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Efficient DNA ligation by selective heating of DNA ligase with a radio frequency alternating magnetic field

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ABSTRACT

We present a simple method for efficient DNA ligation utilizing the heat generation of ferromagnetic particles subjected to an ac magnetic field. We carry out the ligation of DNA fragments with cohesive ends using T4 DNA ligase immobilized on the surface of ferromagnetic particles. When a radio frequency alternating magnetic field is applied, ferromagnetic particles dissipate heat and DNA ligase on the particles is selectively heated up and activated with little influence on the annealing of DNA ends, as a result of which the ligation efficiency increases. We show that the ligation efficiency increases with an increase in the field amplitude.

1. Introduction

The joining of DNA fragments using DNA ligase is an essential process in gene cloning. One of the important parameters for performing DNA ligation efficiently is the temperature [1]. In the case of DNA strands with cohesive ends, ligation is generally performed at 12–16 °C since higher temperatures may reduce the ligation efficiency by melting annealed DNA ends [1–3]. The ligation of blunt-ended DNA is usually carried out at room temperature with a high concentration of T4 DNA ligase [1,4]. The optimal temperature of commonly used DNA ligase is around 37 °C and therefore the above ligation conditions are not optimal for the action of DNA ligase. Several attempts to increase the ligation efficiency by improving thermal conditions for the reaction have been reported. Lund, Duch and Pedersen [2] reported that a high enzyme activity and DNA annealing were balanced by constant temperature cycling, as a result of which the ligation efficiency was increased. Ranjan and Rajagopal [3] demonstrated that DNA fragments with 2-bp overhangs were ligated using T4 DNA ligase at room temperature with high efficiency when the DNA fragments were subjected to heating followed by flash freezing prior to the ligation reaction. It was recently reported that an enzyme immobilized on ferromagnetic particles was heated and activated under a radio frequency alternating magnetic field due to heat dissipation from the particles caused by magnetic hysteresis and eddy currents with no influence on the activity of free enzyme around the particles [5]. This suggests that DNA ligase can be activated utilizing the heat generation of ferromagnetic particles subjected to an ac magnetic field with little effect on the annealing of DNA ends. In this article, we carry out the ligation of DNA fragments with cohesive ends using T4 DNA ligase immobilized on ferromagnetic particles and show that the ligation efficiency is increased by applying an ac magnetic field. The basic concept of our method is illustrated in Fig. 1. DNA ligase is immobilized on the surface of ferromagnetic particles and a reaction solution containing the DNA ligase/ferromagnetic particle hybrids and DNA fragments to be ligated is set at low temperature suitable for the annealing of DNA ends. When a radio frequency alternating magnetic field is applied, the ferromagnetic particles generate heat caused by magnetic hysteresis and eddy currents [6–11] and DNA ligase on the particles is selectively heated up and activated. If we optimize the experimental conditions, the activation of DNA ligase can be carried out almost without melting annealed DNA ends. The present method is so simple that it can easily be combined with other methods for efficient DNA ligation including the addition of molecular crowding agents such as polyethylene glycol [12–14] or hexamine cobalt chloride [15].

2. Materials and method

2.1. Immobilization of T4 DNA ligase on ferromagnetic particles

We immobilized T4 DNA ligase (EC 6.5.1.1, Takara Bio Inc.) on ferromagnetic iron particles, the surface of which had not been modified with any molecules (Spherical Ferromagnetic Iron Powder, Catalog No. 19844-1, Polysciences Inc.). The average diameter of the particles measured by an optical microscope (TE2000-U, Nikon Corp.) was 1.1 ± 0.5 μm. The saturation magnetization, remanent magne-

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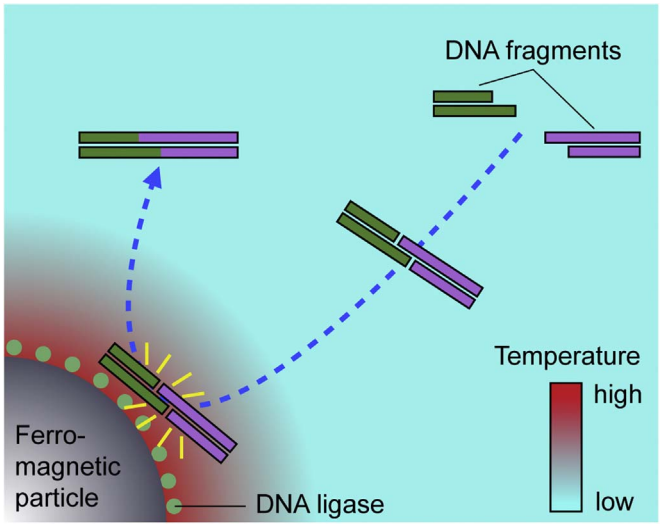

Fig. 1. DNA ligation utilizing heat generation from ferromagnetic particles under a radio frequency alternating magnetic field. DNA ligase is immobilized on the surface of ferromagnetic particles and a reaction solution containing the DNA ligase/ferromagnetic particle hybrids and DNA fragments to be ligated is set at low temperature suitable for the annealing of DNA ends. When a radio frequency alternating magnetic field is applied, the ferromagnetic particles dissipate heat caused by magnetic hysteresis and eddy currents and DNA ligase on the particles is selectively heated up and activated with little influence on DNA annealing, as a result of which the ligation efficiency increases.

tization and coercivity of the particles measured by a vibrating sample magnetometer (7407, LakeShore Cryotronics Inc.) were, respectively, 1.64 MA/m, 0.03 MA/m and 2.30 kA/m, where the remanent magnetization and coercivity were estimated from the hysteresis loop obtained when the maximal intensity of the external magnetic field was set at 1.6 MA/m. One mg of ferromagnetic particles was added to 1 mL of T4 DNA ligase aqueous solution (5.96 μg/mL) and the solution was left for 16 h at 4 °C. After collecting the ferromagnetic particles using a magnet, the supernatant was removed and the collected particles were redispersed in sterilized water. The above washing process was repeated three times to remove T4 DNA ligase molecules remaining in the solution, after which the volume fraction of the particles was set at 1.3×10^{-2}% adjusting the volume of the solvent (water). We estimated the total number of T4 DNA ligase molecules contained in the supernatants removed in the above washing procedure by the Bradford method, from which the average number of T4 DNA ligase molecules immobilized on each ferromagnetic particle was calculated to be about 8.6×10^4. Note that the concentration of T4 DNA ligase in the supernatant removed in the third washing treatment was reduced to an undetectable level, which means that there were hardly any free DNA ligase molecules in the solution after the washing procedure. The concentration of immobilized T4 DNA ligase in the particle solution calculated from the number density of particles, which corresponds to the volume fraction of particles divided by the volume of a particle, and the average number of T4 DNA ligase molecules immobilized on each particle was 1.60 μg/mL.

2.2. Preparation of DNA

DNA fragments of 1,114-bp were synthesized by PCR amplification using plasmid DNA (pUC19) purchased from Takara Bio Inc. (catalog No. 3219) as a template and primers, the base sequences of which were 5′-GCACTGCATAATTCTCTTAC-3′ and 5′-GGCTTTACACTTTATGCTTC-3′, and then isolated by agarose gel electrophoresis and phenol extraction. Note that the DNA fragments we synthesized had no phosphate group at their both ends since each primer used in the PCR had hydroxyl group instead of phosphate group at the 5′ end. The prepared DNA fragments were digested with

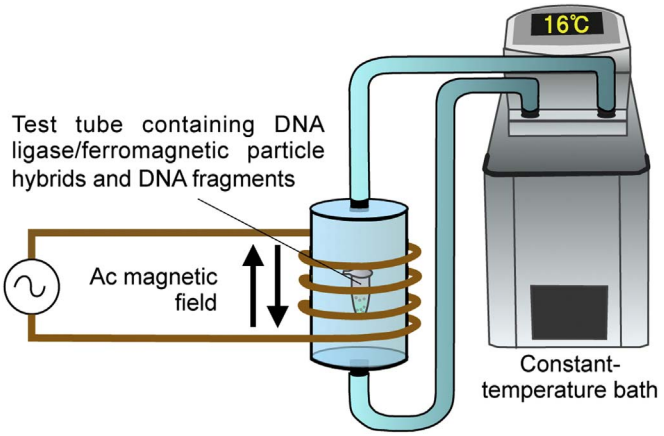

Fig. 2. Schematic representation of the experimental system. A test tube containing reaction solution for DNA ligation is placed in a cylindrical container filled with circulating water, the temperature of which is regulated at 16 °C, from a constant-temperature bath and DNA ligation reaction is carried out under an ac magnetic field generated by a coil and a radio frequency power supply for 10 min. The total amount of ligated DNA is estimated by agarose gel electrophoresis, from which the ligation efficiency is calculated.

restriction enzyme AatII (EC 3.1.21.4, Takara Bio Inc.) producing 611- and 499-bp DNA fragments with 4-bp overhangs and then the DNA was recovered by ethanol precipitation.

2.3. DNA ligation with T4 DNA ligase immobilized on ferromagnetic particles

We carried out the ligation of 611- and 499-bp DNA fragments with cohesive ends using T4 DNA ligase immobilized on ferromagnetic particles, where the ligation occurred through the cohesive ends created by the digestion since the other ends had no phosphate group as mentioned in the previous section. The outline of our experimental system is shown in Fig. 2. Five μL of T4 DNA ligase/ferromagnetic particle hybrid-dispersed solution, 2 μL of T4 DNA ligase buffer (Takara Bio Inc.), which consisted of 660 mM Tris-HCl (pH 7.6), 66 mM $MgCl_2$, 100 mM DTT and 1 mM ATP, 5 μL of aqueous solution containing 0.4 mM each of the DNA fragments, and 8 μL of sterilized water were mixed in a test tube, which was placed in a cylindrical container filled with circulating water, the temperature of which was regulated at 16 °C, from a constant-temperature bath (LTB-400, AS ONE CO.). Immediately after mixing the above solutions, an ac magnetic field of 0.34 MHz was applied to the mixture solution using a coil and radio frequency power supply (LI8310, Ameritherm Inc.). The amplitude of the ac magnetic field was changed from 15 to 30 kA/m. 10 min after the application of the ac magnetic field, the ligation reaction was terminated by heating the solution up to 75 °C for 30 min using an electric hot plate (NA-1, AS ONE CO.). The reaction solution was subjected to agarose gel electrophoresis and the total amount of ligated DNA was estimated using ImageQuant LAS 4000 (GE Healthcare Life Sciences), from which the ligation efficiency was calculated (see Supplementary material for details).

3. Results and discussion

We carried out DNA ligation using free and immobilized T4 DNA ligase in the absence of a magnetic field at 16 °C, where the enzyme concentration (final concentration after mixing the solutions for the reaction) in both cases was 0.40 μg/mL, and found that the total amounts of ligated DNA in the cases of free and immobilized T4 DNA ligase were, respectively, 75 ± 11 and 82 ± 4 fmol. This suggests that DNA ligase was still active after having been immobilized on the ferromagnetic particles although the ligation efficiency was slightly decreased compared to that in the case of free DNA ligase. The surface

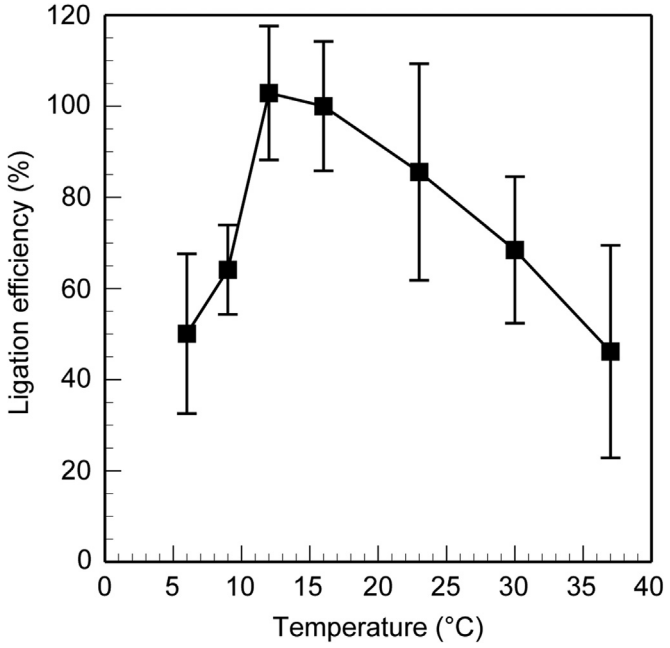

Fig. 3. Dependence of the efficiency of DNA ligation using T4 DNA ligase immobilized on ferromagnetic particles in the absence of a magnetic field on the ambient temperature. The ordinate axis represents the ligation efficiency, which is normalized by that at 16 °C. The standard deviations are obtained from 6 independent experiments.

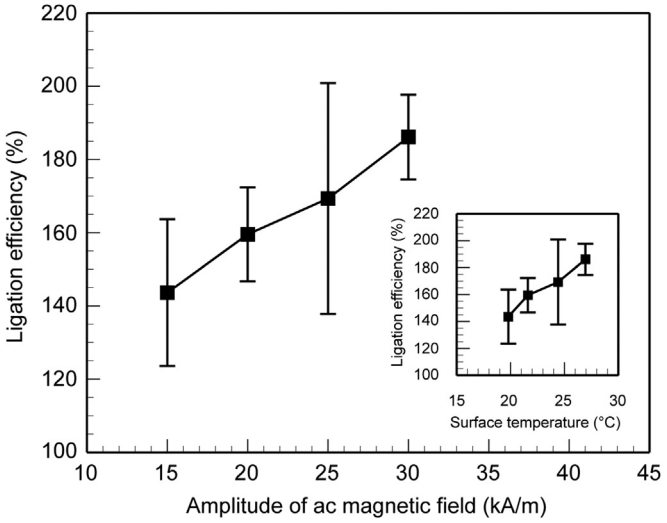

Fig. 4. Dependence of the efficiency of DNA ligation using T4 DNA ligase immobilized on ferromagnetic particles under an ac magnetic field of 0.34 MHz on the amplitude of the magnetic field. The ambient temperature is 16 °C. The ordinate axis represents the ligation efficiency under an ac magnetic field, which is normalized by that in the absence of a magnetic field. The inset shows the ligation efficiency under the ac magnetic field as a function of the average surface temperature of ferromagnetic particles, noting that the surface temperature increases with an increase in the field amplitude. The standard deviations are obtained from 6 independent experiments.

of iron particles used in the present study was not modified with any molecules and the particles were negatively charged in water. Therefore it is supposed that some domains in T4 DNA ligase were positively charged and DNA ligase molecules were attached to the particles by electrostatic force as in the case of the attachment of α-amylase, chitinase and lipase to magnetic particles [5,16,17]. We next carried out DNA ligation with free T4 DNA ligase under an ac magnetic field of 0.34 MHz and confirmed that there was no appreciable effect of the magnetic field on the ligation efficiency even in the case of the maximal field amplitude in the present study (30 kA/m).

The dependence of the efficiency of DNA ligation using T4 DNA ligase immobilized on ferromagnetic particles in the absence of a magnetic field on the ambient temperature is shown in Fig. 3, where the ordinate axis represents the ligation efficiency normalized by that at 16 °C. Note that the total amount of DNA ligated at 16 °C was 75 fmol. The ligation efficiency reached maximum in the temperature range of 12–16 °C. The activity of T4 DNA ligase increases with an increase in the temperature up to its optimal temperature (37 °C). However, higher temperatures dissociate DNA fragments joined by base pairing at their overhanging ends, which decreases the ligation efficiency. The dependence of the ligation efficiency on the temperature shown in Fig. 3 is the result of competition between the above two factors. When the ac magnetic field was applied, DNA ligase immobilized on the ferromagnetic particles was selectively heated caused by heat dissipation from the particles and therefore the ligation efficiency increased. Fig. 4 shows the dependence of the efficiency of DNA ligation using T4 DNA ligase immobilized on ferromagnetic particles under an ac magnetic field of 0.34 MHz at 16 °C on the amplitude of the magnetic field, where the ordinate axis represents the ligation efficiency normalized by that in the absence of a magnetic field. Note that the total amount of DNA ligated in the absence of a magnetic field was 75 fmol. The ligation efficiency was increased with an increase in the field amplitude. In the absence of a magnetic field, the efficiency of DNA ligation using DNA ligase immobilized on the ferromagnetic particles was slightly lower than that using free DNA ligase as mentioned above. However, the rate of increase in the efficiency of DNA ligation using immobilized DNA ligase in the presence of the ac magnetic field was much higher than the rate of decrease in the efficiency caused by the immobilization. In our previous study [5], we investigated the heat generation from the same ferromagnetic iron particles under an ac magnetic field and it is estimated that the average surface temperature of the particles in the present case increases from 20 to 27 °C as the field amplitude is increased from 15 to 30 kA/m. The inset in Fig. 4 shows the ligation efficiency under the ac magnetic field as a function of the average surface temperature of ferromagnetic particles.

Although the ferromagnetic particles and T4 DNA ligase on the particles were heated by the ac magnetic field, the average temperature of the reaction solution as a whole was kept suitably low for the annealing of DNA ends (16 °C). Indeed, no appreciable increase in the temperature of the reaction solution was detected since the volume fraction of the heat-dissipating ferromagnetic particles in the solution (final volume fraction after mixing the solutions for the reaction) was as low as 3.2×10^{-3}%. We analyzed heat transfer between ferromagnetic particles and the solvent fluid surrounding the particles in order to understand the thermal conditions in the present experiment in more detail. Since the volume fraction of the particles was very low in the present experiment, the particles can be assumed to be thermally isolated from each other. The steady-state temperature distribution around a spherical particle obtained by solving the heat conduction equation is:

$$T = \frac{a}{r}(T_s - T_f) + T_f \tag{1}$$

where r, a, T_s, and T_f are, respectively, the distance from the center of a particle, the radius of the particle, the surface temperature of the particle and the ambient temperature, which corresponds to the average temperature of the reaction solution (16 °C) in the present experiment. The volume fraction of regions around particles, the temperature of which is higher than a certain threshold, T_c, obtained from Eq. (1) is given by

$$\phi = \phi_p \left\{ \left(\frac{T_s - T_f}{T_c - T_f} \right)^3 - 1 \right\} \tag{2}$$

where ϕ_p is the volume fraction of particles. When the amplitude of the

ac magnetic field was 30 kA/m, which corresponds to the maximum field amplitude in the present study, the particle's surface temperature estimated above was 27 °C, in which case, the volume fraction of the regions around the particles heated to 17 °C or higher (volume fraction of fluid regions where the temperature increase is higher than 1 °C) calculated by Eq. (2) is as low as 4.2% and therefore we suppose that the annealing of DNA ends was efficiently carried out almost without being affected by heat dissipation from the magnetic particles. However, in the present case, phosphodiester bonds between DNA fragments are formed by DNA ligase immobilized on the surface of magnetic particles and therefore annealed DNA fragments have to travel through the higher temperature region to the particle's surfaces (see Fig. 1). Now we compare DNA ligation under an ac magnetic field and that in the absence of a magnetic field, noting that the temperature of small regions including magnetic particles is locally increased under an ac magnetic field at the ambient temperature of 16 °C in the former case, whereas the temperature is uniform throughout the reaction solution in the latter case. When the surface temperature of magnetic particles in the former case is equal to the ambient temperature in the latter case, the activities of DNA ligase in those cases are equal to each other since DNA ligase is immobilized on the surface of magnetic particles. Comparing the ligation efficiency under the ac magnetic field as a function of the particle's surface temperature (inset in Fig. 4) and the temperature dependence of the ligation efficiency in the absence of a magnetic field (Fig. 3), we found that the ligation efficiency under the ac magnetic field was always higher than that in the absence of a magnetic field at the same temperature. The ligation efficiency under the ac magnetic field increased with an increase in the particle's surface temperature, while the ligation efficiency under a zero magnetic field in the same temperature range (20–27 °C) decreased with an increase in the ambient temperature. Therefore the difference between the ligation efficiencies in those cases increased with an increase in the temperature. The present result clearly shows that DNA ligase immobilized on ferromagnetic particles is activated by heating with an ac magnetic field having much less effect on the annealing of DNA ends than the case of uniform heating. If the field amplitude is increased further, although the activity of DNA ligase may increase, the effect of heat dissipation from ferromagnetic particles on DNA annealing may become more significant at the same time and therefore, it is supposed that the ligation efficiency may reach the maximum at a higher field amplitude.

In summary, we carried out the ligation of DNA fragments with cohesive ends using T4 DNA ligase immobilized on ferromagnetic particles and found that the ligation efficiency was increased under a radio frequency alternating magnetic field caused by heat generation from the particles. In the present method, DNA ligase is immobilized on ferromagnetic particles and therefore, if DNA ligation is carried out under moderate temperature conditions, DNA ligase on particles after the reaction may be recovered using a magnet and reused. Note that the inactivation temperature of T4 DNA ligase has been experimentally estimated to be 38 °C, where the inactivation temperature is defined as the temperature at which the concentration of active enzyme becomes half of the total enzyme concentration [18]. In the present case, although T4 DNA ligase immobilized on the ferromagnetic particles was heated under the ac magnetic field, the particle's surface temperature, ranging from 20 to 27 °C, was much lower than the above inactivation temperature since the ambient temperature was as low as 16 °C. We will be analyzing the reusability of T4 DNA ligase immobilized on ferromagnetic particles in detail. Furthermore, magnetic particles can be manipulated using an alternating magnetic field or a gradient magnetic field without any difficulty [19–23]. So if we use DNA ligase/ferromagnetic particle hybrids, DNA ligation can be carried out at a specific position in micro reactors or micro total analysis systems (μ-TASs). In the case of blunt-end ligation or the ligation of DNA fragments with 2-bp overhangs, the optimal experimental conditions may be different from those in the present case, that is, the ligation of DNA fragments with 4-bp overhangs. We will be systematically investigating the dependence of the ligation efficiency on the experimental parameters such as the amplitude and the frequency of the ac magnetic field, the number density of particles, and the ambient temperature for various types of ligations. We believe that our ligation method could be useful for efficient cell transformation. We will also be analyzing the transformation efficiency of recombinant DNA prepared with the present ligation method.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.10.006.

Appendix B. Supplementary material

Supplementary material associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.10.006.

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