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Quantitative Detection of *Toxoplasma gondii* by Competitive Polymerase Chain Reaction of the Surface Specific Antigen Gene-1

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Abstract

A quantitative competitive (QC)-PCR was developed to assay the cell number of *Toxoplasma gondii* (*T.gondii*), an intracellulary infective protozoan, by estimating copy numbers of specific gene. The truncated surface specific antigen gene-1 (SAG1) was used as a competitor of the QC-PCR targeting SAG1 of *T.gondii*. The determined amount of competitor SAG1 DNA was coamplified with Fukaya strain genomic DNA. QC-PCR products were electrophoresed in an agarose gel and stained with ethidium bromide. The band intensities of QC-PCR products were measured using a gel densitometer. A good relationship was obtained between the cell numbers of *T.gondii* and the band intensities of QC-PCR products. From 1 to 10^4 copies of SAG1 could be quantitated in 2 μg genomic DNA (*T.gondii* DNA was included in carrier DNA). This method is more simple and sensitive than cell counting methods for *T.gondii* and may be useful for investigating the organ distributions and kinetics of *T.gondii* infection.

Key words: *Toxoplasma*; QC-PCR; SAG1.

Introduction

Toxoplasma gondii (*T. gondii*) can cause life-threatening diseases in developing human fetuses (McCabe and Remington, 1983) and in immunocompromized individuals, particularly patients with AIDS (Koppel *et al.*, 1985; Mills, 1986; Navia *et al.*, 1986). For most healthy individuals, however, the infection is mild with few or no clinical symptoms. Therefore, rapid and accurate diagnosis is important. Serologically-based diagnosis techniques for patients who have serious immune dysfunction due to AIDS or through immunosuppressive drug therapies may prove unreliable because of impaired antibody production.

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Because of its extraordinarily high sensitivity, the polymerase chain reaction (PCR) was used recently to detect small amounts of *T. gondii* DNA (Burg *et al.*, 1989; Holliman *et al.*, 1990; Savva *et al.*, 1990; Holliman *et al.*, 1992; Bretagne *et al.*, 1993; Wastling *et al.*, 1993; Hohlfeld *et al.*, 1994). However, quantitation of *T. gondii* by PCR is unreliable because the amount of PCR product increases exponentially with each cycle of amplification. Therefore, minute differences in any of the parameters that affects the efficiency of amplification can alter dramatically the product yield. Both the kinetics and efficiency of amplification of a target template depend on the starting abundance of the template and on the sequence match of the primers and target template and may also be affected by inhibitors present in the specimen (Dickover *et al.*, 1990; Noonan *et al.*, 1990). In the quantitative competitive PCR (QC-PCR) method for DNA quantitation a competitive DNA template matched to the target sequence of interest, but differing from it by virtue of an introduced internal deletion, is used in competitive titration and PCR steps, providing stringent

internal control. The quantitation is based on determination of the relative, not absolute, amounts of the different amplified products derived from the wild-type and competitive template after electrophoretic separation (Gilliland *et al.*, 1990; Piatak *et al.*, 1993). This report describes the successful establishment of a quantitative method to estimate the copy numbers of *T. gondii* by QC-PCR.

Materials and methods

Experimental animals and parasites

The Fukaya strain of *T. gondii*, which is avirulent, obtained from Dr. Y. Suzuki, Jikei Medical University, Tokyo, Japan. Cysts of Fukaya were prepared from C57BL/6 (B6) mice that were infected with 5 cysts of the Fukaya strain by the oral route 6 weeks earlier. Brain material was obtained by continuous density gradient centrifugation using Percoll (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) (Cornlissen *et al.*, 1981). The pooled brains of 3 mice were homogenized in 5 ml of phosphate buffered saline (PBS). Then, 5 ml of brain suspension containing *T. gondii* cysts was mixed with a 45% Percoll suspension and the mixture was centrifuged for 15 min. at 1250 g in a swinging bucket rotor (CD 100R, Tomy Seiko, Tokyo, Japan). The recovery of cysts from crude brain suspension was around 90%. Tachyzoites of *T. gondii* of Fukaya strain were prepared by culturing spleen cells and mesenteric lymph node cells of BALB/c mice infected with 15 Fukaya cysts for one week.

Construction of competitive cDNA of SAG1 (competitor SAG1)

The cDNA fragment of SAG1, which was from restricted digestion of the PCR product of the genomic SAG1 of *T. gondii* using the restriction enzymes (Nde I at 451 bp site and Xho I at 1210 bp site), constructed in the pET-21b(+) (Novagen, Madison, WI. USA) plasmid (pET-21b(+)SAG1 to be constructed for obtaining recombinant SAG1 protein by using 6-His. column) was digested wih the restriction enzymes, Sac II and Sac I at bp positions 511 and 665. The small fragment of 154 bps was deleted and the cohesive termini of the larger fragment were treated with cloned Klenow Fragment (Takara Co., Kyoto, Japan) to obtain blunt end insert DNA. After that, the blunt ends of the SAG1 in the pET-21b(+) were ligated with T4 DNA ligase (Takara) (Fig. 1). The competitor SAG1 plasmid was transfected to *E. coli*. HB101 cells. After 48 hours culture, several transformed clones were selected and mini-preparations of the plasmid with the truncated DNA were done. The truncated pET-21b(+) SAG1 DNA samples were amplified by PCR. One clone (molecular size 605 bps) was chosen as the competitor. The smaller molecular size product of pET-21b(+) SAG1 was confirmed to be the truncated SAG1, in which 154 (511-665) bps were deleted, with an ABI 373A DNA sequencer (ABI Applied Biosystems, Foster, CA., USA).

Competitive coamplification of the pET-21b(+) SAG1 with competitor SAG1 DNA

The sequences of the primers were based on the published sequence for the *T. gondii* SAG1 gene (Burg *et al.*, 1988). One of those (P-1) was of the same as that sequence and the other (P-2) was the

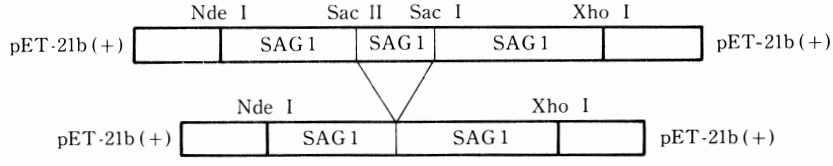

Fig. 1 Construction of competitor SAG1.
SAG1 constructed in pET-21b(+) was cut with restricted enzymes (Sac II. Sac I) at positions 511 and 665 base pairs. The cohesive ends were treated with larger fragment *E.coli*. DNA polymerase I. The blunt ends were ligated with T4 DNA ligase.

antisense. The primer, P-1 (5'-TCGGATCCCCCT-CTTGTTGC), corresponds to nucleotides 452-471 of the SAG1 gene; the other primer, P-2 (5'-CTCCAGTTTCACGGTACAGT), to nucleotides 1191-1210. In the competitive PCR, we added the competitor SAG1 DNA to PCR reaction solution (normally in a final volume of 50 μl). The competitor SAG1 DNA bound competitively to the oligos with the pET-21b(+) SAG1 because we used the same oligos in this system. Besides the pET-21b(+) SAG1 and the competitor SAG1 DNA to be amplified, each reaction mixture also contained PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.005% Tween 20, 0.005% NP-40), 0.2 mM each deoxynucleotide triphosphate, 0.2 μM each of the two oligos and 1 unit of replitherm (Epicentre Technologies Corp. Madison, WI. USA). The amplification reaction cycle was performed as follows; 94°C for 3 min., 55°C for 2 min. and 72°C for 2 min. for first one cycle and then at 94°C for one min., 55°C for 3 min., 72°C for 2 min. for 35 cycles and for last one cycle at 94°C one min., 55°C for 2 min. and 72°C for 5 min.

Quantitation of T. gondii *tachyzoites by QC-PCR*

In preliminary experiments, ten week old Balb/c mice were perorally infected with 15 cysts of Fukaya strain. The infected mice were sacrificed at day 14 after infection. Spleen cells and mesenteric lymph node cells from infected mice were cultured in RPMI culture medium in 5% CO_2 incubator for one week. Fukaya tachyzoites from *in vitro* cultured spleen cells and mesenteric lymph node cells were separated with low speed centrifugation at 500 g from host cells and counted. The DNA of varying numbers of *T. gondii* tachyzoites with carrier cell (human B lymphoma cells) was prepared. Five hundred μl digestion buffer (100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K) was added to cell pellets. The pellets were digested in a shaking water bath at 50°C for overnight. DNA was extracted 2 times with phenol, 2 times with phenol/chloroform/isoamyl alcohol and 2 times with chloroform/isoamyl alcohol. The prepared DNA was treated with 20 μg/ml pancreatic RNase for one hour at 37°C and was quantitated with Genequant (Pharmacia LBK Biochrom Ltd., Cambridge, UK). The competitor SAG1 DNA was diluted serially when a determined tachyzoite number (1×10^3) was used, otherwise when the concentration of the competitor SAG1 DNA was constant (5.6×10^{-4} ng or 5.6×10^{-5} ng), the genomic DNA of *T. gondii* was diluted serially to 1×10^4, 1×10^3, 1×10^2, 1×10^1, 1×10^0. After coamplification of genomic DNA and the competitor SAG1 DNA by PCR, gel electrophoresis of the PCR products were measured with an IPLab Gel densitometer (Signal Analytical Corp., Vienna, VA., USA). The standard curve was obtained using the corresponding SOFTWARE (Signal Analytical Corp.).

Results

Amplification of T. gondii *SAG1 by conventional PCR*

The minimum detectable cell number of *T. gondii* was assayed by conventional PCR. *T. gondii* tachyzoites cultured *in vitro* were collected and counted with a microscope. The DNA of a determined number of *T. gondii* was prepared and then the DNA of a varying number (1000, 100, 10, 1) of *T. gondii* were added to PCR mixture as templates.

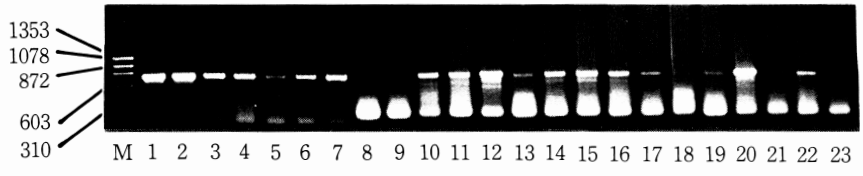

Fig. 2 Conventional PCR amplification of one copy SAG1 gene. Varying numbers of Fukaya tachyzoites (10^3, 10^2, 10^1, 10^0) were amplified by conventional PCR. M is the molecular size marker (øX174/*Hae* III digest). Lanes 1 and 2 represent 10^3 tachyzoites. Lanes 3 and 4 represent 10^2. Lanes 5, 6 and 7 represent 10^1. Lanes 8 to 23 represent 10^0.

The genomic SAG1 was amplified by 35 cycles of PCR reaction. The data in Fig. 2 demonstrate that one *T. gondii* tachyzoite cell could be detected. Although the band intensities of PCR products generally decreased with *T. gondii* numbers, the band intensities of PCR products from same *T. gondii* tachyzoites varied somewhat (Fig. 2). The specific bands of genomic SAG1 were obtained in 11 out of 16 samples that each contained the DNA of one *T. gondii* tachyzoite and 10^3 carrier cells (human B lymphoma cells).

Coamplification of the competitor SAG1 and the pET-21b(+) SAG1

The competitor SAG1 and the pET-21b(+) SAG1 were coamplified. After electrophoresis of PCR products, two bands were seen. The molecular sizes of the two bands were 759 and 605 bps (Fig. 3a). The DNA sequence of a smaller PCR product showed 154 base pairs of SAG1 to be deleted. Different amounts of DNA of the competitor SAG1 (2.8×10^{-2}, 2.8×10^{-3}, 2.8×10^{-4}, 2.8×10^{-5}, 2.8×10^{-6} ng) and constant pET-21b(+) SAG1 (7.5×10^{-4} ng) were coamplified. The intensities of PCR products of the competitor SAG1 were decreased according to the amount of the competitor SAG1 DNA, however, those of the pET-21b(+) SAG1 increased gradually in spite of their being the same amount of DNA (Fig. 3b). The results also indicate that the avidities of the pET-21b(+) SAG1 and the truncated pET-21b(+) SAG1 DNA combining to oligos were almost the same and that the amplification efficiencies of both the pET-21b(+) SAG1 and the competitor SAG1 were almost the same.

Coamplification of the SAG1 gene of Fukaya strain of T. gondii *and the competitor SAG1*

To determine whether the SAG1 gene of Fukaya strain of *T. gondii* could be detected well in the presence of host genomic DNA derived from 10^3 human B lymphoma (ARH) cells, constant amounts of genomic DNA of *T. gondii* (1000 cells) with different amounts of the competitor SAG1 DNA (5.6×10^{-1}, 5.6×10^{-2}, 5.6×10^{-3}, 5.6×10^{-4}, 5.6×10^{-5}, 5.6×10^{-6} ng) were coamplified. After gel electrophoresis the band intensities of PCR products of Fukaya genomic SAG1 DNA increased gradually while the DNA concentration of the competitor

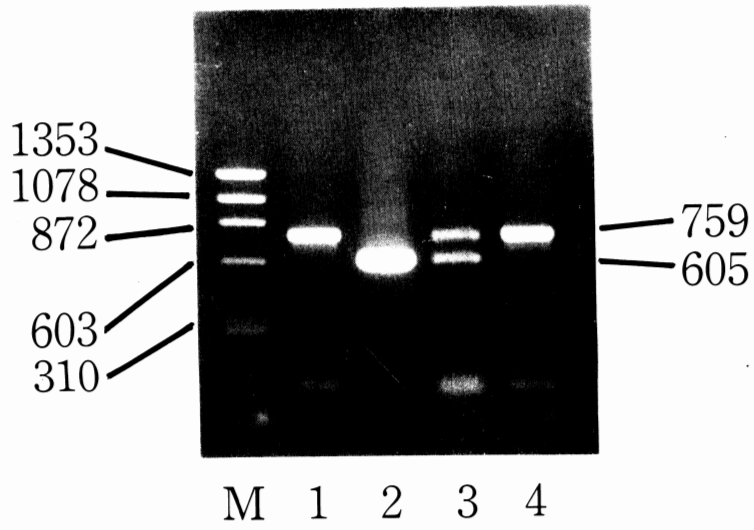

Fig. 3a Different molecular sizes of genomic SAG1 and competitor SAG1 after amplification by PCR. M is molecular size marker (øX174/*Hae* III digest). Lane 1 is pET-21b(+) SAG1. Lane 2 is competitor SAG1. Lane 3 is coamplified pET-21b(+) SAG1 and competitor SAG1. Lane 4 is genomic SAG1.

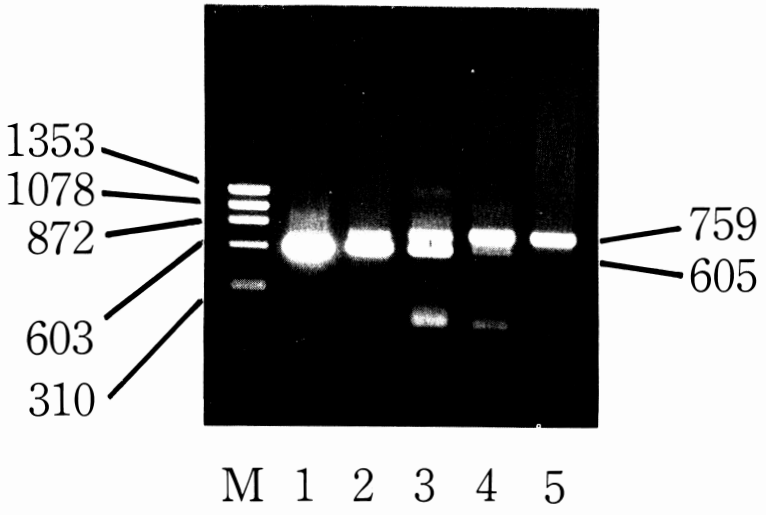

Fig. 3b T/C (template/competitor) ratio change after coamplification of different amounts of DNA of competitor SAG1 and a constant amount of pET-21b(+) SAG1 (7.5×10^{-4}). M is the molecular size marker (øX174/*Hae* III digest). The amounts of competitor SAG1 are 2.8×10^{-2} ng, 2.8×10^{-3} ng, 2.8×10^{-4} ng, 2.8×10^{-5} ng and 2.8×10^{-6} ng from lane 1 to 5, respectively.

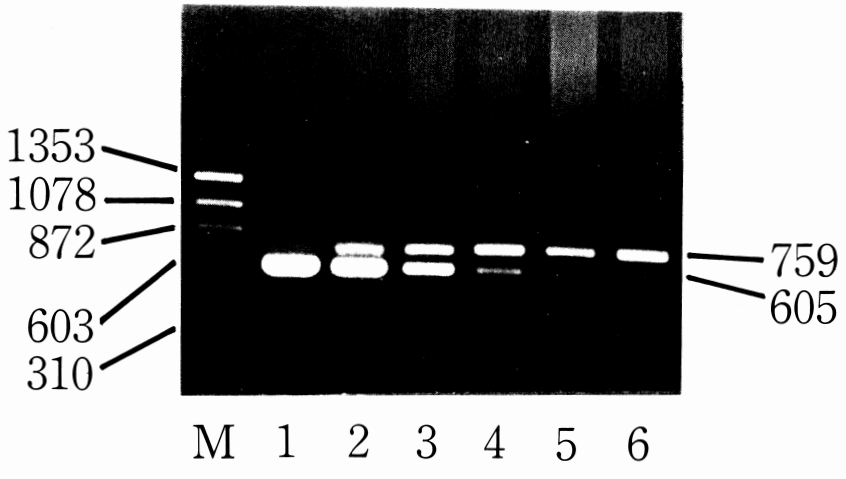

Fig. 4a T/C ratio change after coamplification of different amounts of DNA of competitor SAG1 and a constant amount of genomic DNA in 1×10^3 Fukaya tachyzoites. The amounts of competitor SAG1 are 5.6×10^{-1} ng, 5.6×10^{-2} ng, 5.6×10^{-3} ng, 5.6×10^{-4} ng, 5.6×10^{-5} ng and 5.6×10^{-6} ng from lane 1 to 6, respectively.

SAG1 decreased (Fig. 4a). Otherwise, when a constant concentration of the competitor SAG1 (5.6×10^{-5} ng) with different amounts of Fukaya genomic DNA (10^4, 10^3, 10^2, 10^1, 10^0 *T. gondii* tachyzoites) were coamplified, the intensities of PCR products of genomic SAG1 increased relative to *T. gondii* numbers while these of competitor SAG1 decreased gradually (Fig. 4b and 4c).

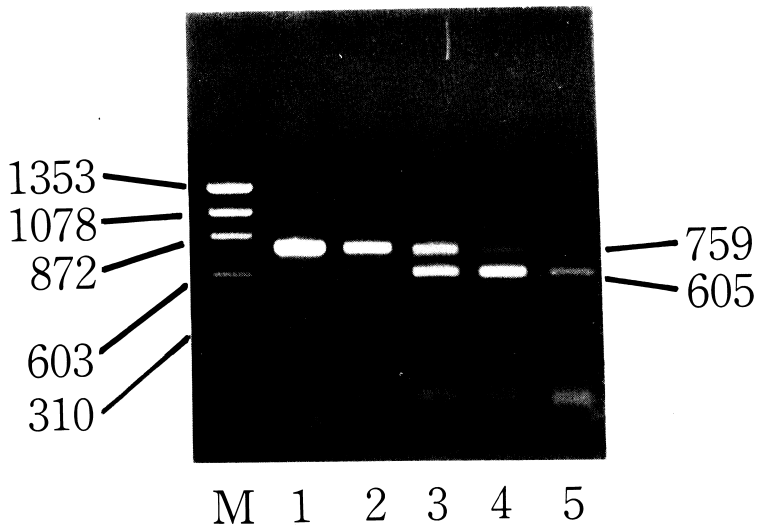

Fig. 4b T/C ratio change after coamplification of a constant amount of competitor SAG1 (5.6×10^{-5} ng) DNA and different amounts of Fukaya genomic DNA. The amounts of Fukaya genomic DNA were derived from 10^4, 10^3, 10^2, 10^1 and 10^0 tachyzoites in lanes 1 to 5 (intensities of bands of Fukaya genomic DNA/competitor SAG1 DNA are 1422/314, 1363/402, 622/516, 366/705, 310/717) respectively.

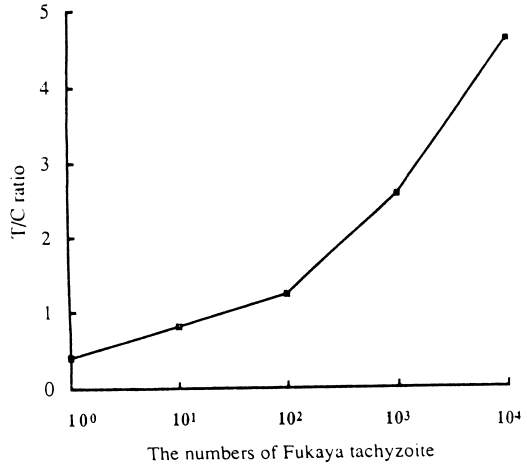

Fig. 4c The standard curve from Fig. 4b. The PCR products from Fig. 4b were electrophoresed. The intensities of the bands of PCR products were analyzed using a densitometer. The ratio of Fukaya genomic SAG1 to truncated SAG1 was calculated (T/C ratio). The Fukaya tachyzoites numbered 10^0 to 10^4.

Discussion

In recent years, PCR has been used intensively to detect *T. gondii* in AIDS (Burg *et al.*, 1988; Holliman *et al.*, 1990; Lebech *et al.*, 1992; Parmley *et al.*, 1992; Cristina *et al.*, 1993; Dupouy-Camet *et al.*, 1993; Filice *et al.*, 1993; Schoondermark-van de Ven *et al.*, 1993a), congenital toxoplasmosis (Grover *et al.*, 1990; Cazenave *et al.*, 1992; Schoondermark-Van de Ven *et al.*, 1993b; Hohlfeld *et al.*, 1994), cardiac transplant recipients (Holliman *et al.*, 1992) and ocular toxoplasmosis patients (Aouizerate *et al.*, 1993) using repetitive B1 genes (Schoondermark-Van de Ven *et al.*, 1991; Gross *et al.*, 1992; Parmley *et al.*, 1992; Filice *et al.*, 1993; Joss *et al.*, 1993; Schoondermark-van de Ven *et al.*, 1993a; Wastling *et al.*, 1993), the SAG1 gene (Savva *et al.*, 1990; Holliman *et al.*, 1992; Dupouy-Camet *et al.*, 1993; Wastling *et al.*, 1993) ribosomal DNA (Cazenave *et al.*, 1992; Aouizerate *et al.*, 1993; Guay *et al.*, 1993) small subunit ribosomal RNA (MacPherson and Gajadhar, 1993) and the TGR1E gene (Cristina *et al.*, 1993). However these conventional PCR meth-

ods could not be used to quantitate gene copy numbers because many parameters may affect the efficiency of amplification and its supersensitivity. Before establishing QC-PCR, conventional PCR was performed to determine how few *T. gondii* could be detected. As shown in Figure 2, our results illustrate that, in conventional PCR, only one copy of the SAG1 gene may be detected. This was as sensitive as the results of previously studies (Guay *et al.*, 1993; Joss *et al.*, 1993). There were, however, different band intensities of PCR products derived from amplification of the same amount of DNA representing the same number of *T. gondii* in the experiment. This difference in the quantity of PCR products may be resulted from inhibitory factors in the host genomic DNA specimen that could not be removed by proteinase K digestion or phenol/chloroform extraction. Previous studies showed that PCR based on SAG1 was not sensitive compared to that based on the B1 gene and gave more false negative results (Wastling *et al.*, 1993). In the present study, when the number of *T. gondii* exceeded 10, despite the difference in band intensities in conventional PCR products, no false negative was observed. Five negative results out of 16 samples in the PCR of one *T. gondii* may be caused by an inhibitor in host genomic DNA and this inhibition was obvious in the samples where the target DNA comprised a small fraction of total DNA (Abbott *et al.*, 1988).

To overcome the variability in the efficiency of PCR amplification, the competitor SAG1 (same as pET-21b(+) SAG1 except for the deletion of 154 bps (Fig. 3a)) was used as an internal control and competitor that binds competitively to the 2 oligos, DNA polymerase and dNTPs. The quantitation of *T. gondii* SAG1 by QC-PCR seems reasonable, as shown in Fig. 4b.

After replacing the pET-21b(+) SAG1 with the genomic DNA of *T. gondii* of the Fukaya strain, and with varying amounts of genomic DNA or DNA of the competitor SAG1, a good relationship was also observed between the band intensities of PCR products and the amount of genomic SAG1 or the truncated pET-21b(+) SAG1 DNA. By establishing a standard curve after measuring the band intensities of PCR products of both genomic and the competitor DNA using an IPLab Gel densitometer and calculating the ratio of the band intensities of the template DNA and the competitor DNA, the numbers of *T. gondii* could be determined.

Quantitation of *T. gondii* in tissues has been described by many researchers. Techniques have included limiting dilution of parasitic focus formation and direct microscopic counting of parasites from peritoneal lavage and brain tissue, however, these estimation methods of *T. gondii* seem unreliable because *T. gondii* is an intracellular protozoan. Thus, the present method of quantitating *T. gondii* with QC-PCR may help detection both cell free tachyzoites and intracellular *T. gondii*.

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