Affymetrix®

User Manual

DMET™ Plus Premier Pack

For DMET Plus Cartridge Arrays

P/N 702687 Rev. 4

For research use only.
Not for use in diagnostic procedures.

Contents

About the DMET™ Plus Premier Pack

The therapeutic efficacy of any given drug is influenced by a number of different factors that in part include age, weight, and concurrent drug use. These factors may vary between patients. In addition, fixed parameters such as gender and human genome sequence variation can contribute as well [2-4]. This genetic variation, which includes both single nucleotide polymorphisms (SNPs) as well as more complex structural variations in the form of insertions, duplications and deletions, underlies every individual's response to drugs.

Many of the enzymes involved in drug metabolism are genetically polymorphic. Consequently, their activity may differ depending upon an individual's genotype. For example, drugs may be metabolized more slowly in individuals who are carriers of a genetic polymorphism that results in decreased or null activity of a given enzyme. These individuals are at particular risk for adverse drug reactions or therapeutic failure [4]. Conversely, drug therapy could be ineffective if the drug is metabolized too rapidly due to other genetic polymorphisms that can be present. Genetically determined variation particularly impacts drugs with narrow therapeutic indices, hence increasing the risk for the development of adverse drug effects.

Comprehensive genotyping could be helpful when choosing the right drug at the optimal dosage for individual patients. This is the vision of individualized drug therapy or personalized medicine [2, 5]. According to a recent study conducted by the Federal Drug Administration [1], approximately one-quarter of the prescriptions written in the United States in 2006 contained pharmacogenetic labeling recommendations.

The Drug Metabolizing Enzymes and Transporters (DMET™) Plus Premier Pack (DMET Plus) offers the greatest representation of genetic diversity across the known ADME markers. It enables faster discovery and measurement of genetic variation associated with drug response than with traditional non-multiplex methods. The polymorphisms represented on this array were chosen by virtue of their functional significance as documented in the scientific literature. These polymorphisms have been publicly reviewed and prioritized by a panel of experts made up from both the pharmaceutical industry and academia. DMET Plus genotypes 1,936 high-value drug metabolism and transporter markers in ~230 genes. These markers have been evaluated across a minimum of 1,200 individuals from multiple populations including Caucasian, African, and Asian.

Content and Assay

DMET Plus offers the greatest representation of genetic diversity across the known ADME markers. The 1,936 markers included in the DMET™ Plus Panel include common and rare variations, short insertion or deletion alleles, and analysis of triallelic SNPs. In addition to known biomarkers such as common variants in CYP2D6, CYP2C19 and other cytochrome P450 genes, DMET Plus contains over 1,000 variants in drug transporters that can be used in clinical research studies to discover novel genetic associations. The transporter gene family, which includes genes such as MDR1, ABCB2, ABCG2 among other important genes, represents one of the most active areas of investigation for next generation drug targets [8-11]. DMET Plus also performs quantitative assessment of genes with whole-gene deletions (including GSTT1[12,13], GSTM1 [14], CYP2D6 [15], CYP2A6, and UGT2B17) and reports allele names in both genotyping reports and translation reports. Several recent studies have identified adverse drug reactions correlate to these classes of genetic markers [16-19].

The DMET Plus Panel includes a set of 315 markers that were selected in collaboration with the PharmaADME group (www.pharmaADME.org) along with leading pharmaceutical companies and academic leaders. These key markers have been demonstrated in the literature to have a known effect on drug metabolism [6, 20-22]. Allelic frequencies for the key markers in the DMET Plus assay are below 9% on average [6], although several more common variants are also present [23]. Consequently, many of these variants cannot be adequately interrogated by common SNP or tagging approaches that typically interrogate markers with an average minor allele frequency (MAF) of 20% or greater. Caldwell et al. (2008) recently used an earlier research version of the DMET product to identify a novel common genetic variant in the CYP4F2 gene that correlated closely with effective warfarin requirements in three independent United States population groups [23]. This association would have been missed if a tagging SNP approach had been employed in the study as advocated by other clinical researchers [24].

Many of the known genetic markers that influence the metabolism of commonly prescribed drugs are not assayed by conventional microarray SNP methods due to the presence of pseudogenes or other closely related genomic sequences. DMET Plus is capable of processing these complex markers by making use of a pre-amplification step in the processing of genomic DNA. Some markers are first pre-amplified using multiplex polymerase chain reaction (mPCR) prior to joining the other markers in the DMET Plus assay flow. Genomic sequences that contain the polymorphic markers of interest are preferentially amplified through the use of highly selective Molecular Inversion Probe (MIP) [25, 26] amplification. The resulting target DNA is then labeled and hybridized to the DMET Plus Array to obtain genotypes using a single color detection format.

Data Analysis

DMET™ Console software (part of the DMET Plus Premier Pack) translates the results and converts the genetic profiles into a more conventional format known as *star nomenclature* [7]. The star nomenclature format is routinely used in pharmacogenomics research studies. The automated analysis takes only a few minutes to complete, and provides pharmaceutical researchers with familiar data that can be easily integrated into existing workflows. DMET Console software also features easy-to-use controls for marker content to ensure that the genetic markers that are reported in clinical studies are consented to and compliant with Informed Consent and other IRB oversight.

DMET genotyping software enables single-sample processing by relying on preset and analytically validated cluster boundaries established for each of the DMET variants. Taken together, all of the specialized features of DMET Plus make it a powerful new tool to enable the vision of personalized medicine become a reality.

References

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Before You Start

Laboratory Requirements

To prevent sample contamination from PCR products, the protocol is performed in three separate areas:

- mPCR Staging Area
  - A separate laboratory, or
  - A fume hood in the Pre-Amp Lab
- Pre-Amp Lab
- Post-Amp Lab

For more information on laboratory requirements and the equipment required to perform this protocol, refer to the *DMET™ Plus Premier Pack Site Preparation Guide*, P/N 702735.

Reagents Required

From Affymetrix — DMET™ Plus Premier Pack (P/N 901268)

One DMET™ Plus Premier Pack is sufficient to process 48 reactions: 45 samples and 3 genomic DNA controls.

- DMET Plus Reagent Kit — P/N 901267
  - Box 1: DMET Plus Pre-Amp Kit (P/N 901273)
  - Box 2: DMET Plus Labeling Kit (P/N 901271)
  - Box 3: DMET Plus Hyb-Stain Kit (P/N 901269)
  - Box 4: DMET Plus Panel Kit (P/N 901272)
- Wash Solution A (3 bottles)
- Wash Solution B (2 bottles)
- DMET Plus Array (48 arrays; P/N 901317)

From Other Suppliers

Table 2.1 Reagents Required from Other Suppliers

Description	Supplier	Part No.
AccuGENE® Water or other molecular biology grade water	Lonza Group LTD	51200
QIAGEN® Multiplex PCR Kit	QIAGEN	206143
Streptavidin, R-Phycoerythrin Conjugate (SAPE), 1 mL	Life Technologies	S866
TITANIUM™ Taq Polymerase, 100 µL	Clontech	639208 (24 rxns) 639209 (500 rxns)
TE Buffer, pH 8.0	TekNova	T0223

This protocol also requires the use of reagents contained in the following kit.

Table 2.2 Quant-iT™ PicoGreen® dsDNA Assay Kit required for the DMET Plus Premier Pack Protocol

Description	Supplier	Part Number
Quant-iT PicoGreen dsDNA Assay Kit	Life Technologies	P7589

Normalize Samples

All genomic DNA samples should be normalized to a single concentration of 60 ng/µL using 1X TE buffer. The controls included in every DMET Plus Kit are already normalized to a working concentration.

IMPORTANT: We strongly recommend you determine your sample concentration using the Quant-iT PicoGreen assay by Life Technologies. Sample concentration determined by UV absorbance is often inaccurate and can yield very different results.

Working with Enzymes

Enzymes in the DMET Plus Kit are temperature sensitive and may lose activity as their temperature rises. For best results:

- Keep at –20°C until used.
- Handle tubes by the cap only. Do not touch the sides of the tubes as the heat from your fingers will raise the reagent temperature.
- Spin down the tubes so that the contents are uniform.
- When preparing master mixes, always add reagents in the order shown in the table.

Pipettes and Pipetting Recommendations

The types of pipettes specified for use throughout this protocol are:

- Single channel, manual
- 12-channel, manual or electronic
- Optional: 24-channel, manual or electronic

General Pipetting Recommendations

General pipetting recommendations are as follows:

- Many of the reagents in the DMET Plus Kit are in very viscous solutions. For best results:
  - Pipet slowly to allow enough time for the correct volume of solution to enter the pipette tip.
  - Avoid excess solution on the outside of pipette tips.
- To ensure full volume transfer, check pipette tips after each pick up and dispense.
- To avoid the formation of air bubbles, dispense liquids at the bottom of each well.
- Always use the type and volume of pipette specified in the protocol.

Electronic Pipetting Recommendations

Follow the instructions provided with the pipettes for the dispense/mix program that:

- Allows reagents to be aspirated and dispensed at a set volume.
- Mixes automatically upon dispensing, wherein the mix volume can be different from the dispense volume.

Two options are available for tracking the number of mixes when using Rainin EDP3-Plus electronic pipettes: the counter option, or the beep option (pipette beeps after each mix). We recommend using the beep option, since the counter does not start at zero with each use. Instead, it counts pipette operations sequentially. Refer to the instructions provided with your pipettes for more information.

Quality Control Gel Recommendations

We recommend running two quality control gels during the protocol. Knowing in advance that a sample will not provide data will save arrays. The purpose of each gel is described below.

- Gel 1: Run to identify any samples that did not amplify. No bands are visible for samples that have not amplified.
- Gel 2: Run after fragmentation to confirm acceptable fragment size.

Thermal Cycler Requirements and Programs

To run the DMET Plus Premier Pack Protocol at a throughput of 48 assays/day, you will need 2 thermal cyclers: 1 in the Pre-Amp Lab; 1 in the Post-Amp Lab.

This protocol has been optimized using the GeneAmp® PCR System 9700 Thermal Cyclers listed in Table 2.3 below.

Table 2.3 Thermal cyclers validated for use with the DMET Plus Premier Pack Protocol

Manufacturer/ Distributor	Item	Part Number (U.S.)
Pre-Amp Lab — Use one of the thermal cyclers listed below.		
Life Technologies Thermal Cyclers	GeneAmp® PCR System 9700 Thermal Cycler, 96-well, Silver	N8050001
	GeneAmp® PCR System 9700 Thermal Cycler, 96-well, Gold-plated	4314878
Post-Amp Lab — Use one of the thermal cyclers listed below.		
Life Technologies Thermal Cyclers	GeneAmp® PCR System 9700 Thermal Cycler, 96-well, Silver	N8050001
	GeneAmp® PCR System 9700 Thermal Cycler, 96-well, Gold-plated	4314878

Thermal Cycler Programs

Use only the thermal cyclers listed in Table 2.3. We recommend that you enter and store the following programs prior to processing samples. Program details are located in Appendix B, *Thermal Cycler Programs* on page 64.

Pre-Amp Lab

- DMET Plus mPCR
- DMET Plus Anneal
- DMET Plus Assay

Post-Amp Lab

- DMET Plus Clean Up
- DMET Plus Frag
- DMET Plus Label
- DMET Plus Denature

DMET™ Plus Premier Pack Protocol

The DMET Plus Premier Pack protocol is presented in stages. The stages are:

Preparing a Plate of Genomic DNA and a Batch Registration File

Genomic DNA Preparation

To perform the DMET Plus Premier Pack assay, you will need to prepare a plate of genomic DNA (gDNA). The protocol is written for processing 48 reactions: 45 gDNA sample, plus 3 gDNA controls.

As part of gDNA plate preparation:

1. Determine sample concentrations (you should use the *Quant-iT™ PicoGreen® dsDNA Assay Kit* from Life Technologies).
2. Normalize all gDNA samples to a single concentration of 60 ng/µL using 1X TE buffer.

   The gDNA controls included in the DMET Plus Premier Pack Reagent Kit are already normalized to a working concentration.

IMPORTANT: **We strongly recommend you determine your sample concentration using the Quant-iT PicoGreen assay by Life Technologies. Sample concentration determined by UV absorbance is often inaccurate and can yield very different results.**

Batch Registration File Preparation

A template for recording sample information is provided in Affymetrix GeneChip® Command Console (AGCC). In AGCC we refer to this template as a *Batch Registration File.* We recommend entering your sample information into a batch registration file as you prepare your genomic DNA plate. Then just prior to scanning your arrays, you will add the array barcodes to this file, and upload the information into AGCC.

Instructions on completing the sample information spreadsheet are listed in Appendix A, *Registering Samples in Affymetrix GeneChip® Command Console* on page 56.

Stage 1 — mPCR

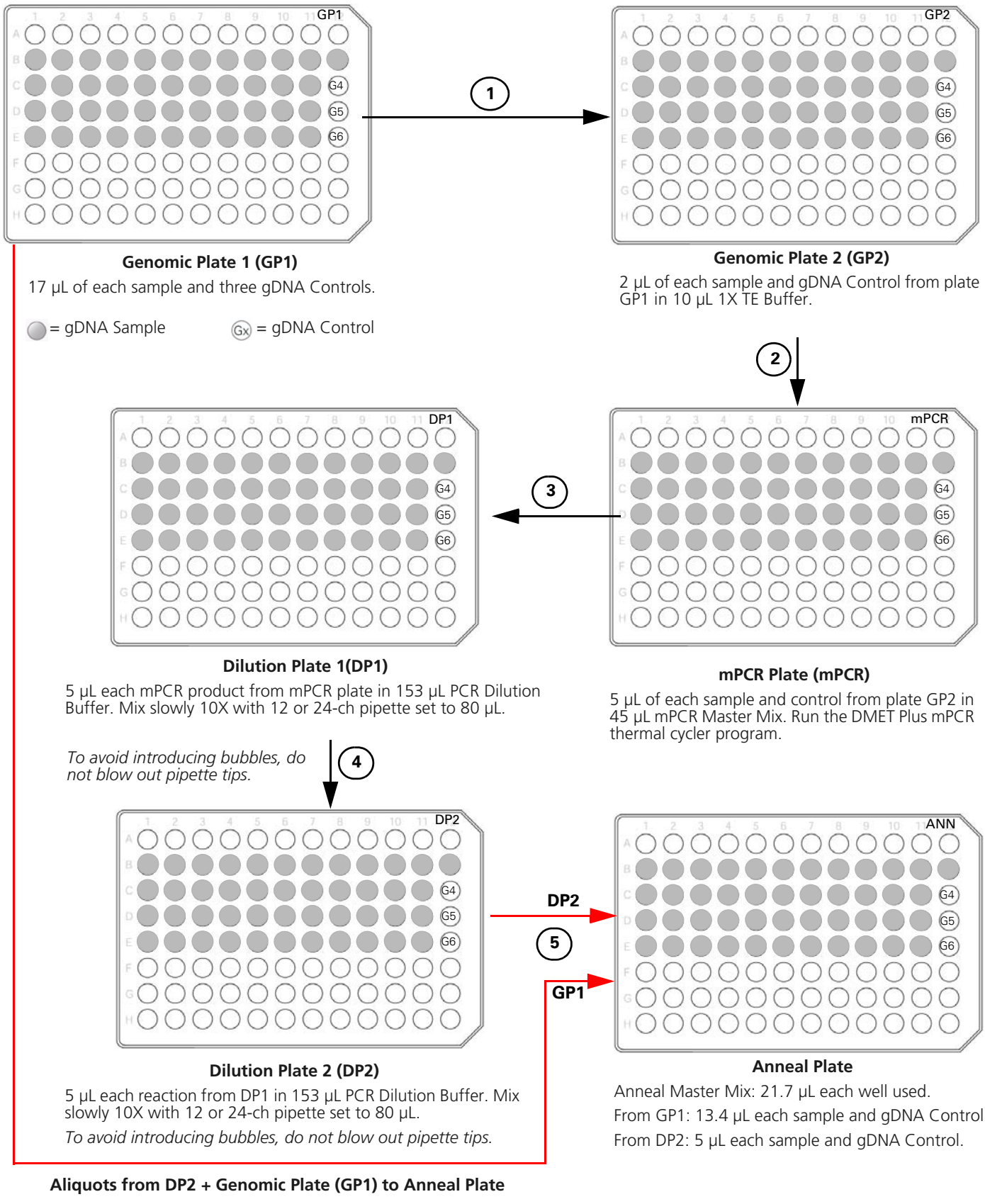
About this Stage

The workflow for this stage is illustrated on page 11.

Location and Duration

- Pre-Amp Lab and mPCR Staging Area
  - Preparation and running of mPCR plate on the thermal cycler is conducted in the Pre-Amp Lab
  - Dilution of mPCR products is conducted in the mPCR Staging Area
- Hands-on time: approximately 1.5 hr
- Thermal cycler time: 2 hr

mPCR Stage Workflow



Genomic Plate 1 (GP1)

17 µL of each sample and three gDNA Controls.

● = gDNA Sample Gx = gDNA Control

Genomic Plate 2 (GP2)

2 µL of each sample and gDNA Control from plate GP1 in 10 µL 1X TE Buffer.

mPCR Plate (mPCR)

5 µL of each sample and control from plate GP2 in 45 µL mPCR Master Mix. Run the DMET Plus mPCR thermal cycler program.

Dilution Plate 1(DP1)

5 µL each mPCR product from mPCR plate in 153 µL PCR Dilution Buffer. Mix slowly 10X with 12 or 24-ch pipette set to 80 µL.

To avoid introducing bubbles, do not blow out pipette tips.

Dilution Plate 2 (DP2)

5 µL each reaction from DP1 in 153 µL PCR Dilution Buffer. Mix slowly 10X with 12 or 24-ch pipette set to 80 µL.

To avoid introducing bubbles, do not blow out pipette tips.

Anneal Plate

Anneal Master Mix: 21.7 µL each well used.

From GP1: 13.4 µL each sample and gDNA Control.

From DP2: 5 µL each sample and gDNA Control.

Aliquots from DP2 + Genomic Plate (GP1) to Anneal Plate

Equipment and Materials Required

The following equipment and materials are required to perform this stage. Quantities shown are for processing 45 samples and 3 gDNA controls.

In the Pre-Amp Lab

Table 3.1 Equipment and Materials Required in the Pre-Amp Lab for *Stage 1 — mPCR*

Quantity	Item
2	Aluminum block, 96-well, chilled in 4 °C refrigerator
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
1	Marking pen, extra fine point, permanent
As required	MicroAmp® Clear Adhesive Films
1	Microcentrifuge
3	PCR plate, 96-well
1 of each	Pipettes: ▪ single-channel P20 ▪ single-channel P1000 ▪ 12-channel P20 (manual or electronic) ▪ 12-channel P200 (manual or electronic) ▪ Optional: 24-channel P20 (manual or electronic)
As required	Pipette tips for the pipettes listed above
2	Reagent reservoir, 50 mL
45	Genomic DNA samples to be tested
1	Thermal cycler, 96-well GeneAmp® PCR System 9700 (gold or silver block)
1	Tube, 5 mL
1 or 2	Required: Vortexer with plate attachment Optional: Vortexer with tube attachment

In the mPCR Staging Area

Table 3.2 Equipment and Materials Required in the mPCR Staging Area for *Stage 1 — mPCR*

Quantity	Item
3	Aluminum block, 96-well, chilled in 4 °C refrigerator
1	Ice container, rectangular, filled with ice
1	Marking pen, extra fine point, permanent
As required	MicroAmp Clear Adhesive Films
2	PCR plate, 96-well
1 of each	Pipettes: ▪ 12-channel P20 ▪ 12-channel P200 (manual or electronic) ▪ Optional: 24-channel P20 (manual or electronic) ▪ Optional: 24-channel P100 (manual or electronic)
As required	Pipette tips for the pipettes listed above
1	Reagent reservoir, 50 mL

DMET Plus Premier Pack Kit Components Required

Table 3.3 Reagents required from the DMET Plus Premier Pack Reagent Kit

From the DMET Plus Panel Kit box:
DMET Plus mPCR Primer Mix
1X TE Buffer
DMET Plus gDNA Control 4
DMET Plus gDNA Control 5
DMET Plus gDNA Control 6
PCR Dilution Buffer

NOTE: Genomic controls may be retired from time to time dictated by availability. For example, in March 2011 Genomic Controls 1, 2, and 3 were retired. Typically new controls are taken from the Accuracy plate. Genomic controls after March 2011 are now labeled G4, G5, and G6.

Other Reagents Required

Table 3.4 Reagents required from the QIAGEN® Multiplex PCR Kit

Number of Tubes	QIAGEN Multiplex PCR Kit Component
2	2X QIAGEN Multiplex PCR Master Mix
1	Q-Solution, 5X
1	RNase-free water

Thaw Reagents

Location: Pre-Amp Lab

To thaw the reagents:

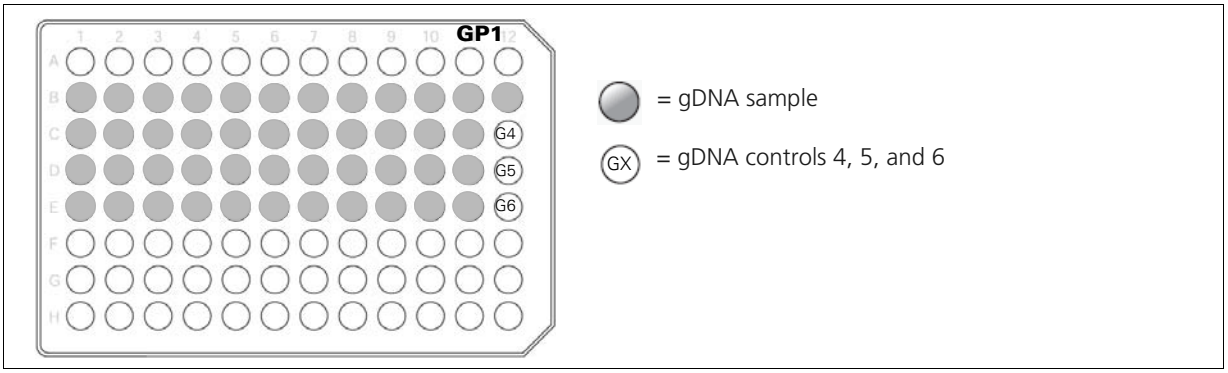
1. Place the following reagents on the bench top at room temperature and thaw:
   - From the DMET Plus Premier Pack Kit:
     - mPCR Primer Mix
     - gDNA Controls 4, 5, 6
     - 1X TE Buffer
     - PCR Dilution Buffer

   From the QIAGEN Multiplex PCR Kit:
     - Two tubes 2X QIAGEN Multiplex PCR Master Mix
     - Q-Solution, 5X
     - RNase-free water
2. Once thawed, place all reagents on ice until ready to use.
   ***Exception**:* Leave PCR Dilution Buffer buffer at room temperature. Do not place it on ice.

Prepare Genomic Plate 1 (GP1)

In this step, you will prepare what is referred to as Genomic Plate 1 (GP1). This plate consists of 45 genomic DNA (gDNA) samples and 3 gDNA Controls at a concentration of 60 ng/µL. Controls are included in the DMET Plus Premier Pack Kit.

Figure 3.1 Plate layout for plate GP2 (identical to layout of plate GP1)



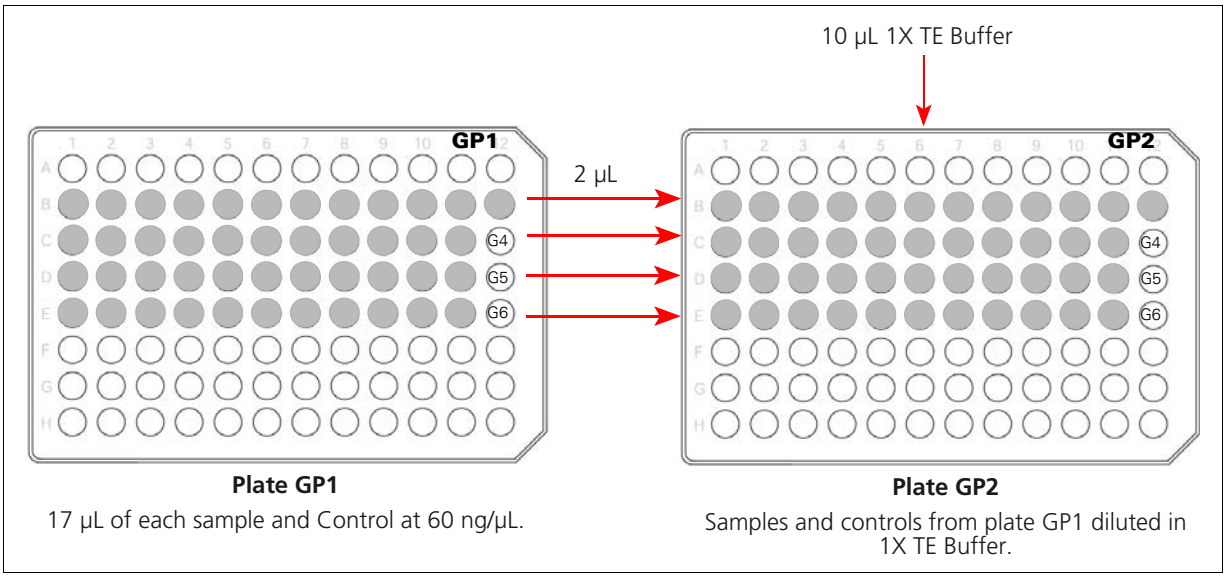
Location: Pre-Amp Lab

To prepare plate GP1:

1. Using a permanent marker, label a 96-well PCR plate with the designation *GP1* (Figure 3.1).
2. Vortex the gDNA samples; then spin down at 2000 rpm for 30–60 sec.
3. Following the layout in Figure 3.1, aliquot 17 µL of:
   A. Each gDNA sample to be processed.
      All samples should be quantitated and normalized to 60 ng/µL.
   B. Each gDNA control to wells C12, D12 and E12.
4. Seal the plate, and spin down at 2000 rpm for 30–60 sec.
5. Place in an aluminum block on ice.
   The plate can be stored at 4 °C until ready to use.

Prepare Genomic Plate 2 (GP2)

Figure 3.2 Plate layout for plate GP2 (identicalto layout of plate GP1)



Location: Pre-Amp Lab

To prepare plate GP2:

1. Briefly vortex the 1X TE Buffer; then spin down.
2. Pour the buffer into a reagent reservoir.
3. Using a permanent marking pen, label a 96-well PCR plate *GP2*.
4. Place in an aluminum block on ice.
5. Using a 12-channel P20 pipette:
   - **A.** Aliquot 10 µL of 1X TE Buffer into each well of rows B, C, D, and E of plate GP2.
   - **B.** Transfer 2 µL of each sample and genomic control on plate GP1 to the corresponding well of plate GP2 (Figure 3.2).
   - **C.** Pipet up and down 3 times to rinse tips.

     Final volume of each well is 12 µL; final concentration of each well is 10 ng/µL.Tightly seal plate GP2.
6. Vortex the center of the plate at high speed for 3 sec.
7. Spin down at 2000 rpm for 30–60 sec, and return to the aluminum block.
8. Seal plate GP1 and store on ice or at 4 °C.

   Aliquots from this plate are taken again during *Stage 2 — Anneal*.

Prepare the mPCR Master Mix

Location: Pre-Amp Lab

To prepare the mPCR Master Mix:

1. Gently vortex the QIAGEN Multiplex PCR Master Mix; then spin down and place on ice.
   Or mix 10 times using a single-channel P1000 pipette set to 750 µL.
2. To prepare the 5X Q-Solution:
   A. Alternate between vortexing and spinning down until clear (no precipitate).
   B. Place on ice.
3. Spin down the RNase-free water and mPCR Primer Mix; then place on ice.
4. Using a permanent marking pen, label a 5 mL tube *mPCR*.
5. To the mPCR tube, add the reagents listed in Table 3.5 in the order shown.
6. Using a single-channel P1000 pipette set to 900 µL, mix by pipetting up and down 5 times.
7. Place the master mix on ice until ready to use.

Table 3.5 mPCR Master Mix

Reagent	1 Reaction	48 Reactions (> 20% extra)
QIAGEN Multiplex PCR Master Mix	25 µL	1500 µL
mPCR Primer Mix (3 µM)	5 µL	300 µL
5X Q-Solution	5 µL	300 µL
RNase-free Water	10 µL	600 µL
TOTAL	**45 µL**	**2700 µL**

Prepare and Incubate the mPCR Plate

Location: Pre-Amp Lab

To prepare and incubate the mPCR Plate:

1. Using a permanent marking pen, label a 96-well PCR plate *mPCR*.
2. Place the plate in an aluminum block on ice.
3. Transfer the mPCR Master Mix to a reagent reservoir.
4. Aliquot 45 µL of mPCR Master Mix to each well of rows B, C, D and E of plate mPCR using a 12-channel P200 pipette,
5. Using a 12- or 24-channel P20 pipette:
   A. Transfer 5 µL of each sample and control from plate GP2 to the corresponding well of plate mPCR (Figure 3.3 on page 17).
   B. Mix up and down 3 times to rinse pipette tips (total volume each well: 50 µL).
6. Tightly seal plate mPCR.
7. Vortex the center of the plate at high speed for 3 sec; then spin down at 2000 rpm for 30–60 sec.
8. Place plate mPCR on a thermal cycler and run the program *DMET Plus mPCR* (Figure 3.4 on page 17).
9. Seal plate GP2 and keep on ice until the mPCR stage is successfully completed.
10. While the program is running, allow the PCR Dilution Buffer to thaw to room temperature.

Figure 3.3 Transferring samples and controls from plate GP2 to plate mPCR

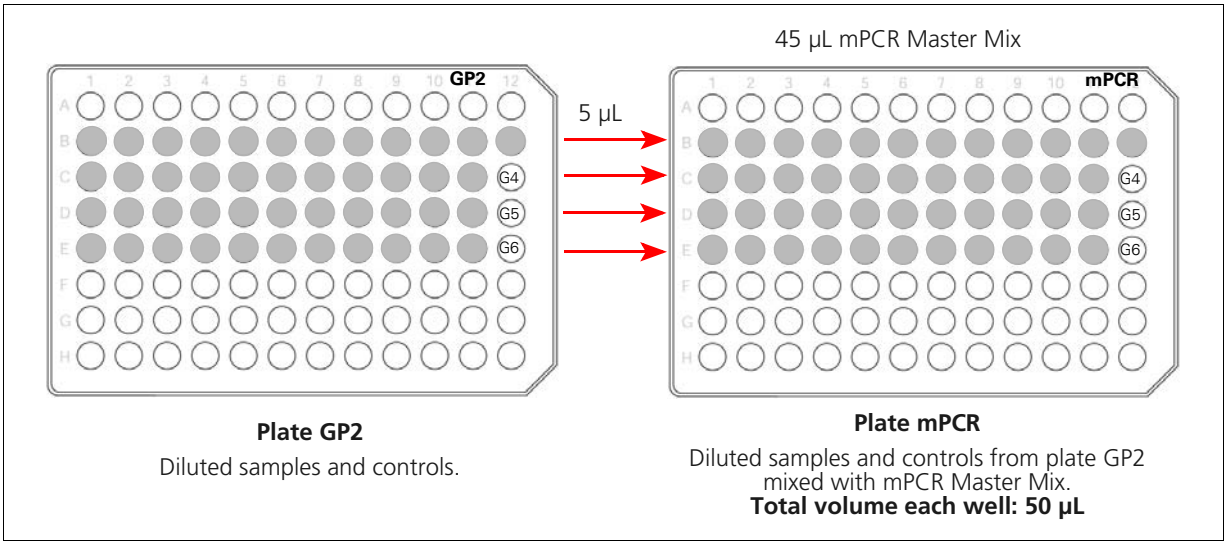
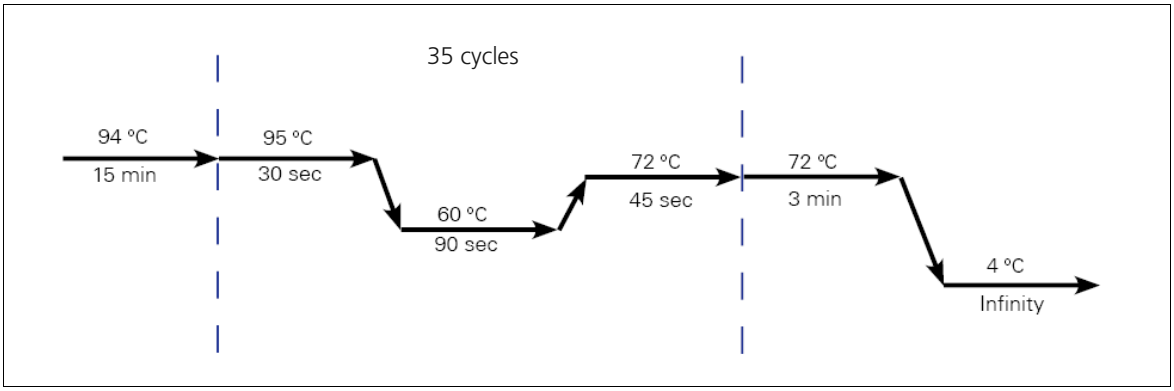


Figure 3.4 DMET Plus mPCR thermal cycler program



Dilute the mPCR Products

The mPCR products will be diluted twice. A new plate is used for each dilution.

Location: mPCR Staging Area

Prepare the Dilution Plates

To prepare the dilution plates:

1. When the DMET Plus mPCR program is finished, remove the mPCR plate from the thermal cycler, cool on ice for 2 min, and spin down at 2000 rpm for 30–60 sec.
2. Keeping the plate sealed, transfer the plate and the PCR Dilution Buffer to the mPCR Staging Area.
3. Place plate mPCR in an aluminum block on ice.
4. Using a permanent marking pen, label two 96-well PCR plates as follows:
   A. Label one plate DP1 (dilution plate 1)
   B. Label one plate DP2 (dilution plate 2)
5. Place plates DP1 and DP2 in aluminum blocks on ice.
6. Mix the PCR Dilution Buffer by inverting the bottle 10 times.

7. Pour the buffer into a reagent reservoir.
8. Aliquot 153 µL of PCR Dilution Buffer to each well of rows B, C, D and E of plates DP1 and DP2 using a 12-channel P200 pipette.

   To avoid introducing bubbles, do not blow out the pipette tips (dispense to the first stop only).

Aliquot mPCR Product to Plates DP1 and DP2

To aliquot mPCR product to plates DP1 and DP2:

1. Remove the adhesive film from plate mPCR.
2. For plate DP1, use a 12- or 24-channel P20 pipette and:
   - **A.** Transfer 5 µL of each mPCR product from plate mPCR to the corresponding well of plate DP1.

     Total volume each well: 158 µL
   - **B.** Pipet up and down 3 times to rinse the tips.
   - **C.** Set a 12-channel P200 or a 24-channel P100 pipette to 80 µL, and slowly mix by pipetting up and down 10 times.

     IMPORTANT: On the final mix, do not blow out the pipette tips (dispense to the first stop only). Blowing out pipette tips (dispensing to the second stop) will introduce bubbles.
3. For plate DP2, use a 12- or 24-channel P20 pipette and:
   - **A.** Transfer 5 µL of each mPCR product from plate DP1 to the corresponding well of plate DP2.

     Total volume each well: 158 µL
   - **B.** Pipet up and down 3 times to rinse the tips.
   - **C.** Set a 12-channel P200 or a 24-channel P100 pipette to 80 µL, and slowly mix by pipetting up and down 10 times.

     IMPORTANT: On the final mix, do not blow out the pipette tips (dispense to the first stop only). Blowing out pipette tips (dispensing to the second stop) will introduce bubbles.
   - **D.** Tightly seal plate DP2 and keep in an aluminum block on ice until ready to use.

     The mPCR products are now diluted 1000-fold on plate DP2.
4. Discard plate DP1.
5. Continue to *Stage 2 — Anneal*.

Stage 2 — Anneal

About this Stage

During this stage, genomic DNA samples and controls, mPCR products from *Stage 1 — mPCR*, assay panel probes, and reagents (Anneal Master Mix) are combined in an *Anneal Plate*.

The Anneal Plate is then placed on a thermal cycler and the program, *DMET Plus Anneal*, is run. Because the samples must be left to anneal for 16 to 18 hr, this stage is typically performed at the end of the day, and the program is allowed to run overnight.

Location and Duration

- Pre-Amp Lab and mPCR Staging Area
- Hands-on time: approximately 45 min
- Thermal cycler time: 16 to 18 hr

Input Required from Previous Stage

Input Required from Previous Stage
Plate GP1
Plate DP2

Equipment and Materials Required

The following equipment and materials are required to perform this stage.

In the Pre-Amp Lab

Table 3.6 Equipment and Materials Required in Pre-Amp Lab for *Stage 2 — Anneal*

Quantity	Item
2	Aluminum block, 96-well, chilled in 4 °C refrigerator
1	Centrifuge, plate
1	Eppendorf tube, 1.5 mL
1	Ice container, rectangular, filled with ice
1	Marking pen, extra fine point, permanent
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge

Table 3.6 Equipment and Materials Required in Pre-Amp Lab for *Stage 2 — Anneal*

Quantity	Item
1	PCR plate, 96-well
1 each	pipette: ▪ Single-channel P20 ▪ Single-channel P1000 ▪ 12-channel P200 ▪ 12- or 24-channel P20
1	Reagent reservoir, 50 mL
1	Thermal cycler, 96-well GeneAmp PCR System 9700 (gold or silver block)
1	Vortexer

In the mPCR Staging Area

Table 3.7 Equipment and Materials Required in mPCR Staging Area for *Stage 2 — Anneal*

Quantity	Item
2	Aluminum block, 96-well, chilled in 4 °C refrigerator
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
1 each	pipette: ▪ 12- or 24-channel P20

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Pre-Amp Kit box:
Pre-Amp Water
Buffer A
Enzyme A

From the DMET Plus Panel Kit box:
DMET MIP Panel

Thaw Reagents

Location: Pre-Amp Lab

To thaw the reagents:

1. If the Pre-Amp Lab and mPCR Staging Area are in the same room, put on fresh gloves now.
2. Place the following reagents on the bench top at room temperature and thaw; then place on ice.
   - Pre-Amp Water
   - DMET MIP Panel
   - Buffer A
3. To thaw Enzyme A:
   A. Place on the bench top and allow to thaw *only as long as required to defrost*.
      - Time to defrost varies based on lab temperature (typically 5 min or less).
      - You can spin down to help thaw. Do NOT vortex.
   B. Once defrosted, place on ice until ready to use.
4. Using a permanent marking pen, label a 96-well PCR plate *ANN*.

Prepare the Anneal Master Mix

Location: Pre-Amp Lab

IMPORTANT: Enzyme A is extremely temperature sensitive. To avoid denaturing, keep the Anneal Master Mix on ice until ready to use. Minimize warming by hand contact.

To prepare the Anneal Master Mix:

1. Label the Eppendorf tube *Ann*.
2. Place on ice until ready to use.
3. To the tube labeled Ann, add the reagents listed in Table 3.8 in the order shown.
4. Using a single-channel P1000 pipette set to 900 µL, mix the cocktail by pipetting up and down 5 times.
5. Transfer the Anneal Master Mix to a reagent reservoir.
6. Keep on ice until ready to use.
7. Store the remaining Pre-Amp Water at 4°C.

Table 3.8 Anneal Master Mix

Reagents	1 Reaction	48 Reactions (~ 25% extra)
Pre-Amp Water	16.6 µL	996
Buffer A	5 µL	300 µL
Enzyme A	0.0625 µL	3.8 µL
Total Volume	**21.7 µL**	**1299.8 µL**

Transfer Samples from Plate GP1 to the Anneal Plate

Location: Pre-Amp Lab

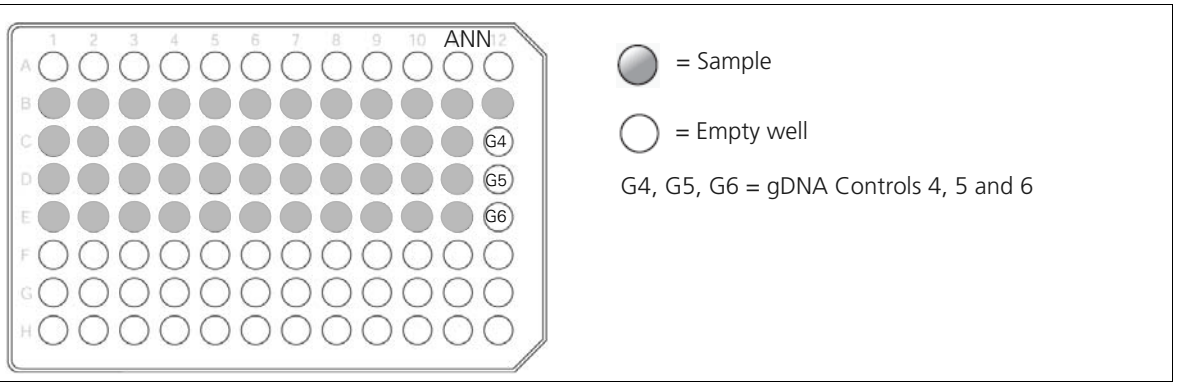
To transfer samples from plate GP1 to the Anneal Plate:

1. Spin down plate GP1 at 2000 rpm for 30–60 sec.
2. Place plates ANN and GP1 in aluminum blocks on ice.
3. Referring to Table 3.9 and Figure 3.5 on page 22 load the Anneal Plate as follows:
   A. Aliquot 21.7 µL of Anneal Master Mix to each well of rows B, C, D and E on the Anneal Plate using a 12-channel P200 pipette.
   B. Aliquot 13.4 µL of each sample and control from plate GP1 to the corresponding well on the Anneal Plate using a 12- or 24-channel P20 pipette, (pipet up and down 3 times to rinse the tips).
4. Tightly seal the Anneal Plate with a clear adhesive film.
5. Transfer the Anneal Plate to the mPCR Staging Area.

Table 3.9 Loading the Anneal Plate

Component	gDNA Sample and Control Wells
Sample or control from plate GP1	13.4 µL
Anneal Master Mix	21.7 µL
Total Volume	**35.1 µL**

Figure 3.5 Anneal Plate layout



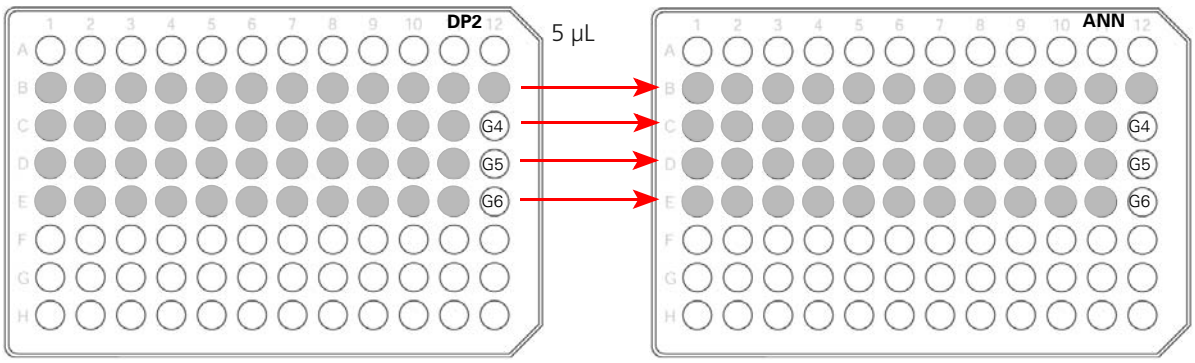
Add Diluted mPCR Product to the Anneal Plate

Location: mPCR Staging Area

To add diluted mPCR product to the Anneal Plate:

1. Add 5 µL of diluted mPCR product from plate DP2 to the corresponding wells of the plate ANN using a 12- or 24-channel P20 pipette (Figure 3.6).
   Total volume each well: 40.1 µL
2. Tightly seal the Anneal Plate and transfer it to the Pre-Amp Lab.

Figure 3.6 Transferring diluted mPCR product from plate DP2 to the Anneal Plate



Transfer 5 µL of each diluted mPCR product from plate DP2 to the Anneal Plate.

Anneal and Add DMET MIP Panel

Location: Pre-Amp Lab

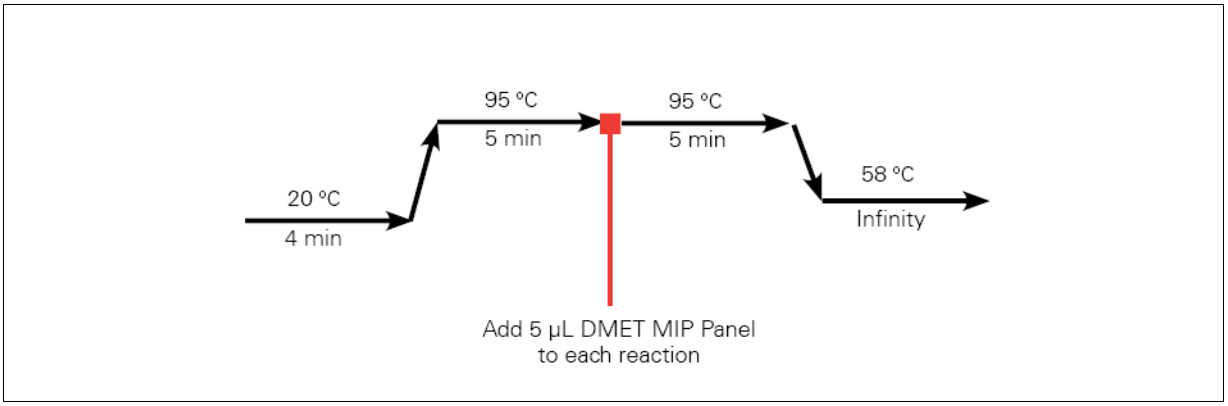
To anneal and add the DMET MIP Panel:

1. Vortex the center of the Anneal Plate at high speed for 3 sec.
2. Spin down at 2000 rpm for 30–60 sec.
3. Start the thermal cycler program, *DMET Plus Anneal* (Figure 3.7).
4. When the temperature reaches 20°C, load the Anneal Plate and close the lid.
5. At the end of the first 95 °C hold, press *Pause* on the thermal cycler.
6. Remove the Anneal Plate and place in an aluminum block on ice for 2 min.
7. While the Anneal Plate is cooling, aliquot the DMET MIP Panel to one strip of 12 tubes, 25 µL in each tube.
8. Using a 12-channel P20 pipette, add the DMET MIP Panel as follows:
   - **A.** Add 5 µL DMET MIP Panel to each reaction on the Anneal Plate.
   - **B.** Pipet up and down 3 times to rinse the tips.

     Use new pipette tips for each addition. Total volume each well: 45.1 µL
9. Tightly seal the Anneal Plate, vortex at high speed for 3 sec, and spin down at 2000 rpm for 30–60 sec.
10. Place the plate back on the thermal cycler and press *Resume*.
11. Incubate the samples for 16 to 18 hr.

    Optimal incubation time is 16 to 18 hours. Do not incubate samples for more than 18 hr.

Figure 3.7 DMET Plus Anneal thermal cycler program



Stage 3 — Gap Fill Through Amplification

About this Stage

During this stage, Gap Fill Mix is added to each reaction. Then the samples are transferred from the Anneal Plate to an Assay Plate.

The Assay Plate is then placed on a thermal cycler and the program *DMET Plus Assay* is started. During the first 42 min of this program, three additional reagents are added to the Assay Plate, one reagent at a time. Prior to each addition, the plate is removed from the thermal cycler and cooled on ice for 2 min.

The reagents added during thermal cycling are:

1. dNTP Mix
2. Exo Mix
3. Universal Amp Mix

Location and Duration

- Pre-Amp Lab
- Hands-on time: 2.5 hours
- Thermal cycler time: 1 hr 27 min

Input Required from Previous Stage

Item
Anneal Plate

Equipment and Materials Required

Table 3.10 Equipment and Materials Required for *Stage 3 — Gap Fill Through Amplification*

Quantity	Item
2	Aluminum block, chilled in 4 °C refrigerator
1	Anneal Plate from previous stage
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge
1 each	Pipettes: ▪ Single-channel P200 ▪ 12-channel P10 ▪ 12-channel P20 ▪ 12-channel P200 ▪ Optional: 24-channel; P20
As required	Pipette tips
1	Plate, 96-well PCR
2	Reagent reservoirs, 50 mL

Table 3.10 Equipment and Materials Required for *Stage 3 — Gap Fill Through Amplification*

Quantity	Item
1	Thermal cyclers, 96-well GeneAmp PCR System 9700 (gold or silver block)
1	Tube, Eppendorf 1.5 mL
4	Tube strips with caps, PCR 12-well

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Pre-Amp Kit box:
Gap Fill Mix 1
Gap Fill Mix 2
Exo Mix
Cleavage Enzyme
Universal Amp Mix
dNTP Mix

Other Reagents Required

Reagent
TITANIUM™ *Taq* Polymerase

Thaw The Reagents

IMPORTANT: Leave the Exo Mix, Cleavage Enzyme, Gap Fill Mixes 1 and 2, and the TITANIUM Taq Polymerase at –20 °C until ready to use.

To thaw the reagents:

1. Place the dNTP Mix and the Universal Amp Mix on the bench top at room temperature and thaw.
2. Spin down and keep on ice until ready to use.

Prepare The Gap Fill Mix

To prepare the Gap Fill Mix:

1. Place a chilled aluminum block on ice.
2. Spin down Gap Fill Mixes 1 and 2.
3. Label the 1.5 mL Eppendorf tube and one strip of 12 PCR tubes with the letter *G*.
4. To prepare the Gap Fill Mix:
   A. *Slowly* aliquot 190 µL Gap Fill Mix 2 to the Eppendorf tube.
   B. *Slowly* add 10 µL Gap Fill Mix 1 to the Eppendorf tube.
      Both solutions are 50% glycerol, so pipet slowly.
   C. Using a P200 set to 150 µL, mix well by pipetting slowly up and down 15 times.

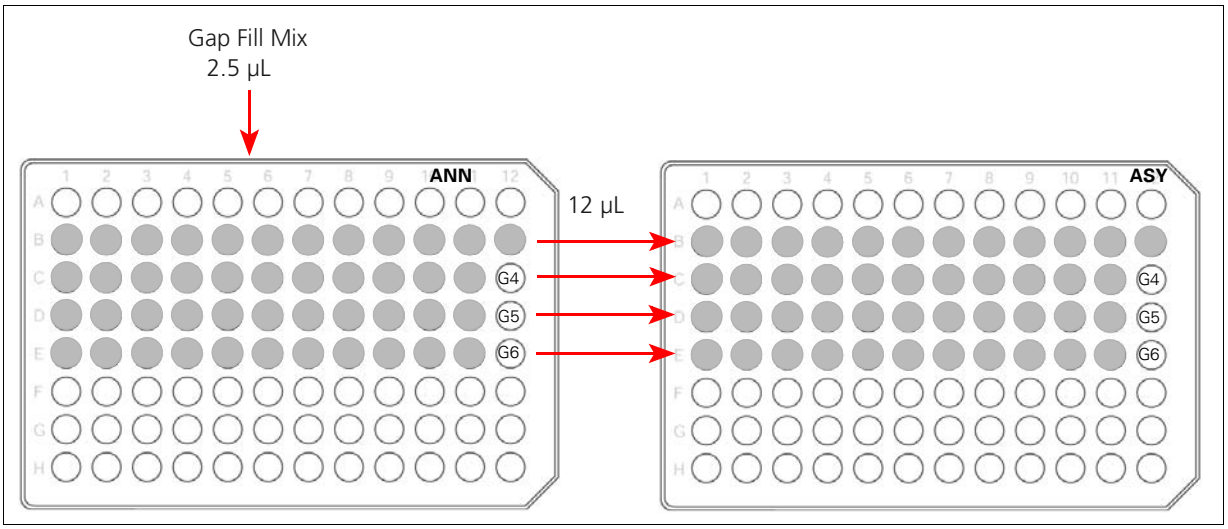
5. Aliquot 14 µL of Gap Fill Mix to each strip tube.
6. Cap and spin down the strip tubes.
7. Place the strip tubes in the aluminum block on ice.

Add Gap Fill Mix

To add the Gap Fill Mix:

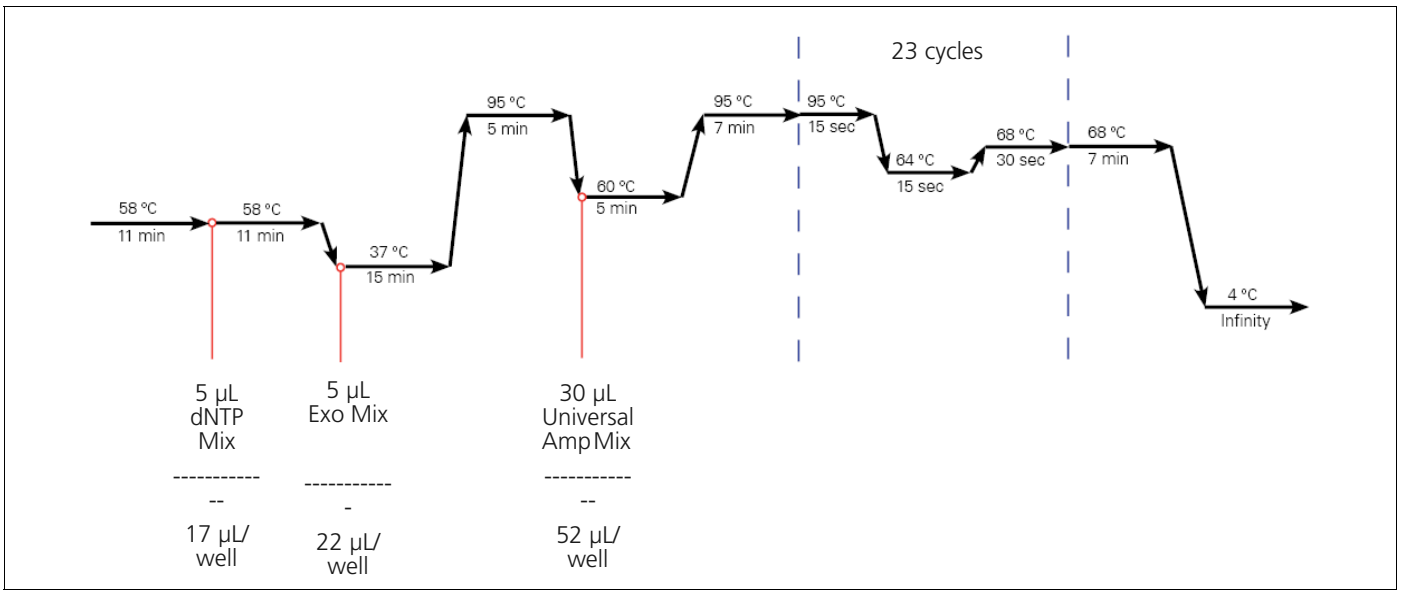
1. Remove plate ANN from the thermal cycler, place in a cold block on ice for 2 min; then spin down at 2000 rpm for 30–60 sec.
2. Using a 12-channel P10 pipette, add 2.5 µL Gap Fill Mix to each reaction (pipet up and down 3 times to rinse tips).
3. Tightly seal the plate, vortex at high speed for 3 sec, then spin down at 2000 rpm for 30–60 sec.
4. Label a fresh 96-well PCR plate *ASY*.
5. Transfer 12 µL each reaction to the Assay Plate using a 12- or 24-channel P20 pipette.
6. Tightly seal the plate; then spin down at 2000 rpm for 30–60 sec.
7. Start the *DMET Plus Assay* program, wait for the thermal cycler to reach 58 °C; then load the plate and allow the program to run (Figure 3.9 on page 28).

Figure 3.8 Preparing the Assay Plate



NOTE: The ANN plate can be stored at –20 °C short term and can be used in the case of sample loss downstream.

Figure 3.9 DMET Plus Assay thermal cycler program



Prepare and Add the dNTP Mix

To prepare and add the dNTP Mix:

1. Vortex the dNTP Mix; then spin down.
2. Label one strip of 12 tubes with the letter *D*.
3. Aliquot 25 µL to each tube.
4. Cap and spin down the strip tubes.
5. Place the strip tubes in an aluminum block on ice.
6. To add the dNTP Mix to the Assay Plate:
   A. After 11 min at 58 °C, press *Pause* on the thermal cycler and remove the Assay Plate.
   B. Place the plate in an aluminum block on ice for 2 min.
   C. Spin down at 2000 rpm for 30–60 sec.
   D. Using a 12-channel P20 pipette, add 5 µL dNTP Mix to each reaction (pipet up and down 3 times to rinse tips).

      Total volume each well: 17 µL
7. Tightly seal the plate, vortex at high speed for 3 sec; then spin down at 2000 rpm for 30–60 sec.
8. Place the plate back on the thermal cycler and press *Resume*.

Prepare and Add the Exo Mix

To prepare and add the Exo Mix:

1. Remove the Exo Mix from the –20 °C freezer and spin down.
2. Label one strip of 12 tubes with the letter *E*.
3. Aliquot 25 µL to each tube.
4. Cap and spin down the strip tubes.
5. Place the strip tubes in an aluminum block on ice.
6. To add the Exo Mix to the Assay Plate:

A. When the thermal cycler temperature reaches 37 °C, press *Pause* and remove the Assay Plate.
B. Place the plate in an aluminum block on ice for 2 min.
C. Spin down at 2000 rpm for 30–60 sec.
D. Using a 12-channel P20 pipette, add 5 µL Exo Mix to each reaction (pipet up and down 3 times to rinse tips).
   Total volume each well: 22 µL

7. Tightly seal the plate, vortex at high speed for 3 sec; then spin down at 2000 rpm for 30–60 sec.
8. Place the plate back on the thermal cycler and press *Resume*.

Prepare and Add the Universal Amp Mix

To prepare and add the Universal Amp Mix:

1. During the 5 min at 95 °C period on the thermal cycler, prepare the Universal Amp Mix as follows:
   A. Remove the Cleavage Enzyme and TITANIUM *Taq* Polymerase from the –20 °C freezer.
   B. Vortex and spin down the Universal Amp Mix.
      Do not vortex the Cleavage Enzyme and TITANIUM *Taq* Polymerase.
   C. Spin down the Cleavage Enzyme and TITANIUM *Taq* Polymerase.
   D. Add 25 µL of Cleavage Enzyme to the Universal Amp Mix tube.
   E. Add 70 µL TITANIUM *Taq* Polymerase to the Universal Amp Mix tube.
   F. Set a P1000 pipette to 900 µL and mix by pipetting up and down 10 times.
   G. Pour the Universal Amp Mix into a reagent reservoir on ice.
2. To add the Universal Amp Mix to the Assay Plate:
   A. When the thermal cycler temperature reaches 60 °C, press *Pause* and remove the Assay Plate.
   B. Place the plate in an aluminum block on ice for 2 min.
   C. Spin down at 2000 rpm for 30–60 sec.
   D. Using a 12-channel P200 pipette, add 30 µL Universal Amp Mix to each reaction (pipet up and down 3 times to rinse tips).
      Total volume each well: 52 µL
3. Tightly seal the plate, vortex at high speed for 3 sec; then spin down at 2000 rpm for 30–60 sec.
4. Place the plate back on the thermal cycler and press *Resume*.
5. When the program has ended, transfer the sealed Assay Plate to the Post-Amp Lab and place on ice.

IMPORTANT: To prevent contamination from PCR products, the Assay Plate must remain tightly sealed until it has been transferred to the Post-Amp Lab.

Stage 4 — PCR Clean-up and First QC Gel

About this Stage

During this stage, you will transfer the sealed Assay Plate to the Post-Amp Lab. There you will add PCR Clean Up Mix to each reaction, place the plate on a thermal cycler, and run the program *DMET Plus Clean Up*. You will then take an aliquot from each sample and run a QC gel to check the PCR products.

Location and Duration

- Post-Amp Lab
- Hands-on time: 20 min
- Thermal cycler time: 30 min

Input Required from Previous Stage

Item
Assay Plate with amplified DNA

Equipment and Materials Required

Table 3.11 Equipment and Materials Required for *Stage 4 — PCR Clean-up and First QC Gel*

Quantity	Item
2	Aluminum block, chilled in 4 °C refrigerator
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge
1 each	Pipettes: ▪ Single-channel P20 ▪ 12-channel P10 ▪ 12-channel P20
As required	Pipette tips
1	Thermal cycler, 96-well GeneAmp PCR System 9700 (gold or silver block)
4	Tube strips with caps, PCR 12-well

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Labeling Kit box:
PCR Clean Up Mix

QC Gel Materials Required

Table 3.12 QC Gel Materials Required

Quantity	Item	Vendor and Part Number
8 µL/rxn	1X TE Buffer or Molecular Biology Grade Water	TekNova, P/N T0223
1	NEB Low Molecular Weight Ladder	New England Biolabs, P/N N3233S
2 µL/rxn	2X Loading buffer	Sigma, P/N G2526
1	3% Agarose gel	Bio-Rad Precast ReadyAgarose™ Wide-Mini Gel, P/N 161-3040

Transfer Assay Plate to Post-Amp Lab

If you have not already done so, seal and transfer plate ASY to the Post-Amp Lab and place on ice.

IMPORTANT: To prevent contamination from PCR products, the Assay Plate must remain tightly sealed until it has been transferred to the Post-Amp Lab.

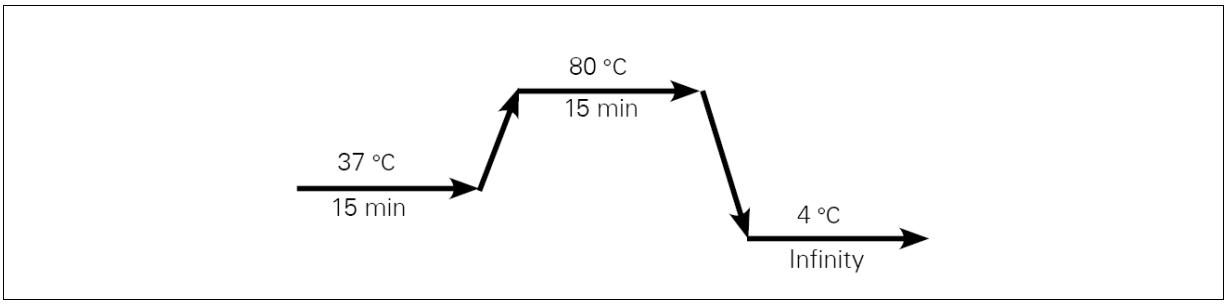
Prepare and Add the PCR Clean Up Mix

To prepare and add the PCR Clean Up Mix:

1. Spin down the tube of PCR Clean Up Mix.
2. Label one strip of 12 tubes *PCM*.
3. Aliquot 15 µL to each strip tube.
4. Cap and spin down the strip tubes.
5. Place the strip tubes in an aluminum block on ice.
6. Using a 12-channel P20 pipette, add 2.5 µL PCR Clean Up Mix to each reaction (pipet up and down 3 times to rinse tips).

   Total volume each well: 54.5 µL
7. Tightly seal the plate, vortex at high speed for 3 sec; then spin down at 2000 rpm for 30–60 sec.
8. Place plate ASY on a thermal cycler and run the *DMET Plus Clean Up* program (Figure 3.10).

Figure 3.10 DMET Plus Clean Up thermal cycler program



Prepare and Run the First QC Gel

The first quality control gel is used to check for PCR product.

Prepare the Gel Materials

While the DMET Plus Clean Up program is running, prepare the materials required for the first QC gel.

Prepare the QC Gel 1 Plate

To prepare the gel plate:

1. When the DMET Plus Clean Up program is finished, remove plate ASY from the thermal cycler and spin it down at 2000 rpm for 30–60 sec.
2. Label one fresh PCR plate *Gel1* (the gel plate) and load it as follows:
   - **A.** Pour 1X TE Buffer or molecular biology-grade water into a reagent reservoir.
   - **B.** Aliquot 8 µL of 1X TE Buffer or water to the appropriate wells.
   - **C.** Add 2 µL of Loading Buffer.
   - **D.** Transfer 2 µL of each reaction from the plate ASY (after PCR Clean Up), pipetting up and down 3 times to rinse the tips.
3. Reseal plate ASY and keep in aluminum block on ice.
   Total volume left in each well: 52.5 µL
4. Tightly seal the gel plate, vortex, and spin down.
5. Load 10 µL of each reaction and the ladder onto a 3% agarose gel.
6. Run the gel at 120 V for 20 min.
7. Examine the gel to ensure PCR products are between 100–150 bp.
   Figure 3.11 illustrates good results for each sample.

Figure 3.11 A good first quality control gel. PCR products are between 100-150 bp

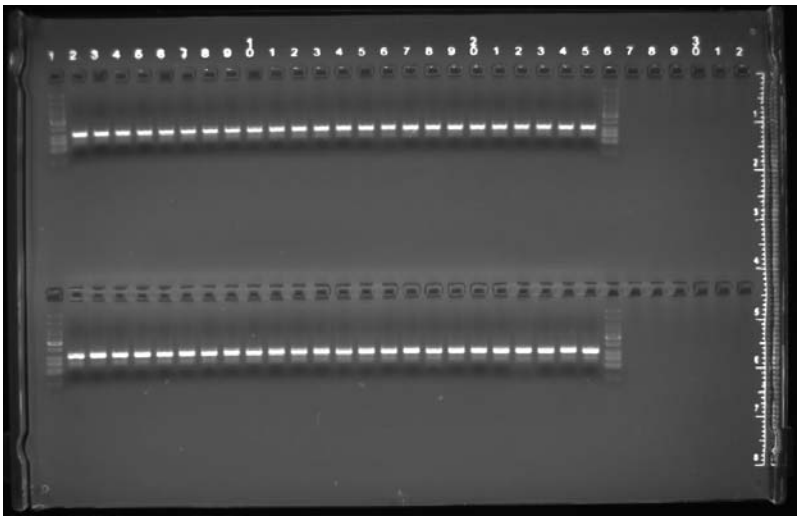
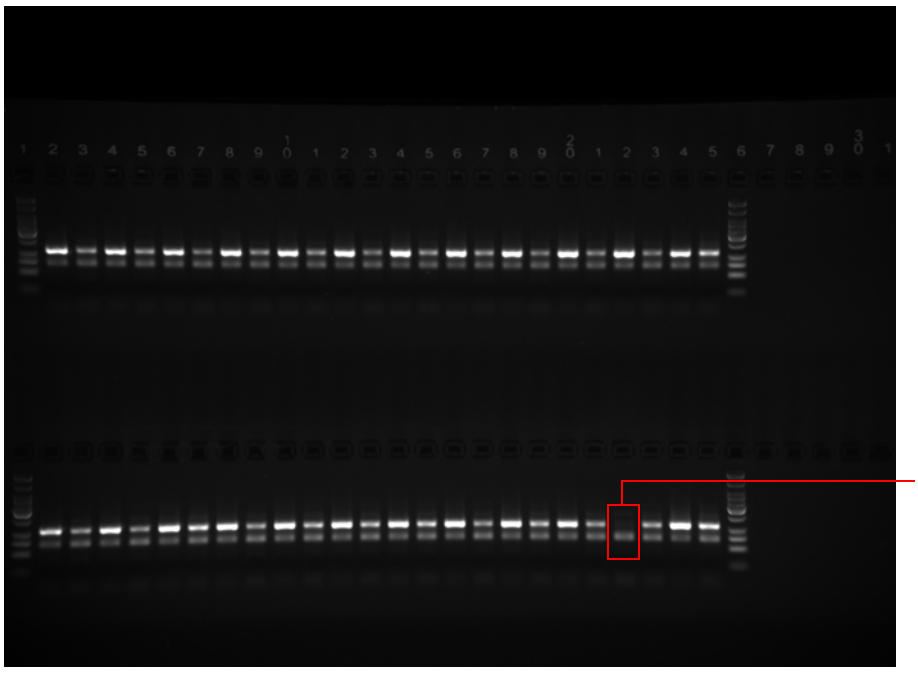


Figure 3.12 illustrates poor PCR results. The sample from well D9 has no product of interest. Uneven band intensities in the other lanes indicate varying amounts of PCR product generated in different wells of the Assay Plate.

Figure 3.12 A poor first quality control gel



Stage 5 — Fragmentation and Second QC Gel

About this Stage

During this stage, smaller DNA fragments are generated to improve sample hybridization onto the DMET Plus Arrays. The DNA fragment size is then checked on the second QC gel.

Location and Duration

- Post-Amp Lab
- Hands-on time: 45 min
- Thermal cycler time: 30 min

Input Required from Previous Stage

Item
Assay Plate with cleaned amplified DNA

Equipment and Materials Required

Table 3.13 Equipment and Materials Required for *Stage 5 — Fragmentation and Second QC Gel*

Quantity	Item
2	Aluminum blocks, chilled in 4 °C refrigerator
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge
1 each	Pipettes: ▪ Single-channel P200 ▪ Single-channel P1000 ▪ 12-channel P20 ▪ 12-channel P200 ▪ Optional: 24-channel; P20
As required	Pipette tips
2	Plate, 96-well PCR
2	Reagent reservoirs, 50 mL
1	Thermal cycler, 96-well GeneAmp PCR System 9700 (gold or silver block)
1	Tube, Eppendorf 1.5 mL
4	Tube strips with caps, PCR 12-well

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Labeling Kit box:
Post-Amp Water
Fragmentation Buffer
Fragmentation Reagent

Gel Materials Required

Table 3.14 Gel Materials Required

Quantity	Item	
1	NEB Low Molecular Weight Ladder	New England Biolabs, P/N N3233S
2 µL/rxn	2X Loading buffer	Sigma, P/N G2526
1	3% Agarose gel	Bio-Rad Precast Ready Agarose Wide-Mini Gel, P/N 161-3040

Thaw the Reagents

To thaw the reagents:

1. Place the following reagents on the bench top at room temperature and thaw:
   - Post-Amp Water
   - Fragmentation Buffer
2. Once thawed, place on ice.

Prepare and Run the Fragmentation Reaction

To prepare and run the fragmentation reaction:

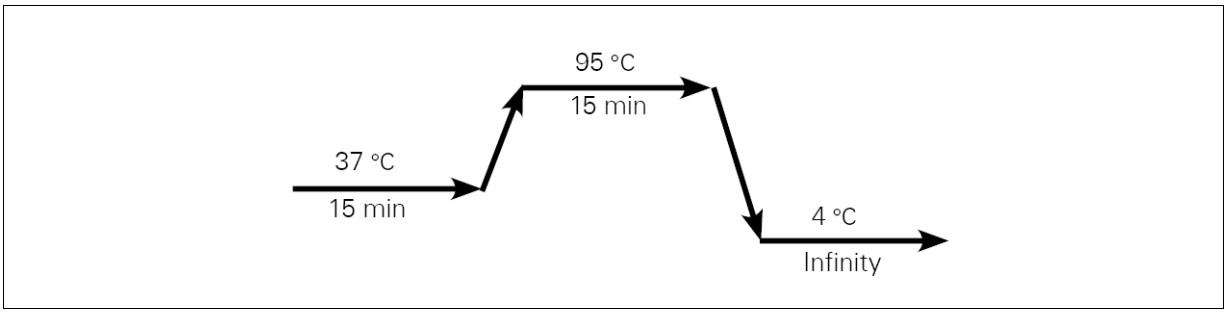
1. Label one fresh PCR plate *Frag/Label*.
2. Transfer 25 µL of each reaction from the plate ASY to plate Frag/Label.
3. Label a 1.5 mL Eppendorf tube *Frag*, and place on ice.
4. Label a strip of 12 tubes *F*, and place on ice.
5. Keeping all reagents and the Frag tube on ice:
   A. Add the Fragmentation Buffer and Post-Amp Water to the Frag tube (Table 3.15 on page 36).
   B. Cool the tube on ice for 5 min.
   C. Remove the Fragmentation Reagent from the freezer, spin down for 3 sec, and immediately place on ice.
   D. Add the Fragmentation Reagent to the Frag tube.
   E. Vortex the master mix at high speed for 3 sec.
   F. Spin down for 3 sec and immediately place on ice.

Table 3.15 Fragmentation Master Mix

Reagent	1 Reaction	48 Reactions (>20% extra)
Post-Amp Water	8.9 µL	536 µL
Fragmentation Buffer	1 µL	60 µL
Fragmentation Enzyme	0.0675 µL	4.1 µL
TOTAL	**10 µL**	**600 µL**

6. Working quickly and on ice:
   A. Aliquot 45 µL of Fragmentation Master Mix to each strip tube.
   B. Using a 12-channel P20 pipette, add 10 µL Fragmentation Master Mix to each reaction, pipetting up and down 3 times to rinse the tips.
      Total volume each well: 35 µL
   C. Tightly seal the plate, vortex the plate at high speed for 3 sec, and spin down at 2000 rpm for 30–60 sec.
7. Place the plate on a thermal cycler and run the *DMET Plus Frag* program (Figure 3.13).

Figure 3.13 DMET Plus Frag thermal cycler program



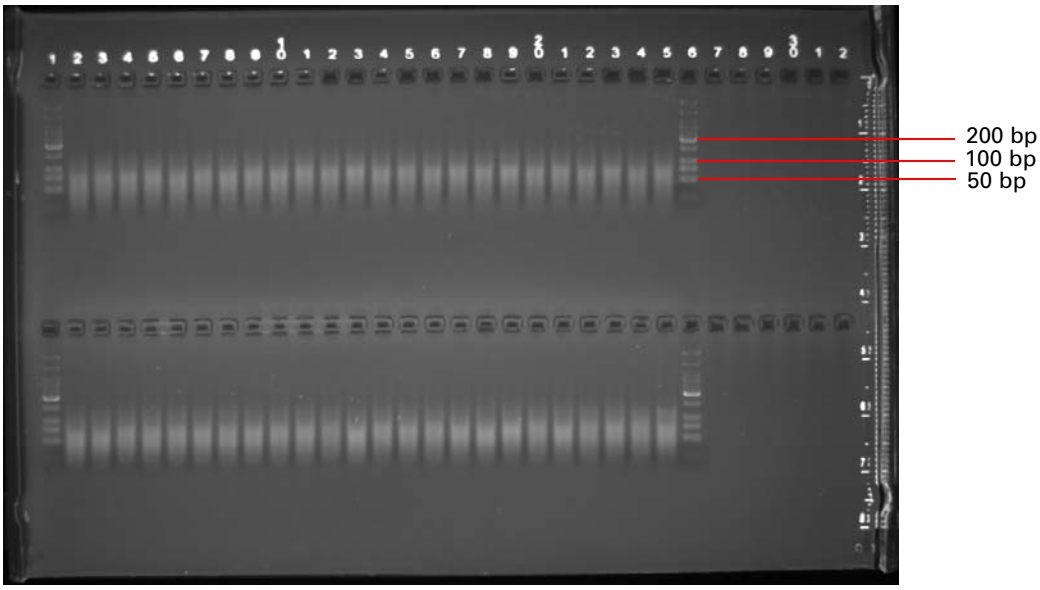
Prepare and Run the Second Quality Control Gel

Run the second quality control gel to check the fragmentation reaction.

To prepare and run the second quality control gel:

1. Label a 96-well PCR plate *Gel2* (the gel plate).
2. Aliquot 10 µL of each reaction from the Frag/Label plate to the gel plate.
3. Reseal plate Frag/Label.
4. Add 2 µL of Loading Buffer to each reaction, pipetting up and down 3 times to rinse the tips.
5. Seal the gel plate, vortex at high speed for 3 sec, and spin down at 2000 rpm for 30–60 sec.
6. Load 10 µL of each reaction and the ladder onto a 3% agarose gel.
7. Run the gel at 120 V for 24 min.
8. Examine the gel to ensure that fragments are < 120 bp, with the smear centered around 50 bp. Figure 3.14 on page 37 illustrates good QC gel results.

Figure 3.14 A good second quality control gel. Fragments < 120 bp, with smear centered around 50 bp



Stage 6 — Labeling

About this Stage

During this stage, you will prepare a Labeling Master Mix, add it to each sample on the Frag/Label Plate, place the plate on a thermal cycler, and run the program DMET Plus Label.

Location and Duration

- Post-Amp Lab
- Hands-on time: 20 min
- Thermal cycler time: 1:15 hr

Input Required from Previous Stage

Item
Frag/Label Plate containing fragmented DNA

Equipment and Materials Required

Table 3.16 Equipment and Materials Required for *Stage 3 — Gap Fill Through Amplification*

Quantity	Item
2	Aluminum block, chilled in 4 °C refrigerator
1	Centrifuge, plate
1	Ice container, rectangular, filled with ice
As required	MicroAmp Clear Adhesive Films
1	Microcentrifuge
1 each	Pipettes: ▪ Single-channel P200 ▪ Single-channel P1000 ▪ 12-channel P10 ▪ 12-channel P20 ▪ 12-channel P200 ▪ Optional: 24-channel; P20
As required	Pipette tips
2	Plate, 96-well PCR
2	Reagent reservoirs, 50 mL
1	Thermal cycler, 96-well GeneAmp PCR System 9700 (gold or silver block)
1	Tube, Eppendorf 1.5 mL
4	Tube strips with caps, PCR 12-well

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Labeling Kit box:
Post-Amp Water
DNA Labeling Reagent
5X TdT Buffer
TdT Enzyme

Thaw the Reagents

To thaw the reagents:

1. Place the following reagents on the bench top and thaw at room temperature:
   - Post-Amp Water
   - DNA Labeling Reagent
   - 5X TdT Buffer
2. Once thawed, keep all reagents on ice until ready to use.

Prepare and Run the Labeling Reaction

IMPORTANT: The fragmentation and labeling reactions are done in the same plate (Frag/Label Plate). If you did not run the second QC gel, you must remove 10 µL from each well before proceeding with the labeling reaction (total volume in each well should be 25 µL).

To prepare and run the labeling reaction:

1. Mark the following consumables as indicated:
   - 1.5 mL Eppendorf tube with *Label*, and place on ice.
   - Strip of 12 tubes with an *L*, and place on ice.
2. To the Eppendorf tube, add the reagents listed in Table 3.17 below in the order shown.
3. Vortex the master mix at high speed for 3 sec.
4. Spin for 3 sec at high speed and place on ice.

Table 3.17 Labeling Master Mix

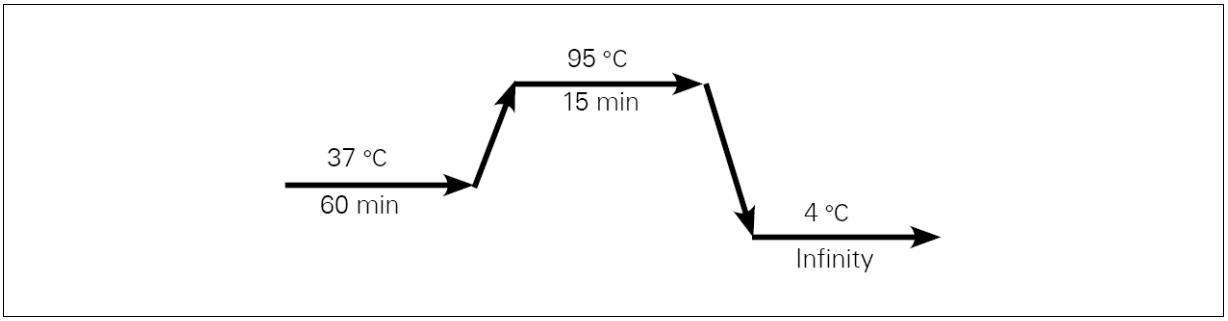
Reagent	1 Reaction	48 Reactions (>20% extra)
Post-Amp Water	0.4 µL	24 µL
5X TdT Buffer	7 µL	420 µL
DNA Labeling Reagent	0.9 µL	54 µL
TdT Enzyme	1.7 µL	102 µL
TOTAL	**10 µL**	**600 µL**

5. Aliquot 45 µL of Labeling Master Mix to each strip tube.
6. Using a 12-channel P20 pipette, add 10 µL Labeling Master Mix to each reaction, pipetting up and down 3 times to rinse the tips.

Total volume each well: 35 µL

7. Tightly seal the plate, vortex the at high speed for 3 sec, and spin down at 2000 rpm for 30–60 sec.
8. Place the plate on a thermal cycler and run the *DMET Plus Label* program (Figure 3.15).

Figure 3.15 DMET Plus Label thermal cycler program



Stage 7 — Hybridization

About this Stage

During this stage, each reaction is denatured then loaded onto a DMET™ Plus Array – one sample per array. The arrays are then placed into a hybridization oven that has been preheated to 49 °C. Samples are left to hybridize for 16 to 18 hours.

To help ensure the best results, carefully read the information below before you begin this stage.

IMPORTANT:

- **Be sure to equilibrate the arrays to room temperature; otherwise, the rubber septa may crack and the array may leak.**
- **An accurate hybridization temperature is critical for this assay. Therefore, we recommend that your hybridization ovens be serviced at least once per year to ensure that they are operating within specifications.**

Location and Duration

- Post-Amp Lab
- Hands-on time: approximately 1 hr
- Thermal cycler time: 10 min
- Hybridization time: 16 to 18 hours

Input Required from Previous Stage

Item
Frag/Label Plate containing fragmented and labeled DNA

Equipment and Consumables Required

The following equipment and consumables are required for this stage.

Table 3.18 Equipment and Consumables Required for *Stage 7 — Hybridization*

Quantity	Item
1	Aluminum block, chilled to 4 °C (do not freeze)
1 per sample	DMET Plus Array
1	GeneChip® Hybridization Oven 640
1	Ice bucket, filled with ice
As required	MicroAmp Clear Adhesive Film

Table 3.18 Equipment and Consumables Required for *Stage 7 — Hybridization*

Quantity	Item
1 each	Pipettes: ▫ Single channel P200 ▫ 12-channel P20 ▫ 12-channel P200
As required	Pipette tips for pipettes listed above; full racks
1	Plate, 96-well PCR
1	Plate centrifuge
1	Plate holder
1	Solution basin, 55 mL
1	Thermal cycler, 96-well GeneAmp PCR System 9700 (gold or silver block)
2 per array	Tough-Spots®, 1/2 in. diameter

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Hyb-Stain Kit box:
Hybridization Solution
Oligo Control Reagent (OCR)

Preheat the Hybridization Ovens

To preheat the hybridization ovens:

1. Turn each oven on and set the temperature to 49 °C.
2. Set the rpm to 35.
3. Turn the rotation on and allow to preheat.

Thaw Reagents

To thaw the reagents:

1. Place the Hybridization Solution and Oligo Control Reagent tubes on the bench top at room temperature and thaw.
2. Once thawed, place the Oligo Control Reagent on ice.

Prepare the Arrays

To prepare the arrays:

1. Unwrap the arrays and place on the bench top.
2. Allow the arrays to warm to room temperature (10 to 15 min).
3. For each array:
   A. Mark the array with a meaningful designation (e.g., a number) to ensure that you know which sample is loaded onto each array.
   B. Insert a 200 µL pipette tip into the upper right septum.

IMPORTANT: To ensure that the data collected during scanning is associated with the correct sample, number the arrays in a meaningful way. It is critical that you know which sample is loaded onto each array.

Figure 3.16 Preparing the arrays for hybridization

Prepare the Hybridization Master Mix

To prepare the Hybridization Master Mix:

1. Add 50 µL of Oligo Control Reagent directly to the Hybridization Solution tube.
2. Mix well by inverting the tube 10 times.
3. Pour the Hybridization Master Mix into a reagent reservoir and place on ice.

Prepare the Hyb Plate and Denature the Samples

To prepare the Hyb Plate and denature the samples:

1. Label a fresh 96-well plate with *Hyb* and place on ice.
2. Spin down the Frag/Label plate at 2000 rpm for 30 sec, and place in an aluminum block on ice.
3. Using a 12-channel P200 pipette:
   A. Wet the pipette tips by aspirating and dispensing three times.
   B. Aliquot 92 µL of Hybridization Master Mix to the appropriate wells of the Hyb Plate.

IMPORTANT: Any time fresh pipette tips are used, aspirate and dispense three times to wet the tips prior to aliquoting Hybridization Master Mix.

4. Using a 12- or 24-channel P20 pipette, transfer 8 µL of each reaction from the Frag/Label Plate to the Hyb Plate (pipet up and down 3 times to rinse tips).

   Total volume each well: 100 µL

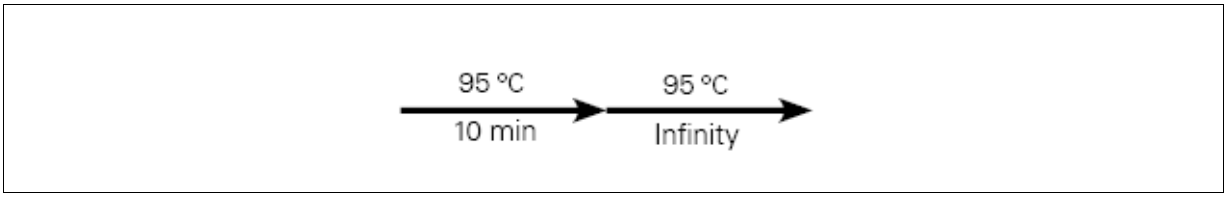
Table 3.19 Hybridization Cocktail Components

Component	Volume
Hybridization Master Mix	92µL
Reaction from Frag/Label Plate	8 µL
Total Volume Each Well	**100 µL**

5. Tightly seal the Hyb Plate, vortex, then spin down at 2000 rpm for 30 sec.
6. Start the *DMET Plus Denature* program, wait for the thermal cycler to reach 95 °C; then load the plate and allow the program to run.

NOTE: The Frag/Label plate can be stored at –20 °C for up to one week if repeat hybridizations are necessary.

Figure 3.17 DMET Plus Denature thermal cycler program



Generate and Upload a Sample Batch Registration File

To generate and upload a sample batch registration file:

1. Generate and enter information into a sample batch registration file.
2. Scan the array barcodes.
3. Upload the sample and array information to Affymetrix GeneChip Command Console (AGCC).

For more information, see *Generating a Sample Batch Registration File* on page 56.

Load the Samples onto Arrays

To load the samples onto arrays:

1. At the end of the 10 min incubation, remove the Hyb Plate and place it in an aluminum block on ice for 2 min.
2. Spin the plate down at 2000 rpm for 30–60 sec.
3. Working 16 arrays at a time:
   A. Aspirate 95 µL from one well and inject it onto an array.
   B. Remove the pipette tip from the upper right septum of each array.
   C. Repeat steps A and B until 16 arrays are loaded.
   D. Cover both septa with large Tough-Spots as shown in Figure 3.18.

      Using larger Tough-Spots that overlap the window makes them easier to remove later on.

Figure 3.18 Covering the septa with Tough-Spots® that overlap the window

E. Place the arrays into hybridization oven trays.

F. Load the trays into the hybridization oven.

G. Repeat these steps until all of the samples are loaded onto arrays and placed in the oven.

4. Allow the arrays to incubate at 49 °C and 35 rpm for 16 to 18 hr.
5. Seal and store the Frag/Label plate at –20 °C for 1 to 2 days.
6. Place remaining Hybridization Master Mix in an Eppendorf tube and store at –20 °C until all arrays have been successfully scanned.
7. To prepare for washing and staining, move the Stain Buffer and Hold Buffer from –20 °C to 4 °C.

IMPORTANT: **Arrays must rotate in the oven for 16 to 18 hours at 49 °C and 35 rpm (18 hr maximum before you begin *Stage 8 — Washing, Staining and Scanning Arrays*).**

Stage 8 — Washing, Staining and Scanning Arrays

About this Stage

During this stage, you will wash, stain and scan the arrays. The instruments you will use include the:

- Fluidics Station 450 to wash and stain arrays
- GeneChip® Scanner 3000 7G to scan arrays

After one set of arrays has been washed and stained, you will remove them from the fluidics station and load them onto the scanner. Then you will reload the fluidics station to wash and stain the next set of arrays.

The fluidics station and scanner are controlled by one of the software applications listed below. For detailed information on these applications, refer to the appropriate user's guide.

- Affymetrix GeneChip® Operating Software (GCOS)
  Affymetrix *GeneChip® Operating Software User's Guide*
- Affymetrix GeneChip® Command Console (AGCC)
  Affymetrix GeneChip® Command Console™ User's Guide

NOTE: The instructions for this stage are based on the use of AGCC software.

Location and Duration

- Post-Amp Lab
- Hands-on and wash time: approximately 6 hr
- Scanning time: approximately 6 to 7 hr

Materials Required from Previous Stage

Item
Samples that have been hybridized onto DMET Plus Arrays

Equipment and Consumables Required

The following equipment and consumables are required for washing, staining and scanning arrays.

Table 3.20 Equipment and Consumables Required for *Stage 8 — Washing, Staining and Scanning Arrays*

Quantity	Item
1	GeneChip® Scanner 3000 7G
2 or more	GeneChip® Fluidics Station 450
1	Ice bucket, filled with ice
As required	Kimwipes®
1	Pipette, single channel P200
1	Pipette, single channel P1000
As required	Pipette tips for pipettes listed above; full racks
1 tube/array	Eppendorf tubes, Natural, 1.5 mL
1 tube/array	Eppendorf tubes, Amber, 1.5 mL
2 per array	Tough-Spots®, 3/8 in. diameter

DMET Plus Premier Pack Reagent Kit Components Required

From the DMET Plus Hyb-Stain Kit box:
Stain Buffer
Hold Buffer

From the DMET Plus Premier Pack Reagent Kit box:
Wash Solution A
Wash Solution B

Other Reagents Required

Other Reagents Required
Streptavidin Phycoerythrin (SAPE)

Prepare the Reagents:

To prepare the reagents:

1. If not already thawed, place the Stain Buffer and Hold Buffer on the bench top and allow to thaw.
2. Invert the Stain and Hold Buffer tubes 5 times each.
3. Keep on ice until ready to use.

Prime the Fluidics Station

To prime the fluidics station:

1. Turn on the fluidics station and:
   - **A.** Place Wash Solns A and B in the designated positions.
   - **B.** Fill the dH_2O container.
   - **C.** Empty the waste container.
2. On the computer used to control the fluidics station:
   - **A.** Double-click the icon Affymetrix *Launcher* (on the desktop; Figure 3.19).
   - **B.** In the Launcher window, double-click **AGCC Fluidics Control**.
     The AGCC Fluidics Station window is displayed (Figure 3.20).

Figure 3.19 Launching the AGCC fluidics station control software

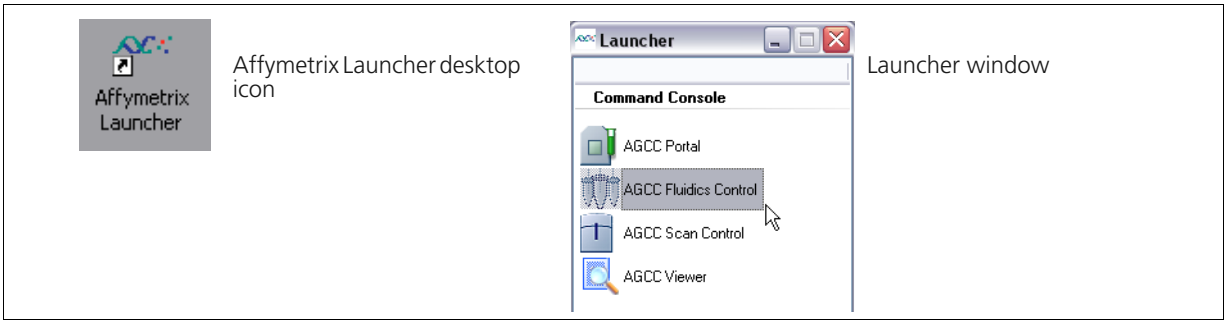
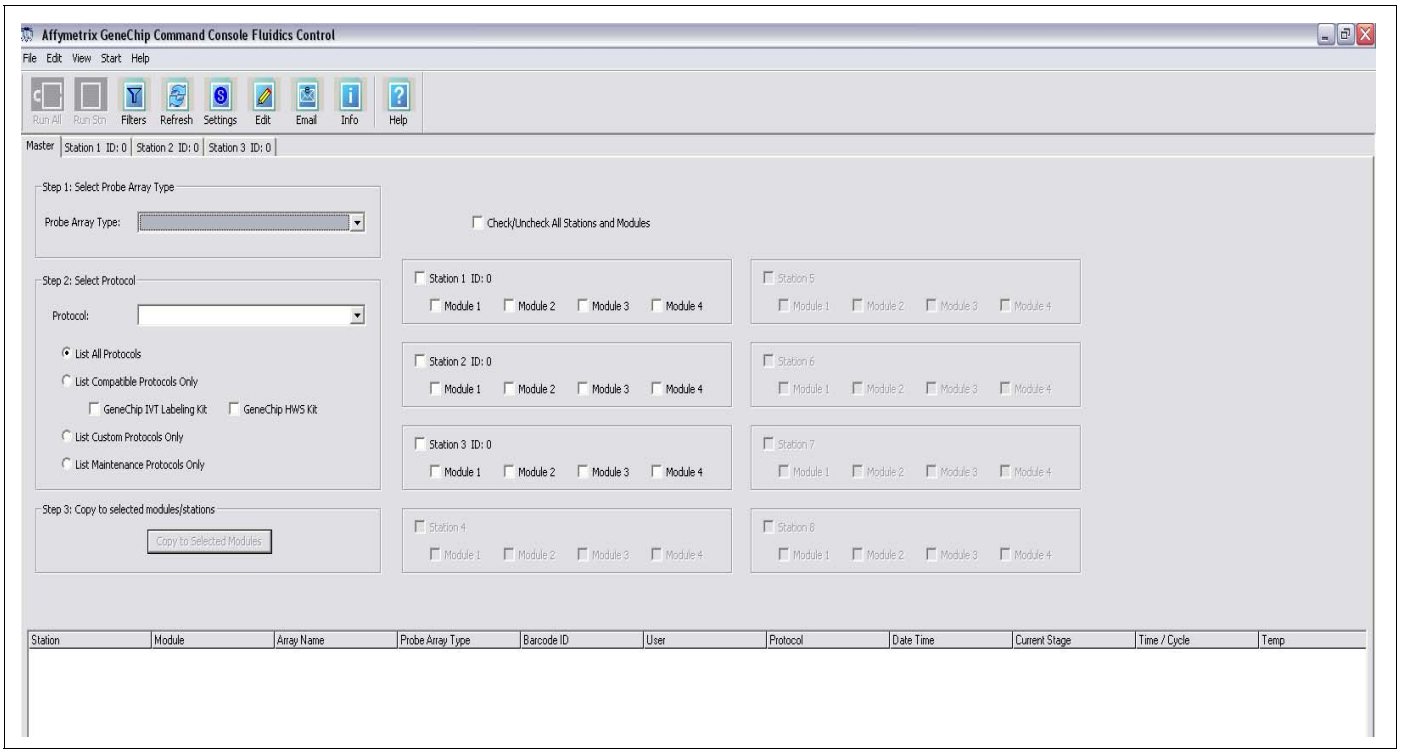


Figure 3.20 Affymetrix GeneChip® Command Console Fluidics Control window



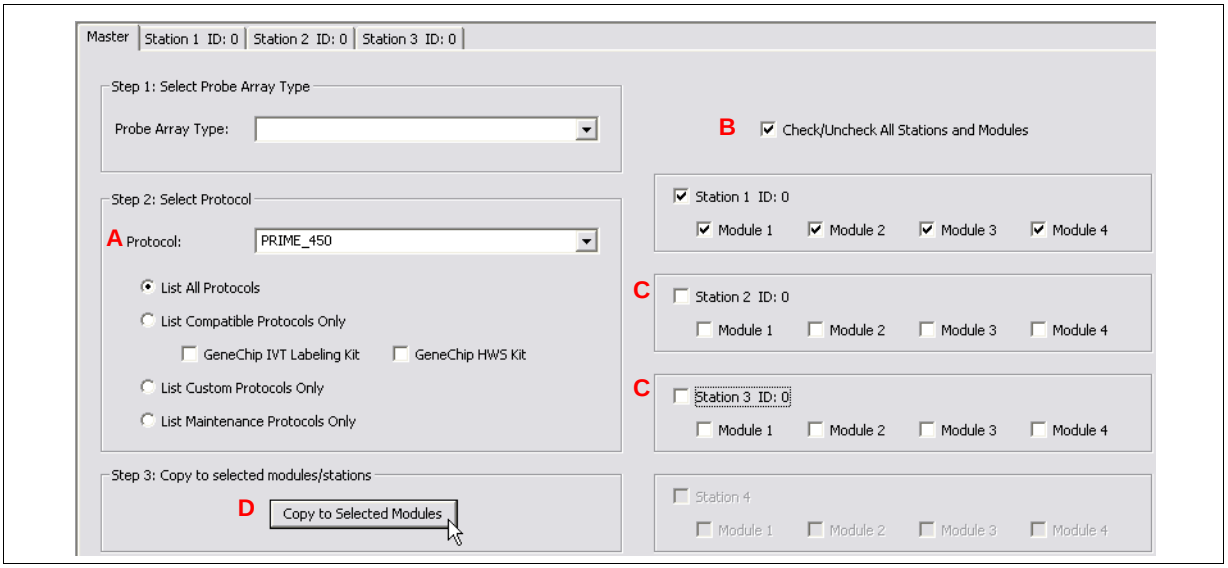
3. To configure the software (Figure 3.21 on page 49):
   - **A.** Open the Protocol drop-down menu and select **PRIME_450**.

B. Select the **Check/Uncheck All Stations and Modules** check box.

C. Deselect any fluidics stations or modules that will not be used.

D. Click **Copy to Selected Modules**.

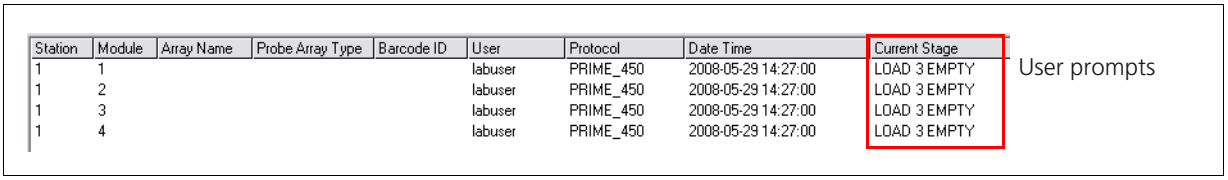
Figure 3.21 Configuring the software to prime the fluidics stations



4. Click the **Run All** button.

5. Follow the prompts displayed in the *Current Stage* column (lower half of the window; Figure 3.22). Prompts are also displayed in the fluidics station window. For example, you will be prompted to load three empty vials into positions 1, 2 and 3. Once the vials are loaded and locked in place, priming will begin. Progress is also displayed in the Current Stage and Time/Cycle columns of the control window.

Figure 3.22 Progress portion of the fluidics station control window



Prepare the SAPE Stain Solution

To prepare the SAPE Stain Solution:

1. Add 90 µL of SAPE to the Stain Buffer tube.

2. Invert the tube 5 times to mix (do not place on ice).

IMPORTANT: SAPE Stain Solution is light sensitive. Keep protected from light.

Wash and Stain the Arrays

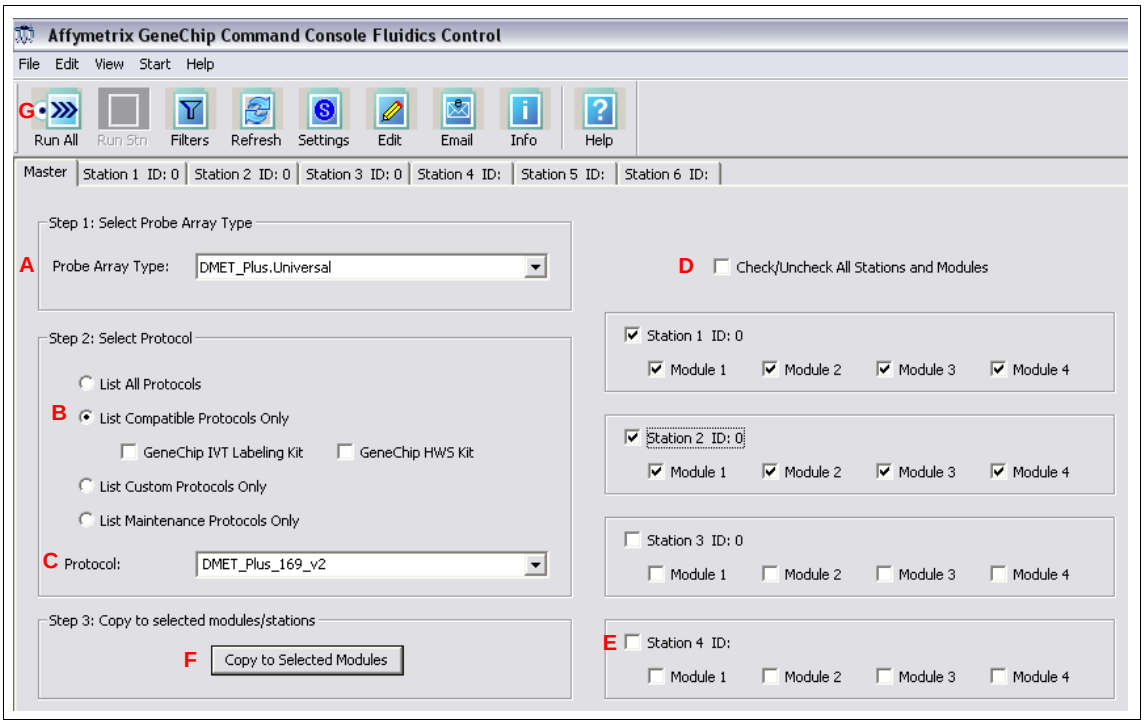
Prompts displayed in the fluidics station window are shown in *italics*.

Setup the Software and the Fluidics Station

To setup the software and the fluidics station:

1. Configure the software as follows:
   - **A.** Open the Probe Array Type drop-down menu and select **DMET_Plus.Universal**.
   - **B.** Select the button for **List Compatible Protocols Only**.
   - **C.** Open the Protocol drop-down menu and select **DMET_Plus_169_v2.**
   - **D.** Select the **Check/Uncheck All Stations and Modules** check box.
   - **E.** Deselect any fluidics stations or modules that will not be used.
   - **F.** Click **Copy to Selected Modules**.
   - **G.** Click the **Run All** button.

Figure 3.23 Configuring the software to wash and stain DMET Plus Arrays

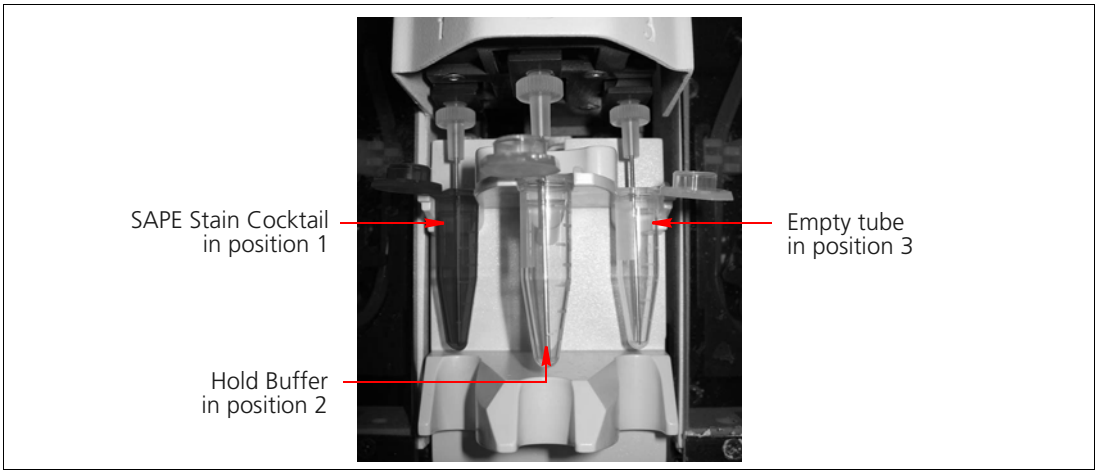


2. Remove eight arrays from the hybridization oven.

> **IMPORTANT:** **Leave remaining arrays in the hybridization oven until ready to wash. Be sure to rebalance the trays every time you remove some arrays.**

3. Remove the Tough-Spots from the arrays.
4. When *Load Cartidge* is displayed, place each array on a fluidics station and lock in to place.
5. When *Load Vials 1 & 2* is displayed, aliquot and load the reagents as follows:
   - **A.** Amber tube with 300 µL of SAPE Stain Solution in position 1.
   - **B.** Clear tube with 300 µL of Hold Buffer in position 2.
   - **C.** Empty tube in position 3.
   - **D.** Lock the tubes in place.

Figure 3.24 Reagent positions on the Fluidics Station



6. If the scanner is not turned on:
   A. In the Launch window, double-click **AGCC Scan Control**.
   B. Turn the scanner on.
      The scanner must warm up for 10 min before scanning arrays.

Sample Registration

If you have not already done so, you need to prepare a file containing sample information, and upload this information to AGCC prior to scanning your arrays. For more information, see Appendix A, *Registering Samples in Affymetrix GeneChip® Command Console* on page 56.

Remove the Arrays from the Fluidics Station

To remove the arrays from the fluidics station:

1. When *Eject and Inspect Cartridge* is displayed, remove and inspect each array for bubbles.
   The display on the fluidics station will read *Reload Cartridge to Debubble or Engage Washblock.*

If …	Then …
no bubbles are visible	engage the washblock and proceed to Step 2.
bubbles are visible	place the array back on the fluidics station and engage the washblock. The array is drained and refilled with Hold Buffer. Repeat this process as many times as necessary to remove all bubbles.

2. When *Remove Vials 1 & 2* is displayed, remove and discard the vials from positions 1 and 2.
   Leave the empty tube in position 3.
3. When *Load Clean Vials* is displayed, load empty vials in positions 1 and 2 and resume the script.
4. When *Remove Vials 1 & 2* is displayed, remove the vials from positions 1 and 2.
   Protocol Done is displayed.

Prepare and Load the Arrays Onto the Scanner

To prepare the arrays for scanning:

1. Place the arrays face down and carefully cover the septa with Tough-Spots (Figure 3.25).

   ! IMPORTANT: Do not allow edges of Tough-Spots to overlap the large center circle of the array. If tags overlap, the array may get stuck in the scanner. Press firmly to ensure that the Tough-Spot is securely affixed to the array.

2. Inspect the windows for dust, lint or other blemishes.
3. If necessary, clean the array window using compressed air.
4. Scan the array barcodes into the Batch Registration file prepared for this run; then upload the file to AGCC.
   For more information, see *Upload the Batch Registration File to AGCC* on page 58.
5. Immediately load the arrays onto the scanner carousel, starting at position 1.
   The carousel holds 48 arrays.

Figure 3.25 Covering the array septa with Tough-Spots prior to scanning. Do not overlap the window

Wash and Stain the Remaining Arrays

To process the remaining arrays, repeat the steps listed under *Wash and Stain the Arrays* on page 50.

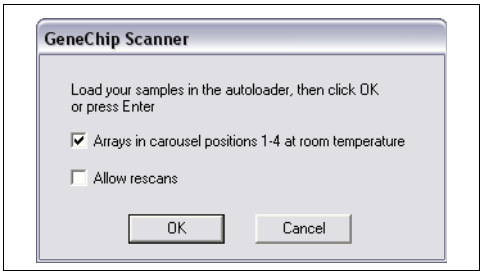
IMPORTANT: Load new tubes of Stain and Storage Cocktail for each array. Leave the empty tube in position 3.

Scan the Arrays

To scan the arrays:

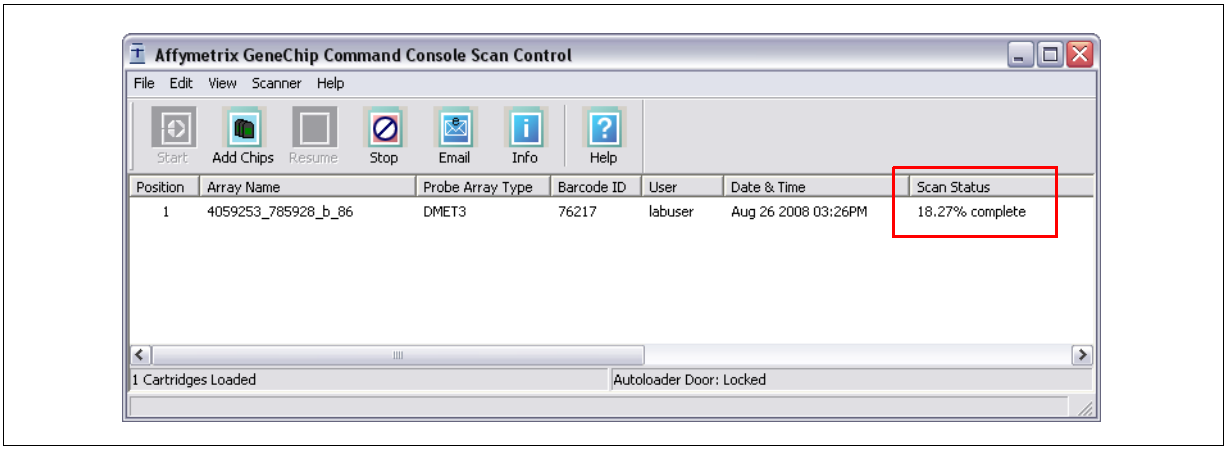
1. Open the AGCC Scan Control software.
2. Click the **Start** icon.
3. Select the check box "**Arrays in carousel positions 1-4 at room temperature**" (Figure 3.26).
   If the arrays are not at room temperature, do not select. The scanner will warm the arrays to room temperature (~ 10 min).
4. If any arrays in the carousel are to be rescanned, select the check box "**Allow rescans**".

Figure 3.26 Prompt displayed before scanning begins



Scanning begins. The status is shown in the Scan Status column (Autofocus, X% complete; Scan complete; Figure 3.27).

Figure 3.27 Scan status



Adding Arrays During an AutoLoader Run

To add arrays while an AutoLoader run is in progress:

1. Click the **Add Chips** icon. 

The GeneChip Scanner window appears (Figure 3.28).

Figure 3.28 Adding additional arrays during a run

2. Click **Add after Scan**.

> **IMPORTANT:** Do not use the Add Now feature. Use only the Add after Scan feature when working with Universal Tag Arrays.

3. When the status on the scanner reads **Autoloader Door Unlocked**, open the scanner and add the arrays.
4. Close the scanner.
5. When the following message is displayed, click **OK**.

Figure 3.29 Press Resume message

6. Click **OK**; then click the **Resume** icon.
7. If any arrays in the carousel are to be rescanned, select the check box "**Allow rescans**".

Shut Down the Fluidics Station

To shut down the fluidics station:

1. Remove Wash Solns A and B and replace with distilled H_2O (dH_2O).
2. Place tubing in dH_2O.
3. Place empty tubes in positions 1, 2 and 3.
4. Select the **All Modules** button.
5. Copy to selected modules and fluidics stations.
6. Run the protocol called **Shutdown_450**.
7. Turn off the fluidics station.

> **IMPORTANT:** To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, a weekly bleach protocol is highly recommended.

Shutdown the Scanner

To shutdown the scanner, push the power button.

Rescanning Arrays

Guidelines for Rescanning Arrays

IMPORTANT: We strongly recommend that arrays be rescanned if necessary within 6 to 24 hours of the first scan. Scanning arrays after 24 hours can result in degraded data.

Guidelines for rescanning arrays are as follows.

Table 3.21 Guidelines for Rescanning Arrays

If …	Then …
there is no image when viewing the .dat file (white screen)	rewash the array with fresh Wash Solution A and rescan. See *Rewash Arrays* below.
lint, dust or bubbles are present	clean the array window or rewash the array as appropriate and rescan. See *Rewash Arrays* below.
data quality is poor	rescan the array.
the image does not grid properly (.cel file is missing)	do not rescan the array. Manually apply the grid to the array image and generate the missing.cel file.

Rewash Arrays

To rewash arrays:

1. Insert a P-200 pipette tip in the upper-right septum of the array.
2. Pipet out the Hold Buffer from the array.
3. Pipet fresh Wash Solution A through the lower left septum as follows:
   A. Pipet up and down 5 times to fill and drain the array.
   B. Repeat the fill and drain with fresh Wash Solution A two more times.
   C. Remove Wash Solution A from the array.
4. Slowly refill the array with fresh Hold Buffer (approximately 100 µL), ensure there are no air bubbles and rescan.

Rescan Arrays

To rescan an array:

1. Load the array into the autoloader.
2. In AGCC Scan Control, click the **Start** icon.
3. Select the check boxes:
   - **Arrays in carousel positions 1-4 at room temperature** (if not at room temperature, do not select this checkbox)
   - **Allow rescans**
4. Click **OK**.

Registering Samples in Affymetrix GeneChip® Command Console

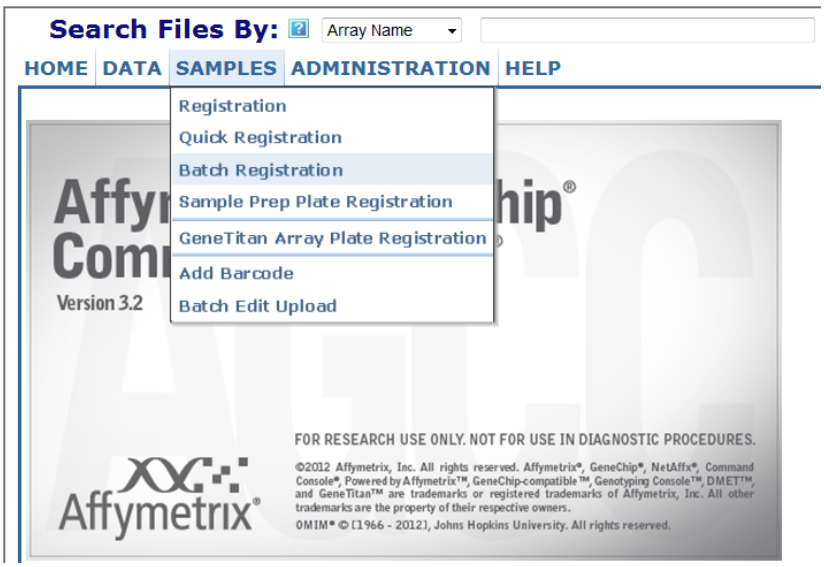
IMPORTANT: We strongly recommend that you *batch register* your sample and array information ***prior to washing and scanning***. If you accidentally wash and scan your arrays without first registering them, the sample file (.ARR files) will not include two of the attributes required by DMET™ Console: sample type and consented markers. Before you can genotype the .CEL files, you will have to manually edit these files to include the required information.

Generating a Sample Batch Registration File

To generate a sample batch registration file:

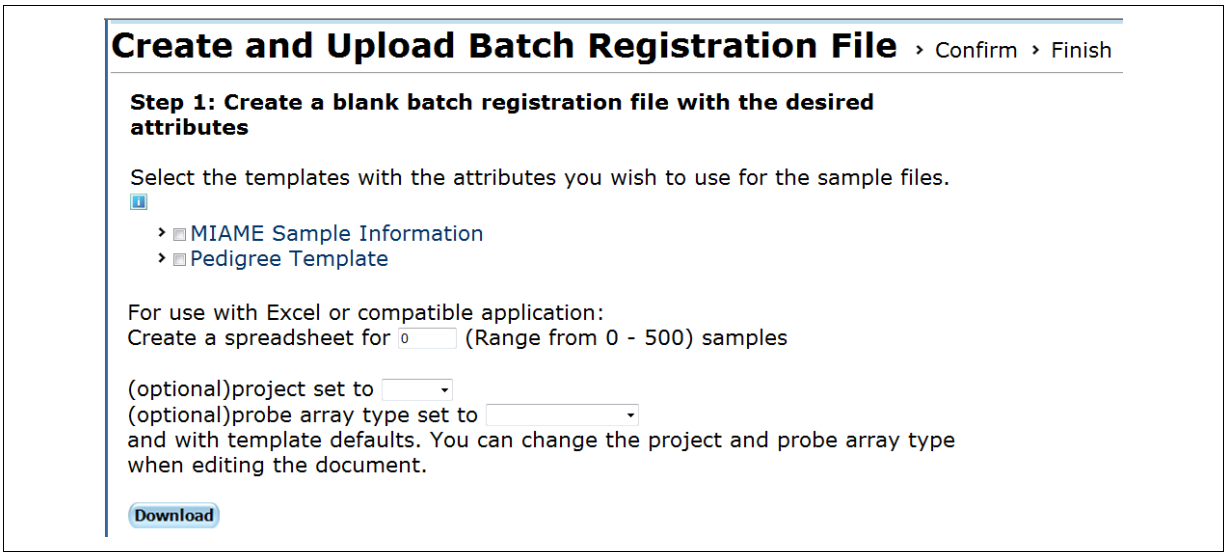
1. From the Launcher, open **AGCC Portal**.
2. Hold the cursor over **Samples** tab and select **Batch Register** from the drop-down menu (Figure A.1).

Figure A.1 Selecting Batch Register to open a spreadsheet template



3. Create a blank Batch Registration File by selecting (Figure A.2):
   - **A.** A template (this example uses the template included with DMET Console).
   - **B.** The file type (TSV or Excel).
   - **C.** The number of samples to be recorded on the spreadsheet.
   - **D.** Optional: A project name from the drop-down menu.
   - **E.** Optional: The array type from the drop-down menu (select DMET_Plus).

Figure A.2 Creating a blank batch sample registration file

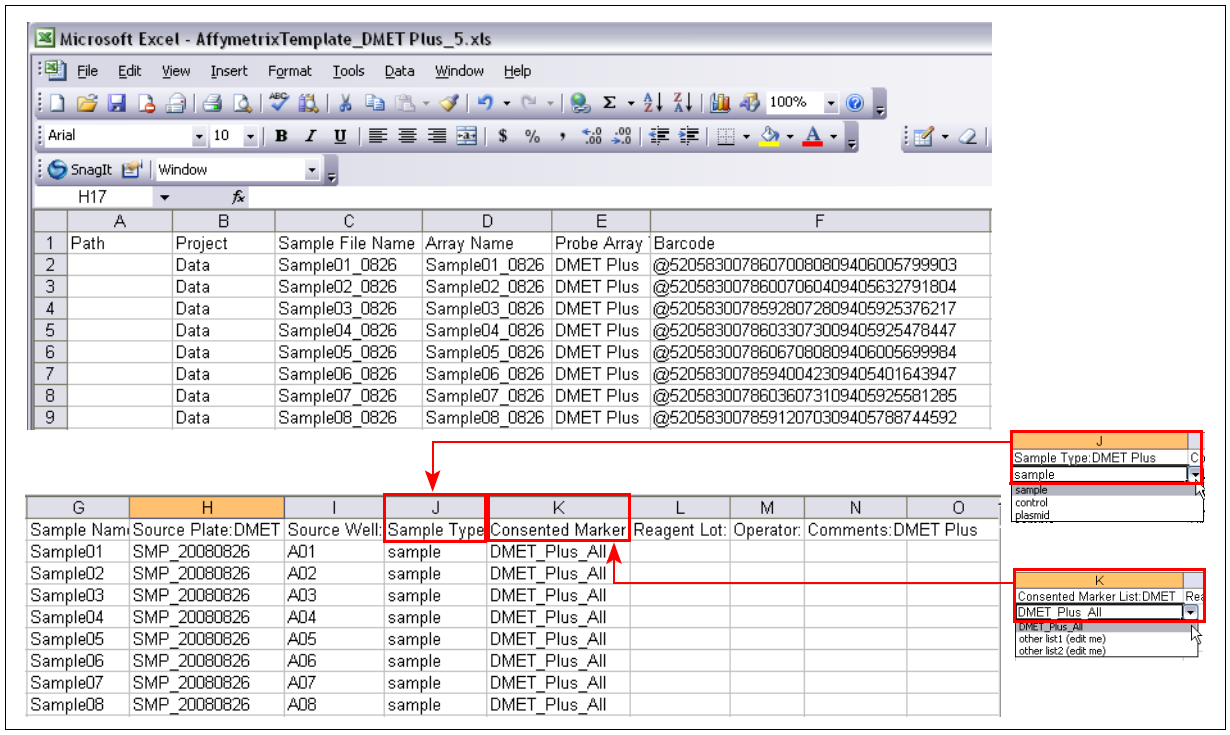


4. Click **Download**.
   A blank registration file is displayed.
5. Enter your sample information (Figure A.3 on page 58).
   Required fields:
   - Path or Project
   - Sample File Name (name that AGCC will assign to the .ARR file)
   - Probe Array Type (click in an empty cell to open the drop-down menu)
   - Sample Name
   - Sample Type (click in an empty cell to open the drop-down menu)
     - Samples designated as types **sample** and **control** are processed using the same settings.
   - Consented Marker List (click in an empty cell to open the drop-down menu)

     Because DMET™ Console also supports filtering out markers after CHP files are created, we recommend that you select DMET_Plus_All. This value allows all markers to be genotyped. Select a more restricted marker list only if you are certain that you will never want to access results from the markers that are excluded.

6. Open **File > Save As** and:
   A. Select the location where the file will be stored.
   B. Enter a name for the file.

Figure A.3 Enter information into the batch registration file



	A	B	C	D	E	F
1	Path	Project	Sample File Name	Array Name	Probe Array	Barcode
2		Data	Sample01_0826	Sample01_0826	DMET Plus	@520583007860700808094060057999903
3		Data	Sample02_0826	Sample02_0826	DMET Plus	@520583007860070604094056327918O4
4		Data	Sample03_0826	Sample03_0826	DMET Plus	@520583007859280728094059253762l7
5		Data	Sample04_0826	Sample04_0826	DMET Plus	@520583007860330730094059254784l7
6		Data	Sample05_0826	Sample05_0826	DMET Plus	@520583007860670808094060056999l4
7		Data	Sample06_0826	Sample06_0826	DMET Plus	@520583007859400423094054016439l7
8		Data	Sample07_0826	Sample07_0826	DMET Plus	@520583007860360731094059255812l5
9		Data	Sample08_0826	Sample08_0826	DMET Plus	@520583007859120703094057887445l2

	G	H	I	J	K	L	M	N	O
1	Sample Nam	Source Plate:DMET	Source Well:	Sample Type	Consented Marker	Reagent Lot:	Operator:	Comments:DMET Plus	
2	Sample01	SMP_20080826	A01	sample	DMET_Plus_All				
3	Sample02	SMP_20080826	A02	sample	DMET_Plus_All				
4	Sample03	SMP_20080826	A03	sample	DMET_Plus_All				
5	Sample04	SMP_20080826	A04	sample	DMET_Plus_All				
6	Sample05	SMP_20080826	A05	sample	DMET_Plus_All				
7	Sample06	SMP_20080826	A06	sample	DMET_Plus_All				
8	Sample07	SMP_20080826	A07	sample	DMET_Plus_All				
9	Sample08	SMP_20080826	A08	sample	DMET_Plus_All				

Upload the Batch Registration File to AGCC

To upload the batch registration file to AGCC:

1. Open the batch registration file created for this set of samples.
2. Scan the array barcodes into this file.
3. In the Batch Sample Registration window, Step 3 (Figure A.4 on page 59):
   A. If custom barcodes have been affixed to the arrays, select the check box **Allow Custom Barcodes**.
   B. Click **Browse**; then navigate to and open the batch registration file.
   C. Click **Upload**.
   D. Click **Save**.
   The message "**Batch Array Registration is complete.**" is displayed (Figure A.5 on page 59).

IMPORTANT: You must click Save once you have uploaded the batch registration file. If you do not click Save, the information is not uploaded.

Figure A.4 Uploading a sample batch registration file

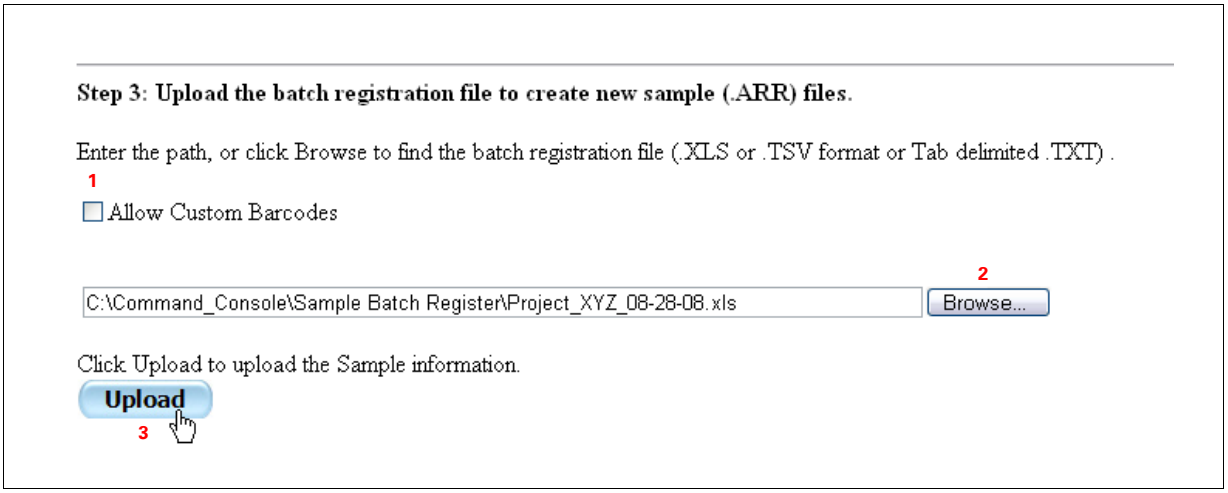
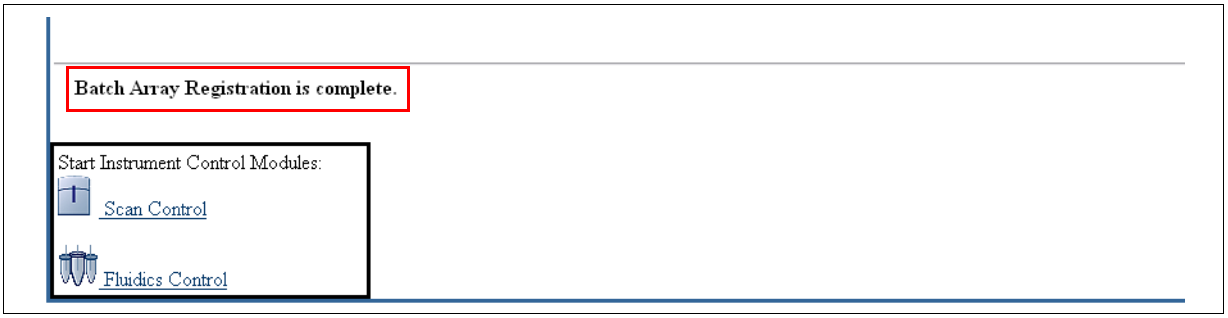


Figure A.5 Batch registration complete message



About Batch Registration Files

A batch registration file is a file that contains sample attributes, such as the sample name, source plate, source well and sample type. Your options for creating this file include:

- Using the pre-defined template provided with DMET Console (DMET.TEMPLATE).
- Editing a template.
- Creating your own template.

Using the Predefined Template — DMET.TEMPLATE

If the predefined template is not already installed on the computer with AGCC, you must first copy it to this computer. Obtain the DMET Console software from Affymetrix. The .ZIP file containing the installer also includes the file DMET.TEMPLATE. Copy this file to the Templates folder used by AGCC (usually located at C:\Command_Console\Templates).

Creating or Editing a Template

Required Attributes

You can edit existing templates to add and modify sample attributes.

IMPORTANT: Two attributes are required by DMET Console for genotyping: Sample Type and Consented Marker List.

Sample Type: required values are sample or control

Consented Marker List: DMET Console includes one predefined marker list: DMET_Plus_All.

Additional consented marker lists can be added. Follow these guidelines:

- Prepare a custom marker list as an excel spreadsheet or tab-delimited file. Save the file.
- Import the marker list into AGCC.
- In your existing template, add the name of the custom marker list to the Control Vocabulary field for Consented Marker List. *The name you enter in this field must exactly match the name of your custom marker list file*.

If DMET Console does not find a file that exactly matches the value chosen for Consented Marker List, the data will not be genotyped. Markers not included in your custom list will be masked as *NotAvailable* in the CHP files. Refer to the *DMET Console User Manual* for more information.

Editing Templates

To edit a template:

1. From the AGCC Portal, open **Administration > Templates > Edit**.
2. Select the template you would like to edit (for example, you can edit DMET.Template to suit your project or study requirements).
3. Delete, edit, or add attributes.

   Remember that DMET Console requires the attributes Sample Type and Consented Marker List. See *Required Attributes* above for more information.
4. Click **Save**.

Creating Templates

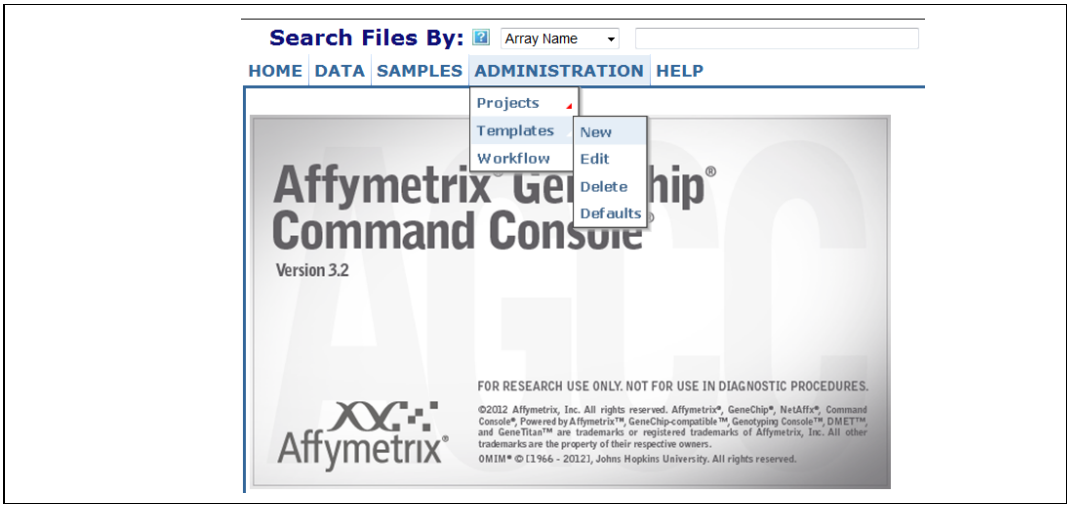
You can create and edit templates in AGCC Portal. To help with template creation, the following fields are automatically included when creating a template:

Path	**Probe Array Type**	**Barcode**
Project	**Sample File Name**	**Array Name**

To create your own template:

1. From the AGCC Portal, open **Administration > Templates > New**.

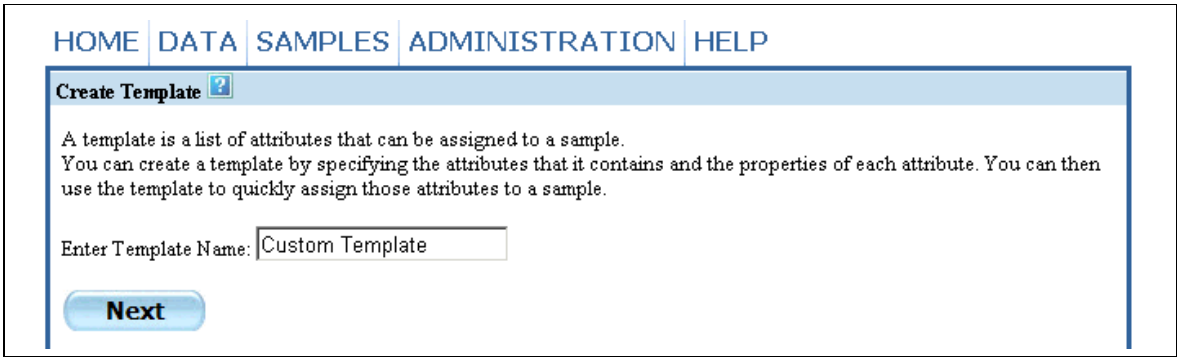
Figure A.6 Creating a new template for sample batch registration



2. Enter a name for the template; then click **Next**.

   The template name is appended to each attribute that you add; therefore, you may want to keep the name as short as possible (see Figure A.10 on page 63).

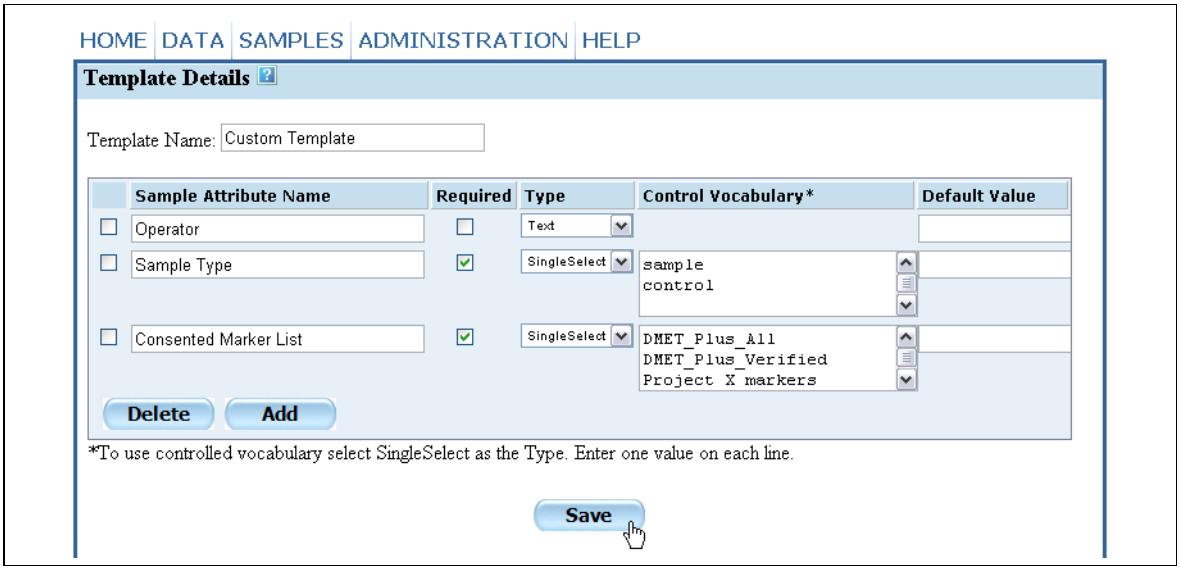
Figure A.7 Entering a name for the new template



3. Add attributes to the template by clicking **Add** and defining the attribute.

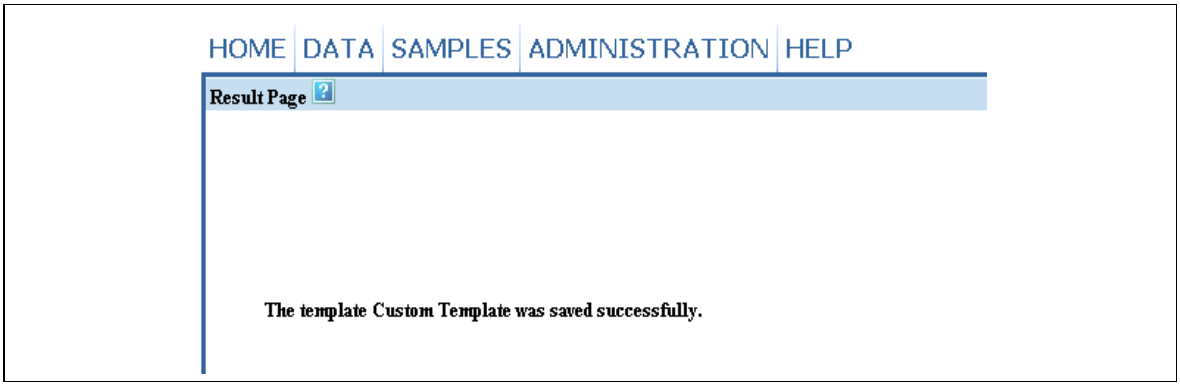
   Remember that DMET Console requires the attributes *Sample Type* and *Consented Marker List*. See *Required Attributes* on page 60 above for more information.

Figure A.8 Adding attributes to a new template



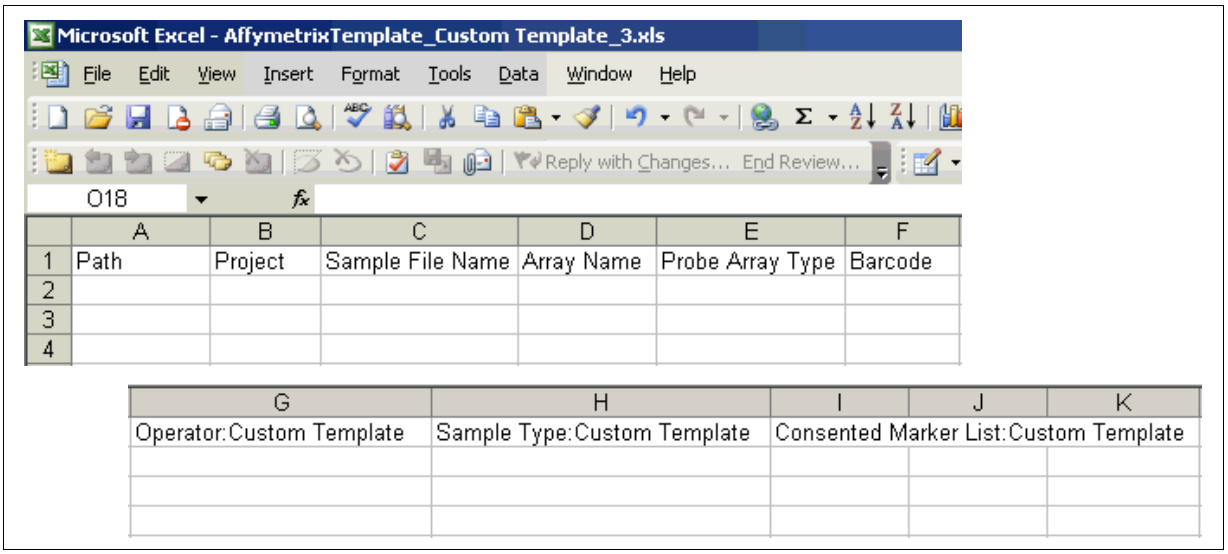
4. When finished adding attributes, click **Save**.

Figure A.9 Adding attributes to a new template



The template as described in the instructions above would look like Figure A.10 when selected for use as an Excel spreadsheet.

Figure A.10 Example of a custom template



	A	B	C	D	E	F
1	Path	Project	Sample File Name	Array Name	Probe Array Type	Barcode
2						
3						
4						

G	H	I	J	K
Operator:Custom Template	Sample Type:Custom Template	Consented Marker List:Custom Template		

Thermal Cycler Programs

Seven thermal cycling programs are used throughout the DMET™ Plus Premier Pack protocol (DMET Plus protocol). This appendix describes each of the programs required for the protocol.

Thermal Cyclers

To run the DMET Plus protocol at a throughput of 48 assays/day, you will need 4 thermal cyclers: 2 in the Pre-Amp Lab; 2 in the Post-Amp Lab.

Pre-Amp Lab Thermal Cycler Programs

Set up the thermal cyclers in the Pre-Amp Lab to run the following programs:

- DMET Plus mPCR
- DMET Plus Anneal
- DMET Plus Assay

Post-Amp Lab Thermal Cycler Programs

Set up the thermal cyclers in the Post-Amp Lab to run the following programs:

- DMET Plus PCR Clean Up
- DMET Plus Frag
- DMET Plus Label
- DMET Plus Denture

Setting the Ramp Speed and Volume for Each Program

IMPORTANT: **Be sure to set the correct ramp speed and volume for each thermal cycler program.**

Ramp Speed

Max = Ramp speed for GeneAmp® PCR System 9700 Thermal Cycler (gold or silver block).

Setting Ramps Speeds and Volumes

The following instructions are for programming a GeneAmp PCR System 9700 thermal cycler (gold or silver block).

To set the ramp speed and volume for each program:

1. Press **Run** (F1).
2. Use the arrow pad to select the program.
3. Press **Start** (F1).
4. Press the down arrow to move to the ramp speed.
5. Press **Max** (F3).
6. Press the up arrow to move to the reaction volume and enter the volume appropriate volume:
   - DMET Plus mPCR: **50**
   - DMET Plus Anneal: **40**

- DMET Plus Assay: **52**
- DMET Plus Clean Up: **55**
- DMET Plus Frag: **35**
- DMET Plus Label: **35**
- DMET Plus Denature: **100**

7. Press **Start** (F1) to start the program.

DMET Plus mPCR Thermal Cycler Program

About the DMET Plus mPCR Program

The *DMET Plus mPCR* program consists of three holds and 1 cycle.

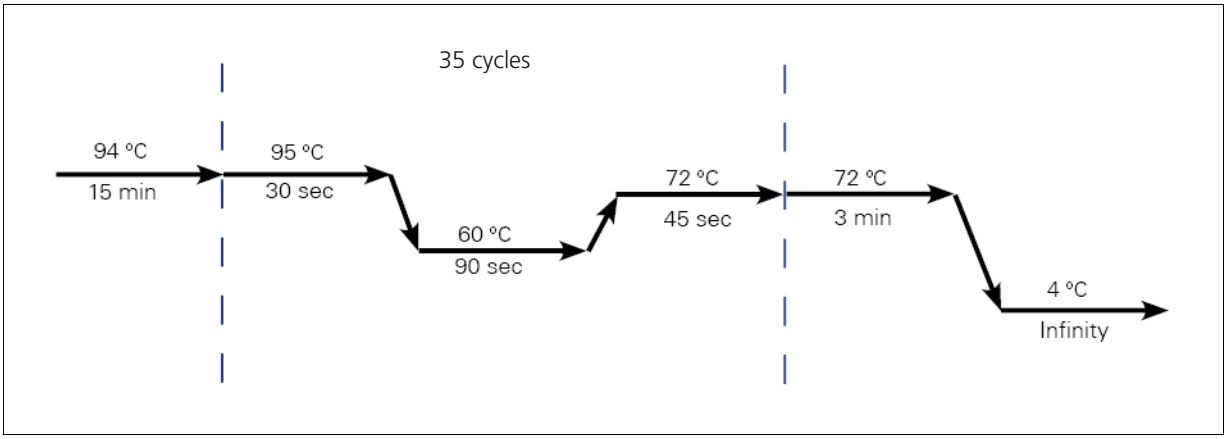
Ramp speed and volume:

- **Ramp speed:**
  - GeneAmp PCR System 9700 (gold or silver block) = Max
- **Volume**: 50 µL

Table B.1 Stages of the DMET Plus mPCR thermal cycler program

Stage	Temperature	Time	Cycles
Activation	94°C	15 min	—
Denature	95°C	30 sec	35 cycles
Anneal	60°C	90 sec	
Extend	72 °C	45 sec	
Finish	72 °C	3 min	—
	4°C	Infinity	—

Figure B.1 DMET Plus mPCR thermal cycler program



DMET Plus Anneal Thermal Cycler Program

About the DMET Plus Anneal Program

The *DMET Plus Anneal* program consists of four holds and no cycles.

Ramp speed and volume:

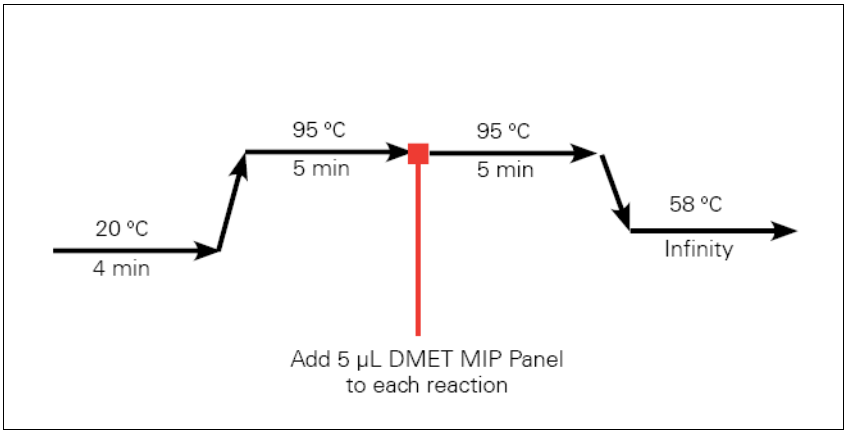
- **Ramp speed**
  - GeneAmp PCR System 9700 (gold or silver block) = Max
- **Volume**: 40 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 64.

Table B.2 Stages of the DMET Plus Anneal thermal cycler program

Stage	Temperature	Time
Enzyme A	20°C	4 min
Denature	95°C	5 min
Denature	95°C	5 min
Anneal	58°C	Infinity

Figure B.2 DMET Plus Anneal thermal cycler program



DMET Plus Assay Thermal Cycler Program

About This Program

The DMET Plus Assay thermal cycler program consists of 8 holds and 1 cycle.

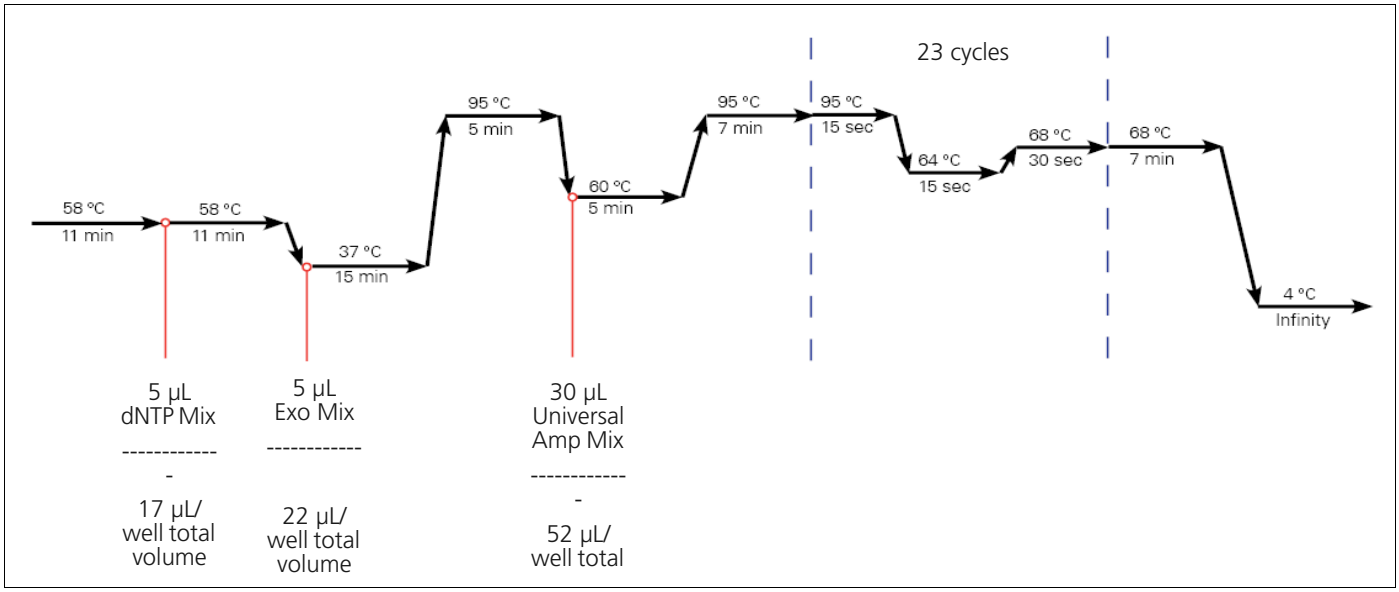
- **Ramp speed**
  - GeneAmp PCR System 9700 (gold or silver block) = Max
- **Volume**: 52 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 64.

Table B.3 Stages of the DMET Plus Assay thermal cycler program

Stage	Temperature	Time	Cycles
Gap Fill	58°C	11 min	—
dNTP Mix addition	58°C	11 min	—
Exo Mix addition	37°C	15 min	
Denature	95°C	5 min	
Universal Amp Mix addition	60°C	5 min	
Denature	95°C	7 min	
Denature	95°C	15 sec	23 cycles
Anneal	64°C	15 sec	
Extend	68°C	30 sec	
Finish	68°C	7 min	
	4°C	Infinity	

Figure B.3 DMET Plus Assay thermal cycler program



DMET Plus Clean Up Thermal Cycler Program

About the DMET Plus Clean Up Program

The DMET Plus Clean Up program consist of 2 holds and no cycles.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

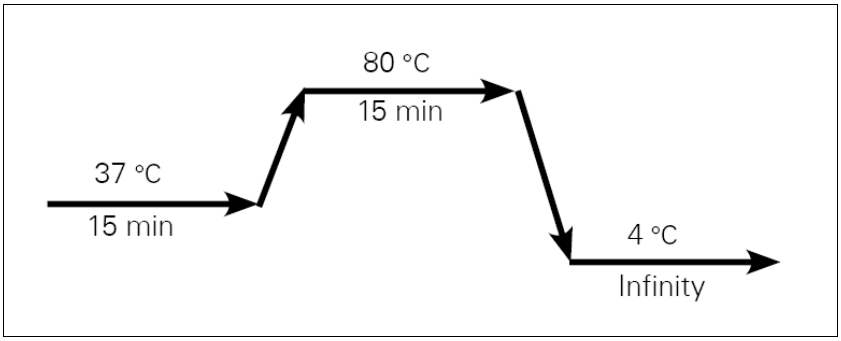
Volume: 55 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 64.

Table B.4 Stages of the DMET Plus Clean Up thermal cycler program

Stage	Temperature	Time
1	37 °C	15 min
2	80 °C	15 min
Hold	4°C	Infinity

Figure B.4 DMET Plus Clean Up thermal cycler program



DMET Plus Frag Thermal Cycler Program

About the DMET Plus Frag Program

The *DMET Plus Frag* program consists of 2 holds and no cycles.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

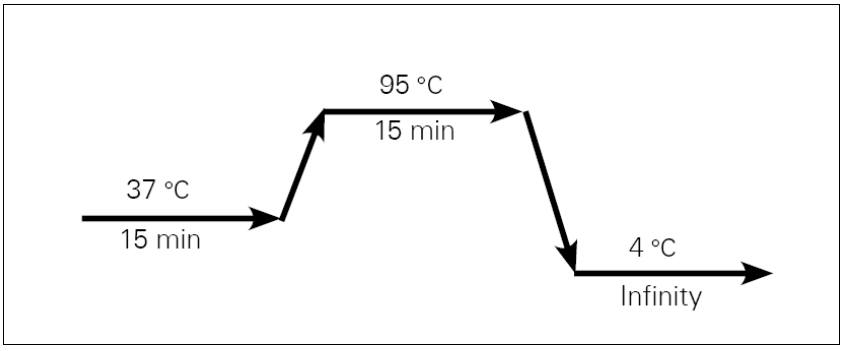
Volume: 35 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See *Setting the Ramp Speed and Volume for Each Program* on page 64.

Table B.5 Stages of the DMET Plus Frag thermal cycler program

Stage	Temperature	Time
1	37°C	15 min
2	95°C	15 min
Hold	4°C	Infinity

Figure B.5 DMET Plus Frag thermal cycler program



DMET Plus Label Thermal Cycler Program

About the DMET Plus Label Program

The *DMET Plus Label* program consists of 2 holds and no cycles.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

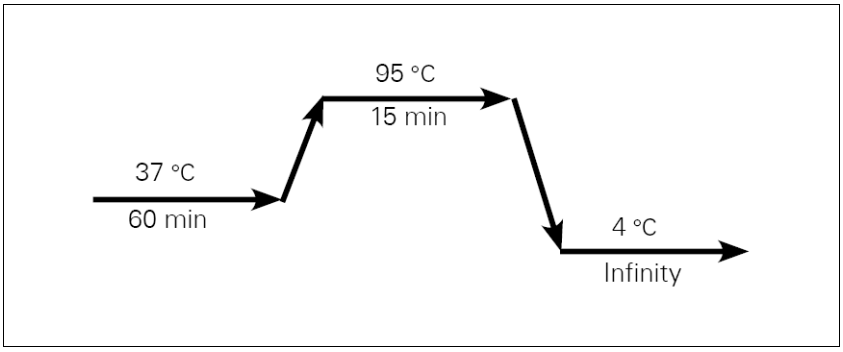
Volume: 35 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See ***Setting the Ramp Speed and Volume for Each Program* on page 64**.

Table B.6 Stages of the DMET Plus Label thermal cycler program

Stage	Temperature	Time
1	37°C	60 min
2	95°C	15 min
Hold	4°C	Infinity

Figure B.6 DMET Plus Label thermal cycler program



DMET Plus Denature Thermal Cycler Program

About the DMET Plus Denature Program

The *DMET Plus Denature* program consists of 2 holds and no cycles.

Ramp speeds:

- GeneAmp PCR System 9700 (gold or silver block) = Max

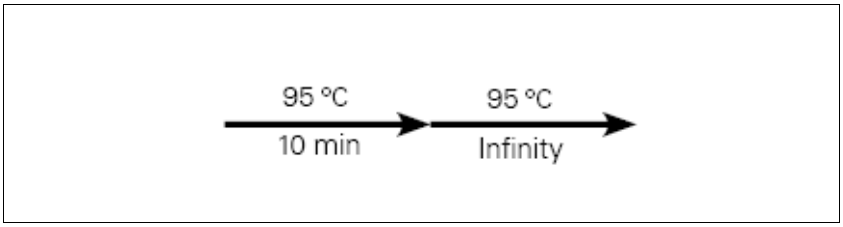
Volume: 100 µL

IMPORTANT: The ramp speed and volume must be set the first time you use the program. See ***Setting the Ramp Speed and Volume for Each Program* on page 64**.

Table B.7 Stages of the DMET Plus Denature thermal cycler program

Stage	Temperature	Time
Denature	95°C	10 min
Standby	95°C	Infinity

Figure B.7 DMET Plus Denature thermal cycler program



Equipment, Software, Consumables and Reagents List

This appendix lists the equipment, software, consumables and reagents required to perform the DMET™ Plus Premier Pack protocol (DMET Plus protocol).

From Affymetrix

Affymetrix Equipment and Software Required

Table C.1 Affymetrix Equipment and Software Required

✓	Item	Part Number
❒	GeneChip® Fluidics Station 450 — two or more units required	00-0079
❒	GeneChip® Hybridization Oven 645	00-0331
❒	One of the following:	
	GeneChip® 3000 Scanner 7G with workstation and autoloader	00-0215
	GeneChip® 3000 7G System for WGA	00-0362
❒	GeneChip® Command Console Software	Latest version
❒	DMET™ Console	Latest version

Affymetrix Reagents and Arrays Required

Table C.2 DMET™ Plus Kit

✓	Item	Part Number
❒	48 reactions — Components contained in this kit are listed below.	901268
	Box 1 – DMET™ Plus Pre-Amp Kit ▪ Enzyme A ▪ Buffer A ▪ Pre-Amp Water ▪ Gap Fill Mix 1 ▪ Gap Fill Mix 2 ▪ Exo Mix ▪ Universal Amp Mix ▪ Cleavage Enzyme ▪ dNTP Mix	
	Box 2 – DMET™ Plus Labeling Kit ▪ PCR Clean Up Mix ▪ Post-Amp Water ▪ Fragmentation Buffer ▪ Fragmentation Reagent ▪ DNA Labeling Reagent ▪ 5X TdT Buffer ▪ TdT Enzyme	
	Box 3 – DMET™ Plus Hyb-Stain Kit ▪ Oligo Control Reagent ▪ Hybridization Solution ▪ Stain Buffer ▪ Hold Buffer	
	Box 4 – DMET™ Plus Panel Kit ▪ DMET mPCR Primer Mix ▪ DMET MIP Panel ▪ 1X TE Buffer ▪ PCR Dilution Buffer ▪ DMET gDNA Control 4 ▪ DMET gDNA Control 5 ▪ DMET gDNA Control 6	
	Wash Solutions (shipped separately) ▪ Wash Solution A (3 bottles) ▪ Wash Solution B (2 bottles)	
	Arrays (shipped separately) ▪ DMET Plus Arrays (48 arrays)	

From Other Suppliers — Equipment and Consumables Required

When performing the pre-PCR stages of the DMET Plus protocol, great care should be taken to avoid sample contamination with PCR products.

mPCR Staging Area and Pre-Amp Lab

The mPCR Staging Area is located in the Pre-Amp Lab.

mPCR Staging Area

The mPCR Staging Area is under a hood or in a PCR cabinet that is located in the Pre-Amp Area. The equipment and consumables listed in Table C.3 should be dedicated to this location.

Table C.3 mPCR Staging Area — Dedicated Equipment and Consumables Required

✓	Item	Vendor	Part Number
EQUIPMENT			
❒	Choose one of the following: ▪ Laminar Flow Cabinet, 6 ft (ESCO, SVE-6A) ▪ PCR Cabinet	Laminar Cabinet: ESCO SVE-6A or equivalent PCR Cabinet: C.B.S. Scientific P-048-02 or equivalent	
❒	Aluminum blocks, Biocooler 96-well (3 blocks required)	Bio-Smith	81001
❒	Ice Bucket, 4 to 9 liters (Magic Touch Icewares)	Fisher Scientific	—
❒	Pipettes: ▪ Choose one of the following – manual only: ▫ Pipette, 12-channel, 2–20 µL ▫ Pipette, 24 channel, 2–20 µL ▪ 12-channel, 20–200 µL (manual or electronic) ▪ Optional: 24-channel, 10–100 µL (manual or electronic)	Rainin	 L12-20 L24-20 L12-200 L24-100
❒	Optional: Plate sealer	Any vendor	—
CONSUMABLES			
❒	Markers, fine point, permanent	Any vendor	—
❒	MicroAmp® Clear Adhesive Film	Life Technologies	4306311
❒	Pipette tips: ▫ 20 µL filter tips ▫ 100 µL filter tips ▫ 200 µL filter tips	Rainin	 GP-L10F GP-L100F GP-L200F
❒	Plate, 96-well PCR, half-skirt, 0.2 mL	E&K Scientific	289196
❒	Reagent reservoir, 50 mL	Rainin	RV-050

Pre-Amp Lab

Table C.4 Pre-Amp Lab Equipment and Consumables Required

✓	Item	Vendor	Part Number
EQUIPMENT			
❒	Aluminum blocks, Biocooler 96-well (2 blocks required)	Bio-Smith	81001
❒	Centrifuge, plate	Eppendorf	5804 or 5810
❒	Freezer, –20°C; deep freeze; manual defrost; 17 cu ft	Any vendor	—
❒	Ice Bucket, 4 to 9 liters (Magic Touch Icewares)	Fisher Scientific	—
❒	Microfuge (for tubes and strip tubes)	Any vendor	—
❒	Pipettes One each of the following is required: ▫ Single-channel, 2–20 µL ▫ Single-channel, 20–200 µL ▫ Single-channel, 100–1000 µL ▫ 12-channel, 0.5–10µL ▫ 12-channel, 2–20 µL (manual or electronic) ▫ 12-channel, 20–200 µL (manual or electronic) Optional: ▫ 24-channel P20 (manual or electronic)	Rainin	 L-20 L-200 L-1000 L12-10 L12-20 L12-200 L24-20
❒	Refrigerator, 4 °C	Any vendor	—
❒	Thermal cycler, GeneAmp® PCR System 9700 (gold/silver block)	Life Technologies	▪ 4314878 (gold block) ▪ N8050001 (silver block)
❒	Vortex Required: One vortex with platform for plates. Optional: Additional vortex with tube attachment	VWR	58815-234 with platform
CONSUMABLES			
❒	Markers, fine point, permanent	Any vendor	—
❒	MicroAmp Clear Adhesive Film	Life Technologies	4306311
❒	Plate, 96-well PCR, half-skirt, 0.2 mL	E&K Scientific	289196

Table C.4 Pre-Amp Lab Equipment and Consumables Required

✓	Item	Vendor	Part Number
❒	Pipette tips: ▫ 20 µL filter tips ▫ 200 µL filter tips ▫ 1000 µL filter tips	Rainin	 GP-L10F GP-L200F GP-L1000F
❒	Reagent reservoir, 50 mL	Rainin	RV-050
❒	Tube, Eppendorf 1.5 mL, Safe-Lock natural	VWR	21008-959
❒	Tube, 5 mL, graduated transport (Axygen)	VWR	89005-596
❒	Select 8- or 12-strip tubes. Eight tubes can be easier to work with. ▫ Tubes, strip of 8, clear polypropylene ▫ Caps for 8-strip tubes ▫ Tubes, strip of 12, clear polypropylene ▫ Caps for 12-strip tubes	E&K Scientific	 28008 490018 690012 490012

Post-Amp Lab

Table C.5 Post-Amp Lab Equipment and Consumables Required

✓	Item	Vendor	Part Number
EQUIPMENT			
❒	Aluminum blocks, Biocooler 96-well (2 blocks required)	Bio-Smith	81001
❒	Centrifuge, plate	Eppendorf	5804 or 5810
❒	Freezer, –20°C; deep freeze; manual defrost; 17 cu ft	Any vendor	—
❒	Ice Bucket, 4 to 9 liters (Magic Touch Icewares)	Fisher Scientific	—
❒	Microfuge (for tubes and strip tubes)	Any vendor	—
❒	Pipettes One each of the following is required: ▫ Single-channel, 2–20 µL ▫ Single-channel, 20–200 µL ▫ Single-channel, 100–1000 µL ▫ 12-channel, 0.5–10µL ▫ 12-channel, 2–20 µL (manual or electronic) ▫ 12-channel, 20–200 µL (manual or electronic) Optional: ▫ 24-channel P20 (manual or electronic)	Rainin	 L-20 L-200 L-1000 L12-10 L12-20 L12-200 L24-20
❒	Refrigerator, 4 °C	Any vendor	—
❒	Thermal cycler, GeneAmp PCR System 9700 (gold/silver block)	Life Technologies	▪ 4314878 (gold block) ▪ N8050001 (silver block)
❒	Vortex Required: One vortex with platform for plates. Optional: Additional vortex with tube attachment	VWR	58815-234 and 58815-216

Table C.5 Post-Amp Lab Equipment and Consumables Required

✓	Item	Vendor	Part Number
CONSUMABLES			
❒	Kimwipes	Any vendor	—
❒	Markers, fine point, permanent	Any vendor	—
❒	MicroAmp Clear Adhesive Film	Life Technologies	4306311
❒	Plate, 96-well PCR, half-skirt, 0.2 mL	E&K Scientific	289196
❒	Plateholder, 96-well PCR	Any vendor	—
❒	Pipette tips: ▫ 20 µL filter tips ▫ 200 µL filter tips ▫ 1000 µL filter tips	Rainin	 GP-L10F GP-L200F GP-L1000F
❒	Reagent reservoir, 50 mL	Rainin	RV-050
❒	Tough-Spots®: ▫ 3/8 in. (9.5 mm) diameter ▫ 1/2 in. (13 mm) diameter	Diversified Biotech	 SPOT-1000 SPOT-1100
❒	Tube, Eppendorf: ▫ 1.5 mL, Safe-Lock natural ▫ 1.5 mL, Safe-Lock amber	VWR	 21008-959 21008-960
❒	Tube, 5 mL, graduated transport (Axygen)	VWR	89005-596
❒	Select 8- or 12-strip tubes. Eight tubes can be easier to work with. ▫ Tubes, strip of 8, clear polypropylene ▫ Caps for 8-strip tubes ▫ Tubes, strip of 12, clear polypropylene ▫ Caps for 12-strip tubes	E&K Scientific	 28008 490018 690012 490012
❒	Vortex Required: One vortex with platform for plates. Optional: Additional vortex with tube attachment	VWR	 58815-234 and 58815-216
QC Gel Materials for use with the Bio-Rad Wide Mini-Sub Cell GT			
❒	Buffers — select one of the following: ▫ 1X TE Buffer ▫ AccuGENE® Water or other molecular biology grade water	 TekNova Lonza Group LTD	 T0223 51200
❒	Buffer, 2X Loading	Sigma	G2526
❒	Gel, Wide-Mini 2 x 32 wells, 3% agarose with ethidium bromide	Bio-Rad	161-3040
❒	Ladder, Low Moledular Weight	New England Biolabs	N3233S

From Other Suppliers — Reagents Required

Table C.6 Reagents Required from Other Suppliers

✓	Item	Vendor	Part Number
❒	AccuGENE® Water or other molecular biology grade water	Lonza Group LTD	51200
❒	QIAGEN® Multiplex PCR Kit	QIAGEN	206143
❒	Streptavidin, R-phycoerythrin conjugate (SAPE)	Life Technologies	S866 (1 mL)
❒	TITANIUM™ *Taq* Polymerase	Clontech	639208 (100 rxns) 639209 (500 rxns)
❒	TE Buffer, pH 8.0	TekNova	T0223
❒	Quant-iT™ PicoGreen® dsDNA Assay Kit	Life Technologies	P7589

Supplier Contact List

Table C.7 Supplier Contact List

Supplier	Web Site Address (www not required for some addresses)
Affymetrix	www.affymetrix.com
Bio-Rad	bio-rad.com
Bio-Smith	biosmith.com
C.B.S. Scientific	www.cbsscientific.com
Clontech	www.clontech.com
Diversified Biotech	divbio.com
E&K Scientific	eandkscientific.com
Eppendorf	eppendorf.com
ESCO	www.escoglobal.com
Fisher Scientific	www.fishersci.com
Life Technologies	www.lifetechnologies.com
Lonza	www.lonza.com
New England Biolabs	www.neb.com
QIAGEN	www.qiagen.com
Rainin	www.rainin.com
TekNova	teknova.com
VWR	vwr.com