

SNP Genotyping Using the Sequenom MassARRAY iPLEX Platform

Stacey Gabriel,¹ Liuda Ziaugra,¹ and Diana Tabbaa¹

¹Broad Institute of MIT and Harvard, Cambridge, Massachusetts

ABSTRACT

The method for SNP genotyping described in this unit is based on the commercially available Sequenom MassARRAY platform. The assay consists of an initial locus-specific PCR reaction, followed by single base extension using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Using MALDI-TOF mass spectrometry, the distinct mass of the extended primer identifies the SNP allele. *Curr. Protoc. Hum. Genet.* 60:2.12.1-2.12.18. © 2009 by John Wiley & Sons, Inc.

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INTRODUCTION

Many high-throughput single-nucleotide polymorphism (SNP) genotyping technologies are currently available. Each offers a unique combination of scale, accuracy, throughput, and cost. However, the state of SNP genotyping is in flux, with no single technology or platform that can satisfy all users and study designs. The Sequenom MassARRAY platform has several attractive features for users desiring an accurate custom SNP genotyping assay (Gabriel et al., 2002) with modest multiplexing and minimal assay setup costs due to unmodified oligonucleotide primers. It utilizes a homogeneous reaction format with a single extension primer to generate allele-specific products with distinct masses, multiplexed PCR reactions, a single termination mix and universal reaction conditions for all SNPs, small reagent volumes, and a throughput of >100,000 genotypes/day/system.

The method described in this unit is based on the commercially available Sequenom MassARRAY platform and reflects modifications made by the authors in establishing a high-throughput genotyping laboratory utilizing four MassARRAY systems. The assay (Tang et al., 1999) is based on primer extension and offers two levels of specificity. First, a locus-specific PCR reaction (see Basic Protocol 2) takes place, followed by a locus-specific primer extension reaction (iPLEX assay; see Basic Protocol 3) in which an oligonucleotide primer anneals immediately upstream of the polymorphic site being genotyped (see Basic Protocol 1 for preparation of probe and genomic DNA). In the iPLEX assay, the primer and amplified target DNA are incubated with mass-modified dideoxynucleotide terminators (Fig. 2.12.1). The primer extension is made according to the sequence of the variant site, and is a single complementary mass-modified base. Through the use of MALDI-TOF mass spectrometry, the mass of the extended primer is determined. The primer's mass indicates the sequence and, therefore, the alleles present at the polymorphic site of interest. Sequenom supplies software (SpectroTYPER) that automatically translates the mass of the observed primers into a genotype for each reaction.

PRE-PCR: DNA AND OLIGO POOL PREPARATION

This protocol describes preparation of working DNA and oligonucleotide plates for genotyping on the Sequenom platform.

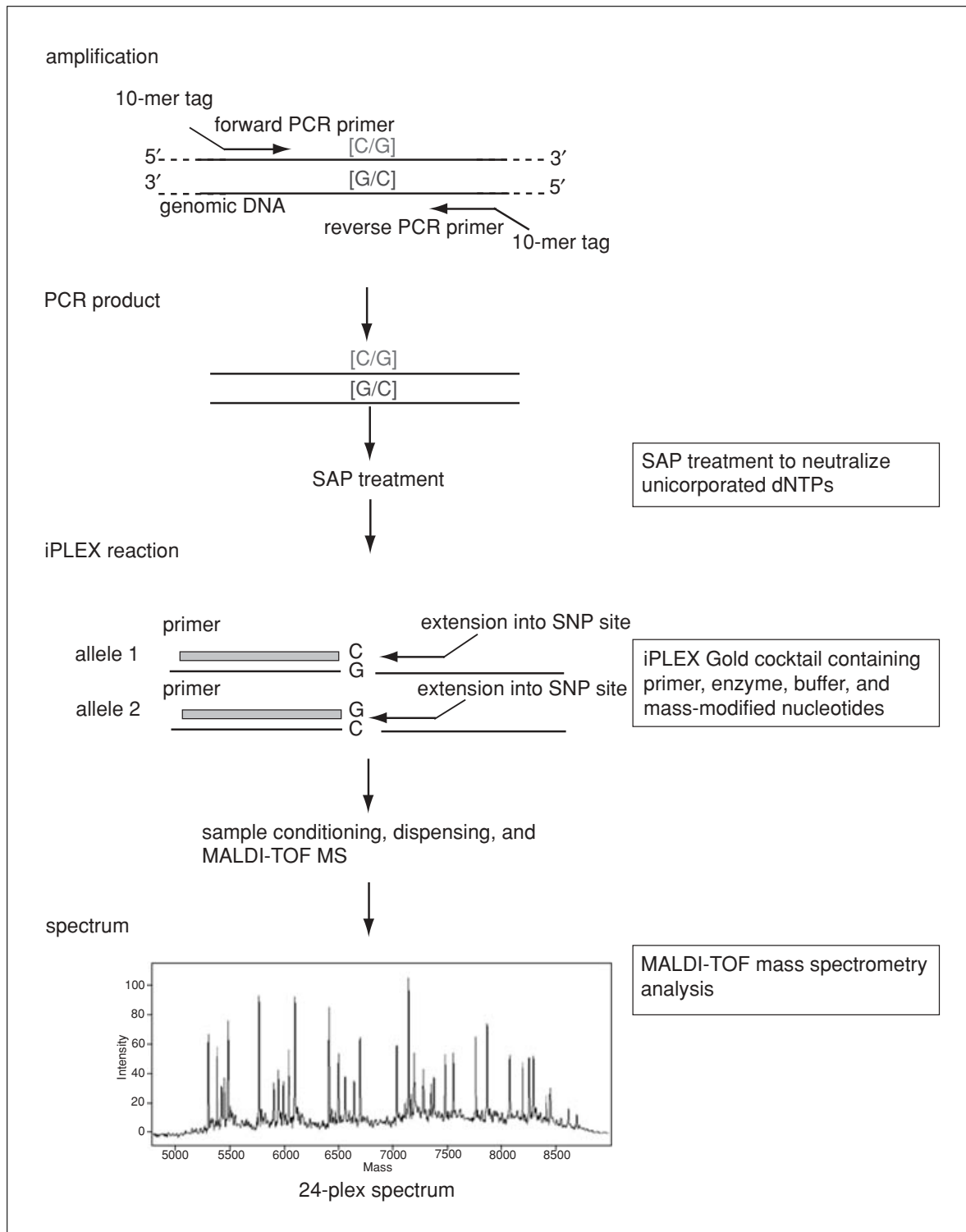


Figure 2.12.1 The MassEXTEND iPLEX reaction. A schematic of the genotype reaction of a C-to-G SNP.

DNA

Genomic DNA, whole-genome amplification (WGA) DNA, or cDNA samples are diluted to 2.5 to 5 ng/ μ l concentration in TE buffer at a concentration of 0.25 \times or less (the UV A_{260}/A_{280} ratio should be between 1.7 and 2.0). The DNA is divided into aliquots (also see Basic Protocol 2, step 1) at 2 μ l/well into a 384-well PCR reaction plate from a deep-well PCR source plate (Marsh Biomedical). Packard or Tecan 8-tip robotics can be used for DNA dilution and transfer into the deep-well source plate. 96- or 384-tip robots can be used to transfer the 2- μ l DNA aliquot into the wells of the 384-well PCR reaction plate. DNA needs to be amplified by PCR prior to performing an extension reaction of the SNP of interest. Keep DNA-containing plates at 4°C.

Oligonucleotides

Sequenom's MassARRAY Designer software automatically designs PCR and extension primers (probes) for each SNP to be investigated (see Background Information). All oligos for PCR and iPLEX reactions are ordered unmodified, with standard purification (e.g., Integrated DNA Technologies). Forward and reverse PCR primers are ordered in 96-well deep-well plates, mixed by the vendor at final, equimolar 240 μ M concentrations. Collapsed PCR primers undergo further dilution after the pooling to a working concentration of 1 μ M each. Probes (for iPLEX extension) are ordered unmixed in 96-well deep-well plates at 250 to 450 μ M. Lab technicians or automation robots perform PCR and extension oligo pooling according to the assay pool plexes and oligo plate maps. The PCR assay pool plexes consist of the multiplexed forward and reverse PCR oligonucleotide primers for each reaction present together in one multiplexed assay pool. The Single Base Extension pool plexes consist of the multiplexed oligonucleotide primers, which anneal adjacent to the polymorphic site for each reaction present together in the multiplexed assay pool. By pooling locus-specific primers, many individual loci of DNA with their corresponding SNP sites may be analyzed in a one-well reaction. Due to the inverse relationship between peak intensity and analyte mass, extension primers in iPLEX assays are adjusted by concentration to ensure that the extension primers are as equal in intensity as possible. The primers are sorted into three groups based on mass, with the highest mass group diluted to 15 μ M, the middle group to 10 μ M, and the low mass group to 5 μ M. Extension oligo pools are spotted on SpectroCHIPs and run on detectors to verify uniformity of intensities of low/medium/high mass probes and accuracy of the manual pooling procedure (probes' masses must match expected spectra). Any oligos of low-peak intensity are spiked in by adding volume to equalize the probe peak intensities. For example, if a given peak is about half the intensity of the other peaks, an equal volume is spiked in to double the peak intensity (see Fig. 2.12.2 and below). Store diluted working oligos at 4°C and concentrated stocks at -20°C .

Adjusting Oligo Concentration within Each Assay Pool

1. Divide the available volume of the highest mass oligo Bin by 3 to get the volume for the lowest mass Bin 1, and divide by 1.5 to get the volume for the second Bin 2.
2. Determine the final volume by multiplying the volume of Bin 3 (highest mass oligos) by the initial oligo concentration and dividing by 15 μ M.
3. Multiply the volumes obtained for each bin by the number of oligos in each bin and add these values together. Subtract this total from the final volume to get the volume of water (if any) to add.
4. Add 5 μ l of the oligo pool mix to 95 μ l water. Mix well.
5. Aliquot 20 μ l to several wells of a 384-well PCR plate (one pool per row). Define the plate in the Sequenom Typer Plate Editor database as to which pools are in which wells. Add resin, and run on the Mass Spectrometer as done for sample plates.

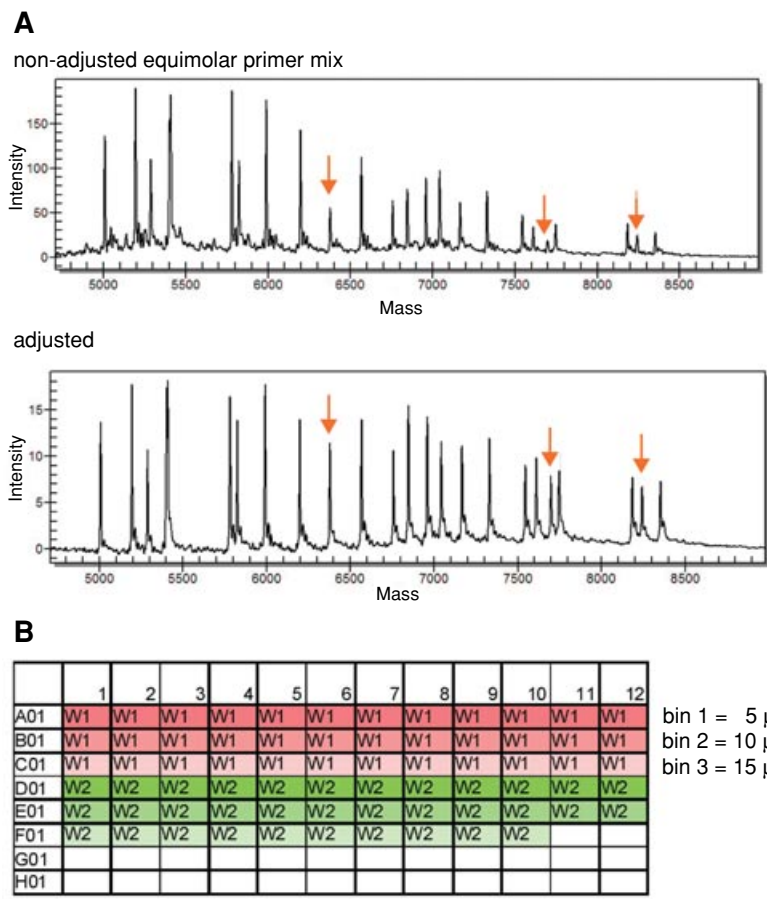


Figure 2.12.2 Adjustment of extend oligo concentrations within each pool. **(A)** Spectra of adjusted and non-adjusted oligos in an assay pool (figure from Sequenom's iPLEX Gold Application Guide). The arrows indicate obvious peak intensity differences before and after primer concentration adjustment. **(B)** Sorting of the oligos into three bins, with oligo masses increasing within each pool from left to right by column and then downwards by row within the plate array. The three bins within each separate pool are shown with varying color intensity for distinction. Bin 1, being the darkest, is pooled at the lowest concentration, bin 2 is pooled at medium concentration, and bin 3, being the lightest, is pooled at the highest concentration.

- Examine the relative peak heights in Typer over several wells to determine if any extend oligos need to be spiked in amount (generally done for peaks that have less than half the intensity of other peaks). Add a corresponding amount of that particular extend oligo to the pool.

For example, add an equal amount of the original amount to double the concentration of that oligo.

BASIC PROTOCOL 2

SNP Genotyping Using Sequenom MassARRAY iPLEX Platform

2.12.4

AMPLIFICATION OF TARGET LOCI BY PCR

This procedure describes the steps involved in amplifying a specific fragment of genomic DNA for the purpose of genotyping that fragment on the Sequenom platform. The goal of an optimal multiplex PCR reaction is to evenly amplify many individual loci of DNA with minimal nonspecific byproducts. Purified amplicons are then used as templates for the primer extension reaction (see Basic Protocol 3). This protocol describes setup of reaction plates, either when one primer pool is applied across 384 DNA samples (Fig. 2.12.3A), or when four primer pools are used for 96 DNA samples in offset quadrants on 384-well PCR plates (Fig. 2.12.3B). Due to the configuration of the Multimek robot described

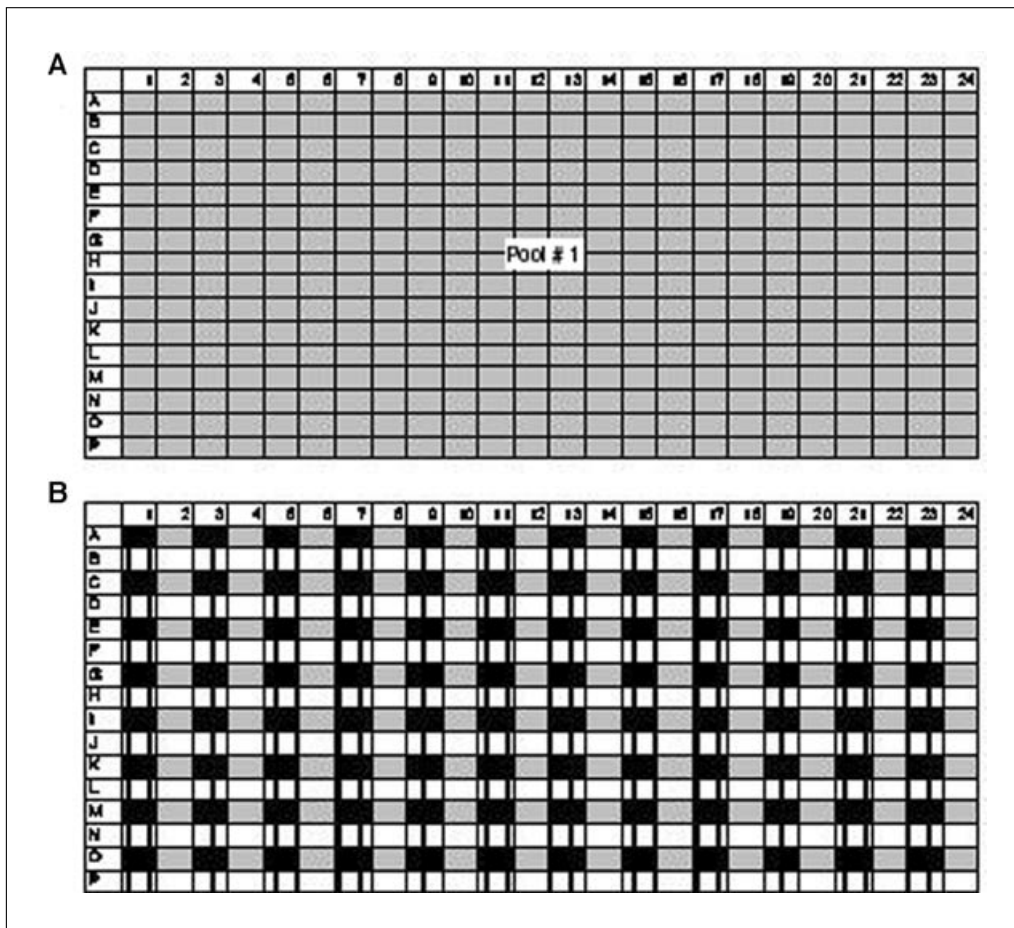


Figure 2.12.3 Recommended configurations for setup of 384-well PCR plate. (A) Same assay pool for all wells, different DNA in the wells; (B) Same 96 DNA samples arrayed in offset quadrant wells, four different assay pools.

below, the “tight-quadrant” format requires specialized automation (or manual transfer) for transfer of primer pools to the 384 plate.

This protocol is intended for assaying at least one 384-well plate, and can be scaled up to encompass many plates.

NOTE: As with all PCR methodology, exercise great care to ensure a lack of contamination of the preparations with unwanted DNA. A separate “pre-PCR” area should be reserved for setting up PCR (see *APPENDIX 2D* for further discussion of special considerations for PCR experiments).

Materials

- DNA source plate: 384-well deep-well PCR plate containing 2.5 ng/μl DNA of interest (see Basic Protocol 1; store at 4°C)
- 100 mM dNTPs (Applied Biosystems; store at –20°C)
- 25 mM MgCl₂ (QIAGEN; store at –20°C)
- Ultrapure PCR-grade H₂O (Invitrogen)
- 5 U/μl HotStarTaq Plus DNA polymerase with 10× PCR buffer (QIAGEN; store at –20°C)
- Forward and reverse primers: 1 μM each in multiplex pool (see Basic Protocol 1 and Background Information; store at 4°C)
- 384-well PCR reaction plate (Eppendorf twin.tec)
- 96-tip or 384-tip pre-PCR Tomtec or Hydra-type (Matrix Technologies) robotic workstation (dedicated to DNA)

96-tip pre-PCR Multimek robotic pipettor with stacker (Beckman-Coulter) and disposable aerosol-barrier “Beckman-style” tips (LabSource)
96-well ABGene Thermo-Fast skirted plates (AB-0800)
1.5-ml microcentrifuge tubes *or* 15- or 50-ml conical polypropylene centrifuge tubes (e.g., Falcon)
MicroAmp adhesive plate sealers (Applied Biosystems)
Pre-PCR tabletop centrifuge with microtiter plate carriers
ABI Viper dual *or* Thermo Scientific Hybaid 384-well blocks, PC-controlled thermal cyclers

Add DNA to reaction plates

1. Create program for robotic workstation (Tomtec or Hydra-type dedicated to DNA) to dispense 2 μ l of DNA sample per well of 384-well PCR plate, using either of the configurations shown in Figure 2.12.3: 384 DNA samples to 384-well PCR plate applying one pool per plate *or* 96 DNA samples to 384-well PCR plate with offset quadrants, applying four pools. Dispense the DNA in the selected pattern

Add PCR master mix to reaction plate

2. Create program on pre-PCR Multimek automated pipettor that allows addition of 4 μ l of master mix from a 96-well ABGene plate into each well of the 384-well reaction plate.

The V-bottom ABGene source plate allows for lower source plate volumes.

An example layout of a Multimek with plate stackers is shown in Figure 2.12.4.

3. Calculate the volumes needed for the number of wells in the plate for each assay pool from Table 2.12.1, corresponding to the dispensed DNA pattern chosen from Figure 2.12.3 (also see step 1).
4. Combine PCR master mix components in the amounts and in the order shown in Table 2.12.1 in 1.5-ml microcentrifuge tubes or 15- to 50-ml conical polypropylene centrifuge tubes, depending on the number of plates and assays to be run. For the offset quadrants, make four mixes using four primer pools. Aliquot the appropriate volume per well into the 96-well ABGene source plate.

IMPORTANT NOTE: *If running more than twelve 384-well plates, it is necessary to prepare two plates of master mix, because the ABGene plates should not be used to hold >220 μ l per well. Wear gloves when working in pre-PCR area.*
5. Calibrate the Multimek pipettor according to the manufacturer’s instructions. Make sure that the wash bucket is full of water and the waste bucket is empty before beginning. Put on aerosol-resistant tips.
6. Put 384-well PCR plates containing the source DNA (see step 1) in the plate carriers and place in the left-most stacker.
7. Place PCR mix, in an ABGene plate, in Position 1 on the robot deck.
8. Open the Start menu, select Programs, and then open the Stacknet Software. Press Start when the Stacknet window opens up.
9. From the Stacknet window, select Program, and then select the Computer radio button.
10. In the Computer menu, press Load, then select the program that was created to add 4 μ l of master mix (see step 2). Press Start.

The program will “error out” if the robot cannot pick up any more plates.

The total reaction volume per well will be 6 μ l: 4 μ l of master mix and 2 μ l of 2.5 ng/ μ l source DNA.

Table 2.12.1 PCR Master Mix^a

	H ₂ O	PCR buffer (10 ×)	MgCl ₂	dNTP mix	Primer mix (1 μM)	HotStarTaq Plus	Total	Amount per well of 96-well plate
1 well	2.222	0.825	0.429	0.132	0.66	0.132 ^b	4.4 ^c	
1 plate	1194.55	443.52	230.63	70.96	354.82	70.96	2365.44	22 μl

^aReagent volumes in microliters.

^bFor iPLEX Gold >24 plex reaction, increase Taq to 0.220 μl per well.

^cOnly 4 μl is added to each well.

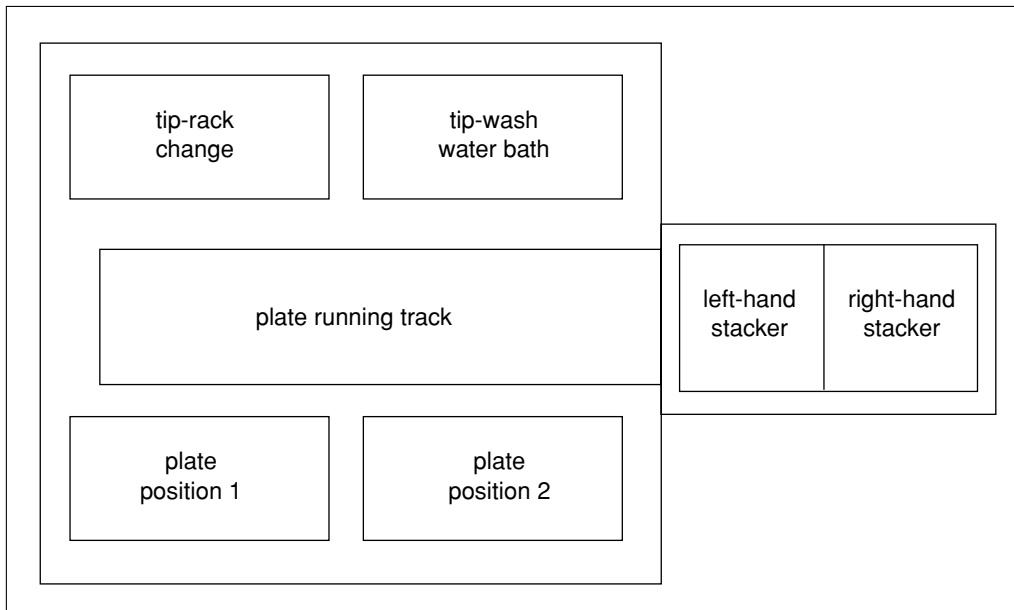


Figure 2.12.4 96-tip head Beckman Multimek with stacker deck layout. This configuration is used throughout the protocol.

11. Empty the waste bucket and place the wash tube into a new water bottle at the end of the run. Remove the reaction plates from the stacker, and carefully remove them from the carriers. Seal plates carefully with MicroAmp sealers. Dispose of the used ABGene plate. Vortex the plates on a plate vortexer.
12. Centrifuge the plates 1 min at $425 \times g$, room temperature, in a pre-PCR tabletop centrifuge with microtiter plate carriers, to bring the solutions to the bottom of the wells.

Perform thermal cycling (PCR reaction)

13. Place the plates in the ABI or Hybaid 384-well block. Set up lid mode as Constant at 100°C. Enter 6 μl volume. Perform PCR with the following cycling program (runs ~2.5 hr):

1 cycle:	5 min	94°C	(initial denaturation)
45 cycles:	20 sec	94°C	(denaturation)
	30 sec	56°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	3 min	72°C	(final extension)
Final step:	indefinite	4°C	(hold).

14. Centrifuge the plates as in step 12 to bring the solutions to the bottoms of the wells, then store at 4°C until ready for SAP cleanup procedure (Support Protocol 1).

POST-PCR: SAP REACTION CLEANUP

Shrimp alkaline phosphatase (SAP) dephosphorylates unincorporated dNTPs by cleaving the phosphate groups from the 5' termini. Treatment with SAP is performed in order to remove remaining, nonincorporated dNTPs from amplification products.

This procedure is performed on a post-PCR 96-tip SpectroPREP Multimek robot. The Multimek dispenses 2 µl of a SAP cocktail into each individual well of the 384-well post-PCR reaction plate. After SAP cocktail is added, the plate is removed from the robot and centrifuged. Treated plates are placed in a 37°C incubator for 50 min, and right after this are placed in an 85°C incubator for an additional 20 min to inactivate SAP enzyme. Alternatively, incubation can take place on a 384-well block thermal cycler.

Materials

- 1 × SAP buffer (Sequenom)
- 1.7 U/µl shrimp alkaline phosphatase (SAP; Sequenom; store at 0°C)
- PCR products in 384-well PCR plates (see Basic Protocol 2)
- 1.5-ml microcentrifuge tubes *or* 15- or 50-ml conical polypropylene centrifuge tubes (e.g., Falcon)
- 96-well ABGene plates (AB-0800)
- 96-tip post-PCR Multimek robotic pipettor with stacker (Beckman-Coulter)
- MicroAmp adhesive plate sealers (Applied Biosystems)
- Post-PCR tabletop centrifuge with microtiter plate carriers
- 37° and 85°C Precision incubators (VWR) *or* ABI *or* Hybaid thermal cycler with 384-well blocks

Set up SAP reaction

1. Combine SAP reaction components in the amounts and in the order shown in Table 2.12.2 in 1.5-ml microcentrifuge tubes or in 15- to 50-ml conical polypropylene centrifuge tubes, depending on the number of plates and assays to be run.
2. Based on the number of plates to be run, aliquot the correct amount of SAP reaction cocktail per well of a 96-well ABGene plate.
3. Place the 384-well reaction plates onto carriers and load them onto the stackers in the post-PCR Multimek SpectroPREP robot. Place the 96-well ABGene plate with the SAP cocktail on to the Multimek in Position 1.
4. Select the SAP program from the StakNet Control Program to add 2 µl of SAP cocktail to each well.

Once the 2 µl of cocktail is dispensed, the robot then mixes the 6 µl of post-PCR product that was already in each of the wells with the 2 µl of added SAP cocktail, creating a homogeneous mixture.

5. Remove the reaction plates from the stacker and carefully remove them from the carriers. Seal the plates carefully with MicroAmp sealers. Dispose of the used ABGene plate. Vortex the plates on a plate vortexer.

Table 2.12.2 SAP Reaction Cocktail^a

	Water	10× SAP buffer	SAP (1.7 U/µl)	Total amount of mix
1 well	1.53	0.17	0.3	2
1 plate	1107.72	123.08	217.2	1448

^aReagent volumes in microliters.

6. Centrifuge the plates 1 min at $425 \times g$, room temperature, in a post-PCR tabletop centrifuge with microtiter plate carriers, to bring solutions to the bottom of the wells.

Incubate SAP reactions

Using incubators

- 7a. Incubate the plates in a 37°C Precision incubator for 50 min, then immediately transfer to an 85°C incubator and incubate for 20 min.

IMPORTANT NOTE: *Do not stack plates in the incubators; spread them in one layer only.*

- 8a. Remove the plates from the 85°C incubator and cool to room temperature. Centrifuge as in step 6, then store at 4°C until ready to process for primer extension procedure (see Basic Protocol 3).

Using thermal cycler

- 7b. Place the plates in the ABI or Hybaid 384-well block. Execute the following program:

1 cycle:	40 min	37°C
1 cycle:	10 min	85°C
Final step:	indefinite	4°C.

- 8b. Centrifuge as in step 6, then store at 4°C until ready to process for primer extension procedure (see Basic Protocol 3).

PRIMER EXTENSION

The primer extension or iPLEX reaction is a universal method for detecting single-base polymorphisms or small insertion/deletion polymorphisms in amplified DNA. After PCR cleanup (Support Protocol 1), a primer extension reaction cocktail (containing extend primer, buffer, enzyme, and mass-modified ddNTPs) is added to the amplification products. During the iPLEX reaction, the primer is extended by one mass-modified nucleotide depending on the allele and the design of the assay.

This protocol describes the reaction plate setup when either one primer pool is applied across 384 DNA samples or four primer pools are used for 96 DNA samples in offset quadrants on 384-well PCR plates (see Fig. 2.12.3 A and B). This protocol uses a Multimek robot. The reaction cocktail is easy to scale up for any number of PCR plates.

Materials

- Ultrapure PCR-grade H₂O (Invitrogen)
- iPLEX enzyme (Sequenom)
- 10× iPLEX buffer (Sequenom)
- iPLEX Extension Mix (Sequenom)
- Extend primers: from 5 to 10 μM each in multiplex pool
- PCR products in 384-well PCR plates, cleaned up by SAP reaction (see Support Protocol 1)
- 1.5-ml microcentrifuge tubes *or* 15- or 50-ml conical polypropylene centrifuge tubes (e.g., Falcon)
- 96-well ABGene plates
- 96-tip post-PCR Multimek SpectroPREP (Sequenom) with stacker
- MicroAmp adhesive plate sealers (Applied Biosystems)
- Post-PCR tabletop centrifuge with microtiter plate carriers
- ABI or Hybaid Thermal cycler with 384-well blocks

BASIC PROTOCOL 3

Genotyping

2.12.9

Table 2.12.3 Primer Extend Master Mix with Sequenom Termination Mixes

	H ₂ O	iPLEX buffer (10 ×)	iPLEX extension mix	Probe mix in 3 bins (5 μM to 15 μM)	iPLEX enzyme	Total	Amount per well of 96-well plate
1 well	0.4926	0.222	0.2	1.0444	0.041	2	
1 plate	346.79	156.29	140.8	735.26	28.86	1408	13 μl

^aReagent volumes in microliters.

Set up primer extension reaction

1. Calculate the amount of cocktail needed based on the number of wells, and combine primer extension reaction components in the amounts and in the order shown in Table 2.12.3 in 1.5-ml microcentrifuge tubes or in 15- to 50-ml conical polypropylene centrifuge tubes, depending on the number of plates and assays to be run.

Add primer extension cocktails to reaction plates

2. Aliquot cocktail into 96-well ABGene plate in the configuration corresponding to the PCR plate layout (see Basic Protocol 2 and Fig. 2.12.3). Make four mixes for the offset plate pattern.
3. Place the 384-well reaction plates (from Support Protocol 1) on to carriers, and load them on to the stackers.
4. Place the ABGene plate with the primer extension cocktail (from step 2) on to the Multimek in Position 2.
5. Select the iPLEX Cocktail Addition program from the StakNet Control Program and run the program to add 2 μl of primer extension mix to each well of the reaction plate.
6. Remove the reaction plates from the stacker and carefully remove them from the carriers. Seal plates carefully with MicroAmp sealers. Dispose of the used ABGene plate. Vortex the plates on a plate vortexer.
7. Centrifuge the plates 1 min at 425 × *g*, room temperature, in the post-PCR centrifuge with microtiter plate carriers to bring the solutions to the bottom of the wells.

Perform thermal cycling (primer extension reaction)

8. Place plates in the ABI or Hybaid 384-well block. Perform primer extension with the following cycling program:

1 cycle:	30 sec	94°C	(initial denaturation)
40 cycles:	5 sec	94°C	(denaturation)
	5 cycles:	5 sec	52°C (annealing)
		5 sec	80°C (extension)
1 cycle:	3 min	72°C	(final extension)
Final step:	indefinitely	4°C	(hold).

Note that the 5 cycles sit within the 40 cycles.

9. Clean up the primer extension productions (see Support Protocol 2).

PRIMER EXTENSION REACTION RESIN CLEANUP

This cleanup step is important to optimize mass spectrometry analysis of the extended reaction products. SpectroCLEAN (Sequenom) is a cationic resin pretreated with acid reagents. A slurry of the resin is added directly to primer extension reaction products to remove salts such as Na⁺, K⁺, and Mg²⁺ ions. If not removed, these ions can result in high background noise in the mass spectra. This procedure takes ~1.5 hr for ten plates.

Materials

Ultrapure PCR-grade H₂O (Invitrogen)
SpectroCLEAN resin (Sequenom; store at room temperature)
Post-primer extension products in 384-well plates (see Basic Protocol 3), kept at 4°C

Clean plastic bottle with cap
Turbulator nonmagnetic mixing device (ACME Automation), firmly positioned on the Multimek deck (e.g., Position 1)
96-tip post-PCR Multimek SpectroPREP (Sequenom) with stacker
Disposable “Beckman-style” tips for Multimek
Post-PCR tabletop centrifuge with microtiter plate carriers
TiterTop plate sealers (LabSource)
Plate rotator (VWR)

Make resin slurry

1. Add 80 ml of ultrapure water to a clean plastic bottle reserved for resin. Transfer resin contents from two whole containers (28 g each) of SpectroCLEAN resin into the bottle with the water.
2. Cap the bottle and swirl it gently a few times to disperse all resin particles as evenly as possible in the suspension. Wait a little bit and swirl again, making sure that all entrapped air is gone.
3. Swirl again and pour enough of the resin suspension into a Turbulator to just cover the bottom. Apply pressurized air, letting it go through the resin slurry. Slowly add more of the resin, mixing thoroughly. Remove air bubbles with a clean pipet tip.

The Turbulator is a nonmagnetic mixing device. Operating via a vacuum diaphragm pulse technique, it pulses ~4 times a second with a fully adjustable amplitude, thereby creating a mild to major effect with 96 little geysers that can protrude above the surface of the reservoir liquid. The system includes a high-quality vacuum pump, replaceable diaphragm, and tubing. Make sure Turbulator is firmly positioned on Multimek deck, for example, at Position 1 (Fig. 2.12.4).

Calibrate SpectroPREP Multimek and load/unload tips

4. Calibrate the instrument by opening the Communication Engine icon on the computer desktop designated for Multimek use and selecting Calibrate under the Multimek column.
5. Load or unload tips by double-clicking on the Method Editor icon and selecting tip load or unload. Make sure that the tip rack is placed towards the back to load tips or forward to unload tips. Click OK. Go to Script and select RUN.

Add resin

6. Begin an application by going to Start and selecting the StackNet Stacker Network Control option under Programs.
7. Select Start on the StackNet Plate Process Network Control screen. Select Program followed by Computer, then Load.

- Centrifuge post-primer extension plates 1 min at $425 \times g$, room temperature, in post-PCR tabletop centrifuge with microtiter plate carriers to bring solutions to the bottom of the wells.
- Carefully unseal plates. Place the plates on metal carriers. Stack the plates in the right-hand stacker and hit the START button in the application program window.

The Multimek will aspirate 16 μ l of resin suspension (containing 7 mg resin) and add this volume to each well of the 384-well reaction plates in a quadrant pattern. Make sure that the water pump is running well and that enough water is present in the tip wash station.

- After resin transfer, take the plates off the stacker and seal with TiterTop seal.

Inspect plates after resin addition to be sure that all wells have gotten the same amount of resin.

Rotate plates

- Place the plates on a plate rotator, balanced in pairs. Rotate for 30 min.
- After the rotation is done, turn off the rotator and remove the plates. Centrifuge the plates 3 min at $425 \times g$, room temperature, in post-PCR tabletop centrifuge.

Centrifuged plates are ready to be spotted on SpectroCHIPs (see Basic Protocol 4). The resin remains at the bottom of the well. The purified product will be removed from the supernatant by the SpectroPoint. They can be stored at 4°C for later spotting and detection (stable up to 1 month). Rotate the plates again after cold storage.

BASIC PROTOCOL 4

SPOTTING PRIMER EXTENSION PRODUCTS ON SpectroCHIPs

This protocol describes the arraying of the extended/desalted analyte products from 384-well microtiter plates on 384-sample SpectroCHIPs. In order to incorporate oligonucleotides with the appropriate matrix for MALDI-TOF (3-hydroxypicolinic acid), a small volume (~25 nl) is arrayed onto existing matrix spots on the silica chip. This process uses the capillary action of slotted pins and contact dispensing for nanovolumes.

Materials

- 100% and 50% ethanol
- Plates from primer extension, cleaned up with resin and centrifuged (see Support Protocol 2)
- 3-point calibrant (Sequenom)
- SpectroPOINT nanoliter sample dispensing instrument with appropriate software (Sequenom)
- SpectroCHIPs (Sequenom)
- Adhesive plate sealers (TiterTop from LabSource)

- Open the Spectropoint software on the PC.
- Precondition the main head and single pin with 100% ethanol by clicking the Precondition tab. Select 30 min. Empty the trough by clicking the appropriate button and dumping 100% ethanol in the trough.
- Empty and refill the sonicator with 50% ethanol from the fill tank. Click on the button within the Precondition tab to fill the trough.
- Wash the main head ten times by selecting CLEAN MAIN HEAD while 10 cycles are selected.
- Spin down resined plates (see Support Protocol 2) and load onto microtiter stage two at a time.

6. Load chips on to the pintool scout plate in the appropriate order. Label the chip packages to correspond with plate names and keep in order.

Only two plates can be loaded onto the deck at a time. Label the chip packages as the plates are loaded onto the deck to ensure the names are correct.
7. Fill the calibrant well with 80 μ l of 3-point calibrant.
8. Home the pintool XYZ axis by selecting HOME MACHINE.

This is important to do at least once a day to prevent drift.
9. Under the Run Setup tab, select which chips are to be spotted.

The main spots and calibrant spot differently so it is best to spot each separately at different speeds.
10. Select “analyte only” and press the Start button on the lower left of the screen to begin the run.
11. After the analyte is spotted on the chip, select “calibrant only.” A speed of 120 works well. Make sure the load offset (height of the pins) is enough to keep the pins out of the resin. Check that the pins are not hitting the resin.
12. Press the Start button on the lower left of the screen to begin the run.
13. Once the chip is spotted, cover the plate with adhesive sealer and remove any extra calibrant from the calibrant well. Rinse the calibrant well thoroughly with clean water.

DETECTION OF PRIMER EXTENSION PRODUCTS BY MASS SPECTROMETRY

This protocol describes detection of extended products using a MassARRAY Compact mass spectrometer (Sequenom; manufactured by Bruker Instruments) and Sequenom real-time detection software.

The chip is placed into the mass spectrometer and each spot is then shot with a laser under vacuum by the matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) method. A laser beam serves as desorption and ionization source in MALDI mass spectrometry. The matrix plays a key role in this technique by absorbing the laser light energy and causing part of the illuminated substrate to vaporize. A rapidly expanding matrix plume carries some of the analyte into the vacuum with it and aids the sample-ionization process. The matrix molecules absorb most of the incident laser energy, minimizing sample damage and ion fragmentation (i.e., soft ionization).

Once the sample molecules are vaporized and ionized, they are transferred electrostatically into a time-of-flight mass spectrometer (TOF-MS), where they are separated from the matrix ions, individually detected based on their mass-to-charge (m/z) ratios, and analyzed. High transmission and sensitivity, along with theoretically unlimited mass range, are among the inherent advantages of TOF instruments. Detection of an ion at the end of the tube is based on its flight time, which is proportional to the square root of its m/z .

Materials

- Compact mass spectrometer (Sequenom)
- Spotted SpectroCHIPs (see Basic Protocol 4)
- Scout plate (Sequenom)
- Tweezers
- Router.exe, FlexControl, ServerControl, SpectroCaller 3.4 (Sequenom), SpectroAcquire 3.4, and Typer ChipLinker software

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1. Start the Compact mass spectrometer according to the manufacturer instructions. Take the scout plate that is used to hold the chips out by hitting the Target IN/OUT button on the front of the machine.

There is also an eject button in the FlexControl software labeled “carrier” that will probe out. The Target button will flash as this occurs. Only after the light starts blinking should the door be opened.

2. Open the door and take the scout plate out. The scout plate for the Compact holds two chips. Remove any previously detected chips, load two chips onto the scout plate, and place their holders on top of the machine in the order they were loaded onto the scout plate. Check that the chips are seated correctly on the scout plate. Handle the chips using tweezers without touching the chip pads on the top surfaces.
3. Send the scout plate into the compact by hitting the Target IN/OUT button.
4. Open software in this exact order: Router.exe, FlexControl, ServerControl, Spectro-Caller3.4, SpectroAcquire3.4, Typer ChipLinker.
5. Use the Chiplinker software to select which chips are to be run. Select the plate definition for the chips to be detected.
6. Fill in the Experiment Name field with the plate name or a descriptive field for experiments or tests.

The Chip Barcode field can be filled in to have the date or any other desired information.

7. Click Add to add them to the list below. Then click Create to create the files needed for SpectroAcquire.
8. Copy and paste the Chip Barcode entries into the “chip” field on the Auto Run Setup tab of the SpectroAcquire window. Click Barcode Report and this will check that your input to the chip field matches a created file.

A separate window will pop up with either OK or Error.

9. On the Auto Run tab, click Start Run.

Allow 30 to 60 min for detection of one SpectroCHIP. Detection time correlates to the complexity of the multiplex reaction.

The resulting spectra are analyzed by SpectroTyper software, which combines the base caller with a clustering algorithm.

COMMENTARY

Background Information

The MassARRAY Designer software can automatically design both PCR and MassEXTEND primers for multiplexed assays. The software checks for and avoids primer combinations and nontemplate extension products that could possibly result in non-specific extension. The new 3.1 version incorporates an Exchange Plex function allowing users to balance multiplex levels of SNP groups to minimize the number of reactions. The software has a proven design efficiency of >95% of all confirmed SNPs.

Sequenom RealSNP software is used to mark sequences for proximal SNP primer design avoidance, scan PCR primers against a specified genome to validate that only a unique

amplification product will be produced that would contain the target for an extend probe primer, and for validation of multiplex designs with respect to unintended amplification products that could lead to false positives during mass spectra analysis. The output file can be uploaded to the Sequenom database and may also be used for primer ordering.

Typically, the input FASTA file includes 500 base pairs of specific genomic sequences, 250 upstream and downstream from the SNP of interest. The SNP is designated by brackets (e.g., [A/G]). The PCR primers (~30-mers) include a universal 10-mer sequence tag, which aids in even amplification of multiplex reactions. The following are standard parameters for multiplex assays design; however, the

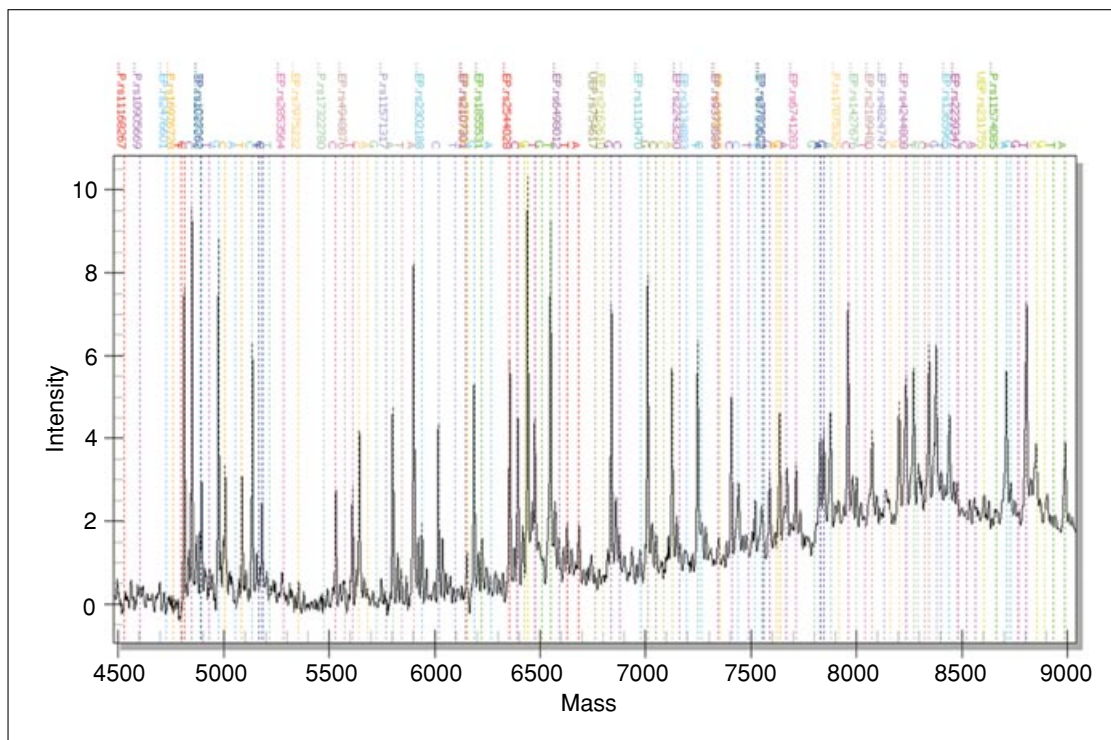


Figure 2.12.5 Example of a 36-plex reaction. The positions of all probes and possible analyte peaks are labeled. Mass is in daltons (Da). For the color version of this figure go to <http://www.currentprotocols.com>.

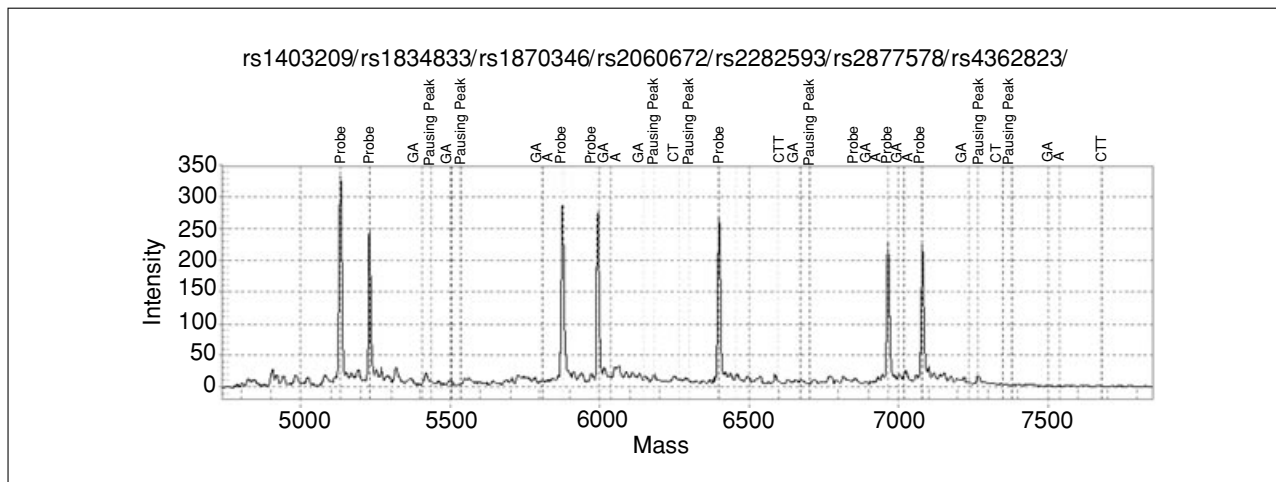


Figure 2.12.6 Lack of primer extension. In this spectrum, only unextended probes are observed. This should also be observed in the negative control well.

software is capable of designing as many as 40-plex reactions or single plex reactions:

1. MassEXTEND.
2. Multiplex Level: maximum = 24; minimum = 1.
3. Design filter: 2 alleles.
4. Length of the amplicon (bp) for SNP capture: minimum = 80; optimal = 100; maximum = 120 with the hME-10 primary and secondary tags.
5. Extend primer (probe) design length is 17 to 30 (bp) with T_m of 45° to 100°C by NN (Nearest Neighborhood algorithm).

6. Peak masses: minimum Separation by 30 Da with an upper limit of 8500 Da.

Anticipated Results

Genotype calls are made in real time during chip detection. Traces are available for viewing immediately after detection using a suite of software tools, SpectroTYPER-RT (Sequenom). The software allows simultaneous visualization of all assays in a multiplex reaction (Fig. 2.12.5) and offers flexibility in narrowing down on one or a few assays. A

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successful iPLEX reaction can contain up to 40 multiplexed reactions. The design software can more easily combine assays into the higher plexes when more SNP sequences are available as input. If the Real SNP design checks for proximal SNPs and specificity of primers are employed, good results are usually obtained for all of the assays in the plex group. The software also allows user overwriting of the automatic calls. Visualization of the spectra can aid troubleshooting to identify the most likely errors in the process. Some common areas of troubleshooting are described below.

Critical Parameters and Troubleshooting

PCR reaction

Although it is difficult to troubleshoot an individual PCR reaction within the context of the multiplex PCR reaction, one sign of a weak PCR reaction is a large excess of unextended primer. Although some small amount of unextended primer can often be observed in all reactions, a large unextended primer peak with very small analyte peaks can indicate a weak PCR. In these cases, the assay can often be

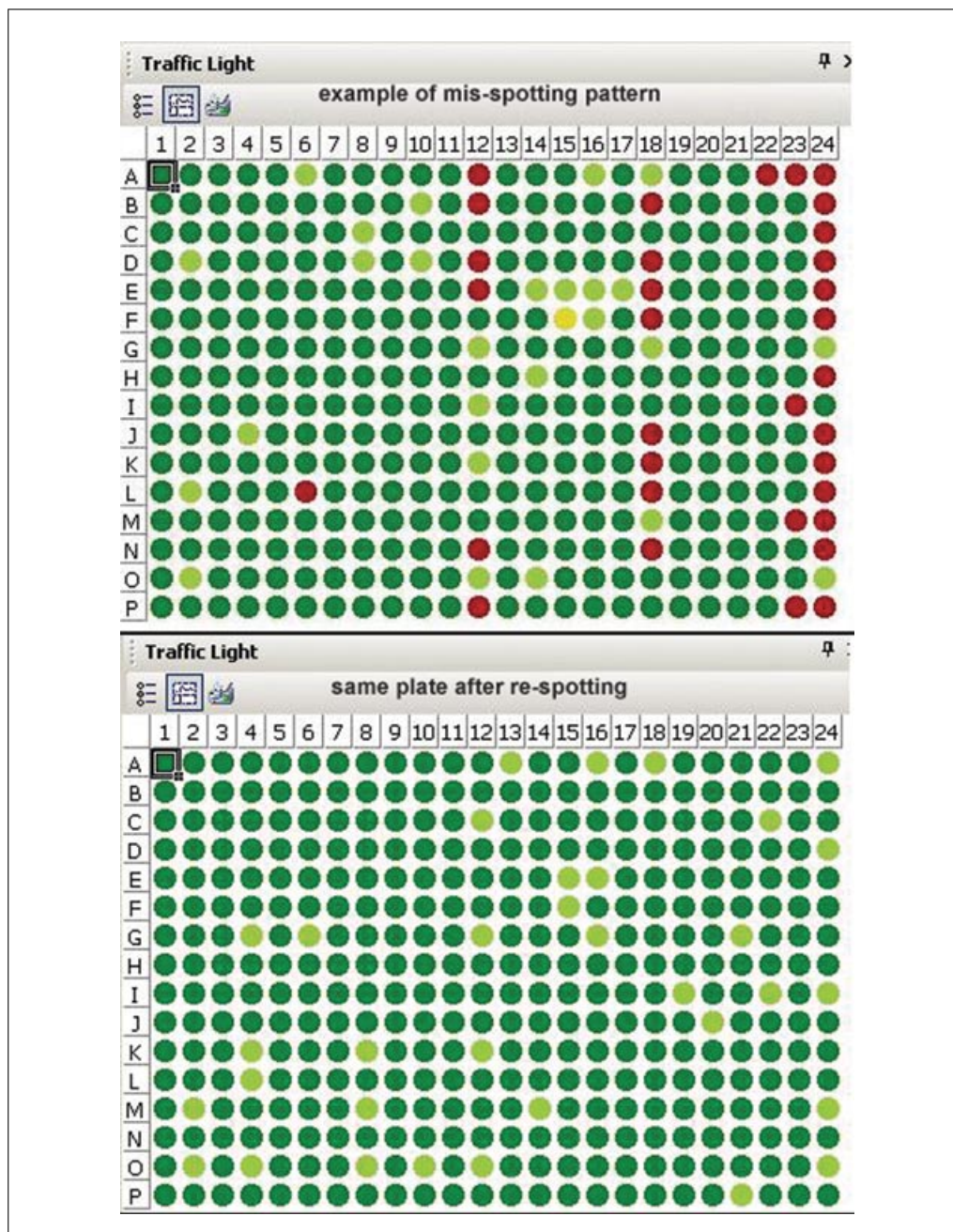


Figure 2.12.7 Example of a plate mis-spotting pattern corrected by re-spotting. For the color version of this figure go to <http://www.currentprotocols.com>.

rescued by genotyping in a lower multiplex pool.

PCR cleanup

If the SAP PCR cleanup (see Support Protocol 1) fails, excessive quantities of dNTP will be present in the iPLEX reaction. The dNTP would be likely to interfere with incorporation of the terminating ddNTPs, and would most likely result in long extension of the primer beyond the position of the SNP. In this case, few or no analyte peaks would be observed.

iPLEX reaction

Failure of the iPLEX chemistry would result in spectra with no extension, so only unextended primer peaks would be observed (Fig. 2.12.6). Failure in dispensing the iPLEX probe would result in a complete lack of peaks. If the samples are mis-spotted, there would also be a complete lack of peaks. Mis-spotting patterns are often seen in columns of six (corresponding to the pins of the SpectroPOINT), and are corrected by respotting (Fig. 2.12.7).

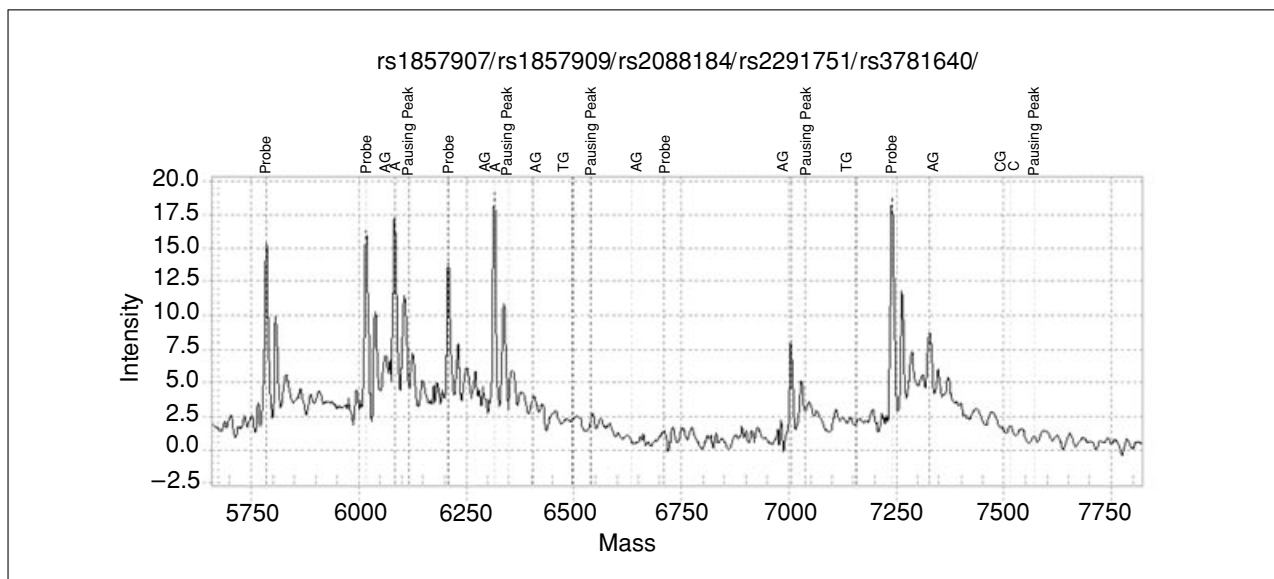


Figure 2.12.8 Spectrum with excessive background noise. This is typical of a failure to complete the resin cleanup step after primer extension.

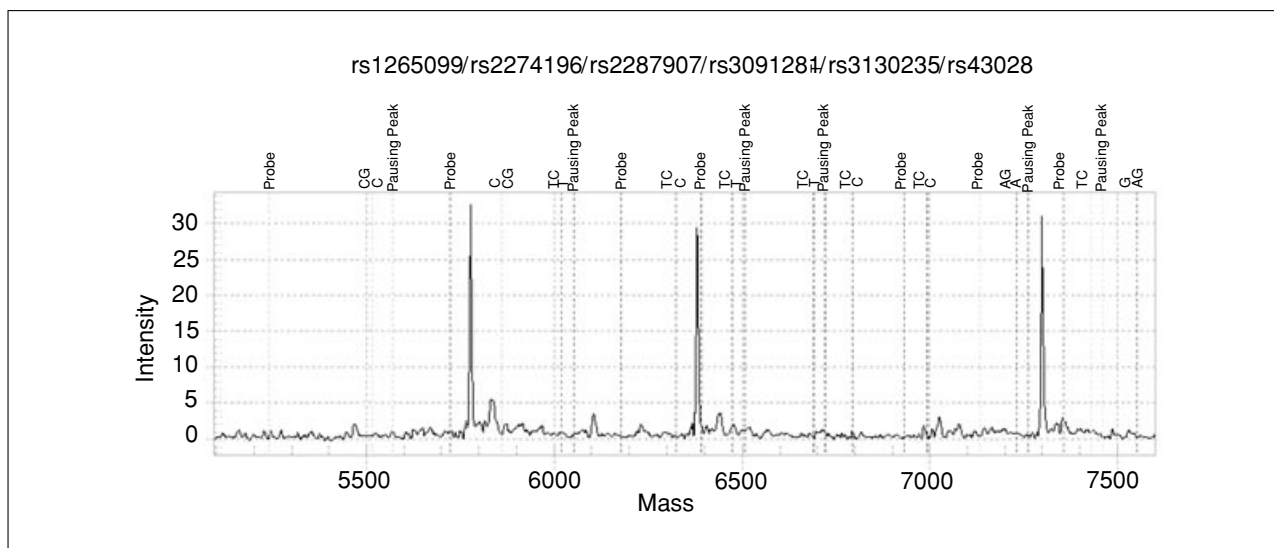


Figure 2.12.9 Misalignment of peaks. In this spectrum, the positions of the observed peaks are unaligned with the expected positions. This could result from incorrect plate identification or the addition of an incorrect primer mix.

iPLEX clean up

Failure in *iPLEX* cleanup results in excessively high background in spectra, due to the salt in the reaction (Fig. 2.12.8).

Chip Spotting/mass spectrometry

The SpectroTYPER software uses the assay design information to calculate the expected position of the correct analyte peaks in the spectra. If a plate is loaded incorrectly, or if a plate is spotted on a mislabeled chip, the resulting spectra will contain unaligned peaks (Fig. 2.12.9).

Other considerations

Because the Sequenom MassARRAY platform requires a multistep process, checks should be put in place to allow verification of plate identity. One example of a simple routine plate check is to code plates in such a way that negative control wells, and duplicate DNA samples (where possible), are in unique positions. Checking for expected results in these wells (i.e., no extension in the water wells or concordance among duplicates) can help iden-

tify a plate mixup that has occurred earlier in the process.

Literature Cited

- Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J., and Altshuler, D. 2002. The structure of haplotype blocks in the human genome. *Science* 296:2225-2229.
- Tang, K., Fu, D.J., Julien, D., Braun, A., Cantor, C.R., and Koster, H. 1999. Chip-based genotyping by mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* 96:10016-10020.

Key References

- International Multiple Sclerosis Genetics Consortium, Hafler, D.A., Compston, A., Sawcer, S., Lander, E.S., Daly, M.J., De Jager, P.L., de Bakker, P.I., Gabriel, S.B., Mirel, D.B., Ivinson, A.J., Pericak-Vance, M.A., Gregory, S.G., Rioux, J.D., McCauley, J.L., Haines, J.L., Barcellos, L.F., Cree, B., Oksenberg, J.R., and Hauser, S.L. 2007. Risk alleles for multiple sclerosis identified by a genomewide study. *N. Engl. J. Med.* 357:851-862. Epub 2007 Jul 29.
- Useful reference using the iPLEX platform.*