This guide includes instructions for:

- Genotyping
- Allelotyping
Dear Customer,

Thank you very much for your purchase of a MassARRAY System from SEQUENOM. We are sure that you will be thrilled with this system and that with proper care and use, it will provide you with years of quality service. To help you maintain your investment SEQUENOM Inc. offers a comprehensive service agreement that will provide both instrument maintenance as well as instrument service.

We have prepared for you a number of manuals that go into detail on each of the individual system components to help familiarize you with this system. These manuals explain not only general system operation but parameters to test system suitability as well. Because we want you to be comfortable with your new system we recommend that you take the time to review each of the manuals and then store them close to your system for easy reference.

Your MassARRAY System is protected by a limited warranty that you will find detailed on page 187 of this manual. It is important that you fully understand the coverage provided to you under this warranty as well as your rights and responsibilities.

At SEQUENOM, we are focused on your total satisfaction and will be happy to answer any questions or concerns that you may have.

SEQUENOM, Inc.
San Diego, California

“SEQUENOM's mission is to be the leading provider of genomic systems and knowledge for personalized medicine and the life science industry.”

---

Corporate Headquarters
3595 John Hopkins Court
San Diego, CA 92121
Tel. (858) 202-9000
Fax (858) 202-9001
Sales (877) 4GENOME

European Office
Mendelssohnstrasse 15 D
D-22761 Hamburg Germany
Tel. 49.40.899676.0
Fax 49.40.899676.10

US East Coast Office
142-F North Road, Suite 150
Sudbury, MA 01776
Tel. (978) 371-9830
Fax (978) 371-9844

www.sequenom.com

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Section 1
Introduction

This section contains a general description of the homogeneous MassEXTEND (hME) reaction. It also includes information about designing hME assays. The information in this section pertains to both genotyping and allelotyping.

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Introduction

The liquid handler is an automated, 96-channel pipettor used to transfer, add, and mix reagents to process Homogeneous MassEXTEND (hME) reactions.

The hME reaction is a universal method for detecting insertions, deletions, substitutions, and other polymorphisms in amplified DNA.

![Diagram of the hME reaction process]

**Figure 1: Homogeneous MassEXTEND reaction**

**Amplified DNA**

\[
\begin{array}{c}
\text{dATP} \\
\text{dCTP} \\
\text{dTTP} \\
\text{dGTP}
\end{array}
\]\n
SAP treatment to neutralize unincorporated dNTPs

**Addition of hME reaction cocktail** (containing primer, enzyme, buffer, ddA, ddC, ddG, and dT)

**Thermocycling of hME reaction:** enzymatic addition of nucleotides into the diagnostic site

**Sample preparation (desalting)** using Clean Resin

**MALDI-TOF mass spectrometry analysis**

**Arbitrary Scale**

**m/z**

Unextended primer

Allele 1

Allele 2

**Version 1 Revision 5**

September 19, 2002
The first step in processing hME reactions is to neutralize unincorporated dNTPs in amplification products using shrimp alkaline phosphatase (SAP). The SAP cleaves a phosphate from the unincorporated dNTPs, converting them to dNDPs and rendering them unavailable to future reaction.

Next, hME reaction cocktail (primer, enzyme, buffer, dNTPs/ddNTPs) is added to the amplification products.

The amplification products and hME reaction cocktail are thermocycled to process the hME reaction, which involves the enzymatic addition of nucleotides into the diagnostic site.

During the hME reaction, the primer is extended by a specific number of nucleotides depending on the allele and the design of the assay. In the reaction mixture, all four nucleotides A, T, C, and G are present as either dNTPs or ddNTPs (for regular SNP assays, usually three nucleotides are present as ddNTPs and one as dNTP). The incorporation of a ddNTP terminates the extension of the primer.

Using a DNA polymerase that incorporates both ddNTPs and dNTPs at the same rate, the hME reaction produces allele-specific extension products of different masses depending on the sequence analyzed (see Table 1 and Figure 2).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>ddC terminated extension product</th>
<th>Length of product [bp]</th>
<th>Calculated mass [Da]</th>
</tr>
</thead>
<tbody>
<tr>
<td>unextended primer</td>
<td>Extension Primer</td>
<td>23</td>
<td>7082.6</td>
</tr>
<tr>
<td>Allele 1</td>
<td>Extension Primer + ddC</td>
<td>24</td>
<td>7355.8</td>
</tr>
<tr>
<td>Allele 2</td>
<td>Extension Primer +dT +ddC</td>
<td>25</td>
<td>7660.0</td>
</tr>
</tbody>
</table>

Prior to mass spectrometry, the products of the hME reaction are desalted and transferred onto a SpectroCHIP by the MassARRAY nanodispenser (nanodispenser) or MassARRAY piezodispenser (piezodispenser). The SpectroCHIP is then analyzed by the MassARRAY genotype analyzer (genotype analyzer).

Figure 2: Spectrum of the ddC terminated extension products depicted in Figure 1 and Table 1
Allelotyping

In addition to the genotyping of individual, amplified DNA, the MassARRAY system can be used to estimate the relative frequencies of alleles in a pool of multiple, amplified DNAs (referred to as “allelotyping”).

Note: Allelotyping requires the purchase of an upgrade to the MassARRAY system (some instrument upgrades, different reagent kits, SpectroCHIPs, and software are required). For information about purchasing an upgrade to your MassARRAY system, contact SEQUENOM at (877) 4GENOME [(877) 443-6663] in the US. For more information about contacting SEQUENOM, see Appendix J “SEQUENOM, Inc. Contact Information” on page 185.

Prior to amplification and the hME reaction, proper DNA isolation, quantitation, and pooling are critical to obtaining good results.

- **Isolation** We recommend the PUREGENE™ Genomic DNA Purification Kit (from Gentra Systems, Inc.). The isolated DNA must have \( \frac{A_{260}}{A_{280}} = 1.7-2.0 \).

- **Quantitation** We recommend PicoGreen dsDNA reagent (from Molecular Probes, Inc.); note that a spectrofluorometer is required. Instead of using PicoGreen, you may simply use an ultraviolet spectrophotometer to quantitate the DNA, however the concentration and purity estimates may not be as accurate.

- **Pooling** Before amplification, the DNA samples must be pooled together. The population size (i.e. the number of individual DNAs) of a pool depends on your needs and resources. In general, it is easier to process populations that can be broken down into multiples of 96—this reflects the commercially available microplate formats of 96 or 384 wells. We recommend populations of 300 or more, for example 384, because it gives greater statistical power.

With minor modifications, the same hME reaction used for genotyping is used for allelotyping. The modifications involve different amounts of DNA in PCR (see the Allelotyping section of this manual for more information).

The hME reaction products are dispensed to allelotyping SpectroCHIPs. The hME reaction product for each pooled sample is dispensed four times to SpectroCHIPs. For 96 reactions, the product of each reaction is dispensed to four wells (or pads) on a 384-well SpectroCHIP. For 384 reactions, the product of each reaction is dispensed to one well on four 384-well SpectroCHIPs. The redundancy in dispensing is necessary for better statistical analysis of the results.

The spectra acquired by the genotype analyzer are analyzed by the MassARRAY Typer (Typer) software which displays spectra and calculates the relative frequencies of alleles.

For more information and instructions, see the Allelotyping section of this manual.
For optimum results, design your hME reactions using MassARRAY Assay Design (SEQUENOM’s assay-design software). If you are not able to design assays using Assay Design or you do not have Assay Design, follow the guidelines below.

1. Choose the SNP or mutation of interest.

2. Design the extension primer:
   - Design an extension primer so the 3’ end is immediately adjacent to the polymorphic site.
   - Extension primers should be between 16 and 25 nucleotides long (approximately 4800 to 7500 Da). Tm should be 60°C or higher.
   - For each possibility, choose the appropriate termination mix from Table 2, “Selection of termination mix”
   - Pick an extension primer and termination mix combination which terminates at the polymorphic site for one allele (yielding an extended primer of just one nucleotide, that is, a terminator) and as soon thereafter for the alternative allele (usually yielding an extended primer of 2 nucleotides, including terminator—longer may be acceptable). The mass of the extended primer should be within the mass range of 5000 to 8500 Da.

   **Table 2: Selection of termination mix**

<table>
<thead>
<tr>
<th>SNP (Biallelic)</th>
<th>Termination Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/C</td>
<td>CGT (40 Da)</td>
</tr>
<tr>
<td>A/G</td>
<td>ACT (32 Da)</td>
</tr>
<tr>
<td>A/T</td>
<td>CGT (25 Da)</td>
</tr>
<tr>
<td>C/G</td>
<td>ACT (56 Da)</td>
</tr>
<tr>
<td></td>
<td>AGT (24 Da)</td>
</tr>
<tr>
<td>C/T</td>
<td>ACG (31 Da)</td>
</tr>
<tr>
<td>G/T</td>
<td>ACT (41 Da)</td>
</tr>
<tr>
<td>small ins/del</td>
<td>- dependent on sequence -</td>
</tr>
</tbody>
</table>

   Numbers in parentheses are the mass differences between a correct termination and a false termination (i.e., premature termination caused by pausing of the polymerase).

3. Design the amplification primers:
   - Optimal amplicon size containing the polymorphic site should be 70 to 150 bp. Fragments longer than 400 bp should be avoided.
   - To avoid confusion in the mass spectrum, make sure that the mass of the amplification primer is different from the extension primer and its extension products. It is recommended that you add a generic 10-mer tag to the 5’ end of each amplification primer.
4. Special considerations for multiplexing the hME reaction:

   • If multiplexing hME reactions, be sure to group reactions by termination mix.
   • For best results, make sure that all extension primers and all possible extension products, including possible pausing peaks differ by at least 50 Da (see Appendix B “Selection of Appropriate Termination Mixes” on page 59).
   • In multiplexed hME, the preferred mass range for analytes is 5000 to 8500 Da.

For the rationale behind Table 2, “Selection of termination mix”, and three examples illustrating the importance of choosing the appropriate termination mix, see Appendix B “Selecting Appropriate Termination Mixes” on page 149. Also, see Appendix C “Calculating the Mass of DNA Molecules” on page 107 for two examples of how to calculate expected masses of DNA molecules (Appendix C also includes a table of the masses of common nucleotides).

Components

The following MassARRAY components are used to process hME reactions.

   • Liquid handler (automated, 96-channel pipettor)
   • Liquid handler controller PC
   • Universal plate holders
   • Clean Resin plates, spoon, and scraper

Reagents and SpectroCHIPS

The hME Starter Kit, Allelotyping Starter Kit, MassEXTEND Packs, and Clean Resin Kit contain the reagents and SpectroCHIPS required to perform hME reactions.

For more information about the hME Starter Kit and the MassEXTEND Packs see the “Homogeneous MassEXTEND Assay Product Description” (included with the hME Starter Kit).

For more information about the Allelotyping Starter Kit, see the literature included with the kit.

Other Essential Components

The following components are not provided with the MassARRAY system, but are required for processing hME reactions.

   • Thermal cycler capable of processing 384-well microplates
   • Rotator capable of holding a microplate (for example, Fisher Scientific model 346)
   • Plate centrifuge (for example, Eppendorf Centrifuge 5804)
   • Twelve-channel pipettor and reservoirs
Consumables

The following are required consumables for processing hME reactions on the liquid handler.

- 20µL tips for the liquid handler (VWR International #BK717254)
- 384-Well microplates (in the United States: Marsh Biomedical Products, Inc. #SP 0401 Sequen)
- 96-Well, polypropylene, skirted microplates (in the United States: Marsh Biomedical Products, Inc. #AB-0800)
- 96-Well, polystyrene microplates (96-Well Plate Vee Bottom, Sarstedt, Inc. #82.1583)
- Disposable plate sealing film (Microseal™ ‘A’ Film, MJ Research Inc. #MSA-5001)
- Adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626)
- 1.5 mL tubes
- Autoclaved Type 1 water (>18.2 MΩ/cm resistivity)
- Reagent-grade isopropanol (70%) for liquid handler cleaning

Safety

Protective laboratory gloves and safety glasses are required when handling any reagent.

DNA Sample Requirements for hME

For genotyping, you must isolate, quantitate, and amplify the genomic DNA samples to be used with the MassARRAY system. (See “Chapter 2 Isolating and Quantitating DNA” on page 9 and “Chapter 3 Amplifying DNA for Genotyping” on page 13.)

For allelotyping, you must isolate, quantitate, pool, and amplify the genomic DNA samples. (See “Chapter 6 Isolating and Quantitating DNA” on page 85, “Chapter 7 Pooling DNA Samples” on page 89, “Chapter 8 Amplifying DNA for Allelotyping” on page 91.)
Section 2
Genotyping

This section contains instructions on using the liquid handler to process genotyping hME reactions.

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Chapter 2

Isolating and Quantitating DNA

Introduction

Before conducting any reaction to determine a genetic variation such as a single nucleotide polymorphism (SNP) with the MassARRAY system, you must have stocks of highly pure DNA to use as templates for amplification using the polymerase chain reaction (PCR).

Depending on the application, genomic DNA or cDNA is used as a template to amplify the region surrounding the genetic variation(s) of interest. Amplicons for use with the MassARRAY system are typically 70-150 bp in length. Since the amplicons are relatively short, only genomic DNA is discussed in this chapter—in most cases, the information in this chapter is also applicable to cDNA. If you wish to use cDNA templates, it is assumed you have a cDNA generation method already optimized.

After isolation, and before PCR, the genomic DNA must be quantitated. We recommend quantitation be done twice—first, with an ultraviolet (UV) spectrophotometer for an initial estimate; then with PicoGreen® dsDNA quantitation reagent.

Finally, after isolation and quantitation, we recommend diluting a large aliquot of the DNA to a working dilution of 50 ng/µL.

Isolating DNA

DNA Quality

To produce large quantities of PCR amplicons for use with the MassARRAY system, it is recommended that the genomic-DNA template be highly pure. The ratio of ultraviolet (UV) spectrophotometer readings at 260 nm and 280 nm wavelengths should be between 1.7 and 2.0 (i.e., $A_{260}/A_{280} = 1.7-2.0$). Ratios in this range indicate the presence of nucleic acid with low amounts of contaminating protein. In general, we have found genomic DNA of this quality is suitable for downstream applications, such as PCR, following isolation from samples such as whole blood, buffy coat, or cultured cells.

Isolation Kits

Out of the several commercially available DNA-isolation kits we have tested, we found the PUREGENE™ Genomic DNA Purification Kit from Gentra Systems, Inc. to perform best. In our testing, we isolated genomic DNA from whole blood, with 1-10 mL of starting material. All kits isolated genomic DNA well enough to produce PCR amplicons suitable for use with the MassARRAY system. The kit from Gentra Systems had the highest average yield of genomic DNA per mL of blood processed, along with the best average $A_{260}/A_{280}$ ratio.

We recommend the use of the kit from Gentra Systems. For convenience, the kit may be purchased through SEQUENOM (SEQUENOM part number 01257). More information about the PUREGENE Genomic DNA Purification Kit can be found at Gentra Systems’ Web site (www.gentra.com) or by calling their technical services at (800) 866-3039 in the United States.

Note: Genomic DNA may be isolated using any method you wish—it is not absolutely necessary you use the kit mentioned above. The only requirement is that whatever method you use yields highly pure DNA suitable for PCR.
Isolating and Quantitating DNA

Quantitating DNA

Isolation Protocol

Overview of the procedure for using the PUREGENE™ Genomic DNA Purification Kit on whole blood

For reference, “Appendix E Using the PUREGENE™ Genomic DNA Purification Kit” on page 165 contains the protocols, excerpted from Genomic DNA Purification Kit Instructions provided by Gentra Systems, Inc., for isolating genomic DNA from whole blood. For more information about protocols for using the PUREGENE Genomic DNA Purification Kit, see Genomic DNA Purification Kit Instructions included with the kit.

Note: DNA isolated with the PUREGENE Genomic DNA Purification Kit is stable for at least 9 years at 4 °C. For long term storage, the DNA may be stored at -20 °C, but, it is recommended that you avoid repeated freezing and thawing to reduce DNA damage. For more information about DNA stability, see Gentra Systems’ Web site (www.gentra.com).

Warning: Certain chemicals used in PUREGENE reagents may be hazardous: Tris(hydroxymethyl) aminomethane, ethylenediaminetetraacetic acid, sodium dodecyl sulfate, ammonium chloride, and ammonium acetate. These chemicals may be harmful if swallowed and contact with the eyes and skin should be avoided. In case of contact, wash with large amounts of water and seek medical attention. Wear protective clothing.

Material Safety Data Sheets (MSDS) are available from Gentra Systems, Inc. (for contact information, see “Contacting Gentra Systems” on page 170).

Quantitating DNA

With an Ultraviolet Spectrophotometer

Initial quantitation should be conducted with a UV spectrophotometer at wavelengths of 260 nm and 280 nm. The ratio of absorbance readings at the two wavelengths should be between 1.7 and 2.0 (i.e., A_{260}/A_{280} = 1.7-2.0).

Calculate the amount of DNA present by assuming one optical density (O.D.) unit, at 260 nm, to be equal to 50 µg/mL of double-stranded DNA (dsDNA). Use the following formula:

\[ \text{O.D. units} \times \text{dilution factor}^* \times 50 \text{µg/mL} = \text{amount of DNA} \]

(*For example, if the DNA is diluted 1:10, then the dilution factor is 10.)

Note that quantitating using a UV spectrophotometer, at 260 nm, does not distinguish between single-stranded DNA, RNA, and dsDNA. A UV spectrophotometer should be used to obtain an initial estimate of genomic-DNA concentration and purity. Then, we
recommend the use of PicoGreen dsDNA reagent (PicoGreen) to obtain a better estimate of concentration and purity.

With PicoGreen

PicoGreen from Molecular Probes, Inc. is a fluorescent nucleic acid stain. Because it is specific for dsDNA, it should provide better quantity estimates than a UV spectrophotometer and also has better lower-range quantitation sensitivity.

To use PicoGreen, you must have a spectrofluorometer with fluorescein excitation and emission wavelengths of 502 nm and 523 nm, respectively.

To order PicoGreen or for more information, visit Molecular Probes’ Web site (www.probes.com) or contact Molecular Probes at (541) 465-8300 in the United States.

Note: Quantitation may be done with a UV spectrophotometer alone. However, we recommend the use of PicoGreen dsDNA reagent, in addition to the UV spectrophotometer, for more accurate concentrations of DNA for use with the MassARRAY system.

PicoGreen Protocol

For reference, “Appendix F Using the PicoGreen® dsDNA Quantitation Reagent” on page 171 contains product information, including an experimental protocol, provided by Molecular Probes, Inc.

Warning: Molecular Probes, Inc. does not have data about the mutagenicity or toxicity of PicoGreen dsDNA quantitation reagent. However, because the reagent binds to nucleic acids, it should be treated and handled as a potential mutagen. Also, the reagent is in DMSO solution, which is known to facilitate the entry of organic molecules into tissues. Use double gloves when handling PicoGreen dsDNA quantitation reagent.

Working Dilution

After quantitation, we recommend diluting a large aliquot of the DNA to a stock concentration of 50 ng/µL. This concentration of stock can be used for both allelotyping and genotyping studies by diluting it to working concentrations.

Allelotyping requires 25 ng of DNA per reaction; first pool the DNA and then dilute the pooled DNA (at 50 ng/µL) 1:2 with nanopure water. Genotyping requires 2.5 ng of DNA per reaction; dilute the stock 1:20 with nanopure water.
Notes:
Chapter 3

Amplifying DNA for Genotyping

Introduction

This chapter covers amplifying genomic DNA for use with the MassARRAY system, using the polymerase chain reaction (PCR). DNA must be amplified prior to using the MassARRAY system.

Important: The polymerase chain reaction (PCR) is a patented process. You must have a license to perform PCR amplification. Purchase of MassARRAY system components or MassEXTEND reagents does not confer a license to perform PCR. Information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Centre Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

The sections in this chapter contain recommended protocols for amplifying DNA using PCR. Included are a protocol for a single PCR reaction (i.e. a single well) and two protocols for performing 384 PCR reactions (i.e. a whole 384-well microplate).

Note: This chapter contains recommended protocols. SEQUENOM does not guarantee these protocols to improve results. If you have amplification protocols in place, use them—note that you must ultimately have 5µL of amplification product for each sample.
Single Reaction

The following is the recommended protocol for a single PCR reaction.

**To perform a single PCR reaction**

1. Prepare a PCR cocktail as described in the following table.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (HPLC grade)</td>
<td>N/A</td>
<td>2.24 µL</td>
</tr>
<tr>
<td>10X HotStar Taq PCR buffer, containing 15mM MgCl₂ (QIAGEN)</td>
<td>1X, 1.5 mM MgCl₂‡</td>
<td>0.50 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1 mM†</td>
<td>0.20 µL</td>
</tr>
<tr>
<td>dNTP mix (GIBCO), 25 mM each</td>
<td>200 µM each</td>
<td>0.04 µL</td>
</tr>
<tr>
<td>Enzyme HotStar Taq Polymerase (5U/µL from QIAGEN)</td>
<td>0.1 U/reaction</td>
<td>0.02 µL</td>
</tr>
<tr>
<td>Forward and reverse PCR primer mix (1 µM each primer)</td>
<td>200 nM</td>
<td>1.00 µL</td>
</tr>
<tr>
<td>Genomic DNA* (2.5 ng/µL)</td>
<td>2.5 ng/reaction</td>
<td>1.00 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td></td>
<td><strong>5.00 µL</strong></td>
</tr>
</tbody>
</table>

‡ Final concentration of MgCl₂ is 2.5 mM (1.5 mM from the 10X HotStar Taq PCR buffer + 1.0 mM MgCl₂)

* The TE concentration in the DNA solution should be 0.25X TE or less

**Note:** Do not use PCR enhancers, such as Q-Solution.

2. Thermocycle the PCR cocktail as follows:

   1. 95°C 15 minutes
   2. 95°C 20 seconds
   3. 56°C 30 seconds
   4. 72°C 1 minute
   5. 72°C 3 minutes
   6. 4°C forever

45 cycles
384 Reactions

There are two recommended protocols for 384 PCR reactions, covering these alternative pipetting schemes:

- Same assay for all wells, different DNA in the wells.
- Same DNA in all wells, different assays for the wells.

To perform 384 PCR reactions (same assay, different DNA)

Note: These instructions cover performing PCR for a whole 384-well microplate of reactions in which the same assay will be applied to different DNA.

1. Prepare a PCR cocktail as described in the following table.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume for 384 Reactions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (HPLC grade)</td>
<td>N/A</td>
<td>1120 µL</td>
</tr>
<tr>
<td>10X HotStar Taq PCR buffer, containing 15mM MgCl₂ (QIAGEN)</td>
<td>1X, 1.5 mM MgCl₂‡</td>
<td>250 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1 mM‡</td>
<td>100 µL</td>
</tr>
<tr>
<td>dNTP mix (GIBCO), 25 mM each</td>
<td>200 µM each</td>
<td>20 µL</td>
</tr>
<tr>
<td>Enzyme HotStar Taq Polymerase (5U/µL from QIAGEN)</td>
<td>0.1 U/reaction</td>
<td>10 µL</td>
</tr>
<tr>
<td>Forward and reverse PCR primer mix (1 µM each primer)</td>
<td>200 nM</td>
<td>500 µL</td>
</tr>
<tr>
<td>Total Volume:</td>
<td></td>
<td>2000 µL</td>
</tr>
</tbody>
</table>

† Volumes include 30% overhang to account for possible pipetting loss
‡ Final concentration of MgCl₂ is 2.5 mM (1.5 mM from the 10X HotStar Taq PCR buffer + 1.0 mM MgCl₂)

Note: Do not use PCR enhancers, such as Q-Solution.

2. Into a 384-well microplate (Marsh Biomedical Products, Inc. #SP 0401 Sequen), dispense 4 µL of the PCR cocktail into each well.

3. To each well of the 384-well microplate, add 1 µL of the appropriate genomic DNA (concentration of 2.5 ng/µL).

The TE concentration in the DNA solution should be 0.25X TE or less.
4. Thermocycle the 384-well microplate as follows:

1. 95°C 15 minutes
2. 95°C 20 seconds
3. 56°C 30 seconds
4. 72°C 1 minute
5. 72°C 3 minutes
6. 4°C forever

**To perform 384 PCR reactions**
(same DNA, different assays)

**Note:** These instructions cover performing PCR for a whole 384-well microplate of reactions in which different assays will be applied to the same DNA.

**Note:** The current configuration of the liquid handler allows a maximum of 96 different assays to be applied to a microplate. For a 384-well microplate of reactions, each assay is applied to four wells.

1. Prepare a PCR cocktail as described in the following table.

   *Table 5: PCR cocktail for 384 reactions (same DNA, different assays)*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume for 384 Reactions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (HPLC grade)</td>
<td>N/A</td>
<td>1120 µL</td>
</tr>
<tr>
<td>10X HotStar Taq PCR buffer, containing 15mM MgCl₂ (QIAGEN)</td>
<td>1X, 1.5 mM MgCl₂ ‡</td>
<td>250 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1 mM‡</td>
<td>100 µL</td>
</tr>
<tr>
<td>dNTP mix ( Gibco), 25 mM each</td>
<td>200 µM each</td>
<td>20 µL</td>
</tr>
<tr>
<td>Enzyme HotStar Taq Polymerase (5U/µL from QIAGEN)</td>
<td>0.1 U/reaction</td>
<td>10 µL</td>
</tr>
<tr>
<td>Genomic DNA* (2.5 ng/µL)</td>
<td>2.5 ng/reaction</td>
<td>500 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td></td>
<td><strong>2000 µL</strong></td>
</tr>
</tbody>
</table>

† Volumes include 30% overhang to account for possible pipetting loss
‡ Final concentration of MgCl₂ is 2.5 mM (1.5 mM from the 10X HotStar Taq PCR buffer + 1.0 mM MgCl₂)
* The TE concentration in the DNA solution should be 0.25X TE or less

**Note:** Do not use PCR enhancers, such as Q-Solution.

2. Into a 384-well microplate (Marsh Biomedical Products, Inc. #SP 0401 Sequen), dispense 4 µL of the PCR cocktail into each well.
3. To each well of the 384-well microplate, add 1 µL of the appropriate forward and reverse primer mix (1 µM each primer).

4. Thermocycle the 384-well microplate as follows:
   1. 95°C 15 minutes
   2. 95°C 20 seconds
   3. 56°C 30 seconds
   4. 72°C 1 minute
   5. 72°C 3 minutes
   6. 4°C forever
   45 cycles
Notes:
Chapter 4

Single-Plate Processing of Homogeneous MassEXTEND Reactions

Introduction

This chapter covers single-plate processing of Homogeneous MassEXTEND (hME) reactions using the liquid handler. In single-plate processing, a single sample microplate is processed. (For four-plate, high-throughput processing, see “Chapter 5 High-Throughput Processing of Homogeneous MassEXTEND Reactions” on page 41.)

Note: The term sample microplate refers to any 384-well microplate of amplification products (“sample”) on which you want to perform the hME reaction.

The main steps in processing are:

1. Neutralize unincorporated dNTPs in the amplification products
2. Prepare and add hME reaction cocktail
3. Thermocycle the hME reaction
4. Clean up the hME reaction product

Detailed instructions for each of these main steps are provided in this chapter.

Before You Begin

Before you use the liquid handler to process an hME reaction, you should:

1. Change the tips if necessary:

   Change the tips at the beginning of each day (see “Changing Tips” on page 127).

2. Check the wash system tanks:

   Fill the supply tank and empty the drain tank if necessary (see “Checking the Wash System Tanks” on page 130).

3. If you perform both single-plate and high-throughput (i.e. four-plate) processing, remove all but one of the universal plate holders on the liquid handler deck.

   See “To prepare the liquid handler deck for single-plate processing” below. (Note: If you perform only single-plate processing, skip this section. Its instructions are only applicable if your liquid handler is currently configured for high-throughput processing.)
To prepare the liquid handler deck for single-plate processing

- Remove the universal plate holders from positions 1, 2, and 4.

Important: Leave the universal plate holder on position 3.

To remove a universal plate holder, simply lift it off the liquid handler deck.

There should be a universal plate holder only on position 3:
The first step in processing the hME reaction is to neutralize remaining, unincorporated dNTPs in the amplification products. Shrimp alkaline phosphatase (SAP) enzyme is used to neutralize unincorporated dNTPs. The SAP cleaves a phosphate from the unincorporated dNTPs, converting them to dNDPs and rendering them unavailable to future reaction.

**To neutralize unincorporated dNTPs**

1. In a 1.5 mL tube, prepare the SAP enzyme solution as described in the following table.

   **Table 6: SAP Enzyme Solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for Single Reaction</th>
<th>Volume for 384-Well Microplate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanopure water</td>
<td>1.53 µL</td>
<td>881.3 µL</td>
</tr>
<tr>
<td>hME Buffer</td>
<td>0.17 µL</td>
<td>97.9 µL</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase (SAP)</td>
<td>0.30 µL</td>
<td>172.8 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>2.00 µL</strong></td>
<td><strong>1152.0 µL</strong></td>
</tr>
</tbody>
</table>

   † Volumes for a 384-well microplate include 50% overhang to account for possible pipetting loss

2. Hold the 1.5 mL tube, containing the SAP enzyme solution, to a shaker for five seconds to mix the solution.

3. Centrifuge the 1.5 mL tube of SAP enzyme solution for ten seconds at 5000 RPM.

   **Note:** If you do not have a minifuge, skip this step.

4. In a new 96-well, polystyrene microplate (96-Well Plate Vee Bottom, Sarstedt, Inc. #82.1583), pipette 92 µL of SAP enzyme solution into each well of row H. See the following illustration.
5. Using a twelve-channel pipettor, draw from the wells in row H and distribute 11 µL to each well in rows A-G. See the following illustration.

![Diagram of a 96-well polystyrene microplate (partial view; Sarstedt #82.1583)](image1)

**Note:** The SAP enzyme solution is moderately viscous. Use care when pipetting to minimize loss of solution due to adhesion to the pipettor tips.

When you are done distributing the SAP enzyme solution, each well in rows A-G should have 11 µL of the SAP enzyme solution. The wells in row H should have 15 µL. (Note: These volumes are approximate; actual volumes may be slightly less due to pipetting loss.)

6. Raise the safety shield on the liquid handler.

To raise the safety shield, on the left side of the liquid handler, press and hold the top end of the release lever. (For more detailed instructions on raising the safety shield, see “To raise the safety shield” on page 122.)

7. Place the microplate of the SAP solution on position 1 of the liquid handler deck.

![Diagram of the liquid handler deck](image2)

**Note:** If you perform high-throughput processing in addition to single-plate processing, you may have a plate positioner located on position 5 of the liquid handler deck. This does not affect single-plate processing.

**Caution**
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.
8. Onto the universal plate holder (position 3), place the 384-well microplate containing the amplification products. See the following illustration.

![Diagram of the liquid handler deck](Diagram of the liquid handler deck)

**Note:** Orient the microplate so well A1 is to the **upper left**

**Important:** The sample microplate containing the amplification products must be the original one that was thermocycled during amplification. The amplification products should not have been transferred to a non-thermocycled microplate.

9. Lower the safety shield on the liquid handler.

To lower the safety shield, on the left side of the liquid handler, press and hold the bottom end of the release lever. (For more detailed instructions on lowering the safety shield, see “To lower the safety shield” on page 122.)

10. On the liquid handler controller PC, run the **hME SAP ADDITION** method.

2 µL of SAP enzyme solution is added to each well in the 384-well sample microplate.

**Note:** For instructions on running a method, see “Running a Method” on page 124.

11. When the method is done, raise the safety shield on the liquid handler.

12. Remove the sample microplate from the universal plate holder.

To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

**Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.
13. Seal the sample microplate with plate sealing film.

Make sure the edges of the plate sealing film are well-sealed.

14. Thermocycle the sample microplate as follows:

1. 37º C for 20 minutes.
2. 85º C for 5 minutes.
3. 4º C forever.

**Note:** If you have a programmable thermal cycler, enter the preceding program and name it hME SAP. When processing other hME reactions in the future, simply run the hME SAP program for this step.

15. While the sample microplate is thermocycling, begin preparing the hME reaction cocktail. Proceed to “Preparing and Adding the hME Reaction Cocktail” on the next page.
After unincorporated dNTPs have been neutralized in the amplification product (see the preceding section) the next step is to prepare the hME reaction cocktail.

**To prepare the hME reaction cocktail**

1. In a 1.5 mL tube, prepare the hME reaction cocktail as described in the following table.

   **Note:** Add the reagents in the order in which they appear in the table.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Concentration</th>
<th>Final Reaction Concentration (in 9 µL)</th>
<th>Volume for Single Reaction</th>
<th>Volume for One Sample Microplate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (NanoPure grade)</td>
<td>N/A</td>
<td>N/A</td>
<td>1.728 µL</td>
<td>915.70 µL</td>
</tr>
<tr>
<td>Appropriate hME EXTEND Mix (containing buffer and d/ddNTPs)</td>
<td>10X buffer with 2.25 mM d/ddNTPs</td>
<td>Together with PCR buffer, the reaction happens in 1X buffer 50 µM d/ddNTP each</td>
<td>0.200 µL</td>
<td>105.98 µL</td>
</tr>
<tr>
<td>hME primer(s)</td>
<td>100 µM each</td>
<td>~600 nM</td>
<td>0.054 µL</td>
<td>28.62 µL</td>
</tr>
<tr>
<td>MassEXTEND Enzyme‡ (Thermo Sequenase)</td>
<td>32 U/µL</td>
<td>0.063 U/µL</td>
<td>0.018 µL</td>
<td>9.54 µL</td>
</tr>
</tbody>
</table>

   Total Volume: 2.000 µL 1059.84 µL

   † Volumes are for a 384-well microplate and include 38% overhang to account for possible pipetting loss

   ‡ Keep the MassEXTEND Enzyme at -20°C until you are ready to add it to the reaction cocktail.
2. Into a new, 96-well polystyrene microplate (Sarstedt #82.1583 96-Well Plate Vee Bottom), dispense 85 µL of hME reaction cocktail into each well of row H. See the following illustration.

![Diagram of a 96-well polystyrene microplate](image)

Caution
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.

3. Using a twelve-channel pipettor, draw from the wells in row H and distribute 10 µL to each well in rows A-G. See the following illustration.

![Diagram of a 96-well polystyrene microplate](image)

When you are done distributing the hME reaction cocktail, each well in rows A-G should have 10 µL of the hME reaction cocktail. The wells in row H should have 15 µL. (Note: These volumes are approximate; actual volumes may be slightly less due to pipetting loss.)

4. Proceed to “To add the hME reaction cocktail” on the next page.
To add the hME reaction cocktail

1. Place the 96-well microplate, containing the hME reaction cocktail, on position 2 of the liquid handler deck.

2. Remove the sample microplate from the thermal cycler and remove the plate sealing film.

3. Place the sample microplate onto the universal plate holder on the liquid handler deck.

4. Lower the safety shield on the liquid handler.

Diagram of the liquid handler deck

Note: Orient the microplate so well A1 is to the upper left

For more information about placing microplates onto the universal plate holder, see “Using the Universal Plate Holder” on page 123
5. On the liquid handler controller PC, run the **hME COCKTAIL MIX ADDITION** method.

2 µL of hME reaction cocktail is added to each well of the sample microplate.

| Note: For instructions on running a method, see “Running a Method” on page 124. |

6. When the **hME COCKTAIL MIX ADDITION** method is done, raise the safety shield.

7. Proceed to “Thermocycling the hME Reaction” on the next page.
After you have added the hME reaction cocktail to the sample microplate (see the preceding section), the next step is to process the hME reaction by thermocycling.

**To thermocycle the hME reaction**

1. Remove the sample microplate from the universal plate holder.

   To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

   **Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.

2. Seal the sample microplate with plate sealing film.

   Make sure the edges of the plate sealing film are well-sealed.

3. Thermocycle the sample microplate as follows:

   1. 94º C for 2 minutes.
   2. 94º C for 5 seconds.
   3. 52º C for 5 seconds.
   4. 72º C for 5 seconds.
   5. 4º C forever.

   **Note:** If you have a programmable thermal cycler, enter the preceding program and name it **hME Extend**. When processing other hME reactions in the future, simply run the **hME Extend** program for this step.

4. Proceed to “Cleaning Up the hME Reaction Products” on the next page.

   **Note:** The cleanup of hME reaction products involves adding resin to the sample microplate. If you are not ready to add resin, you may store the sample microplate. Seal the microplate with plate-sealing film and store it at either -20 or 4 C. **(Caution:** When you are ready to add resin, let the microplate thaw to room temperature before adding the resin.)
Cleaning Up the hME Reaction Products

After the hME reaction has been processed by thermocycling (see the preceding section), the next step is to clean up the hME reaction products with Clean Resin (resin). This cleanup step is important to optimize mass spectrometry analysis of the hME reaction products.

**Note:** If you have a Clean Resin dispenser, do not follow the instructions in this section (i.e. skip the rest of this chapter). Instead, use the resin dispenser to add resin to your microplates of hME reaction products. For instructions on using the resin dispenser, see the SpectroCLEAN Resin Dispenser User’s Guide.

There are two different methods of cleaning up the hME reaction products:

**Automated**

The liquid handler is used to add resin to the sample microplate.

See “Cleaning Up the hME Reaction Products (Automated Only)” below.

**Manual**

Resin is added to the sample microplate manually. The liquid handler is used only to add water to the sample microplate before the resin is added manually.

See “Cleaning Up the hME Reaction Products (Manual Only)” on page 36.

---

**To prepare a microplate of resin**

**Note:** Perform these steps on a clean plastic sheet. The excess resin that is scraped off the dimple plate will fall to the plastic sheet. You can return the excess resin to its container for future use.

1. Using the elongated spoon, transfer resin from its container onto a 96-well dimple plate.

**Important:** There are two types of dimple plate: 96-well and 384-well. Be sure to use the 96-well plate. See the illustration to the right.

---

**Clean Resin Dispenser**

An instrument that quickly dispenses resin to two 384-well microplates unattended (takes approximately one minute per plate). For more information, contact SEQUENOM, Inc. at (877) 4GENOME (1-877-443-6663).

**Elongated Spoon**

This is a plastic spoon with an elongated handle. It is supplied with the MassEXTEND Assay Starter Kit. Use it to scoop resin out of its container.
2. Use the scraper to spread resin into the wells of the dimple plate.

![Scraper](image)

**Scraper**
The scraper is a flat, piece of plastic supplied with MassEXTEND Assay Starter Kit. It fits the dimensions of the dimple plates and is used to removed excess resin.

---

2. Use the scraper to spread resin into the wells of the dimple plate.

Sweep the scraper back and forth across the dimple plate to spread the resin

**Note:** Make sure there is resin in each well

3. Scrape excess resin off the dimple plate using the scraper.

![Scraper](image)

Return the excess resin to its container.

4. Proceed to “To transfer the resin to a 96-well microplate” below.

### To transfer the resin to a 96-well microplate

1. Place a clean, 96-well polypropylene microplate (Marsh Biomedical Products, Inc. #AB-0800), upside-down, over the dimple plate. Align the microplate so its wells fit directly over the corresponding wells in the dimple plate.

![96-well microplate](image)

Make sure the microplate rests against this small, rounded post on the dimple plate; this aligns the wells in the microplate with the wells in the dimple plate.
2. Holding the 96-well microplate and the dimple plate together, flip them over so the resin falls out of the dimple plate into the wells of the microplate.

![Diagram of the liquid handler deck](image)

Flip them over so the dimple plate is on top

Tap the resin out of the dimple plate. Make sure all resin falls out into the microplate.

3. Proceed to “To add the resin to the 384-well sample microplate” below.

**To add the resin to the 384-well sample microplate**

1. When the sample microplate is done thermocycling, remove it from the thermal cycler and remove the plate sealing film.

2. If they are still on the liquid handler deck, remove the microplates of SAP enzyme solution and hME reaction cocktail.

![Diagram of the liquid handler deck](image)

If they are still on the liquid handler deck, remove the microplates of SAP enzyme solution and hME reaction cocktail; discard the microplates.
3. Place the sample microplate onto the universal plate holder (position 3) on the liquid handler deck.

4. Place the 96-well microplate of resin on position 4 of the liquid handler deck.
5. Place a reservoir of 100 mL of nanopure water on position 2 of the liquid handler deck.

![Diagram of the liquid handler deck](image)

**Note**: You may use the lid from a box of tips for the liquid handler as the reservoir. The lid is slightly smaller than the deck position dimensions. Be sure to center it on the deck position.

6. Lower the safety shield on the liquid handler.

7. On the liquid handler controller PC, run the **hME CATION CLEAN-UP** method.

   The nanopure water is added to the resin. Then, 16 µL of the resin/water solution is added to each well of the sample microplate. After the resin/water solution is added, the contents of the microplate wells are mixed by repeatedly aspirating and dispensing (25 times).

   **Note**: For instructions on running a method, see “Running a Method” on page 124.

8. When the **hME CATION CLEAN-UP** method is done, raise the safety shield.

9. Go on to “To centrifuge the hME reaction products.”

   **To centrifuge the hME reaction products**

   1. Remove the sample microplate from the universal plate holder.

      To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

      **Important**: When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.

   2. Centrifuge the sample microplate for three minutes at 1600 RPM.
3. The hME reaction products are now ready for transfer to a SpectroCHIP, using the MassARRAY nanodispenser or MassARRAY piezodispenser.

You are done processing the hME reaction. Do not proceed to “Cleaning Up the hME Reaction Products (Manual Only)” on the next page.

Note: If you are not ready to transfer the hME reaction products to a SpectroCHIP at this time, you can store the microplate of reaction products at -20°C until you are ready. Place adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626) on the microplate before storing. Make sure the edges of the sealing foil are well-sealed. Do not store the microplate for more than two weeks.

If you have stored a microplate of hME reaction products, thaw and then centrifuge the microplate (for three minutes at 1600 RPM) before transferring the reaction products to a SpectroCHIP.
Cleaning Up the hME Reaction Products (Manual Only)

Important: This section covers only manual cleanup of hME reaction products.

For automated cleanup of hME reaction products, do not follow the instructions in this section; instead see “Cleaning Up the hME Reaction Products (Automated Only)” on page 30.

For information about the differences between automated and manual cleanup of hME reaction products, see “Cleaning Up the hME Reaction Products” on page 30.

First, spread resin on the 384-well dimple plate. Next, add nanopure water to each well of the sample microplate. Then, add the resin to the hME reaction products. Finally, rotate and centrifuge the hME reaction products. See the following steps for detailed instructions.

Important: You must wear gloves when handling all equipment, components, and reagents. Wear gloves when handling resin, dimple plates, and microplates.

To prepare a plate of resin

Note: Perform these steps on a clean plastic sheet. The excess resin that is scraped off the dimple plate will fall to the plastic sheet. You can return the excess resin to its container for future use.

1. Using the elongated spoon, transfer resin from its container onto the 384-well dimple plate.

Important: There are two types of dimple plate: 96-well and 384-well. Be sure to use the 384-well plate. See the illustration to the right.

2. Use the scraper to spread resin into the wells of the dimple plate.

Note: Make sure there is resin in each well.

---

Elongated Spoon
This is a plastic spoon with an elongated handle. It is supplied with the MassEXTEND Assay Starter Kit. Use it to scoop resin out of its container.

Scraper
The scraper is a flat, piece of plastic supplied with MassEXTEND Assay Starter Kit. It fits the dimensions of the dimple plates and is used to removed excess resin.
3. Scrape excess resin off the dimple plate using the scraper.

Return the excess resin to its container.

4. Let the resin stand in the dimple plate for at least 20 minutes.

While letting the resin stand in the dimple plate, add water to the sample microplate. See the next set of steps.

To add water to the 384-well sample microplate

1. When the sample microplate is done thermocycling, remove it from the thermal cycler and remove the plate sealing film.

2. Raise the safety shield on the liquid handler.

3. Place the sample microplate onto the universal plate holder on the liquid handler deck.
4. Place a reservoir containing 40-50 mL of nanopure water on position 4 of the liquid handler deck.

![Diagram of the liquid handler deck](attachment:diagram.png)

Place a reservoir of nanopure water on position 4

Note: You may use the lid from a box of tips for the liquid handler as the reservoir. The lid is slightly smaller than the deck position dimensions. Be sure to center it on the deck position.

5. Lower the safety shield on the liquid handler.

6. On the liquid handler controller PC, run the **hME H2O ADDITION** method.

   16 µL of nanopure water is added to each well of the sample microplate.

   **Note:** For instructions on running a method, see “Running a Method” on page 124.

7. When the **hME H2O ADDITION** method is done, raise the safety shield.

8. Remove the sample microplate from the universal plate holder (position 3).

   To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

   **Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.

9. Check the microplate wells for air bubbles.

   If air bubbles are present, centrifuge the microplate (for 30 seconds at 1600 RPM, or until the air bubbles are gone).

10. Proceed to “To add resin to the sample microplate” on the next page.
To add resin to the sample microplate

1. Place the sample microplate, upside-down, onto the dimple plate.

2. Holding the sample microplate and the dimple plate together, flip them over so the resin falls out of the dimple plate into the wells of the microplate.

3. Proceed to “To rotate and centrifuge the hME reaction products” on the next page.

Dimple plate

384-well sample microplate (upside-down)

Make sure the microplate rests against this small, rounded post on the dimple plate; this aligns the wells in the microplate with the wells in the dimple plate

Flip them over so the dimple plate is on top

384-well microplate and dimple plate

Tap the dimple plate so the resin falls out into the microplate. Make sure all the resin in the dimple plate falls out into the microplate wells.
To rotate and centrifuge the hME reaction products

1. Rotate the sample microplate on a rotator for five minutes, at room temperature.
   
   The rotator must rotate the microplate 360° about its long axis.

2. Centrifuge the sample microplate for three minutes at 1600 RPM.

3. The hME reaction products are now ready for transfer to a SpectroCHIP, using the MassARRAY Nanodispenser or MassARRAY Piezodispenser.

Note: If you are not ready to transfer the hME reaction products to a SpectroCHIP, you can store the microplate at -20°C until you are ready. Place adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626) on the microplate before storing. Make sure the edges of the sealing foil are well-sealed. Do not store the microplate for more than two weeks.

If you have stored a microplate of hME reaction products, thaw and then centrifuge the microplate (for three minutes at 1600 RPM) before transferring the reaction products to a SpectroCHIP.
Chapter 5

High-Throughput Processing of Homogeneous MassEXTEND Reactions

**Requirement**
High-throughput processing requires Multimek High Throughput Upgrade (part number 11233). For more information, contact SEQUENOM at (877) 4GENOME [(877) 443-6663].

**Introduction**
This chapter covers high-throughput processing of Homogeneous MassEXTEND (hME) reactions using the liquid handler. In high-throughput processing, four sample microplates are processed at one time.

**Note:** The term **sample microplate** refers to any 384-well microplate of amplification products ("sample") on which you want to perform the hME reaction.

The main steps in processing are:

1. Neutralize unincorporated dNTPs in the amplification products
2. Prepare and add hME reaction cocktail
3. Thermocycle the hME reaction
4. Clean up the hME reaction products

Detailed instructions for each of these main steps are provided in this chapter.

**Two-Plate Option**

Rather than processing four sample microplates at a time, you may process two microplates at a time. Throughout this chapter alternative instructions, or additional information, for two-plate processing is provided as **Two-Plate Option** notes. See the following example.

**Two-Plate Option:**

To process two microplates, follow the four-plate instructions in this chapter as you would normally. When you encounter a step with a **Two-Plate Option** note, follow the instructions in the note.

**Important:** If you are processing four sample microplates, simply ignore all **Two-Plate Option** notes.
Before You Begin

Before you use the liquid handler to process any hME reaction, you should:

1. Change the tips if necessary:
   
   Change the tips at the beginning of each day (see “Changing Tips” on page 127).

2. Check the wash system tanks:
   
   Fill the supply tank and empty the drain tank if necessary (see “Checking the Wash System Tanks” on page 130).

3. Make sure all four universal plate holders are on the liquid-handler deck.
   
   See “To prepare the liquid-handler deck for high-throughput processing” below.

To prepare the liquid-handler deck for high-throughput processing

1. A plate positioner must be on position 5.

![Diagram of the liquid-handler deck]

There must be a plate positioner here (on position 5).

There is an indentation at the back of the plate positioner. Orient the plate positioner so the indentation is on this side (toward the back of the liquid handler).

There are two holes in the bottom of the plate positioner that line up with the positioning pegs on the liquid-handler deck. Seat the plate positioner flat on the liquid-handler deck with the positioning pegs fitting into these holes.
2. There should be a universal plate holder on each position 1-4.

![Diagram of the liquid-handler deck](image)

Positions 1-4 should each have a universal plate holder.

3. To place a universal plate holder on a deck position, see the following illustration.

![Diagram of universal plate holder](image)

This block on the liquid-handler deck enforces the proper orientation of the universal plate holder.

Place the universal plate holder on the deck position, in the orientation shown here.

Positioning post on the liquid-handler deck.
The first step in processing the hME reaction is to neutralize remaining, unincorporated dNTPs in the amplification products. Shrimp alkaline phosphatase (SAP) enzyme is used to neutralize unincorporated dNTPs. The SAP cleaves a phosphate from the unincorporated dNTPs, converting them to dNDPs and rendering them unavailable to future reaction. Complete the following procedure.

**To neutralize unincorporated dNTPs**

1. In a 15 mL tube, prepare the SAP enzyme solution as described in the following table (in the Volume for Four Sample Microplates column).

   **Note:** Add the reagents in the order in which they appear in the table.

   **Table 8: SAP Enzyme Solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for Single Reaction</th>
<th>Volume for Two Sample Microplates†</th>
<th>Volume for Four Sample Microplates†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanopure water</td>
<td>1.53 µL</td>
<td>1762.6 µL</td>
<td>3525.2 µL</td>
</tr>
<tr>
<td>hME Buffer</td>
<td>0.17 µL</td>
<td>195.8 µL</td>
<td>391.6 µL</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase (SAP)</td>
<td>0.30 µL</td>
<td>345.6 µL</td>
<td>691.2 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>2.00 µL</strong></td>
<td><strong>2304.0 µL</strong></td>
<td><strong>4608.0 µL</strong></td>
</tr>
</tbody>
</table>

† Volumes are for 384-well microplates and include 50% overhang to account for possible pipetting loss

2. Hold the 15 mL tube, containing the SAP enzyme solution, to a shaker for five seconds to mix the solution.

3. Into a new 96-well, polystyrene microplate (96-Well Plate Vee Bottom, Sarstedt, Inc. #82.1583), pipette 184 µL of SAP enzyme solution into each well of rows D and H. See the following illustration.

   **Diagram of a 96-well polystyrene microplate (Sarstedt #82.1583)**

   Note: The wells in rows D and H will be used as reservoirs from which you will distribute SAP enzyme solution to the rest of the wells in the microplate.

   Dispense 184 µL of SAP enzyme solution into each well of these rows.
Two-Plate Option: Instead of dispensing 184 µL of SAP enzyme solution into the wells of both rows D and H, dispense 184 µL of SAP enzyme solution into the wells of only row H.

4. Using a twelve-channel pipettor, draw from the wells in row H and distribute 44 µL to each well in rows G-E. Then, draw from the wells in row D and distribute 44 µL to each well in rows C-A. See the following illustration.

Caution
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.

When you are done distributing the SAP enzyme solution, each well in rows A-C and E-G should have 44 µL of the SAP enzyme solution. The wells in rows D and H should have 52 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.)

Two-Plate Option: Draw from the wells in row H and distribute 22 µL to each of the other wells (in rows A-G). After distributing the SAP enzyme solution, each well in rows A-G should have 22 µL of SAP enzyme solution. And each well in row H should have 30 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.)

Note: The SAP enzyme solution is moderately viscous. Use care when pipetting to minimize loss of solution due to adhesion to the pipettor tips.

5. Raise the safety shield on the liquid handler.

For instructions on raising the safety shield, see “To raise the safety shield” on page 122.
6. Place the microplate of SAP solution on position 5 of the liquid-handler deck.

![Diagram of the liquid-handler deck](image)

**Note:** Orient the microplate so well A1 is to the upper left.

7. Place the sample microplates onto the liquid-handler deck. See the following illustrations.

![Diagram of the liquid-handler deck](image)

**Note:** Orient the microplates so well A1 is to the upper left.

**Important:** The microplates containing the amplification products must be the originals thermocycled during amplification. The amplification products should not have been transferred to non-thermocycled microplates.

**Two-Plate Option:** Place the two sample microplates on positions 1 and 2. Leave positions 3 and 4 empty.

8. Lower the safety shield on the liquid handler.

To lower the safety shield, on the left side of the liquid handler, press and hold the bottom end of the release lever. (For more detailed instructions on lowering the safety shield, see “To lower the safety shield” on page 122.)
9. On the liquid handler controller PC, run the **hME FOUR PLATE SAP ADDITION** method.

When you run the method, 2 µL of SAP enzyme solution is added to each well in the sample microplates.

<table>
<thead>
<tr>
<th>Two-Plate Option:</th>
<th>Run the <strong>hME TWO PLATE SAP ADDITION</strong> method instead of the <strong>hME FOUR PLATE SAP ADDITION</strong> method.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Note:</strong></td>
<td>For instructions on running a method, see “Running a Method” on page 124.</td>
</tr>
</tbody>
</table>

10. When the method is done, raise the safety shield on the liquid handler.

11. Remove the microplate of SAP enzyme solution from position 5 and discard it.

12. Remove the sample microplates from the universal plate holders on the liquid-handler deck.

To remove a sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

| **Important:**     | When removing a microplate from a universal plate holder, be careful not to splash any sample out of the microplate. |

13. Seal the sample microplates with plate sealing film.

Make sure the edges of the plate sealing film are well-sealed.

14. Thermocycle each sample microplate as follows:

1. 37°C for 20 minutes.
2. 85°C for 5 minutes.
3. 4°C forever.

| **Note:** | If you have a programmable thermal cycler, enter the preceding program and name it **hME SAP**. When processing other hME reactions in the future, simply run the **hME SAP** program for this step. |

15. While the sample microplates are thermocycling, begin preparing the hME reaction cocktail. Proceed to “Preparing and Adding the hME Reaction Cocktail” on the next page.
Preparing and Adding the hME Reaction Cocktail

How Many Cocktails to Prepare and Add
The number of hME reaction cocktails you must prepare and add depends on the number of assays you want to run. Each assay requires a different reaction cocktail. For example, if you want to run the same assay on all four sample microplates then you must prepare only one reaction cocktail and add it to all four sample microplates. Alternatively, if you want to run four assays (one on each microplate) then you must prepare and add four reaction cocktails.

You can prepare and add:
- **One hME reaction cocktail**: the same cocktail is added to all four sample microplates.
- **Two hME reaction cocktails**: one cocktail is added to two sample microplates, a different cocktail to the other two microplates.
- **Four hME reaction cocktails**: four different cocktails are added, each to a different sample microplate.

See the following illustrations.

**One hME Reaction Cocktail**

*Same hME reaction cocktail to all four sample microplates*

**Two hME Reaction Cocktails**

*One hME reaction cocktail to two sample microplates, a different cocktail to the other two microplates*
The basic procedure for preparing and adding hME reaction cocktail is the same regardless of how many reaction cocktails you are preparing and adding. At the points where the instructions differ, follow only the instruction for the number of reaction cocktails you are preparing and adding. The differing instructions are contained in the following types of notes:

**One hME Reaction Cocktail:** This is an example of a *One hME Reaction Cocktail* note. It contains instructions that should be followed when preparing one hME reaction cocktail and adding it to the sample microplates.

**Two hME Reaction Cocktails:** This is an example of a *Two hME Reaction Cocktails* note. It contains instructions that should be followed when preparing two hME reaction cocktails and adding them to the sample microplates.

**Four hME Reaction Cocktails:** This is an example of a *Four hME Reaction Cocktails* note. It contains instructions that should be followed when preparing four hME reaction cocktails and adding them to the sample microplates.

Proceed to “To prepare the hME reaction cocktail” on page 50.

---

**Two-Plate Option**

For processing only two sample microplates, *do not follow the instructions in this section*. Instead, see “Preparing and Adding the hME Reaction Cocktail (Two-Plate Option Only)” on page 60.
To prepare the hME reaction cocktail

1. In 15 mL tubes, prepare hME reaction cocktail as described in the following table.

Add the reagents in the order in which they appear in the table. (See the notes after the table for specific instructions for the different number of reaction cocktails you may prepare.)

Table 9: hME reaction cocktail

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Concentration</th>
<th>Final Reaction Concentration (in 9 µL)</th>
<th>Volume for Single Reaction</th>
<th>Volume for Four Sample Microplates†</th>
<th>Volume for Two Sample Microplates†</th>
<th>Volume for One Sample Microplate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (NanoPure grade)</td>
<td>N/A</td>
<td>N/A</td>
<td>1.728 µL</td>
<td>3662.80 µL</td>
<td>1831.40 µL</td>
<td>915.70 µL</td>
</tr>
<tr>
<td>Appropriate hME EXTEND Mix (containing buffer and d/ddNTPs)</td>
<td>10X buffer with 2.25 mM d/ddNTPs</td>
<td>Together with PCR buffer, the reaction happens in 1X buffer 50 µM d/ddNTP each</td>
<td>0.200 µL</td>
<td>423.92 µL</td>
<td>211.96 µL</td>
<td>105.98 µL</td>
</tr>
<tr>
<td>hME primer(s)</td>
<td>100 µM each</td>
<td>~600 nM</td>
<td>0.054 µL</td>
<td>114.48 µL</td>
<td>57.24 µL</td>
<td>28.62 µL</td>
</tr>
<tr>
<td>MassEXTEND Enzyme‡ (Thermo Sequenase)</td>
<td>32 U/µL</td>
<td>0.063 U/µL</td>
<td>0.018 µL</td>
<td>38.16 µL</td>
<td>19.08 µL</td>
<td>9.54 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td></td>
<td></td>
<td>2.000 µL</td>
<td>4239.36 µL</td>
<td>2119.68 µL</td>
<td>1059.84 µL</td>
</tr>
</tbody>
</table>

† Volumes are for 384-well microplates and include 38% overhang to account for possible pipetting loss
‡ Keep the MassEXTEND Enzyme at -20°C until you are ready to add it to the reaction cocktail.

One hME Reaction Cocktail: Refer to the “Volume for Four Sample Microplates” column. Prepare only one tube.

Two hME Reaction Cocktails: Refer to the “Volume for Two Sample Microplates” column. Prepare two tubes, each containing one of the two different hME reaction cocktails. (Important: Keep track of which hME reaction cocktail each tube contains.)

Four hME Reaction Cocktails: Refer to the “Volume for One Sample Microplate” column. Prepare four tubes, each containing one of the four different hME reaction cocktails. (Important: Keep track of which hME reaction cocktail each tube contains.)

In the notes above, when you are instructed to prepare multiple tubes, it means you should follow the “recipe” in the specified table column multiple times—once for each tube of different reaction cocktail you wish to prepare.
2. Pipette hME reaction cocktail, from the tube or tubes you prepared in the previous step, to new microplates (96-well polystyrene; Sarstedt #82.1583 96-Well Plate Vee Bottom). See the following notes and illustration.

<table>
<thead>
<tr>
<th>One hME Reaction Cocktail:</th>
<th>Prepare one cocktail microplate. Into each well of rows D and H, pipette 170 µL of the hME reaction cocktail.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two hME Reaction Cocktails:</td>
<td>Prepare two cocktail microplates, one for each hME reaction cocktail. Into one microplate, pipette 170 µL of the first hME reaction cocktail into each well of row H. Into the other microplate, do the same thing with the second hME reaction cocktail. Pipette 170 µL into each well of row H. (Important: Keep track of which microplate contains which hME reaction cocktail.)</td>
</tr>
<tr>
<td>Four hME Reaction Cocktails:</td>
<td>Prepare four cocktail microplates, one for each hME reaction cocktail. Match up each tube of hME reaction cocktail with one of the microplates. For each tube and microplate, do the following: pipette 85 µL of the hME reaction cocktail into each well of row H. (Important: Keep track of which microplate contains which hME reaction cocktail.)</td>
</tr>
</tbody>
</table>

3. Label each microplate to keep track of which reaction cocktail it contains.

Use whatever method you routinely employ for labeling microplates, for instance writing directly on the microplate or placing adhesive labels. Be sure not to disturb or contaminate the reaction cocktail in the wells. If using adhesive labels, be sure they do not cover any well.
4. Using a twelve-channel pipettor, distribute the hME reaction cocktail across the wells in each cocktail microplate. See the following notes and illustration.

<table>
<thead>
<tr>
<th>One hME Reaction Cocktail:</th>
<th>Draw from the wells in row D and distribute 40 µL to each well in rows A-C. Then draw from the wells in row H and distribute 40 µL to each well in rows E-G.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>When you are done distributing the cocktail, each well in rows A-C and E-G should have 40 µL and each well in rows D and H should have 50 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Two hME Reaction Cocktails:</th>
<th>Do the following for each of the two cocktail microplates: Draw from the wells in row H and distribute 20 µL to each well in rows A-G.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>When you are done distributing the cocktail, each well in rows A-G should have 20 µL and each well in row H should have 30 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Four hME Reaction Cocktails:</th>
<th>Do the following for each of the four cocktail microplates: Draw from the wells in row H and distribute 10 µL to each well in rows A-G.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>When you are done distributing the cocktail, each well in rows A-G should have 10 µL and each well in row H should have 15 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.)</td>
</tr>
</tbody>
</table>

**Caution**
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.

**Diagram of a 96-well polystyrene microplate (Sarstedt #82.1583), showing the pipetting scheme for the case in which you are preparing only one hME reaction cocktail for all four sample microplates (i.e. One hME Reaction Cocktail option)**
5. Discard the 15 mL tube or tubes in which you prepared the hME reaction cocktail.

6. The next stage of processing involves adding the hME reaction cocktail to the sample microplates. There are three different ways to add hME reaction cocktail depending on the number of reaction cocktails you have. See the following notes.

<table>
<thead>
<tr>
<th>One hME Reaction Cocktail:</th>
<th>See “To add the same hME reaction cocktail to the sample microplates” on page 53.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two hME Reaction Cocktails:</td>
<td>See “To add two different hME reaction cocktails to the sample microplates” on page 55.</td>
</tr>
<tr>
<td>Four hME Reaction Cocktails:</td>
<td>See “To add four different hME reaction cocktails to the sample microplates” on page 57.</td>
</tr>
</tbody>
</table>

**To add the same hME reaction cocktail to the sample microplates**

Complete these steps only if you want to add the same hME reaction cocktail to the four sample microplates.

1. Place the 96-well microplate, containing the hME reaction cocktail, on position 5 of the liquid-handler deck.

   ![Diagram of the liquid-handler deck](image)

   **Note:** Orient the microplate so well A1 is to the upper left

2. Wait for the hME SAP thermocycling program to complete on all four sample microplates.

   When thermocycling is complete remove the plate sealing film from the microplates.
3. Place the sample microplates on positions 1-4 of the liquid-handler deck.

4. Lower the safety shield on the liquid handler.

5. On the liquid handler controller PC, run the **hME FOUR PLATE COCKTAIL MIX ADDITION** method.

   2 µL of hME reaction cocktail is added to each well of the 384-well sample microplate.

   **Note:** For instructions on running a method, see “Running a Method” on page 124.

6. When the **hME FOUR PLATE COCKTAIL MIX ADDITION** method is done, raise the safety shield.

7. Remove the microplate of hME reaction cocktail and discard it.

8. Remove the sample microplates from the liquid-handler deck.

9. Proceed to “Thermocycling the hME Reaction” on page 70.
To add two different hME reaction cocktails to the sample microplates

Complete these steps only if you want to add two different hME reaction cocktails to the sample microplates—one cocktail to two of the sample microplates, the other cocktail to the other two sample microplates.

**Note:** You will repeat these steps twice. The sample microplates are processed two at a time.

1. Place one of the 96-well microplates, containing hME reaction cocktail, on position 5 of the liquid-handler deck.

2. Wait for all of the sample microplates to complete thermocycling.

When thermocycling is complete remove the plate sealing film from the microplates.

3. Place two of the sample microplates on positions 1 and 2 of the liquid-handler deck.

**Important:** Make sure the two sample microplates you place on the liquid-handler deck are those to which you want to add the particular hME reaction cocktail currently on position 5.
4. Lower the safety shield on the liquid handler.

5. On the liquid handler controller PC, run the **hME TWO PLATE COCKTAIL ADD. 1 SOURCE** method.

2 µL of hME reaction cocktail is added to each well of the sample microplates.

**Note:** For instructions on running a method, see “Running a Method” on page 124.

6. When the **hME TWO PLATE COCKTAIL ADD. 1 SOURCE** method is done, raise the safety shield.

7. Remove the microplate of hME reaction cocktail and discard it.

8. Remove the sample microplates from the liquid-handler deck.

9. Repeat these steps for the remaining hME reaction cocktail microplate and sample microplates.

10. Proceed “Thermocycling the hME Reaction” on page 70.
To add four different hME reaction cocktails to the sample microplates

Complete these steps only if you want to add four different hME reaction cocktails to the sample microplates—each reaction cocktail added to one of the sample microplates.

Note: You will repeat this procedure twice. The sample microplates are processed two at a time.

1. Remove the universal plate holders from positions 3 and 4. Set them aside.

2. Place two of the 96-well microplates, containing hME reaction cocktail, on positions 3 and 4 of the liquid-handler deck.

3. Wait for all of the sample microplates to complete thermocycling.

When thermocycling is complete remove the plate sealing film from the microplates.
4. Place two of the sample microplates on positions 1 and 2 of the liquid-handler deck.

5. Lower the safety shield on the liquid handler.

6. On the liquid handler controller PC, run the **hME TWO PLATE COCKTAIL MIX ADDITION** method.

   2 µL of hME reaction cocktail is added to each well of the sample microplates.

   **Note**: For instructions on running a method, see “Running a Method” on page 124.

7. When the **hME TWO PLATE COCKTAIL MIX ADDITION** method is done, raise the safety shield.
8. Remove the microplates of hME reaction cocktail and discard them.

9. Remove the sample microplates from the liquid-handler deck.

10. Repeat these steps for the remaining two hME reaction cocktail microplates and sample microplates.

11. Replace the universal plate holders (removed in step 1) onto positions 3 and 4.

The following illustration shows how to place universal plate holders.

12. Proceed to “Thermocycling the hME Reaction” on page 70.
Preparing and Adding the hME Reaction Cocktail (Two-Plate Option Only)

**Important:** This section applies only to two-plate processing. If you are performing regular high-throughput processing (i.e. four-plate processing) do not follow the instructions in this section. Instead, see “Preparing and Adding the hME Reaction Cocktail” on page 48.

After unincorporated dNTPs in the amplification products have been neutralized (see “Neutralizing Unincorporated dNTPs in the Amplification Products” on page 44) the next step is to prepare the hME reaction cocktail and add it to the sample microplates.

There are two different ways to prepare and add hME reaction cocktail depending on whether the same hME reaction cocktail or different cocktail is to be added to the sample microplates.

You can prepare and add:

- **One hME reaction cocktail:** the same cocktail is added to both sample microplates.
- **Two hME reaction cocktails:** one cocktail is added to one sample microplate, a different cocktail to the other microplate.

See the following illustrations.

**One hME Reaction Cocktail**

![Illustration of one hME reaction cocktail](image)

**Two hME Reaction Cocktails**

![Illustration of two hME reaction cocktails](image)
The basic procedure for preparing and adding hME reaction cocktail is the same regardless of how many reaction cocktails you are preparing and adding. At the points where the instructions differ, follow only the instruction for the number of reaction cocktails you are preparing and adding. The differing instructions are contained in the following types of notes:

<table>
<thead>
<tr>
<th>Type of Note</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>One hME Reaction Cocktail</td>
<td>This is an example of a One hME Reaction Cocktail note. It contains instructions that should be followed when preparing one hME reaction cocktail and adding it to the sample microplates.</td>
</tr>
<tr>
<td>Two hME Reaction Cocktails</td>
<td>This is an example of a Two hME Reaction Cocktails note. It contains instructions that should be followed when preparing two hME reaction cocktails and adding them to the sample microplates.</td>
</tr>
</tbody>
</table>

Proceed to “To prepare the hME reaction cocktail” on page 62.
To prepare the hME reaction cocktail

1. In 15 mL tubes, prepare hME reaction cocktail as described in the following table.

Add the reagents in the order in which they appear in the table. (See the notes after the table for specific instructions for the different number of reaction cocktails you may prepare.)

Table 10: hME reaction cocktail

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Concentration</th>
<th>Final Reaction Concentration (in 9 µL)</th>
<th>Volume for Single Reaction</th>
<th>Volume for Two Sample Microplates†</th>
<th>Volume for One Sample Microplate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (NanoPure grade)</td>
<td>N/A</td>
<td>N/A</td>
<td>1.728 µL</td>
<td>1831.40 µL</td>
<td>915.70 µL</td>
</tr>
<tr>
<td>Appropriate hME EXTEND Mix (containing buffer and d/ddNTPs)</td>
<td>10X buffer with 2.25 mM d/ddNTPs</td>
<td>Together with PCR buffer, the reaction happens in 1X buffer 50 µM d/ddNTP each</td>
<td>0.200 µL</td>
<td>211.96 µL</td>
<td>105.98 µL</td>
</tr>
<tr>
<td>hME primer(s)</td>
<td>100 µM each</td>
<td>~600 nM</td>
<td>0.054 µL</td>
<td>57.24 µL</td>
<td>28.62 µL</td>
</tr>
<tr>
<td>MassEXTEND Enzyme‡ (Thermo Sequenase)</td>
<td>32 U/µL</td>
<td>0.063 U/µL</td>
<td>0.018 µL</td>
<td>19.08 µL</td>
<td>9.54 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td></td>
<td></td>
<td>2.000 µL</td>
<td>2119.68 µL</td>
<td>1059.84 µL</td>
</tr>
</tbody>
</table>

† Volumes are for 384-well microplates and include 38% overhang to account for possible pipetting loss
‡ Keep the MassEXTEND Enzyme at -20°C until you are ready to add it to the reaction cocktail.

One hME Reaction Cocktail: Refer to the “Volume for Two Sample Microplates” column. Prepare only one tube.

Two hME Reaction Cocktails: Refer to the “Volume for One Sample Microplate” column. Prepare two tubes, each containing one of the two different hME reaction cocktails.
(Important: Keep track of which hME reaction cocktail each tube contains.)

In the Two hME Reaction Cocktails note above, when you are instructed to prepare two tubes, it means you should follow the “recipe” in the specified table column twice—once for each tube of different reaction cocktail you wish to prepare.
2. Pipette hME reaction cocktail, from the tube or tubes you prepared in the previous step, to new microplates (96-well polystyrene; Sarstedt #82.1583 96-Well Plate Vee Bottom). See the following notes and illustration.

<table>
<thead>
<tr>
<th>One hME Reaction Cocktail:</th>
<th>Into a new microplate, pipette 170 µL of the hME reaction cocktail into each well of row H.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two hME Reaction Cocktails:</td>
<td>Prepare two cocktail microplates, one for each hME reaction cocktail. Match up each tube of hME reaction cocktail with one of the microplates. For each tube and microplate, do the following: pipette 85 µL of the hME reaction cocktail into each well of row H.</td>
</tr>
<tr>
<td></td>
<td><em>(Important: Keep track of which hME reaction cocktail is contained by each microplate.)</em></td>
</tr>
</tbody>
</table>

3. Label each microplate to keep track of which reaction cocktail it contains.

Use whatever method you routinely employ for labeling microplates, for instance writing directly on the microplate or placing adhesive labels. Be sure not to disturb or contaminate the reaction cocktail in the wells. If using adhesive labels, be sure they do not cover any well.
4. Using a twelve-channel pipettor, distribute the hME reaction cocktail across the wells in each cocktail microplate. See the following notes and illustration.

**Caution**
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.

| One hME Reaction Cocktail: | Draw from the wells in row H and distribute 20 µL to each well in rows A-G. When you are done distributing the cocktail, each well in rows A-G should have 20 µL and each well in row H should have 30 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.) |
| Two hME Reaction Cocktails: | Do the following for each of the two cocktail microplates: Draw from the wells in row H and distribute 10 µL to each well in rows A-G. When you are done distributing the cocktail, each well in rows A-G should have 10 µL and each well in row H should have 15 µL. (Note: these volumes are approximate; actual volumes may be slightly less due to pipetting loss.) |

Drawing from the wells in row H, distribute either 20 µL (One hME Reaction Cocktail) or 10 µL (Two hME Reaction Cocktails) of hME reaction cocktail into each of the other wells.

5. Discard the 15 mL tube or tubes in which you prepared the hME reaction cocktail.
6. The next stage of processing involves adding the hME reaction cocktail to the sample microplates. There are two different ways to add hME reaction cocktail to sample microplates. Which method you should use depends on the number of hME reaction cocktails you want to add to the sample microplates. See the following notes.

<table>
<thead>
<tr>
<th>One hME Reaction Cocktail:</th>
<th>See “To add the same hME reaction cocktail to the sample microplates” below.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two hME Reaction Cocktails:</td>
<td>See “To add two different hME reaction cocktails to the sample microplates” on page 67.</td>
</tr>
</tbody>
</table>

**To add the same hME reaction cocktail to the sample microplates**

Complete these steps only if you want to add the same hME reaction cocktail to both sample microplates.

1. Place the 96-well microplate, containing hME reaction cocktail, on position 5 of the liquid-handler deck.

2. Wait for both sample microplates to complete thermocycling.

When thermocycling is complete remove the plate sealing film from the microplates.

Diagram of the liquid-handler deck

- Place the hME reaction cocktail on position 5
- Note: Orient the microplate so well A1 is to the upper left

**Diagram of the liquid-handler deck**
3. Place the sample microplates on positions 1 and 2 of the liquid-handler deck.

![Diagram of the liquid-handler deck]

**Note:** Orient the microplates so well A1 is to the upper left.
Place the two sample microplates onto positions 1 and 2.

4. Lower the safety shield on the liquid handler.

5. On the liquid handler controller PC, run the **hME TWO PLATE COCKTAIL ADD. 1 SOURCE** method.

2 µL of hME reaction cocktail is added to each well of the sample microplates.

**Note:** For instructions on running a method, see “Running a Method” on page 124.

6. When the **hME TWO PLATE COCKTAIL ADD. 1 SOURCE** method is done, raise the safety shield.

7. Remove the microplate of hME reaction cocktail and discard it.

8. Remove the sample microplates from the liquid-handler deck.

9. Proceed to “Thermocycling the hME Reaction” on page 70.
To add two different hME reaction cocktails to the sample microplates

Complete these steps only if you want to add two different hME reaction cocktails to the sample microplates—each reaction cocktail added to one of the sample microplates.

1. Remove the universal plate holders from positions 3 and 4. Set them aside.

2. Place the 96-well microplates, containing hME reaction cocktail, on positions 3 and 4 of the liquid-handler deck.

3. Wait for both sample microplates to complete thermocycling.

When thermocycling is complete remove the plate sealing film from the microplates.
4. Place the sample microplates on positions 1 and 2 of the liquid-handler deck.

![Diagram of the liquid-handler deck](image)

- **Note:** Orient the microplates so well A1 is to the upper left.
- Place the two sample microplates onto positions 1 and 2.
- Turn the corner hold-downs onto the microplates' skirts to secure them.

**Important:** The reaction cocktail from each hME reaction cocktail plate will be dispensed to the microplate below it (i.e., reaction cocktail from position 3 goes to the microplate on position 1 and reaction cocktail from position 4 goes to position 2). Be sure to match up the sample microplates and hME reaction cocktail plates correctly. For example, if the reaction cocktail for a given sample microplate is on position 3, then you must place the sample microplate on position 1.

5. Lower the safety shield on the liquid handler.

6. On the liquid handler controller PC, run the hME TWO PLATE COCKTAIL MIX ADDITION method.

2 µL of hME reaction cocktail is added to each well of the sample microplates.

**Note:** For instructions on running a method, see “Running a Method” on page 124.

7. When the hME TWO PLATE COCKTAIL MIX ADDITION method is done, raise the safety shield.
8. Remove the microplates of hME reaction cocktail and discard them.

8. Remove the microplates of hME reaction cocktail and discard them.

9. Remove the sample microplates from the liquid-handler deck.

10. Replace the universal plate holders (removed in step 1) onto positions 3 and 4.

The following illustration shows how to place universal plate holders.

11. Proceed to “Thermocycling the hME Reaction” on the next page.
After you have added the hME reaction cocktail to the sample microplates (see “Preparing and Adding the hME Reaction Cocktail” on page 48), the next step is to process the hME reaction by thermocycling.

**To thermocycle the hME reaction**

1. Seal the sample microplates with plate sealing film.

   Make sure the edges of the plate sealing film are well-sealed.

2. Thermocycle each sample microplate as follows:

   1. 94°C for 2 minutes.
   2. 94°C for 5 seconds.
   3. 52°C for 5 seconds.
   4. 72°C for 5 seconds.
   5. 4°C forever.

   **Note:** If you have a programmable thermal cycler, enter the preceding program and name it hME Extend. When processing other hME reactions in the future, simply run the hME Extend program for this step.

3. Once you have started thermocycling the sample microplates, proceed to “Cleaning Up the hME Reaction Products” on the next page.

   **Note:** The cleanup of hME reaction products involves adding Clean Resin to the sample microplates. If you are not ready to add Clean Resin, you may store the sample microplates after they have completed thermocycling. Seal the microplates with plate-sealing film and store them at either -20 or 4°C. **(Caution:** When you are ready to add Clean Resin, let the microplates thaw to room temperature before adding resin.)
After the hME reaction has been processed by thermocycling (see the preceding section), the next step is to clean up the hME reaction products with Clean Resin (resin). This cleanup step is important to optimize mass spectrometry analysis of the hME reaction products.

**Note:** If you have a Clean Resin dispenser, do not follow the instructions in this section (i.e., skip the rest of this chapter). Instead, use the resin dispenser to add resin to your microplates of hME reaction products. For instructions on using the resin dispenser, see the *SpectroCLEAN Resin Dispenser User’s Guide*.

There are two different methods of cleaning up the hME reaction products:

**Automated**

The liquid handler is used to add resin to the sample microplates.

See “Cleaning Up the hME Reaction Products (Automated Only)” below.

**Manual**

Resin is added to the sample microplates manually. The liquid handler is used only to add water to the sample microplates before the resin is added manually.

See “Cleaning Up the hME Reaction Products (Manual Only)” on page 77.

**Note:** The cleanup process is carried out two microplates at a time.

**Important:** This section covers only automated cleanup of hME reaction products.

For manual cleanup of hME reaction products, do not follow the instructions in this section; instead, see “Cleaning Up the hME Reaction Products (Manual Only)” on page 77.

First, prepare microplates of resin. Next, using the liquid handler, add the resin to each well of the sample microplates. Finally, rotate and centrifuge the sample microplates. See the following steps for instructions.

**Important:** You must wear gloves when handling all equipment, components, and reagents. Wear gloves when handling the resin, dimple plates, and microplates.
To prepare a microplate of resin

**Note:** Perform these steps on a clean plastic sheet. The excess resin that is scraped off the dimple plate will fall to the plastic sheet. You can return the excess resin to its container for future use.

1. Using the elongated spoon, transfer resin from its container onto the 96-well dimple plate.

   **Important:** There are two types of dimple plate: 96-well and 384-well. Be sure to use the 96-well dimple plate. See the illustration to the right.

   ![96-Well dimple plate: 96 wells arranged in an 8 X 12 grid](image)

2. Use the scraper to spread resin into the wells of the dimple plate.

   **Note:** Make sure there is resin in each well

3. Scrape excess resin off the dimple plate using the scraper.

   **Note:** Return the excess resin to its container.
4. Place a clean, 96-well, polypropylene microplate (Marsh Biomedical Products, Inc, #AB-0800), upside-down, over the dimple plate. Align the microplate so its wells fit directly over the corresponding wells in the dimple plate.

5. Holding the 96-well microplate and the dimple plate together, flip them over so the resin falls out of the dimple plate into the wells of the microplate.

Tap the resin out of the dimple plate. Make sure all the resin falls out into the microplate.

6. Repeat these steps three more times to prepare a total of four microplates of resin.

**Two-Plate Option:** Repeat these steps only once to prepare a total of two microplates of resin.

When you have prepared the microplates of resin, proceed to “To add the resin to the sample microplates.”

**To add the resin to the sample microplates**

1. If the sample microplates are still thermocycling, wait for the thermocycling to complete.

2. Remove the plate sealing film from each sample microplate.
3. Place two sample microplates onto positions 1 and 2 of the liquid-handler deck.

4. Remove the universal plate holders from positions 3 and 4. Set them aside.

**Note:** Orient the microplates so well A1 is to the upper left

If there are no universal plate holders on positions 3 and 4, skip this step.
5. Place two microplates of resin on positions 3 and 4 of the liquid-handler deck.

![Diagram of the liquid-handler deck]

5. Place two microplates of resin on positions 3 and 4. 

**Note:** Orient the microplates so well A1 is to the upper left.

6. Place a reservoir of 100 mL of nanopure water on the plate positioner.

![Diagram of the liquid-handler deck]

6. Place a reservoir of 100 mL of nanopure water on position 5 (the plate positioner).

**Note:** You may use the lid from a box of tips for the liquid handler as the reservoir.

7. Lower the safety shield on the liquid handler.

8. On the liquid handler controller PC, run the hME TWO PLATE CATION CLEAN-UP method.

Water is added to the resin, and then 16 µL of resin/water solution is added to each well of the 384-well sample microplates.

**Note:** For instructions on running a method, see “Running a Method” on page 124.

9. When the hME TWO PLATE CATION CLEAN-UP method is done, raise the safety shield.

10. Remove the microplates of resin from positions 3 and 4 and discard them.
11. Remove the sample microplates from positions 1 and 2 and set them aside.

12. Repeat these steps, from step 3, to add resin to the remaining two sample microplates.

**Two-Plate Option:** Do not repeat the steps. Proceed to the next step below.

13. Replace the universal plate holders (removed in step 4) onto positions 3 and 4.

The following illustration shows how to place universal plate holders.

14. Proceed to “To centrifuge the sample microplates.”

**To centrifuge the sample microplates**

1. Centrifuge each sample microplate for three minutes at 1600 RPM.

2. The hME reaction products (in the sample microplates) are now ready for transfer to a SpectroCHIP, using the MassARRAY nanodispenser or MassARRAY piezodispenser.

**You are done processing the hME reaction.** Do not proceed to “Cleaning Up the hME Reaction Products (Manual Only)” on the next page.

**Note:** If you are not ready to transfer the hME reaction products to a SpectroCHIP at this time, you can store a microplate of reaction products at -20°C until you are ready. Place adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626) on the microplate before storing. Make sure the edges of the sealing foil are well-sealed. Do not store the microplate for more than two weeks.

If you have stored a microplate of hME reaction products, thaw and then centrifuge the microplate (for three minutes at 1600 RPM) before transferring the reaction products to a SpectroCHIP.
Cleaning Up the hME Reaction Products (Manual Only)

Note: This section covers only manual cleanup of hME reaction products.

For automated cleanup of hME reaction products, do not follow the instructions in this section; instead, see “Cleaning Up the hME Reaction Products (Automated Only)” on page 71.

For information about the differences between automated and manual cleanup of hME reaction products, see “Cleaning Up the hME Reaction Products” on page 71.

First, using the liquid handler, add water to the sample microplates (containing the hME reaction products). Next, add the resin to the sample microplates. Finally, rotate and centrifuge the sample microplates. See the following for detailed instructions.

Important: You must wear gloves when handling all equipment, components, and reagents. Wear gloves when handling the resin, dimple plates, and microplates.

To add water to the sample microplates

1. If the sample microplates are still thermocycling, wait for the thermocycling to complete.

2. Remove the plate sealing film from each 384-well sample microplate.

3. Raise the safety shield on the liquid handler.

4. Place the four sample microplates on positions 1-4 of the liquid-handler deck.

Two-Plate Option: Place the two sample microplates on positions 1 and 2. Leave positions 3 and 4 empty.
5. Place a reservoir containing 75 mL of nanopure water on position 5 of the liquid-handler deck.

- **Diagram of the liquid-handler deck**

**Note:** You may use the lid from a box of tips for the liquid handler as the reservoir.

**Note:** There may be some space between the positioning posts and the reservoir (i.e. the reservoir may be slightly smaller than the area inside the positioning posts). In this case, center the reservoir on the plate positioner.

**Two-Plate Option:** The diagram above shows four sample microplates. However, if you are processing only two microplates, positions 3 and 4 are empty.

6. Lower the safety shield on the liquid handler.

7. On the liquid handler controller PC, run the hME FOUR PLATE WATER ADDITION method.

   16 µL of nanopure water is added to each well of the four 384-well sample microplates.

**Two-Plate Option:** Run the hME TWO PLATE WATER ADDITION method instead of hME FOUR PLATE WATER ADDITION.

For instructions on running a method, see “Running a Method” on page 124.

8. When the hME FOUR PLATE WATER ADDITION method is done, raise the safety shield.

**Two-Plate Option:** When the hME TWO PLATE WATER ADDITION method is done, raise the safety shield.
9. Remove the sample microplates from the liquid-handler deck.

   To remove a sample microplate from a universal plate holder, turn the corner hold-
   downs away from the microplate’s skirt. Then, lift the microplate off the universal
   plate holder.

   **Important:** When removing a microplate from a universal plate holder, be careful not
   to splash any sample out of the microplate.

10. Check each microplate’s wells for air bubbles.

    If air bubbles are present, centrifuge the microplate (for 30 seconds at 1600 RPM, or
    until the air bubbles are gone).

11. Proceed to “To prepare a dimple plate.”

    **To prepare a dimple plate**

    **Note:** Perform these steps on a clean plastic sheet. The excess resin that is
    scraped off the dimple plate will fall to the plastic sheet. You can return the
    excess resin to its container for future use.

    1. Using the elongated spoon, transfer resin from its container onto a 384-well dimple
       plate.

       **Important:** There are two types of dimple plate: 96-well and 384-well.
       Be sure to use a 384-well plate. See the illustration to the right.

    2. Use the scraper to spread resin into the wells of the dimple plate.

       Sweep the scraper back and forth across the dimple plate to spread the resin

       **Note:** Make sure there is resin in each well
3. Scrape excess resin off the dimple plate using the scraper.

```
Scrape away
from the small,
rounded post
```

Return the excess resin to its container.

4. Repeat steps 1-3 three times to prepare a total of four dimple plates.

**Two-Plate Option:** Repeat steps 1-3 only once to prepare a total of two dimple plates.

5. Let the resin stand in the dimple plates for at least 20 minutes.

After 20 minutes, proceed to “To transfer the resin to the sample microplates.”

**To transfer the resin to the sample microplates**

1. Place one of the sample microplates, upside-down, onto one of the dimple plates you filled in the preceding procedure.
2. Holding the sample microplate and the dimple plate together, flip them over so the resin falls out of the dimple plate into the wells of the microplate.

Tap the dimple plate so the resin falls out into the microplate. Make sure all the resin in the dimple plate falls out into the microplate wells.

3. Repeat steps 1 and 2 for each of the three other sample microplates.

For each of the three other sample microplates, use one of the remaining, filled dimple plates.

**Two-Plate Option:** Repeat steps 1 and 2 once, for the other sample microplate. For the other sample microplate use the remaining dimple plate you prepared.

4. When you have added resin to each of the sample microplates, proceed to "To rotate and centrifuge the sample microplates."

**To rotate and centrifuge the sample microplates**

1. Rotate each sample microplate on a rotator for five minutes, at room temperature.

The rotator must rotate the microplate 360º about its long axis.

2. Centrifuge each 384-well sample microplate for three minutes at 1600 RPM.

3. The hME reaction products, contained in the sample microplates, are now ready for transfer to a SpectroCHIP, using the MassARRAY Nanodispenser or MassARRAY Piezodispenser.

**Note:** If you are not ready to transfer the hME reaction products to a SpectroCHIP, you can store a microplate at -20º C until you are ready. Place adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626) on the microplate before storing. Make sure the edges of the sealing foil are well-sealed. Do not store the microplate for more than two weeks.

If you have stored a microplate of hME reaction products, thaw and then centrifuge the microplate (for three minutes at 1600 RPM) before transferring the reaction products to a SpectroCHIP.
Notes:
Section 3
Allelotyping

This section contains instructions on using the liquid handler to process allelotyping hME reactions.

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Processing of Homogeneous MassEXTEND
Reactions for Allelotyping.................................95
Isolating and Quantitating DNA

Introduction

Before conducting any reaction to determine a genetic variation such as a single nucleotide polymorphism (SNP) with the MassARRAY system, you must have stocks of highly pure DNA to use as templates for amplification using the polymerase chain reaction (PCR).

Depending on the application, genomic DNA or cDNA is used as a template to amplify the region surrounding the genetic variation(s) of interest. Amplicons for use with the MassARRAY system are typically 70-150 bp in length. Since the amplicons are relatively short, only genomic DNA is discussed in this chapter—in most cases, the information in this chapter is also applicable to cDNA. If you wish to use cDNA templates, it is assumed you have a cDNA generation method already optimized.

After isolation, and before PCR, the genomic DNA must be quantitated. We recommend quantitation be done twice—first, with an ultraviolet (UV) spectrophotometer for an initial estimate; then with PicoGreen® dsDNA quantitation reagent. If possible, quantitate the DNA the same day as it is isolated.

Finally, after isolation and quantitation, we recommend diluting a large aliquot of the DNA to a working dilution of 50 ng/µL.

Isolating DNA DNA Quality

To produce large quantities of PCR amplicons for use with the MassARRAY system, it is recommended that the genomic-DNA template be highly pure. The ratio of ultraviolet (UV) spectrophotometer readings at 260 nm and 280 nm wavelengths should be between 1.7 and 2.0 (i.e., $A_{260}/A_{280} = 1.7-2.0$). Ratios in this range indicate the presence of nucleic acid with low amounts of contaminating protein. In general, we have found genomic DNA of this quality is suitable for downstream applications, such as PCR, following isolation from samples such as whole blood, buffy coat, or cultured cells.

Isolation Kits

Out of the several commercially available DNA-isolation kits we have tested, we found the PUREGENE™ Genomic DNA Purification Kit from Gentra Systems, Inc. to perform the best. In our testing, we isolated genomic DNA from whole blood, with 1-10 mL of starting material. All kits isolated genomic DNA well enough to produce PCR amplicons suitable for use with the MassARRAY system. The kit from Gentra Systems had the highest average yield of genomic DNA per mL of blood processed, along with the best average $A_{260}/A_{280}$ ratio.

We recommend the use of the kit from Gentra Systems. For convenience, the kit may be purchased through SEQUENOM (SEQUENOM part number 01275). More information about the PUREGENE Genomic DNA Purification Kit can be found at Gentra Systems’ Web site (www.gentra.com) or by calling their technical services at (800) 866-3039 in the United States.

Note: Genomic DNA may be isolated using any method you wish—it is not absolutely necessary you use the kit mentioned above. The only requirement is that whatever method you use yields highly pure DNA suitable for PCR.
Isolating and Quantitating DNA

Quantitating DNA

Isolation Protocol

Overview of the procedure for using the PUREGENE™ Genomic DNA Purification Kit on whole blood

For convenience, “Appendix E Using the PUREGENE™ Genomic DNA Purification Kit” on page 165 contains the protocols, excerpted from Genomic DNA Purification Kit Instructions provided by Gentra Systems, Inc., for isolating genomic DNA from whole blood. For more information about protocols for using the PUREGENE Genomic DNA Purification Kit, see Genomic DNA Purification Kit Instructions included with the kit.

Note: DNA isolated with the PUREGENE Genomic DNA Purification Kit is stable for at least 9 years at 4°C. For long term storage, the DNA may be stored at -20°C, but, it is recommended that you avoid repeated freezing and thawing to reduce DNA damage. For more information about DNA stability, see Gentra Systems’ Web site (www.gentra.com).

Warning: Certain chemicals used in PUREGENE reagents may be hazardous: Tris(hydroxymethyl) aminomethane, ethylenediaminetetraacetic acid, sodium dodecyl sulfate, ammonium chloride, and ammonium acetate. These chemicals may be harmful if swallowed and contact with the eyes and skin should be avoided. In case of contact, wash with large amounts of water and seek medical attention. Wear protective clothing.

Material Safety Data Sheets (MSDS) are available from Gentra Systems, Inc. (for contact information, see “Contacting Gentra Systems” on page 170).

Quantitating DNA With an Ultraviolet Spectrophotometer

Initial quantitation should be conducted with a UV spectrophotometer at wavelengths of 260 nm and 280 nm. The ratio of absorbance readings at the two wavelengths should be between 1.7 and 2.0 (i.e., A260/A280 = 1.7-2.0).

Calculate the amount of DNA present by assuming one optical density (O.D.) unit, at 260 nm, to be equal to 50 µg/mL of double-stranded DNA (dsDNA). Use the following formula:

\[
\text{O.D. units} \times \text{dilution factor}^* \times 50 \mu\text{g/mL} = \text{amount of DNA}
\]

(*For example, if the DNA is diluted 1:10, then the dilution factor is 10.)

Note that quantitating using a UV spectrophotometer, at 260 nm, does not distinguish between single-stranded DNA, RNA, and dsDNA. A UV spectrophotometer should be used to obtain an initial estimate of genomic-DNA concentration and purity. Then, we
recommend the use of PicoGreen dsDNA reagent (PicoGreen) to obtain a better estimate of concentration and purity.

With PicoGreen

PicoGreen from Molecular Probes, Inc. is a fluorescent nucleic acid stain. Because it is specific for dsDNA, it should provide better quantity estimates than a UV spectrophotometer and also has better lower-range quantitation sensitivity.

To use PicoGreen, you must have a spectrofluorometer with fluorescein excitation and emission wavelengths of 502 nm and 523 nm, respectively.

To order PicoGreen or for more information, visit Molecular Probes’ Web site (www.probes.com) or contact Molecular Probes at (541) 465-8300 in the United States.

Note: Quantitation may be done with a UV spectrophotometer alone. However, we recommend the use of PicoGreen dsDNA reagent, in addition to the UV spectrophotometer, for more accurate concentrations of DNA for use with the MassARRAY system.

PicoGreen Protocol

For reference, “Appendix F Using the PicoGreen® dsDNA Quantitation Reagent” on page 171 contains product information, including an experimental protocol, provided by Molecular Probes, Inc.

Warning: Molecular Probes, Inc. does not have data about the mutagenicity or toxicity of PicoGreen dsDNA quantitation reagent. However, because the reagent binds to nucleic acids, it should be treated and handled as a potential mutagen. Also, the reagent is in DMSO solution, which is known to facilitate the entry of organic molecules into tissues. Use double gloves when handling PicoGreen dsDNA quantitation reagent.

Working Dilution

After quantitation, we recommend diluting a large aliquot of the DNA to a stock concentration of 50 ng/µL. This concentration of stock can be used for both allelotyping and genotyping studies by diluting it to working concentrations.

Allelotyping requires 25 ng of DNA per reaction; first pool the DNA and then dilute the pooled DNA (at 50 ng/µL) 1:2 with nanopure water. Genotyping requires 2.5 ng of DNA per reaction; dilute the stock 1:20 with nanopure water.
Notes:
Chapter 7
Pooling DNA Samples

Introduction
SEQUENOM has developed a technology referred to as allelotyping for obtaining relative allele frequencies for genetic variations, such as SNPs, from populations in a single reaction. To perform allelotyping, you must pool together the DNA samples before amplification.

Caution: Use standard PCR preparation precautions when pooling DNA samples.

Population Size
Population sizes depend on your needs and resources. In general, we suggest using populations that can be broken down into multiples of 96, which reflects the commercially available microplate formats of 96- and 384-well microplates. This makes it easier to process samples.

We recommend populations of 300 or more individuals, such as 384 (4 X 96), because it gives greater statistical power.

Note: It is not absolutely required that population size be 300 or more. However, the greater the population size, the greater the statistical power.

Creating a Pool
In a DNA pool, it is critical that each individual DNA sample be present in equimolar amounts relative to the other DNA samples. This is best accomplished by accurately quantitating DNA before pooling (see “Quantitating DNA” on page 86). After quantitation, dilute each DNA sample to a uniform, stock concentration (50 ng/µL recommended; see “Working Dilution” on page 87). After quantitating and diluting to a uniform concentration, complete the following steps to create a pool.

To create a pool
1. Requantitate each DNA sample to confirm a concentration of 50 ng/µL (if the concentration is not 50 ng/µL see the note below).

Note: When you requantitate stock DNA, you may find the concentration to be lower or higher than 50 ng/µL. If so, when adding the DNA to the pool, add more or less accordingly. Then, when diluting the pool to the working concentration, adjust the amount of nanopure water accordingly. For example, if you find a stock DNA to be at a concentration of 40 ng/µL, add 125 µL to the pool (assuming a total pool volume of 30 mL and 300 individual DNAs in the pool). Adding the extra amount of the DNA to the pool will correct its concentration in the working dilution to 25 ng/µL. Since the volume added is 25 µL more than the amount you would normally add, subtract 25 µL from the volume of nanopure water with which you dilute the pool to the working concentration.
2. Add the individual DNA samples to the same tube in equal volumes.

For example, if you wish to make a 30 mL (total volume) pool comprised of 300 individual DNA samples, using 50 ng/µL stock concentrations of the individual DNA samples, add 100 µL of each individual DNA sample to a single tube.

**Note:** To minimize the effects of pipetting error, add the DNA to the tube using the same calibrated pipette with filter tips. Also, it is important you use good pipetting technique.

3. Dilute the pool 1:2 (with nanopure water) to a concentration of 25 ng/µL.

This is the working concentration. At 25 ng/µL, the DNA pool is ready for use in PCR reactions.

For a single PCR reaction (i.e. a single well), you need 1 µL of 25 ng/µL pooled DNA. For 384 PCR reactions (i.e. a whole 384-well microplate), you need 500 µL of 25 ng/µL pooled DNA. The 500 µL includes a 30% overhang to account for possible pipetting loss.

**Note:** Optionally, after diluting the pool to the working concentration, you may requantitate the pool to verify that it is at 25 ng/µL.

4. If you must store the DNA pool, store it at 4°C.
**Introduction**

This chapter covers amplifying genomic DNA for allelotyping with the MassARRAY system, using the polymerase chain reaction (PCR). DNA must be amplified prior to using the MassARRAY system.

**Important:** The polymerase chain reaction (PCR) is a patented process. You must have a license to perform PCR amplification. **Purchase of MassARRAY system components or MassEXTEND reagents does not confer a license to perform PCR.** Information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Centre Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

This chapter contains recommended protocols for amplifying DNA using PCR. Included are a protocol for a single PCR reaction (i.e. a single well) and two protocols for performing 384 PCR reactions (i.e. a whole 384-well microplate).

**Note:** This chapter contains recommended protocols. SEQUENOM does not guarantee these protocols to improve results. If you have amplification protocols in place, use them—note that you must ultimately have 5 µL of amplification product for each sample.
The following is the recommended protocol for a single PCR reaction.

**To perform a single PCR reaction**

1. Prepare a PCR cocktail as described in the following table.

   **Table 11: PCR cocktail (single reaction)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (HPLC grade)</td>
<td>N/A</td>
<td>2.24 µL</td>
</tr>
<tr>
<td>10X HotStar Taq PCR buffer, containing 15mM MgCl₂ (QIAGEN)</td>
<td>1X, 1.5 mM MgCl₂ †</td>
<td>0.50 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1 mM †</td>
<td>0.20 µL</td>
</tr>
<tr>
<td>dNTP mix (GIBCO), 25 mM each</td>
<td>200 µM each</td>
<td>0.04 µL</td>
</tr>
<tr>
<td>Enzyme HotStar Taq Polymerase (5U/µL from QIAGEN)</td>
<td>0.1 U/reaction</td>
<td>0.02 µL</td>
</tr>
<tr>
<td>Forward and reverse PCR primer mix * (1 µM each primer)</td>
<td>200 nM</td>
<td>1.00 µL</td>
</tr>
<tr>
<td>Pooled genomic DNA** (25 ng/µL)</td>
<td>25 ng/reaction</td>
<td>1.00 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td></td>
<td>5.00 µL</td>
</tr>
</tbody>
</table>

† Final concentration of MgCl₂ is 2.5 mM (1.5 mM from the 10X HotStar Taq PCR buffer + 1.0 mM MgCl₂)
* For positive control, use the appropriate positive control forward and reverse PCR primers. For more information, see Appendix D “Using Positive Control for Allelotyping” on page 161.
** The TE concentration in the DNA solution should be 0.25X TE or less

**Note:** Do not use PCR enhancers, such as Q-Solution.

2. Thermocycle the PCR cocktail as follows:

   1. 95°C 15 minutes
   2. 95°C 20 seconds
   3. 56°C 30 seconds 45 cycles
   4. 72°C 1 minute
   5. 72°C 3 minutes
   6. 4°C forever
When allelotyping, typically, each well of a 384-well microplate contains the same pooled DNA. Different assays are performed on the wells.

**To perform 384 PCR reactions (same pooled DNA, different assays)**

**Note:** These instructions cover performing PCR for a whole 384-well microplate of reactions in which different assays will be applied to the same pooled DNA.

1. Prepare a PCR cocktail as described in the following table.

   **Table 12: PCR cocktail for 384 reactions (same DNA, different assays)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume for Single Reaction</th>
<th>Volume for 384 Reactions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (HPLC grade)</td>
<td>N/A</td>
<td>2.24 µL</td>
<td>1120 µL</td>
</tr>
<tr>
<td>10X HotStar Taq PCR buffer, containing 15mM MgCl₂ (QIAGEN)</td>
<td>1X, 1.5 mM MgCl₂‡</td>
<td>0.50 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1 mM‡</td>
<td>0.20 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>dNTP mix (GIBCO), 25 mM each</td>
<td>0.04 µL</td>
<td>20 µL</td>
<td></td>
</tr>
<tr>
<td>Enzyme HotStar Taq Polymerase (5U/µL from QIAGEN)</td>
<td>0.1 U/reaction</td>
<td>0.02 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Pooled genomic DNA* (25 ng/µL)</td>
<td>25 ng/reaction</td>
<td>1.00 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td>4.00 µL</td>
<td>2000 µL</td>
<td></td>
</tr>
</tbody>
</table>

† Volumes include 30% overhang to account for possible pipetting loss
‡ Final concentration of MgCl₂ is 2.5 mM (1.5 mM from the 10X HotStar Taq PCR buffer + 1.0 mM MgCl₂)
* The TE concentration in the DNA solution should be 0.25X TE or less

**Note:** Do not use PCR enhancers, such as Q-Solution.

2. Into a 384-well microplate (Marsh Biomedical Products, Inc. #SP 0401 Sequen), dispense 4 µL of the PCR cocktail into each well.

**Note:** To use the allelotyping positive control, leave well A1 empty. You will add a positive control PCR cocktail to well A1 in step 4.
3. To each well of the 384-well microplate, add 1 µL of the appropriate forward and reverse primer mix (1 µM each primer).

**Note:** To use the allelotyping positive control, leave well A1 empty. You will add a positive control PCR cocktail to well A1 in step 4.

4. (Optional; if you are not using the positive control, skip this step) Prepare a positive control PCR cocktail directly in well A1.

Follow the “recipe” for a single reaction as described in Table 11. For the forward and reverse PCR primers, use the appropriate positive control forward and reverse PCR primers. Add the reagents directly to well A1.

For more information about positive control for allelotyping, see Appendix D “Using Positive Control for Allelotyping” on page 161.

5. Thermocycle the 384-well microplate as follows:
   1. 95°C 15 minutes
   2. 95°C 20 seconds
   3. 56°C 30 seconds
   4. 72°C 1 minute
   5. 72°C 3 minutes
   6. 4°C forever

45 cycles
Chapter 9

Processing of Homogeneous MassEXTEND Reactions for Allelotyping

Introduction

This chapter covers processing of Homogeneous MassEXTEND (hME) reactions, for allelotyping, using the liquid handler.

Note: The term sample microplate refers to any 384-well microplate of amplification products (“sample”) on which you want to perform the hME reaction.

The main steps in processing are:

1. Neutralize unincorporated dNTPs in the amplification product
2. Prepare and add hME reaction cocktail
3. Thermocycle the hME reaction
4. Clean up the hME reaction product

Detailed instructions for each of these main steps are provided in this chapter.

Before You Begin

Before you use the liquid handler to process an hME reaction, you should:

1. Change the tips if necessary:
   Changed the tips at the beginning of each day (see “Changing Tips” on page 127).

2. Check the wash system tanks:
   Fill the supply tank and empty the drain tank if necessary (see “Checking the Wash System Tanks” on page 130).

3. Make sure there is a plate positioner, with a universal plate holder, on deck position 5; also make sure all other plate positions are empty.
   See “To prepare the liquid-handler deck for allelotyping processing” below.
To prepare the liquid-handler deck for allelotyping processing

The liquid-handler deck should be configured as shown here:
- Plate positioner on position 5 with a universal plate holder on it. (Be sure to orient the universal plate holder as shown here; see step 1 below for more information.)
- Other deck positions empty.

For instructions on configuring the liquid-handler deck as shown here, see the steps below.

1. A plate positioner must be on position 5, and a universal plate holder must be on the plate positioner.
2. If there is a universal plate holder on position 1, 2, 3, or 4, remove it from the liquid-handler deck.

Diagram of the liquid-handler deck

Remove any universal plate holders from positions 1-4

To remove a universal plate holder, simply lift it off the liquid-handler deck
Neutralizing Unincorporated dNTPs in the Amplification Product

The first step in processing the hME reaction is to neutralize remaining, unincorporated dNTPs in the amplification products. Shrimp alkaline phosphatase (SAP) enzyme is used to neutralize unincorporated dNTPs. The SAP cleaves a phosphate from the unincorporated dNTPs, converting them to dNDPs and rendering them unavailable to future reaction.

**To neutralize unincorporated dNTPs**

1. In a 1.5 mL tube, prepare the SAP enzyme solution as described in the following table.

   **Note:** Add the reagents in the order in which they appear in the table.

   Table 13: SAP Enzyme Solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for Single Reaction</th>
<th>Volume for 384-Well Microplate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanopure water</td>
<td>1.53 µL</td>
<td>881.3 µL</td>
</tr>
<tr>
<td>hME Buffer</td>
<td>0.17 µL</td>
<td>97.9 µL</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase (SAP)</td>
<td>0.30 µL</td>
<td>172.8 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>2.00 µL</strong></td>
<td><strong>1152.0 µL</strong></td>
</tr>
</tbody>
</table>

   † Volumes for a 384-well microplate include 50% overhang to account for possible pipetting loss

2. Hold the 1.5 mL tube, containing the SAP enzyme solution, to a shaker for five seconds to mix the solution.

3. Centrifuge the 1.5 mL tube of SAP enzyme solution for ten seconds at 5000 RPM.

   **Note:** If you do not have a minifuge, skip this step.

4. In a new 96-well, polystyrene microplate (96-Well Plate Vee Bottom, Sarstedt, Inc. #82.1583), pipette 92 µL of SAP enzyme solution into each well of row H. See the following illustration.

   **Note:** The wells in row H will be used as reservoirs from which you will distribute SAP enzyme solution to the rest of the wells in the microplate

   **Dispense 92 µL of SAP enzyme solution into each well of this row**

   *Diagram of a 96-well polystyrene microplate (Sarstedt #82.1583)*
5. Using a twelve-channel pipettor, draw from the wells in row H and distribute 11 µL to each well in rows A-G. See the following illustration.

Diagram of a 96-well polystyrene microplate
(partial view: Sarstedt #82.1583)

Drawing from the wells in row H, distribute 11 µL of SAP enzyme solution into each of the other wells

Note: The SAP enzyme solution is moderately viscous. Use care when pipetting to minimize loss of solution due to adhesion to the pipettor tips.

When you are done distributing the SAP enzyme solution, each well in rows A-G should have 11 µL of the SAP enzyme solution. The wells in row H should have 15 µL. (Note: These volumes are approximate; actual volumes may be slightly less due to pipetting loss.)

6. Raise the safety shield on the liquid handler.

To raise the safety shield, on the left side of the liquid handler, press and hold the top end of the release lever. (For more detailed instructions on raising the safety shield, see “To raise the safety shield” on page 122.)

7. Place the microplate of the SAP solution on position 1 of the liquid-handler deck.

Diagram of the liquid-handler deck

Caution
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.

Note: Orient the microplate so well A1 is to the upper left
8. Onto the universal plate holder at position 5, place the 384-well microplate containing the amplification products ("sample microplate"). See the following illustration.

![Diagram of the liquid-handler deck](image)

**Caution:** If you must transfer the amplification products from the original microplate to another microplate, transfer it to a microplate that has been “shrunk” by thermocycling. Do not transfer it to a non-thermocycled microplate.

9. Lower the safety shield on the liquid handler.

To lower the safety shield, on the left side of the liquid handler, press and hold the bottom end of the release lever. (For more detailed instructions on lowering the safety shield, see “To lower the safety shield” on page 122.)

10. On the liquid handler controller PC, run the **ALLELOTYPING SAP ADDITION** method.

2 µL of SAP enzyme solution is added to each well in the 384-well sample microplate.

**Note:** For instructions on running a method, see “Running a Method” on page 124.

11. When the method is done, raise the safety shield on the liquid handler.

12. Remove the 384-well sample microplate from the universal plate holder on position 5 (plate positioner).

To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

**Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.
13. Seal the 384-well sample microplate with plate sealing film.

Make sure the edges of the plate sealing film are well-sealed.

14. Centrifuge the 384-well sample microplate at 1000 RPM for 1 minute.

15. Thermocycle the 384-well sample microplate as follows:

   1. 37°C for 20 minutes.
   2. 85°C for 5 minutes.
   3. 4°C forever.

   **Note:** If you have a programmable thermal cycler, enter the preceding program and name it hME SAP. When processing other hME reactions in the future, simply run the hME SAP program for this step.

16. Remove and discard the microplate of SAP enzyme solution.

17. While the sample microplate is thermocycling, begin preparing the hME reaction cocktail. Proceed to “Preparing and Adding the hME Reaction Cocktail” on the next page.
After unincorporated dNTPs have been neutralized in the amplification product (see the preceding section) the next step is to prepare and add the hME reaction cocktail.

The typical protocol is to perform different assays on the same pooled DNA, i.e. each well of a 384-well microplate contains the same DNA pool and different assays are performed on each well. The following instructions cover how to prepare and add hME reaction cocktail for this protocol.

**To prepare the hME reaction cocktail (different assays, same pooled DNA)**

1. In a 1.5 mL tube, prepare the hME reaction cocktail as described in the following table.

   **Note:** Add the reagents in the order in which they appear in the table.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Concentration</th>
<th>Final Reaction Concentration (in 9 µL)</th>
<th>Volume for Single Reaction</th>
<th>Volume for One Sample Microplate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (NanoPure grade)</td>
<td>N/A</td>
<td>N/A</td>
<td>1.564 µL</td>
<td>828.92 µL</td>
</tr>
<tr>
<td>Appropriate hME EXTEND Mix</td>
<td>10X buffer with 2.25 mM d/ddNTPs</td>
<td>Together with PCR buffer, the reaction happens in 1X buffer 50 µM d/ddNTP each</td>
<td>0.400 µL</td>
<td>212.00 µL</td>
</tr>
<tr>
<td>MassEXTEND Enzyme† (Thermo Sequenase)</td>
<td>32 U/µL</td>
<td>0.063 U/µL</td>
<td>0.036 µL</td>
<td>19.08 µL</td>
</tr>
<tr>
<td>hME primer(s)</td>
<td>(Added to each well separately; see step 3 below)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Volume‡: 2.000 µL</td>
<td>1060.00 µL</td>
</tr>
</tbody>
</table>

* Volumes are for a 384-well microplate and include 38% overhang to account for possible pipetting loss

† Keep the MassEXTEND Enzyme at -20° C until you are ready to add it to the reaction cocktail.

‡ Total volume does not include the volume contributed by the hME primer(s).
2. Distribute 2 µL of reaction cocktail to each well of four, 96-well microplates (96-Well Plate Vee Bottom, Sarstedt, Inc. #82.1583). See below.

Pour the reaction cocktail into a pipetting reservoir. Using a twelve-channel pipettor, draw from the reservoir and pipette 2 µL of reaction cocktail to each well of the four microplates. See the following illustration.

Caution
When pipetting, be sure to pipette into the centers of microplate wells—droplets must not be placed so they adhere to well-walls. Also, make sure there are no air bubbles in the wells. Centrifuge a microplate at 1600 RPM for one minute to remove air bubbles and collect liquid at the center of wells.

All wells should have 2 µL of reaction cocktail

Diagram of four 96-well polystyrene microplates (Sarstedt #82.1583)
3. Into each well of the four 96-well microplates (which you just filled with reaction cocktail), add 2 µL of the appropriate 5.4 µM hME primer.

Be sure to add the correct hME primer to each well. Each well in the four 96-well microplates corresponds (or maps) to one well in the 384-well sample microplate. How the wells map depends on where each 96-well microplate will be placed on the liquid-handler deck when transferring the reaction cocktail to the 384-well sample microplate. The following illustration shows how the 96-well microplate wells are mapped to the 384-well microplate wells.

The wells of the 96-well microplates are mapped to every other well in every other row of the 384-well microplate. The mapping of 96-well microplate 3 begins at well A1 of the 384-well microplate. The mapping of microplate 4 begins at well A2. Microplate 1 begins at well B1. And microplate 2 begins at well B2.

Note: Since you are performing different assays on the pooled DNA samples, every well of the 96-well microplates should receive different hME primer.
4. At this point, you should have four, 96-well microplates of reaction cocktail. These are referred to as “cocktail microplates.”

5. Centrifuge each microplate at 1000 RPM for one minute.

6. Proceed to “To add the hME reaction cocktail” below.

To add the hME reaction cocktail

1. Make sure the liquid-handler deck is configured as shown below.

The liquid-handler deck should be configured as shown here:
- Plate positioner on position 5 with a universal plate holder on it. (Note: the universal plate holder must be oriented as shown here; for more information see page 96.)
- Other deck positions empty.

For instructions on configuring the liquid-handler deck as shown here, see “To prepare the liquid-handler deck for allelotyping processing” on page 96.

2. Place the four 96-well cocktail microplates onto the liquid-handler deck.

Caution: Place each 96-well microplate on its appropriate deck position, as determined when you prepared the cocktail microplates (page 104 step 3).

3. Remove the sample microplate from the thermal cycler and remove the plate sealing film.
4. Place the 384-well sample microplate onto the universal plate holder on the plate positioner.

5. Lower the safety shield on the liquid handler.

6. On the liquid handler controller PC, run the **ALLELOTYPING-384 COCKTAIL MIX ADDITION** method.

   2 µL of hME reaction cocktail is added to each well of the sample microplate.

   **Note:** For instructions on running a method, see “Running a Method” on page 124.

7. When the **ALLELOTYPING-384 COCKTAIL MIX ADDITION** method is done, raise the safety shield.
8. Remove and discard the 96-well microplates of hME reaction cocktail.

9. Proceed to “Thermocycling the hME Reaction” on the next page.

**Note:** As an alternative to the procedures for preparing and adding the hME reaction cocktail, there is a way to automate all pipetting operations rather than having to hand-pipette cocktail microplates. The following liquid handler methods are used: ALLELOTYPING OLIGO ADDITION, ALLELOTYPING COCKTAIL ADDITION TO OLIGOS, and ALLELOTYPING 384-TO-384 COCKTAIL ADDITION.

Note that this requires your hME primers to be in 96-well microplates suitable for use on the liquid handler. If your primers are supplied in some other format by your oligonucleotide supplier, you will still have to hand-pipette them to 96-well microplates before using the methods mentioned above.

For more information contact your SEQUENOM customer service representative.
After you have added the hME reaction cocktail to the 384-well sample microplate (see the preceding section), the next step is to process the hME reaction by thermocycling.

**To thermocycle the hME reaction**

1. **Remove the 384-well sample microplate from the universal plate holder on the plate positioner.**

   To remove the 384-well sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

   **Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.

2. **Seal the 384-well sample microplate with plate sealing film.**

   Make sure the edges of the plate sealing film are well-sealed.

3. **Centrifuge the 384-well sample microplate at 1000 RPM for one minute.**

4. **Thermocycle the 384-well sample microplate as follows:**

   1. 94º C for 2 minutes.
   2. 94º C for 5 seconds.
   3. 52º C for 5 seconds.
   4. 72º C for 5 seconds.
   5. 4º C forever.

   **Note:** If you have a programmable thermal cycler, enter the preceding program and name it **hME Extend**. When processing other hME reactions in the future, simply run the **hME Extend** program for this step.

5. **Proceed to “Cleaning Up the hME Reaction Products” on the next page.**

   **Note:** The cleanup of hME reaction products involves adding Clean Resin to the sample microplate. If you are not ready to add Clean Resin, you may store the sample microplate. Seal the microplate with plate-sealing film and store it at either -20 or 4 ºC. (Caution: When you are ready to add Clean Resin, let the microplate thaw to room temperature before adding the resin.)
After the hME reaction has been processed by thermocycling (see the preceding section), the next step is to clean up the hME reaction products with Clean Resin (resin). This cleanup step is important to optimize mass spectrometry analysis of the hME reaction products.

There are two different methods of cleaning up the hME reaction products:

### Automated

The liquid handler is used to add resin to the sample microplate.

See “Cleaning Up the hME Reaction Products (Automated Only)” below.

### Manual

Resin is added to the sample microplate manually. The liquid handler is used only to add water to the sample microplate before the resin is added manually.

See “Cleaning Up the hME Reaction Products (Manual Only)” on page 114.

---

**Important:** This section covers only automated cleanup of hME reaction products.

For manual cleanup of hME reaction products, do not follow the instructions in this section; instead see “Cleaning Up the hME Reaction Products (Manual Only)” on page 114.

First, prepare a microplate of resin. Next, using the liquid handler, add the resin to each well of the sample microplate. Finally, rotate and centrifuge the hME reaction products. See the following steps for instructions.

**Important:** You must wear gloves when handling all equipment, components, and reagents. Wear gloves when handling the resin, dimple plates, and microplates.

### To prepare a microplate of resin

**Note:** Perform these steps on a clean plastic sheet. The excess resin that is scraped off the dimple plate will fall to the plastic sheet. You can return the excess resin to its container for future use.

1. Using the elongated spoon, transfer resin from its container onto a 96-well dimple plate.

**Important:** There are two types of dimple plate: 96-well and 384-well. Be sure to use the 96-well plate. See the illustration to the right.

---

**Elongated Spoon**

This is a plastic spoon with an elongated handle. It is supplied with the MassEXTEND Assay Starter Kit. Use it to scoop resin out of its container.
2. Use the scraper to spread resin into the wells of the dimple plate.

3. Scrape excess resin off the dimple plate using the scraper.

4. Proceed to “To transfer the resin to a 96-well microplate” below.

**To transfer the resin to a 96-well microplate**

1. Place a clean, 96-well polypropylene microplate (Marsh Biomedical Products, Inc. #AB-0800), upside-down, over the dimple plate. Align the microplate so its wells fit directly over the corresponding wells in the dimple plate.
2. Holding the 96-well microplate and the dimple plate together, flip them over so the resin falls out of the dimple plate into the wells of the microplate.

Tap the resin out of the dimple plate. Make sure all the resin falls out into the microplate.

3. Proceed to “To add the resin to the 384-well sample microplate” below.

**To add the resin to the 384-well sample microplate**

1. When the 384-well sample microplate is done thermocycling, remove it from the thermal cycler and remove the plate sealing film.

2. Place the 384-well sample microplate onto the universal plate holder at position 5 (plate positioner) on the liquid-handler deck.

---

**Diagram of the liquid-handler deck**

- **Sample Microplate**
- **Wash station**
- **Turn the corner hold-downs onto the microplate's skirt to secure the microplate**

**Note**: Orient the microplate so well A1 is to the **upper left**

For more information about placing microplates onto the universal plate holder, see “Using the Universal Plate Holder” on page 123
3. Place the 96-well microplate of resin on position 4 of the liquid-handler deck.

4. Place a reservoir of 100 mL of nanopure water on position 2 of the liquid-handler deck.

5. Lower the safety shield on the liquid handler.

6. On the liquid handler controller PC, run the ALLELOTyping CATION CLEANUP method.

   The water is added to the resin. Then, 16 µL of the resin/water solution is added to each well of the sample microplate.

   **Note:** For instructions on running a method, see “Running a Method” on page 124.

7. When the ALLELOTyping CATION CLEANUP method is done, raise the safety shield.

8. Go on to “To centrifuge the hME reaction products.”
To centrifuge the hME reaction products

1. Remove the sample microplate from the universal plate holder.

   To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

   **Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.

2. Centrifuge the sample microplate for three minutes at 1600 RPM.

3. The hME reaction products are now ready for transfer to a SpectroChip, using the MassARRAY nanodispenser or MassARRAY piezodispenser.

   **You are done processing the hME reaction.** Do not proceed to “Cleaning Up the hME Reaction Products (Manual Only)” on the next page.

**Note:** If you are not ready to transfer the hME reaction products to a SpectroCHIP at this time, you can store the microplate of reaction products at -20º C until you are ready. Place adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626) on the microplate before storing. Make sure the edges of the sealing foil are well-sealed. Do not store the microplate for more than two weeks.

   If you have stored a microplate of hME reaction products, thaw and then centrifuge the microplate (for three minutes at 1600 RPM) before transferring the reaction products to a SpectroCHIP.
First, spread resin on the 384-well dimple plate. Next, add nanopure water to each well of the sample microplate. Then, add resin to the hME reaction products. Finally, rotate and centrifuge the hME reaction products. See the following steps for detailed instructions.

**Important:** You must wear gloves when handling all equipment, components, and reagents. Wear gloves when handling the resin, dimple plates, and microplates.

### To prepare a plate of resin

**Note:** Perform these steps on a clean plastic sheet. The excess resin that is scraped off the dimple plate will fall to the plastic sheet. You can return the excess resin to its container for future use.

1. Using the elongated spoon, transfer resin from its container onto the 384-well dimple plate.

   **Important:** There are two types of dimple plate: 96-well and 384-well. Be sure to use the 384-well plate. See the illustration to the right.

2. Use the scraper to spread resin into the wells of the dimple plate.

   **Note:** Make sure there is resin in each well.
3. Scrape excess resin off the dimple plate using the scraper.

Return the excess resin to its container.

4. Let the resin stand in the dimple plate for at least 20 minutes.

While letting the resin stand in the dimple plate, add water to the sample microplate. See the next set of steps.

To add water to the 384-well sample microplate

1. When the sample microplate is done thermocycling, remove it from the thermal cycler and remove the plate sealing film.

2. Raise the safety shield on the liquid handler.

3. Place the 384-well sample microplate onto the universal plate holder at position 5 (plate positioner) on the liquid-handler deck.
4. Place a reservoir containing 40-50 mL of nanopure water on position 4 of the liquid-handler deck.

![Diagram of the liquid-handler deck](image)

5. Lower the safety shield on the liquid handler.

6. On the liquid handler controller PC, run the **ALLELOTYPING H2O ADDITION** method.

   16 µL of nanopure water is added to each well of the sample microplate.

   **Note:** For instructions on running a method, see “Running a Method” on page 124.

7. When the **ALLELOTYPING H2O ADDITION** method is done, raise the safety shield.

8. Remove the sample microplate from the universal plate holder (position 3).

   To remove the sample microplate, turn the corner hold-downs away from the microplate’s skirt. Then, lift the microplate off the universal plate holder.

   **Important:** When removing the microplate from the universal plate holder, be careful not to splash any sample out of the microplate.

9. Check the microplate wells for air bubbles.

   If air bubbles are present, centrifuge the microplate (for 30 seconds at 1600 RPM, or until the air bubbles are gone).

10. Proceed to “To add resin to the sample microplate” on the next page.
To add resin to the sample microplate

1. Place the sample microplate, upside-down, onto the dimple plate.

2. Holding the sample microplate and the dimple plate together, flip them over so the resin falls out of the dimple plate into the wells of the microplate.

3. Proceed to “To centrifuge the hME reaction products” on the next page.

To rotate and centrifuge the hME reaction products

1. Rotate the sample microplate on a rotator for five minutes, at room temperature.

   The rotator must rotate the microplate 360° about its long axis.
2. Centrifuge the sample microplate for three minutes at 1600 RPM.

3. The hME reaction products are now ready for transfer to a SpectroCHIP, using the MassARRAY Nanodispenser or MassARRAY Piezodispenser.

Note: If you are not ready to transfer the hME reaction products to a SpectroCHIP, you can store the microplate at -20°C until you are ready. Place adhesive sealing foil (Marsh Biomedical Products, Inc. #AB-0626) on the microplate before storing. Make sure the edges of the sealing foil are well-sealed. Do not store the microplate for more than two weeks.

If you have stored a microplate of hME reaction products, thaw and then centrifuge the microplate (for three minutes at 1600 RPM) before transferring the reaction products to a SpectroCHIP.
This section contains information about general procedures and maintenance that you may need to perform while using the liquid handler to process either genotyping or allelotyping reactions.

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Chapter 10
Routine Procedures

Introduction

This chapter contains general or routine procedures you may need to perform while using the liquid handler. For instructions on processing hME reactions, see “Chapter 4 Single-Plate Processing of Homogeneous MassEXTEND Reactions” on page 19 or “Chapter 5 High-Throughput Processing of Homogeneous MassEXTEND Reactions” on page 41.

The following topics are covered in this chapter:

- Raising and Lowering the Safety Shield
- Using the Universal Plate Holder
- Running a Method
- Changing Tips
- Checking the Wash System Tanks

These are the most common, routine tasks you may need to perform. For information about other parts of the liquid handler hardware or software see the manuals supplied by Beckman Instruments, Inc. (“Multimek™ 96 Installation and Maintenance Manual” and “Multimek™ Pro Software Reference Manual”).
Raising and Lowering the Safety Shield

The safety shield ensures you do not accidentally place your hands on or near the deck while the liquid handler is operating.

**To raise the safety shield**

**Important:** Do not raise the safety shield while a method is running. If you raise the safety shield while a method is running, the method is aborted. If a method is aborted, you must reset the liquid handler.

To reset the liquid handler, on the control keypad (on the front of the liquid handler), press the **Reset** button.

- On the left side of the liquid handler, press and hold the top of the release lever and raise the safety shield all the way up until it stops.

![Pressing top of release lever to raise safety shield](image.png)

**To lower the safety shield**

- On the left side of the liquid handler, press and hold the bottom of the release lever and lower the safety shield all the way down until it stops.

![Pressing bottom of release lever to lower safety shield](image.png)
Thermocycling tends to slightly bow plastic microplates. For the liquid handler to properly transfer material to a 384-well microplate, the microplate must be flat. A universal plate holder is used to flatten bowed microplates.

**Using the Universal Plate Holder**

**To place a microplate onto a universal plate holder**

1. Place the microplate onto the universal plate holder so that well A1 is to the upper left.

2. Turn the corner hold-downs onto the microplate’s skirt to secure the microplate to the universal plate holder.

**To remove a microplate from a universal plate holder**

- Turn the corner hold-downs away from the microplate’s skirt and lift the microplate off the universal plate holder.
Routine Procedures
Running a Method

Running a Method

The operation of the liquid handler is controlled by the Method Editor software on the liquid handler controller PC.

The automated pipetting operations needed to process hME reactions are separated into methods, or programs. For instance, the hME SAP Addition method transfers the shrimp alkaline phosphatase (SAP) enzyme from its 96-well microplate to a 384-well sample microplate.

At several points in this manual, you are instructed to run a method on the liquid handler controller PC. The steps for running the various methods are identical, except for selecting the particular method to be run.

To run a method

1. If it is not already running, start the Method Editor on the liquid handler controller PC. Be sure to lower the safety shield on the liquid handler before starting the Method Editor (see the following note).

Important: If you start the Method Editor while the safety shield is raised, an error occurs. You must reset the liquid handler. To reset the liquid handler, on the control keypad (on the front of the liquid handler), press the Reset button.

To start the Method Editor, double-click the Method Editor icon on the Windows desktop. (Or, on the Windows Taskbar, click Start, point to Multimek Pro, and then click Method Editor.)

The Multimek Pro - Method Editor window appears.

2. Click the tool.

The Open Method dialog box appears.

Note: the actual methods listed in this dialog box may differ from those shown in this illustration.
3. Click the method you want to run and then click OK.

The Multimek Pro - Setup Method - <method name> dialog box appears (where <method name> is the name of the method you selected).

4. Click OK.

You are returned to the Multimek Pro - Method Editor window. It now shows, in a flowchart-like diagram, the steps in the method you selected.
5. On the **Script** menu, select **Run**.

The **Multimek Pro - Run-Time Engine - <method name>** on COM1 window appears (where `<method name>` is the name of the method you selected).

6. Click the **Run** button to start the method.

   **Pause**: Pauses the method after the current step is completed. Click either **Run** or **Pause** to continue.

   **Stop**: Stops all movement. You cannot continue once you have stopped a method. You can only run the method again from the beginning.

---

**Caution**: Do not stop a method when it is washing tips at the wash station. An error will occur and the liquid handler will lock in the wash cycle.

If this happens, turn off power to the liquid handler.

---

Then turn it back on. On the control keypad (on the front of the liquid handler) press **Reset**.

7. When the method is done, on the **File** menu, select **Exit**.

You are returned to the **Multimek Pro - Method Editor** window. You may leave the program running.
Changing Tips

At the beginning of each day of use, change the tips on the liquid handler. First, unload the tips currently on the liquid handler head. Then load new tips. For instructions see the following steps.

**Note:** Always load a full box of tips. Loading a partial box may change tip height and affect results.

- **To unload tips**
  1. Raise the safety shield on the liquid handler.
     
     For instructions on raising the safety shield, see “To raise the safety shield” on page 122.
  2. Remove the plate positioner from position 5.

![Diagram of the liquid-handler deck](image)
3. Place the auto tip load station on position 5 of the liquid-handler deck. See the following illustration.

![Auto tip load station diagram]

**Important:** Make sure you orient the auto tip load station so that the hooks, on top, point forward toward you.

There are two holes in the bottom of the auto tip load station. Firmly seat the auto tip load station flat on the liquid-handler deck with the positioning pegs fitting into these holes.

4. Place an empty tip box in the auto tip load station.
   
   Make sure the empty tip box is seated flat and push it to the **front** of the auto tip load station.

5. Lower the safety shield on the liquid handler.
   
   For instructions on lowering the safety shield, see “To lower the safety shield” on page 122.

6. On the liquid handler controller PC, run the **Tip Unload** method.
   
   The tips are unloaded into the empty tip box.

7. Raise the safety shield and remove the box of unloaded tips.

8. Proceed to “To load tips.”
To load tips

1. Take the lid off a box of new tips and place the box of new tips in the auto tip load station.

   Make sure the box of new tips is seated flat and push it to the back of the auto tip load station.

2. Lower the safety shield on the liquid handler.

   For instructions on lowering the safety shield, see “To lower the safety shield” on page 122.

3. On the liquid handler controller PC, run the Tip Load method.

   The new tips are loaded onto the liquid handler head.

4. Raise the safety shield on the liquid handler.

5. Remove the auto tip load station from the liquid-handler deck. See the following illustration.

![Diagram of the liquid-handler deck]
6. Replace the plate positioner onto position 5.

   Orient the plate positioner so the indentation is on this side (toward the back of the liquid handler).

   There are two holes in the bottom of the plate positioner that line up with the positioning pegs on the liquid-handler deck. Seat the plate positioner flat on the liquid-handler deck with the positioning pegs fitting into these holes.

   ! [Diagram of the liquid-handler deck]

   **Checking the Wash System Tanks**

   Before processing an hME reaction on the liquid handler, check the wash system tanks. See the following illustration.

   ! [Diagram of the liquid-handler deck]

   **To fill the water supply tank**

   1. Fill the water supply tank with Type I water.

      If you must move the water supply tank to fill it, lift off the lid and pull out the tubing. To keep the tubing clean, rest the end of the tubing in a clean jar or on a clean, absorbent pad (“lab diaper”). Replace the tubing when you have filled the water supply tank.
2. Switch the power controller unit to Manual. This will run water through the wash system.

![Diagram of the liquid-handler deck]

Note: Typically, the power controller unit is located on the floor on, or near, the console drive unit.

Run water through the wash system until you see it emerging from the wash station wells.

![Diagram of the liquid-handler deck]

Note: It is important that you run water through the wash system after you have filled the water supply tank. This makes sure there is water in the wash station wells. A tip may be damaged if it attempts to aspirate water from an empty wash station well.

3. Switch the power controller unit back to Auto.

To empty the drain tank

1. Lift off the lid and pull out the tubing to the drain tank.

2. Empty the drain tank.

3. Place the tubing back in the drain tank and replace the lid.
Notes:
Introduction

This chapter contains information about routine maintenance that is required to keep the liquid handler running optimally. It also includes troubleshooting information.

For information about other parts of the liquid handler hardware or software not covered here, see the manuals supplied by Beckman Instruments, Inc. (“Multimek™ 96 Installation and Maintenance Manual” and “Multimek™ Pro Software Reference Manual”).

Daily Maintenance

Check air pressure daily. Check the air pressure at two points: the air compressor and the liquid handler. The air compressor, supplying air to the liquid handler, should be set to 90-95 psi. The liquid handler should be set to 80-85 psi. See the following instructions.

➤ Check and adjust air pressure

1. Check the gauge on the air compressor.

   It should read 90-95 psi. Adjust the pressure if necessary.

2. The pressure gauge on the liquid handler is located underneath its hood, to the back and left. It should read 80-85 psi.

3. If you must adjust the liquid handler air pressure, turn off the liquid handler first.

4. Raise the safety shield.
5. Reach in and adjust the air pressure by turning the adjustment knob, above the pressure gauge.

Pull the knob out to adjust the air pressure. Turn the knob clockwise to increase pressure, counter-clockwise to decrease pressure.

6. Close the safety shield.

7. Turn on the liquid handler and press Reset on the control keypad (on the front of the liquid handler).

**Weekly Maintenance**

**Clean the wash system**

Perform once a week on Monday or before first use of instrument during the week.

1. Remove the tubing from the water supply tank and empty the tank.

2. Remove the tubing from the drain tank, empty the tank, and replace the tubing.

3. Pour approximately 500 mL or more of 70% isopropanol into the water supply tank.

   Pour the isopropanol so that you coat the sides of the supply tank.

4. Turn over the water supply tank lid and pour some isopropanol into it. Swirl the lid to coat it with the isopropanol.

5. On the power controller unit, switch to Manual. This will run the isopropanol through the wash system.

![Power controller unit](image)
6. To make sure the water tubing aspirates isopropanol from the supply tank, tip the supply tank so the isopropanol collects in one area and dip the end of the tubing into the isopropanol.

7. When the isopropanol is flushed through the wash system and starts to emerge into the drain tank, switch the power controller unit back to Auto.

8. Let the entire system incubate with the isopropanol for 15 minutes.

9. On the power controller unit, switch to Manual to run the rest of the isopropanol through the wash system.

10. When all of the isopropanol is taken from the supply tank, switch the power controller unit to Auto.

11. Dispose of the isopropanol from the drain tank to a flammable waste container.

12. Rinse the supply tank (including its lid) several times with Type I water until all of the isopropanol is gone.

13. Refill the supply tank with Type I water.

14. On the power controller unit, switch to Manual to run water through the wash system.

   Keep running water through the wash system until all isopropanol is flushed from the wash system.

15. Replace the tubing into the supply and drain tanks and replace the tank lids.

16. Over weekends, release the tubing clamps on the console drive unit so water does not sit in the wash station.

\[\textbf{Clean the Labware Deck}\]

Perform once a week.

- If chemicals or residue are visible on the liquid-handler deck, wipe the deck with water and then with 70% isopropanol.

\[\textbf{Note:}\] Never use alcohol to clean the safety shield.
Check oil waste
Perform once a week.
1. If the oil waste bottle on the air compressor is more than half-full, empty it.
2. Dispose of the dirty oil properly.

Troubleshooting
- If liquid handler tips are not loading tightly and volumes are inaccurate, check that there is adequate air supply and pressure (see “Check and adjust air pressure” on page 133).
- If the liquid handler gives controller errors, try recalibrating the instrument: open the Communications Engine icon; click Multimek, and then Calibrate (to 130 steps).

Note: When solving controller errors, it helps SEQUENOM support personnel to know the specific type of error that occurred. In the Multimek Pro - Runtime Engine, on the File menu, select Show Error. Note the error information that is shown.

- If air bubbles are repeatedly introduced by a transfer operation at any stage of processing, contact SEQUENOM.

Ordering and Service Information
Beckman Instruments, Inc.
Biotechnology Development Center
2500 N. Harbor Blvd.
Fullerton, CA 92834-3100
USA
SEQUENOM, Inc.
3595 John Hopkins Court
San Diego, CA 92121
USA
Phone: 1-858-202-9000
Fax: 1-858-202-9001
SEQUENOM GmbH
Mendelssohnstr. 15D
D-22761 Hamburg
Germany
Phone: +49-40-899-676-0
Fax: +49-40-899-676-10

References
1. Multimek™ 96 Installation and Maintenance Manual, #147211; Beckman Instruments, Inc.
Section 5
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Appendix A

Designing and Evaluating Homogeneous MassEXTEND (hME) Assays

Introduction

The following flowcharts describe a recommended procedure for designing and evaluating both uniplex and multiplexed homogeneous MassEXTEND (hME) assays. This is a high-level description and it does not contain detailed, low-level steps. It is intended as a general guide to the sequence of tasks involved in designing and evaluating hME assays.

After the flowcharts, there are notes that elaborate on some of the steps. Not all steps have a note. If a step has a note, the note number is indicated as a superscripted number, at the end of the step text.

For information about the operation of a MassARRAY system component mentioned in these flowcharts or the notes following the flowcharts, see the user’s guide for the component.
Designing and Evaluating Homogeneous MassEXTEND (hME) Assays

This flowchart describes the process of designing and evaluating hME assays. (Note: At the “Evaluate the assays” step refer to the sub-flowchart on page 141.)

- Design assays using MassARRAY Assay Design\(^1\)
- Order oligonucleotides\(^2\) (Importer)
- Reorder unacceptable oligos
- Is the quality of the oligos acceptable?\(^3\) (Oligo Check)
- Prepare an assay evaluation plate with negative and positive controls and run it through the MassARRAY system\(^5\)
- Evaluate the assays
- Perform a trial run using acceptable assays on actual DNA samples of interest\(^8\)
- Assess the stability of assays\(^9\)
Evaluating Assays

This flowchart describes the process for evaluating each assay after you have run the evaluation plate through the MassARRAY system.

Repeat this process to evaluate each individual assay. In the case of a multiplex, evaluate individually each assay grouped into the multiplex.

Start here

Did the negative control produce a call in Typer? Yes 

Is the assay part of a multiplex? No Discard the assay and redesign it, or drop the assay altogether

No 

Yes 

Pull out the assay from the multiplex and evaluate the assay by itself. If it is acceptable, consider it for use as a uniplex assay.

Did the positive control produce a call in Typer? No 

Yes 

Amplify the positive control DNA using the amplification primers for only the assay in question

Check the amplification product on an agarose gel

Does the agarose gel show expected amplification product? No Redesign the amplification primers, or drop the assay altogether

Yes 

Perform hME on the amplification product using the hME primers for only the assay in question?

Does the positive control produce a call in Typer? No Redesign the hME primers, or drop the assay altogether

Yes 

Consider the assay for use as a uniplex assay

The assay is acceptable
**Notes**

1. **Design Assays Using MassARRAY Assay Design**

   Design assays using MassARRAY Assay Design

   For assays that are not designed at the multiplex level you choose, try to redesign them at a lower multiplex level. If MassARRAY Assay Design (Assay Design) is not able to design an assay, use other design methods.

   **Note:** This applies to uniplex assays as well. If Assay Design is unable to design an assay, use other design methods.

   See *SpectroDESIGNER User’s Guide* for information about designing assays with Assay Design ("SpectroDESIGNER" is the former name of Assay Design).

2. **Order Oligonucleotides**

   Order oligonucleotides (Importer)

   Using MassARRAY Importer (Importer), create oligonucleotide (oligo) files, which you can give to your oligo supplier to order oligos. Order oligos from a reputable supplier that, preferably, conducts mass-spectrometry-based quality control on each oligo.

   See *SpectroIMPORTER User’s Guide* for information about creating oligo files ("SpectroIMPORTER" is the former name of Importer).

3. **Is the Quality of the Oligos Acceptable?**

   Is the quality of the oligos acceptable? (Oligo Check)

   The oligos you order from your supplier must be checked for quality using MALDI-TOF mass spectrometry. Either check the oligos yourself, using MassARRAY Oligo Check (Oligo Check), or have your oligo supplier check them before sending them to you.

   If you check the oligos yourself, reorder any unacceptable oligos. Unacceptable oligos derive from synthesis failures, which include, for example, incomplete synthesis ("n-1"), wrong base composition (leading to wrong mass), high depurination, and incomplete deprotection.

   See *SpectroCHECK User’s Guide* for information about checking oligos using Oligo Check ("SpectroCHECK" is the former name of Oligo Check).

   **Note:** In a high-throughput environment, you may not want to wait for reordered oligos to arrive from your supplier. In that case, proceed with evaluating and running the assays for which you have acceptable oligos. Set aside those assays for which you had to reorder oligos. Evaluate them at a later time, when corresponding oligos are available.
4 Adjust hME Primer Concentrations (optional and only for multiplexes)

For best multiplexing results you may have to adjust the concentrations of hME primers to even out peak heights (intensities) in the mass spectrum.

The peaks in the mass spectrum for a multiplex may not have comparable heights. Variations in peak height may stem from incorrect concentrations or different desorption/ionization behavior in MALDI.

For each multiplex, prepare a mix of all the hME primers needed. Using MALDI-TOF mass spectrometry (MassARRAY Genotype Analyzer), analyze a 1 µM dilution of the hME primer mix. Check whether the hME primer peaks in the mass spectrum have comparable heights. If all peaks are at least 50% the height of the highest peak, they are acceptable. If any peak is less than 50% the height of the highest peak, add more of the hME primer having the short peak. See the following illustration.

**Note:** The following illustration (and the illustrations on the next page) depict only two peaks for the sake of simplicity. Actual spectra may include more peaks. Compare all peaks to the highest peak and adjust the concentration of any primers having “short” peaks.

---

Note: This is not an actual spectrum. It is only a diagram representing part of a spectrum for illustrative purposes.
The following examples provide general guidelines on bringing up the peak height of a primer. They are only rough, initial guidelines. In the examples, primer A has the highest peak in the spectrum. Primer B is the “short” primer in question.

Note: Each primer may behave differently and may have to be adjusted differently.

After adding more primer for the short peaks to the hME primer mix, re-analyze a 1 µM dilution of the hME primer mix. The peak heights should be comparable.

Once the concentrations of the hME primers have been adjusted in the primer mix to even out peak heights, use the adjusted primer mix in actual assay runs.
**Prepare an Assay Evaluation Plate and Run It**

Prepare an assay evaluation plate with negative and positive controls (positives and negatives) for the assays (“assay evaluation plate”).

For both negative and positive control, run through the complete hME reaction procedure, including amplification. For negative control, perform the amplification reactions but do not add genomic DNA. For positive control, add genomic DNA in the amplification reactions.

The MassARRAY liquid handler transfers hME reaction cocktail from each well of a 96-well microplate to four wells in the assay evaluation plate. For example, hME reaction cocktail from well A1 in the 96-well microplate is transferred to wells A1, A2, B1, and B2 of the assay evaluation plate. In effect, there are four wells in the assay evaluation plate for each assay. See the following illustration.

In each four-well group in the assay evaluation plate, two wells should be negative control and two wells should be positive control.

Note: Rather than combining positive and negative controls on a single plate, you may wish to prepare separate positive- and negative-control plates to avoid possible airborne contamination during pipetting.
Did the Negative Control Produce a Call?

Negative controls should not produce a call in MassARRAY Typer (Typer). Calls in negative controls derive from either hairpin/dimer extension or primer impurities, which may lead to wrong calls on actual DNA samples.

If a negative control produces a call and the assay in question is part of a multiplex, pull the assay out of the multiplex and re-evaluate it separately, as a uniplex assay. Perform a uniplex hME reaction, using the hME primers for only the assay in question and then run it on another assay evaluation plate. If the assay is acceptable, consider it for use as a uniplex assay.

When you pull an assay out of a multiplex, you can still use the multiplex, but without the assay in question. For example, if you pull an assay out of a quadruplex, you can still use the remaining triplex.

If an assay produces a call in negative control and it is not part of a multiplex, the assay is unacceptable. Discard it and redesign the assay. If you do not want to redesign the assay, or cannot redesign it, drop the assay.

Perform hME Using the hME Primers for Only the Assay in Question

If the agarose gel shows expected amplification product for the assay in question, then perform hME on the amplification product using the hME primers for only that assay.

Next, run the hME reaction product through the MassARRAY system. In MassARRAY Typer (Typer), does the assay produce a call? If it does produce a call, consider the assay for use as a uniplex assay. If it does not produce a call, redesign the hME primers or drop the assay altogether.
8 Perform a Trial Run

Run the acceptable assays on actual DNA samples of interest. If you had to pull an unacceptable assay out of a multiplex, run the remaining acceptable assays as a multiplex without the pulled assay. Also, if you had to pull an assay out of a multiplex and found it acceptable as a uniplex assay, you can include it in the trial run as a separate uniplex assay.

For the trial run, in MassARRAY Typer, create a new plate and apply the acceptable assays to it. Be sure not to include any unacceptable assays in the plate.

9 Assess the Stability of Assays

During analysis of actual DNA samples, it is recommended you collect and compare the data obtained from an assay until it is determined to perform well and be stable.

To assess the stability of assays, pay particular interest to the following two issues:

- **Are all three genotypes seen?** After a high enough number of DNA samples have been analyzed, are all three genotypes seen? If you do not see all three genotypes, it may be due to allelic dropout. If you suspect allelic dropout, consider redesigning the assay.

- **Are peak heights stable?** For heterozygous samples, how are the peak heights distributed and how consistent is the distribution? If the peak heights for the alleles vary, relative to each other, over different samples, then the assay is not stable.

Consider redesigning those assays that appear unstable.
Notes:
Appendix B

Selecting Appropriate Termination Mixes

Introduction

It is critical to select the appropriate termination mix as listed in Table 15, “Selecting a termination mix”.

Table 15: Selecting a termination mix

<table>
<thead>
<tr>
<th>SNP (Biallelic)</th>
<th>Termination Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/C</td>
<td>CGT (40 Da)</td>
</tr>
<tr>
<td>A/G</td>
<td>ACT (32 Da)</td>
</tr>
<tr>
<td>A/T</td>
<td>CGT (25 Da)</td>
</tr>
<tr>
<td>C/G</td>
<td>ACT (56 Da) AGT (24 Da)</td>
</tr>
<tr>
<td>C/T</td>
<td>ACG (31 Da)</td>
</tr>
<tr>
<td>G/T</td>
<td>ACT (41 Da)</td>
</tr>
<tr>
<td>small ins/del</td>
<td>- dependent on sequence-</td>
</tr>
</tbody>
</table>

Numbers in parentheses are the mass differences between a correct termination and a false termination (i.e., premature termination caused by pausing of the polymerase).

Occasionally, inappropriate extension products can occur by pausing of the polymerase after incorporation of one non-terminating nucleotide (i.e. dNTP), resulting in a prematurely terminated extension primer. The mass difference between this falsely terminated and a correctly terminated MassEXTEND reaction at the polymorphic site is sometimes too small to resolve consistently and can lead to miscalls if an inappropriate termination mix is used.

The mass differences between a false termination (i.e., one caused by pausing) and a correct termination must therefore be maximized to avoid making miscalls. Table 16 on page 150 shows the mass differences that can result. It is best to avoid mass differences of ≤ 24 Da.
Table 16: Mass differences for pairs of nucleotides

<table>
<thead>
<tr>
<th></th>
<th>Deoxyribonucleotides</th>
<th>Dideoxyribonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dC</td>
<td>dT</td>
</tr>
<tr>
<td>Deoxyribonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddC</td>
<td>NA</td>
<td>31</td>
</tr>
<tr>
<td>ddT</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>ddA</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>ddG</td>
<td>24</td>
<td>9</td>
</tr>
</tbody>
</table>

*Note: Differences in masses between a dideoxy- and a deoxy-nucleotide of the same base are not shown since a termination mix never contains both.*

The problems that can result are illustrated in the examples on the following pages.
**Examples**  

A/G polymorphism

**Extension primer**

5’-cgta\textsubscript{c}gctgccg\textsubscript{t}ggaacg\textsubscript{t}t\textsubscript{a/g}ctgtaagtgc\textsubscript{t}ctgctattgc\textsubscript{t}catgc\textsubscript{t}cg\textsubscript{t}g\textsubscript{t}c\textsubscript{g}tc-3’

3’-tcgatcg\textsubscript{a} gag\textsubscript{c}gc\textsubscript{t} gc\textsubscript{t} ga\textsubscript{c}gc\textsubscript{a}c\textsubscript{c} t\textsubscript{a/t} g\textsubscript{a} g\textsubscript{a}a\textsubscript{g}t\textsubscript{a} cat\textsubscript{a} g\textsubscript{c} t\textsubscript{g} ca\textsubscript{t} g\textsubscript{a}-5’

**template**

- For the extension primer shown, there are two possible termination mixes, the dA (ddC/ddG/ddT) termination mix and the dG (ddA/ddC/ddT) termination mix.

- For the dA (ddC/ddG/ddT) termination mix, the mass difference between false termination (pausing) after incorporation of dA and termination by ddG is 0 Da—completely indistinguishable and therefore unacceptable. In this case, one would be unable to differentiate between a correct termination with ddG (denoting the G allele) and false termination caused by the polymerase pausing after incorporation of dA; the A allele would be miscalled as the G allele. For the dG (ddA/ddC/ddT) termination mix, the mass difference between ddG and ddA is 32 Da, the maximum mass difference possible for this polymorphism using the termination mixes. Therefore, choose the dG (ddA/ddC/ddT) termination mix.

**Table 17: dG (ddA/ddC/ddT) termination mix**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extension primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cgtactgccg\textsubscript{t}ggaacg\textsubscript{t}t\textsubscript{a/g}</td>
<td>18</td>
<td>5515.6</td>
</tr>
<tr>
<td>Correct products:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cgtactgccg\textsubscript{t}ggaacg\textsubscript{t}t\textsubscript{a/g} (+ddA)</td>
<td>19</td>
<td>5812.8</td>
</tr>
<tr>
<td>G allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cgtactgccg\textsubscript{t}ggaacg\textsubscript{t}t\textsubscript{a/g} (+ddG+ddC)</td>
<td>20</td>
<td>6118.0</td>
</tr>
<tr>
<td>False products:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pausing) G allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cgtactgccg\textsubscript{t}ggaacg\textsubscript{t}t\textsubscript{a/g} (+ddG)</td>
<td>19</td>
<td>5844.8</td>
</tr>
</tbody>
</table>
C/G polymorphism

**Extension primer**

5' - cgtactgccccgtggaacgt
5' - agctagcttttgcgcgtacctgcctggggaacgt[c/g]gggtgaagtgcctattgcatcgtcgagtc-3'
3' - tcgatcgaacgcatgcacggaccttgca[g/c]cccacttcagcagtaacgtacgacgctcag-5'
template

- For the extension primer shown, there are two possible termination mixes, the dC (ddA/ddG/ddT) termination mix and the dG (ddA/ddC/ddT) termination mix.
- For the dC (ddA/ddG/ddT) termination mix, the mass difference between false termination (pausing) after incorporation of dC and termination by ddG is 24 Da; although discernible, one should check the other termination mix to see if a larger mass difference is possible.
- For the dG (ddA/ddC/ddT) termination mix, the mass difference between dG and ddC is 56 Da, the maximum mass difference possible for this polymorphism. **However**, since the polymorphic site is followed by 3 Gs, the difference between correct allelic products would be quite large: 7120.6 Da - 5788.8 Da = 1331.8 Da. This large mass difference can lead to different behavior in the mass spectrometer and should be avoided. Therefore, choose the dC (ddA/ddG/ddT) termination mix.

**Table 18: dC (ddA/ddG/ddT) termination mix**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extension primer</td>
<td>cgtactgccccgtggaacgt</td>
<td>18</td>
</tr>
<tr>
<td>Correct products:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>cgtactgccccgtggaacgt (+dC+ddG)</td>
<td>20</td>
</tr>
<tr>
<td>G allele</td>
<td>cgtactgccccgtggaacgt (+ddG)</td>
<td>19</td>
</tr>
<tr>
<td>False products: (pausing) C allele</td>
<td>cgtactgccccgtggaacgt (+dC)</td>
<td>19</td>
</tr>
</tbody>
</table>

**Table 19: dG (ddA/ddC/ddT) termination mix**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extension primer</td>
<td>cgtactgccccgtggaacgt</td>
<td>18</td>
</tr>
<tr>
<td>Correct products:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>cgtactgccccgtggaacgt (+dC)</td>
<td>19</td>
</tr>
<tr>
<td>G allele</td>
<td>cgtactgccccgtggaacgt (+ddGdGdG+dT)</td>
<td>23</td>
</tr>
<tr>
<td>False products: (pausing) G allele</td>
<td>cgtactgccccgtggaacgt (+dG)</td>
<td>19</td>
</tr>
</tbody>
</table>

* The product size of the two alleles is quite different which can lead to different desorption/ionization behavior of the two molecules in the mass spectrometer.
C/T polymorphism

- In this example the forward extension primer is not favorable as it would have five consecutive dTs at the 3’ end of the primer.
- For the reverse extension primer the complementary strand has to be considered. Choosing the complementary strand, the C/T polymorphism is now treated like an A/G polymorphism. In this case there are two possible termination mixes, the dA (ddC/ddG/ddT) and the dG (ddA/ddC/ddT) termination mix.
- For the dA (ddC/ddG/ddT) termination mix, the mass difference between false termination (pausing) after incorporation of dA and termination by ddG is 0 Da—completely indistinguishable and therefore unacceptable. In this case one would be unable to differentiate between a correct termination with ddG (denoting the G allele) and false termination caused by pausing of the polymerase after incorporation of dA (A allele). Additionally, the mass difference between the two possible correct products would be very high (7660 Da - 5805.8 Da = 1854.2 Da), which should be avoided to prevent different desorption/ionization behavior in the mass spectrometer.
- For the dG (ddA/ddC/ddT) termination mix, the mass difference between dG and ddA is 32 Da, the maximum mass difference possible for this polymorphism. In this case, the site of the extension products differ by only one base (329.2 Da) which is preferable.
- Therefore the reverse primer in combination with the dG (ddA/ddC/ddT) termination mix is chosen in this example.

Table 20: dG (ddA/ddC/ddT) termination mix

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extension primer</td>
<td>gcaatagcgacttcacag</td>
<td>18</td>
</tr>
<tr>
<td>Correct products:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>gcaatagcgacttcacag (+dG+ddA)</td>
<td>20</td>
</tr>
<tr>
<td>T allele</td>
<td>gcaatagcgacttcacag (+ddA)</td>
<td>19</td>
</tr>
<tr>
<td>False products:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pausing) C allele</td>
<td>gcaatagcgacttcacag (+dG)</td>
<td>19</td>
</tr>
</tbody>
</table>
Notes:
Appendix C

Using Positive Control for Genotyping

This appendix contains information about using the positive control for genotyping available from SEQUENOM. If you want to use the positive control, read this appendix before you begin processing sample microplates.

Note: The term sample microplate refers to any microplate of genomic DNA samples.

Introduction

The positive control consists of a synthetic, control template and a control EXTEND primer. The control template is used in place of amplified, genomic DNA. The control primer is used in hME reaction cocktail in place of hME primer.

When using the positive control, a sample microplate is processed as usual with a couple alterations in the procedures.

First, during amplification of the genomic DNA samples, four wells in the sample microplate are left empty and designated positive control wells. After amplification, a solution of the synthetic template is placed in the empty positive control wells.

Second, when preparing hME reaction cocktail, an additional reaction cocktail is prepared in which the control primer is used instead of regular hME primer to produce a positive control reaction cocktail. Regular hME reaction cocktail is added to the amplified DNA samples. The positive control reaction cocktail is added to the wells containing the synthetic, control template (i.e. the positive control wells).

The rest of the steps in processing the sample microplate proceed as usual. When analyzing results in MassARRAY Typer (Typer), the positive control wells should produce the expected mass for the particular EXTEND mix, i.e. termination mix, used in the positive control reaction cocktail (see Table 23, “Expected masses for positive control,” on page 160).

How to Use this Appendix

The next two sections (“Preparing and Adding the Positive Control Template” and “Preparing and Adding Positive Control Reaction Cocktail”) describe how the usual processing procedures must be modified to use the positive control. Follow the instructions they contain.
Preparing and Adding the Positive Control Template

This section contains information about modifying the procedures in “Chapter 3 Amplifying DNA for Genotyping” to use the positive control.

When preparing a sample microplate for amplification, designate a group of four wells as positive control wells and leave them empty.

The sample microplate is divided into 96 four-well groups. Choose one of these four-well groups as the positive control wells. For more information about the four-well groups, see “Four-Well Groups” on page 158.

Amplify the samples in the sample microplate as usual. After amplification, prepare a solution of positive control template as described in the following table. Prepare the solution in a 1.5 mL tube.

Table 21: Positive control template solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control template</td>
<td>15 µL</td>
</tr>
<tr>
<td>hME buffer</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Nanopure water</td>
<td>7.5 µL</td>
</tr>
</tbody>
</table>

Total Volume: 25 µL

Pipette 5 µL of the positive control template solution to each of the four positive control wells in the sample microplate.

Begin processing the sample microplate as usual.
This section contains information about modifying the procedures under “Preparing and Adding the hME Reaction Cocktail” on page 25 to use the positive control. Basically, one of the wells in the reaction cocktail microplate must contain positive control reaction cocktail instead of regular hME reaction cocktail. See below.

Before preparing the hME reaction cocktail, prepare 10 µL of positive control reaction cocktail as described in the following table. Prepare the positive control reaction cocktail directly in the appropriate well of the reaction cocktail microplate; to determine which well to use, see “Reaction Cocktail Microplate to Sample Microplate Mapping” on page 159.

### Table 22: Positive control reaction cocktail

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (NanoPure grade)</td>
<td>6.4 µL</td>
</tr>
<tr>
<td>Appropriate hME EXTEND Mix (containing buffer and d/ddNTPs)†</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Positive control EXTEND primer</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>MassEXTEND Enzyme (Thermo Sequenase)</td>
<td>0.1 µL</td>
</tr>
<tr>
<td><strong>Total Volume:</strong></td>
<td><strong>10.0 µL</strong></td>
</tr>
</tbody>
</table>

† Any hME EXTEND Mix may be used; see “Expected Masses” on page 160 for the expected masses of positive control for each possible hME EXTEND Mix.

After pipetting the positive control reaction cocktail to the appropriate well, distribute the regular hME reaction cocktail across the microplate as usual (but be sure to skip the well containing the positive control reaction cocktail.)

**Note:** Normally, when preparing the microplate of reaction cocktail, you use the wells in row H as reservoirs from which you distribute the reaction cocktail across the rest of the plate. If you used one of the wells in row H for the positive control reaction cocktail, use the well above it in row G as a reservoir from which you distribute the reaction cocktail to the rest of the wells in the same column.

Proceed with processing as usual—adding the reaction cocktail to the sample microplate and processing the sample microplate as usual.

When analyzing results in Typer, the positive control wells should produce the expected masses for the particular hME EXTEND mix used in the positive control reaction cocktail. See “Expected Masses” on page 160.
**Four-Well Groups**

Before amplifying the genomic DNA samples in the sample microplate, you must choose a group of four wells to be used as positive control wells. These wells are left empty during amplification. After amplification, they are filled with a solution of positive control template. The following illustration shows the four-well groups you can designate as positive control wells.

You may not “break apart” any four-well group. All wells in a four-well group must be used as positive control wells. It is not necessary that you fill all four wells with positive control template, however you may not use any of them for regular genomic DNA sample. This is due to the MassARRAY liquid handler (liquid handler) configuration. When reaction cocktail is transferred to the sample microplate, positive control reaction cocktail will be transferred to all four wells in the group. If you had regular DNA sample in any of the wells, your results would not be valid because the incorrect reaction cocktail would be added to the sample.

**Note:** The four-well grouping scheme is required by the configuration of the liquid handler. Reaction cocktail always resides in a 96-well microplate. In the case of 384-well sample microplates, there are four times as many sample wells as reaction cocktail wells. Each well in the reaction cocktail microplate must be mapped to four wells in the sample microplate. During processing, the liquid handler aspirates reaction cocktail from one well in the reaction cocktail microplate and dispenses the cocktail to four wells in the sample microplate. For more information, see “Reaction Cocktail Microplate to Sample Microplate Mapping” on page 159.
The well, in the reaction cocktail microplate, into which you pipette the positive control reaction cocktail must be the one that corresponds to the wells in the sample microplate containing the positive control template. Each well in the reaction cocktail microplate corresponds to four wells in the sample microplate. That is, the reaction cocktail in one well of the 96-well microplate of reaction cocktail will be transferred to four wells in the 384-well sample microplate. See the following illustration.

In the preceding illustration, the one-to-four mapping for only wells A1 and B1 is shown. Each well in the microplate of reaction cocktail maps to four wells in the sample microplate. The following illustration shows the complete mapping between reaction cocktail wells and sample wells. Overlaid on a sample microplate is a grid that delineates the wells into four-well groups. Each four-well group is labeled with the well in the reaction cocktail microplate that corresponds to it. For example, well G6 in the reaction cocktail microplate corresponds to wells M11, M12, N11, and N12 in the sample microplate.
Expected Masses

The following table lists the expected masses of the extended positive control primer for the different hME EXTEND mixes that may be used in the positive control reaction cocktail.

Table 23: Expected masses for positive control

<table>
<thead>
<tr>
<th>hME EXTEND Mix</th>
<th>MALDI Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unextended control primer</td>
<td>5131.4 Da</td>
</tr>
<tr>
<td>hME EXTEND Mix 15, 19, 21, or 26</td>
<td>5757.8 Da</td>
</tr>
<tr>
<td>hME EXTEND Mix 17, 20, 22, 24, 25, 27, or 28</td>
<td>5444.6 Da</td>
</tr>
<tr>
<td>hME EXTEND Mix 18 or 23</td>
<td>6704.4 Da</td>
</tr>
<tr>
<td>hME EXTEND Mix 16</td>
<td>8911.8 Da</td>
</tr>
</tbody>
</table>
This appendix contains information about using the positive control for allelotyping available from SEQUENOM. If you want to use the positive control, read this appendix before you begin processing sample microplates.

**Note:** The term sample microplate refers to any microplate of pooled, genomic DNA samples.

**Introduction**

The positive control consists of four different sets of oligonucleotides, each set consisting of a forward PCR primer, reverse PCR primer, and EXTEND primer. Each set corresponds to a different triple-termination mix: ACG, ACT, AGT, or CGT referred to respectively as the ACG control, ACT control, AGT control, and CGT control.

**Note:** Unlike positive control for genotyping, no synthetic, control DNA template is provided. A positive control reaction is performed using your own DNA pool as template.

**Positive Control Primer Sets**

The components of each positive control set are listed in the following table.

<table>
<thead>
<tr>
<th>Termination Mix</th>
<th>Primer Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACG</td>
<td>ACG-Control-F</td>
<td>ACG forward PCR primer</td>
</tr>
<tr>
<td></td>
<td>ACG-Control-R</td>
<td>ACG reverse PCR primer</td>
</tr>
<tr>
<td></td>
<td>ACG-Control-E</td>
<td>ACG EXTEND primer</td>
</tr>
<tr>
<td>ACT</td>
<td>ACT-Control-F</td>
<td>ACT forward PCR primer</td>
</tr>
<tr>
<td></td>
<td>ACT-Control-R</td>
<td>ACT reverse PCR primer</td>
</tr>
<tr>
<td></td>
<td>ACT-Control-E</td>
<td>ACT EXTEND primer</td>
</tr>
<tr>
<td>AGT</td>
<td>AGT-Control-F</td>
<td>AGT forward PCR primer</td>
</tr>
<tr>
<td></td>
<td>AGT-Control-R</td>
<td>AGT reverse PCR primer</td>
</tr>
<tr>
<td></td>
<td>AGT-Control-E</td>
<td>AGT EXTEND primer</td>
</tr>
<tr>
<td>CGT</td>
<td>CGT-Control-F</td>
<td>CGT forward PCR primer</td>
</tr>
<tr>
<td></td>
<td>CGT-Control-R</td>
<td>CGT reverse PCR primer</td>
</tr>
<tr>
<td></td>
<td>CGT-Control-E</td>
<td>CGT EXTEND primer</td>
</tr>
</tbody>
</table>

*This is the concentration of the primer in a mixture of forward and reverse primers.

**Note:** All primers are supplied at 10 µM concentration. You must dilute them to working concentrations before adding them to your reactions. Working concentration for a PCR primer is 1µM*. Working concentration for an EXTEND primer is 5.4 µM.
In the 384-well sample microplate, containing your DNA pools, select a well as the positive control well (well A1 recommended). For the PCR, use the appropriate positive control PCR primers in the positive control well. When processing the hME reaction, use the appropriate positive control EXTEND primer in the positive control well.

When analyzing spectra in MassARRAY Typer (Typer), the positive control should produce peaks at the expected masses (see “Expected Masses” below).

The following tables list the expected masses of the EXTEND primer, a pausing peak, and analytes for each control.

### Table 25: ACG control

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Expected Mass</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACG-Control-E</td>
<td>CAGAACACTTAGACCCCCACC</td>
<td>6000.00</td>
<td>EXTEND Primer</td>
</tr>
<tr>
<td>dT Pause</td>
<td>CAGAACACTTAGACCCCCACTG</td>
<td>6304.10</td>
<td>Pausing peak</td>
</tr>
<tr>
<td>G</td>
<td>CAGAACACTTAGACCCCCACC</td>
<td>6273.10</td>
<td>Analyte</td>
</tr>
<tr>
<td>A</td>
<td>CAGAACACTTAGACCCCCACTG</td>
<td>6617.30</td>
<td>Analyte</td>
</tr>
</tbody>
</table>

### Table 26: ACT control

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Expected Mass</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT-Control-E</td>
<td>GTTGGCAGCTGTTTGTTTCTG</td>
<td>6440.20</td>
<td>EXTEND Primer</td>
</tr>
<tr>
<td>dG Pause</td>
<td>GTTGGCAGCTGTTTGTTTCTG</td>
<td>6769.40</td>
<td>Pausing peak</td>
</tr>
<tr>
<td>T</td>
<td>GTTGGCAGCTGTTTGTTTCTA</td>
<td>6737.40</td>
<td>Analyte</td>
</tr>
<tr>
<td>C</td>
<td>GTTGGCAGCTGTTTGTTTCTG</td>
<td>7057.60</td>
<td>Analyte</td>
</tr>
</tbody>
</table>

### Table 27: AGT control

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Expected Mass</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT-Control-E</td>
<td>CCAGAGGGGTTCCTCTGG</td>
<td>5596.70</td>
<td>EXTEND Primer</td>
</tr>
<tr>
<td>dC Pause</td>
<td>CCAGAGGGGTTCCTGGGC</td>
<td>5885.90</td>
<td>Pausing peak</td>
</tr>
<tr>
<td>T</td>
<td>CCAGAGGGGTTCCTGGGA</td>
<td>5893.90</td>
<td>Analyte</td>
</tr>
<tr>
<td>G</td>
<td>CCAGAGGGGTTCCTGGCA</td>
<td>6183.10</td>
<td>Analyte</td>
</tr>
</tbody>
</table>
A control pool of DNA is not used in positive control for allelotyping. Rather, one of your own DNA pools is used as template. The relative frequencies of alleles will vary according to the DNA pool you use. I.e., the positive control should produce peaks at the expected masses, however the relative frequencies of the allele peaks will vary according to the DNA pool used.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Expected Mass</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGT-Control-E</td>
<td>ATGAACACTTCAGCAAACAGG</td>
<td>6432.30</td>
<td>EXTEND Primer</td>
</tr>
<tr>
<td>dA Pause</td>
<td>ATGAACACTTCAGCAAACAGGA</td>
<td>6745.50</td>
<td>Pausing peak</td>
</tr>
<tr>
<td>T</td>
<td>ATGAACACTTCAGCAAACAGGT</td>
<td>6720.40</td>
<td>Analyte</td>
</tr>
<tr>
<td>A</td>
<td>ATGAACACTTCAGCAAACAGGAC</td>
<td>7018.60</td>
<td>Analyte</td>
</tr>
</tbody>
</table>
Notes:
Appendix E

Using the PUREGENE™
Genomic DNA Purification Kit

Introduction

This appendix contains protocols for using the PUREGENE Genomic DNA Purification Kit to purify DNA from whole blood. These protocols are excerpted from Genomic DNA Purification Kit Instructions provided by Gentra Systems, Inc.

For technical support on the PUREGENE Genomic DNA Purification Kit, contact Gentra Systems, Inc. (see “Contacting Gentra Systems” on page 170).

Note: Gentra Systems, Inc. offers kits for purifying DNA from a variety of sources other than whole blood (e.g. Buffy coat, bone marrow, cultured cell, buccal cell, body fluids, and solid tissue). For information about these kits and the protocols for using them, contact Gentra Systems, Inc. (see “Contacting Gentra Systems” on page 170). Whatever, the source, you must ultimately have at least 25 ng of DNA for allelotyping and 2.5 ng for genotyping.

Warning: Certain chemicals used in PUREGENE reagents may be hazardous: Tris(hydroxymethyl) aminomethane, ethylenediaminetetraacetic acid, sodium dodecyl sulfate, ammonium chloride, and ammonium acetate. These chemicals may be harmful if swallowed and contact with the eyes and skin should be avoided. In case of contact, wash with large amounts of water and seek medical attention. Wear protective clothing.

Material Safety Data Sheets (MSDS) are available from Gentra Systems, Inc. (for contact information, see “Contacting Gentra Systems” on page 170).

Whole Blood Sample Collection and Handling

1. If possible, collect whole blood and bone marrow in EDTA to reduce DNA degradation. However, other anticoagulants such as ACD (citrate) and heparin may also be used successfully.

2. Store fresh samples at 4°C for not more than 5 days to obtain optimum results.

3. Frozen samples are stable at -80°C for at least two years. Before use, thaw quickly in a 37°C waterbath and keep sample on ice until use.

Purifying DNA from 300 µL Whole Blood

Standard 45 minute protocol, includes RNase treatment

Expected yield: 5-15 µg DNA

Cell Lysis

1. Add 300 µL whole blood (or bone marrow) to a 1.5 mL microfuge tube containing 900 µL RBC Lysis Solution. Invert to mix and incubate 10 minutes at room temperature; invert again at least once during the incubation.

2. Centrifuge for 20 seconds at 13,000-16,000 x g. Remove supernatant with a pipette leaving behind the visible white cell pellet and about 10-20 µL of the residual liquid.
3. Vortex the tube vigorously to resuspend the cells in the residual liquid; this greatly facilitates cell lysis in Step 4 below.

4. Add 300 µL **Cell Lysis Solution** to the resuspended cells and pipet up and down to lyse the cells. Usually no incubation is required; however, if cell clumps are visible after mixing, incubate at 37 °C or room temperature until the solution is homogeneous. Samples are stable in **Cell Lysis Solution** for at least 18 months at room temperature.

**RNase Treatment (Optional)**

1. Add 1.5 µL **RNase A Solution** to the cell lysate.

2. Mix the sample by inverting the tube 25 times and incubate at 37 °C for 15 minutes.

**Protein Precipitation**

1. Cool the sample to room temperature.

2. Add 100 µL **Protein Precipitation Solution** to the cell lysate.

3. Vortex vigorously at high speed for 20 seconds to mix the **Protein Precipitation Solution** uniformly with the cell lysate.

4. Centrifuge at 13,000-16,000 x g for 3 minutes. The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, repeat Step 3 followed by incubation on ice for 5 minutes and then repeat Step 4.

**DNA Precipitation**

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5 mL microfuge tube containing 300 µL **100% Isopropanol** (2-propanol).

2. Mix the sample by inverting gently 50 times.

3. Centrifuge at 13,000-16,000 x g for 1 minute; the DNA will be visible as a small white pellet.

4. Pour off supernatant and drain tube briefly on clean absorbent paper. Add 300 µL **70% Ethanol** and invert the tube several times to wash the DNA pellet.

5. Centrifuge at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol. *Pellet may be loose so pour slowly and watch pellet.*

6. Invert and drain the tube on clean absorbent paper for 5 seconds.

**DNA Hydration**

1. Add 100 µL **DNA Hydration Solution** (100 µL will give a concentration of 100 µg/mL if the total yield is 10 µg DNA).

2. Vortex 5 seconds at medium speed to mix.

3. Incubate sample at 65 °C for 5 minutes to accelerate rehydration.
4. Vortex 5 seconds at medium speed to mix and pulse spin briefly to collect sample at the bottom of the tube.

5. Store DNA at 4 C. For long term storage, place sample at -20 C or -80 C.

**Expected yield: 50-150µg DNA**

**Cell Lysis**
1. Add 3 ml whole blood (or bone marrow) to a 15 mL tube containing 9 mL RBC Lysis Solution. Invert to mix and incubate 10 minutes at room temperature. Invert again at least once during the incubation.

2. Centrifuge at 2,000 x g for 10 minutes. Remove supernatant leaving behind the white cell pellet and about 100-200 µL of the residual liquid.

3. Vortex the tube vigorously to resuspend the cells in the residual liquid. This greatly facilitates cell lysis in Step 4 below.

4. Add 3 mL Cell Lysis Solution to the resuspended cells and pipet up and down to lyse the cells. Usually no incubation is required; however, if cell clumps are visible, incubate at 37 C until the solution is homogeneous. Samples are stable in Cell Lysis Solution for at least 18 months at room temperature.

**RNase Treatment (Optional)**
1. Add 15 µL RNase A Solution to the cell lysate.

2. Mix the sample by inverting the tube 25 times and incubate at 37 C for 15 minutes.

**Protein Precipitation**
1. Cool sample to room temperature.

2. Add 1 mL Protein Precipitation Solution to the cell lysate.

3. Vortex at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate.

4. Centrifuge at 2,000 x g for 10 minutes. The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, repeat Step 3, followed by incubation on ice for 5 minutes and then repeat Step 4.

**DNA Precipitation**
1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 15 mL tube containing 3 mL 100% Isopropanol (2-propanol).

2. Mix the sample by inverting gently 50 times until the white threads of DNA form a visible clump.

3. Centrifuge at 2,000 x g for 3 minutes. The DNA will be visible as a small white pellet.
4. Pour off supernatant and drain tube briefly on clean absorbent paper. Add 3 mL 70% Ethanol and invert tube several times to wash the DNA pellet.

5. Centrifuge at 2,000 x g for 1 minute. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.

6. Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 minutes.

**DNA Hydration**

1. Add 250 µL DNA Hydration Solution (250 µL will give a concentration of 400 µg/mL if the total yield is 100 µg DNA).

2. Rehydrate DNA by incubating at 65 C for 1 hour and overnight at room temperature. Tap tube periodically to aid in dispersing the DNA.

3. For storage, sample may be centrifuged briefly and then transferred to a 1.5 mL microfuge tube.

4. Store DNA at 4 C. For long term storage, store at -20 C or -80 C.

Expected yield: 150-500 µg DNA

**Cell Lysis**

1. Add 10 mL whole blood (or bone marrow) and 30 mL RBC Lysis Solution to a 50 mL centrifuge tube. Invert to mix and incubate 10 minutes at room temperature; invert again at least once during the incubation.

2. Centrifuge for 10 minutes at 2,000 x g. Remove supernatant leaving behind the visible white cell pellet and about 200-400 µL of the residual liquid.

3. Vortex the tube vigorously to resuspend the cells in the residual liquid. This greatly facilitates cell lysis in Step 4 below.

4. Add 10 mL Cell Lysis Solution to the resuspended cells and pipet up and down to lyse the cells. Usually no incubation is required; however, if cell clumps are visible, incubate at 37 C until the solution is homogeneous. Samples are stable in Cell Lysis Solution for at least 18 months at room temperature.

**RNase Treatment (Optional)**

1. Add 50 µL RNase A Solution to the cell lysate.

2. Mix the sample by inverting the tube 25 times and incubate at 37 C for 15-60 minutes.

**Protein Precipitation**

1. Cool sample to room temperature.

2. Add 3.33 mL Protein Precipitation Solution to the cell lysate.
3. Vortex at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate.

4. Centrifuge at 2,000 x g for 10 minutes. The precipitated proteins will form a tight, dark brown pellet. If the protein pellet is not tight, repeat Step 3, followed by incubation on ice for 5 minutes and then repeat Step 4.

**DNA Precipitation**

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 50 mL centrifuge tube containing 10 mL 100% Isopropanol (2-propanol).

2. Mix the sample by inverting gently 50 times until the white threads of DNA form a visible clump.

3. Centrifuge at 2,000 x g for 3 minutes. The DNA will be visible as a white pellet.

4. Pour off supernatant and drain tube on clean absorbent paper. Add 10 mL 70% Ethanol and invert tube several times to wash the DNA pellet.

5. Centrifuge at 2,000 x g for 1 minute. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.

6. Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 minutes.

**DNA Hydration**

1. Add 1 mL DNA Hydration Solution (1 mL will give a concentration of 300 µg/ml if the total yield is 300 µg DNA).

2. Rehydrate DNA by incubating at 65 C for 1 hour and overnight at room temperature. Tap tube periodically to aid in dispersing the DNA.

3. For storage, sample may be centrifuged briefly and then transferred to a 1.5 mL microfuge tube.

4. Store DNA at 4 C. For long term storage, store at -20 C or -80 C.
Using the PUREGENE™ Genomic DNA Purification Kit

Contacting Gentra Systems

Gentra Systems, Inc.
13355 10th Avenue North, Suite 120
Minneapolis, Minnesota 55441 USA
Telephone: (763) 543-0678    Toll-Free: (800) 866-3039 in the United States
Fax: (763) 543-0699

Ordering by Phone
To place an order, call, toll-free in the United States: (800) 866-3039

Ordering Online
To place an order online, visit www.gentra.com.

Technical Assistance by Phone
Monday - Friday
7 AM to 6 PM (Central Standard Time in the United States)
Telephone: (763) 543-0678    Toll-Free: (800) 866-3039 in the United States

Technical Assistance by E-Mail
E-mail: techservice@gentra.com

Technical Assistance on Evenings or Weekends
Evening or weekend inquiries may be left on voice-mail at the above numbers or sent by e-mail to the above address. They will receive priority attention the following business day.
Appendix F

Using the PicoGreen® dsDNA Quantitation Reagent

Introduction

This appendix contains the experimental protocol provided for PicoGreen dsDNA quantitation reagents and kits by Molecular Probes, Inc. This material is excerpted from PicoGreen dsDNA Quantitation Reagent and Kits product information sheet (from Molecular Probes, Inc.). This information is provided here for easy reference. For more information about kits, reagents, storage, handling, and disposal, see the literature provided with the PicoGreen reagent or contact Molecular Probes, Inc. (see “Contacting Molecular Probes, Inc.” on page 176)

Note: For technical support on the PicoGreen dsDNA quantitation reagent, contact Molecular Probes, Inc. (see “Contacting Molecular Probes, Inc.” on page 176).

Warning: Molecular Probes, Inc. does not have data about the mutagenicity or toxicity of PicoGreen dsDNA quantitation reagent. However, because the reagent binds to nucleic acids, it should be treated and handled as a potential mutagen. Also, the reagent is in DMSO solution, which is known to facilitate the entry of organic molecules into tissues. Use double gloves when handling PicoGreen dsDNA quantitation reagent.

Experimental Protocol

Assay Buffer Preparation

The simple buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE), is used below for diluting the PicoGreen reagent, for diluting DNA samples and in the assay itself. Because the PicoGreen dye is an extremely sensitive detection reagent for dsDNA, it is imperative that the TE solution used is free of contaminating nucleic acids. The 20X TE buffer, which is included in the PicoGreen dsDNA Quantitation Kits, is certified to be nucleic acid–free and DNase-free. Prepare the 1X TE working solution, by diluting the concentrated buffer 20-fold with sterile, distilled, DNase-free water.

Reagent Preparation

On the day of the experiment, prepare an aqueous working solution of the PicoGreen reagent by making a 200-fold dilution of the concentrated DMSO solution in TE. For example, to prepare enough working solution to assay 20 samples in 2 mL volumes, add 100 µL PicoGreen dsDNA quantitation reagent to 19.9 mL TE. We recommend preparing this solution in a plastic container rather than glass, as the reagent may adsorb to glass surfaces. Protect the working solution from light by covering it with foil or placing it in the dark, as the PicoGreen reagent is susceptible to photodegradation. For best results, this solution should be used within a few hours of its preparation.

DNA Standard Curve

1.1 Prepare a 2 µg/mL stock solution of dsDNA in TE. Determine the DNA concentration on the basis of absorbance at 260 nm (A 260) in a cuvette with a 1 cm pathlength; an A 260 of 0.04 corresponds to 2 µg/mL dsDNA solution. For a standard
curve, we commonly use bacteriophage lambda or calf thymus DNA, although any purified dsDNA preparation may be used. The lambda DNA standard, provided at 100 µg/mL in the PicoGreen Kits, can simply be diluted 50-fold in TE to make the 2 µg/mL working solution. For example, 30 µL of the DNA standard mixed with 1.47 mL of TE will be sufficient for the standard curve described below. It is sometimes preferable to prepare the standard curve with DNA similar to the type being assayed; e.g., long or short linear DNA fragments when quantitating similar-sized restriction fragments or plasmid when quantitating plasmid DNA. However, we have found that most linear dsDNA molecules yield approximately equivalent signals, regardless of fragment length. Our results have shown that the PicoGreen assay remains linear in the presence of several compounds that commonly contaminate nucleic acid preparations, although the signal intensity may be affected (Table 29). Thus, to serve as an effective control, the dsDNA solution used to prepare the standard curve should be treated the same way as the experimental samples and should contain similar levels of such compounds. To create a five-point standard curve from 1 ng/mL to 1 µg/mL, proceed to step 1.2. For a low-range standard curve from 25 pg/mL to 25 ng/mL, prepare a 40-fold dilution of the 2 µg/mL DNA solution to yield a 50 ng/mL DNA stock solution and proceed to step 1.5.

Table 29: Effects of several compounds that commonly contaminate nucleic acid preparations on the signal intensity of the PicoGreen dsDNA quantitation assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>%Signal Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>50 mM</td>
<td>3% decrease</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>30 mM</td>
<td>3% increase</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>200 mM</td>
<td>30% decrease</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>5 mM</td>
<td>8% decrease</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>50 mM</td>
<td>33% decrease</td>
</tr>
<tr>
<td>Urea</td>
<td>2M</td>
<td>9% increase</td>
</tr>
<tr>
<td><strong>Organic Solvents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>0.1%</td>
<td>13% increase</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10%</td>
<td>12% increase</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2%</td>
<td>14% increase</td>
</tr>
<tr>
<td><strong>Detergents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium dodcyl sulfate</td>
<td>0.01%</td>
<td>1% decrease</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1%</td>
<td>7% increase</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>2%</td>
<td>16% decrease</td>
</tr>
</tbody>
</table>

* The compounds were incubated at the indicated concentrations with PicoGreen reagent in the presence of 500 ng/mL calf thymus DNA. All samples were assayed in a final volume of 200 µL in 96-well microplates using a CytoFluor® microplate reader. Samples were excited at 485 nm and fluorescence intensity was measured at 520 nm.
Using the PicoGreen® dsDNA Quantitation Reagent
Experimental Protocol

Table 29: Effects of several compounds that commonly contaminate nucleic acid preparations on the signal intensity of the PicoGreen dsDNA quantitation assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>%Signal Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgC</td>
<td>0.1%</td>
<td>19% increase</td>
</tr>
<tr>
<td>Other Compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>2%</td>
<td>8% increase</td>
</tr>
<tr>
<td>Agarose</td>
<td>0.1%</td>
<td>4% increase</td>
</tr>
</tbody>
</table>

* The compounds were incubated at the indicated concentrations with PicoGreen reagent in the presence of 500 ng/mL calf thymus DNA. All samples were assayed in a final volume of 200 µL in 96-well microplates using a CytoFluor® microplate reader. Samples were excited at 485 nm and fluorescence intensity was measured at 520 nm.

1.2 For the high-range standard curve, dilute the 2 µg/mL DNA stock solution into disposable cuvettes (or plastic test tubes for transfer to quartz cuvettes) as shown in Table 30. Then add 1.0 mL of the aqueous working solution of PicoGreen reagent (prepared in Reagent Preparation) to each cuvette. Mix well and incubate for 2 to 5 minutes at room temperature, protected from light.

Table 30: Protocol for preparing a high-range standard curve.

<table>
<thead>
<tr>
<th>Volume (µL) of 2 µg/mL DNA Stock</th>
<th>Volume (µL) of TE</th>
<th>Volume (µL) of Diluted PicoGreen Reagent</th>
<th>Final DNA Concentration in PicoGreen Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0</td>
<td>1000</td>
<td>1 µg/mL</td>
</tr>
<tr>
<td>100</td>
<td>900</td>
<td>1000</td>
<td>100 ng/mL</td>
</tr>
<tr>
<td>10</td>
<td>990</td>
<td>1000</td>
<td>10 ng/mL</td>
</tr>
<tr>
<td>1</td>
<td>999</td>
<td>1000</td>
<td>1 ng/mL</td>
</tr>
<tr>
<td>0</td>
<td>1000</td>
<td>1000</td>
<td>blank</td>
</tr>
</tbody>
</table>

1.3 After incubation, measure the sample fluorescence using a spectrofluorometer or fluorescence microplate reader and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm). To ensure that the sample readings remain in the detection range of the fluorometer, the instrument’s gain should be set so that the sample containing the highest DNA concentration yields a fluorescence intensity near the fluorometer’s maximum. To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.
1.4 Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus DNA concentration (see Figure 3).

1.5 For the low-range standard curve — from 25 pg/mL to 25 ng/mL — dilute the 50 ng/mL DNA stock solution (prepared in step 1.1) into disposable cuvettes (or plastic test tubes for transfer to quartz cuvettes) as shown in Table 31. Then add 1.0 mL of the aqueous working solution of PicoGreen reagent (prepared in Reagent Preparation) to each cuvette. Mix well and incubate for 2 to 5 minutes at room temperature, protected from light. Continue with steps 1.3 and 1.4. Adjust the fluorometer gain to accommodate the lower fluorescence signals.

![Figure 3: Dynamic range and sensitivity of the PicoGreen dsDNA quantitation assay. Calf thymus DNA was added to cuvettes containing PicoGreen dsDNA quantitation reagent diluted in 10 mM Tris-HCl, 1mM EDTA, pH 7.5 (TE). The samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm using a spectrofluorometer. Fluorescence emission intensity was then plotted versus DNA concentration; the inset shows an enlargement of the results obtained with DNA concentrations between zero and 750 pg/mL.]

<table>
<thead>
<tr>
<th>Volume (µL) of 50 ng/mL DNA Stock</th>
<th>Volume (µL) of TE</th>
<th>Volume (µL) of Diluted PicoGreen Reagent</th>
<th>Final DNA Concentration in PicoGreen Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0</td>
<td>1000</td>
<td>25 ng/mL</td>
</tr>
<tr>
<td>100</td>
<td>900</td>
<td>1000</td>
<td>2.5 ng/mL</td>
</tr>
<tr>
<td>10</td>
<td>990</td>
<td>1000</td>
<td>250 pg/mL</td>
</tr>
<tr>
<td>1</td>
<td>999</td>
<td>1000</td>
<td>25 pg/mL</td>
</tr>
<tr>
<td>0</td>
<td>1000</td>
<td>1000</td>
<td>blank</td>
</tr>
</tbody>
</table>

Table 31: Protocol for preparing a low-range standard curve.
Sample Analysis

2.1 Dilute the experimental DNA solution in TE to a final volume of 1.0 mL in disposable cuvettes or test tubes. You may alter the amount of sample diluted, provided that the final volume remains 1.0 mL. A higher dilution of the experimental sample may serve to diminish the interfering effect of certain contaminants. However, extremely small sample volumes should be avoided because they are difficult to pipet accurately. See Eliminating Single-Stranded Nucleic Acids from Samples (below) for information on eliminating RNA and ssDNA from the sample.

2.2 Add 1.0 mL of the aqueous working solution of the PicoGreen reagent to each sample. Incubate for 2 to 5 minutes at room temperature, protected from light.

2.3 Measure the fluorescence of the sample using instrument parameters that correspond to those used when generating your standard curve (see step 1.3). To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.

2.4 Subtract the fluorescence value of the reagent blank from that of each of the samples. Determine the DNA concentration of the sample from the standard curve generated in DNA Standard Curve.

2.5 The assay may be repeated using a different dilution of the sample to confirm the quantitation results.

Eliminating Single-Stranded Nucleic Acids from Samples

We have found that dsDNA can be quantitated in the presence of equimolar concentrations of single-stranded nucleic acids with minimal interference. Table 32 shows the concentrations of RNA or ssDNA that, for a given dsDNA concentration, result in less than a 10% change in the signal intensity using the PicoGreen assay protocol. Fluorescence due to PicoGreen reagent binding to RNA at high concentrations can be eliminated by treating the sample with DNase-free RNase. The use of RNase A/RNase T1 with S1 nuclease will eliminate all single-stranded nucleic acids and ensure that the entire sample fluorescence is due to dsDNA.1

Table 32: Sensitivity of the PicoGreen dsDNA assay for quantitating dsDNA in the presence of single-stranded nucleic acids.

<table>
<thead>
<tr>
<th>[dsDNA]*</th>
<th>[RNA] (amount relative to dsDNA)</th>
<th>[ssDNA] (amount relative to dsDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg/mL</td>
<td>10 µg/mL (10X)</td>
<td>300 ng/mL (0.3X)</td>
</tr>
<tr>
<td>500 ng/mL</td>
<td>500 ng/mL (1X)</td>
<td>50 ng/mL (0.1X)</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>100 ng/mL (10X)</td>
<td>30 ng/mL (2X)</td>
</tr>
<tr>
<td>5 ng/mL</td>
<td>50 ng/mL (10X)</td>
<td>15 ng/mL (3X)</td>
</tr>
<tr>
<td>100 pg/mL</td>
<td>1 ng/mL (10X)</td>
<td>1 ng/mL (10X)</td>
</tr>
<tr>
<td>50 pg/mL</td>
<td>500 pg/mL (10X)</td>
<td>500 pg/mL (10X)</td>
</tr>
</tbody>
</table>

* For several concentrations of dsDNA, we show the concentration of RNA or ssDNA that results in no more than a 10% increase in the sample’s signal intensity.

References

Using the PicoGreen® dsDNA Quantitation Reagent
Contacting Molecular Probes, Inc.

**Molecular Probes, Inc.**
PO Box 22010
Eugene, OR 97402-0469
Phone: (541) 465-8300  Fax: (541) 344-6504

**Online**
For the most up-to-date information, visit www.probes.com.

**Customer Service in the United States**
7:00 am to 5:00 pm (Pacific Time)
Phone: (541) 465-8338  Fax: (541) 344-6504  order@probes.com

**Toll-Free Ordering for USA and Canada**
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**Technical Assistance in the United States**
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 465-8353  Fax: (541) 465-4593  tech@probes.com

**Molecular Probes Europe BV**
PoortGebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378  Fax: +31-71-5233419

**Customer Service in Europe, Africa, and the Middle East**
9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850  Fax: +31-71-5233419
euorder@probes.nl

**Technical Assistance in Europe, Africa, and the Middle East**
9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431  Fax: +31-71-5241883
eurotech@probes.nl
Appendix G

Troubleshooting for Allelotyping

Introduction

If your pool frequency differs from genotyped frequency by 6% or greater, this appendix lists possible causes. Additionally, some possible solutions are listed. Note that these are only suggestions and are not guaranteed to correct the problem.

Possible Causes

1. DNA quantitation was not accurate.
   Try requantititating the DNA.

2. Pipetting errors occurred when the pools were made.
   Try re-making the pools; take particular care to use good pipetting technique.

3. Preferential amplification of one allele over another in amplification of pooled DNA.
   Try optimizing the amplification reaction.

4. Population not in Hardy-Weinberg equilibrium, i.e., all individuals are genotyped as heterozygotes.
   Discard the assay.

5. Large unextended MassEXTEND primer peak is present in spectra.
   Try redesigning the assay using the opposite primer.

6. “Pausing” peak present in spectra.
   Try redesigning the assay using the opposite primer.

7. Large heterozygote-skewing occurs in individual heterozygotes; 75% or greater for one allele.
   Try redesigning the assay.

8. Extension has occurred off a secondary structure of the MassEXTEND primer itself (e.g. hairpin).
   Test whether this is occurring by running a negative control (i.e. run the MassEXTEND reaction without amplification product).

9. Extension has occurred off a dimer formed by the MassEXTEND primer.
   Test whether this is occurring by running a negative control (i.e. run the MassEXTEND reaction without amplification product).
10. The weighted frequency error is greater than 1.5%.

This indicates the assays may be unstable. If the whole microplate of pooled samples has a weighted frequency error greater than 1.5%, try re-running the pooled samples. If only certain assays have a weighted frequency error greater than 1.5%, try redesigning those assays.

The weighted frequency error can be found in Allelotype Reports, in the FREQ_ERROR column. For information about Allelotype Reports, see MassARRAY Typer Software Guide.

11. Standard deviation of 2.5% or greater between the frequency from the same assay run at different times or in different wells.

Try redesigning the assays and/or optimizing the amplification reaction.

12. Assay primers map to the human genome at more than one site using BLAST.

Try redesigning the assays.
Appendix H

Calculating the Mass of DNA Molecules

Mass Calculation of DNA Molecules

Table 33: Masses of common nucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Mass in Dalton</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyadenosine</td>
<td>dA C₁₀H₁₄O₅N₅P</td>
<td>313.2 Da</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>dC C₉H₁₄O₆N₃P</td>
<td>289.2 Da</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>dG C₁₀H₁₄O₆N₅P</td>
<td>329.2 Da</td>
</tr>
<tr>
<td>Deoxythymidine</td>
<td>dT C₁₀H₁₃O₇N₂P</td>
<td>304.2 Da</td>
</tr>
<tr>
<td>Dideoxyadenosine</td>
<td>ddA C₁₀H₁₄O₅N₅P</td>
<td>297.2 Da</td>
</tr>
<tr>
<td>Dideoxycytidine</td>
<td>ddC C₉H₁₄O₅N₃P</td>
<td>273.2 Da</td>
</tr>
<tr>
<td>Dideoxyguanosine</td>
<td>ddG C₁₀H₁₄O₅N₅P</td>
<td>313.2 Da</td>
</tr>
<tr>
<td>Dideoxythymidine</td>
<td>ddT C₁₀H₁₃O₇N₂P</td>
<td>288.2 Da</td>
</tr>
<tr>
<td>Adenosine</td>
<td>A C₁₀H₁₂O₆N₅P</td>
<td>329.2 Da</td>
</tr>
<tr>
<td>Cytidine</td>
<td>C C₉H₁₂O₇N₃P</td>
<td>305.2 Da</td>
</tr>
<tr>
<td>Guanosine</td>
<td>G C₁₀H₁₂O₇N₅P</td>
<td>345.2 Da</td>
</tr>
<tr>
<td>Uridine</td>
<td>U C₉H₁₁O₇N₂P</td>
<td>306.2 Da</td>
</tr>
</tbody>
</table>

The following are two examples for calculating the expected mass of specific DNA molecules.

Unmodified extension primer: 5’-CCT GTA GTG GTA ACA TC-3’

(primers are usually 5’-OH, without 5’-phosphate-group)

Mass calculation:

\[
\left(4 \times \text{dA} + 4 \times \text{dC} + 4 \times \text{dG} + 4 \times \text{dT}\right) - \text{Phosphate} + \text{OH} + \text{H} = \\
\left(4 \times 313.2 \text{ Da} + 4 \times 289.2 \text{ Da} + 4 \times 329.2 \text{ Da} + 4 \times 304.2 \text{ Da}\right) - 62 \text{ Da} = 5185.4 \text{ Da}
\]

As the DNA molecule will get protonated during the MALDI-TOF analysis, an additional mass of 1 Da has to be added. This means the expected mass of this primer in the mass spectrum is 5186.4 Da.
MassEXTEND extension product: 5'–CCT GTA GTG GTA ACA TC G^dd–3'

(containing all dNs except the last base, which is a ddN)

Mass calculation:

Primer mass + ddG =

\[
\begin{align*}
((4 \times 313.2 \text{ Da}) + (4 \times 289.2 \text{ Da}) + (4 \times 329.2 \text{ Da}) + (5 \times 304.2 \text{ Da})) - 62 \text{ Da} &+ 313.2 \\
5185.4 \text{ Da} + 313.2 \text{ Da} & = 5498.6 \text{ Da}
\end{align*}
\]

Again, the molecule will get protonated during the MALDI-TOF process. The expected mass of the extension product in the mass spectrum is 5499.6 Da.
QC Analysis of Oligonucleotides by MALDI TOF Mass Spectrometry

Because oligonucleotide purity is critical to the success of all DNA-based assays, SEQUENOM recommends the use of quality-controlled oligonucleotides.

**Note:** Do not use HPLC-purified oligonucleotides with the MassARRAY system.

Mass analysis of oligonucleotides is a highly efficient and accurate quality control, since it yields both the mass of the oligonucleotide and gives a good indication of its quality. Mass spectrometry can be used to determine the length of the oligonucleotide, its deprotection and level of purity. SEQUENOM strongly advises the use of a reliable facility for checking the quality of your oligonucleotides and comparing them against the manufacturer-provided quality control (QC) documentation.

In the absence of such facility, the MassARRAY system can be used for QC of your oligonucleotides.

**To use the MassARRAY System for Oligonucleotide QC**

1. The oligonucleotide should be diluted to a concentration of about 10 pmol/µl.

2. Pipette 7 µl of each oligonucleotide solution in a well of an AB0800 MTP (where MTP refers to a microtiterplate).

3. Place the MTP on the MassARRAY nanodispenser or MassARRAY piezodispenser and start dispensing the oligonucleotide solution onto a SpectroCHIP.

4. Analyze the respective SpectroCHIP positions with a MassARRAY genotype analyzer (genotype analyzer). Only analyze the patches which are loaded with the oligonucleotide solution. For more information about using a genotype analyzer, see either MassARRAY Typer Software Guide (autoflex) or MassARRAY Typer Software Guide (Biflex); note: which manual you should use depends on the particular model of genotype analyzer you have—autoflex or Biflex.

5. Be sure to carefully record the SpectroCHIP serial number and the used wells.

6. After analysis store the partially used SpectroCHIP in a desiccator for further use.

**Guidelines for Spectra Interpretation**

In general, the MALDI mass spectrum of a pure oligonucleotide shows one or more distinct peaks with high resolution and signal-to-noise ratio. The spectrum may also display signals at half or double the expected oligonucleotide mass. These signals are due to doubly charged or dimerized molecular ions and are not diagnostic of oligonucleotide quality.
Peaks that signify inferior oligonucleotide quality are:

- Low mass adducts with m/z M-300 Daltons. Additional signals in front (about –300 Da, see Table 34 for correct masses and Figure 32) of the oligonucleotide (not complete removal of n-1 synthesis products).

Table 34: Masses for checking Oligonucleotide quality.

<table>
<thead>
<tr>
<th>Mass shift [Da]</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n-A</td>
<td>-313.2</td>
</tr>
<tr>
<td>n-C</td>
<td>-289.2</td>
</tr>
<tr>
<td>n-G</td>
<td>-329.2</td>
</tr>
<tr>
<td>n-T</td>
<td>-304.2</td>
</tr>
<tr>
<td>Na(^+)-adduct</td>
<td>+23</td>
</tr>
<tr>
<td>K(^+)-adduct</td>
<td>+39</td>
</tr>
<tr>
<td>NH(_4)(^+)-adduct</td>
<td>+17</td>
</tr>
<tr>
<td>Depurination (-G)</td>
<td>-151</td>
</tr>
<tr>
<td>Depurination (-A)</td>
<td>-135</td>
</tr>
</tbody>
</table>

Figure 32: Example for a spectrum of an oligonucleotide with additional signals from n-1 synthesis products.
- A signal with the mass of the unmodified oligonucleotide (incomplete modification). In case of biotin-modified oligonucleotides, see Figure 33.

Figure 33: Spectra of oligonucleotide with and without biotin modification.

**Note:** Modified oligonucleotides should always be ordered HPLC (or equivalent) purified.

- Excessive sodium and/or potassium adducts with m/z M+n23 and M+n39 respectively. Excessive adduction with alkali cations results in broad peaks, poor resolution, and signal-to-noise ratio.
- Adducts at about M+100 Da resulting from incomplete deprotection.
- Signals arising from excessive depurination during synthesis detected at m/z ~M-150 Da.

**Attention:** Depurination also occurs in the condensed phase in the MALDI matrix. This is noted in MALDI preparations that are not promptly analyzed.

- Presence of additional inexplicable peaks may indicate cross-contamination or carry over during the synthesis or purification process.

*Please contact your oligonucleotide supplier if your oligonucleotide sample does not meet high QC standards.*
Notes:
## Contact Information

### Corporate Headquarters
3595 John Hopkins Court  
San Diego, CA 92121  
Telephone: (858) 202-9000  
Fax: (858) 202-9001

### Sales and Support
Telephone: (877) 4GENOME (1-877-443-6663)  
Fax: (858) 202-9220

### U.S. East Coast
142-F North Road, Suite 150  
Sudbury, MA 01776  
Telephone: (978) 371-9830  
Fax: (978) 371-9844

### Europe
Mendelssohnstrasse 15D, D-22761  
Hamburg, Germany  
Telephone: (+49) 40-899676-0  
Fax: (+49) 40-899676-10
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