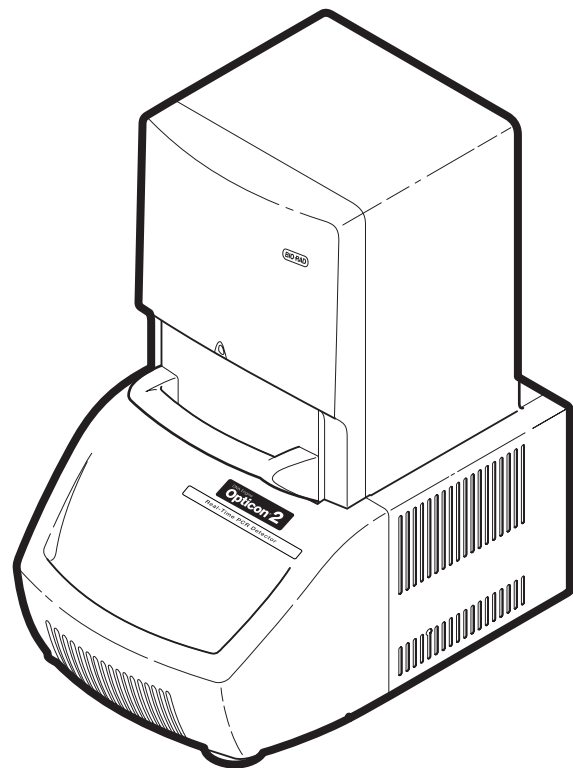# DNA Engine Opticon® 2 System

For Real-Time PCR Detection

Operations Manual

Supports Opticon Monitor™ Version 3.1

CFB-3220   DNA Engine Opticon 2 System

BIO·RAD

DNA Engine Opticon® 2 System

For Real-Time PCR Detection

CFB-3220 Opticon™ 2 system

Operations manual

Supports Opticon Monitor™ Version 3.1

BIO·RAD

Table of Contents

Explanation of Symbols

CAUTION: Risk of Danger! Wherever this symbol appears, always consult note in this manual for further information before proceeding. This symbol identifies components that pose a risk of personal injury or damage to the instrument if improperly handled.

CAUTION: Risk of Electrical Shock! This symbol identifies components that pose a risk of electrical shock if improperly handled.

CAUTION: Hot Surface! This symbol identifies components that pose a risk of personal injury due to excessive heat if improperly handled.

Safety Warnings

Warning: Operating the DNA Engine Opticon 2 system before reading this manual can constitute a personal injury hazard. Only qualified laboratory personnel trained in the safe use of electrical equipment should operate this instrument.

Warning: Do not open or attempt to repair the DNA Engine Opticon 2 tower or base. Doing so will void your warranties and can put you at risk for electrical shock. Return the Opticon 2 system to the factory (US customers) or an authorized distributor (all other customers) if repairs are needed.

Warning: The sample block can become hot enough during the course of normal operation to cause burns or cause liquids to boil explosively. Wear safety goggles or other eye protection at all times during operation.

Warning: The DNA Engine Opticon 2 system incorporates neutral fusing, which means that live power may still be available inside the machines even when a fuse has blown or been removed. Never open the Opticon 2 base: you could receive a serious electrical shock. Opening the base will also void your warranties.

Safe Use Guidelines

The DNA Engine Opticon 2 system is designed to operate safely under the following conditions:

- Indoor use

- Altitude up to 2000 m

- Ambient temperature 15–31°C

- Humidity 10–80%, noncondensing

- Transient overvoltage per Installation Category II, IEC 664

- Pollution degree 2, in accordance with IEC 664

Electromagnetic Interference

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 device has been tested and found to comply with the EMC standards for emissions and susceptibility established by the European Union at time of manufacture.

This digital apparatus does not exceed the Class A limits for radio noise emissions from digital apparatus set out in the Radio Interference Regulations of the Canadian Department of Communications.

LE PRESENT APPAREIL NUMERIQUE N'EMET PAS DE BRUITS RADIOELEC-TRIQUES DEPASSANT LES LIMITES APPLICABLES AUX APPAREILS NUMERIQUES DE CLASS A PRESCRITES DANS LE REGLEMENT SUR LE BROUIL-LAGE RADIOELECTRIQUE EDICTE PAR LE MINISTERE DES COMMUNICATIONS DU CANADA.

FCC Warning

Warning: Changes or modifications to this unit not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.

Note: This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radiofrequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his own expense.

Note Regarding FCC Compliance: Although this design of instrument has been tested and found to comply with Part 15, Subpart B of the FCC Rules for a Class A digital device, please note that this compliance is voluntary, for the instrument qualifies as an "Exempted device" under 47 CFR § 15.103(c), in regard to the cited FCC regulations in effect at the time of manufacture.

This instrument system is labeled, "For Research Use Only".

1 Introduction

1.1 Meet the DNA Engine Opticon® 2 System

Thank you for purchasing a DNA Engine Opticon 2 real-time PCR detection system from Bio-Rad Laboratories, Incorporated. Designed by a team of molecular biologists and engineers, the Opticon™ 2 system will meet your needs for a sensitive, easy-to-use, and compact real-time PCR detection system. Some of the DNA Engine Opticon 2 system's many features include:

• A DNA Engine® Peltier thermal cycler delivers superior thermal accuracy and well-to-well thermal uniformity

• A 96-well sample block accepts standard consumables (low-profile 96-well microplates and low-profile 0.2 ml strip tubes)

• An integrated heated lid permits oil-free cycling

• Long-lived LEDs excite fluorescent dyes in the 470–505 nm range

• Sensitive optics detect fluorophores with emission spectra in the 523–543 nm range (SYBR Green I, FAM) in the first channel, and 540–700 nm in the second channel

• Intuitive Opticon Monitor™ software facilitates experimental setup, run initiation, run monitoring, and data analysis

• Dual modes of temperature control include calculated control, for maximum speed and accuracy, or block control, for adapting protocols optimized in other cyclers

• Compact footprint measuring 34 cm wide x 47 cm deep x 60 cm high, allows the Opticon 2 unit to fit comfortably on any lab bench

• The Opticon 2 detector is available separately or as an upgrade for existing DNA Engine thermal cyclers

1.2 Using This Manual

This manual contains instructions for operating your DNA Engine Opticon 2 system safely and productively:

• Chapter 2 describes the physical characteristics of the Opticon 2 system

• Chapter 3 presents the basics of installing and operating the Opticon 2 system

• Chapter 4 discusses the chemistry and sample-vessel compatibility of the Opticon 2 system

• Chapters 5-8 step you through the use of the Opticon Monitor software including how to enter and run protocols and how to analyze collected data

• Chapter 9 explains the proper maintenance of the Opticon 2 system

• Chapter 10 gives calibration information for the Opticon 2 system

1.3 Important Safety Information

Safe operation of the DNA Engine Opticon 2 system begins with a complete understanding of how the instrument works. Please read this entire manual before attempting to operate the Opticon 2 system. Do not allow anyone who has not read this manual to operate the instrument.

Warning: The DNA Engine Opticon 2 system can generate enough heat to inflict serious burns and could deliver strong electrical shocks if not used according to the instructions in this manual. Please read the safety warnings and guidelines at the beginning of this manual on pages v and vi, as well as the warnings throughout the manual, and exercise all precautions outlined in them.

2 Layout and Specifications

2.1 Instrument Features

Protocol-indicator light

Green trigger handle
(door mechanism)

Hand hold for
opening drawer

Front air vents

Optical tower

Air vents
(also on other side)

Cycler drawer

Figure 2-1. Isometric view of instrument

DAQ (data acquisition)
cable port

Serial cable port

Air vents

Power module

Power switch (fuses)

Power cord jack
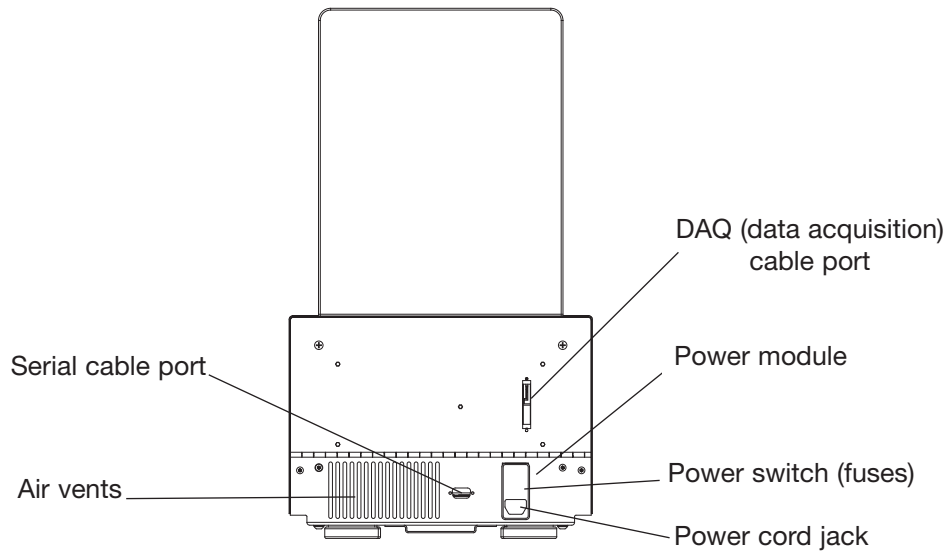
Figure 2-2. Back view of instrument

2.2 Specifications

Thermal range	0° to 105°C, but not more than 30°C below ambient temperature
Thermal accuracy	±0.3°C of programmed target at 90°C, NIST-traceable
Thermal homogeneity	±0.4°C well-to-well within 30 sec of arrival at 90°C
Ramping speed	Up to 3.0°C/sec
Sample capacity	96-well microplate (low-profile) or 96 x 0.2 ml tubes (low-profile)
Line voltage	100–240 VAC*
Frequency	50–60 Hz*
Power	850 W maximum
Fuses	Two 6.3 A, 250 V Type S505, fast acting (user changeable) Two 8.0A, 250 V Type S505, fast acting (inaccessible to the user)
Weight	29 kg (excluding computer and monitor)
Dimensions (W x D x H)	34 x 47 x 60 cm (excluding computer and monitor)
Fluorescence excitation range	470–505 nm
Fluorescence detection ranges	Channel 1: 523–543 nm Channel 2: 540–700 nm

*No adjustment by the user is necessary to use electrical power within these ranges. But all users should employ the proper IEC-320 cord set to connect the instrument to a power outlet, see Chapter 3.

2.2 Gradient Specifications

Accuracy	±0.3°C of target at end columns within 30 sec (NIST-traceable)
Column uniformity	±0.4°C, in column, well–to–well, within 30 sec of target attainment
Calculator accuracy	±0.4°C of actual column temperature (NIST-traceable)
Lowest programmable gradient temperature	30°C
Highest programmable gradient temperature	105°C
Gradient range (temperature differential)	from 1°C up to 24°C

2.3 Computer Specifications

These are the minimum specifications for the computer provided with the Opticon 2 system.

Networking	Ethernet
Operating system	Windows XP Professional
Display	15" flat-panel, resolution 1024 x 768
Memory	256 MB RAM
Storage	40 GB hard drive; 48X CD-RW
Data acquisition board	National Instruments PCI-6036E 200kS/s (samples per second)

Note: Specifications are subject to change without notice.

3 Installation and Operation

3.1 Unpacking the Opticon™ 2 System

Please follow these instructions for unpacking the DNA Engine Opticon® 2 system to reduce the risk of personal injury or damage to the instrument.

⚠️ **Important:** DO NOT lift the instrument out through the top of the box.

Important: DO NOT use the green handle to lift the instrument at any time.

- Cut the band securing the cardboard cover to the support base.

- Open the top of the cardboard cover.

- Remove the top foam insert.

- Remove the accessory box (contents listed below).

- Lift the cardboard cover up and off of the instrument.

- Firmly grasp the sides of the instrument from beneath to support the weight of the cycler and the optical tower. Carefully lift the instrument off of the shipping support. Do not lift the instrument by the green handle or the cycler drawer.

3.1.1 Packing Checklist

After unpacking the Opticon 2 system, verify that you have received the following:

1. One DNA Engine Opticon 2 unit (Opticon 2 detector with DNA Engine® thermal cycler)

2. One computer with keyboard, mouse, monitor, cables, and installed software (Opticon Monitor™ and Windows XP)

   - One serial cable for connecting the Opticon 2 unit's serial port (Figure 2-2) to the computer serial port

   - One data acquisition cable for connecting the Opticon 2 unit's DAQ port (Figure 2-2) to the data acquisition card in the computer

3. One Opticon 2 accessory pack including:

   - One power cord for the Opticon 2 unit

   - Two spare fuses

   - *DNA Engine Opticon® 2 System for Real-Time PCR Detection Operations Manual* (this document)

   - Opticon Monitor software CD ROM

   - Consumables samples including 0.2 ml low-profile strip tubes in opaque white (TLS-0851) and optical flat caps for 0.2 ml tubes and plates (TCS-0803).

If any of these components are missing or damaged, contact Bio-Rad or the authorized distributor from whom you purchased the DNA Engine Opticon 2 system to obtain a replacement. Please save the original packing materials in case you need to return the Opticon 2 system for service. See Appendix A for shipping instructions.

3.2 Setting Up the Opticon 2 System

The Opticon 2 system requires a location with three power outlets to accommodate the Opticon 2 unit, the computer, and the monitor. A location with network access (Ethernet 10/100 BaseT) is recommended if you wish to transfer setup and analysis files between the computer running the Opticon 2 unit and other computers.

The Opticon 2 system requires only minimal assembly. Insert the power cord plug into its jack at the back of the instrument, just below the power switch (see Figure 2-2 for the location of the jack), then plug the power cord into a standard 110 V or 220 V electrical outlet. Both the Opticon 2 unit and the monitor will accept 220 V automatically, but you must set the voltage on the computer. See "Power Supply Requirements", section 3.2.2 for more information.

Before powering up the computer, be sure that the Opticon 2 unit is connected to the computer. There are two cables that connect the Opticon 2 unit to the computer. Connect the serial cable to the serial cable port on the Opticon 2 unit (see Figure 2-2) and serial port #2 on the computer. Connect the data acquisition cable to the DAQ port on the Opticon 2 unit (see Figure 2-2) and the data port on the computer.

Note: The DAQ cable has high-density connectors; take care not to bend any of the pins.

3.2.1 Environmental Requirements

For reasons of safety and performance, ensure that the area where the DNA Engine Opticon 2 system is installed meets the following conditions:

- Indoor use
- Nonexplosive environment
- Normal air pressure (altitude below 2000 m)
- Ambient temperature 15°– 31°C
- Relative humidity above 10% and up to 80%, noncondensing
- Installation Category II (portable equipment)
- Pollution Degree 2 Environment (normally only nonconductive pollution)
- Unobstructed access to air that is 31°C or cooler
- Protection from excessive heat and accidental spills. (Do not place the DNA Engine Opticon 2 system near such heat sources as radiators, and protect it from danger of having water or other fluids splashed on it, which can cause electrical short circuits.)

3.2.2 Power Supply Requirements

The DNA Engine Opticon 2 unit requires 100–240 VAC, 50–60 Hz and a grounded outlet. Supply voltage fluctuations should not exceed ±10%. The Opticon 2 unit can use universal input voltage sources (90–264 VAC) without adjustment, so there is no voltage-setting switch. The monitor can also accept either 110 or 220 V power without adjustment.

⚠️ **Important! For 220 V operation of the computer, the red voltage-setting switch located on the back of the computer, near the power cord jack, must display 230 V rather than 115 V.**

The Opticon 2 unit is equipped with a power-entry module that accepts cord sets with an IEC 60320-1 type C13 connector (this is the same standard configuration used by many computer manufacturers for their equipment). All cord sets used with the Opticon 2 unit must be rated to carry at least 10 A at 125 V or 250 V, the latter specification depending upon the supply voltage used. Additionally, the cord set must meet all other applicable national standards — thus at a minimum, the cord set should carry the mark of a nationally recognized testing agency appropriate to your nation.

Note: Do not cut the supplied 120 V power cord and attach a different connector. Use a one-piece molded connector of the type specified above.

3.2.3 Air Supply Requirements

The DNA Engine Opticon 2 unit requires a constant supply of air that is 31°C or cooler in order to remove heat from the heat sink. Air is taken in from the lower vents located on the sides of the instrument and exhausted from the upper vents on both sides (see Figure 2-1). If the air supply is inadequate or too hot, the instrument can overheat, causing performance problems and even automatic shutdowns.

3.2.3.1 Ensuring an Adequate Air Supply

• Do not block air intake vents (see Figure 2-1).

Position the DNA Engine Opticon 2 unit at least 10 cm from vertical surfaces and other thermal cyclers or heat-generating equipment (greater distances may be required; see below).

• Do not allow dust or debris to collect in the air intake vents.

• Do not place unit on any papers, including bench protectors ("diapers").

3.2.3.2 Ensuring That Air Is Cool Enough

• Do not position two or more Opticon 2 units (or other instruments) so that hot exhaust air blows directly into the air intake vents.

• Confirm that the Opticon 2 unit receives air that is 31°C or cooler by measuring the temperature of air entering the machine through its air intake vents.

Place the Opticon 2 unit where you plan to use it, and turn it on. Try to reproduce what will be typical operating conditions for the instrument in that location, particularly any heat-producing factors (e.g., nearby equipment running, window blinds open, lights on). Run a typical protocol for 30 min to warm up the Opticon 2 unit, then measure the air temperature at the air intake vents. If more than one instrument is involved, measure the air temperature for each.

If the air intake temperature of any instrument is warmer than 31°C, consult Table 3-1 for possible remedies. After implementing possible remedies, verify that the temperature of the air entering the air intake vents has been lowered, using the procedure outlined above.

Table 3-1. Troubleshooting Air Supply Problems

Cause	Possible Remedies
Air circulation is poor.	Provide more space around instrument or adjust room ventilation.
Ambient air temperature is high.	Adjust air conditioning to lower ambient air temperature.
Instrument is in warm part of room.	Move instrument away from, or protect instrument from, such heat sources as radiators, heaters, other equipment, or bright sunlight.
Instruments are crowded.	Arrange machines so that warm exhaust air does not enter intake vents.

3.3 Turning the Opticon 2 System and Computer On

Locate the power switch on the back, left side of the Opticon 2 unit just above the power cord (see Figure 2-2). To turn the Opticon 2 unit on, press the switch so that the side marked "I" is depressed. The thermal cycler requires several minutes to warm up after the instrument is powered up. To turn the Opticon 2 unit off, depress the "0" side of the power switch.

Be sure that the Opticon 2 unit is connected to the computer and turned on before launching Opticon Monitor software. The blue protocol-indicator light on the front of the Opticon 2 unit (see Figure 2-1) is illuminated only during a protocol run.

Press the power button on the front of the computer once to turn the computer on. Select Shutdown from the Start menu to turn the computer off. Press the power button on the front of the monitor once to turn it on, and press it again to turn the monitor off.

3.4 Opening and Closing the Cycler Drawer

To gain access to the Opticon 2 unit's thermal-cycling block, first squeeze the green trigger handle (Figure 3-1, step 1) and allow the spring-loaded door to lift up (step 2). Then, use the hand hold on the drawer front to slide the cycler drawer out toward you, exposing the 96-well sample block (step 3).
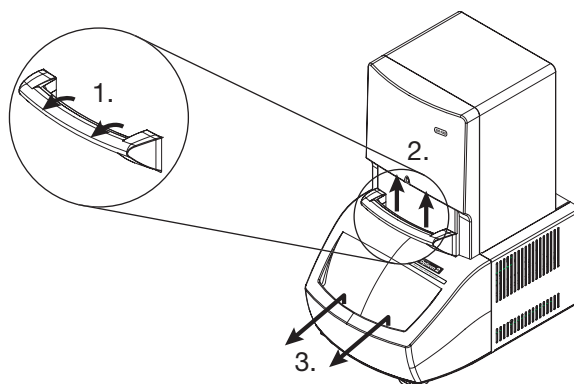
Figure 3-1. Opening the Opticon 2 system

To return the Opticon 2 unit to the closed position, slide the cycler drawer back into the instrument and lower the green handle, firmly pressing down to secure the drawer. It is not necessary to squeeze the trigger handle when closing the instrument.

Note: Do not open the cycler drawer while the blue protocol-indicator light is illuminated. Opening the door, particularly during a scan of the plate, may interrupt the software's control of the protocol.

3.5 Loading Sample Vessels Into the Block

⚠ **Important! Do not use full-height 0.2 ml tubes or full-height unskirted microplates.** Refer to section 4.3, "Selecting the Correct Sample Vessel" and section 4.5. "Sample Vessel and Sealing Selection Chart for Optical Assays" for tube and microplate recommendations.

To ensure uniform heating and cooling of samples, sample vessels must be in complete contact with the block. Adequate contact is ensured by doing the following:

- Ensure that the block is clean before loading samples (see Chapter 9 for cleaning instructions).

- Firmly press strips of 0.2 ml low-profile tubes, or a 96-well low-profile microplate into the block wells (see section 4.3**,** "Selecting the Correct Sample Vessel").

- We strongly recommend that oil not be used to thermally couple sample vessels to the block.

Tip: Spin down reactions in tubes or microplates before loading into the thermal-cycler block. Air bubbles in samples, or liquid on the plate deck, can adversely affect results.

4 Compatible Chemistries, Sample Vessels, and Sealing Options

4.1 Optical System

The Opticon™ 2 system uses an array of 96 LEDs to sequentially illuminate each of the 96 wells in the cycler block. The LEDs efficiently excite fluorescent dyes with excitation spectra in the 470–505 nm range. The Opticon 2 system uses two photomultiplier tubes (PMTs) for fluorescence detection. The first PMT is optimized to detect dyes with emission spectra in the 523–543 nm range, such as SYBR Green I and FAM. The second PMT is optimized for dyes with emission spectra of 540–700 nm.

The Opticon 2 detector is calibrated at the factory and requires no further calibration before use. See Chapter 10 for instructions on how to test the detector's calibration and how to recalibrate, if required.

4.2 Compatible Chemistries

The Opticon 2 detector is compatible with popular dye chemistries including SYBR Green I, molecular beacons, hydrolysis probes (TaqMan probes), Scorpions probes, the Amplifluor system, and LUX fluorogenic primers. In addition to performing real-time quantification and DNA melting profiles, the Opticon 2 system is useful as a temperature-controlled fluorimeter for a number of applications including ligand binding and protein structure studies. If you have questions regarding the compatibility of a particular chemistry with the Opticon 2 detector, contact Bio-Rad technical support at 888-652-9253.

4.2.1 SYBR Green I

SYBR Green I (available from Molecular Probes, Inc. of Eugene, Oregon) is a double-stranded-DNA binding dye. Because the fluorescence of SYBR Green I is greatly enhanced upon binding dsDNA, it is an ideal dye for the detection of amplification products. The maximum absorbance of SYBR Green I is ~497 nm and the emission maximum is ~520 nm.[1]

SYBR Green I offers several advantages for detection of nucleic acids in real time. Because SYBR Green I binds to all dsDNA, it does not have to be customized for individual templates. Therefore, protocols can be adapted quickly and at relatively low cost. Furthermore, because multiple SYBR Green I molecules bind to a single amplification product, SYBR Green I is very sensitive. On the other hand, because SYBR Green I binds to all dsDNA, false positive signals from primer-dimers, secondary structure, or spurious priming can interfere with accurate quantification. Measuring fluorescence at elevated temperatures may help reduce the detection of nonspecific products.[2] Performing a melting curve analysis can help to identify optimal annealing and plate-read temperatures, to avoid generating false positives. Melting curve analysis can also be used to analyze product homogeneity after a run.

[1]Molecular Probes, Inc.

[2]Morrison, T.B., J.J. Weis and C.T. Wittwer. 1998. *Biotechniques* 24:954-962.

Bio-Rad recommends using buffers containing 5% dimethyl sulfoxide (DMSO) with a concentration of 1x or less SYBR Green I with the Opticon 2 detector. For additional information on optimizing protocols using SYBR Green I with thermostable enzymes available from Bio-Rad, contact Bio-Rad technical support at 888-652-9253.

4.2.2 Molecular Beacons

Molecular beacons are dual-labeled oligonucleotide probes designed to form stem-loop structures in the absence of target. In the hairpin configuration, the fluorophore at one end of the molecule is brought into close proximity with a quenching moiety at the other end of the molecule. When the fluorophore is excited in this configuration, it transfers energy to the quencher rather than emitting that energy as light. A "dark" quencher is often used, so the energy transferred from the fluorophore is emitted in the infrared, rather than the visible range. If a second fluorophore is used as a quencher, the transferred energy is emitted as light at the quenching fluorophore's characteristic wavelength.

Molecular beacons are designed such that the loop, which is usually 15–30 nucleotides long, is complementary to the target sequence. The arms flanking the loop, which are usually 5–7 nucleotides long, are designed such that they are complementary to each other, and thus favor formation of a stem structure. A fluorophore is attached to the end of one arm, and a quencher is attached to the other. Molecular beacons must be carefully designed so that, at the annealing temperature of the reaction, hairpins form in the absence of template but annealing of the loop sequence to the target is energetically favorable in the presence of template. When the loop of a molecular beacon hybridizes to the target sequence, the conformational change of the probe separates the fluorophore and the quencher. When the fluorophore is excited, it now emits light at its characteristic wavelength.

One advantage of molecular beacons is that, unlike SYBR Green, molecular beacons specifically detect the target of interest. Great sensitivity, including detection of single nucleotide polymorphisms (SNPs), is possible with carefully designed molecular beacons under optimized reaction conditions (temperature, buffer). The major disadvantage is that each probe must be carefully and uniquely designed for the detection of a specific target.

Molecular beacons are a technology patented by the Public Health Research Institute of New York, NY, and are available from a number of licensed suppliers. When designing molecular beacons for use with the Opticon 2 detector, fluorophores with excitation and emission spectra falling within the Opticon 2 detector's excitation (470–505 nm) and detection (523–543 nm, 540–700 nm) ranges, such as FAM, should be used. Either dark quenchers or a quenching fluorophore may be used with the Opticon 2 detector.

4.2.3 Hydrolysis Probes (TaqMan Probes)

TaqMan probes are a patented technology available from a number of licensed suppliers. They are oligonucleotide probes whose fluorescence is dependent on the amplification of a target sequence. TaqMan probes are designed to anneal to the target sequence between the forward and reverse primer-binding sequences. A reporter fluorophore is attached to the 5' end of the probe and a quencher to the 3' end.

When the intact probe anneals to the target sequence (and when it is not bound), excitation of the reporter is quenched because of its proximity to the 3' quencher. As extension proceeds, the 5' exonuclease activity of the polymerase cleaves the probe, separating the reporter from the quencher. Liberated reporter molecules accumulate as the number of cycles increases, so the increase in fluorescence is proportional to the amount of amplified product.

One advantage of TaqMan probes, particularly for quantification, is that fluorescence is dependent not only on the presence of a specific target, but also on amplification of that target. Like molecular beacons, however, TaqMan probes must be individually designed for specific targets. See section 4.2.2, "Molecular Beacons", for recommendations on the use of specific fluorophores and quenchers with the Opticon 2 detection system.

4.2.4 Scorpions Probes

Scorpions probes (available from licensed suppliers) contain an amplification primer linked, through an intermediary portion containing an amplification stopper, to a target-specific probe. The sequences flanking the probe sequence are complementary to each other, and can form a hairpin structure. This hairpin structure brings a fluorophore and a quencher into close proximity.

During amplification, extension of the target sequence proceeds from the primer portion of the Scorpions probe. During denaturation, the hairpin loop opens. As the reaction cools following denaturation, the target-specific Scorpions probe sequence binds to the amplified, complementary target sequence, so the original hairpin structure does not re-form. Thus the fluorophore and quencher are separated, and fluorescence emission can be detected. The amplification stopper prevents read-through during amplification, which could lead to opening of the hairpin, and thus fluorescence detection, in the absence of target.

Because the Scorpions probe is integrated into the product, there is a direct relationship between the number of targets generated and the amount of fluorescence.

See section 4.2.2, "Molecular Beacons", for recommendations on the use of specific fluorophores and quenchers with the Opticon 2 detection system.

4.2.5 Amplifluor Universal Detection System

The Amplifluor system (available from Intergen Company of Purchase, NY) makes use of a universal primer that emits a fluorescence signal only following incorporation of the primer into an amplification product. The universal primer consists of an 18-base primer tail ("Z sequence") coupled to a hairpin sequence labeled with a fluorophore and a quencher. First, the target is amplified using target-specific primers, one of which has the Z sequence added to its 5' end. In the following round of amplification, the complement to the Z sequence is incorporated into the product. The universal primer then anneals to the complement of the Z sequence and extension proceeds. In the next cycle, extension proceeds through the universal primer, incorporating it into the amplification product. In the process, the hairpin is unfolded, separating the fluorophore and quencher and emitting a fluorescence signal that is proportional to the amount of amplified product.

See section 4.2.2, "Molecular Beacons", for recommendations on the use of specific fluorophores and quenchers with the Opticon 2 detection system.

4.2.6 LUX Fluorogenic Primers

LUX (Light Upon eXtension) fluorogenic primers (available from Invitrogen Corporation, Carlsbad, CA) come as a primer set. One primer is labeled with a single fluorophore, while the other primer is unlabeled. The labeled primer is designed to form a hairpin loop that causes an intrinsic quenching of the fluorophore. When the primer binds to the target and extension begins, the primer is incorporated into the double-stranded PCR product and the fluorescence emitted by the fluorophore increases. These primers are sensitive and target-specific, and allow melting curve analyses to be performed. Although the primers have to be designed specifically for the target of interest, there is no requirement for additional probes, as there is with the TaqMan system.

See section 4.2.2, "Molecular Beacons", for recommendations on the use of specific fluorophores and quenchers with the Opticon 2 detection system.

4.3 Selecting the Correct Sample Vessel

⚠ Important: Do not use full-height 0.2 ml tubes or full-height unskirted microplates. Full-height 0.2 ml tubes and most unskirted microplates do not provide sufficient clearance between the sample block and lid-heater assembly. Do not force the cycler drawer closed.

For proper clearance in the Opticon 2 unit, the distance from the bottom of a tube/plate to the cap rim cannot exceed 17.5 mm. In general, fully skirted 96-well microplates, such as Microseal® and Hard-Shell® microplates from Bio-Rad, provide sufficient clearance when sealed with either domed or flat optical caps (see section 4.5, "Sample Vessel and Sealing Selection Chart for Optical Assays"). If unskirted microplates are used, low-profile plates, such as the MLL-series Multiplate™ microplates from the Bio-Rad line, are required.

Low-profile 0.2 ml strip tubes, such as Bio-Rad TLS-series tubes, are recommended for small numbers of samples. Full-height 0.2 ml tubes do not provide sufficient clearance.

4.3.1 Vessels Optimized for Fluorescence Detection and qPCR

For optimal sensitivity in fluorescence-detection assays, we recommend thin-walled 0.2 ml tube strips and microplates with opaque, white wells. Bio-Rad offers PCR plates and tubes with opaque white wells or clear wells designed for fluorescence detection assays and optimized to ensure a precise fit in the Opticon 2 system's sample block (see section 4.5, "Sample Vessel and Sealing Selection Chart for Optical Assays").

Microplates and tubes with black wells may be useful in applications requiring very low levels of background, but signal strength is significantly reduced when plates and tubes with black wells are used.

Note: In-factory calibration of the Opticon 2 detector is performed with both clear and opaque white microplates.

4.4 Sealing Sample Vessels

Steps must be taken to prevent the evaporation of water from reaction mixtures during PCR, to avoid changing the concentration of reactants. Only a layer of wax, such as Chill-out™ liquid wax, will completely prevent evaporation from sample vessels. Nonetheless, an adequate degree of protection can be achieved by sealing with optical caps and cycling the samples using the heated lid to prevent condensation/refluxing.

4.4.1 Sealing with Optical Caps and the Heated Lid

The heated inner lid maintains the upper part of sample vessels at a higher temperature than the reaction mixture. This prevents condensation of evaporated water vapor onto the vessel walls, so solution concentrations are unchanged by thermal cycling. The heated lid also exerts pressure on the tops of vessels loaded into the sample block. This pressure helps to maintain a vapor-tight seal as well as to firmly seat tubes or microplates in the block, for the most efficient transfer of heat to and from the samples.

Optical caps must be used along with the heated lid to prevent evaporative losses. Ultra-clear, optical cap strips (available from Bio-Rad) provide high light transmission for fluorescence detection and vapor-tight sealing. Tight-fitting caps do the best job of preventing vapor loss.

Note: When tubes are cooled to below-ambient temperatures, a ring of condensation may form in tubes above the liquid level but below the top of the sample block. This is not a cause for concern since it occurs only at the final cool-down step, when the PCR is finished.

4.4.2 Sealing with Chill-out™ Liquid Wax

Clear Chill-out liquid wax (available from Bio-Rad) may be used to seal sample vessels for optical assays. Clear Chill-out liquid wax is the same easy-to-use alternative to oil as the standard, red-colored Chill-out wax, but clear Chill-out wax provides excellent light transmission for optimal performance in real-time PCR. Chill-out liquid wax provides 100% prevention of condensation and vapor loss. At room temperature and above, this overlay is transparent and can be applied by pipette. Chill-out liquid wax solidifies below 10°C. Use only a small amount of Chill-out liquid wax; 1–3 drops (15–50 µl) is usually sufficient. (**Note:** Include this volume in the total volume when setting up a calculated-control protocol). Be sure to use the same amount of wax in all reaction wells, to ensure a uniform thermal profile.

4.5 Sample Vessel and Sealing Selection Chart for Optical Assays

The following reaction vessels and sealing options are recommended for use with the DNA Engine Opticon® 2 system and are available from Bio-Rad. To place an order, call 888-729-2165 or fax 888-729-2166.

Product	Catalog Number	Product Highlights
Vessels		
Low-profile 0.2 ml tubes strips of 8	TLS-0801 (clear) TLS-0851 (opaque white)	• Ideal for small numbers of samples • Use white wells for maximum signal • Use clear wells for uniform signal and for sample viewing
Low-profile Multiplate™ 96-well unskirted microplates	MLL-9601 (clear) MLL-9651 (opaque white)	• Ideal for fewer than 96 samples — microplates can be cut to size • Use white wells for maximum signal • Use clear wells for uniform signal and for sample viewing
Microseal® 96-well skirted microplates	MSP-9601 (clear) MSP-9651 (opaque white)	• Use white wells for maximum signal • Use clear wells for uniform signal and for sample viewing
Hard-Shell® 96-well skirted microplates	HSP-9601 white shell/clear wells HSP-9661 black shell/clear wells HSP-9611 red shell/clear wells HSP-9621 yellow shell/clear wells HSP-9631 blue shell/clear wells HSP-9641 green shell/clear wells HSP-9655 white shell/white wells HSP-9665 black shell/white wells HSP-9615 red shell/white wells HSP-9625 yellow shell/white wells HSP-9635 blue shell/white wells HSP-9645 green shell/white wells	• Ideal for 96 samples • Plate remains absolutely flat during PCR for uniform light collection • Colored shells are good for color coding
Sealing Options		
Optical flat caps strips of 8	TCS-0803	• Ultra-clear for high light transmission • PCR volumes >5 µl
Chill-out™ liquid wax optical grade	CHO-1411	• Replaces mineral oil overlay • High light transmission • PCR volumes >2 µl

4.6 Reaction Volume Recommendations

Reaction volumes of 20–100 µl are recommended for most applications. Nonetheless, it is beneficial to empirically optimize reagent concentrations and sample volumes with the Opticon 2 system, because the sensitivity of the optical system often allows a cost-saving reduction in reagent concentrations. Volumes as low as 10 µl can be used, although sensitivity may be slightly reduced.

The maximum recommended sample volume is 100 µl. Volumes exceeding 100 µl do not maintain adequate contact with the wells of the sample block resulting in nonuniform heating and cooling within the sample.

The reaction volume is used to calculate the temperature of the samples during a calculated-control run (see section 6.4.3, "Choosing Temperature and Lid Control Modes"). Therefore, thermal accuracy is optimized when all samples contain identical volumes.

5 Introduction to Opticon Monitor™ Software

5.1 Introduction

Opticon Monitor software controls all operations on the Opticon 2 system. This chapter will introduce you to Opticon Monitor software and discuss the basics of launching and navigating the software. Chapter 6 describes experimental setup and programming, Chapter 7 discusses run initiation and status, and Chapter 8 focuses on data analysis. This manual documents version 3.1 of the Opticon Monitor software.

5.2 How Opticon Monitor Software Works

Opticon Monitor software is structured such that different types of operations are accessed from different screens. Operations can be categorized into three stages: experimental setup, data acquisition and monitoring, and data analysis.

1. Experimental setup and programming. Setup and programming operations are accessed from the Master File window. The master file consists of a plate file, which specifies the parameters (e.g., well contents) of the plate, and a protocol file, which specifies the experimental conditions (e.g., incubation temperatures and timing of fluorescence measurements). Users can create new files or use existing files, in their current form or after editing.

2. Run initiation and status. After a plate and a protocol setup have been created or selected, a run can be initiated. The user may stop the run at any time and may skip protocol steps, if desired. The Status window appears automatically upon run initiation and can be used to monitor the progress of the run and the ongoing thermal profile of the sample, block, and/or lid. Data collection can be monitored during the run by viewing a plot of fluorescence intensity vs. cycle number.

3. Data analysis. Depending on the type of data being collected (as specified in the master file) data can be analyzed using the Quantitation, Melting Curve, and/or Analysis windows. Starting copy number can be quantified either during or after the run by using the functions in the Quantitation window to set the threshold line for determining C_T values, view and adjust an automatically generated standard curve, and interpolate samples with unknown template concentrations against the curve. If a melting analysis was performed, the melting profile, including fluorescence intensity vs. temperature and/or the negative first derivative (–dI/dt) of fluorescence intensity vs. temperature, can be viewed in the Melting Curve window. Genotyping and Expression analyses are available in the Analysis window.

5.3 Launching and Navigating Opticon Monitor Software

Opticon Monitor software must be installed on the computer before the Opticon 2 system is connected. The Opticon 2 system comes with Opticon Monitor software installed.

To launch Opticon Monitor software, choose *Programs* from the Windows *Start* menu, and then *Opticon Monitor 3.1*. Upon first launching the software, the Opticon Monitor window will display a new master file template (Figure 5-1). On subsequent launches, the most recently viewed master file template will appear. You can also launch Opticon Monitor software by double clicking on an existing Opticon Monitor master (.mast), plate (.plate), protocol (.prot), or data (.tad) file, in which case the chosen file will be displayed.
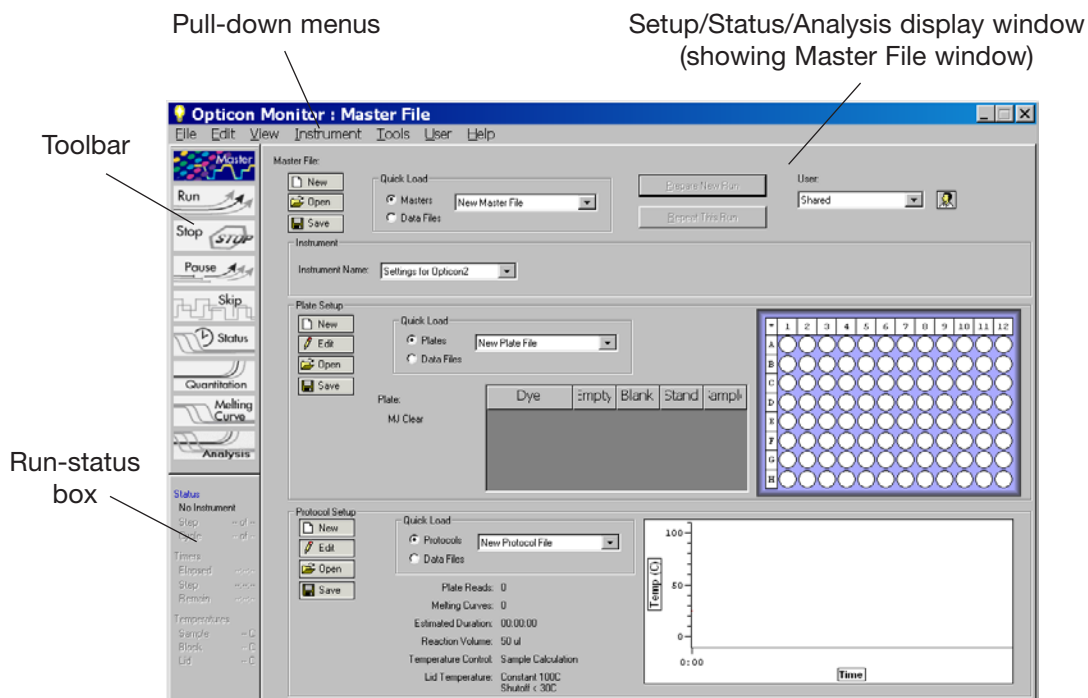
Figure 5-1. Parts of the Opticon Monitor window

5.4 Parts of the Opticon Monitor Window

The Opticon Monitor window includes the following panels:

The pull-down menus provide access to many functions, including exporting data, creating calibration files for specific dyes, and viewing message and error logs. Specific menu items will be described in the following chapters, in conjunction with descriptions of the items' specific functions.

The Setup/Status/Analysis display window displays options and data related to the different types of operations Opticon Monitor performs (i.e., experimental setup, run monitoring, and data analysis).

The toolbar (Figure 5-2) contains buttons that serve as the primary means of controlling a run and navigating between the setup/programming, run/status, and data analysis windows. Only those buttons with a blue background are available at a given time. For example, the Melting Curve button is gray, meaning you cannot navigate to the Melting Curve analysis window, unless a data file that includes melting curve data has been opened.
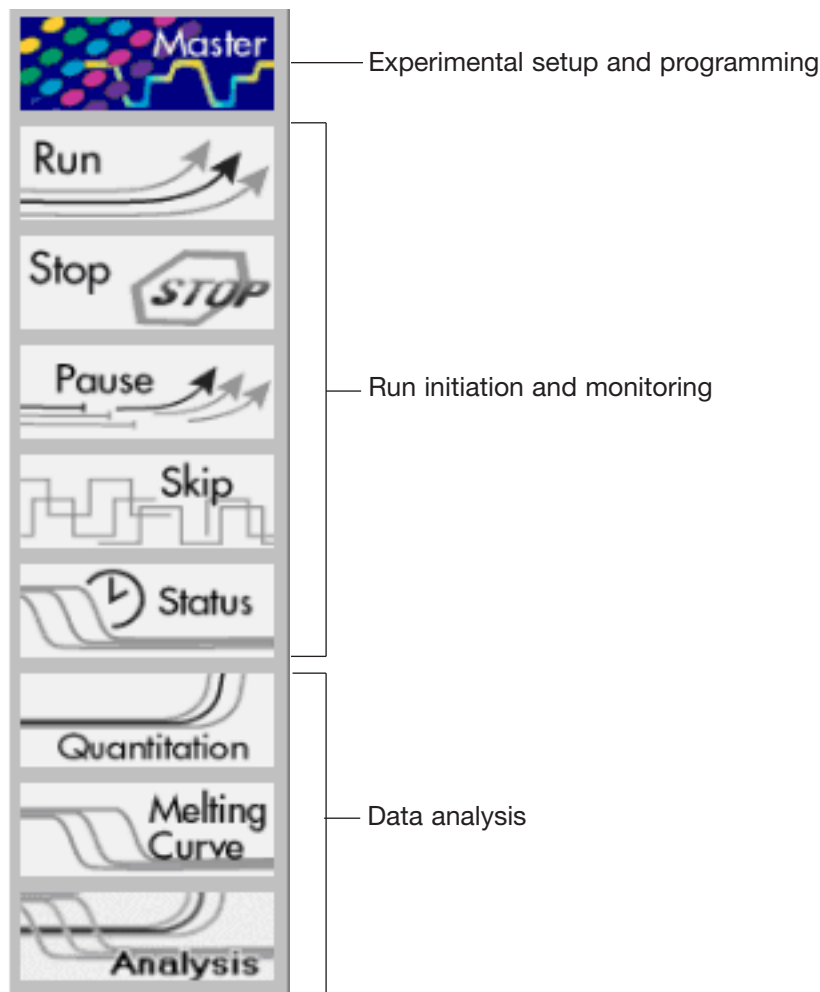
Figure 5-2. The toolbar

The run-status box indicates the current state of the thermal cycler. If no instrument is attached to the computer, the panel reads No Instrument, and other status information is grayed out (see Figure 5-3). If an instrument is connected, the run-status box indicates whether a protocol is running, and gives additional information, as described in Chapter 7.

Figure 5-3. The run-status box

5.5 Exiting Opticon Monitor Software

Exit Opticon Monitor software by selecting *Exit* from the *File* menu, or by clicking the close button in the upper-right corner of the title bar. If a protocol is running, you should stop the protocol before exiting Opticon Monitor software

5.6 Opticon Monitor File Extensions

When saving files, the Opticon Monitor software automatically adds one of the following file extensions:

.mast Master file: Controls a run by specifying which plate and protocol files to apply during the run.

.plate Plate file: Specifies the contents of the 96 wells, any descriptive well labels that were assigned, the calibration files for the plate type and dyes used, and the amounts of any quantitation standards for use in generating a standard curve.

.prot Protocol file: Specifies the order and parameters of protocol steps including temperature incubations, plate reads, temperature gradients, goto steps, and melting curves.

.tad Data file: Contains the fluorescence and temperature data collected during the run, and any selected options and analysis parameters.

5.7 Which Version of Opticon Monitor Software Are You Running?

To determine which version of Opticon Monitor software is currently installed on your computer, choose *About Opticon Monitor* from the *Help* menu. The About window will display the Opticon Monitor version number. This manual documents Opticon Monitor software version 3.1.

5.8 Viewing Logs

To view a record of operations performed by Opticon Monitor software, select *Message log* from the *View* pull-down menu.

To view a list of error messages, select *Error log* from the *View* pull-down menu.

6 Experimental Setup and Programming

Contents continued on following page

6.1 Overview of Experimental Setup and Programming

Before running a protocol on the Opticon 2 system, you must tell the system what each well of the microplate contains, the sequence and duration of temperature incubations to use in the run, and when during the run the detector should collect fluorescence data. These instructions are given in plate and protocol files. The plate file specifies the contents of the wells, any descriptive well labels that the user assigns, the calibration files for the plate type and dyes used, and the quantities of any standards to be used in generating a standard curve. The protocol file specifies the order and parameters of temperature incubations, plate reads, temperature gradients, cycling (goto) steps, and melting procedures to be used in the experimental run. The protocol file also specifies the reaction volume, temperature control method, and heated lid settings.

Plate files and protocol files are created independently of each other. This design allows you to run different saved protocols with the same plate setup, or the same protocol with different saved plate setups, without having to recreate the plate and protocol files for each new combination. The master file links a specific plate file and a specific protocol file for use in the current run.

This chapter describes:

- The parts of the Master File, Plate Setup, and Protocol Setup windows

- How to create new plate and protocol files and how to assign them to a master file

- How to assign existing plate and protocol files, with or without modifications, to a master file

- How to reuse and edit existing master files

6.2 The Master File Window

Upon launching Opticon Monitor software (see Chapter 5), the Opticon Monitor display window will show either a new master file template (first launch), or the last master file used (subsequent launches). All setup and programming operations can be accessed from the Master File window. If another window is showing, click the *Master* button on the toolbar to view the Master File window.
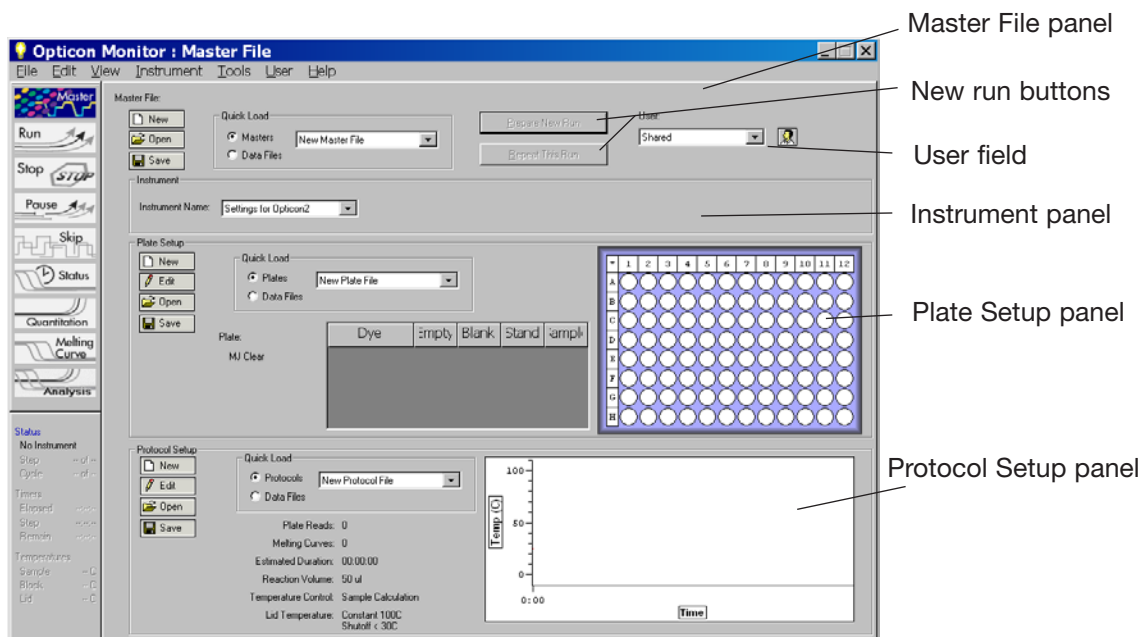
Figure 6-1 The Master File window

If the Master File window is displaying a master file from a previously run experiment, click on the *Prepare New Run* button in the top panel of the Master File window to clear the master-, plate-, and protocol-file templates. If you want to use the same, or a slightly modified plate setup and/or protocol for another run, follow the instructions in the section "Reusing Master Files" at the end of this chapter.

6.2.1 Parts of the Master File Window

The Master File window contains the following panels:

The Master File panel is used to create *New* master files, *Open* previously created files, and *Save* files — by clicking on the appropriate button on the left side of the panel. You can also open a saved master file by selecting it from the pull-down menu in the Quick Load field (see section 6.2.4, "Using the Quick-Load Menus"). The Master File panel also includes buttons for repeating a run or preparing a new run, described above, and a field for managing users, described in the next section.

The Instrument panel is used to select which instrument will be used to run the experiment if more than one instrument is connected to the computer. See section 6.2.3, "Selecting an Instrument" for more information.

The Plate Setup panel is used to assign plate files to the master file. You can create a *New* file, *Open* a previously created file, *Edit* the file, and *Save* the file by clicking on the appropriate button on the left side of the panel. You can also open a file by selecting it from the pull-down menu in the Quick Load field (see section 6.2.4, "Using the Quick Load Menus"). Once a plate file is created or opened, a summary of the contents is displayed in the table and the plate diagram in the Plate Setup panel. See section 6.3.15, "Exiting the Plate Setup" for more information.

The Protocol Setup panel is used to assign protocol files to the master file. You can create a *New* file, *Open* a previously created file, *Edit* the file, and *Save* the file by clicking on the appropriate button on the left side of the panel. You can also open a file by selecting it from the pull-down menu in the Quick Load field (see section 6.2.4, "Using the Quick-Load Menus"). Once a protocol file is created or opened, a summary of the protocol and a graphical representation will appear in the Protocol Setup panel. See section 6.4.8, "Exiting the Protocol Setup" for more information.

6.2.2 Managing Users

The User field in the Master File panel specifies default storage locations for master, plate, protocol, and data files. Adding a user creates a folder with the user's name and creates folders within that folder for saving the four types of files. If no specific user is specified, new files are saved in the Shared folder.

6.2.2.1 Adding New Users

To create a new user folder, click the user icon [icon] in the Master File window.
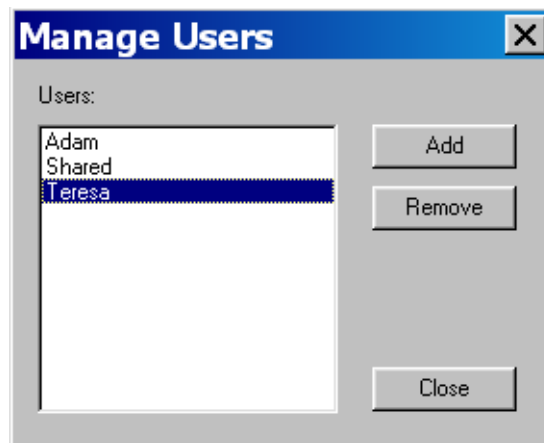
Figure 6-2. Managing users

In the Manage Users window that appears, select *Add*. Enter the new user's name in the New User window that appears and then click *OK*. The new user's name will appear in the Users list.

6.2.2.2 Specifying a User

To select a user, choose a name from the drop-down list in the User field. Alternatively, click on the user icon, highlight a user name in the *Mange Users* window that appears, and then click *OK*. The user's folder will become the default save location for newly saved files.

6.2.2.3 Removing Users

To remove a user from the Opticon Monitor software, select the user's name in the *Manage Users* window and then click *Remove*. You will be asked to confirm deletion of the user, as **all data associated with the user will also be deleted.**

6.2.3 Selecting an Instrument

If more than one instrument is connected to the computer, use the drop-down list in the Instrument panel to select the instrument you wish to run. If the instrument you want to use does not appear on the drop-down list, make sure it is attached to the computer and then select *Scan For Instruments* from the *Instrument* pull-down menu. The instrument will then be listed on the drop-down list.

If you want to setup an experiment on a computer that is not currently attached to an instrument, choose *Settings for [an instrument]* from the drop-down list.

To re-initialize an instrument, for example if it were not properly connected to the computer upon launching Opticon Monitor software, select *Reinitialize Instrument* from the *Instrument* pull-down menu.

If you want the currently selected instrument to be automatically selected upon launching Opticon Monitor software, select *Set Default Instrument* from the *Instrument* pull-down menu.

The setup and analysis options available on other screens depend on what instrument is selected in the *Instrument* panel of the Master File window. This manual will document and illustrate the options available for the Opticon 2 system. When Opticon 2 is selected in the Instruments panel, no more than two dyes may be assigned to a well.

6.2.4 Using the Quick-Load Menus

The Quick-Load menus provide rapid access to any existing plate, protocol, master, or data files.

If the *Masters*, *Plates*, or *Protocols* option is selected in the respective panels, all of the available plate, protocol, or master files (but not data files) that have been saved to the Shared folder or individual user folders are displayed in the drop-down list. The files are listed along with their associated user as shown below.
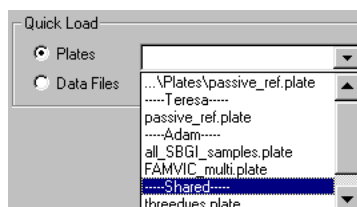
Figure 6-3. Quick-load drop-down menu

Scroll to locate the desired file in the drop-down menu, and select the file.

If the *Data Files* option is selected, all of the data files are listed in the drop-down menu. Selecting a data file will apply the plate, protocol, or master file that was used to generate that data file to the current master file.

6.3 Creating a Plate File

6.3.1 What Is a Plate File?

A plate file describes the experimental microplate. Opticon Monitor software uses the information in this file to interpret the raw data collected by the fluorescence detectors. The plate file specifies: (1) the type of microplate and dyes used in the experiment, so that the software can apply the appropriate calibration files to the data; (2) the "contents" classification (see section 6.3.6, "Specifying Well Contents") of individual wells, which tells the software what wells to use in different types of calculations, such as background subtraction and standard-curve generation; (3) the quantities and units of any standards; and (4) well descriptions to help the user identify the specific reaction components.

6.3.2 Accessing the Plate Setup Window

To create a new plate file, click the *New* button in the Plate Setup panel of the Master File window. Alternatively, you may alter the currently loaded plate file by clicking the *Edit* button. In either case, the screen shown below will appear.
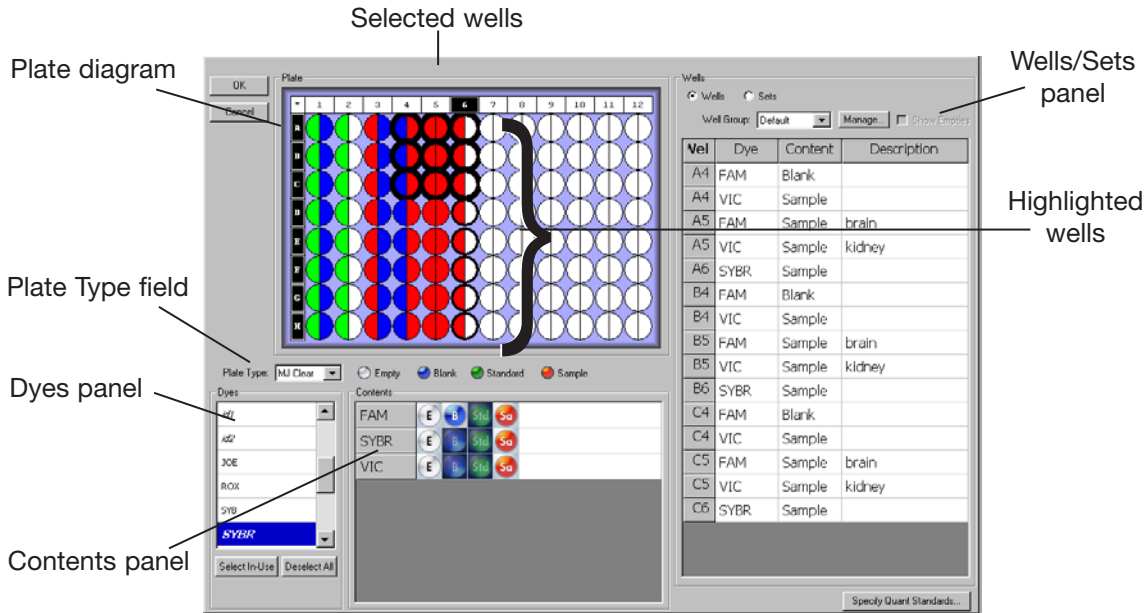
Figure 6-4. Plate Setup window

6.3.3 Parts of the Plate Setup Window

The Plate Setup window includes the following panels; these will be described in more detail in subsequent sections of this chapter:

The Plate diagram is a representation of the 96 wells that may be used in the experiment. The wells in the Plate diagram are color-coded according to the contents specified for each dye: white for empty, blue for blank, green for standards, red for sample, and orange for passive reference (see section 6.3.6, "Specifying Well Contents" and section 6.3.7, "Viewing Well Contents in the Plate Diagram"). A legend indicating which color corresponds to each type of reaction appears below the plate diagram. Note that the legend for passive reference only appears if this option is selected from the *Plate* pull-down menu.

The Plate Type field is used to indicate the type and well color (white or clear) of the vessels that will be used in the experiment.

The Dyes panel, in the lower left corner of the window, is used to select dye(s) to display in the Wells panel. All calibrated dyes are listed in this panel.

The Contents panel is used to assign contents to wells selected in the Plate diagram and to enter the descriptions that appear in the Wells panel. A dye must be listed in the Contents panel for the user to identify which wells contain that dye and what type of reaction contents have been specified for it.

The right-hand display panel displays either the Wells panel or the Sets panel, depending on which radio button is selected at the top of the panel. When *Wells* is selected, the panel displays a table listing the contents and descriptions of selected wells/dyes. Wells can also be assigned to Well groups in the Wells panel. When *Sets* is selected, buttons for creating and editing well sets appear, and the panel displays a table listing all sets that have been defined. Wells or sets can be sorted by clicking on the column headers in the table.

6.3.4 Beginning Plate Setup — Specifying the Plate Type and Dyes

The Opticon 2 system must be calibrated for both the type of reaction vessel and the dyes that will be used in an experiment. The system is precalibrated with settings for plates (or tubes) and many popular dye chemistries. Plate calibration files exist for white or clear wells. Dye calibration files exist for FAM, SYBR Green I, HEX, TAMRA, TET, and VIC dyes.

To begin a new plate setup, first select the type of vessel used from the drop-down list in the Plate Type field.

Next, select the dye(s) that will be used in the experiment, using the Dyes panel. Selecting a dye lists the dye in the Contents panel, allowing you to specify well contents for the dye. The content classifications and descriptions assigned to selected dyes in selected wells are listed in the Wells panel.

If you no longer want a dye to appear in the Contents and Wells panels, click on the name of the dye in the Dyes panel. If that dye has already been assigned to some wells, its name will be emboldened in the Dyes panel. To remove all dyes from the Contents and Wells panels, click on the *Deselect All* button at the bottom of the Dyes panel. Click the *Select In Use* button to list in the Contents and Wells panels all dyes that have been assigned to wells.

Note that only two dyes can be used in a given well. If you assign more than two dyes to a single well, you will not be able to exit the Plate Setup window unless you correct the error. On the other hand, many dyes can be used in a single plate, for example by using FAM and VIC in column 1 and SYBR Green and HEX in column 2.

Also be aware that although the software allows you to combine any two dyes in a well, it is difficult to discriminate signals from dyes with largely overlapping emission spectra. You should consider the spectra of dyes when designing your experiment.

6.3.4.1 Using Uncalibrated Dyes

Selecting a dye from the Dyes panel tells the software which dye-calibration files to apply to the fluorescence measurements. If the desired calibration file is not present for the dye/plate combination being used, it may be possible to use the setting for one of the precalibrated dyes, if one has an emission spectrum similar to that of the dye you want to use. Of course, any dye used must be excited at wavelengths between 470–505 nm. Please contact Bio-Rad if you would like calibration recommendations when using non-precalibrated dyes.

If it is not possible to use one of the precalibrated settings, it will be necessary to perform a calibration by selecting *Dye Calibration* from the *Tools* pull-down menu. Follow the directions in the Dye Calibration Wizard to create a calibration file(s) for the dye(s) being used (see Chapter 10).

6.3.5 Selecting and Deselecting Wells

To select wells, either for specifying contents for an experiment or, later, to view the data for a specific well, use the Plate diagram.

Move the cursor over an individual well, row letter, or column number to highlight the well or wells with a thin outline and darken the corresponding well coordinates (see wells in column 6 in Figure 6-4). Clicking on a highlighted well will select it. Selected wells appear heavily outlined (see wells A4 through C6 in Figure 6-4).

Select an individual well by clicking on the well.

Select a different well by clicking on that well. Previously selected wells will be deselected.

Select multiple wells by holding down the left mouse button and dragging the cursor over the wells to be selected, or hold down the control key and click on the individual wells you wish to select.

Select all wells in a column by clicking on the numbered column header. Select multiple columns by holding down the control key while clicking on the numbered column headers.

Select all wells in a row by clicking on the lettered box at the start of the row. Select multiple rows by holding down the control key while clicking on the lettered boxes.

Select all wells in the plate by clicking on the asterisk in the upper-left corner of the plate diagram.

Deselect a single one of several selected wells by holding down the control key while clicking on the well.

Deselect all wells by clicking on any selected well.

6.3.6 Specifying Well Contents

Well "contents" tell the software what type of reaction each dye will indicate in each well. The software uses this information when performing calculations, to identify which wells provide the fluorescence data needed for the calculations. The different content classifications and their uses are:

- Empty: Nothing has been added to the well. Fluorescence measurements are not used. Undefined wells are considered empty.

- Blank: The well contains an incomplete set of reactants (e.g., buffer only). Fluorescence intensity measurements from blank wells can later be subtracted from measurements taken from other wells.

- Quantitation Standard: The well contains a user-specified standard of known quantity (see section 6.3.13, "Specifying Quantitation Standards"). Fluorescence intensity readings from all quantitation standards are automatically used to plot a standard curve of log quantity vs. cycle number.

- Sample: The well contains an experimental sample. If quantitation standards are also used, the quantities of samples are automatically calculated by interpolating from the standards.

- Passive Reference: The dye is present in the well as a reference, to compensate for variability in concentration or volume of the samples. The fluorescence measured for the other dye in the well will automatically be divided by the fluorescence measured for the passive-reference dye. This designation is only available if *Passive Reference* has been selected from the *Plate* pull-down menu (see section 6.3.8, "Using Passive References").

The defined contents for a well are dye-specific, so the contents of a well can be defined as a sample reaction using one dye and a blank reaction for another dye.

To specify contents:

1. Select the dye(s) for which you want to specify contents using the Dyes panel. The selected dye(s) will appear in the Contents panel adjacent to the Dyes panel. You may find it easiest to specify contents for all wells containing one dye, and then deselect that dye before selecting and specifying contents for another dye.

2. Select the well or group of wells for which contents are to be specified, using one of the methods described in the section "Selecting and Deselecting Wells".

3. Indicate the type of reaction each dye is used for in the selected wells by clicking on one of the buttons to the right of the dye's name in the Contents panel: *E* for empty, *B* for blank, *St* for quantitation standard, *Sa* for sample, or *PR* for passive reference (if this option has been selected in the *Plate* pull-down menu, see section 6.3.8, "Using Passive References").

If you decide you no longer want the fluorescence data from a particular well to be used in the calculations for which it is specified, you can return to the Plate Setup window, select the well, and redefine its contents. This can be done even after the experiment has been run.

## 6.3.7 Viewing Well Contents in the Plate Diagram

Once the dyes in a set of wells has been designated for use as blanks, standards, samples, or reference, the corresponding wells in the Plate diagram will be color coded according to the contents: white for empty, blue for blank, green for standards, red for sample, and orange for passive reference. A legend appears below the Plate diagram.

Each well in the diagram is partitioned to show the contents assigned to different dyes in that well (see Figure 6-5). A dye must be selected in the Dyes panel before its contents will be displayed in the Plate diagram. The number of partitions is determined by either the number of dyes selected (in the default view) or the number of dyes the well can hold (if *Fixed Well Sections* is selected from the *Plate* pull-down menu). Note that if you change the display mode in the Plate Setup window, the plate diagram in the Plate Setup panel of the Master File window will change accordingly.

6.3.7.1 The Default Plate-Diagram Display

In the default Plate view, each well in the Plate diagram is partitioned into the same number of sections as there are dyes selected in the Dyes panel. For example, if a single dye is selected from the Dyes panel, the wells in the Plate diagram will not be partitioned and will show only contents assigned to the selected dye; if three dyes are selected in the Dyes panel, each well in the Plate diagram will be divided into thirds, as shown in Figure 6-5A. When multiple dyes are selected, the content classification for each dye is represented in the same section of each well, e.g., FAM in the top left section, SYBR in the top right, and VIC in the lower section. The dyes are displayed alphabetically, moving clockwise around the circle. Note that since a maximum of two dyes may be assigned to a given well in the Opticon 2 system, if three dyes are selected, one dye in each well must be designated as "Empty".

A. Default Plate diagram
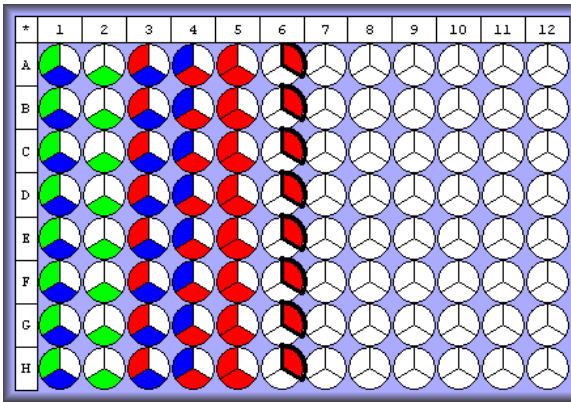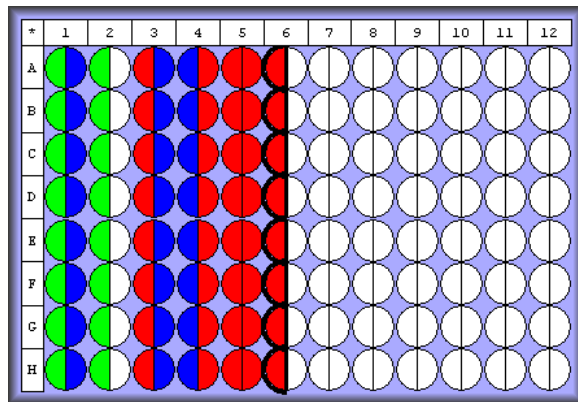
B. Fixed Well Sections

Figure 6-5. The two display modes for the Plate diagram. For both plates, FAM and VIC dyes were assigned to columns 1–5 and SYBR Green was assigned to column 6. The contents for SYBR Green are highlighted in both displays. Remember that the color represents the content classification, not the dye.

6.3.7.2 Fixed Well Sections

Because the default display option partitions wells into the number of selected dyes, the display can become complicated if many dyes are viewed at once. In these cases, it may be easier to use the Fixed-Well-Sections display option.

When *Fixed Well Sections* is selected from the *Plate* pull-down menu, the wells of the plate diagram are partitioned into sections corresponding to the maximum number of dyes that can be added to the well — two for the Opticon 2 system, four for the Chromo4™ system.

If only one dye is assigned to a well in the Opticon 2 system, its content classification is displayed in the left half of the circle when *Fixed Well Sections* is selected. If two dyes are assigned to a well, they are ordered alphabetically, e.g., FAM in the left half, VIC in the right half. Therefore, if a dye is used in singleplex reactions in some wells and multiplex reactions in other wells, its contents may be represented in different halves of different wells. For example, in the plate diagram in Figure 6-5B, the contents for VIC are displayed in the right half of wells in column 1 and in the left half of the wells in column 2 (compare to the plate diagram on the left, in which the contents for VIC are always displayed in the bottom section).

6.3.7.3 Highlighting Contents for a Dye

To view the content classifications for a single dye, first click *Deselect All* at the bottom of the Dyes panel, and then select the dye whose contents you wish to view. Click on the dye again to deselect it.

You can also highlight the wells containing a specific dye when multiple dyes are selected. To do so, position the cursor over the name of the dye in the Contents panel: the well sections corresponding to that dye will be highlighted with a dark line in the Plate diagram (see column 6 in Figure 6-5 A and B).

6.3.8 Using Passive References

If you wish to use a passive reference to standardize fluorescence across wells, select *Passive References* from the *Plate* pull-down menu. The orange circle for passive reference will then appear in the plate-diagram legend, and *PR* will be added to the buttons in the Contents panel. Any dye can be used as a passive reference, but only one dye can serve as a passive reference in a given well.

When a dye is designated as a passive reference in a well, the fluorescence measured for the other dye in that well is automatically divided by the fluorescence measured for the passive-reference dye. If, after you run the experiment, you decide you don't want this calculation to be made, return to the Plate Setup window and reassign the well contents for that dye. Make sure you select all wells that contain the passive reference and change the contents to E (Empty — see section 6.3.6, "Specifying Well Contents").

6.3.9 Assigning Well Descriptions

To aid in sample identification, you can enter additional descriptions for individual wells or groups of wells, e.g., the target or the template source. This may be done most easily while you are specifying contents, but it can be done at any time.

Begin by selecting well(s) for which you want to enter descriptions, using the Plate diagram. Next, select the dyes for which you want to enter descriptions, using the Dyes panel. Finally, click on the white rectangular field in the line bearing the dye's name in the Contents panel, and type a description. The well label will be applied to the selected well or wells and appear in the Description column in the table in the Wells panel (see next section).

Alternatively, you can enter a description by clicking on an individual cell in the Description column of the table in the Wells panel and typing directly into the cell. You can also copy and paste a well description from one Description field in the table to another by using control+c to copy and control+v to paste. Finally, you can copy descriptions from an Excel file. When doing this, be careful to copy the labels into the correct column of the Wells information table.

6.3.10 Viewing Well Information in the Wells Panel

The panel on the right side of the Plate Setup window displays either the Wells panel or the Sets panel, depending on which radio button has been selected at the top of the panel. To view the information entered for selected dyes in selected wells, select *Wells*. The well-information table will then be displayed (see Figure 6-4).

To list well information in the table, you must first select well(s) in the Plate diagram and also select dye(s) in the Dyes panel. The columns in the well-information table list the Well coordinates, which of the selected Dye(s) have been assigned to each well, the Contents assigned to each selected dye, and any Description that has been entered. To list dyes with contents classified as "Empty", select *Show Empties* above the Wells table. This option is not available if *Fixed Well Sections* is selected: dyes assigned the "Empty" classification are not listed when *Fixed Well Sections* is selected.

The listing of wells in the plate information table can be sorted by *Well*, *Dye*, *Content*, or *Description* by clicking on the appropriate column heading.

The well-information table can also be used to enter additional descriptions of a well's contents. See the previous section for more information.

6.3.11 Defining Well Groups

The Well Groups function adds an extra level of organization to a plate, to simplify viewing of experiments that include many reaction types and to allow independent analysis of multiple experiments in the same plate.

Defining well groups is especially useful if two experiments with different quantitation standards are run simultaneously. By default, Opticon Monitor software uses all wells classified as standards to generate a standard curve. If the Manage Well Groups function is used to define groups of wells, then when one of those groups is selected, the Standards graph will include only the standards present in that particular group.

You can create, manage, and select well groups from the Plate Setup, Quantitation, Melting Curve, and/or Analysis windows. To define well groups from the Plate Setup window, select *Wells* at the top of the right-hand panel, and then click on the *Manage* button at the top of the Wells panel. The Well Groups Manager window, displaying a list of any defined groups and a plate diagram, will open.
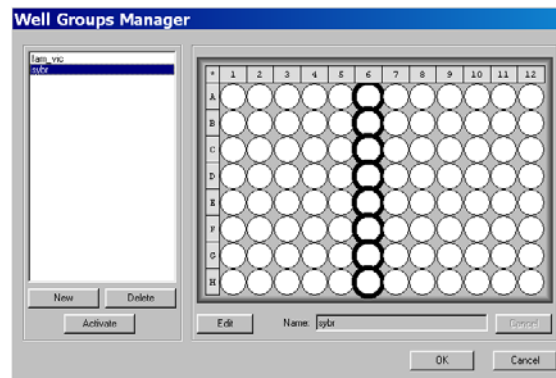
Figure 6-6. The Well Group Manager window
with one group selected

To create a new group, click the *New* button at the bottom, left corner of the window. Select wells to include in the group using the plate diagram, enter a name in the Name field, and then click *Apply*. The newly defined group will appear in the list on the left side of the window.

You may define additional groups by clicking *Add New*. The same well may be added to multiple groups.

To edit an existing group, highlight its name in the list in the Well Groups Manager window, click *Edit*, and proceed as for new groups. To delete a group, highlight its name and click *Delete*.

To close the Well Groups Manager window, click *OK* or highlight one well group and click *Activate*. If you choose *Activate,* all the wells that are not in the activated group are not selectable — they are gray with an X through them — in the Plate diagram in the Opticon Monitor window. If you choose *OK*, the Opticon Monitor window will display the entire microplate (or whatever group was displayed when the Manager window was opened).
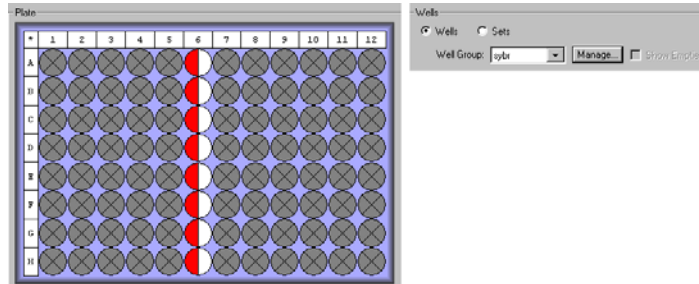

Figure 6-7. Plate diagram with one group activated

To activate a different group, you may either re-select *Manage* and proceed as above, or simply select a defined group from the Well-group drop-down menu in the Wells panel. To display the entire microplate, choose *default* from the drop-down menu.

6.3.12 Creating Well Sets

If sets of replicate wells are defined in the Plate Setup window, Opticon Monitor software will calculate the mean and standard deviation of the fluorescence measured at each cycle in each of the wells in the set. You can then plot the mean and standard deviation in the Graphs panel of the Quantitation window, and you can view calculations based on the mean, maximum, and minimum C_T values (see Chapter 8). In addition, defining well sets, specifically a calibrator set, is required for relative expression analyses (discussed in the next section and in Chapter 8).

To create a well set, first select the *Sets* radio button at the top of the right-hand panel. This displays the Sets panel.
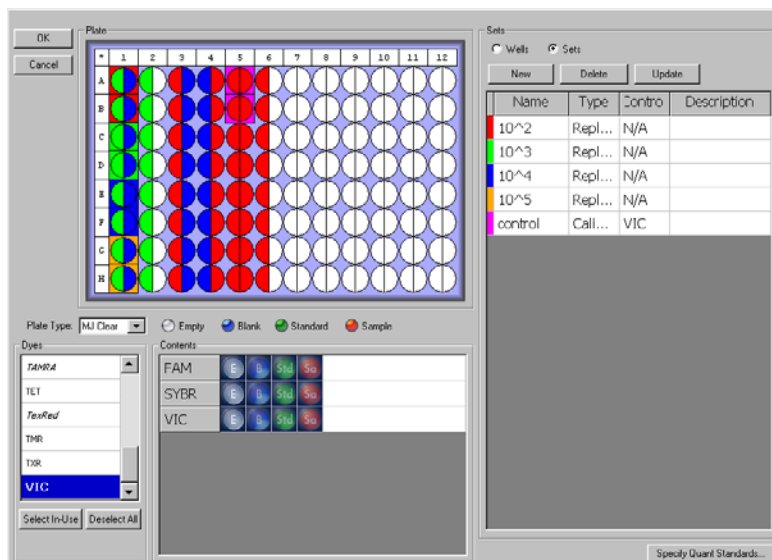

Figure 6-8. Plate Setup window displaying Sets panel

To add a new set, click *New*. You may select wells to include in the set either before or after clicking *New*, using the plate diagram. When you click *New*, a new row with the name New Set will be added to the Sets table in the Sets panel. The bar to the left of the New Set row in the Sets table will be given a new color, and a box of the same color will be drawn around that set's wells in the Plate diagram (if wells were selected before clicking the *New* button). Please note the following:

• You may create well sets before or after assigning contents to the wells, but the same content must be assigned to every well in the set.

• If you select wells after creating the set, you must click *Update* to assign the new wells to the set.

• If you change the content assignment of wells after a set has been created, you must click *Update* to accept the changes.

• Well sets are not dye-specific. You do not have to create sets independently for each dye.

To name a set, click in the Name field that says *New Set*, highlight the words, and type in a new name. If you don't change the name, the next new set will be named "New Set 2". You can further define the set by clicking in the Description field and typing a description of the set.

By default, a new set is assigned the type "Replicate". You can change the type to Calibrator by clicking in the Type field and choosing *Calibrator* from the drop-down menu. Calibrators are used in expression analyses using the ^3C_T and $^{33}C_T$ methods (see Chapter 8). When you specify a set as a calibrator, you can select which dye is used for indicating the internal control by selecting the dye from the drop-down menu in the Control column. The internal-control dye is used only in $^{33}C_T$ calculations.

To edit a well set, highlight the row containing the set's name in the Sets table, and then select wells to include in the set using the Plate diagram. Remember to hold down the control key while selecting new wells if you want to keep the previously selected wells in the set. Click *Update* to accept the changes to the set.

To delete a well set, highlight the set's row in the Sets table, then click *Remove*.

6.3.13 Specifying Quantitation Standards

If you are using quantitation standards, after you have designated which wells contain the standards, click the *Specify Quant Standards* button in the bottom, right corner of the Plate Setup window. A pop-up window will appear listing all of the wells to which quantitation standards have been assigned, and indicating which dye(s) in that well have reactions using standards. You can sort the items by *Well*, *Dye*, or *Amount* (once amounts have been entered) by clicking on the appropriate column heading.
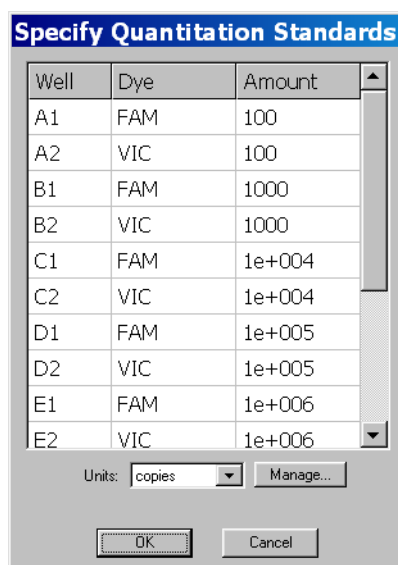

Figure 6-9. The Specify Quantitation Standards window

Enter the amount of each standard in the Amount fields. Alternatively, you can cut and paste multiple cells from an Excel worksheet. Numbers may be entered as integers or using scientific notation (e.g., 1e+004). Note that numbers greater than 1,000 and numbers entered in the form 1E4 will be converted to the form 1e+004.

After entering quantities, specify the Units of the standards by choosing *ng*, *pg*, *moles*, *molecules*, *ge* (genome equivalents), or *copies* from the drop-down menu. You can define additional units by selecting the *Manage* button.

Select *OK* to apply the Standard quantities to the plate file, or click *Cancel* to undo any changes to the Standard quantities. A standard curve will automatically be generated during data analysis, using the values supplied.

To define additional units, first click the *Manage* button in the Specify Quantitation Standards pop-up window. Next, select *Add* in the Manage Standards window that appears, and then type the desired unit name in the Add Quantitation Standard Name window. To remove unit designations, highlight the designation in the Standards list in the Manage Standards window and click the *Remove* button.

6.3.14 Pasting Plate Information From Microsoft Excel

If on a previous run you exported a plate file to Microsoft Excel (see section 8.6.1, "Exporting Data"), you may paste the plate information from the Excel file into the Opticon Monitor plate file. First, open the Excel file and select the type of plate information you want to paste (Well Name, Dye, Contents, Description, and/or Std [Standard] Quantity). Include the column headings to have these automatically applied in the Opticon Monitor software. Copy the selected cells to the clipboard (Control-c). Next, return to the Opticon Monitor screen and, in the Plate Setup window, select *Paste from Excel* from the *Edit* menu. The following window will appear:
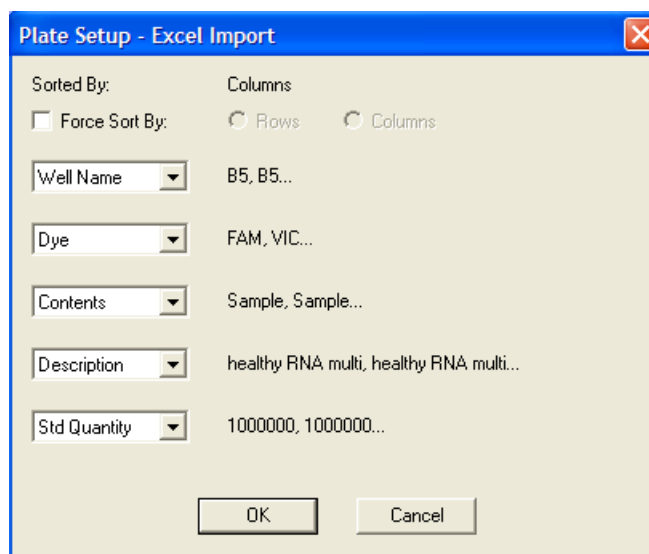

Figure 6-10. Pasting plate data from Excel

If you copied the column headings specifying the type of plate information, these headings will automatically appear in the drop-down menus in the Plate Setup – Excel Import dialog box. If the headings were not selected, or if you want to reclassify the data, select the type of plate information from the drop-down menu. Note that the data in each column must match the format for the different plate information types (Well Name, Dye, Contents, Description, or Std Quantity). Click *OK* to enter the plate information into the plate information table. Note that other plate parameters, such as plate type, are not specified by the *Paste from Excel* command.

6.3.15 Exiting the Plate Setup

Once you have finished entering plate file parameters, click the *OK* button in the upper, left corner of the Plate Setup window to return to the Master File window. Alternatively, if you wish to discard the plate file information and return to the Master File window, click *Cancel*.

When you click *OK*, a summary diagram and table of the newly assigned plate contents will appear in the Plate Setup panel of the Master File window. The plate diagram will use the same display (default or fixed-well-sections) as was last used in the Plate Setup window. The table will list all the dyes that have been assigned to wells and the number of wells assigned each content type (empty, blank, standard, sample). To highlight in the plate diagram the wells that contain a particular dye, position the cursor over the name of the dye in the table. To highlight only the wells that are assigned a particular content for a particular dye, place the cursor over the appropriate cell in the table.
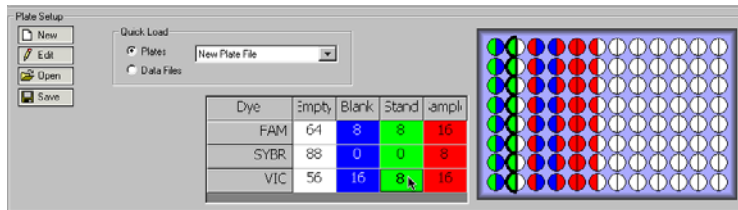

Figure 6-11. Plate Setup summary in Master File window.
VIC standards are highlighted.

6.3.16 Saving a Plate File

To save the newly created plate file, click the *Save* button in the Plate Setup panel of the Master File window. Enter an appropriate name in the File name field of the save window. Click the *Save* button to save the .plate file.

6.4 Creating a Protocol File

The protocol file contains the thermal-cycling parameters for an experimental run, and specifies when during the run the Opticon 2 detector will measure fluorescence. Protocol steps are entered and edited in the Protocol Setup window. A listing and graphical representation of the protocol are displayed for easy review.

Click the *New* button in the Protocol Setup panel of the Master File window to create a new protocol file.
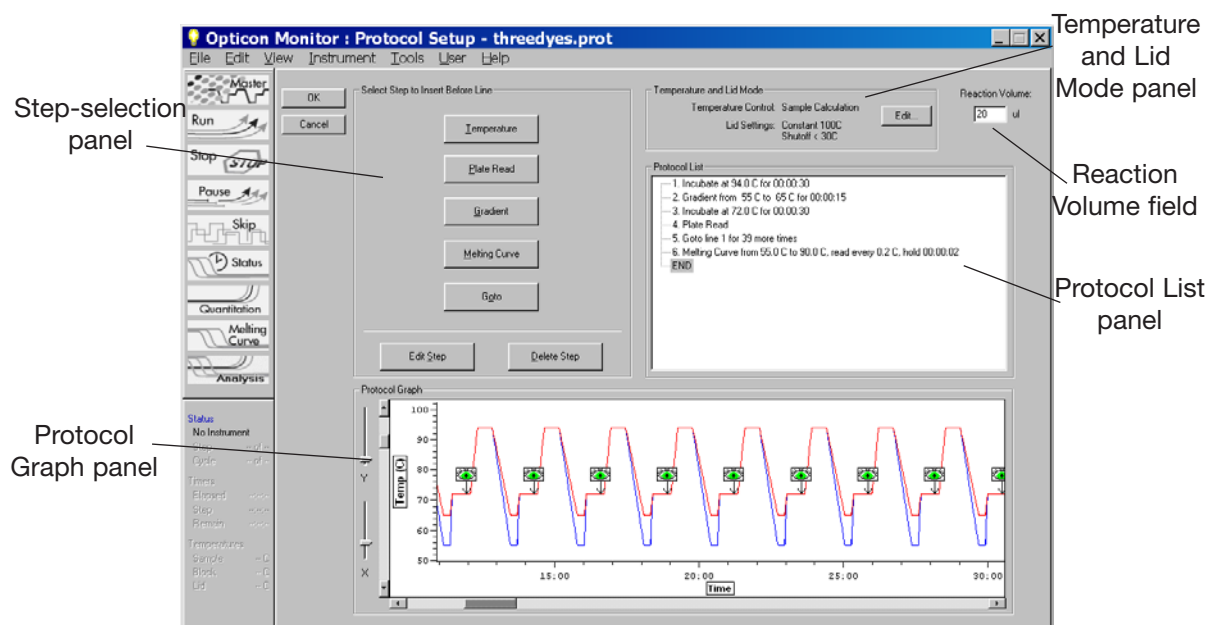

Figure 6-12. The Protocol Setup window

6.4.1 Parts of the Protocol Setup Window

The Protocol Setup window is made up of the following panels, which will be described in more detail in subsequent sections of this chapter:

The step-selection ("Select Step to Insert Before Line") panel is used to add or edit different kinds of steps to the protocol. The long name is intended to remind you that new steps are inserted before the highlighted step in the protocol list.

The Temperature and Lid Mode panel is used to select options for controlling the temperature of the sample holder and for heating the lid.

The Reaction Volume field is for entering the volume of the wells, for use in temperature-control calculations.

The Protocol List panel displays the protocol steps in numbered order.

The Protocol Graph panel displays a graphical illustration of the protocol, showing temperature increases and decreases as well as plate reads. Each cycle of the protocol is represented.

6.4.2 Reaction Volume

For the Opticon Monitor software to accurately calculate and control the sample temperature, the user must enter the volume of reactions (in μl, including the volume of any oil or wax overlay) in the Reaction Volume field in the upper, right-hand corner of the Protocol Setup window. See section 4.6, "Reaction Volume Recommendations" for additional information.

6.4.3 Choosing Temperature and Lid Control Modes

Click the *Edit* button in the Temperature and Lid Mode panel to display the Protocol Options window, and to specify the methods of temperature and lid control to be used in the run.
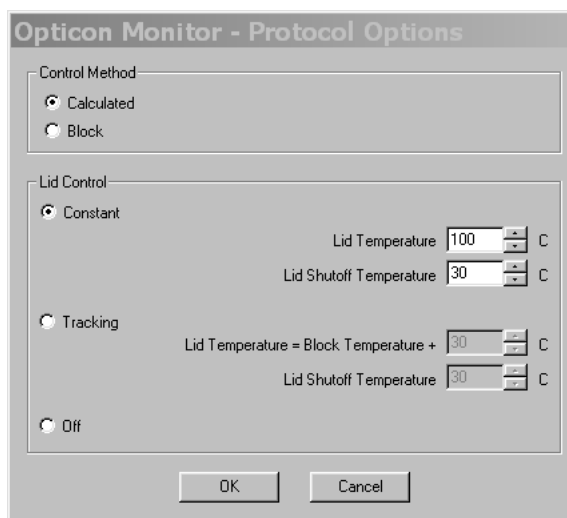

Figure 6-13. The Protocol Options window for choosing temperature and lid control modes

6.4.3.1 Temperature Control Modes

The Opticon 2 system can control block temperature in two different ways. Each method has implications for the speed and accuracy of sample heating.

1. *Calculated* control is the default method of temperature control. It is the preferred method for most protocols, yielding consistent, reliable, and fast programs.

    With calculated control, the user-entered incubation periods specify how long the samples, rather than the block, should remain at the target temperature. The system estimates sample temperatures based on the block's thermal profile, the rate of heat transfer through the sample tube, and the sample volume. Brief, precise block-temperature overshoots are used to bring samples to the correct temperature rapidly. Since this estimate is based on known quantities and the laws of thermodynamics, sample temperatures are controlled much more accurately than with block temperature control.

Because the samples are held at the target temperature for the user-specified duration when using calculated control, these durations can be shortened significantly compared to when using block control. Typically, optimal hold times for denaturation steps range from 5–30 seconds when using the calculated control method. Annealing and extension steps can also be shortened, but the periods for these will be reaction-specific. In addition to offering the simple convenience of spending less time running reactions, shorter protocols minimize false priming and help to preserve enzyme activity.

Calculated control also makes transferring protocols easier, because the instrument automatically compensates for vessel type and reaction volume.

2. When *Block* control is selected, the user-entered incubation periods specify how long the block should remain at the target temperature, without regard to sample temperature. Under block control, the temperature of samples always lags behind the temperature of the block, and that lag varies with sample volume and vessel type (typical lag time is 10–30 seconds). Therefore, the sample temperature is less precisely controlled in block-control mode than in calculated-control mode. Block control is used chiefly to run protocols developed on thermal cyclers that do not have the calculated-control option.

6.4.3.2 Lid Control Modes

When a sample is heated, condensation can form on the tube cap or the plate sealer. This changes the volume of the sample, the concentration of components, and thus the kinetics of the enzymatic reaction. Use of a heated lid minimizes condensation by keeping the upper surface of the reaction vessel at a temperature slightly greater than that of the sample itself.

The Opticon 2 system provides three options for controlling lid temperature: *Constant*, *Tracking*, or *Off*.

• The Constant method keeps the inner lid at a specified temperature (°C). This is the default method of control. To use constant lid-temperature control, select Constant and enter a Lid Temperature between 30°C and 110°C or use the arrows to scroll to the desired temperature. For low-profile tubes, a constant temperature of up to 5°C above the highest temperature in the protocol is recommended. You can also specify a sample-block temperature below which the heated lid will turn off. Enter a Lid Shutoff Temperature between 1°C and 50°C or use the arrows to scroll to the desired temperature.

- The *Tracking* method offsets the temperature of the heated inner lid a specified number of degrees relative to that of the sample block. Tracking is useful for protocols that have long incubations in the range of 30–70°C, where maintaining a high lid temperature may be counterproductive. An offset of 5°C above block temperature is adequate for most protocols. To use tracking lid-temperature control, select *Tracking* and enter the number of degrees, from 1°C to 45°C, that you wish the lid temperature to be maintained above the block temperature, using the format Lid Temperature = Block Temperature + __°C. You can also use the arrows to scroll to the desired temperature. To specify a sample-block temperature below which the heated lid will turn off, enter a Lid Shutoff Temperature between 1°C and 50°C or use the arrows to scroll to the desired temperature.

  Note: Because there is no active cooling of the lid, a decrease in the lid temperature may not be observed during rapid cycling. Note also that the lid heats more slowly than the sample block due to its additional thermal mass.

- When *Off* is selected in the lid control panel, no power is applied to the heated lid. In this mode, condensation will occur at a rate consistent with the incubation temperature and the type of seal being used. This option is not recommended unless you are using a wax overlay.

Click the *OK* button to apply the temperature and lid control settings to the protocol, or choose *Cancel* to close the window without changing the settings. The Temperature Control and Lid Settings will appear in the appropriate fields of the Temperature and Lid Mode panel in the Protocol Setup window.

6.4.4 Entering a New Protocol

To create a protocol, simply select the steps you want to perform using the buttons in the Select Step to Insert Before Line panel. Clicking on one of the buttons opens a window that prompts you to enter specific parameters.

As you insert steps into a protocol, descriptions of the steps will appear in the Protocol List panel, and a graphical representation of each step's temperature and time period (in minutes) will appear in the Protocol Graph panel. To view the graphical display more clearly, use the sliders to the left of the display panel to zoom in and out on the X- and Y-axes. Use the scroll bars on the axes to position the graph appropriately.

Before beginning to enter a new protocol, note that the END step is highlighted in the Protocol List panel. Opticon Monitor software adds new steps before the step that is highlighted.

The following sections will describe the types of protocol steps you may enter, using the protocol below as an example.

6.4.4.1 Example Protocol

1. Incubate at 94°C for 30 seconds to denature dsDNA.

2. Optimize annealing temperature by incubating at a range of 55°C to 65°C across the twelve columns of the sample block, for 15 seconds.

3. Incubate at 72°C for 30 seconds to allow extension.

4. Read the fluorescence intensity of the Blank, Quantitation Standard, and Sample wells.

5. Sequentially repeat steps 1-4, 39 more times, then proceed to step 6.

6. Identify and determine the purity of reaction products by melting profile — raise the temperature from 55°C to 90°C, and read the fluorescence 2 seconds after every 0.2°C increase in temperature.

6.4.4.2 Temperature Step

A temperature step specifies incubation temperature and duration. The Opticon 2 system ramps the sample to this temperature at its maximum rate unless ramp-modifying instructions are added to the program (see the description of "Manual Ramp Rate" later in this section).

Click the *Temperature* button to enter a temperature incubation step (e.g., step 1 or step 3 from our example) into a protocol.
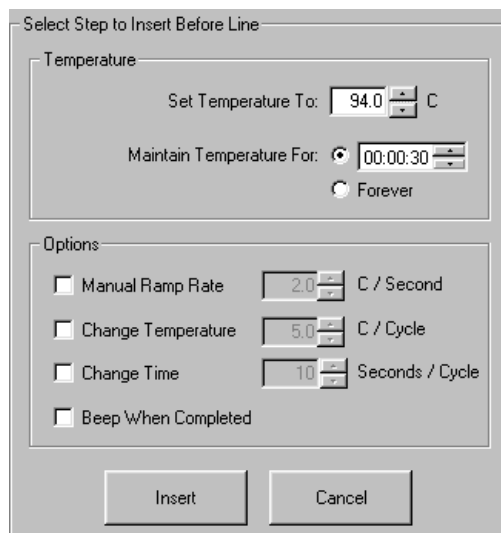

Figure 6-14. Temperature step window

Enter the desired temperature, from 0°C to 105°C, into the Set temperature to field or use the arrows to scroll to the desired temperature — 94°C in step 1 of our example.

Enter the desired incubation time, to a maximum of 18 hours, in the Maintain for field. Click in the *hour*, *minute*, or *second* field and either enter a time period or use the arrows to scroll to the desired time — 00:00:30 in step 1 of our example. Alternatively, you can select *Forever* to maintain the desired temperature indefinitely. A forever incubation step at the end of a protocol can be useful for holding reaction products at a sub-ambient temperature (we recommend 10°C) until they can be processed. In the graphical representation of the protocol, a forever incubation is indicated with an infinity sign ( ).

Click the *Insert* button to add the temperature step to the protocol without further modifications. The temperature step should appear as step 1 in the Protocol List panel, and a graphical representation of the temperature and duration should appear in the Protocol Graph panel. Note that the END step is again highlighted indicating that the next step will be added above the END step and therefore after step 1.

You can also choose to modify a temperature step before inserting it into the protocol. Available options include:

Manual ramp rate: Set a slower-than-maximum rate of heating or cooling. A slower-than-maximum ramp rate ranging from 0.1°C to 2.5°C per second can be specified.

Change temperature: Modifies a temperature step to allow a per-cycle increase or decrease of temperature (0.1°C to 10.0°C per cycle) each time the step is executed. This feature is useful when annealing stringency is a consideration, such as in a touchdown program.

In a touchdown program, the temperature selected for the annealing step begins higher than the calculated annealing temperature and incrementally decreases with each cycle, first reaching, and eventually falling below, the calculated annealing temperature. Thus the reaction begins at a temperature favoring high stringency in hybridization and increments to a lower stringency. The higher initial stringency favors the desired product by creating a high signal-to-noise ratio in the early amplification cycles.

Change time: Modifies a temperature step to allow a per-cycle lengthening or shortening of the hold time of the step (by 1–60 sec/cycle) each time the step is executed. This capability is useful for slowly increasing (typically by 2 to 5 seconds per cycle) the hold time during an extension step. The number of bases that the polymerase must incorporate during the extension step increases in later cycles because there are more template molecules and/or fewer active polymerase molecules. Allotting extra time for extension can increase the likelihood that synthesis will be completed.

Beep when completed: Modifies a temperature step so the instrument will beep when the target temperature is reached.

6.4.4.3 Gradient Step

The temperature gradient feature allows you to optimize denaturing or annealing conditions by incubating at several different temperatures simultaneously. The range of temperatures that can be achieved from left to right across the 96-well sample block can be as small as 1.0°C or as great as 24.0°C. The maximum programmable temperature is 105.0°C; the minimum programmable temperature is 30.0°C.

Click the *Gradient* button to insert a gradient step into a protocol.
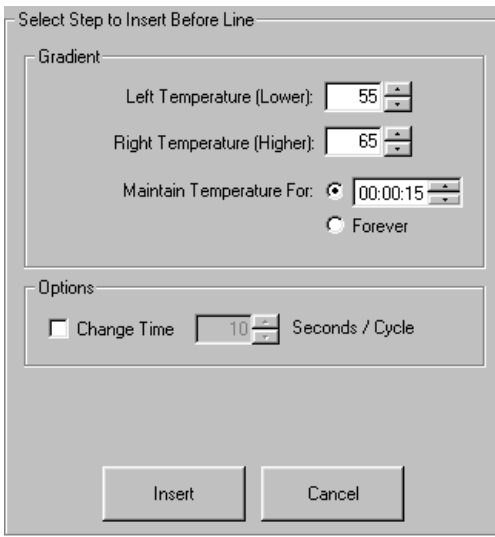

Figure 6-15. Gradient step window

The minimum gradient temperature is assigned to the left column (column 1) of the sample block and can range from 30°C to 104°C. In our example, the Left Temperature (Lower) field is set to 55. The maximum gradient temperature is assigned to the right column (column 12) and can range from 31°C to 105°C. In our example, the Right Temperature (Higher) field is set to 65. The minimum temperature difference between the far left and far right columns is 1°C and the maximum difference is 24°C. A warning will appear if you attempt to enter a difference exceeding 24°C.

After entering the range of temperatures for the gradient, enter the desired incubation time in the Maintain Temperature for field by clicking in the hour: minute: or second: field and either entering a time period or using the arrows to scroll to the desired time — 00:00:15 in step 2 of our example. Alternatively, you can select *Forever* to maintain the desired temperature gradient indefinitely.

Click *Insert* to add the gradient step to the protocol without further modifications. The gradient step should appear as step 2 in the Protocol List panel. Note that the END step is again highlighted indicating that the next step will be added above the END step and therefore after step 2.

You can also choose to modify the gradient step before inserting it into the protocol by choosing the *Change Time* option (see section 6.4.4.2, "Temperature Step" for an explanation of this option).

6.4.4.4 Gradient Calculator

To accurately predict the temperature of each of the twelve columns across the block during a gradient incubation, select *Gradient Calculator* from the *Tools* menu.
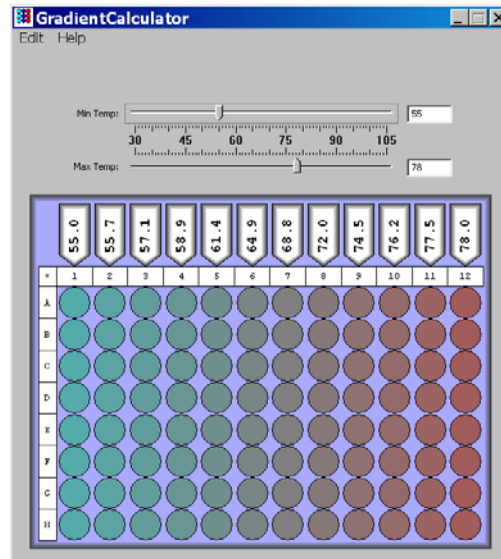

Figure 6-16. Gradient calculator

Set the minimum and maximum temperatures of the gradient step, using the sliders above the plate diagram. The arrow above each column will then display the predicted temperature of the column, and the wells in the diagram will be colored to reflect the gradient.

Note that the gradient temperature distribution is not linear but, rather, has a broader spread in temperature between the center columns of wells. This is a natural consequence of the geometry of the Peltier-Joule heaters that underlie the block. Although not evenly distributed, the temperatures displayed are within ±0.4°C of the actual temperature attained by each well in a given column.

The predicted temperature for the column that yields the best results can be accurately transferred to a temperature step in a non-gradient protocol. Select *Copy* from the *Edit* menu to copy the temperatures to the clipboard for pasting into another program.

6.4.4.5 Plate Read Step

Insertion of a plate read step directs the Opticon 2 system to measure the fluorescence of the wells. The plate read begins immediately after the programmed end of the previous incubation step, step 3 in our example. The Opticon 2 system performs the plate read at the current incubation temperature, and then initiates the next step, step 5 in our example.

To insert a plate read step, click the *Plate Read* button. In our example, the plate read step should appear as step 4 in the Protocol List panel (step 3 is a temperature step, like step 1). A plate read appears as an eye icon in the graphical protocol display.

6.4.4.6 Adding Multiple Temperature, Gradient, or Plate Read Steps

To add additional temperature steps, gradient steps, or plate reads to your protocol, click the appropriate button and follow the directions for the specific step as outlined above.

6.4.4 7 Goto Step

The goto step abbreviates a protocol that has many repeating steps. When a goto step is executed, the protocol program returns to the step specified by the goto step, and repeats that step and all subsequent steps back to the goto step. After it has looped back to the goto step a specified number of times, the protocol program moves on to the step that follows the goto step.

Note: You cannot nest goto steps. In other words, a protocol loop cannot contain another goto step within this loop. You cannot include melting curve steps (see next section) within a goto loop.

Step 5 of our example protocol indicates that the protocol should return to step 1, repeat steps 1-4 39 additional times for a total of 40 cycles, and then proceed to step 6. To add the goto step, click the *Goto* button in the protocol creation panel.

Figure 6-17. Goto step window

Enter the line number of the step to which the protocol program should return. You may also select the number of the step using the drop-down menu. In our example, enter *1* in the Go to line field, and *39* in the How many more times? field. Click the *Insert* button to add the goto step to the protocol.

6.4.4.8 Melting Curve Step

A melting curve can be generated to identify specific fragments and/or to assess the homogeneity of a sample. Melting profiles are influenced by several factors, including the number and concentration of discrete fragments produced, the length and G+C content of each fragment, and buffer conditions.

Melting curves are often useful for verifying the identity of amplification products, as well as distinguishing positive internal controls from amplified products. In these cases, simply specify a melting curve after a cycling run, and the instrument will perform both procedures automatically. The Opticon 2 system can also be programmed to run a melting curve independently of a cycling protocol.

To add a melting curve step, click the *Melting Curve* button in the protocol creation panel.
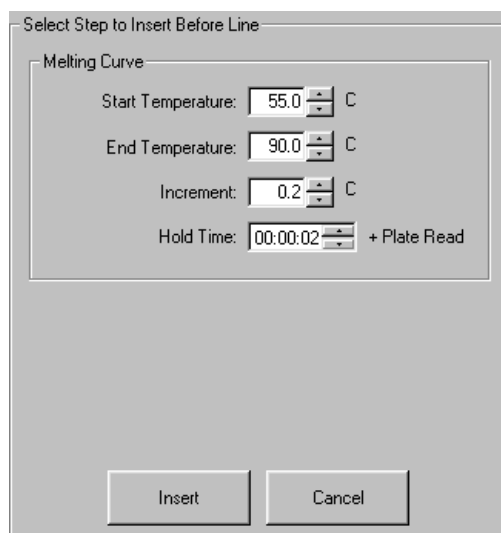
Figure 6-18. Melting Curve step window

Enter a Start temperature (0.0°C to 99.0°C), and an End temperature (1.0°C to 100.0°C). In our example, the melting curve starts at 55°C and ends at 90°C.

Next, specify when during the melting curve step the Opticon 2 detector will measure fluorescence. Designate a temperature Increment between reads of 0.1°C to 10°C and a Hold Time (1 sec to 1 hour) for which the temperature increment should be maintained before the fluorescence is read. A temperature increment of 0.2°C and a hold time of 1–2 sec is often recommended.

Click the *Insert* button to add a melting curve step to the protocol. Only one melting curve step can be performed per protocol.

6.4.5 Editing a Protocol Step

To edit a protocol step, first click on the step in the Protocol List panel to highlight it, then click the *Edit Step* button in the protocol-editing panel. The existing parameters for the step will appear. After making the desired changes, click the *Replace* button to enter the edited step into the protocol, or click *Cancel* to leave the step unmodified.

6.4.6 Deleting a Protocol Step

To delete a protocol step, click on the step in the Protocol List panel to highlight it, then click the *Delete Step* button in the protocol-editing panel to remove the step from the protocol. The remaining protocol steps will automatically renumber.

6.4.7 Inserting a Protocol Step Between Existing Steps

To insert a protocol step between existing steps, highlight the step in the Protocol List panel that will follow the newly inserted step. All protocol steps are added immediately before the step that is highlighted in the Protocol List panel. Next, click the button corresponding to the type of step you would like to add.

6.4.8 Exiting the Protocol Setup

Once you have finished entering protocol file parameters, click the *OK* button in the upper, left corner of the Protocol Setup window to return to the Master File window. A graphical representation of the protocol and a summary of the total number of plate reads, melting curves, and the estimated run duration will appear in the Protocol Setup panel of the Master File window.
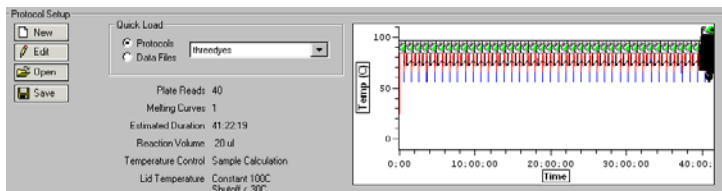

Figure 6-19. Protocol Setup panel of the Master File window

If you wish to discard the protocol file information and return to the Master File window, click *Cancel*.

6.4.9 Saving a Protocol File

To save the newly created protocol file, click the *Save* button in the Protocol Setup panel of the Master File window. Enter an appropriate name in the File name field of the Save window. Click the *Save* button to save the .prot file.

6.5 Saving a Master File

To save a master file, click the *Save* button in the Master File panel of the Master File window. Enter an appropriate name in the File name field of the save window. Click the *Save* button to save the .mast file.

You can also choose not to save this collection of component files and proceed directly to the run (see Chapter 7 for information on initiating a run).

6.6 Assigning Existing Plate and Protocol Files to a Master File

To assign existing plate and protocol files to a new or existing master file, either click the *Open* button in the panel of the Master File window corresponding to the type of file you wish to assign, or use the Quick Load menus to assign existing plate/protocol files to a master file (see section 6.2.4,"Using the Quick-Load Menus").

Clicking *Open* will display all the plate/protocol files in either the Shared folder or, if a user has been selected, in that user's folder. Select the desired file or, if the desired file has been saved to an alternate location, use the Windows browse screen to locate the file, and then click *Open*. The plate/protocol file will be applied to the master file, and a corresponding summary will appear in the Master File window.

To view the newly assigned plate or protocol file and/or make any necessary modifications, click the *Edit* button in the appropriate panel of the Master File window. The Plate Setup or Protocol Setup window will open, allowing you to modify the file parameters. Select *OK* to retain any modifications and return to the Master File window, or select *Cancel* to return to the Master File window without modifying the plate/protocol file. Select *Save* to save any changes to the plate/protocol file under the same or a newly assigned file name.

Click the *Save* button in the Master File panel to save any changes to the master file.

Important Note: If a component plate or protocol file is edited and saved under the same name, the edited file will replace the original file in all master files to which that file has been assigned.

6.7 Reusing Master Files

A new master file need not be created for every run. Existing master files may be reused without modifying the plate or protocol files, or the master file may be edited to accommodate changes such as a different arrangement of samples in the plate.

If a run has just completed, or if a data file for a previously completed run has just been displayed, and you wish to use the same master file, click on the *Repeat This Run* button. To clear the master file template, click on the *Prepare New Run* button. To display an existing master file, click the *Open* button in the Master File panel or use the Quick Load menu to open an existing master file. To access a recently used master file, select the desired master file from the list of *Recent Master Files* under the *File* menu.

Selecting *Open* will display all the master files in the Shared folder or, if a user has been selected, in that user's folder. Select the desired file or use the Windows browse screen to locate the file if it has been saved to a different location, and then click *Open*. The master file will be applied to the master file template and the corresponding plate and protocol file summaries will appear in the Master File window.

To use the master file without any changes, proceed to Chapter 7, "Run Initiation and Status".

You can also modify the master file before initiating a run, by editing the assigned plate or protocol files (*Edit* button), by substituting files (*Open* button or Quick Load), or by creating new component files (*New* button).

Click the *Save* button if you want to save any changes to the master file.

7 Run Initiation and Status

7.1 Running a Protocol

Before initiating a run, check that the appropriate master file is displayed. Click the *Master* button on the toolbar to access the Master File window if it is not currently displayed. If a run has just ended or a data file for a previously completed run has just been displayed, select the *Prepare New Run* button to clear the master file template, or select the *Repeat This Run* button to use the same master file (see Chapter 6 for instructions on how to create and edit master files).

Check the Status section in the run-status box in the bottom-left corner of the window to verify that the instrument is ready to begin a run (Figure 7-1, left). If the Status section indicates that No Instrument is attached (Figure 7-1, center) or that the Door is Open (Figure 7-1, right), correct the problem before continuing.
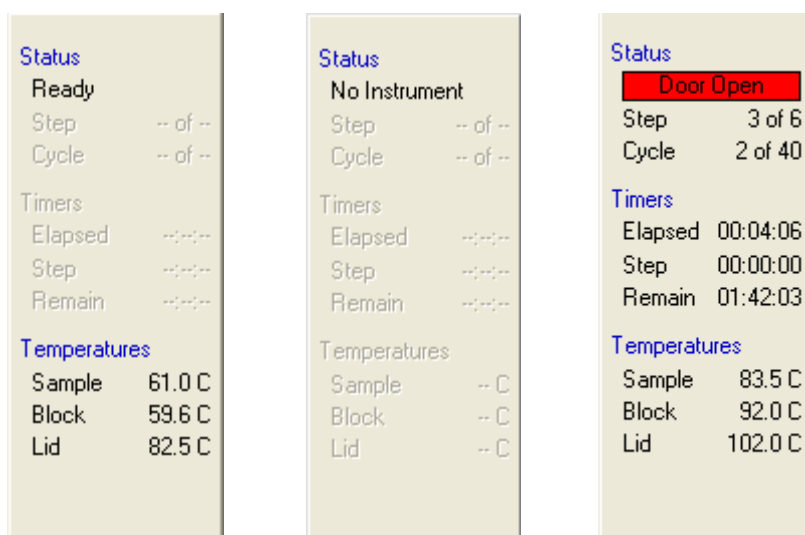
Figure 7-1 The run-status box indicates whether the instrument is ready.

Initiate the run by clicking the *Run* button on the toolbar. A Windows browse screen will appear asking you to name the file to which the data will be saved. Click *Save* to accept the default filename, or enter an appropriate file name and then click the *Save* button. The data file will be saved as a .tad (acquired data) file. The file will be saved in the data folder of the selected user (see "Specifying a User", section 6.2.2.2).

The default data filename can be set using the *Filename Preferences* command under the *User* menu. The fields that can be included in the default filename are: username, year (two format choices), month (two format choices), day (two format choices), time (six format choices), and count, which adds an incremental count to the filename. In addition to these variable fields, the default file name can include constant characters, for example an underscore between fields. The fields and constant characters must be typed into the Data Filename Format field in the Filename Format window as in the following example:

<year1>_<month2><day1>_<time4>

This setting will result in a default filename formatted as follows:

2004_March15_102013PM

7.2 Interrupting a Run

Click the *Stop* button on the toolbar to halt the run at any time. The *Skip* button can be used to skip to the next step in the protocol file. **Note that if you click *Skip* while a Goto loop is being executed, the program will skip out of the loop and implement the next protocol step. Pressing *Skip* does not skip to the next step within the loop.** For example, if step 8 of a protocol says to goto step 2 for 39 times, and you press *Skip* during the 20th execution of step 5, the program will skip to step 9 of the protocol, not step 6.

7.3 Monitoring Run Status

7.3.1 Protocol Information on the Toolbar

A summary of run information is displayed on the bottom half of the toolbar.

Figure 7-2 The run-status box during a run

The run-status box includes three sections:

Status: If a run is in progress, this section indicates the current step and cycle number. This section also indicates when a plate read or an infinite incubation step is in progress, as well as if the instrument is open or disconnected (see Figures 7-1 and 7-2).

Time: This section displays an estimate of the how long the experiment has been running, how much longer the current step will last, and how long before the entire run is finished

Temperature: displays the current sample, block, and lid temperatures.

7.3.2 The Status Window

The Status window allows you either to monitor the system's progression through the protocol, by viewing the Thermal Cycler Status screen, or to monitor real-time data collection, by viewing the Optical Read Status screen. The Thermal Cycler Status screen is automatically displayed in the Status window when the run is initiated. To access the Status window after another screen has been displayed, click the *Status* button on the toolbar. The tabs in the lower-left corner of the Status window toggle the display between the *Thermal Cycler Status* and *Optical Read Status* screens.

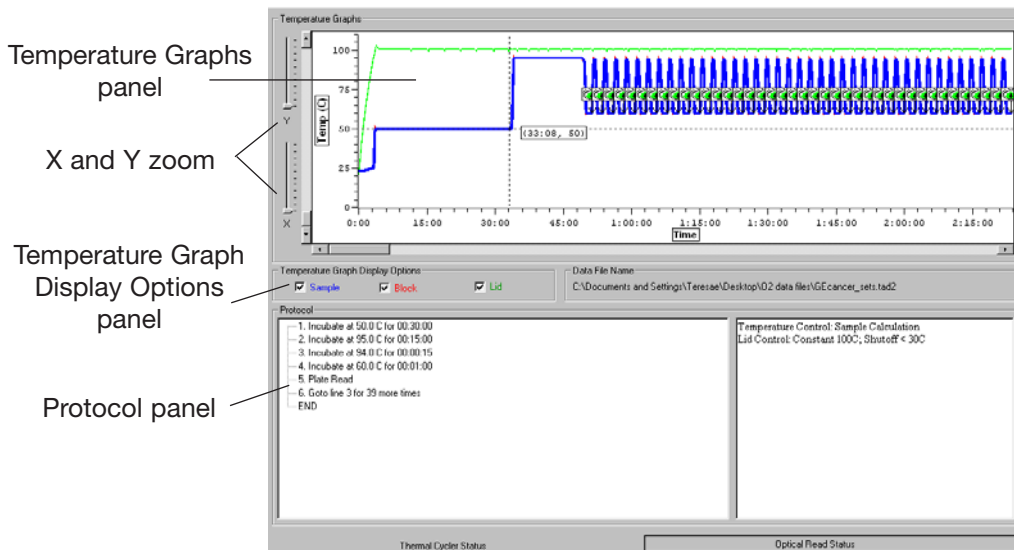### 7.3.2.1 Thermal Cycler Status Screen

Figure 7-1. Thermal Cycler Status screen

Thermal profiles are the instrument are displayed in the Temperature Graphs panel at the top of the Thermal Cycler Status screen. Select the appropriate boxes from the Temperature Graph Display Options panel to display a graph of the *Sample*, *Block*, and/or *Lid* temperatures over time. To clearly view the graph, use the X and Y zoom sliders to the left of the graph, or right click and drag a box around the area of the graph that you wish to magnify. The protocol is listed in the bottom panel of the window, with the current step highlighted. The END step is highlighted if the run has finished.

7.3.2.2 Optical Read Status Screen

Click the *Optical Read Status* tab to monitor real-time data collection in real time.
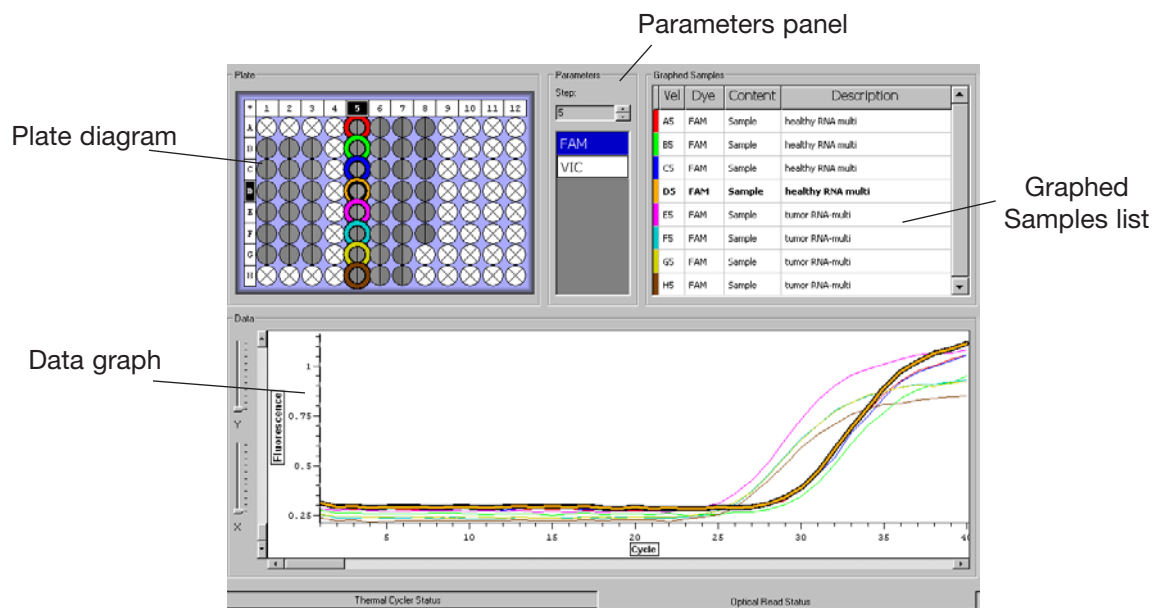
Figure 7-2. Optical Read Status screen

A graph of fluorescence versus cycle number can be displayed for selected dyes in selected wells. Use the Plate diagram to select the wells you want to be displayed in the graph (see section 6.3.5, "Selecting and Deselecting Wells", for additional information), and use the Parameters panel to the right of the Plate diagram to select the dye(s) you want displayed. Note that no data will appear in the Data graph until you select a well and a dye.

Use the Step field in the Parameters panel to display the signal intensity data for a particular step, if a plate read is included in more than one step of the protocol. This can be particularly useful if, for example, you wish to monitor a plate read in step 3 and later a melting curve in step 6.

Selected wells will appear outlined in color. The color outlining the well corresponds to the color of the well coordinates/descriptions in the Graphed Samples list and to the color of the fluorescence intensity trace in the Data graph.

Deselect all wells by clicking on any selected well. To deselect a subset of wells, hold down the control key, and click on the well(s) you wish to deselect. The well(s) will no longer appear outlined in color, and the corresponding fluorescence intensity trace will be removed from the Data graph.

Deselect a previously selected dye by clicking on its name in the Parameters panel. Data for that dye will no longer appear in the Data graph for any of the selected wells.

To highlight the results for a particular sample in the plate diagram, Data graph, and the Graphed Samples list, do one of the following:

Move the cursor over a well on the Plate diagram. The column number and row letter coordinates of the well will be highlighted in the Plate diagram. The well will also be highlighted in the Graphed Samples list, and its trace will be thickened on the Data and Standards graph.

Move the cursor over a particular trace on the graph to thicken the trace and display the x and y coordinates corresponding to the position of the cursor on the trace. The corresponding well in the Plate diagram and the well coordinates in the Graphed Samples list will also be highlighted

Select a well from the Graphed Samples list to highlight the well coordinates in the Plate diagram and thicken its trace on the Data graph.

7.4 Performing Data Analysis During a Run

Opticon Monitor software allows users to analyze data while a protocol is running. Quantitation, Analysis, and Melting Curve windows can be used to view the data collected so far in the current run (see Chapter 8 for more details about these windows). It is also possible to analyze data collected during a previous run while a current run is in progress. To analyze previously gathered data during a run, select the *File->Open->Data File* path you would normally use to open a data file. The Status, Quantitation, Melting Curve and Analysis windows will display the data from the previous run and data analysis can be performed as usual in these windows. The Protocol Info in the run-status box (in the lower-left corner of the window) will continue to display the status of the current run. To close the data set and return to the current protocol running, select the *Reattach to Run* option located under the *File* menu.

It is also possible to open a second Opticon Monitor session while a run is in progress — simply follow the same steps used to open the initial session.

8 Data Analysis

Contents continued on following page

8.1 Analysis Features in Opticon Monitor Software

The analysis options available in Opticon Monitor software include absolute quantitation using standard curves; relative quantitation using ^3C_T and $^{33}C_T$; genotyping by clustering of samples on a scatter plot of C_T vs C_T; and melting curve analysis and calculation of melting temperature (T_m). This chapter describes how to perform these analyses.

8.2 Basic Principles of Real-Time Quantitation

Real-time fluorescence detection of PCR products allows one to calculate the initial quantity of template present in different samples. This is done by counting the number of PCR cycles required to reach a defined level of fluorescence (the threshold fluorescence). Because fluorescence intensity reflects the amount of product, samples that initially have more template will require fewer PCR cycles to reach the threshold than samples with less template. This strong relationship can be defined mathematically: the number of cycles required to reach threshold (i.e., the threshold-cycle or C_T value) is inversely proportional to the log of the initial template number. Therefore, C_T values can be used to calculate initial template quantities.

To calculate the initial quantity of template in a sample, one must first define the fluorescence threshold at which different samples and references will be compared. The simplest way to do this is by first plotting fluorescence vs. cycle number, and then positioning a threshold line on the graph at a point where the signals surpass background levels and begin to increase exponentially. The C_T for an individual sample is then defined as the cycle at which the sample's fluorescence trace crosses the threshold line.

The C_T values obtained in different samples are compared to the C_T of references to calculate either absolute or relative quantities of the template. If quantitation standards with a range of known amounts of template are used as references, one can generate a linear standard curve of the log of template quantity vs. C_T. The absolute quantity of initial template in unknown samples can then be interpolated from the standards, using the samples' C(t) values. If quantitation standards are not available, the quantity of template in one sample can be calculated relative to another based on the difference in the C_T values, ^3C_T.

Opticon Monitor software automatically generates standard curves for absolute quantitation when standards are specified in the plate setup. The software can also estimate reaction efficiency and calculate ^3C_T values for relative expression analysis. The following sections will describe these functions in more detail.

8.3 Viewing Data and Performing Absolute Quantitation

The Quantitation window displays graphs of fluorescence intensity data and an auto-matically generated standard curve (if quantitation standards were specified during plate setup). In this window, you may adjust the data analysis options, position the threshold line, adjust the standard curve, and calculate the quantity of sample ini-tially present in a reaction. If a run is in progress or has just completed, click the *Quantitation* button on the toolbar to view the quantitation data.

To access a previously generated data file, select *Open* and then *Data File* from the *File* menu. Select the desired file from the standard browsing window. To view a recently created or viewed data file, select the desired file from the *Recent Data Files* list accessible from the *File* menu. When the data file loads, the Quantitation window is displayed.

The Quantitation window has two screens: the Graphs screen and the Quantity Calculations screen. Toggle between these screens by clicking on the tabs at the bottom of the window. The features of these two screens are described in the following sections.

8.3.1 The Graphs Screen

The Graphs screen is the default Quantitation screen. The main purpose of this screen is to set parameters, including the fluorescence threshold, for accurately calculating template quantities. This screen can also be used to view graphs of the fluorescence data measured at each cycle of the protocol and to depict the relative intensities of fluorescence measured in all the wells in the plate during a particular cycle.
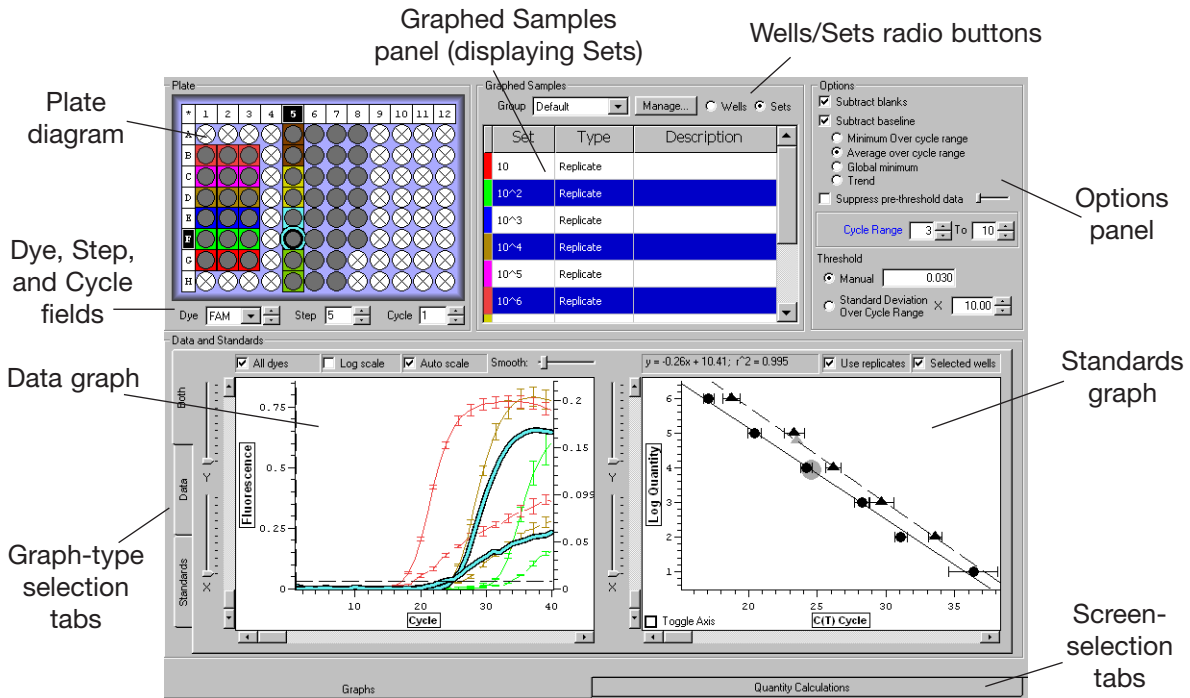

Figure 8-1. The Graphs screen of the Quantitation window

8.3.1.1 Parts of the Graphs Screen

The Graphs screen includes the following panels and fields. These will be discussed in more detail in subsequent sections of this chapter:

The Plate diagram is a representation of the wells in the microplate. It is used to select the wells for which data will be displayed in the Data graph. It can also depict the fluorescence intensity measured in each well and show well sets.

The Dye, Step, and Cycle fields below the Plate diagram are used to select the primary dye for which fluorescence data will be displayed, the protocol step for which data will be displayed (if the protocol included more than one plate-read step), and the cycle, if any, for which you wish to highlight data.

The Graphed Samples panel lists selected wells, when the *Wells* options is selected, and lists all well sets when *Sets* is selected. When *Sets* is selected, this panel can be used to select sets for which summary data will be displayed in the Data graph, or to select all wells in a given set. You can also manage well groups in this panel.

The Options panel is used to specify whether blanks should be subtracted from samples and standards, to select a baseline subtraction method, and to select a method for positioning the threshold line.

The bottom panel displays a *Data* graph of fluorescence (or log fluorescence) versus cycle number, a *Standards* graph of log quantity versus C_T cycle, or *Both* depending on which tab is clicked on the left side of the panel.

8.3.2 Using the Plate Diagram to View Fluorescence Intensity

The Plate diagram can display the approximate fluorescence intensity that was measured in each well during each amplification cycle. The shading of a well in the Plate diagram correlates with the signal intensity measured in that well for the specific Dye, Step, and Cycle specified in the fields below the diagram.
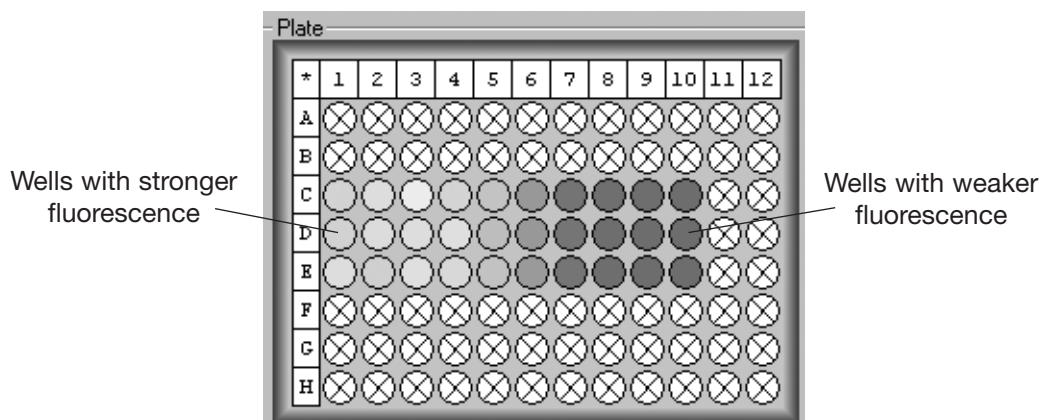

Figure 8-2. Well Intensities displayed in Plate diagram

Dark grey wells indicate weak or no signal, whereas white or light grey wells indicate strong signal. Normally, if you use the arrows adjacent to the Cycle field to scroll through the cycles, the wells will get lighter as the cycle number increases, indicating that the fluorescence intensity is increasing. By scrolling to the last cycle, you can get an indication of the endpoint fluorescence intensity in each well.

Use the Dye field to view the fluorescence intensity for a specific dye in both the Plate diagram and the Data graph (see the next section). To display the fluorescence data for a particular step, if a plate read was performed at more than one step of the protocol, enter the step number in the Step field or use the arrows to scroll to the desired step. Use the Cycle field to view (in the Plate diagram) the signal intensity measured during a specific cycle, as described above. You can also use the Cycle field to mark a particular cycle in the Data graph with a vertical dotted line.

8.3.3 The Data Graph

The Data graph plots the fluorescence measured at each cycle in selected wells. Additionally, the Data graph can plot the average of the fluorescence measured at each cycle in all the wells of selected sets. The standard deviation of the fluorescence in well sets is plotted as error bars on the average fluorescence trace.

Click the *Data* tab on the left side of the graph panel to display a large graph of fluorescence vs. cycle number. Note that no graph will be displayed until at least one well is selected.
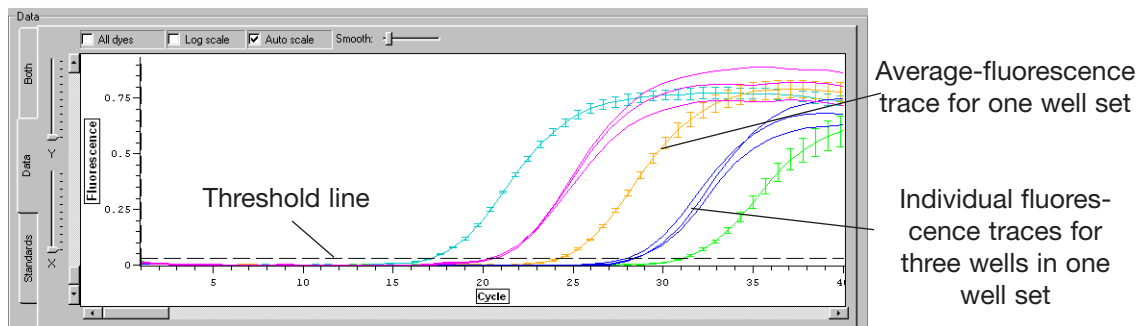

Figure 8-3. Fluorescence data graph

To plot data for individual wells, use the Plate diagram to select the wells (see section 6.3.5, "Selecting and Deselecting Wells"). To plot the mean and standard deviation of fluorescence for a set of wells, choose Sets in the Graphed Samples panel and then click on the name of the set. If you no longer wish to plot the summary data, click on the well set again. To plot unaveraged data for every well in a particular set, click on the colored tab (in the Graphed Samples panel) to the left of the set you wish to display.

Selected wells will appear outlined in color in the Plate diagram. The color that out-lines a well is the same as that used to color that well's fluorescence-intensity trace in the Data graph. When *Wells* is selected in the Graphed Samples panel, each well for which data are plotted is colored differently, and the color appears on the tab to the left of the well's coordinates in the Graphed Samples list. When *Sets* is selected in the Graphed Samples panel, the data for all wells in a given set are plotted with the same color, and that color appears on the tab to the left of the set's name in the Graphed Samples panel.

The fluorescence intensity data for the dye that is selected in the Dye field below the Plate diagram will be displayed in the Data graph. If the *All dyes* option is selected in the field at the top of the Data graph panel, the intensity data for the dye selected in the Dye field will be drawn with a solid line and the data for the other dye will be drawn as a dotted line of the same color.

Select the *Auto scale* option to adjust the scale of the y-axis to accommodate the fluorescence data displayed.

To display the log of fluorescence vs. cycle number, which may be useful when setting the threshold line (see section 8.3.5, "Positioning the Threshold Line"), select the *Log scale* option at the top of the Data graph panel.
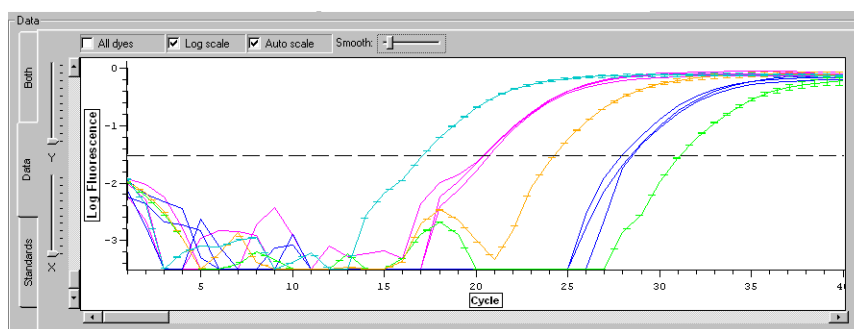

Figure 8-4. Plot of log fluorescence

To expand regions of the graph, right click and drag a box around the area of the graph that you wish to magnify. Alternatively, use the X and Y sliders to the left of the graph to zoom along the x- and y-axes. The scroll bars along the axes can be used to position the region of interest in the display panel. Double-right-click on the graph panel to zoom out to full view.

Moving the cursor over a data trace will thicken the trace and display the C_T value along with the x and y coordinates corresponding to the current position of the cursor over the trace. In addition, the coordinates of the corresponding well will be highlighted in the Plate diagram, and the well information will be emboldened in the Graphed Samples list, if *Wells i*s selected. Moving the cursor over a well in the Plate diagram or a description in the Graphed Samples list will thicken the corresponding trace in the Data graph, highlight the well coordinates in the Plate diagram, and embolden the name in the Graphed Samples list (the C_T will not be shown on the graph, however).

The *Smooth* slider bar, located at the top of the graph panel, allows the user to smooth the data display by creating a running average over a variable number of points. For example, 3-point smoothing takes each particular data point, along with the point before and after it, and takes an average of the three. This average is then plotted on the graph in place of the original data point. The default Smooth setting is 3. The user can turn off all smoothing by setting the slider all the way to the left. Smoothing of the data for each dye is done independently, after selecting the dye in the Dye field under the Plate diagram. **Note that smoothing data can change the C_T value and therefore affect quantity calculations.**

8.3.4 Adjusting the Data Graph

You can choose from the following items in the Options panel of the Quantitation window, to adjust the data graph:

- *Subtract blanks*: If this option is selected, the fluorescence measured in all wells designated as blanks will be averaged and subtracted from the fluorescence measured in all wells designated as samples or quantitation standards, on a cycle-by-cycle basis. Note that this generally is not necessary for accurate quantitation.

- *Subtract baseline*: If this option is selected, a baseline fluorescence value (or set of values in the case of the *Trend* option), will be subtracted from the fluorescence data collected in each well. The value is calculated from the signals measured in each well and thus will vary from well to well. A baselining option must be selected before a standard curve will be plotted.

  There are four options for defining the baseline signal value for a well:

  - *Minimum over cycle range*: The baseline signal value for each well is defined as the minimum fluorescence value measured in that well over the range of cycles specified in the Cycle range boxes.

  - *Average over cycle range*: The baseline signal value for each well is defined as the average of the measured fluorescence in that well, calculated from the range of cycles specified in the Cycle range boxes.

  - *Global minimum*: The baseline signal is defined as the lowest fluorescence signal measured in the well, over all cycles.

  - *Trend*: Opticon Monitor software looks for an initial trend of increasing or decreasing fluorescence and calculates a baseline to subtract from the signals that will eliminate this trend. When this option is selected, *Global minimum* is used to set the baseline for the data from cycles beyond the trend.

- *Suppress pre-threshold data*: This option can be used to specify the earliest cycle for which data is displayed on the Data graph. If the option is selected and the slider is positioned all the way to the right, the fluorescence data for selected wells will be plotted only for the cycle at which the threshold fluorescence is surpassed (the threshold cycle) and for subsequent cycles. Moving the slider to the

left increases the number of cycles preceding the threshold cycle that are displayed. When the slider is positioned all the way to the left, the earliest cycle for which fluorescence data are plotted will be 10 cycles before the threshold cycle.

- Threshold: These two options are for positioning the threshold line, which determines each fluorescence curve's threshold cycle (C_T) for use in quantitation of starting copy number (see next section, "Positioning the Threshold Line").

8.3.5 Positioning the Threshold Line

The threshold line, sometimes called the C_T line, appears as a dashed horizontal line on the Data graph. Normally, this line should be positioned such that, on a graph of baseline-subtracted data, it intersects the fluorescence traces at a point where the signal intensities surpass background levels and begin to increase exponentially. The threshold line should be positioned independently for each dye.

To adjust the position of the threshold line, select one of the Threshold options in the Options panel, or left-click and drag the threshold line to the desired position on the Data graph. If no threshold line appears on the graph, select *Manual* from the Threshold options and enter a value that is less than the maximum fluorescence value displayed on the y-axis of the graph. Next, drag the threshold line to the desired position.

Options for setting the threshold line include:

- *Manual*: The threshold line can be set manually by entering a threshold value for fluorescence intensity or by dragging the threshold line to the desired position on the graph.

- *Standard deviation over cycle range*: The threshold line can be set to a user-defined multiple of standard deviation above the mean fluorescence across the Cycle range specified by the user (in the panel above the Threshold options). A maximum of 10 standard deviations is allowed.

It is often useful to consider both the Data and the Standards graphs (if standards were used) when establishing the position of the threshold line. The best option for setting the threshold line can often be determined by observing the effects of each option on the fit of the quantitation standards to a linear standard curve (see the next section for more information). The effect the position of the threshold line has on the standard curve can easily be visualized by dragging the line up and down.

Viewing the log of fluorescence vs. cycle number, by selecting the *Log scale* option above the Data graph, may allow you to more accurately set the threshold line. In the log scale view, the threshold line should be placed in the linear portion of the curve, not low on the curve as it is in the fluorescence vs. cycle number view.

8.3.6 The Standards Graph

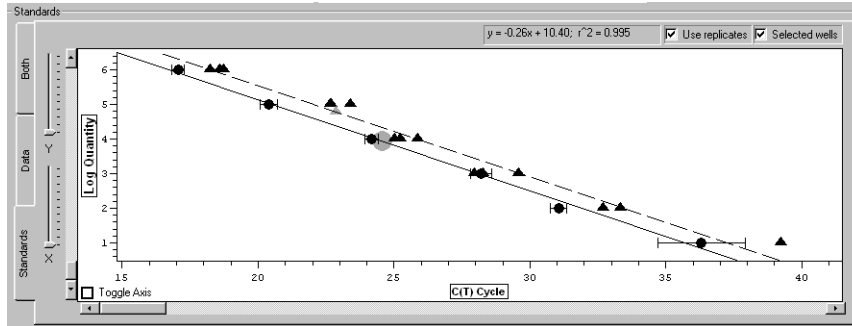To display a large Standards graph, click the *Standards* tab on the left-hand side of the Graphs panel.

Figure 8-5. The Standards graph

Standard curves are automatically generated using the information provided in the Specify Quantitation Standards window during creation of the Plate file. The Standards graph(s) plots the base-10 logarithm of initial quantity (ng, copies, etc.) versus the C_T value for wells specified as standards for the dye selected in the Dye field (below the Plate diagram). A line is automatically fitted to these data, and the equation for this line is displayed above the Standards graph. If the *All dyes* option is selected above the Data graph, the standards data for the dye selected in the Dye field will be fitted with a solid line, while the data for the second dye will be fitted with a dashed line. The line equation for the dye selected in the Dye field will be the only equation displayed. Note that *All dyes* can only be selected when the Data graph is showing.

If well sets were created in the Plate Setup window, the mean C_T value of the replicate standards can be plotted, with error bars representing the standard deviation, by selecting *Use replicates* above the Standards graph. You must select and deselect *Use replicates* for each dye individually; selecting this option only affects the dye selected in the Dye field. To plot summary data for another dye, choose that dye in the Dye field and then click *Use replicates*.

Written above the Standards graph is the equation describing the best-fit linear standard curve, and the r-square value of that line, for the dye selected in the Dye field. These items can help guide the positioning of the threshold. To view the equation and r-square value for another dye, change which dye is selected in the Dye field.

The line equation is of the form y = mx + b, where m is the slope of the line and b is the y-intercept. Theoretically, if perfect doubling of the template was achieved during each cycle, the slope of the line would be –0.30. When the threshold line is properly positioned, the slope should be close to this value.

The r^2 (r^2) value is the square of the correlation coefficient, often called the coefficient of determination. This number indicates what proportion of the variation in one variable is explained by variation in the other variable. In other words, the r^2 value tells how close to the regression line the data points lie. The value of r^2 can vary between 0 and 1, with values closer to 1 signifying a good fit. An r^2 value of 0.999 indicates that the linear standard curve explains 99.9% of the variation in the data. Ideally, when the threshold line is properly positioned, the r^2 value will be greater than 0.990.

Choose the *Selected wells* option to plot quantitation data from wells selected in the Plate diagram or Graphed Samples list. Sample data points are plotted with gray symbols. If a well set is selected in the Graphed Samples list, and if *Use replicates* is selected above the Standards graph, the mean fluorescence of the sample set will be plotted with error bars representing the standard deviation.

The *Toggle Axis* box in the bottom left corner of the Standards graph allows you to switch the x and y axes of the graph, if you have a preference for how the data are viewed. The default graph shows C_T on the x-axis and log quantity on the y-axis. Checking the *Toggle Axis* box switches the axes so that C_T is on the y-axis. Note that the r^2 value and the slope in the equation describing the standard curve also change. When log quantity is plotted along the x-axis, a slope of –3.32 indicates perfect doubling, and the y-intercept (b) indicates the sensitivity.

If a Standards graph is not automatically displayed when the Quantitation window is opened, check that the threshold line has been appropriately set on the Data graph, and that the quantitation standards have been defined in the Specify Quantitation Standards window (see section 8.3.7, "Changing the Values of Quantitation Standards").

8.3.6.1 Adjusting the Standard Curve

If desired, you can adjust the standard curve by deselecting outlying points. Moving the cursor over a data point will increase the size of the point and highlight the corresponding well in the plate diagram, Graphed Samples list, and Data graph. To exclude a point from the standard curve, click on the point and it will turn red, indicating that it is no longer being used in the calculation of the line. The standard curve will automatically be recalculated. Click the point again to include the point in the calculation of the curve; the point will again appear black.

Note: If a deselected point is part of a well set, it will still be included in the average when Use Replicates is selected. To exclude the well from the average, you must remove it from the set, in the Plate Setup window. (See section 6.3.12 "Creating Well Sets".)

8.3.7 Changing the Values of Quantitation Standards

If, during creation of the plate file, a mistake was made in entering the values of quantitation standards, or quantitation standards were not specified, you can change or add quantitation standards during the data-analysis phase.

To change the values of the standards, first click the *Master* button on the toolbar. Click the *Edit* button in the Plate Setup panel and make the desired changes to the quantitation standards, as described in section 6.3.13. Click *OK* to return to the Master File window, and then click on the *Quantitation* button on the toolbar to continue analyzing data with the modified standards.

8.3.8 Managing Well Groups

By default, Opticon Monitor software uses all wells classified as standards to generate a standard curve. In some cases, such as when two experiments with different quantitation standards are run simultaneously, you may only want to use a subset of the standards, to generate a standard curve for quantitation of a subset of the samples. In these cases, the Manage Well Groups function can be used to define subsets of wells. When this is done, the Standards graph will include only the standards present in the selected group.

To define well groups, click the *Manage* button in the Graphed Samples panel. A window listing any defined groups will appear (groups may also be defined in the Plate Setup, Melting Curve, and Analysis windows). Follow the instructions in section 6.3.11, "Defining Well Groups" to create a new group or to delete or edit an existing group.

To select a previously defined group, use the Group drop-down menu in the Graphed Samples panel. When a group is selected, all wells that are not members of that group will appear as empty (i.e., gray with an X through them) in the Plate diagram. The Standards graph will only plot data from wells in the selected group that are designated as standards. If no wells are designated as standards in the selected group, no standard curve will be drawn, and no quantity calculations will be performed on any wells.

To view data from all wells that have been assigned contents (i.e., all non-empty wells), choose *Default* from the Group drop-down menu in the Graphed Samples panel.

8.3.9 The Quantity Calculations Screen

The second screen of the Quantitation window, the Quantity Calculations screen, displays tables of data, calculations, and summary statistics for well sets and individual wells. Click the *Quantity Calculations* tab to display this screen.

Efficiency	C(t)	copies	Avg C(t)	Max C(t)	Min C(t)	C(t) SD	Avg copies	Max copies	Min copies
73.9024%	22.3704	N/A	22.3938	22.9334	22.1056	0.381895	100000	100000	100000
86.3306%	32.6177	N/A	32.5566	32.6855	32.3355	0.157079	100	100	100
92.3221%	35.3488	N/A	35.8337	37.8399	34.8251	1.41862	10	10	10

Well	Dye	Content	Description	Efficiency	C(t)	copies
A5	VIC	Sample	healthy RNA multi	47.9968%	24.0292	23411.7
A5	FAM	Sample	healthy RNA multi	106.954%	27.2296	946.499
B5	FAM	Sample	healthy RNA multi	118.064%	27.3124	900.119
B5	VIC	Sample	healthy RNA multi	45.1865%	23.4567	33692.9
C5	FAM	Sample	healthy RNA multi	98.2233%	27.2426	939.084
C5	VIC	Sample	healthy RNA multi	104.042%	22.9583	46257.5
D5	FAM	Sample	healthy RNA multi	150.833%	27.3092	901.871
D5	VIC	Sample	healthy RNA multi	108.879%	24.0919	22497.7
E5	FAM	Sample	tumor RNA-multi	109.307%	23.4203	9543.4
E5	VIC	Sample	tumor RNA-multi	92.6098%	23.2842	37599.2
F5	FAM	Sample	tumor RNA-multi	160.984%	23.5831	8646.09
F5	VIC	Sample	tumor RNA-multi	36.4068%	22.5734	59083.8

Figure 8-6. The Quantity Calculations screen

Use the radio buttons at the top of the screen to *Show all wells and sets* or to *Show selected only. F*or the latter option, wells and sets are selected in the Graphs screen. Wells can be sorted as desired, by clicking on any of the column headers.

The Wells table lists for each dye in each (selected) well:

* The Well coordinates

* The Dye

* The Content classification (standard, sample, blank)

* Any Description entered during plate setup

* The approximate reaction Efficiency, which is determined as follows: A line is fitted to three points — the point where the fluorescence trace crosses the threshold line and the points corresponding to the fluorescence intensity at the next two cycles. The slope of this line is the approximated reaction efficiency. The efficiency can also be listed as a percentage (100% efficiency means perfect doubling occurred and the slope = 2), by selecting *Efficiency as %* at the top of the screen.

* The C_T value, the cycle number at which the fluorescence intensity trace for the well intersects the threshold line on the Data graph

* The initial quantity of template, either user-specified (for standards) or calculated by interpolating from the standards (for samples), given in the units specified during plate setup

The Sets table lists, for each dye in each (selected) set (Use the slider at the bottom of the table to see hidden columns):

- The name of the well Set

- The Dye used in each set

- The Type of set (replicate or calibrator)

- The Content classification (standard, sample, blank) of wells in the set

- Any Description given to the set

- The reaction Efficiency, calculated as described above, of the average-fluorescence trace for the set, i.e., the trace drawn through the mean of the fluorescence measurements at each cycle

- The C_T value of the average fluorescence trace

- The template quantity calculated for the C_T value of the average fluorescence trace (in units specified during plate setup)

- The average (Avg) of the individual C_T values of wells in the set

- The maximum (Max) of the individual C_T values of wells in the set

- The minimum (Min) of the individual C_T values of wells in the set

- The standard deviation (SD) of the individual C_T values of wells in the set

- The template quantity calculated for the mean, maximum, and minimum individual C_T values of wells in the set

- The standard deviation of the template quantities calculated from the individual C_T values of wells in the set

Click the *Copy to Clipboard* button to copy the quantity calculations to the clipboard for pasting into word processing or spread sheet programs.

8.4 Melting Curve

Because the melting temperature of nucleic acids is affected by length, G+C content, and the presence of base mismatches, among other factors, products can often be distinguished by their melting temperature (T_m). A melting curve analysis can be used to verify that only the intended target was amplified, often eliminating the need for electrophoresis.

To generate a melting profile, you must use an indicator chemistry whose fluorescence depends on the presence of double-stranded DNA (e.g., SYBR Green I). To generate a melting curve, sample fluorescence is measured repeatedly as the temperature is gradually increased. A decrease in fluorescence is observed as melting progresses, with a sharp decrease occurring at the melting temperature. This sharp decrease appears as a peak in a plot of the negative first derivative of the fluorescence intensity over time.

If a run is in progress or has just completed, click the *Melting Curve* button on the toolbar to analyze the melting curve data. To access a previously generated data file, select *Open* and then *Data File* from the *File* menu. Select the desired file from the standard browse window. To view a recently created data file, select the desired file from the *Recent Data Files* list accessible from the *File* menu. Once the data file loads, click the *Melting Curve* button on the toolbar to analyze melting curve data.

8.4.1 Display

The Display screen is the default screen in the Melting Curve analysis window. In this window, you can display a graph of fluorescence intensity (I) versus temperature (T), –dI/dT versus temperature, or both, for wells selected in the Plate diagram or for sets selected in the Graphed Samples panel (when *Sets* is selected).
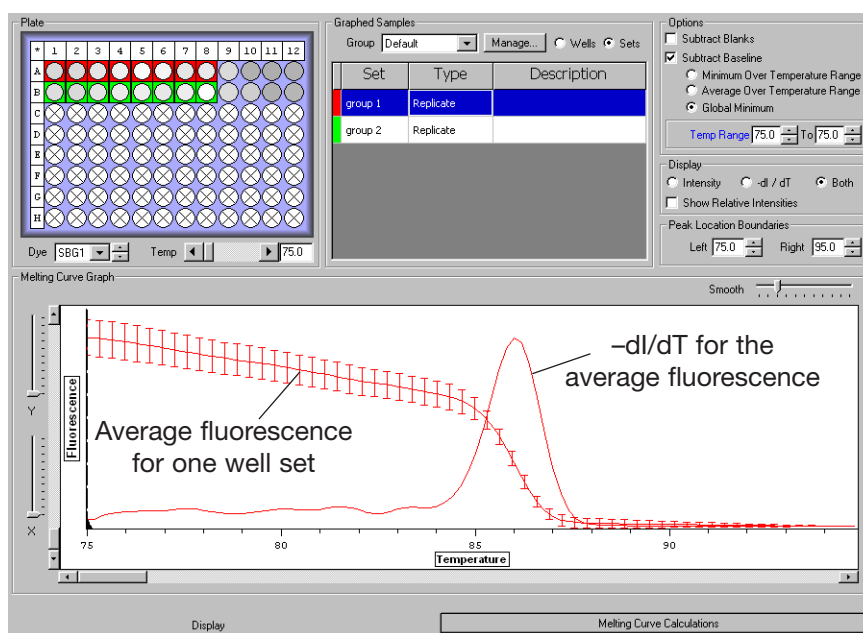
Figure 8-7. Melting curve analysis

8.4.1.1 Selecting Dyes, Wells, and/or Sets

Use the Dye field below the Plate diagram to choose the dye for which you wish to display melting curve data.

To display a list of all sets that have been defined, select *Sets* at the top of the Graphed Samples panel. Select a set by clicking on its row in the Graphed Samples list, and a mean melting curve will be constructed by averaging the fluorescence measurements at each temperature step for all wells in the set. Error bars representing the standard deviation at each point will appear on the graph (see Figure 8-7).

To plot the individual melting curves for all wells in a set, click on the colored tab to the left of the set's row in the Graphed Samples list. Use the Plate diagram to select individual wells to be included in the graph. See section 6.3.5, "Selecting and Deselecting Wells" for additional information.

Selected wells appear outlined in color. The color outlining the well corresponds to the colored tab in the Graphed Samples list and to the color of the trace in the melting curve graph. If *Wells* is selected in the Graphed Samples panel, each selected well will be represented by a different color; if *Sets* is selected, all wells in a set will be represented by the same color.

Deselect all wells by clicking on any selected well. To deselect a subset of wells, hold down the control key, and click on the well(s) you wish to deselect. The well(s) will no longer appear outlined in color, and the corresponding trace(s) will be removed from the graph. Deselect a set by clicking on its row in the Graphed Samples list. To deselect all individual wells in a set, click the colored tab to the left of the set's row in the Graphed Samples list.

8.4.1.2 Choosing Background-Subtraction Options

You can adjust the following data analysis options in the Options panel of the Melting Curve window:

- *Subtract Blanks*: If this option is selected, the fluorescence measured in all wells designated as blanks (blue) will be averaged and subtracted, as background, from the fluorescence measured in all wells designated as samples (red) or quantitation standards (green).

- *Subtract Baseline*: If this option is selected, the baseline signal, an absolute fluorescence value, will be subtracted from the fluorescence data collected in each well. This value is based on the signals measured in each well and therefore will vary from well to well.

    There are three ways to define the baseline signal value for a well:

    - *Minimum over temperature range*: The baseline signal value is defined as the minimum fluorescence value measured in the range of cycles specified in the Temp. Range field.

    - *Average over temperature range*: The baseline signal value is defined as the average of the measured fluorescence calculated from the range of cycles specified in the Temp. Range field.

    - *Global minimum*: The baseline signal is defined as the weakest fluorescence signal measured in the well. This value will be set to zero.

8.4.1.3 Display Options

Use the Display panel to choose how the melting curve data are graphed.

• Select *Intensity* to graph fluorescence intensity versus temperature.

• Select *–dI/dT* to graph the negative first derivative of the fluorescence intensity versus temperature. When sets are graphed, the –dI/dT curve is the negative first derivative of the mean fluorescence intensity trace of the set vs. temperature.

The dotted vertical line, drawn in the same color as the corresponding trace, marks the maximum –dI/dT value, the temperature at which the rate of change in fluorescence is the greatest. This is defined as the melting temperature (T_m) of the product.

The dotted horizontal line indicates the sharpness of the –dI/dT curve as the number of degrees Celsius over which the curve spans (i.e., the curve width) at half of the maximum -dI/dT value calculated for the well (i.e., the FWHM, or full width half max).

• Select *Both* to simultaneously display the Intensity and the -dI/dT graphs.

• Select *Show Relative Intensities* to display the relative intensities of the signals for the selected wells. If this option is not selected, the melting curves of all wells will be auto-scaled, so that the peaks are the same height.
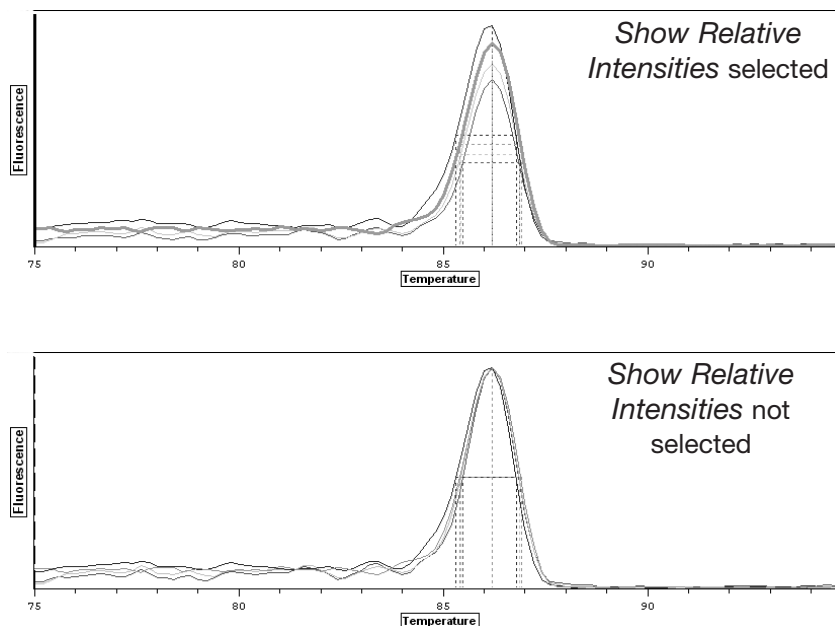
Figure 8-8. Melting curve with and without *Show Relative Intensities* selected

The Peak Location Boundaries field allows you to limit the range of temperatures over which Opticon Monitor software looks for peaks in the –dI/dT trace, thereby limiting the range of possible calculated melting temperatures (see Figure 8-9 below). This can be particularly useful for excluding unwanted peaks, or for determining the melting temperature for a second, smaller peak (e.g., when genotyping heterozygotes). You can set *Left* and *Right* peak location boundaries by entering the temperature or by using the arrows to scroll to the desired temperature. Alternatively, drag the peak location boundary guides to the desired location on the graph.

Without peak location boundaries

With peak location boundaries
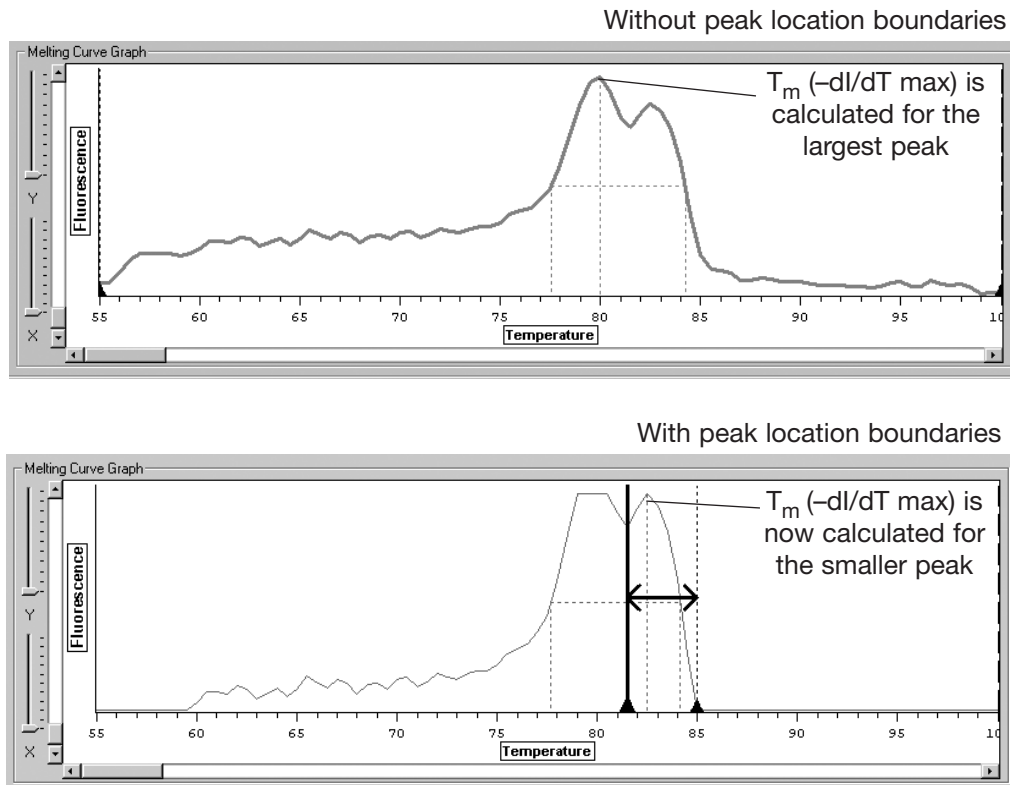
Figure 8-9. Using peak boundary markers to define peak regions

The Temp. field, located below the Plate diagram, can be used to highlight the results associated with a particular temperature, by drawing a dotted vertical line on the graph at the designated temperature. Either use the scroll bar to scroll to the desired temperature or enter the desired temperature in the box located to the right of the scroll bar. This field can also be used to display fluorescence intensity information in the Plate diagram (see section 8.3.2, "Using the Plate Diagram to View Fluorescence Intensity").

The *Smooth* slider allows you to change the number of points that are used in calculating a running average to smooth the melting-curve graph. This can be particularly useful for resolving peaks when many reads have been collected over small increments in temperature, resulting in a choppy graph. The default setting for well-resolved data is 3.

8.4.2 Melting Curve Calculations

Click on the *Calculations* tab at the bottom of the Melting Curve window to display the melting curve calculations screen. This screen displays data tables for individual wells and well sets.

| | Show All Wells and Sets | Show Selected Only | | | | | | | | Copy to Clipboard |

Sets

Content	Description	FWHM	dI/dT ma	Tm	Avg Tm	Max Tm	Min Tm	SD Tm
Sample		6.845	0.07847	86	86	86.2	86	0.09682
Sample		7.063	0.07548	86.2	86	86.2	86	0.09682

Wells

Well	Dye	Content	Description	FWHM	–dI/dT max	Tm
B12	SBG1	Sample		48.16	0.0009487	83.8
A12	SBG1	Sample		3.39	0.0008159	85.8
A1	SBG1	Sample		5.128	0.07364	86
A4	SBG1	Sample		7.038	0.08454	86
A5	SBG1	Sample		7.119	0.09298	86
A6	SBG1	Sample		7.053	0.08682	86
A7	SBG1	Sample		7.034	0.07491	86
A2	SBG1	Sample		6.623	0.07321	86.2
A3	SBG1	Sample		6.966	0.07876	86.2
A8	SBG1	Sample		7.045	0.07309	86.2
B1	SBG1	Sample		5.653	0.06706	86.2
B5	SBG1	Sample		7.199	0.08161	86.2
B6	SBG1	Sample		7.283	0.07621	86.2

| Graphs | Calculations |

Figure 8-10. Melting curve calculations screen

Use the radio buttons at the top of the screen to either *Show all wells and sets* or *Show selected only*.

For each (selected) well, the Wells table lists:

- The Well coordinates

- The Dye used

- The Content classification (blank, standard, etc.)

- A Description of the well contents, if one was entered during creation of the plate file

- FWHM (Full Width Half Maximum): The width, in °C, of the –dI/dT peak, at half of the maximum –dI/dT value calculated for the well. This describes the sharpness of the peak

- The –dI/dT max, the peak value of the negative-first-derivative trace (between peak location boundaries, if specified)

- The T_m (–dI/dT Max): the temperature at which the peak –dI/dT occurs; this is the melting temperature of the main amplification product

For each (selected) well set, the Sets table lists:

- The name of the Set

- The Dye used

- The Type of set (replicate or calibrator)

- The Content classification (blank, standard, etc.)

- A Description of the well contents, if one was entered during creation of the plate file

- FWHM (Full Width Half Maximum): the width, in °C, of the peak of the –dI/dT of the average fluorescence, measured at half of the maximum –dI/dT value calculated for the set.

- The –dI/dT max, the peak value of the negative-first-derivative trace for the set

- The T_m (–dI/dT Max): the temperature at which the peak –dI/dT occurs. This is the melting temperature of the main amplification product

- The Avg T_m (–dI/dT Max): the mean of the melting temperatures calculated for the individual wells in the set

- The Max T_m (–dI/dT Max): the highest of the melting temperatures calculated for the individual wells in the set

- The Min T_m (–dI/dT Max): the lowest of the melting temperatures calculated for the individual wells in the set

- The SD T_m (–dI/dT Max): the standard deviation of the melting temperatures calculated for the individual wells in the set

Click the *Copy to Clipboard* button to copy the melting curve calculations to the clipboard for pasting into word-processing or spreadsheet programs.

8.5 Additional Analysis Options

Opticon Monitor software has two types of data-analysis options in addition to absolute quantitation and melting curve analysis: expression analyses, using delta-C_T or delta-delta-C_T calculations, and genotyping, using scatter plots. Expression and genotyping analyses are performed in the Analysis window, which is accessed by clicking the *Analysis* button on the side toolbar. Each analysis will be described in more detail in this section.

8.5.1 Relative-Expression Analysis

8.5.1.1 Relative Expression Using Delta-C_T and Delta-Delta-C_T

Because a sample's C_T value is dependent on both the efficiency of amplification and the amount of target initially present in the sample, the C_T and efficiency of two reactions can be used to calculate the relative initial quantity of target. If the efficiency of both reactions is 100%, that is, there is perfect doubling of product with each amplification cycle, the expression ratio between samples is equal to $2^{-\Delta C_T}$, where ΔC_T is the difference between the C_T values of the two reactions.

If a multiplex experiment is performed, the amount of target in different samples can be standardized to an endogenous reference before the relative expression of the target is calculated. In this case, assuming the efficiencies of the two reactions is equal and perfect doubling occurs, the relative expression ratio is equal to $2^{-\Delta\Delta C_T}$. The $\Delta\Delta C_T$ is calculated by first comparing the C_T of the target gene in each sample to that of the endogenous reference, yielding the ΔC_T of the sample, and then calculating the difference in ΔC_T between two samples.

Opticon Monitor software can calculate relative expression ratios using either ΔC_T or $\Delta\Delta C_T$. In both cases, equal efficiency and perfect doubling are assumed. When performing these analyses, it is good practice to examine the reaction efficiency of each target to verify that they are roughly equal. Reaction efficiencies are listed in the Calculations table accessed by clicking on the *Calculations t*ab at the bottom of the Analysis window.

8.5.1.2 Expression-Analysis Options in Opticon Monitor Software

The Graphs screen is the default screen in the Analysis window. In this screen, you select the type of analysis you want to perform, select the wells and/or sets to be analyzed, and view a graph of the analysis results.

To perform relative-expression analyses, select *Expression* from the drop-down menu in the Analysis Type field in the upper-right corner of the Analysis window. The following window will be displayed:
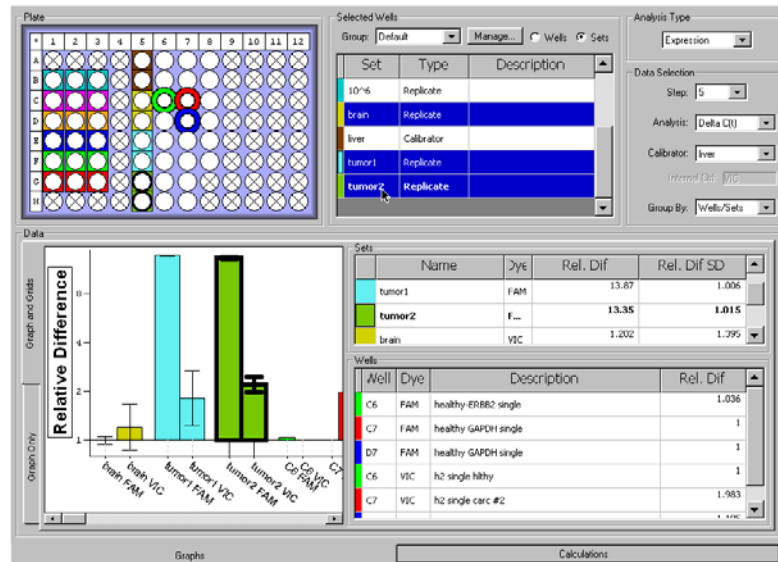
Figure 8-11. Expression analysis using the ^3C_T method. Results from one well set are highlighted.

Select the type of analysis you want to perform — *Delta C_T* or *Delta Delta C_T* from the drop-down list in the Analysis field in the Data Selection panel of the Analysis window.

To perform a relative-expression analysis, you must specify which sample or well set you will compare other samples to. This sample/set is called the calibrator. The calibrator is specified in the Plate Setup window, in the Type column of the Sets table. If you did not specify a calibrator during initial plate setup, you may do so at any time by returning to the Plate Setup windrow. The default Type is *Replicate*. To make a set a calibrator, choose *Calibrator* from the drop-down list in the Type column. If the calibrator includes more than one sample well, the average C_T will be used in the relative-expression calculation.

When performing $^{33}C_T$ analysis, in addition to specifying a calibrator sample or well set, you must specify which indicator dye is used for the internal control. This is done in the Control column of the Sets table in the Plate Setup window. Once a set is designated as a calibrator in the Type column, the internal-control indicator dye can be selected from the drop-down list in the Control column.

To continue expression analysis:

If more than one calibrator was specified in the Plate Setup window, select the Calibrator you wish to use for the current analysis from the drop-down list in the Data Selection panel of the Analysis window. If you are performing $^{33}C_T$ analysis, the indicator dye chosen for the internal control will automatically be listed below the Calibrator field.

To calculate the expression level of a target in samples or well sets relative to the calibrator, select wells from the Plate diagram or well sets from the table in the Selected Wells panel (when *Sets i*s selected). The relative expression (2^{-3C_T} or 2^{-33C_T}) calculated for selected wells or sets is plotted in the Data panel in the lower left corner of the window. If sets are plotted, error bars representing the standard deviation are shown. You can choose to group the bars in the graph by *Wells/Sets* or by *Dye* by selecting the appropriate item from the drop-down list in the Data Selection panel.

The relative expression for each selected well or set is listed in the Wells and Sets tables, respectively. The Sets table also lists the standard deviation of the relative difference. This information is also listed in the tables on the Calculations screen, accessed by clicking the *Calculations* tab at the bottom of the Analysis window. The relative difference listed in these tables is calculated as either 2^{-3C_T} or 2^{-33C_T}, depending on whether *Delta C_T* or *Delta Delta C_T* is selected from the drop-down list in the Analysis field in the Data Selection panel. Note that the relative expression of the calibrator is, by definition, equal to 1.

You can hide the Wells and Sets panels and view a full-width graph by clicking on the *Graph Only* tab on the left side of the Data panel. To view the Wells and Sets panels again, click on the *Graph and Grid* tab.
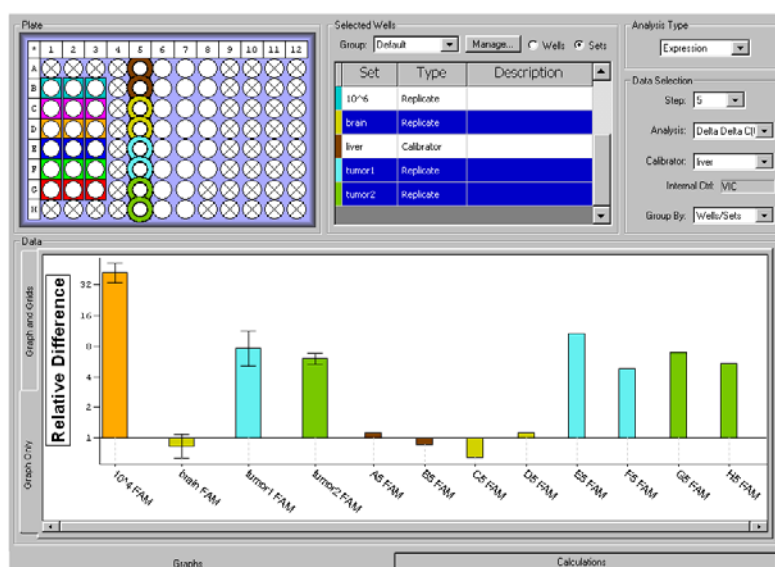
Figure 8-12. Expression analysis using the $^{33}C_T$ method; *Graph Only* tab selected

8.5.1.3 Relative Expression Calculations Screen

Clicking the *Calculations* tab at the bottom of the Analysis window displays the Calculations screen. The tables on this screen list analysis calculations for the Analysis Type selected in the Analysis Graphs screen, for well sets and individual wells.

Sets

Content	Description	copies	Efficiency	Rel. Dif	Avg Rel. Di	Max Rel. Di	Min Rel. Di	Rel. Dif
Sample		N/A	2.12247	1	1.02091	1.28123	0.813485	1.254
Standard		N/A	2.18189	67.8532	65.099	67.665	61.4345	1.042
Standard		N/A	2.36804	35.0492	28.2084	41.4796	22.6104	1.314

Wells

Well	Dye	Content	Description	copies	Efficiency	Rel. Dif
A5	FAM	Sample	healthy RNA multi	946.499	2.06954	1.28123
B5	FAM	Sample	healthy RNA multi	900.119	2.18064	0.813485
C5	FAM	Sample	healthy RNA multi	939.084	1.98223	0.60442
D5	FAM	Sample	healthy RNA multi	901.871	2.50833	1.26623
E5	FAM	Sample	tumor RNA-multi	9543.4	2.09307	10.7169
F5	FAM	Sample	tumor RNA-multi	8646.09	2.60984	5.84926
G5	FAM	Sample	tumor RNA-multi	7926.44	2.14148	8.33566
H5	FAM	Sample	tumor RNA-multi	8364.8	1.95938	8.16863
B1	FAM	Standard	10^6	1e+006	2.49355	47.9259
B2	FAM	Standard	10^6	1e+006	2.14118	32.7229
B3	FAM	Standard	10^6	1e+006	2.394	40.2967
C1	FAM	Standard	10^5	100000	2.52542	115.847

Figure 8-13. Calculations screen showing relative expression analysis

When *Relative Expression* is selected on the Graphs screen, the Calculations tables list the reaction efficiency and relative difference calculated for each well and well set. For well sets, the efficiency and relative difference are calculated using the C_T of the average-fluorescence trace, i.e., the trace drawn through the average of the fluorescence measured at each cycle for the wells in the set. The Sets table also lists the average of the relative differences calculated for each of the individual wells in the set, as well as the standard deviation and the relative difference for the maximum and minimum C_Ts in the set.

Click the *Copy to Clipboard* button to copy the quantity calculations to the clipboard for pasting into word processing or spread sheet programs.

8.5.2 Genotyping With Scatter Plots

In addition to their use in quantitation, C_T values can be used for genotyping of samples. Genotyping analysis can be performed on data from multiplex experiments in which different alleles were amplified using distinct primer sets and indicator dyes. To genotype, the samples are sorted into groups defined by which dyes emitted above-background signals following amplification: dye 1, dye 2, both dyes, or neither dye. Opticon Monitor software can sort the data by creating scatter plots of $C_{T(dye1)}$ vs. $C_{T\{dye2\}}$ or endpoint-fluorescence of dye 1 vs. endpoint-fluorescence of dye 2.

To create a scatter plot, select *Genotyping* from the drop-down menu in the Analysis Type field in the upper-right corner of the Analysis window. The window will display the following screen:
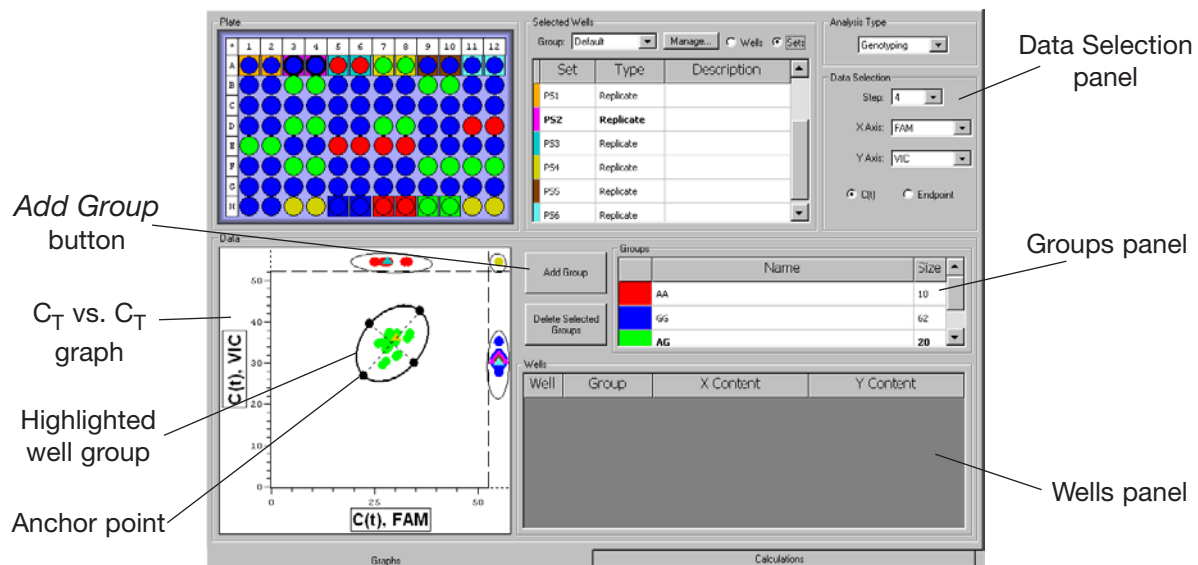
Figure 8-14. Genotyping analysis

In the Data Selection panel, select which protocol step you want to analyze, which dye you want displayed on each axis, and whether you want to compare the fluorescence data based on C_T or Endpoint. The graph in the lower left-hand corner plots data from all wells in the plate and from all defined sets. As shown in Figure 8-15, both endpoint and C_T plots can cluster data into groups based on which dye(s) had above-background signals.

A. Endpoint vs. endpoint

B. C$_T$ vs. C$_T$

Samples that emitted a signal from VIC only

Samples that emitted a signal from both dyes

Samples that emitted no signal
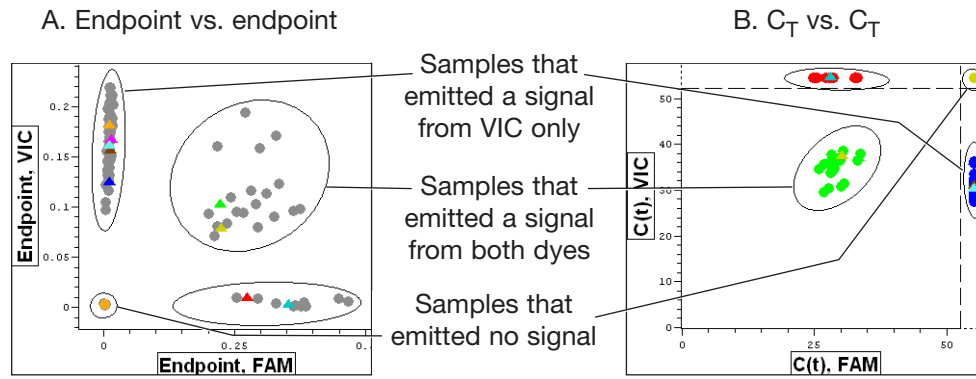
Figure 8-15. Genotyping scatter-plot options

The graph in Figure 8-15A plots the endpoint fluorescence data from each well in a two-color experiment, with the fluorescence from the FAM channel plotted along the x-axis and the fluorescence from the VIC channel plotted along the y-axis. The software has plotted the relative fluorescence intensities for each dye, as measured at the end of the run.

The graph in Figure 8-15B plots the C$_T$ values obtained in the same experiment as in the graph in Figure 8-15A. The C$_T$ for FAM is plotted along the x-axis and the C$_T$ for VIC is plotted along the y-axis. The dashed lines at the top and right edges of the graph are separators that mark the last cycle of the experimental protocol. The fluorescence of those samples plotted above or to the right of these lines did not rise above threshold for at least one of the dyes during this experiment.

8.5.2.1 Identifying Individual Wells and Sets in the Scatter Plot

When the scatter plot is first constructed, the data from wells are plotted as black circles and, if *Sets* is selected in the Selected Wells panel, the data from sets is plotted as triangles colored as indicated by the tabs next to the set names. Positioning the mouse cursor over a symbol on the scatter plot highlights the corresponding well in the Plate diagram or the corresponding set in the Graphed Samples panel. Positioning the cursor over a well in the Plate diagram or an item in the Graphed Samples panel enlarges the corresponding symbol in the scatter plot. Selecting a well in the Plate diagram colors the symbols representing other wells gray in the scatter plot, while the selected well's symbol remains black.

8.5.2.2 Defining Subsets of Data in the Scatter Plot

Using the scatter plot, you can identify subsets of data based on the clustering pattern of the data points (Figure 8-15). For example, in genotyping, samples with the same genotype will tend to cluster in a similar C$_T$ range. Users can select samples that cluster together and assign particular group names, e.g., the genotype, to the samples located in these clusters.

To group a cluster of data points, click on the *Add Group* button in the center of the Analysis window. This converts the mouse pointer to a set of cross hairs. Move the cross hairs to the periphery of a clustered data set in the scatter plot. Left-click the mouse to set an anchor point on one side of the data set, drag the cross hairs to the opposite edge of the data set, and left-click the mouse to mark the end of the set. Dragging the cross hairs perpendicular to the ellipse that was just created causes the ellipse to radiate outward. Expand and contract the radius to include the data points that you wish to analyze. When you are satisfied with the area that you have defined, left-click a final time to set the group.

If no wells have been selected in the Plate diagram, data points in the scatter plot will turn from black to a particular color (red, green, blue, etc.) when they are included in a set. If wells have been selected, then only the symbols corresponding to selected wells will change color when they are added to a set — the others will remain gray until they are selected or all wells are deselected in the Plate diagram.

When a group has been created, it will be listed in the Groups panel as a "New Group", along with the number of data points that are in the group. To rename the group, highlight the group name, type the new name and then press the *Enter* key on the keyboard. To delete a group, highlight the group name and then click the *Delete Selected Groups* button.

Defined groups are color coded — the graphed data points included in the set and. the sample wells that correspond to these data points (in the Plate diagram) will be assigned the same color. This color will appear in the left-hand column in the Groups panel. You can change the color of a group by right-clicking on the color and selecting a different color from the palette that appears.

When a group is selected by clicking on a group name from the Groups panel, its member wells will be listed in the Wells panel along with their descriptions. Wells can be sorted by *Well*, *Group*, *X content*, or *Y Content* by clicking on the appropriate column header. Reverse the sort order by clicking the header again. Clicking the *Wells* header multiple times allows you to sort by column or row, in forward or reverse order.

8.5.2.3 Editing a Group

You can alter the size, shape, and position of an ellipse after it has been drawn. To change the dimensions of the ellipse, click on one of the four anchor points that lie on the ellipse. (These will appear when you position the mouse pointer on the ellipse, as in Figure 8-14). Position the pointer at the intersection of the axes with the ellipse.) Dragging the anchor points can alter both the shape and size of the ellipse. To move the ellipse without changing its dimensions, click on the ellipse (between anchor points) and drag it to a new location.

8.5.2.4 Calculations Table

Clicking the *Calculations* tab at the bottom of the Analysis window displays a table listing the group assignment, as well as the C_Ts and reaction efficiencies associated with each dye, for individual wells and well sets. The maximum, minimum, and standard deviation of the individual C_Ts of wells in each set is also listed. Clicking the *Copy to Clipboard b*utton allows you to paste the information in the table into spreadsheet programs.

Figure 8-16. Calculations table showing genotyping analysis

8.6 Exporting and Printing Data

8.6.1 Exporting Data

For customized data analysis, Opticon Monitor software provides the option to write all the fluorescence data collected during a run to a comma-separated-values (CSV) file, which can be opened in other programs, such as Microsoft Excel. The Export command creates a CSV file listing all fluorescence measurements (for selected wells) that were collected during all plate read and melting curve steps in an experimental run.

To export data for selected wells, choose *Export* (under the *Quantitation*, *Melting Curve*, or *Analysis* menu, depending on which window is being viewed — the exported data will be the same). Wells can be selected in the window that appears.
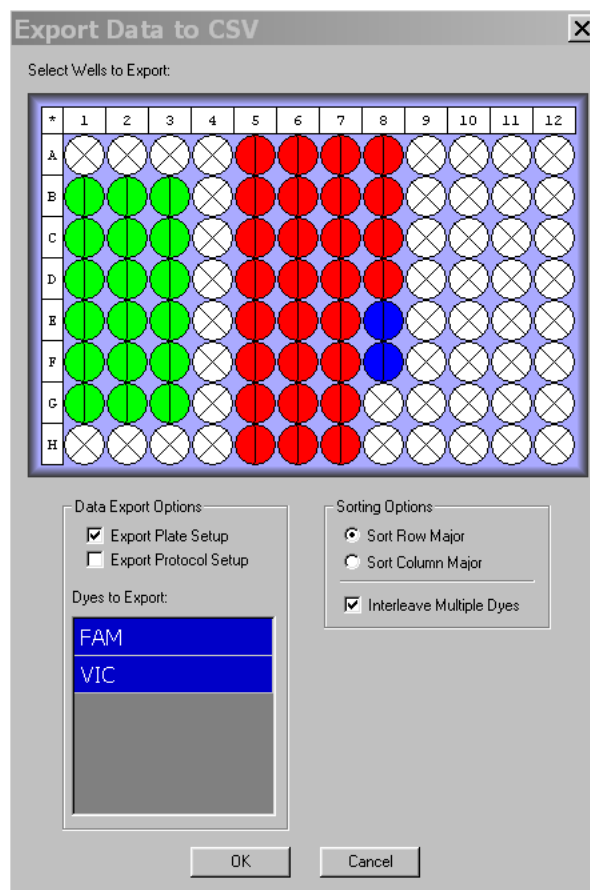
Figure 8-17. Exporting data

You can also select the dye(s) for which data will be exported by selecting from the Dyes to Export list (selected dyes are highlighted in blue). You can choose to sort the wells by row or by column; and can choose whether to *Interleave Multiple Dyes* (i.e., to list data for all dyes in each well together), or to first list the data for each well for one dye and then list the data for each well for the second dye.

In addition to exporting fluorescence data, you can choose to export the plate and/or protocol setup information by checking *Export Plate Setup* and/or *Export Protocol Setup*. The plate information that is exported includes the well number, dye used, well contents, and well descriptions. If the plate setup is exported, the information can later be pasted into a different master file to easily reconstruct the plate file (see section 6.3.14, "Pasting Plate Information from Microsoft Excel).

By default, the exported CSV file also lists analysis options (i.e., Subtract Blanks, Subtract Baseline, Baseline Start cycle, Baseline End cycle, and Suppress Pre-Threshold) that were chosen on the analysis screen for each protocol step at which data were collected.

8.6.2 Copying Results to Clipboard

The *Copy to Clipboard* command (under the *Quantitation*, *Melting Curve*, or *Analysis* menu, depending on which window is being viewed) allows the user to paste run information and graphical data into a variety of applications for presentation or storage. This command can also be implemented by clicking on the *Copy to Clipboard* button on the Calculations screen. The following items can be copied using *Copy to Clipboard* from the specified windows:

8.6.2.1 From the Quantitation Window

The *Data Graph* (a plot of fluorescence — or log fluorescence — vs. C_T cycle), *Standards Graph*, *Microtitre plate* (the Plate diagram), or *Quantity calculations* can be copied to the clipboard and pasted into several applications. Selecting *Quantity Calculations* from the *Copy to Clipboard* menu generates CSV tables to be pasted subsequently into an Excel spreadsheet. The tables have the same content as the Quantity Calculations screen, including information for both well sets and individual wells. See section 8.3.9, "The Quantity Calculations Screen", for more details on these tables. The tables can also be copied to the clipboard by clicking on the *Copy to Clipboard* button on the Quantity Calculations screen.

8.6.2.2 From the Melting Curve Window

The *Data Graph* (the plot of fluorescence intensity or –dI/dT or both), *Microtitre plate* (the Plate diagram) or *Melting Temp Calculations* can be copied to the clipboard. Selecting *Melting Temp Calculations* generates a CSV file with information identical to that shown on the Melting Curve Calculations screen, including both well sets and individual wells. See section 8.4.2, "Melting Curve Calculations", for more details of this table. This table can also be copied to the clipboard by clicking on the *Copy to Clipboard* button on the Melting Curve calculations screen.

8.6.2.3 From the Analysis Window

The *Data Graph* (the plot of C_T or Endpoint fluorescence or the plot of relative expression levels), *Microtitre Plate*, *Calculations* or *Analysis List* can be copied to the clipboard. Selecting *Calculations* generates a CSV file with information identical to that shown on the Calculations screen, including both well sets and individual wells. Selecting *Analysis List* generates a CSV file listing the Group Name, and the Dye 1/Dye 2 contents (standard, sample, etc.) for each sample belonging to a Group in the scatter plot (as defined by the user — see section 8.5.2.2, "Defining Subsets of Data in the Scatter Plot"). Selecting *Analysis List* does not copy the relative expression calculations.

8.6.3 Creating and Printing Reports

Opticon Monitor software can assemble a report of the real-time run and send it directly to a networked printer or save it as an HTML file. This can be done by selecting *Print HTML Report* from the *Quantitation*, *Analysis*, or *Melting Curve* pull-down menu in the appropriate window.

If *Print HTML Report* is selected from the *Quantitation* menu, the software creates a report that contains: the user's name; the name and location of the data file on the Opticon computer's hard drive; an indication of the active dye; the position of the C_T line; and an indication of whether the C_T level was set manually or "via signal-to-noise". The report will also include the Plate diagram with selected wells highlighted; the Data graph of fluorescence (or log fluorescence) versus C_T cycle; and the Standards graph along with the equation detailing the fit of the data. The Plate diagram and the graphs will appear exactly as they do when *Print HTML Report* is selected, i.e., with or without error bars on the Standards graph, showing *All Dyes* or only the selected dye, etc.

The report also includes tables similar to those on the Calculations screen (see section 8.3.9). The tables will include all wells and sets or only selected wells and sets, depending on what radio button has been selected on the Calculations screen. When P*rint HTML Report* is selected, a window opens, allowing you to select which columns from each table you want to include in the report (see Figure 8-18).
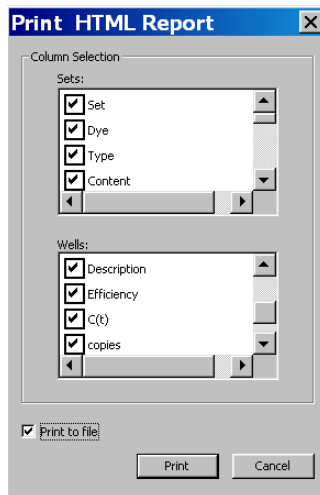
Figure 8-18. Printing from the Quantitation window

If *Print HTML Report* is selected from the Analysis window, the software creates a file that contains: the user's name; the name and location of the data file on the Opticon computer's hard drive; the dye graphed on each axis of the genotyping graph, and the plate type chosen during plate setup. The report also includes the Plate diagram as it appears when *Print HTML Report* is selected, as well as the Data graph that is showing (genotyping or relative expression). Note that only the small version of the Data graph will be included in the report, even if the *Graph Only* tab has been selected in the Analysis window.

The report printed from the Analysis window will also include tables similar to those appearing in the Calculations screen. You can select what columns to include when the Print HTML Report window opens.

If *Print HTML Report* is selected from the Melting Curve menu, the software creates a report that contains: the user's name; the name and location of the data file on the Opticon computer's hard drive; and an indication of Display Type, i.e., whether intensity, -dI/dT, or both are plotted in the graph. The report will also include the Plate diagram with selected wells highlighted; and the Data graph of fluorescence intensity and/or -dI/dT vs. temperature. Finally, the report will include a tables similar to those that appear on the Calculations screen. You can select what columns to include when the Print HTML Report window opens.

To save a report to the computer's hard drive, select mark the *Print to File* box near the bottom of the Print HTML Report window. When you then click *Print*, a Save window will open. Specify a name and location for the HTML file. The Plate diagram and graphs will be saved as separate .bmp files in the same location. Click *Save* to save the file.

To print the report directly to a printer, make sure the *Print to File* box is unmarked before you click *Print*.

9 Maintenance

9.1 Cleaning the DNA Engine Opticon® 2 Unit

9.1.1 Cleaning the Chassis and Block

Clean the outside of the Opticon™ 2 unit with a damp, soft cloth or tissue whenever something has been spilled on it, or when the chassis is dusty. A mild soap solution may be used if needed.

Clean block wells with swabs moistened with water, 95% ethanol, or a 1:100 dilution of bleach in water. If using bleach, swab wells with water afterward to remove all traces of bleach. Clean spilled liquids out of the block as soon as possible: dried fluids can be difficult to remove. Do not clean the block with caustic or strongly alkaline solutions (e.g., strong soaps, ammonia, or bleach at a higher concentration than specified above). These will damage the block's protective anodized coating, and possibly lead to electrical shorting.

⚠ **Caution: Do not pour any cleaning solution into the block's wells and then heat the block, in an attempt to clean it. Severe damage to the block, the heated lid, and the chassis can result.**

9.1.2 Cleaning the Air Vents

With the Opticon 2 unit turned off, clean the air intake and exhaust vents with a soft-bristle brush, a damp cloth, or a vacuum cleaner whenever dust is visible (see Figure 2-1). If these vents become clogged with dust and debris, airflow to the heat sink will be hampered, causing performance problems related to overheating.

Tip: To prevent problems with overheating, check regularly for dust buildup.

9.1.3 Cleaning the Optics

The optical components of the detector should not be cleaned by the user. Disassembly of the optical tower will void your warranty.

Should you suspect difficulty with the optics, please contact the customer service staff of Bio-Rad at 888-652-9253, or your local distributor.

9.2 Changing the Fuses

The circuits in the DNA Engine Opticon 2 unit are protected by four fuses, two external, user-changeable 6.3 A, 250 V Type S505, fast acting, 5 x 20 mm fuses and two user-inaccessible 8 A fuses. When a fuse blows, an error message will appear in the Opticon Monitor™ software indicating that it can not communicate with the instrument.

Warning: The DNA Engine Opticon 2 unit incorporates neutral fusing, which means that live power may still be available inside the unit even when a fuse has blown or been removed. **Never disassemble the thermal cycler base.** You could receive a serious electrical shock. Disassembling the base will also void your warranty.

To change the 6.3 A fuses:

1. Turn the instrument off. Disconnect the power cord from the back of the instrument.

2. Turn the computer off and disconnect the serial and DAQ cables from the Opticon 2 unit. The Opticon 2 unit also draws power from the computer.

3. Insert one corner of a small flat-head screwdriver just under the fuse cover, located immediately below the power switch and just above the power cord jack (see Figure 2-2), and gently pry the cover loose. Pull the cover straight out as far as it will go, then push it downward to expose the 6.3 A fuses.

4. Remove both fuses and replace them with new ones (it is impossible to determine visually which fuse is blown). You may also test the fuses with an ohmmeter to determine which is defective and replace just that one.

5. Gently press the fuse cover back in place, and reconnect the power cord and the computer.

6. Once the fuse has been replaced and the power restored, the DNA Engine Opticon 2 unit will resume the run, but will not communicate with the Opticon Monitor software. Restart the software to halt the run, since no data will be collected.

10 Instrument Calibration

10.1 Introduction

The Opticon™ 2 detector is calibrated at the factory to accommodate a variety of fluorescent dye chemistries and PCR plates. Calibrations may be added, edited, or removed via the *Dye Calibration* interface from the *Instrument* menu. Nonetheless, it is strongly recommended that the factory calibrations not be removed or changed as this may adversely affect the performance of the instrument.

10.2 Testing Calibration

An accurately calibrated instrument will detect the same fluorescence intensity in every well when reading samples of uniform composition.

To determine if the Opticon 2 detector is accurately calibrated:

1. Prepare a test plate by accurately pipetting 50 µl of a carefully prepared dye solution into each of the 96 wells of a PCR plate (see step 13 in the "Recalibrating" section below for recommended concentrations for several popular dyes.)

2. Program the following protocol:

   • Temperature step of 30°C for 30 sec

   • Plate Read step

   • Goto line 1 for a total of 2 more times

   • End.

3. Analyze the fluorescence data for all 96 wells using the Optical Read Status screen. If the instrument is accurately calibrated, the fluorescence data should appear as relatively straight lines that are tightly clustered — within the accuracy of pipetting. Several test plates should be measured to determine the error resulting from pipetting.

4. If the fluorescence data do not appear as tightly clustered straight lines, follow the instructions below for recalibrating the instrument.

10.3 Recalibration

1. Select *Dye Calibration* from the *Instrument* menu.

2. The Dye Calibration Wizard will launch and provide basic instructions for creating new calibrations.

3. Dye calibrations are performed by first scanning a blank plate (an empty microplate that is sealed with clear optical caps), followed by scanning the same plate containing the fluorophore of interest (also sealed with clear optical caps).

Differences in the collected signals from the "empty plate" and the "dye plate" are used to determine color separation matrices that resolve the dye of interest from mixtures. It is strongly recommended that the same plate be used for both the empty plate read and the dye plate read, as variations from plate to plate can be significant and interfere with obtaining an optimal color separation matrix.

4. Follow the instructions provided by the Wizard and begin by selecting or entering a Plate type into the appropriate field on the first screen. This plate type description will identify the type of PCR plate used for this particular calibration (e.g., White, Clear).
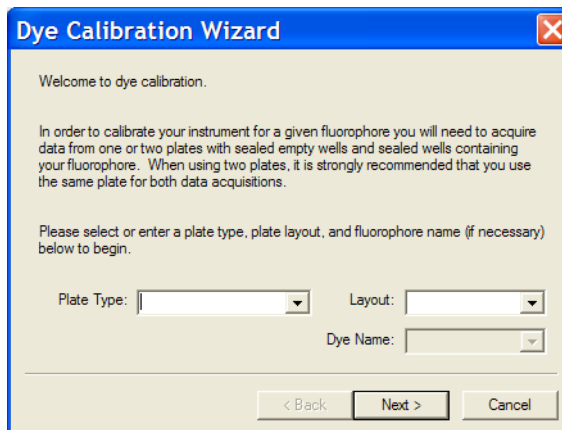
Figure 10-1. Dye Calibration Wizard

5. After selecting a plate type, select *FULL* from the Layout drop-down list. This is the only Layout option users should select when running a calibration.

6. Next, enter the dye to be calibrated or select the dye from the Dye name drop-down list (e.g., FAM, VIC).

7. Proceed to the next screen by clicking on the *Next* button.

8. If a calibration already exists for this particular dye/plate combination, a warning message will appear. If you wish to continue, click on the *Yes* button.

9. Insert the sealed empty plate, close the door and click on the *Go* button. The Opticon 2 system will begin the calibration procedure.
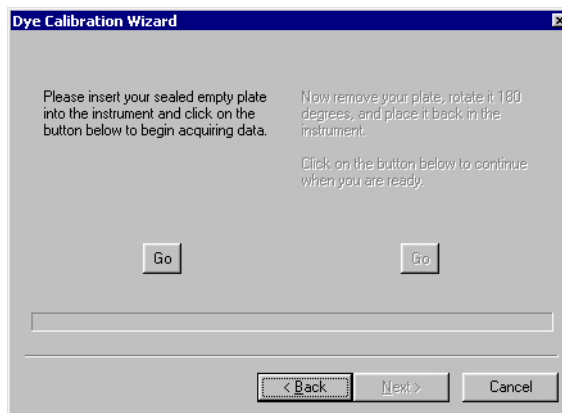
Figure 10-2. Beginning calibration

10. Remove the plate and rotate it 180 degrees when prompted (the left half of the screen is grayed out and the right half is activated). Close the door and click on the *Go* button to continue.
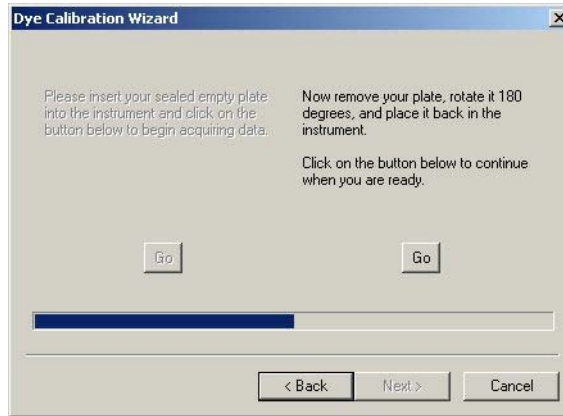
Figure 10-3. Continuing calibration

11. After the empty plate has been scanned, you will be able to view the acquired data and check it for anomalies that may indicate a problem with the plate (e.g., unusually high signal in certain wells). See Figure 10-4, below:
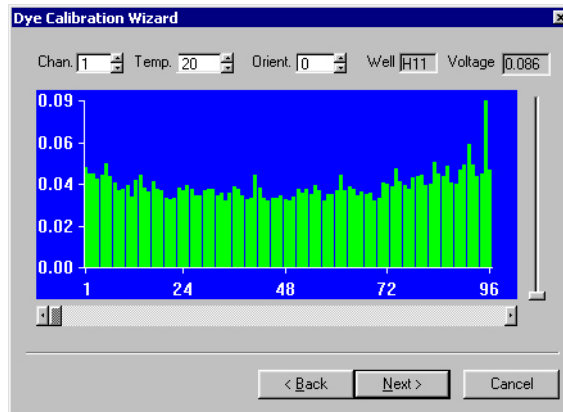
Figure 10-4. Fluorescence data for empty plate

12. Click on the *Next* button to proceed to the dye plate section.

13. Remove the empty plate from the instrument. Remove the optical caps and fill each well with 50 µl of dye diluted in reaction buffer. The fluorophore should be present at a relatively high concentration to ensure the signal obtained is well-above background. Use the following concentrations as a starting point:

• FAM 0.30 µM

• TET 0.40 µM

• VIC 0.50 µM

• HEX 0.60 µM

• TMR 2.0 µM

14. Seal the filled dye plate with clear optical caps and place the plate in the instrument.

15. Click *Go* to continue the calibration (see Figure 10-5).
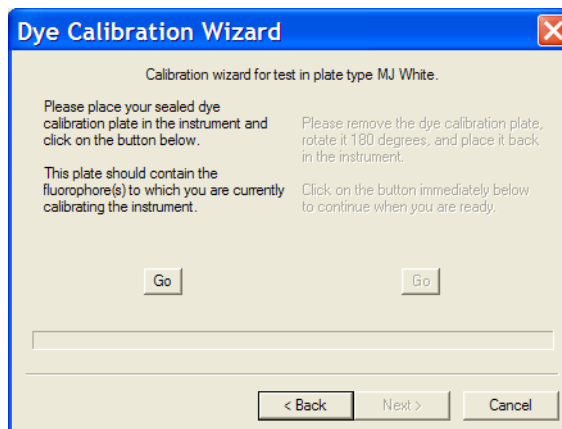
Figure 10-5. Continuing with fluorescent dyes.

16. Remove the plate and rotate it 180 degrees when prompted. Close the door and click *Go* to continue (see Figure 10-3).

17. After the dye plate has been scanned, you will be able to view the acquired data and again check it for anomalous readings. Ideally, the average signal on at least one of the channels should be at least 10x greater than the blank plate signals for that channel. This insures that the dye is providing the vast majority of the total signal and will insure a robust color separation matrix.
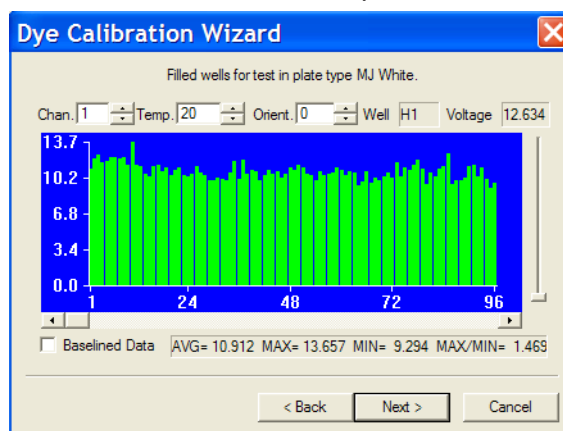
Figure 10-6. Fluorescence data for dye plate

18. Use the display spinners above the bar graph to select the data for channel (Chan)*1* or channel *2*, or the ratio of the two channels, *1/2*. The graph will display the data for the dye plate. The values should be highly consistent from well to well. If this is not the case, you may need to rescan the dye plate.

19. If the results are satisfactory, click *Next* to proceed to the final screen.

20. Add comments (if desired) to the calibration (e.g., record the date).

21. Click *Finish* to save and complete the calibration process.
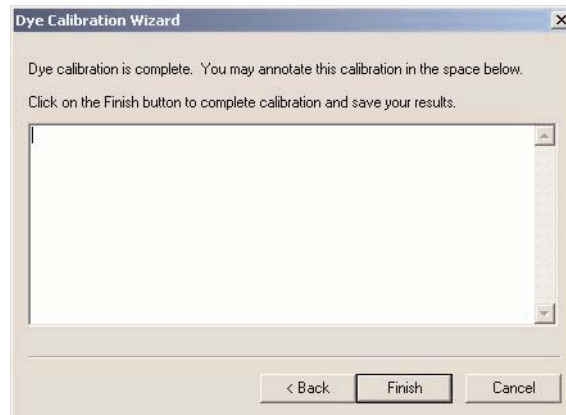
Figure 10-7. Comments window

22. To run another calibration with the same plate type, click the *Yes* button when the Opticon Monitor software asks if you want to calibrate another dye.
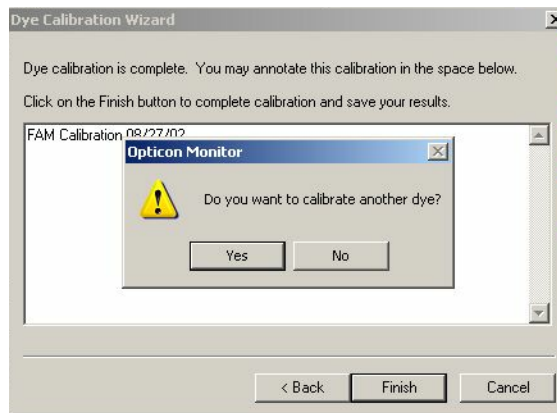
Figure 10-8. Running a second calibration
with the same plate type

23. To run another calibration with a different plate type, close the Wizard and restart the calibration process from the beginning.

A Appendix A: Warranties

The DNA Engine Opticon® 2 system (CFB-3220, which comprises CFD-3220 and PTC-200) thermal cyclers are warranted against defects in materials and workmanship. For specific Warranty information, contact your local Bio-Rad office. If any defects should occur during the warranty period, Bio-Rad will replace the defective parts without charge. However, the following defects are specifically excluded:

1. Defects caused by improper operation or by improper packaging of returned goods.

2. Repair or modifications done by anyone other than Bio-Rad Laboratories.

3. Use with tubes, plates, or sealing materials not specified by Bio-Rad Laboratories for use with the DNA Engine Opticon 2 system.

4. Deliberate or accidental misuse.

5. Damage caused by disaster.

6. Damage due to use of improper solvent or sample.

The warranty does not apply to fuses.

For inquiries or to request repair service, contact Bio-Rad Laboratories after confirming the model and serial number of your instrument.

For technical service, call your local Bio-Rad office or, in the United States, call 1-800-4BIORAD (1-800-424-6723), or visit us on the Web at **discover.bio-rad.com**.

B Appendix B: License Agreement

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Bio-Rad Laboratories, Inc., 1000 Alfred Nobel Drive, Hercules, California, 94547, U.S.A., declares that the product

PTC0200, The DNA Engine® Thermal Cycler

to which this declaration relates, is in conformity to the following standards or normative documents.

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13 September, 2004
 date of issue

Brad Crutchfield
Vice President

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