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# Hot Cell-Direct PCR Aimed at Specific Cell Detection

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Additional information is available at the end of the chapter

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## Abstract

Since the polymerase chain reaction (PCR) was proposed, it has become an essential method in the field of biological gene analysis, providing a method to amplify DNA sequences of interest. To detect and/or analyze genes in cells, the gene or expressed gene must first be extracted before PCR. This procedure takes time and may result in the loss of samples. In order to avoid such drawbacks, two methods, hot cell-direct PCR and reverse transcription-PCR (RT-PCR), were invented, to detect genes in cells. Using hot cell-direct PCR, specific genes in microbial cells such as *invA* in *Salmonella enterica* have been easily detected and applied to discriminate Archaea from bacteria. As hot cell-direct PCR and RT-PCR are fairly simple processes, they can be applied to detect genes in single cells. We developed an original compact disc (CD)-shaped microfluidic device with microchambers for single-cell isolation and a detection system for expressed genes in isolated single cells in a microchamber on the device. We succeeded in the detection of PCR and RT-PCR products in individual cells and successfully detected *S. enterica* cells by hot cell-direct PCR. Expressed genes in Jurkat cells—human leukemia T cells—were analyzed by this method.

**Keywords:** hot cell-direct PCR, RT-PCR, cell, gene, expressed gene

## 1. Introduction

Almost three decades ago, in 1987, the polymerase chain reaction (PCR) was first proposed by Mullis and Faloona [1]. In PCR, specific DNA sequences are amplified through repeated cycles of temperature changes that denature the DNA sequence, bind primer sequences for the target sequence (annealing) and elongate the reaction catalyzed by DNA polymerase [1]. Since non-heat-resistant DNA polymerase was used initially, polymerase had to be added after each

reaction cycle because the enzyme denatured and became inactivated. However, after the use of heat-resistant DNA polymerase, such as Taq polymerase, PCR prevailed widely, allowing for the amplification of various genes [2].

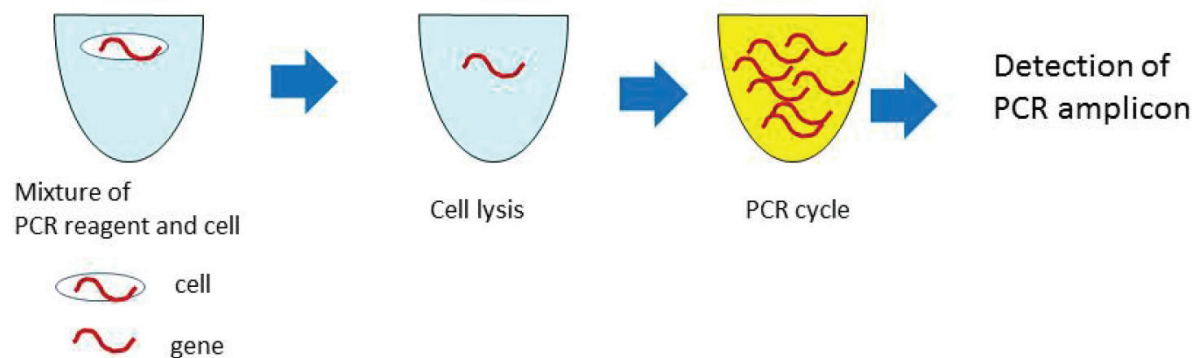
Since 1987, PCR has been applied to various biological fields of study. In the medical field, it was used to detect and identify pathogenic bacteria [3–7], in diagnostics of genetic or familial diseases [8–10], in prenatal diagnosis of fetal genes [11–13] and in risk assessment of hypertension [14, 15]. In forensic medicine, it was utilized to identify humans [16–20].

In the field of food science, PCR was used to detect food-borne bacteria and viruses and to discriminate beef, pork and chicken [21–23]. It was also useful to discriminate genetically engineered crops. More recently, in the field of environmental sciences, it was utilized to conduct a metagenomic analysis of soil, water and other samples to estimate microbes in the environment [24–26].

Usually genes in a living cell must be extracted from cell lysates in order to perform PCR. In the extraction process, DNA from cells is condensed, thus avoiding the negative effect of substances in cells on PCR. However, it takes time to lyse cells and extract DNA from the lysates, and these procedures are troublesome. Moreover, a small amount of DNA might be lost in the procedure. Recently, it was shown that PCR of a crude sample of lysate could be performed without extraction [27]. This implies that the substances in the lysate did not seriously affect PCR.

To establish an easy and rapid PCR procedure, we attempted to execute PCR in a single container in which target cells were lysed. As the simplest method, we proposed that in the PCR reagent mixture, that cells be lysed by heat treatment and that subsequent PCR heat cycles be repeated without the addition of any reagent to the same container (**Figure 1**). This method was performed with only a heat-changing procedure after placing cells and the PCR reagent mixture together in one container. We named the process hot cell-direct PCR [28]. This method can avoid contamination during extraction [29].

On the other hand, the detection of an expressed gene (m-RNA) in cells is important in gene detection as well as genomic DNA, because biological phenomena are closely related to the expression level and phase of a vast number of gene expression events. Conventional detection



**Figure 1.** Scheme of hot cell-direct PCR.

of m-RNA was performed as follows. After the lysis of target cells, total m-RNA was extracted from the obtained lysate. The m-RNA was reverse-transcribed to complementary DNA (c-DNA) from total RNA by using reverse transcriptase and the obtained c-DNA was amplified by PCR. This procedure is referred to as reverse transcription PCR (RT-PCR), which has been applied to analyze gene expression in biological phenomena. RT-PCR has to be performed very carefully, and experience and skills are necessary because RNA molecules are easily degraded by RNase.

In our study, we examined whether it is possible or not for PCR to express an m-RNA gene without extracting m-RNA from cells, as is done for genomic DNA. This would avoid contamination of RNase by researchers and allow RT-PCR to be performed without any trained skills. To perform such a procedure, every reagent necessary for RT-PCR should be initially mixed with a suspension of target cells, which must be lysed by heat treatment. Reverse transcription and PCR should be performed in the same tube. This method needs heat-stable reverse transcriptase different from conventional RT-PCR. We developed a method that utilized heat-stable reverse transcriptase and demonstrated that without extracting m-RNA from the cell lysate, that RT-PCR could be performed efficiently compared to conventional RT-PCR [30]. We named the method hot cell-direct RT-PCR. This method allowed for the amplification of genes from a small number of copies both by hot cell-direct PCR and RT-PCR and even from the lysate of a single cell.

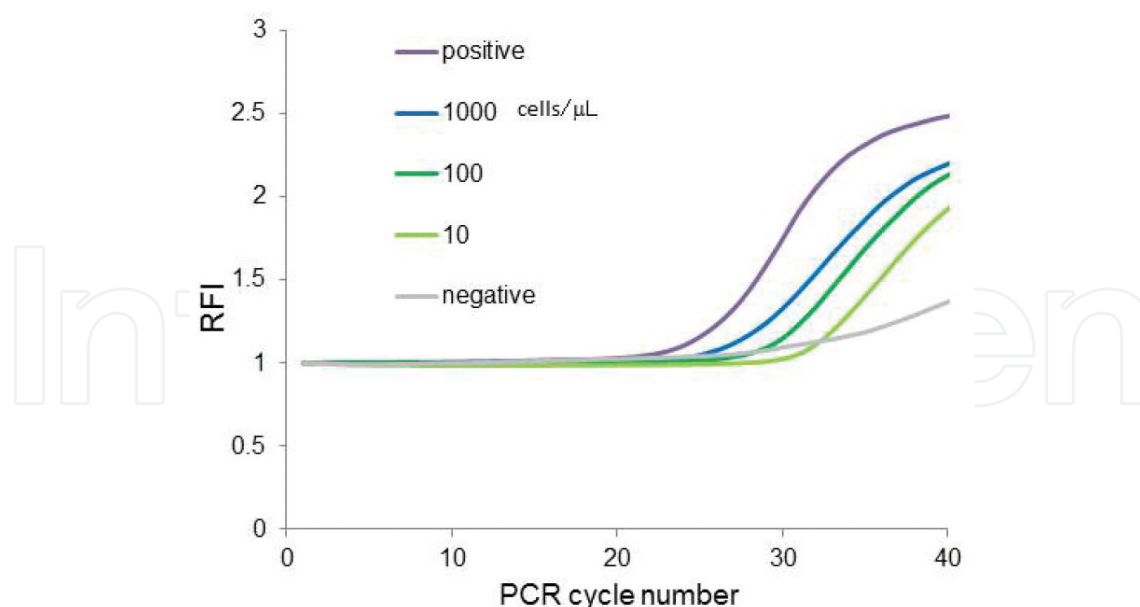
In this chapter, we review these methods and describe some examples, including application of hot cell-direct PCR to detect pathogenic bacteria by amplification of a specific bacterial gene and the discrimination of microbial species. Using hot cell-direct RT-PCR, it may be possible to detect expressed genes in eukaryotic cells.

## 2. Hot cell-direct PCR of microbial cells

In general, conventional methods to detect and identify bacteria require multiple subculture steps, such as enrichment culture, and selective culture. Collected samples are first multiplied in nutrition-rich medium, then cultured in selective medium, taking several days to detect and identify bacterial species in samples [31]. This needs not only time but also a lot of culture medium and labor. If the target bacterium has a specific gene, amplification of that gene by PCR and simultaneous detection of the PCR product will reduce the time and medium required to detect and identify bacteria from the sample. Using hot cell-direct PCR, bacteria will be easily detected in the lysate without the need to extract DNA from the cell lysate and allowing for the easy detection of bacterial species [29]. We applied hot cell-direct PCR to detect a food-borne pathogen in food and to discriminate bacteria and Archaea in an environmental sample.

### 2.1. Detection of *Salmonella enterica*

*Salmonella enterica* is widely known as notorious food-borne pathogen and responsible for human salmonellosis [32]. *S. enterica* is often transmitted to humans through chicken meat or



**Figure 2.** Hot cell-direct PCR of *S. enterica* cells in real-time PCR system.

eggs, because chickens sometimes carry *S. enterica* in their intestines. The incubation period after infection may last from 72 h to several days and infection can cause abdominal pain, diarrhea, vomiting or fever in a patient. This pathogen is currently detected by a culture method and suspicious foods are tested by cultures that take several days to grow and test. However, in order to avoid the spread of infection, a rapid method to detect the pathogen is required. Since *S. enterica* is known to have a specific gene *invA*, which is the gene coding for the invading factor into a host cell, a detection method based on amplification of *invA* by PCR exists [33]. To amplify *invA*, a commercialized PCR detection kit (Cycleavag salmonella detection kit, TAKARA, Osaka, Japan) was used. The kit contains a fluorescent probe (cycling probe) to detect the PCR product of *invA*, and fluorescence caused by the probe increases as the PCR product increases, similar to the TaqMan probe. This kit is promising tool for hot cell-direct PCR.

This kit contains a primer/probe mix, Taq polymerase, RNaseH, buffer and a dNTP mixture. The primer/probe mix is a mixture of primers and probes to detect the *invA* gene. The probe for the *invA* gene was labeled with 6-carboxyfluorescein (FAM) and a quencher. The increase in fluorescence from FAM occurred after an amplification of the *invA* gene. After the probe was hybridized to the PCR product, it was cut by RNaseH. Then, the fluorescence intensity is increased by uncoupling the quencher from the probe.

We examined hot cell-direct PCR of *S. enterica* using this kit, as follows [29]. First, a suspension of bacterial (*S. enterica*) cells was mixed with all the kit's reagents in a microtube and the mixture was heated at 95°C for 2 min to lyse cells. In the same tube, PCR (DNA denaturation 95°C × 5 s, annealing of primer 55°C × 10 s and elongation of DNA 72°C × 30 s) cycles were performed without the addition of any reagent. These processes only involved change in temperature of the reaction mixture. Through PCR, fluorescence of the FAM probe increased and was measured using a real-time PCR system (ABI 7500).

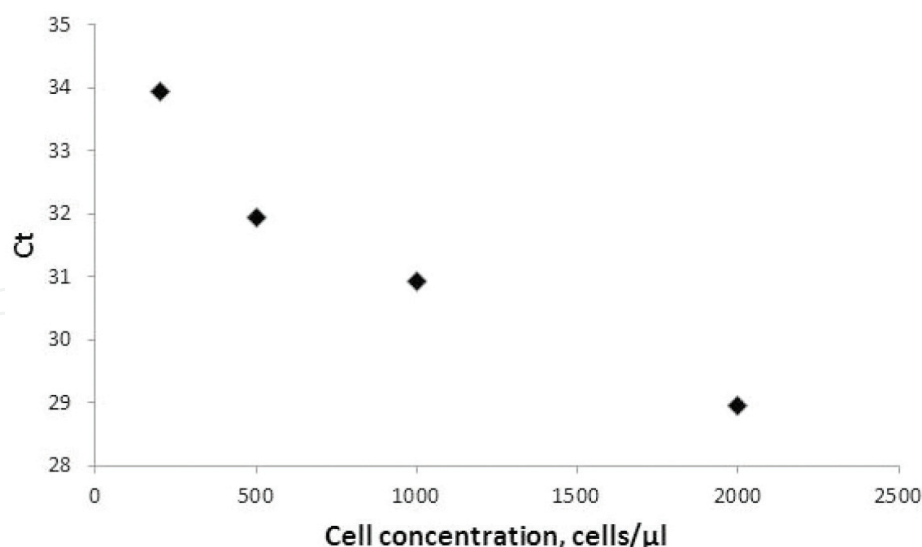


Hot cell-direct PCR was using a range of concentrations (10–1000 cells/ $\mu$ L) of *S. enterica* suspension (**Figure 2**). At each concentration, fluorescent intensity increased with each PCR cycle. In real-time PCR, relative fluorescent intensity (RFI) is unity (value 1) at the start of PCR and RFI increases as the number of PCR cycle proceeds. The number of PCR cycles with a recognizable increase in RFI depends on the concentration of amplicon of the target gene with higher concentrations showing a smaller number of cycles while lower concentrations have a larger number of cycles. As shown in **Figure 2**, a lower concentration of *S. enterica* showed a larger number of cycles with a recognizable increase in RFI. In addition to this experiment, selectivity of the reaction was confirmed under the coexistence of *Escherichia coli* cells. A cell suspension of *S. enterica* (1000 cells/ $\mu$ L) and various concentrations of *E. coli* (1000–1,000,000 cells/ $\mu$ L) were mixed and hot cell-direct PCR of *invA* was examined. At any concentration of *E. coli*, and the same number of cycles resulted in a recognizable increase in RFI curves. The lysate of both *S. enterica* and *E. coli* did not have an effect on the PCR of *invA* and hot cell-direct PCR of this specific gene is a useful and easy way to detect *S. enterica*.

## 2.2. Detection of *Bacillus cereus*

*Bacillus cereus* is a food-borne bacterium that infects humans mainly from cooked carbohydrates such as rice and pasta, with symptoms being vomiting and diarrhea. Conventionally *B. cereus* in food has been tested by a culture-based method that takes 7 days to identify the bacterium. Such a long period required for its identification has hampered efforts to avoid the spreading of *B. cereus* infection. Thus, a rapid and reasonable method to detect and identify *B. cereus* is required. The diarrheal syndrome is caused by enterotoxins produced by *B. cereus* with known enterotoxins being HBL, NHE and CytK1. HBL is the gene product of *hblA*, *hblC* and *hblD* and NHE is that of *nheA*, *nheB* and *nheC* [34]. Among these enterotoxins, the toxicity of *B. cereus* is correlated with the amount of secreted NHE. Thus, NHE is regarded as an important enterotoxin related to food-borne disease. Ester et al. reported that NHE is produced by most of strains of *B. cereus* and that the carrying rate of the NHE gene in *B. cereus* is in 99.69% of strains. Thus, detection of the NHE gene will demonstrate its existence in most *B. cereus* strains.

Yang et al. reported the detection of *B. cereus* targeting one of the NHE gene, *nheB* [35]. They reported a PCR primer for the amplification of *nheB*. Since we have succeeded in hot cell-direct PCR of *invA* in *S. enterica*, we tried to perform hot cell-direct PCR of this specific *B. cereus* gene [36]. There is no commercialized kit for the amplification of *nheB*. We performed PCR of *B. cereus* utilizing the primer set reported by Yang et al., forward primer (SGF3: GCACTTATGG-CAGTATTTGCAGC) and reverse primer (SFR3: GCATCTTTTAAGCCTTCTGGTC), to amplify *nheB* of two *B. cereus* strains. After PCR using these primers, the *nheB* amplicon was observed by electrophoresis. To develop the hot cell-direct PCR procedure of *nheB*, the TaqMan probe to detect the PCR amplicon is necessary. Since there is no report on the TaqMan probe of *nheB*, we designed probes. The *nheB* amplicon is 152 bp long. From the amplicon sequence, we designed probes using the “Primer Express Software Version 3.0 (Applied Biosystems) software”, in which the sequences had a 30–80% of GC contents, and where the length was 15–30 bp and contained no GGGG nor AAAAAA. The selected sequence was termed probe 1 (5'-ATTATGCCGCTCATACGTATGCAGCTG-3'). To construct the TaqMan probe, the 5' end



**Figure 3.** Ct in hot cell-direct PCR of *B. cereus*.

was labeled with FAM and the 3' end was labeled with Dark Quencher. Probe 1 was examined to detect the *nheB* amplicon by the real-time PCR system. With the use of probe 1, as PCR cycles proceeded, fluorescence of the sample solution increased. Using the primers (SGF3 and SGR3) and probe 1, hot cell-direct PCR of *B. cereus* was performed. The *B. cereus* suspension was mixed with *Ex Taq HS* (TAKARA, Japan), dNTP mixture, probe 1, primers and *Ex Taq*<sup>TM</sup> buffer (TAKARA). The sample mixture was heat-treated at 95°C × 5 min (lysis), and PCR cycle of 95°C × 30 s, 55°C × 30 s and 72°C × 30 s was repeated. By measuring fluorescence using real-time PCR, the sample containing *B. cereus* cells showed an increase in fluorescent as the number of PCR cycles increased. In real-time PCR, cycle number increase, there is a recognizable increase in RFI, defined as Ct. The Ct with an RFI value of 1.2 was examined with various concentrations of *B. cereus* cells (100, 500, 1000 and 2000 cells/μL). At a lower concentration of cells, a larger Ct was observed (**Figure 3**). These results demonstrated that *B. cereus* can be detected by hot cell-direct PCR as *S. enterica* and that hot cell-direct PCR can be performed using TaqMan probe as well as a cycling probe.

### 2.3. Discrimination of Archaea and Bacteria

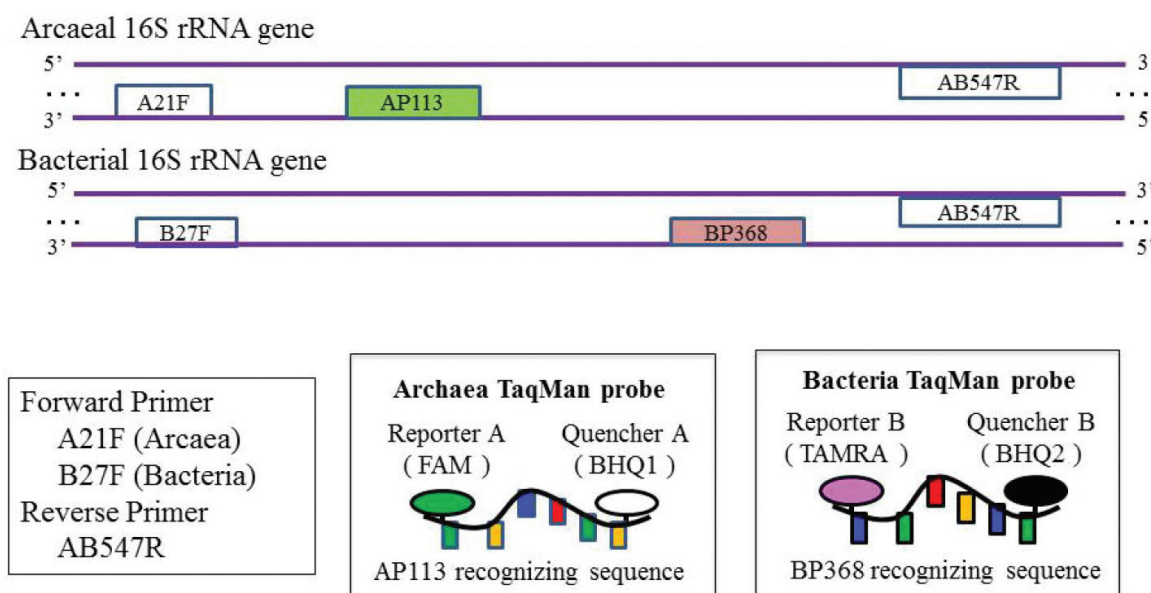
All living organisms on earth are classified into prokaryotes (including Archaea and bacteria) and eukaryotes. In an environmental community, less than 1% of the total numbers of prokaryotes have species that are able to be cultured [37]. Most Archaea species grow in habitats with extremely limited conditions, including temperature, altitude, salinity, pH, and anaerobic conditions and thus are difficult to cultivate in the laboratories. Not only archaeal species but also some bacterial and eukaryotic species coexist in the same environment [38].

Molecular analysis of environmental samples has clear advantages for discovering and characterizing microbial diversity and understanding interactions between microbes and with abiotic environmental factors. The culture-independent analysis of the small subunit ribosomal RNA (ssu rRNA) gene, 16S rRNA for Archaea and bacteria and 18S rRNA for

Eukarya in environmental samples or by metagenomic analysis provide valuable information about microbial diversity. The advantages of utilizing the ssu rRNA gene are: (1) ssu rRNA genes exist and the stability of their sequences are conserved in all living organisms; (2) nucleotide length (about 1600 bp for 16S rRNA) is sufficient for phylogenetic analysis; (3) conserved regions and variable regions are distributed throughout the gene, and are comparable to relatively closely-related species; (4) it is easy to obtain universal or specific primers for sequence analysis; (5) a huge number of sequence data are available. Fundamental techniques for molecular analysis of *Microsphaera* are based on PCR, electrophoresis and sequencing.

There are nine variable regions in 16S rRNA: V1 at nucleotide position 69–99 (*E. coli*), V2 (137–242), V3 (433–497), V4 (576–682), V5 (822–879), V6 (986–1041), V7 (1117–1173), V8 (1243–1294) and V9 (1435–1465). On the other hand, consensus sequences such as positions 515–533, 1390–1407 and 1492–1507 of *E. coli* are suitable for universal PCR primers of all living organisms [38, 39].

Differentiation of archaeal and bacterial species in an environmental sample has been possible using hot cell-direct PCR with two sets of PCR primers and two different fluorescence-labeled TaqMan probes. The primers and probes were designed with specific sequences in conserved regions of the 16S rRNA gene of Archaea and bacteria, respectively [40]. For example, the following primers and probes were used to analyze geothermal microflora in our study: A21F: 5'-TTCCGGTTGATCCYGCCGGA as the forward primer for Archaea, B27F: 5'-AGAGTTTGATCCTGGCTCAG as the forward primer for bacteria, and AB547R: 5'-TTACCGCGGCKGCTGGCAC as the reverse primer for Archaea and bacteria. TaqMan probes were designed with FAM and BHQ1 for Archaea: FAM-AP113 (5'-ACGGCTCAGTAACACGTGGCTAA)-BHQ1, and 5'-carboxytetramethyl rhodamine (TAMRA) and BHQ2 for bacteria: (TAMRA)-BP368 (5'-ACTCCTACGGGAGGCA GCAGTAGG)-BHQ2 (**Figure 4**).



**Figure 4.** Primers and probes for the discrimination of Archaea and bacteria.



In this method, the cells in a sample were lysed, and then DNA was amplified by PCR with a TaqMan probe and analyzed sequentially by real-time PCR in one tube. The reaction mixture consisted of the cell-containing sample, primer sets and TaqMan probes for Archaea and bacteria, Premix Taq DNA polymerase, and buffer. PCR was performed with a Real-time PCR system using the following program: an initial lysis step at 95°C for 5 min; 30 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. The fluorescence intensity of FAM and TAMRA were analyzed sequentially. As model Archaea and bacteria, *Metallosphaera sedula* TH-2 and *E. coli* B were examined. The sample containing *M. sedula* with A21F primer and AP113 probe only showed an increase in RFI of FAM and no increase with other primers or probes. In contrast, the *E. coli* sample with B27F primer and BP368 probe showed an increase in RFI.

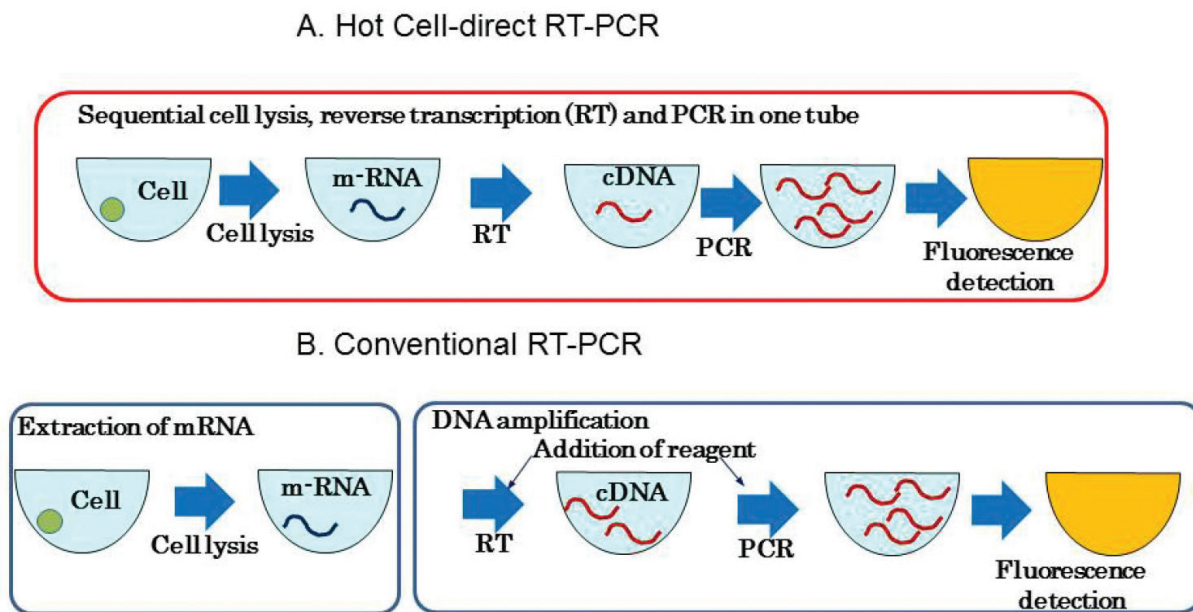
The RFI of FAM and TAMRA increased significantly in the reaction mixture that contained Archaea and bacteria, respectively [40]. In the Microsphaera analysis, a combination of unicellular fractionation and this method should be useful for differentiating Archaea and bacteria at the single cell level.

The most important aspect for the sensitive and robust amplification of a PCR product is the selection of a suitable primer set. A number of primers were designed and investigated for ssu rDNA, but there was no perfect match yet, especially for Archaea. Much effort is still required to evaluate primers [41].

### 3. Hot cell-direct RT-PCR of gene expression

Hot cell-direct PCR is a promising method to amplify the genes of microbes and to detect microbial species. Furthermore, we aimed to apply this method to detect expressed genes as well as specific genes in cells. Usually the analysis of an expressed gene is carried out after m-RNA extraction from lysed cells. Extracted m-RNA is reverse-transcribed into complementary DNA (c-DNA). The obtained c-DNA is amplified by PCR and the amplicon is analyzed by electrophoresis or real-time PCR (**Figure 5 (B)**). This method is called reverse transcription PCR (RT-PCR) because PCR is carried out after the reverse transcription of m-RNA. RT-PCR needs the extraction of the expressed gene (m-RNA) from lysed cells, and this process is similar to gene extraction of PCR. However, extraction of m-RNA is more difficult than DNA extraction, because RNA molecules are easily degraded by RNase in saliva and sweat which is secreted from humans around the working area. Such degradation of RNA molecules through the extraction process should be avoided when performing RT-PCR. Thus, RT-PCR has to be performed by a highly trained worker under sterile conditions. If the extraction process is omitted, such problems can be eliminated.

We investigated RT-PCR without the extraction of m-RNA from lysed cells as in hot cell-direct PCR and aimed to establish hot cell-direct RT-PCR, which was performed only by heat treatment after preparation of the RT-PCR reagent mixture with target cells [30]. Reverse transcription in RT-PCR is catalyzed by reverse transcriptase (RTase). Generally speaking, lysis buffer containing detergent is used to lyse cells and in the buffer cells are lysed by heat treatment. RTase used in general RT-PCR is inactivated by detergents and heat treatment, like most proteins. Therefore, RNA molecules were extracted from lysed cells and washed after lysis.



**Figure 5.** (A) Hot cell-direct RT-PCR and (B) Conventional RT-PCR.

Reagent for reverse transcription containing RTase was added to the washed RNA and reverse transcription was performed. In order to achieve our purpose, we considered the use of heat-stable RTase in solution. Heat-stable RTase can be added before cell lysis and lysis and reverse transcription can be accomplished in the same tube without extracting m-RNA (**Figure 5 (A)**).

As a candidate of heat-stable RTase, we discovered heat-stable DNA polymerase with reverse transcriptase activity, which can act as RTase and DNA polymerase, namely Tth DNA polymerase, which has good reverse transcription activity in the presence of  $Mn^{2+}$  [42]. As a suitable reagent for Tth DNA polymerase, we examined the Tth polymerase kit (Roche Applied Science). The kit contains Tth DNA polymerase (5 U/mL), 0.1% TritonX-100 and 25 mM  $Mn(OAc)_2$ . To avoid the degradation of RNA molecules by RT-PCR, we used RNase inhibitor. We examined several genes expressed in eukaryotic cells. In this review, mainly describe our investigation using Jurkat cells. Jurkat cells are known human T-cell lymphocytes. Among the expressed genes, we examined the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, because it is a housekeeping gene; that is, expression of the gene is constantly observed. We examined RT-PCR of *GAPDH* in Jurkat cells using a double dye probe designed for the detection of *GAPDH* (Nippon EGT, Japan) [30].

At first, cell lysis was confirmed with various concentrations of a detergent (TritonX-100). To the Tth DNA polymerase kit, TritonX-100 was added at a concentration of 0.001, 0.01, 0.1 and 1% and mixed with proliferated Jurkat cells (1000 cells/ $\mu$ L). The mixture was heat treated at 94°C for 10 min to lyse cells. The lysate was reverse transcribed (42°C for 15 min) and PCR was performed as follows: 95°C for 30 s to denature the DNA, and 60°C for 60 s of annealing and elongation. After the reaction, PCR products were confirmed by electrophoresis. The band of the *GAPDH* amplicon was observed at all concentrations of TritonX-100. It was predicted that a higher concentration of detergent would degrade the cell membrane more strongly than a lower concentration. However, the lowest concentration of sample indicated the thickest

band and a higher concentration of detergent resulted in a thinner band. The detergent in Tth DNA polymerase (0.1%) was enough to lyse cells and no additional detergent was necessary to obtain the PCR product of the *GAPDH* gene.

At first, reverse transcription was carried out at 42°C, in which most of the reaction was performed using non heat-stable reverse transcriptase. In our study, the reaction temperatures examined were 42, 50, 60 and 70°C to optimize the reaction using Tth DNA polymerase. Relative to initial fluorescence intensity was measured by PCR cycles with a real-time PCR system. PCR cycle number (Ct) with an RFI of 1.3 at various temperatures was compared (Figure 6). At the same concentration of *GAPDH* m-RNA, more efficient reverse transcription will produce a higher concentration of its cDNA and reduce the number of PCR cycle numbers at the same RFI. At 42°C, Ct was 22 and it was 18, 17 and 19 at 50, 60 and 70°C, respectively. The smallest Ct was observed at 60°C. Then 60°C appeared to be the most efficient temperature for reverse transcription catalyzed by Tth DNA polymerase [43].

Conventionally RT-PCR is performed using the extracted m-RNA from lysed cells. To confirm the efficiency of hot cell-direct RT-PCR, we compared hot cell-direct RT-PCR and conventional PCR, at the same concentration of cell suspension. In conventional RT-PCR, total RNA was extracted from a given concentration cell suspension after cell lysis. In hot cell-direct RT-PCR, the same number of cells was used as that used in conventional RT-PCR. In this study, a cell concentration of 1000, 500, 250 and 125 cells/μL was compared in both RT-PCR methods. The number of genes doubled in one cycle of PCR amplification.

When the sample with a certain concentration of gene needs the N cycle to reach the desired Ct, another sample with half the concentration of that gene needs N + 1 cycles to reach that Ct. When RT-PCR is performed for a sample of total RNA, the same phenomenon will be observed as in hot cell-direct RT-PCR. In the case of total RNA, the Ct of 1000, 500, 250 and 125 cells/μL was 16, 17, 18 and 19, respectively. The observed result agreed with the expected one. As in hot cell-direct RT-PCR, Ct was almost the same as in RT-PCR using total RNA. These results demonstrate that in hot cell-direct RT-PCR, the cell lysate did not affect RT-PCR and that the

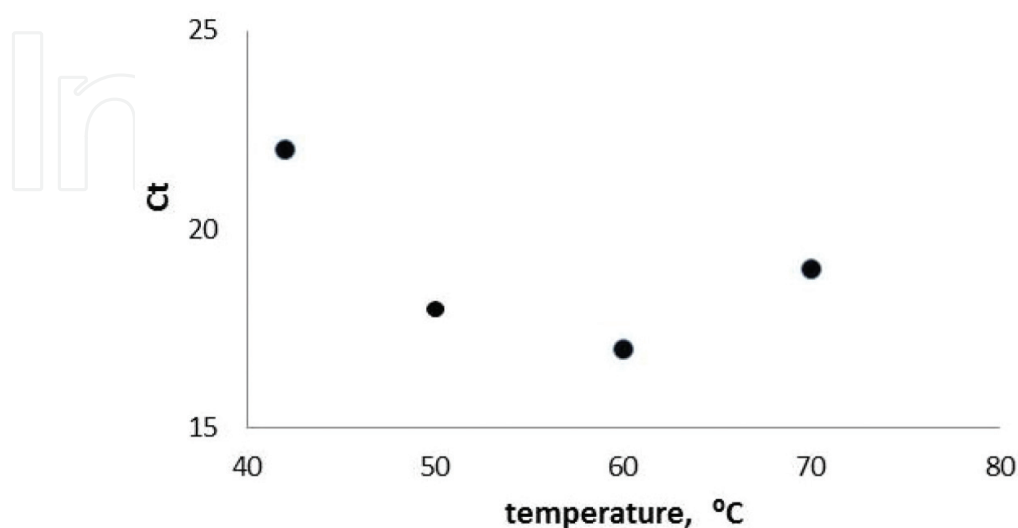


Figure 6. Ct in hot cell-direct RT-PCR of Jurkat cells at various RT temperature.

same amount of PCR amplicon was produced as RT-PCR with total RNA, without needing to extract m-RNA from the cell lysate. We examined hot cell-direct RT-PCR of other genes ( $\beta$ -actin and IL-2) expressed in Jurkat cells by real-time PCR and amplification of expressed genes was also observed, as for the *GAPDH* gene. Thus, hot cell-direct RT-PCR is useful and very easy to detect expressed genes.

#### 4. Application of hot cell-direct PCR to single-cell analysis

Recently, single-cell analysis has drawn attention to the analysis of characteristics of each cell in a single-cell population, because it has been suggested that each cell has different characteristics, even in the same population and with the same genes. The most traditional single-cell isolation technique is micromanipulation. Patient training and experience of the operator are necessary conditions to perform micromanipulation. As the operator takes only one cell at a time, it is a low throughput method. On the other hand, a fluorescence-activated cell sorter (FACS) is a high throughput method to analyze cells. It can separate cells one by one rapidly and classify them depending on their shape and size with the assistance of fluorescent staining. However, this is not appropriate to identify a gene or expressed gene in a single cell. To characterize a single cell, single-cell isolation has been investigated with the use of microfluidic devices. The microfluidic method is a promising method to isolate cells from a large number of cells easily and rapidly. We have developed an original compact disk (CD)-shaped microfluidic device with microchannels and microchambers for single-cell isolation and a rapid and easy single-cell isolation method on the device. On this CD-shaped device, after applying the cell suspension in the microchannel, cells are easily isolated in microchambers on the channel by the rotation of the device, without the need for any micropump. By applying the cell suspension to many microchannels, cells can be isolated in a large number of microchambers simultaneously. On one device, 24 microchannels are arranged and on one microchannel 300 microchambers are arranged. In total, 7200 microchambers on one device are available for single-cell isolation (**Figure 7**) [28, 44]. This device was fabricated using heat-resistant material, silicone and glass, and can be used in heating reactions such as PCR. On the device, single-cell isolation and hot-cell direct PCR or RT-PCR can be achieved very easily. After PCR, fluorescence of each microchamber should be measured and the existence of the target gene is reflected by the increase in fluorescence. By hot cell-direct RT-PCR on the device, gene analysis can be performed easily. In our study, detection of *S. enterica* cells was based on hot cell-direct PCR of the *invA* gene. As an application of the detection of expressed gene analysis, detection of *GAPDH* in isolated Jurkat cells is shown.

##### 4.1. Detection of *Salmonella enterica*

As a way to detect food-borne bacteria, application of our device to *S. enterica* was investigated. *S. enterica* was detected after PCR of *invA* as described above. When a cell trapped in a microchamber is *S. enterica*, fluorescence increases, caused by amplification of *invA* after hot cell-direct PCR. When the entrapped cell is not *S. enterica*, fluorescence of the chamber does not



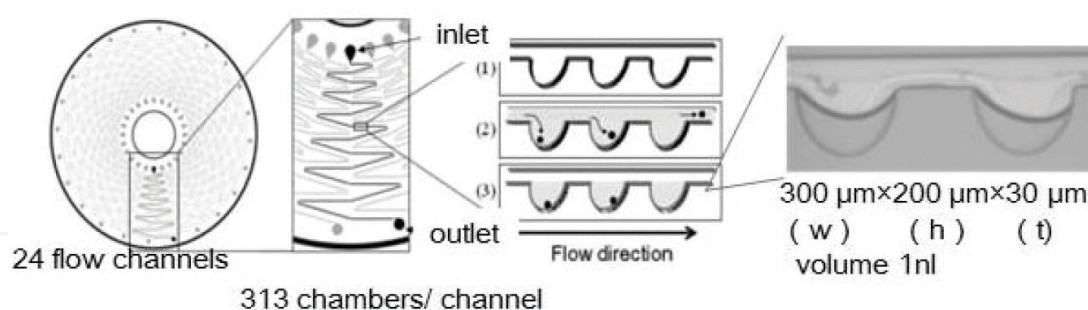


Figure 7. CD-shaped microfluidic device.

increase. Therefore, only the microchamber entrapping *S. enterica* cell shows an increase in fluorescence.

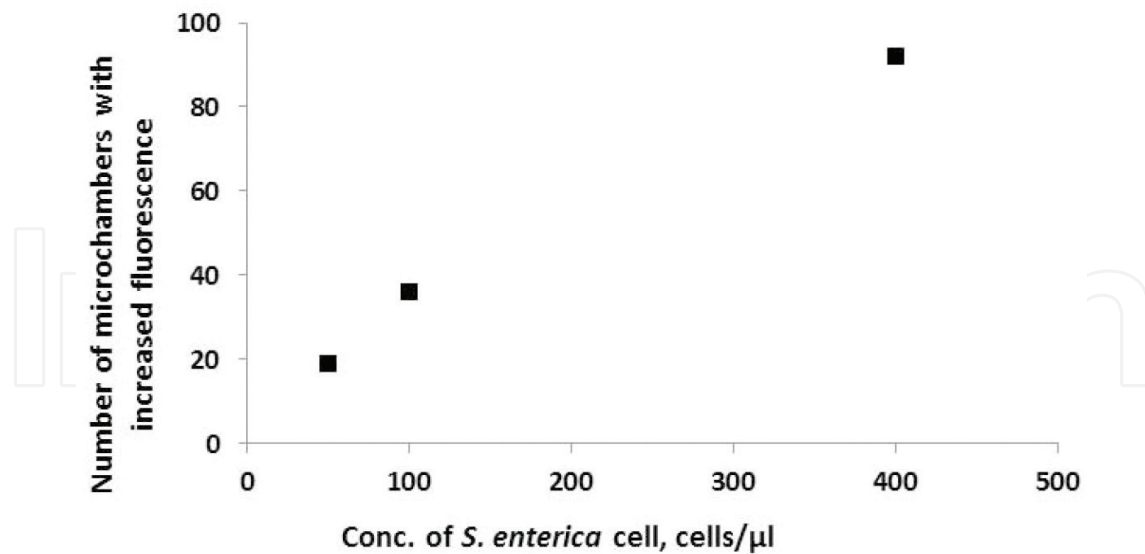
The isolation of microparticles such as bacterial cells into microchambers on the device depends on a Poisson distribution [29]. The isolation of *S. enterica* cells was confirmed by applying a cell suspension (50–400 cells/ $\mu\text{L}$ ) to a microchannel. As the cell concentration increased, the number of microchambers entrapping a cell increased, and at a concentration range of 50–200 cells/ $\mu\text{L}$ , the number of cells observed in a microchamber was one or zero. However, at a concentration of 400 cells/ $\mu\text{L}$ , the number of cells was occasionally two or more. At the same concentration range, *S. enterica* cells were applied to the device and hot cell-direct PCR was performed according to the same temperature change protocol in a microtube. The fluorescence of each microchamber was measured using an epifluorescent microscope customized to observe the microchambers on the device [28].

*S. enterica* cells mixed with PCR reagent were applied to the inlet of the microchannel and isolated in microchambers by rotation of the device. Before hot cell-direct PCR, fluorescence of each microchamber was measured under a fluorescent microscope. Thereafter, the device was set to a thermal cycler, which has a customized stage for the device to perform temperature changes. In the thermal cycler, cells were lysed by heat at  $95^{\circ}\text{C}$  for 2 min and followed by 40 PCR cycles. After completing the PCR cycles, the fluorescence of each microchamber was measured again. Then the fluorescence ratio of after to before hot cell-direct PCR was evaluated.

At the range of 50–400 cells/ $\mu\text{L}$ , the microchambers showed increased fluorescence and the number of microchambers with increased fluorescence depended on the applied concentration of *S. enterica* cells (Figure 8). A single *S. enterica* cell was confirmed by hot cell-direct PCR on the device. When *E. coli* cells (1000 cells/mL) were mixed with *S. enterica* cells, almost the same increase in fluorescence was observed. This method can be applied to single-cell detection of a specific cell type.

Since *S. enterica* is known to infect chicken meat, we investigated the detection of *S. enterica* from ground chicken on the CD-shaped microfluidic device by hot cell-direct PCR. After the sample of ground chicken was suspended in buffered peptone water (BPW), the sample solution from meat was filtered through a filter bag. Using the filtrate, sample detection limit was examined and 40,000 cells/mL (40 cells/ $\mu\text{L}$ ) could be determined but not a lower concentration. After sampling through the filter bag, the filtrate was cultured in medium for 4–8 h at





**Figure 8.** Dependence of number of microchambers with increased fluorescence on concentration of *S. enterica* cells.

30°C and from the cultured medium *S. enterica* cells were collected by centrifugation and the detected on the device. After culture, 30 cells/g were detected in the sample [28].

Conventionally, *Salmonella* sp. is detected with a culture-based method that takes several days to identify the bacterial species. However, the hot cell-direct PCR method using the microfluidic device takes a very short amount of time (8–12 h including enrichment culture) and a small amount of reagent. This method can be applied to detect various bacteria when primers and TaqMan probe for the specific gene are available. We therefore consider this method to be promising for microbial research.

#### 4.2. Analysis of expressed gene in single Jurkat cells

Analysis of expressed genes in single cells has garnered much interest. For example, differences in gene expression of cells at an early stage of development are very interesting, but it is not easy to assess because the number of cells is limited. In general, biological phenomena are analyzed based on cell populations. However, phenotypic variation has been observed in one population of cloned cells by FACS. This suggests that individual cloned cells have different phenotypes and gene expression is different from cell to cell. To investigate actual differences in gene expression of individual cells, evaluation of gene expression in an isolated single cell is necessary. The m-RNA extracted from a single cell is so little that it cannot be amplified and detected by RT-PCR in a tube-based analysis. On the other hand, a microfluidic method can concentrate cells in a small volume of reagent after isolating the target cell, allowing for the detection of an expressed gene after RT-PCR of the gene in the cell. If the evaluation and analysis of gene expression of an isolated single cell is enabled by hot cell-direct RT-PCR on the microfluidic device above, it may provide a solution. We investigated the possibility of analyzing an expressed gene in single cells by using Jurkat cells by hot cell-direct RT-PCR on the CD-shaped microfluidic device for single-cell isolation.

Initially, *GAPDH* gene expression was examined. Proliferated Jurkat cells were harvested and suspended in the RT-PCR reagent mentioned above and the mixture was applied to the microfluidic device. The concentration of cells in the suspension was 200–400 cells/ $\mu$ L and the cells were isolated in microchambers. On the device, cell lysis (95°C for 10 min), reverse transcription (60°C for 15 min) and PCR cycles were executed according to the hot cell-direct RT-PCR temperature program. The number of microchambers showing higher fluorescence after RT-PCR corresponded to the value evaluated using a Poisson distribution. After 40 PCR cycles, the before-to-after PCR fluorescence ratio of microchambers was evaluated and compared. The chamber entrapping no cells did not show any increase in fluorescence and the fluorescence ratio was almost 1.0, little variation was observed. The chamber entrapping a cell showed a remarkable increase in fluorescence while the fluorescence ratio showed a wide range ( $2.30 \pm 0.41$ ) [45]. This variation in the fluorescence ratio of the chamber entrapping a single cell was larger than that of the chamber with no cell. This result indicates the possibility that the gene expression level of a single cell was different in each cell. This heterogeneity in gene expression was considered to be caused by different cell cycle phases, although the *GAPDH* gene is known as a housekeeping gene and is constitutively expressed. Furthermore, we are now investigating the expression of other genes besides the *GAPDH* gene in cultured human cells.

## 5. Conclusion

Hot cell-direct PCR and RT-PCR are effective methods to detect genes and expressed genes very easily, as described above. Specific cell detection is possible by using the CD-shaped device, allowing for rapid and easy biological assays.

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