CYP2D6 Allele–specific Copy Number Analysis

QuantStudio™ 3D workflow for digital analysis

Pub. No. MAN0011114 **Rev.** B.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *QuantStudio™ 3D Digital PCR System User Guide* (Pub. No. MAN0007720). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Introduction

The important drug metabolizing gene CYP2D6 is highly polymorphic and exhibits copy number variation (CNV). Characterizing an individual's diplotype requires both SNP genotyping and CNV analysis. Reported CYP2D6 star (*) allele haplotypes fall into three functional categories (full, reduced, or no activity). The combination of alleles in an individual's diplotype is predictive of their drug metabolizer phenotype (Table 3 on page 8 and Table 4 on page 9). If an individual carries a CYP2D6 duplication and is heterozygous for alleles from different functional categories, identifying the specific duplicated allele may be required to accurately predict the drug metabolizer phenotype (Table 3 on page 8). A number of star alleles are documented to be duplicated in a subset of the population (Table 2 on page 8). The nature of the duplicated allele cannot always be identified using SNP genotype and CNV results alone, but may be identified by allele–specific copy number variation (ASCNV) analysis using TaqMan® Drug Metabolism Genotyping Assays and digital PCR (dPCR) using the QuantStudio™ 3D Digital PCR System.

This quick reference provides instructions specific to this application, and has been optimized for use withTaqMan® Drug Metabolism Genotyping Assays that target star allele–defining SNPs in the major duplicated alleles. For detailed instructions on dPCR, TaqMan® Copy Number Assays and TaqMan® SNP Genotyping Assays see page 8.

Required materials and equipment not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
QuantStudio™ 3D Digital PCR System	Contact your local sales office
QuantStudio™ 3D AnalysisSuite™ Cloud Software	**thermofisher.com/analysissuite**
TaqMan® Drug Metabolism Genotyping Assay	See Table 1 on page 3.
QuantStudio™ 3D Digital PCR 20K Chip Kit v2 Includes: • QuantStudio™ 3D Digital PCR 20K Chip v2 • QuantStudio™ 3D Digital PCR Chip Lid v2 • QuantStudio™ 3D Digital PCR Sample Loading Blade • Immersion Fluid • Immersion Fluid Tip	A26316
QuantStudio™ 3D Digital PCR Master Mix v2 1.5 mL	A26358
SpeI-HF™ (with 10X CutSmart™ Buffer) 500 units (20 U/ µL)	New England Biolabs R3133S

Before you begin

See *Pharmacogenomics Experiments Application Guide* (Pub. No. MAN0009612) for detailed instructions.

1. Purify and quantify gDNA samples.
2. Run SNP genotyping experiments to detect key CYP2D6 variants using TaqMan® Drug Metabolism Genotyping Assays.
3. Analyze genotypes using TaqMan® Genotyper Software or the Applied Biosystems™ qPCR Analysis Module.
4. Run copy number experiments using the CYP2D6 exon 9 target assay (Hs00010001_cn) to detect full length alleles (but not null-activity, exon 9 CYP2D7 conversion alleles such as CYP2D6*36).
5. Analyze copy numbers using CopyCaller™ Software.
6. Translate SNP genotypes and copy numbers to star allele results using AlleleTyper™ Software.
7. Identify samples that carry duplications of CYP2D6 and that are heterozygous for star alleles of different functional categories. See Table 1 on page 3.

Workflow

"Select the sample and assay" on page 3

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"Digest the gDNA" on page 3

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"Prepare and load the sample on the chip" on page 4

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"Run the digital PCR" on page 5

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"Analyze the data" on page 5

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"Export the results" on page 7

Methods

1 Select the sample and assay

For samples that carry a duplicated CYP2D6 allele and are heterozygous for star alleles of different functional categories, identify a TaqMan® Drug Metabolism Genotyping Assay, according to the following table, that distinguishes one of the star alleles from the other. Run at least one of the recommended assays per sample in a dPCR experiment.

Table 1 TaqMan® Drug Metabolism Genotyping Assay to key SNPs in the major duplicated alleles

Diplotype	Recommended assay	Detected allele	SNP	VIC™ dye[1]	FAM™ dye[1]
*1/*10	C__11484460_40	*10	100C>T	A (T)	G (C)
	C__27102414_10	*10	4180G>C	C (G)	G (C)
*2/*10	C__11484460_40	*10	100C>T	A (T)	G (C)
	C__27102425_10	*2	2850C>T	A (T)	G (C)
*2A/*10	C__11484460_40	*10	100C>T	A (T)	G (C)
	C__27102425_10	*2A	2850C>T	A (T)	G (C)
	C__32407252_30	*2A	–1584C>G	G (C)	C (G)
*35/*10	C__11484460_40	*10	100C>T	A (T)	G (C)
	C__27102425_10	*35	2850C>T	A (T)	G (C)
	C__27102444_80	*35	31G>A	C (G)	T (A)
*1/*17	C___2222771_A0	*17	1023C>T	G (C)	A (T)
	C__27102425_10	*17	2850C>T	A (T)	G (C)
	C__27102414_10	*17	4180G>C	C (G)	G (C)
*2/*17	C___2222771_A0	*17	1023C>T	G (C)	A (T)
*2A/*17	C__32407252_30	*2A	–1584C>G	G (C)	C (G)
	C___2222771_A0	*17	1023C>T	G (C)	A (T)
*35/*17	C__27102444_80	*35	31G>A	C (G)	T (A)
	C___2222771_A0	*17	1023C>T	G (C)	A (T)
*4/*10	C__27102431_D0	*4	1846G>A	C (G)	T (A)
*4/*17	C__27102431_D0	*4	1846G>A	C (G)	T (A)
	C___2222771_A0	*17	1023C>T	G (C)	A (T)
	C__27102425_10	*17	2850C>T	A (T)	G (C)

[1] The SNP alleles provided in the VIC™ dye and FAM™ dye columns refer to the genome (+) strand base; the (–) strand base is given in parentheses and is equivalent to the CYP2D6 cDNA alleles.

2 Digest the gDNA

The purpose of the restriction digest is to separate tandem, duplicated genes that would otherwise amplify in the same well. This prevents undercounting the number of copies in a digital PCR reaction.

2 Digest the gDNA *(continued)*

a. For each sample, mix reagents in a 1.5-mL microcentrifuge tube or 96-well sample plate according to the following table.

Note: It is also recommended to include a tube for a no template control (NTC); run at least one NTC chip per assay in dPCR.

Reagent	Quantity
gDNA (5 ng/µL)[1]	20 µL (100 ng)
CutSmart™ Buffer, 10X	5 µL
SpeI-HF™ restriction endonuclease; use according to manufacturer's instructions.	1 unit
Nuclease-free Water	Bring to final volume of 50 µL
Total	**50 µL**

[1] See: "Before you begin" on page 2

Note: The SpeI-HF™ enzyme cuts the CYP2D6 gene within intron 7 to separate duplicated gene copies but does not digest within the amplicon sequences of the assays in Table 1 on page 3.

b. *(96-well sample plate only)* Cover the plate with MicroAmp™ adhesive film.

c. Vortex sample, then centrifuge for 30 seconds at 201 x *g*.

d. Incubate sample at 37°C for 60 minutes, then heat inactivate at 80°C for 20 minutes.

e. Vortex sample, then centrifuge for 30 seconds at 201 x *g*.

Digested samples can be stored at –20°C until needed.

3 Prepare and load the sample on the chip

For materials and information required to perform dPCR see *QuantStudio™ 3D Digital PCR System User Guide* (Pub. No. MAN0007720). In this procedure, you will prepare the dPCR reactions for the samples that you intend to load on the QuantStudio™ 3D Digital PCR Chips.

Note: The volumes of the example protocol have been adjusted so that two chips are run for each sample. We recommend running duplicate chips for each sample and assay combination.

a. Prepare PCR Reaction Mix in a low DNA bind 0.5-mL microcentrifuge tube as described in the following table.

Material	Stock concentration	Volume		Final concentration
		Per chip[1]	1 sample / 2 chips[1]	
QuantStudio™ 3D Digital PCR Master Mix v2	2X	8.7 µL	17.4 µL	1X
TaqMan® Drug Metabolism Genotyping Assay	20X	0.87 µL	1.74 µL	1X
SpeI-HF™ digested gDNA	2 ng/µL	1.75 µL	3.5 µL	200 pg/µL
Nuclease-free Water	—	6.1 µL	12.2 µL	—
Total PCR Reaction Mix volume	**—**	**17.24 µL**	**34.84 µL**	**—**

[1] Volumes include 20% overage.

b. Vortex sample, then briefly centrifuge.

c. Load 14.5 µL of reaction per QuantStudio™ 3D Digital PCR Chip.

4 Run the digital PCR

See *QuantStudio™ 3D Digital PCR System User Guide* (Pub. No. MAN0007720) for instructions on operating the instrument.

For use with ProFlex™ PCR System

	Stage 1		Stage 2			Stage 3	Cover temp.	Reaction volume
Temp.	45°C	98°C	95°C	53.5°C	57.5°C	10°C	70.0°C	1 nL[1]
Time (min:sec)	01:00	02:30	01:00	00:10	00:30	Hold		
Hold / Cycles	1X (Hold)		40X (Cycles)			1X (Hold)		

[1] Use 33 nL for device firmware older than v 1.1.4.

Load the QuantStudio™ 3D Digital PCR Chips into the thermal cycler, then start the run.

IMPORTANT! If not using the "3D" default protocol from ProFlex™ v 1.1.4 firmware, you must create a thermal protocol.

Note: The reaction volume (20 µL) on the instrument display does not refer to the reaction volume of the chip and should not be changed.

5 Analyze the data

See *QuantStudio™ 3D Digital PCR System User Guide* (Pub. No. MAN0007720) for a detailed description.

a. Image the thermal cycled QuantStudio™ 3D Digital PCR Chip on the QuantStudio™ 3D Digital PCR Instrument.

b. Export EDS experiment files to the USB drive, and upload them to the cloud for analysis using the AnalysisSuite™ Cloud Software.

c. Analyze the data using the AnalysisSuite™ Cloud Software. Assign sample and assay information for each chip and analyze data.

Note: Assign identical sample names for duplicate chips in order to obtain an average % FAM™ value.

d. Review each chip using the **Review Calls** tab.

If the clusters are not well separated, manual calling may be required.

e. Identify the sample chips that require manual calling.

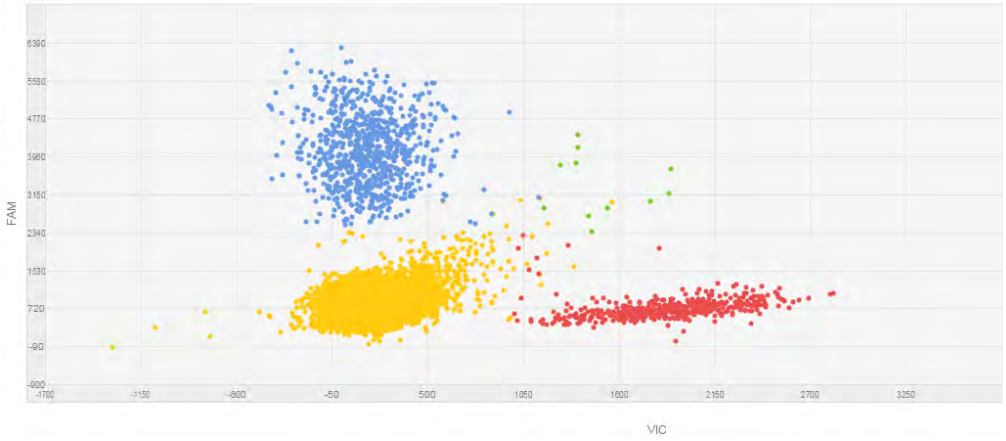

Figure 1 Sample chip that requires manual calling

In the scatter plot each dot represents one through hole on the dPCR chip: Red = VIC™ labeled allele, Blue = FAM™ labeled allele, Green = both a VIC™ allele and a FAM™ allele present, Yellow = no amplification

f. Review No Template Control (NTC) chips run with the same assay.

5 Analyze the data *(continued)*

g. Select all NTC data points using the lasso tool and select **Undetermined**.

To better overlay NTC data points of NTC and sample chips, highlight data points on the NTC chips in a different color by manually designating them **Undetermined**.

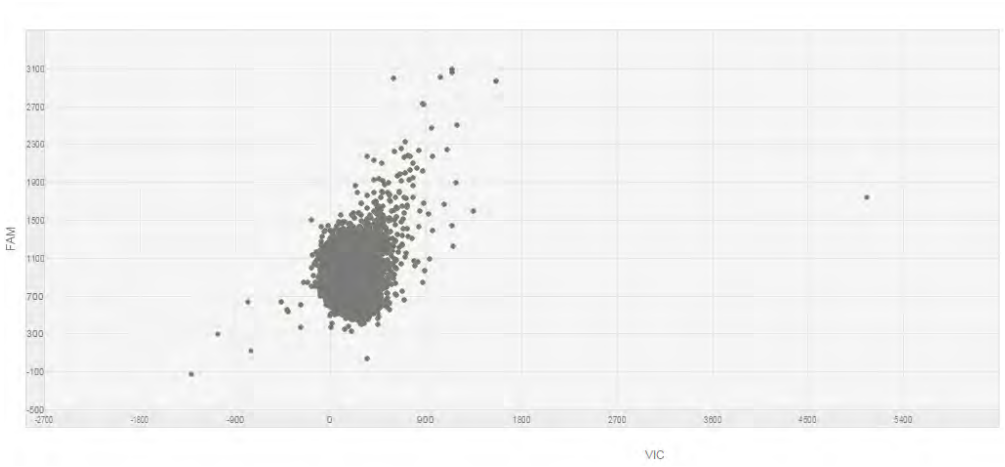

Figure 2 No Template Control chip

h. Hold down the control key and select the sample chip requiring manual calling to see where the data points overlap.

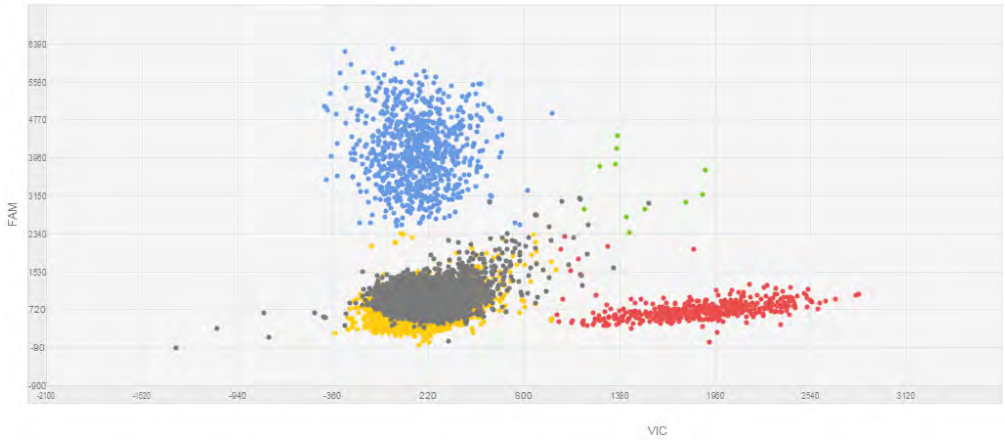

Figure 3 Overlap view of NTC and sample chip

i. Identify ambiguous data points that are on the same trajectory as the NTC. Circle them using the lasso tool, then select **No Amp** (yellow) to change calls so they are not used in the data analysis.

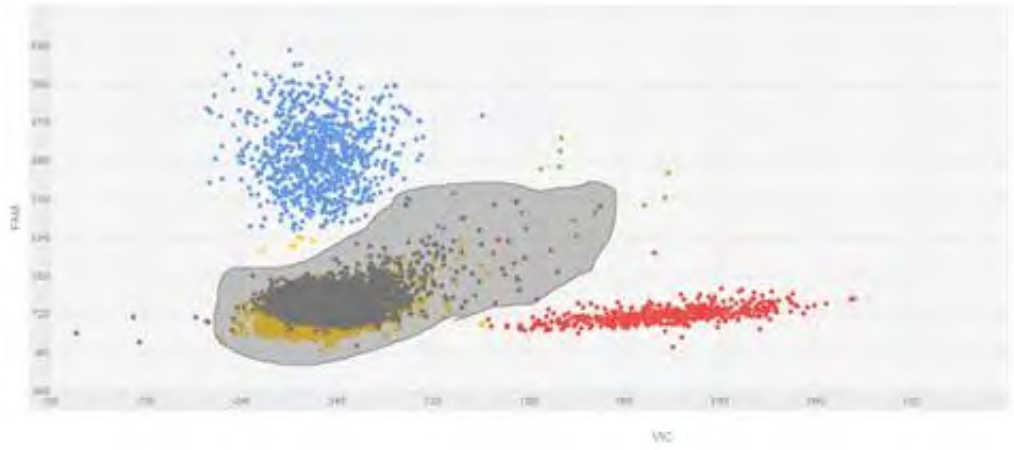

Figure 4 NTC and ambiguous data points selected with the lasso tool

5 Analyze the data *(continued)*

j. Review the data.

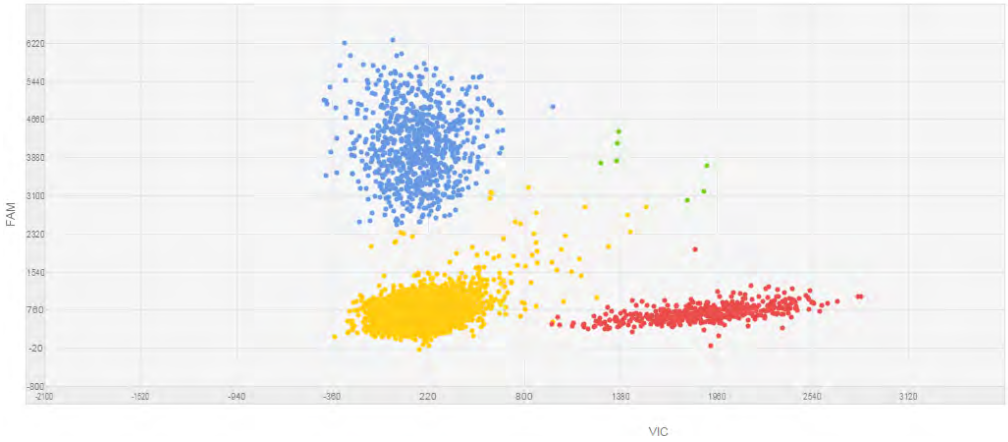

Figure 5 Sample chip after manual call

In the AnalysisSuite™ Cloud Software **See Results** tab, the averaged percent FAM™ allele value is displayed for each assay/sample tested (when replicate chips are given the same name). The values for each replicate can be viewed in the **Replicates** tab. For a 3-copy heterozygous sample, the % FAM™ value in the **Target/Total** column should be close to 33% or 66% for a given allele, equal to a 1:2 or 2:1 FAM™/VIC™ SNP allele ratio. Use the dye/allele information in Table 1 on page 3 to identify the duplicate allele in a sample. For example, if a sample has the diplotype *2A/*4x2, the 1846G>A assay (C_27102431_D0) will give a 66% FAM™ result as the duplicated 1846A variant allele is labeled with FAM™ dye.

Color	Assay	Sample ▲	Target/Total
	C__27102425_10	*1/*2Ax2	32.064%
	C__27102431_D0	*1/*4	49.355%
	C__27102431_D0	*2A/*4x2	67.063%

Figure 6 Example of the **Results** tab

6 **Export the results**

Export the results using the **Export** tab.

Troubleshooting

Observation	Possible cause	Recommended action
A 1:2 allele ratio was expected for a 3-copy sample, but the ratio was closer to 1:1	The restriction digest was incomplete and tandem copies of CYP2D6 were not separated.	Re-do the digest with either an increase in the incubation time or the amount of restriction enzyme used.
	The initial copy number evaluation was inaccurate and the sample is copy number 2 (or more rarely, copy number 4).	Review qPCR CNV data analysis and consider quantifying CYP2D6 copy number by dPCR.
	A hybrid allele with a CYP2D7 exon 9 conversion is present; For example, if a sample typed as *2x2/*10 is really *2x2/*36-*10, the *2 2850C>T and *10 100C>T assays will give 1:1 (2:2) ratios (as *36 contains the 100T and 2850C bases).	Check for the presence of a *36-like allele by running CNV experiments using assays that target CYP2D6 intron 2 (Hs04502391_cn) or intron 6 (Hs04083572_cn) in addition to the exon 9 assay.
A 1:2 allele ratio was expected for a 3-copy sample, but the ratio was closer to 1:3	The restriction digest was incomplete and tandem copies of CYP2D6 were not separated.	Repeat the digest with either an increase in the incubation time or the amount of restriction enzyme used.

Observation	Possible cause	Recommended action
A 1:2 allele ratio was expected for a 3-copy sample, but the ratio was closer to 1:3 *(continued)*	The initial copy number evaluation was inaccurate and the sample is copy number 4 and there are 3 copies of the duplicated allele.	Review qPCR CNV data analysis and consider quantifying CYP2D6 copy number by dPCR.
	A hybrid allele with a CYP2D7 exon 9 conversion is present; e.g. if a sample typed as *4/*10x2 is really *36-*4/*10x2, the *4 1846G>A assay will give a 1:3 ratio (as *36 does not contain the *4 1846A base).	Check for the presence of a *36-like allele by running CNV experiments using assays that target CYP2D6 intron 2 (Hs04502391_cn) or intron 6 (Hs04083572_cn) in addition to the exon 9 assay.
C___2222771_A0 does not give good cluster separation	The thermal profile may not be optimal for this assay.	Decrease annealing and extension temperatures by 4 °C.

Related documentation

PDF versions of this guide and the following publications are available at **thermofisher.com**.

Document	Pub. No.
Pharmacogenomics Experiments Application Guide	MAN0009612
QuantStudio™ 3D Digital PCR System User Guide	MAN0007720

Reference tables

Table 2 CYP2D6 alleles that can be duplicated

Haplotype[1]	Major SNPs	Enzyme function	Activity score
*1	Reference	Full	1.0
*2	2850C>T; 4180G>C	Full	1.0
*2A	**–1584C>G**; 2850C>T; 4180G>C	Full	1.0
*4	100C>T; **1846G>A**; 4180G>C	None	0
*9	2615_2617delAAG	Reduced	0.5
*10	**100C>T**; 4180G>C	Reduced	0.5
*17	**1023C>T**; 2850C>T; 4180G>C	Reduced	0.5
*35	–1584C>G; **31G>A**; 2850C>T; 4180G>C	Full	1.0

[1] Information from **pharmvar.org**

Table 3 Predicted CYP2D6 metabolizer phenotypes

Predicted metabolizer phenotype	Activity score	Genotypes
Ultra rapid	>2.0	More than two copies of functional alleles
Extensive	1.0–2.0	Two full or reduced function alleles or one full function allele plus either one nonfunctional or one reduced function allele
Intermediate	0.5	One reduced and one nonfunctional allele
Poor	0	No functional alleles

Table 4 CYP2D6 predicted phenotypes in individuals carrying 2 or 3 gene copies

	1X Full	1X Reduced	1X None	2X Full	2X Reduced	2X None
1X Full	Extensive	Extensive	Extensive	Ultra rapid	Extensive[1]	Extensive
1X Reduced	Extensive	Extensive	Intermediate	Ultra rapid[1]	Extensive	Intermediate[2]
1X None	Extensive	Intermediate	Poor	Extensive	Extensive[2]	Poor

[1] Heterozygous for full and reduced function alleles
[2] Reduced and no activity alleles

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Revision history: Pub. No. MAN0011114

Revision	Date	Description
B.0	27 June 2018	• Updated compatible thermal cyclers. • Changed centrifuge speed unit from rpm to relative centrifugal force (x *g*). • Added QuantStudio™ 3D Digital PCR 20K Chip Kit v2 to list of required materials not supplied. • Explained purpose of restriction digest. • Adjusted Reaction Mix ingredient volumes to account for overage. • Clarified no–template control step of data analysis. • Updated for general style, formatting, and branding.
A.0	12 August 2015	New document.