the product of the PCR of HGB GH20-21 primers in the both SP01 and SP03. The product of the PCR employing the four primers shows bands at 110, 204, 250, 268 bp length, suggesting that the most extracted DNA strands are less than 300bp long.

SP01, DNA extracted from one section of 20 μm thickness by means of DEXPAT **SP03**, DNA extracted from one section of 20 μm thickness by means of DEXPAT



Marker	HBG	HBG	Marker	HBG	HBG
9	PC03-04	PC03, 04	9	PC03-04	PC03, 04
	HBG	GH20, 21		HBG	GH20, 21
		GH20-21			GH20-21

Material and method

Paraffin-embedded gastric wall sections with carcinoma (SP01) and without carcinoma (SP03) and tiny pieces of mucosal tissue with carcinoma (SP02) and without carcinoma (SP04) were prepared. Paraffin sections were 2.3x1.5 cm in SP01, 2.5x1.0 cm in SP03, 2.3x2.0 mm in SP02, and 2.0x1.2 mm in SP04, as shown in Fig. 1.

Each one section of 3, 10 and 20 μm thickness of the paraffin-embedded tissue (SP01 and SP03) was cut into a 1.5 ml microtube. Each one section of 3, 10, 20, 50, 100 and 200 μm thickness of the paraffin-embedded tissue (SP02 and SP04) was cut into a 1.5 ml microtube.

From two 20 μm thick sections of paraffin-embedded gastric mucosa with and without adenocarcinoma resected one years before (1998), 6 years before (1993), 11 years before (1988), 16 years before (1983), and 21 years before (1978), DNA was extracted in order to see the nature of the DNA in the paraffin-embedded tissue that was stored long time.

DNA extraction by means of TaKaRa DEXPAT

According to the operating manual of TaKaRa DEXPAT™¹, the microtubes with a paraffin section and 0.5 ml TaKaRa DEXPAT™ solution were heated by means of heat block at 100°C for 10 min. Before the heating, a small hole was made on the cap of the microtubes by means of a needle, to avoid the open of the cap by a high pressure that yields in boiling. After the heating, the solutions were sedimentated at 12,000 rpm for 10 min at room tempera-

ture. Three hundred to 400 μl of supernatant under paraffin layer was removed by using a micropipette from the microtube to a new microtube (Fig. 3). The supernatant is the template DNA solution for the PCR (Fig. 4).

DNA concentration by means of microconcentrator

Three hundred μl of the supernatant DNA solution was concentrated about 10 times by means of microconcentrator (Microcon, Amicon INC.). According to the manual of the microconcentrator², a trace amount of glycerin that maintains moist ultrafiltration membrane of device was removed by spin-rinse with 0.1N NaOH and H₂O. The device was immersed by Tris-EDTA buffer until its usage. Before usage, after spin-rinse of TE buffer, the device was spin-rinsed once by H₂O. Three hundred μl of the supernatant DNA solution was applied to the device, was dry-uped, and was diluted in 20 to 30 μl of TE buffer (Fig. 3).

The concentrated DNA solution was employed for the PCR (Fig. 5).

Phenol extraction, ethanol sedimentation and about 10 times concentration of the DNA extracted

Three hundred μl of DNA solution extracted from two 20 μl thick sections of paraffin-embedded tissue of stomach with and without adenocarcinoma was processed though phenol extraction and ethanol sedimentation and was concentrated about 10 times.

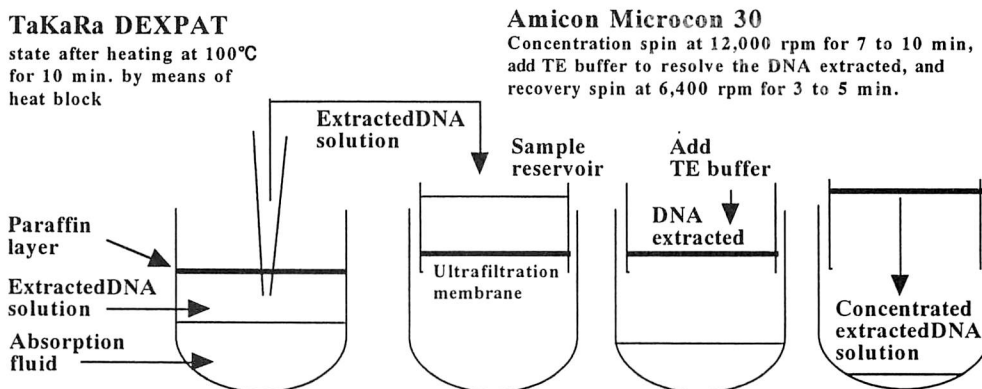
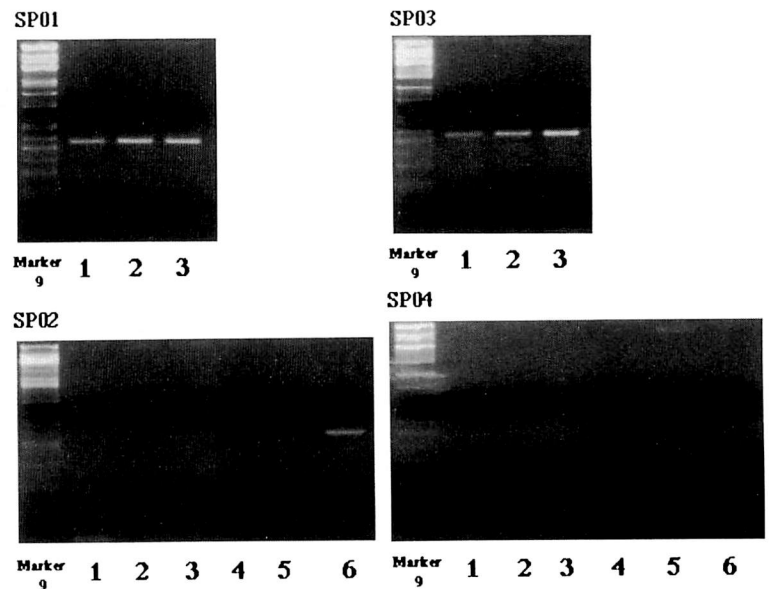


Figure 3. Extraction of DNA by means of TaKaRa DEXPAT™ and concentration by means of Amicon MICROCON

Figure 4. PCR of HBG PC03-04 primers, employing the DNA extracted solution by means of TaKaRa DEXPAT™

In each of SP01, SP02, SP03 and SP04, the template DNA solution was 5 μ l of the solution
 1: DNA extracted from a 3 μ m thick section
 2: DNA extracted from a 10 μ m thick section
 3: DNA extracted from a 20 μ m thick section
 4: DNA extracted from a 50 μ m thick section
 5: DNA extracted from a 100 μ m thick section
 6: DNA extracted from a 200 μ m thick section



PCR of human β -globin (HBG) gene

Primers employed were PC03 and 04, and GH20 and 21. The amplified DNA of the PCR employing PC03 and 04 is 110 bp long and that of the PCR employing GH20 and 21 is 408 bp or 204 bp long. PCR employing the 4 primers yields 110, 204, 250, 268 and 408 bp long bands of amplified DNA.

One hundred μ l of PCR solution includes 10 μ l of 10x Buffer, 8 μ l of dNTP mixed solution, 0.5 μ l of TaKaRa Ex Taq, 2 μ l of each primers and autoclaved H₂O. The PCR was performed according to the following protocol, predenature for 5 min at 94°C, 30 cycles of denature for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 72°C, and post-extension for 5 min at 72°C. Twenty μ l of the PCR product was charged on 4% agar gel electrophoresis stained by ethidium bromide.

Measurement of DNA in the extracted DNA solution

The amount of DNA extracted from the sections of SP01 and SP02 by means of TaKaRa DEXPAT™ and concentrated by means of Amicon Microcon microconcentrator was measured by a spectrophotometer before and after phenol extraction and ethanol sedimentation of DNA. The DNA amount extracted the paraffin sections of gastric wall tissue stored long time was also measured. The ratio (A₂₆₀/A₂₈₀) of the absorption at 260 nm (A₂₆₀) versus the absorption at 280 nm (A₂₈₀) was calculated for an index of protein contamination. The absorption at 260 nm (A₂₆₀) represented the amount of DNA.

Result

The length of DNA strands extracted was examined by means of PCR employing HBG primers and the DNA solution extracted from one 20 μ m thick section of the SP01 and of the SP03 (Fig. 1). Five μ l of the supernatant DNA solution was applied to the PCR of HBG PC03 and 04 primers, of HBG GH20 and 21 primers, and of HBG PC03, 04, GH20 and 21 primers. The PCR employing the

extracted DNA solutions from the sections of the SP01 and of the SP03 indicated the same results, as shown in Fig. 2. In the PCR of HBG PC03 and 04, the amplified DNA was of 110 bp long DNA. In the PCR of HBG GH20 and 21, the amplified DNA showed an obvious band at 204 bp length and a trace band at 408 bp length. In the PCR of the four primers, the amplified DNA showed bands at 110, 204, 250 and 268 bp length in a smear. The DNA strands extracted from the paraffin sections were of less than 268 bp length, although a trace amounts of DNA of longer than 408 bp was recognized.

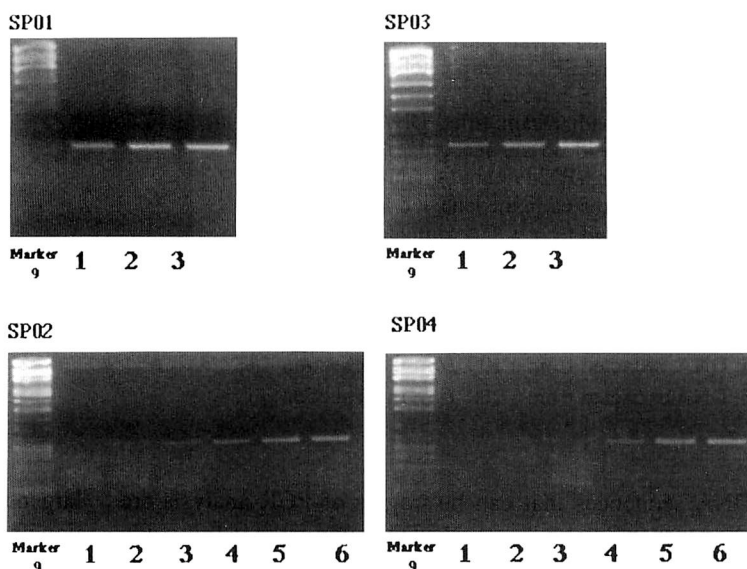
It was examined by means of the PCR employing HBG PC03 and 04 primers whether each extracted DNA solution included an enough amount of DNA for the PCR analysis. The products of PCR, which employed the extracted DNA solutions extracted from one 3, 10 and 20 μ m thick sections of the SP01 and SP03 paraffin-blocks as the template DNA solution, revealed a band of amplified DNA at 110 bp length on the agar gel electrophoresis, suggesting that these extracted DNA solutions included an amount of DNA enough for the PCR, as shown in Fig. 4 SP01 and SP03. But the extracted DNA solutions extracted from one of 3, 10, 20, 50, 100 and 200 μ m thick sections of the SP02 and SP04 paraffin-blocks did not include an amount of DNA enough for the PCR, as shown in Fig. 4 SP02 and SP04, although the PCR of that from one 200 μ m thick section of the SP02 paraffin-block yielded a band of the amplified DNA at 110 bp length.

The about 10 times concentrated DNA solutions of the extracted DNA solutions from the sections of the SP01 and SP03 paraffin-blocks included an amount of DNA enough for the PCR, revealing a band of amplified DNA at 110 bp length (Fig. 5, SP01 and SP03). Concatamer formation was not seen (Fig. 5).

Employing the about 10 times concentrated DNA solutions of the extracted DNA solutions from the sections of the SP02 paraffin-block, the PCR amplified DNA enough to reveal a band at 110 bp length on the agar gel electrophoresis in the lanes of 20, 50, 100 or 200 micrometers thick sections in Fig. 5 SP02. From the lane of 20

Figure 5. PCR of HBG PC03-04 primes, employing the about 10 times concentrated DNA solution

In each of SP01, SP02, SP03 and SP04, the template DNA solution was 5 μ l of the solution
 1; DNA extracted from a 3 μ m thick section
 2: DNA extracted from a 10 μ m thick section
 3: DNA extracted from a 20 μ m thick section
 4: DNA extracted from a 50 μ m thick section
 5: DNA extracted from a 100 μ m thick section
 6: DNA extracted from a 200 μ m thick section



μ m thick section to that of 200 μ m thick section, the band of the amplified DNA became stronger, probably reflecting the DNA amount of the concentrated DNA solutions. The Fig. 5 SP04 showed the same tendency as the Fig. 5 SP02. But the band of the amplified DNA at the 110 bp length was seen in the lanes of the 50, 100 and 200 μ m thick sections.

The A260/A280 of the SP01 and SP02 concentrated DNA solutions was from 0.465 to 0.610, as indicated in the table 1. Contamination of a large amount of protein in the solutions was indicated. The A260/A280 of the DNA solutions after phenol extraction and ethanol sedimentation was from 1.493 to 1.740, indicating a quite low degree of the protein contamination. The DNA in the solutions was from 50 ng/ μ l x 50 to 1218 ng/ μ l x 50. From the 10 to 20 μ m thick sections (SP01 and SP02) the largest amount of DNA was extracted. The volume of DNA extracted from one 10 μ m thick section indicated the lowest amount of DNA.

The nature of DNA in the SP01 and SP02 solutions after phenol extraction and ethanol sedimentation was examined by means of the PCR for HBG (Fig. 6). As shown in Fig. 4, three bands of amplified DNA at 110, 250 and

268 bp were recognized in SP01. In the SP02, a band of amplified DNA was recognized at 110 bp in the lane 4, 5 and 6.

The DNA solutions extracted from paraffin-embedded tissue of stomach with and without adenocarcinoma included shorter than 250 bp and longer than 110 bp strands of DNA, as shown in Fig. 7.

The DNA extraction fluid extracted from two 20 μ m thick sections of the paraffin-embedded gastric tissue stored long time included a large amount of DNA. But the amplified DNA bands of the PCR of HBG (Fig. 7) did not run parallel with the amount of the DNA in table 2.

Discussion

It is quite important to see the nature of DNA extracted from paraffin section, because fixation of tissue, procedurs to make paraffin block, and storage duration of the paraffin-block effect on the nature of the DNA, especially in the length of the DNA strands. As shown in Fig. 2, the DNA extracted DNA comprised dominantly strands of less 268 bp length, although the product of the PCR employing HBG GH20-21 primers suggested a small amount of DNA strands longer than 408 bp length. Because most

Table 1. The content of DNA in 300 micron liters extracted DNA solution of DEXPAT treatment

		The concentrated solution#1 A260/A280#3	Phenol extraction and ethanol extraction#2 A260/A280#3	DNA(ng/ μ l)
SP01	3 micron meter thick	0.465	1.571	561
	10 micron meter thick	0.474	1.740	1218
	20 micron meter thick	0.500	1.579	532
SP02	3 micron meter thick	0.610	1.493	203
	10 micron meter thick	0.583	1.515	50
	20 micron meter thick	0.550	1.545	431
	50 micron meter thick	0.585	1.507	303
	100 micron meter rthick	0.581	1.523	303
	200 micron meter thick	0.466	1.539	397

#1: The about 10 times concentrated DNA solution by means of the microconcentrater

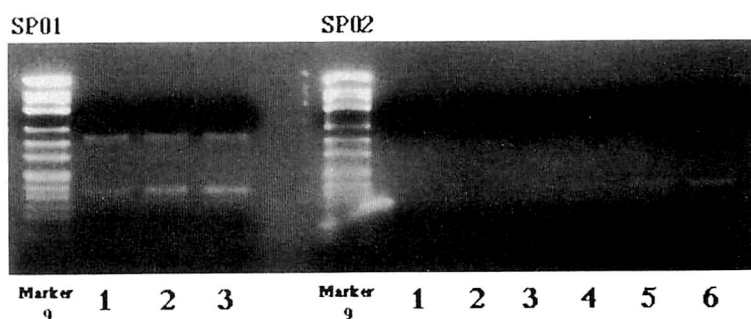
#2: The DNA slution extracted from the concentrated solution (#1) by means of phenol extraction and ethanol extraction

#3; Ratio of the absorption at 260 nm versus the absorption at 280 nm by spectrophotometry

Figure 6. PCR of HBG gene, employing primers, PC03 and 04, and GH20 and 21, and DNA solutions of SP01 and SP01 after phenol extraction and ethanol extraction in table 1

In SP 01, bands of amplified DNA are seen at 110, 250 and 260 bp are recognized in the clear background. In SP02 faint but gradual increase of bands are recognized in the lane 4, 5 and 6.

- 1; DNA extracted from a 3 μm thick section
- 2: DNA extracted from a 10 μm thick section
- 3: DNA extracted from a 20 μm thick section
- 4: DNA extracted from a 50 μm thick section
- 5: DNA extracted from a 100 μm thick section
- 6: DNA extracted from a 200 μm thick section



DNA sequences that can be targets of PCR analysis are shorter than 200 bp length, the DNA extracted in this study was proved to be enough for PCR analysis. The PCR employing HBG PC03, PC04, GH20 and GH21 is useful to see the length of the extracted DNA.

Usually 0.1 μg of template DNA is applied to PCR. The amount of DNA extracted could be measured. And the template DNA amount could be adjusted to 0.1 μg DNA per 5 μl. But in a surgical pathology laboratory there would not be an apparatus to measure a quite small amount of DNA. Then, it is useful to see the enough amount of tissue for PCR by estimating the areas where target cells exist under microscope.

It was shown in Fig. 4 SP03 that the concentration of DNA extracted from a section of 2.5 cm x 1.0 cm x 3 μm largeness is enough for the PCR. But the concentration of DNA extracted from a section of 2.0 mm x 1.2 mm x 200 μm largeness was not enough for the PCR. The concentration of DNA extracted from a SP02 section of 2.3 mm x 2.0 mm x 200 μm largeness, about two times larger than the SP04 section, was enough for the PCR. The minimum

largeness of the tissue section, from which the concentration of DNA extracted by means of TaKaRa DEXPAT™ is enough for the PCR was shown to be between 2.0 mm x 1.2 mm x 200 μm and 2.3 mm x 2.0 mm x 200 μm.

An extremely small amount of DNA can be the template DNA for 2 times PCR employing the same primer set or the inner primers. Such an extremely small amount DNA can be gotten by means of micro-resection method. Depending on the areas where the target cells exist, an adequate sensitivity of the PCR must be employed. On the other hand, this study indicated that the about 10 times concentrated solution of the extracted DNA solution from one 100 μm thick section of gastric mucosa biopsy material more than 2 mm x 2 mm largeness is enough for PCR analysis. Therefore, the section of 100 μm thickness with target cells areas larger than 2 mm x 2 mm is needed in the PCR. Recently, Taq DNA polymerase that can amplify more the target sequence of DNA than TaKaRa Ex Taq DNA polymerase employed in this study has been introduced so that thinner section would be enough for such Taq DNA polymerase.

Figure 7. The nature of DNA in the paraffin-embedded tissue stored long time

One band of amplified DNA at 110 bp and two bands of comcatamer of primers at about 20 and 40 bp were recognized in each lane. In the lanes of 1988 Ca and 1988 Non-Ca, additional weak bands at the longer length were seen.

Ca: The DNA solution extracted from the section with adenocarcinoma was employed as a template DNA solution. **Non-Ca:** The DNA solution extracted from the section without adenocarcinoma was employed as a template DNA solution.

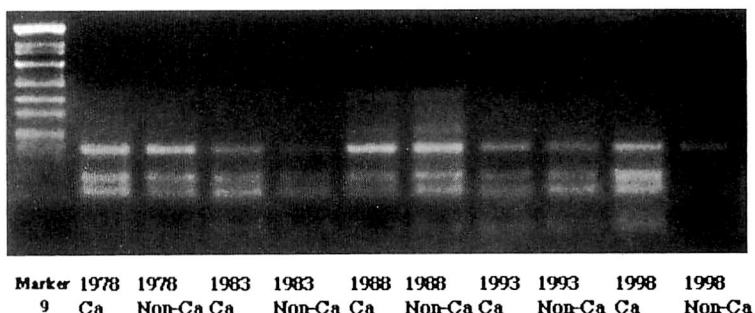


Table 2. The content of DNA in the extraction fluid from two 20 μm thick sections of gastric wall sections with and without adenocarcinoma, which were stored for 1 year (1998), 6 years (1993), 11 years (1988), 16 years (1983) and 21 years (1978).

	1978		1983		1988		1993		1998	
	Ca ^{#1}	Non-Ca ^{#2}	Ca	Non-Ca	Ca	Non-Ca	Ca	Non-Ca	Ca	Non-Ca
A260/A280 ^{#3}	1.559	1.552	1.564	1.477	1.400	1.494	1.588	1.564	1.569	1.577
DNA (ng/μl)	2232	2247	2378	2547	2575	2482	1959	2282	2061	2187

#1: The DNA extraction fluid from the sections with adenocarcinoma.

#2: The DNA extraction fluid from the sections without adenocarcinoma.

#3; Ratio of the absorption at 260 nm versus the absorption at 280 nm by spectrophotometry

Concatamer formation was not seen in the PCR employing the about 10 times concentrated DNA solution, suggesting that the about 10 times concentration is not the overconcentration to yield concatamer in the PCR.

The complete spine-rinse of a trace of glycerin in ultrafiltration membrane of the microconcentrator² is quite important in the concentration of the template DNA solution for PCR, because an amount of DNA enough for the template DNA solution is absorbed even by the trace amount of glycerin. Although the routine method can concentrate DNA solution and remove substances other than DNA, the easy and rapid procedure of DNA concentration by means of the Amicon Microcon microconcentrator is important in practise.

The amount of DNA can be estimated by photoabsorption at 260 nm in spectrophotometry. Because of the contamination of a large amount of protein as quite low ratio of A₂₆₀/A₂₈₀ in Table 1, the exact amount of DNA in the DNA extraction fluid gotten by TaKaRa DEXPAT™ could not be measured, although the bands of the amplified DNA in the PCR employing the DNA extraction fluid suggested its quality enough for PCR, as shown in Figures 2, 3 and 4.

The DNA in the DNA extraction fluid processed through phenol extraction and ethanol sedimentation revealed high values of A₂₆₀/A₂₈₀ ratios and indicated its enough amount. The amount of DNA extracted (Table 1) suggested that the largest amount of DNA can be extracted from the 10 to 20 μ m thick sections. Gradual increase of DNA extracted according to the thickness of the section was proved by the PCR in Fig. 5 and 6, although the amount of

DNA measured were of the almost same amount in SPO2. Probably a large amount of short-stranded DNA was extracted from thin sections in SPO2. Then, the DNA extracted from paraffin-embedded sections must be examined by means of PCR for HBG in order to see whether the DNA includes long stranded DNA enough for the PCR analysis.

The DNA extracted from paraffin sections of stomach with and without adenocarcinoma included shorter than 250 bp and longer than 110 bp strands of DNA enough for usual PCR analysis, as shown in Fig. 7. In the not-saturated PCR amplification of the target DNA, the amount of amplified DNA runs parallel with that of the target DNA in the template DNA solution. The stain of the bands of the amplified DNA by ethidium bromide has a quality to suggest the amount of the amplified DNA. Therefore, it suggested a large amount of the short-stranded DNA in the DNA extraction fluid that the bands of the amplified DNA in Fig. 7 did not run parallel with the amount of the DNA in the corresponding the extraction fluid in Table 2. On the other hand, it was suggested that there was an amount of the shorter than 250 bp and longer than 110bp long-stranded DNA in the paraffin-embedded tissue. The storage duration of the paraffin-blocks did not effect on the amount of the such long-stranded DNA.

References

- 1, Operation manual of TaKaRa DEXPAT™, Code No. 9091, TaKaRa Biocheical (in Japanese)
- 2, Operating manual of MICROCON, Microconcentrators, Publication 1-394 H, AMICON, Inc, Beverly, USA