# Improvement of the dot-blot-SNP technique for efficient and cost-effective genotyping



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# Improvement of the dot-blot-SNP technique for efficient and cost-effective genotyping

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## Abstract

**Although the dot-blot-SNP technique is a laborsaving, cost-effective method for SNP genotyping of a large number of plants, the synthesis of 5'-digoxigenin(DIG)-labeled oligonucleotides for use as probes is still costly. We developed two probe-labeling methods for this technique, one being digoxigenin labeling of oligonucleotides by PCR (PCR-DIG labeling) and the other being hybridization using a bridge probe and a 5'-DIG-labeled oligonucleotide (bridge hybridization). Bridge hybridization detected allele-specific signals under hybridization conditions similar to those for the 5'-DIG-labeled oligonucleotides and biotin-labeled oligonucleotides, while signals were detected only under a lower stringency condition by PCR-DIG labeling. As a method for genotyping using many markers at one time, two methods, i.e., PCR using mixed primer pairs and hybridization using mixed probes, were examined with successful results. Eighty-five SNP markers designed for genotyping of rice cultivars detected allele-specific signals, the genotyping results corresponding to the previously reported ones.**

**Keywords: SNP analysis, Dot-blotting, Large-scale genotyping, Cultivar identification**

## Introduction

Two methods, i.e., PCR-DIG-labeling and bridge hybridization (Fig. 1), were examined for detecting SNPs. 5'-biotin-labeled oligonucleotides were also used for comparison with 5'-DIG-labeled oligonucleotides. For preparation of the PCR-DIG-labeled oligonucleotide probe, a template oligonucleotide of 49 nt consisting of a 17-nt probe sequence inserted between 4-nt sequences (GGCC) recognized by *Hae*III, and 12-nt sequences for annealing of primers at both ends (5'-GATACAGTGTAC-3' and 5'-TGTCACAGTGAC-3') was synthesized. The 49-bp oligonucleotide was amplified and labeled by PCR with primers (5'-GATACAGTGTACGG-3' and 5'-GTCACTGTGACAGG-3') using PCR-DIG-labeling mix (Roche Diagnostics, Switzerland). The PCR product was cleaved by *Hae* III. After denaturation in boiling water, equal volumes of the cleaved products of the DIG-labeled oligonucleotide and the unlabeled oligonucleotide as a competitive probe without removal of both end sequences were added to hybridization buffer. For bridge hybridization, a 48-nt oligonucleotide consisting of a 17-nt sequence of one allele of an SNP marker, a 6-nt spacer sequence (TATATT), and a 25-nt bridge sequence, which is an arbitrary sequence (5'-ACGAAGCCTCTTAATTGCGAATGTA -3'), was prepared as a bridge probe. A 48-nt oligonucleotide having a 17-nt sequence of the other allele of the SNP marker, the 6-nt spacer sequence, and a 25-nt bridge sequence (5'-TGAAAAGAAGCTTCATCCTTCTCGT-3') was also prepared as a competitive probe. A 5'-DIG-labeled oligonucleotide having a sequence complementary to the 25-nt bridge sequence of the bridge probe (5'-TACATTCGCAATTAAGAGGCTTCGT-3' or 5'-ACGAGAAGGATGAAGCTTCTTTTCA-3') was added to hybridization buffer. Signals were detected as previously described (Shirasawa et al. 2006).

SNP marker S10844, whose 'Nipponbare'-type allele is contained in 'Nipponbare', 'Koshihikari', 'Akihikari', and 'Kasalath' and whose variant-type allele is contained in 'Hatsuboshi', 'Kihou', 'Hitomebore', and 'Kirara397' (Shirasawa et al. 2006), was used for evaluation of the labeling methods. Under the highest stringency condition tested, i.e., hybridization and washing with 0.1 × SSC/0.1% SDS at 50˚C, allele-specific signals were detected by 5'-DIG labeled oligonuceotides, but PCR-DIG-labeled oligonucleotides, bridge hybridization, and 5'-biotin-labeled oligonucleotides showed no signal (Fig. 2). On the other hand, non-specific signals were high in the analysis of the variant-type allele under the lowest stringency condition, i.e., hybridization and 1 × SSC/0.1% SDS washing at 40˚C, by 5'-DIG-labeled oligonucleotides and bridge hybridization. Allele-specific signals were detected by PCR-DIG-labeled oligonucleotides and 5'-biotin-labeled oligonucleotides under this hybridization condition. Under intermediate stringency conditions, i.e., hybridization and 0.1 × SSC/0.1% SDS washing at 40˚C or hybridization and 1 × SSC/0.1% SDS washing at 50˚C, allele-specific signals for both alleles were clearly detected by bridge hybridization and 5'-DIG-labeled oligonucleotides, and weakly detected by 5'-biotin-labeled oligonucleotides, but not detected by PCR-DIG-labeled oligonucleotides. Seven SNP markers, which are necessary for distinguishing all the 17 Japonica cultivars used in this study (Table 1), were prepared with these two labeling methods and biotin labeling. Although all the labeling methods detected allele-specific signals, the optimum hybridization conditions were different not only between the SNP markers but also between the labeling methods (Table 2). Bridge hybridization yielded higher signals than PCR-DIG-labeled oliginucleotides.

The dot-blot-SNP technique is suitable for genotyping of many plant individuals, but not for analyzing a small number of plants. For identification of cultivars using a few plants, analysis with multiple SNP markers is desirable. Using bridge

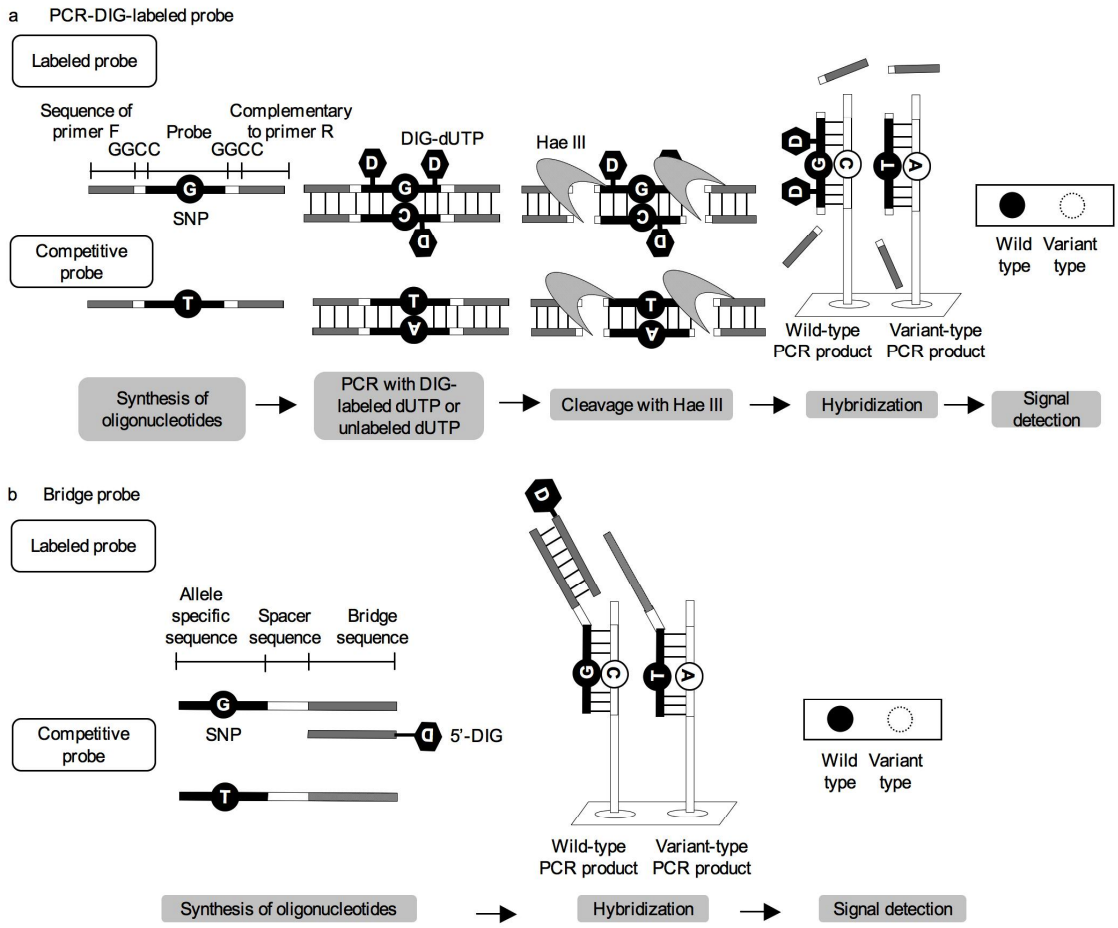


**Fig. 1.** Scheme of PCR-DIG labeling and bridge hybridization. a: PCR-DIG-labeling. Probe sequences were amplified and labeled by PCR with DIG-dUTP using an oligonucleotide as a template. After cleavage with *Hae*III, the DIG-labeled probe was hybridized with dot-blotted PCR products together with an unlabeled competitive probe. Black boxes indicate sequences used as probes, and white boxes and gray boxes show recognition sites of *Hae*III and sequences for primer annealing in PCR-DIG labeling, respectively. b: Bridge hybridization. A bridge probe having an allele-specific sequence (black box), a spacer sequence (white box) and a bridge sequence (gray box) was hybridized with dot-blotted PCR products together with a competitive bridge probe and a 5'-DIG-labeled oligonucleotide having a sequence complementary to the bridge sequence.

hybridization probes and 5'-biotin-labeled oligonucleotides, we tested two methods of dot-blot hybridization with multiple SNP markers, one being multiplex PCR followed by dot-blot-SNP analysis with different membranes for each probe and the other being dot-blotting of PCR products amplified with each primer pair onto two membranes followed by hybridization with mixtures of multiple probes. After multiplex PCR using the primer pairs to amplify 300-bp DNA fragments for the seven SNP markers shown in Table 2, bridge hybridization detected allele-specific signals in both alleles of S0651, R1744, E61310, C53863, and E2439, but no signal in E1919. Analysis of variant-type alleles in R2702 showed many false signals (Fig. 3). 5'-biotin-labeled oligonucleotide probes yielded similar results, but slightly lower signal intensities.

Using the other method, i.e., separate PCR and hybridization with multiple probes, allele-specific signals without non-specific signals were detected in four of the seven SNP markers in bridge hybridization (Fig. 4). Non-specific signals were high in analyses of the both alleles of S0651 and E2439 and the 'Nipponbare' allele of R2702. Raising the hybridization temperature enabled detection of allele-specific signals of these SNP markers, but such a hybridization condition reduced the allele-specific signals of R1744 and E61310. In hybridization with 5'-biotin-labeled oligonucleotide probes, intensities of allele-specific signals were lower than those in bridge hybridization, but non-specific signals were also low, enabling easier genotyping.

Oligonucleotides for 45 loci having SNPs between Japonica rice cultivars (Shirasawa et al. 2007) were prepared and labeled by the PCR-DIG-labeling method. For detection of allele-specific signals, hybridization and washing conditions were optimized (Supplementary Table 1). 5'-biotin-labeled oligonucleotides for 20 loci were also designed (Supplementary Table 2). Genotypes of 17 Japonica rice cultivars were analyzed by dot-blot-SNP using the 45 PCR-DIG-labeled oligonucleotides and the 20 5'-biotin-labeled oligonucleotides, the results corresponding to the genotyping by PCR-RF-SSCP reported by Shirasawa et al. (2007). Bridge probes for 20 SNP markers were also prepared for graphical genotyping of low-temperature tolerant lines developed by a cross between 'Hitomebore' and 'Lijiangxintuanheigu' (Supplementary Table 3), and all of them yielded allele-specific signals.

Although the most powerful tools for large-scale SNP analysis are considered to be the microarray techniques based on allele-specific hybridization (Hacia et al. 1998) or primer extension (Syvänen 2001, Shen et al. 2005), these techniques require costly

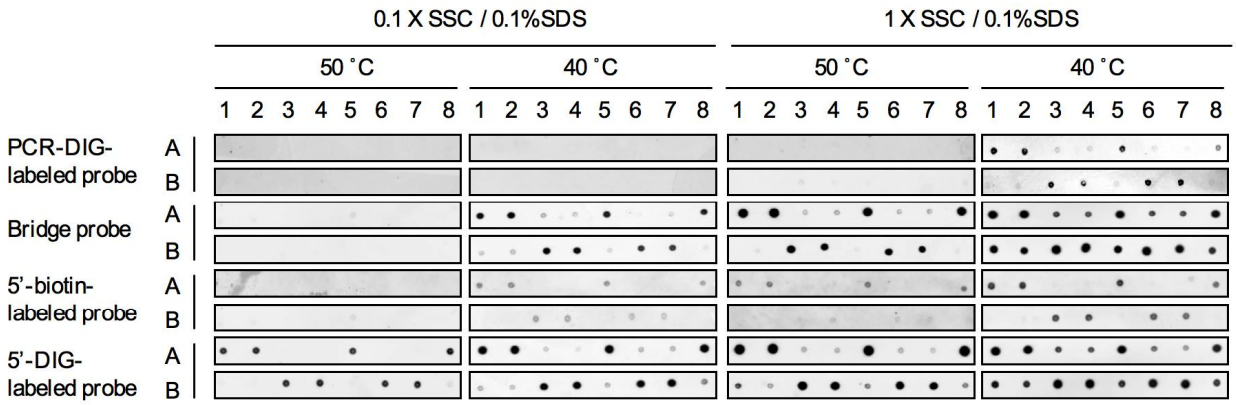


**Fig. 2**. Dot-blot-SNP analysis using the three types of oligonucleotide probes under different hybridization conditions. Used SNP marker was S10844 (probe sequences, CAGTAGAGCTGTGGAGA and CAGTAGAGTTGTGGAGA; primer sequences CTCTTCTACGCCAGGTTCCAA and GCACAGTAACCAGATCAACAG, Shirasawa et al. 2006). The probes were hybridized with PCR products of eight cultivars. A, 'Nipponbare'-type allele; B, variant-type allele. 1, 'Nipponbare'; 2, 'Koshihikari'; 3, 'Hatsuboshi'; 4, 'Kihou'; 5, 'Akihikari'; 6, 'Hitomebore'; 7, 'Kirara397'; 8, 'Kasalath'. Hybridization and washing were performed at 50˚C or 40˚C, and second washing was carried out with 0.1 × SSC/0.1% SDS or 1 × SSC/0.1% SDS. 5'-DIG-labeled oligonucleotides were used for control experiment.

Table 1 A set of SNP markers required for identification of the 17 cultivars

| Marker name | Chr | bp | 1. Nipponbare | 2. Akihikari | 3. Hatsuboshi | 4. Hatsunishiki | 5. Hinohikari | 6. Hitomebore | 7. Itadaki | 8. Kihou | 9. Kirara397 | 10. Koganebare | 11. Koshihikari | 12. Nourin 1 | 13. Nourin 6 | 14. Nourin 8 | 15. Nourin 22 | 16. Rikuu 132 | 17. Sasanishiki |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E1919 | 1 | 4905189 | A | B | B | B | A | B | B | A | B | A | B | B | A | A | A | B | B |
| S0651 | 4 | 24229543 | A | A | B | A | A | A | B | B | A | B | A | A | A | A | A | A | A |
| R1744 | 7 | 259886 | A | B | A | A | A | A | A | A | A | A | A | A | B | B | B | B | A |
| E61310 | 7 | 24283242 | A | B | B | B | B | B | A | A | A | A | B | B | B | A | B | B | A |
| C53863 | 7 | 27391821 | A | A | A | A | B | A | A | A | A | A | B | B | A | A | A | A | A |
| E2439 | 10 | 14680293 | A | A | A | B | A | A | B | A | B | A | A | B | A | A | A | B | B |
| R2702 | 11 | 30616183 | A | B | B | A | B | B | B | B | A | A | B | B | B | A | A | B | B |

A, 'Nipponbare'-type allele; B, variant-type allele.

special equipment, which is not available in most laboratories or small research institutes. Furthermore, these techniques are not suitable for genotyping of a small number of loci, less than 50, of a large number of plants, more than 1,000, which are used in conventional crossbreeding programs. Despite being a classical technique, the dot-blot-SNP technique (Shirasawa et al. 2006) may be the most laborsaving, cost-effective technique among various SNP assays for analysis of a large number of individuals. Although hybridization and signal detection in the dot-blot-SNP technique is somewhat laborious, the labor and time for each sample are small because more than 1,000 samples can be analyzed at one time. This technique requires no special equipment and is usable in any small laboratory. One problem with this technique, however, is the high cost for synthesizing the 5'-DIG-labeled oligonucleotides.

Although PCR-DIG-labeled oligonucleotides detected no signal under the same hybridization condition as the 5'-DIG-labeled oligonucleotides, optimizing the hybridization condition enabled detection of allele-specific signals. Oligonucleotides for PCR-DIG-labeled probes can be prepared at low cost, but the DIG-labeling process entails some labor. Bridge hybridization was found to be nearly as sensitive as direct hybridization of 5'-DIG-labeled oligonucleotides. Bridge hybridization is a complicated technique using three oligonucleotides for hybridization, i.e., a bridge probe having the sequence of an allele to be detected, a bridge probe having the sequence of the other allele as a competitive probe, and the 5'-DIG-labeled oligonucleotide that hybridizes only with the former bridge probe. However, one pair of 5'-DIG-labeled oligonucleotides can be applied to any SNP markers. Preparation of only a pair of unlabeled 48-nt oligonucleotides is required for producing a new SNP marker. Bridge hybridization is considered to be the most cost-effective and laborsaving method among the four labeling methods tested when analyzing SNPs at many loci.

Both multiplex PCR followed by hybridization with different oligonucleotide probes and separate PCR followed by hybridization

Table 2 Nucleotide sequences and optimum hybridization conditions of the seven SNP markers used for identification of the 17 rice cultivars

| Marker name | Forward primer sequence (5'-3') / Reverse primer sequence (5'-3') | Probe sequence of 'Nipponbare' type (5'-3') / Probe sequence of variant type (5'-3') | 5'-biotin-labeling | | | PCR-DIG-labeling | | | Bridge hybridization | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Temp.[a] | Washing buffer | Ratio[b] | Temp. | Washing buffer | Ratio | Temp. | Washing buffer | Ratio |
| E1919 | AGCTGCTCGAAGTGAAGGAG | GTCATCCCaATTCCAGT | 50 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 50 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| | GCTTCAAACTTTATCGGAAC | GTCATCCCgATTCCAGT | 50 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 50 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| S0651 | GCCTGGGGAATCAAGAAAAT | TATTGTGCcGGTGTTGA | 50 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 50 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| | AAACAATGACCAGGTACTGG | TATTGTGCtGGTGTTGA | 50 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 50 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| R1744 | CGCGTGGAAAATGAGAGAGT | GTACATACaTTGTGTAT | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:1 | 40 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| | TGAGATAAATAGGATCGACC | GTACATACgTTGTGTAT | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:1 | 40 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| E61310 | CTGTAGCGTCGTTAAGCACG | TAAATGATaGAAATTCT | 30 | 0.1 x SSC/ 0.1%SDS | 1:1 | 30 | 1 x SSC/ 0.1%SDS | 1:5 | 30 | 0.1 x SSC/ 0.1%SDS | 1:1 |
| | ACCAGAAAAGCGCACAAAGT | TAAATGATgGAAATTCT | 30 | 0.1 x SSC/ 0.1%SDS | 1:1 | 30 | 1 x SSC/ 0.1%SDS | 1:1 | 40 | 0.1 x SSC/ 0.1%SDS | 1:10 |
| C53863 | GAGCTGTTCACGCTCACGTA | GGTGTATTaCAGCGATT | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 0.5 x SSC/ 0.1%SDS | 1:5 |
| | TGTCGCAGTGTAAAATCTGC | GGTGTATTtCAGCGATT | 40 | 1 x SSC/ 0.1%SDS | 1:5 | 40 | 1 x SSC/ 0.1%SDS | 1:1 | 50 | 0.5 x SSC/ 0.1%SDS | 1:10 |
| E2439 | TCGGAGCTCCAGTTCCGAGC | AGGGCTTTcGCCTCGGC | 50 | 0.1 x SSC/ 0.1%SDS | 1:5 | 40 | 0.1 x SSC/ 0.1%SDS | 1:1 | 50 | 0.1 x SSC/ 0.1%SDS | 1:5c |
| | GCCATGGTGTGTTTGCTCTA | AGGGCTTTgGCCTCGGC | 50 | 0.1 x SSC/ 0.1%SDS | 1:5 | 50 | 1 x SSC/ 0.1%SDS | 1:1 | 50 | 0.1 x SSC/ 0.1%SDS | 1:5c |
| R2702 | AGGATTTCTTCAACGGCA | CTGCTGGGgAAGTTTGA | 50 | 1 x SSC/ 0.1%SDS | 1:10 | 50 | 1 x SSC/ 0.1%SDS | 1:1 | 50 | 0.5 x SSC/ 0.1%SDS | 1:5 |
| | TTCAGGATACCATTGGCATC | CTGCTGGGcAAGTTTGA | 50 | 1 x SSC/ 0.1%SDS | 1:10 | 50 | 1 x SSC/ 0.1%SDS | 1:5 | 50 | 0.5x SSC/ 0.1%SDS | 1:5 |

a: Hybridization and washing temperature, b: Ratio of a labeled probe to an unlabeled competitive probe, c: Long bridge sequences (25 nt) were used for this marker

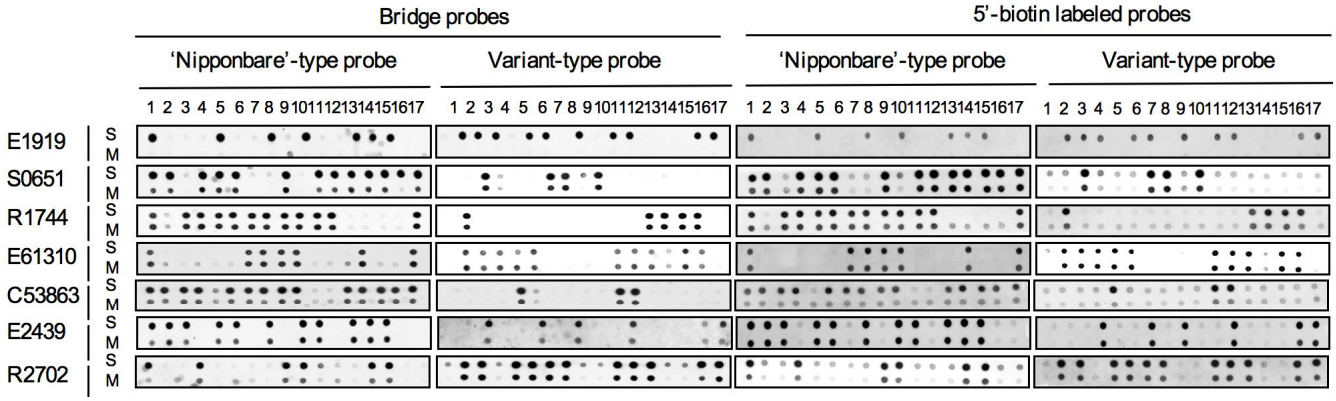


**Fig. 3**. Dot-blot-SNP analysis using multiplex PCR. DNA fragments of the 17 cultivars (1 to 17, Table 1) were amplified by PCR with a single primer pair (S) or by multiplex PCR with a mixture of the seven primer pairs (M) for E1919, S0651, R1744, E61310, C53863, E2439, and R2702. Both bridge hybridization markers and 5'-biotin-labeled oligonucleotides were used.

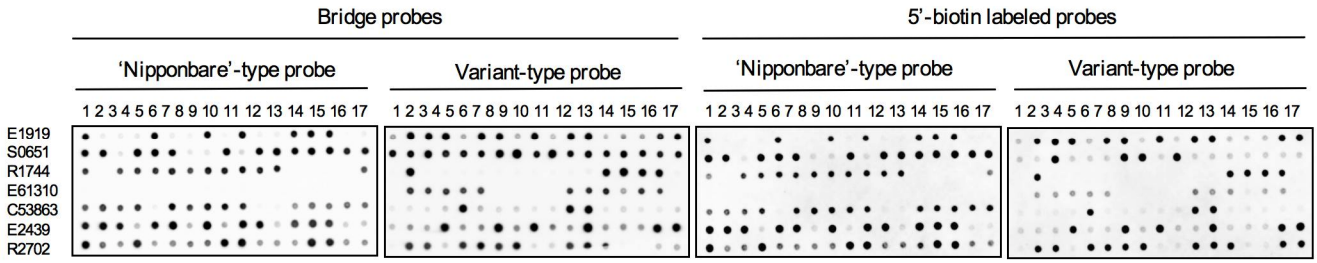


**Fig. 4**. Genotyping of the 17 cultivars using mixed probes of dot-blot-SNP markers. 'Nipponbare'-type probes of seven SNP markers, i.e., E1919, S0651, R1744, E61310, C53863, E2439, and R2702, were mixed and hybridized separately with variant-type probe mixture. The 17 cultivars are shown as '1' to '17' (Table 1).

with multiple probes detected allele-specific signals and were found to be useful techniques for analysis of a small number of samples with many SNP markers. In multiplex PCR using seven primer pairs, five markers detected allele-specific signals by both bridge hybridization and 5'-biotin labeling. The inability to detect allele-specific signals is considered to be due to the low ability of some primer pairs to amplify the target sequences under the thermal cycling condition used for multiplex PCR. Hybridization with mixed probes also yielded allele-specific signals with four SNP markers, while three markers detected non-specific background signals. Further addition of the competitive oligonucleotides may reduce the background signals. Since optimum hybridization conditions are different between probes, choosing SNP markers having similar hybridization conditions would yield better results in hybridization with multiple probes.

The remaining laborious step of dot-blot-SNP analysis that should be improved is preparation of genomic DNAs from a large number of plants for PCR templates. DNAs prepared by the CTAB method (Murray and Thompson 1980) or the method of Edwards et al. (1991) are reliable PCR templates, but these methods require much time. In the method of Wang et al. (1993), only maceration of a plant tissue in alkaline solution and dilution of the solution are required. Although this method is highly simple, but success of DNA amplification has been found to be inconsistent (Shirasawa et al. 2006, our unpublished results). Blotting DNA from a plant tissue onto FTA® cards (Whatman, UK) can be used as a simple method for preparation of the PCR templates (Roy and Nassuth 2005), although it is costly. Recently we found a 1-mm leaf disk to be directly usable as a PCR template for dot-blot-SNP analysis (Shiokai et al. 2009).

The SNP markers developed in the present study can be used for genotyping of plants and identification of Japonica rice cultivars. Since DNA polymorphisms are infrequent between the genomes of Japonica rice cultivars (Nasu et al. 2002; Monna et al. 2006; Shirasawa et al. 2004, 2007), these markers may also be useful for QTL analysis and marker-assisted selection using progeny of a hybrid between Japonica rice cultivars. These dot-blot-SNP markers together with the 100 markers reported in our previous paper (Shirasawa et al. 2006) are good additional DNA markers for Japonica rice.

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**Supplementary Data**

**Supplementary Table 1** Nucleotide sequences and hybridization conditions of PCR-DIG-labeled dot-blot-SNP markers

**Supplementary Table 2** Nucleotide sequences and hybridization conditions of 5'-biotin-labeled dot-blot-SNP markers

**Supplementary Table 3** Nucleotide sequences and hybridization conditions of bridge hybridization markers

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Supplementary table 1 Nucleotide sequences and hybridization conditions of PCR-DIG-labeled dot-blot-SNP markers

| Marker name | Chr. | SNP position | Forward primer sequence (5'-3')<br>Reverse primer sequence (5'-3') | Probe sequence of 'Nipponbare' type (5'-3')<br>Probe sequence of variant type (5'-3') | Temperature of hybridization and second washing[a] | Second washing buffer |
|---|---|---|---|---|---|---|
| S0063 | 1 | 1017259 | AGTTAACCGCCAGGCTTACA<br>TCCATGTGCAGTCACTGTGC | TTGAAACCaTATACATC<br>TTGAAACCgTATACATC | 40 | 1xSSC/0.1%xSDS |
| C0122 | 1 | 28629759 | GGTCATCTATGCTAGCACAC<br>ATCCTCTGGAACTTGCTTGC | GACTACAGcACAATTGC<br>GACTACAGtACAATTGC | 40 | 1xSSC/0.1%xSDS |
| R0655 | 1 | 34355286 | CCCTGTAACAAGGAGTTGAT<br>ACCTCCTTGAAGTTCTGCTGTCT | TGAAAAATaCTATCAAC<br>TGAAAAATgCTATCAAC | 40 | 1xSSC/0.1%xSDS |
| C63989 | 1 | 34611539 | AACACGTAGACTAACTCGGTGGA<br>ACCTTCTACTGCTCCATTAG | TGGATACAgCATTGGCT<br>TGGATACAaCATTGGCT | 40 | 1xSSC/0.1%xSDS |
| S4284 | 1 | 39470663 | GTGTGCAGCTATTGTGTAGA<br>GTACAATTGCTGACATGTGG | TATTAAGCgTATGCTAT<br>TATTAAGCcTATGCTAT | 40 | 1xSSC/0.1%xSDS |
| C30021 | 1 | 40211213 | CTAAGTTCCTTGGGCGACAA<br>GTCCACTCATTTATGCAGCC | TGAGTTACtGCCATTTG<br>TGAGTTACcGCCATTTG | 40 | 1xSSC/0.1%xSDS |
| S13049 | 2 | 2924042 | ATCTGTGAAATGTCCGACCT<br>TAGGACAAAGCCAGGAAGGA | CTAGTTCAtGAAATTAG<br>CTAGTTCAgGAAATTAG | 40 | 1xSSC/0.1%xSDS |
| C60715 | 2 | 2882443 | ACACACAAACGGTTGGTTCA<br>CGGCTCTAGCGGTCAACGAG | AGCATACAtTGTAACCA<br>AGCATACAcTGTAACCA | 40 | 1xSSC/0.1%xSDS |
| R0910 | 2 | 3142223 | TATGCGTGTCTGCATGTCAA<br>GCACAGCATGAAAGCAATGG | GTCAATGGcGTCTGCGG<br>GTCAATGGaGTCTGCGG | 40 | 1xSSC/0.1%xSDS |
| E10780 | 2 | 17696138 | GGACTGAGGTGAGAGATCTTGAG<br>TCATCCGTATTTGAGTCGGG | AAAATCATgAGCTGCAA<br>AAAATCATaAGCTGCAA | 40 | 1xSSC/0.1%xSDS |
| E31585 | 2 | 23267087 | CTACCACTAACATCTGAACC<br>TGAGTTAGTAGCACAGGGCCTTA | ATTGATTTaATAAGCTA<br>ATTGATTTgATAAGCTA | 40 | 1xSSC/0.1%xSDS |
| S20768 | 2 | 25028486 | TTGTAATGCCAACTGCTGAC<br>CCTGCAAAGCAGAACTGTTGTA | ATCAGTTAtTACAACTG<br>ATCAGTTAcTACAACTG | 40 | 1xSSC/0.1%xSDS |
| S13818 | 2 | 33206902 | ACTGGCTTGCAATGCTTTCT<br>ACTAAGCTCGACAGGACCAC | AATTAACAtGATAAGAA<br>AATTAACAcGATAAGAA | 40 | 1xSSC/0.1%xSDS |
| C12409 | 2 | 36572369 | CTTACCTCACGGAAGGTCATAAG<br>TCTGCAGCTATTGACCACTG | TATGGATCgAAGTAGAA<br>TATGGATCaAAGTAGAA | 40 | 1xSSC/0.1%xSDS |
| E11192 | 2 | 31563824 | CTGTGTGCTGATTTATTCGG<br>GGAGGAGGGTGTACTCTTGCTC | TTACACCTgAAGTGATT<br>TTACACCTcAAGTGATT | 40 | 1xSSC/0.1%xSDS |
| E60742 | 3 | 33298583 | GGCCCAGTATTGACTTTGTTTC<br>CATACCGAATGATAATGCTC | TCGGATTCaTCAGTCTT<br>TCGGATTCtTCAGTCTT | 50 | 0.1xSSC/0.1%xSDS |
| E61946 | 4 | 1039701 | AAGGTAATGGTGCAGTAGCTCAG<br>TTGCTGTAGCGCTGTTGCCG | ATAGTTTCcGACATCTA<br>ATAGTTTCaGACATCTA | 40 | 1xSSC/0.1%xSDS |
| E1294 | 4 | 14861640 | GGTCCCTCAAGGTCATCAAA<br>ACTCAAGAGTTCCTGTAAGC | TCCAGTTGaCAAGCAAC<br>TCCAGTTGgCAAGCAAC | 40 | 1xSSC/0.1%xSDS |
| S0651 | 4 | 24229551 | GCCTGGGGAATCAAGAAAAT<br>AAACAATGACCAGGTACTGG | TATTGTGCcGGTGTTGA<br>TATTGTGCtGGTGTTGA | 40 | 1xSSC/0.1%xSDS |
| S3010 | 4 | 32432915 | TGATGCATGCGTGTTCTCTT<br>CCTTGCCTTGGAGGTGATTC | GATGCCTCcGGAGAAAT<br>GATGCCTCgGGAGAAAT | 40 | 1xSSC/0.1%xSDS |
| S14881 | 6 | 17611605 | GATGACCGGTCCTGTTGCTT<br>GCGCATAAGGAGGCAATTAG | CTCCTCCGaCCAGGAGC<br>CTCCTCCGgCCAGGAGC | 40 | 1xSSC/0.1%xSDS |
| C30223 | 6 | 20197088 | GAAGCACTAGAAGCCCCCTATTA<br>AGACAGTTGGAACTATTGCC | TCATGGGAcCCTTCCTG<br>TCATGGGAtCCTTCCTG | 40 | 1xSSC/0.1%xSDS |
| E61502 | 6 | 22378340 | GTCAGCGCTTGTGTACGCGT<br>CTATAGTGCACATGGATGCTAAGG | CTATTACTgACTTCGAA<br>CTATTACTaACTTCGAA | 30 | 1xSSC/0.1%xSDS |
| C12560 | 6 | 24422125 | CTACAGGTATGCTACTGCAA<br>GCATCTAACCTTGCGGATAG | ACACTAGGtTCAGTACT<br>ACACTAGGcTCAGTACT | 40 | 1xSSC/0.1%xSDS |
| E60075 | 7 | 5490161 | GTCGTATGTTGCAGGAACCA<br>GTATTGGAGCACAACATGCC | AATGTTGTgAAGCACAT<br>AATGTTGTcAAGCACAT | 50 | 0.1xSSC/0.1%xSDS |
| E50426 | 7 | 9023318 | CGCACCATTTAGCAGTTTGA<br>TGCAAGTACTATAGACACTC | GTTGCATAgAACCTATT<br>GTTGCATAtAACCTATT | 40 | 1xSSC/0.1%xSDS |
| E51255 | 7 | 13578072 | GGTGTACTAGTGTCCAGAAG<br>CTACAGGTTCCCATCTGCTC | CATTGAACaAACCCATG<br>CATTGAACgAACCCATG | 40 | 1xSSC/0.1%xSDS |
| C11630 | 7 | 28357612 | ATGTCTTGGTGGTCTTGTAGCAC<br>TTGCTGCAGTGTCTGGTGTA | GCTTCTGCgAACATTAT<br>GCTTCTGCcAACATTAT | 50 | 1xSSC/0.1%xSDS |
| C10985 | 7 | 28362096 | CGAAGAACGCATGTTGGTTA<br>ATGTTTCCATATTCTTCAGG | GGATGGTAtGGATGAAC<br>GGATGGTAcGGATGAAC | 40 | 1xSSC/0.1%xSDS |
| E30622 | 7 | 29014196 | TAGGACATTGGGTGAGCTTG<br>ACCAAATAGTCTGCACATGC | ATTCAGTGcTGATACGA<br>ATTCAGTGtTGATACGA | 40 | 1xSSC/0.1%xSDS |
| R3089 | 7 | 18997374 | ATCGCCTCCGCTTCTGCTTT<br>TGATGTCTCCAATCTGCAGA | ATCAACTAcGCTATTTA<br>ATCAACTAaGCTATTTA | 40 | 1xSSC/0.1%xSDS |
| S15651 | 8 | 5365093 | CCAACAGGTATGGCAATATG<br>CTATTTACACGAGGCATTGTCG | ATAATTGCgTGATCCCT<br>ATAATTGCcTGATCCCT | 40 | 1xSSC/0.1%xSDS |
| S2092 | 8 | 5221699 | CTCCTCTTCCTTATTTCTGC<br>GCACGAGGATGCACATGTGG | AATTTCCCgGGCCTCAA<br>AATTTCCCaGGCCTCAA | 40 | 1xSSC/0.1%xSDS |
| S4853 | 8 | 5226393 | CTTGCTTGTGCCTGCACCTA<br>AACAGTTTGTGATTGCCTCG | TAGGAGTAtATAATTTC<br>TAGGAGTAcATAATTTC | 40 | 1xSSC/0.1%xSDS |
| E20920 | 8 | 6037745 | ATCAGCGACCGCCAATGTCG<br>GTTCCACTATTAGCTCCGTCTTAC | TCGATCTCcTTCTGCCT<br>TCGATCTCtTTCTGCCT | 40 | 1xSSC/0.1%xSDS |
| C52909 | 8 | 20314028 | GGCTGGTGCGCTGTACATAA<br>TGATGCAGCAGTCAAACACA | CCCTGCATgATTGAGAT<br>CCCTGCATaATTGAGAT | N: 50, V: 40 | 1xSSC/0.1%xSDS |
| E61986 | 8 | 6240593 | CTCACTCGGTGCATGGAATG<br>CCTGATAGTGCCAGATTAAT | ACCGTCACtAGCTGAAA<br>ACCGTCACcAGCTGAAA | 40 | 1xSSC/0.1%xSDS |
| E4156 | 8 | 28163769 | TCTGCAGGATCAGCATGAGG<br>ACCATGTACATTAGCGTACC | CAATCCACaGTATTTGA<br>CAATCCACtGTATTTGA | 40 | 1xSSC/0.1%xSDS |
| S13017 | 9 | 12729050 | GGACCTGTTCACTTTGGTGC<br>ATTCTACCGGTCCAAGTCGTC | ATCGTAGCcAAAATAAA<br>ATCGTAGCtAAAATAAA | 40 | 1xSSC/0.1%xSDS |
| S0065 | 10 | 8699830 | GTGGGATGTGGGACTCAAAC<br>GTCTAACTCCCTAGAAACAG | AAAATATCaTTAGTCCA<br>AAAATATCtTTAGTCCA | 40 | 1xSSC/0.1%xSDS |
| S14938 | 11 | 256540 | GGTAGATTATTATGAGGACTGAC<br>TGGCAATAGACATAGGCTAG | GCTGTCTGgTTCCGGGG<br>GCTGTCTGaTTCCGGGG | 40 | 1xSSC/0.1%xSDS |
| E11325 | 11 | 2256140 | AAGCCTGTTCTACTACCACC<br>GCCAATAGACGATCGGGCGC | AAAATTTTgGTAAGGTT<br>AAAATTTTaGTAAGGTT | 30 | 1xSSC/0.1%xSDS |
| E0935 | 11 | 9119726 | TTGCAAGAACTTCCCCTGAT<br>CTAAGCCAGATTGAGGACAT | ATCATTGAcCATACAAT<br>ATCATTGAtCATACAAT | 30 | 1xSSC/0.1%xSDS |
| E3876 | 11 | 25197076 | AGCATTTGGCTCGTAGCAAT<br>CCTCCAATATGCTTGCAGGC | TCAAATGCaTCGACGAG<br>TCAAATGCgTCGACGAG | 50 | 0.1xSSC/0.1%xSDS |
| R2664 | 11 | 25335264 | ACTGGGACACACGCTACAAGATA<br>ATAGGAAAGTAATGCGGTGC | GGTAGCTAaCAGCGATC<br>GGTAGCTAcCAGCGATC | 40 | 1xSSC/0.1%xSDS |

a: 'N' represents a 'Nipponbare' type and 'V' does a variant type.

Supplymentary table 2 Nucleotide sequences and hybridization conditions of 5'-biotin-labeled dot-blot-SNP markers

| Marker name | Chr. | SNP position | Forward primer sequence (5'-3')<br>Reverse primer sequence (5'-3') | Probe sequence of 'Nipponbare' type (5'-3')<br>Probe sequence of variant type (5'-3') | Temperature of hybridization and second washing | Second washing buffera |
|---|---|---|---|---|---|---|
| C0101 | 1 | 4851683 | CTCCATCTCCCATCCAGGTA<br>GGAACTACATATTCAGATCC | CTTGCTGTcGTTAGTGT<br>CTTGCTGTtGTTAGTGT | 40 | 1xSSC/0.1%xSDS |
| C10106 | 1 | 8793184 | CAGTGCTGTACTAGGCTTAG<br>AACAGAGTCTGGCTTCTGCTG | AGCAGTAAcCAGTGCTG<br>AGCAGTAAaCAGTGCTG | 40 | 1xSSC/0.1%xSDS |
| C1456 | 1 | 26469268 | GGATGTGTTAAAGGAGCCAG<br>AGCACTCTTGGTGCAACCAG | AGCGTGCAtGGCGGAAC<br>AGCGTGCAcGGCGGAAC | 50 | 0.1xSSC/0.1%xSDS |
| S10045 | 1 | 30926011 | GGACGTTGCCATGGAAGTCT<br>CTGTGCAGGTACACAACAGAAAG | TTCATCTTgTTAGTGTT<br>TTCATCTTaTTAGTGTT | 40 | 1xSSC/0.1%xSDS |
| C53722 | 2 | 17816703 | CAGCATGGAGGTGGCATGAT<br>TAATGGGCAGATCACGACAG | GAGGGCAAaGAGCTGGA<br>GAGGGCAAgGAGCTGGA | 40 | 1xSSC/0.1%xSDS |
| E60475 | 2 | 18828120 | GACAGTGACACAGTGACACC<br>CCACTGAAATGGGTAACTCTGTC | GACAACTTtTCTAATAGC<br>GACAACTTaGCTAATAGC | 40 | 1xSSC/0.1%xSDS |
| C30024 | 3 | 5901203 | GATGCTAGTGGTGCTGTCCG<br>CACGCGTTTATCACTAGTGA | ATAAACTGtATTTTTTT<br>ATAAACTGaATTTTTTT | 40 | 1xSSC/0.1%xSDS |
| C0820 | 4 | 6896396 | AAGGTTCATCAGTAACCATC<br>TGTTCCTGGGCTCAATTAAC | AAATGCTCaTTGAAGGA<br>AAATGCTCtTTGAAGGA | 40 | 1xSSC/0.1%xSDS |
| C0106 | 4 | 16667671 | AACAGCATGAGAATAAAAACGC<br>TATGCGGTTCTGCTGTATTG | CATACATAaACAAAAT<br>CATACATAtcaACAAAAT | 40 | 1xSSC/0.1%xSDS |
| S21013 | 4 | 20120616 | CACTCAGCTGGTTGAGGATG<br>GGATGCGTATTGGACCATCT | CCATAATGgTGGACACT<br>CCATAATGaTGGACACT | 40 | 1xSSC/0.1%xSDS |
| E20943 | 5 | 16554327 | GGGTTCCTCCCTCGTGTATT<br>TTTGCTACTTGAAGAACCAC | TCTGTTGGcCAATTGAT<br>TCTGTTGGgCAATTGAT | 40 | N:1xSSC-V:0.1xSSC/0.1%xSDS |
| S5407 | 6 | 4430027 | GGATAGTAAGTGCTAGCAAA<br>TTGGAGTAGGGCATTGTTCA | TACACGAAGAAGAATAACA<br>TACACGAAGAAgaaGAATAACA | 40 | 1xSSC/0.1%xSDS |
| E61502 | 6 | 22377949 | CGCTAAACAGCTCCACATTG<br>GTACACAAGCGCTGACAAAATG | GTGGAGTGgAGTGGAGG<br>GTGGAGTGaAGTGGAGG | 40 | 1xSSC/0.1%xSDS |
| E0211 | 6 | 22841785 | GAAGGCTGCGGCAGATAATA<br>ACCGTTGACCAAAATGGCTA | gtcacttACCATCATTA<br>cagtcACCATCATTAGG | 40 | 1xSSC/0.1%xSDS |
| R1804 | 7 | 3324222 | CGCAAGTACAGCATGCATTC<br>GACCCCCTATAGCTTTGATTTCTC | GTGTATTTgACTATCTG<br>GTGTATTTtACTATCTG | 50 | 1xSSC/0.1%xSDS |
| R1488 | 7 | 5290923 | AGATAAGCAGAGGAAGGTCC<br>CTAGAGCATGAGAACTGACC | AAGATGGCtGAAGTATC<br>AAGATGGCcGAAGTATC | 50 | 1xSSC/0.1%xSDS |
| C61928 | 7 | 19182659 | ATATTAGTCGTCCTAACAACTG<br>CACGGTCCAAGTACCAAGGC | GATAAGAGagTGGTTAGG<br>GATAAGAGTGGTTAGG | 40 | 1xSSC/0.1%xSDS |
| S4853 | 8 | 5227221 | AAAACCGGAAGGAGTAATAGGG<br>GAATACTGCTTCCATCCCAG | ATAATAATcATATTGTT<br>ATAATAATtATATTGTT | 30 | 1xSSC/0.1%xSDS |
| S3680 | 8 | 18344367 | TGACCAGTTCAGATAACCTG<br>GGAGGTATGTCTTCCACATGCTA | AAAACTACaTTTTAGCA<br>AAAACTACgTTTTAGCA | 40 | 1xSSC/0.1%xSDS |
| E0349 | 9 | 17110873 | CGAAGTGCTAATCACATCTG<br>CCATGGATATCATGGATCTC | ATGCAGGCcTCTCATAT<br>ATGCAGGCtTCTCATAT | 40 | 1xSSC/0.1%xSDS |

a: 'N' represents a 'Nipponbare' type and 'V' does a variant type.

Supplymentary table 3 Sequences and hybridization conditions of bridge hybridization

| Marker name | Chr. | SNP position | Forward primer sequence (5'-3')<br>Reverse primer sequence (5'-3') | Probe sequence of 'Nipponbare' type (5'-3')<br>Probe sequence of variant type (5'-3') | Temperature of hybridization and second washing | Second washing buffer | Ratio[a] | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Nipponbare' type | Variant type |
| NLSNP1 | 1 | 19714968 | GTACGTCATCCATAGTCCGATTC<br>AAAAATGCGTGGACGTTAGC | CGTGCATGtGTGAGGCG<br>CGTGCATGcGTGAGGCG | 50 | 0.1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP45 | 2 | 977553 | CTGGGACTGGATGAGACGTT<br>CGGAGCTGGATCAGACCTAC | TGCTGCCGgCGAACCGC<br>TGCTGCCGaCGAACCGC | 50 | 0.1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP4 | 2 | 17056398 | GTTACCGTGGGTACCAGCACTAT<br>GGCTTGCAGAAGAGTGAAGAAT | TCTTTGAAtGCTTGATT<br>TCTTTGAAgGCTTGATT | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP7 | 3 | 9640009 | GCTGGGTGATAGAGCTACCTTC<br>CTTGGCACACAGAACAAAGA | ACTTGCTCcATCTATTT<br>ACTTGCTCtATCTATTT | 40 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP8 | 3 | 21926296 | GAACGGAGGAAGTACATGAGAAAC<br>AGGATGCATGAGGGGATATCTA | TAGAGTCAcGTAGAGAT<br>TAGAGTCAtGTAGAGAT | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP11 | 3 | 33369280 | CCAGATTTTGTCTTGGCCCTAA<br>GGTGGATGCAAATAGGTACAAC | ATCAGTACaTGCTAAGT<br>ATCAGTACgTGCTAAGT | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP36 | 4 | 28171432 | GGTACCTTGGTTACTTGGGCTA<br>CTCCTCCTCTCTCTTGGGGTAT | CTGGTacACTGGGCTAG<br>CTtGGTtACTGGGCTAG | 50 | 0.1xSSC/0.1%SDS | 1:10 | 1:5 |
| NLSNP67 | 6 | 5476999 | GGCGGTAATAGTGGGACTGA<br>CCGGTATGATGGATGGTTTC | ATGCAAGGcCAACCTCT<br>ATGCAAGGtCAACCTCT | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP13 | 6 | 6458563 | ACTCCACGAGGTAGTCCTCGAC<br>GGCCCATCTACAGTATACACACC | TTCGCTTGcTTCCAGAC<br>TTCGCTTGtTTCCAGAC | 40 | 1xSSC/0.1%SDS | 1:50 | 1:5 |
| NLSNP15 | 6 | 18992003 | GAATGCTTACCGAGATTCCATC<br>GACTACATGAGGTCCAGCTATGC | CATCGGTAgAGCTAGAC<br>CATCGGTAcAGCTAGAC | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP70 | 6 | 21979144 | ACAAGCCTCTGGTGCTTTTT<br>TGCAGGCCACTGACTTAACA | GTTGAATTcAATTTTTA<br>GTTGAATTtAATTTTTA | 40 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP18 | 8 | 20864104 | CAGTTAACCACTTCGACAGAAGG<br>GGGAGGGATTTGCAGATTAAC | AATAGATAtCCACAAGC<br>AATAGATAcCCACAAGC | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP88 | 9 | 8418691 | CAGGCTTCCTGTTCTTCGTC<br>TTCGCTACTGGGTCCACTC | CAACTACAaCAACTTGG<br>CAACTACAgCAACTTGG | 50 | 1xSSC/0.1%SDS | 1:10 | 1:5 |
| NLSNP89 | 9 | 11464861 | CTCGCCATGTTCAAAGAAGC<br>CTGGGTGTGAGAGACGAACA | AAGAAGCTgAGCGACGC<br>AAGAAGCTaAGCGACGC | 50 | 0.1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP20 | 9 | 13754905 | GATAAAGCAGAGAGGGGAAGATG<br>CCCTCATTTGCACATTCAGA | TGGCACTCcGAGGCTCC<br>TGGCACTCtGAGGCTCC | 50 | 0.1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP93 | 10 | 9665031 | CGTGTGGGAGGGAAAAATAA<br>AGGACTTCCTCCGTCTCAAA | CGACAAAAcCCTATAAT<br>CGACAAAAgCCTATAAT | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP21 | 10 | 12100820 | ATATTTCAGCAGTGGGTTGTGG<br>CGACTCTTTCTTCTCTCCATCTTC | GGTGTCGAtGCTGTGCT<br>GGTGTCGAgGCTGTGCT | 50 | 0.1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP94 | 10 | 16148652 | AAATTGGCAGCAGTGTCCTT<br>TTGGACAGAGCCAACCTACA | ACCAAGCAtGCATCTGC<br>ACCAAGCAaGCATCTGC | 50 | 0.1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP96 | 11 | 6180907 | CAGCGCGTGGTAACTATGAC<br>ATCTCATCCCCTCCTTACCC | CCAGTCCAtATCTGGAT<br>CCAGTCCAgATCTGGAT | 50 | 1xSSC/0.1%SDS | 1:5 | 1:5 |
| NLSNP29 | 12 | 24186101 | ACTGATGACAGGTGAGACCAAGT<br>TACGTGCTCCGTACGTATGCTAT | GGTTCACTaTACTATTA<br>GGTTCACTgTACTATTA | 40 | 1xSSC/0.1%SDS | 1:5 | 1:5 |

a: Ratio of competitive probe of labeled probe,