 

# Asymmetric PCR Using the Primers Anchored on the Surface of Magnetic Nanoparticles

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Asymmetric PCR is generally used to produce ssDNA, which could function as probes for detecting various kinds of genes. An asymmetric PCR technique based on magnetic nanoparticles (MNPs) has been developed in this work. One of the two amplification primers was bound to the MNP surface by modifying its 5′-end and leaving the 3′-end available for DNA polymerase activity, while the other was unbound. The results obtained showed that PCR could proceed successfully when the concentration ratio of the unbound primer to the MNP-bound primer was from 1:100 to 1:10. Plenty of target ssDNA connected with MNPs (MNP–ssDNA complexes) along with some dsDNA with one strand connected to MNPs could be generated. In virtue of their immobilization on the MNP surface, asymmetric PCR products could be easily isolated by the external magnetic field. This new surface asymmetric amplification process can be used not only to produce MNP–ssDNA complexes, but also to provide an alternative route to isolate products of asymmetry PCR.

In general, it is possible to produce plenty of target ssDNA by PCR when the molar amount of the two amplification primers is unequal. Such technique is called asymmetric PCR.[1–6] The principle is that only one primer takes part in the amplification reaction when the other primer added in limited amount has been used up, and then an excess of ssDNA will be produced. In initial cycles, the amplification product is dsDNA and the amount of dsDNA increases exponentially. After the limiting primer is exhausted, the product becomes ssDNA and follows a linear growth. In the end, the amount of ssDNA is 10–20 times more than that of dsDNA.[2] Nowadays, asymmetric PCR has become an important technique to generate ssDNA.[2,22]

The nanotechnology advance in life science has led to the rapid development of nano-biotechnology, which has been attracting much attention in the past few years for its extensive application in the fields of biology and medicine. For instance, surface-functionalized MNPs with superparamagnetism have been applied in protein and enzyme immobilization, immunoassay, RNA and DNA purification, cell isolation, and target drug.[7–10] Because the solid-phase DNA amplification can potentially be used in generating DNA chips for genomic study,[11–17] some groups have developed several kinds of solid phase DNA amplification-celine Adessi group,[18] Lockley and his co-worker[19] and the Sheila group[20] have tried PCR on the surface of glass, nylon, and Au nanoparticles, respectively. However, so far no work about PCR based on MNPs has been reported. If PCR could proceed on the surface of MNPs, a new method to produce DNA chips and to isolate PCR products can be established. Therefore, it is very significant to study PCR based on MNPs.

Recently, our group has studied symmetric PCR on the surface of nanoparticles in detail. Our results indicated that PCR could proceed when two amplification primers were anchored on the surface of nanoparticles. However, can asymmetric PCR proceed when only one of the two amplification primers is immobilized on the surface of nanoparticles? In this work, we studied the regularity of the asymmetric PCR proceeding on the MNP surface. One of the two amplification primers was anchored on the MNP surface, while the other was unbound. Then the two primers underwent PCR at different concentrations. The result indicates that DNA templates are able to hybridize to the unbound primer and the MNP-bound primer. Primers are elongated with the DNA polymerase to produce a copy of the hybridized template. One strand of this copy is covalently attached to the MNP surface to which the primer is originally attached. When the concentration of MNP-bound primer is higher than the unbound one, the amplified ssDNA is MNP–ssDNA. We can isolate and purify the newly synthesized DNA conveniently in an external magnetic field and the MNP–ssDNA complexes can be used as probes which will be widely applied in generating DNA chip, detecting DNA sequence and preparing gene missile.

## Experimental

**Materials.** Primers were designed with 5′-disulfide-modified. Their sequences were as follows:

Forward primer1 (F1): HO–$(CH_2)_6$–S–S–$(CH_2)_6$–O–$(PO_3)$-5′-TCT TTA TAG TCC TGT CGG GTT TCG<3′,

Reverse primer1 (R1): HO–$(CH_2)_6$–S–S–$(CH_2)_6$–O–$(PO_3)$-5′-TAT TGG GCG CTC TTC CGC TTC CTC<3′,

Forward primer2 (F2): HO–$(CH_2)_6$–S–S–$(CH_2)_6$–O–$(PO_3)$-5′-CGC TCT TCC GCT TCC TC<3′,

Reverse primer2 (R2): HO–$(CH_2)_6$–S–S–$(CH_2)_6$–O–$(PO_3)$-5′-AGC ACC GCC TAC ATA CCT C<3′.

All of these primers were synthesized by Sangon Biologic Engineering Technology and Service Co., Ltd (China). *Plasmid pBluescript SK*$^+$ (pSK) was used as a DNA template and was extracted from *Escherichia coli* according to the kit of Promega: Cat# A7270 Wizard® plus Maxipreps DNA Purification system Lot# 157324. All other reagents were of analytical grade commercially available.

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**Preparation of MNPs and Attachment of Primers to the MNP Surface.** MNPs preparation and primer immobilization on the MNP surface were essentially as described previously.[21]

**PCR on the MNP Surface.** The 30 μL mixture for PCR contained 1 × Reaction Buffer (50 M KCl, 10 mM Tris-HCl pH 9.0 at 25 °C, 0.1% TritonX-100, 1.5 mM $MgCl_2$), 200 μM each of four dNTPs, 1 μL of PSK, 1U of Taq DNA polymerase, and about 25 pmol of each of MNP-bound primers (MNP-F1 and MNP-R1). After 3 min of the initial denaturation at 94 °C, amplification was then carried out in 30 cycles of 50 s at 94 °C, 50 s at $T$ °C ($T$ = 52, 56, 60, or 65), 30 s at 72 °C. In the last cycle, the elongation step at 72 °C was prolonged to 4 min.

**Asymmetric PCR on the MNP Surface.** Except for one of the MNP-bound primers being replaced by 0.1–5 pmol unbound primer, the 30 μL mixture for asymmetric PCR contained all reaction components in the same amount as described above. After 3 min of the initial denaturation at 94 °C, amplification was then carried out in 45 cycles of 50 s at 94 °C, 50 s at 65 °C, and 30 s at 72 °C. In the last cycle, the elongation step at 72 °C was prolonged to 4 min.

**Agarose Gel Electrophoresis.** PCR products connecting with MNPs were separated magnetically from the amplification reaction mixture using a magnet and were washed three times with sterilized water to remove excess dNTPs, polymerase, buffer components, and unbound products. After the PCR products were resuspended in 30 μL sterilized water, 2-mercaptoethanol was added till the final concentration was 3% (v/v). The mixture was incubated at 37 °C for 8 h to cleave the S–S bonds between MNP and PCR products. After centrifuging at 4000 rpm for 5 min, the supernatant was analyzed by electrophoresis using 2% agarose gel in 1 × TBE buffer. After electrophoresis, the DNA bands were visualized through an UV transilluminator.

## Results and Discussion

**Preparation of MNPs and Attachment of Primers to the MNP Surface.** The monodispersed core–shell MNPs were prepared by coating nano-size $\gamma$-$Fe_2O_3$ with silica in reverse microemulsion.[21] The TEM image shown in Fig. 1 indicates that the average diameter of particles is about 50 nm and that the dispersion of round particles is uniform. After core–shell MNPs were coated with 3-mercaptopropyloxysilane (MPTS), 5′-disulfide-modified primers were bound to thiol-derivatised surface of MNPs via disulfide bonds.[21] The Raman spectrum shown in Fig. 2 confirmed that the primer had been anchored on the MNP surface. If one compares these results with the Raman spectra of the MNP surface modified with thiols (shown as Supporting Material 1), the peak of thiol at 2167 $cm^{-1}$ apparently decreases and the S–S peak at 440 $cm^{-1}$ occurs, as shown in Fig. 2. This indicates that a reaction between the thiols loaded on the surface of MNPs and the 5′-disulfide-modified oligoDNA has taken place by way of the thiol/disulfide exchange. The appearance of the skeleton vibration of deoxyribose at 964 $cm^{-1}$ and the stretching vibration of the $^{-}O$–P–$O^{-}$ at 802 $cm^{-1}$ also proves that the primer has been successfully anchored on the MNP surface. The structure of the core–shell MNPs connecting with the primer is shown in Fig. 3. The linker of 36 atoms between the primer and MNPs can improve the efficiency of the primer annealing to the template.[20]

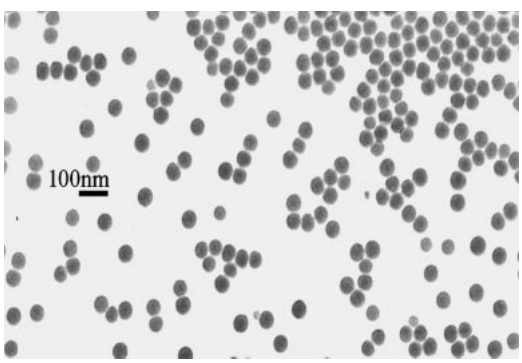


Fig. 1. TEM image of $\gamma$-$Fe_2O_3$ coated with silica.

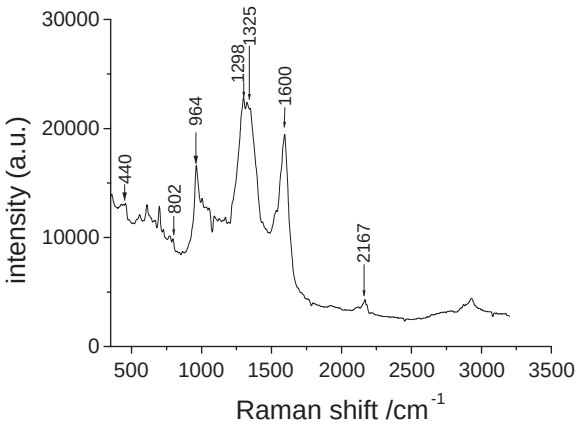


Fig. 2. Raman spectrum of the MNP surface modified with 5′-disulfide-modified primer.

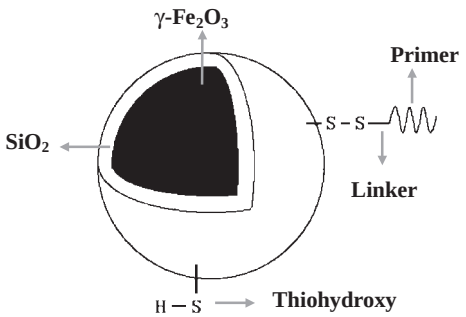


Fig. 3. The structure of core–shell MNPs connecting with primer.

**Agarose Gel Analysis of the PCR on the MNP Surface.** The expected dsDNA fragment of about 277 bp has been obtained by PCR using primer pair MNP-F1 and MNP-R1 at different annealing temperatures (Fig. 4). It is obvious that the bands become sharper with the increase of the annealing temperature. This indicates the nonspecific amplification product is decreasing and that the specificity of PCR product is increasing along with the increase of annealing temperature. We also can conclude that PCR could proceed on the MNP surface and that the annealing temperature affected the amplification: that is, the higher annealing temperature, the better the specificity of PCR product. The reason may be that, when primers were anchored on the MNP surface, their Brownian motion was restrained. At a lower annealing temperature (such as 52 °C or 56 °C), the MNP-bound primers could not hybridize with templates efficiently, which decreased the specificity of the prod-

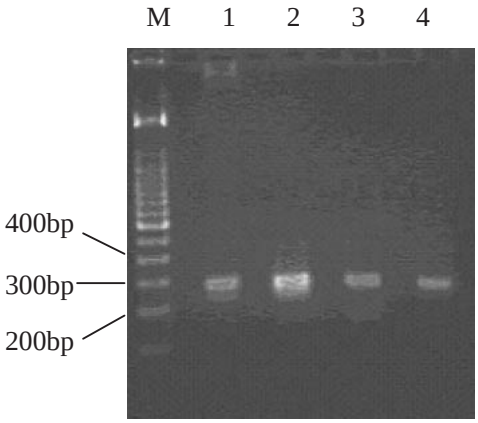


Fig. 4. Electrophoretic analysis of PCR using MNP-F1 and MNP-R1. M: 100 bp molecular size marker; Lanes 1–4: Annealing temperatures was 52 °C, 56 °C, 60 °C, and 65 °C, respectively.

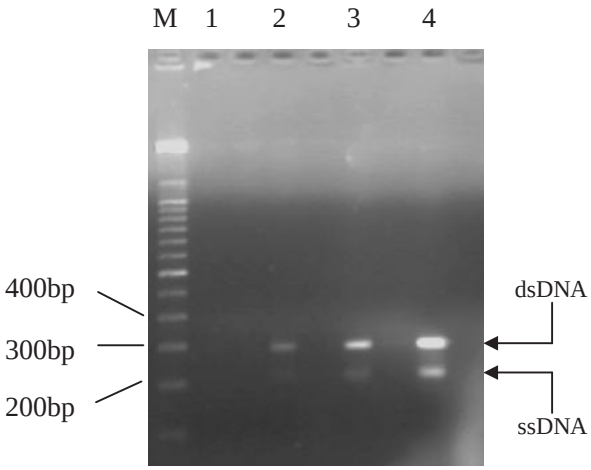


Fig. 5. Electrophoretic analysis of asymmetric PCR using MNP-F1 and R1. M: 100 bp molecular size marker; Lanes 1–4: The amount of MNP-F1 was about 25 pmol, the amount of R1 was 0.1, 0.25, 0.4, and 0.5 pmol, respectively.

ucts. So it is necessary to choose a higher annealing temperature (such as 60 °C or 65 °C) to increase the specificity of the PCR product. This rule could also be suitable for asymmetric PCR using one MNP-bound primer. Thus we chose 65 °C as annealing temperature in the following asymmetric PCR in this work.

**Agarose Gel Analysis of the Asymmetric PCR on the MNP Surface.** An asymmetric PCR was performed using forward primer F1 anchored on the MNP surface (MNP-F1) and reverse primer R1 (Fig. 5). The amount of MNP-F1 was about 25 pmol, and the amounts of R1 were 0.1, 0.25, 0.4, and 0.5 pmol, respectively, for the four reactions. Our result shows that the product of asymmetric PCR shown in Fig. 5 is different from that shown in Fig. 4. There are two bands in the lanes 2–4 of Fig. 5. The upper band is double stranded DNA target of about 277 bp. The lower one verified by sequencing is single stranded of target dsDNA (the data are shown as Supporting Material 2). Due to the efficiency of ethidium bromide staining of ssDNA being lower than that with dsDNA, the fluorescence intensity of the single-stranded band is weaker than that of dsDNA, though the amount of ssDNA is greater than that of dsDNA.[22] There is no band in lane 1. The reason may be that the products were not enough to be detected by electrophoresis when the amount R1 was too few. The band intensities in the lane 2–4 became stronger along with the increase of R1, which suggests that the amount of both dsDNA and ssDNA increases. The result shown in Fig. 5 demonstrates that asymmetric PCR can successfully proceed when the forward primer is anchored on the MNP surface and both dsDNA and ssDNA can be attained.

To further study the regularity of the asymmetric PCR based on MNPs, we also tested whether asymmetric PCR could proceed when the reverse primer was anchored on the MNP surface. Meanwhile, the scope of the concentration ratio of the unbound primer to the MNP-bound primer was enlarged (Fig. 6). The amount of MNP-R1 was about 25 pmol, and that of F1 was 0.1, 0.25, 0.4, 0.5, 1, and 5 pmol, respectively for the six reactions. In the lanes 2–4, the upper band is 277 bp target dsDNA and the lower one is the expected ssDNA product. It is obvious that the variation of the bands in lanes 1–4 is consistent with the one in Fig. 5. This suggests that asymmetric PCR could proceed in the same way when the reverse primer is anchored on the MNP surface. Enlarging the scope of the concentration ratio, the intensities of the two bands change differently. As the concentration of F1 primer increases, the intensity of the dsDNA band becomes much stronger. However, the band of ssDNA becomes stronger first, then becomes weaker till it fades away at last. This process indicates that the amount of dsDNA increased along with the increase of the limiting primer, but the amount of ssDNA increased firstly and decreased later. As the amount of ssDNA has become less than the minimum that could be detected by electrophoresis, the band of ssDNA does not appear any more. All the results shown in Fig. 5 and Fig. 6 demonstrate that the asymmetric PCR can successfully proceed when one of the two primers is anchored

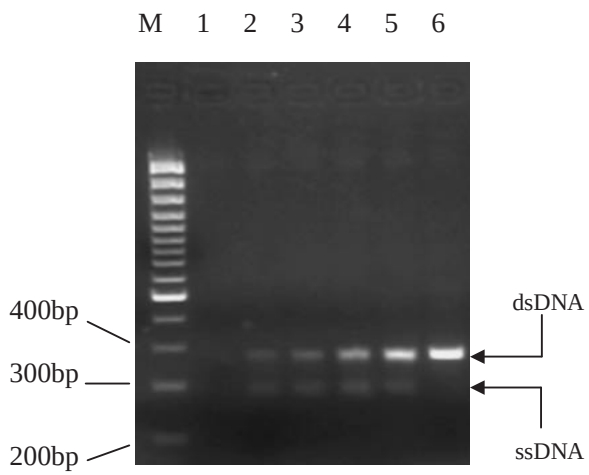


Fig. 6. Electrophoretic analysis of asymmetric PCR using MNP-R1 and F1. M: 100 bp molecular size marker; Lanes 1–6: The amount of MNP-R1 was about 25 pmol, the amount of F1 was 0.1, 0.25, 0.4, 0.5, 1, and 5 pmol, respectively.

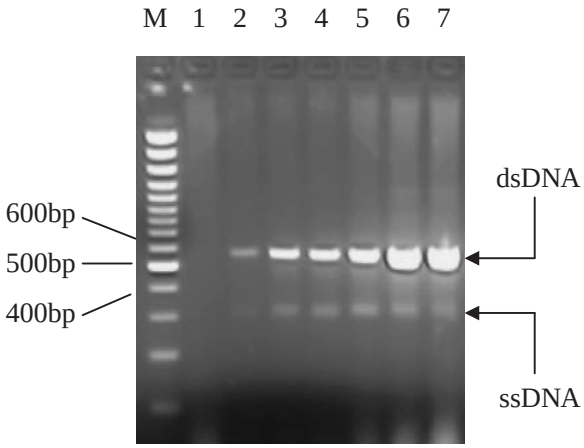


Fig. 7. Electrophoretic analysis of asymmetric PCR using MNP-F2 and R2. M: 100 bp molecular size marker; Lanes 1–7: The amount of MNP-F2 was about 25 pmol, the amount of R2 was 0.1, 0.25, 0.4, 0.5, 1, 2.5, and 5 pmol, respectively.

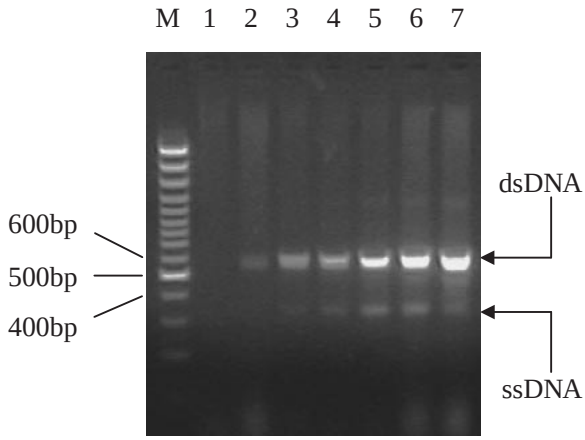


Fig. 8. Electrophoretic analysis of asymmetric PCR using MNP-R2 and F2. M: 100 bp molecular size marker; Lanes 1–7: The amount of MNP-R2 was about 25 pmol, the amount of F2 was 0.1, 0.25, 0.4, 0.5, 1, 2.5, and 5 pmol, respectively.

on the MNP surface. The amount of dsDNA increases gradually along with the limiting primer increasing, but the amount of ssDNA increases firstly and decreases later. The concentration ratio can be called an optimal concentration ratio when the product of ssDNA is the highest. The optimal ratio was 1:50 when primer F1 and R1 were used.

In order to confirm the regularity of asymmetric amplification based on MNPs, we used pSK as a template and we designed F2/R2 as primers. Figure 7 shows the result of asymmetric PCR using MNP-F2 and R2. The amount of MNP-F2 was about 25 pmol, and the amount of R2 was 0.1, 0.25, 0.4, 0.5, 1, 2.5, and 5 pmol, respectively for the seven PCR reactions. The upper band is 588 bp target dsDNA, and the lower band is corresponding to the anticipated ssDNA. There are no bands in the lane 1, which might suggest that the amount of amplification products were not enough to be detected by agarose gel. In the lanes 2–7, the intensity of the band of dsDNA is quite strong and the ssDNA band is firstly enhanced and later weakened along with gradually increase of R2. The amount of ssDNA was the most when the concentration ratio of the primer connecting with MNP to the limiting primer was about 1:25. The result of asymmetric PCR using MNP-R2 and F2 shown in Fig. 8 is consistent with the ones in Fig. 7. Results in Fig. 7 and Fig. 8 further verified the regularity of the asymmetric PCR based on MNPs: either of the two primers is anchored on the MNP surface, asymmetric PCR can successfully proceed. When the concentration ratio of the unbound primer to the primer anchored on the MNP surface is from 1:100 to 1:10, plenty of target ssDNA will be generated by asymmetric PCR, and the amount of ssDNA is the most at the optimal concentration ratio.

## Conclusion

A large amount of target MNP–ssDNA can be generated by an optimized asymmetric PCR when the primer anchored on the MNP surface used at the higher concentration. Due to different system of asymmetric PCR has different optimal concentration ratio, we must optimize the concentration ratio to obtain the target MNP–ssDNA by asymmetric PCR. On account of their immobilization on the surface of MNP, products of asymmetric PCR can be isolated by external magnetic field. This technique not only provided an efficient solution to produce MNP–ssDNA complexes more easily and quickly but also find a new method to isolate the asymmetric PCR products. The MNP–ssDNA complexes could be prepared as probes and widely used in genomic study.

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## Supporting Information

The Raman spectrum of the MNP surface modified with thiols and the sequencing result of ssDNA generated by the asymmetric PCR on the MNP surface were contained in the material. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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