



iPLEX CHEMISTRY

Single Nucleotide Polymorphism Detection with the iPLEX[®] Assay and the MassARRAY[®] System

Efficient, scalable, and cost-effective SNP genotyping and somatic mutation analysis.

INTRODUCTION

The MassARRAY[®] System from Agena Bioscience™ is a scalable platform with a suite of research applications for quantitative and qualitative nucleic acid analysis, enabling flexible assay design and fast time-to-results (Figure 1). Capable of easily interrogating from 10 to as many as 400 single nucleotide polymorphisms (SNPs) per sample, and analyzing from just a few samples to hundreds of samples per day, the MassARRAY System can be used on its own or in conjunction with next-generation sequencing (NGS) platforms to dramatically accelerate genetic analysis.

Since its introduction in 2000, the MassARRAY System has been widely adopted for a variety of genomic applications including gene fine-mapping, validation of genome-wide association studies, linkage studies, and routine genetic analysis of SNPs and somatic mutations.

A variety of biomarkers can be analyzed from a full spectrum of biological samples including blood, plasma, buccal swabs, and even compromised formalin-fixed, paraffin-embedded (FFPE) samples, with as little as 5 ng input DNA.

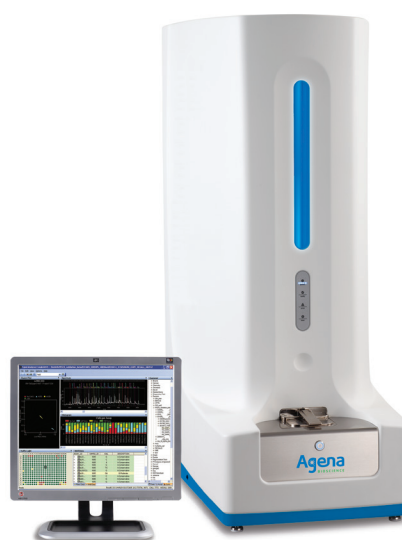


Figure 1: The MassARRAY System includes the MassARRAY Analyzer mass spectrometer and integrated data analysis software.

SNP genotyping on the MassARRAY System combines the robust multiplexed primer extension chemistry of the iPLEX[®] assay with highly sensitive matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to precisely, rapidly, and cost-effectively analyze from hundreds to over 100,000 genotypes per day. With a high level of accuracy and reproducibility (>99% call rates with >99.7% accuracy

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on validated assays) and allele detection frequencies as low as 5%, the iPLEX single base extension (SBE) chemistry enables highly stringent calling thresholds and superior performance at high multiplexing levels.¹

This application note describes the iPLEX SNP genotyping and somatic mutation workflow and outlines advances in the protocol that enable:

- Flexible, scalable, and sensitive genotyping in 24-, 96-, or 384-well formats.
- Cost-effective and accurate multiplexing of up to 40 different SNPs in a single reaction well.
- Optimized thermal cycling methods and universal cycling parameters that reduce workflow time to just one day.

STREAMLINED WORKFLOW

ASSAY DESIGN

The iPLEX workflow begins by using Assay Design Suite (ADS) software to design the PCR and iPLEX extension primers for each SNP or somatic mutation of interest from dbSNP rs numbers or dbSNP data in FASTA format (Figure 2). Depending upon the complexity of the assay, a single multiplex reaction can contain up to 40 markers. All oligos for PCR amplification and iPLEX extension reactions are ordered unmodified, with standard purification, minimizing assay setup costs. Alternatively, predesigned or custom designed panels containing the necessary premixed primers and reagents are available for purchase directly from Agena Bioscience.

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Once the PCR and extension primers have been designed and acquired, genomic DNA or cDNA is amplified in 96- or 384-well microtiter plates using reagents supplied in the iPLEX reagent kits. After PCR, excess nucleotides are dephosphorylated by shrimp

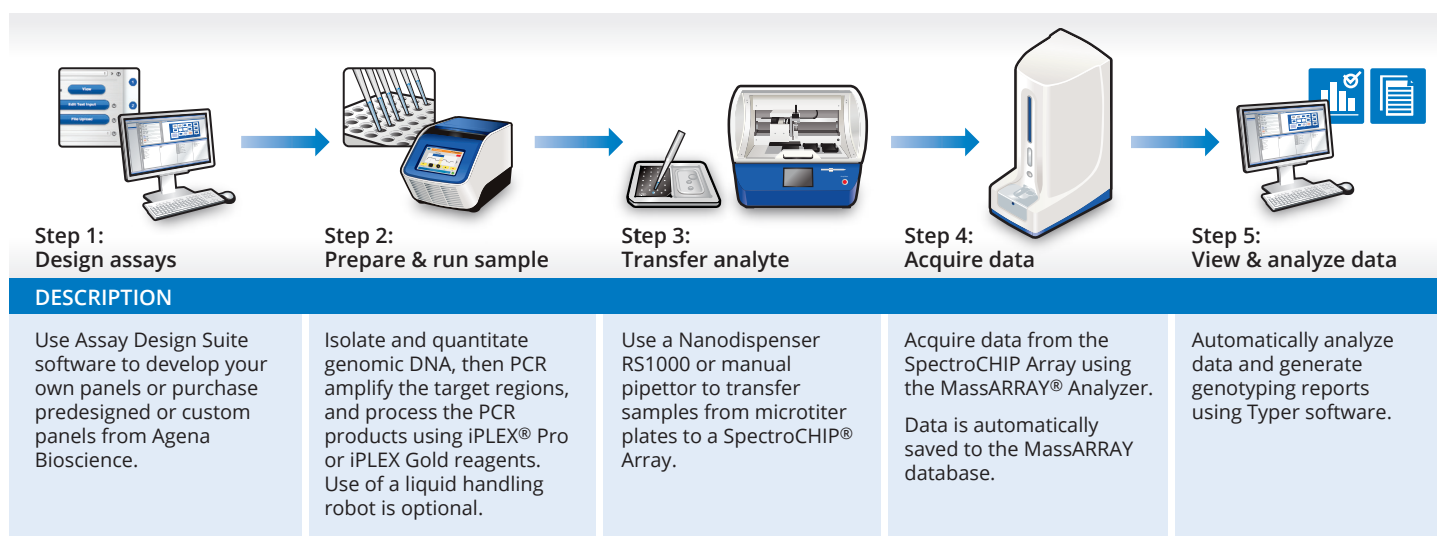


Figure 2: iPLEX genotyping workflow on the MassARRAY System.

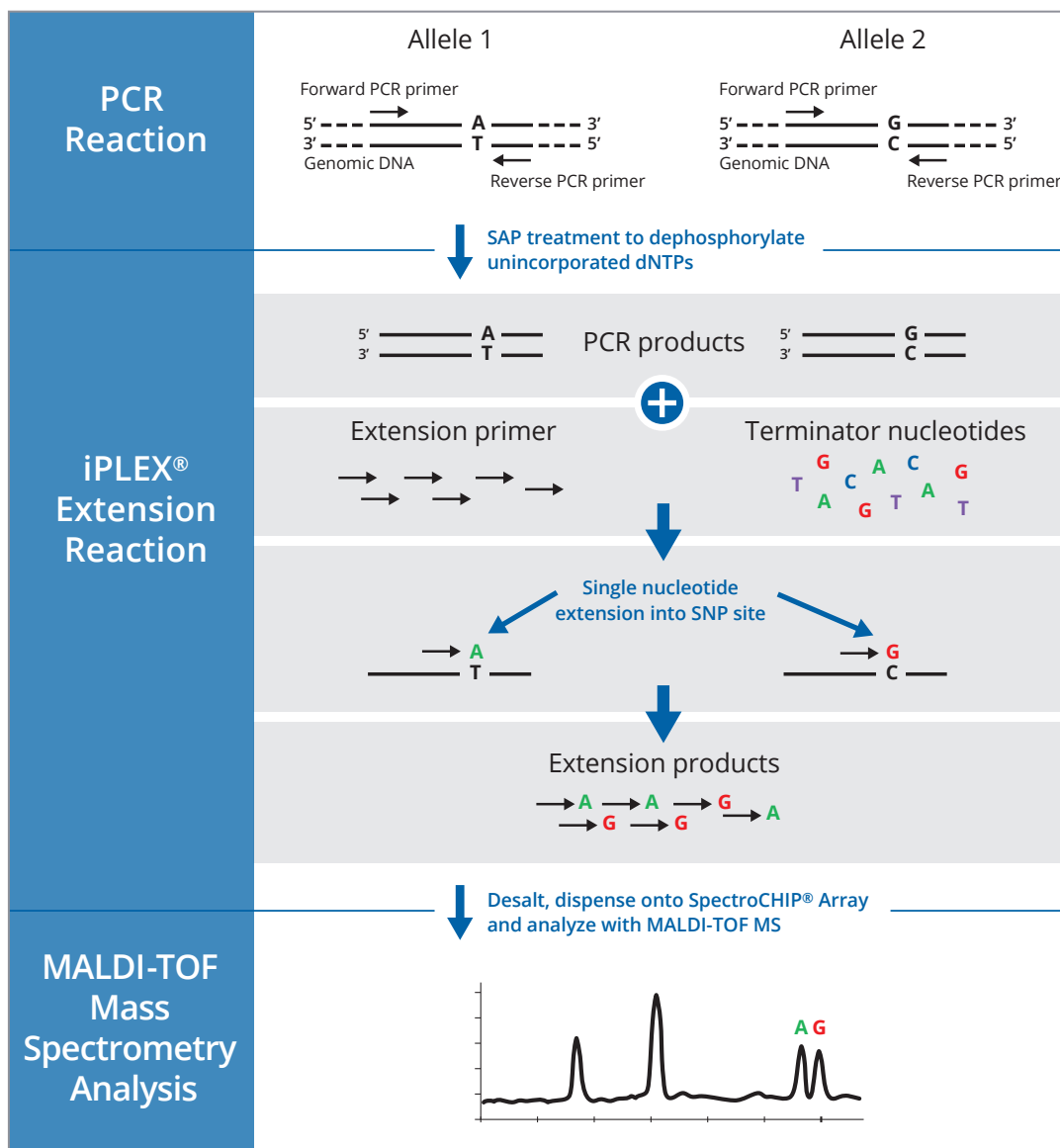


Figure 3: Overview of iPLEX genotyping assay chemistry.

alkaline phosphatase (SAP). This is followed by the iPLEX single base extension reaction in which a mix of oligonucleotide extension primers, designed to anneal to the amplified DNA fragments, is added together with an extension enzyme and mass-modified dideoxynucleotide terminators. The extension primers anneal directly adjacent to each SNP site to be assayed, and are

extended and terminated by a single complementary base into the genotyping target site (Figure 3).

The extension products (analytes) are desalted using Clean Resin and then transferred from the microtiter plate via manual dispensation or an automated nanodispenser onto a SpectroCHIP® Array, where they crystallize with a pre-spotted MALDI matrix.

DATA ANALYSIS

The SpectroCHIP Array is loaded into the MassARRAY Analyzer, where the analyte crystals are irradiated by a laser, inducing desorption and ionization. The positively charged molecules accelerate into a flight tube towards a detector. Separation occurs by time-of-flight, which is proportional to the mass of the individual molecules. After each laser pulse, the detector records the relative time of flight for each extension product and the results are displayed automatically using Typer software.

The entire process from laser firing to signal detection takes less than 50 minutes to analyze 384 samples.

iPLEX chemistry incorporates mass-modified terminators into the SBE reaction, providing sufficient mass for allele separation and more sensitive detection using MALDI-TOF MS.^{2,3} The MassARRAY Analyzer is designed to detect DNA within a mass range of approximately 4,500 Da to 9,000 Da and can easily distinguish between analytes separated by 16 Da, the smallest difference between mass-modified terminators (Figure 4). The number of multiplexes is limited only by the peaks that can be resolved accurately within this mass range.

Typer software automatically generates reports that identify the SNP alleles (homozygous or heterozygous) in each sample. Because genotype calls are made in real time during MALDI-TOF analysis, simultaneous visualization of all assays in a multiplex reaction are available immediately at the end of each run. Data is saved automatically in the MassARRAY database and can be retrieved at any time for future analysis.

The entire workflow is highly reproducible, can be completed in an 8-hour day, and generates SNP genotypes with greater than 99.7% accuracy. For the complete iPLEX assay protocol, see the iPLEX Reagents User Guide.⁴

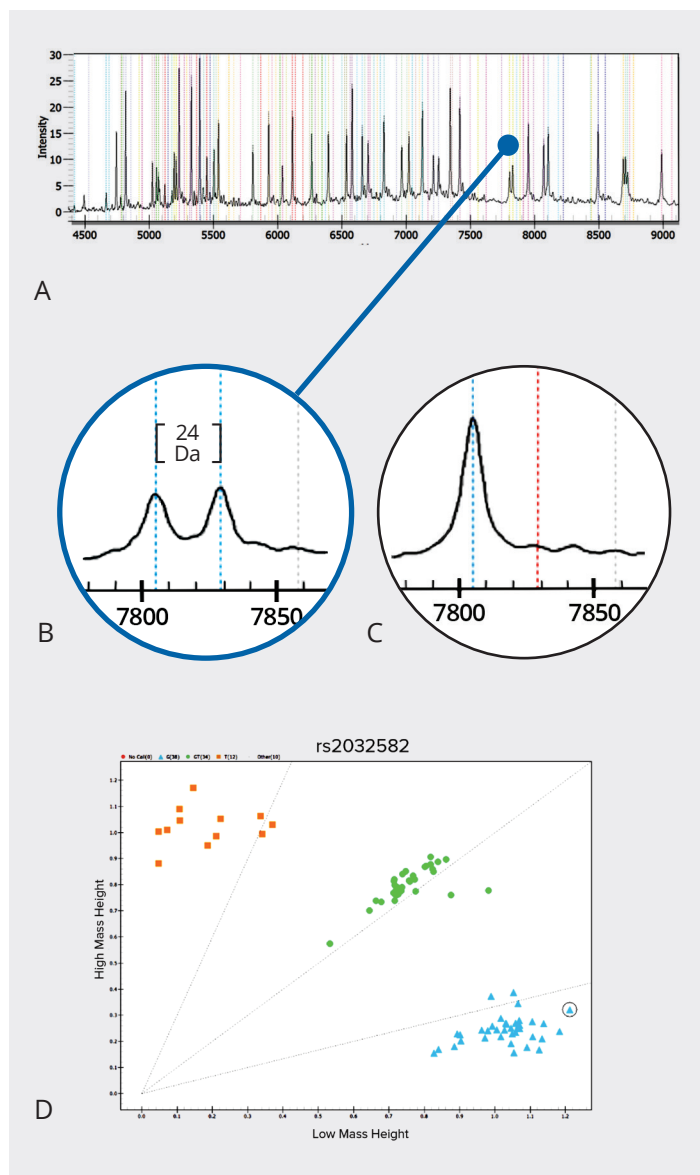


Figure 4: Highly accurate genotyping data with the MassARRAY System. The MassARRAY spectrum (A) demonstrates a single 36-plex assay. The highlighted peaks (B) indicate 24 Da separation in heterozygous alleles of the SNP in question compared to the single peak homozygous genotype (C) found in the same SNP. The cluster plot (D) displays the SNP genotypes of all samples assayed.

ENHANCEMENTS IN MassARRAY PROCESSING

The following process improvements have been evaluated and incorporated into the MassARRAY workflow to reduce processing time and increase sample throughput and performance.

VERITI® THERMAL CYCLER SPEEDS UP PCR

Although the iPLEX workflow requires minimal hands-on time (< 30 minutes), the overall processing time can take up to 10 hours to complete using standard thermal cyclers. Agena Bioscience has developed an accelerated workflow that uses a fast ramping thermal cycler, enabling the entire workflow to be completed in a single eight-hour shift. The Veriti Thermal Cycler 96- and 384-well formats from Applied Biosystems take advantage of a 4°C/second ramping rate, which is twice as fast as the GeneAmp® PCR System 9700 (GeneAmp 9700), significantly decreasing the time required for the DNA amplification and primer extension steps in the iPLEX reaction. When compared to the GeneAmp 9700, Agena Bioscience scientists were able to reduce the iPLEX workflow time by up to two hours using the Veriti Thermal Cycler 384-well format, without negatively impacting data quality in two panels (OncoFOCUS™ Panel and iPLEX ADME PGx Pro Panel).⁵

REDUCED ASSAY TIME INCREASES THROUGHPUT

Thermal cycling optimization with two of Agena Bioscience's blood group typing Hemo ID™ modules has resulted in a reduction in the

number of PCR and SBE cycles, reducing total assay time even further. Using the Veriti Thermal Cycler 96- and 384-well formats, 35 cycles of PCR, a 26-minute SAP reaction, and 100 cycles of extension PCR, Agena Bioscience scientists were able to reduce the turnaround time of the standard iPLEX assay for these two modules from 8.5 hours to just 5 hours, with no reduction in data quality. With these new parameters and one fast ramping thermal cycler, one MassARRAY Analyzer (384-format) can potentially analyze up to 96,000 assays a year with these Hemo ID modules.

Other Agena Bioscience panels may also benefit from similar cycling parameter optimization to reduce assay time. Contact Agena Bioscience Customer Support to determine if these parameters can be adapted for your panel of interest.

ROBUST ASSAY DESIGN SUITE SOFTWARE REDUCES GENOTYPING ERRORS

Assay Design Suite software offers several functionalities that increase the likelihood of assay success while reducing potential genotyping errors. ADS uses the dbSNP database to map proximal SNPs in the target sequence to the genome, preventing primers from being designed over other SNPs and ensuring that they bind and amplify unique regions. All combinations of multiplexed primers are automatically screened for cross-binding possibilities, resulting in high specificity and reduced calling errors.

FLEXIBILITY OF SCALE WITH VERSATILITY OF APPLICATION

Two iPLEX reagent options are available. iPLEX Gold is intended for standard genotyping, while iPLEX Pro is available for more demanding assays that require higher performance and sensitivity, such as in diagnostic and clinical applications. Reagent sets are available in 24-, 96-, and 384-well formats, making the system scalable for low- to high-throughput applications.

The iPLEX assay and the MassARRAY System provide efficient, scalable, and cost-effective genotyping and somatic mutation detection for a variety of biological

samples. From targeted discovery using tens to thousands of multiplexed assays to validation of select markers against thousands of samples, the iPLEX assay enables a variety of genomic studies. From basic research, NGS follow-up, and association studies, to applied and clinical genetic testing in pharmacogenetics and oncology, iPLEX chemistry and the MassARRAY System provide the flexibility, high multiplexing capability, and low detection frequencies required for successful genotyping and somatic mutation studies.

REFERENCES

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2. Ross P, Hall L, Smirnov I, Haff L. (1998) High level multiplex genotyping by MALDI-TOF mass spectrometry. *Nat Biotechnol* 16(13):1347-51.
3. Storm N, Darnhofer-Patel B, van den Boom D, Rodi CP. (2003) MALDI-TOF mass spectrometry based SNP genotyping. *Methods Mol Biol* 212:241-262.
4. Agena Bioscience, iPLEX Reagents User Guide, USG-CUS-071.
5. Agena Bioscience. (2015) Fast ramping thermal cyclers enable an 8 hour MassARRAY workflow.

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