Available online at www.sciencedirect.com
ScienceDirect
Journal of Biotechnology 128 (2007) 41–49
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

A proportional analysis method using non-kinetic real-time PCR

Tetsushi Suyama *, Nana Matsuura, Takahiro Kanagawa [1]

National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

Received 2 May 2006; received in revised form 7 September 2006; accepted 21 September 2006

Abstract

Prompted by increasing interest in proportional analysis of genetic types, we developed a simple assay technique for determining the ratio of a specific target gene in the total genes that can be amplified with the same PCR primer. The key feature of this method is that the following two tasks are performed in a single-tube real-time PCR system: task 1, PCR amplification of the total genes including the target using a labeled PCR primer, with concurrent monitoring of the total copy number of the PCR product; task 2, detection of the signal of the target gene at each cycle of amplification, using a labeled nucleotide probe. In principle, the ratio of the target gene to the total genes is represented by the signal detected in 'task 2' at the cycle in which the PCR product reached a prescribed copy number (assessed by 'task 1').

Keywords: Real-time PCR; Ratio analysis; Fluorescence quenching; Quenching probe or primer; QProbe- or QPrimer-PCR; PD-PCR

1. Introduction

The variation ratios among genes with similar sequences contained in a nucleotide sample are currently estimated by two methodologies: quantification of each component in the mixture using a real-time PCR method (Higuchi et al., 1993; Heid et al., 1996; Wittwer et al., 1997; Kurata et al., 2001); and enumeration of the components in the mixture after PCR amplification using a 'non-real-time' method, e.g., quantitative dot blot hybridization, restriction fragment length polymorphism analysis, denaturing gradient gel electrophoresis, etc. (Saiki et al., 1986; Theophilus et al., 1989; Liu et al., 1997). Real-time PCR uses equipment that can fluorescently monitor the amount of product

Abbreviations: BODIPY FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid; bps, base pairs; dATP, 2′-deoxyadenosine-5′-triphosphate; dCTP, 2′-deoxycytidine-5′-triphosphate; dGTP, 2′-deoxyguanosine-5′-triphosphate; DNA, deoxyribonucleic acid; dUTP, 2′-deoxyuridine-5′-triphosphate; Mbps, $10^6 \times$ bps; PCR, polymerase chain reaction; PD-PCR, proportion-deductive PCR; QPrimer, quenching primer; QProbe, quenching probe; R_G and R_R, quenching ratio (%) of green and red fluorescence, respectively; $R_{G,T\%}$, the R_G value at the cycle in which the R_R reached T%; R.S.Ds., relative standard deviations; rRNA, ribosomal ribonucleic acid; TAMRA, tetramethylrhodamine; templates A and B, model templates for this study

* Corresponding author. Tel.: +81 298 61 6026; fax: +81 298 61 6400.
E-mail address: t.suyama@aist.go.jp (T. Suyama).
[1] Present address: Faculty of Bioenvironmental Science, Kyoto Gakuen University, 1-1 Nanjo, Sogabe, Kameoka, Kyoto 621-8555, Japan.

0168-1656/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2006.09.022

during each PCR cycle, and the initial copy number of the template is calibrated versus the PCR kinetics, using the cycle number at which the PCR product reaches a prescribed amount. Kinetic real-time PCR is a very useful technique for its wide dynamic range of calibration. However, this method requires two separate quantifications (one for the target and another for the total) for a single assay of genetic ratio, using a separate calibration curve for each quantification employing specific external standards. Moreover, the efficiency of PCR amplification is easily influenced by all components of the assay system, and the fluctuation in PCR efficiency often causes large errors in quantification (Heid et al., 1996). 'Non-real-time' methods require differentiation, identification, and quantification of the target gene in PCR products, and in most cases, these processes are time-consuming and require special skills.

A 16S rRNA gene is the most frequently used target for microbial quantification and community analysis (Zhang and Fang, 2006). This target is present in all bacteria, and its sequence contains conserved and variable regions between different taxonomy levels. The universal primers or probes for amplifying or detecting a certain range of bacteria (e.g., all bacteria, specific phylum, or specific genus) can target the conserved regions, and the primers or probes for determining a specific group of bacteria (e.g., specific genus, species, or strain) can target the variable regions of the 16S rRNA gene sequences. 16S rRNA-based community analysis has been utilized for various purposes, including quality control and improvement of food fermentation (Giraffa, 2004); health-control of gastrointestinal tract in humans (Vaughan et al., 2005) and farm animals (Castillo et al., 2006); water and marine product hygiene (Thompson et al., 2004; Wullings and van der Kooij, 2006); diagnosis at medical facilities (Horz et al., 2005; Sakamoto et al., 2004); and characterization of microbial consortia which have degrading activity for specific chemical compounds (Lee et al., 2005; Limpiyakorn et al., 2005; Liu et al., 2006; Nakatsu et al., 2005; Yu et al., 2005). Most such work has been conducted using the kinetic real-time PCR or 'non-real-time' methods referred to above.

Here, we introduce a new simple method for estimating the ratio of a target DNA to the total (designated proportion-deductive PCR, or PD-PCR) that is applicable for ratio analysis of 16S rRNA genes. When a mixture of templates is amplified with the same primer, the ratio of the product from each component in the mixture reflects the initial ratio of the component. No matter how many copies of initial templates are contained in unknown DNA samples, the initial ratio of a specific template can be estimated by measuring the copy number of its product at the cycle in which the total PCR product is increased to a prescribed copy number. This method does not involve a kinetic comparison, and the quantification is not in principle influenced by the PCR efficiency.

We used a model system composed of two templates (templates A and B) and measured the ratio '(template A)/(templates A plus B)' for evaluating PD-PCR (Fig. 1). The templates A and B were PCR products of 16S rRNA genes with approx. 1500 bps amplified from bacteria (*Microlunatus phosphovorus* NM1 and *Escherichia coli* K12, respectively) phylogenetically distant from each other. Using a labeled primer as the indicator for total PCR product and a labeled probe as the indicator for the product from the specific template, PD-PCR can be performed using real-time PCR equipment. As the indicators, we used fluorescently labeled nucleotides whose fluorescence intensity quantitatively quenches under hybridization to the DNA with complementary sequences (Kurata et al., 2001). A nucleotide probe labeled with BODIPY FL (green fluorescence dye) was designed so that the probe would hybridize to a unique region of the plus strand of the template A sequence and report the abundance of the template A product by quenching of green fluorescence (QProbe, Fig. 1, step at 60 °C). Also, a pair of primers amplifying approx. 330 bps of the templates A and B, containing the target region of the QProbe in the template A, was designed. The reverse primer was labeled with TAMRA (red fluorescence dye), which reports the yield of total PCR product by quenching of red fluorescence (QPrimer, Fig. 1, step at 72 °C). Practically, the measurement of absolute copy numbers of the PCR products is not needed for estimation of the genetic ratio, but we used the calibration curves showing the relationships between the fluorescence data and the initial ratio of the templates. The ratio of the specific template is in principle determined by a single-tube reaction using only one calibration curve, and the experimental procedure can be conducted as easily as the usual real-time PCR assays.