

# Chapter 14

## Genotyping Single Nucleotide Polymorphisms by Multiplex Minisequencing Using Tag-Arrays

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### Abstract

The need for multiplexed methods for SNP genotyping has rapidly increased during the last decade. We present here a flexible system that combines highly specific genotyping by minisequencing single-base extension with the advantages of a microarray format that allows highly multiplexed and parallel analysis of any custom selected SNPs.

Cyclic minisequencing reactions with fluorescently labeled dideoxynucleotides (ddNTPs) are performed in solution using multiplex PCR product as template and detection primers, designed to anneal immediately adjacent and upstream of the SNP site. The detection primers carry unique Tag-sequences at their 5' ends and oligonucleotides complementary to the Tag-sequence, cTags, are immobilized on a microarray. After extension, the tagged detection primers are allowed to hybridize to the cTags, and the fluorescent signals from the array are measured and the genotypes are deduced by cluster analysis of the incorporated labels. The "array of arrays" format of the system, accomplished by a silicon rubber grid to form separate reaction chambers, allows either 80 or 16 samples to be analyzed for up to 200 or 600 SNPs, respectively on a single microscope slide.

**Key words:** Single nucleotide polymorphism, SNP, genotyping, fluorescent minisequencing, microarray, multiplex PCR, "array of arrays."

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### 1. Introduction

As a result of the successful Human Genome Project we have access to a complete nucleotide sequence of the 3 billion nucleotides that constitute our genome (1–3). Another large effort, the Human Haplotype Mapping (HapMap) project has generated detailed information on over 5 million single nucleotide polymorphisms (SNPs) and the linkage disequilibrium patterns between the SNP alleles in the human genome (4, 5). The Human Genome Project,

and particularly the HapMap project, have been accompanied by a rapid development in genotyping technology, and today there are commercially available systems for SNP genotyping on a very large, and even genome-wide scale. These genotyping systems are enabled by recent developments in technology for production of microarrays and for fluorescence scanning of the arrays in clever combination with known reaction principles for genotyping (6). One of the most frequent reaction principles in multiplex genotyping systems today is minisequencing or single-base primer extension, in which a DNA polymerase is allowed to extend a detection primer by a single nucleotide at the position of the SNP (7). The most spectacular example of single-base extension is the Infinium II assay from Illumina (8). The system, presented in this chapter, combines the highly specific genotyping principle of minisequencing with the advantages of a microarray format that allows medium to highly multiplex and parallel analysis. The system is flexible and can be designed for any panel of SNPs. It is particularly useful for establishing genotyping panels for other organisms than humans, where common predefined assays are not available (9, 10). The specificity of the single-base extension reaction allows quantitative analysis of SNP genotypes in genomic DNA for analysis of copy number variation or genotyping pooled DNA samples (11), and in RNA for allele-specific gene expression analysis (12, 13) or relative quantification of alternatively spliced transcripts (14). Recently quantitative SNP analysis has been scaled up for genome-wide detection of loss of heterozygosity and copy number variation (15).

The Tag-array minisequencing system utilizes generic capture oligonucleotides (“cTags”) that are immobilized on a microarray. Multiplex cyclic minisequencing reactions with fluorescently labeled dideoxynucleotides (ddNTPs) are performed in solution using minisequencing primers designed to anneal immediately adjacent and upstream of the SNP site. The primers carry 5'-Tag-sequences complementary to one of the arrayed cTags, and the SNPs are separated by hybridizing the extended minisequencing primers to their corresponding cTags with known locations on the array. The incorporated fluorescently labeled ddNTPs allow deduction of the genotypes of each SNP based on measurement of the signal intensities by fluorescence scanning of the arrays (11, 16). The use of generic Tag-sequences enables universal, non-SNP specific array designs. The “array of arrays” format described below is accomplished by a silicon rubber grid creating separate reaction chambers for multiple samples on a single microscope slide. Either 80 or 16 samples can be simultaneously analyzed for up to 200 or 600 SNPs, respectively (10, 17, 18) (**Fig. 14.1**).

The procedure described in detail in **Section 3** outlines (1) selection of appropriate SNPs, (2) design of oligonucleotides for PCR, immobilization on the microarray, and SNP genotyping, (3) preparation of microarrays, (4) manufacturing of the silicon

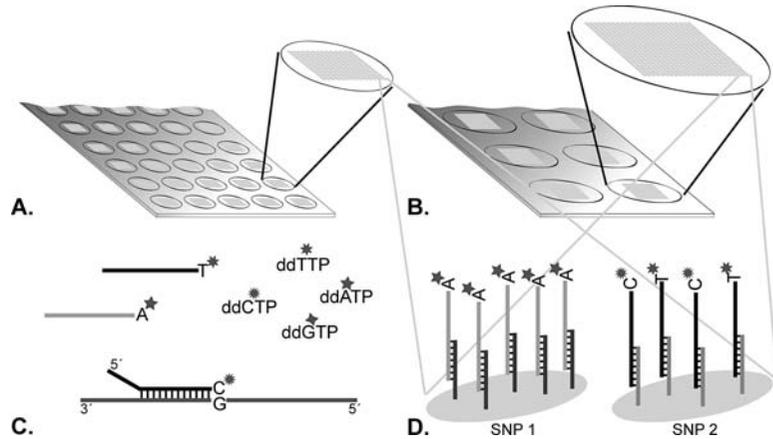


Fig. 14.1. Principle of Tag-array minisequencing. Schematic cut views of two arrayed slides with the subarrays in either 384-well (A) or 96-well (B) format. One of the subarrays for each slide containing up to 200 (A) or 600 (B) cTags is shown enlarged. The principle of the minisequencing reaction is illustrated with a minisequencing primer carrying a 5'-Tag-sequence annealed to its target, and extended with a labeled ddCTP at the position of the SNP (C). The Tag-sequences of the extended minisequencing primers are allowed to hybridize to their complementary cTags arrayed as spots in the subarrays (D). The genotypes are deduced by measuring the fluorescence of the incorporated nucleotides. Part of one subarray, with the result for two SNPs, is shown. This sample is homozygous (A/A) for SNP 1 and heterozygous (C/T) for SNP 2.

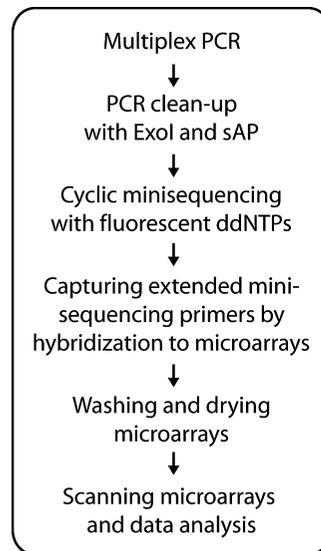


Fig. 14.2. Flow chart illustrating the main steps of the procedure for genotyping SNPs by minisequencing using Tag-arrays.

rubber grid, (5) the genotyping reaction, and (6) data analysis and genotype calling. The main steps of the assay are illustrated in Fig. 14.2. Several alterations and modifications of the method are possible and a number of suggestions are given in Section 4. The

protocol is provided under the assumption that the instrumentation, reagents, and consumables specified in **Section 2** are to be used, but other equivalent procedures are also feasible.

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## 2. Materials

### 2.1. Instrumentation and Software

1. Access to arraying instrument or purchased customized arrayed slides. We use a ProSys 5510A instrument (Cartesian Technologies Inc., Huntingdon, UK) with Stealth Micro Spotting Pins (TeleChem International Inc., Sunnyvale, CA).
2. PCR instrument.
3. Multichannel pipette and/or a pipetting robot (optional).
4. Centrifuge for microtiter plates (recommended).
5. Minisequencing reaction rack (**Fig. 14.3**).
6. Hybridization oven at 42°C.

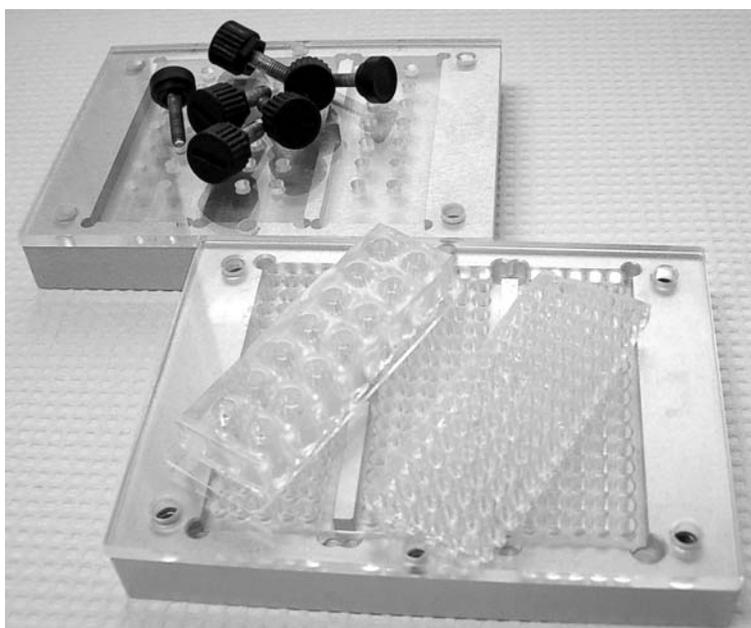


Fig. 14.3. The arrayed slide is covered with a silicon rubber grid to give separate reaction chambers and placed in a custom-made heat conducting aluminum rack. A 384-well (*right*) and a 96-well (*left*) format silicon rubber grid is shown on top of a reaction rack. A plexiglas cover with drilled holes through which the reaction chambers are accessible is tightly screwed on top of the assembly, thus securing correct positioning of the silicon grid during hybridization.

7. Array scanner and software for signal quantification. We use the ScanArray Express system (PerkinElmer Lifesciences, Boston, MA).
8. Software for cluster analysis and genotype calling. We use the SNPSnapper software (Juha Saharinen, <http://www.bioinfo.helsinki.fi/SNPSnapper/>).

## **2.2. Reagents and Consumables**

All reagents and consumables should be of standard molecular biology grade. Use sterile distilled or deionized water.

### 2.2.1. Oligonucleotides Primers and Nucleotides

1. PCR primers.
2. Minisequencing primers with 5'-Tag-sequences.
3. cTags with a 3' 15-T residue spacer and a 3'-amino group.
4. Reaction control – four oligonucleotides differing at one internal nucleotide position and a minisequencing primer (with a 5'-Tag-sequence) complementary to the oligonucleotide templates.
5. Spot control – fluorescently labeled cTag oligonucleotide.
6. Hybridization control – two fluorescently labeled Tag-oligonucleotides.
7. Print control – fluorescently labeled oligonucleotide designed to hybridize to any cTag.
8. Fluorescent dideoxynucleotides (Texas Red-ddATP, TAMRA-ddCTP, R110-ddGTP and Cy5-ddUTP; PerkinElmer Life Sciences). Keep light-protected working aliquots at +4°C and store stock solutions at –20°C.

### 2.2.2. Enzymes

1. DNA polymerase. We use Smart-Taq Hot DNA polymerase (Naxo, Tartu, Estonia).
2. Exonuclease I (Fermentas, Vilnius, Lithuania).
3. Shrimp Alkaline Phosphatase (GE Healthcare, Uppsala, Sweden).
4. DNA polymerase compatible with fluorescently labeled ddNTPs. We use KlenThermase DNA polymerase (GENE-CRAFT, Lüdinghausen, Germany).

### 2.2.3. Buffer Solutions

1. Standard PCR reagents or reagents optimized for multiplex PCR.
2. 2x Printing buffer: 300 mM phosphate buffer pH 8.5. Store at room temperature up to 1 month.
3. Blocking solution: 50 mM ethanolamine, 100 mM Tris-HCl, pH 9.0 and 0.1 % SDS. Prepare directly before use. Ethanolamine is highly corrosive and should be handled according to safety instructions.

4. Washing solutions; (I) 4xSSC, (II) 2xSSC and 0.1% SDS, and (III) 0.2xSSC. (20xSSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0. 10% SDS: 10% w/v sodium dodecyl sulfate)
5. 1 M Tris-HCl, pH 9.5.
6. 50 mM MgCl<sub>2</sub>.
7. 1 % v/v Triton X-100.
8. Hybridization solution: 6.25x SSC

#### 2.2.4. Consumables

1. 384- or 96-well v-bottomed microtiter plates (ABgene, Epsom, UK).
2. CodeLink™ Activated Slides (GE Healthcare, Uppsala, Sweden).
3. Elastosil RT 625 A and B (polydimethyl siloxan; Wacker-Chemie, München, Germany).

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### 3. Methods

#### 3.1. SNP Selection

SNPs can be identified either experimentally or in databases, for example dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP>). Database searches for SNPs may be aimed at genes of interest, candidate chromosomal regions or randomly distributed SNPs with known allele frequencies, depending on the aim of the project, *see also* **Note 1**.

#### 3.2. Oligonucleotide Design

The length of the PCR fragments should be short, optimally around 100–150 bp. Design PCR primers flanking the SNPs of interest using available software. Primer3 is freely available on the internet (<http://frodo.wi.mit.edu/primer3/input.htm>). *See also* **Note 2**.

##### 3.2.1. PCR Primers

Each PCR primer pair should be tested in an *in silico* PCR to verify that a specific DNA fragment is being amplified (e.g. <http://genome.brc.mcw.edu/cgi-bin/hgPcr>). *See also* **Note 3**.

##### 3.2.2. Minisequencing Primers

Minisequencing primers are designed to anneal immediately adjacent and upstream of the SNP position. The minisequencing primers should be approximately 20 bases long, and have a melting temperature of 55–60°C to ensure specificity in the cyclic primer extension reaction. The 5' end of each minisequencing primer should contain Tag-sequences complementary to the cTags that are printed on the microarray. The Tags should be 20 bases long, have similar melting temperature and not be complementary to either each other, to the gene specific part of the minisequencing primers or to the human genome (16). The

Affymetrix GeneChip<sup>®</sup> Tag Collection can be used as source for Tag-sequences (Affymetrix, Santa Clara, CA). Minisequencing primers for both forward and reverse DNA strands are often helpful as controls for the genotyping results (*see* **Note 4**).

### 3.2.3. Complementary Tag-Sequences

The complementary Tag-sequences (cTags) have 15 3' T-residues as a spacer and a 3'-amino group to enable covalent attachment of the cTags to the microarray slides.

### 3.2.4. Control Oligonucleotides

We recommend the use of control oligonucleotides for each step of the genotyping procedure (*18*).

To control for the spotting procedure, a fluorescently labeled cTag must be included on the array. A control oligonucleotide designed to hybridize to any cTag (5'-AAA AAA AAA ANN NNN NNN NN- Fluorophore -3') is recommended for testing the printing of some subarrays or microarrays from each batch.

As minisequencing reaction control, a minisequencing primer that is complementary to four synthesized single-stranded oligonucleotide templates differing at one nucleotide position to mimic the four possible alleles of a SNP is useful. Add the control templates to the minisequencing reaction mixture at a final concentration of 1.5 nM. A corresponding cTag must be included on the array. To control the hybridization reaction, a fluorescently labeled oligonucleotide complementary to an arrayed cTag is used. Optimally, use two differently labeled hybridization control oligonucleotides, and add them in an alternating pattern over the microarray to allow assessment that no leaking between wells has occurred.

## 3.3. Preparation of Microarrays

Dissolve the cTags in Printing buffer to a final concentration of 25  $\mu\text{M}$ , *see also* **Note 5**. If not used directly or if to be reused, store the cTags at  $-20^{\circ}\text{C}$ , but limit freeze-thawing cycles to a maximum of ten. Prepare the arrays by contact printing of the cTag oligonucleotides onto CodeLink<sup>™</sup> Activated slides using the ProSys 5510A instrument with SMP3 pins. These pins deliver 1 nl of the cTag-solution to the slides to form spots with a diameter of 125–150  $\mu\text{m}$  and with a center-to-center distance of for example 200  $\mu\text{m}$  (*see* **Note 6**). For the possibility of using the “array of arrays” format, print spots in a subarray pattern corresponding to the spacing of wells in a 384-well microtiter plate, (*see* **Note 7**; **Fig. 14.1**). After arraying, mark the position of some of the subarrays on the back side of the slides using a diamond-pen.

### 3.3.1. Microarray Printing

### 3.3.2. Post-Printing Processing of the Microarray Slides

Process the slides according to the instructions of the manufacturer. The protocol for CodeLink<sup>™</sup> Activated Slides is given below.

1. Prepare an incubation chamber with 75% relative humidity. Add as much solid NaCl to water as needed to form a 1-cm deep slurry at the bottom of a plastic container with an air-tight lid.
2. After printing, keep the arrays in the incubation chamber for 4–72 h.
3. Prepare the blocking solution and preheat it to 50°C.
4. Deactivate the excess of amine-reactive groups by immersing the arrayed slides into the blocking solution for 30 min at 50°C.
5. Rinse twice with dH<sub>2</sub>O. Immerse the slides in washing solution I for 30 min at 50°C (at least 10 ml per slide should be used). Rinse again with dH<sub>2</sub>O.
6. Spin dry the slides for 5 min at 900 rpm. Store the slides desiccated at room temperature until use.

### 3.3.3. Quality Control of Printing Procedure

For each batch of printed slides it is useful to analyze a few subarrays by hybridization to control the quality of the spots. After blocking the slides, hybridize the 3'-fluorescently labeled print-control oligonucleotide to some subarrays at 300 nM concentration in 6.25x SSC for 5 min with subsequent washing and scanning as described below.

### 3.4. Preparation of Reusable Silicon Rubber Grid

A grid of silicon rubber reaction chambers is prepared using inverted v-bottomed microtiter plates as mould (**Fig. 14.3**).

1. Add the two Elastosil RT 625 components in a 50 ml Falcon tube in a mass ratio of 9:1 (i.e. 46.8 g of A and 5.2 g of B) and rotate and turn the tube manually until the components are fully mixed (~ 30 min; *see Note 8*).
2. Pour the mixture onto an inverted v-bottomed 384-well microtiter plate, leaving about 1–2 mm of the tip of the wells uncovered. Allow the silicon rubber to harden at least over night at room temperature (*see Note 9*).
3. Remove the silicon rubber grid from the plate, and use a scalpel to cut the silicon rubber into pieces of the same size as microscope slides, with the wells matching the printed subarrays.
4. The silicon rubber grid is reusable, wash it in 10% chlorine, rinse with water and allow to dry after each use.

### 3.5. Genotyping

#### 3.5.1. Multiplex PCR and Clean-Up

1. Amplify DNA samples and non-template control samples by multiplex PCR according to an optimized protocol. The success of the amplification may be verified on a 2% agarose gel for a subset of the samples.
2. For each sample, pool the multiplex PCR products (*see Note 10*).
3. Prepare a master-mix of the exonuclease (ExoI) and alkaline phosphatase (sAP) reagents for clean-up of the PCR products (**Table 14.1**; *see Note 11*).

**Table 14.1**  
**PCR clean-up reagents**

Reagent	Volume per reaction ( $\mu\text{l}$ )	Final concentration
PCR products	7.1	
50 mM $\text{MgCl}_2$	1.6	7.61 mM <sup>a</sup>
1 M Tris-HCl pH 9.5	0.5	0.05 M
20 U/ $\mu\text{L}$ Exonuclease I	0.3	0.57 U/ $\mu\text{l}$
1 U/ $\mu\text{L}$ Shrimp Alkaline Phosphatase	1.0	0.10 U/ $\mu\text{l}$
Total volume	10.5	

<sup>a</sup> The true final concentration of  $\text{MgCl}_2$  is higher depending on the contribution from the PCR products.

4. Add 3.4  $\mu\text{l}$  of the clean-up mixture in a final volume of 10.5  $\mu\text{l}$ .
5. Incubate at 37°C for 45 min.
6. Inactivate the enzymes by heating to 85°C for 15 min.

### 3.5.2. Cyclic Minisequencing

1. Prepare a master-mix with minisequencing reagents (Table 14.2). The fluorophores in this mixture are light-sensitive (*see* Note 12).

**Table 14.2**  
**Minisequencing reagents**

Reagent	Volume per reaction ( $\mu\text{l}$ )	Final concentration
PCR products after clean-up	10.50	
100 nM each pooled Minisequencing primers	1.50	10 nM
100 $\mu\text{M}$ Fluorescently labeled ddNTPs <sup>a</sup>	4 $\times$ 0.015	0.10 $\mu\text{M}$
1 % Triton X-100	0.30	0.02 %
25 U/ $\mu\text{l}$ KlenThermase	0.04	0.067 U/ $\mu\text{l}$
H <sub>2</sub> O	2.60	
Total volume	15.00	

<sup>a</sup>Texas Red-ddATP, TAMRA-ddCTP, R110-ddGTP, Cy5-ddUTP.

2. After the clean-up step, add 4.5  $\mu\text{l}$  of minisequencing reaction mixture in a final volume of 15  $\mu\text{l}$ .
3. Perform the minisequencing reactions in a thermal cycler using an initial 3 min denaturation step at 96°C followed by for example 33 cycles of 20 s at 95°C and 20 s of 55°C in a thermocycler (*see* **Note 13**).

### 3.5.3. Capture by Hybridization

1. Position a silicon rubber grid over the arrayed slide according to the diamond-pen markings. Place the arrayed slides into the custom-made aluminum reaction rack and tighten the Poly-methyl methacrylate (PMMA or “plexiglas”) cover (**Fig. 14.3**). Preheat the assembly to 42°C on a heat block (*see* **Note 14**).
2. Add 7  $\mu\text{l}$  of the hybridization solution to each minisequencing reaction to a final volume of 22  $\mu\text{l}$ . It is recommended to include hybridization control oligonucleotides at 0.25 nM concentrations in the hybridization mixture.
3. Transfer 20  $\mu\text{l}$  of each sample to a separate reaction chamber on the microscope slide. A multichannel pipette is convenient for this step.
4. Hybridize for 2 h at 42°C in a humid and dark environment accomplished for example by placing a wet tissue on the plexiglas lid and covering it with plastic film and aluminum foil.

### 3.5.4. Washing

1. Prepare the three washing solutions. Preheat solution II to 42°C.
2. After hybridization, take the slides from the reaction rack and immediately rinse with Solution I, at room temperature.
3. Wash the slides twice for 5 min with Solution II at 42°C, and twice for 1 min with Solution III, at room temperature, in 50 ml Falcon tubes.
4. Spin dry the slides for 5 min at 900 rpm, and store them protected from light.

### 3.5.5. Fluorescence Scanning

If allowed by the used scanner, balance the signal intensity from each laser channel so that no signals are saturated and the signals from the four fluorophores are as equal as possible. Balancing is feasible if a reaction control with signals from all four fluorophores has been included. **Figure 14.4** shows an example of a scanned array.

### 3.6. Data Analysis and Genotype Assignment

A quantification program such as the one supplied with the Scan-Array Express instrument handles the scanning images and quantifies the signals from each spot. The raw-data is collected in an Excel sheet. Subtract the background, measured either around the spots or at negative control spots, i.e., spotted cTags

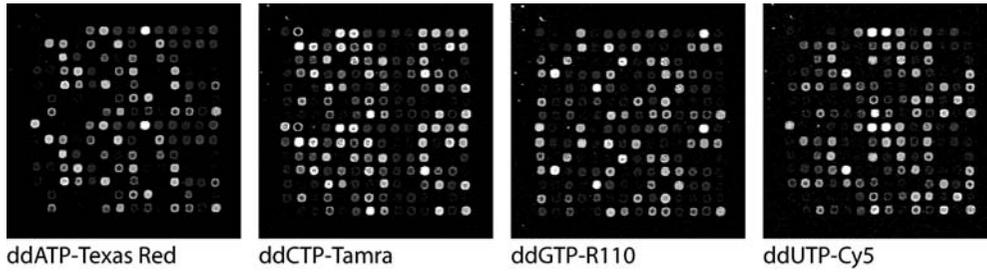


Fig. 14.4. Scanning results from genotyping one sample for 45 SNPs in one subarray, after cyclic minisequencing with ddATP, ddCTP, ddGTP, and ddUTP labeled with Texas Red, TAMRA, R110, and Cy5, respectively. Each cTag was spotted as horizontal duplicates and both polarities of the SNPs were analyzed, which together with the spots for the controls resulted in 196 spots (18).

without corresponding tagged primers, from the signals measured in each channel.

Assign the genotypes of the SNPs in each sample by calculating the ratios between the signals from one of the alleles and the sum of the signals from both the alleles;  $\text{Signal Allele1} / (\text{Signal Allele 1} + \text{Signal Allele2})$ . A scatter plot with this ratio on the horizontal axis and the sum of the signals from both alleles on the vertical axis may be used for assigning the genotypes (Fig. 14.5). This scatter plot should give three distinct genotype clusters, with the homozygote samples clustering at each side and the heterozygotes in the middle. The ratios may vary between SNPs, depending on the sequence surrounding it, the type of nucleotide incorporated, and the signal intensity of the fluorophores, *see also Note 15*.

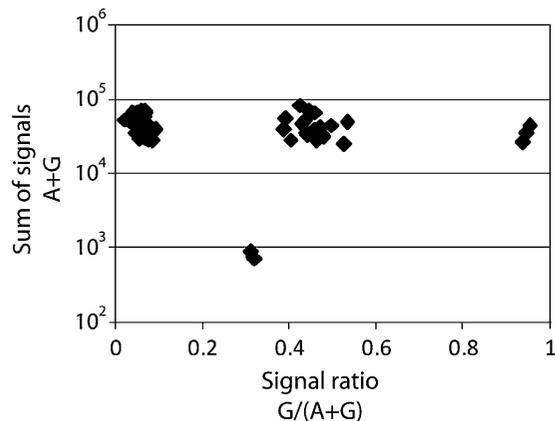


Fig. 14.5. Scatter plot for one SNP with a G/A variation analyzed in 80 samples, i.e., one slide with a 384-well format and  $5 \times 16$  subarrays. The sum of the fluorescence signals from both alleles in each sample is plotted on the vertical axis. The ratio between the signal from one allele divided by the sum of the signals from both alleles is plotted on the horizontal axis. The three distinct clusters represent the three genotypes, where in this example three non-template control samples fall below the clusters.

When using the ScanArray Express or QuantArray program for signal analysis, or if the signal quantitation output files have been converted to fit their format, the genotyping results can be visualized using the SNPSnapper software customized for this method. The SNPSnapper software can be used for exporting the SNP genotypes together with allele fractions and ratios to a text file for further analysis.

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## 4. Notes



1. Some of the SNPs in the databases may not be polymorphic in the population from which the study samples originate. The SNP allele frequencies in a particular population may be determined by analyzing pooled DNA samples using quantitative minisequencing in microtiter plates, or in the microarray format (11, 19).
2. A touchdown PCR procedure may be used (20). One strategy when designing primers for multiplex PCR is to aim at as similar primer melting temperature and G/C content as possible. Complementary 3' sequences in the primers can be avoided by designing primers with the same 3' terminal nucleotides (21). Other options are to introduce common tails on the 5' ends of all PCR primers, and subsequent amplification with one common primer for all the fragments at an elevated temperature (22), or the use of universal 5'-sequences making the PCR primers eligible for the same reaction conditions (23).
3. We recommend excluding SNPs located in repetitive elements identified by the RepeatMasker program (<http://www.repeatmasker.org>).
4. To avoid strong hairpin-loop structures, evaluate the complete minisequencing primer, including the Tag-sequence. Secondary structures that involve the 3' end of a primer may lead to mis-incorporation of nucleotides. A primer design software that predicts secondary structures (mfold: <http://mfold.bioinfo.rpi.edu/> or NetPrimer <http://www.premierbiosoft.com/net-primer/netprimer.html>) can be used.
5. The array may also be manufactured with immobilized minisequencing primers. In this assay variant the genotyping reaction is performed directly on the array surface (24). This may be useful when SNPs located less than 20 bp apart are to be genotyped.
6. Microarrays may be purchased from a commercial supplier or manufactured in-house. There are several different slide types

and attachment chemistries. Some of them have been tested in our system (25).

7. The number of spots in each subarray can be varied by changing the subarray pattern from a 384- to a 96-wells format, thus the number of SNPs to be analyzed per subarray is increased, but the maximum number of samples that can be analyzed simultaneously is decreased (26).
8. Elastosil RT601 may be used instead of RT625 to give a slightly harder silicon rubber to decrease deformation of the wells when the rack lid is tightened. If large subarrays, utilizing all available surface, are printed, deformation of the wells may cause the cTags at the corners of the subarray to be covered by the silicon. The softer, RT625, silicon sticks better to the glass surface, and decreases the risk of leakage between wells.
9. Depending on the number of SNPs to be interrogated, an inverted 96-well microtiter plate may be used as silicon rubber mould to allow larger subarrays (26).
10. Instead of multiplex PCR, single fragment PCR can be used with subsequent pooling of the amplified fragments, possibly after concentration using ethanol precipitation or spin dialysis. This is especially recommended when genotyping SNPs in cDNA (RNA) for detection of allele-specific gene expression (12). Also if a large number of multiplex PCR products are pooled, it may be advantageous to concentrate the pool prior to the subsequent steps.
11. Alkaline phosphatase inactivates the remaining dNTPs and exonuclease I degrades the single-stranded PCR primers, which would disturb the subsequent minisequencing reactions.
12. Cy5-ddUTP can be used at a 1.5–2-fold higher concentration than the other ddNTPs to compensate for its lower incorporation efficiency. Instead of using four differently labeled nucleotides in the same reaction, depending on the available microarray scanner, a single label or two labels may be used in four or two separate reactions respectively (27).
13. If the obtained fluorescent signals are weak, the number of cycles may be increased. We have used up to 99 cycles, but increasing the number of cycles often also increases the background signals.
14. Background problems can arise if the hybridization chamber is not kept humid which causes drying out of the samples on the slide.
15. The flanking sequence as well as the fluorophores attached to the dideoxynucleotides affect the efficiency and sequence

specificity of nucleotide incorporation by the DNA polymerase. The different properties of the fluorophores, such as molar extinction coefficients, emission spectra and quantum yield, as well as unspecific background may also affect the obtained signal intensities and signal ratios (11).

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