SEQUENOM®
Application Note

iPLEX™ Assay: Increased Plexing Efficiency and Flexibility for MassARRAY® System Through Single Base Primer Extension with Mass-Modified Terminators

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Introduction

SEQUENOM presents here a newly developed genotyping assay termed iPLEX™ for use with the MassARRAY® platform. Relative to the standard multiplexing assay for homogenous MassEXTEND® (hME)[1], the iPLEX genotyping assay has been modified with regards to:

- Assay design
- PCR amplification conditions
- Primer extension conditions and components (terminators, DNA polymerase, cycling)
- Dispensing speed for analyte transfer onto the SpectroCHIP® bioarray
- Parameters for Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) data acquisition
- Genotype calling algorithms
- TYPER software for data analysis

These changes now allow for routine multiplexing of assays up to the 29-plex level.

Overview

Figure 1 outlines the steps involved in the iPLEX assay. The most significant difference relative to the existing hME genotyping assay is that all reactions for the iPLEX assay are terminated after a single base extension (SBE). hME uses 1 base in conjunction with 2-3 base extension products for genotyping in order to create large mass separations between allele-specific products[2]. The use of single base primer extension reactions coupled with MALDI-TOF MS for multiplexed genotyping has previously been shown to be feasible using standard ddNTP terminators[3].

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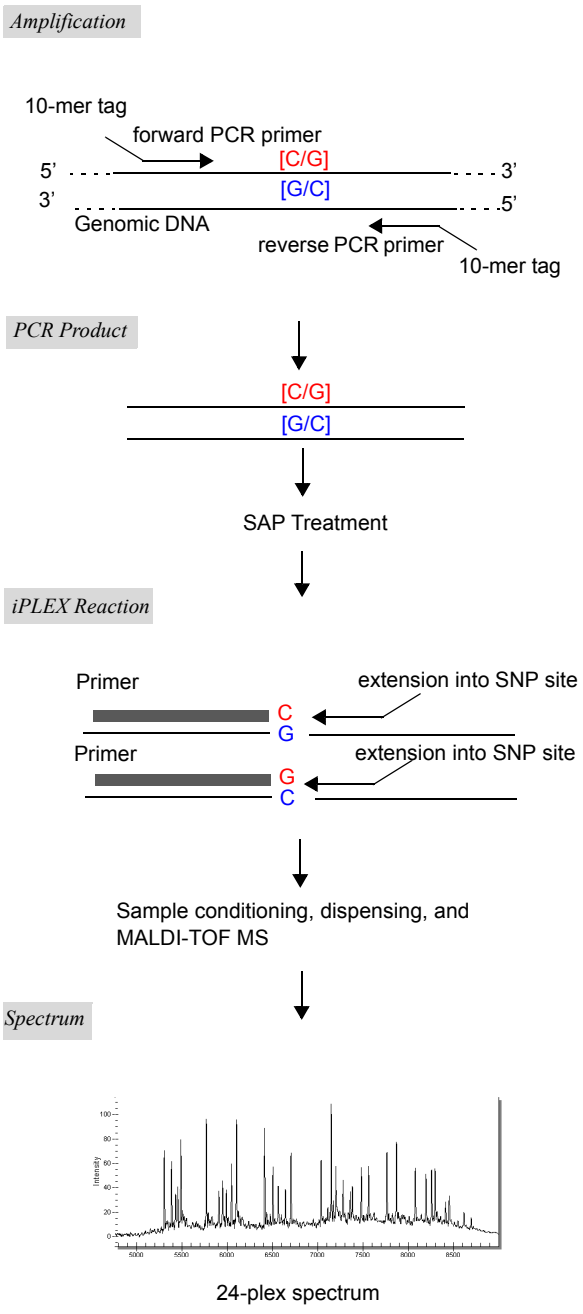


Figure 1 *iPLEX Assay* (The scheme depicts a single assay)

However, the mass separation of SBE products is small, 9-40 daltons (Da), making routine use for high-throughput genotyping prohibitive. The iPLEX assay alleviates this issue by incorporating mass-modified terminators. Table 1 shows the mass differences between the SBE products for the iPLEX assay. No two alleles for the iPLEX assay are within 15Da of each other. However, if samples are not properly desalted, sodium (22Da) and potassium (38Da) adducts are of concern since they can complicate accurate heterozygote allele discrimination for A/C (24Da) and C/G (40Da) SNPs. Yet, even when such adducts are present they normally have much smaller peak areas relative to their parent-signal and this property can be used to distinguish between them.

TABLE 1. Mass Differences Between the iPLEX Products

Terminator	A	C	G	T
A	0	-24	16	55.9
C	24	0	40	79.9
G	-16	-40	0	39.9
T	-55.9	-79.9	-39.9	0

A significant advantage of the iPLEX assay is that the allele-bias observed in many hME assays is greatly reduced. Figure 2 shows a comparison of allele-specific bias between the iPLEX assay and hME for 22 assays with comparable data. The reduction of the mean allele-specific bias between heterozygous alleles from 60:40 in hME to 52:48 for the iPLEX assay allows for the use of more stringent calling thresholds. Because of these differences, comparable or better accuracy and call rates from a 12-plex hME reaction can now be made for a 24-plex iPLEX reaction.

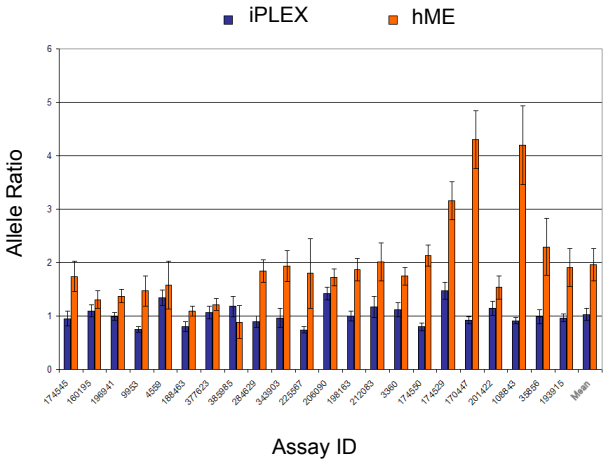


Figure 2 Comparison of Allele-specific Bias Between the iPLEX Assay and hME for 22 Assays

Note: An allele ratio of 1 indicates equal peak areas for both heterozygous alleles.

Numerous other changes are associated with the iPLEX assay compared to hME. Assay design of iPLEX reactions uses different masses than hME and therefore requires a modified Assay Design software (see "Assay Design"). Design efficiency is greatly enhanced because a common termination mix is used. This effect is illustrated in Figure 3, which plots the percent of successful 15-plex designs on the y-axis vs. the number of input SNPs on the x-axis. As shown, the iPLEX assay successfully multiplexes all assays into the maximal number of 15-plexes with a limited number of input SNPs compared to hME, which requires more input files to maximize plex level design efficiency. In addition, computational power and time are greatly reduced for iPLEX assay designs.

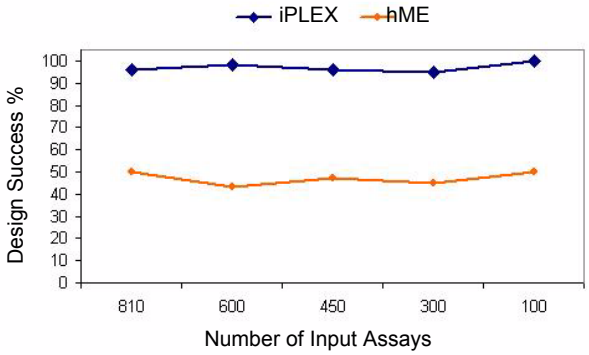


Figure 3 Comparison of Successful 15-plex Designs for the iPLEX Assay vs. hME

In order to achieve the highest multiplexing levels, an option in the Assay Design software has been incorporated. This option allows non-templated nucleotides to be added to the 5'-end of extend primers, allowing greater flexibility in their masses. This takes place in conjunction with all standard algorithms of Assay Design[4] (optimal primer designs, minimized primer-primer interactions and maximized plex level).

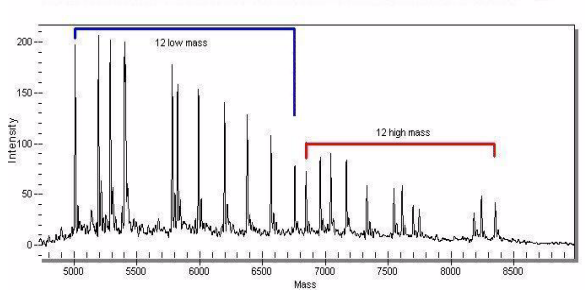
The PCR conditions for the iPLEX assay have been optimized for amplification of multiplexed reactions. Relative to the most recent application note for 12-plex reactions[1], the amount of DNA template and DNA polymerase have been increased within the context of a 5ul reaction.

SAP treatment to dephosphorylate unincorporated dNTPs has not changed for the iPLEX assay. It is essential that the PCR reaction and SAP are mixed or gently vortexed upon addition of the SAP. We have found that this maximizes dNTP dephosphorylation. Functional dNTPs can extend in the primer extension reactions causing contaminant peaks that greatly complicate data interpretation.

The post-PCR primer extension reaction of the iPLEX assay uses a modified termination mix, DNA polymerase

and cycling conditions. The specifics of these new conditions are described in the "Materials and Methods" section. In addition, it has been determined that an essential step for successful iPLEX reactions are concentration adjustments of extension primers. A current fundamental characteristic of the MassARRAY MALDI-TOF MS is the inverse relationship between analyte signal-to-noise ratio and increased mass of the analyte. The larger the extension product, the smaller the signal-to-noise ratio. This effect is illustrated in Figure 4A for the extension primers of one of the 24-plexes from this study.

A. Unadjusted extension primers of one of the 24-plexes



B. Adjusted extension primers of one of the 24-plexes

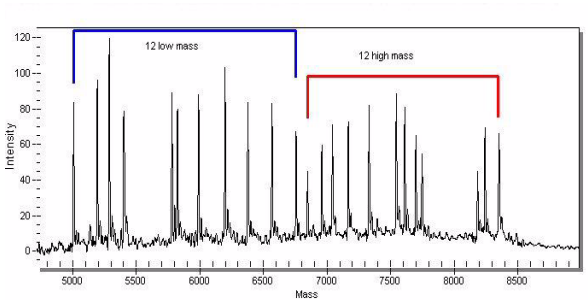


Figure 4 Extend Primer Concentration Adjustments

To compensate for this effect it is suggested to load 2x the concentration of the oligos that occupy the large mass portion of the multiplex reaction. In the context of a multiplexed assay, such as the 24-plexes presented here, the primer concentration for the assays with the twelve highest masses are doubled relative to the assays with the twelve lowest masses (0.625uM:1.25uM). An example of the effect of doubling the concentration of the high mass oligos is shown in Figure 4B. In general, the peak heights are equilibrated. This is important for multiplexing using the MassARRAY system to ensure that adequate signal-to-noise ratios and peak areas are generated from the high mass extension products. More in-depth extension primer adjustment methods are presented in the *iPLEX Application Guide*. These are suggested if implementation is feasible. Minimally, we recommend doubling the concentration for the high mass primers as just discussed; all data presented herein were generated using this method.

Another important facet to the iPLEX assay is the cycling of the primer extension reactions. Increased cycle numbers with shorter cycling time yield higher extension rates and higher calling rates per assay. The specifics of the new cycling are defined in the "Materials and Methods" section. SEQUENOM suggests using 200 short cycles based on our optimization experiments. Data for 100 vs. 200 vs. 300 cycles is presented to help users determine what is acceptable for their own needs.

Desalting, dispensing, and conducting MALDI-TOF MS analysis of iPLEX reactions is similar to that for multiplexed hME reactions[1]. Desalting calls for 6mg of Clean Resin. Dispensing and MALDI-TOF MS data acquisition of iPLEX reactions require specific optimization by trained personnel. Software upgrades are required for data acquisition and analysis (see the "MALDI-TOF MS Analysis" section).

The study presented in this application note covers the evaluation of three 24-plex reactions used to genotype seven DNAs for which genotypes have already been determined. Results for these assays have previously been described for 12-plex hME reactions[1]. This allowed for the comparison of genotype concordance and for the gauging of calling accuracy of iPLEX reactions run at the 24-plex level.

Materials and Methods

Assay Design

Assay designs for iPLEX reactions require modified Assay Design software to allow for SBE designs with the modified masses associated with the new termination mix. Figure 5 shows Assay Design with standard settings for use with the iPLEX assay.

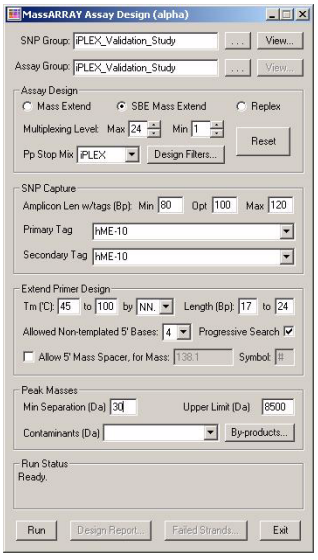


Figure 5 Assay Design

In this new version of Assay Design, primer design algorithms are the same, but multiplexing efficiency and flexibility are enhanced since all SBE products use the same termination mix, making multiplexing of any set of assays easier. The smaller mass separation compared to hME also contributes to the greater efficiency, as does the new non-templated base addition functionality. SEQUENOM recommends using eXTEND in conjunction with Assay Design for genotyping with the MassARRAY system, including the iPLEX assay. The eXTEND suite is available at RealSNP.com under Tools. It has several functionalities that increase the likelihood of assay success and reduce the likelihood of genotyping errors. These include mapping proximal SNPs (ProxSNP) in your Assay Design input files. The two PCR primers are mapped to the genome to ensure that they bind and amplify a unique region (PreXTEND). After assay design, the combination of all the multiplexed primers are screened for cross-binding possibilities (PleXTEND). Designs for this study were created using a modified version of Assay Design (v3.0) to allow for iPLEX designs. Our previous application note on multiplexing hME reactions analyzed 84 SNPs, designed into 7x12-plexes[1]. For this study we redesigned assays for these 84 SNPs to be run using iPLEX conditions. The 84 assays were designed into 3x24-plexes and a single 12-plex. All input files were run through ProxSNP prior to design and PreXTEND after design. We chose to run the 24-plexes for comparing iPLEX data with that of previous hME data. SNP IDs and primers for the 3x24-plexes are listed in the appendix. Table 2 shows the breakdown of the terminations for the 72 assays.

TABLE 2. Terminations for the 72 Assays

Termination	Number of Assays	Percent
C/T	30	41.67
A/G	11	15.28
A/T	7	9.72
C/A	7	9.72
C/A	10	13.89
G/T	7	9.72

DNA Isolation

DNA was isolated from 10ml of whole blood, collected in citrate-treated tubes, using the Puregene DNA isolation kit (Gentra Systems, www.gentra.com) following the standard protocol for this volume provided with the kit. However, DNA was resuspended in autoclaved, nanopure water instead of TE to a final concentration of 50ng/ul and stored at 4 degrees Celsius prior to use.

PCR Amplification

Perform the following steps for PCR amplification:

1. Combine the items listed in Table 3 below.

TABLE 3. PCR Cocktail Mix

Reagent	Conc. in 5μL	Volume (1rxn)	Volume (384rxns)*
Nanopure H_2O	NA	1.850μL	888μL
PCR Buffer with $MgCl_2$ (10x)	1.25x	0.625μL	300μL
$MgCl_2$ (25mM)	1.625mM	0.325μL	156μL
dNTP mix (25mM)**	500μM	0.100μL	48μL
Primer mix (500nM each)	100nM	1.000μL	480μL
Genomic DNA (5-10ng/μL)	5-10ng/rxn	1.000μL	480μL
Hotstar Taq® (5U/μL)	0.5U/rxn	0.100μL	48μL
Total		5.000μL	2400μL

*Volumes include a 25% overhang
**No more than 5 freeze thaw cycles

2. Gently mix or vortex samples.

3. Cycle the PCR reaction as follows in a standard thermocycler:

- 94º C for 15 minutes
- 94º C for 20 seconds
- 56º C for 30 seconds
- 72º C for 1 minute

45 cycles

- 72º C for 3 minutes
- 4º C forever

SAP Treatment

Perform the following steps to dephosphorylate unincorporated dNTPs:

1. Combine the items listed in Table 4 below.

TABLE 4. SAP Mix

Reagent	Volume (1rxn)	Volume (384rxns)*
Nanopure H_2O	1.330μL	638.4μL
10x SAP buffer	0.170μL	81.6μL
SAP enzyme (1U/μL)	0.500μL	240.0μL
Total	2.000μL	960.0μL

*Volumes include a 25% overhang

2. Add 2μL of the SAP mix to each 5μL PCR reaction.

3. Gently mix or vortex samples.

4. Incubate the SAP treated PCR reaction as follows in a standard thermocycler:

- 37º C for 40 minutes
- 85º C for 5 minutes
- 4º C forever

Adjusting Extension Primers

When conducting multiplexing experiments, adjusting the concentrations of oligos to equilibrate signal-to-noise ratios is highly recommended. As masses increase, signal-to-noise ratios tend to decrease. In extreme cases, signals become indistinguishable from noise, resulting in calling errors. A general method to adjust extension primers is to divide the primers into a low mass group and a high mass group. All primers in the high mass group are doubled in concentration with respect to the low mass group. For example, in a 24-plex, the 12 lowest mass primers would be at a concentration of 0.625μM and the 12 highest mass primers would be at 1.25μM in the final 9ul reaction. Figure 4A-B illustrates 24 extend primers with and without adjustment.

iPLEX Reaction

Perform the following steps for iPLEX primer extension:

1. Combine the items listed in Table 5 below.

TABLE 5. iPLEX Cocktail Mix

Reagent	Conc. in 9μL	Volume (1rxn)	Volume (384rxns)**
Nanopure H_2O	NA	0.755μL	362.40μL
iPLEX Buffer Plus (10x)	0.222X	0.200μL	96.00μL
iPLEX termination mix	1X	0.200μL	96.00μL
Primer mix (7μM: 14μM)*	0.625uM: 1.25uM	0.804μL	385.92μL
iPLEX enzyme	1X	0.041μL	19.68μL
Total		2.000μL	960.00μL

* 7μM and 14μM illustrate the doubled concentration of the high mass primers relative to the low mass primers. Low mass primers should be at 0.625uM and high mass primers at 1.25uM in the final 9uL reaction.

** Volumes include a 25% overhang

2. Gently mix or vortex samples.

3. Cycle the iPLEX reaction using two-step 200 short cycles program as follows in a standard thermocycler:

- 94º C for 30 seconds
- 94º C for 5 seconds
- 52º C for 5 seconds
- 80º C for 5 seconds

(5 cycles; 40 cycles)

- 72º C for 3 minutes
- 4º C forever

For example, an MJ Thermocycler would be programmed as follows:

I. 94º C for 30 seconds
II. 94º C for 5 seconds
III. 52º C for 5 seconds
IV. 80º C for 5 seconds
V. GOTO III, 4 more times
VI. GOTO II, 39 more times
VII. 72º C for 3 minutes
VII. 4º C forever

The 200-short-cycle program uses two cycling loops, one of five cycles that sits inside a loop of 40 cycles. These two loops result in a 200-cycle program. The sample is denatured at 94º C. Strands are annealed at 52º C for 5 seconds and extended at 80º C for 5 seconds. The annealing and extension cycle is repeated four more times for a total of five cycles and then looped back to a 94º C denaturing step for 5 seconds and then enters the 5 cycle annealing and extension loop again. The five annealing and extension steps with the single denaturing step are repeated an additional 39 times for a total of 40. The 40 cycles of the 5 cycle annealing and extension steps equate to a total of 200 cycles (5x40). A final extension is done at 72º C for three minutes and then the sample is cooled to 4º C.

Clean Resin

Desalt the iPLEX reaction products to optimize mass spectrometric analysis. Dilute samples with 16uL of water and use 6mg of resin. Use the 3mg dimple plate twice, or use a 6mg dimple plate (part#11235). Centrifuge at 4000rpm to get the extra amount of resin packed into the bottom of the well.

Note: The volume of water recommended to dilute the samples for iPLEX differs from hME. The iPLEX chemistry contains higher concentrations of surfactants that affect the dispensing performance on the SpectroCHIP.

See “Cleaning Up the hME Reaction Products” in the *MassARRAY Liquid Handler User's Guide* for further instructions.

Dispensing to SpectroCHIP® Bioarrays

Use a nanodispenser to dispense reaction products onto a 384-element SpectroCHIP bioarray. See the “Dispensing MassEXTEND Reaction Products onto SpectroCHIPs” chapter in the *MassARRAY Nanodispenser User's Guide* for further instructions.

Note: A trained individual should optimize your nanodispenser prior to using the iPLEX assay for the first time.

MALDI-TOF MS Analysis

MassARRAY Workstation version 3.4 software must be used to process and analyze iPLEX SpectroCHIP bioarrays. The following table shows the software components and their respective versions, all contained within the MassARRAY Workstation package.

TABLE 6. MassARRAY Workstation Version 3.3 and Software Components

Software	Version
Assay Design	3.0.0
Services	2.0.8
Assay Editor	3.4.0
Plate Editor	3.4.0
TYPER Analyzer	3.4.0
Acquire	3.3.1
Caller	3.4.0

See the “Acquiring Spectra” chapter in the *MassARRAY Typer User's Guide* for instructions on acquiring spectra.

Note: A trained individual should optimize your MALDI-TOF MS prior to using the iPLEX assay for the first time.

Statistical Analysis

Call Rate and Extension Rate were calculated using Microsoft Excel 2000 (Microsoft Corp., www.microsoft.com) and transferred to Minitab for analysis. Peak areas were calculated by TYPER software and transferred to Minitab for analysis. One-way analysis of variance (ANOVA) was conducted using Minitab software (Minitab Inc., www.minitab.com).

Results

Three 24-plex genotyping reactions were run, using the iPLEX conditions specified in the “Materials and Methods” section, over seven DNAs at three unique cycle numbers: 100 vs. 200 vs. 300 cycles. Each genotyping reaction, at each cycle number, was replicated four times per plate and each plate was repeated over three days with independent cocktails. Three responses were measured to analyze the effect of cycle number:

- Call Rate = Percent of genotypes with calls other than low probability or no alleles out of the total number of possible calls. Bad spectra were omitted from the population since they represent issues independent of assay performance such as improperly dispensed analyte.

- Extension Rate = Percent conversion of extend primer converted from unextended primer into allele-specific analyte for any given assay.

- Peak Area = Calculated area under the peak for all allele-specific analytes for any given assay.

The data was analyzed as a whole population for comparative statistics; all means presented are of all 72 assays. A total of n=6048 observations are possible per response (72 assays x 7 DNAs x 4 replicates x 3 repeats). If no data is observed for a well due to mis-dispensing or some other factor then 24 observations are lost. Figure 6A-C shows the results of One-Way ANOVA for the three responses. Figure 6A shows that call rate was highest for 200 cycles at 95.6%. Call rates for 100 and 300 cycles were similar at 94.1% and 94.4% respectively. Despite these close means the difference between the three populations is statistically significant because of the large sample size. The extension rate was enhanced with increased cycle number as would be expected (Figure 6B) where 300 cycles yielded an extension rate of 95.1% compared with 94.0% for 200 cycles and 84.3% for 100 cycles. Peak area was maximal for 200 cycles with a mean peak area of 331 compared with 308 for 100 cycles and 322 for 300 cycles (Figure 6C).

The relationship between call rate, extension rate and peak area is complex. Maximum extension rate does not necessarily correlate with the highest call rate. The relationship of these three responses over the three cycle numbers is shown in Figure 7.

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A. Call Rate (n=3)

One-way ANOVA: 100-call, 200-call, 300-call

```
Source     DF        SS      MS     F      P
Factor      2    0.7748  0.3874  7.67  0.000
Error   18069  912.6966  0.0505
Total   18071  913.4714

S = 0.2247   R-Sq = 0.08%   R-Sq(adj) = 0.07%

                                  Individual 95% CIs For Mean Based on
                                  Pooled StDev
Level        N    Mean   StDev  -----+---------+---------+---------+--
100-call  6048  0.9405  0.2366  (--------*-------)
200-call  6024  0.9557  0.2058                    (-------*-------
300-call  6000  0.9437  0.2306       (-------*-------)
                                  -----+---------+---------+---------+--
                                     0.9380    0.9450    0.9520    0.955

Pooled StDev = 0.2247
```

B. Extension Rate (n=3)

NOVA: 100-ext, 200-ext, 300-ext

```
   DF        SS        MS       F      P
    2   42.2973   21.1486  802.82  0.000
.8069  475.9879    0.0263
.8071  518.2852

:3   R-Sq = 8.16%   R-Sq(adj) = 8.15%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
   N    Mean   StDev    +---------+---------+---------+------
6048  0.8431  0.1929    (*)
6024  0.9398  0.1419                                  (*-)
6000  0.9507  0.1471                                     (
                        +---------+---------+---------+------
                      0.840     0.870     0.900     0.930
```

C. Peak Area (n=3)

NOVA: 100-peak, 200-peak, 300-peak

```
  DF          SS       MS      F      P
   2     1681462   840731  27.23  0.000
8069   557886195    30875
8071   559567656

   R-Sq = 0.30%   R-Sq(adj) = 0.29%

                        Individual 95% CIs For Mean Based on
                        Pooled StDev
   N   Mean  StDev  --------+---------+---------+---------
6048  307.7  172.7  (----*---)
6024  331.0  178.6                      (---*---)
6000  322.5  175.8             (---*----)
                    --------+---------+---------+---------
```

Figure 6 Results of One-Way ANOVA for Three Responses: Call Rate, Extension Rate, and Peak Areas

Figure 7 shows that call rate and peak area reach their apex at 200 cycles whereas extension rate is highest at 300 cycles. Extension rate is a measure of the extended product yield of an assay; whereas peak area also incorporates overall product yield and its measurement on the MALDI-TOF MS. Both are a component of a successful assay that results in a correct genotype call. The mean values obtained for the data set of the three 24-plexes are well above those required for a successful assay. In addition, the call rates for the 72 iPLEX assays are increased relative to that of the 12-plexes run with standard hME over the same DNAs[1].

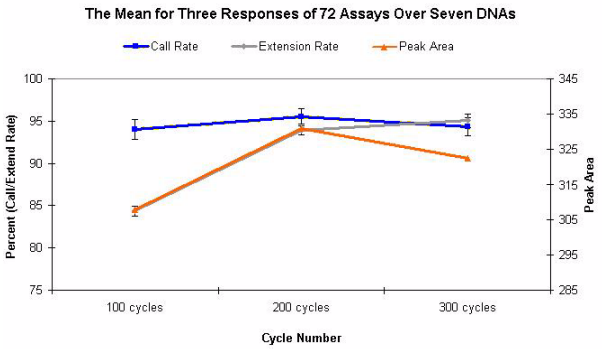


Figure 7 Mean for Three Responses of 72 Assays Over Seven DNAs

Table 7 lists the call rates for all 72 assays at 200 cycles, for each of the three separate runs with the mean and standard deviation for each assay. Only three assays had a mean call rate below 80% over seven unique DNAs and 92% of the assays had a mean call rate above 90% for the three separate runs.

The accuracy of the call rates for each of the 72 assays was also assessed individually, based on the concordance of the genotypes obtained with iPLEX assays to those previously genotyped with hME. Figure 8 shows call rate, call accuracy and call accuracy after filtering poor performing assays. The majority of conflicting calls turned out to be the result of a single assay rs205384 for all cycle numbers. The rs205384 assay had an average call rate of 33.6%, well below the average, indicating a fundamental problem with this assay. Assay rs385985 yielded the lowest average call rate of 19.3%, but did not result in any conflicts. By removing the rs205384 assay from the panel, the calling accuracy after filtering was 99.9% for 100 cycles, 100% for 200 cycles, and 99.8% for 300 cycles. Taken together, the data suggests that 200 cycles is the most robust cycle number for iPLEX assays and therefore SEQUENOM recommends starting with 200 cycles.

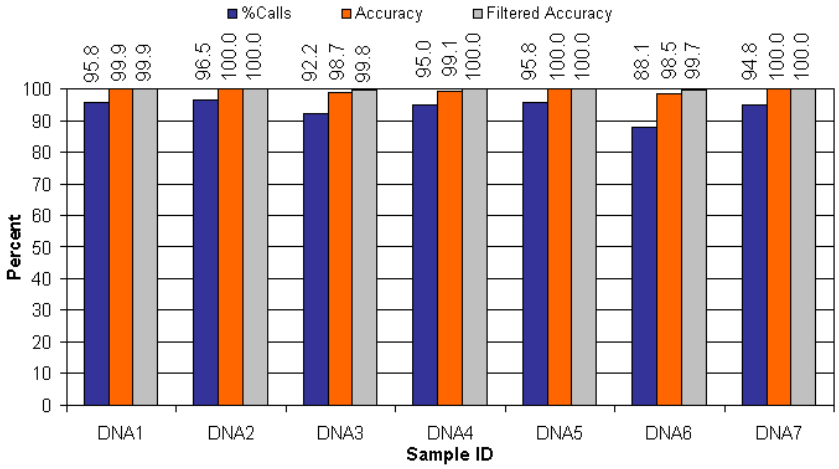
TABLE 7. Genotype Call Rate of 72 Assays From Seven DNAs for 200 Cycles

Assay	Day 1	Day 2	Day 3	Mean	StDev	Assay	Day 1	Day 2	Day 3	Mean	StDev
rs3360	100.000	100.000	100.000	100.000	0.000	rs225567	96.429	100.000	100.000	98.810	2.062
rs3702	96.429	96.429	100.000	97.619	2.062	rs226058	100.000	100.000	100.000	100.000	0.000
rs4559	100.000	100.000	100.000	100.000	0.000	rs226997	96.429	74.074	71.429	80.644	13.734
rs9038	100.000	100.000	96.429	98.810	2.062	rs229591	96.429	100.000	100.000	98.810	2.062
rs9953	100.000	100.000	100.000	100.000	0.000	rs229664	96.429	100.000	100.000	98.810	2.062
rs13526	96.429	100.000	100.000	98.810	2.062	rs230364	96.429	96.429	100.000	97.619	2.062
rs33146	100.000	100.000	96.429	98.810	2.062	rs235122	100.000	100.000	100.000	100.000	0.000
rs33234	100.000	100.000	100.000	100.000	0.000	rs243867	89.286	96.429	100.000	95.238	5.455
rs90951	96.429	100.000	100.000	98.810	2.062	rs244340	96.429	100.000	100.000	98.810	2.062
rs150342	100.000	100.000	100.000	100.000	0.000	rs256655	92.857	92.593	67.857	84.436	14.358
rs151288	100.000	96.429	100.000	98.810	2.062	rs258099	96.429	100.000	100.000	98.810	2.062
rs160195	89.286	89.286	100.000	92.857	6.186	rs259728	100.000	100.000	100.000	100.000	0.000
rs170447	100.000	100.000	100.000	100.000	0.000	rs276901	96.429	100.000	100.000	98.810	2.062
rs174545	100.000	100.000	100.000	100.000	0.000	rs284629	100.000	100.000	100.000	100.000	0.000
rs174550	100.000	100.000	100.000	100.000	0.000	rs289066	89.286	92.857	100.000	94.048	5.455
rs180870	96.429	100.000	100.000	98.810	2.062	rs299531	96.429	92.857	100.000	96.429	3.571
rs180923	100.000	100.000	96.429	98.810	2.062	rs310334	100.000	100.000	100.000	100.000	0.000
rs188463	100.000	100.000	100.000	100.000	0.000	rs332287	100.000	100.000	0*	66.667	57.735
rs193915	100.000	100.000	100.000	100.000	0.000	rs342143	92.857	100.000	96.429	96.429	3.571
rs196941	100.000	100.000	100.000	100.000	0.000	rs343903	100.000	100.000	100.000	100.000	0.000
rs198163	100.000	100.000	100.000	100.000	0.000	rs364823	100.000	100.000	100.000	100.000	0.000
rs198182	100.000	100.000	100.000	100.000	0.000	rs368140	100.000	100.000	100.000	100.000	0.000
rs199461	96.429	89.286	89.286	91.667	4.124	rs375437	100.000	100.000	100.000	100.000	0.000
rs201422	96.429	100.000	100.000	98.810	2.062	rs375938	100.000	100.000	100.000	100.000	0.000
rs204039	96.429	100.000	100.000	98.810	2.062	rs377623	100.000	100.000	100.000	100.000	0.000
rs205384	46.429	22.222	32.143	33.598	12.169	rs385985	35.714	18.519	3.571	19.268	16.085
rs206090	96.429	100.000	100.000	98.810	2.062	rs503455	100.000	100.000	100.000	100.000	0.000
rs206146	89.286	85.185	100.000	91.490	7.649	rs515868	96.429	96.429	100.000	97.619	2.062
rs208596	100.000	100.000	100.000	100.000	0.000	rs538515	96.429	100.000	96.429	97.619	2.062
rs208944	89.286	100.000	92.857	94.048	5.455	rs593218	100.000	100.000	100.000	100.000	0.000
rs210820	100.000	100.000	100.000	100.000	0.000	rs635865	100.000	100.000	96.429	98.810	2.062
rs210827	96.429	100.000	100.000	98.810	2.062	rs680671	100.000	100.000	96.429	98.810	2.062
rs212083	96.429	100.000	100.000	98.810	2.062	rs731396	100.000	100.000	96.429	98.810	2.062
rs221476	96.429	100.000	100.000	98.810	2.062	rs752920	100.000	100.000	100.000	100.000	0.000
rs222774	100.000	92.857	85.714	92.857	7.143	rs773126	92.857	100.000	96.429	96.429	3.571
rs225564	85.714	85.714	96.429	89.286	6.186	rs884606	100.000	100.000	100.000	100.000	0.000

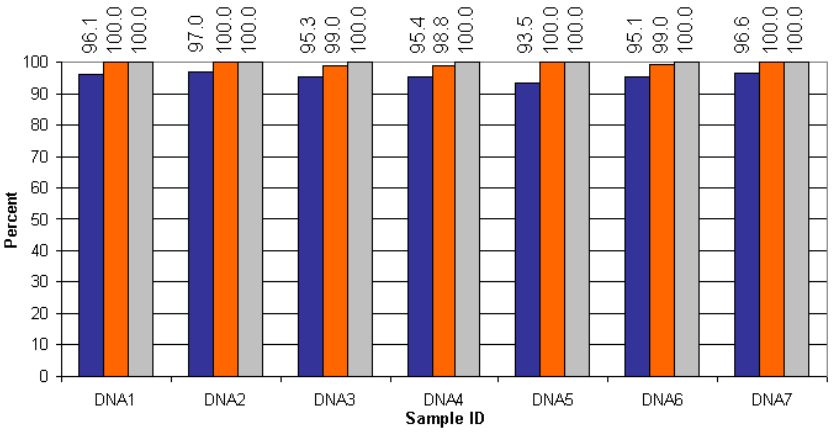
* No alleles observed for all reactions, suggesting PCR failure

Percent Call Rate, Accuracy, and Filtered Accuracy of 72 iPLEX Assays over Seven DNAs for 100, 200, and 300 Cycles

A. 100 Cycles



B. 200 Cycles



C. 300 Cycles

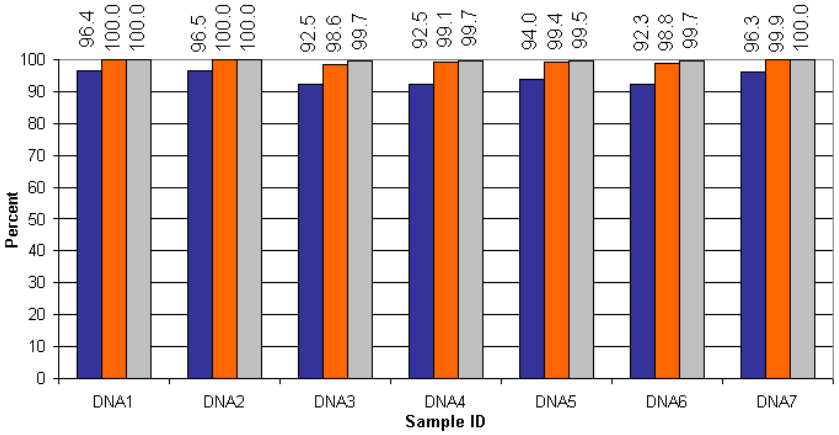


Figure 8 Genotype Call Rate and Concordance of iPLEX and hME Assays

Conclusion

The new iPLEX genotyping reaction offers multiple advances over the existing hME reaction including:

- Successful multiplexing up to the 24-plex level (29 plexes have been achieved with similar results, unpublished data)
- Flexible and efficient assay design allows for multiplexing with small or limited SNP sets
- Improved call rates and accuracy at higher multiplex levels
- Increased throughput of up to 9216 genotypes per chip for a 24-plex
- Significantly reduced cost per genotype (below 5 cents per genotype at the 24-plex level)

These improvements make the iPLEX assay the optimal solution for a broad range of genotyping studies, including fine mapping and microarray data validation. In addition, the single termination mix simplifies study logistics and reaction handling.

iPLEX Requirements

Software

- Assay Design 3.0
- Services 2.0
- Assay Editor 3.4
- Plate Editor 3.4
- TYPER Analyzer 3.4
- Acquire 3.3
- Caller 3.4

Consumables

- iPLEX Reagent Kit (SEQUENOM catalog #10130)
- SpectroCHIP/Clean Resin kit (SEQUENOM catalog #10117)

Contact your SEQUENOM sales or customer support representative for information.

References

[1] Beaulieu, M., Multiplexing the Homogeneous MassEXTEND Assay. Application Note (2003): http://www.sequenom.com/Assets/pdfs/appnotes/Multiplexing_hME_App_Note.pdf.

[2] Storm, N., et al., MALDI-TOF mass spectrometry-based SNP genotyping. *Methods Mol. Biol.* (2003) 212:241-262.

[3] Ross P, et al., High-level multiplex genotyping by MALDI-TOF mass spectrometry. *Nat Biotechnol.* (1998) Dec; 16(13): 1347-51.

[4] Tang, K., et al., Single nucleotide polymorphism analyses by MALDI-TOF MS. *Int. J. Mass. Spec.* (2003) 226:37-54.

Contacts

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Trademarks

Appendix: PCR and Extend Primer Sequences of 3x24-plexes

TABLE 8. 24-plex 1

SNPID	2nd-PCR Primer	1st-PCR Primer	AMP (bp)	Extend Primer
rs208596	ACGTTGGATGTGAGAACATATCACTCTGCC	ACGTTGGATGCATGGGAAACCTTTTCATGC	115	CTCTGCCTTCATCTCCC
rs4559	ACGTTGGATGTGTCACGTAGGCAAAAGCAG	ACGTTGGATGGTGAACGTGTATGTACCTAG	119	CTAAGGTGCCAGCTATA
rs160195	ACGTTGGATGCATGGTATAGTGTGTGTCTG	ACGTTGGATGTCCCACGTTAGAATTGTCTG	104	TGTGTGTCTGTGGCGAG
rs375938	ACGTTGGATGAGGAGAGGTCGGGAAATCAC	ACGTTGGATGCGGGAGACCTCCTTGGTTTG	102	CCTTGGGCTACTCTTCAC
rs193915	ACGTTGGATGTGGCTGCTCCCTGATCCTAA	ACGTTGGATGCATAGCCATCTTGGATACCC	96	CCTGATCCTAACTTCTGA
rs199461	ACGTTGGATGGCAACTTTAAAAACAGCACC	ACGTTGGATGCTCAGTTGTGATAGTTAACC	82	AGCACCAAAGTTACCTATG
rs375437	ACGTTGGATGAAGCATCAAACTCTGTGGTC	ACGTTGGATGACCTGTGGAAGGCACTACTC	119	CTGTGGTCAGATACTGTTT
rs196941	ACGTTGGATGCAAAGTCCAATCCCAGCATC	ACGTTGGATGAGCATCATCTACCTCCAGAC	95	CATCAGTCCCAAAATCTCAC
rs198182	ACGTTGGATGATGCAAGGCAGATATGGCAC	ACGTTGGATGGCTGTCTGTCATGCTCATAG	106	GTTGAAAGGACCTTAAGAAA
rs364823	ACGTTGGATGTCTGTAACGGCTTTTGTGTC	ACGTTGGATGTGGTATTGTTGCAGGGAAAG	117	GAGTTTCCTAGTCTGTTCTAC
rs259728	ACGTTGGATGGCAGATTTAAACGAACTGGG	ACGTTGGATGCCTTATTAGTCAATGTCTATG	102	GGGAAATAATGAACAGTGTGA
rs222774	ACGTTGGATGTTCCATGCAGAGGCTGTTTC	ACGTTGGATGTGTGAACACGCACACCCCAC	85	CAGAGGCTGTTTCCGGAGTTTC
rs310334	ACGTTGGATGTGGTTGAGTCTACCACTGTC	ACGTTGGATGAGACCAGAACAGCAGATGAC	96	CCCTCAATGGCCCTCTTCACTCT
rs9953	ACGTTGGATGCATCTGCTGGTACAGTCCAC	ACGTTGGATGTTCTGCCAAGACAGAATCCC	120	TGCTGGTACAGTCCACACTTCCA
rs150342	ACGTTGGATGAATCTAGAAGACCGGAGACC	ACGTTGGATGAACAGAAGTCACCTACATGG	108	CTGGTTGATTTTTAAGAACTCTA
rs593218	ACGTTGGATGAACCAAGCACTGTAGTCAGG	ACGTTGGATGGACACCCACATGACTATAGC	102	CTGTAGTCAGGAGAATAAGGTGT
rs174550	ACGTTGGATGCCTTCAAAAGTACCAAGGCC	ACGTTGGATGCATTTGGCGGCATGCTGAAG	104	AAGTACCAAGGCCTACAAGGTCTC
rs174545	ACGTTGGATGTGCTCATCAGGTTCAACTGC	ACGTTGGATGTCAGCTCACTGGTCCATTTC	104	GGTTTGCAAATGAGGATGGAGAAG
rs151288	ACGTTGGATGTACTATCTTTGGTAGGAGGC	ACGTTGGATGCAGAGGAAAGGCTGAAATCC	96	ATGGTAATTTGCCATTTTTTTAACC
rs289066	ACGTTGGATGGTCTGTGCTAAATGTCTTTG	ACGTTGGATGGAGCTGAAGAGAGGGAAAGT	97	ATGTCTTTGTATTGCATTTTAGTGG
rs226058	ACGTTGGATGATCTCGTCCCTTGCGCTCAC	ACGTTGGATGAAGACGACCCAAAAGGGTAC	118	CACCTCGTCCCATGCGCCCTACCCTA
rs188463	ACGTTGGATGTCTGCTCAAGACTCAAGGTG	ACGTTGGATGTTCCTCCCCCTCAGAATCTC	105	GTAGAGGAAGGGCAAAGGACAAAGAA
rs884606	ACGTTGGATGTGAGCACAGTAGAAAGAGCC	ACGTTGGATGCTTCTGCCTTTGCACACAGC	116	AGAGTTCAGGCCCTGCCTCCTGGGTTC
rs752920	ACGTTGGATGCATCTGCGAAAAGCCAAACC	ACGTTGGATGCAAGCTAACACTCCTTTGGG	112	ATGTAATAAGTAGCAAGTTGCCTGTGT

TABLE 9. 24-plex 2

SNPID	2nd-PCR Primer	1st-PCR Primer	AMP (bp)	Extend Primer
rs90951	ACGTTGGATGTTCCTCTCCAGTCCCTCCTG	ACGTTGGATGACACAGATGATGACCAGCAG	117	CCCTCCTGCAGCGTCTC
rs204039	ACGTTGGATGTGTTCTTGCTGTCTCATGCC	ACGTTGGATGGGAATTAGCCAGGCAAGAAG	99	TGCCTCTGTGCATGTTG
rs221476	ACGTTGGATGTCATTTAAAGCAGTGTCAGC	ACGTTGGATGGCTACTTATTGGAAGGTGGG	98	GCAGTGTCAGCTGGTTA
rs225564	ACGTTGGATGCTCTCTGTGCAGTTAAATTTG	ACGTTGGATGCTAATGACCAGTGATGATAAG	120	AGACATTTATGTGGCCAA
rs230364	ACGTTGGATGGTATGTAACAAAAGATTGGG	ACGTTGGATGTCTTGTTGCCCTTTCTAATG	103	AAAAGATTGGGAGGAGAT
rs773126	ACGTTGGATGAGCAAGATTCCAAGCCGTTC	ACGTTGGATGGCTCTTCATGTTAGCTCTGC	98	GTTCTGAACTTGCTTCCTC
rs258099	ACGTTGGATGAGGTCATCTCATAACCTAGC	ACGTTGGATGCTAAGACGCAACTCTAGCAC	120	GAGAAATCATGCACAGAGC
rs515868	ACGTTGGATGTGTGAAATGGTCCCCAAATG	ACGTTGGATGTACAGGGTGCAGTCATATAC	99	ATGGTCCCCAAATGGAATTG
rs208944	ACGTTGGATGCCCAACCTTACCATCTTGTC	ACGTTGGATGCCAAATTCCAAGAGCTAGAC	118	GGACAGGGACAAAATGACAA
rs180870	ACGTTGGATGACATTTCTAGAGAAACAGGC	ACGTTGGATGACTGACAGAGATTCCTTGGC	106	CTGAATTTTACATACCTCATC
rs212083	ACGTTGGATGCTCCCAAAAGGAATGACTCG	ACGTTGGATGTGTTGGAAATTCCTTCTGCC	103	CTAGAGACCTCAAAAGAGATA
rs229664	ACGTTGGATGTCTCCCCATACGACCCTCCT	ACGTTGGATGTGAGGACCGGGGCGACCGA	120	GGCCCGGGAAGCAGCCAACACA
rs210827	ACGTTGGATGCCTTCTCCTCGCTTTTCTAC	ACGTTGGATGGACTGGACAATCTAAGAGGG	94	TCCTCGCTTTTCTACCACAGTTC
rs342143	ACGTTGGATGGACTTCGGGACCATCACTTA	ACGTTGGATGACCTGCCAAGAAGCAGGTAC	106	GGACCATCACTTAAAAACTCTTC
rs3702	ACGTTGGATGCCACGTCTTGAGAAGAAGAA	ACGTTGGATGTTCTCTTGGGCTCTAGGTCC	120	TCTTGAGAAGAAGAATACTATGT
rs201422	ACGTTGGATGGTCCCTAATTCAAAGGTCCC	ACGTTGGATGTAGAATGCTACAACCACCGG	109	CCCTAATTCAAAGGTCCCTGCTCA
rs244340	ACGTTGGATGTTAAGGAGCCTAAAGGTTGC	ACGTTGGATGGTAGGGATCAGCCACTTAAC	118	AAGGAGCCTAAAGGTTGCTAGACA
rs206090	ACGTTGGATGAAAGCCACTGCCTGCAGAAC	ACGTTGGATGGGAGTGACCACTAGTCGAAG	118	AGCCACTGCCTGCAGAACCCAGAGT
rs225567	ACGTTGGATGGCACATGTATCCCAGAAGTG	ACGTTGGATGAAGAGTGGAGTGAGAGGATG	116	AATTAAAAAAAGTATAGCAGGGAAG

TABLE 9. 24-plex 2

SNPID	2nd-PCR Primer	1st-PCR Primer	AMP (bp)	Extend Primer
rs276901	ACGTTGGATGCCAACTTCTTAATGCTCTTCC	ACGTTGGATGCTGGGACATATCTGTATGCC	101	TAATGCTCTTCCTATAAACAAGAAAC
rs538515	ACGTTGGATGGCTGATTTTGATGGACTCCG	ACGTTGGATGCCATCCAAAATTGTATTCAG	93	TGATTTTGATGGACTCCGATAATACA
rs299531	ACGTTGGATGTTGTTGGTTCTAGCTCATGG	ACGTTGGATGAGAAGTACCTTCTCATGTCC	104	TTGTTGGTTCTAGCTCATGGATAAGC
rs229591	ACGTTGGATGAGGTTCTTCTTGCAGAAGGC	ACGTTGGATGAGTTTGGGCACCCGCAGATC	114	CTGGTCCCACTGTGCCGACACCTCCTT
rs243867	ACGTTGGATGCCATGGAGCTTTTGGGATAT	ACGTTGGATGGGCAACACAATTATATCCAC	117	ATGGAGCTTTTGGGATATGTTTGTTAC

TABLE 10. 24-plex 3

SNPID	2nd-PCR Primer	1st-PCR Primer	AMP (bp)	Extend Primer
rs170447	ACGTTGGATGAAGACCACCACCCTCTCCATG	ACGTTGGATGGCTGAGATGGTGTTAAAGGG	109	CTCACGCCCCTGCCACC
rs3360	ACGTTGGATGCATCATTCCCAACAACATCC	ACGTTGGATGGTGAGGCTCAATTTTTGCCC	93	AACAACATCCTCTGCCA
rs256655	ACGTTGGATGAGCTCTACTCGGATTTCCAG	ACGTTGGATGGAGGAGAGCCCAGTCCCCA	109	CATGCCTAGGAGAGTAA
rs368140	ACGTTGGATGTTGTGTCTAACCTAACCCCG	ACGTTGGATGAAATGCCACCTATTCCCCAG	103	TCTCTGTTCTGGGCCACG
rs332287	ACGTTGGATGCCCAGCAGTACCCATTATAG	ACGTTGGATGATATGCCTTTGCCAGGAGAC	108	TGGTTCTCTAGAGTCGTG
rs226997	ACGTTGGATGCTTGAGGGTTAGTGTAAGGC	ACGTTGGATGGAGTGCCAGTTCCAAAGATG	102	GGACTGTGTGAATTATGG
rs206146	ACGTTGGATGGCAAAAATCCTTGAAATGCC	ACGTTGGATGTTTCAGTGGACTGAAGTGGG	101	TCACTATCATTCCTCCTTT
rs198163	ACGTTGGATGATCTTACACCACTCTCCGTC	ACGTTGGATGACAGGAACCACAGACCATTG	107	TCTTAGATGCCTTCACTCCA
rs33234	ACGTTGGATGGAAAGGTCAAATACAGCCTC	ACGTTGGATGCAGCTTTCAGCTGGAGGAAC	100	CAAATACAGCCTCTTGCTTC
rs503455	ACGTTGGATGGCAGTGATACTAGAGCTGAG	ACGTTGGATGAGAGTAGTTTCATGGTGCAG	107	CCTGGCTTTAAACAAAGCTG
rs635865	ACGTTGGATGCCCAGTGTGGGTTTTGCTTG	ACGTTGGATGTTGGTCGAGTCCATTCTACG	102	TTGAAAACAGTGCAGCAGGT
rs205384	ACGTTGGATGTTCAGGTTGCGATAGTCCCG	ACGTTGGATGACCTTCCAGAGAATGAGCAG	111	GAGTGCAGAGATGAGGCTGGC
rs731396	ACGTTGGATGTAGGTGTCAGGAAACATACG	ACGTTGGATGTCGAGACTTCCTGGATTACC	105	GTCAGGAAACATACGACCTCAA
rs284629	ACGTTGGATGATCAGGAGGGTGACCAAAAC	ACGTTGGATGCATTCCTCAGGGTTTTCTCC	112	GAGGGTGACCAAAACCACAAGT
rs235122	ACGTTGGATGTGCCCTTGAGCCTCAGCATT	ACGTTGGATGAAGCATGGACGTGCAGAAAG	101	GCCTCAGCATTGCCCTGTACTAC
rs377623	ACGTTGGATGAGGGACAAGTTCCGATATTC	ACGTTGGATGTCCTGCCCTTCTTGATCTTG	108	TAAAAAGGATCAGAGTGCTAGTT
rs385985	ACGTTGGATGGTCTTGATGTCTAGATTTCC	ACGTTGGATGATATCTAACTCCCCTACTTC	80	GATGTCTAGATTTCCTTTAAGATA
rs9038	ACGTTGGATGATGGCAGGGAGATGAAGACG	ACGTTGGATGTTTATCTCAGCCATCCGCAG	101	TTCCAGCCTGTCCCCGCATCTGGCC
rs13526	ACGTTGGATGGTGCACATACTTAATCCCAG	ACGTTGGATGCTGTGCAATGAACCTCCCTA	120	CATACTTAATCCCAGTAATTCAACT
rs33146	ACGTTGGATGATTCTTCATGGCCTCCAACC	ACGTTGGATGTGAGACCTCTGTGAACTCCG	108	GATTTCAGGACTGGGGCAATTTGCT
rs343903	ACGTTGGATGTCAGAACATTGCACTGTGAG	ACGTTGGATGAGCTTACACATTAGGCCTGG	105	TAACATTTTGCCATTTCTGTAAGACT
rs180923	ACGTTGGATGCTTCTGCCTGATCACAGAAC	ACGTTGGATGTCCCCACTTCTGATGGATTG	114	ATGTCTTCCGTCAGGTGAAGGAAAGA
rs210820	ACGTTGGATGCCATCATTCTGAATCCTGCC	ACGTTGGATGGCAAAAATTCAAGGCAACTGG	112	CTTTCTTTTGAATGCACATATTTTATT
rs680671	ACGTTGGATGACTTGTAGCTCACACAGGTC	ACGTTGGATGACTAAGGCCTTTAATGGAGC	89	CACACAGGTCAGAGGACAGGCATCCCA

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