Takara Bio USA, Inc.

SmartChip ND™ Real-Time PCR Cycler and Software User Manual

Cat. Nos. 640290, 640023
(092924)

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I.   Safety and Regulatory Compliance

Location	Mark	Applicable standards
U.S. and Canada		CAN/CSA-C22.2, No. 61010-1-12 + GI1 + GI2 (R2017) + A1 UL 61010-1:2012 R7.19
		This device complies with part 15 of the FCC Rules. Operation is subject to the following two conditions: (1) This device may not cause harmful interference, and (2) this device must accept any interference received, including interference that may cause undesired operation. This Class A digital apparatus complies with Canadian ICES-003. Cet appareil numérique de la classe A est conforme à la norme NMB-003 du Canada.
European Union		EN IEC 61326-1 (2021) Group 1 Class A EN IEC 61000-4-2 (2008) EN IEC 61000-4-3 (2006) + A1 + A2 EN IEC 61010–1:2010 EN IEC 61010–1:2010/AMD1:2016

II. Introduction

A. About this Manual

This manual provides instructions for the installation and use of the SmartChip ND Real-Time PCR Cycler (referred to as the SmartChip ND cycler) and SmartChip® qPCR Software, which is a component of the SmartChip ND Real-Time PCR System (Cat. No. 640290). For operation of the SmartChip ND dispenser component (Cat. No. 640291) of the SmartChip ND Real-Time PCR System, please refer to the SmartChip ND and qPCRStudio Software User Manual.

Please follow directions and pay attention to information designated as follows:

NOTE: Helpful ancillary information

IMPORTANT: Information on proper system operation

WARNING: Instructions for safe operation of Takara Bio instruments

B. Technical Support

Review the information in this manual thoroughly before using the equipment. Also review documentation supplied with any accessory equipment you are using. If you require technical support, you can contact your authorized Takara Bio service technician or Takara Bio directly at technical_support@takarabio.com.

III. Specifications and Requirements

The SmartChip ND cycler performs and analyzes real-time PCR using nanowell chips that contain samples, PCR reagents, and PCR assays.

Table 1. SmartChip ND cycler specifications and requirements.

Category	Specification
Dimensions	Thermal cycler: 70 cm wide, 60 cm deep, 50 cm high Computer/monitor: 60 cm wide, 50 cm deep, 50 cm high
Space requirements	Clearance around the SmartChip ND cycler of at least 100 mm wide by 75 mm deep, with a minimum overhead clearance of 54 mm
Weight	Thermal cycler: 57 kg Computer/monitor: 25 kg
Power supply requirements	100–240 VAC, 8.0/4.0 A (47–63 Hz) power outlets within 2 m Main supply voltage that does not deviate by more than 10% from nominal Will tolerate an overvoltage impulse as per IEC60346-4-443, category II
Fuse type	5 x 20-mm cartridge fuses compliant with appropriate safety ratings 100–120 VAC use 6.3 A/250 VAC time-lag fuse 200–240 VAC use 6.3 A/250 VAC time-lag fuse
Air source	Clean dry air (–40°C dewpoint) at 6.9–8.3 bar (100–200 psi) pressure Tubing and air inlet comprising a push-fit connector for 4-mm diameter plastic tubing If appropriate house air is not available, the following can be used: Compressed High-Purity Nitrogen: North America Cylinder Size K, Europe/Asia Cylinder Size 50 (replace canister when pressure drops below 500 psi [34 bar]) A third option is an oil-free air compressor with a high-quality mist filter Please contact Takara Bio customer support for an approved list
Air inlet type	Regulator output: either suitable for 4-mm OD urethane tubing, or with M5 or 1/8 NPT female port
Environmental conditions	Ambient temperature: 15–30°C Relative humidity, non-condensing: 30–70% Altitude: <2,000 m from sea level Pollution degree: 2 or less

A. SmartChip ND Cycler Safety Warnings

- Except as described in this manual, all maintenance and repair should be performed by your qualified Takara Bio service technician.
- Do not remove the outer covers of the SmartChip ND cycler. Doing so may expose the operator to dangerous electrical and mechanical hazards.
- Do not operate the SmartChip ND cycler if the power cord is damaged.
- If the SmartChip ND cycler is not used as specified by the manufacturer, the protection provided by this equipment may be impaired.
- Use of the SmartChip ND cycler may cause exposure to toxic or biohazardous chemicals, thereby presenting a hazard. Appropriate personal protective equipment (PPE), which should at a minimum include gloves, eye protection, and lab coat, should be worn at all times in the laboratory.
- The SmartChip ND cycler should only be operated inside an appropriate building, not outside or in wet environments.

B. Setup and Installation

Your Takara Bio Service Engineer will unpack and install your SmartChip ND cycler and explain the basic operation of the system.

This manual provides information about installing and using the SmartChip qPCR Software for control of the SmartChip ND cycler and/or analysis of data generated by the SmartChip ND cycler.

IV. System Description

A. The SmartChip ND Cycler Includes:

- Personal computer with monitor
- qPCR Software (required for instrument operation)
- IEC 320 C13 power cord
- 5 m of 4-mm diameter plastic tubing to connect the instrument to your air supply
- SmartChip ND Cycler Installation Kit

B. Back View of the SmartChip ND Cycler

Figure 1. Back view of the SmartChip ND cycler.

The power switch and power cable connection are on the back of the SmartChip ND cycler. The universal serial bus (USB) connector, air inlet, and thermal diagnostic port are on the back left side of the instrument.

- The USB connector connects the instrument to the PC via the provided cable.
- The round air inlet port accepts 4-mm diameter plastic tubing with an inlet pressure ≤8.3 bar (120 psi)

IMPORTANT: The air pressure is displayed in the bottom right corner of the SmartChip qPCR Software windows. If the pressure goes outside the normal range, it will be displayed in red, and an error message will appear. When values are outside of normal, first check the air pressure gauge on the regulator and adjust it if necessary. If the supply pressure is 100–120 psi and the problem persists, contact Takara Bio technical support.

V. SmartChip Technology

SmartChip technology distinguishes Takara Bio's PCR platform from other systems. Each SmartChip has a 72 x 72 array of nanowells and can accommodate up to 5,184 150- or 250-nl real-time polymerase chain reactions (PCR) reactions in a single run. There are three types of chips:

- **SmartChip Custom Panels 150 nl:** contain PCR assays dispensed into the chips at Takara Bio. You add your experimental samples and PCR reagents to these chips using the SmartChip ND.

- **SmartChip MyDesign Chips 150 nl (blank):** You add experimental samples, PCR reagents, and PCR assays to these chips using the SmartChip ND.

- **SmartChip MyDesign Chips 250 nl (blank):** You add experimental samples, PCR reagents, and PCR assays to these chips using the SmartChip ND.

Gene Expression Analysis

For mRNA expression analysis, the SmartChip ND Real-Time PCR System has been tested with cDNA from human brain (Cat No. 637242), human lung (Cat No. 637206), human placenta (Cat No. 637208), and a pooled reference sample (Cat No. 637260) with SmartChip TB Green® Gene Expression Master Mix (Takara Bio, Cat. No. 640210).

NOTE: Other master mixes have not been validated for performance and are not supported. Precipitate may be observed in the SmartChip TB Green Gene Expression Master Mix. This precipitate does not affect the performance of the kit. The precipitate can be dissolved easily by warming to room temperature and mixing for a few minutes. Ensure that the precipitate is fully dissolved before use.

- **SmartChip MyDesign Chips** are provided empty. Use the SmartChip ND to add both PCR assay(s) and experimental cDNA or DNA sample(s) in any of the 14 configurations supported for expression analysis.

- **SmartChip Custom Panels** are designed for targeted expression analysis; they are custom manufactured to your specifications. Choose from commercially available assays or have your assay design of choice predispensed into a chip. We offer SmartChip Custom Panels for expression analysis in six different configurations, designed for analysis of 3–96 samples using 384–12 assays in quadruplicate.

SNP Genotyping

The SmartChip system can be used for SNP genotyping using TaqMan SNP Genotyping Assays (Thermo Fisher Scientific) with Minor Groove Binder (MGB), BHQ*plus* assays (LGC Biosearch), rhAmp assays (IDT), and, with minor modifications, KASP assays (LCG Genomics). Please contact [field support](#) if you have questions about implementing any of these assays.

- **SmartChip MyDesign Chips** are provided empty. Use the SmartChip ND to add SNP genotyping assays, reagent master mix, and experimental DNA samples in any of the 14 configurations supported for SNP genotyping analysis.

- **SmartChip SNP Genotyping Panels** contain TaqMan SNP Genotyping Assays that you have shipped to Takara Bio. They are available in eight configurations to analyze 12–384 samples using a single replicate with 384–12 TaqMan SNP Genotyping Assays. We dispense the assays and send the resulting SmartChip SNP Genotyping Panels with a CD containing files with assay locations, thermal profile, and data analysis parameters. Use the SmartChip ND to add your experimental DNA samples to the SmartChip Panel.
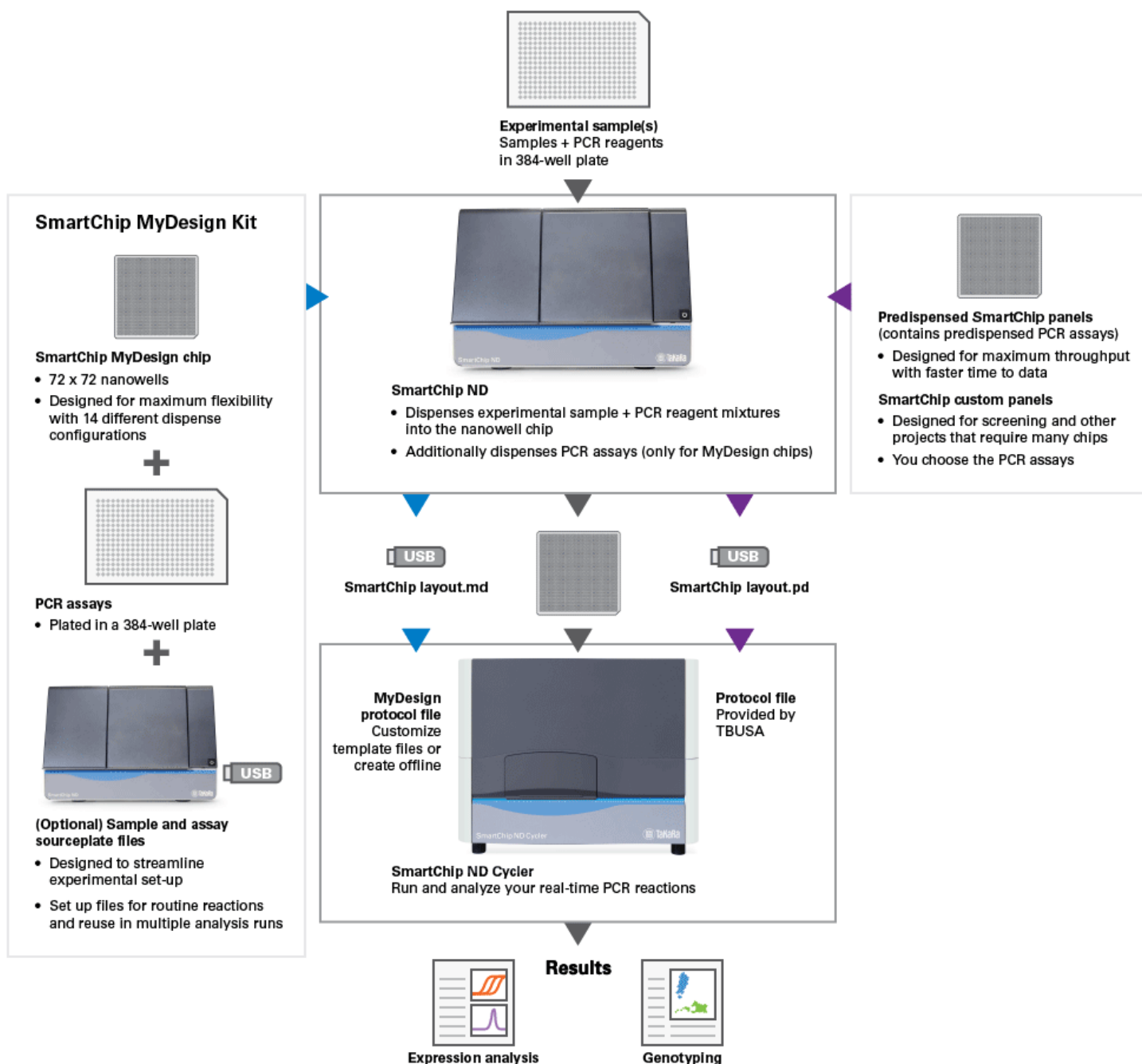
Figure 2. Overview of the SmartChip ND Real-Time PCR System workflow.

VI. SmartChip qPCR Software: Files Needed by the SmartChip ND Cycler

The SmartChip ND Real-Time PCR System (the SmartChip ND dispenser and SmartChip ND Cycler) needs information about your experimental samples, your PCR assays, and how to run and analyze the PCRs. This information comes from different sources depending on whether you are using SmartChip MyDesign Chips that are supplied empty or SmartChip Custom Panels that contain PCR assays pre-dispensed at Takara Bio, and whether you are interrogating a set of samples or PCR assays for the first time or in a subsequent experiment. Table 2 below outline where these files come from.

Table 2. File sources for chip and experiment types.

SmartChip type	Sample names and locations	PCR Assay names and locations	Thermal cycling information	Data analysis parameters
SmartChip MyDesign Chip	SmartChip Layout file from the SmartChip ND dispenser		Use or modify a MyDesign Protocol file installed with the qPCR software or use a protocol file that you previously modified and saved.	
Pre-dispensed SmartChip Custom Panel	SmartChip Layout file from the SmartChip ND		Open the Protocol file supplied on the CD shipped with the SmartChip Custom Panel	

VII. SmartChip qPCR Software: Basics

For instructions on how to install the SmartChip qPCR Software, see Appendix A: Installing SmartChip qPCR Software.

SmartChip qPCR Software controls the SmartChip ND Cycler and analyzes expression of genotyping data. Click on the **Actions** menu to switch between DNA binding dye-based expression, probe-based expression, and genotyping analysis modes.

Data analysis may be performed on the SmartChip ND Cycler computer or offline on a separate computer.

To start using the SmartChip qPCR Software on the SmartChip ND Cycler computer, open the software by selecting the SmartChip qPCR shortcut on the desktop. A window similar to the example below (Figure 4) will be displayed. Actual screen displays will vary based on the application being used and whether you are performing data analysis with a SmartChip ND Cycler attached or offline.
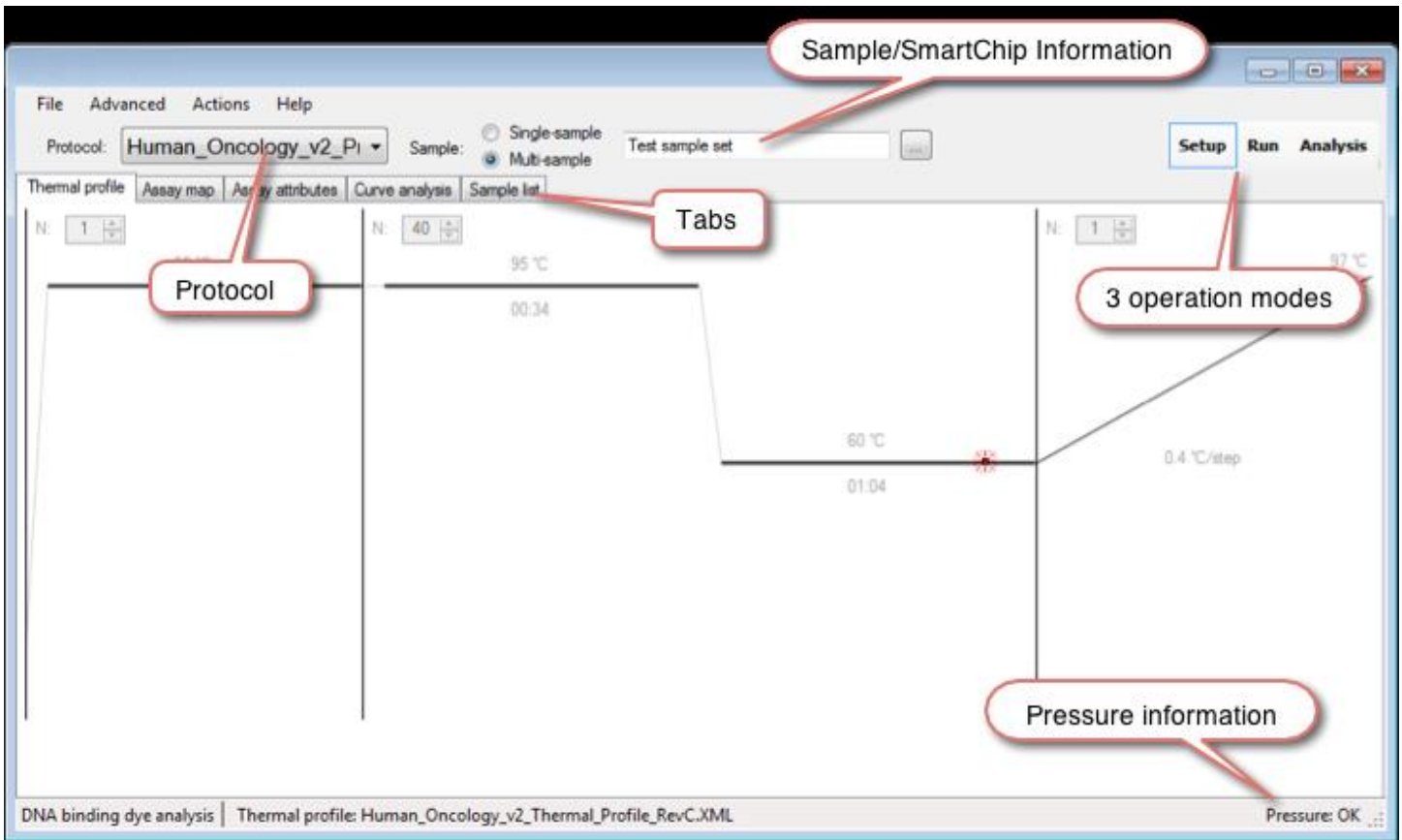
Figure 3. SmartChip qPCR Software window.

A. Convenient qPCR Software Features

Right click: qPCR Software makes use of right-clicks to offer context-sensitive menu options. We encourage you to try right-clicking while using the software to access numerous features.

Column display: On several data and analysis screens, you can choose the information you want to display by right-clicking anywhere within the table (not in the column headers) and choosing **Columns**. This will open a *Selected fields* dialog box (Figure 5) with check boxes next to the information categories. Just click to check or uncheck the columns that you want to display or hide. The selection is remembered the next time the program starts.
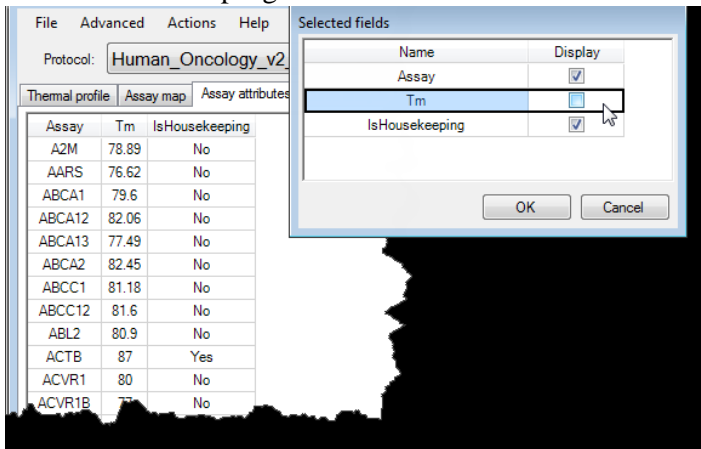
Figure 4. Selected fields dialog box.

Select and Copy: Right-clicking in many data tables also offers the option to select or copy the data as described in Table 3 below. (Also see Section XII.E.)

Table 3. Options to select or copy data from data tables.
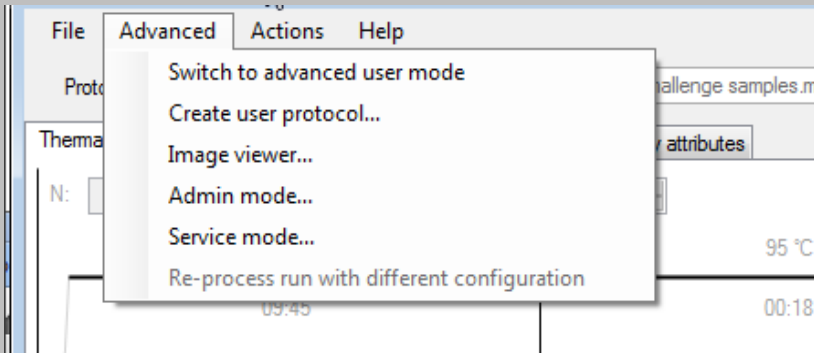
Field	Function
Copy	Copy all selected rows to the clipboard
Copy all	Copy the entire table, including the header row, to the clipboard
Select all	Select all rows
Columns	Launches the Select fields window to customize the columns to be displayed

Scroll: You can often scroll in the qPCR software using the arrow keys on your computer keyboard or the scroll wheel of your mouse.

Zoom/Pan: In many of the data analysis displays, you can zoom/pan using the scroll wheel of your mouse. Right-click to undo. (Also see Appendix F)

B. Major Attributes of the SmartChip qPCR Software Display

Table 4. SmartChip qPCR Software display: drop-down menus and protocols.

Component	Functions
Drop-down menus	**File**: Open, save, or export data, depending on the mode (i.e., **Setup**, **Run**, or **Analysis**) **Advanced**: Some of the operations available are specific for the active mode of operation. Switch between "standard GUI", which displays only the information that is most frequently accessed and "advanced user mode", which displays all of the data collected during real-time PCR. Enter or leave "Admin mode". Enter administrator mode by selecting **Admin mode…** from the drop-down menu and typing the password (case-sensitive) from your SmartChip qPCR Software CD. You need to be in Admin mode to create new user protocols and you must exit Admin mode to run real-time PCR experiments. **Actions**: Switch between DNA Binding Dye-Based Expression, Probe-Based Expression, and Genotyping Analysis modes. **Help**: Access software information, license agreement, this user manual, and quick guides.
Protocol	Select the appropriate protocol for the type of chip you are using.

SmartChip MyDesign Chip: Use one of the protocols installed with the qPCR software, modify one of these generic protocols for your experiment, or select a protocol you have already created and saved.

SmartChip Custom Panel: Run the executable file from the CD provided with your chip to install protocol subfiles into their proper locations.

Table 5. SmartChip qPCR Software display: sample/SmartChip information, modes of operation, tabs, config files, and pressure.

Component	Functions
Sample/SmartChip information	Depending on the Protocol File you have selected, you will see different choices here. **SmartChip MyDesign Chip** **Layout File:** Select the `SmartChip layout.md` file created by the SmartChip ND when the chip was filled. **Pre-dispensed SmartChip Panels or SmartChip Custom Panels** **Single-sample:** Select **Single-Sample** and enter the sample name in the box when you are using a SmartChip Panel to analyze a single sample. **Multi-sample:** Click **Multi-sample** when you are using a SmartChip Custom Panel that contains samples dispensed into the chip using the SmartChip ND. Then select the `SmartChip layout.pd` file created by the SmartChip ND.
Modes of operation	**Setup:** Define or select a protocol to run the real-time PCR, and to enter sample information, or choose a SmartChip layout file that defines samples/assays on the chip. **Run:** Control the instrument and perform an expression analysis or genotyping run. **Analysis:** Perform and view data analyses.
Tabs	Tabs are specific to the active mode of operation (i.e., **Setup**, **Run**, or **Analysis**).
Config files	After a run, the bottom left corner displays the Tab name and config files in use. If an existing *.raw file is opened, the bottom left corner displays "from saved run".
Pressure	The bottom right corner displays the supply pressure. When the chip holder is in scan mode, the bottom right corner will display the Cycler chamber pressure and airflow.

C.   Importing and Exporting Protocols
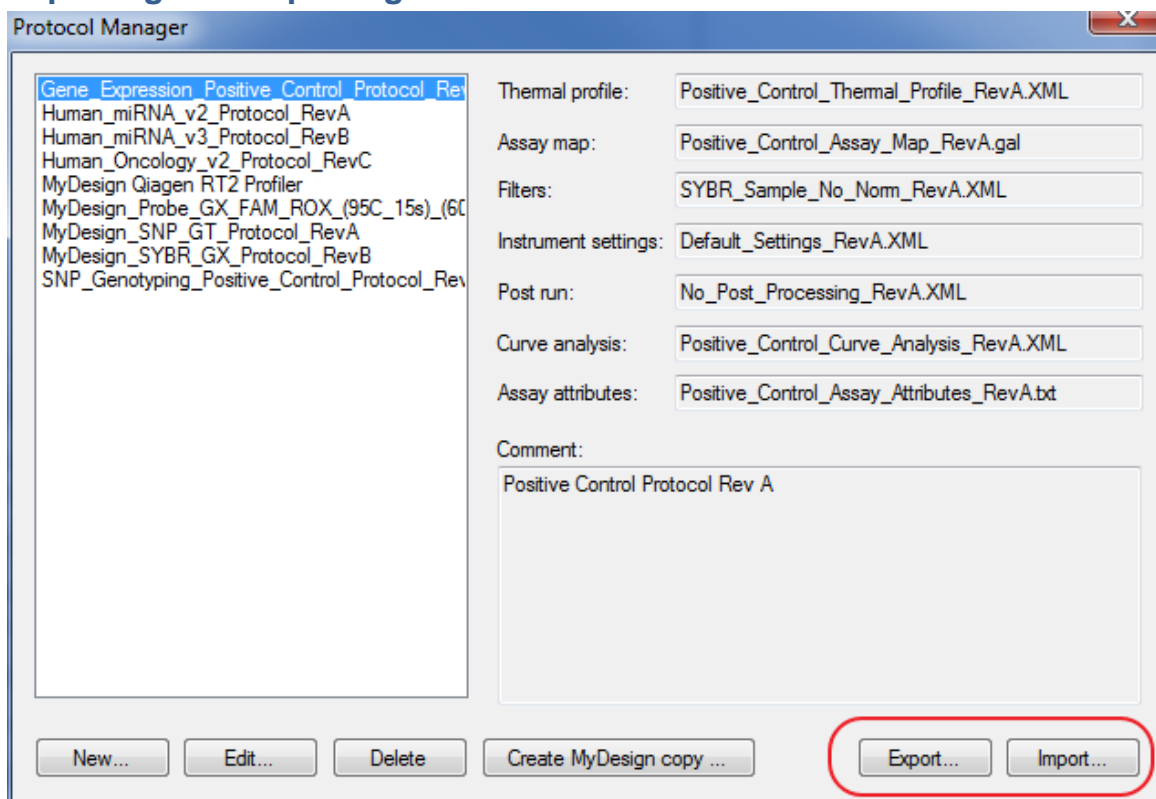
Figure 5. Importing and exporting protocols using the Protocol Manager.

When you are in administrator mode, you can import and export protocols using the Protocol Manager (Figure 5). Exporting a protocol writes all of the subcomponents that comprise a full protocol (i.e., Thermal profile, Assay map, Filters, etc.) to a single .xml file. The exported protocol file can then be imported into qPCR software on a different computer. This makes it easy to transfer all necessary files between computers, for example to analyze data using a computer that is not connected to the SmartChip ND Cycler.

When a protocol file is imported, the qPCR software will place all of the subcomponent files in appropriate folders with their original file names. If files with the same names are found on the target computer, the import function will compare the file contents to determine if they are identical or not. If the files have different content, you will have to make a choice as shown in Figure 6 below.
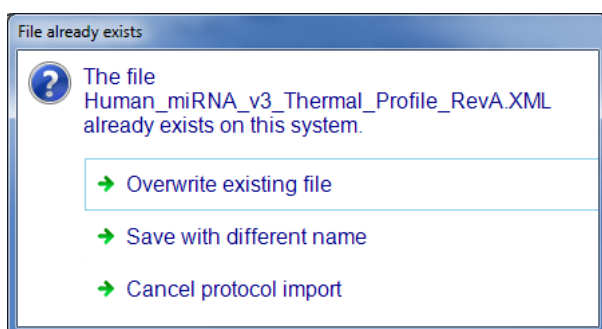
Figure 6. Subcomponent file comparison options. If you choose "Overwrite existing file", any other protocols that include the component you overwrite will be affected. If you choose "Save with different name", a file-save dialogue box will open and you can type in a new name. If you choose "Cancel protocol import", your protocol will not be imported.

VIII. SmartChip qPCR Software: SmartChip ND Real-Time PCR Quick Guide

This flow chart (Figure 7) shows how fast and easy it is to initiate a typical SmartChip run on the SmartChip ND cycler. Simply turn on your computer, open the SmartChip qPCR Software, and follow the steps below. In just a matter of minutes, your SmartChip run will be underway.

Choose the action for the cycler:
- DNA binding dye-based expression
- Probe-based expression analysis
- Genotyping analysis

Program the SmartChip ND cycler for your experiment:
- Select, import, modify, or load a protocol
- Select or enter sample/assay information

Run your reaction:
- Place the SmartChip MyDesign Chip or custom panel into the cycler
- Select a destination folder to store the run data
- Select [OK] to start the run

Analyze the results using qPCR software

Figure 7. SmartChip ND Real-Time PCR flow chart.

IX. Expression Analysis Procedures: Overview

NOTE: These instructions cover real-time PCR that uses both DNA binding dye (e.g., green intercalating dye) or probe hydrolysis [e.g., TaqMan Assays (Thermo Fisher Scientific) or PrimeTime Assays] for detection.

This is an overview—for detailed procedures, start with "Expression Analysis Procedures: Setup Mode" (Section X).

A. Starting the SmartChip ND Cycler and qPCR Software

1. Turn on the SmartChip ND cycler using the power switch on the back left-side of the instrument.
2. From the computer, open the SmartChip qPCR Software. It will initialize the SmartChip ND cycler. The status of the initialization process will be shown in text at the top right of the qPCR Software window. It may take several minutes before the instrument is ready.

B. Entering Information About Your Experiment

1. Pull down the **Actions** menu and, if necessary, switch to **DNA binding Dye-Based Expression Analysis** or **Probe-Based Expression Analysis**. This determines which protocols will be visible in the protocol pull-down menu.
2. Select Setup mode by clicking on the *Setup* tab in the top right corner.
3. In the drop-down menu labeled **Protocol** in the top left corner, select the appropriate protocol for your chip (see Figure 8):
   - **SmartChip MyDesign Chip**
     - Select one of the expression analysis protocols installed with the qPCR software or an appropriate saved protocol from a previous experiment.
     - Customize one of the expression analysis protocols installed with the qPCR software. See Section X.E for instructions.
   - Pre-dispensed **SmartChip Custom Panel**: Double-click the `setup.exe` file on the CD sent with your chip to load files that describe your chip into the qPCR software.
   - Off-the-shelf **SmartChip Panel:** Protocol files for predefined SmartChip Panels are installed with the qPCR software. Choose the protocol for the SmartChip Panel you are running.
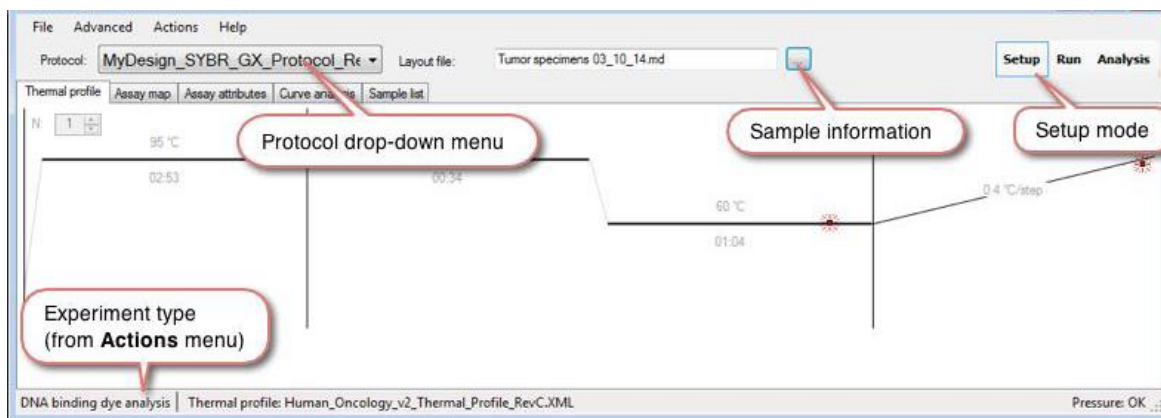
Figure 8. Selecting the appropriate protocol for your chip.

4. Enter your Sample or Sample/PCR Assay information:

- **SmartChip MyDesign Chip:** In the "MyDesign File" field, select the `SmartChip layout.md` file created by the SmartChip ND when the chip was filled.
- Pre-dispensed **SmartChip Custom Panel:**
  - o If you loaded more than one Sample/PCR Reagent Mixture into your chip, click the button for **Multi-sample**. Click the [...] button and, in the *Open* dialog box, select the `SmartChip layout.pd` file created by the SmartChip ND when you added Sample/PCR Reagent Mixtures to your chip.
  - o If your SmartChip Panel contains just one sample loaded using the SmartChip Single Sample NanoDispenser, click the **Single-sample** radio button. Then type your sample name.
- Off-the-shelf **SmartChip Panel:** Click the button for **Single-sample**. In the box, enter the name of your experimental sample.

C.   Starting the PCR Run

Select Run mode to insert the sealed SmartChip Panel or MyDesign Chip into the SmartChip ND Cycler. Start the run using the procedure below.

1. If you entered Admin mode to create a new protocol, be sure to exit Admin mode in order to run your PCR. Note also that Protocol and SmartChip information must be complete before the run will start.
2. Select Run mode by clicking on the [Run] button in the top right corner of the qPCR software interface.
3. Click the [open-drawer] icon) to open the SmartChip ND Cycler drawer.

IMPORTANT: Never open the door manually.

4. The *Chip Info* (Figure 9) dialog box will open. Enter "Chip", "User", and other requested information. This information will be included in the analysis files and Summary report.

To use the barcode reader to enter your chip number, place your cursor in the "Chip" entry field. (You should see a blinking cursor in the box.) Point the barcode reader at the 2-D barcode symbol on the bottom of the chip and pull the trigger to enter the SmartChip code into the *Chip Info* window. (The code is also written in human readable form so that you can double-check that it is entered correctly.)
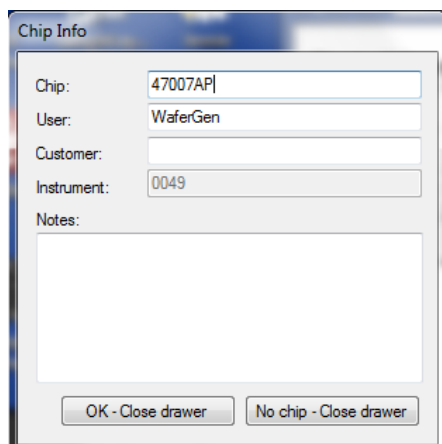
Figure 9. Chip Info dialog box.

5. Peel away the blue backing from your sealed SmartChip Panel or MyDesign Chip to expose the adhesive on the clear seal. Place it in the SmartChip ND Cycler with the beveled edge in the lower right corner as shown in Figure 10. Make sure the chip lays flat in the SmartChip holder.
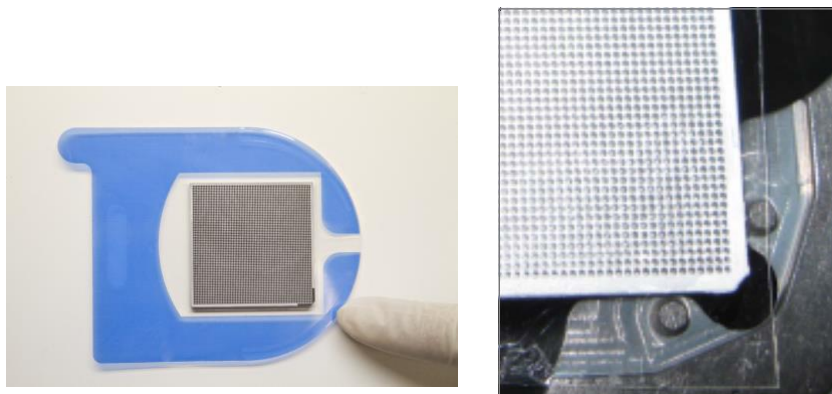
Figure 10. Peel the blue backing from the sealed chip and place it in the SmartChip ND cycler.

IMPORTANT: Always position chips so that when viewed from above, the beveled corner is on the lower right side.

6. Click [OK-With Chip] to close the drawer of the SmartChip ND Cycler.

7. The [Start Run] button) will turn green. Press this button.

8. In the *Choose data folder* dialog box (Figure 11) that appears, select a destination folder and file name options for your run data and click [OK].
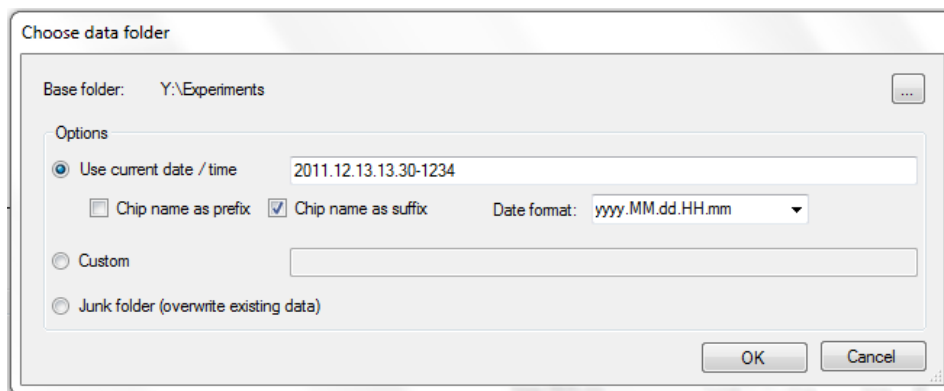
Figure 11. *Choose data folder* **window.**

IMPORTANT: The thermal cycling of the chip will start only after you have selected a destination folder for the run data and pressed [OK].

9. The run will start. An estimated duration will be displayed in the top center of the display.

10. When the run is complete, remove the chip from the SmartChip ND Cycler and dispose of it in a covered waste container. To minimize carryover contamination risk, do not remove the adhesive film.

X. Expression Analysis Procedures: Setup Mode

Setup mode defines the SmartChip ND Cycler parameters needed to perform qPCR with the SmartChip Panel or MyDesign Chip. Parameters include a protocol and a SmartChip layout file (or sample information typed into the **Sample** box for off-the-shelf SmartChip Panels.

A. Overview of Drop-Down Menus in Setup Mode for Expression Analysis
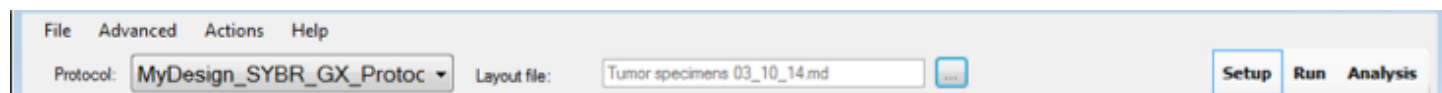
Figure 12. Setup mode top menu bar.

Table 6 describes the selections available from the Setup mode top menu bar (Figure 13).

Table 6. Drop-down menu options in Setup mode for expression analysis.

Menu	Options
File	**Open file**: the choice of file type is determined by which tab is selected.
	Save file under selected tab. You must be in Admin mode to save files.
	Run info displays file, chip, user, customer, and instrument information, as well as notes.
Advanced	**Create user protocol** can be used to modify a protocol's curve analysis settings and save them.
	Image viewer is designed for visual inspection of the data. For more information, see "Image Viewer" in Section XVII.
	Admin mode should be used by trained personnel only. It is used to save changes to curve analysis settings and to modify protocols using the **Protocol Manager**. Changing any of these settings can affect your experimental results. The password to enter Admin mode is printed on the qPCR Software CD.
	Service mode is designed for Takara Bio staff to configure advanced SmartChip ND Cycler settings.
	Switch to advanced user mode to display all of the data collected during real-time PCR or **Switch to standard GUI** to display only information that is most frequently accessed.
Actions	Switch between **DNA binding Dye-Based Expression**, **Probe-Based Expression**, or **Genotyping Analysis** modes.
Help	**About** provides qPCR Software version, copyright, and license agreement information.
	Open User Manual opens this User Manual in pdf format.
	Setting up a run opens a Quick Guide for setting up a run.
	Saving run data opens a Quick Guide for saving run data.
	Loading an existing chip run opens a Quick Guide for loading an existing chip run.
	Reprocessing a chip run opens a Quick Guide for reprocessing an existing chip run.

B.   Overview of Tabs in Setup Mode for Expression Analysis

Each tab in Setup mode corresponds to a file with instructions for a specific SmartChip ND Cycler feature or function. qPCR Software accesses this information through SmartChip Layout files and Protocol files. Table 7 below shows the information organization for different types of chips. Tabs shown in bold text are visible in the standard GUI. Those not shown in bold are displayed only in advanced user mode.

Table 7. Overview of tabs in Setup mode for expression analysis.

Tab	SmartChip MyDesign Chips	SmartChip Custom Panels
Thermal Profile	Protocol	
Filters		
Instrument Settings		Protocol
Curve Analysis		
Post Run Settings		
Assay Map		
Assay Attributes		
Sample List	`SmartChip layout.md`	`SmartChip layout.pd`

C.   Starting the SmartChip ND Cycler and qPCR Software

1. Turn on the SmartChip ND Cycler using the power switch on the back left-side of the instrument.
2. From the computer, open the SmartChip qPCR Software by selecting the **SmartChip qPCR** icon on the desktop, or via **Start > Program Files > qPCR > PCR Instrument**. The software will initialize the SmartChip ND Cycler and show instrument status at the top right of the window. This may take several minutes.

D.   Setup Mode Instructions for Expression Analysis

1. Pull down the **Actions** menu and, if necessary, switch to **DNA binding Dye-Based Expression Analysis** or **Probe-Based Expression Analysis**. (This determines what protocols will be visible in the "Protocol" pull-down menu.)
2. Select Setup mode by clicking on the *Setup* tab in the top right corner.

IMPORTANT: The rest of the instructions for setting up a SmartChip ND Cycler run are different depending on which type of SmartChip nanowell chip you are using.

1. SmartChip MyDesign Chip

If you are running a SmartChip MyDesign Chip that was supplied empty from Takara Bio and contains PCR reactants dispensed into the chip at your facility, follow these instructions.

a. Click the icon next to "Protocol" to pull down the menu, then select an appropriate Protocol file.

- Choose one of the default expression analysis protocols installed with the qPCR software.
- Select an appropriate saved protocol from a previous experiment (Figure 13).
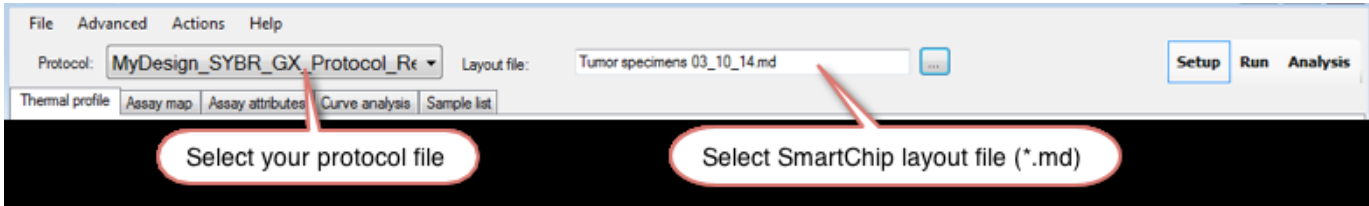
Figure 13. Selecting a saved protocol from a previous experiment for your SmartChip MyDesign Chip.

- If you need to change any of the cycling or analysis parameters in the available protocols, you will need to create a new protocol and select it (see Section X.E).

b. Click the button labeled [**…**] to open the *Open* dialog box. Navigate to the location of the SmartChip Layout file (*.md extension) from the SmartChip ND for your chip. (You will likely access the SmartChip Layout file on a USB memory stick or across a network.) Select the file and click [Open] to accept your choice and close the window.

c. If you entered Admin Mode in order to create a new protocol, be sure to exit Admin mode by deselecting **Admin mode…** under the **Advanced** menu in order to run your SmartChip PCR.

2. (Pre-dispensed) SmartChip Custom Panel

If you are running a SmartChip Custom Panel containing a set of PCR Assays that Takara Bio dispensed into a SmartChip Panel for you, follow these instructions.

a. Insert the CD that was sent with your SmartChip Custom Panel into the computer that controls the SmartChip ND Cycler.

b. Run the setup program from the CD to load your SmartChip Panel Protocol File into the qPCR Software—just double-click on the file named setup.exe.

c. Enter your sample information:

- If your SmartChip Panel contains multiple samples that were loaded using the SmartChip ND, click the **Multi-sample** radio button next to "Sample". Then click the button labeled [**…**] to open the **Open** dialog box and navigate to the location of the SmartChip Layout file (*.pd extension) from the SmartChip ND for your chip. (You will likely access the SmartChip Layout file on a USB memory stick or across a network.) Select the file and click [Open] to accept your choice and close the window (Figure 14).

Figure 14. Selecting the protocol for your pre-dispensed SmartChip Custom Panel.

- If your SmartChip Panel contains just one sample loaded into the panel using the SmartChip Single Sample NanoDispenser, click the **Single-sample** radio button. Then type in the name of the sample you loaded onto your SmartChip Panel.

E. Creating a New Protocol for SmartChip MyDesign Chips (Expression)

There are two ways to create new protocols. If you need to simply modify the curve analysis settings, you should use Method 1: Creating a User Protocol (see below). If you also want to modify other aspects of the protocol, you will need to use Method 2: Protocol Manager (starts on page 31). For either method, you will need to enter Admin mode.

1. Method 1: Creating a User Protocol

a. From the *Curve Analysis* tab (Figure 15), adjust your analysis settings as needed. In order to save your adjusted settings, you will need to enter Admin mode (next step).
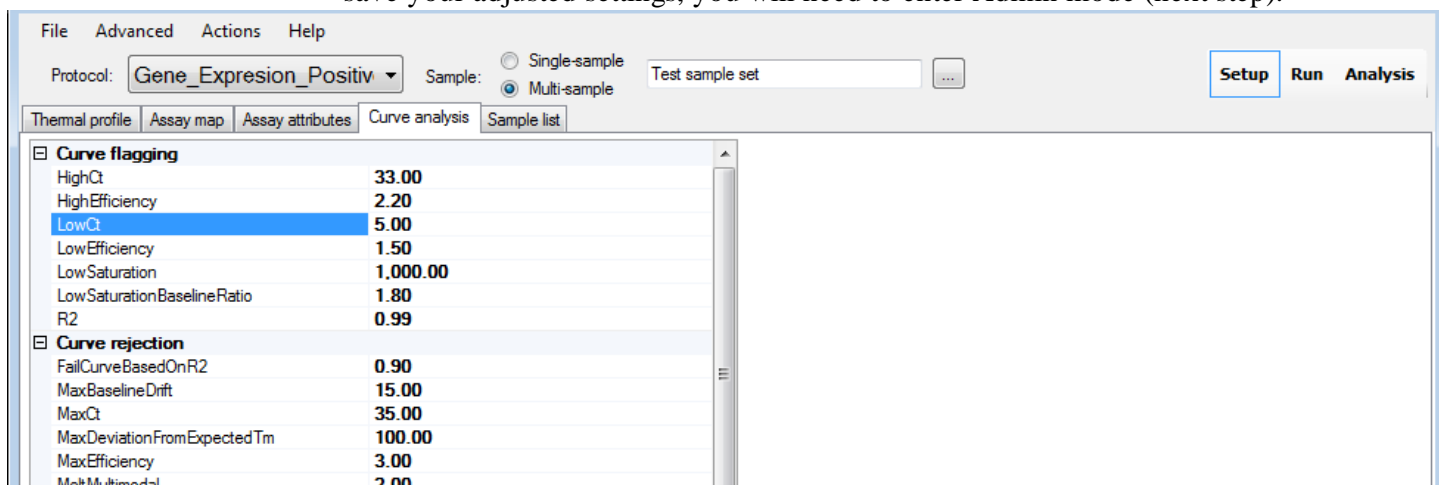
Figure 15. Curve Analysis tab.

b. Enter Admin mode: From the **Advanced** drop-down menu, select **Admin mode…** In the window that appears, type the password (case-sensitive) from your SmartChip qPCR Software CD. If you cannot locate your password, contact Takara Bio technical support (technical_support@takarabio.com) for help.

> **NOTE:** Once you are in Admin mode, you can change your password if desired.

c. Save your adjusted curve analysis settings: From the **File** drop-down menu, select **Save curve analysis settings**. In the new window that opens, choose a logical name and file location for the settings and click [Save].

d. Exit Admin mode: From the **Advanced** drop-down menu, deselect **Admin mode…** (the check mark will disappear.)

e. From the **Advanced** drop-down menu, select **Create user protocol…** In the window that opens (Figure 16), choose the protocol that you want to modify with your newly created curve analysis settings. In the next pull-down menu, choose the curve analysis settings file you created in Step c.
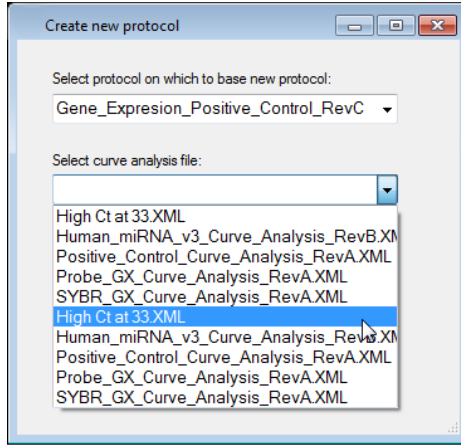
Figure 16. Create new protocol window.

f. Click the [Generate new protocol] button and save the modified protocol file with a logical name (Figure 17).
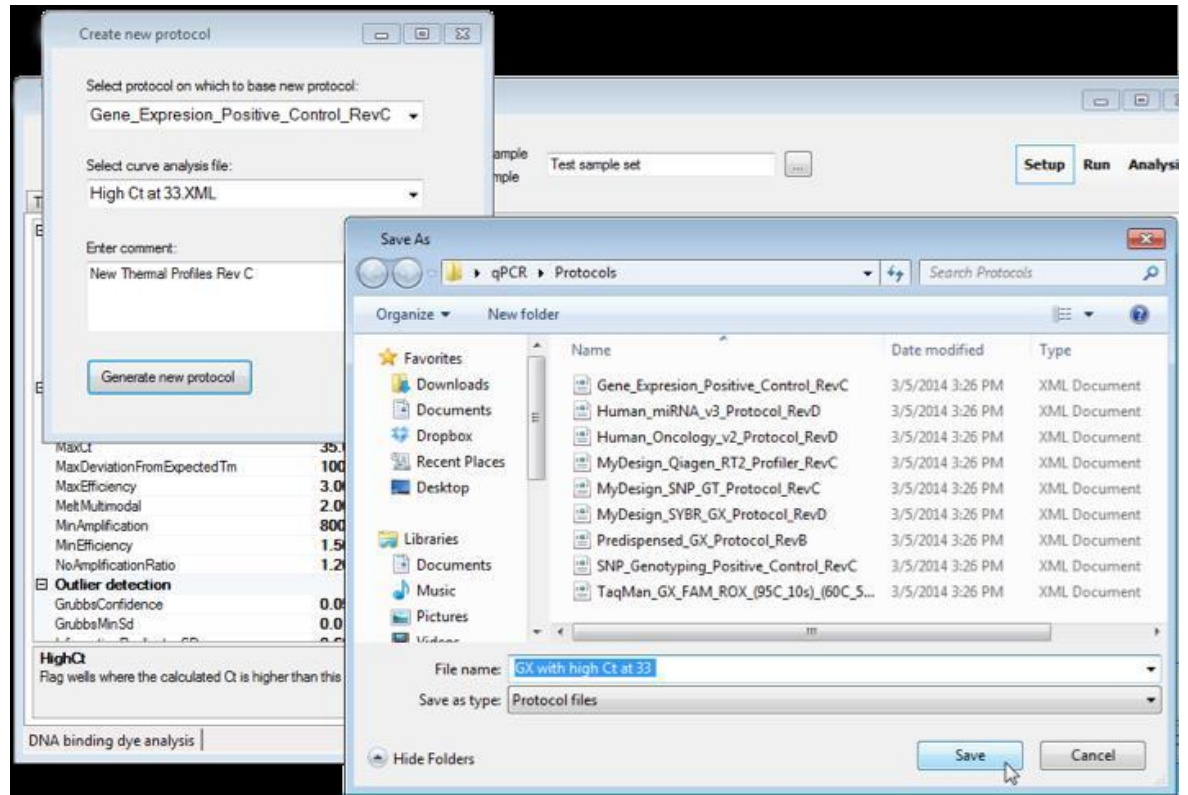
Figure 17. Saving your modified protocol.

2. **Method 2: Protocol Manager**

NOTE: Protocols can be deleted using the Protocol Manager.

a. Enter Admin mode: From the **Advanced** drop-down menu, select **Admin mode…** In the window that appears, type the password (case-sensitive) from your SmartChip qPCR Software CD. If you cannot locate your password, contact Takara Bio technical support for help.

NOTE: Once you are in Admin mode, you can easily change your password if desired.

b. Create a copy of one of the default MyDesign protocols using the Protocol Manager.
    i. From the **Advanced** drop-down menu, select **Protocol Manager**.
    ii. Choose a protocol from the *Protocol Manager* (Figure 18) and click [Edit].
        • For DNA binding dye-based expression analysis, choose 'MyDesign_SYBR_GX_Protocol_RevB'.
        • For probe-based expression analysis, we strongly recommend that you include ROX dye in your PCR Reagent Mix, as described in the SmartChip MyDesign Kit User Manual. Choose 'MyDesign_Probe_GX_FAM_ROX(95C_15s)_45_cycles'.
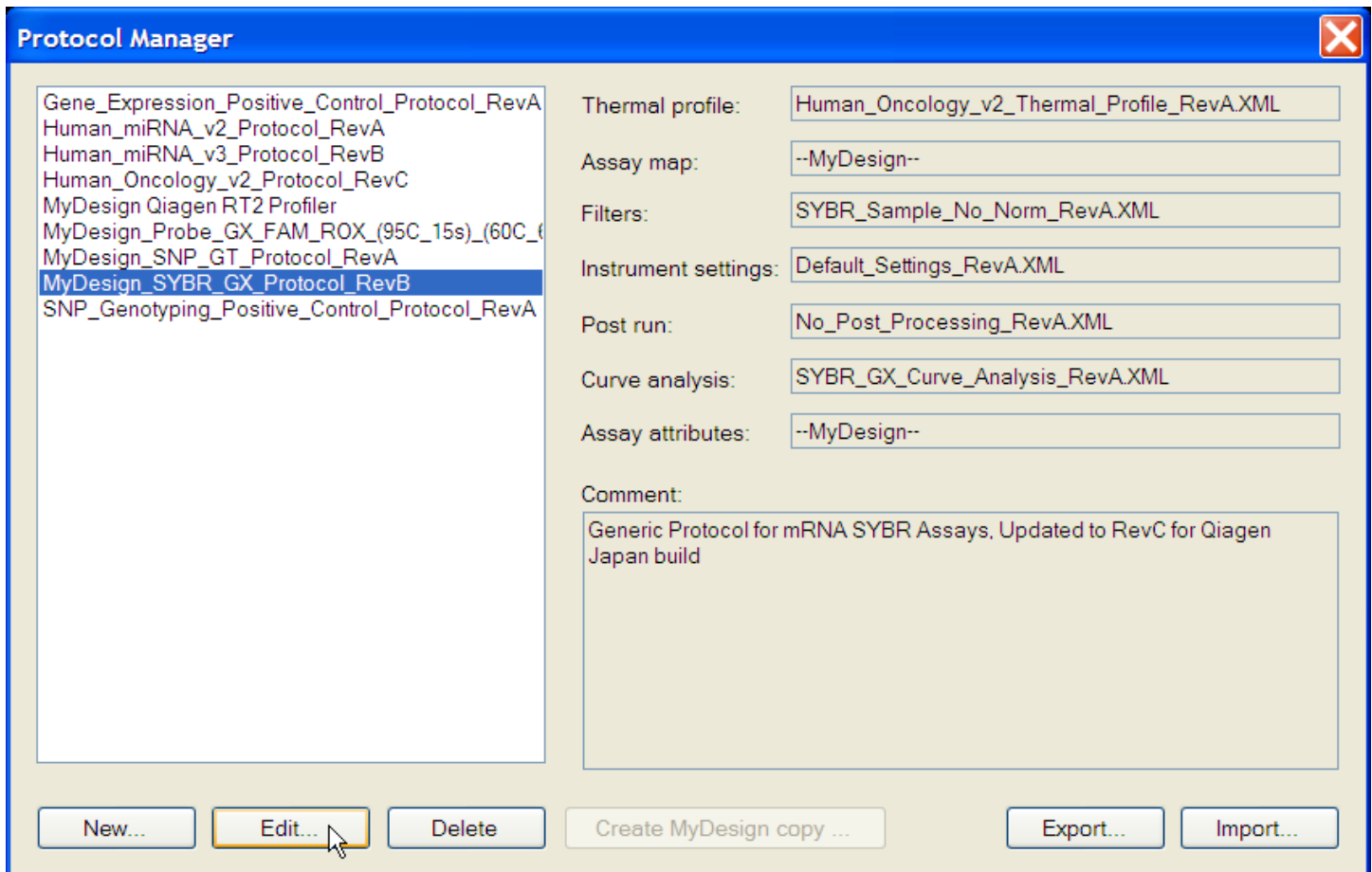
Figure 18. Protocol Manager window.

c. Rename the protocol as desired in the "Protocol name" field. This will open a *Name has changed* window. We recommend that you choose to **Create a new protocol, keep previous** (Figure 19). This preserves a copy of the default MyDesign gene expression profile on your computer. Close the *Protocol Manager* window by clicking the red [X] icon in the upper right corner.
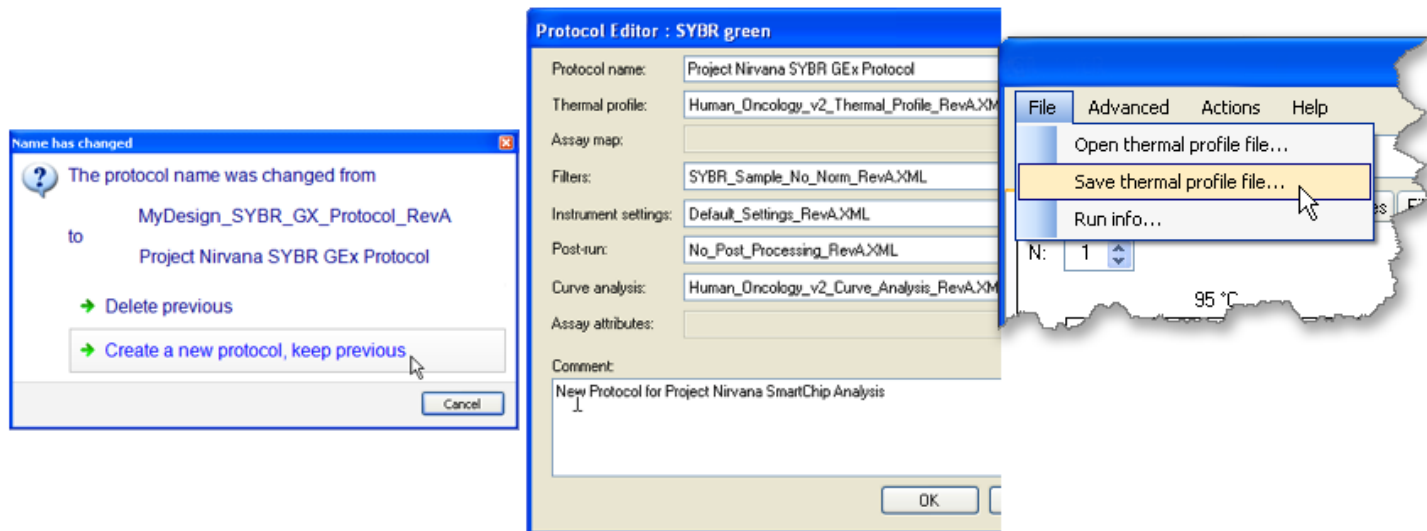
Figure 19. Renaming and saving the new protocol.

d. Adjust the thermal cycling protocol as necessary and save the file:
   i. Make sure that your new protocol is showing in the "Protocol" box. Select the *Thermal profile* tab and change the thermal cycling parameters by typing in new times and/or temperatures. You can also add and delete cycle components using the buttons at the lower left. (For example, you may want to adjust the annealing temperature to accommodate your real-time PCR assays [PCR primer pairs].)
   ii. Save the modified thermal profile by choosing **Save thermal profile…** from the **File** drop-down menu. Name the file logically (e.g., "Project Nirvana thermal profile" in the example in Figure 19) and click [Save].
e. Pull the modified thermal profile into your new protocol file (Figure 20):
   i. From the **Advanced** drop-down menu, select **Protocol Manager**. Choose your new protocol ("Project Nirvana SYBR GEx Protocol" in this example).
   ii. Click [Edit] at the bottom of the *Protocol Manager* window. This will open the *Protocol Editor* window.
   iii. In the "Thermal Profile" pull-down menu, select the thermal profile you just saved ("Project Nirvana thermal profileXML" in this example). Click [OK] to accept your choice and close the *Protocol Editor* window. Click the red [X] icon to close the *Protocol Manager* window.
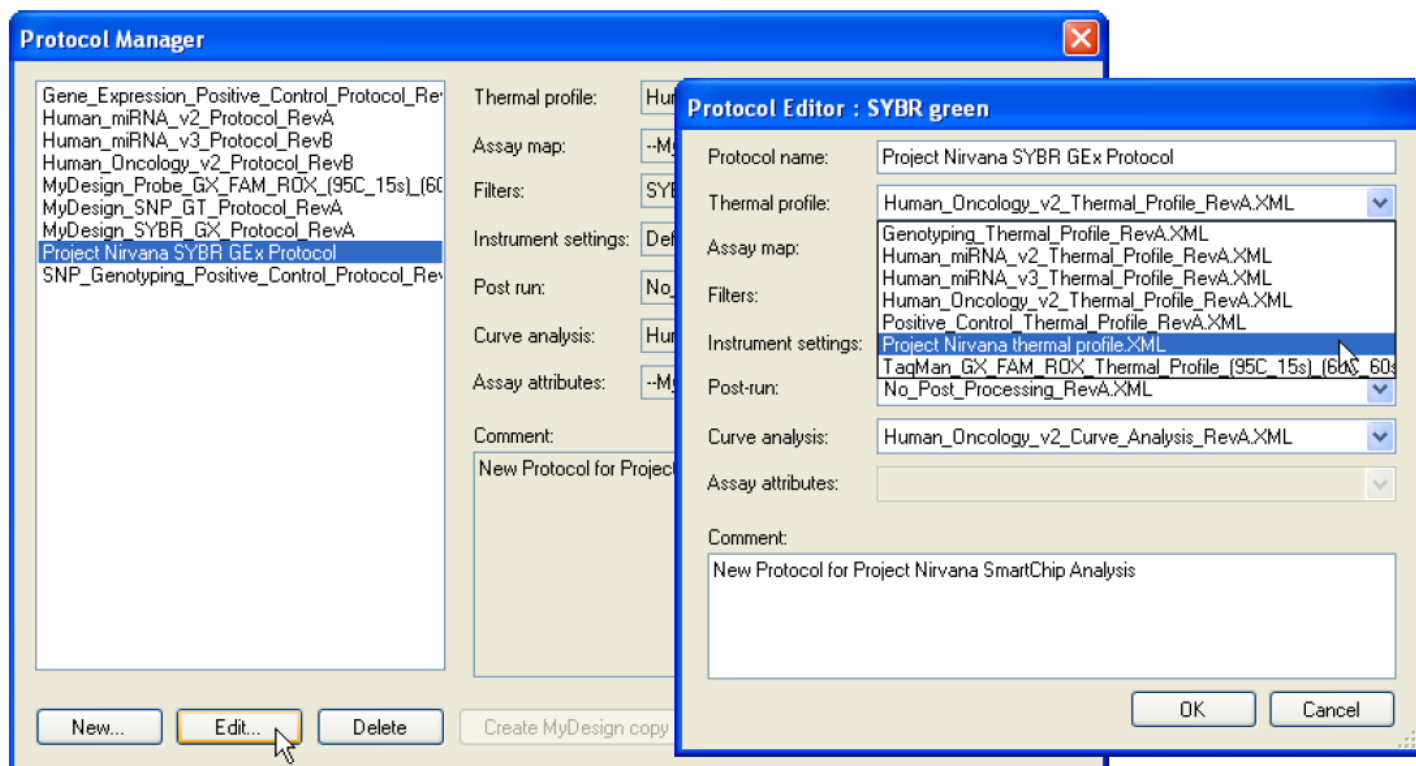
Figure 20. Pulling the modified thermal profile into your new protocol.

f. Exit Admin mode in order to run your experiment. From the **Advanced** drop-down menu, deselect **Admin mode…** (the check mark will disappear).

F. Setup Tabs for Expression Analysis

You can use these Setup Tabs to adjust curve analysis settings or to view the protocol components of a run. You must be in Admin mode to make changes in most of these tabs, and some areas can only be changed in Service mode.

1. Thermal Profile

Select the **Thermal profile** tab to display a graph of the thermal profile (Figure 21). Vertical lines visually separate each cycle. Temperatures and durations for each cycle are displayed. Note that

the ramp times are not shown in the user interface. The ⬕ icon indicates that a fluorescence image capture has started. Each cycle can contain one or more steps, and a cycle can be repeated several times.

IMPORTANT: If you have upgraded your qPCR software, we recommend that you use the updated protocols and associated thermal profiles packaged with the software. If you need custom thermal profiles created using an earlier version of qPCR software, contact Takara Bio technical support at technical_support@takarabio.com for assistance with replicating them precisely.

In Admin mode, you can change the thermal cycling parameters by typing in new times and/or temperatures. You can also add and delete steps and cycles using the buttons at the lower left. Use the [Toggle] button to add or remove a fluorescent imaging event for the selected step.

Save the modified thermal profile by choosing **Save thermal profile…** from the **File** drop-down menu. Name the file and click [Save].
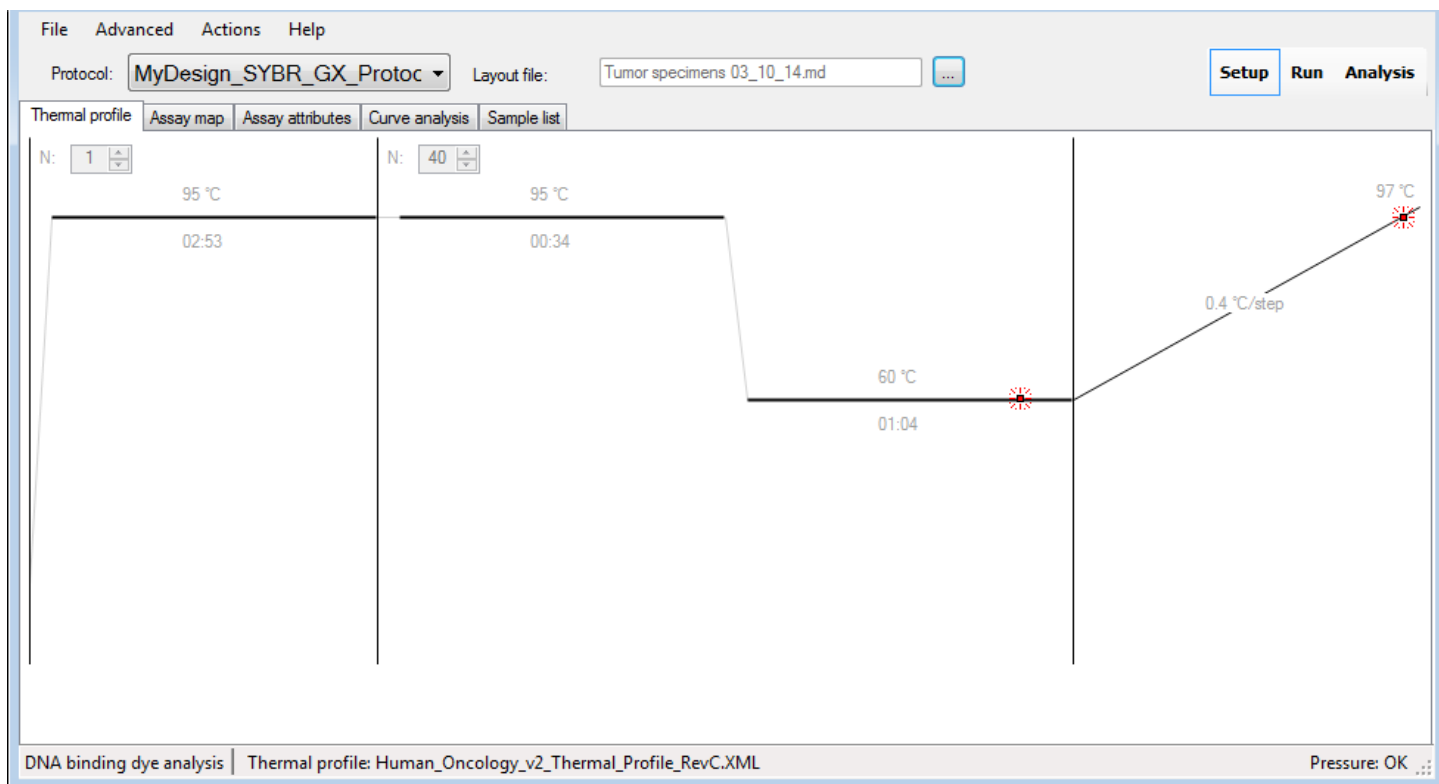
Figure 21. Graph of the thermal profile in the Thermal profile tab.

By default, Thermal Profiles are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data \Wafergen\qPCR\ThermalProfiles`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\ThermalProfiles`

NOTE: Folders may be hidden depending on your Windows File Explorer folder options settings.

2. Sample List Tab

The *Sample list* tab (Figure 22) shows the samples in the SmartChip Panel or MyDesign Chip.

Sample information and locations will be populated when you load the SmartChip Layout file into qPCR software.

Figure 22. Sample list tab.

By default, Sample Layout files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\SampleLayoutFiles`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\SampleLayoutFiles`

3. Assay Map Tab

The *Assay map* tab (Figure 23) lists the Assay map file and contains the names and location of the assays that have been dispensed into the chip. If you are using a SmartChip MyDesign Chip, this tab will contain information from the SmartChip Layout file you selected.

If you are using a pre-dispensed SmartChip Custom Panel, run the setup program provided on the CD sent with your SmartChip Panel to automatically copy Assay map files and other files to their appropriate locations. For pre-dispensed SmartChip Panels, this information comes from the Protocol file you selected.
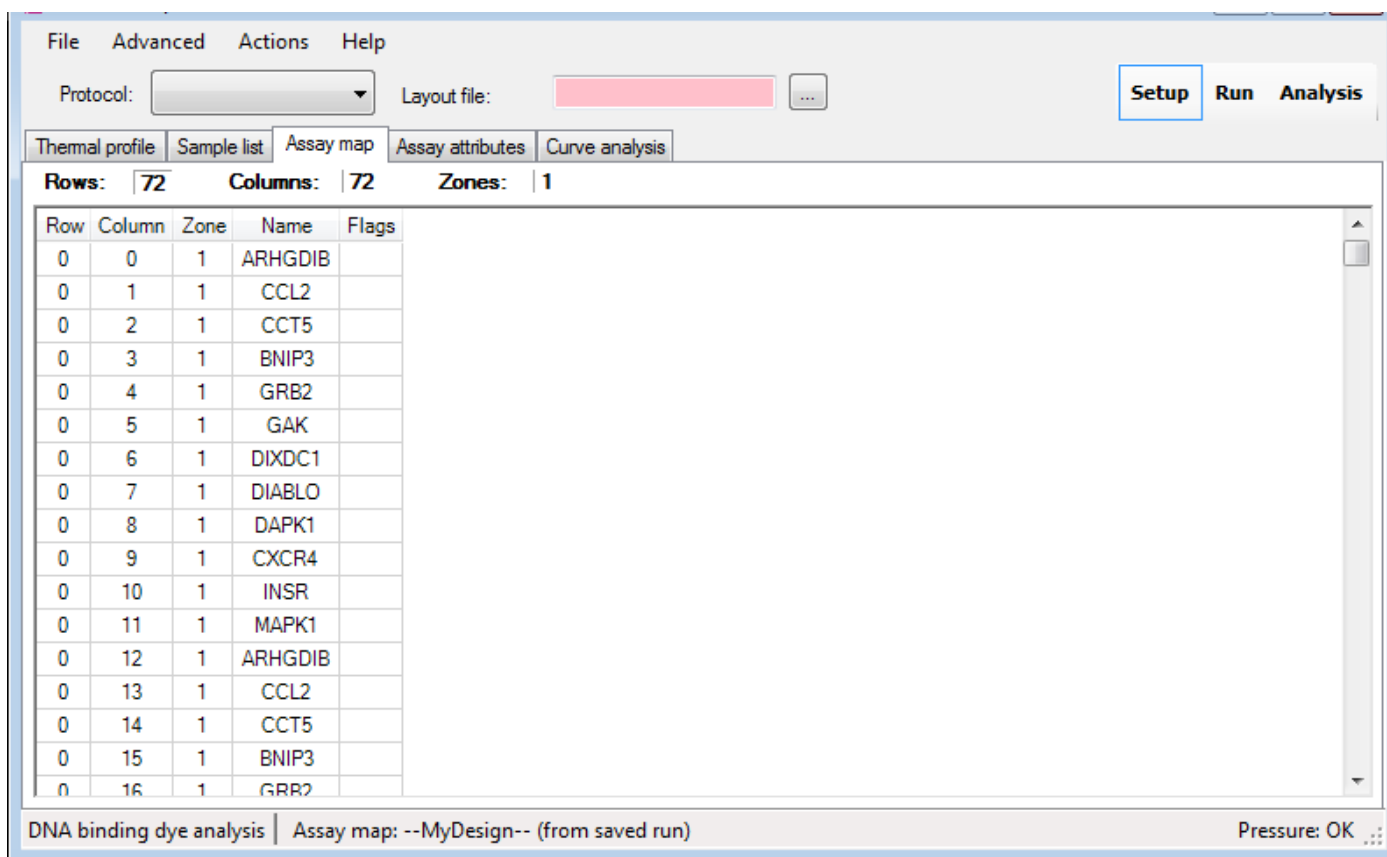
Figure 23. Assay map tab.

By default, SmartChip Assay map files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\AssayMaps`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\AssayMaps`

4. Filters Tab (Displayed in Advanced User Mode Only)

Use the *Filters* tab to view the sample reading dyes.

> **IMPORTANT:** The following descriptions are for INFORMATIONAL USE ONLY. Do not change any of the parameters in the *Filters* tab.
>
> The SmartChip system has been tested with TB Green dye for DNA binding dye–based expression (SmartChip TB Green Gene Expression Master Mix, Takara Bio Cat. No. 640211) and probe-based PCR assays labeled with FAM dye and a non-fluorescent quencher (PrimeTime qPCR Assays [Integrated DNA Technologies, Inc.], TaqMan Gene Expression Assays [Thermo Fisher Scientific]). To use other fluorescent dyes with the system, please contact Takara Bio technical support (technical_support@takarabio.com) for the latest information on compatible PCR assay types and master mixes.
>
> **NOTE:** Precipitate may be observed in the SmartChip TB Green Gene Expression Master Mix. This precipitate does not affect the performance of the kit. The precipitate can be dissolved easily by warming to room temperature and mixing for a few minutes. Ensure that the precipitate is fully dissolved before use.

Figure 24. Filters tab.

By default, Filter files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\Filters`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\Filters`

5. Instrument Settings Tab (Displayed in Advanced User Mode Only)

From the *Instrument settings* tab, you can inspect the advanced run-time parameters.

IMPORTANT: The following descriptions are for INFORMATIONAL USE ONLY. Changes to the *Instrument settings* tab can only be made in Service mode.
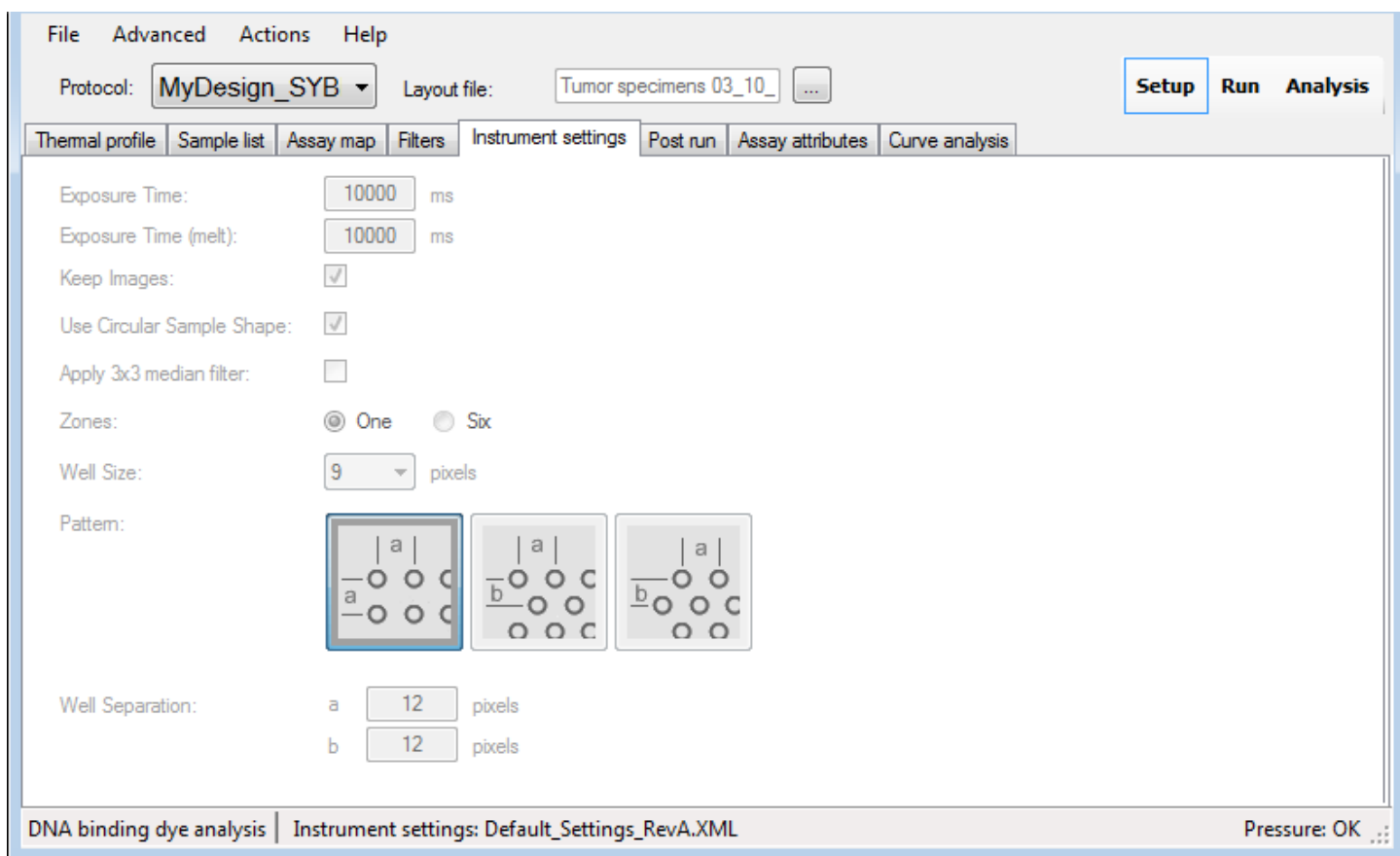
Figure 25. Instrument settings tab.

Table 8. Description of the various instrument settings.

Settings	Description
Exposure Time	10 sec
Exposure Time (melt)	10 sec
Keep Images	Keeping images to re-analyze a run is highly recommended. Reviewing images may allow you to find the reason for any anomalies in the data. An additional 150–300 MB of disk space will be required per run.
Use Circular Sample Shape	Select according to the type of SmartChip nanowell chip used
Apply 3 x 3 Median Filter	Removes speckle noise from images. Best to not use this filter
Zones	One
Well Size	Diameter of the sampling disk
Pattern	Select the pattern that matches the SmartChip nanowell chip
Well Separation	Fixed distance between cells

By default, Instrument Settings are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\InstrumentSettings`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\InstrumentSettings`

6. Post Run Tab (Displayed in Advanced User Mode Only)

From the *Post run* tab (Figure 26), you can view the post-run settings. If the "Generate summary report" box is checked, a summary report will be automatically generated after completion of data processing for a SmartChip run.

The "Filename template" value determines how the summary report file will be named. To configure the Filename template, you must be in Admin mode.

NOTE: To configure external processing, you must have knowledge of DOS command line processing. For help with this, you may contact Takara Bio technical support (technical_support@takarabio.com).
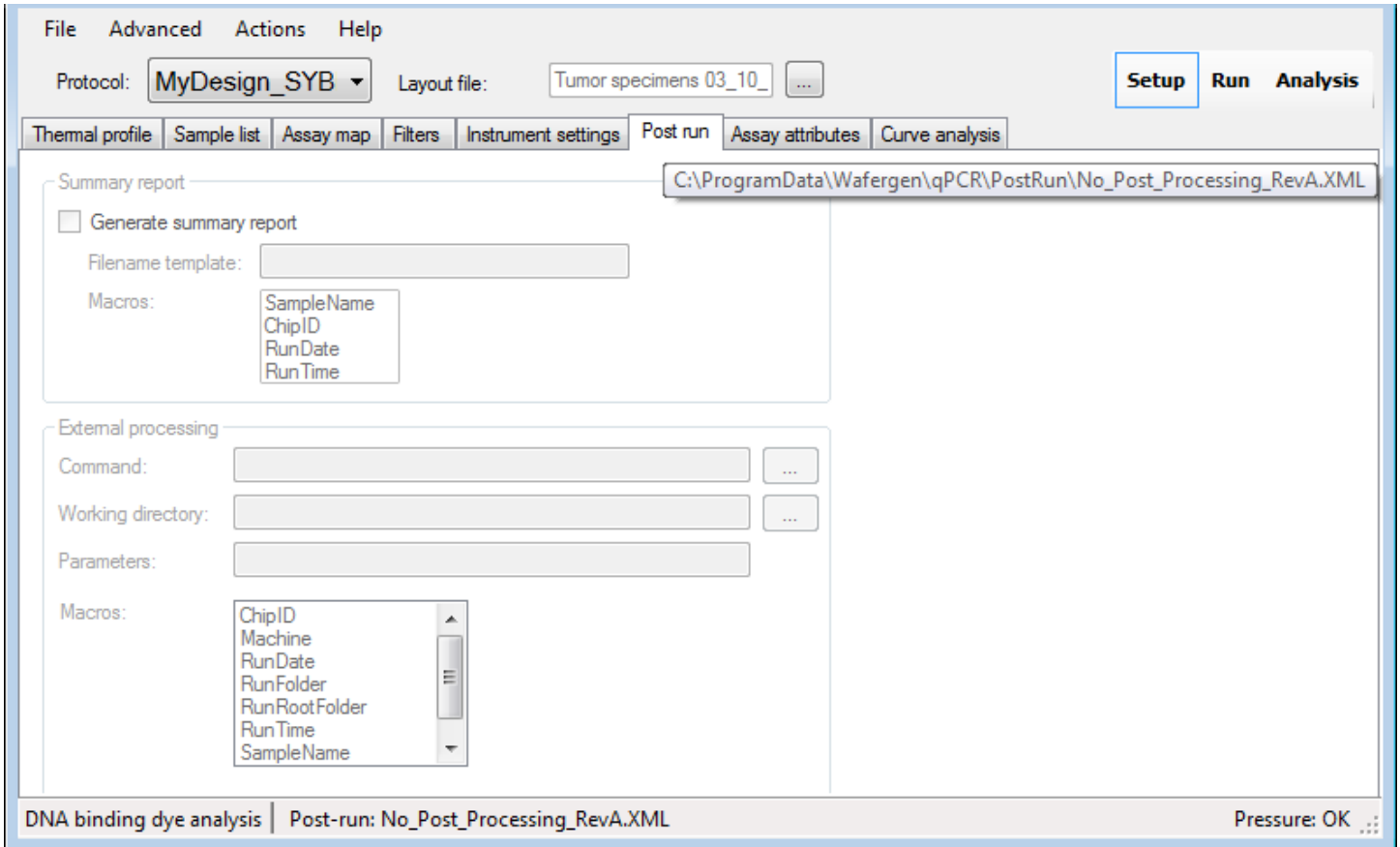
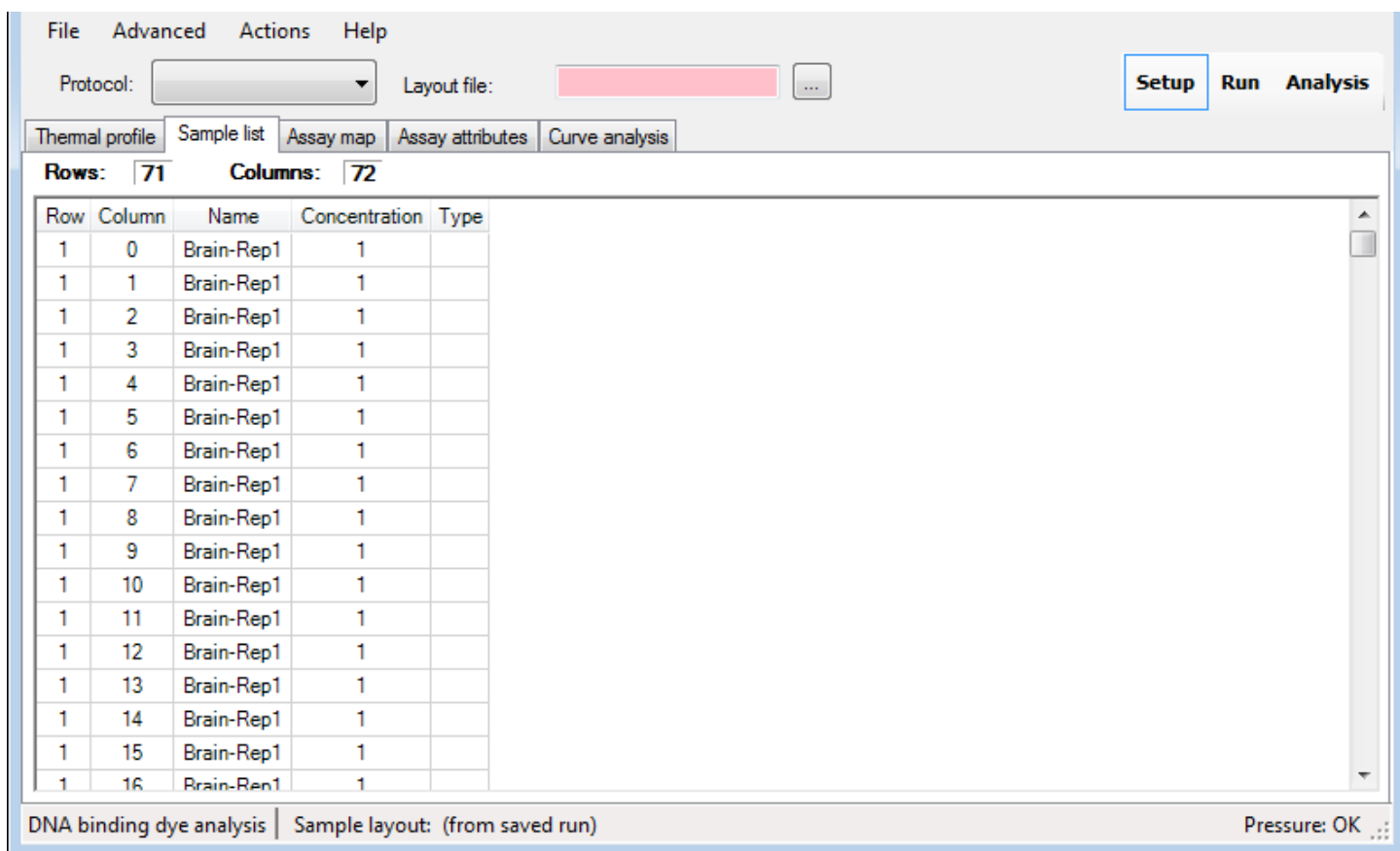**Figure 26. Post run tab.**

By default, post-run files are stored in the following locations:

- Windows XP—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\PostRun`
- Windows 7—`C:\ProgramData\Wafergen\qPCR\PostRun`

7. Curve Analysis Tab
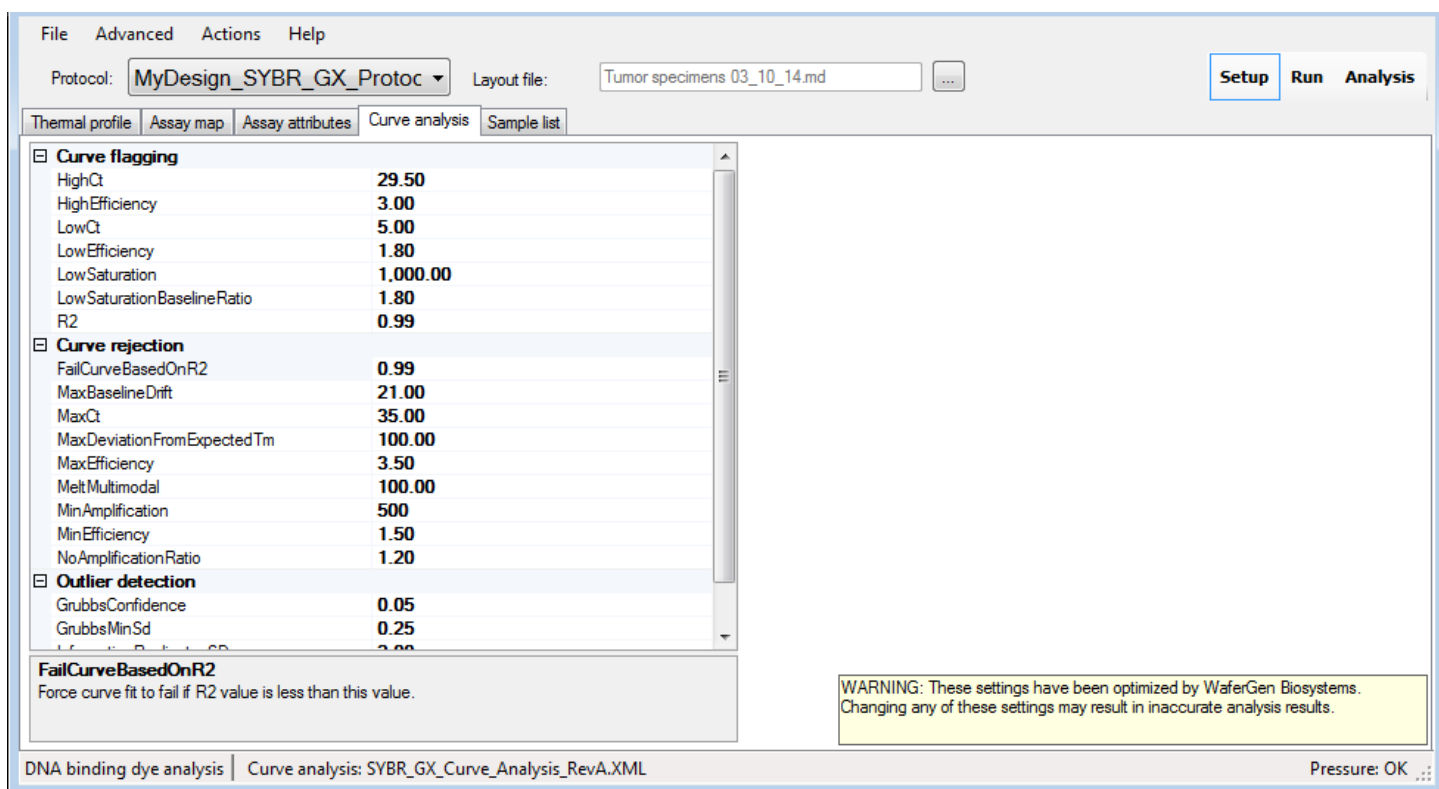
Figure 27. Curve analysis tab.

From the *Curve analysis* tab (Figure 27), you can view and change the curve analysis parameters from a SmartChip run.

Note that some settings are displayed only in Advanced user mode. In addition, some settings are specific for either DNA binding dye-based or probe-based expression analysis, and some are applicable for both types of analyses.

See "Expression Analysis Parameters" in Appendix C for a description of the settings on this tab.

Note that in DNA binding dye-based expression analysis, the C_t will be calculated using the second derivative maximum method. (See setting "UseSecondDerivativeMax: True".) Change this setting to "False" if you want to define C_t as the cycle in which fluorescent signal reaches 10% of the maximum fluorescence of the reaction (based on curve fitting). (This is how C_t was calculated in qPCR software versions prior to version 2.8.0.65.)

If you change any of the parameters, the experimental data will be recalculated according to the new settings the next time you enter Analysis mode.

If you want to use these settings for analysis of subsequent SmartChip runs, first enter Admin mode, then save your settings by choosing **Save Curve Analysis Settings** from the **File** drop-down menu. Then pull the modified curve analysis settings into the protocol file of your choice (using **Create user protocol…** from the **Advanced** drop-down menu) or create a new protocol using the **Protocol Manager** (under the **Advanced** drop-down menu).

By default, Curve analysis files for expression analysis are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\CurveAnalysis`
- **Windows 7**—`C:\Program Data\Wafergen\qPCR\CurveAnalysis`

8. Assay Attributes Tab

The list under the *Assay attributes* tab may contain assay ID, assay name, amplicon T_m (for DNA binding dye-based PCR only), and "IsHousekeeping" status. If there is no information for a given field (as in Figure 29 below), the column will not be displayed.

Assay attributes for SmartChip MyDesign Chips will be populated when you load the SmartChip Layout file into the qPCR software.

If you are using a SmartChip Custom Panel, running the setup program on the CD provided with your chip will automatically copy assay attribute files and other files to their appropriate locations.
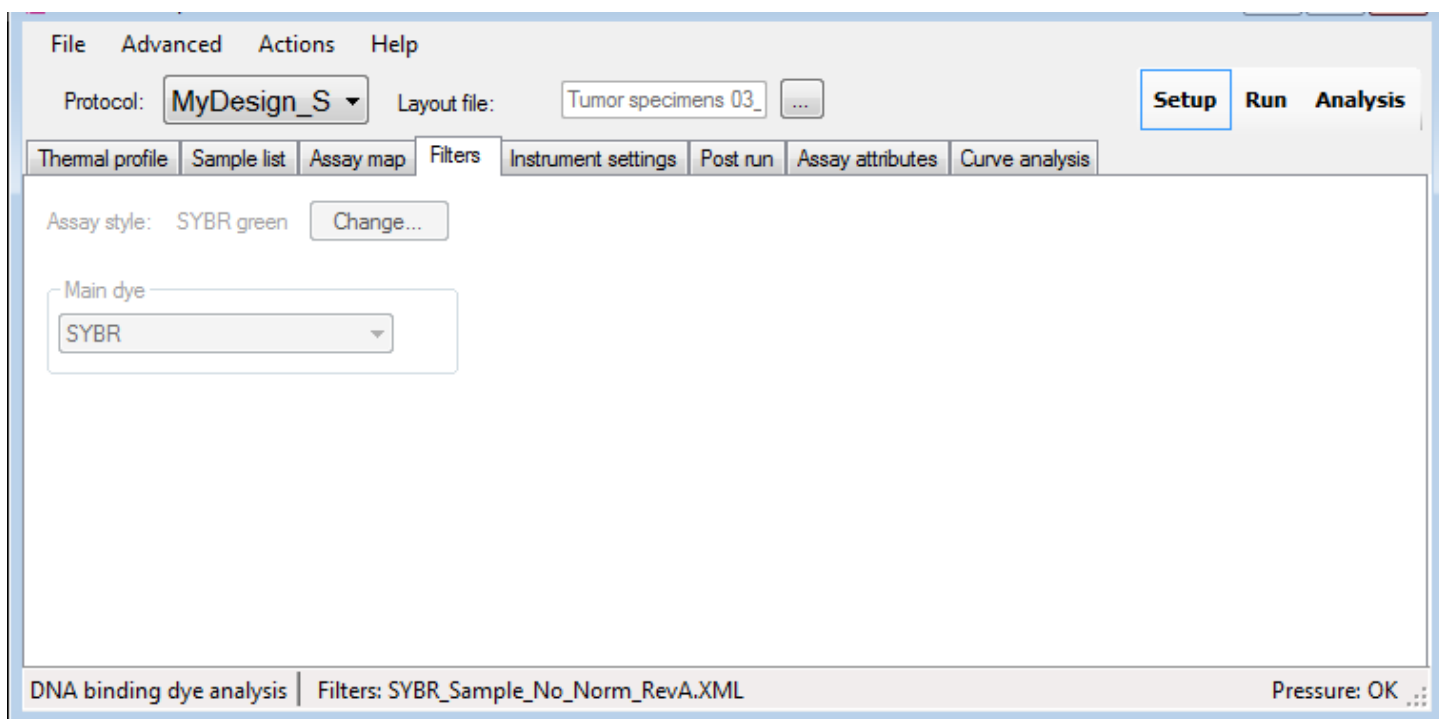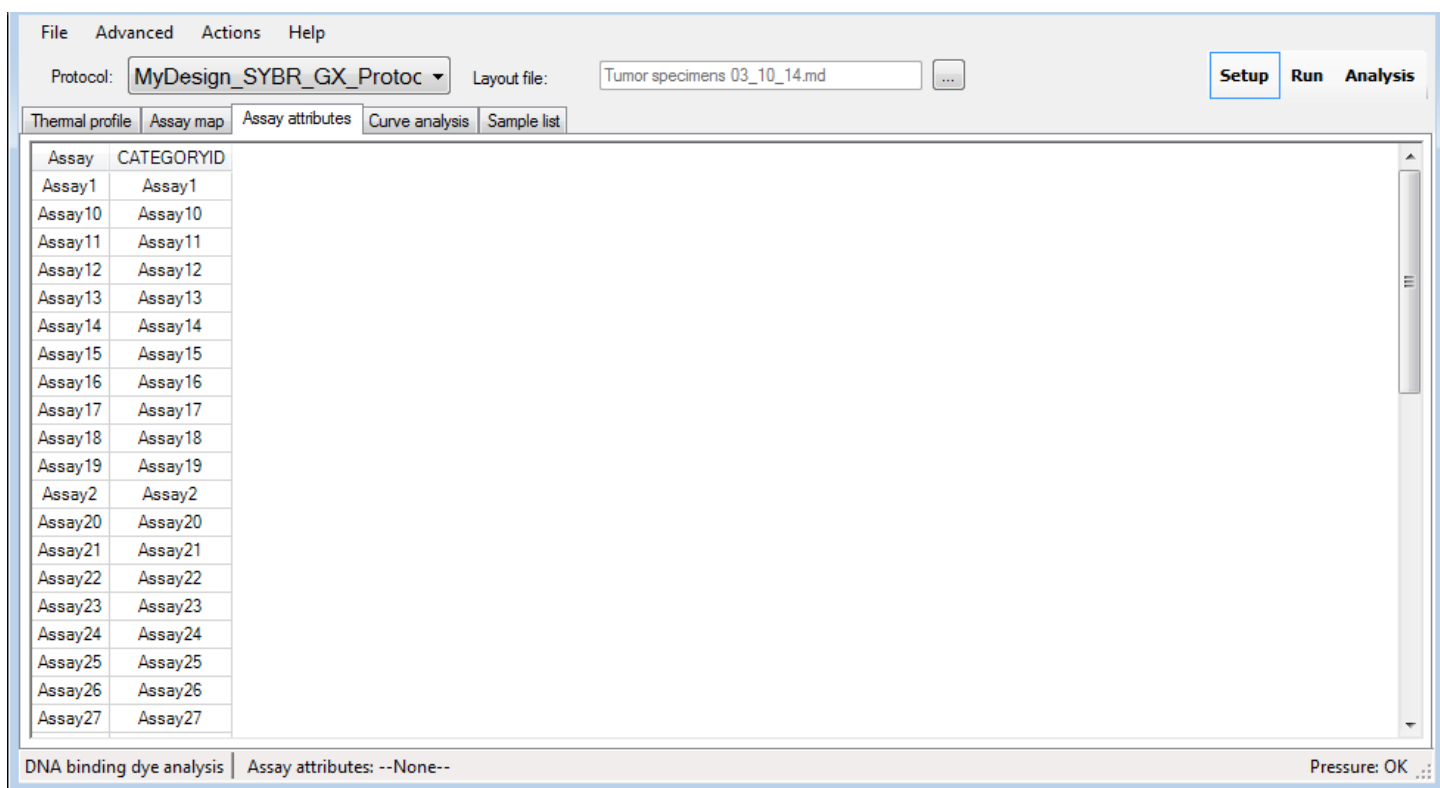

Figure 28. Assay attributes tab.

By default, assay attribute files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\AssayAttributes`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\AssayAttributes`

XI. Expression Analysis Procedures: Run Mode

A. Introduction

Select the *Run* mode to start and monitor the parameters of a SmartChip ND Cycler run. The *Run* window's top navigation bar provides drop-down menus that contain protocol and sample information (Figure 29). The *Run* window also contains one or more panels of run data in graphical format. Right-clicking anywhere on a graph displays a pop-up menu with options for **Copy**, **Save image as**, **Page setup**, **Print**, **Show point values**, **Zoom/Pan**, and **Set scale to default**. An *Event log* (bottom left) lists any errors or abnormal events that occur during a run. *Run info* (bottom right) provides basic information about the run.
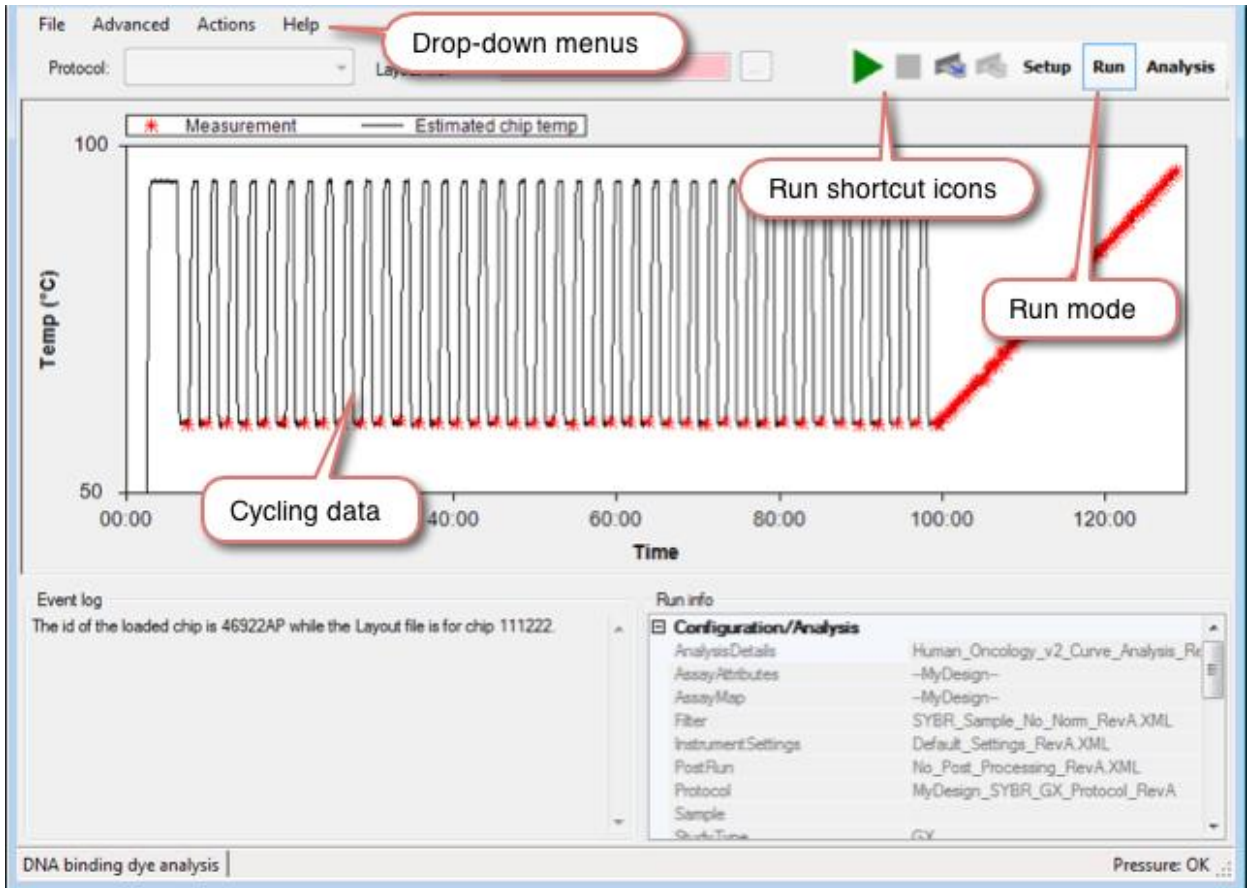
Figure 29. Run window.

B. Overview of Run Mode Drop-Down Menus
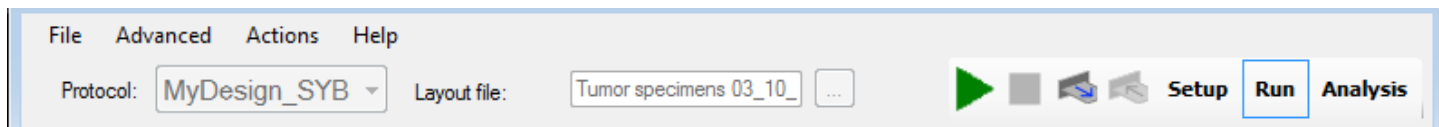
Figure 30. Run mode top menu bar.

Upon switching to Run mode, the top menu options change (Figure 30). Table 9 below describes the selections available from the Run mode top menu bar.

Table 9. Menu options available from the Run mode top menu bar.

Menu	Options
File	**Open** an existing run. Allows inspection of data related to completed experiments. Hint: A quick way of loading a run into the program is to drag a folder from Windows Explorer into the SmartChip gene expression or genotyping application. You will be prompted to choose which raw or analysis file you want to load. **Run info** displays file, user, chip, and instrument information as well as notes.
Advanced	**Switch to advanced user mode** to display all of the data collected during real-time. **Switch to standard GUI** to display only information that is most frequently accessed. **Toggle between Pressure and Temperature graph panel** in graphical format. (See next section for examples.) **Image viewer** is designed for visual inspection of the data. For more information, see "Image Viewer" in Section XVII. **Re-process run with different configuration** will rerun data and generate a new .raw file when the Assay map, Assay attributes, or Sample layout file have changed. If other config files are changed, the run data will no longer be visible. **Admin mode** should be used by trained personnel only. It is used to save changes to curve analysis settings and to modify protocols using the **Protocol Manager**. Changing any of these settings can affect your experimental results. The password to enter Admin mode is printed on your qPCR software CD. You must exit Admin mode in order to run the SmartChip ND Cycler. **Service mode** is designed for Takara Bio staff to configure advanced SmartChip ND Cycler settings.
Actions	Switch between **DNA binding dye-Based Expression**, **Probe-Based Expression**, and **Genotyping Analysis** modes.
Help	**About** provides application version, copyright, and license agreement information. **Open user manual** opens the User Manual in pdf format. **Setting up a run** opens a pop-up box with a Quick Guide for setting up a run. **Saving run data** opens a pop-up box with a Quick Guide for saving run data. **Loading an existing chip run** opens a Quick Guide for loading an existing chip run. **Reprocessing data** allows you to reprocess completed chip runs with different curve analysis parameters.

C. Run Shortcut Icons

These icons are visible only using software installed in instrument mode.

▶ [Run experiment]   ⬑ [Open drawer]

⬛ [Abort run]   ⬋ [Close drawer]

D.   Run Data Panels (Advanced Drop-Down Menu)

The **Advanced** drop-down menu in Run mode offers several options to view run-data graphs. Up to two graphs can be shown at once. Selecting the type of graph toggles between showing and hiding the specified graph.

1.   Pressure/Temperature Graphs

Select **Toggle pressure graph panel** to show or hide the pressure and air flow curves.

Select **Toggle temperature graph panel** to show or hide a detailed graph of the temperature sensor measurements during a run. The stars in the temperature graph represent image measurement acquisitions (Figure 31).
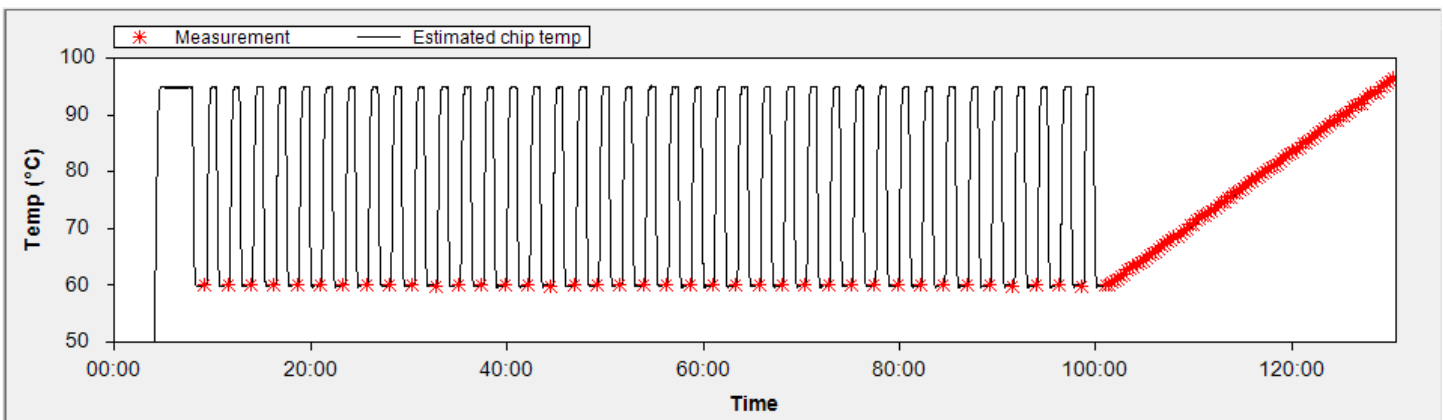
Figure 31. Example temperature graph.

XII. Expression Analysis Procedures: Analysis Mode

A. Introduction

Select *Analysis* mode to view and analyze data from SmartChip runs. The *Analysis* window's top navigation bar has drop-down menus that contain protocol and sample information. After using the **File** drop-down menu to select an analysis file for review, you can use the *Analysis* tabs to view the data in different formats.

The main part of the *Analysis* window (Figure 32) typically shows a table of data on the left with the graphical display on the right. If more than one row of data is highlighted on the left, the graph on the right will display an overlay of all well data selected. For a description of the data analysis method, refer to Appendix C.
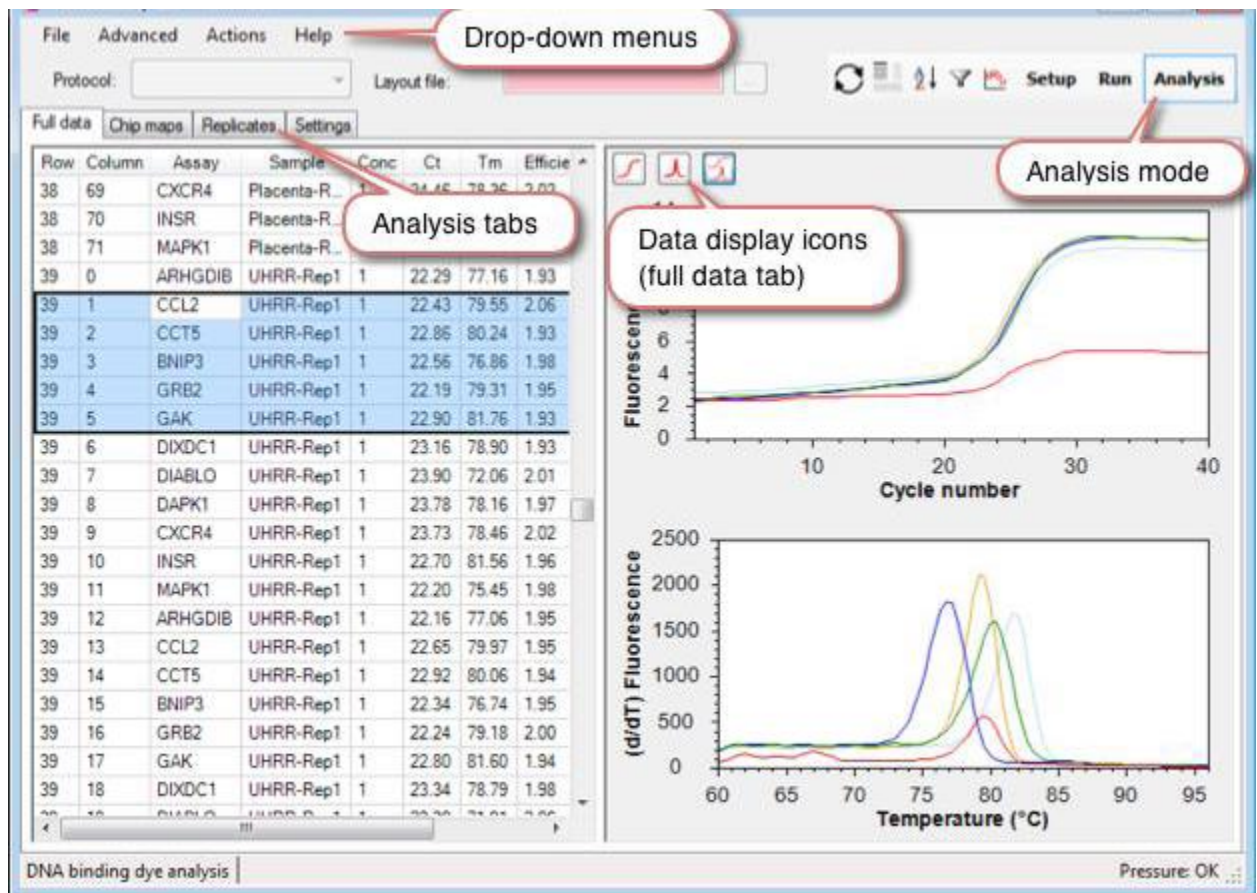
Figure 32. Expression analysis window.

NOTE: SmartChip ND qPCR Software may also be used for offline data analyses. To install SmartChip ND qPCR Software on a laptop or desktop computer that is not controlling a SmartChip ND Cycler, refer to Appendix A.

B. Analysis of DNA Binding Dye-Based and Probe-Based Real-Time PCR

The qPCR software uses similar methods to analyze results from the SmartChip system for DNA binding dye-based and probe-based real-time qPCR experiments. There are, however, some differences; they are described below.

1. DNA Binding Dye-Based Real-Time PCR

Melt curve analysis parameters: We always recommend running a melt curve analysis as the last step of DNA binding dye-based PCR experiments. Since any amplification product will result in fluorescent signal, melt curve results help researchers distinguish meaningful data from experimental artifacts. All analysis related to T_m and melt curves are used only when the SmartChip ND Cycler is in DNA binding dye-based expression analysis mode.

Curve fit model: A sigmoid curve fit model is used in DNA binding dye-based expression analysis mode:

$$f(x) = \frac{D}{1 + e^{-\left(\frac{x-C}{B}\right)}} + A$$

C$_t$ calculation: The C_t is also known as a quantification cycle (Cq) and crossing point (Cp). It is calculated using the second derivative maximum method when "UseSecondDerivativeMax" is set to 'True' in the **Analysis settings**.

In previous versions of qPCR software, C_t was defined as the cycle in which fluorescent signal reaches 10% of the maximum fluorescence of the reaction (based on curve fitting). You can check which method is used in for a given data analysis file on the **Settings** tab in **Analysis mode** or the **Curve analysis** tab in **Setup mode**.

2. Probe-Based Real-Time PCR

ROX normalization: We recommend using ROX dye in probe-based expression analysis. The qPCR software includes several normalization parameters based on ROX fluorescence.

Efficiency measurements in real-time PCR are often a measurement of the PCR assay efficiency and are based on results from amplification of a dilution series of template. In the qPCR software, efficiency is a measure of the fluorescence of reactions from one cycle to the next at or near the calculated C_t value. It is normal for the reported efficiency to be less than two.

Curve fit model: A five parameter logistic curve fit model is used in probe-based expression analysis:

$$f(x) = \frac{D}{\left(1 + \left(\frac{x}{C}\right)^B\right)^E} + A$$

C$_t$ calculation: The C_t is calculated using the second derivative maximum method.

C.   Overview of Analysis Mode Drop-Down Menus
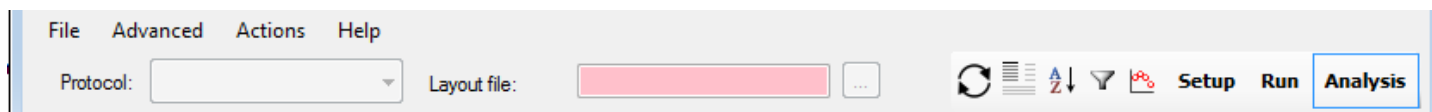
Figure 33. Analysis mode top menu bar.

Table 10 below describes the selections available from the *Analysis* mode top menu bar (Figure 33).

Table 10. Menu options available from the Analysis mode top menu bar

Menu	Options
File	**Open** gene expression files with .raw and .anl file extensions. A .raw run file contains the raw data of the SmartChip Panel wells. An .anl run file contains curve analysis data.
	Save gene expression curve analysis results with .anl file extensions. A summary report will also be generated in a .pdf format.
	Export well data saves well data as a tab-delimited text file.
	Export curves saves curve data as a tab-delimited text file.
	Export replicate data saves replicate data as a tab-delimited text file.
	Export RDML file exports data in RDML format for use in compatible data analysis applications.
	Export qBasePlus file saves data that is compatible with Qiagen's RT² Profiler PCR Array Data Analysis tool. This tool requires data in Excel .xls format. If the computer running the qPCR software does not have Microsoft Excel installed, you will need to export to .csv format, then open the .csv file in Excel and save to .xls format.
	Export StatMiner file saves data that is compatible with StatMiner software.
	Export Bioconductor file saves data that is compatible with Bioconductor software.
	Export calibration data saves calibration (standard curve) data in a tab-delimited format.
	Run info displays file, user, chip, and instrument information as well as notes.
Advanced	**Switch to advanced user mode** to display all of the data collected during real-time PCR.
	Switch to standard GUI to display only information that is most frequently accessed.
	Toggle between Pressure and Temperature graph panel in graphical format. (See next section for examples.)
	Image viewer is designed for visual inspection of the data. For more information, see "Image Viewer" in Section XVII.
	Service mode is designed for Takara Bio staff to configure advanced SmartChip ND Cycler settings.
	Curve analysis settings takes you back to **Setup mode** where you can modify curve analysis settings if desired. The settings will take effect when you go back to the **Analysis mode**.
	Summary report options tailors information to be included in the summary report.
Actions	Switch between **DNA binding Dye-Based Expression**, **Probe-Based Expression**, and **Genotyping Analysis** modes.
Help	**About** provides application version, copyright, and license agreement information.
	Open user manual opens the User Manual in pdf format.
	Setting up a run opens a pop-up box with a Quick Guide for setting up a run.
	Saving run data opens a pop-up box with a Quick Guide for saving run data.
	Loading an existing chip run opens a Quick Guide for loading an existing chip run.

Reprocessing data allows you to reprocess completed chip runs with different curve analysis parameters.

D.   Expression Data Analysis

Select *Analysis* mode to view and analyze the SmartChip Panel run data (Figure 34).
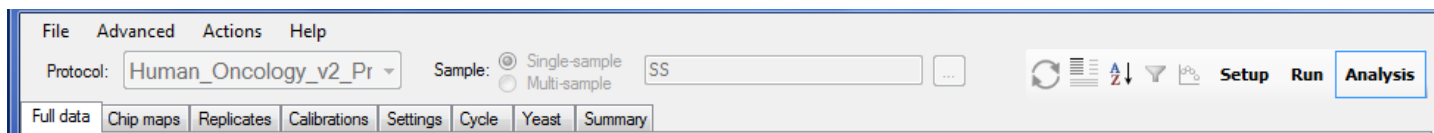
Figure 34. View and analyze SmartChip Panel run data in Analysis mode.

Tabs that are **not** in bold type in Table 11 below are visible in Advanced user mode only.

Table 11. Tabs available in Analysis mode for expression data.

Tab	Function
Full Data	For each well, this tab shows all of the run information, including the assay ID, C_t, T_m (DNA binding dye-based PCR only), R^2, efficiency, multiple peaks in melt curve, F_0 (the value of the theoretical fluorescence at cycle 0, based on the curve fitting), as well as any flags and asymmetry. You can select wells to graphically show the amplification curve and/or melt curve.
Chip Maps	View the distribution of measurements for all wells across the entire SmartChip Panel in both tabular and graphical formats.
Replicates	View replicate curves and outliers in tabular and graphical formats.
Calibrations	If the sample information file contains concentration information and there is a dilution series on the SmartChip Panel, a calibration curve (or standard curve) will be generated.
Settings	Displays the analysis settings (thresholds) that were in effect when the curve analysis was performed.
Cycle	Contains raw fluorescent signal values for each cycle image, organized in a map view.
Yeast	(DNA binding dye-based expression analysis only) Displays the mean C_t values for the Yeast Control samples.
Summary	Displays analysis summary statistics, including overall well data, replicate data, and flagged well data.

E.   Excluding Data from Analysis

1. How to Select Data for Exclusion

You can choose data to exclude from analysis in the *Full Data* and *Replicates* tabs in *Analysis* mode (Figure 35). To do so, click to select a row or rows of data, then right-click to exclude sample(s), assay(s), or both. Right-click anywhere in the data table and choose **Clear all exclusions** to clear them.

Excluding Data from the Full Data tab

Excluding Data from the Replicates tab
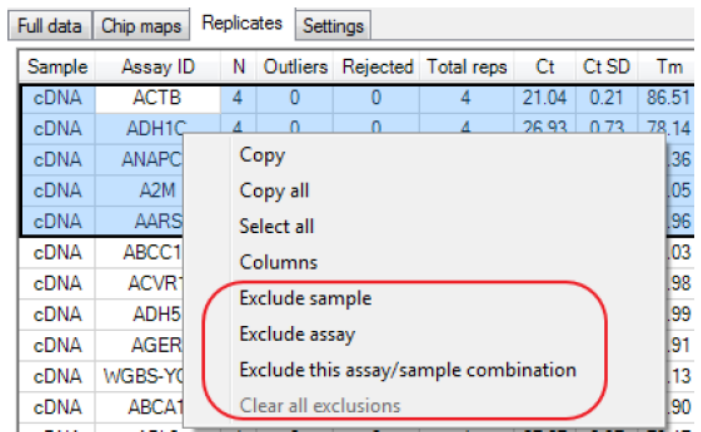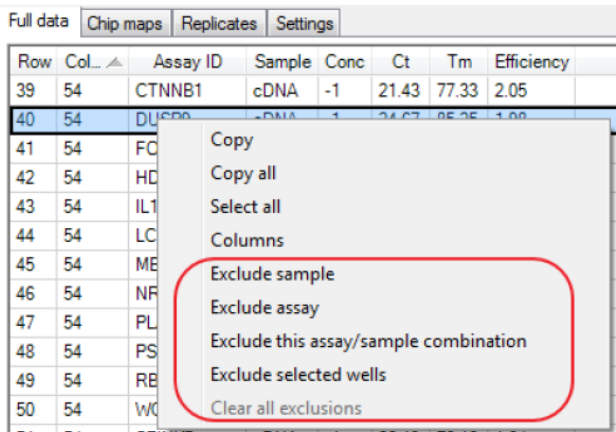
Figure 35. Excluding data from the Full Data and Replicates tabs.

If the experiment included dilutions, from the *Calibrations* tab, you can exclude data directly from the scatter plot by selecting wells by holding down the **[Shift]** key and then clicking and dragging the representative data point icon on the chart. (Figure 36).
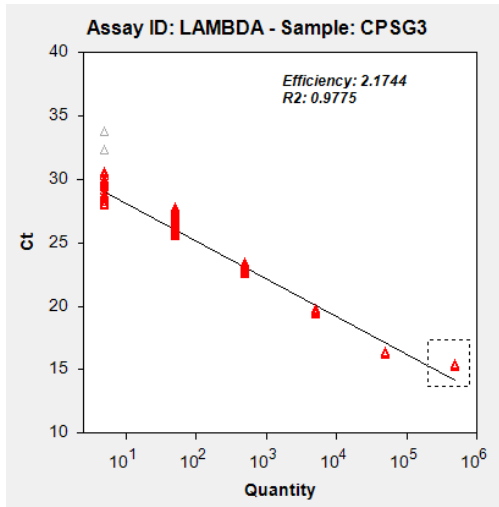
Figure 36. Excluding data from the Calibrations tab.

You will then be prompted to confirm that you want to exclude the selected data points (Figure 37).
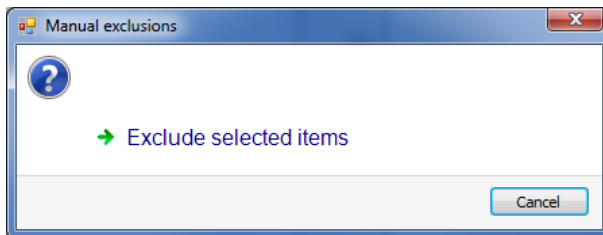
Figure 37. Confirm data exclusion.

2. How Data Exclusion Works

- Statistics, tables, and curve graphs will be updated right away when data are excluded from analysis
- Excluded data will not be exported or the C_t values will be left empty
- When you re-analyze the data, you will be asked if you want to keep the exclusions or not (Figure 38)
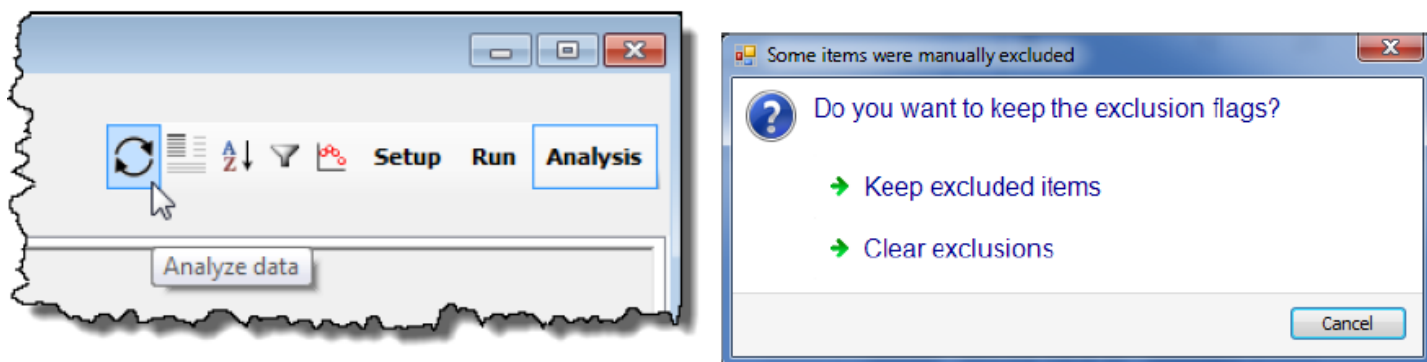
Figure 38. Confirmation of exclusions when re-analyzing data.

- Data that are excluded receive a flag: 'Manually Excluded'
- When there are replicates, all replicates will be flagged as 'Manually Excluded' if one or more of the individual replicate wells have the flag.
- Excluded data points do not contribute to the averages and standard deviations; they are treated as outliers

F.   Saving or Exporting Run Data

NOTE: When the run is complete, the results are automatically stored in the data folder in a file called `[chipID].raw` in the file path defined during Setup, where `[chipID]` will be the value of the chip ID supplied when the chip was loaded into the instrument. This file contains reaction growth curve information. It also contains the parameters that were specified for the run. The file does not contain data based on the curves, such as C_t values. These must be re-computed every time a `.raw` file is loaded.

1. Select *Analysis* mode, which displays computed data based on **Curve analysis** parameters defined in *Setup* mode. If any recalculations are needed based on parameter changes, they will be performed automatically.
2. From the **File** drop-down menu, select **Save** to generate an `.anl` file which will display the curve data without the need for recalculation when opened in the SmartChip qPCR Software after the run.
3. Name the "Summary report" and select [OK]. This will produce a Summary Report PDF file that will provide a brief synopsis of the data from the SmartChip run.
4. To export data, select the **File** drop-down menu and then select one of the **Export** commands: well data, replicate data, RDML file, qbasePLUS file, StatMiner file, Bioconductor file, or calibration data.

G. Loading an Existing SmartChip Run

SmartChip Panel runs can be loaded from `.raw` and `.anl` files. The latter contain calculated curve data, while the former does not.

- To load a run using a `.raw` file, use the **Open** command from within the *Run* mode.
- To load a run using an `.anl` file, use the **Open** command from within the *Analysis* mode.
- Either type of file can be loaded in the UI by dragging the file or the entire run folder onto the SmartChip qPCR application icon. If the folder contains both `.raw` and `.anl` files, you will be given a choice of which one to load.

H. qbasePLUS Export from qPCR Software

SmartChip qPCR Software supports exporting to qbasePLUS from Biogazelle. All fields are tab-delimited text files. Table 12 below is an example of a partial file.

Table 12. Example partial qbasePLUS exported file.

Well	Type	Sample	Gene	C_t	Quantity	Exclusion	Target type
R1C2	UNKN	ss	LAMBDA	20.49427	0		REF
R1C3	UNKN	ss	LAMBDA	20.4356	0		REF
R1C4	UNKN	ss	LAMBDA	20.28875	0		REF
R1C5	UNKN	ss	LAMBDA	20.41708	0		REF

1. Sample Field Handling

If the "Sample" field contains any of the non-case-sensitive strings, such as 'POS', 'NEG', or 'NTC', the exported *Type* column will contain 'POS' or 'NEG'. For all other instances, the *Type* column will contain 'UNKN'. Table 13 below shows an example.

Table 13. Example qbasePLUS exported file with POS and NEG designations.

Well	Type	Sample	Gene	C_t	Quantity	Exclusion	Target type
R1C2	UNKN	ss	LAMBDA	20.4499	0		REF
R1C3	POS	ssPos	LAMBDA	20.99	0		REF
R1C4	NEG	ssNTC	LAMBDA	20.44	0		REF
R1C5	NEG	ssNEG	LAMBDA	20.3	0		REF

2. Flag Field Handling

The *Exclusion* field in the exported qbasePLUS file is set to 'TRUE' for a well if the *Flag* field contains one or more of the following qPCR flags: 'NoAmplification', 'CurveFitFailed', 'MultipleMeltPeaks', 'CtlsOutlier', 'TmAPD', or 'MultiModal'. The tables below show examples.

Table 14. qPCR Row.

Row	Col	Sample	C_t	T_m	…	Flags
1	0	ss	20.4499855	76.061		MultipleMeltPeaks, MultiModal

Table 15. qbasePLUS output based on the qPCR Row in Table XIV above.

Well	Type	Sample	Gene	C_t	Quantity	Exclusion	Target type
R2C1	UNKN	ss	LAMBDA	20.44999	0	TRUE	REF

NOTES: The row and column in qbasePLUS output starts at 1, unlike the qPCR software in which the first row and column start with 0.

The *MultipleMeltPeaks* flag was called *PrimerDimersDetected* in previous versions of the qPCR software.

I. Analysis Tabs

1. Full Data Tab

Once the data are loaded at the end of an experiment or by opening a `.raw` file, you can perform a curve analysis by pressing the [Analyze Data] icon (⟳). Analyzed data for individual wells on the SmartChip Panel are displayed in the *Full data* tab.

The analysis will determine the C_t value for each amplification curve. No C_t values are generated for wells that do not appear to have any observed amplification or those in which the calculated C_t value is considered unreliable, based on the Curve analysis settings.

Figure 39 below is a typical *Full data* tab display window showing the actual curves and data that apply to the various sections of the curve.

Figure 39. Typical Full data tab display.

Data display icons for DNA binding dye-based PCR:

    [Display amplification curves]

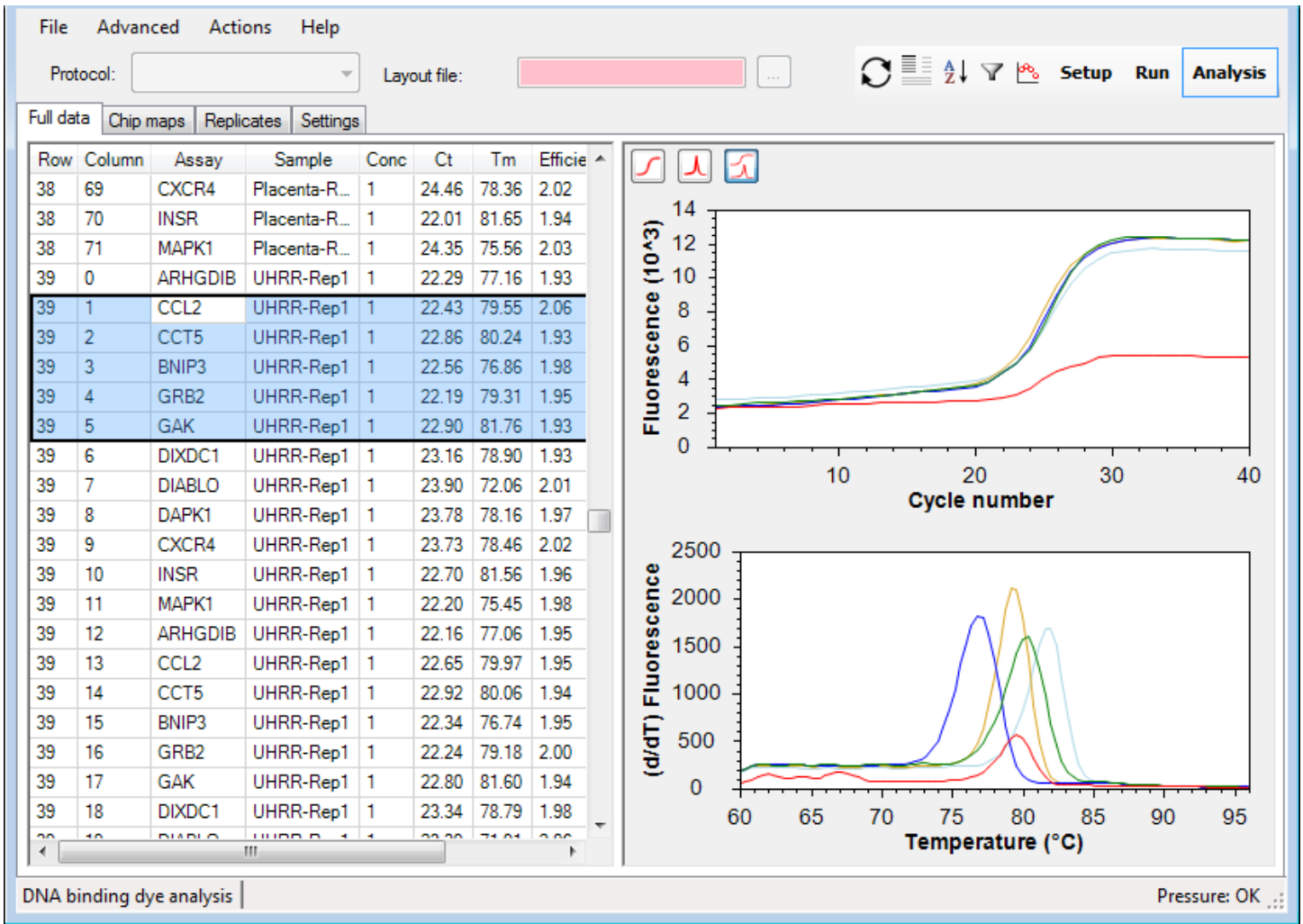   [Display both amplification and melting curves]

    [Display melting curves]

Data display icons for probe-based PCR:

    [Display amplification curves]

    [Display normalization data, if available]

In Table 16 below, the data categories **not** shown in boldface text are displayed only in Advanced user mode.

Table 16. Categories and functions in the Full data tab.

Category	Function
Row, Column	Row/column of the well, relative to the area defined in the Assay Map file. Starting index is 0. Note that gaps in the dispensed area (empty rows) are still counted.
Assay ID	Name of the PCR assay in the well. Wells that have no PCR assays are not listed in the table.
Sample	Name of the sample in the well.
Conc	Concentration of the sample in the well. This is available only if there is a dilution series of samples. A Sample Layout file
Ct	C_t denotes the threshold cycle. This is also known as the quantification cycle (Cq) and crossing point (Cp). **DNA binding dye-based expression analysis**: The C_t is calculated using the second derivative maximum method. In previous versions of qPCR Software (prior to version 2.8.0.65), C_t was calculated as the cycle in which fluorescent signal reaches 10% of the maximum fluorescence of the reaction (based on curve fitting). If you are unsure, you can check which method was used by looking at the **UseSecondDerivativeMax** value (**True** or **False**) in the **Curve Analysis Settings**. **Probe-based expression analysis**: Calculated using the second derivative maximum method.
Tm	(**DNA binding dye-based PCR only**) The amplicon melting temperature (T_m).
Expected Tm	(**DNA binding dye-based PCR only**) Available when running pre-dispensed chips.
Norm init	(**Probe-based PCR only**) The initial fluorescence value of the ROX dye measured at the beginning of the run.
Norm final	(**Probe-based PCR only**) The end fluorescence value of the ROX dye measured at the end of the run.
Norm range	(**Probe-based PCR only**) The fluorescence range of ROX dye measured during a run.
R2	Generalized version of the coefficient of determination (R^2).
Chi2	Normalized sum of squared residuals.
Straight R2	(**DNA binding dye-based PCR only**) Represents the R^2 value of a straight line fit.
Efficiency	PCR efficiency as calculated from the fitted curve.
Multiple peaks	(**DNA binding dye-based PCR only**) Checked if multiple peaks are detected in the melting curve.
Outlier	Indicates outlier wells.
Multimodal melt peaks	(**DNA binding dye-based PCR only**) Indicates if the T_ms of the replicates exhibit a multimodal distribution.
F0	This value indicates the theoretical fluorescence at cycle 0, based on the curve fit.
Flags	All flags associated with the curve. (See Appendix D)
Asymmetry	Normalized value for well symmetry. Zero means totally symmetric; the maximum value is 255.

Table 16 continues on next page.

Table 16. Categories and functions in the Full data tab continued.

Category	Function
A, B, C, D, (E)	A: Fluorescence value of the baseline (represents background).
	B: Number of cycles between baseline fluorescence and saturation.
	C: Inflection point—halfway between baseline fluorescence and saturation.
	D: The increase in fluorescence between baseline and saturation.
	E: Asymmetry parameter—describes the difference between the curves at the base and the top of the exponential phase of amplification (this affects the definitions of all other parameters).
	(**DNA binding dye-based expression analysis**) Data are fit to a sigmoid curve according to the following formula: $$f(x) = \frac{D}{1 + e^{-\left(\frac{x-C}{B}\right)}} + A$$ (**Probe-based expression analysis**) Data are fit to a five-parameter logistic curve according to the following formula: $$f(x) = \frac{D}{\left(1 + \left(\frac{x}{C}\right)^B\right)^E} + A$$
AmpRatio	The ratio of the sigmoid curve parameters.
TmMinusMean	(**DNA binding dye-based PCR only**) Amplicon T_m minus the mean T_m value of replicates
CtMinusMean	C_t value minus the mean C_t value of replicates.
S-DispenseTip	The dispenser tip on the SmartChip ND used to dispense the sample.
S-DropIndex	The SmartChip ND dispense event for the nanowell. (The dispense head has 8 valves that dispense liquids in nanowells. So, for example, an S-DropIndex of 4 means that the nanowell was dispensed during the 4th time the dispense head was positioned and released liquid.)
S-GlobalDropIndex	The number of dispense events used by the SmartChip ND tip to dispense all samples into all nanowells in the chip.
S-SP-Well	The SmartChip ND Source Plate well from which the sample was drawn for dispensing into the chip.
A-DispenseTip	The dispenser tip on the SmartChip ND used to dispense the assay.
A-DropIndex	The SmartChip ND dispense event for the nanowell. (The dispense head has 8 valves that dispense liquids in nanowells. So, for example, an S-DropIndex of 4 means that this nanowell was dispensed during the 4th time the dispense head was positioned and released liquid.)
A-GlobalDropIndex	The number of dispense events used by the SmartChip ND tip to dispense all assays into all nanowells in the chip.
A-SP-Well	The SmartChip ND Source Plate well from which the assay was drawn for dispensing into the chip.

2. Chip Maps Tab

By selecting the *Chip maps* tab, the window displays information across the entire SmartChip Panel, often at the single-well level (Figure 40). If applicable, a histogram of measurements is also displayed.

NOTE: You may want to resize the columns in this window to make them wide enough to read the contents. You can resize the entire grid by dragging the first column wider or taller.
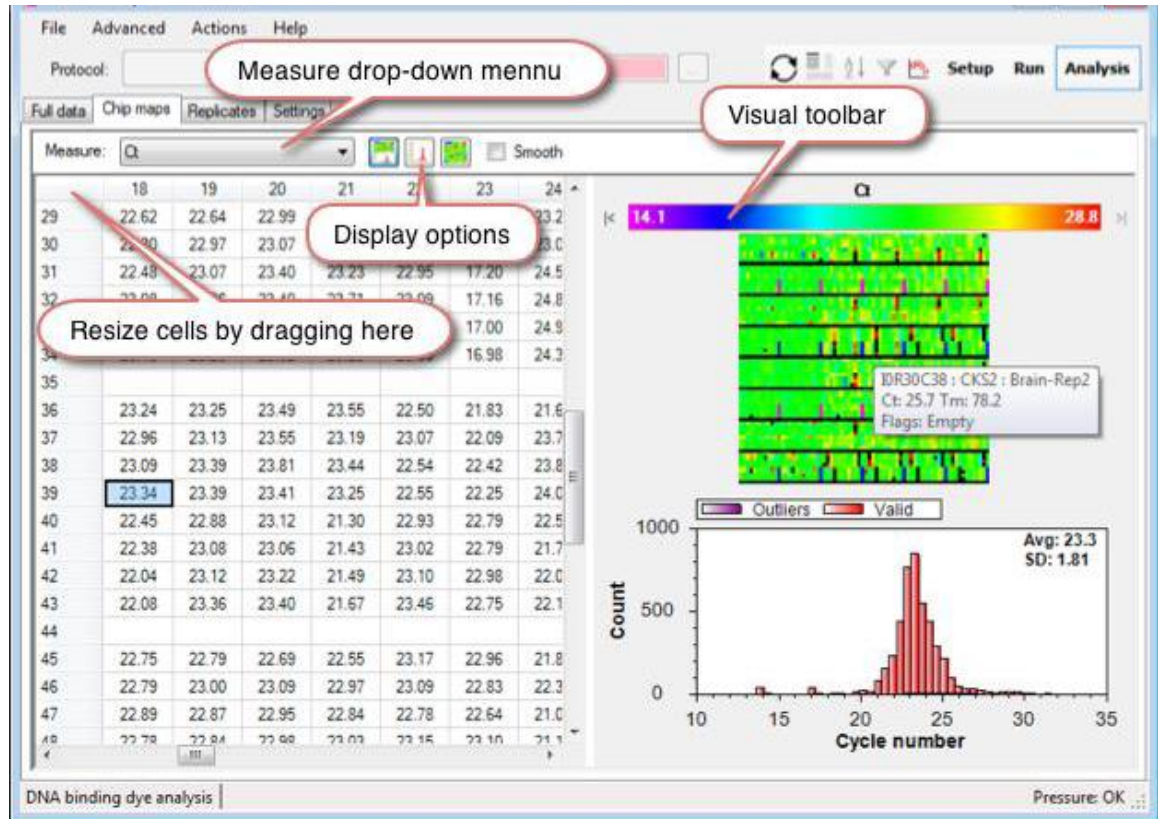

Figure 40. Chip maps tab.

Measure: Use the **Measure** drop-down menu to select the data you want to include. All columns from the *Full data* tab are available, plus the following additional information.

Table 17. Additional information available in the Measure drop-down menu.

| Function	Measure
TmSD	(**DNA binding dye-based PCR only**) The standard deviation of the T_m value for the replicates group to which the selected well/data point belongs.
CtSD	The standard deviation of the C_t values for the replicates group to which the selected well/data point belongs.
Functional Replicates	The number of non-outlier replicates in the replicate group.

NOTE: Replicates will have identical values measured.

Chip map graphic display: When the mouse cursor is positioned at the top of the map, a tool tip displays details for the well under the cursor.

Display options: You can use these icon ( ) to display the map, histogram, or both, respectively.

The visual toolbar:

You can perform the same adjustments with the visual toolbar as with the Image Contrast Toolbar (see page 105).
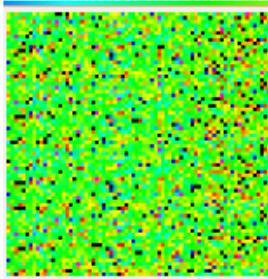
By clicking on a well in the map, the table or the image corresponding to the well is highlighted. It is also possible to select/deselect multiple table items by pressing the **[Shift]** key while mouse-clicking on table cells. The Image Viewer also supports **[Ctrl]**+mouse-click.

3. Replicates Tab

Selecting a row in the table will display all replicate curves in the graph below (Figure 41). Curves that have been declared outliers are drawn with dashed lines. If the *Image Viewer* is open, the selected wells will be outlined in the image. Conversely, clicking on a well in the image will select the corresponding line in the table.

The curve viewing features are slightly different for DNA binding dye-based and probe-based PCR.
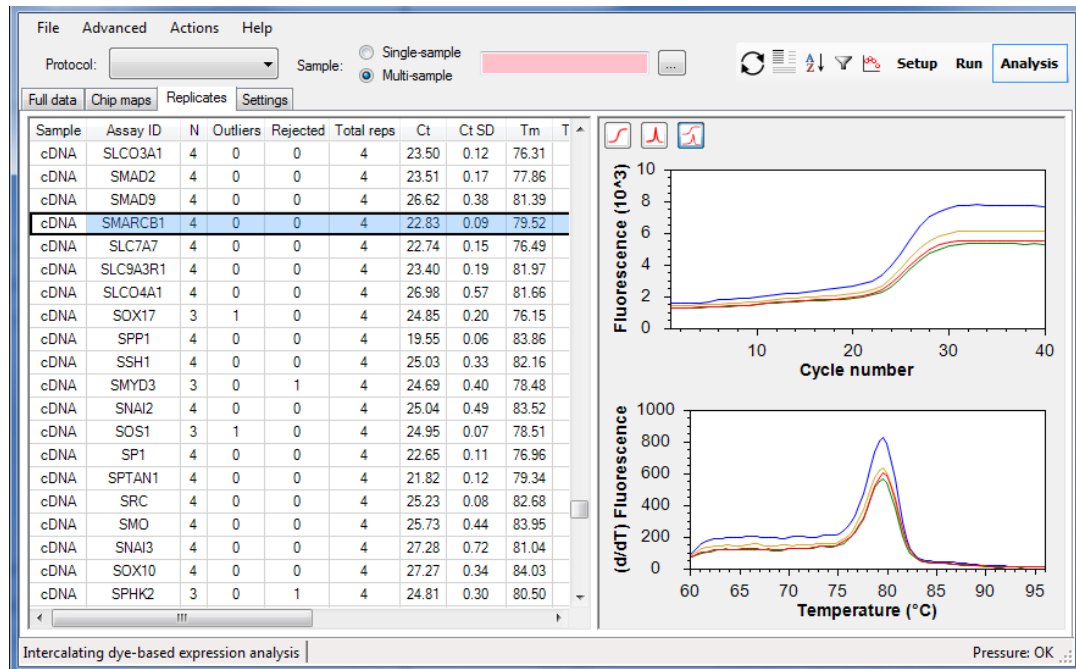

Figure 41. Example display of all replicate curves.

Rejected values and outliers are not included in the averages. Rejected wells are rejected a priori, based on their individual properties, while outliers are excluded because the C_t differs from the average of all other replicates. Dashed lines the amplification or T_m curve windows indicate rejected values or outliers.

The *Replicates* tab takes all data at the nanowell level and summarizes for each PCR assay. The summarized data set includes statistics for all wells that are not rejected or deemed to be outliers at the well level. Table 18 below defines the meaning of the column headings on the *Replicates* tab. The result categories **not** shown in boldface text are visible only in Advanced user mode.

Table 18. Column headings in the Replicates tab.

Result	Description
Sample	Sample name
Assay ID	PCR assay name
N	Number of wells with the same Assay/Sample combination. May be less than the number of wells that have been dispensed if outliers have been detected or wells were rejected.
Outliers	Number of non-rejected wells that fall outside the Grubbs outlier statistical analysis test.
Rejected	Number of rejected (according to curve fit analysis settings) samples within a replicate group (a set of wells that have identical content, i.e., same primer and sample, and if the sample is defined the set must also have the same sample concentration).
Total reps	Total number of replicates with the same Primer/Sample combination.
Ct	Average C_t value of the curves. Only non-outlier, non-rejected wells are included.
Ct SD	Standard deviation of the C_t values.
Tm	(**DNA binding dye-based PCR only**) Average T_m of the curves. Only non-outlier, nonrejected wells are included.
Tm SD	(**DNA binding dye-based PCR only**) The standard deviation of the T_m values.
Efficiency	Average reaction efficiency based on curve fit. Value does not necessarily match the efficiency obtained from a dilution series.
Conc	The original concentration of the sample. (Used for dilution series.)
Multiple peaks	(**DNA binding dye-based PCR only**) Checked if at least one curve has a primer-dimer peak in the melt curve.
Flags	All flags associated with the curve, even benign ones.

4. Calibrations Tab (Displayed in Advanced User Mode Only)

If the sample information file contains concentration information and the chip includes a dilution series, the qPCR software will generate a calibration curve (Figure 42).
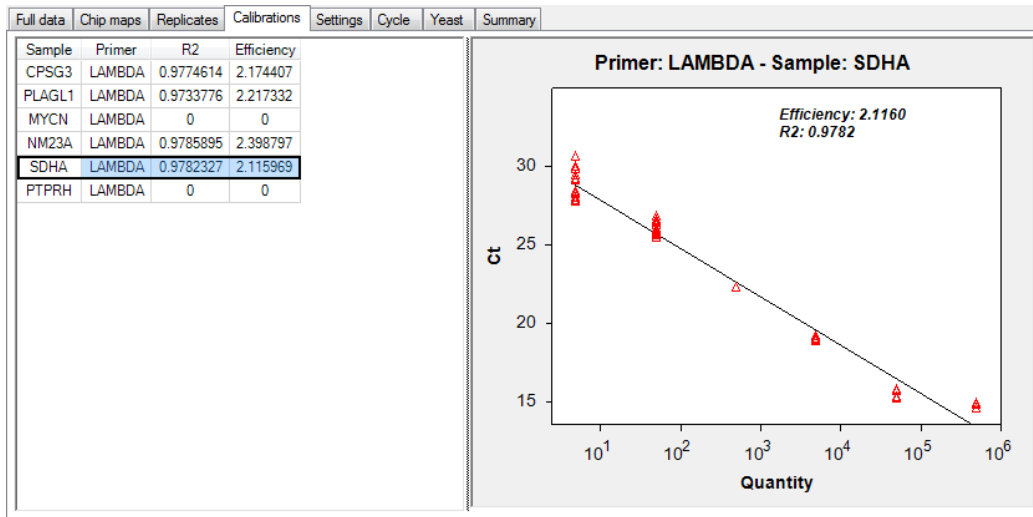

Figure 42. Example calibration curve.

5. Settings Tab

This is a read-only copy of the settings used for analysis of the data. The quickest way to change these settings is to click the [Curve analysis settings] icon ( ) on the top right of the window (Figure 43).
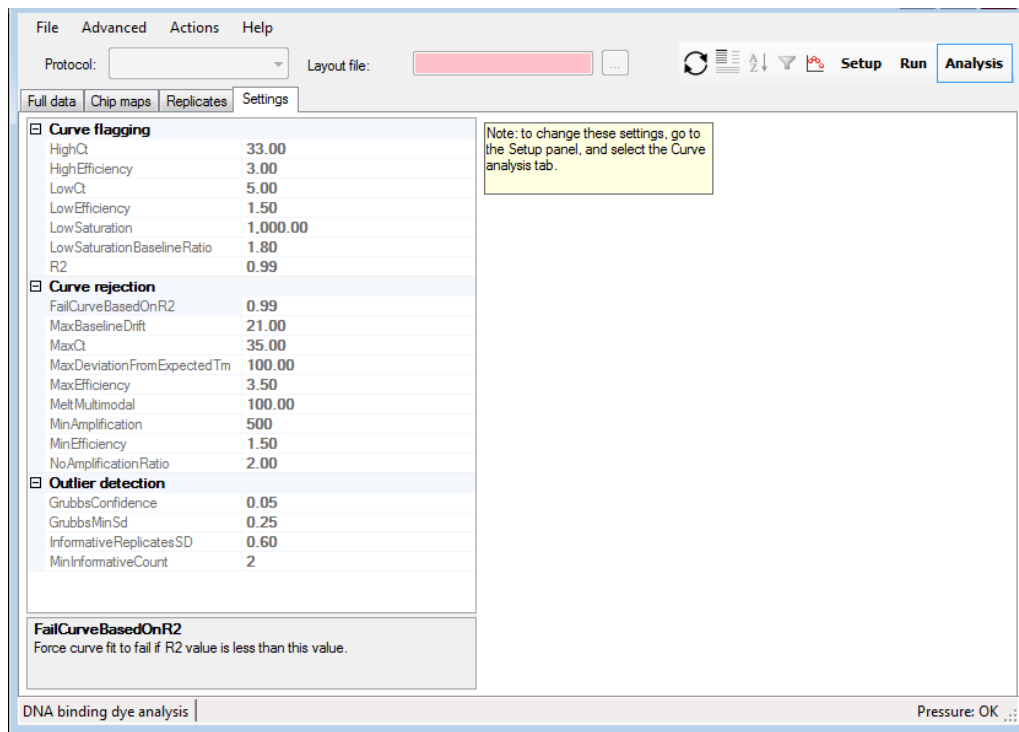

Figure 43. Settings tab.

6. Cycle Tab (Displayed in Advanced User Mode Only)

The *Cycle* tab (Figure 44) contains detailed signal values for each nanowell from every cycle of the thermal protocol, organized in a map view: the location in the table or heatmap matches the location on the chip.

NOTE: You may want to resize the columns in this window to make them wide enough to read the contents. You can resize the entire grid by dragging the first column wider.
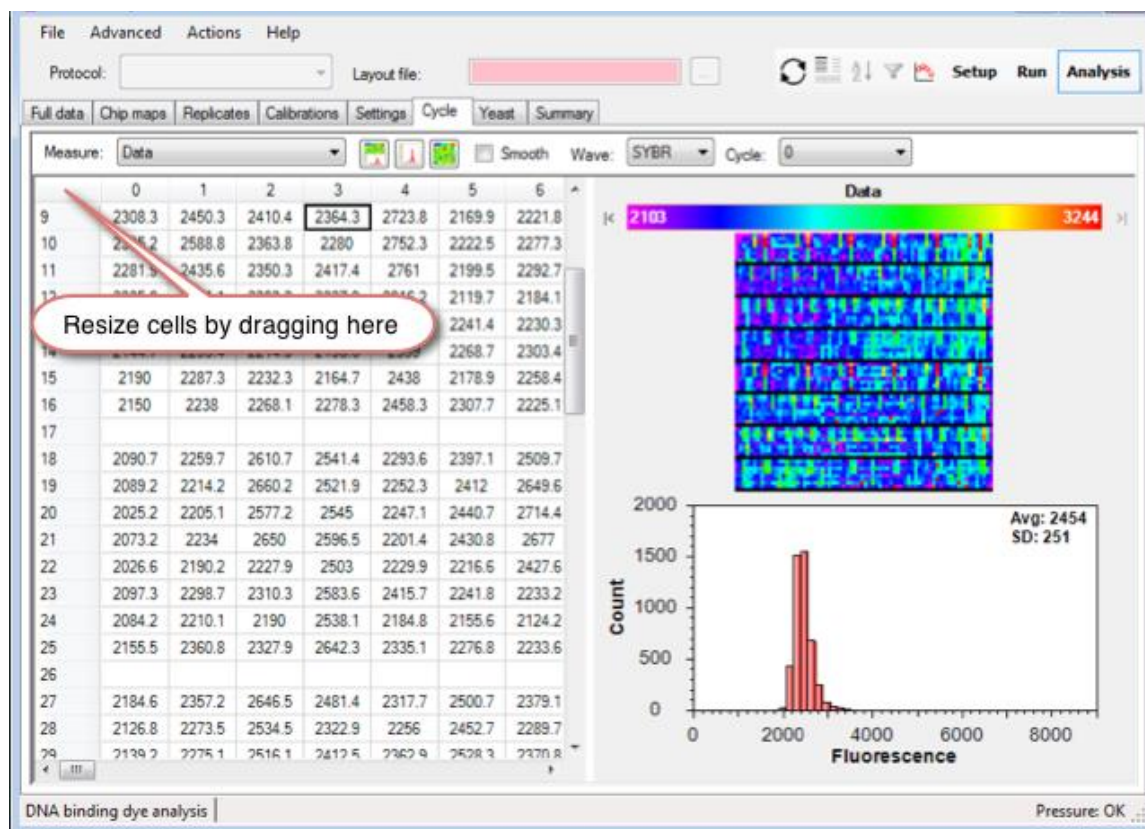

Figure 44. Cycle tab.

Measure: Use the down arrow in the **Measure** drop-down menu to select **Data**, the **fluorescence signal**, or **Asymmetry**.

Smooth: Click the box next to "Smooth" to check the SmartChip run for experimental defects. When "Smooth" is checked, the display will show the 3 x 3 nanowell median fluorescence value.

Wave: Choose which fluorescent dye data to display. Display 'SYBR' or 'FAM' to view expression analysis data from DNA binding dye- or probe-based expression analysis, respectively. All nanowells should contain an equal amount of ROX dye, so displaying 'ROX' data will give you an idea of the uniformity of reaction mixtures in the Source Plates and the dispense volumes from the SmartChip ND.

Cycle: The **Cycle** drop-down menu allows selection of individual cycles. –1 is the reference image taken before cycling starts.

7. Yeast Tab (DNA Binding Dye-Based Analysis; Displayed in Advanced User Mode Only)

The *Yeast* tab (Figure 45) displays the results of yeast control reactions.
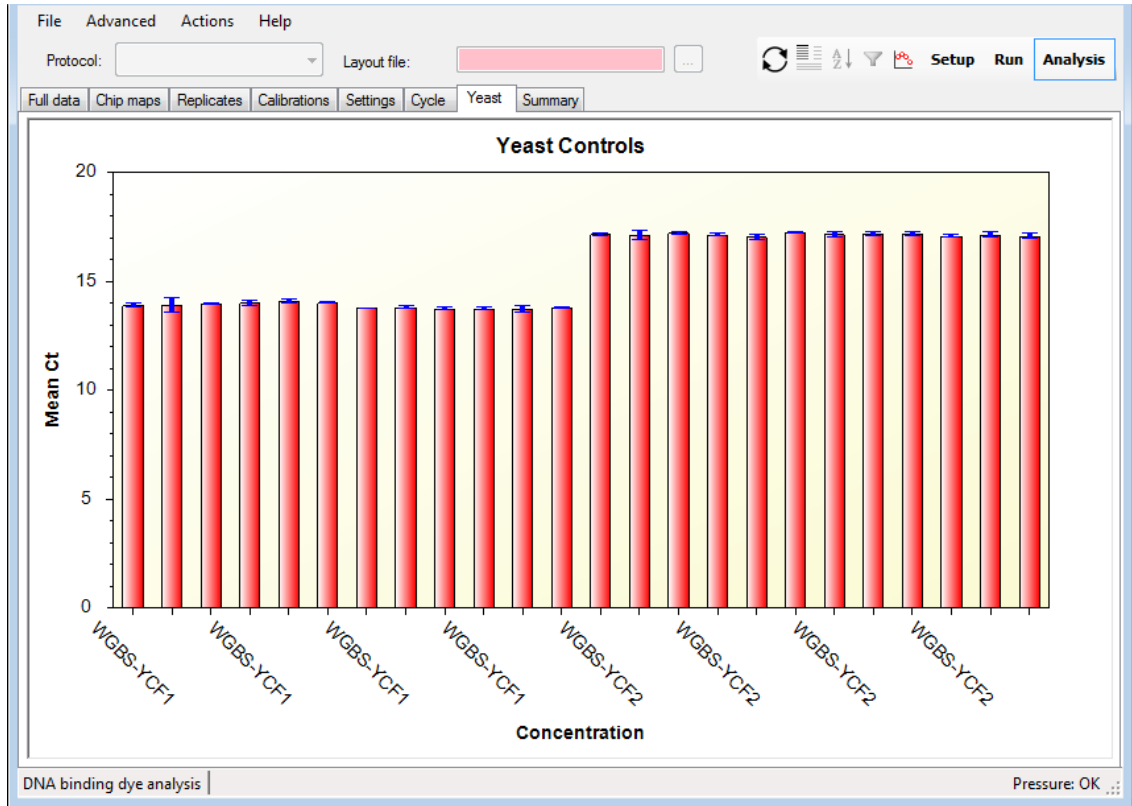

Figure 45. Yeast tab.

8. Summary Tab (Displayed in Advanced User Mode Only)

The *Summary* tab (Figure 46) displays analysis summary statistics, including overall chip data, replicate data, and flagged well data.
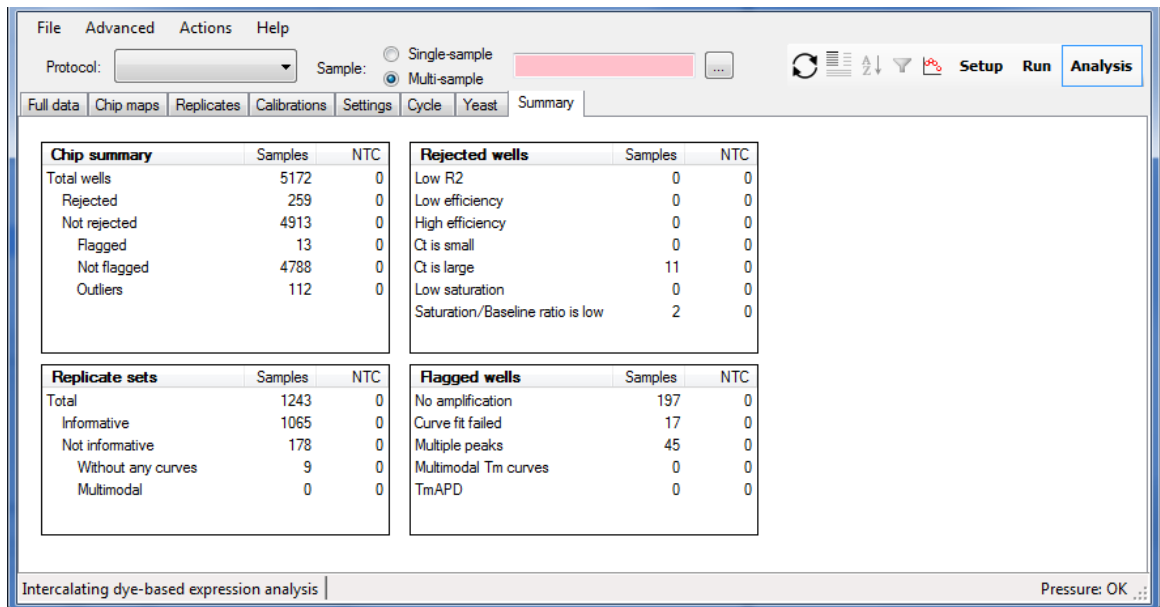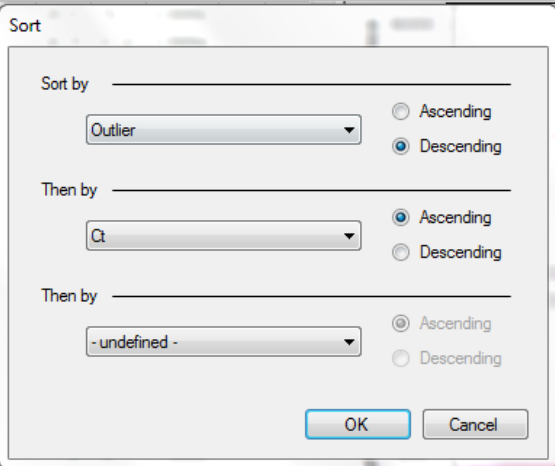

Figure 46. Summary tab.

9. Analysis Tool Icons

The following icons (Table 19) can be found in the *Analysis* mode tool bar, located in the top right corner of the SmartChip qPCR Software.

Table 19. Analysis tool icons.

Tool Icon		Function
	Analyze data	Use this button to re-analyze data. Pressing it starts cluster analysis. In almost all cases, a cluster analysis is started immediately when needed.
	Curve analysis settings	Takes you to the *Curve analysis* tab in **Setup**, where you can review/edit the curve analysis settings. You must be in Admin mode to save your modified settings for use in later experiments.
	Sort	Opens a dialog box with options to sort data by one or more columns simultaneously.

For a more convenient way of sorting by a single column, click on the column header of the *Full data* tab. A second click reverses the sort order.

	Filter	Choose to hide or show data flags. You can also filter (show) data based on flags. (See next page for more information.)
	Show scatter plot	Allows you to quickly review the data to help spot trends. (See page 64 for more information.)

10. Filter Icon

Use the [Filter] icon ⟨filter icon⟩) to obtain finer control over what is displayed in the *Full data* and *Replicates* data tables, based on the flags in each row (Figure 47).

If a filter is in effect, the tool icon is displayed in selected mode.

The filters are automatically cleared when a new analysis file is loaded, a new curve analysis is performed, or a column header is clicked.
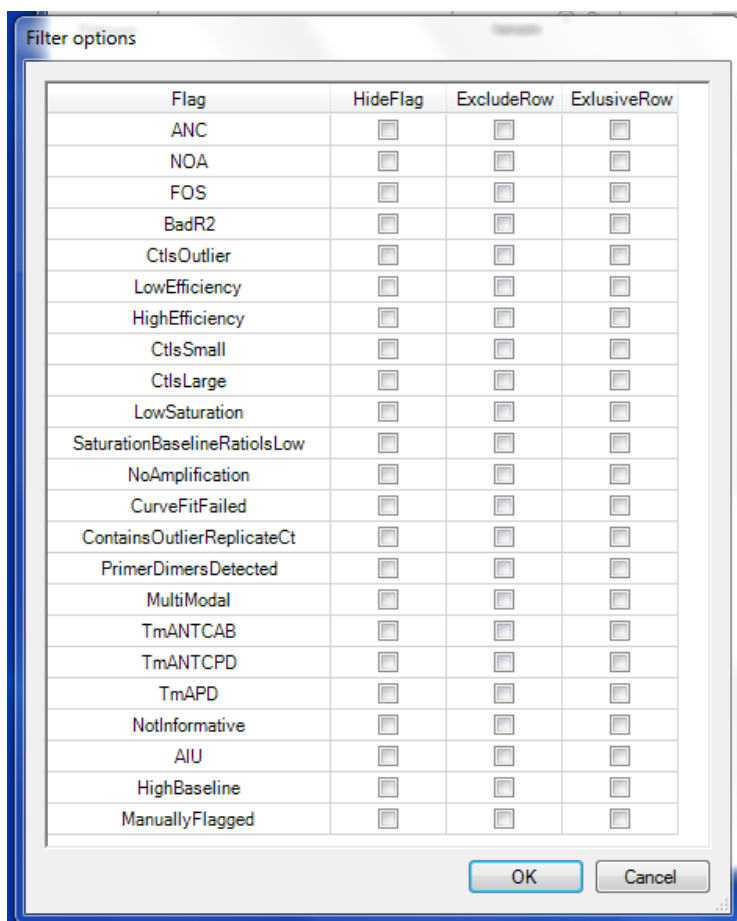
Figure 47. Filter options.

HideFlag: Do not display the selected flag in the **Flags** column of the table.

ExcludeRow: If a particular row contains the selected flag, do not show it in the table.

ExclusiveRow: Only display rows that include the specified flags. If multiple flags are selected, all rows are displayed that contain at least one of the selected flags.

NOTE: Be aware that the link between the *Image viewer* and data table may appear broken when the data are filtered. If you click on a well in the image that is not displayed in the table anymore, that well will not be highlighted.

11. Show Scatter Plot Icon

Use the [Show scatter plot] icon   ) to quickly display and find trends in the data.

The "Replicates" box allows switching between individual or replicate measurements.

The "Bin" setting groups the data points in bins. If you have x data points and your beginning value is set to y, you should have x/y displayed points on the graph. Binning works by sorting the data points by the value in the x-axis and then averaging consecutive items. Note that the "Bin" setting (Figure 48) in the left graph is '50' and is '4' in the right graph. In some cases, setting a higher binning value makes data trends more obvious.
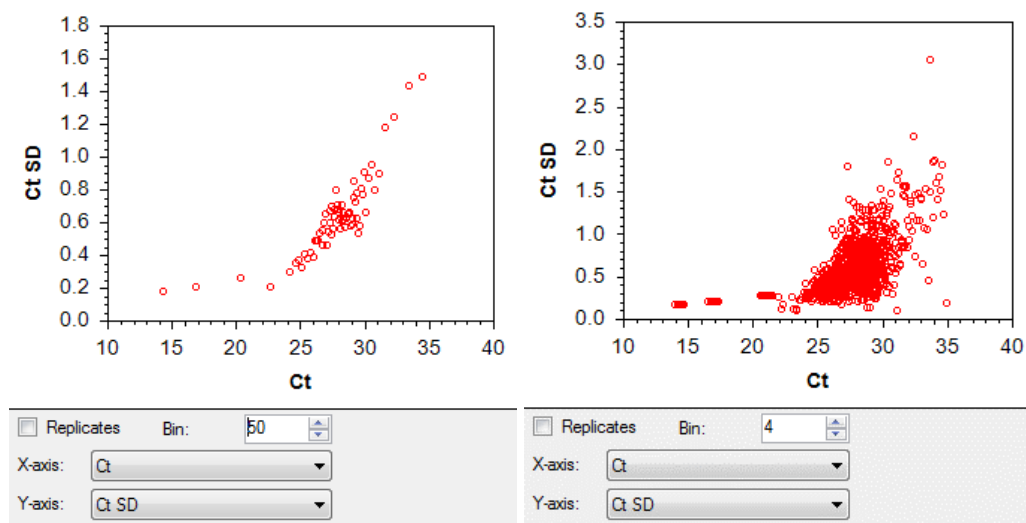
Figure 48. Example scatter plots with different Bin settings.

XIII. Genotyping Procedures: SNP Genotyping Overview
This is an overview. For detailed information, see Section XIV, "Genotyping Procedures: Setup Mode".

A.   Starting the SmartChip ND Cycler and qPCR Software

1. Turn on the SmartChip ND Cycler using the power switch on the back left-side of the instrument.
2. From the computer, open the SmartChip qPCR Software by selecting the **SmartChip qPCR** icon on the desktop, or via **Start > Program Files > qPCR > qPCR Instrument**. The software will initialize the SmartChip ND Cycler. The status of the initialization process will be shown in text at the top right of the qPCR Software window. It may take several minutes before the instrument is ready.

B.   Entering Information About Your Experiment

1. Select Setup mode by clicking on the *Setup* tab in the top right corner (Figure 49).
2. Pull down the **Actions** menu and, if necessary, switch to **Genotyping**.

3. In the drop-down menu in the "Protocol" field (top-left corner), select the appropriate protocol for your chip:

- **SmartChip MyDesign Chip**
  - o Select either one of the MyDesign_SNP_GT protocols or the MyDesign_KASP_GT protocol installed with the qPCR software, or an appropriate saved protocol from a previous experiment.
  - o Log in to Admin mode and customize the protocol for your experiment. See Section XIV.E for instructions.
- Pre-dispensed **SmartChip Custom Panel**: Double-click the setup.exe file on the CD sent with your chip to load files that describe your chip into the qPCR software.

4. Enter your Sample or Sample/PCR Assay information:

- **SmartChip MyDesign Chip:** In the "Layout file:" field, select the SmartChip layout.md file created by the SmartChip ND when the chip was filled.
- Pre-dispensed **SmartChip Custom Panel:**
  - o If you loaded more than one Sample/PCR Reagent Mixture into your chip, click the button for **Multi-sample**. Click the [...] button, and in the *Open* dialog box, select the SmartChip layout.pd file created by the SmartChip ND when you added Sample/PCR Reagent Mixtures to your chip.
  - o If your SmartChip Panel contains just one sample, loaded using the SmartChip Single Sample NanoDispenser, click the "Single-sample" radio button. Then type in your sample name.
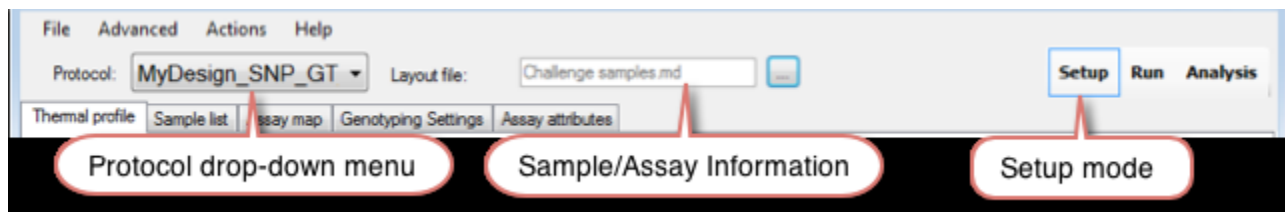
Figure 49. Entering information about your SNP genotyping experiment.

C. Starting the PCR Run

Select *Run* mode to insert the sealed SmartChip Panel or MyDesign Chip into the SmartChip ND Cycler. Start the run using the procedure below. Refer to "Genotyping Procedures: Run Mode" in Section XV for more information about using this menu.

1. If you entered Admin mode to create a new protocol, be sure to exit Admin mode in order to run your PCR. Note also that Protocol and SmartChip information must be complete before the run will start.
2. Select Run mode by clicking on the [Run] button in the top-right corner of the qPCR software interface.
3. Click the [Open drawer] icon (   ) to open the SmartChip ND Cycler drawer.

4. The *Chip Info* (Figure 50) dialog box will open. Enter values for "Chip", "User", and other requested information. This information will be included in the analysis files and Summary report.

To use the barcode reader to enter your chip number, place the mouse cursor in the "Chip" entry field. (You should see a blinking cursor in the box.) Point the barcode reader at the 2-D barcode symbol on the bottom of the chip and pull the trigger to enter the SmartChip code into the *Chip Info* window. (The code is also written in human readable form so that you can double-check that it is entered correctly.
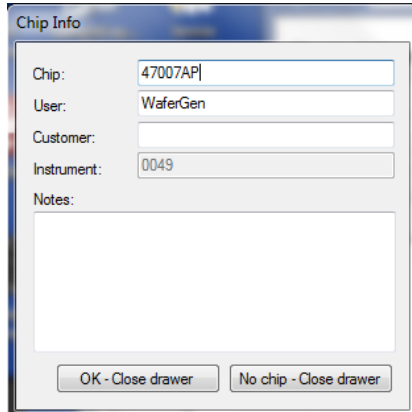
Figure 50. Chip Info dialog box.

5. Peel away the blue backing from your sealed SmartChip Panel or MyDesign Chip to expose the adhesive patches. Place it in the SmartChip ND Cycler with the beveled edge in the lower right corner as shown in Figure 51. Make sure the chip lays flat in the SmartChip holder.
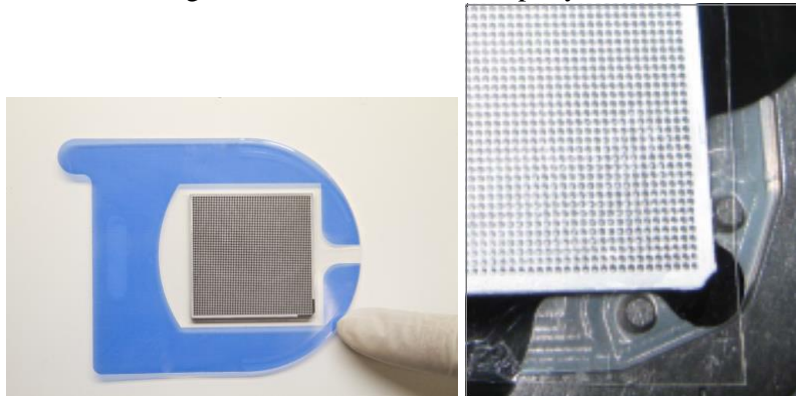
Figure 51. Peel the blue backing from the sealed chip and place it in the SmartChip ND Cycler.

IMPORTANT: Always position chips so that when viewed from above, the beveled corner is on the lower right side.

6. Click [OK-With Chip] to close the drawer of the SmartChip ND Cycler.

7. The [Start Run] icon ( ) will turn green. Press this button.

8. In the "Choose data folder" dialog box (Figure 52) that appears, select a destination folder and file name options for your run data, and click [OK].
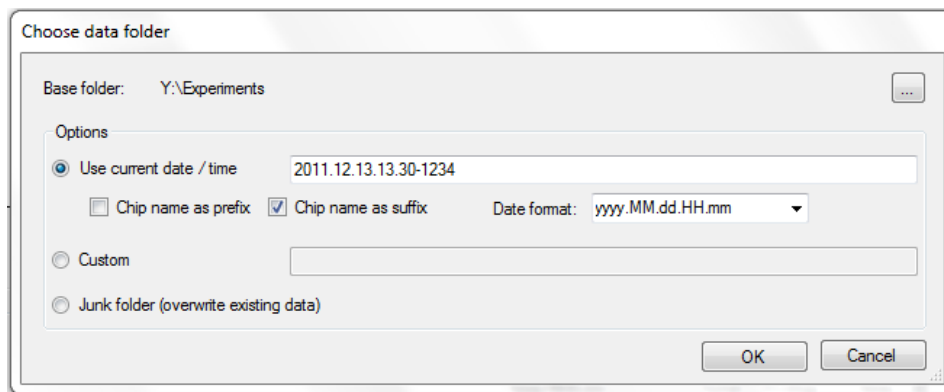
Figure 52. Choose data folder window.

IMPORTANT: The thermal cycling of the chip will start only after you have selected a destination folder for the run data and pressed [OK].

9. The run will start, and an estimated duration will be displayed in the top center of the display.
10. When the run is complete, remove the chip from the SmartChip ND Cycler and dispose of it in a covered waste container. To minimize carryover contamination risk, do not remove the adhesive film.

XIV. Genotyping Procedures: Setup Mode

A. Introduction

Setup mode defines the SmartChip ND Cycler parameters needed to perform qPCR with the SmartChip Panel or MyDesign Chip. Parameters include a protocol and a SmartChip layout file (or sample information typed into the "Sample" box for off-the-shelf SmartChip Panels).

Protocols contain the information required to describe and conduct thermal cycles on the assays found on the SmartChip Panel or MyDesign Chip. The information can include: Thermal Profile, Assay Map, Assay Attributes, Filter Selections, Instrument Settings, Post Run Settings, and Genotyping Settings.

B. Overview of Drop-Down Menus in Setup Mode for SNP Genotyping
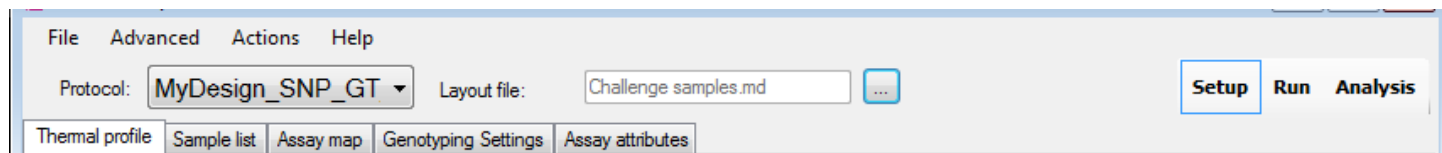
Figure 53. Setup mode top menu bar.

Table 20 below describes the selections available from the *Setup* mode top menu bar (Figure 53).

Table 20. Drop-down menu options in Setup mode for expression analysis.

Menu	Options
File	**Open** file under the selected tab **Save** file under the selected tab. **Run info** displays file, chip, user, customer, and instrument information, as well as notes.
Advanced	**Switch to advanced user mode** to display all of the data collected during genotyping PCR or **Switch to standard GUI** to display only information that is most frequently accessed. **Create user protocol** can be used to modify a protocol's genotyping settings and save them. **Image viewer** is designed for visual inspection of the data. For more information, see "Image Viewer" in Section XVII. **Admin mode** should be used by trained personnel only. It is used to save changes to genotyping settings and to modify protocols using the **Protocol Manager**. Changing any of these settings can affect your experimental results. The password to enter Admin mode is printed on the qPCR Software CD. **Service mode** is designed for Takara Bio staff to configure advanced SmartChip ND Cycler settings.
Actions	Switch between **DNA binding Dye-Based Expression**, **Probe-Based Expression**, or **Genotyping Analysis** modes.
Help	**About** provides qPCR Software version, copyright, and license agreement information. **Open User Manual** opens this User Manual in pdf format. **Setting up a run** opens a Quick Guide for setting up a run. **Saving run data** opens a Quick Guide for saving run data. **Loading an existing chip run** opens a Quick Guide for loading an existing chip run. **Reprocessing a chip run** opens a Quick Guide for reprocessing an existing chip run.

C.   Overview of Tabs in Setup Mode for SNP Genotyping

Each tab in Setup mode corresponds to a file with instructions for a specific SmartChip ND Cycler feature or function. The qPCR Software accesses this information through SmartChip Layout files and Protocol files. Table 21 below shows the information organization for different types of chips. Tabs shown in bold text are visible in the standard GUI. Those not shown in bold are displayed only in advanced user mode.

Table 21. Overview of tabs in Setup mode for expression analysis.

Tab	SmartChip MyDesign Chips	SmartChip Custom Panels
Thermal Profile Filters Instrument Settings **Genotyping Settings** Post Run Settings	Protocol	Protocol
Sample List **Assay Map**	`SmartChip` `layout.md`	
Assay Attributes		`SmartChip` `layout.pd`

Assay Attributes

If you previously defined assay attributes for SmartChip MyDesign Chips in the SmartChip Dispenser Software that runs the SmartChip ND, then this information will be populated when you load the SmartChip Layout file into the qPCR software. Alternatively, you can create a .csv file that contains three columns: Assay name, Dye 1 allele, and Dye 2 allele, using Microsoft Excel or a similar program, as shown in Figure 54 below. Place the file in the following folder, depending on your computer's operating system:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\AssayAttributes`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\AssayAttributes`

	A	B	C	
1	Name	FAM	VIC	
2	Assay1	C	T	
3	Assay2	G	A	
4	Assay3	A	G	
5	Assay4	C	G	
6	Assay5	C	T	
7	Assay6	C	A	
8	Assay7	G	A	
9	Assay8	A	C	

Figure 54. Example .csv file for assay attributes.

Create a custom protocol and import the .csv file with your assay attribute information into the protocol using the Protocol Manager (see Section XIV.E.2).

D. Setup Mode Instructions for SNP Genotyping

The precise instructions for setting up a SmartChip ND Cycler run depend on what type of SmartChip nanowell chip you are using and whether you are using probe-based detection (e.g., TaqMan) or allele-specific PCR (e.g., KASP). Follow the instructions below specific to your chip.

1. SmartChip MyDesign Chip

If you are running a SmartChip MyDesign Chip that was empty when supplied from Takara Bio and PCR reagents were dispensed into the chip at your facility, follow these instructions.

a. Click the icon next to "Protocol" to pull down the menu, then select an appropriate Protocol file:
   * Choose **Protocol > MyDesign SNP_GT** for probe-based discrimination or **Protocol > MyDesign KASP GT** for allele-specific PCR discrimination.
   * Select an appropriate saved protocol from a previous experiment (Figure 55).
   * If you need to change any of the cycling or analysis parameters in the available protocols, you will need to create one and select it. See Section XIV.E for instructions.
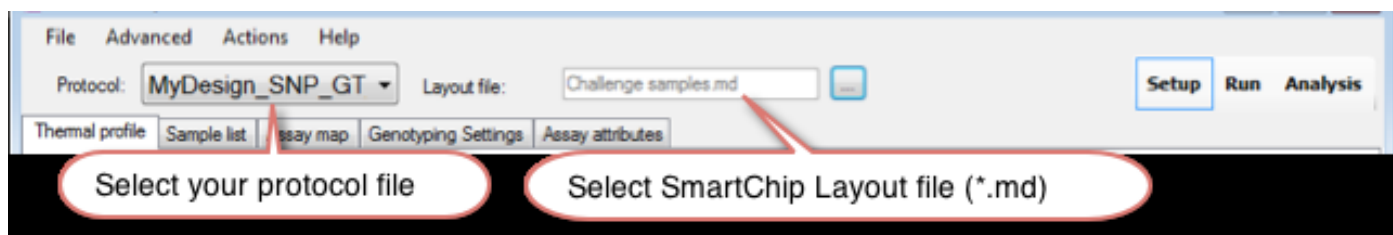
Figure 55. Selecting a saved protocol from a previous experiment for your SmartChip MyDesign Chip.

b. Click the [**…**] button next to the **Layout** file box to open the *Open* dialog box. Navigate to the location of the SmartChip Layout file (.md extension) from the SmartChip ND for your chip. (You will likely access the SmartChip Layout file on a USB memory stick or across a network.) Select the file and click [Open] to accept your choice and close the window.

c. If you entered Admin Mode in order to create a new protocol, be sure to exit Admin mode in order to run your SmartChip PCR.

2. (Pre-dispensed) SmartChip Custom Panel

If you are running a SmartChip Custom Panel containing a set of PCR Assays that Takara Bio dispensed into a SmartChip Panel for you, follow these instructions.

a. Insert the CD that was sent with your SmartChip Custom Panel into the computer that controls the SmartChip ND Cycler.

b. Run the setup program from the CD to load your SmartChip Panel Protocol File into the qPCR Software—just double-click on the file named `setup.exe`.

c. Enter your sample information:
   * If your SmartChip Panel contains multiple samples that were loaded using the SmartChip ND, click the **Multi-sample** radio button next to "Sample". Then click the button labeled [**…**] to open the *Open* dialog box and navigate to the location of the SmartChip Layout file (.pd extension) from the SmartChip ND for your chip. (You will likely access the SmartChip Layout file on a USB memory stick or across a network.) Select the file and click [Open] to accept your choice and close the window (Figure 56).

Figure 56. Selecting the protocol for your pre-dispensed SmartChip Custom Panel.

- If your SmartChip Panel contains just one sample, loaded into the panel using the SmartChip Single Sample NanoDispenser, click the **Single-sample** radio button. Then type in the name of the sample you loaded onto your SmartChip Panel.

IMPORTANT: Once the protocol and SmartChip layout are selected, the information that appears within each tab cannot be changed or modified.

E.   Creating a New Protocol for SmartChip MyDesign Chips (SNP Genotyping)

There are two ways to create new protocols. If you simply need to modify the genotyping settings, you should use Method 1: Creating a User Protocol (see below). If you want to modify other aspects of the protocol, for example to add assay attribute information for allele calling, you will need to use Method 2: Protocol Manager. For either method, you will need to enter Admin mode. The two methods are described below.

1. Method 1: Creating a User Protocol

a. Enter Admin mode: From the **Advanced** drop-down menu, select **Admin mode…** In the window that appears, type the password (case-sensitive) from your SmartChip qPCR Software CD. If you cannot locate your password, contact Takara Bio technical support (technical_support@takarabio.com) for help.

NOTE: Once you are in Admin mode, you can change your password if desired.

b. From the *Genotyping Settings* tab (Figure 57), adjust your analysis settings as needed.
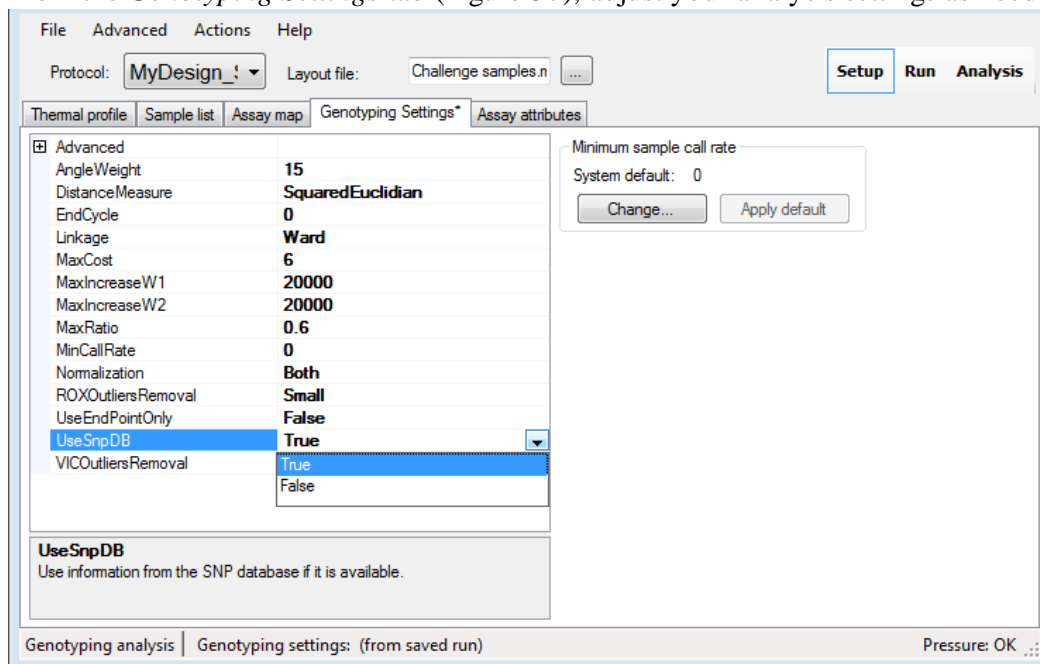
Figure 57. Genotyping Settings tab.

c. Save your adjusted genotyping settings: From the **File** drop-down menu, select **Save genotyping analysis settings**. In the window that opens (Figure 58), choose a logical name and file location for the settings and click [Save].
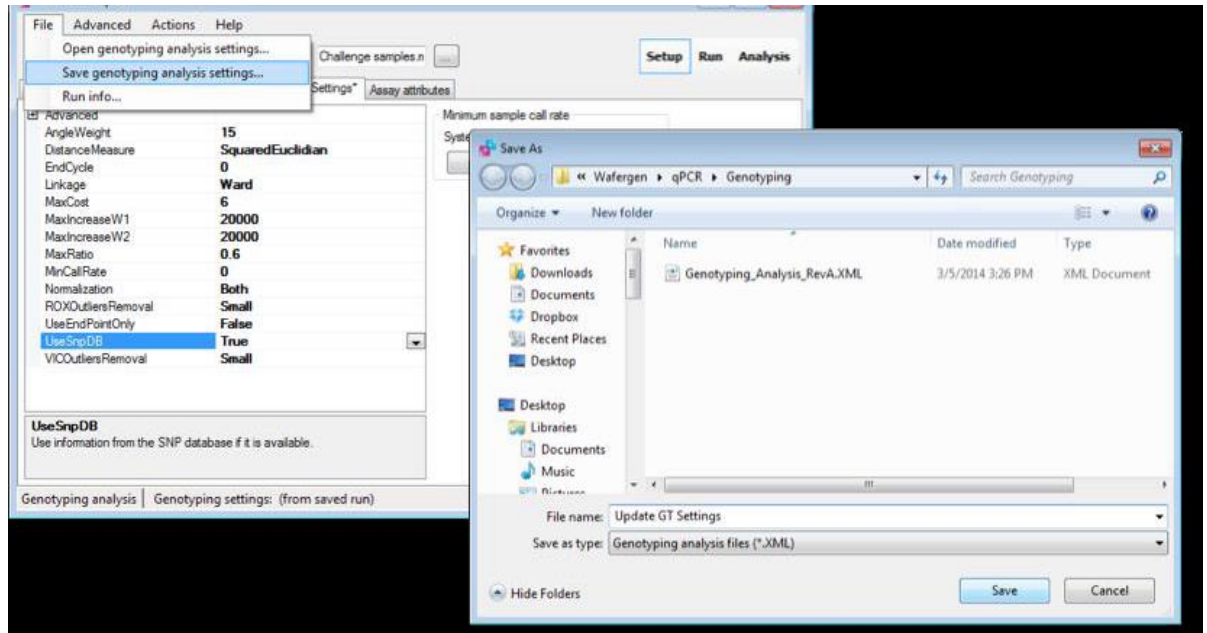
Figure 58. Saving adjusted genotyping analysis settings.

d. Exit Admin mode: From the **Advanced** drop-down menu, deselect **Admin mode…** (the check mark will disappear.)

e. From the **Advanced** drop-down menu, select **Create user protocol…** In the window that opens, choose the protocol that you want to modify with your newly created genotyping settings. In the next pull-down menu, choose the genotyping settings file you created in Step c.

f. Click the [Generate new protocol] button and save the modified protocol file with a logical name (Figure 59).
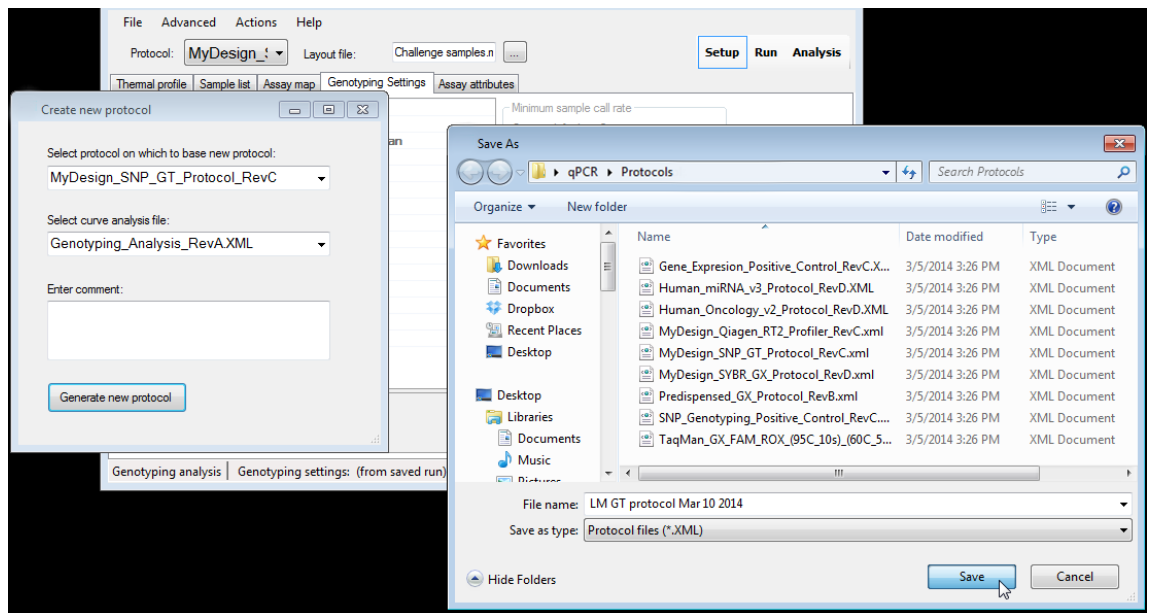
Figure 59. Saving your modified genotyping protocol.

2. Method 2: Protocol Manager

NOTE: Protocols can be deleted using the Protocol Manager.

a. Enter Admin mode: From the **Advanced** drop-down menu, select **Admin mode…** In the window that appears, type the password (case-sensitive) from your SmartChip qPCR Software CD. If you cannot locate your password, contact Takara Bio technical support (technical_support@takarabio.com) for help.

NOTE: Once you are in Admin mode, you can change your password if desired.

b. Create a copy of one of the default MyDesign_SNP_GT protocols using the *Protocol Manager* and click [Edit] at the bottom of the window.
   i. For probe-based detection, choose **MyDesign_SNP_GT_Protocol_RevA**.
   ii. For KASP chemistry, choose **MyDesign_KASP_GT_protocol_RevA**.
c. Rename the protocol as desired in the "Protocol name" field.
d. If you want to import the .csv file with your assay attributes, pull down the "Assay attributes**"** drop-down menu, navigate to the file with your assays, and select it.
e. Click [OK]. This will open a *Name has changed* window. We recommend that you choose to **Create a new protocol, keep previous** (Figure 60). This preserves a copy of the default MyDesign SNP genotyping profile on your computer. Close the *Protocol Manager* window by clicking the red [X] icon in the upper-right corner.
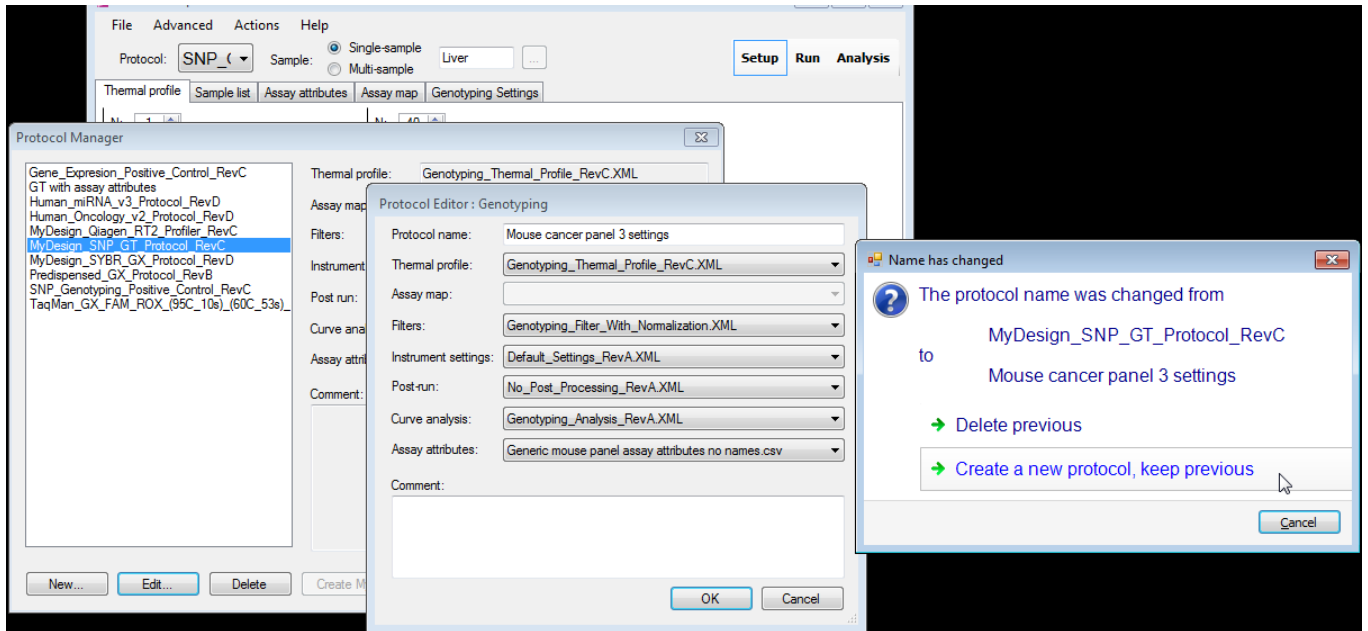
Figure 60. Renaming and saving the new genotyping protocol.

f. If you want to adjust the thermal cycling protocol, do so as follows:

    i. Select the protocol you want to change in the **Protocol** box. Select the *Thermal profile* tab and change the thermal cycling parameters by typing in new times and/or temperatures. You can also add and delete cycle components using the buttons at the lower left.

    ii. Save the modified thermal profile by choosing **File > Save thermal profile…** from the drop-down menu.

g. Pull the modified thermal profile into your new protocol file:

    i. From the **Advanced** drop-down menu, select **Protocol Manager**. Choose the protocol into which you want to import the new thermal profile.

    ii. Click [Edit] at the bottom of the *Protocol Manager* window. This will open the *Protocol Editor* window.

    iii. In the "Thermal Profile" pull-down menu, select the thermal profile you just saved. Click [OK] to accept your choice and close the *Protocol Editor* window. Click the red [X] icon to close the *Protocol Manager* window.

h. Exit Admin mode in order to run your experiment. From the **Advanced** drop-down menu, deselect **Admin mode…** (the check mark will disappear).

F.   Setup Tabs for SNP Genotyping

You can use the Setup Tabs to make protocol adjustments or to take a closer look at the various protocol components of a run. You must be in Admin mode to make changes in most of these tabs, and some areas can only be changed in Service mode.

1. Thermal Profile

Select the *Thermal profile* tab to display a graph of the thermal profile; Figure 61 is an example of a thermal profile of a probe-based cycling reaction. Vertical lines visually separate each cycle. Temperatures and durations for each cycle are displayed. Note that the ramp times are not shown in the user interface. The icon indicates that a fluorescence image capture has started. Each cycle can contain one or more steps, and a cycle can be repeated several times.

IMPORTANT: If you have upgraded your qPCR software, we recommend that you use the updated protocols and associated thermal profiles packaged with the software. If you need custom thermal profiles created using an earlier version of qPCR software, contact Takara Bio technical support (technical_support@takarabio.com) for assistance with replicating them precisely.

In Admin Mode, you can change the thermal cycling parameters by typing in new times and/or temperatures. You can also add and delete steps and cycles using the buttons at the lower left. Use the [Toggle] button to add or remove a fluorescent imaging event for the selected step.

Save the modified thermal profile by choosing **Save thermal profile…** from the **File** drop-down menu. Name the file and click [Save].

Figure 61. Graph of the thermal profile for probe-based SNP detection in the Thermal profile tab.

By default, Thermal Profiles are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data \Wafergen\qPCR\ThermalProfiles`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\ThermalProfiles`

NOTE: Folders may be hidden depending on whether you installed the qPCR software in Instrument or Analysis mode and/or how your Windows OS is configured.

2. Sample List Tab

The *Sample list* tab (Figure 62) shows the samples in the SmartChip Panel or MyDesign Chip.

Sample information and locations will be populated when you load the SmartChip Layout file into the qPCR software.
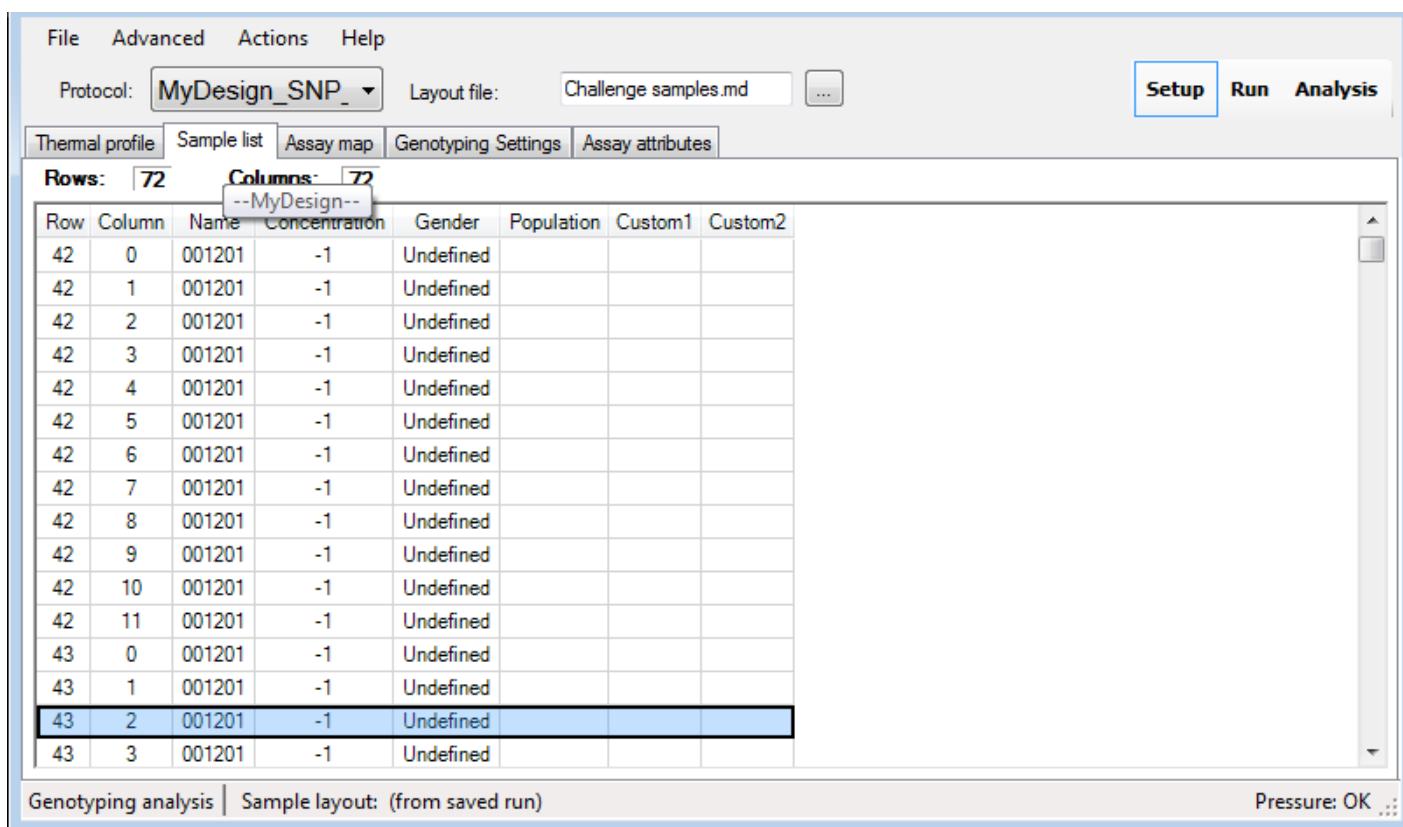
Figure 62. Sample list tab.

By default, Sample Layout files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\SampleLayoutFiles`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\SampleLayoutFiles`

3. Assay Map Tab

The *Assay map* tab (Figure 63) lists the Assay map file and contains the names and location of the assays that have been dispensed into the chip.

If you are using a pre-dispensed SmartChip Custom Panel, run the setup program provided on the CD sent with your SmartChip Panel to automatically copy Assay map files and other files to their appropriate locations.
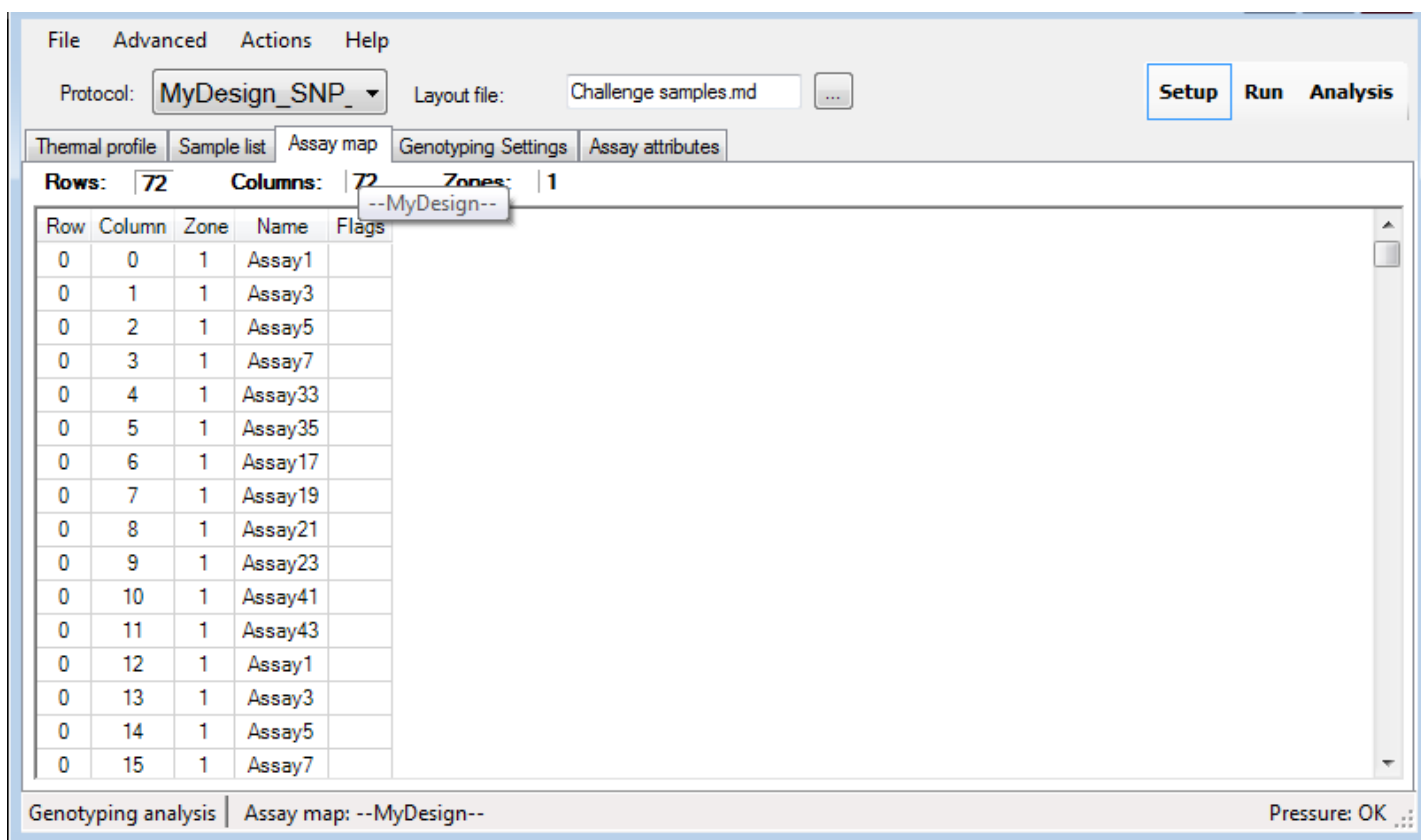
Figure 63. Assay map tab.

By default, SmartChip Assay map files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\AssayMaps`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\AssayMaps`

4. Genotyping Settings Tab

From the *Genotyping Settings* tab (Figure 64), you can view the cluster analysis parameters from a SmartChip SNP genotyping run.

IMPORTANT: We do NOT recommend changing genotyping settings. In order to edit these settings, you must be in Admin mode.

Figure 64. Genotyping Settings tab.

By default, Genotyping Settings files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\GenotypingSettings`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\GenotypingSettings`

5. Assay Attributes Tab

The *Assay attributes* tab contains assay ID, assay name, species, gene symbol, and category ID.

If you defined assay attributes for SmartChip MyDesign Chips in the qPCRStudio Software that runs the SmartChip ND dispenser, then this information will be populated when you load the SmartChip Layout file into the qPCR Software. Alternatively, you can create a .csv file using Excel that contains three columns: Assay name, Dye 1 allele, and Dye 2 allele, as in the table shown in the screenshot below (Figure 66). To use this assay attribute information, add it to your protocol using the **Protocol Manager** (see Section XIV.E.2).

If you are using a SmartChip Custom Panel, running the setup program on the CD provided with your chip will automatically copy assay attribute files and other files to their appropriate locations.
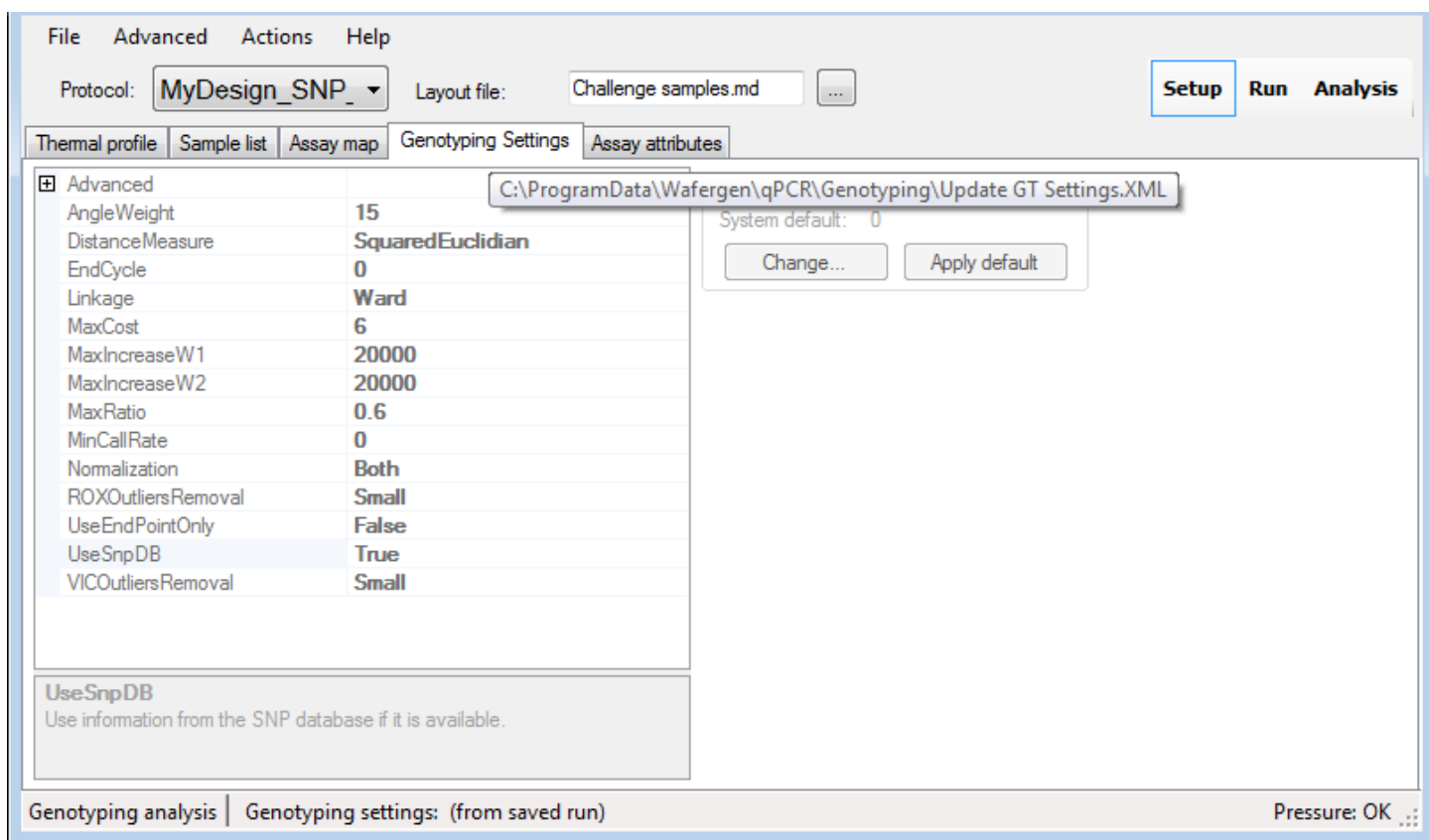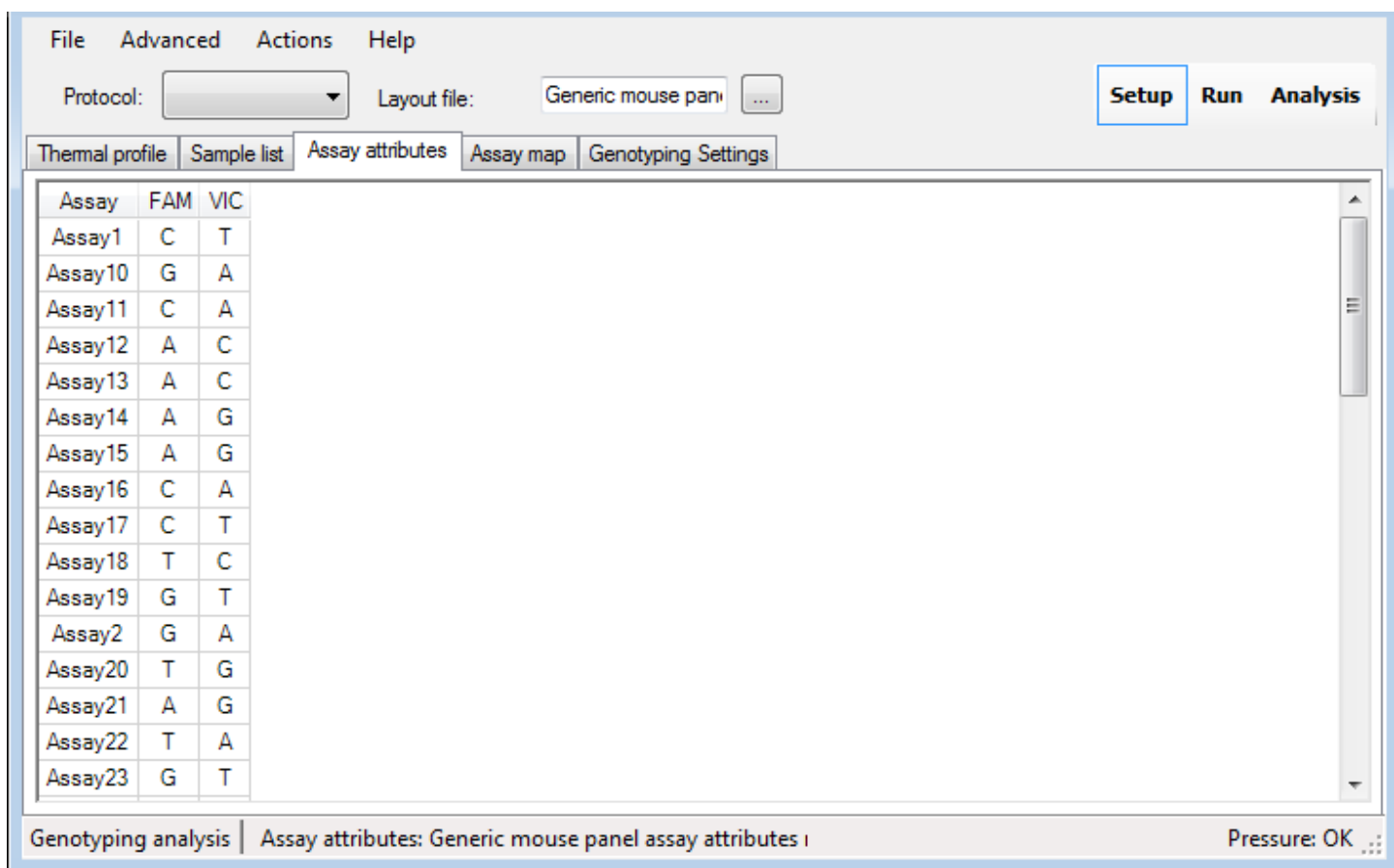
Figure 65. Assay attributes tab.

By default, Genotyping Settings files are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\AssayAttributes`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\AssayAttributes`

6. Filters Tab (Displayed in Advanced User Mode Only)

Use the *Filters* tab (Figure 66) to view the normalization and sample reading dyes.

> **IMPORTANT:** The following descriptions are for **INFORMATIONAL USE ONLY**. Do not change any of the parameters in the *Filters* tab. The SmartChip system has been tested with SmartChip SNP Genotyping Master Mix and either TaqMan SNP Genotyping Assays (Thermo Fisher Scientific) or BHQ*plus* probe (LGC Biosearch). The rhAmp SNP Genotyping Assay chemistries work under the same conditions as the probe-based detection methods. The KASP Assay chemistry (LGC Biosearch) works with minor additions to the reaction mix and a specific cycling protocol.
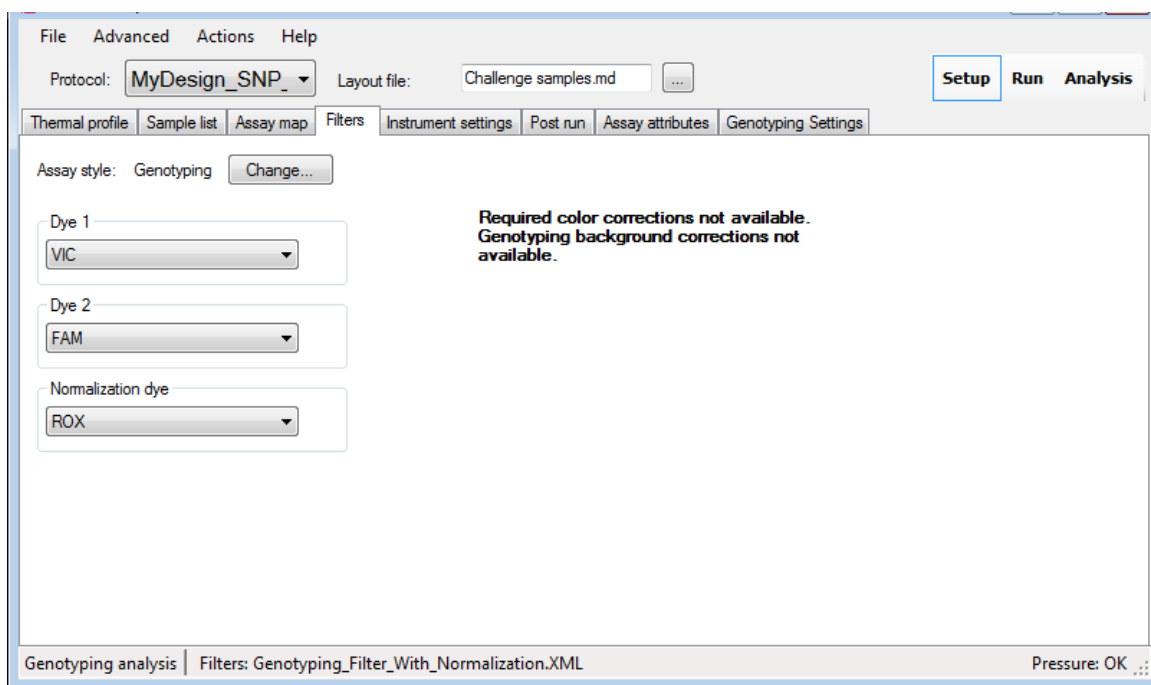
Figure 66. Filters tab.

By default, Filter files are stored in the following locations:

- **Windows XP**—C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\Filters
- **Windows 7**—C:\ProgramData\Wafergen\qPCR\Filters

7. Instrument Settings Tab (Displayed in Advanced User Mode Only)

From the *Instrument settings* tab (Figure 67), you can inspect the advanced run-time parameters.

IMPORTANT: The following descriptions are for informational use only. Changes to the *Instrument settings* tab can only be made in Service mode.
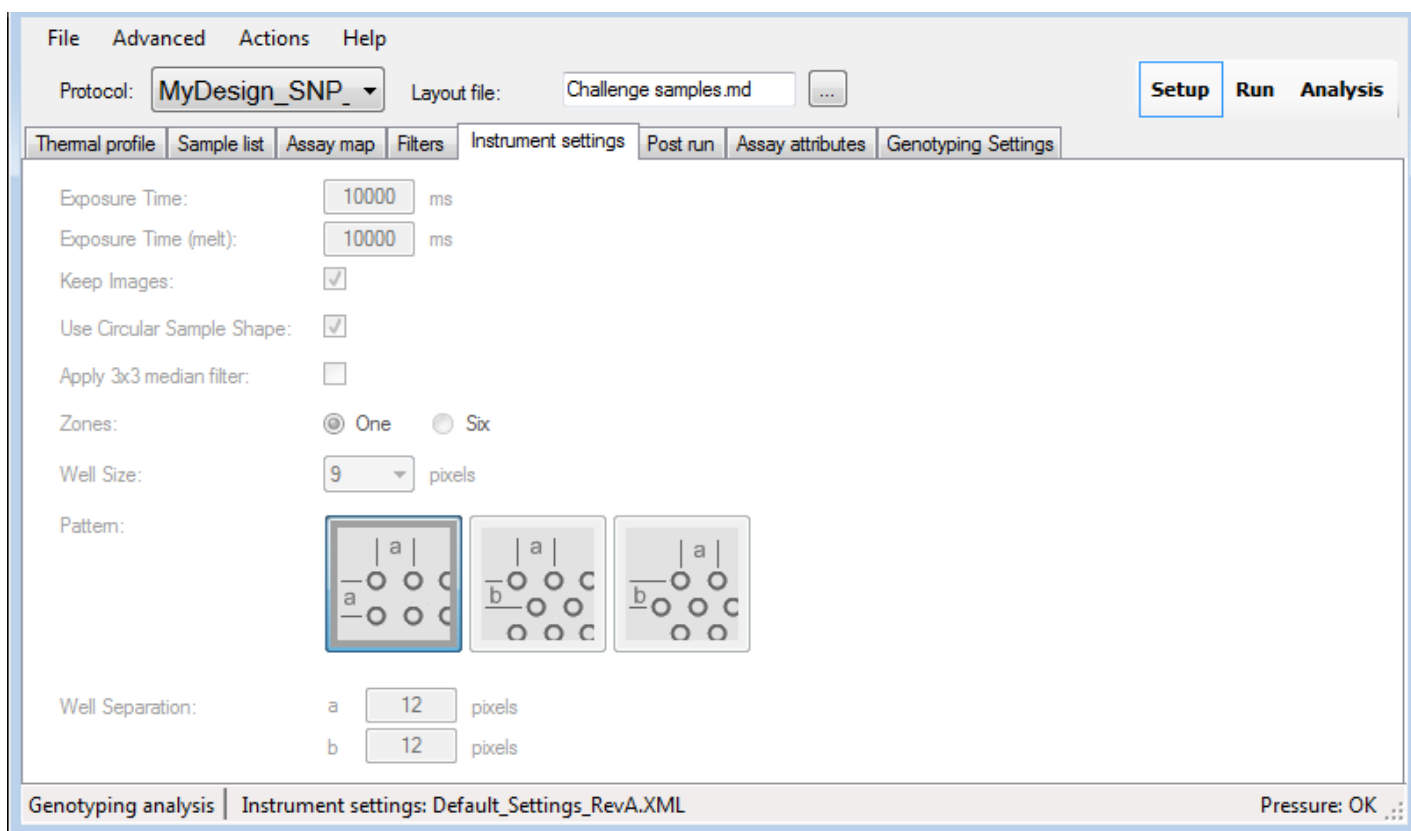
Figure 67. Instrument settings tab.

Table 22. Description of the various instrument settings.

| Settings	Description
Exposure Time	10 sec
Exposure Time (melt)	10 sec
Keep Images	Keeping images to re-analyze a run is highly recommended. Reviewing images may allow you to find the reason for any anomalies in the data. An additional 150–300 MB of disk space will be required per run.
Use Circular Sample Shape	Select according to the type of SmartChip nanowell chip used
Apply 3 x 3 Median Filter	Removes speckle noise from images. Best to not use this filter
Zones	One
Well Size	Diameter of the sampling disk
Pattern	Select the pattern that matches the SmartChip nanowell chip
Well Separation	Fixed distance between cells

By default, Instrument Settings are stored in the following locations:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\InstrumentSettings`
- **Windows 7**—`C:\ProgramData\Wafergen\qPCR\InstrumentSettings`

8. Post Run Tab (Displayed in Advanced User Mode Only)

From the *Post run* tab (Figure 68), you can view the post-run settings. If the "Generate summary report" box is checked, a summary report will automatically be generated after completion of data processing for a SmartChip run.

The "Filename template" determines how the summary report file will be named. To configure the filename template, you must be in Admin mode.

NOTE: To configure external processing, you must have knowledge of DOS command line processing. For help with this, you may contact Takara Bio technical support (technical_support@takarabio.com).
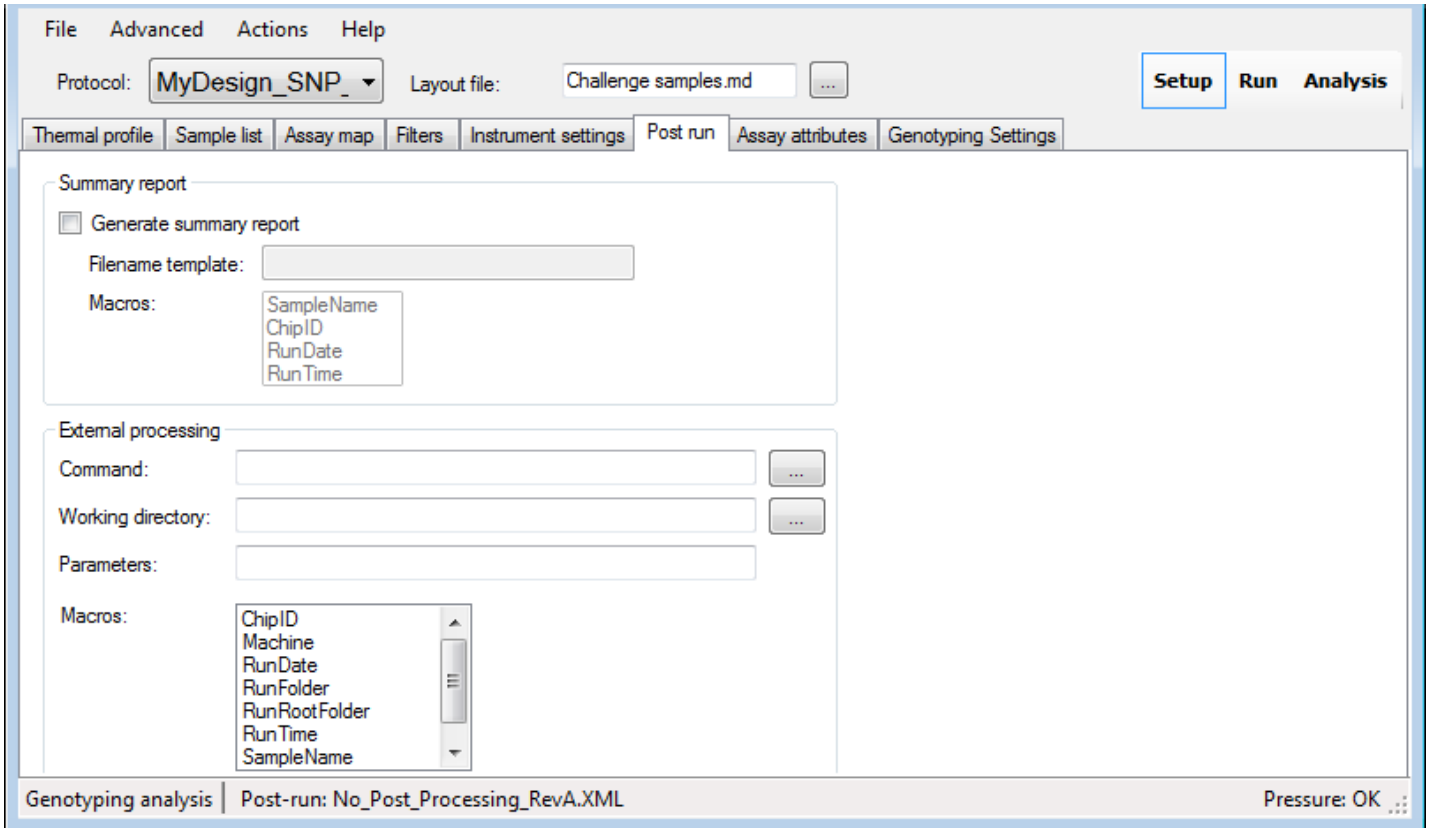
Figure 68. Post run tab.

By default, Post run files are stored in the following locations:

- Windows XP—C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR\PostRun
- Windows 7—C:\ProgramData\Wafergen\qPCR\PostRun

XV. Genotyping Procedures: Run Mode

A. Introduction

Select the **Run** mode to start and monitor the parameters of a SmartChip run. The **Run** window's top navigation bar provides drop-down menus that contain protocol and sample information. Figure 69 below is an example protocol for a probe-based detection experiment). The *Run* window contains one or more panels of run data in graphical format. Right-clicking anywhere on a graph displays a pop-up menu with options for **Copy**, **Save image as**, **Page setup**, **Print**, **Show point values**, **Zoom/Pan**, and **Set scale to default**. An *Event log* (bottom left) lists any errors or abnormal events that occur during a run. *Run info* (bottom right) provides basic information about the run.

Figure 69. Run window for a probe-based detection experiment. The KASP protocol will look different.

B.   Overview of Genotyping Run Mode Drop-Down Menus

Figure 70. Run mode top menu bar.

Upon switching to Run mode, the top menu options change (Figure 70). Table 23 below describes the selections available from the Run mode top menu bar.
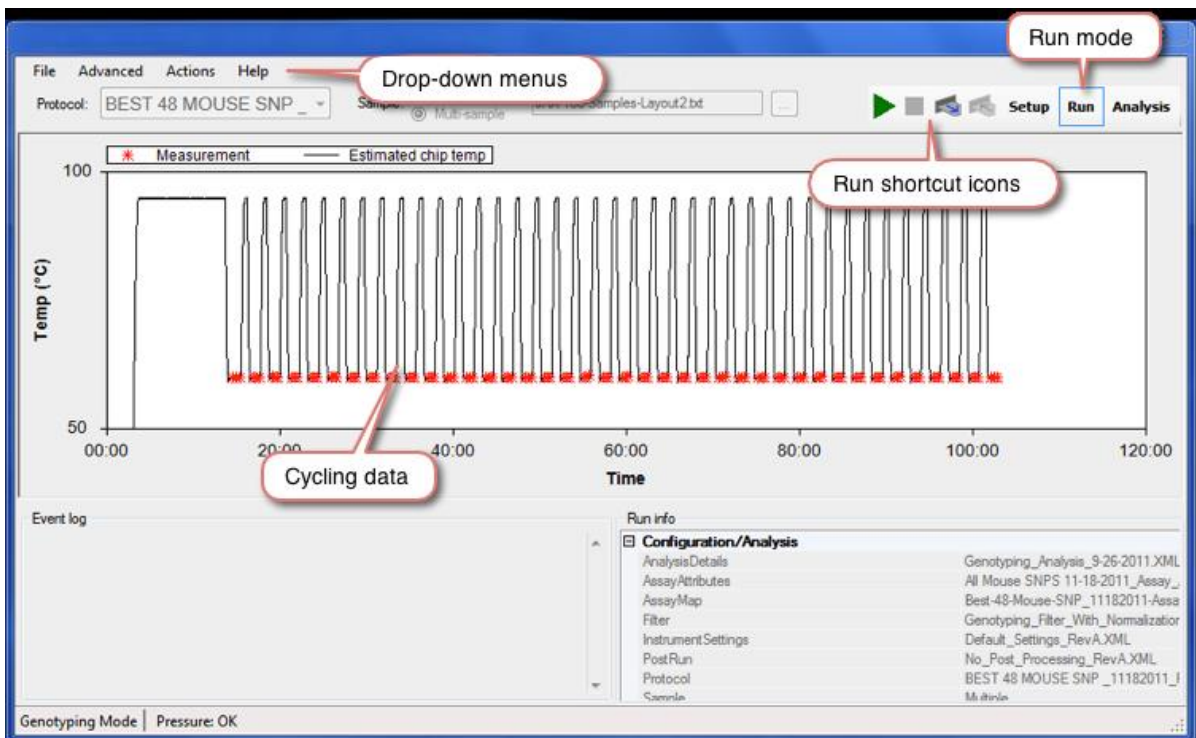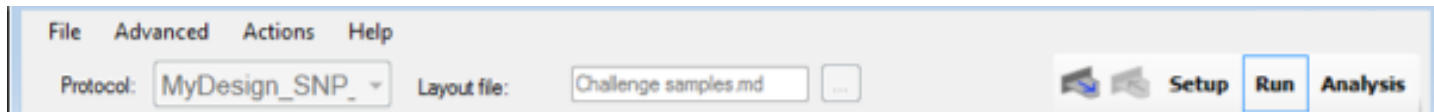
Table 23. Menu options available from the genotyping Run mode top menu bar.

Menu	Options
File	**Open** an existing run. Allows inspection of data related to completed experiments.
	Hint: A quick way of loading a run into the program is to drag a folder from Windows Explorer into the SmartChip qPCR application. You will be prompted to choose which raw or analysis file you want to load.
	Run info displays file, user, chip, and instrument information as well as notes.
Advanced	**Toggle between Pressure and Temperature run data** in graphical format.
	Image viewer is designed for visual inspection of the data. For more information, see "Image Viewer" in Section XVII.
	Re-process run with different configuration will rerun data and generate a new .raw file when the Assay map, Assay attributes, or Sample layout files have changed. If other config files are changed, the displayed run information will be cleared.
	Admin mode should be used by trained personnel only. It is used to save changes to genotyping settings and to modify protocols using the **Protocol Manager**. Changing any of these settings can affect your experimental results. The password to enter Admin mode is printed on your qPCR software CD.
	Service mode is designed for Takara Bio staff to configure advanced SmartChip ND Cycler settings.
	Switch to advanced user mode to display all of the data collected during real-time.
	Switch to standard GUI to display only information that is most frequently accessed.
Actions	Switch between **DNA binding dye-Based Expression**, **Probe-Based Expression**, and **Genotyping Analysis** modes.
Help	**About** provides application version, copyright, and license agreement information.
	Open user manual opens the User Manual in pdf format.
	Setting up a run opens a pop-up box with a Quick Guide for setting up a run.
	Saving run data opens a pop-up box with a Quick Guide for saving run data.
	Loading an existing chip run opens a Quick Guide for loading an existing chip run.
	Reprocessing data allows you to reprocess completed chip runs with different curve analysis parameters.

C.   Run Shortcut Icons

These icons are visible only using software installed in instrument mode.

▶   [Run experiment]   ⬋   [Open drawer]

■   [Abort run]   ⬋   [Close drawer]

D.   Genotyping Run Data Panels (Advanced Drop-Down Menu)

The **Advanced** drop-down menu in Run mode offers several options to view run-data graphs. Up to two graphs can be shown at once. Selecting the type of graph toggles between showing and hiding the specified graph.

1. Pressure Graph

Select **Toggle pressure graph panel** to show or hide the pressure and air flow curves (Figure 71).
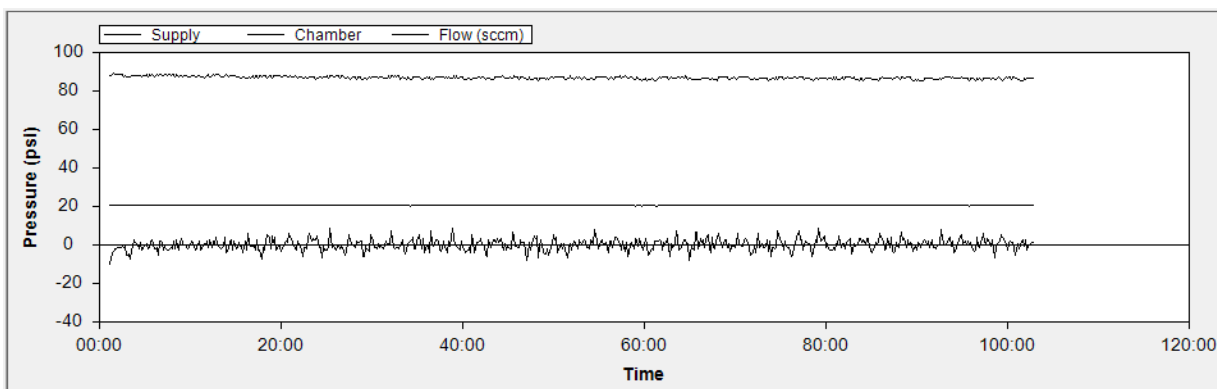

Figure 71. Example pressure graph.

2. Temperature Graph

Select **Toggle temperature graph panel** to show or hide a detailed graph of the temperature sensor measurements during a run (Figure 72). The stars in the temperature graph represent image measurement acquisitions.
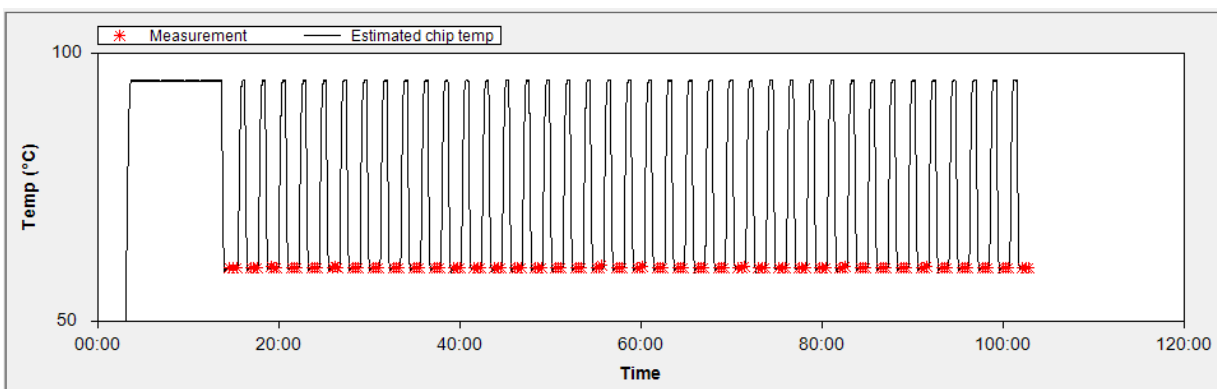

Figure 72. Example temperature graph.

XVI. Genotyping Procedures: Analysis Mode

A. Introduction

Select **Analysis mode** to view and analyze data from SmartChip Panel data. The *Analysis* window's top navigation bar has drop-down menus that contain protocol and sample information. After using the **File** drop-down menu to select an analysis file for review, you can use the *Analysis* tabs to view the data in different formats.

The main part of the *Analysis* window (Figure 73) typically shows a table of data on the left with the graphical display on the right. If more than one row of data is highlighted on the left, the graph on the right will display an overlay of all well data selected. For a description of the data analysis method, refer to Appendix C.
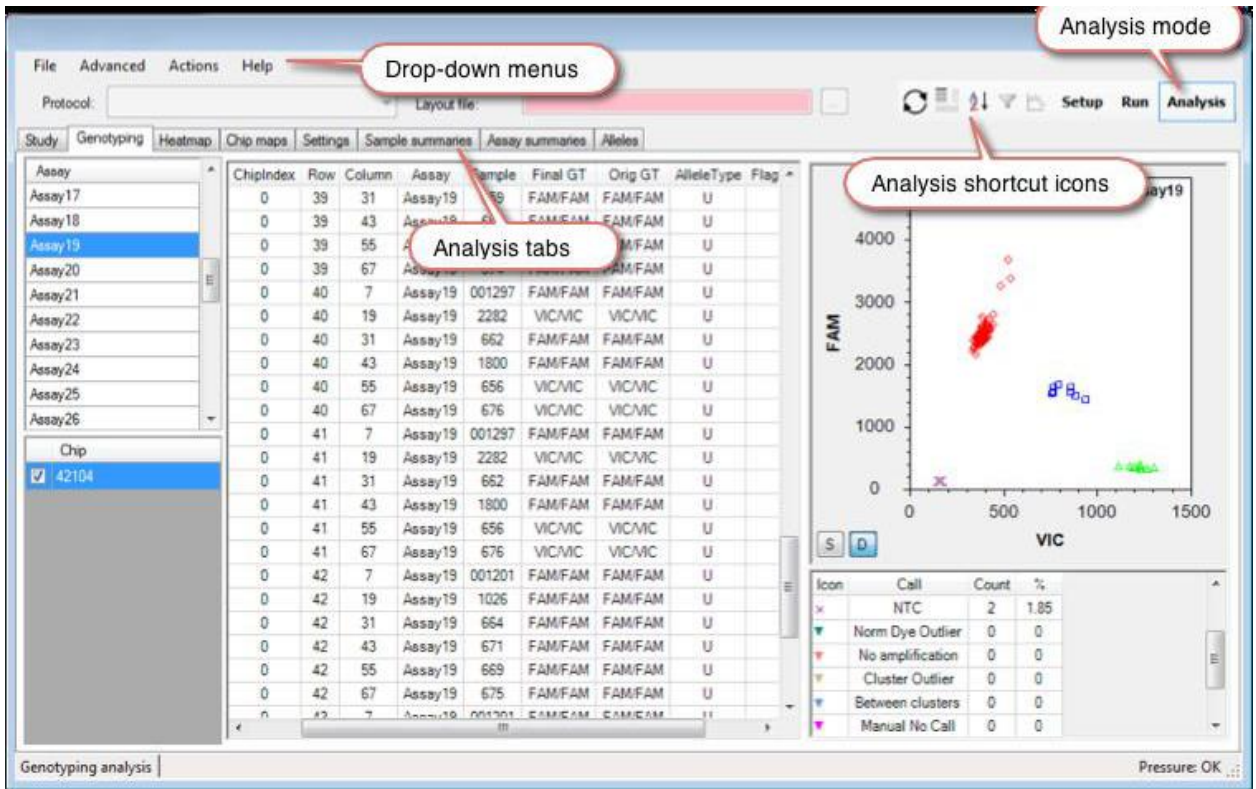

Figure 73. Genotyping analysis window.

NOTE: SmartChip qPCR Software may also be used for offline data analyses. To install SmartChip qPCR Software on a laptop or desktop computer that is not controlling a SmartChip ND Cycler, refer to Appendix A.

B.   Overview of Genotyping Analysis Mode Drop-Down Menus

Figure 74. Genotyping analysis mode top menu bar.

Table 24 below describes the selections available from the Analysis mode top menu bar (Figure 74).

Table 24. Menu options available from the Analysis mode top menu bar

Menu	Options
File	**Open** genotyping files with .sty file extensions. A .sty run file contains previously derived genotype analysis data.
	Save genotyping analysis results with .sty file extensions. A summary report will also be generated in a .pdf format.
	Export well data saves well data as a tab-delimited text file.
	Run info displays file, user, chip, and instrument information as well as notes.
Advanced	**Image viewer** is designed for visual inspection of the data. For more information, see "Image Viewer" in Section XVII.
	Admin mode should be used by trained personnel only. It is used to save changes to genotyping settings and to modify protocols using the **Protocol Manager**. Changing any of these settings can affect your experimental results. The password to enter Admin mode is printed on the qPCR software CD.
	Service mode is designed for Takara Bio staff to configure advanced SmartChip ND Cycler settings.
	Switch to advanced user mode to display all of the data collected during genotyping PCR.
	Switch to standard GUI to display only information that is most frequently accessed.
	Genotyping settings takes you back to the **Genotyping Settings** tab in Setup mode where you can modify analysis settings if desired. The settings will take effect when you go back to the Analysis mode.
	Summary report options tailors information to be included in the summary report.
Actions	Switch between **DNA binding Dye-Based Expression**, **Probe-Based Expression**, and **Genotyping Analysis** modes.
	GT Custom Settings: Change color scheme and symbol size. Toggle FAM and VIC on the x- and y-axes of the graph.
	Re-analyze RAW files: Provides options for re-analyzing data and saving the summary.
Help	**About** provides application version, copyright, and license agreement information.
	Open user manual opens the User Manual in pdf format.
	Setting up a run opens a pop-up box with a Quick Guide for setting up a run.
	Saving run data opens a pop-up box with a Quick Guide for saving run data.
	Loading an existing chip run opens a Quick Guide for loading an existing chip run.
	Reprocessing data allows you to reprocess completed chip runs with different curve analysis parameters.

C. Saving or Exporting Genotyping Run Data

NOTE: When the SmartChip Panel run is complete, the results are automatically stored in the data folder in a file called [chipID].raw, where [chipID] is the chip ID value supplied when the chip was loaded into the instrument. This file contains SNP genotype probe-generated fluorescence information. It also contains the parameters that were specified for the run. You can choose to **Save Allele Calls** if the assay attributes were provided in the protocol.

1. Select **Analysis** mode, which displays computed data. Select **Save** from the **File** drop-down menu to generate the study (.sty) file, which will display the genotyping data without the need for recalculation when opened in the SmartChip qPCR Software following the SmartChip run.
2. Choose whether to save allele calls and/or generate a Summary report by clicking the checkbox in the **Save** dialog box and select [Save].
3. To export data from a study, select **Export well data** from the **File** drop-down menu. The data will be exported as a .txt file, which can be opened as a tab-delimited file.

D. Loading an Existing Genotyping SmartChip Run

SmartChip runs can be loaded from .raw files for single SmartChip run analysis and .sty files for single- or multi-file SmartChip studies. The .sty files contain calculated user data, while the .raw files do not.

- To load a run using a .raw file, use the **Open** command from within the **Run mode**.
- To load a run using an .sty file, use the **Open** command from within the **Analysis mode**.
- Either type of file can be loaded by dragging the file or the entire run folder on top of the SmartChip qPCR Software window. If the folder contains multiple .raw or .sty files, you will be asked which you want to use.
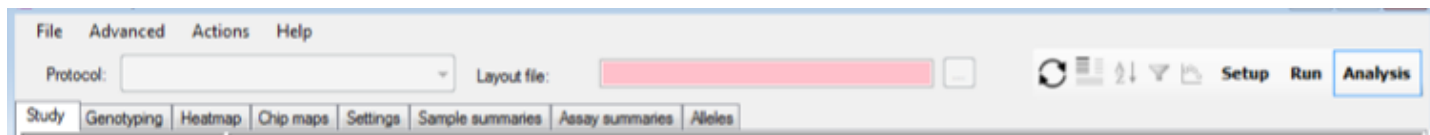
E.   Genotyping Data Analysis

Figure 75. View and analyze SmartChip Panel genotyping run data in Analysis mode.

Table 25. Tabs available in Analysis mode for genotyping data.

Tab	Function
Study	The **Study** tab allows for selection of data from multiple SmartChip runs to be analyzed as a single set or study. Once a study file is saved or selected, the **Study** tab summarizes the run information for each run in the study set.
Genotyping	This tab shows results for all chips, samples, and assays in a study. Assay results information is displayed in a table format, in the genotype clusters graphic, and in the summary data for selected chips and assays. The assay results include SNP genotyping call rate, SNP genotype, and any flags for uncalled genotyping samples. You can either select specific rows in the result table or data points in the cluster graph to review the genotype.
Heatmap	Shows the sample genotype or genotype flags for non-call determination against the assays performed in a study.
Chip maps	View the distribution results for all wells across a selected SmartChip Panel in both tabular and graphical formats. Selected SmartChip Panels in a study are chose from a pull-down menu.
Settings	Displays the analysis settings (thresholds) that were in effect when the cluster analysis for genotyping was performed.
Sample Summaries	In a given study, sample and assay call rates for each sample, each chip, and each assay are displayed. The assays summary table includes overall assay call rates, genotypes, and flagged well data.
Assay Summaries	
Alleles	For each assay, the allele identity of the two dyes used for genotyping are shown.

F.   Analysis Tabs

1. Study Tab

The *Study* tab (Figure 76) allows you to create a new study by selecting SmartChip Panels or MyDesign Chips from one or more SmartChip runs for inclusion in a user-defined study. Choose **Select Raw files**, then select the folder containing the chip data of interest. Click [OK].
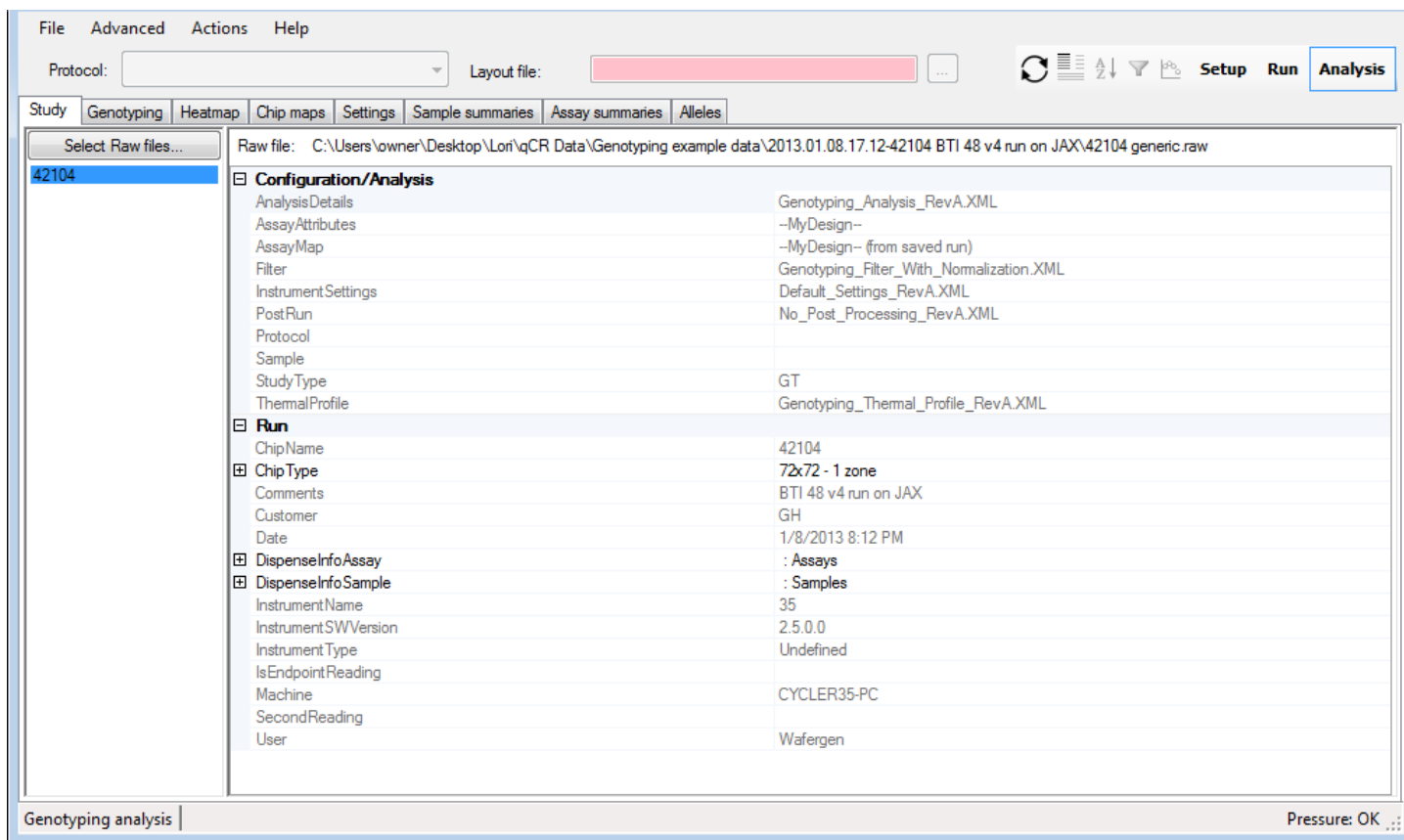

Figure 76. Study tab.

A dialog box will open, showing all the .raw data files available to upload into the study (Figure 77). If multiple .raw data files exist for a particular chip run, all the files will be shown and marked with an asterisk (*). Choose the appropriate files be clicking the box in the "Select" column in the row for the file. Press [OK] when done to create the study workspace. Selecting **File > Save** will save the study as a .sty file for future review.

NOTE: In certain cases where the selected .raw files have been analyzed with different analysis settings, you will be prompted to select an analysis settings file. If .raw files have been gathered with different thermal profiles, number of cycles, or filter settings, the study manager will not allow the study file to be created.

Figure 77. Example list of .raw data files available for upload into a study.

2. Genotyping Tab

Once the data are loaded (at the end of an experiment, by opening a .raw file, or opening a .sty file) the software will automatically analyze the data. Alternatively, you can perform a new analysis by pressing the [Analyze Data] icon ⟳).

The analysis will determine the sample genotype, genotyping cluster plots, and call rates of the genotyping assays. Genotyping calls include: FAM/FAM, VIC/VIC, HET, NTC, or U (no call). No-call wells are also labeled with appropriate flags for troubleshooting purposes. Flags include:

- Norm Dye Outlier: Outlier from ROX normalization
- No amplification: NonNTC wells with signal <NTC
- Cluster Outlier: Identifies points too far from cluster centroid
- Between clusters: Identifies points too close to more than one cluster to call
- Manual No Call: Data that has been removed manually from particular assay/chip
- Sample excluded: Data that has been removed automatically from all assays/chip

No genotype is determined for flagged sample data. The system can flag wells according to criteria outlined in Appendix D.

The "Genotyping" tab is divided into three main sections:

- **Chips and Assays** contains a list of all assays and chips present in the selected file or study. Toggling between selected assays and chips within the section is possible, with subsequent analysis shown in sections two and three.

  NOTE: Toggling **Chips** or **Assays** only affects the displayed data. The full data is still in memory.

- **Genotyping Results** is a tabular list of all genotyping results for the selected chips and assays. See the genotyping results chart below (Figure 78).

- **Data display and data display icons** are a graphical representation of genotyping results for selected chips and assays, along with a data summary of each call type.
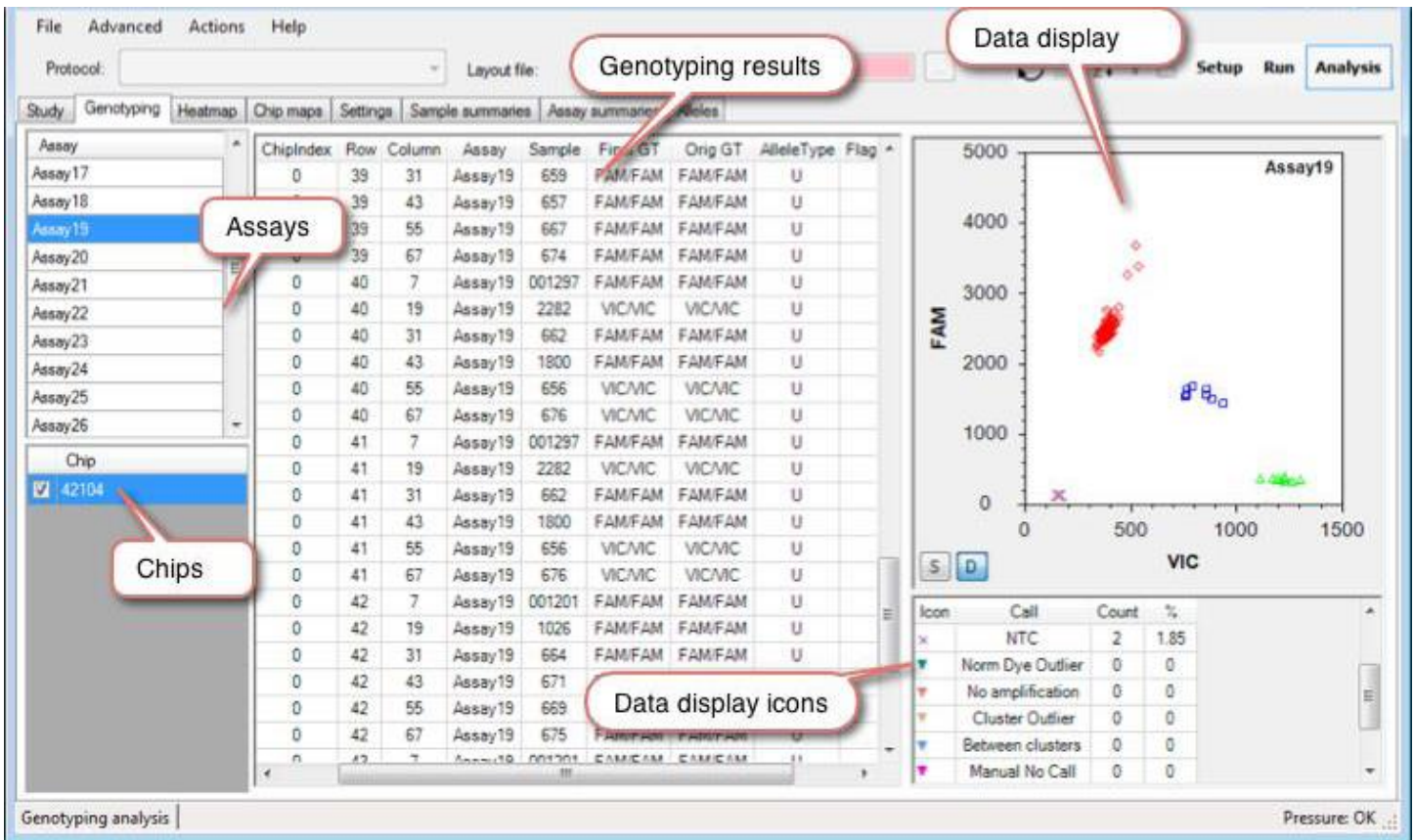

Figure 78. Example of Genotyping Results in the Genotyping tab.

Genotyping result categories in Table 26 below that are NOT shown in boldface text are displayed in Advanced user mode only.

Table 26. Genotyping result categories in the Genotyping tab.

Result	Description
Chip	SmartChip identification number.
Row	Row of the well relative to the area defined in the Assay map file. Starting index is 0.
Column	Column of the well relative to the area defined in the Assay map file. Starting index is 0.
Assay ID	SNP genotyping assay ID number from the Assay map file.
Assay Name	Name of the assay in the well from the Assay attributes file.
Sample	Sample name in the Sample layout file.
Final GT	Genotype of the sample automatically determined by the SmartChip qPCR Software or manually assigned by a user.
Orig GT	The original genotype of the sample determined by the SmartChip qPCR Software.
Rng: VIC	The fluorescence range of the VIC probe measured during a run.
Rng: FAM	The fluorescence range of the FAM probe measured during a run.
Rng: ROX	The fluorescence range of ROX dye measured during a run.
End: VIC	The end fluorescence value of the VIC probe measured at the end of the run.
End: FAM	The end fluorescence value of the FAM probe measured at the end of the run.
End: ROX	The end fluorescence value of the ROX dye measured at the end of the run.
Initial: VIC	The initial fluorescence value of the VIC probe measured at the beginning of the run.
Initial: FAM	The initial fluorescence value of the FAM probe measured at the beginning of the run.
Initial: ROX	The initial fluorescence value of the ROX dye measured at the beginning of the run.
Angle	The angle (in radians) of the determined genotype data point.
Score	Confidence that a point belongs to the assigned cluster; the inverse of Cost. A higher Score is better.
Cost	The normalized distance of a point from the center of a cluster of points. The inverse of Score. A lower Cost is better. If Cost is above MaxCost, then the point cannot be genotyped.
Flags	Indicates the reason for flagged data for which genotype is not automatically determined by the software or manually excluded by the user.
Gender	The gender of the genotype sample as defined in the Sample layout file.
Population	The population of the sample as defined in the Sample layout file.
Custom1	Custom sample information 1, defined by the user during Sample layout file generation using SmartChip ND Software.
Custom2	Custom sample information 2, defined by the user during Sample layout file generation using SmartChip ND Software.
Species	Label associated with the assay coming from Assay attributes file.
Chromosome	Label associated with the assay coming from Assay attributes file.
S-DispenseTip	The dispenser tip on the SmartChip ND used to dispense the sample.
S-DropIndex	The SmartChip ND dispense event for the nanowell. (The dispense head has 8 valves that dispense liquids in nanowells. So, for example, an S-DropIndex of 4 means that the nanowell was dispensed during the fourth time the dispense head was positioned and released liquid.)
S-GlobalDropIndex	The number of dispense events used by the SmartChip ND tip to dispense all samples into all nanowells in the chip.

Table 26 continues on the next page.

Table 26. Genotyping result categories in the Genotyping tab continued.

Result	Description
S-SP-Well	The SmartChip ND Source Plate well from which the sample was drawn for dispensing into the chip.
A-DispenseTip	The dispenser tip on the SmartChip ND used to dispense the assay.
A-DropIndex	The SmartChip ND dispense event for the nanowell. (The dispense head has 8 valves that dispense liquids in nanowells. So, for example, an S-DropIndex of 4 means that this nanowell was dispensed during the fourth time the dispense head was positioned and released liquid.)
A-GlobalDropIndex	The number of dispense events used by the SmartChip ND tip to dispense all assays into all nanowells in the chip.
A-SP-Well	The SmartChip ND Source Plate well from which the assay was drawn for dispensing into the chip.

Data display

The *Data display* panel can be viewed using either default scaling or smart scaling (Figure 79). In general, smart scaling provides a view of the data that is easier to interpret. You can easily switch between the two scaling modes by clicking the [S] or [D] scaling buttons in the data display.
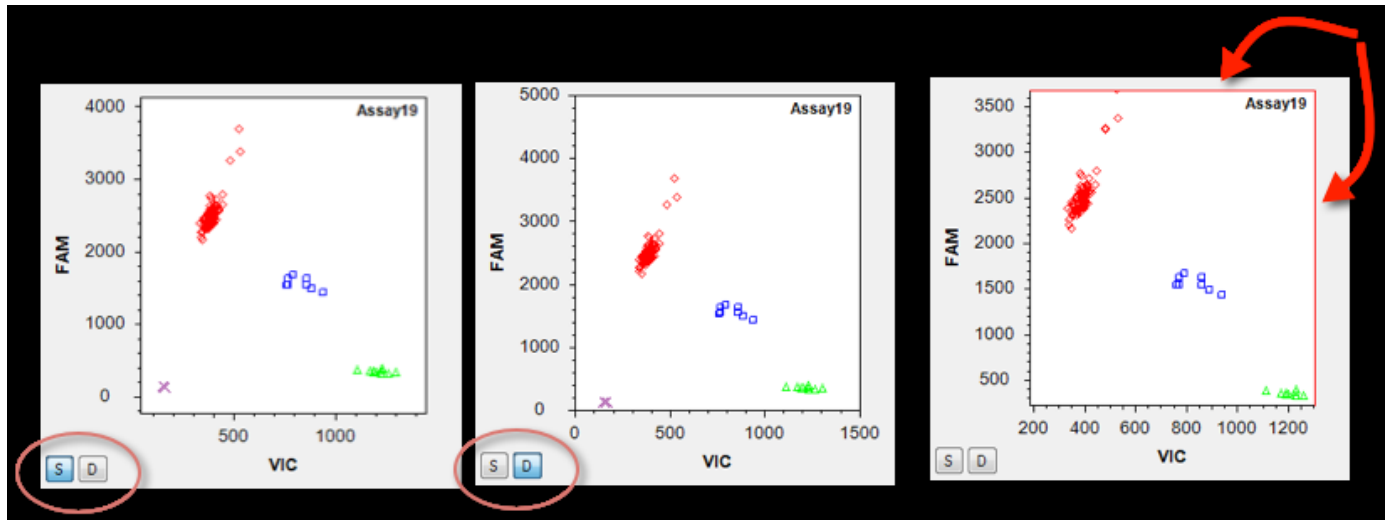

Figure 79. Data display panel.

Position the cursor over the data display and right-click to open a pop-up box with multiple data view options (Table 27).

Table 27. Data view options in the data display.

Function	Description
Copy	Copies the data to a clipboard from which it can be pasted into other files.
Save Image As	Save the image with a different file name.
Page Setup	User-established page layout parameters and printer settings prior to printing an image of the data display (e.g., paper size, source, orientation, and margins).
Print	Prints the current data display.
Show Point Values	Place crossbars over any call to display Assay ID, Sample, Genotype, Score, Fluorescence range, Ending fluorescence value, and Angle.
Un-Zoom	Undo the most recent manual zoom/pan.
Undo All Zoom/Pan	Restore the automatic zoom/pan.
Set Scale to Default	Adjust zoom/pan so that all points are visible.
Polar coordinates	Display points in a polar coordinate system where the x-axis represents the combined fluorescence of VIC and FAM and the y-axis represents the angle defined by VIC and FAM in radians.
Smart Scaling	Adjust zoom/pan to show the vast majority of points and crop extreme outliers.
Show curve legends	Highlights user-selected call information horizontally across the genotyping results list.
Show SNP database	Displays the SNP database when available. SNP databases can be defined in Admin mode.
De-select all wells	De-selects data from any previously selected wells.

Table 28. Data display icons.

Icon	Description	Icon	Description
◇	FAM/FAM—Homozygotes	▼	No amplification—NonNTC wells with signal <NTC
□	HET—VIC/FAM Heterozygotes	▼	Cluster Outlier—Points too far from cluster centroid
△	VIC/VIC—Homozygotes	▼	Between Clusters—Points too close to more than one cluster to call
×	NTC—No Template Control	▼	Manual No Call—Removed manually from particular assay/chip
▼	Norm Dye Outlier—Outlier from ROX normalization	●	Sample excluded—Removed automatically from all assays/chip

Manually edited genotypes will have a solid fill and a corresponding genotype icon. A color-filled icon represents a manually assigned genotype, while an open icon represents the automatically determined genotype (Table 28).

3. Heatmap Tab

Clicking the *Heatmap* tab opens a study heatmap (Figure 80). The heatmap displays the sample genotype across the assays loaded into a study over a single or multiple SmartChip Panel(s). In addition to called genotypes, the heatmap displays icons indicating various flags and exclusions as described in Table XXIX in the previous section. The heatmap is useful for assessing assay- or sample-specific issues with a low call rate.

NOTE: You can scale the display by turning the mouse scroll wheel while pressing the **[Ctrl]** key.
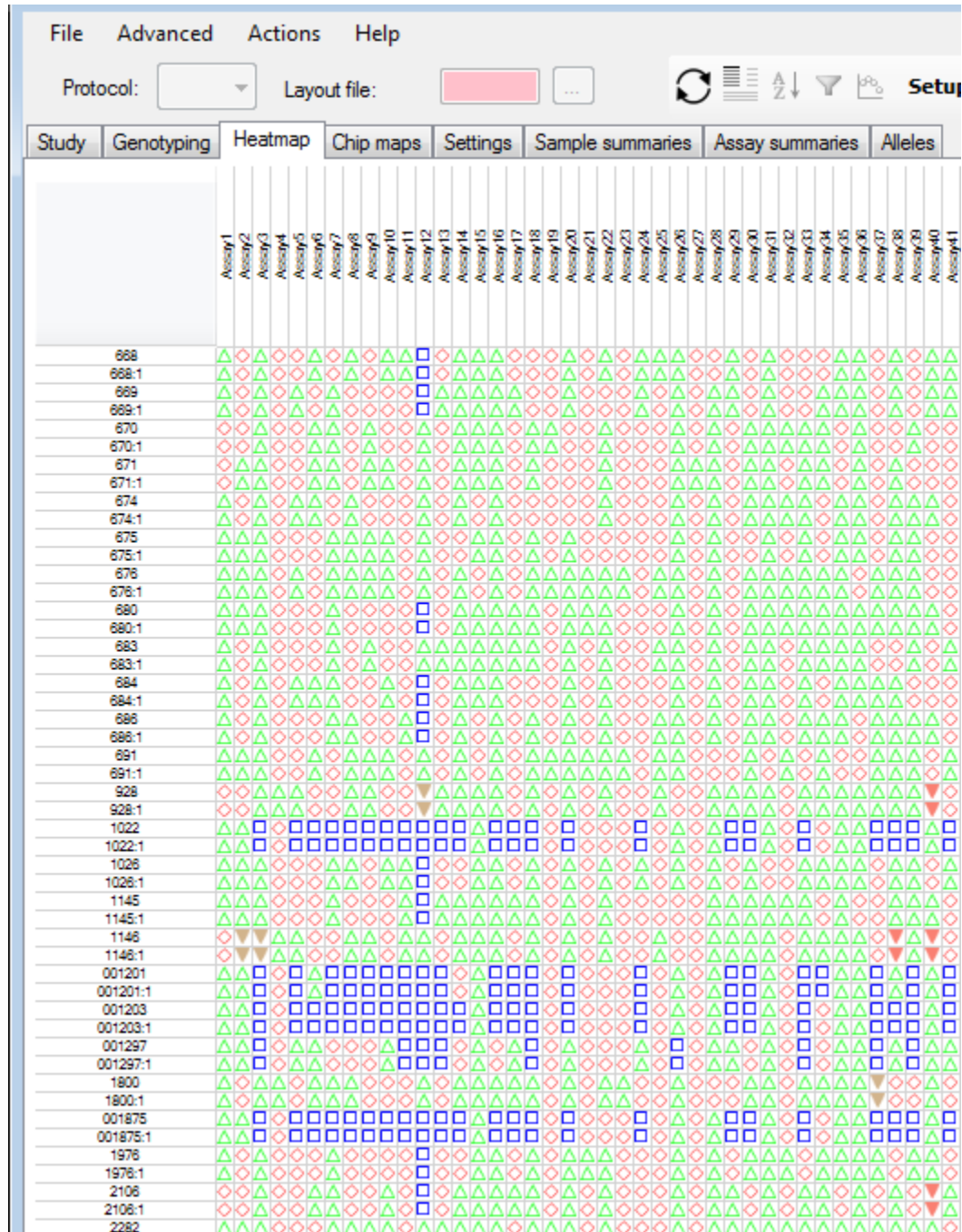
Figure 80. Example genotyping heatmap.

4. Chip Maps Tab

Clicking the *Chip maps* tab opens a chip map showing the distribution of measurements across the SmartChip Panel (Figure 81). Any chip with a study can be selected via the **Chip** pull-down menu. If applicable, a histogram of measurements is also displayed.

NOTE: You may want to resize the columns in this window to make them wide enough to read the contents. You can resize the entire grid by dragging the first column wider or taller.
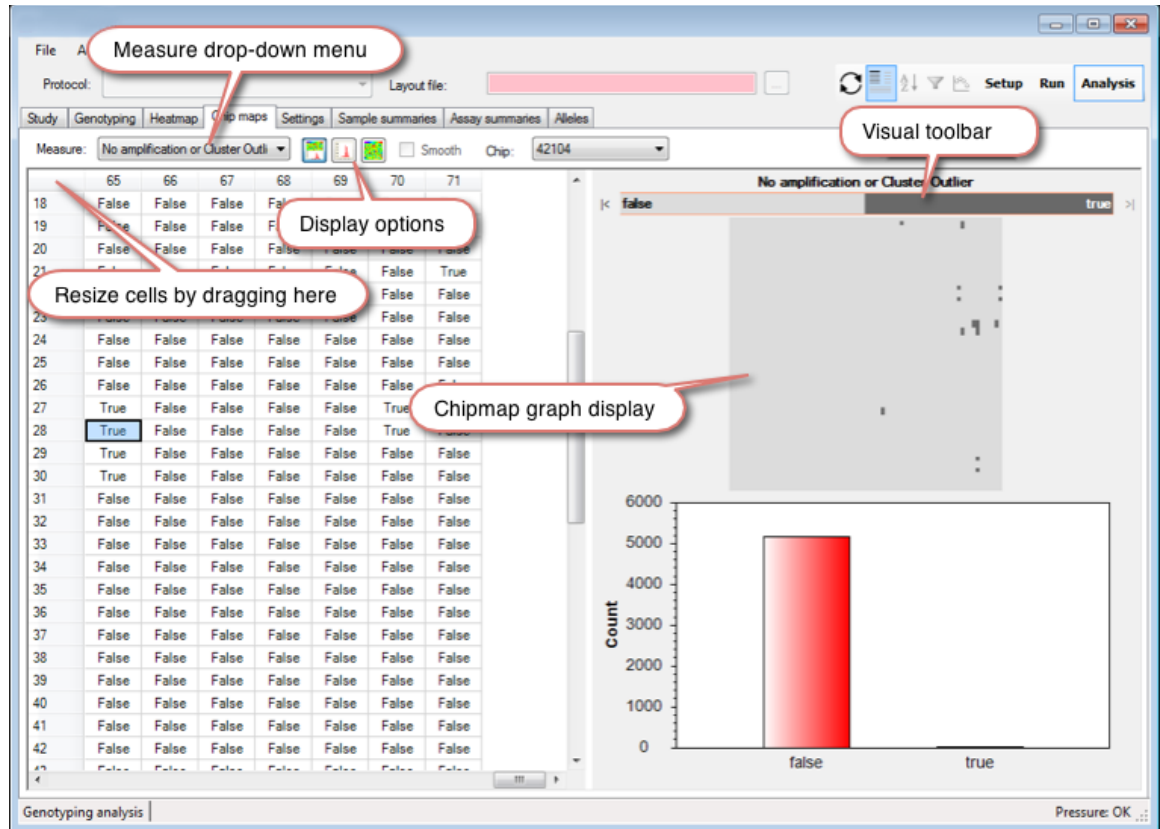
Figure 81. Chip maps tab.

Measure: Use the **Measure** drop-down menu to select the measurement you want displayed for the data: **Assay ID**, **Sample**, **Not called**, **Sample excluded**, **No amplification** or **Cluster Outlier**, **Manual No Call**, or **Norm Dye Outlier**. All columns from the full data table are available, plus the following information in Table 29.

Table 29. Additional information available in the Measure drop-down menu.

| Measurement	Description
Assay ID	Assay ID used for genotyping assays in the SmartChip Panel.
Sample	The Sample layout based on the SmartChip layout file generated by the SmartChip ND and loaded into the qPCR software.
Not called	The combination or sum of all subflags, e.g., Norm dye outlier, No amplification, Cluster Outlier, Between clusters, Manual No Call, and Sample excluded. **True** indicates that a genotype HAS NOT been called; **False** indicates that a genotype HAS been called.
Sample excluded	Individual nanowells in which the data have been excluded automatically or manually.
No amplification or Cluster Outlier	Nanowells in which amplification is not detected or high coast is above MaxCost.
Manual No Call	Nanowell data that was manually flagged and not included in the overall analysis.
Norm Dye Outlier	Nanowell data designated as outliers based on ROX normalization signals.

Chip map graphic display: When the mouse cursor is positioned over the map, a tool tip displays details for the well under the cursor.
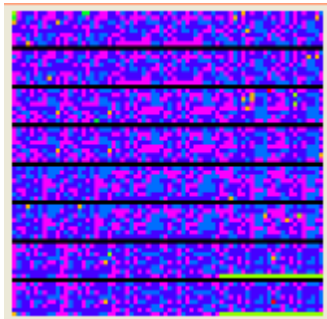
By clicking on a well in the map, the table or image corresponding to the well is highlighted. It is also possible to select/deselect multiple table items by pressing the **[Shift]** key while clicking on table cells. The Image Viewer also supports **[Ctrl]**+**[mouse click]**.
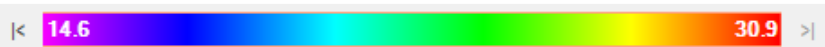
The visual toolbar:
You can perform the same adjustments with the visual toolbar as with the Image Contrast Toolbar (see page 105).

Display options: You can use these icons ( ) to display the map, histogram, or both, respectively.

5. Settings Tab

The *Settings* tab (Figure 82, Table 30) shows SNP genotype analysis settings. We do not recommend changing these settings, but you may opt to modify the minimum sample call rate ("MinCallRate"). If you change any settings, the genotyping data will automatically be re-analyzed the next time the analysis panel is selected. Refer to Appendix C.

Figure 82. Settings tab.

Table 30. Description of functions in the Settings tab.

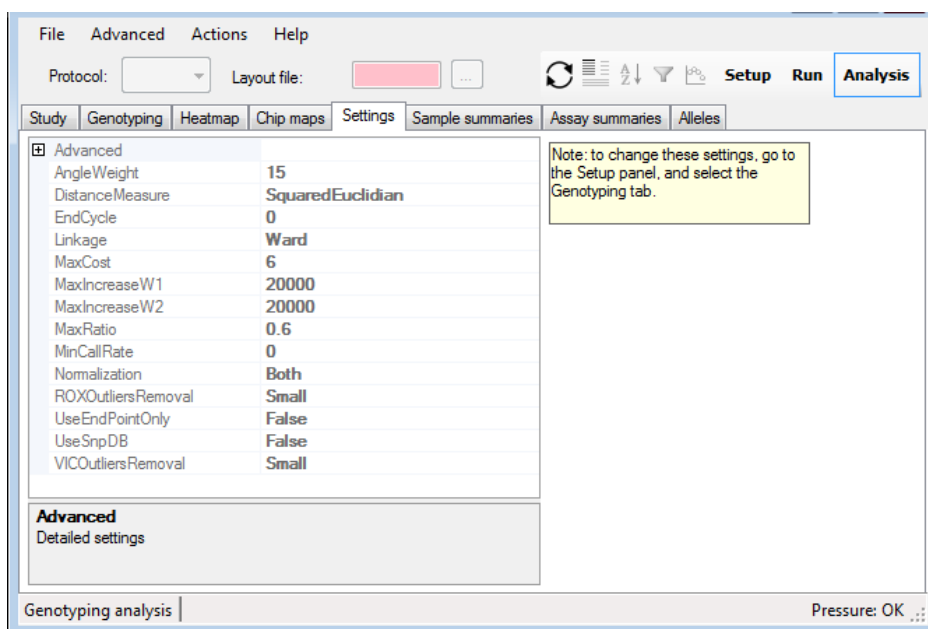| Measurement	Description
Angle Weight	Arithmetic weight of the value of the angle measurement compared to those of VIC and FAM measurements.
DistanceMeasure	One of Euclidian or Squared Euclidian.
EndCycle	The amplification cycle used for end-point analysis. If the value is 0, the last existing amplification cycle is used for data analysis.
Linkage	Method used to link the clusters.
MaxCost	The genotype data point is marked as "Unknown" if its calculated cost function is larger than the default value (6).
MaxIncreaseW1	Maximum fluorescent increase for the VIC probe. If the increase in VIC probe fluorescence is above the default threshold (20,000) the well is considered undetermined. To disable this threshold, set the value to 0.
MaxIncreaseW2	Maximum fluorescent increase for the FAM probe. If the increase in FAM probe fluorescence is above the default threshold (20,000) the well is considered undetermined. To disable this threshold, set the value to 0.
MaxRatio	A genotype data point located between two clusters is marked "Unknown" if the cost ratio of the data point is larger than the default value (0.6).
MinCallRate	If the call rate percentage of a sample falls below the default threshold value (0), the sample is put on the **Exclude** list and all assays for this sample are excluded from data analysis and summary.
Normalization	Select the type of normalization and optional color correction.
ROXOutliersRemoval	Select how ROX outliers are excluded from data analysis and data summary.
VICOutliersRemoval	Select how VIC probe outliers are excluded for data analysis.

6. Sample Summaries Tab

The *Sample summaries* tab (Figure 83) provides data summaries organized by sample, including repeats and samples by chip. A global summary of assay performance, detailing genotypes and call rates for assays, is provided in a table format.

Figure 83. Sample summaries tab.

7. Assay Summaries Tab

The *Assay summaries* tab (Figure 84) provides data summaries organized by assay chip and assay.

Figure 84. Assay summaries tab.

8. Alleles Tab

The *Alleles* tab (Figure 85) shows the genotyping calls, as long as the Assay attributes file defines which nucleotide is associated with a particular dye for each assay.

Figure 85. Alleles tab.

G. Analysis Tool Icons

Table 31. Genotyping analysis tool icons.

Tool Icon		Function
	Analyze data	Manually starts cluster analysis. In almost all cases a cluster analysis is started automatically when needed.
	Genotyping settings	Takes you to a page where you can review the settings.
	Sort	Allows sorting by multiple columns simultaneously.
	Filter	Not applicable in genotyping mode.
	Show scatter plot	Not applicable in genotyping mode.

1. Analyze Data Icon

The [Analyze data] icon ( ) can be used to re-analyze data.

2. Genotyping Settings Icon

The [Genotyping settings] icon ( ) takes you to the *Genotyping settings* tab in Setup mode, where you can review the genotyping settings.

3. Sort Icon

The [Sort] Icon (⧣) opens a dialog box (Figure 86) and allows you to sort the table by one or more columns.
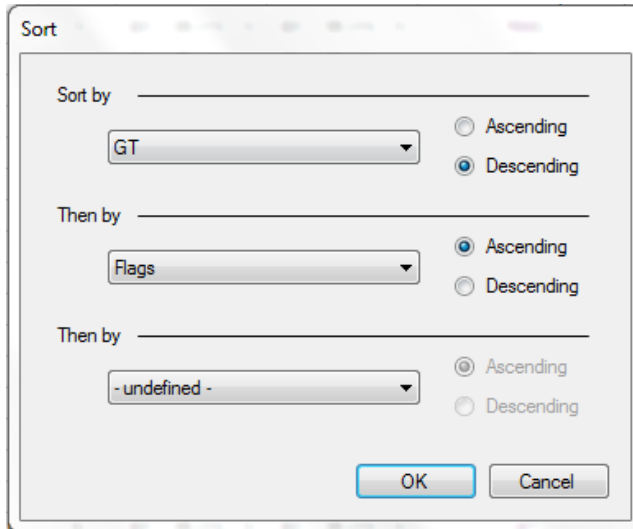
Figure 86. Sorting dialog box.

For a more convenient way of sorting by a single column, click on the column header of the *Full data* tab (Figure 87). A second click reverses the sort order.

Figure 87. Sorting by a single column from the Full data tab.

XVII. Image Viewer

A.   Introduction

The **Image Viewer** is accessible via the **Advanced** drop-down menu in Analysis mode (Figure 88). For genotyping assays, data is collected at three wavelengths or waves. At each PCR cycle, three images (one per wavelength) are collected. These waves can be viewed by selecting the appropriate wave and cycle number.

NOTE: Genotyping .sty files are not associated with images; therefore, no images are available when a .sty file is loaded.
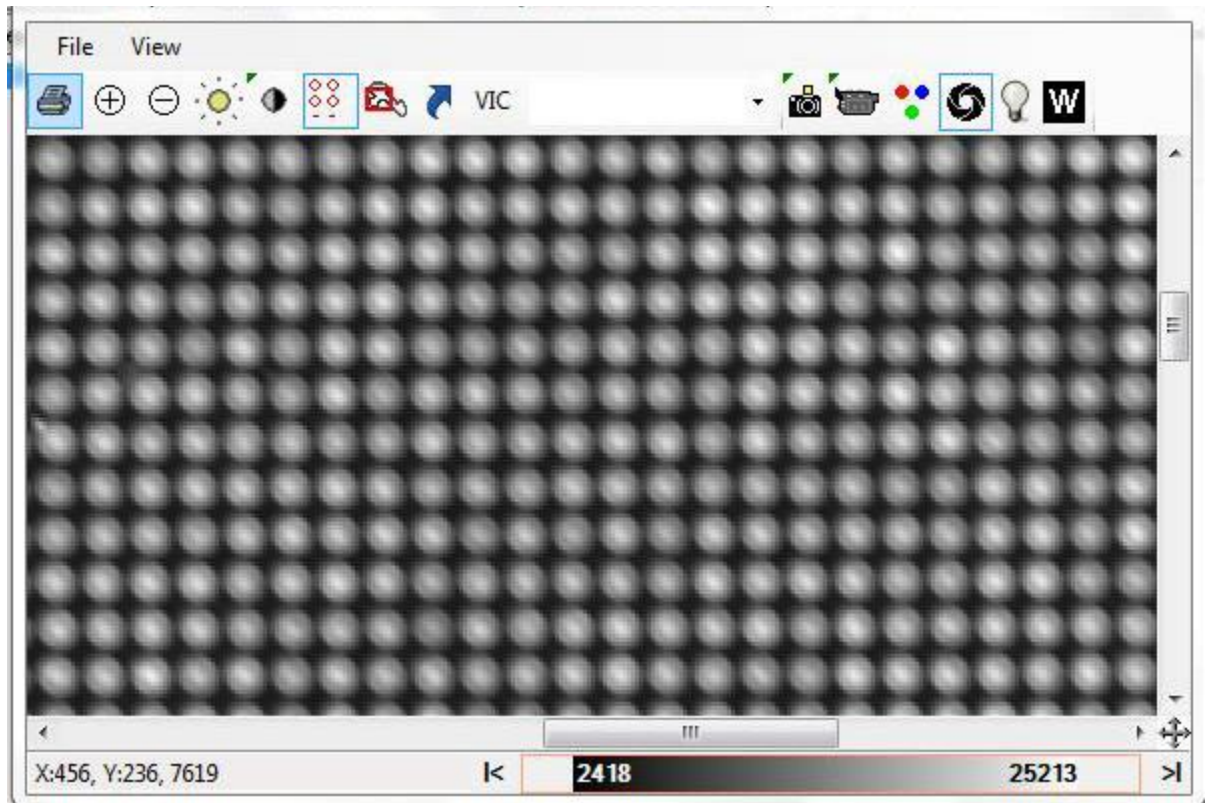
Figure 88. Image Viewer window.

B. Image Viewer Shortcut Icons

🖨	Print	↗	Quick select
⊕ ⊖	Zoom in/zoom out	📷	Snap an image
☀	Image contrast	📷	Start continuous digitization
◑	Auto contrast	⦙⦙	Manual filter selection
⦙⦙	Toggle overlay display	🌀	Enable shutter control (this icon should always be turned on)
📥	Map window	💡	Manual lamp toggle
SYBR	Selected waves: 0-SYBR	F W	Toggle between fluorescence and white image mode
Normalization.tif	Image selector		

NOTE: A green triangle ( ) at the top left of an icon indicates that you can change settings for the icon's action by pressing **[Ctrl] + [icon]**.

Some icons (📷 📷 ⦙⦙ 🌀 💡 F W) are only visible on the computer controlling an instrument, not on remote computers.

1. Zoom Icons

The [Zoom] icons (⊕ ⊖) increase or decrease the size of the Image Viewer. By selecting **[Shift]**+ ⊕ , the Image Viewer window will attempt to resize itself to occupy the same area as the displayed image. Another way of sizing the image is to press the **[Ctrl]** key while turning the scroll wheel on the mouse.

2. Snap Icon

Use the [Snap] icon (📷) to start a single acquisition with the default settings, e.g., exposure time, frames to average (Figure 89).
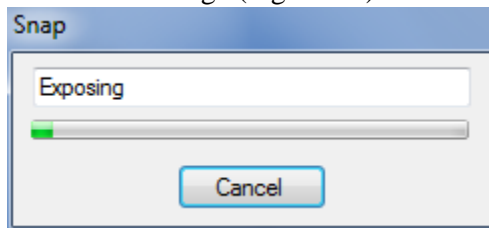
Figure 89. Acquiring an image with the Snap icon.

3. Image Contrast Icon

Fluorescence images generated by the instrument typically have a very large dynamic range. Using the [Image contrast] icon), you can adjust the mapping of 65,535 possible image intensity levels to 256 shades of gray. Drag the control points with the mouse to adjust the mapping (Figure 90).
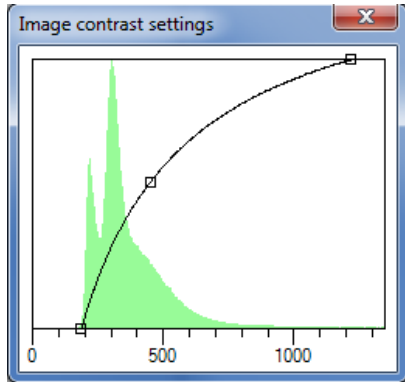
Figure 90. Image contrast settings.

4. Image Contrast Toolbar

The contrast slider toolbar (Figure 91), located at the bottom right of the Image Viewer, can achieve similar effects as those using the Image contrast icon.

Figure 91. Contrast slider toolbar.

Simply click and drag the left and right edges of the grayscale bar or the whole bar itself (Figure 92).
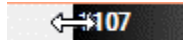
Figure 92. Click and drag the grayscale bar.

Scrolling the mouse wheel while the mouse is over the center of the grayscale bar (Figure 93) changes the shape of the curve and is equivalent to dragging the center control point in the image contrast tool.

Figure 93. Scrolling while the mouse is over the grayscale bar.

The buttons to the left and right of the grayscale bar quickly reset the mapping limits to 0 and 65,535, respectively (Figure 94). Alternatively, you can also double-click on the left or right edges of the bar.

Figure 94. Left and right buttons on the grayscale bar.

Right-click on the toolbar to change the underlying color map (Figure 95).

Figure 95. Change the underlying color map.

5. Auto Contrast Icon

Use the [Auto contrast] icon (● and **[Ctrl]**+ ●) to adjust the upper and lower image contrast and to specify when and how the auto contrast operation is applied (Figure 96).
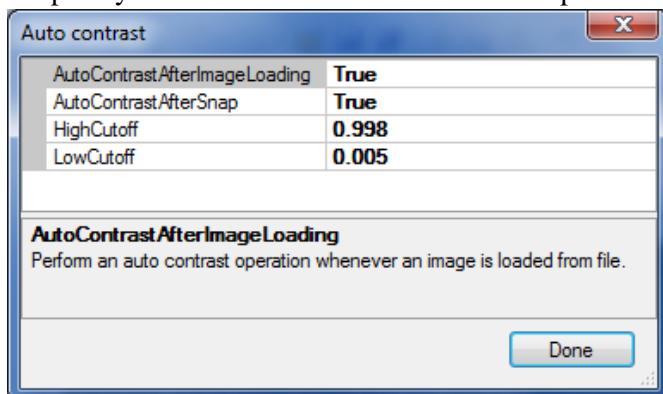
Figure 96. Auto contrast settings.

Table 33. Auto contrast controls and functions.

Control	Function
AutoContrastAfterImageLoading	Perform an auto contrast operation whenever an image is loaded from file.
AutoContrastAfterSnap	Perform an auto contrast operation whenever an image is taken with the camera.
HighCutoff	The high threshold is set such that all pixels above the **HighCutoff** are colored white.
LowCutoff	The low threshold is set such that all pixels below the **LowCutoff** are colored black.

6. Map Window Icon

Use the [Map window] icon ( ) to display the entire image. After selecting this icon, drag or resize the red rectangle to change the viewed portion of the image (Figure 97).
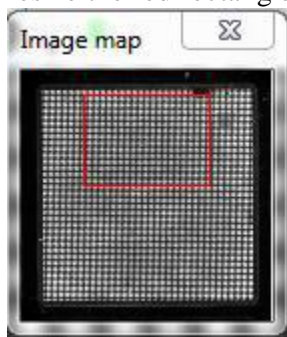
Figure 97. Using the Map window icon to change the viewed portion of the image.

7. Quick Select Icon

Use the [Quick select] icon ( ) for fast switching between images of particular interest (Figure 98).

Normalization
Normalization - white
Reference
Reference - white
First cycle
Last cycle
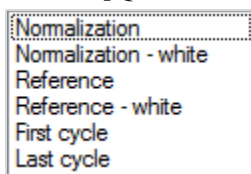
Figure 98. Switching between images with the Quick select icon.

8. Digitize Icon

Selecting the [Digitize] icon ( ) starts a continuous, live digitization procedure (Figure 99). The exposure time can be adjusted.
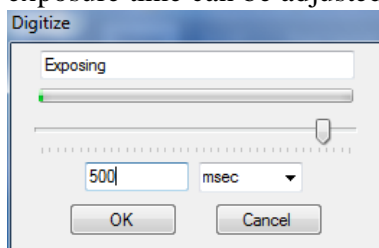
Figure 99. Live digitization procedure.

9. Select Filters Icon

Use the [Select filters] icon ( ) to manually select filter position (Figure 100).

You can move the filter wheels by selecting filters in the wheel drop-down boxes. Use the button to see the physical filter positions. Once a triplet of wheel positions has been defined, it is possible to give it a name by pressing the [New] button. In the future, the triplet can be referred to by this name in the *Filters* tab.

You can turn on white light illumination by clicking the "White light" checkbox. The [Home] button initiates the wheel homing procedure.
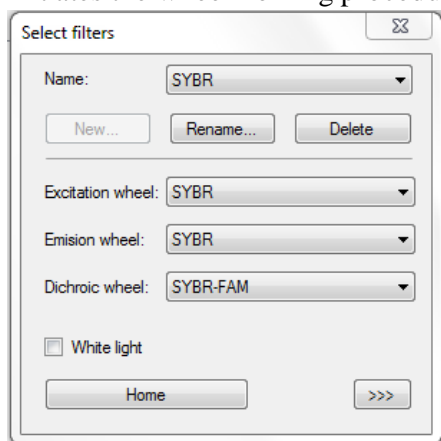
Figure 100. Manually select filter position.

IMPORTANT: Do not change filter settings. Filter settings are preset at the factory based on the hardware of the cycler. The "Name" field at the top (e.g., SYBR) is mapped to the protocol.

XVIII. SmartChip ND Cycler Maintenance

A. Maintenance Schedule

1. Replace the lamp on the SmartChip ND Cycler after 500 hours of service (approximately 250 runs), annually, or if it fails to light.
2. Have Takara Bio staff check and calibrate the SmartChip ND Cycler every 12 months.

B. Maintenance Instructions

1. Changing the O-Ring

The SmartChip qPCR Software displays a warning message if the seal loses its ability to maintain pressure over the SmartChip Panel. This is generally due to dirt or contamination on the sealing surfaces of the two o-rings. There is a large o-ring that seals to the pressure film and a small o-ring that seals the pressure tray to the instrument Figure 101).
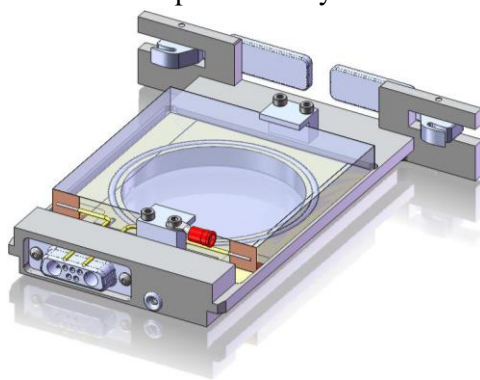
Figure 101. Chip holder.

IMPORTANT: If multiple SmartChip ND Cyclers are connected to the same air line, make sure that the others are not in use. Otherwise, losing air pressure will cause assay failures.

a. Press the [open-drawer] icon ( ) to extend the chip holder out of the SmartChip ND Cycler.
b. Switch off the cycler and disconnect the power and air supply to the instrument, as described below.
   i. Switch off the air pressure valve at the source.
   ii. Disconnect the instant tube fitting at the back of the instrument (hold the strain relief ring in while pulling outward on the tube end). Grip the tube firmly, as there will be residual pressure in the line.
   iii. Manually rotate the chip holder clockwise (i.e., outward) to its maximal position.

   IMPORTANT: Gently move the chip holder to engage the sensor which detects the chip holder in the fully extended position.

c. Remove or loosen the pressure window tray screws:
   i. Remove four flathead screws and the metal panel on the front of the instrument above the doors. Be careful not to drop the screws.
   ii. Loosen two socket head screws, remove the toggle latch cover (black U channel) and open the toggle latches.

d. While wearing gloves, clean the pressure window tray and o-ring:
    i. Slide the pressure window tray outward and remove it from the cycler.
    ii. Clean the glass with anhydrous ethanol and a soft, lint-free cloth or optical wipe. Make sure there is no dust on the surface of the glass.

    IMPORTANT: Do not use general-purpose laboratory wipes, as they are too abrasive and will scratch the glass.

    iii. If there is obvious debris on the exposed o-ring, clean the surface with ethanol and a soft, lint-free cloth.
e. If either or both o-rings show obvious nicks or gouges visible on the surface, replace them:
    i. Get new o-ring(s) from the supplied spare parts kit or order them from Takara Bio.
    ii. Use a toothpick or soft tool to lift the old o-ring from the groove.

    IMPORTANT: DO NOT use metal picks as they may damage the o-ring groove.

    iii. Clean the o-ring groove with ethanol and a cotton swab. Make sure that all lint and dust is removed from the groove.
    iv. Replace the o-ring by moistening the ring with deionized water, placing the ring over the groove, and pressing it down into the groove.

    IMPORTANT: Do not use a wiping motion when installing the o-ring. Wiping can cause the ring to stretch or bunch and not seal properly.

f. Reinstall the pressure window tray:
    i. Close the latches, replace the lock bar, and tighten the socket head screws. As you close the latches, make sure you feel the pressure window tray connecting to the D-Sub electrical connector at the back of the housing.
    ii. Replace the panel and four screws.
g. Reconnect the air line and open the air valve. (This is a reverse of Step b.)
h. Reconnect the electrical power to the cycler but do NOT power it on.

IMPORTANT: Follow the next steps exactly.

i. Reset the qPCR software and power up the cycler:
    i. From the open *Chip info* window, select **NoChip—CloseDrawer**. There will be two error windows that open. Select [OK] in each window.
    ii. Exit the cycler software by selecting the red [X] icon in the upper right corner of the software window.
    iii. Remove the USB cable (which connects the cycler to the computer) from the computer.
    iv. Power the cycler on and plug in the USB cable.

    IMPORTANT: Make sure the chip holder is fully out (see Figure 102, above).

    v. Restart the cycler software and wait for the cycler to finish Homing Wheels.
    vi. Keeping your hands clear of the chip holder and door, go to the *Run* tab and select the [open-drawer] icon.
    vii. Wait 10 sec then select **NoChip—CloseDrawer** from the open *Chip info* window.

2. Cleaning the SmartChip ND Cycler

Do not clean the exterior of this equipment with solvents or harsh chemicals. Only clean using a damp cloth and mild detergents. Always unplug the instrument before wiping it down.

C. Fixing Issues with the Lamp

If the lamp fails to light, it is likely that either you need to replace the lamp or that the lamp thermal switch has been tripped.

IMPORTANT: UNPLUG THE INSTRUMENT BEFORE REPLACING THE LAMP. Determine the cause of the thermal trip and take preventative action, i.e., improve ventilation and make sure that the laboratory temperature is within specifications (15–30°C).

1. Lamp Thermal Switch Tripped

Power cycle the instrument. If the problem persists, replace the lamp.

Follow the instructions for replacing the lamp. After removing the lamp cover, reach into the instrument and press the thermal switch reset button located on the left side of the lamp housing (as you face the back of the cycler).

2. Changing the Lamp on the SmartChip ND Cycler

a. Turn the machine off and remove the power cord.
b. Rotate the instrument to allow access to the rear of the machine. Loosen the four screws on the top right of the rear of the instrument and slip the lamp cover off the back of the instrument.
c. Loosen the locking screw just below the lamp (Figure 104).
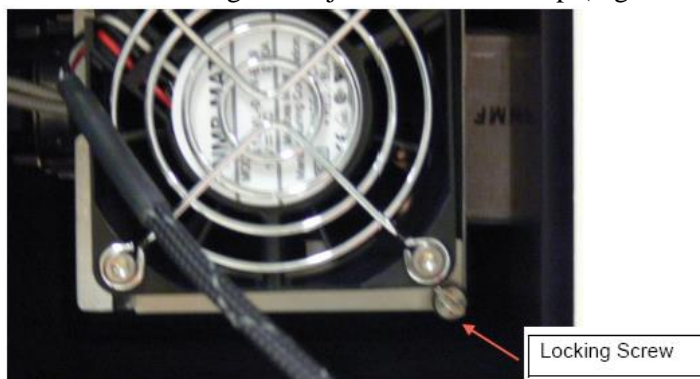
Figure 102. Locking screw below the lamp.

d. Remove the fan and lamp, leaving the wires connected.
   IMPORTANT: The wires are short. Do not overextend them.

e. Eject the lamp by pulling back on the wire lever (Figure 103). Remove the old lamp and discard. Replace with a new lamp supplied by Takara Bio, being careful to not touch the internal parts of

the lamp. The wire lever should be pushed forward (away from the fan) to facilitate installation.
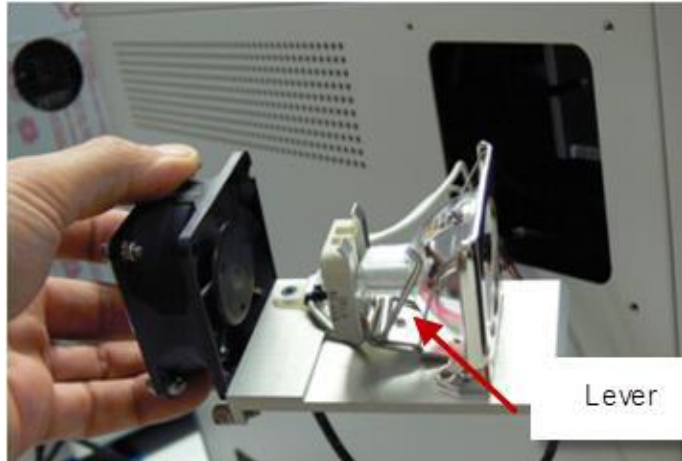
Figure 103. Ejecting the lamp assembly.

f. Now place the fan and lamp assembly back into the instrument, being careful not to trap the white wires (Figure 104).

Figure 104. Replacing the lamp assembly.

g. Once the assembly is in place, tighten the locking screw.
h. Replace the cover and tighten the screws used to secure it.
i. Connect the power and verify that the lamp is functioning by starting a run.

XIX. Troubleshooting

If the SmartChip ND Cycler or SmartChip qPCR Software does not respond as desired or a warning is displayed, please attempt to rectify the problem using Table 34 below. If you cannot solve the problem, please contact Takara Bio technical support (technical_support@takarabio.com).

Table 34. Troubleshooting.

Problem	Possible cause	Solution
Pressure warning, bottom right of software window (numbers turn red) or pop-up window describing the error	Low supply pressure	Make sure the supply pressure is set to between 100 and 120 psi (6.9–8.3 bar).
Chamber pressure <20 psi at start of run.	• Seal failure • Dirty, damaged, or missing o-ring • Hole in sealing film	• Try to reseal (button option in software) • Replace the o-ring in the chamber • Use a new pressure seal
Chip holder does not eject	• Low system pressure • Chip stuck in instrument (does not unload with chip holder)	• Check system pressure • Try loading and unloading the chip holder. Look inside to check that the chip is not dislodged and jammed. If so, unplug the system, disconnect from air pressure, and remove the access panel to clear the jam.
Temperature sensor error or system does not hit target temperatures	Possible sensor failure	Unplug the USB and restart the software and system. If the problem persists, call Takara Bio.
Lamp fails to light	Lamp failed or lamp thermal switch has been tripped	Power cycle the instrument. If the problem persists, check the lamp thermal switch and/or replace the lamp.
Lamp thermal switch tripped	Instrument may be poorly ventilated or temperature in lab may be too high	Improve instrument ventilation and/or lower the temperature in lab. Power cycle the instrument. Push the red reset button until it clicks. If the problem persists, replace the lamp.
Filter wheels fail to home	Communication error	Try re-homing the wheel from the Filters button on the image capture screen. If the problem persists, restart the instrument and software.
Loss of communication	Cable, camera, or board disconnected	Check the USB cable, restart the software, restart the SmartChip ND Cycler, unplug/replug the USB cable.
Software does not start	Orphan process	Use `Windows Task Manager` to end existing `WaferGen.exe` process.

Appendix A: Installing SmartChip qPCR Software

For the computer that controls the instrument, install the qPCR software in **instrument control** mode. In this mode, the software both controls the SmartChip ND Cycler and performs analysis of the results. When installed in instrument control mode, the Windows desktop shortcut is named `SmartChip qPCR Instrument`.

You can also load the software onto a remote computer for the convenience of performing data analysis outside the laboratory, such as at your desk. For this purpose, install the software in **analysis** mode. The Windows desktop shortcut for the software installed in analysis mode is named `SmartChip qPCR Analysis`. In general, instrument control options are not available when the software is installed in analysis mode.

IMPORTANT: Once the SmartChip qPCR Software is installed in either **instrument** or **analysis** mode, it cannot be changed to the other mode unless you uninstall and re-install the software. Please refer to the ReadMe file under `Installation Instructions\` on the SmartChip qPCR Software Installation CD.

1. Log on to the target computer appropriately so that you can install the SmartChip qPCR Software. Depending on the version of the Windows OS running your computer and how it is integrated with a larger network, you may need to log on to the computer in Administrator mode or get help from your IT department in order to install the software.
2. Before installing the SmartChip qPCR Software from your CD, you will first need to install several third-party software components that are also located on the CD. Please refer to the ReadMe file located in the `Installation Instructions\` on the installation CD.
    a. Open the directory called `Install these first\`.
    b. Run `AltovaXML2010R2.exe`, accept the license agreement, and choose to install the complete setup. Please note that revision 2010 rel. 2 is required. AltovaXML provides support for managing .xml files.
    c. Run `dotnetfx35.exe` and accept the license agreement. This is the Microsoft .NET 3.51 updater, which provides required software framework functionality.
    d. Extract the contents of the .zip file to install Apache FOP, which provides tools to write .pdf documents, as follows:
        i. Extract the contents of the .zip file into the `C:\Program Files` directory so that you end up with a folder at `C:\Program Files\fop-0.95`. To do this, enter `C:\Program Files` when the installer prompts you for an installation location. Note that for 64-bit Windows installations, the path is `C:\Program Files (x86)`.
        ii. Verify installation by checking that there is a file called `C:\Program Files\fop-0.95\fop.bat`.
    e. Run `jre-6u21-windows-i586-s.exe`, which installs Sun Microsystems Java support for executing Java.

3. Run the SmartChip qPCR installer:
   a. Run the `setup.exe` program in the `SmartChip qPCR\` directory on the CD.
   b. Read the license agreement and, if you agree, select the [I agree] button.
   c. For system configuration:
      i. If you are installing the software to the computer that will control the SmartChip ND Cycler, select the **Instrument control** button.
      ii. If you are installing the software to a remote computer that is not controlling the SmartChip ND Cycler, select the **Offline analysis** button.
   d. Accept the recommended installation location.
   e. For instructions on how to install FOP, refer to the ReadMe file located in the `Installation Instructions\` on the SmartChip qPCR Software Installation CD.

Appendix B: SmartChip qPCR Software File Locations

SmartChip qPCR Software is compatible with Windows XP and Windows 7. The file path for locating executable files is in the following directory, depending on the operating system:

- **Windows XP and Windows 7, 32-bit**—`C:\Program Files\Wafergen\qPCR`
- **Windows 7, 64-bit**—`C:\Program Files (x86)\Wafergen\qPCR`

The data configuration path is located in the following directory, depending on the operating system:

- **Windows XP**—`C:\Documents and Settings\All Users\Application Data\Wafergen\qPCR`
- **Windows 7** —`C:\ProgramData\Wafergen\qPCR`

Appendix C: Exogenous Controls—Yeast Control Fragments

For gene expression analysis, SmartChip Panels may contain PCR assays for yeast DNA targets. These are considered exogenous controls because your experimental samples are not expected to contain yeast genes. They are labeled: **WGBS-YCF*n***, where n is a number from 1 to 4. The actual number of PCR assays for yeast controls is specific to a particular SmartChip Panel. These assays are designed to detect DNA in a yeast control cocktail that can be added to samples as a positive control for your PCR. After dilution, the effective concentration of each of the yeast targets in a well of the SmartChip Panel is indicated in Table 35 below.

Table 35. Yeast control concentrations per well.

Yeast control	Concentration (copy number/µl)
WGBS-YCF1	5×10^9
WGBS-YCF2	5×10^8
WGBS-YCF3	5×10^7
WGBS-YCF4	5×10^6

A. Expression Curve Analysis

We do not recommend changing **Curve analysis settings**. If you choose to do so anyway, select the [Curve analysis settings] icon ( ) while in **Analysis** mode, or select the *Curve analysis* tab while in **Setup** mode. The parameters are listed in the display table below (Figure 105).

See "Expression Analysis Parameters" in this appendix for a description of the settings.
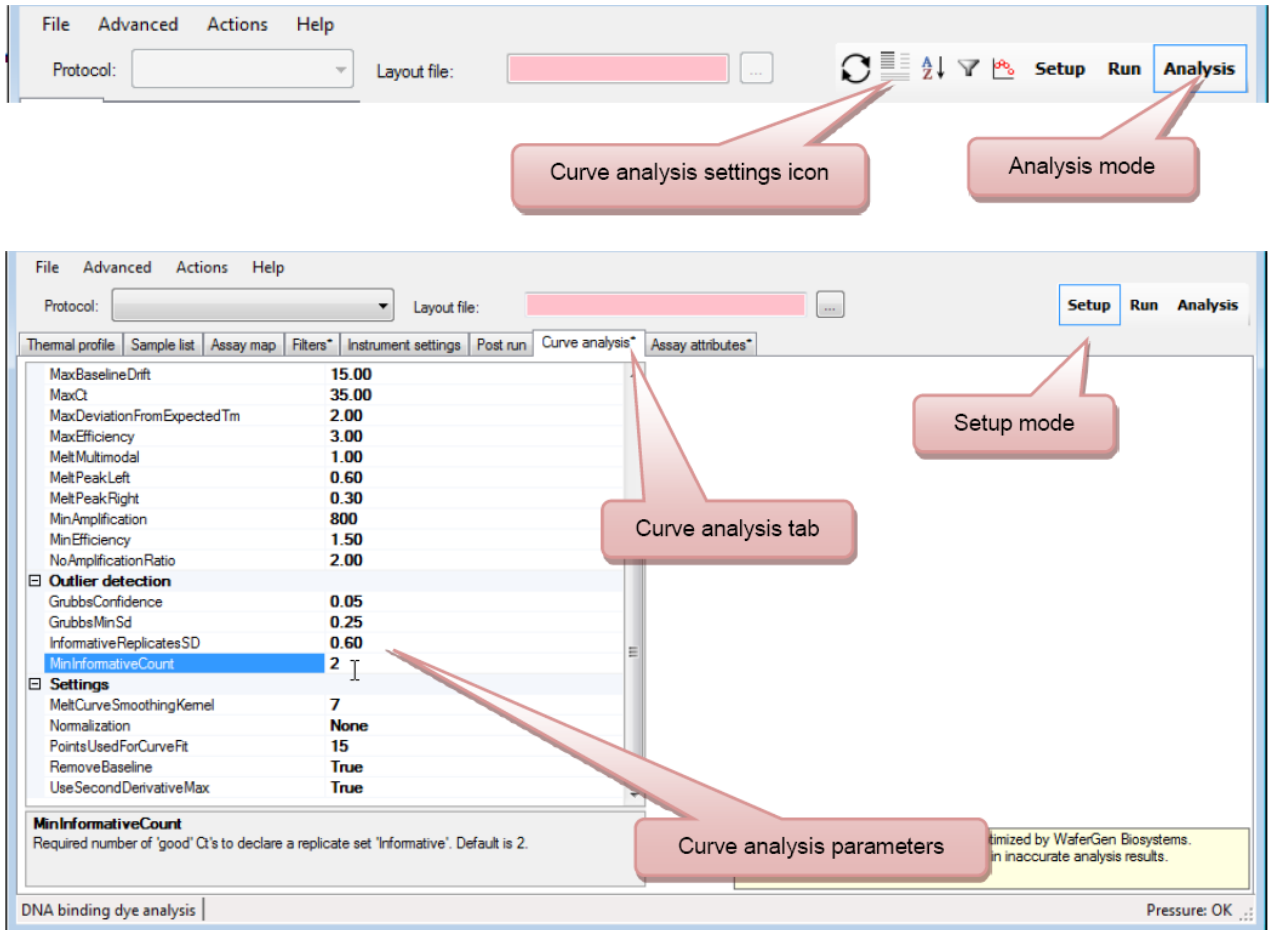
Figure 105. Curve analysis settings.

B. Genotyping Analysis

In general, we do not recommend changing the **Genotyping settings**. You may, however, opt to modify the minimum sample call rate ("MinCallRate"). To do so, select the [Settings] icon ( ) while in **Analysis** mode, or select the *Genotyping settings* tab while in **Setup** mode (Figure 106).
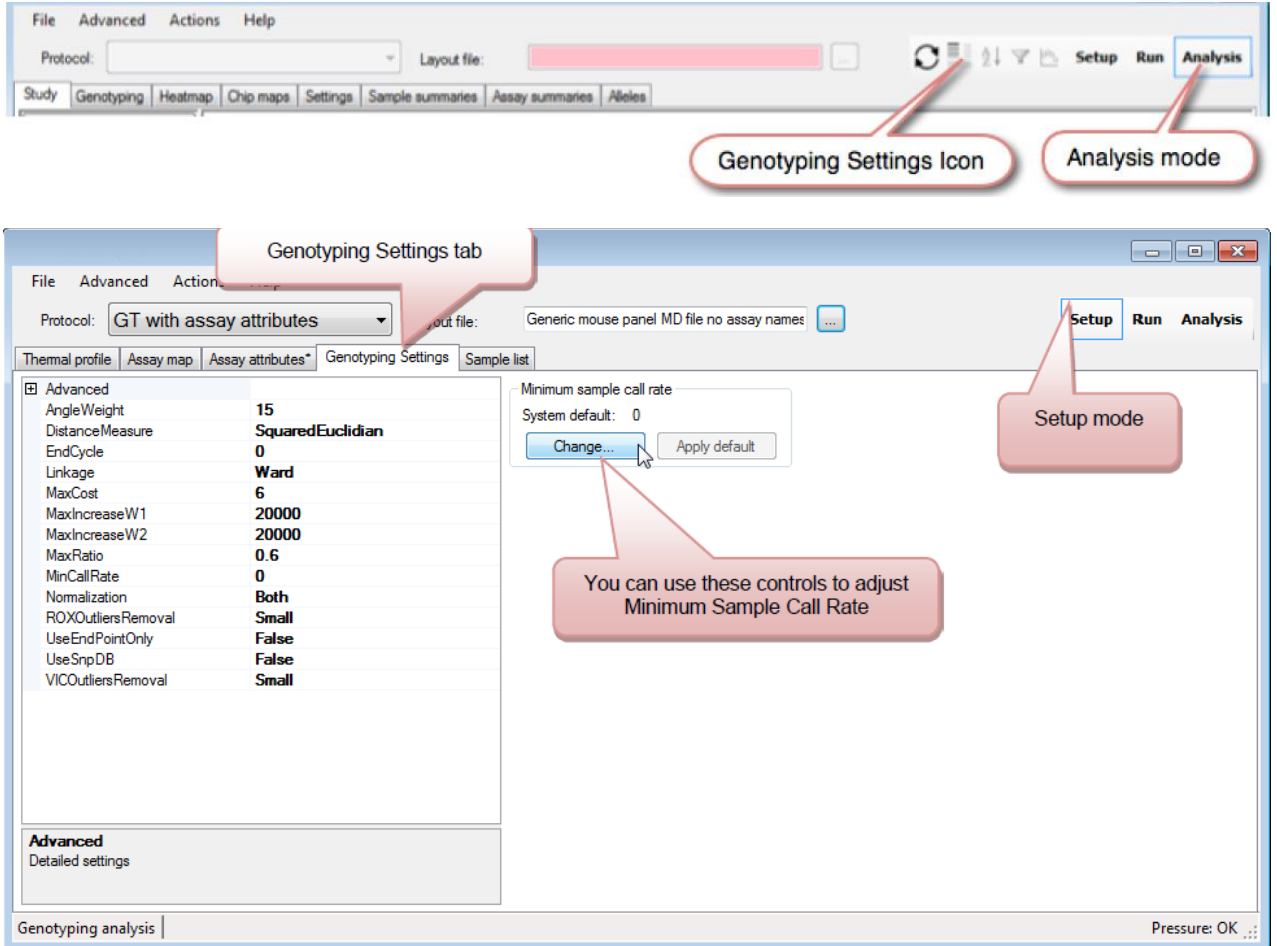
Figure 106. Curve analysis settings.

C. Expression Analysis Parameters

Table 36 below shows the parameters used for qPCR analysis. Some can be adjusted in the *Curve analysis* tab in **Setup** mode. Settings shown in shades of red in the table below can affect expression analysis results, whereas items in gray affect only the assignment flags to your data.

Table 36. Expression analysis parameters.

	Threshold and description	DNA binding dye-based PCR	Probe-based PCR	Affected flag
Curve Flagging	**HighCt:** Flag wells where the calculated C$_t$ value is higher than this value. This value would be lower than **MaxCt**, which would cause the curve fit to fail.	✔	✔	CtIsHigh
	HighEfficiency: Flag wells where the calculated efficiency (based on the curve fit) is higher than this value. This value should be less than **MaxEfficiency** (see below).	✔		HighEfficiency
	LowCt: Flag wells where the calculated C$_t$ value is less than this value. This value should be higher than 4.5.	✔	✔	CtIsSmall
	LowEfficiency: Flag wells where the calculated efficiency (based on the curve fit) is less than this value. This value should be higher than **MinEfficiency**.	✔		LowEfficiency
	LowSaturation: Flag wells where the saturated fluorescence signal is less than this value.	✔	✔	LowSaturation
	LowSaturationBaselineRatio: Flag wells where the ratio of baseline fluorescence versus saturated fluorescence is less than this value. This value can become meaningless when high background fluorescence is present. The ratio is calculated from the curve fit parameters: (D+A)/A, where D and A are parameters in the sigmoidal fit of the amplification curve, further described in the **Analysis Tab** section.	✔		LowSaturation BaselineRatio
	R2: Flag wells where the R^2 of the curve fit is less than this value. Should be higher than **FailCurveBasedOnR2**.	✔	✔	BadR2
Curve Rejection	**FailCurveBasedOnR2**: Force curve fit to fail, if the R^2 of the curve fit is less than this value. Should be smaller than **R2**.	✔	✔	CurveFitFailed
	FailCurveBasedOn StraightR2: Curves where a straight line can be fitted with R^2 higher than or equal to **FailCurveBasedOnStraightR2**.	✔		NoAmplification
	MaxBaselineDrift: Fail a curve fit if the curve parameter ratio (D/E) is less than this value, where D is a parameter in the sigmoidal fit of the amplification curve, further described in the **Analysis Tab** section, and E is a measurement in linear drift of the baseline. Choose values >12. This value can be viewed as actual cycles. If this threshold is triggered for a curve, you need less than **MaxBaselineDrift** cycles in the baseline to achieve the same increase in fluorescence as is provided by the actual growth curve (D).	✔		CurveFitFailed

Table 36 continues on next page.

Table 36. Expression analysis parameters continued.

Threshold and description	DNA binding dye-based PCR	Probe-based PCR	Affected flag
MaxCt: Fail a curve fit if the C_t value is larger than this value. Value should be higher than **HightCt**, which results in a flag. There is no corresponding **MinCt**; however, curve fits will be failed if the C_t value is less than 4.5.	✔	✔	CurveFitFailed
MaxDeviationFrom ExpectedTm: Exclude wells where T_m deviates by more than this value from the expected T_m. This only applies if the assay attribute file contains a T_m column.	✔		TmAPD
MaxEfficiency: Fail a curve fit if the calculated efficiency (based on the curve fit) is higher than this value. Value should be larger than **HighEfficiency**, which results in a flag.	✔		CurveFitFailed
MeltMultimodal: Flag the replicate group if the maximum distance between clusters is more than this threshold during a K-means cluster analysis of the melt peaks. If replicates are spread out across the chip, we expect a larger variation of T_m values, and this threshold should be set to a higher value. If replicates are in neighboring wells, this threshold can be made tighter.	✔		MultiModal
MeltPeakLeft: Flag wells where the ratio between the main peak of the melting curve and the major peak to the left of the main peak is larger than this threshold. Flagged wells are treated similarly to outliers: they are not included in the averages.	✔		MultipleMeltPeaks (previously called PrimerDimers Detected)
MeltPeakRight: Flag wells where the ratio between the main peak of the melting curve and the major peak to the right of the main peak is larger than this threshold. Flagged wells are treated similarly to outliers: they are not included in the averages.	✔		MultipleMeltPeaks
MinAmplification: Wells in which saturated fluorescence minus baseline fluorescence is less than this value are considered to have no amplification. (Baseline: the average fluorescence of the first three cycles. Saturated: the average fluorescence of the cycle with the highest fluorescence and the cycle following it.) Use this threshold to weed out wells that have signal due to crosstalk. See also: **NoAmplificationRatio**.	✔	✔	NoAmplification
MinEfficiency: Fail a curve fit if the calculated efficiency (based on curve fit) is smaller than this value. Value should be less than **LowEfficiency**, which results in a flag.	✔		CurveFitFailed

(Curve Rejection — row group label, left margin)

Table 36 continues on next page.

Table 36. Expression analysis parameters continued.

	Threshold and description	DNA binding dye-based PCR	Probe-based PCR	Affected flag
Curve Rejection	**NoAmplificationRatio**: Wells in which the ratio of baseline fluorescence versus saturated fluorescence is less than this value are considered to have no amplification. (Baseline: the average fluorescence of the first three cycles. Saturated: the average fluorescence of the cycle with the highest fluorescence and the cycle following it.) Note: this value can become meaningless when high background fluorescence is present. Use this ratio to weed out wells that only have signal due to crosstalk. See also: **MinAmplification**.	✔	✔	NoAmplification
Outlier Detection	**GrubbsConfidence**: Confidence value for Grubb's test. Higher values result in a higher number of outliers. Note: The test is not very sensitive to this value. Changing from 0.01 to 0.1, in some cases, might not result in more outliers. For large numbers of replicates, it is possible to set this value >0.1.	✔	✔	Outlier
	GrubbsMinSd: Minimum SD to trigger outlier detection. The Grubbs outlier test is only performed if the SD of the replicates is larger than this threshold. It prevents wells from being discarded simply because the other wells in the replicate set are closely grouped together. Do not set this value to 0.	✔	✔	Outlier
	InformativeReplicatesSD: A result is informative if the standard deviation of the C_t values of replicates is less than this threshold and the number of valid replicates is at least as many as the **MinInformative Count**. Applies to replicates.	✔	✔	NotInformative (replicates)
	MinInformative Count: Flag wells when data from fewer replicates than this value provide informative data.	✔	✔	NotInformative
Settings	**MeltCurveSmoothing Kernel**: Size of a triangular filter that is used to smooth the melting curve. Higher values result in smoother looking curves, while lower values result in sharper peaks and may also allow separation of closely spaced peaks. Choose a value of 5 or 7.	✔		
	Normalization: Options are: • No normalization (not recommended unless the master mix does not include ROX) • Normalize by ROX signal in the first cycle (not recommended) • Normalize by ROX signal in each cycle (recommended)		✔	
	PointsUsedForCurveFit: Number of points used in final curve fit. (Default is 15; use a value between 13 and 17.)	✔		
	RemoveBaseline: Add a linear term to the curve fit to account for the baseline drift. If set to True, the number of points used for the curve fit is increased by five.	✔		

1. **Efficiency Calculations**

The qPCR software calculates efficiency as the ratio of fluorescence values in individual reaction wells from one PCR cycle using data from cycles in proximity to the C_t. This usually results in efficiency values below 2. In probe-based PCR, assay efficiency is typically calculated by amplifying a dilution series of template and plotting the data as a standard curve. If your experiment includes a sample dilution series, the software will generate a standard curve from the data. Find it on the *Calibrations* tab in **Analysis** mode.

2. **Classification of Average C_t Values as Informative (or Not)**

The qPCR software uses statistics to help users understand whether the averages of replicate C_t values are meaningful or not. Two settings are very important in this determination: "InformativeReplicatesSD" and "MinInformative Count". You can adjust these settings in the *Curve analysis* tab in **Setup** mode.

The "InformativeReplicatesSD" setting is helpful in situations in which the C_t values from replicate reactions are widely distributed (have a large SD), but there are no obvious outliers. This is an indication that the data are not reliable.

The "MinInformative Count" setting helps with interpretation of a similar result, in which the C_t values from replicate reactions are widely distributed, but two or three replicates yield similar values. If the "MinInformative Count" value is set too low, unreliable data may appear to be informative because the software will essentially throw out a lot of "inconvenient" data, keeping only the two to three C_t values that are similar and result in a low SD. The default value for the "MinInformative Count" setting is 2. If your experiment includes four or more replicates for a given sample/assay pair, you may want to increase it.

Appendix D: Flags

A. Expression Analysis Flags

In gene expression mode, well reactions can be marked with flags. Table 37 below describes the flags with no associated threshold.

Table 37. Expression analysis flags and descriptions.

Flag	Description
ANC	Amplification in negative control, i.e, non-NTC wells with a signal <NTC.
NOA	No amplification in positive control.
CurveFitFailed	There was amplification, but the curve fit algorithm did not converge; the shape of the curve is somewhat unusual. An extreme case of a **BadR2**.
ContainsOutlierReplicateCt	One of the replicates has a C_t value that is very different from the average of the others (i.e., a replicate of this well has the flag **CtIsOutlier**).
AIU	Amplification of UNPRIMED well.
TmANTCAB	Authentic binding in NTC*.
TmANTCPD	Non-authentic binding in NTC (e.g., have a C_t and T_m in an NTC well)*.

*The last two flags depend on T_m information from the gene database.

B. Genotyping Flags

In genotyping mode, well reactions can be marked with flags. Table 38 below describes the flags with no associated threshold.

Table 38. Genotyping flags and descriptions.

Flag	Description
Norm Dye Outlier	Outlier from ROX normalization.
No amplification	NonNTC wells with signal <NTC.
Cluster Outlier	Identifies points too far from the cluster centroid.
Between Clusters	Identifies points too close to more than one cluster to call.
Manual No Call	Data that has been removed manually from the particular assay/chip.
Sample excluded	Data that has been removed automatically from all assays/chip.

Appendix E: Summary Reports

A. Expression Analysis Summary Reports

In gene expression mode, the **Analysis** mode contains a Summary report which can be viewed by using the *Summary* tab (Figure 107). The first time you load the Summary report, you will need to name it.
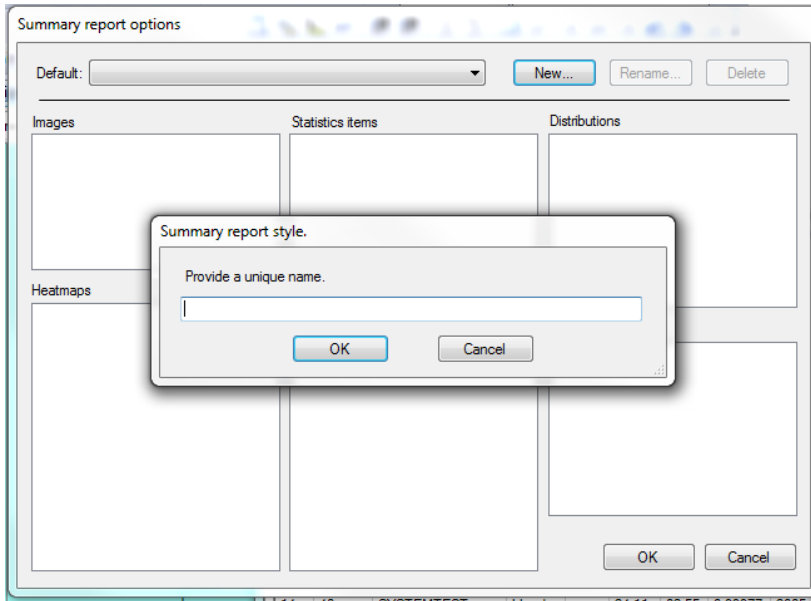
Figure 107. Summary report window for gene expression.

Choose the items to be included in the *Summary report style* dialog box by using the **Advanced** drop-down menu and choosing the desired Summary report options (Figure 108).
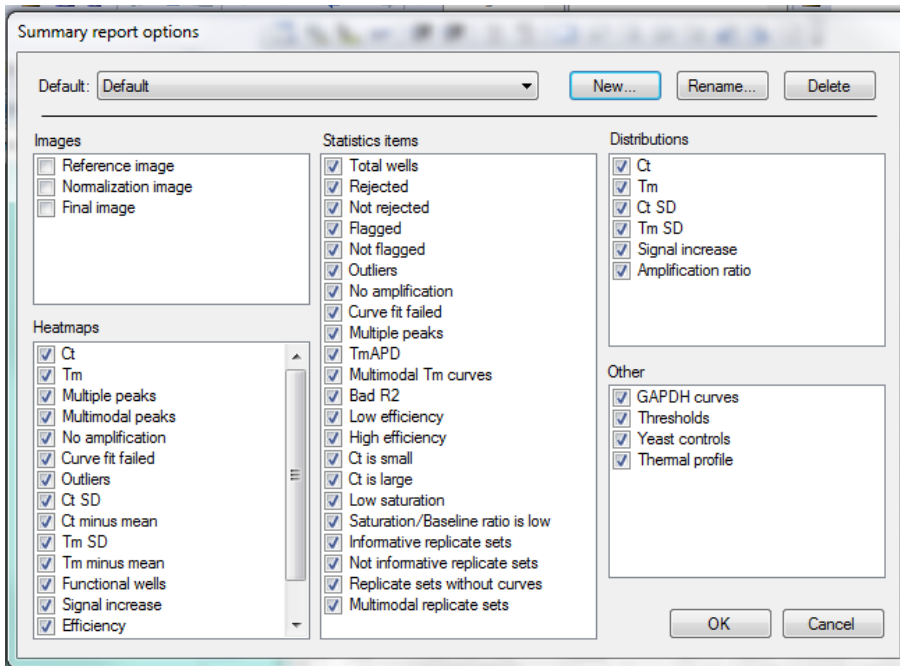
Figure 108. Gene expression Summary report options.

Data Exclusion and Flag Assignment Strategy

The following chart (Figure 109) is a breakdown of replicate group statistics. All amplification curves are represented on the chart. Each curve is first examined on its own merits; some may be rejected at this stage or flagged for your review. Curves that were not rejected based on individual characteristics are then evaluated in the context of replicate reactions. Outliers may be rejected at this stage, based on comparison with results from replicate reactions, or they may simply be flagged.

	Based on Individual Curves	Based on Data from Replicates
Data Rejection	Curve fit algorithm does not converge No Amplification Primer Dimer TmAPD MaxCt MinCt MaxEfficiency MinEffciency Minamplification MaxBaselineDrift R2 MaxDeviationFromExpectedTm	Ct outlier Tm Multimodal
Warning Flags	Bad R2 High Ct	NotInformative

Figure 109. Breakdown of replicate group statistics.

B. Chip Summary

Table 39. Chip summary.

Result	Description
Total wells	Total number of defined wells that have both a sample and primer defined. (Number of Rejected wells + Number of Not rejected wells = Total wells)
Rejected	Number of wells that have been rejected based on the **Curve rejection analysis** setting.
Not rejected	Number of wells not rejected.
Flagged	Number of flagged wells (not counting outlier flags).
Not flagged	Number of wells with no flags (not counting outlier flags).
Outliers	Number of wells that have the 'CtIsOutlier' flag.
No amplification	Number of wells that have the 'NoAmplification' flag. Wells without amplification are excluded from all other calculations.
Curve fit failed	Number of wells that have the 'CurveFitFailed' flag.
Multiple peaks	Number of wells where multiple peaks were detected in the melting curve. Curves flagged this way are not included in the calculation of average C_t values and average T_m values.
Multimodal Tm curves	Number of wells in replicate groups where the T_m values have multimodal distribution.
TmAPD	Number of wells where T_m deviates by more than this value from the expected T_m.
ad R2	Curve fit is poor: R^2 is below **Curve flagging/R2**.
Low efficiency	The efficiency is lower than **Curve flagging/LowEfficiency**. This value is based on the efficiency that is calculated from the curve fit.
High efficiency	The efficiency is higher than **Curve flagging/HighEfficency**. This value is based on the efficiency that is calculated from the curve fit.
Ct is small	The C_t value is less than **Curve flagging/LowCt** and possibly not very accurate.
Ct is large	The C_t value is higher than **Curve flagging/HighCt** and possibly not very accurate. This problem may be addressed by using more cycles.
Low saturation	Curve has been fitted, but the saturation value of the curve is below **Curve flagging/LowSaturation**. Possibly not enough light, the exposure time was too short, or there was not enough dye/primer.
Saturation/Baseline ratio is low	Curve has been fitted, but the ratio of saturation vs. baseline is below **Curve flagging/LowSaturationBaselineRatio**.
Informative replicate sets	Number of replicate sets in which the standard deviation of the C_t values of replicates is less than this threshold and the number of valid replicates is at least as many as the "MinInformative Count". Applies to replicates only.
Not informative replicate sets	Number of replicate sets that are not informative.
Replicate sets without curves	Number of replicate sets where none of the replicates produced a valid amplification curve.
Multimodal replicate sets	Number of replicate sets in which the T_m values have a multimodal distribution.

C. Genotyping Summary Reports

In genotyping mode, the **Analysis** mode contains *Sample summary* reports and *Assay summary* reports, both of which can be viewed by using their respective tabs. The first time you load a Sample summary or Assay summary report, you will need to name it (Figure 110).
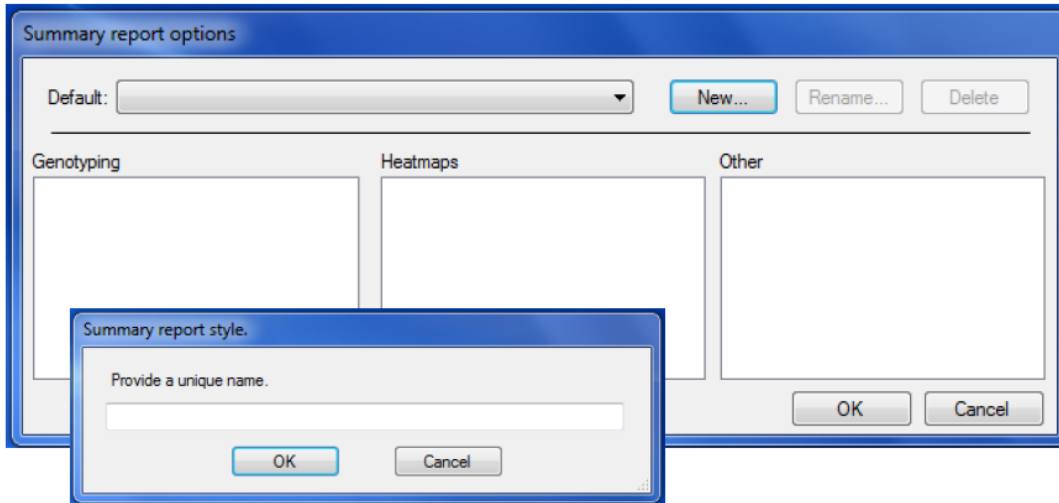

Figure 110. Initial view of Sample summary report or Assay summary report window for genotyping.

Choose the items to be included in the *Summary report style* dialog box by using the **Advanced** drop-down menu and choosing the desired Summary report options (Figure 111).
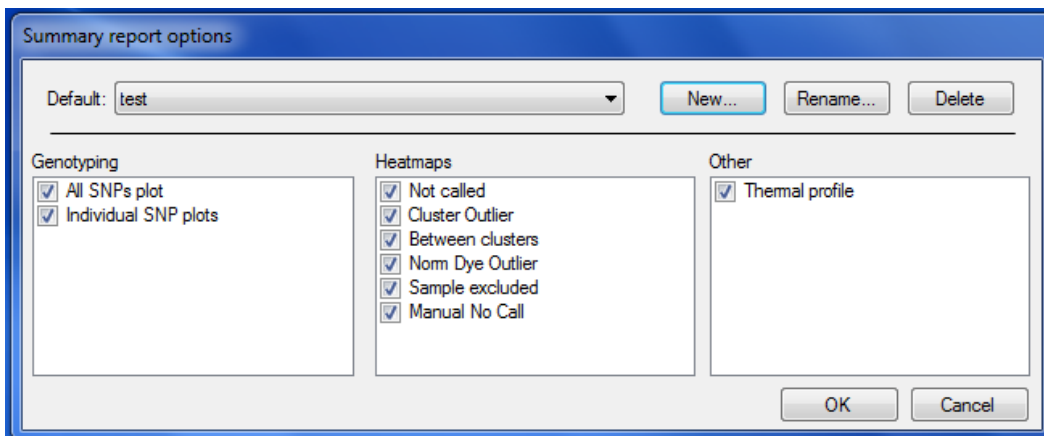

Figure 111. Choosing the items to be included in the Summary report style box.

Appendix F: Curve Graphs

In expression analysis, views of graphs can be zoomed in or out by selecting a sub-area of the graph with the mouse. Another way of zooming is by scrolling the mouse wheel while the mouse cursor is on top of the graph. You may also pan across the graph with the mouse by holding down the [Ctrl] key.

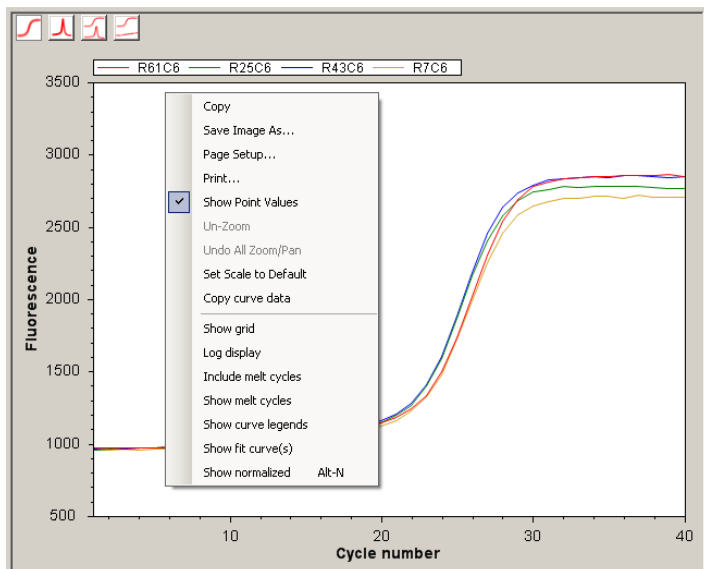Right-clicking on graph windows provides context-sensitive options for viewing the data (Figure 112, Table 40).

Figure 112. Context-sensitive options for viewing the data on curve graphs.

Table 40. Descriptions of viewing options for curve graphs.

Viewing option	Description
Copy	Copy a bitmap of the graph to the clipboard.
Save Image As…	Save an image of the graph to a file. Various file formats are supported.
Page Setup…	Define print parameters.
Print…	Print the graph.
Show Point Values	When the mouse cursor hovers over a line in the graph, a tool tip with additional information is displayed.
Un-Zoom	Undo previous zoom operations.
Undo All Zoom/Pan	Undo all manual zoom/pan operations.
Set Scale to Default	Adjust the scales so that all the data points are visible.
Copy curve data	Copy the data points for all displayed lines to the clipboard. This is useful for copying data to Excel.
Show grid	Display a grid in the background.
Log display	Display the y-axis on a log scale.
Include melt cycles	Display the entire fluorescence curve, including the melt cycles.
Show melt cycles	Display melt cycles only.
Show fit curve(s)	Display the amplification curve resulting from the fitted curve parameters.
Show normalized	Show the curve in a normalized way (baseline at 0, saturation at 100).
Show curve legends	Display a legend with row/column information.

Appendix G: Glossary

C_t (threshold cycle): Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_t value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated to create a fluorescent signal above background fluorescence. Other terms used to describe this are crossing point (C_p) and quantitation cycle (C_q).

Dew point: The dew point is the temperature at which a given parcel of humid air must be cooled, at constant barometric pressure, for water vapor to condense into water. The condensed water is called dew. The dew point is a saturation temperature; it is associated with relative humidity. A high relative humidity indicates that the dew point is closer to the current air temperature. Relative humidity of 100% indicates the dew point is equal to the current temperature and the air is maximally saturated with water. When the dew point remains constant and temperature increases, relative humidity will decrease.

Dynamic range: The range of initial template concentrations over which C_t values are obtained. If endogenous control is used for the delta-delta C_t quantitation method, dynamic ranges of target and control should be comparable. In absolute quantitation, interpolation within this range is accurate, but extrapolation beyond the dynamic range should be avoided. The larger the dynamic range, the greater the ability to detect samples with high and low copy number in the same run.

Melting curve (dissociation) analysis: Every dsDNA fragment has a melting point (T_m), i.e., the temperature at which 50% of the DNA is single stranded. The T_m depends on the length of the DNA, sequence order, GC content, and Watson-Crick pairing. When fragments are heated in the presence of fluorogenic DNA-binding dyes, a sudden decrease in fluorescence occurs when the T_m is reached due to dissociation of DNA strands and release of the dye. This fluorescent peak is determined from the inflection point of the melting curve or the melting peak of the derivative plot, which is the negative first-derivative of the melting curve. High-resolution melting curve analysis can be achieved on the SmartChip ND Cycler.

No-template controls (NTC, minus-sample control): The NTC includes all of the RT-PCR reagents except the RNA template. No product should be synthesized in the NTC or NAC; if product is amplified, it indicates contamination (fluorescent or PCR products) or the presence of amplifiable genomic DNA in the RNA sample. NTC is not equivalent to H_2O controls, and H_2O controls are not used in qPCR experiments.

Real-time PCR: The continuous collection of fluorescent signal from polymerase chain reactions throughout cycles.

Standard curve: Obtained by plotting C_t values against log-transformed concentrations of serial ten-fold (log) dilutions of the target nucleic acid. A standard curve is obtained for quantitative PCR, and the range of concentrations included should cover the expected unknown concentration range. It is used to find out the dynamic range of the target (and/or normalizer), to calculate the slope (therefore, efficiency), r and R^2 coefficients, precision (standard deviation), and sensitivity (y-intercept), and also help with quantitation.

Ideally, the slope of a standard curve should be -3.32, $R^2 > 0.99$, and the y-intercept should be around 30–35 (C_t). For proper evaluation of PCR efficiency, a minimum of three points (ideally five to seven) in triplicates over 5 to 7 logs (1/10 dilutions) of template concentration is necessary. Otherwise, even when the reaction efficiency is 100%, mathematical manipulation is influenced by standard deviations, and efficiency calculation may result in a value between 70 and 170%. A poor standard curve is usually due to reaction assembly error (including calibration issues) or the presence of inhibitors in the reaction.

Standard deviation (SD): Precision of the real-time PCR values (C_t) are assessed by the standard deviation of the C_t values obtained from replicates. If the SD is >0.250, the power to discriminate between a two-fold dilution is less than 95%.

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