



Method B: Bacteroidales in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay

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Note that this method will be updated following its validation in marine and fresh ambient waters.

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Participant Laboratories

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Introduction

Bacteria of the Bacteroidales order are commonly found in the feces of humans and other warm-blooded animals. Although these organisms can be persistent in the environment, the presence of Bacteroidales in water is an indication of fecal pollution and the possible presence of enteric pathogens.

Method B describes a quantitative polymerase chain reaction (qPCR) procedure for the detection of DNA from Bacteroidales bacteria in ambient water matrices based on the amplification and detection of a specific region of the 16S ribosomal RNA gene from these organisms. Results can be obtained by this method in 3-4 hours, allowing same-day notification of recreational water quality. Recent epidemiological studies at fresh water recreational beaches (Reference 17.7) have demonstrated similar or improved positive correlations between Bacteroidales DNA measurements by this method and swimming-associated gastrointestinal (GI) illness rates.

In Method B, water samples are filtered to collect Bacteroidales on polycarbonate membrane filters. Following filtration, total DNA is solubilized from the filter retentate using a bead beater. Bacteroidales target DNA sequences present in the clarified homogenate are detected by the real time polymerase chain reaction technique using TaqMan® Universal Master Mix PCR reagent and the TaqMan® probe system. The TaqMan® system signals the formation of PCR products by a process involving enzymatic hydrolysis of a fluorogenically-labeled oligonucleotide probe when it hybridizes to the target sequence.

Method B uses an arithmetic formula, the comparative cycle threshold (C_T) method, to calculate the ratio of Bacteroidales 16S rRNA gene target sequences (target sequences) recovered in total DNA extracts from water samples relative to those in similarly-prepared extracts of calibrator samples containing a known quantity of Bacteroidales cells. Mean estimates of the absolute quantities of target sequences in the calibrator sample extracts are then used to determine the absolute quantities of target sequences in the water samples. C_T values for sample processing control (SPC) sequences added in equal quantities to both the water filtrate and calibrator samples before DNA extraction are used to normalize results for potential differences in DNA recovery or to signal inhibition or fluorescence quenching of the PCR analysis caused by a sample matrix component or possible technical error.

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1.0 Scope and Application

- 1.1** Method B describes a quantitative polymerase chain reaction (qPCR) procedure for the measurement of the 16S ribosomal RNA (16S rRNA) target gene sequences from all known bacteria of the order Bacteroidales in water. This method is based on the collection of Bacteroidales on membrane filters, extraction of total DNA using a bead beater, and detection of Bacteroidales target sequences in the supernatant by real time polymerase chain reaction using TaqMan® Universal Master Mix PCR reagent and the TaqMan® probe system. The TaqMan® system signals the formation of PCR products by a process involving the enzymatic hydrolysis of a labeled fluorogenic probe that hybridizes to the target sequence.
- 1.2** Bacteroidales are commonly found in the feces of humans and other warm-blooded animals. Although DNA from these organisms can be persistent in the environment, its presence in water is an indication of fecal pollution and the possible presence of enteric pathogens.
- 1.3** The Method B test is recommended as a measure of ambient fresh and marine recreational water quality. Epidemiological studies have been conducted at fresh water and marine water beaches that may lead to the potential development of criteria that can be used to promulgate recreational water standards based on established relationships between health effects and water quality measurements by this method. The significance of finding Bacteroidales DNA target sequences in recreational water samples stems from the direct relationship between the density of these sequences and the risk of gastrointestinal illness associated with swimming in the water that have been observed thus far in the epidemiological studies (Reference 17.7).
- 1.4** This method assumes the use of an ABI sequence detector as the default platform. The Cepheid Smart Cycler® may also be used. The user should refer to the platform specific instructions for these instruments in the Appendices. Users should thoroughly read the method in its entirety before preparation of reagents and commencement of the method to identify differences in protocols for different platforms.

2.0 Summary of Method

The method is initiated by filtering a water sample through a membrane filter. Following filtration, the membrane containing the bacterial cells and DNA is placed in a microcentrifuge tube with glass beads and buffer, and then agitated to extract the DNA into solution. The clarified supernatant is used for PCR amplification and detection of target sequences using the TaqMan® Universal Master Mix PCR reagent and probe system.

3.0 Definitions

- 3.1** Bacteroidales: all genera and species of the order Bacteroidales for which 16S rRNA gene nucleotide sequences were reported in the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank>) at the time of method development.

- 3.2** Target sequence: A segment of the 16S rRNA gene containing nucleotide sequences that are homologous to both the primers and probe used in the Bacteroidales qPCR assay and that are common only to species within this order.
- 3.3** Sample processing control (SPC) sequence (may also be referred to as reference sequence): A segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta* (*O. keta*), containing nucleotide sequences that are homologous to the primers and probe used in the SPC qPCR assay. SPC sequences are added as part of a total salmon DNA solution in equal quantities to all water sample filtrate and calibrator samples prior to extracting DNA from the samples.
- 3.4** DNA standard: A purified, RNA-free and spectrophotometrically quantified and characterized *Bacteroides thetaiotaomicron* strain ATCC #29741 genomic DNA preparation. DNA standards are used to generate standard curves for determination of performance characteristics of the qPCR assays and instrument with different preparations of master mixes containing TaqMan® reagent, primers and probe as described in Section 9.6. Also used for quantifying target sequences in calibrator sample extracts as described in Section 12.2.
- 3.5** Calibrator sample: Samples containing constant added quantities of *B. thetaiotaomicron* strain ATCC #29741 cells and SPC sequences that are extracted and analyzed in the same manner as water samples. Calibrator sample analysis results are used as positive controls for the Bacteroidales target sequence and SPC qPCR assays and as the basis for target sequence quantification in water samples using the $\Delta\Delta C_T$ or ΔC_T comparative cycle threshold calculation method as described in Section 12.4. Analysis results of these samples provide corrections for potential daily or weekly method-related variations in Bacteroidales cell lysis, target sequence recovery and PCR efficiency. QPCR analyses for SPC sequences from these samples are also used to correct for variations in total DNA recovery in the extracts of water sample filtrates that can be caused by contaminants in these filtrates as described in Section 12.4 and/or to signal potentially significant PCR inhibition caused by these contaminants as described in Section 9.8..
- 3.6** $\Delta\Delta C_T$ comparative cycle threshold calculation method ($\Delta\Delta C_T$ method): A calculation method derived by Applied Biosystems (Reference 17.1) for calculating the ratios of target sequences in two DNA samples (e.g., a calibrator and water sample) that either controls (ΔC_T method) or normalizes ($\Delta\Delta C_T$ method) for differences in total DNA recovery from these samples using qPCR analysis C_T values for a reference (SPC) sequence that is initially present in equal quantities prior to DNA extraction.
- 3.7** Amplification factor (AF): A measure of the average efficiency at which target or SPC sequences are copied and detected by their respective primer and probe assays during each thermal cycle of the qPCR reaction that is used in the comparative cycle threshold calculation methods. AF values can range from 1 (0 % of sequences copied and detected) to 2 (100 % of sequences copied and detected) and are calculated from a standard curve as described in Section 12.2.

4.0 Interferences

Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration. These materials can also interfere with DNA recovery and interfere with the PCR analysis by inhibiting the enzymatic activity of the Taq DNA polymerase, and/or inhibiting the annealing of the primer and probe oligonucleotides to sample target DNA enzyme or quenching of hydrolyzed probe fluorescence.

5.0 Safety

- 5.1** The analyst/technician must know and observe the normal safety procedures required in a microbiology and/or molecular biology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.
- 5.2** Where possible, facial masks should be worn to prevent sample contamination.
- 5.3** Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

- 6.1** Separated, and dedicated workstations for reagent preparation and for sample preparation, preferably with HEPA-filtered laminar flow hoods and an Ultraviolet (UV) light source, each having separate supplies (*e.g.*, pipettors, tips, gloves, etc.). *Note:* The same workstation may be used for the entire procedure provided that it has been cleaned with bleach and UV sterilized as specified in section 11.6.1 between reagent and sample preparation. Under ideal conditions, two dedicated workstations are used for sample preparation: one for preparing samples with high target sequence DNA concentrations (*e.g.*, DNA standards and calibrator samples) and one for preparing samples with expected low target sequence DNA concentrations (*e.g.*, filter blanks and ambient water samples).
- 6.2** Balance capable of accuracy to 0.01 g
- 6.3** Extraction tubes: semi-conical, screw cap microcentrifuge tubes, 2.0-mL (*e.g.*, PGC 506-636 or equivalent)
- 6.4** Glass beads, acid washed, 212 - 300 µm (*e.g.*, Sigma G-1277 or equivalent)
- 6.5** Autoclave, capable of achieving and maintaining 121°C [15 lb pressure per square inch (PSI)] for minimally 15 minutes
- 6.6** Workstation for water filtrations, preferably a HEPA-filtered laminar flow hood with a UV light source. This can be the same as used for sample preparation, Section 6.1
- 6.7** Sterile bottles/containers for sample collection
- 6.8** Membrane filtration units (filter base and funnel) for 47 mm diameter filters, sterile glass, plastic (*e.g.* Pall Gelman 4242, or equivalent), stainless steel, or disposable plastic (*e.g.*, Nalgene CN 130-4045 or CN 145-0045, or equivalent), cleaned and bleach treated (rinsed with 10% v/v bleach, then 3 rinses with reagent-grade water), covered with aluminum foil or Kraft paper, and autoclaved or UV-sterilized if non-disposable
- 6.9** Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air can be used.
- 6.10** Flask, filter, vacuum, usually 1 L, with appropriate tubing
- 6.11** Filter manifold to hold a number of filter bases
- 6.12** Flask for safety trap placed between the filter flask and the vacuum source
- 6.13** Anaerobic chamber (BD BBL GasPak 150 Jar System or equivalent)
- 6.14** Disposable gas generator pouches (BD BBL Gas Pak Plus Hydrogen or equivalent)

- 6.15** Forceps, straight or curved, with smooth tips to handle filters without damage, 2 pairs
- 6.16** Polycarbonate membrane filters, sterile, white, 47 mm diameter, with 0.45 μm pore size (*e.g.*, GE Osmotics Inc. 04CP04700 or equivalent)
- 6.17** Graduated cylinders, 100-1000-mL, cleaned and bleach treated (rinsed with 10% v/v bleach, then 3 rinses with reagent-grade water), covered with aluminum foil or Kraft paper and autoclaved or UV-sterilized
- 6.18** Petri dishes, sterile, plastic or glass, 100 \times 15 mm with loose fitting lids
- 6.19** Disposable loops, 1 and 10 μL
- 6.20** Sterile 1cc syringes
- 6.21** Sterile ½" needles, 18 gauge
- 6.22** Permanent ink marking pen for labeling tubes
- 6.23** Visible wavelength spectrophotometer capable of measuring at 595 nm
- 6.24** Single or 8-place mini bead beater (*e.g.*, Biospec Products Inc. # 3110BX or equivalent).
- 6.25** Microcentrifuge capable of 12,000 \times g
- 6.26** Micropipettors with 10, 20, 200 and 1000 μL capacity. Under ideal conditions, each workstation should have a dedicated set of micropipettors (one micropipettor set for pipetting reagents not containing cells or reference DNA and one set for reagents containing reference DNA and for test samples).
- 6.27** Micropipettor tips with aerosol barrier for 10, 20, 200 and 1000 μL capacity micropipettors.
Note: All micropipetting should be done with aerosol barrier tips. The tips used for reagents not containing DNA should be separate from those used for reagents containing DNA and test samples. Each workstation should have a dedicated supply of tips.
- 6.28** Microcentrifuge tubes, low retention, clear, 1.7-mL (*e.g.*, GENE MATE C-3228-1 or equivalent)
- 6.29** Test tube rack for microcentrifuge tubes, use a separate rack for each set of tubes
- 6.30** Conical centrifuge tubes, sterile, screw cap, 50-mL
- 6.31** Test tubes, screw cap, borosilicate glass, 16 \times 125 mm
- 6.32** Pipet containers, stainless steel, aluminum or borosilicate glass, for glass pipets
- 6.33** Pipets, sterile, T.D. bacteriological or Mohr, disposable glass or plastic, of appropriate volume (disposable pipets preferable)
- 6.34** Vortex mixer (ideally one for each work station)
- 6.35** Dedicated lab coats for each work station
- 6.36** Disposable powder-free gloves for each work station
- 6.37** Refrigerator, 4°C (ideally one for reagents and one for DNA samples)
- 6.38** Freezer, -20°C or -80°C (ideally one for reagents and one for DNA samples)
- 6.39** Ice, crushed or cubes for temporary preservation of samples and reagents
- 6.40** Printer (optional)
- 6.41** Data archiving system (*e.g.*, flash drive or other data storage system)

- 6.42** UV spectrophotometer capable of measuring wavelengths of 260 and 280 nm using small volume capacity (*e.g.*, 0.1 mL) cuvettes or NanoDrop® (ND-1000) spectrophotometer (or equivalent) capable of the same measurements using 2 µL sample volumes
- 6.43** ABI 7900 or ABI 7500 Sequence Detector
- 6.43.1** Optical 96 well PCR reaction tray (*e.g.*, Applied Biosystems N801-0560 or equivalent)
- 6.43.2** Optical adhesive PCR reaction tray tape (*e.g.*, Applied Biosystems 4311971 or equivalent) or MicroAmp™ caps (*e.g.*, Applied Biosystems N8010534 or equivalent)
- 6.43.3** ABI 7900 sequence detector
- 6.44** Cepheid Smart Cycler®
- 6.44.1** Smart Cycler® 25 µL PCR reaction tubes (*e.g.*, Cepheid 900-0085 or equivalent)
- 6.44.2** Rack and microcentrifuge for Smart Cycler® PCR reaction tubes. *Note:* Racks and microcentrifuge are provided with the Smart Cycler® thermocycler
- 6.44.3** Cepheid Smart Cycler® System Thermocycler

7.0 Reagents and Standards

Note: The Bacteroidales stock culture (Section 7.9), Salmon DNA/extraction buffer (Section 7.13), and DNA extraction tubes (Section 7.19), may be prepared in advance.

7.1 Purity of Reagents: Molecular-grade reagents and chemicals shall be used in all tests

7.2 Control Culture

- *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) ATCC #29741

7.3 Sample Processing Control (SPC) DNA (source of SPC control sequences)

- Salmon testes DNA (*e.g.*, Sigma D1626 or equivalent)

7.4 Phosphate Buffered Saline (PBS)

7.4.1 Composition:

Monosodium phosphate (NaH ₂ PO ₄)	0.58 g
Disodium phosphate (Na ₂ HPO ₄)	2.50 g
Sodium chloride	8.50 g
Reagent-grade water	1.0 L

7.4.2 Dissolve reagents in 1 L of reagent-grade water in a flask and dispense in appropriate amounts for dilutions in screw cap bottles or culture tubes, and/or into containers for use as reference matrix samples and rinse water. Autoclave after preparation at 121°C [15 lb pressure per square inch (PSI)] for 15 minutes. Final pH should be 7.4 ± 0.2.

7.5 Chopped Meat Carbohydrates Broth (CMCB) *Note:* Formulation is provided only to ensure that the appropriate medium is used for analyses, laboratories should use commercially prepared tubes.

Note: Pre-mixed powder forms of this medium are not available

7.5.1 Composition:

Chopped Meat Pellets	10.2 g
Pancreatic Digest of Casein	30.0 g
Yeast extract	5.0 g
Glucose	4.0 g
Dipotassium Phosphate	5.0 g
Cellobiose	1.0 g
Maltose	1.0 g
Starch	1.0 g
L-Cysteine HCl	0.5 g
Resazurin	0.001 g
Vitamin K ₁	1.0 mg
Hemin	5.0 mg
Reagent-grade water	1.0 L

7.5.2 This medium is manufactured in a pre-reduced (oxygen-free) environment and sealed to prevent aerobiosis. The medium is pre-sterilized and ready for inoculation.

7.6 CDC Anaerobe 5% Sheep Blood (BAP) *Note:* Formulation is provided only to ensure that the appropriate medium is used for analyses, laboratories should use commercially prepared plates.

7.6.1 Composition:

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	20.0 g
Yeast Extract	5.0 g
Hemin	0.005 g
Vitamin K ₁	0.01 g
L-Cystine	0.4 g
Reagent-grade water	1 L
Defibrinated Sheep Blood	5%

7.7 Sterile glycerol (used for preparation of *B. thetaiotaomicron* stock culture as described in section 7.9)

7.8 Preparation of *B. thetaiotaomicron* (ATCC #29741) stock culture

Rehydrate lyophilized *B. thetaiotaomicron* per manufacturer's instructions (for ATCC stocks, suspend in 0.5 mL of sterile chopped meat carbohydrate broth (CMCB) and mix well to dissolve lyophilized culture. Using a sterile syringe and needle aspirate suspension and inoculate 10 mL of CMCB. Incubate at 35.0°C ± 0.5°C for 24-72 hours. After incubation remove septum and transfer liquid to a sterile tube by pipetting. While pipetting the suspension use extreme care to remove as much liquid as possible without siphoning any particulates. Centrifuge suspension at

6000 x g for 5 minutes to create a cell pellet. Using a sterile pipet, discard supernatant. Resuspend pellet in 10 mL of fresh sterile CMCB without the particulates containing 15% glycerol and dispense in 1.5-mL aliquots in microcentrifuge tubes. Freeze at -20°C (short term storage) or -80°C (long term storage). *Note:* Aliquots of suspension may be plated to determine CFU concentration as described in Section 11.1. It is advisable to verify the *B. thetaiotaomicron* culture by using commercially available test kits (e.g., Vitek® or API®)

7.9 PCR-grade water (e.g., OmniPur water from VWR EM-9610 or equivalent). Water must be DNA/DNase free.

7.10 Isopropanol or ethanol, 95%, for flame-sterilization

7.11 AE Buffer, pH 9.0 (e.g., Qiagen 19077 or equivalent) (*Note:* pH 8.0 is acceptable)

Composition:

10 mM Tris-Cl (chloride)

0.5 mM EDTA (Ethylenediaminetetraacetic acid)

7.12 Salmon DNA/extraction buffer

7.12.1 Composition:

Stock Salmon testes DNA (10 µg/mL) (Section 7.3)

AE Buffer (Section 7.12)

7.12.2 Preparation of stock Salmon testes DNA: Each bottle of Salmon DNA contains a specific number of units. Note the units. Add an equal volume of PCR-grade water to dissolve the Salmon testes DNA, and stir, using a magnetic stir bar at low to medium speed, until dissolved (2-4 hours if necessary). The solution at this point will be equivalent to 50 µg Salmon testes DNA/mL. Dilute using PCR grade water to a concentration of ~ 10 µg/mL. Determine concentration of Salmon testes DNA stock by OD₂₆₀ reading in a spectrophotometer. An OD₂₆₀ of 1 is approximately equal to 50 µg/mL (one Unit). This is your Salmon testes DNA stock solution. Unused portion may be aliquoted and frozen at -20°C.

Note: For example, if the bottle contains 250 mg of DNA, using sterilized scissors and sterilized forceps, cut a piece of DNA to weigh approximately 20 mg (approximately 304 Units), and place in a sterile weigh boat. After weighing, place the DNA into a sterile 50 mL tube and add 20 mL PCR grade water. Cap tightly, and resuspend by 2-4 hours of gentle rocking. The concentration should be about 1 mg/mL. Remove three, 10 µL aliquots and dilute each to 1 mL with PCR grade water. Check absorbance (OD₂₆₀) and calculate DNA using the assumption 1 Unit DNA is equal to 1 OD₂₆₀, which is then equivalent to 50 µg/mL DNA. Adjust this stock to 10 µg/mL based on calculated initial concentration of 1 mg/mL by diluting with PCR grade water. Aliquot portions of the adjusted DNA stock and freeze.

7.12.3 Dilute Salmon testes DNA stock with AE buffer to make 0.2 µg/mL Salmon DNA/extraction buffer. Extraction buffer may be prepared in advance and stored at 4° C for a maximum of 1 week.

Note: Determine the total volume of Salmon DNA/extraction buffer required for each day or week by multiplying volume (600 µL) × total number of samples to be analyzed including controls, water samples, and calibrator samples. For example, for 18 samples, prepare enough Salmon/DNA extraction buffer for 24 extraction tubes (18÷6 = 3, therefore, 3 extra tubes for water sample filtration blanks (method blanks) and 3 extra

tubes for calibrator samples). Note that the number of samples is divided by 6 because you should conduct one method blank for every 6 samples analyzed. Additionally, prepare excess volume to allow for accurate dispensing of 600 µL per tube, generally 1 extra tube. Thus, in this example, prepare sufficient Salmon/DNA extraction buffer for 24 tubes plus one extra. The total volume needed is $600 \mu\text{L} \times 25 \text{ tubes} = 15,000 \mu\text{L}$. Dilute the Salmon testes DNA working stock 1:50, for a total volume needed ($15,000 \mu\text{L} \div 50 = 300 \mu\text{L}$) of 10 µg/mL Salmon testes DNA working stock. The AE buffer needed is the difference between the total volume and the Salmon testes DNA working stock. For this example, $15,000 \mu\text{L} - 300 \mu\text{L} = 14,700 \mu\text{L}$ AE buffer needed.

7.13 Bleach solution: 10% v/v bleach (or other reagent that hydrolyzes DNA) (used for cleaning work surfaces)

7.14 Sterile water (used as rinse water for work surface after bleaching)

7.15 TaqMan® Universal PCR Master Mix (*e.g.*, Applied Biosystems 4304437 or equivalent)
Composition:

AmpliTaq Gold® DNA Polymerase

AmpErase® UNG

dNTPs with dUTP

Passive Reference 1 (ROX™ fluorescent dye)

Optimized buffer components (KCl, Tris, EDTA, MgCl₂)

7.16 Bovine serum albumin (BSA), fraction V powder *e.g.*, Sigma B-4287 or equivalent)
Dissolve in PCR-grade water to a concentration of 2 mg/mL.

7.17 Primer and probe sets: Primer and probe sets may be purchased from commercial sources. Primers should be desalted, probes should be HPLC purified.

7.17.1 Bacteroidales primer and probe set (References 17.4 and 17.6):

Forward primer: 5'-GGGGTTCTGAGAGGAAGGT

Reverse primer: 5'-CCGTCATCCTCACGCTACT

TaqMan® probe: 5'-FAM-CAATATTCTCACTGCTGCCTCCCGTA-TAMRA

7.17.2 Salmon DNA primer and probe set (Reference 17.4):

Forward primer: 5'-GGTTTCCGCAGCTGGG

Reverse primer (Sketa 22) : 5'-CCGAGCCGTCCTGGTC

TaqMan® probe: 5'-FAM-AGTCGCAGGCCGGCACCGT-TAMRA

7.17.3 Preparation of primer/probes: Using a micropipettor with aerosol barrier tips, add PCR grade water to the lyophilized primers and probe from the vendor to create stock solutions of 500 µM primer and 100 µM probe and dissolve by extensive vortexing. Pulse centrifuge to coalesce droplets. Store stock solutions at -20°C.

7.18 DNA extraction tubes

Note: It is recommended that tube preparation be performed in advance of water sampling and DNA extraction procedures.

Prepare 1 tube for each sample, and 1 extra tube for every 6 samples (*i.e.*, for method blank) and minimum of 3 tubes per week for calibrator samples. Weigh $0.3 \pm 0.01 \text{ g}$ of glass beads (Section

6.4) and pour into extraction tube. Seal the tube tightly, checking to make sure there are no beads on the O-ring of the tube. Check the tube for proper O-ring seating after the tube has been closed. Autoclave at 121°C (15 PSI) for 15 minutes.

7.19 Purified, RNA-free and spectrophotometrically quantified and characterized *B. thetaiotaomicron* genomic DNA preparations used to generate a standard curve (see Section 11.2).

7.20 RNase A (*e.g.*, Sigma Chemical #R-6513) or equivalent

7.20.1 Composition:

RNase A

Tris-Cl

NaCl

7.20.2 Dissolve 10 mg/mL pancreatic RNase A in 10 mM Tris-Cl (pH 7.5), 15 mM NaCl. Heat to 100°C for 15 minutes. Allow to cool to room temperature. Dispense into aliquots and store at - 20°C. For working solution, prepare solution in PCR-grade water at concentration of 5 µg/µL.

7.21 DNA extraction kit (Gene-Rite #K102-02C-50 DNA-EZ® RW02 or equivalent)

8.0 Sample Collection, Handling, and Storage

8.1 Sampling procedures are briefly described below. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these procedures should not be analyzed.

8.2 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge or bank adjacent to surface water. Composite samples should not be collected, since such samples do not display the range of values found in individual samples. The sampling depth for surface water samples should be 6-12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to provide head space for proper mixing before analyses.

8.3 Storage Temperature and Handling Conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are tightly closed and are not totally immersed in water during transit.

8.4 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 hours between collection and initiation of filtration. *This section will be updated based on results of holding time study.*

9.0 Quality Control

- 9.1** Each laboratory that uses Method B is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records sample storage and retrieval. Additional recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 17.2.
- 9.2** **Media sterility check** — The laboratory should test media sterility by incubating one unit (tube or plate) from each batch of medium (5% BAP/ CMCB) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check every day that samples are analyzed.
- 9.3** **Method blank** (water sample filtration blank) — Filter a 50 mL volume of sterile PBS before beginning the sample filtrations. Remove the funnel from the filtration unit. Using two sterile or flame-sterilized forceps, fold the filter on the base of the filtration unit and place it in an extraction tube with glass beads as described in Section 7.20. Extract as in Section 11.5. Absence of fluorescence growth curve during PCR analysis of these samples (reported as “0” on Smart Cycler® and “undetermined” on ABI model 7900) indicates the absence of contaminant target DNA (see Data Quality Acceptance, below). **Prepare one method blank filter for every six samples.**
- 9.4** **Positive controls** — The laboratory should analyze positive controls to ensure that the method is performing properly. Fluorescence growth curve (PCR amplification trace) with an appropriate cycle threshold (C_T) value during PCR indicates proper method performance. On an ongoing basis, the laboratory should perform positive control analyses every day that samples are analyzed. In addition, controls should be analyzed when new lots of reagents or filters are used.
- 9.4.1** Calibrator samples will serve as the positive control. Analyze as described in Section 11.0. *Note:* Calibrator samples contain the same amount of extraction buffer and starting amount of Salmon DNA as the test samples, hence *B. thetaiotaomicron* calibrator DNA extracts (Section 11.3) will be used as a positive control for both Bacteroidales and SPC qPCR assays.
- 9.4.2** If the positive control fails to exhibit the appropriate fluorescence growth curve response, check and/or replace the associated reagents, and reanalyze. If positive controls still fail to exhibit the appropriate fluorescence growth curve response, prepare new calibrator samples and reanalyze (see Section 9.7).
- 9.5** **No template controls** — The laboratory should analyze “No Template Controls” (NTC) to ensure that the Master Mix PCR reagents are not contaminated. On an ongoing basis, the laboratory should perform NTC analyses every day that samples are analyzed. If greater than one-third of the NTC reactions for a PCR master mix exhibit true positive logarithmic amplification traces with C_T values below 45 (not from chemical degradation of probe with linear kinetics that exhibit rising baseline) or if any one NTC reaction has a C_T value lower than 35, the analyses should be repeated with new Master Mix working stock preparations.
- 9.6** **DNA standards and standard curves** — Purified, RNA-free and spectrophotometrically quantified *B. thetaiotaomicron* genomic DNA should be prepared as described in Section 11.2. Based on reported values for its size, the weight of a single *B. thetaiotaomicron* genome can be estimated to be ~6.7 fg and there are six 16S rRNA gene copies per genome in this species (<http://cmr.jcvi.org/tigr-scripts/CMR/shared/Genomes.cgi>). The concentration of 16S rRNA gene copies per μ L in the standard *B. thetaiotaomicron* genomic DNA preparation can be determined from this information and from its spectrophotometrically determined total DNA concentration by the formula:

Concentration of 16S rRNA gene copies per μ L =	Total DNA concentration (fg/ μ L)	\times	6 16S rRNA gene copies
	6.7 fg/genome		genome

A composite standard curve should be generated from multiple analyses of serial dilutions of this DNA standard using the Bacteroidales primer and probe assay and subjected to linear regression analysis as described in Section 12.2. From that point on, it is recommended that additional standard curves be generated from duplicate analyses of these same diluted standard samples with each new lot of TaqMan® master mix reagents or primers and probes to demonstrate comparable performance by these new reagents. The r^2 values from regressions of these curves should ideally be 0.99 or greater. Comparable performance is assessed by their slopes and y-intercepts which should be consistent with those from the initial composite analyses (e.g., within the 95% confidence range of the average values). Note: The 95% confidence ranges for these parameters in the initial composite standard curve can be generated using the Regression Analysis Tool which can be accessed from the “Data Analysis” selection under the “Tools” menu in Excel. Subsequent regressions can be performed by plotting the data using the Chart Wizard in Excel and using the “add trend line” selection in the Chart menu and “display equation on chart” selection under Options to obtain slope and y-intercept values as illustrated in Section 12.2.

In the event that the slope value from a subsequent standard curve regression is outside of the acceptance range, the diluted standards should be re-analyzed. If this difference persists, new working stocks of the reagents should be prepared and the same procedure repeated. If the differences still persist, the amplification factor values used for calculations of target cell numbers, as described in Section 8, should be modified based on the new slope values. If the slopes are within acceptance range but Y-intercepts are not within acceptance range of this previous average, new serial dilutions of the DNA standard should be prepared and analyzed as described above.

- 9.7 Calibrator samples** — The cell concentration of each cultured *B. thetaiotaomicron* stock suspension used for the preparation of calibrator sample extracts should be determined as described in Section 11.1. A minimum of nine calibrator sample extracts should initially be prepared from three different freezer-stored aliquots of these stocks as described in Section 11.2. Dilutions of each of these calibrator sample extracts equivalent to the anticipated dilutions of the test samples used for analysis (e.g., 1, 5 and/or 25 fold) should be analyzed with the Bacteroidales primer and probe assay. The average and standard deviation of the C_T values from these composite analyses should be determined. From that point on, a minimum of three fresh calibrator sample extracts should be prepared from an additional frozen aliquot of the same stock cell suspension at least weekly and preferably daily before analyses of each batch of test samples. Dilutions of each new calibrator sample extract equivalent to the initial composite dilutions (e.g., 1, 5 and/or 25 fold) should be analyzed using the Bacteroidales primer and probe assay. The average C_T value from these analyses should not be significantly different from the laboratory's average values from analyses of the initial calibrator sample extracts from the same stock cell suspension (i.e., not greater than three standard deviations). If these results are not within this acceptance range, new calibrator extracts should be prepared from another frozen aliquot of the same stock cell suspension and analyzed in the same manner as described above. If the results are still not within the acceptance range, the reagents should be checked by the generation of a standard curve as described in Section 9.6.

- 9.8 Salmon DNA Sample Processing Control (SPC) sequence analyses** — While not essential, it is good practice to routinely prepare and analyze standard curves from serial dilutions of Salmon

DNA working stocks in a manner similar to that described for the *B. thetaiotaomicron* DNA standards in Section 9.6. At this time rRNA gene operon copy numbers per genome have not been reported in the literature for the salmon species *O. keta*. Therefore log-transformed total DNA concentration values or dilution factor values can be substituted for target sequence copy numbers as the x-axis values in these plots and regression analyses.

In general, target DNA concentrations in test samples can be calculated as described in Section 12. However, the Salmon DNA PCR assay results for each test sample's 5 fold dilution should be within 3 C_T units of the mean of the 5 fold diluted calibrator (and/or method blank) sample results. Higher C_T values may indicate significant PCR inhibition or poor DNA recovery possibly due to physical, chemical, or enzymatic degradation. Repeat the Bacteroidales and Salmon DNA PCR assays of any samples whose 5 fold dilution exhibits a Salmon DNA PCR assay C_T value greater than 3 C_T units higher than the mean of the calibrator sample results using a 5 fold higher dilution (net dilution: 25 fold) of the extracts. The Bacteroidales PCR result from assaying the original 5-fold dilution of the sample can be accepted if its Salmon DNA assay C_T value is lower than that of the corresponding 25 fold dilution of the sample. This pattern of results is indicative of poor recovery of total DNA in the extract rather than PCR inhibition. The poor DNA recovery is compensated for by the calculation method. Contrarily, if the Salmon PCR assay C_T value of the 25-fold dilution of the sample is lower than that of the 5 fold dilution of the sample, then the Bacteroidales PCR assay result from the 25 fold dilution of the sample is considered more accurate. However the Bacteroidales PCR results should be reported as questionable if the Salmon DNA assay's result is still not within 3 C_T units of the mean C_T result of the 25 fold dilution of the three calibrators.

10.0 Calibration and Standardization of Method-Related Instruments

- 10.1** Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.
- 10.2** Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check columns for breaks.
- 10.3** Spectrophotometer should be calibrated each day of use using OD calibration standards between 0.01 - 0.5. Follow manufacturer instructions for calibration.
- 10.4** Micropipettors should be calibrated at least annually and tested for accuracy on a weekly basis. Follow manufacturer instructions for calibration.
- 10.5** Follow manufacturer instructions for calibration of real-time PCR instruments.

11.0 Procedure

Note: *B. thetaiotaomicron* cell suspensions (Section 11.1), and *B. thetaiotaomicron* DNA standards (Section 11.2) may be prepared in advance. Calibrator samples (Section 11.3) should be prepared at least weekly.

- 11.1** Preparation of *B. thetaiotaomicron* cell suspensions for DNA standards and calibrator samples
 - 11.1.1** Thaw a *B. thetaiotaomicron* (ATCC #29741) stock culture (Section 7.8) and streak for isolation on CDC anaerobe 5% sheep blood agar (BAP) plates. Incubate plates at 35.0°C ± 0.5°C for 24 ± 2 hours under anaerobic conditions.
 - 11.1.2** Pick an isolated colony of *B. thetaiotaomicron* from the BAP plates and suspend in 1 mL of sterile phosphate buffered saline (PBS) and vortex.

11.1.3 Use 10 µL of the 1-mL suspension of *B. thetaiotaomicron* to inoculate a 10-mL CMCB tube. Place the inoculated tube and one uninoculated tube (sterility check) in an anaerobe chamber with a Gas Pak and incubate at 35.0°C ± 0.5°C for 24 ± 2 hours. *Note:* It is advisable to verify that the selected colony is *Bacteroides* by using biochemical test strips or individual biochemical tests.

11.1.4 After incubation remove septum and transfer liquid to a sterile tube by pipetting. While pipetting the suspension use extreme care to remove as much liquid as possible without siphoning any of the chopped meat.

11.1.5 Centrifuge the CMCB containing *B. thetaiotaomicron* for 5 minutes at 6000 × g.

11.1.6 Aspirate the supernatant and resuspend the cell pellet in 10 mL PBS.

11.1.7 Repeat the two previous steps twice and suspend final *B. thetaiotaomicron* pellet in 5 mL of sterile PBS. Label the tube as *B. thetaiotaomicron* undiluted stock cell suspension, noting cell concentration after determination with one of the following steps.

11.1.8 Determination of calibrator sample cell concentrations based on one of the three options below.

- Option 1: Spectrophotometric absorbance – Remove three 0.1-mL aliquots of undiluted cell suspension and dilute each with 0.9 mL of PBS (10⁻¹ dilution). Read absorbance at 595 nm in spectrophotometer against PBS blank (readings should range from 0.05 to 0.3 OD). Calculate cells/mL (Y) in undiluted cell suspension using the formula below where X is the average 595 nm spectrophotometer reading.

$$Y = (1 \times 10^9 \text{ cells / mL} \times X) / 0.19$$

- Option 2: Hemocytometer counts – Serially dilute 10 µL of undiluted cell suspension with PBS to 10⁻¹, 10⁻², and 10⁻³ dilutions and determine cell concentration of 10⁻² or 10⁻³ dilutions in a hemocytometer or Petroff Hauser counting chamber under microscope.
- Option 3: plating – *Note:* BAP plates should be prepared in advance if this option is chosen. For enumeration of the *B. thetaiotaomicron* undiluted cell suspension, dilute and inoculate according to the following.
 - A) Mix the *B. thetaiotaomicron* undiluted cell suspension by shaking or vortexing the 5 mL tube a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted cell suspension to 99 mL of sterile PBS use care not to aspirate any of the particulates in the medium, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “A”. A 1.0-mL volume of dilution “A” is 10⁻² mL of the original undiluted cell suspension.
 - B) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “A” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “B”. A 1.0-mL volume of dilution “B” is 10⁻³ mL of the original undiluted cell suspension.
 - C) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “B” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “C”. A 1.0-mL volume of dilution “C” is 10⁻⁴ mL of the original undiluted cell suspension.
 - D) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “C” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25

times. This is cell suspension dilution “D”. A 1.0-mL volume of dilution “D” is 10^{-5} mL of the original undiluted cell suspension.

- E) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “D” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “E”. A 1.0-mL volume of dilution “E” is 10^{-6} mL of the original undiluted cell suspension.
- F) Prepare BAP (Section 7.6). Ensure that agar surface is dry. *Note:* To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.
- G) Each of the following will be conducted in triplicate, resulting in the evaluation of nine spread plates:
 - Pipet 0.1 mL of dilution “C” onto surface of BAP plate [10^{-5} mL (0.00001) of the original cell suspension].
 - Pipet 0.1 mL of dilution “D” onto surface of BAP plate [10^{-6} mL (0.000001) of the original cell suspension].
 - Pipet 0.1 mL of dilution “E” onto surface of BAP plate [10^{-7} mL (0.0000001) of the original cell suspension].
- H) For each spread plate, use a sterile bent glass rod or spreader to distribute inoculum over surface of medium by rotating the dish by hand or on a rotating turntable.
- I) Allow inoculum to absorb into the medium completely
- J) Invert plates and incubate in an anaerobe chamber at $35.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 4 hours.
- K) Count and record number of colonies per plate. Refer to the equation below for calculation of undiluted cell suspension concentration.

$$\text{CFU/mL}_{\text{undiluted}} = \frac{\text{CFU}_1 + \text{CFU}_2 + \dots + \text{CFU}_n}{V_1 + V_2 + \dots + V_n}$$

Where:

CFU/mL _{undiluted}	=	<i>B. thetaiotaomicron</i> CFU/mL in undiluted cell suspension
CFU	=	number of colony forming units from BAP plates yielding counts within the ideal range of 30 to 300 CFU per plate
V	=	volume of undiluted sample in each BAP plate yielding counts within the ideal range of 30 to 300 CFU per plate
n	=	number of plates with counts within the ideal range

Table 1. Example Calculations of *B. thetaiotaomicron* Undiluted Cell Suspension Concentration

Examples	CFU / plate (triplicate analyses) from BAP plates			<i>B. thetaiotaomicron</i> CFU / mL in undiluted suspension ^a
	10 ⁻⁵ mL plates	10 ⁻⁶ mL plates	10 ⁻⁷ mL plates	
Example 1	275, 250, 301	30, 10, 5	0, 0, 0	(275+250+30) / (10 ⁻⁵ +10 ⁻⁵ +10 ⁻⁶) = 555 / (2.1 x 10 ⁻⁵) = 26,428,571 = 2.6 x 10⁷ CFU / mL
Example 2	TNTC ^b , TNTC, TNTC	TNTC, 299, TNTC	12, 109, 32	(299+109+32) / (10 ⁻⁶ +10 ⁻⁷ +10 ⁻⁷) = 440 / (1.2 x 10 ⁻⁶) = 366,666,667 = 3.7 x 10⁸ CFU / mL

^aCell concentration is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

^b Too numerous to count

- 11.1.9** Divide remainder of undiluted cell suspension (approximately 5 mL) into 6×0.5 mL aliquots for DNA standard preparations and $100-200 \times 0.01$ mL (10 μ L) aliquots for calibrator samples and freeze at -20°C . Note: Cell suspension should be stirred while aliquoting. It is also recommended that separate micropipettor tips be used for each 10 μ L aliquot transfer and that the volumes in each tip be checked visually for consistency.

11.2 Preparation of *B. thetaiotaomicron* genomic DNA standards

- 11.2.1** Remove two 0.5 mL undiluted *B. thetaiotaomicron* cell suspensions (Section 11.1.8) from freezer and thaw completely.
- 11.2.2** Transfer cell suspensions to extraction tubes with glass beads.
- 11.2.3** Tightly close the tubes, making sure that the O-rings are seated properly.
- 11.2.4** Place the tubes in bead beater and shake for 60 seconds at the maximum rate (5000 rpm).
- 11.2.5** Remove the tubes from the bead beater and centrifuge at $12,000 \times g$ for one minute to pellet the glass beads and debris.
- 11.2.6** Using a 200 μ L micropipettor, transfer 350 μ L of supernatants to sterile 1.7 mL microcentrifuge tubes. Recover supernatants without disrupting the glass beads at the tube bottom.
- 11.2.7** Centrifuge crude supernatants at $12,000 \times g$ for 5 minutes and transfer 300 μ L of clarified supernatant to clean, labeled 1.7 mL low retention microcentrifuge tubes, taking care not to disturb the pellet.
- 11.2.8** Add 1 μ L of 5 μ g/ μ L RNase A solution to each clarified supernatant, mix by vortexing and incubate at 37°C for 1 hour.

Note: Sections 11.2.9 - 11.2.15 may be substituted with an optional method if a DNA purification procedure is chosen other than the DNA-EZ purification kit. In such a case, manufacturer instructions should be followed rather than these steps. Continue onward from Section 11.2.16.

- 11.2.9** Add 0.6 mL of binding buffer solution from a DNA-EZ purification kit to each of the RNase A-treated extracts and mix by vortexing. *Note:* In general, a minimum concentration of 5×10^8 cells is required for this step.

- 11.2.10** If using the DNA-EZ purification kit, perform the following steps. Insert one DNAsure™ column from the DNA-EZ purification kit into a collection tube (also provided with kit) for each of the two extracts. Transfer the extract and binding buffer mixtures from Section 11.2.9 to a DNAsure™ column and collection tube assembly and centrifuge for 1 min at 12,000 × g.
- 11.2.11** Transfer each of the DNAsure™ columns to new collection tubes. Discard previous collection tubes and collected liquid.
- 11.2.12** Add 500 µL EZ-Wash Buffer from the DNA-EZ purification kit to each of the DNAsure™ columns and centrifuge at 12,000 × g for 1 minute. Discard the liquid in the collection tube.
- 11.2.13** Repeat Section 11.2.12.
- 11.2.14** Transfer each of the DNAsure™ columns to a clean, labeled 1.7-mL low retention microcentrifuge tube and add 50 µL of DNA elution buffer to each column. Centrifuge for 30 seconds at 12,000 × g. Repeat this procedure again to obtain a total DNA eluate volume of ~100 µL from each column.
- 11.2.15** Pool the two eluates to make a total volume of approximately 200 µL.
- 11.2.16** Transfer entire purified DNA eluate volume from each column to a clean and sterile microcuvette for UV spectrophotometer and read absorbance at 260 and 280 nM. (Note: the cuvette should be blanked with DNA elution buffer before reading sample.) If necessary, the sample may be diluted with elution buffer to reach minimum volume that can be accurately read by the spectrophotometer (see manufacturer's recommendation) however, this may reduce the DNA concentration to a level that can not be accurately read by the spectrophotometer. If available, readings can be taken of 2 µL aliquots of the sample with a NanoDrop™ Spectrophotometer.
- 11.2.17** Sample is acceptable as a standard if ratio of OD₂₆₀/OD₂₈₀ readings is ≥ to 1.75.
- 11.2.18** Calculate total DNA concentration in sample by formula:
$$\text{OD}_{260} \text{ reading} \times 50 \text{ ng}/\mu\text{L DNA}/1(\text{OD}_{260})$$
- 11.2.19** Transfer sample back to labeled 1.7 mL non-retentive microcentrifuge tube and store at -20°C.

11.3 Extraction of *B. thetaiotaomicron* calibrator samples

- 11.3.1** A minimum of three calibrator extracts should be prepared during each week of analysis.

Note: To prevent contamination of water sample filtrates and filter blanks, this procedure should be performed at a different time, and, if possible, in a different work station than the procedures in Sections 11.1 and 11.2 above and Section 11.5 below.

- 11.3.2** Remove one tube containing a 10 µL aliquot of *B. thetaiotaomicron* undiluted stock cell suspension (Section 11.1.8) from the freezer and allow to thaw completely on ice.

Note: If using BioBalls for calibrators add a single BioBall™ to each of 3, 100 mL sterile PBS blanks, filter (Section 11.4), and extract according to (Section 11.5).

- 11.3.3** While cell stock is thawing, using sterile (or flame-sterilized) forceps, place one polycarbonate filter (Section 6.14) in an extraction tube with glass beads. Prepare one filter for each sample to be extracted in this manner.

- 11.3.4** Dispense 590 µL of Salmon DNA/extraction buffer (Section 7.12) into three extraction tubes with glass beads and filters. Prepare one tube for each of the three calibrator samples to be extracted in this manner. Label tubes appropriately.
- 11.3.5** When *B. thetaiotaomicron* suspension has thawed, transfer 990 µL AE buffer (Section 7.11) to the 10 µL *B. thetaiotaomicron* stock cell tube and mix thoroughly by vortexing. Pulse microcentrifuge tube briefly (1-2 sec.) to coalesce droplets in tube.
- 11.3.6** Immediately after vortexing the *B. thetaiotaomicron* suspension, spot 10 µL onto the polycarbonate filter in a calibrator sample tube.
- 11.3.7** Tightly close the tube, making sure that the O-ring is seated properly.
- 11.3.8** Repeat Sections 11.3.6 and 11.3.7 for the other two filters to prepare three calibrator samples with *B. thetaiotaomicron*.
- 11.3.9** Place the tubes in the mini bead beater and shake for 60 seconds at the maximum rate (5000 rpm).
- 11.3.10** Remove the tubes from the mini bead beater and centrifuge at 12,000 × g for one minute to pellet the glass beads and debris.
- 11.3.11** Using a 200 µL micropipettor, transfer the crude supernatant to the corresponding labeled sterile 1.7-mL microcentrifuge tube. Transfer 400 µL of supernatant without disrupting the debris pellet or glass beads at the tube bottom. *Note:* The filter will normally remain intact during the bead beating and centrifugation process. Generally, 400 µL of supernatant can be easily collected. Collect an absolute minimum of 100 µL of supernatant.
- 11.3.12** Centrifuge at 12,000 × g for 5 minutes and transfer clarified supernatant to a clean, labeled 1.7 mL tube, taking care not to disturb the pellet. *Note:* Cell pellet may not be visible in calibrator samples.
- 11.3.13** Label the tubes as undiluted or 1x *B. thetaiotaomicron* calibrator extracts. Label additional 1.7 mL tubes for 5 and 25 fold dilutions. In appropriately labeled tubes, using a micropipettor, add a 50 µL aliquot of each 1x *B. thetaiotaomicron* calibrator extract and dilute each with 200 µL AE buffer (Section 7.11) to make 5 fold dilutions. In appropriately labeled tubes using a micropipettor, add a 50 µL aliquot of each 5 fold dilution and dilute each with 200 µL AE buffer to make 25 fold dilutions. Store all diluted and undiluted extracts in refrigerator.
- 11.3.14** If the extracts are not analyzed immediately, refrigerate. For long term storage, freeze at -80°C.

11.4 Water sample filtration

Note: It is required that one water sample filtration blank (method blank) be prepared for every 6 water samples (Section 9.3) analyzed by the same procedure.

- 11.4.1** Place a polycarbonate filter (Section 6.16) on the filter base, and attach the funnel to the base so that the membrane filter is now held between the funnel and the base.
- 11.4.2** Shake the sample bottle vigorously 25 times to distribute the bacteria uniformly, and measure 100 mL of sample into the funnel.
- 11.4.3** Filter 100 mL of water sample. After filtering the sample, rinse the sides of the funnel with 20-30 mL of sterile PBS (Section 7.4) and continue filtration until all liquid has been pulled through the filter. Turn off the vacuum and remove the funnel from the filter base.

- 11.4.4** Label an extraction tube with glass beads (Section 7.19) to identify water sample. Leaving the filter on the filtration unit base, fold into a cylinder with the sample side facing inward, being careful to handle the filter only on the edges, where the filter has not been exposed to the sample. Insert the rolled filter into the labeled extraction tube with glass beads. Prepare one filter for each sample filtered in this manner.
- 11.4.5** Cap the extraction tube. Tubes may be frozen at -20°C or -80°C until analysis.
- 11.5** DNA extraction of water sample filtrates and method blanks
- 11.5.1** Using a 1000 µL micropipettor, dispense 590 µL of the Salmon DNA/extraction buffer (Section 7.13) to each labeled extraction tube with glass beads containing water sample or method blank filters from Section 11.4.4. Extract the method blank last.
- 11.5.2** Tightly close the tubes, making sure that the O-ring is seated properly.
- 11.5.3** Place the tubes in the mini bead beater and shake for 60 seconds at the maximum rate (5000 rpm).
- 11.5.4** Remove the tubes from the mini bead beater and centrifuge at 12,000 × g for 1 minute to pellet the glass beads and debris. *Note:* To further prevent contamination, a new pair of gloves may be donned for steps 11.5.5, 11.5.6, and 11.5.7 below.
- 11.5.5** Using the 200 µL micropipettor, transfer 400 µL of the supernatant to a corresponding labeled sterile 1.7-mL microcentrifuge tube, taking care not to pick up glass beads or sample debris (pellet). *Note:* The filter will normally remain intact during the bead beating and centrifugation process. Generally, 400 µL of supernatant can easily be collected. Collect an absolute minimum of 100 µL of supernatant. Recover the method blank supernatant last.
- 11.5.6** Centrifuge crude supernatant from Section 11.5.5 for 5 minutes at 12,000 × g. Transfer 350 µL of the clarified supernatant to another 1.7-mL tube, taking care not to disturb pellet. *Note:* Pellet may not be visible in water samples. Recover the method blank supernatant last.
- 11.5.7** Label the tubes from Section 11.5.6 as undiluted or 1x water sample extracts with sample identification. These are the water sample filter extracts. Also label tubes for method blanks. Label additional 1.7 mL tubes for 5 and 25 fold dilutions. In appropriately labeled tubes, using a micropipettor, add a 50 µL aliquot of each 1x water sample extract and dilute each with 200 µL AE buffer (Section 7.11) to make 5 fold dilutions. In appropriately labeled tubes using a micropipettor, add a 50 µL aliquot of each 5 fold dilution and dilute each with 200 µL of AE buffer to make 25 fold dilutions. Dilute the method blank supernatant last.
- 11.5.8** Store all diluted and undiluted extracts in refrigerator. *Note:* Use of 5 fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while analyses of 25 fold dilutions may unnecessarily sacrifice sensitivity.
- 11.5.9** If the extracts are not analyzed immediately, refrigerate. For long term storage, freeze at -80°C.
- 11.6** Preparation of qPCR assay mix
- 11.6.1** To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach to contact the work surface for a minimum of

15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes. After decontamination, discard gloves and replace with new clean pair.

- 11.6.2** Remove primers and probe stocks from the freezer and verify that they have been diluted to solutions of 500 µM primer and 100 µM probe.
- 11.6.3** Prepare working stocks of *B. thetaiotaomicron*, and Salmon DNA (Sketa 22) primer/probe mixes by adding 10 µL of each *B. thetaiotaomicron* or Salmon DNA (Sketa 22) primer stock and 4 µL of respective probe stock to 676 µL of PCR grade water, and vortex. Pulse centrifuge to create a pellet. Use a micropipettor with aerosol barrier tips for all liquid transfers. Transfer aliquots of working stocks for single day use to separate tubes and store at 4° C.
- 11.6.4** Using a micropipettor, prepare assay mix of the *B. thetaiotaomicron*, and Salmon DNA (Sketa 22) reactions in separate, sterile, labeled 1.7 mL microcentrifuge tubes as described in **Table 2**.

Table 2. PCR Assay Mix Composition

Reagent	Volume/Sample (multiply by # samples to be analyzed per day)
Sterile H ₂ O	1.5 µL
BSA	2.5 µL
TaqMan® master mix	12.5 µL
Primer/probe working stock solution	3.5 µL

^a 16 samples plus and 1 extra - see Section 11.7

Note: This will give a final concentration of 1 µM of each primer and 80 nM of probe in the reactions. Prepare sufficient quantity of assay mix for the number of samples to be analyzed per day including calibrators and negative controls plus at least two extra samples. Prepare assay mixes each day before handling of DNA samples.

- 11.6.5** Vortex the assay mix working stocks; then pulse microcentrifuge to coalesce droplets. Return the primer/probe working stocks and other reagents to the refrigerator.

11.7 ABI 7900 and ABI 7500 (non-Fast) qPCR assay preparation (Reference 17.1)

Transfer 20 µL of mastermix containing Bacteroidales primers and probe to wells of a 96-well PCR reaction tray equal to number of samples to be analyzed including calibrator and negative control samples. (*Note:* The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else).

Example: For the analysis of 18 recreational water samples, 51 wells will require the addition of assay mix with *B. thetaiotaomicron* primers and probe as follows: 18 samples, two replicates each (36), 3 method blanks, two replicates each (6), 3 no template controls, one replicate each (3), and 3 calibrators, 2 replicates each (6) = 51 wells.

- 11.7.1** Transfer 20 µL of mastermix containing *B. thetaiotaomicron* primers and probe to wells of a 96 well PCR reaction tray equal to number of samples to be analyzed including calibrator and negative control samples. Pipette into the center of the wells, taking care to not touch the well walls with the pipette tip. (*Note:* The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else).

- 11.7.2** When all wells are loaded, cover tray loosely with aluminum foil or plastic wrap and transfer to refrigerator or directly to the PCR preparation station used for handling DNA

samples (Section 6.1). *Note:* All aliquoting of assay mixes to reaction trays must be performed each day before handling of DNA samples.

- 11.7.3** Transfer 5 µL each of the diluted (or undiluted) DNA extracts of method blanks and water samples (Section 11.5.7), and then corresponding dilutions of calibrator samples (Section 11.3.13), to separate wells of the PCR reaction tray containing Bacteroidales assay mix. *Note:* Record positions of each sample.
- 11.7.4** Transfer 5 µL each of the diluted (or undiluted) DNA extracts of method blanks and water samples (Section 11.5.7), and corresponding diluted calibrator samples (Section 11.3.13) to separate wells of the PCR reaction tray containing Salmon DNA assay mix. Record positions of each sample.
- 11.7.5** Transfer 5 µL aliquots of AE buffer to wells of PCR reaction tray containing *B. thetaiotaomicron* master mix that are designated as no-template controls. Record positions of these samples.
- 11.7.6** Tightly cap wells of PCR reaction tray containing samples or cover tray and seal tightly with optical adhesive PCR reaction tray.
- 11.7.7** Run reactions in ABI 7900 or ABI 7500 (non-Fast) sequence detector. For platform-specific operation see Appendix A.

11.8 Smart Cycler® qPCR assay preparation

- 11.8.1** Label 25 µL Smart Cycler® tubes with sample identifiers and assay mix type (see Section 11.8.8 for examples) or order tubes in rack by sample number and label rack with assay mix type. It is recommended that the unloaded open Smart Cycler® tubes be irradiated under ultraviolet light in a PCR cabinet for 15 minutes. Using a micropipettor, add 20 µL of the Bacteroidales assay mix (Section 11.6.5) to labeled tubes. Avoid generating air bubbles, as they may interfere with subsequent movement of the liquid into the lower reaction chamber. The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else. Repeat procedure for Salmon DNA (Sketa 22) assay mix.
- 11.8.2** Add 5 µL of AE buffer to no-template control tubes and close tubes tightly.
- 11.8.3** Close the other PCR tubes loosely and transfer to refrigerator or directly to the PCR preparation station used for handling DNA samples (Section 6.1). *Note:* All aliquoting of assay mixes to reaction tubes must be performed each day before handling of DNA samples.
- 11.8.4** Transfer 5 µL each of the diluted (or undiluted) DNA extracts of method blanks and water samples (Section 11.5.7), and then corresponding dilutions of calibrator samples (Section 11.3.13) to tubes containing *B. thetaiotaomicron* and Salmon DNA (Sketa 22) mixes. Close each tube tightly after adding sample. Load the method blank PCR assays last. Label the tube tops as appropriate.
- 11.8.5** When all Smart Cycler® tubes have been loaded, place them in a Smart Cycler® centrifuge, and spin for 2-4 seconds.
- 11.8.6** Inspect each tube to verify that the sample has properly filled the lower reaction chamber. A small concave meniscus may be visible at the top of the lower chamber, but no air bubbles should be present. (If the lower chamber has not been properly filled, carefully open and reclose the tube, and re-centrifuge). Transfer the tubes to the thermocycler.
- 11.8.7** For platform-specific operation see Appendix B.
- 11.8.8** Suggested sample analysis sequence for Smart Cycler®

Example: For analyses on a single 16-position Smart Cycler®, calibrator samples and water samples will need to be analyzed in separate runs and a maximum of 6 water samples (or 2 replicates of 3 samples) can be analyzed per run, as described in **Tables 3** and **4**, below.

Table 3. Calibrator PCR Run - 14 Samples

Sample Description*	Quantity	PCR Assay Master Mix
3 Calibrators × 2 replicates (1, 5, or 25 fold dilution)	6	<i>B. thetaiotaomicron</i>
3 Calibrators × 2 replicates (1, 5, or 25 fold dilution)	6	Salmon DNA
No template controls (reagent blanks)	2	<i>B. thetaiotaomicron</i>

* Diluted equivalently to the water samples

Table 4. Water Sample PCR Run - 14 Samples

Sample Description*	Quantity	PCR Assay Master Mix
Water samples, (1, 5, or 25 fold dilution)	6	<i>B. thetaiotaomicron</i>
Method blank, (1, 5, or 25 fold dilution)	1	<i>B. thetaiotaomicron</i>
Water samples, (1, 5, or 25 fold dilution)	6	Salmon DNA
Method blank, (1, 5, or 25 fold dilution)	1	Salmon DNA

* Use of five-fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while 25 fold dilutions analyses may unnecessarily sacrifice sensitivity.

12.0 Data Analysis and Calculations

12.1 Overview: This section describes a method for determining the ratio of the target sequence quantities recovered from a test (water filtrate) sample compared to those recovered from identically extracted calibrator samples using an arithmetic formula, referred to as the $\Delta\Delta C_T$ comparative cycle threshold calculation method. The $\Delta\Delta C_T$ relative quantitation method also normalizes these ratios for differences in total DNA recovery from the test and calibrator samples using qPCR analysis C_T values for a reference sequence provided by the SPC DNA. These ratios are converted to absolute measurements of total target sequence quantities recovered from the test samples by multiplying them by the average total number of target sequences that are normally recovered from a constant number of target organisms that are added to all calibrator samples. The complete procedure for determining target sequence quantities in water samples is detailed below.

12.2 Generation of C_T value vs. target sequence number standard curve: Three replicate serial dilutions of a DNA standard, prepared as described in Section 9.6 should be prepared to give concentrations of 4×10^4 , 4×10^3 , 4×10^2 , 2×10^2 and 1×10^2 16S rRNA gene sequences per $5 \mu\text{L}$ (the standard sample volume added to the PCR reactions) and the replicates of each dilution pooled. *Note:* A procedure for the determinations of target sequence concentrations in the DNA standard is also provided in Section 9.6.

Aliquots of each of these dilutions should be stored at 4°C in low retention microcentrifuge tubes and can be reused for repeated qPCR analyses. QPCR analyses of these diluted standards using the Bacteroidales primer and probe assay should be performed at least three separate times in duplicate. C_T values from these composite analyses should be subjected to regression analysis

against the log₁₀-transformed target sequence numbers per reaction as described in Section 9.6 with example results illustrated in **Figure 1**.

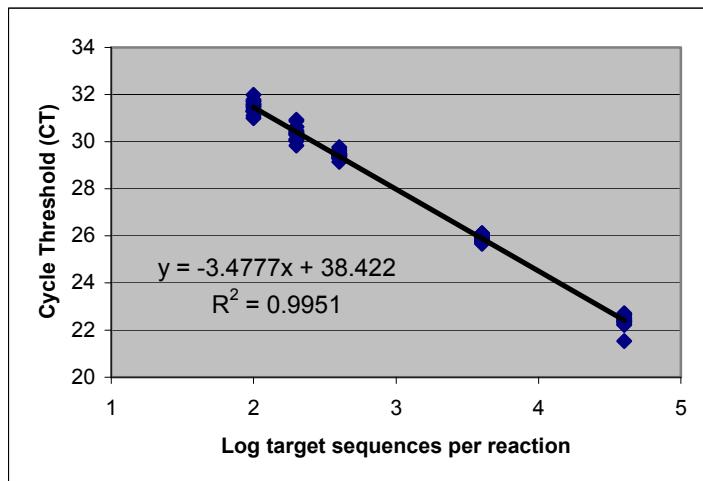


Figure 1: Example plot and regression analysis of qPCR analysis cycle threshold values vs. log target sequences per reaction.

Amplification factors (AF) used for subsequent comparative cycle threshold calculations (Section 12.4) can be calculated from the slope value of this curve by the formula $AF = 10^{(1 / (-\text{slope}))}$. An example calculation using the slope value from the example regression is shown below:

$$AF = 10^{(1 / 3.4777)} = 1.94$$

12.3 Calculation of average target sequence recovery in calibrator sample extracts: A minimum of nine calibrator sample extracts should initially be prepared from at least three different freezer-stored aliquots of each cultured *B. thetaiotaomicron* stock suspension that is prepared as described in Section 11.1. Dilutions of each of these calibrator sample extracts equivalent to the anticipated dilutions of the test samples used for analysis (*e.g.*, 1, 5 and/or 25 fold) should be analyzed with the Bacteroidales primer and probe assay. The average C_T value from these analyses should be interpolated on the standard curve generated from the DNA standard (Section 12.2) to determine the average number of target sequences per 5 μ L of extract used in the reactions. An example calculation using an average calibrator extract C_T value of 25.21 is shown below:

$$\text{Average calibrator target sequences}/5 \mu\text{L extract} = 10^{(25.21-38.44) / -3.477} = 6382$$

Note: Four places should be kept from this calculation for the following calculation (*i.e.*, 6382.6983). Dividing this value by 5 gives the average calibrator target sequences/ μ L extract which can be multiplied by the total volume of the extract at the applicable dilution level (*e.g.*, 600 μ L of original extract volume \times 5 = 3000 μ L for a 5 fold diluted sample) to determine the average total quantity of target sequences recovered in the calibrator sample extracts. An example of this calculation using the average calibrator target sequences/reaction value determined immediately above is shown below:

$$\text{Average target sequences} = \frac{6382 \text{ target sequences}}{\text{x } 3000 \mu\text{L total extract volume}}$$

$$\begin{array}{rcl} \text{Calibrator extract} & & 5 \mu\text{L extract} \\ & = & 3,829,619 \end{array}$$

12.3.1 Calculation of average target sequence recovery per Bacteroidales cell in calibrator sample extracts (optional):

In previous studies, measurements of recreational water quality by the qPCR method have been reported as Bacteroidales calibrator cell equivalents (Reference 17.7). Calculations performed to obtain this reporting unit are identical to those described in Section 12.4 except that the ratios of target sequences obtained as described in Sections 12.4.1 - 12.4.4 are multiplied by the estimated quantities of Bacteroidales cells added to the calibrator samples rather than by the average target sequences recovered per calibrator extract as described in Section 12.4.5. While the use of this reporting unit is no longer recommended because of the false impression it creates concerning the cell concentration detection limit of the qPCR method, it still may be of value for comparing previous results with those of future studies.

A prerequisite for making such comparisons is to determine that the ratio of the numbers of target sequences recovered in calibrator sample extracts to the numbers of Bacteroidales cells added to these samples is consistent in different studies. For the purpose of determining this ratio, it is recommended that the cell concentrations of the cultured *B. thetaiotaomicron* stock suspension used for the preparation of calibrator samples in each laboratory be determined by at least two of the three alternative methods described in Section 11.1 to establish the degree of agreement between these enumeration methods. The recommended quantity of cells that are added to each calibrator sample is 100,000. Dividing the average target sequences recovered per calibrator extract (determined as described in Section 12.3) by this number provides the ratio of target sequence numbers to cell numbers. An example of this calculation using the average target sequences/ calibrator extract value determined in Section 12.3 is shown below:

$$\text{Ratio of target sequence numbers to cell numbers} = 3,829,619 / 100,000 = 38.29$$

- 12.4 Calculation of target sequence quantities in test samples:** A minimum of three fresh calibrator samples should be extracted and analyzed at least on a weekly basis and preferably on a daily basis in association with each batch of water sample filtrates. QC analysis of the analysis results from these calibrator extracts should be performed as described in Section 9.7. C_T values from the Bacteroidales target sequence and salmon DNA Sample Processing Control (SPC) qPCR assays for both the calibrator and test samples are used in the $\Delta\Delta C_T$ comparative cycle threshold calculation method to determine the ratios of target sequences in the test and calibrator sample extracts and these ratios are converted to absolute measurements of total target sequence quantities recovered from the test samples as specified below and illustrated in **Table 5**.

- 12.4.1** Subtract the SPC assay C_T value ($C_{T,SPC}$) from the target assay C_T value ($C_{T,target}$) for each calibrator sample extract to obtain ΔC_T value and calculate the average ΔC_T value for these calibrator samples.
- 12.4.2** Subtract the SPC assay C_T value ($C_{T,SPC}$) from the target assay C_T value ($C_{T,target}$) for each water sample filtrate extract to obtain ΔC_T values for each of these test samples. *Note:* If multiple analyses are performed on these samples, calculate the average ΔC_T value.
- 12.4.3** Subtract the average ΔC_T value for the calibrator samples from the ΔC_T value (or average ΔC_T value) for each of the test samples to obtain $\Delta\Delta C_T$ values.

12.4.4 Calculate the ratio of the target sequences in the test and calibrator samples using the formula: $AF^{\Delta(-\Delta C_T)}$, where AF = amplification factor of the target organism qPCR assay, determined as described in Section 12.2.

12.4.5 Multiply the ratio of the target sequences in the test and calibrator samples by the average target sequences/calibrator extract, determined as described in Section 12.2, to determine absolute numbers of total target sequences/ extract for each of the test samples. *Note:* This calculation can be applied without modification to the analyses of diluted extracts if both the test sample and calibrator extracts are equally diluted and equal volumes of diluted extracts are analyzed.

Table 5. Example Calculations (Amplification factor = 1.94)

Target sequences in Sample	Sample Type	$C_{T,target}$	$C_{T,SPC}$	ΔC_T	$\Delta\Delta C_T$	Measured Target Sequences in Test Sample Extract $(1.94^{-\Delta\Delta C_T} \times \text{avg. target sequences}/\text{calibrator})$
3,829,619	Calibrator	25.21	30.45	-5.24	----	----
Unknown	Test	32.53	30.65	1.88	7.12	$0.0089 \times 3,829,619 = 34,198$

12.4.6 The geometric mean of the measured target sequences and associated coefficients of variation in multiple water samples can be determined from individual sample C_T values using the following procedure:

- 12.4.6.1** Use ΔC_T value for each individual water sample extract and the mean calibrator ΔC_T value to calculate the measured target sequence numbers in each water sample extract, as described in Section 12.4.
- 12.4.6.2** Calculate the \log_{10} of the measured target sequence numbers in each water sample ($\log N$)
- 12.4.6.3** Calculate the mean (M) and standard deviation (S) from the values of $\log N$ obtained in the previous step for all of the water sample extracts.
- 12.4.6.4** Calculate the geometric mean as 10^M .
- 12.4.6.5** The implied coefficient of variation (CV) is calculated, based on the log normal distribution, as the square root of $10^{V/0.434} - 1$, where $V = S^2$.

12.5 Reporting Results: Where possible, duplicate analyses should be performed on each sample. Report the results as Bacteroidales (16S rRNA gene) target sequences per volume of water sample filtered.

13.0 Sample Spiking Procedure

[This section will be updated after validation study]

14.0 Method Performance

[This section will be updated after validation study]

14.1 Accuracy (Bias)

The 16S rRNA gene of Bacteroidales, which contains the target sequence amplified and detected by the primers and probe of the Bacteroidales qPCR assay, is present in multiple copies in the genome of the Bacteroidales order. The number of 16S rRNA gene copies per genome has not been ascertained for all of the Bacteroidales order which the Bacteroidales qPCR assay can amplify and detect. Hence, the use of *B. thetaiotaomicron* cells as a calibrator for relative quantitation purposes and *B. thetaiotaomicron* DNA as a standard for absolute quantitation purposes creates an inherent bias potentially affecting the accuracy of the quantitation depending on the species composition of the Bacteroidales present in a water sample.

The Bacteroidales qPCR method makes the assumption that the Bacteroidales cells present in the water sample contain the same number of genomes and 16S rRNA gene copies as the *B. thetaiotaomicron* calibrator cells which have been grown in culture media to a late-log or stationary phase in batch culture. This assumption has not been validated and if untrue may bias the accuracy of the results in a systematic manner. Bacterial cells contain more than one complete genome during growth and cell division phases of their life cycle. The number of genomic copies depends on their growth rate and cell division time. More than one cell division cycle is often required to complete replication of the genome during rapid log-phase growth and cell division. The 16S rRNA genes are replicated early in the cell cycle maximizing the number of 16S rRNA gene copies present in cells during log phase growth. This facilitates the enhanced ribosome production needed for the high level of protein translation needed during rapid cell growth and division.

[This section will be updated after validation study]

15.0 Pollution Prevention

- 15.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.
- 15.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

16.0 Waste Management

- 16.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.
- 16.2 Samples, reference materials, and equipment known or suspected to have viable Bacteroidales attached or contained must be sterilized prior to disposal.
- 16.3 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

17.0 References

- 17.1** Anonymous. 1997. User Bulletin #2. ABI Prism 7700 Sequence Detection System. Foster City, CA, Applied Biosystems.
- 17.2** Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). *Microbiological Methods for Monitoring the Environment: Water and Wastes*, EPA-600/8-78-017. Cincinnati, OH: U.S. Environmental Protection Agency, 1978.
- 17.3** Dick, L.K., and K.G. Field. 2004. Rapid Estimation of Numbers of Fecal Bacteroidetes by use of a Quantitative PCR Assay for 16S rRNA Genes. *Appl. Environ. Microbiol.* 70:5695-5697.
- 17.4** Haugland, R.A., S.C. Siefring, L.J. Wymer, K.P. Brenner, and A. P. Dufour. 2005. Comparison of *Enterococcus* Density Measurements by Quantitative Polymerase Chain Reaction and Membrane Filter Analysis at Two Freshwater Recreational Beaches. *Water Research* 39:559-568.
- 17.5** Siefring, S., M. Varma, E Atikovic, L. Wymer, and R.A. Haugland. 2007. Improved Real-Time PCR Assays for the Detection of Fecal Indicator Bacteria in Surface Waters With Different Instrument and Reagent Systems. *J. Water Health* 6:225-237.
- 17.6** US EPA. 2010. Method A: Enterococci in Water by Taqman® Quantitative Polymerase Chain Reaction (qPCR) Assay. EPA-821-R-10-004. April 2010.
- 17.7** Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P. & Dufour, A.P. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming associated gastrointestinal illness. *Environmental Health Perspectives* 114: 24-28.

18.0 Acronyms

$\Delta\Delta C_T$	comparative cycle threshold calculation method
AF	amplification factor
BAP	CDC anaerobe tryptic soy agar with 5% sheep blood
BSA	bovine serum albumin
CC	cubic centimeter
CMCB	chopped meat carbohydrate broth
CFU	colony forming units
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
NTC	no template control
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SPC	sample processing control
TNTC	too numerous to count
UV	ultraviolet (light)

Appendix A:
ABI 7900 Sequence Detector Operation

Appendix A - ABI 7900 and ABI 7500 (non-Fast) Sequence Detector Operation

1.0 ABI 7900 Sequence Detector Operation

- 1.0.1 Turn on the ABI Model 7900 sequencer and then the computer. Launch the **SDS 2.2.2** software program by double clicking on its icon on the computer desktop or from the Computer Programs menu. The computer will establish communication with the 7900 instrument and if the connection is successful, the software will display the Connected icon in the status bar when a plate document is opened.
- 1.0.2 Under File menu, select **New**.
- 1.0.3 In resulting **New Document** window that appears, change container selection from **384 well clear plate** to **96 well clear plate** using drop down menu. Accept default selections of **Absolute Quantification** and **Blank Template**. Click **OK** to display a new plate document.
- 1.0.4 Click, hold and drag mouse over all PCR reaction tray wells containing samples in upper left window. Selected wells will be outlined with a bold line and their position numbers should appear in the results table in the lower left window. To unselect wells, repeat above process while holding down control key.
- 1.0.5 Above right hand window, click on **Setup** tab.
- 1.0.6 Click on **Add Detector** button at the bottom of the setup screen.
- 1.0.7 Click on **New** in the pop-up window that appears. Another pop-up window will appear. Under **Name**, type in a name for the detector that will be used by this method (*e.g.*, "Method B"). Under **Group** select Default. Under **Reporter** select FAM. Under **Quencher** select TAMRA. Click on **OK** to close second pop-up window. This step only needs to be performed before the initial analysis run of the method. The detector that is named is selected in all subsequent analysis runs as indicated in step 1.7)
- 1.0.8 In pop-up window that was opened in step 1.6, select the desired detector under Names menu (*e.g.*, Method B) and click on **Copy to Plate Document** button. Click on **Done** button to return to setup screen.
- 1.0.9 Click on **Use** box next to FAM detector in right hand window. This box should become marked with an X. Name and color code for FAM detector should appear in each of the selected well positions in the upper left window and a data column for this detector should be created for each of the selected well positions in the results table in the lower left window.
- 1.0.10 Click on **Instrument** tab right hand window.
- 1.0.11 In instrument screen, change **sample volume** to 25 μL and choose **9600 emulation**.
- 1.0.12 Still in instrument screen, click on **Connect**, then click on **Open/Close** button in lower right hand "Real Time" window to open PCR reaction tray holder door on instrument.
- 1.0.13 Insert PCR reaction tray with prepared reactions in holder.
- 1.0.14 Click on **Open/Close** button to close PCR reaction tray holder door on instrument.
- 1.0.15 Click on **Start** button in lower right hand "Real Time" window to start thermal cycling in instrument.
- 1.0.16 Name run file at prompt.

- 1.0.17 At termination of the run, instrument-calculated cycle threshold values should automatically appear for each well position and detector entry in the lower left hand results table window.
- 1.0.18 At termination of the run success complete, choose **Analysis Settings** from the toolbar. In that box enter a value for the **Manual Ct Threshold** (see 1.18). Click on **OK**. Click on **Analyze** from the toolbar. You should see Ct values in the Results Table.
- 1.0.19 The instrument-selected threshold line is indicated by the bold red line on the plot and the value is listed below the window. This can be manually adjusted by either click-hold and dragging the line up or down or entering new values below. *Note:* Based on results thus far with the instrument, the threshold value can be adjusted from the default value of 0.2 to 0.03, however, this should be done only if the threshold line remains above the background values (seen before the growth curves) for all of the samples.
- 1.0.20 Calculated C_T values for each of the sample tray positions in the lower left hand “Results Table” will automatically be updated following adjustments of the threshold line. Once the threshold is adjusted to the desired level, select “Print Report” under the “File” menu. Check or uncheck desired report items by clicking on their associated boxes and the click on “Print” button. *Note:* Minimum report should have both detector boxes, *i.e.*, “FAM” checked which will show C_T values for all selected tray positions for this detector.
- 1.0.21 Export data by clicking on **File** from the toolbar. From the drop down menu choose **Export**. In the box you will see **Look in:** and here you choose a directory to send the exported file too. Click on **Export**. **Save changes to document?** will appear, click on **Yes**. Click **OK**.

1.1 ABI 7500 (non-Fast) Sequence Detector Operation

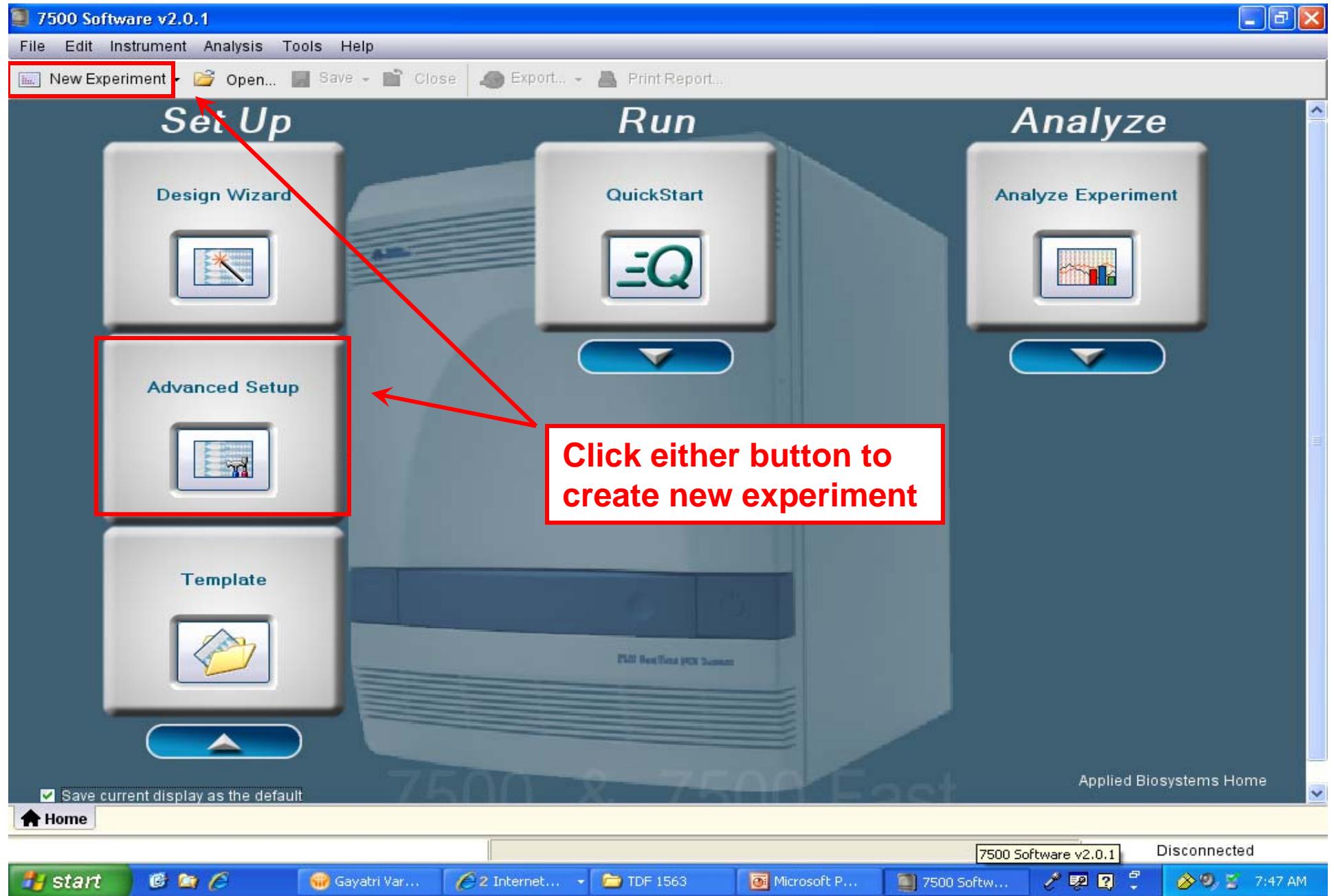
- 1.1.1 Turn on the ABI Model 7500 sequencer and then the computer. Launch the software program by double clicking on its icon on the computer desktop or from the Computer Programs menu. The computer will establish communication with the 7500 instrument. See “How to Set Up a New Experiment Using the ABI 7500” for screen shots. (*Note:* All screen shots were taken for Method A, a qPCR method for *Enterococcus*. Red instructional text in boxes in the screen shots has been changed for Method B. Please carefully read the text here to verify the correct parameters for this method.)
- 1.1.2 Click on either the **New Experiment** or **Advanced Setup** button to create a new experiment, which will pull up the Experiment Menu.
- 1.1.3 From the **Setup** menu, select **Experimental Properties** to select the experiment type and give the experiment a name. Enter the experiment name in the asterisked box.
 - 1.1.3.1 Click on **7500 (96 Wells)** to select the instrument type. *Note:* This protocol is not designed for the 7500 Fast.
 - 1.1.3.2 Scroll down to access more experiment properties options. Click on **Quantitation – Standard Curve** to select the experiment type
 - 1.1.3.3 Click on **TaqMan® Reagents** to select the reagents used.
- 1.1.4 From the **Setup** menu, select **Plate Setup**. Click on the **Define Targets and Samples** tab to define the reporter-quencher dye for each target and also to enter the sample identifications (*e.g.*, Bact 5X).
 - 1.1.4.1 Click on **Add New Target** and enter the name of the target. Under the Reporter heading, click on the drop down menu to select the reporter dye

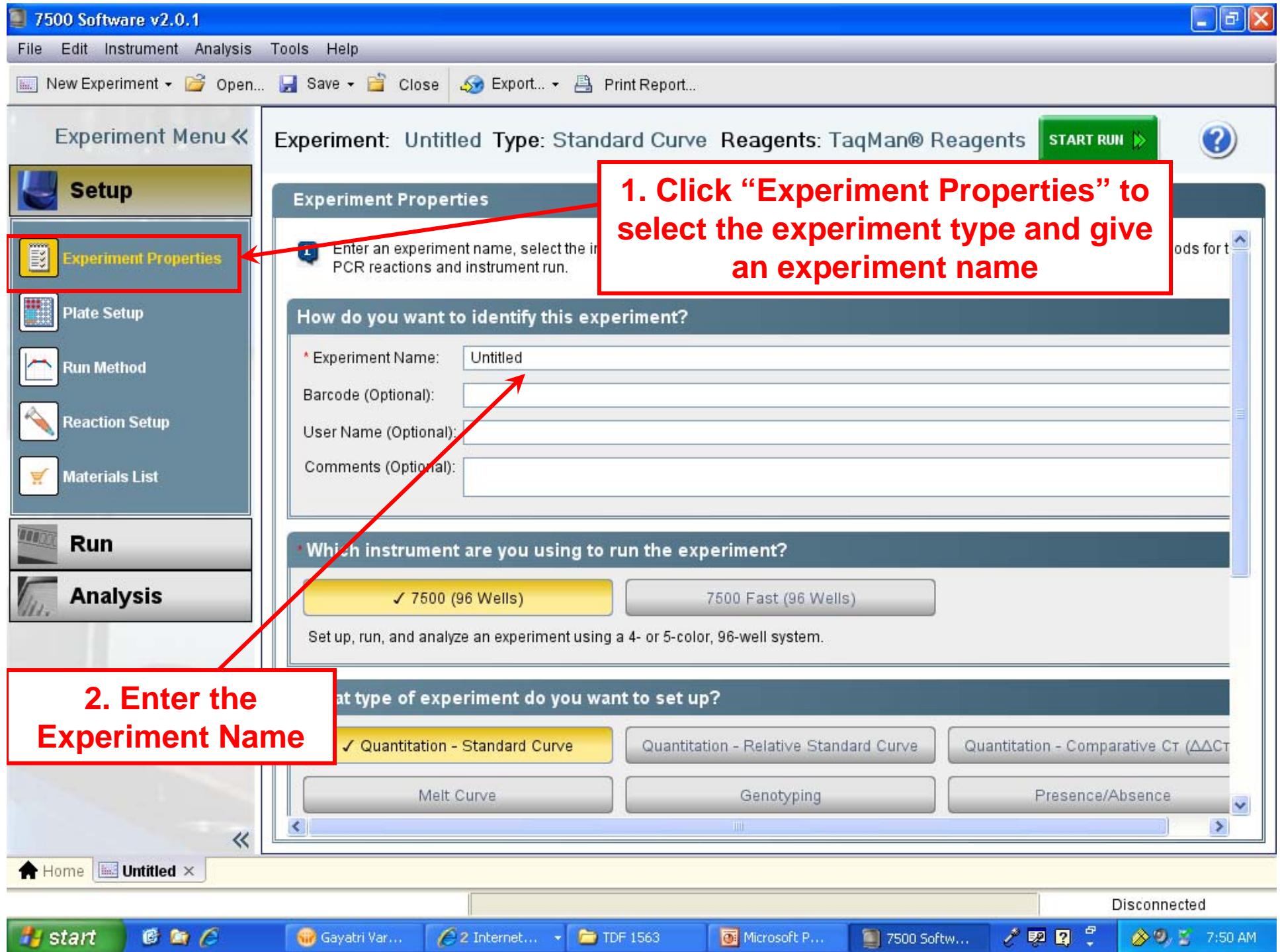
- FAM.** Under the Quencher heading, click on the drop down menu to select **TAMRA** as the quencher.
- 1.1.4.2** Repeat the process in Section 1.1.4.1 to add more than one target, *e.g.*, Sketa 5x.
- 1.1.4.3** In the **Define Samples** section, click on **Add New Sample** and enter the sample name in the Sample 1 box.
- 1.1.4.4** Click on the **Assign Targets and Sample** tab to assign the Target, sample ID and sample type to the wells.
- 1.1.4.5** Drag the mouse over the desired cells to assign the Target, Task and Sample.
- 1.1.4.6** While the cells are highlighted, in the **Assign target(s) to the selected wells** section, check the box under **Assign** for the appropriate Target, then under **Task**, select **U** for unknown, **S** for standard or **N** for negative control.
- 1.1.4.7** In the **Assign sample(s) to the selected wells** section, check the box under **Assign** to label the highlighted cells as Sample 1 (*e.g.*).
- 1.1.4.8** Repeat the process in Section 1.1.4.4, dragging the mouse over the appropriate cells, and then assigning them as Unknowns, Standards or Negative Controls, as appropriate. Note that for the Standards, in the **Assign target(s) to the selected wells** section, if there are 4 different values, each cell will need to be highlighted separately, and a quantity (*e.g.* 40000.0) typed in the **Quantity** box.
- 1.1.4.9** Click on **Print Report** at the top of the screen to print a plate layout for loading master mix and sample extracts.
- 1.1.4.9.1** Select **Plate Layout** option by clicking on the box next to it.
- 1.1.4.9.2** Click **Print Report** to print the plate layout.
- 1.1.5** From the **Setup** menu, select **Run Method** to set up the thermo cycling profile.
- 1.1.5.1** In the **Graphical View** tab, change the **Reaction Volume Per Well** from the default 50 μ L to 25 μ L by typing in 25.
- 1.1.5.2** Under **Cycling Stage** section, change the **Number of Cycles** to 45.
- 1.1.5.3** Check that the default settings of Temperature and Time for the two Holding Stages and the Cycling Stage are correct. Specifically, these should be: Holding Stage 1: 50.0°C for 2:00 minutes, Holding Stage 2: 95.0°C for 10:00 minutes, and Cycling Stage: 95.0°C for 0:15 seconds. (*Note:* The purpose of Holding Stage 1 is to eliminate potential PCR carryover products in the reactions using the Amp-Erase® UNG enzyme provided in the TaqMan® Universal PCR Master Mix. The purpose of Holding Stage 2 is to deactivate the Amp-Erase® UNG enzyme.)
- 1.1.5.4** The second step of the Cycling Stage is defaulted at 60.0°C for 1 minute – change the time to 2:00 minutes. (*Note:* This sets the detection of the fluorescence signal to occur at the end of the second step in each cycle.)
- 1.1.5.5** Click on **Save** at the top of the screen to save the run before actually running the experiment.

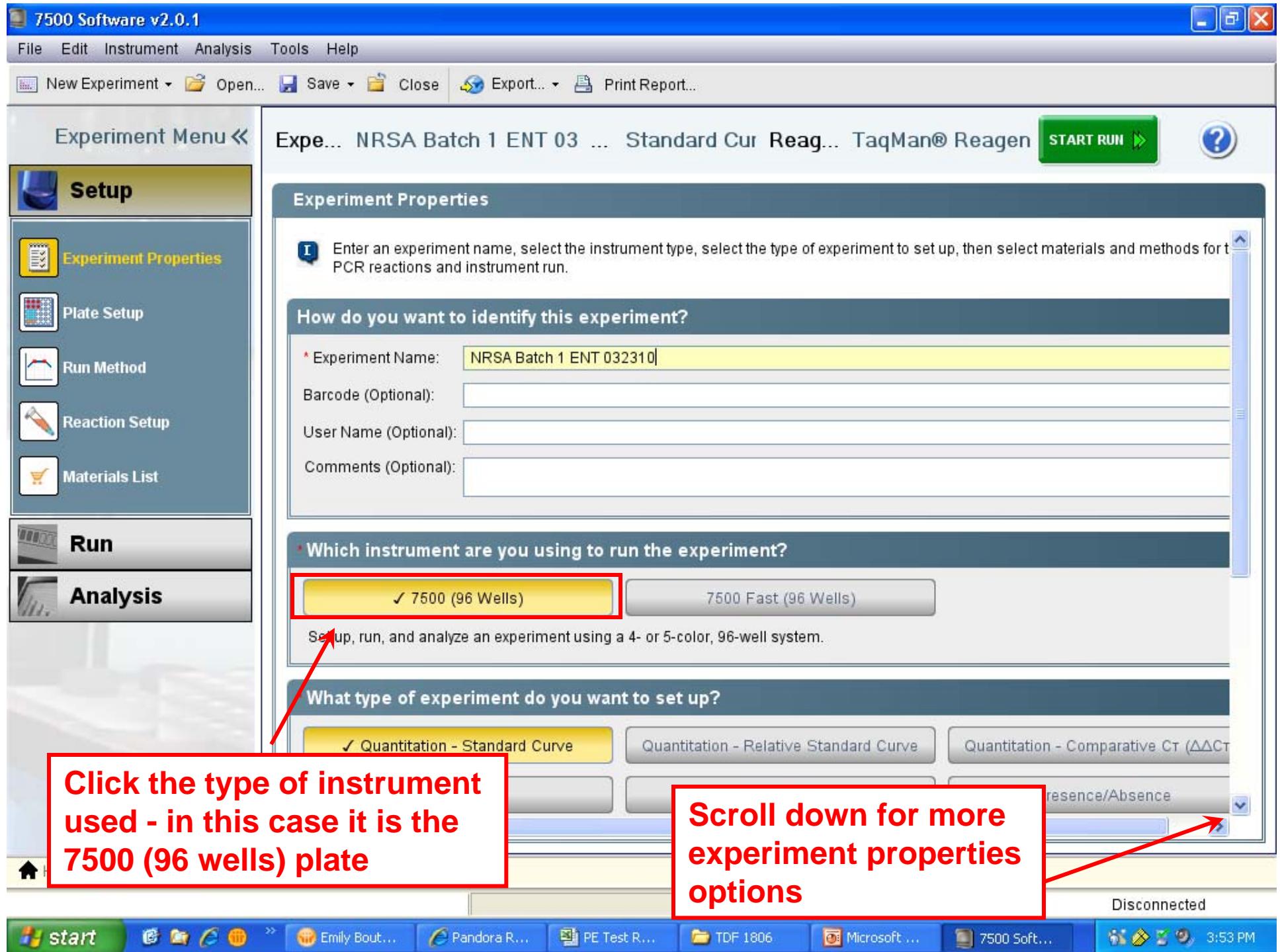
- 1.1.5.6** The Save screen will open so that you may select the folder to save the Run Data File. Type in the Experiment Name (if you did not already do so at the beginning of the Experiment Setup) and click on **Save**. If you had previously typed in an Experiment Name and there is no change, click on **Save**. (Note: You must save to the hard drive (rather than a flash drive).)
- 1.1.6** From the **Experiment** menu, select **Run** to monitor the run in real time.
- 1.1.6.1** The Run Status screen will open - click on **Start Run**. When the run has successfully started, the **Start Run** button will turn change from green to red.
- 1.1.7** When the run has completed, from the **Setup** menu, select **Analysis** to export your results. See “How to Export Results from the ABI 7500 Software” for screen shots.
- 1.1.7.1** The **Analysis Settings for Experiment name** screen will open.
- 1.1.7.2** Under the **C_T Settings** tab, click on the Target desired to be set from Default to Manual Threshold setting (generally, this would be the Bact).
- 1.1.7.3** In the **C_T Settings for Bact** section, uncheck the boxes next to **Use Default Settings**, and **Automatic Threshold**. Enter the desired Threshold (e.g., 0.025). If more than one target is present, highlight the next target (e.g., Sketa) and repeat this step.
- 1.1.7.4** Click on **Apply Analysis Settings** to save the changes.
- 1.1.8** Click on **Export** at the top of the screen to save the experiment results data and to save the selected plots required in the report. Note that the threshold value will have changed on the Amplification plot curve graphic.
- 1.1.8.1** The Export Data screen will open. In the **Select data to export** section, check all of the boxes (*i.e.* Sample Setup, Raw Data, Amplification Data, Results, and Multicomponent Data).
- 1.1.8.2** For **Select one file or separate files**, select **One file** to export all the data into one file with multiple tabs.
- 1.1.8.3** For **Export File Name**, enter the name of the Experimental data file to be exported, and for **File Type**, choose *.xls.
- 1.1.8.4** For **Export File Location**, click on **Browse** to select the folder in which you wish to export your file. Always save your file to the hard drive.
- 1.1.8.5** Click on **Start Export**. When this is completed, an Export Completed screen will open – click on **Close Export Tool** to complete this task.
- 1.1.9** How to interpret your results. See “How to Interpret Results from the ABI 7500” for screen shots.
- 1.1.9.1** After exporting your data, from the **Experiment** menu, select **Analysis**.
- 1.1.9.1.1** From the **Analysis** menu, select **Amplification Plot** to view the results of the selected samples at different plot settings.
- 1.1.9.1.2** Drag the mouse over the desired cells to select the samples to be plotted in the amplification plot.
- 1.1.9.1.3** In the **Amplification Plot** section, in the **Plot Settings** tab, select the **Plot Type** (from the drop down menu) and the **Graph Type** (from the drop down menu).

- 1.1.9.1.4** If the plot requires the display of the Threshold and Baseline, in the **Options** tab, check the boxes next to **Threshold** and **Baseline**. If you want to see the results in tabular format, click on **View Well Table**, and scroll down or sideways for more samples and analysis parameters.
- 1.1.9.1.5** The toolbar above the graph can be used to zoom in and out, print, save the file as *.jpg, and change the plot properties.
- 1.1.9.2** For the Standard Curve, select **Standard Curve** from the **Analysis** menu to view the results of the same sample (assuming they have the same target) and standards in the plot.
- 1.1.9.2.1** In the **Plot Settings** tab, click the drop down menu arrow beside **Target** to select a different target (if the standards were analyzed in more than one target), and click the drop down menu arrow beside **Plot Color** to change the plot color.
- 1.1.9.2.2** The standard curve parameter display below the plot (*i.e.* Target, Slope, Y-Inter(cept), R², and Eff% (percent efficiency) is useful for data analysis.
- 1.1.9.3** For Multicomponent plots, click on **Multicomponent Plot** from the **Analysis** menu to view the variation in the fluorescence of the dyes used in the experiment over the ascending repeat of the 45 thermo cycles based on well, target, or dye.
- 1.1.9.3.1** Click on the drop down menu arrow next to **Plot Color** in the **Plot Settings** tab to select the parameter for the basis of the graph (*e.g.*, well, target, or dye).
- 1.1.9.4** For raw data plots, select **Raw Data Plot** from the **Analysis** menu to view the variation in the emission of fluorescence of the dyes in different filters present over the ascending repeat of the 45 thermo cycles.
- 1.1.9.4.1** In the **Options** tab, drag the pointer on the cycle scale to the desired cycle number to view the fluorescence variation through filters of the samples at that cycle.
- 1.1.9.5** For quality controls, click on **QC Summary** from the **Analysis** menu to view the analysis summary of the samples.
- 1.1.9.6** To view all the plots, click on **Multiple Plots View** from the **Analysis** menu to see all of the plots for the samples in a single window. Each plot will have its own drop down menu as in Sections 1.1.9.1.5..
- 1.1.10** To print the report, click on **Print Report** at the top of the screen.
- 1.1.10.1** Check all of the boxes (*i.e.*, Experimental Summary, Results Summary, Plate Layout, Amplification Plot (3 boxes), Standard Curves, Results Table, and QC Summary).
- 1.1.10.2** Click on **Print Report** at the bottom of the screen

How to Set Up a New Experiment Using the ABI 7500







7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save Close Export... Print Report...

Experiment Menu ↪

Experiment Properties

START RUN ↪

?

Setup

- Experiment Properties
- Plate Setup
- Run Method
- Reaction Setup
- Materials List

Run

Analysis

Experiment: Untitled Type: Standard Curve Reagents: TaqMan® Reagents

Which instrument are you using to run the experiment?

✓ 7500 (96 Wells) 7500 Fast (96 Wells)

Set up, run, and analyze an experiment using a 4- or 5-color, 96-well system.

What type of experiment do you want to set up?

✓ Quantitation - Standard Curve Quantitation - Relative Standard Curve Quantitation - Comparative CT ($\Delta\Delta C_t$)

Melt Curve Genotyping Presence/Absence

Use standards to determine the absolute quantity of target nucleic acid sequence in samples.

Which reagents do you want to use to detect the target sequence?

✓ TaqMan® Reagents SYBR® Green Reagents Other

The PCR reactions contain primers designed to amplify the target sequence and a TaqMan® probe designed to detect amplification of the target sequence.

Which ramp speed do you want to use in the instrument run?

Standard (~ 2 hours to complete a run)

Click to select experiment type

Home Untitled ×

Disconnected

start Fw: qPCR ... Internet... Microsoft P... 7500 Softw... untitled - Paint 8:04 AM

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save Close Export... Print Report...

Experiment Menu ↺

Setup

- Experiment Properties
- Plate Setup
- Run Method
- Reaction Setup
- Materials List

Run

Analysis

Experiment: Untitled Type: Standard Curve Reagents: TaqMan® Reagents **START RUN ➔**

Experiment Properties

* Which instrument are you using to run the experiment?

7500 (96 Wells) 7500 Fast (96 Wells)

Set up, run, and analyze an experiment using a 4- or 5-color, 96-well system.

* What type of experiment do you want to set up?

Quantitation - Standard Curve Quantitation - Relative Standard Curve Quantitation - Comparative CT ($\Delta\Delta Ct$)

Melt Curve Genotyping Presence/Absence

Use standards to determine the absolute quantity of target nucleic acid sequence in samples.

* Which reagents do you want to use to detect the target sequence?

TaqMan® Reagents SYBR® Green Reagents Other

The PCR reactions contain primers designed to amplify the target sequence and a TaqMan® probe designed to detect amplification of the target sequence.

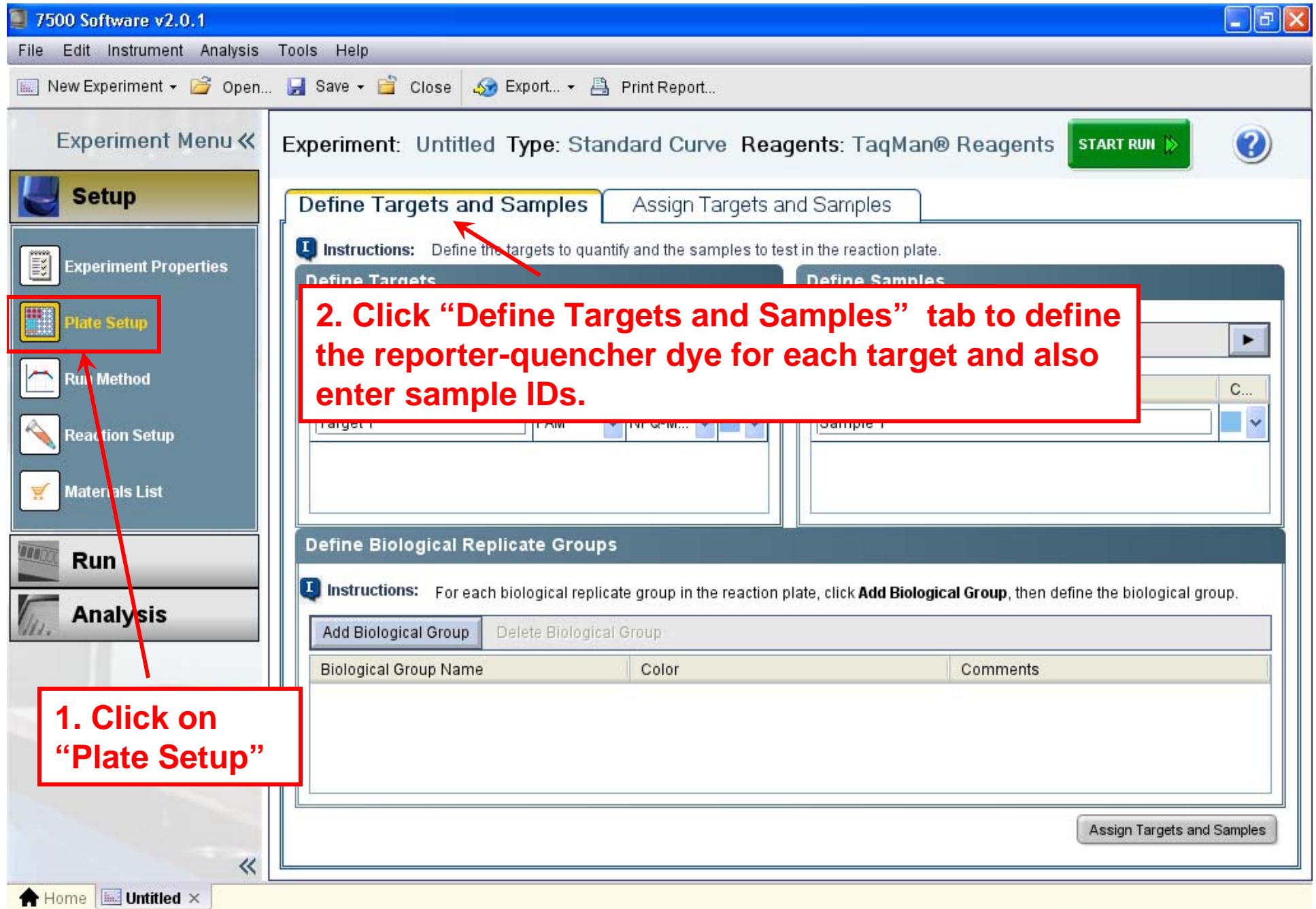
Click on TaqMan® Reagents to select the reagents used

want to use in the instrument run?

Standard (~2 hours to complete a run)

Home Untitled × Disconnected

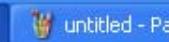
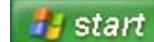
start Fw: qPCR ... Internet... Microsoft P... 7500 Softw... untitled - Paint 8:04 AM



1. Click on
“Plate Setup”

2. Click “Define Targets and Samples” tab to define
the reporter-quencher dye for each target and also
enter sample IDs.

Disconnected



7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save Close Export... Print Report...

Experiment Menu

Setup

Experiment Properties

Plate Setup

Run Method

Reaction Setup

Materials List

Run

Analysis

Experiment: Untitled Type: Standard Curve Reagents: TaqMan® Reagents START RUN ?

Define Targets and Samples

Instructions: Define the targets to quantify in plate.

Define Targets

Add New Target Add Saved Target Save Target ►

Target Name	Reporter	Quencher	C...
Target 1	FAM	NFQ-M...	

Define Biological Replicate Groups

Instructions: For each biological replicate group in the reaction plate, click **Add Biological Group**, then define the biological group.

Add Biological Group Delete Biological Group

Biological Group Name Color Comments

Assign Targets and Samples

1. Click to Add New Target

2. Enter the Target Name

3. Select the Reporter dye FAM from the drop down menu

4. Select the Quencher TAMRA from the drop down menu

Home Untitled × Disconnected

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7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu < Expe... NRSA Batch 1 ENT 03 ... Standard Cur Reag... TaqMan® Reagen START RUN ?

Setup

Experiment Properties
Plate Setup
Run Method
Reaction Setup
Materials List

Run

Analysis

Define Targets and Samples Assign Targets and Samples

I Instructions: Define the targets to quantify and the samples to test in the reaction plate.

Define Targets

Target Name	Reporter	Quencher	C...
ENT 5X	FAM	TAMRA	
SKETA 5X	FAM	TAMRA	

Define Samples

Sample Name	C...
Sample 1	
Sample 2	
Sample 3	

Define Biological Replicate Groups

I Instructions: For each biological replicate group in the reaction plate, click **Add Biological Group**, then define the biological group.

Add Biological Group	Delete Biological Group	
Biological Group Name	Color	Comments

Change this to Bact 5X

You can add more than one target-usually per target DNA (Bact, SKETA)

Assign Targets and Samples

Home Untitled > Emily Bouthiett... Internet Ex... TDF 1806 Microsoft Pow... 7500 Software... 8:47 AM

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>

Emily Bouthiett...

Internet Ex...

TDF 1806

Microsoft Pow...

7500 Software...



7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save Close Export... Print Report...

Experiment Menu < NRSA Batch 1 ENT 03 ... Standard Cur Reag... TaqMan® Reagen START RUN ?

Setup

- Experiment Properties
- Plate Setup
- Run Method
- Reaction Setup
- Materials List

Run

Analysis

Define Targets and Samples Assign Targets and Samples

I Instructions: Define the targets to quantify.

Define Targets

Add New Target Add Saved Target Save Target ►

Target Name	Reporter	Quencher	C...
ENT 5X	FAM	TAMRA	

Select to Add a New Sample

Add New Sample Add Saved Sample ►

Sample Name C...

Sample 1

Define Biological Replicate Groups

I Instructions: For each biological replicate group in the reaction plate, click **Add Biological Group**, then define the biological group.

Add Biological Group Delete Biological Group

Biological Group Name	Color	Comments

Assign Targets and Samples

Home Untitled ×

Disconnected

start Internet Ex... TDF 1806 Microsoft Pow... 7500 Software... 8:24 AM

Experiment Menu <

- Setup**
- Experiment Properties
- Plate Setup
- Run Method
- Reaction Setup
- Materials List

- Run**
- Analysis**

Experiment: Untitled Type: Standard Curve Reagents: TaqMan® Reagents

**Define Targets and Samples** **Assign Targets and Samples**

To set up standards: Click "Define and Set Up Standards."

To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for each target assignment, then

Click to designate the target, sample ID and sample type to the wells

Assign	Target	Task
<input type="checkbox"/>	Target 1	

Mixed Unknown Standard Negative

Define and Set Up Standards

Assign sample(s) to the selected wells.

Assign	Sample
<input type="checkbox"/>	Sample 1

Assign sample(s) of selected well(s) to biological replicates

Layout

View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Wells... View Legend

	1	2	3	4	5	6	7	8	9
A									
B									
C									

Wells: 0 Unknown 0 Standard 0 Negative Control

96 Em

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment NRSA Batch 1 ENT 032310 Ty... Standard Curve Reagen... TaqMan® Reagents START RUN

Define Targets and Samples Assign Targets and Samples

I Instructions: To set up standards: Click "Define and Set Up Standards." To set up unknowns: Select wells, assign target(s), select "U" (Unknown). To set up negative controls: Select wells, assign target(s), then select "N" (Negative Control).

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
<input type="checkbox"/>	ENT 5X	U S N	
<input type="checkbox"/>	SKETA 5X	U S N	

* Mixed U Unknown S Standard N Negative Control

Define and Set Up Standards

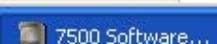
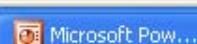
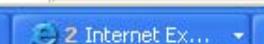
Assign sample(s) to the selected wells.

Assign	Sample
<input type="checkbox"/>	Sample 1
<input type="checkbox"/>	Sample 2

Assign sample(s) of selected well(s) to biological group

Wells: U 0 Unknown S 0 Standard N 0 Negative Control 96 Empty

Drag the mouse over the desired cells to assign the Target, Task and Sample ID



Disconnected

8:58 AM

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open Save Close Export Print Report...

Experiment NRSA Batch 1 ENT 032310 Ty... Start

Define Targets and Samples Assign Targets and

I Instructions: To set up standards: Click "Define and Set Up Standards". To set up unknowns: Select wells, assign target(s), se To set up negative controls: Select wells, assign target(s)

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
<input checked="" type="checkbox"/>	ENT 5X		
<input type="checkbox"/>	SKETA 5X		

* Mixed Unknown Standard Negative Control

Define and Set Up Standards

Assign sample(s) to the selected wells.

Assign	Sample
<input checked="" type="checkbox"/>	Sample 1
<input type="checkbox"/>	Sample 2

Assign sample(s) of selected well(s) to biological group

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Wells View Legend

	1	2	3	4	5	6	7
A	Sample 1						
B							
C							
D							

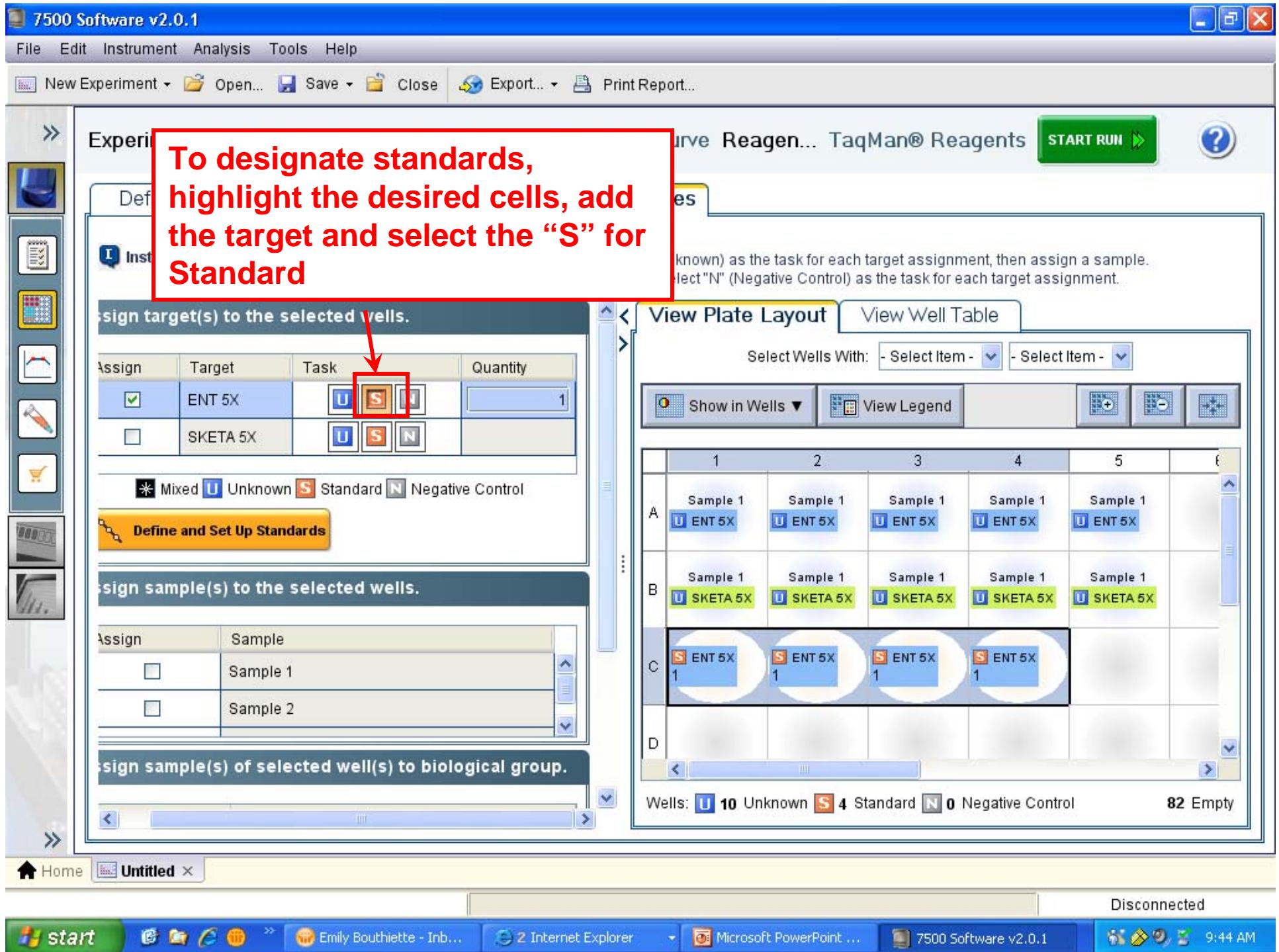
Wells: 5 Unknown 0 Standard 0 Negative Control 91 Empty

While the cells are highlighted, check the desired Target and designate whether the sample is an U (Unknown), S (Standard) or N (Negative Control)

Assign the sample that is to be analyzed in the highlighted cell(s)

Home Untitled Disconnected

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Type in a Quantity for each standard - if there are 4 different values, highlight each cell independently and fill in the quantity

Print Report...

Standard Curve Reagen... TaqMan® Reagents START RUN ?

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
<input checked="" type="checkbox"/>	ENT 5X	U S N	40000.0
<input type="checkbox"/>	SKETA 5X	U S N	

* Mixed U Unknown S Standard N Negative Control

Define and Set Up Standards

Assign sample(s) to the selected wells.

Assign	Sample
<input type="checkbox"/>	Sample 1
<input type="checkbox"/>	Sample 2

Assign sample(s) of selected well(s) to biological group.

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Wells View Legend

	1	2	3	4	5	6
A	Sample 1 U ENT 5X					
B	Sample 1 U SKETA 5X					
C	S ENT 5X 4E4	S ENT 5X 1	S ENT 5X 1	S ENT 5X 1		
D						

Wells: U 10 Unknown S 4 Standard N 0 Negative Control 82 Empty

Home Untitled Disconnected

start

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu < Ex... NRSA 2009 Batch34 ENT C ... Standard Cu Rea... TaqMan® Reager START RUN ?

Setup

- Experiment Properties
- Plate Setup
- Run Method
- Reaction Setup
- Materials List

Run

Analysis

Print Report...

Define Targets and

To set up To set up assign a To set up negative controls. Select wells, assign target(s), then select 'N' (Negative Control) as the task for each target assignment.

Instructions:

Assign target(s) to the selected wells.

Assign	Target	Task
	Enter	*
	Sketa	U S N

* Mixed U Unknown S Standard N Negative Control

Define and Set Up Standards

Assign sample(s) to the selected wells.

Assign	Sample
	Cal-99
	Cal-100

Assign sample(s) of selected well(s) to biological replicates

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Wells... View Legend

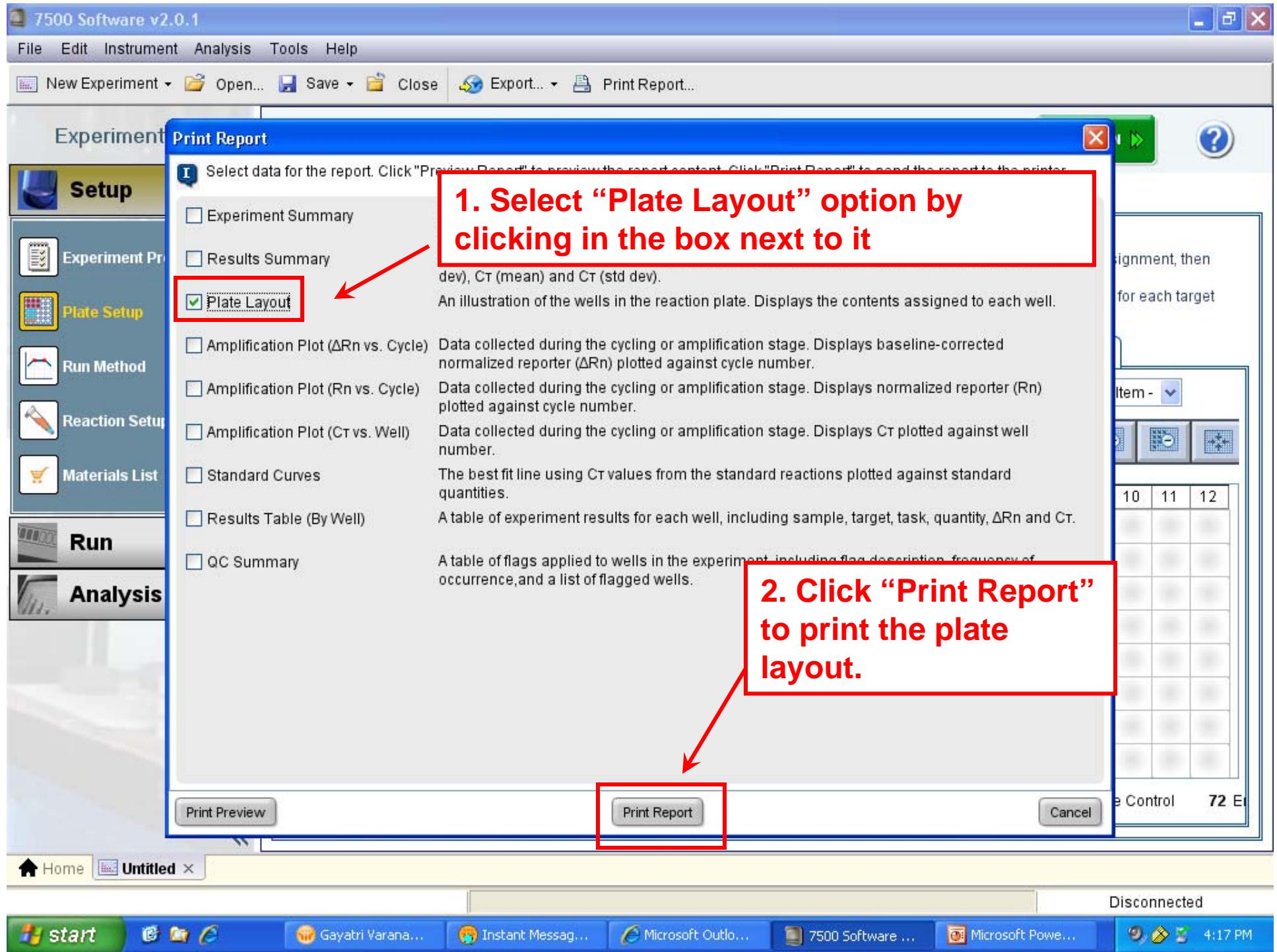
1 2 3 4 5 6 7 8 9 10 11 12

A	B	C	D	E	F	G	H	1	2	3	4	5	6	7	8	9	10	11	12
U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Wells: U 15 Unknown S 8 Standard N 1 Negative Control 72 E

Home Untitled × Disconnected

start Gayatri Varanasi - In... Instant Messaging Co... Microsoft Outlook We... 7500 Software v2.0.1 4:11 PM



7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu < Expe... N RSA Batch 1 ENT 03 ... Standard Cur Reag... TaqMan® Reagen START RUN > ?

Setup

Experiment Properties

Plate Setup

Run Method

Reaction Setup

Materials List

Run

Analysis

Select “Run Method” to set up the thermo cycling profile

Instructions: To set up unknowns: Select wells, assign target(s), select “U” (Unknown) as the task for each target assignment, then assign a sample. To set up negative controls: Select wells, assign target(s), then select “N” (Negative Control) as the task for each target assignment.

Set(s) to the selected wells.

Target	Task	Quantity
ENT 5X	U S N	100
SKETA 5X	U S N	

ed U Unknown S Standard N Negative Control

and Set Up Standards

ole(s) to the selected wells.

Sample
Sample 1
Sample 2

ole(s) of selected well(s) to biological group.

View Plate Layout View Well Table

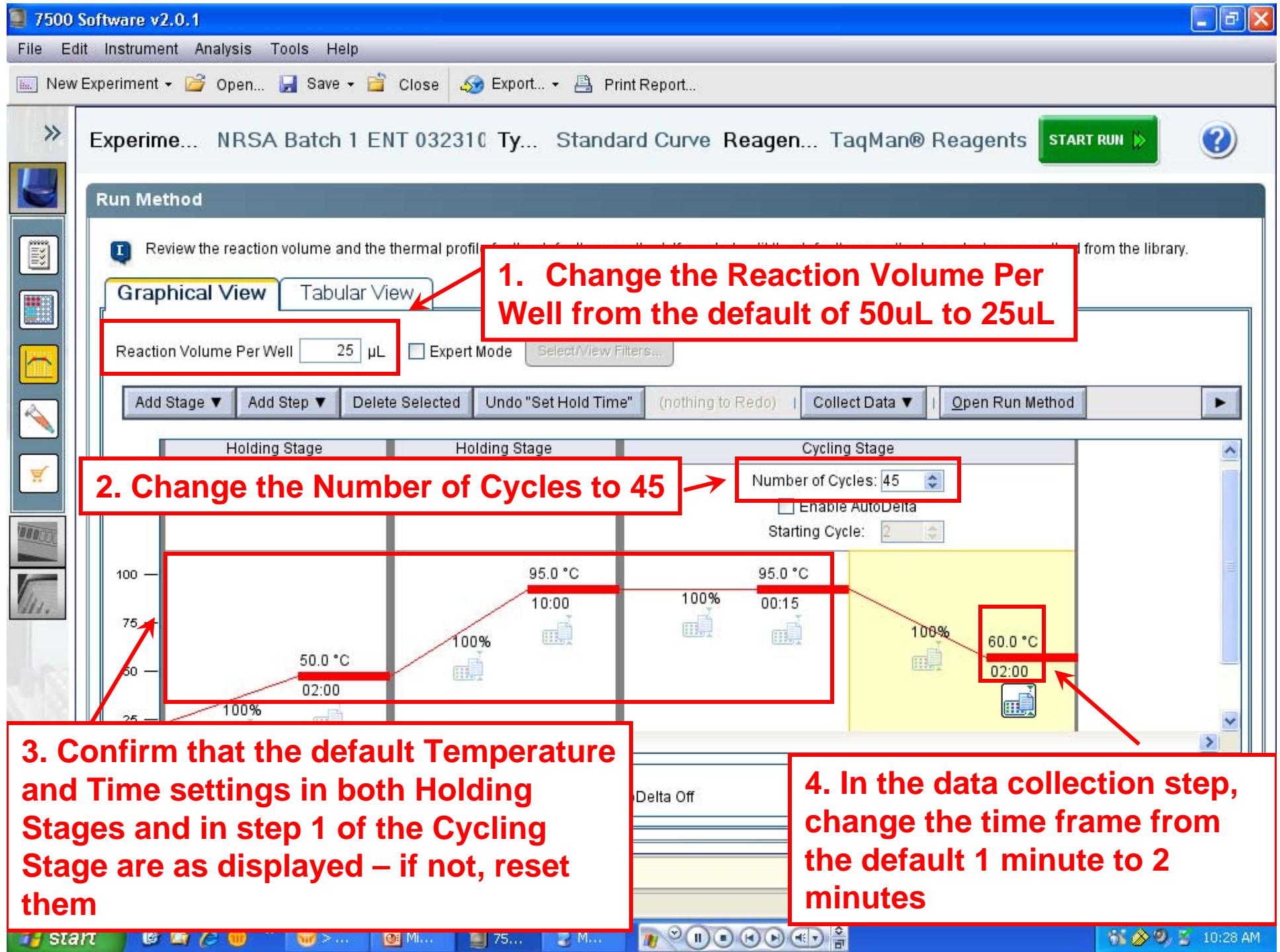
Select Wells With: - Select Item - - Select Item -

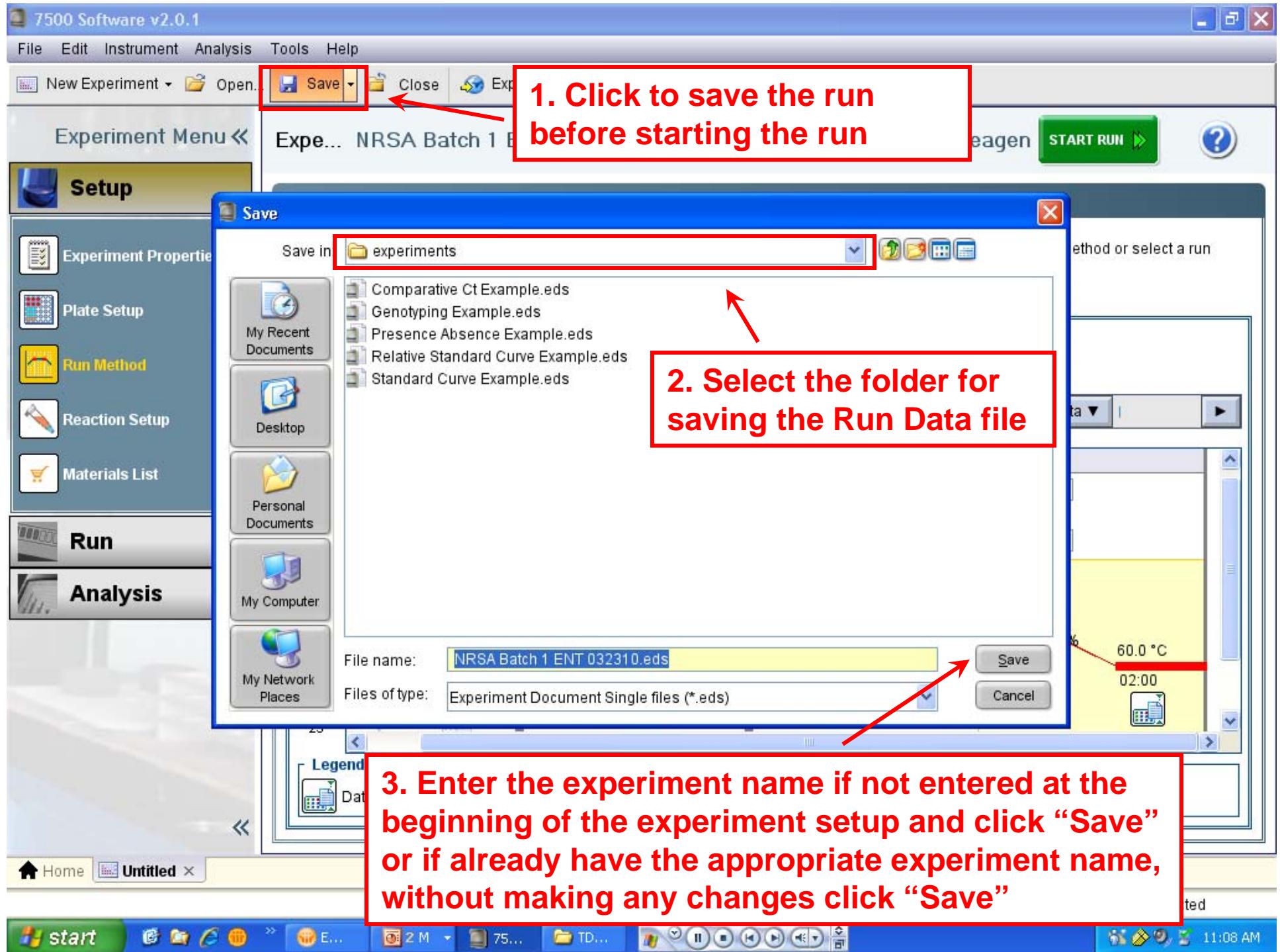
Show in Wells... View Legend

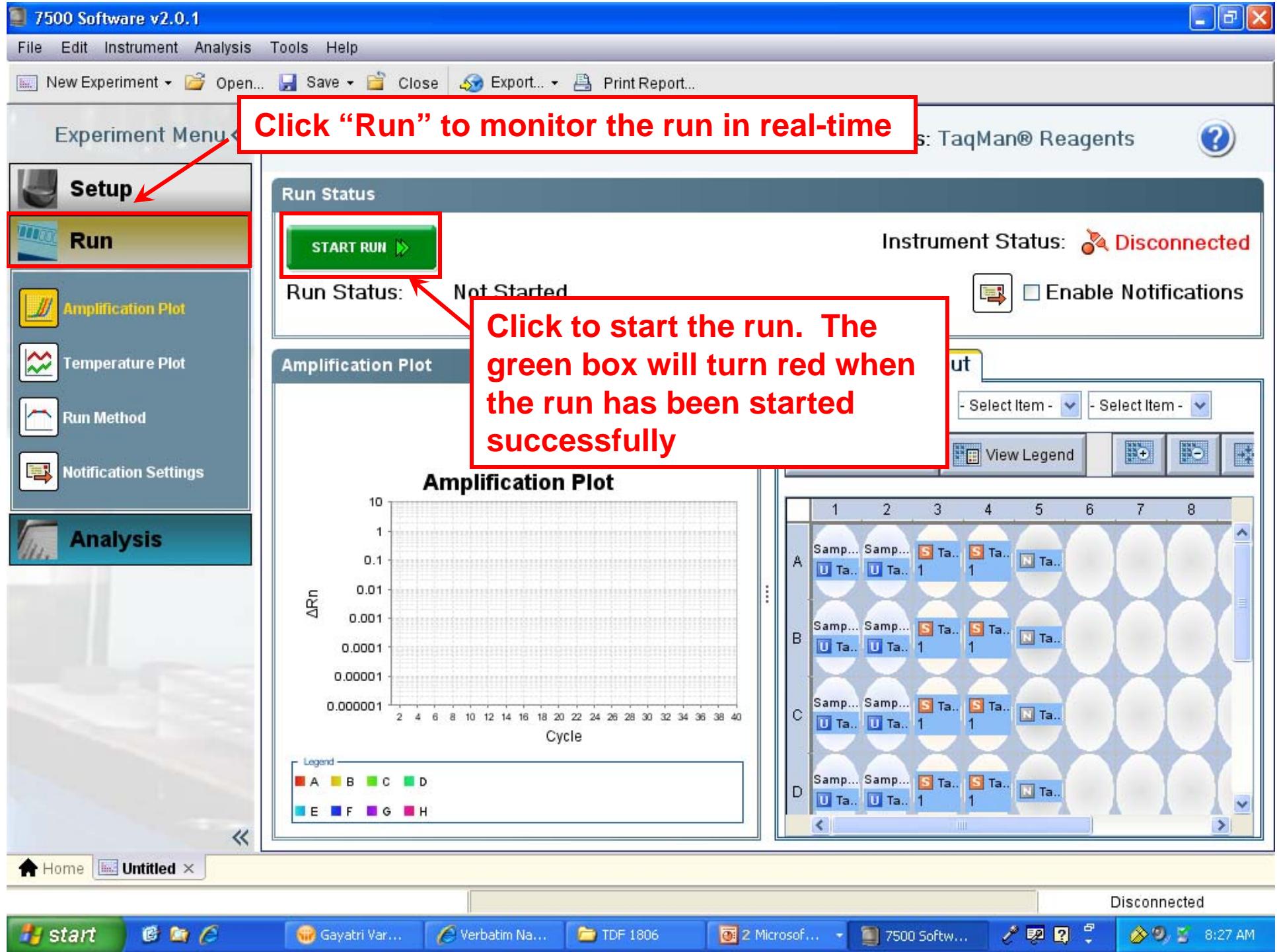
1	2	3	4	5
A Sample 1 U ENT 5X	Sample 1 U ENT 5X			
B Sample 1 U SKETA 5X	Sample 1 U SKETA 5X			
C S ENT 5X 4E4	S ENT 5X 4E3	S ENT 5X 400	S ENT 5X 100	
D				

Wells: U 10 Unknown S 4 Standard N 0 Negative Control 82 E

Home Untitled x Disconnected



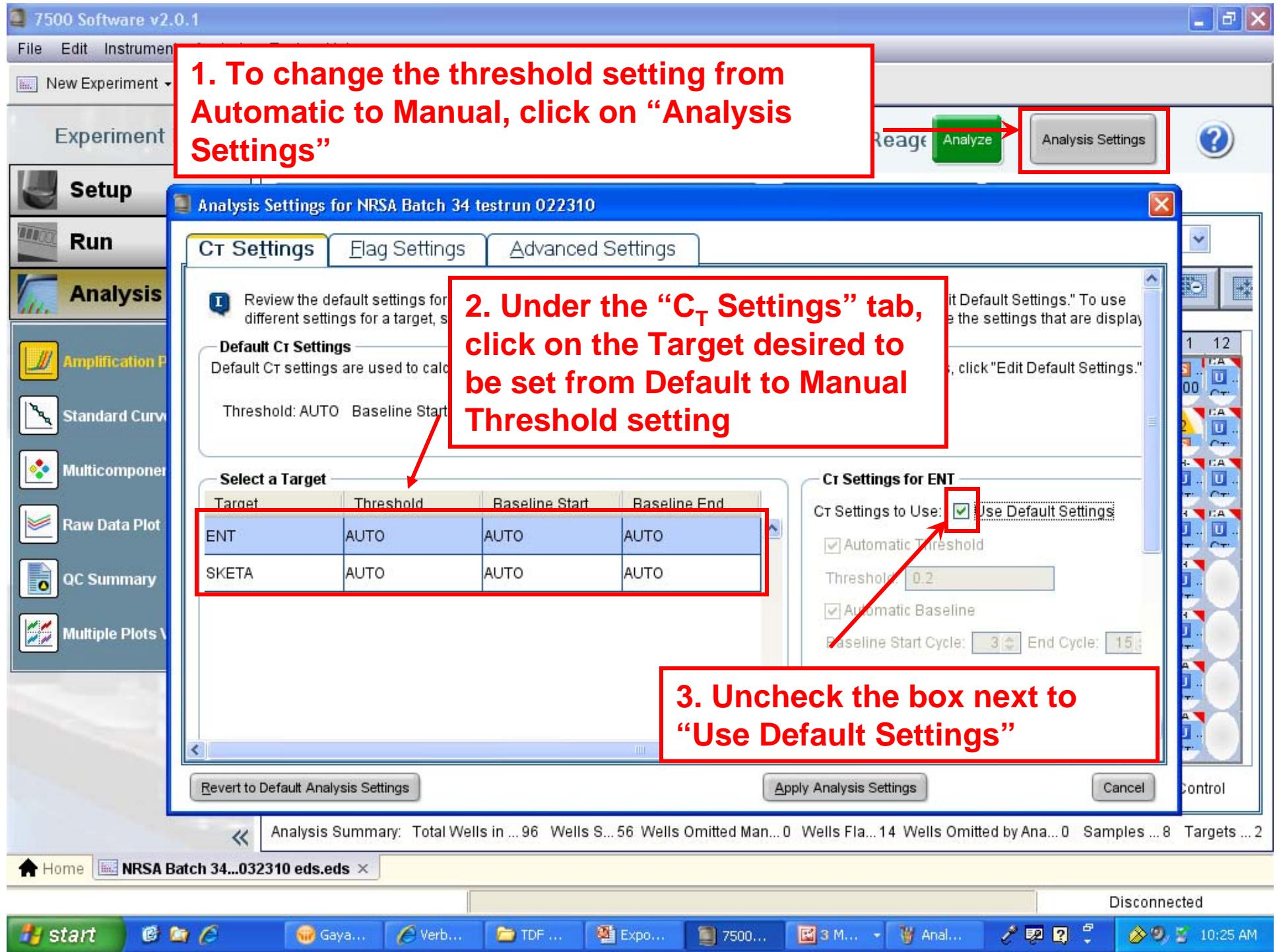




How to Export Results from the ABI 7500 Software

The screenshot shows the ABI 7500 Software interface. On the left, the **Experiment Menu** is open, displaying various options: Setup, Run, **Analysis**, Amplification Plot, Standard Curve, Multicomponent Plot, Raw Data Plot, QC Summary, and Multiple Plots View. The **Analysis** option is highlighted with a red box and has a red arrow pointing to it from the text instructions. The main workspace displays an **Amplification Plot** with a Y-axis of ΔR° on a logarithmic scale from 0.000001 to 10, and an X-axis of Cycle from 0 to 28. The plot shows multiple colored curves representing different samples. Below the plot are **Plot Settings** and **Options** sections. To the right of the plot is a **View Plate Layout** window showing a 12x8 grid of wells labeled A through H. The wells contain various symbols and numbers, such as 'U' for unknown, 'S' for standard, and numerical values like 100, 60, and 35. Below the plate layout, text indicates the number of wells: **Wells: U 40 Unknown S 16 Standard N 0 Negative Control**. At the bottom of the software window, there is an **Analysis Summary** and a status bar indicating **Disconnected**.

Click on the “Analysis” tab in the Experiment Menu to access the “Analysis Settings”



7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu < ... NRSA Batch 34 testrun ...Standard C Rea... TaqMan® Reage Analyze Analysis Settings ?

Setup

Run

Analysis

Amplification P
Standard Curve
Multicomponent
Raw Data Plot
QC Summary
Multiple Plots V

Analysis Settings for NRSA Batch 34 testrun 022310

CT Settings Flag Settings Advanced Settings

I Review the default settings for analysis of targets in this experiment. To edit the default settings, click "Edit Default Settings." To use different settings for a target, select the target from the table, deselect "Use Default Settings," then change the settings that are displayed.

Default CT Settings

Default CT settings are used to calculate the CT for targets without custom settings. To edit the default settings, click "Edit Default Settings."

Threshold: AUTO Baseline Start Cycle: AUTO Baseline End Cycle: AUTO [Edit Default Settings](#)

Select a Target

Target	Threshold	Baseline Start	Baseline End
ENT	AUTO	AUTO	AUTO
SKETA	AUTO	AUTO	AUTO

CT Settings for ENT

CT Settings to Use: Use Default Settings Automatic Threshold

Threshold: 0.2

Automatic Baseline

Baseline Start Cycle: 3 End Cycle: 15

Uncheck the box next to "Automatic Threshold" to enter the desired threshold

Revert to Default Analysis Settings Apply Analysis Settings Cancel

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 14 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

Home NRSA Batch 34...032310 eds.eds

Disconnected

start Gaya... Verb... TDF... Expo... 7500... 3 M... Anal... 10:33 AM

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu NRSA Batch 34 testrun Standard C Re... TagMan® Reader Analyze Analysis Settings ?

Analysis Settings for NRSA Batch 34 testrun 022310

CT Settings Flag Settings Advanced Settings

I Review the default settings for analysis of targets in this experiment. To edit the default settings, click "Edit Default Settings." To use different settings for a target, select the target from the table, deselect "Use Default Settings," then change the settings that are displayed.

Default CT Settings

Default CT settings are used to calculate the CT for targets without custom settings. To edit the default settings, click "Edit Default Settings."

Threshold: AUTO Baseline Start Cycle: AUTO Baseline End Cycle: AUTO Edit Default Settings

Select a Target

Target	Threshold	Baseline Start	Baseline End
ENT	0.2	AUTO	AUTO
SKETA	0.2	AUTO	AUTO

CT Settings for the 2 Selected Targets

CT Settings to Use: Use Default Settings
 Automatic Threshold
Threshold: **0.025**
 Automatic Baseline
Baseline Start Cycle: 3 End Cycle: 15

Enter the desired Threshold.

*If more than one target is present, highlight the next target and repeat this process

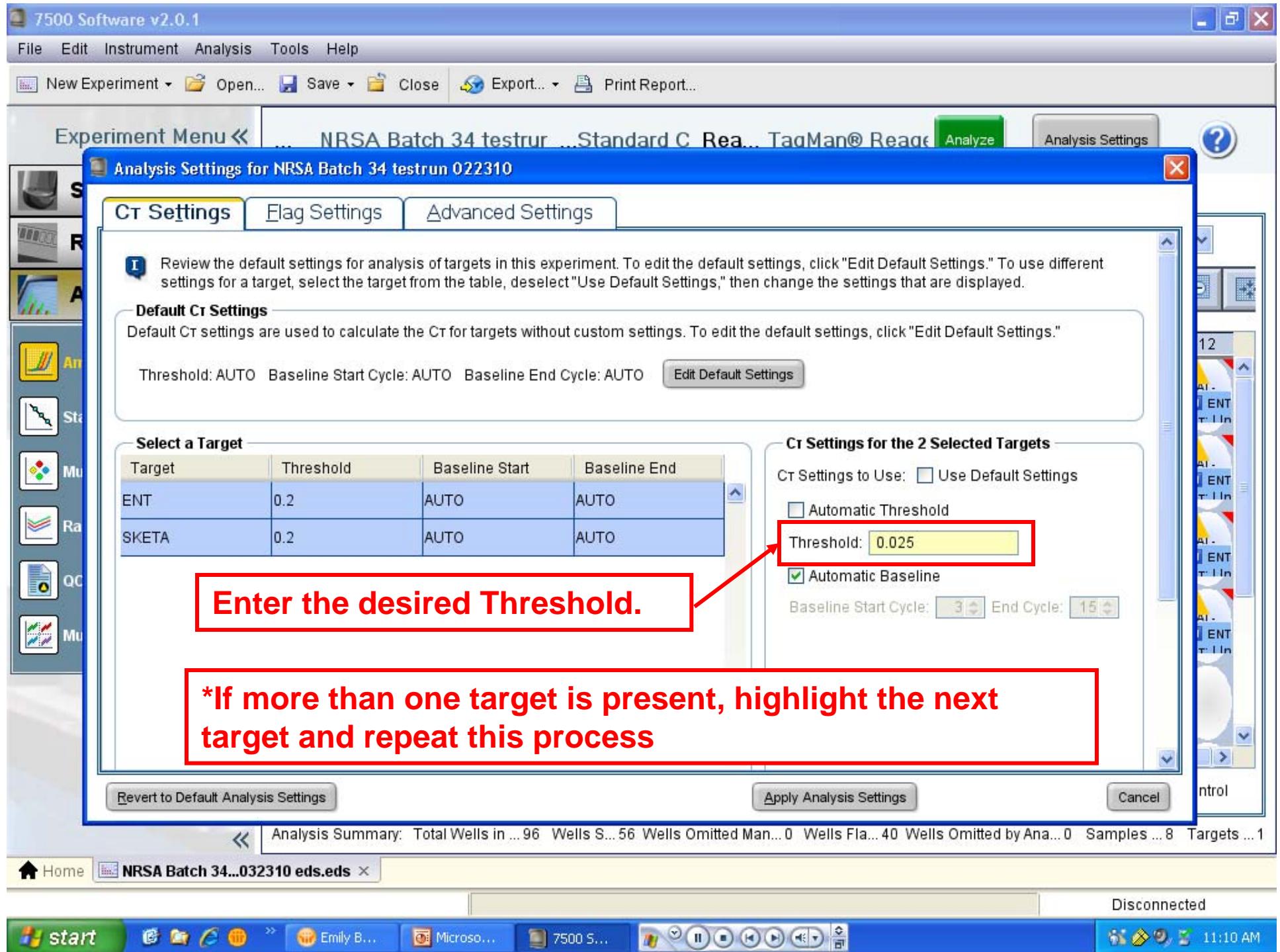
Revert to Default Analysis Settings Apply Analysis Settings Cancel

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 40 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 1

Home NRSA Batch 34...032310 eds.eds Disconnected

start

11:10 AM



7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu < ... NRSA Batch 34 testrun ...Standard C Rea... TaqMan® Reage Analyze Analysis Settings ?

Setup

Run

Analysis

Amplification P
Standard Curve
Multicomponent
Raw Data Plot
QC Summary
Multiple Plots V

Analysis Settings for NRSA Batch 34 testrun 022310

CT Settings Flag Settings Advanced Settings

I Review the default settings for analysis of targets in this experiment. To edit the default settings, click "Edit Default Settings." To use different settings for a target, select the target from the table, deselect "Use Default Settings," then change the settings that are displayed.

Default CT Settings

Default CT settings are used to calculate the CT for targets without custom settings. To edit the default settings, click "Edit Default Settings."

Threshold: AUTO Baseline Start Cycle: AUTO Baseline End Cycle: AUTO Edit Default Settings

Select a Target

Target	Threshold	Baseline Start	Baseline End	Use Default Settings
ENT				<input type="checkbox"/>
SKETA				<input checked="" type="checkbox"/>

CT Settings for SKETA

Automatic Baseline

Baseline Start Cycle: 3 End Cycle: 15

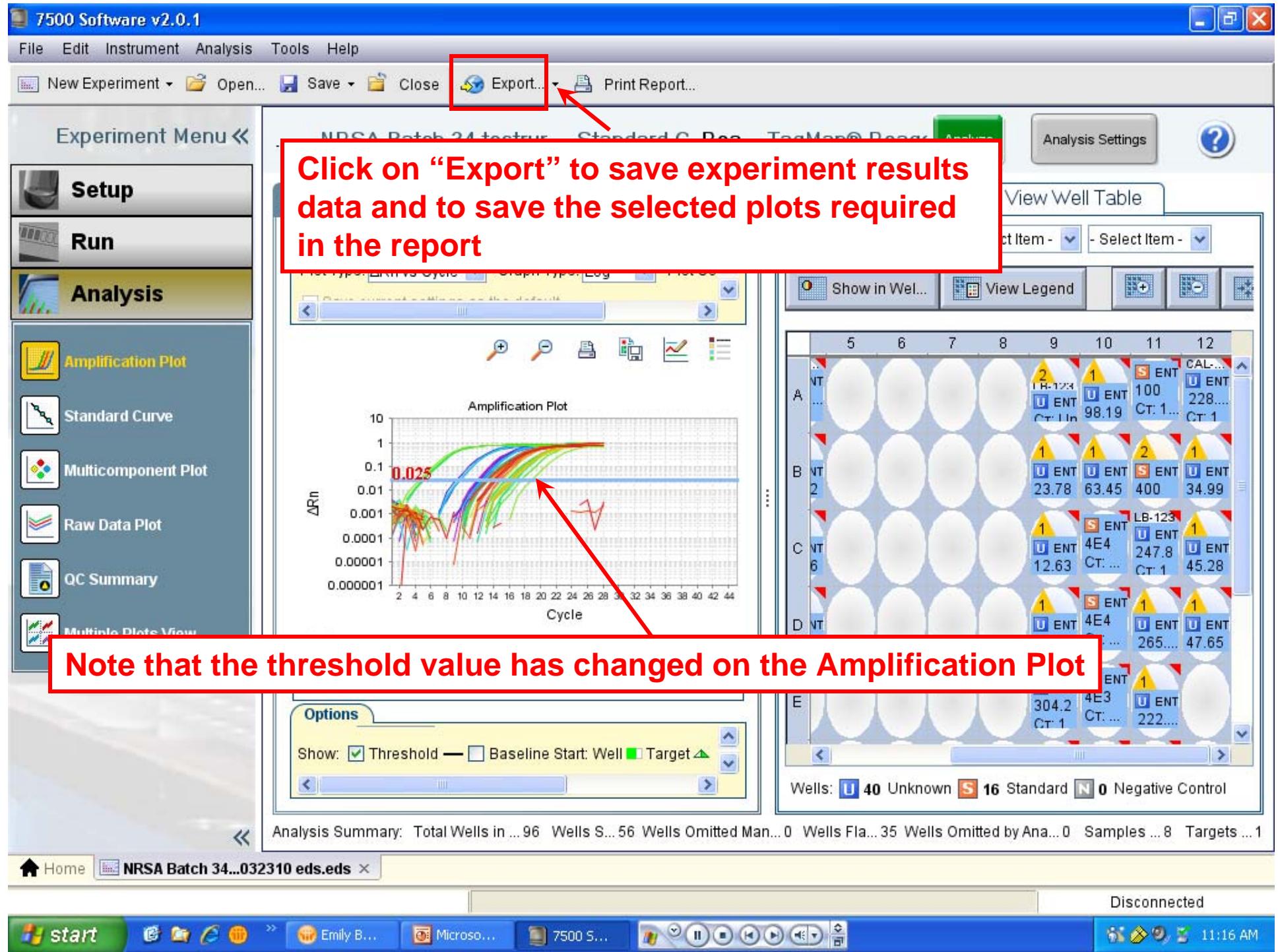
Revert to Default Analysis Settings Apply Analysis Settings Cancel

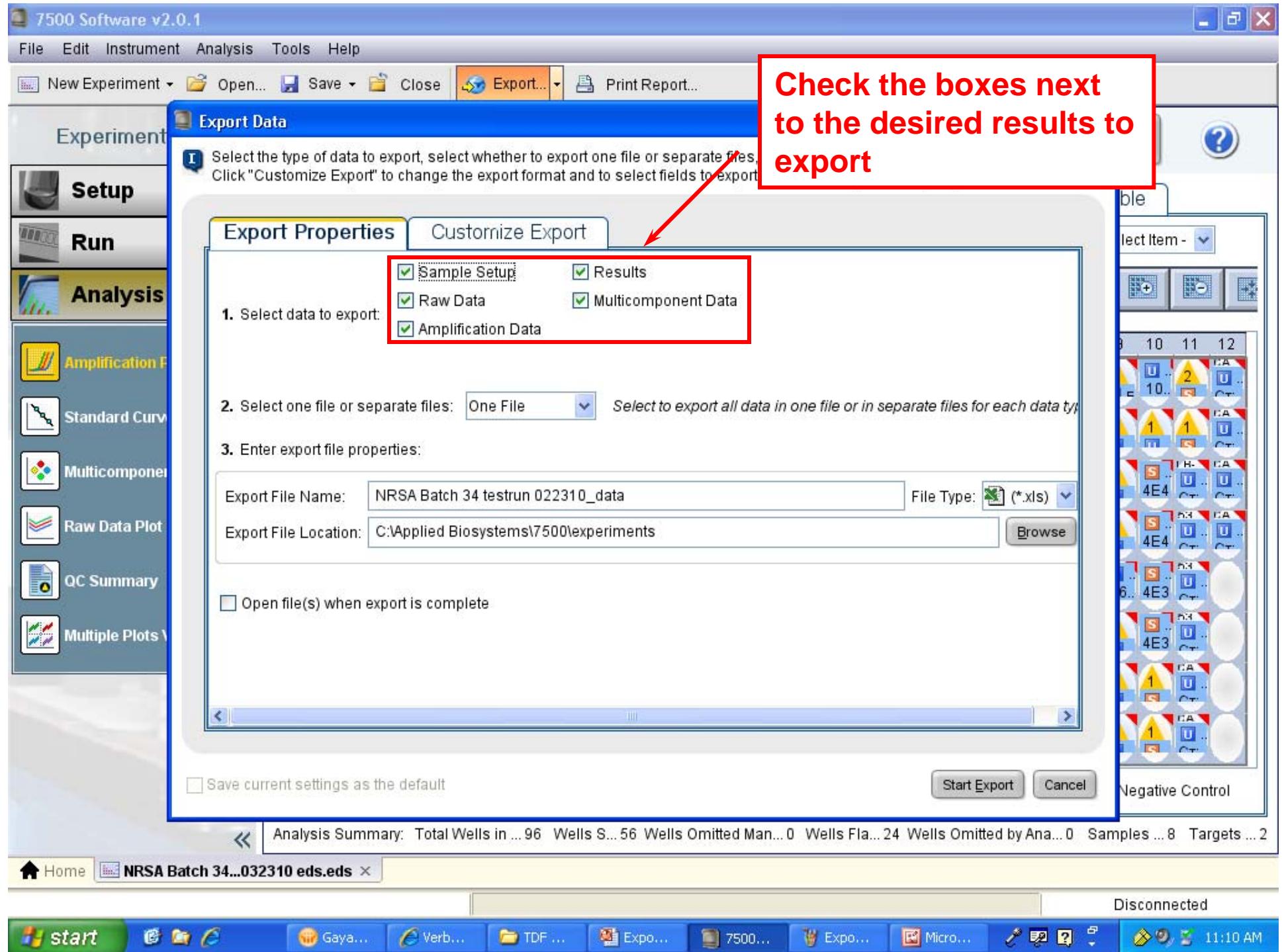
Select “Apply Analysis Settings” to save the changes

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 14 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

Home NRSA Batch 34...032310 eds.eds Disconnected

start Gaya... Verb... TDF... Expo... 7500... 4 M... Anal... 10:40 AM





7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment

Setup

Run

Analysis

Amplification F...

Standard Curve

Multicomponent

Raw Data Plot

QC Summary

Multiple Plots V...

Export Data

Select the type of data to export, select whether to export one file or separate files, then enter export file properties. (Optional) Click "Customize Export" to change the export format and to select fields to export. Click "Start Export" to export your data.

Export Properties Customize Export

1. Select data to export:

Sample Setup Results
Raw Data Multicomp
Amplification Data

2. Select one file or separate files:

One File (selected)
One File
Separate Files

3. Enter export file properties:

Export File Name: NRSA Batch 34 testrun 022310_data
File Type: (*.xls)
Export File Location: C:\Applied Biosystems\7500\experiments
Browse

Open file(s) when export is complete

Save current settings as the default

Start Export Cancel

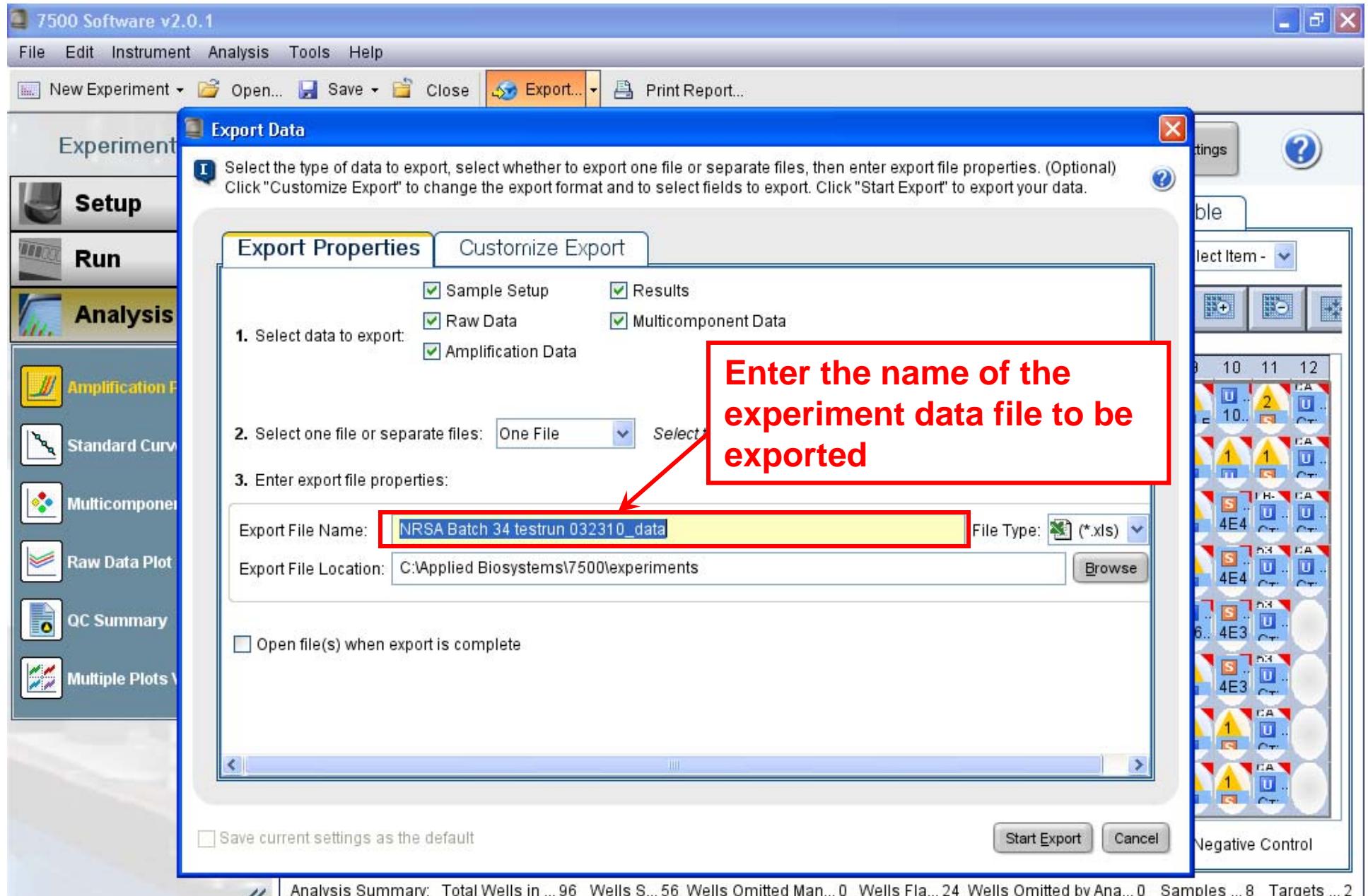
Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 24 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

Home NRSA Batch 34...032310 eds.eds Disconnected

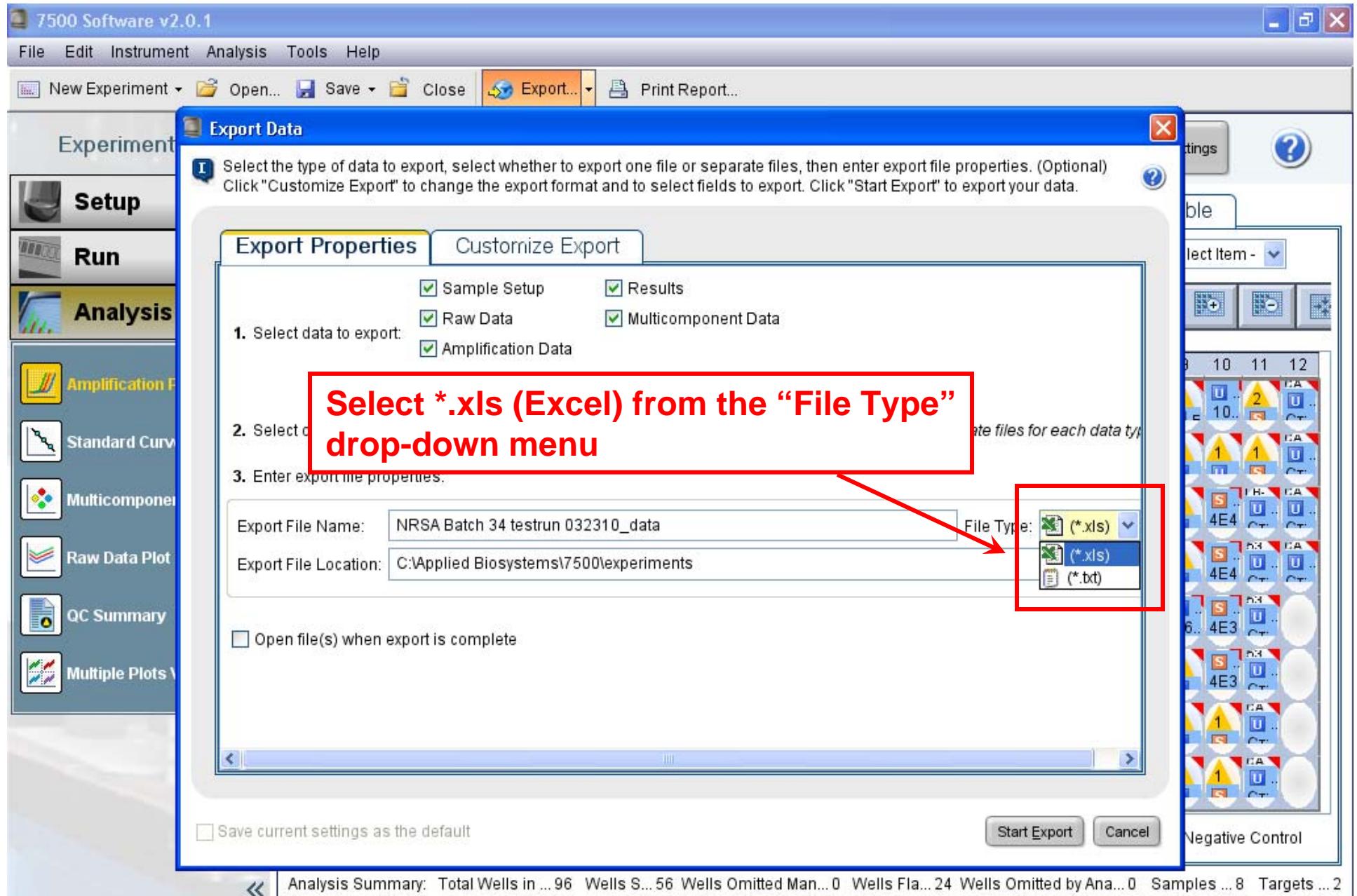
start Gaya... Verb... TDF... Expo... 7500... Expo... Micro... 11:13 AM

From the drop-down menu select “One File” to export all data into a single file with multiple tabs

Negative Control

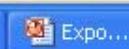


Enter the name of the experiment data file to be exported



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7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment

Setup

Run

Analysis

Amplification F...

Standard Curve

Multicomponent

Raw Data Plot

QC Summary

Multiple Plots V...

Export Data

Select the type of data to export, select whether to export one file or separate files, then enter export file properties. (Optional) Click "Customize Export" to change the export format and to select fields to export. Click "Start Export" to export your data.

Export Properties **Customize Export**

1. Select data to export:

Sample Setup Results
 Raw Data Multicomponent Data
 Amplification Data

2. Select one file or separate files: One File Select to export all data in one file or in separate files for each data type

3. Enter export file properties:

Export File Name: NRSA Batch 34 testrun 022310_data File Type: (*.xls)
Export File Location: C:\Applied Biosystems\7500\experiments **Browse**

Open file(s) when export is complete

Click on “Browse” to select the folder / location to export the File

Save current settings as the default

Start Export Cancel

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 24 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

Home NRSA Batch 34...032310 eds.eds

Disconnected

start Gaya... Verb... TDF... Expo... 7500... Expo... Micro... 11:10 AM

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment

Setup

Run

Analysis

Amplification F...

Standard Curve

Multicomponent

Raw Data Plot

QC Summary

Multiple Plots V...

Export Data

Select the type of data to export, select whether to export one file or separate files, then enter export file properties. (Optional) Click "Customize Export" to change the export format and to select fields to export. Click "Start Export" to export your data.

Export Properties Customize Export

1. Select data to export:

Sample Setup Results
 Raw Data Multicomponent Data
 Amplification Data

2. Select one file or separate files: One File Select to export all data in one file or in separate files for each data type

3. Enter export file properties:

Export File Name: NRSA Batch 34 testrun 032310_data File Type: (*.xls)
Export File Location: C:\Applied Biosystems\7500\experiments

Open file(s) when export is complete

Save current settings as the default

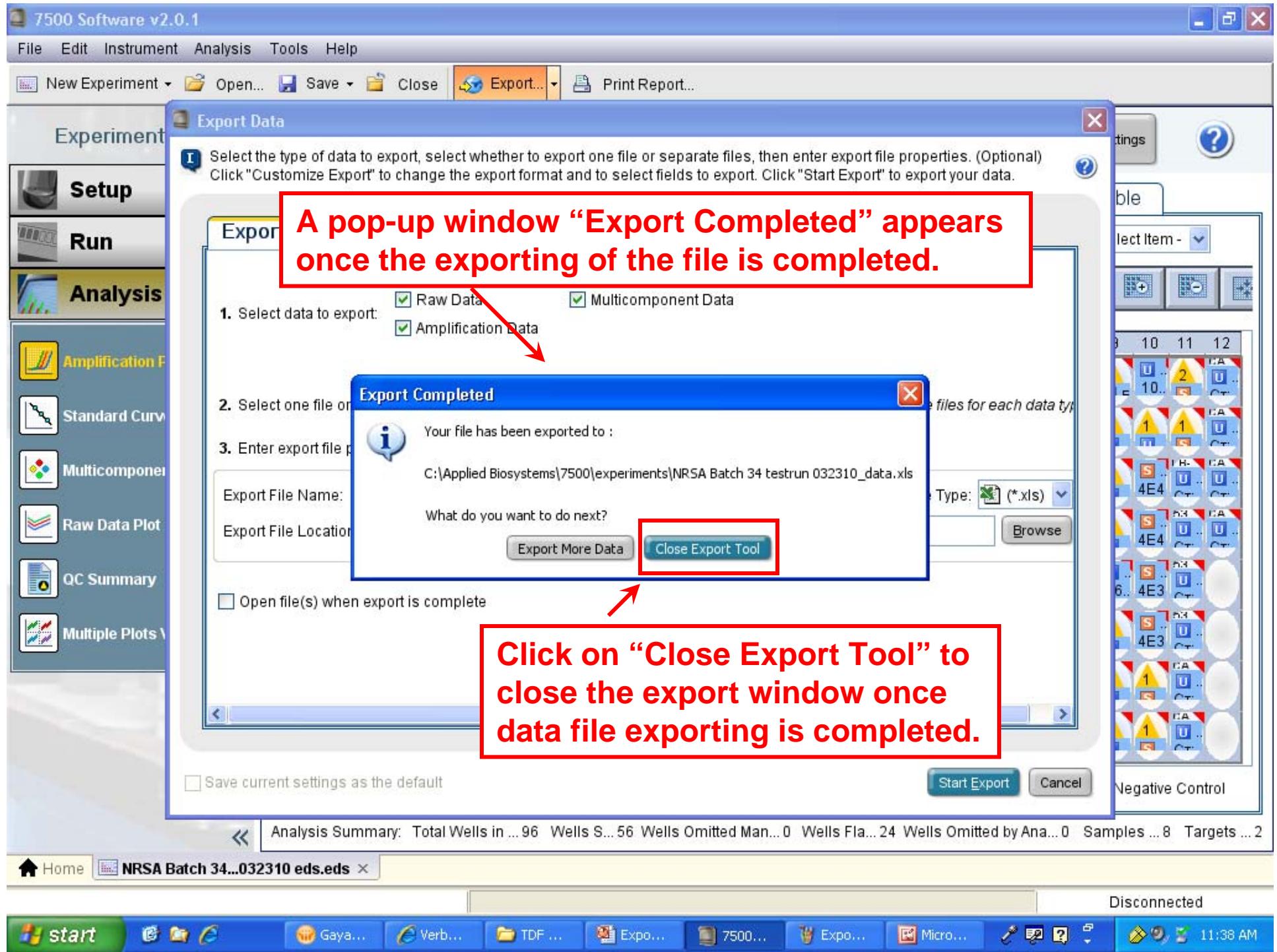
Click on “Start Export” to export the data to the determined location

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 24 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

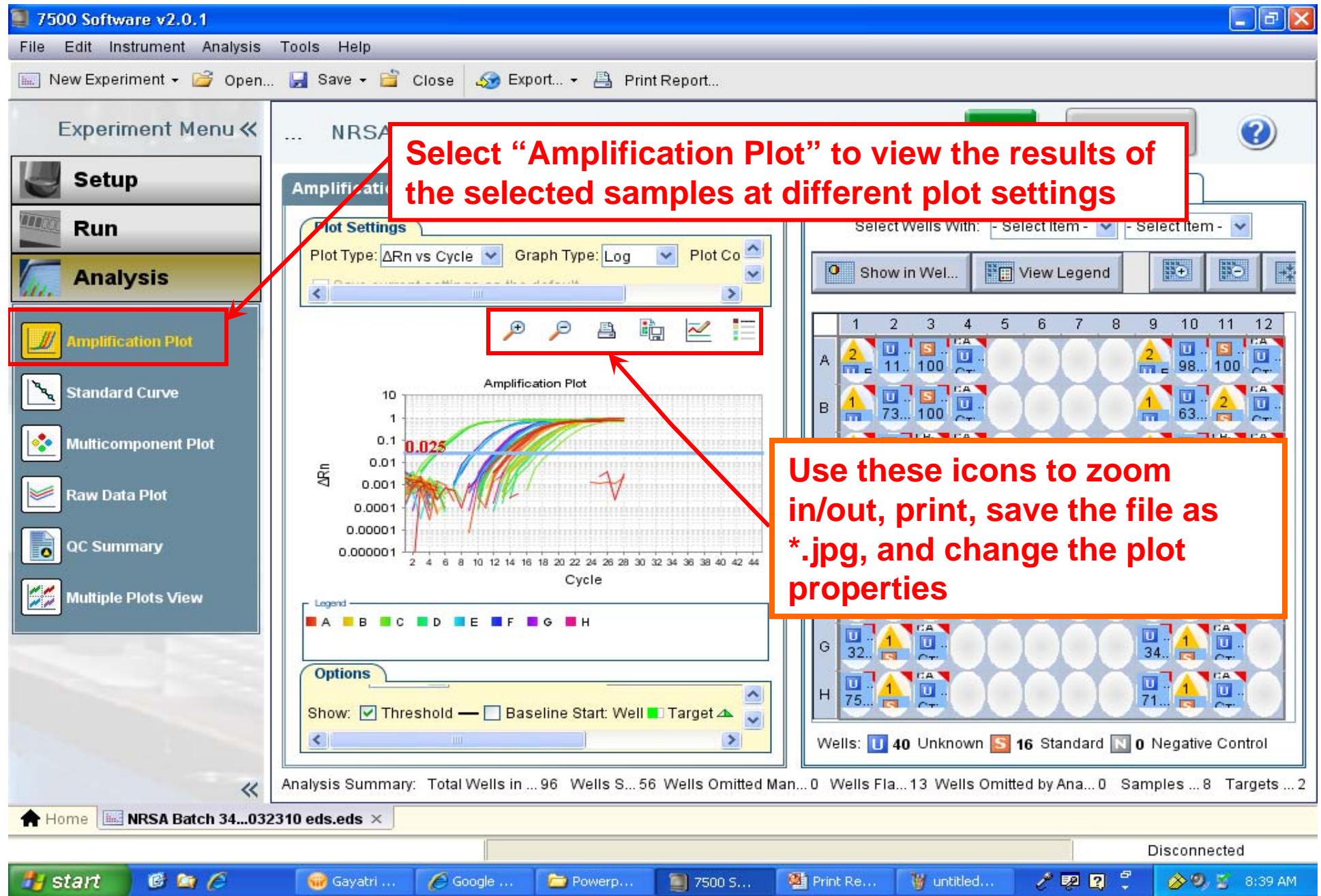
Home NRSA Batch 34...032310 eds.eds

Disconnected

start Gaya... Verb... TDF... Expo... 7500... Expo... Micro... 11:36 AM



How to Interpret Results From the ABI 7500



Analysis Menu- Amplification Plot

2. Select the “Plot Type” from the drop-down menu

3. From the “Graph Type” drop down menu select either Log or Linear

1. Drag the mouse over the desired cells to select the samples to be plotted in the amplification plot

The screenshot shows the 7500 Software interface. On the left, the Analysis menu is open, with 'Analysis' selected. In the center, the 'Amplification Plot' window is active. It displays an 'Amplification Plot' with the Y-axis labeled 'ΔRn' and the X-axis labeled 'Cycle'. The plot shows multiple colored curves representing different samples. Below the plot are 'Plot Settings' and 'Options' sections. The 'Plot Settings' section includes dropdown menus for 'Plot Type' (set to 'ΔRn vs Cycle') and 'Graph Type' (set to 'Log'). The 'Options' section includes settings for 'Target' and 'Threshold'. To the right of the plot is a 'View Plate Layout' window showing a 96-well plate grid with various sample names and IDs. A red box highlights the 'Graph Type' dropdown in the central plot window, and another red box highlights the 'Select Wells With' dropdown in the plate layout window. A large red box on the far right encloses the entire 'View Plate Layout' window.

Analysis Menu- Amplification Plot

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open

Experiment Menu < n® Reagents Analyze Analysis Settings ?

Setup

Run

Analysis

Amplification Plot

Amplification Plot

Plot Settings

Plot Type: ΔRn vs Cycle Graph Type: Log Plot Co

Amplification Plot

Cycle

E F G H

Options

Show: Threshold Baseline Start: Well Target

Analysis Summary: Total Wells in ... 96 Wells

2. Select “View Well Table” to view the results in a tabular format

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Ta... Group By Expand All Collapse

#	Well	Omit	Flag	Sample ...	Target
1	A1		2	LB-123	ENT
2	A2			CAL-99 25X	ENT
3	A3				ENT
4	A4			CAL-100 5x	SKETA
5	A5				
6	A6				
7	A7				
8	A8				
9	A9				
10	A10				
11	A11				
12	A12				
13	B1		1	LB-123	ENT
14	B2			CAL-99 25X	ENT
15	B3				ENT
16	B4			CAL-100 5x	SKETA
17	B5			537238	ENT
18	B6			CAL-100 25X	ENT
19	B7				ENT
				CAL-98 25X	SKETA

3. Scroll down or sideways for more samples and analysis parameters

Disconnected

Home NRSA Batch 34...032310 eds.eds

start Gayatri ... Google ... Powerp... 7500 S... Print Re... untitled... 9:06 AM

Analysis Menu- Standard Curve

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save Close Export... Print Report...

Experiment Menu < ... NRSA Batch 34 testrur ...Standard C Re... TaqMan® Reader Analyze Analysis Settings ?

Select “Standard Curve” to view the results of the samples (if same target) and standards in the plot

Save current settings as the default

Amplification Plot

Standard Curve

Multicomponent Plot

Raw Data Plot

QC Summary

Multiple Plots View

Standard Curve

Target: ENT Slope: -3.496 Y-Inter: 21.433 R²: 0.963 Eff%: 93.222

Legend: Standard (Red Square), Unknown (Blue Square), Unknown (Flagged) (Green Square)

Wells: U 40 Unknown S 16 Standard N 0 Negative Control

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 13 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

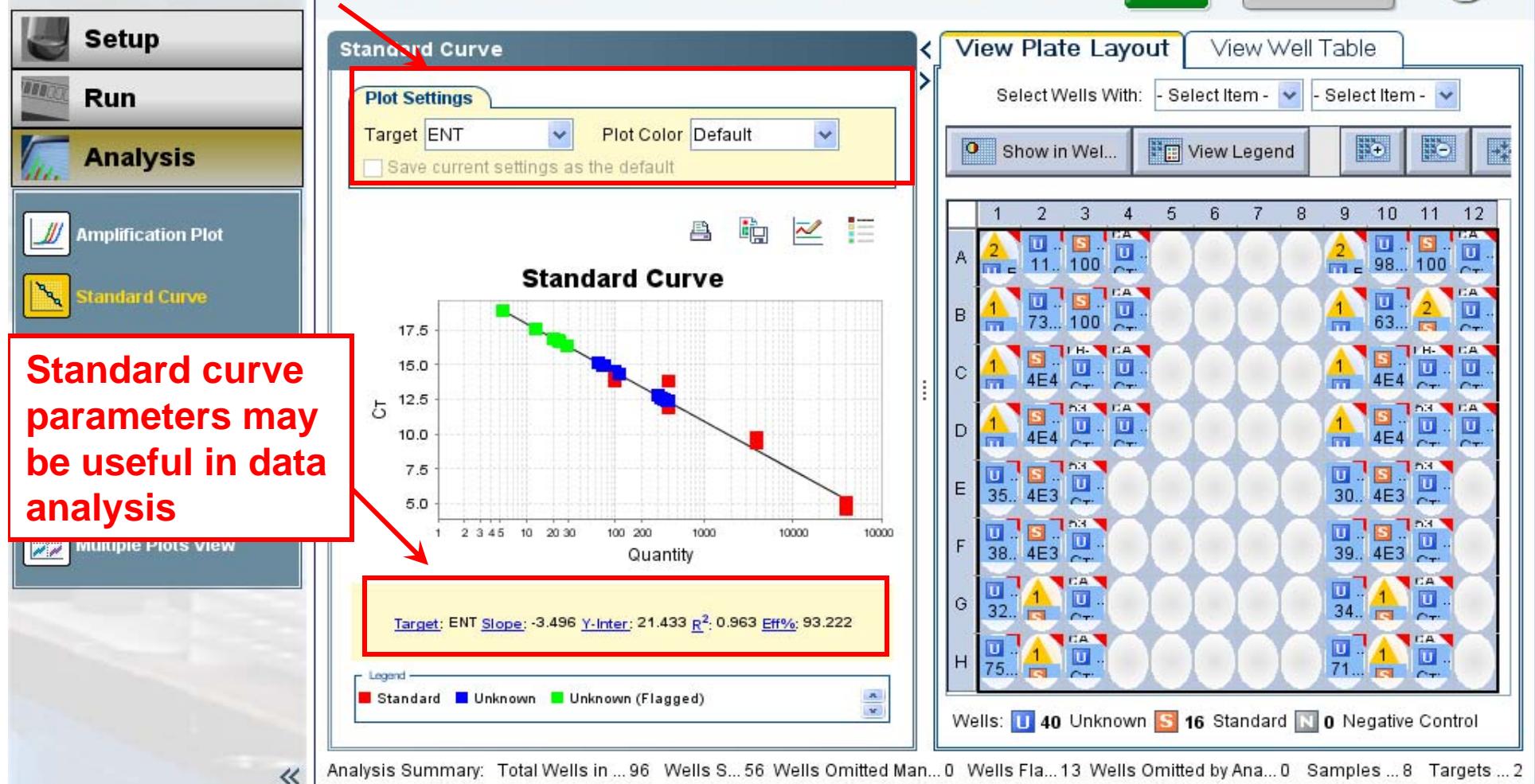
Home NRSA Batch 34...032310 eds.eds Disconnected

start Gayatri ... Google ... Powerp... 7500 S... Print Re... untitled... 9:31 AM

The screenshot shows the 7500 Software interface. On the left, the 'Analysis' option in the 'Experiment Menu' is highlighted. A red box surrounds the 'Standard Curve' icon in the 'Analysis' section of the menu. Another red box highlights the text 'Select “Standard Curve” to view the results of the samples (if same target) and standards in the plot'. The main window displays a 'Standard Curve' plot with a logarithmic x-axis ('Quantity') and a linear y-axis ('Ct'). The plot shows data points for Standards (red squares), Unknowns (blue squares), and Unknowns Flagged (green squares). Below the plot, a legend identifies these symbols. To the right of the plot is a 12x8 grid representing a 96-well plate, with wells labeled A through H and 1 through 12. The grid contains various symbols representing different sample types. At the bottom, an 'Analysis Summary' provides a quick overview of the analysis results.

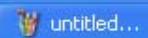
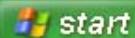
Analysis Menu – Standard Curve

Click here to select a different Target (if the standards were analyzed in more than one target) or change the Plot Color



Home NRSA Batch 34...032310 eds.xls

Disconnected



9:31 AM

Analysis Menu – Multicomponent Plot

7500 Software v2.0.1

Click on the “Plot Color” drop down menu to select the parameter based on which the graph required to be plotted

Experiment Menu <> ... NRSA Batch 34 testrur ...Standard C Rea... TaqMan® Reage Analyze Analysis Settings ?

Multicomponent Plot

Plot Settings

Plot Color Dye

Well Target

Dye

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Well... View Legend

Fluorescence

Multicomponent Plot

1,100,000
1,000,000
900,000
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000

1 2 3 4 5 6 7 8 9 10 11 12

A 2 11 100 1A
1 73 100 1A
C 1 4E4 1H 1A
D 1 4E4 1H 1A
E 35 4E3 1H 1A
F 38 4E3 1H 1A

B 1 63 2 1A
1 4E4 1H 1A
S 30 4E3 1H 1A
U 34 4E3 1H 1A
U 71 1 1A

Unknown S 16 Standard N 0 Negative Control

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 13 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

Home NRSA Batch 34...032310 eds.eds Disconnected

start Gayatri ... Google ... Powerp... 7500 S... Print Re... untitled... 10:34 AM

Select “Multicomponent Plot” to view the variation in the fluorescence of the wells, targets or dyes used over the ascending repeat of the 45 thermo cycles

Analysis Menu – Raw Data Plot

Select “Raw Data Plot” to view the variation in the emission of fluorescence of the dyes in different filters present over the ascending repeat of the 45 thermo cycles

The screenshot shows the 7500 Software interface. On the left, the Analysis menu is highlighted. In the center, the Raw Data Plot window is open, displaying a line graph of fluorescence amplitude across five filters for each of the first four cycles. A legend at the bottom identifies the filters: A (red), B (yellow), C (green), D (light green), E (blue), F (dark blue), G (purple), and H (pink). Below the graph, an 'Options' section includes a dropdown for 'Show Cycle' with a slider from 1 to 28. To the right, the View Plate Layout window shows a grid of wells labeled A through D and 1 through 12, each containing a sample name and concentration. A red box highlights the 'Raw Data Plot' button in the Analysis menu, and another red box highlights the 'Show Cycle' dropdown in the Raw Data Plot options.

Raw Data Plot

Raw Data Plot

Amplitude

Filter

Legend: A B C D E F G H

Show Cycle 28

1 5 10 15 20 25 28

Analysis Summary: Total Wells in ... 96 Wells S... 56 Wells Omitted Man... 0 Wells Fla... 13 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

View Plate Layout

View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Well... View Legend

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	11...	100...	U...								
B	1	73...	100...	U...								
C	1	4E4...	U...	U...	U...	U...	U...	U...	U...	U...	U...	U...
D	1	4E4...	U...	U...	U...	U...	U...	U...	U...	U...	U...	U...

start Gayatri ... Google ... Powerp... 7500 S... Print Re... untitled... 10:45 AM

Analysis Menu – QC Summary

7500 Software v2.0.1

File Edit Instrument Analysis Tools Help

New Experiment Open... Save... Close Export... Print Report...

Experiment Menu < ... NRSA Batch 34 testrur ... Standard C Rea... TaqMan® Reage Analyze Analysis Settings ?

QC Summary

Flag Summary

Total ... 96	Processe... 56	Manually Omitte... 0	Targets... 2
Wells... 56	Flagged ... 13	Analysis Omitte... 0	Sample... 8

Flag Details

Fl...	Name	F...	Wells
AMP...	Amplification in negative con...	0	
BAD...	Bad passive reference signal	0	
OFF...	Fluorescence is offscale	0	
HIG...	High standard deviation in re...	11	B1, B9, B1...
NOA...	No amplification	2	A1, A9
NOI...	Noise higher than others in ...	0	
SPIKE	Noise spikes	0	
NOS...	No signal in well	0	
OUTL...	Outlier in replicate group	1	B11

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Well... View Legend

Wells: U 40 Unknown S 16 Standard N 0 Negative Control

... 56 Wells Omitted Man... 0 Wells Fla... 13 Wells Omitted by Ana... 0 Samples ... 8 Targets ... 2

Disconnected

QC Summary

Multiple Plots View

Select “QC Summary” to view the analysis summary of the samples in the experiment

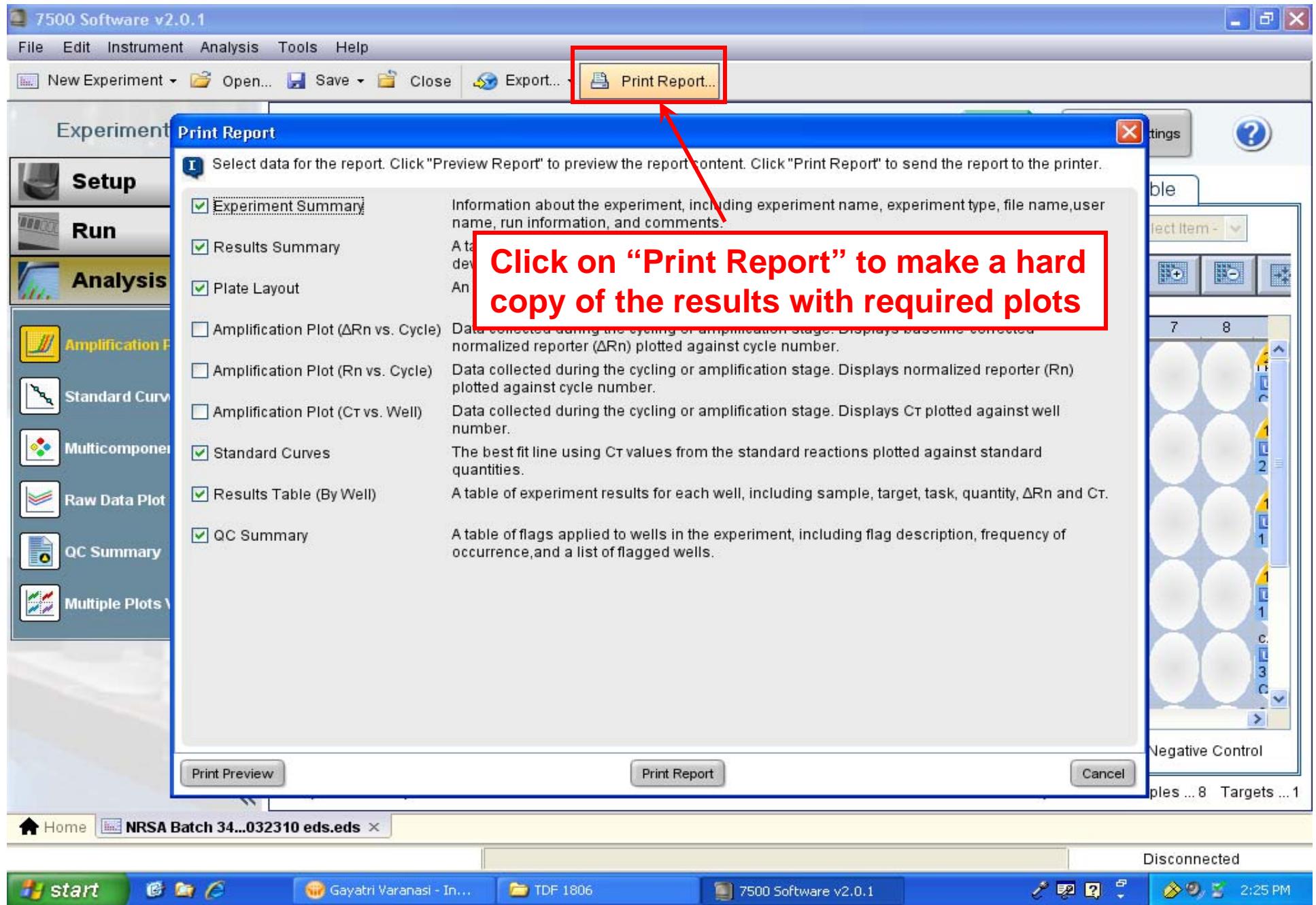
Analysis Menu – Multiple Plots View

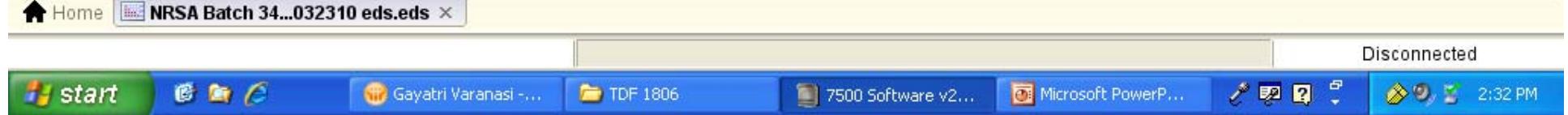
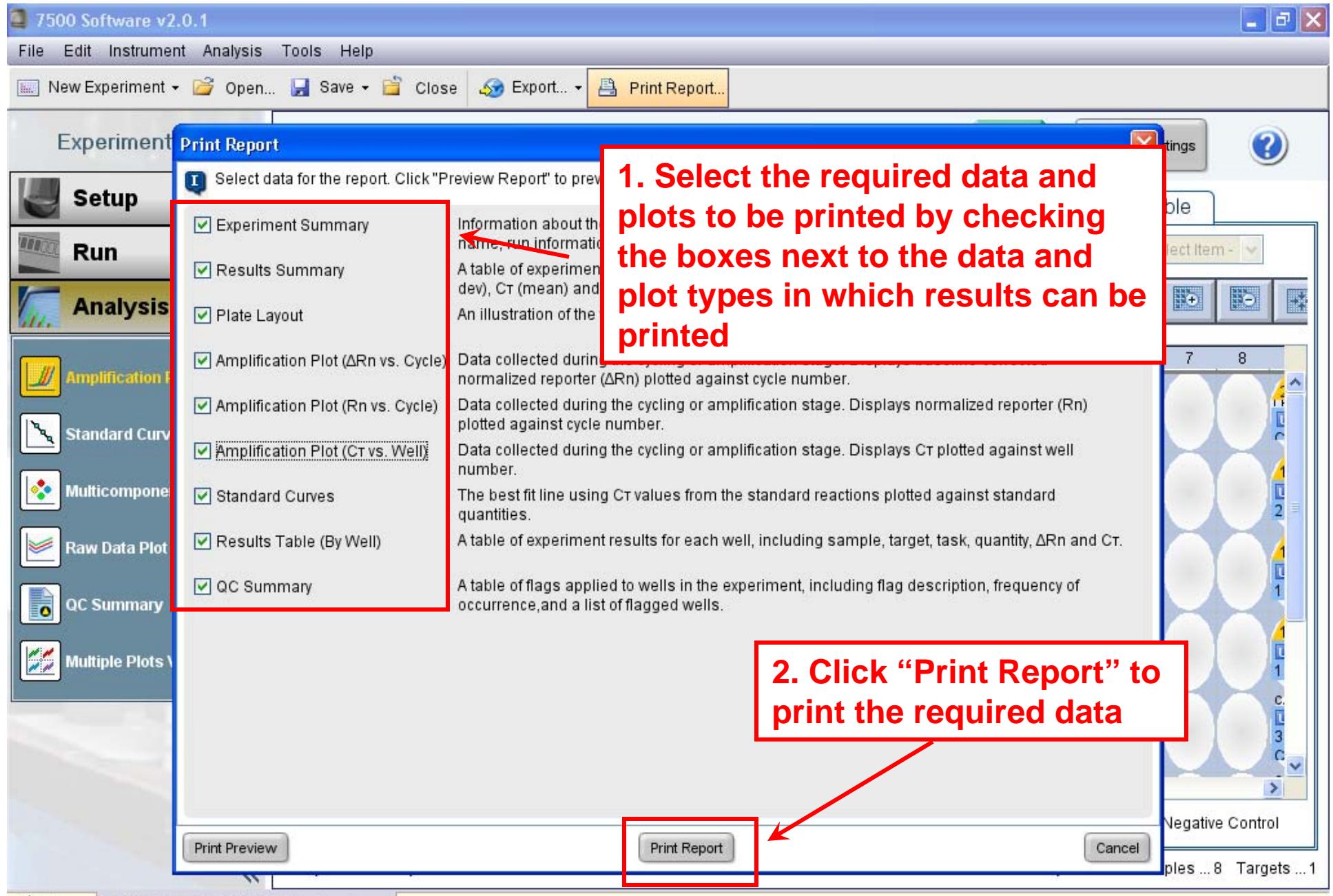
Select “Multiple Plot View” to view all the plots for the samples in one window

The screenshot shows the Bio-Rad iQ5 Real-Time PCR Software interface. On the left, the Experiment Menu is open, with the Analysis option selected. A red box highlights the "Multiple Plots View" option under Analysis. A red arrow points from this highlighted option to the main window. The main window displays the "Multiple Plots View" interface, which contains four sub-plots: "Amplification Plot - ΔRn...", "Standard Curve", "Multicomponent Plot", and "Raw Data Plot". Each plot has a dropdown menu icon at the top and a toolbar below it. To the right of the plots is the "View Plate Layout" panel, which shows a grid for wells A1 through H12, with buttons for "Show in Well..." and "View Legend". A status bar at the bottom provides analysis summary information.

Each plot has a drop down menu located on the top to access various parameters required to displayed and also has the toolbar feature with various options of printing, saving, and other parameters

Analysis – To Print a Data Report





Appendix B:
Cepheid Smart Cycler® Operation

Appendix B - qPCR analysis on Cepheid Smart Cycler®

1.0 Smart Cycler® Operation

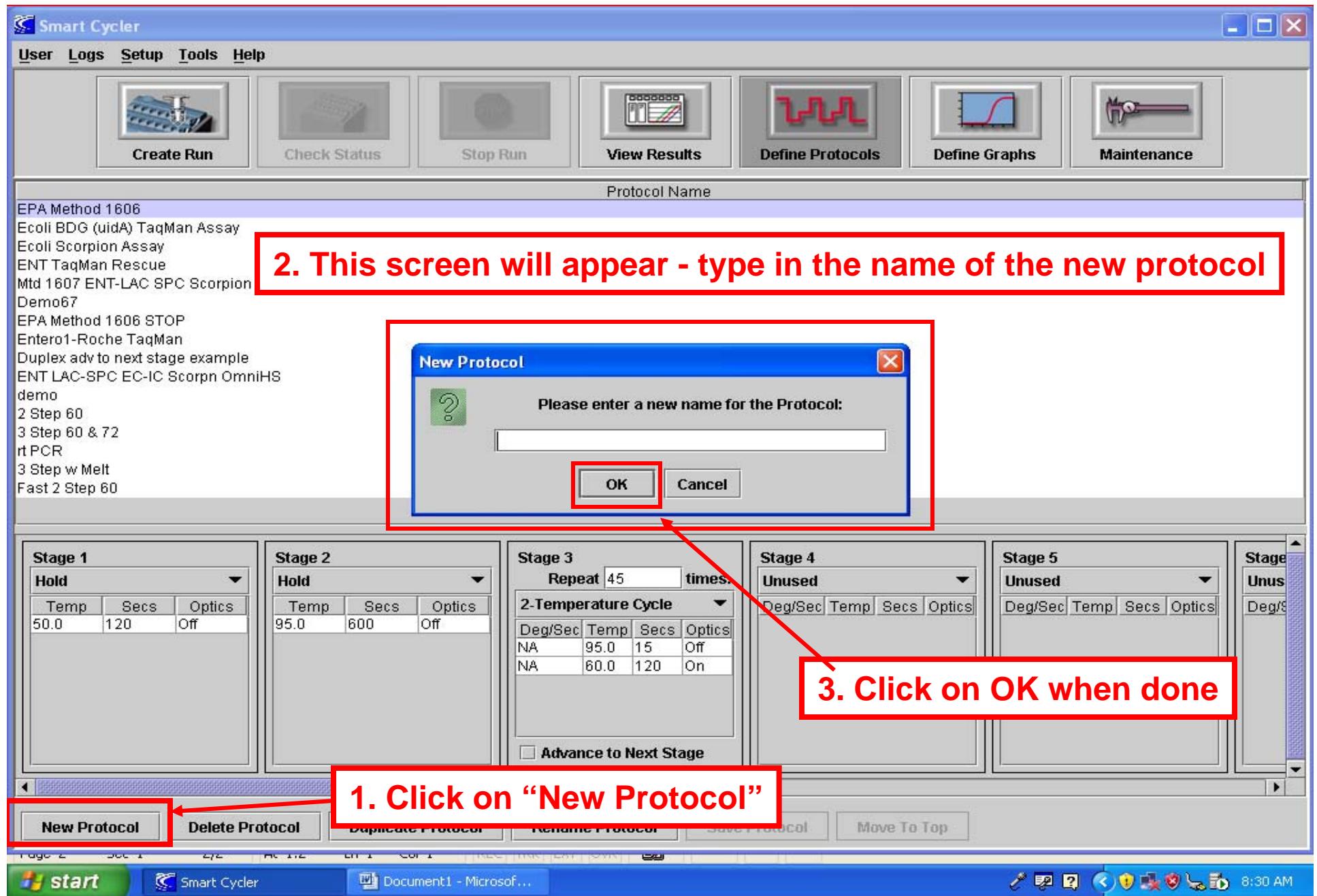
- 1.1 This protocol is intended to provide only information about critical instrument settings required to perform EPA Method B. Further details concerning the operation of the instrument can be obtained from the Smart Cycler® Operation Manual, Cepheid Part #D0190, Rev. D.
- 1.2 Turn on the Smart Cycler®; then the computer.
- 1.3 Double-click on the **Smart Cycler®** icon on the computer desktop.
- 1.4 The following steps for defining a protocol are only required before the initial run of the instrument. The protocol that is defined in these steps is used in all subsequent runs of the instrument.
 - 1.4.1 Click on the **Define Protocols** icon to go to **Define Protocols** screen.
 - 1.4.2 Click on the **New Protocol** button to open the **Protocol Name?** dialog. Enter "EPA Method B" for the new protocol name, and click **OK**. The protocol stages are defined in the series of boxes at the bottom of the **Define Protocol** screen. Make sure the new protocol is highlighted and begin to choose state settings. To define **Stage 1**, click on its drop-down box to display the menu of stage types; then select **Hold**. In the **Temp** column, enter 50.0, and in **Secs** column, enter 120, leaving the **Optics** setting as the default **Off** setting. (*Note:* This stage is performed to eliminate potential PCR carryover products in the reactions using the Amp-Erase® UNG enzyme provided in the TaqMan® Universal PCR Master Mix). Note that the optical reading cannot be programmed during a Hold stage.
 - 1.4.3 To define **Stage 2**, click on its drop-down box to display the menu of stage types, and again select **Hold**. In the **Temp** column, enter 95.0, and in **Secs** column enter 600. Again, leave the **Optics** setting on **Off**. (*Note:* This stage is performed to deactivate the Amp-Erase® UNG enzyme).
 - 1.4.4 To define **Stage 3**, select **2-Temperature Cycle** from its drop down menu. For the first step, enter 95.0 in the first row of the **Temp** column and 15 in the **Secs** column, **Optics** column **Off**. For the second step, enter 60.0 in the second row of the **Temp** column, 120 in the **Secs** column, and click on the **Optics** cell to select **On** from the drop-down menu. *Note:* This sets the detection of the fluorescence signal to occur at the end of the second step in each cycle. Enter 45 in the **Repeat** field at the top of the **Stage 3** box to specify that it should be repeated for 45 cycles. Click the **Save Protocol** button.
 - 1.4.5 To display primary curve graphs, click **Define Graphs**. Highlight **FAM** in **Graph** column. Check the box for **Automatically add to new runs**. Under **Graph Type** choose **Optics** from the pull down menu. Under **Channels** check the box for **Ch 1 (FAM)**. Under **Show** check the boxes for **Primary Curve**, **Threshold (Vertical)** and **Threshold (Horizontal)**. Under **Axes** check the box for **Fluorescence vs. Cycle**. At the bottom of the screen, click on **Save Graph**.
- 1.5 Click on the **Create Run** icon to open the **Create Run** screen. For each new run, enter a unique name in the **Run Name** field. (*Note:* The software does not allow duplicate run names).
- 1.6 Enter any additional information about the run in the **Notes** field. Click the arrow in the **Dye Set** box to display a drop-down menu of the possible selections. Select **FTTC25**. (*Note:* This selects the dye set: FAM, TET, Tex Red, Cy5, and a 25 µL reaction).

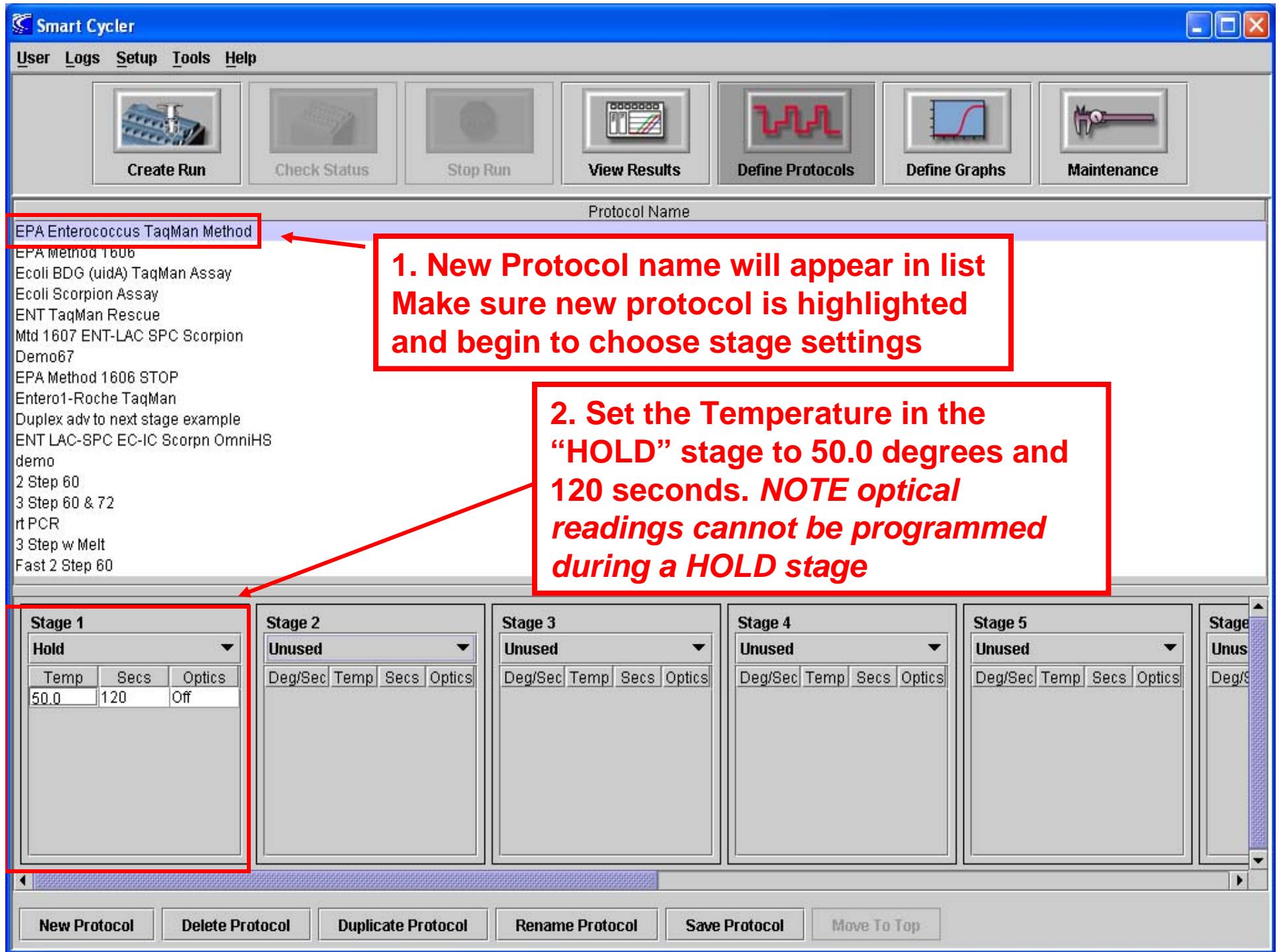
- 1.7** Click the **Add/Remove Sites** button. The **Select Protocols and Sites...** dialog will appear. Highlight (click on) the “EPA Method B” protocol developed prior to the first run (Section 1.4.2) in the **Protocols** list. In the **Sites** list, highlight the sites on the instrument to be used with this protocol in the current run by clicking on them with the shift key held down (*Note:* Sites refer to the I-core modules in the Smart Cycler® processing block in which reaction tubes will be placed; a total of 16 are possible per block. When using multiple blocks, the site numbers will be preceded by the block letters, *e.g.*, A, B, C); then click the right pointing arrow to transfer the selected sites and protocol to the **Selections** table.
- 1.8** Click on the **OK** button to save the selections, and return to the **Create Run** screen.
- 1.9** Place the loaded Smart Cycler® reaction tubes in the I-core module slots, selected above for current run. The tubes should snap into place. Either the front or back of the caps can face the front of the processing block.
- 1.10** In one of the **View** menus that is shown, select **Analysis Settings**. The displayed table includes one row for each of the four possible dye channels defined in the dye set. Click on the cell in the **FAM** row under the **Usage** column heading, and select **Assay** from the drop down menu. Set the **Usage** cells for all other dyes to **Unused** in the same manner. (*Note:* All assays in this protocol use FAM as the reporter dye). All other cells in this table should be left at default settings (See Smart Cycler® Operation Manual).
- 1.11** In the other **View** menu that is shown, select the **Results Table**. Enter the sample identification information for each site in the **Sample ID** column (additional information can be entered into the **Notes** column.). Leave the other columns as default settings (see Smart Cycler® Operation Manual.).
- 1.12** Click on the **Start Run** button. The orange LEDs on the Smart Cycler® processing block should turn on, and the software will automatically switch to the **View Results** screen.
- 1.13** To display the real time temperature profiles for all sites, click **Temperature** in either of the **View** menus. To display real time growth curves for all samples (*i.e.*, the fluorescence signal vs. cycle), click **FAM** in the other **View** menu.
- 1.14** At the end of the run, it is recommended to check the cycle threshold values calculated by the instrument for each sample by opening the **Results Table** window by clicking on this selection in the upper **View** menu. It is also recommended to inspect the growth curves in the **FAM** window which can be opened in the same manner from the lower **View** menu. The default threshold fluorescence value is shown in this window as a single horizontal red line and the cycle thresholds for each site are shown as vertical red lines. To view the data for individual sites in this window, click on that site number in the table to the right of the graph. If the default threshold fluorescence line is well above all of the growth curve lines prior to visible amplification, the threshold fluorescence value can be changed to a lower value. This is done by reopening the **Analysis Settings** window from the upper **View** menu and entering a new value in the **Manual Thresh Fluor Units** cell in the **FAM** row. Conversely if the default threshold fluorescence line is below any of the growth curve lines prior to visible amplification, the threshold fluorescence value should be changed to a higher value in the same manner. Previous studies have indicated that a threshold value of 8 works well for most analyses. Click on the **Update Analysis** button to view the new threshold line in the **FAM** window. The cycle threshold values will be automatically updated in the **Results Table**.
- 1.15** Once the threshold fluorescence value is adjusted to an optimal value, click the **Save Run** button. (*Note:* The Smart Cycler® Software does not give a prompt to save changes before printing or exporting. Therefore, it is possible to make changes to the Results Table or Analysis Settings, and immediately print or export the data, then close the run without saving the changes. In this case,

the data saved in the Smart Cycler® database will not match the printed or exported data. If no changes are made in the threshold fluorescence value, the run data is automatically saved as it is when the program is closed or a new run is created).

- 1.16 To set up automatic export of raw data, (see “How To Set Up Automatic Export of Raw Data” for screen shots) from the main menu, click on **Setup**, then **System Defaults**, then **Export Settings**. From the Export Settings dialog box, check the box beside Results Table and Analysis Settings. Click the **Browse** button to select the folder where you want your raw data saved. Click on the radio button next to Automatic export on run completion, then click the **OK** button.
- 1.17 To manually save the **Results Table and Analysis setting** containing the instrument-calculated cycle threshold values for each sample (see “How to Manually Export Raw Data” for screen shots), click the **Export** button to display the **Export Data** dialog box. Check the box next to the heading **Export Results Table and Analysis Settings** by clicking on it. Leave all other boxes unchecked. Click on **Export**. A box labeled **Export Data** will appear with the run name in the file name box. Click on the drop down menu to the right of Save In: at the top left of the screen to change the directory where you want your data saved. Always save to the hard drive. Click on **Save**. Data are exported as comma-delimited text (*.cvs) files in MS Excel-compatible files to the Export folder in the Smart Cycler® folder: C:\Smart Cycler®\Export. Analysts will later save the file as an XLS file.
- 1.18 To archive a run in the Smart Cycler® program, click on **Tools**. From the drop down menu click on **Data Management** and then **Archive Runs**. Click **Proceed**. Select the run to be archived by clicking on its name in the database list. Click **OK** and then **Proceed**. A box labeled **Archive Run** will appear on the screen. There will be a line labeled **Save in:**. Input which directory you would like the run to be saved in. There will be a line **File Name:**. Enter a file name. Click **Save**. Click **OK**.
- 1.19 To print run data (see “How to Print Run Data” for screen shots), after the run is completed, right click on the graph area, and select **Print**, then **Print Graph with Results Table**. An Optics Graph print preview screen will pop up, click **Print**. The screen will switch back to the post run screen. To print the generated report, click **Report**. A Run Report print preview screen will pop up, click **Print**.
- 1.20 To set up a new analysis (see “How to Set Up a New Run” for screen shots), from the post run screen, click **Create Run** at the top left. The results of your just completed will disappear and an **Add/Remove Sites** button will appear in the middle of the screen – click it. A Select Protocols and Sites screen will pop up, select **EPA Method B** in the Protocols box, and highlight the amount of sites needed for the assay (A1-A16) in the Sites box. Click the right arrow to the right of the sites box to move the sights to the Selections box. After double checking that the correct protocol is highlighted, click **OK**. Add the Run Name in the Run Name box. The Site ID, Protocol, Sample ID, Sample Type, Notes (etc.) box will pop up. Enter the Sample ID for each site. Under this box, find the Usage column and click on **Assay** to select **Unused** for Cy3, TxR and Cy5 (leave Assay for FAM). In the same box, find the Manual Thresh Fluor Units, and click on the units for FAM to change it from 30.0 to 8.0 (leave the units at 30.0 for Cy3, TxR and Cy5). After loading the Smart Cycler with tubes, click **Start Run**. A red light should appear on sites that are in use.

How to Set Up a New Experiment Using the Smart Cycler® (Software Version 2.0)







User Logs Setup Tools Help



Create Run



Check Status



Stop Run



View Results



Define Protocols



Define Graphs



Maintenance

Protocol Name

EPA Enterococcus TaqMan Method

EPA Method 1606

Ecoli BDG (uidA) TaqMan Assay

Ecoli Scorpion Assay

ENT TaqMan Rescue

Mtd 1607 ENT-LAC SPC Scorpion

Demo67

EPA Method 1606 STOP

Enter01-Roche TaqMan

Duplex adv to next stage example

ENT LAC-SPC EC-IC Scorp OmniHS

demo

2 Step 60

3 Step 60 & 72

rt PCR

3 Step w Melt

Fast 2 Step 60

In Stage 2, set the temperature to 95.0 degrees
and 600 seconds

Stage 1		
Hold		
Temp	Secs	Optics
50.0	120	Off

Stage 2		
Hold		
Temp	Secs	Optics
95.0	600	Off

Stage 3		
Unused		
Deg/Sec	Temp	Secs

Stage 4		
Unused		
Deg/Sec	Temp	Secs

Stage 5		
Unused		
Deg/Sec	Temp	Secs

Stage	Unus
	Deg/S

New Protocol

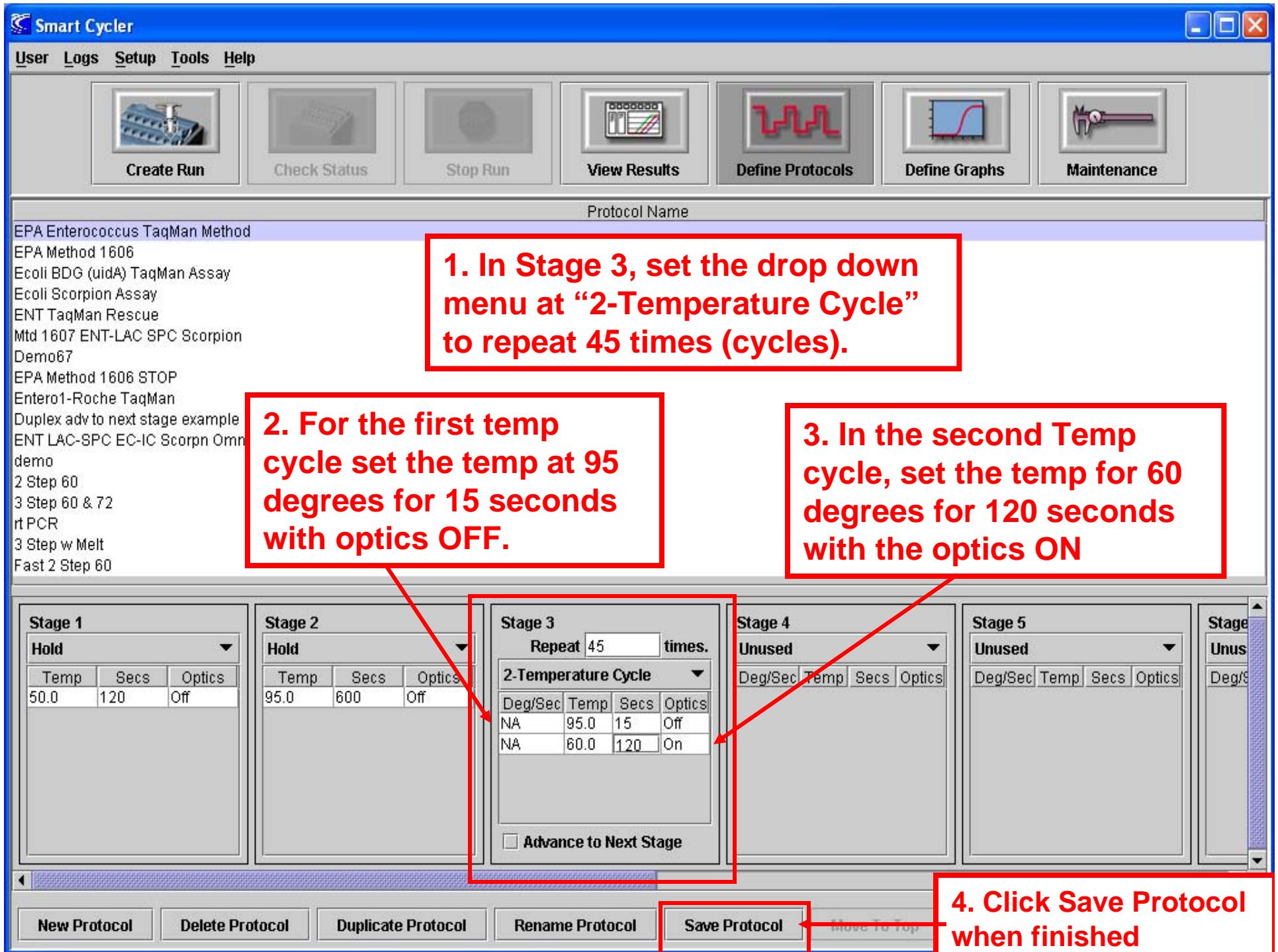
Delete Protocol

Duplicate Protocol

Rename Protocol

Save Protocol

Move To Top



How To Set Up Automatic Export of Raw Data

The screenshot shows the Smart Cycler software interface. The 'Setup' menu is open, and the 'System Defaults' option is selected. A red arrow points from the text in the bottom right corner to the 'Export Settings' button in the 'System Defaults' menu.

Run Name: NRSA 2009 Batch 25E ENT 091809

User: Default User

Started: Sep 18, 2009 01:30 PM
Finished: Sep 18, 2009 03:37 PM

Status: Done

Notes: NRSA 2009 ENT Analysis of Batch 25E

Dye Set: FCTC25

Protocols:

Protocol	Lot Number
EPA Method 160...	

Number of Sites: 30

User Administration

- General
- Analysis Settings
- Automatic Backup
- Export Settings** (highlighted)
- Melt Settings
- Access Options

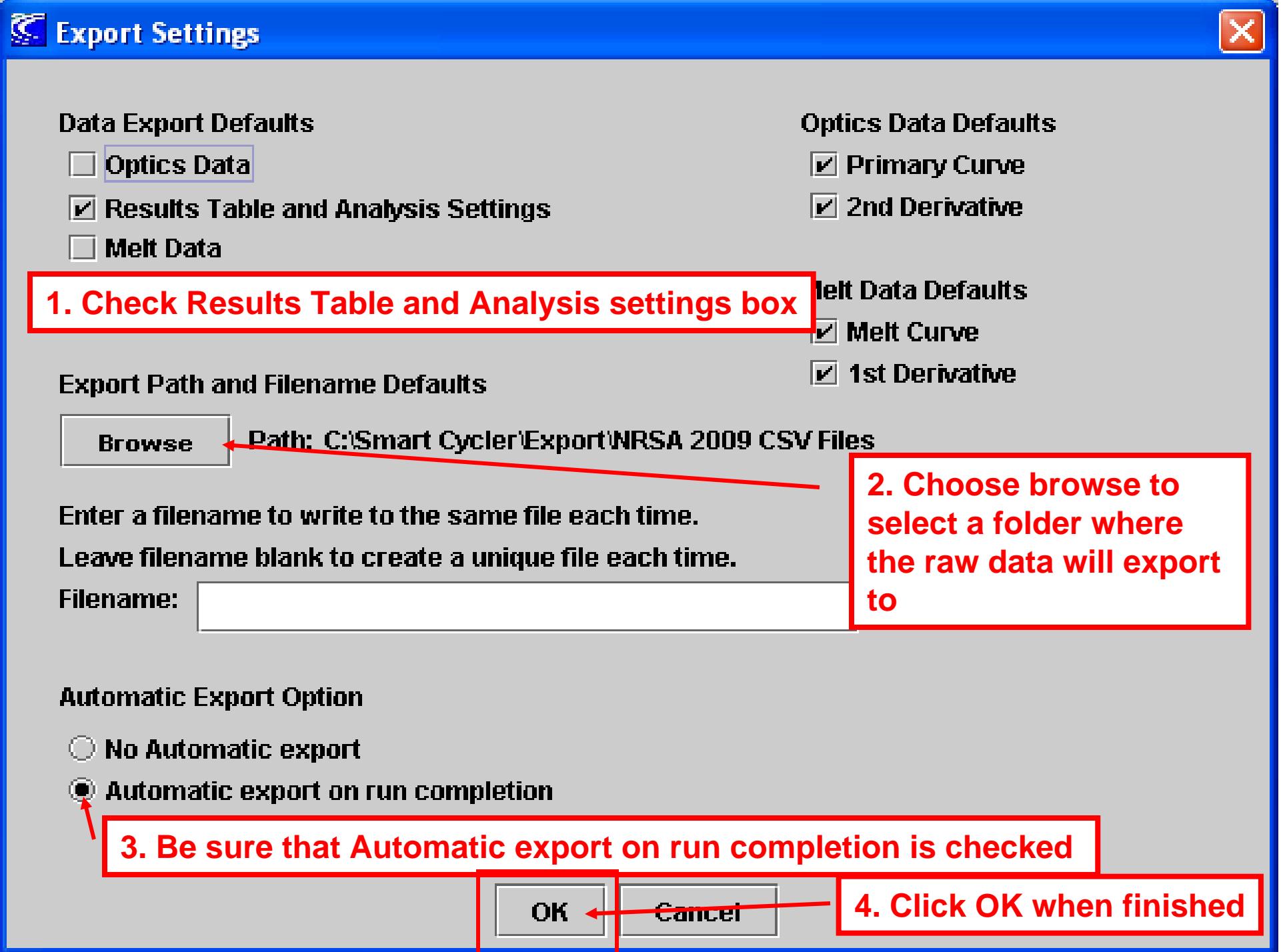
Results Table

Ch	Dye	Usage	Bkgnd Sub	Bkgnd Min Cycle	Bkgnd Max Cycle	Curve Analysis	Thresh Setting	Manual Thresh Fluor Units	Auto Thresh #SD's
1	FAM	Assay	ON	5	40	Primary Curve	Manual	8.0	NA
2	Cy3	Unus...	ON	5	40	Primary Curve	Manual	30.0	NA
3	TxR	Unus...	ON	5	40	Primary Curve	Manual	30.0	NA
4	Cy5								5

Stop Run **View Results** **Define Protocols** **Define Graphs** **Maintenance**

Save Run **Export** **Report** **Select Graphs** **View Another Run** **Delete Run(s)** **Update Analysis** **Import Std Curve** **Compare Run**

To set up automatic export of Raw Data into computer, click on "SETUP", then "System Defaults" then "Export Settings"



How To Manually Export Raw Data

Smart Cycler

User Logs Setup Tools Help

Create Run Check Status Stop Run View Results Define Protocols Define Graphs Maintenance

Run Name: NRSA 2009 Batch 25E ENT 091809

User: Default User

Started: Sep 18, 2009 01:30 PM
Finished: Sep 18, 2009 03:37 PM

Status: Done

Notes: NRSA 2009 ENT Analysis of Batch 25E

Dye Set: FCTC25

Protocols:

Protocol	Lot Number
EPA Method 160...	

Number of Sites: 30

Views

Results Table

Analysis Settings

Protocols

Standard - Tet
Temperature
Intercalate
Melt
FAM
Cy3
Texas Red
Cy5
Standard - FAM

Site ID Protocol Sample ID Sample Type Notes Status FAM Std/Res FAM Ct

A1 EPA Met... LB-96 UNKN ENT 5X OK NEG 0.00
A2 EPA Met... 538480 UNKN ENT 5X OK POS 35.00
A3 EPA Met... 538248 UNKN ENT 5X OK POS 35.15
A4 EPA Met... 537352 UNKN ENT 5X OK POS 34.07
A5 EPA Met... 538574 UNKN ENT 5X OK POS 39.79
A6 EPA Met... 538658 UNKN ENT 5X OK POS 36.69
A7 EPA Met... 537351 UNKN ENT 5X OK POS 37.20
A8 EPA Met... 538651 UNKN ENT 5X OK POS 33.64
A9 EPA Met... 538655 UNKN ENT 5X OK POS 28.93
A10 EPA Met... 537353 UNKN ENT 5X OK POS 29.32
A11 EPA Met... 538673 UNKN ENT 5X OK POS 33.75
A12 EPA Met... 538143 UNKN ENT 5X OK POS 36.14
A13 EPA Met... 538045 UNKN ENT 5X OK POS 36.04

Views

Results Table

Analysis Settings

Protocols

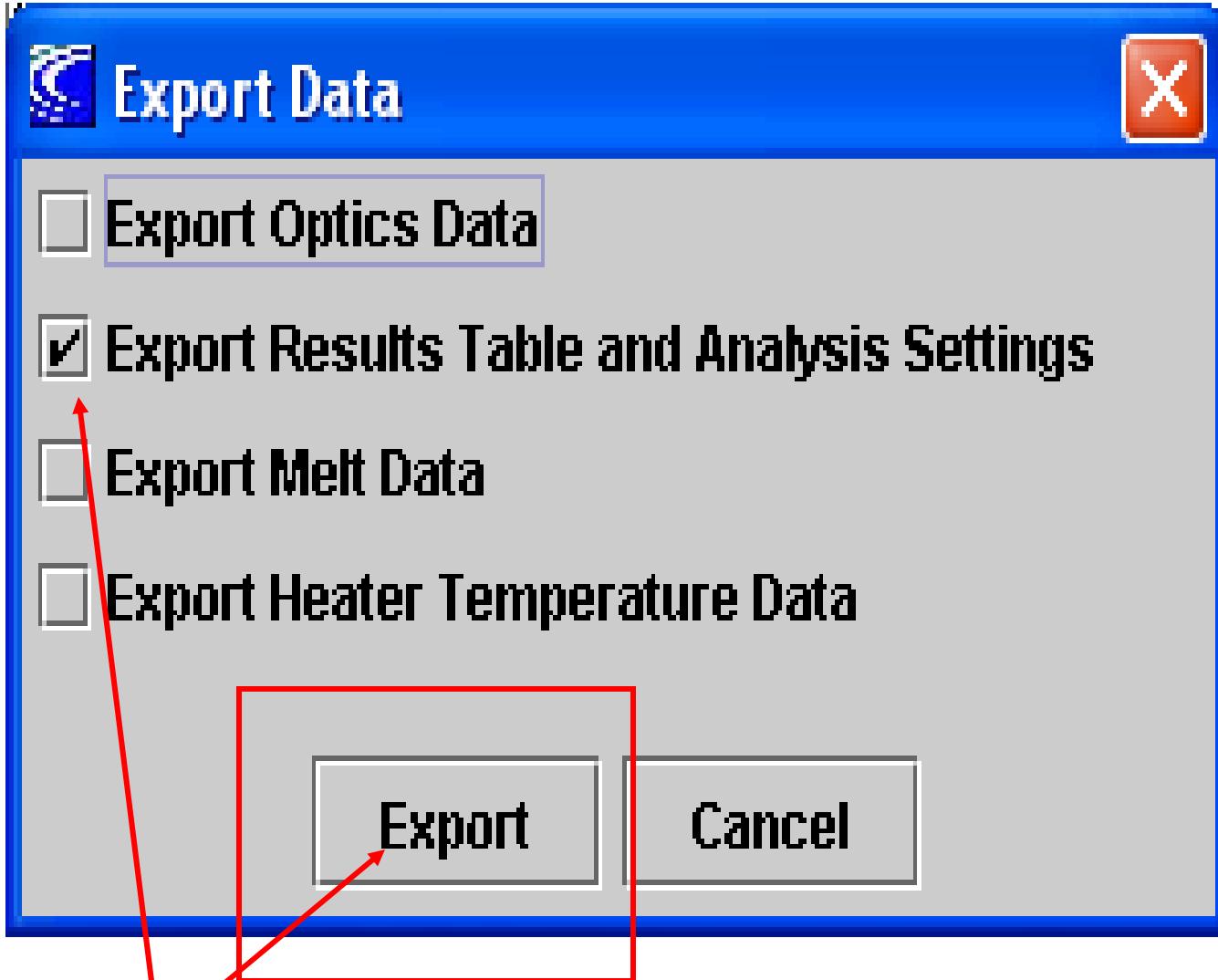
Standard - Tet
Temperature
Intercalate
Melt
FAM
Cy3
Texas Red
Cy5
Standard - FAM

Ch # Dye Name Usage Bkgnd Sub Bkgnd Min Cycle Bkgnd Max Cycle Curve Analysis Thresh Setting Manual Thresh Fluor Units Auto Thresh #SD's A

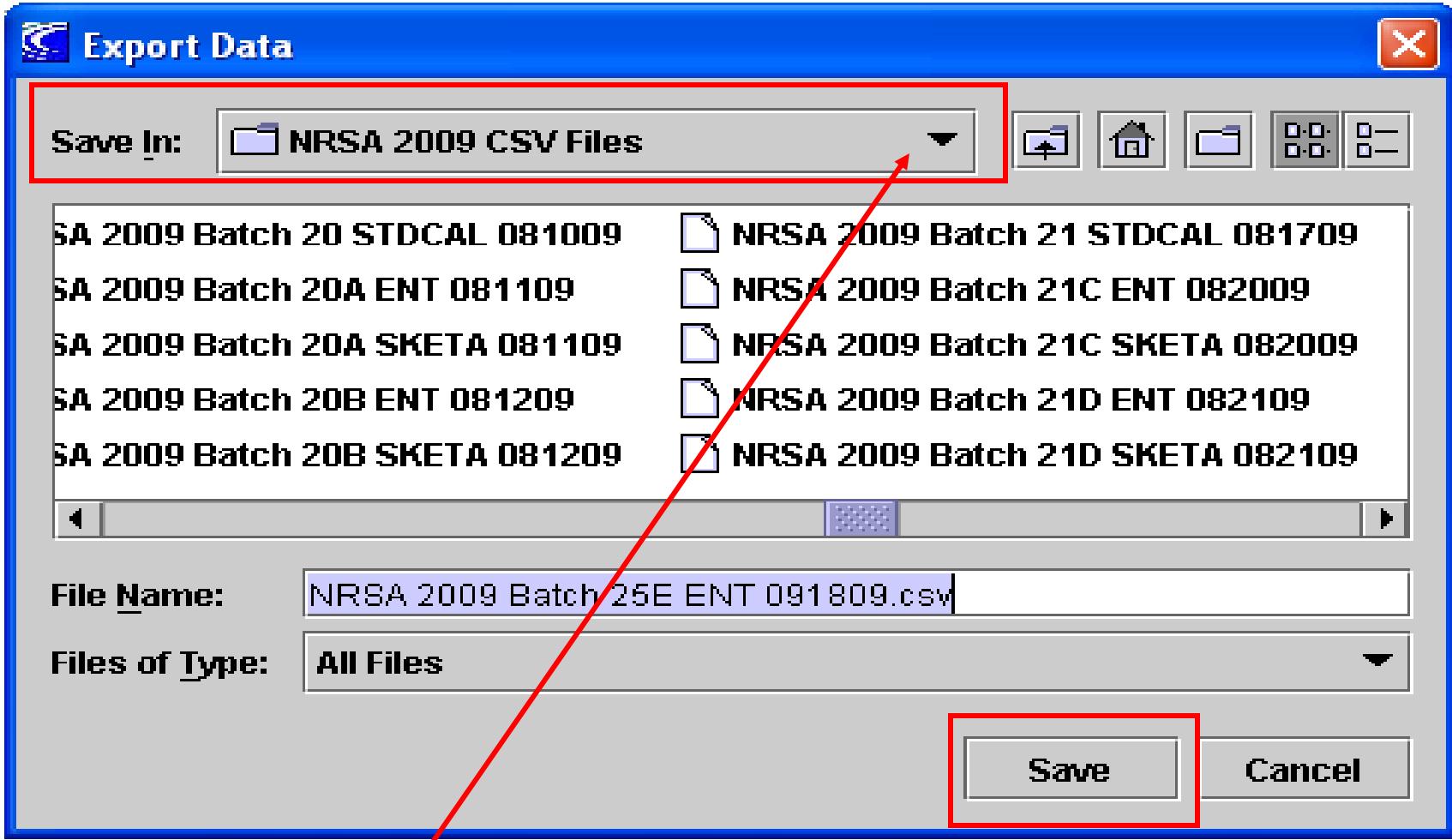
1 FAM Assay ON 5 40 Primary Curve Manual 8.0 NA 5
2 Cy3 Unus... ON 5 40 Primary Curve Manual 30.0 NA 5
3 TxR Unus... ON 5 40 Primary Curve Manual 30.0 NA 5
4 Cy5 Unus... ON 5 40 Primary Curve Manual 30.0 NA 5

Save Run **Export** Report Select Graphs View Another Run Delete Run(s) Update Analysis Import Std Curve Compare Run

Once run is finished, click Export

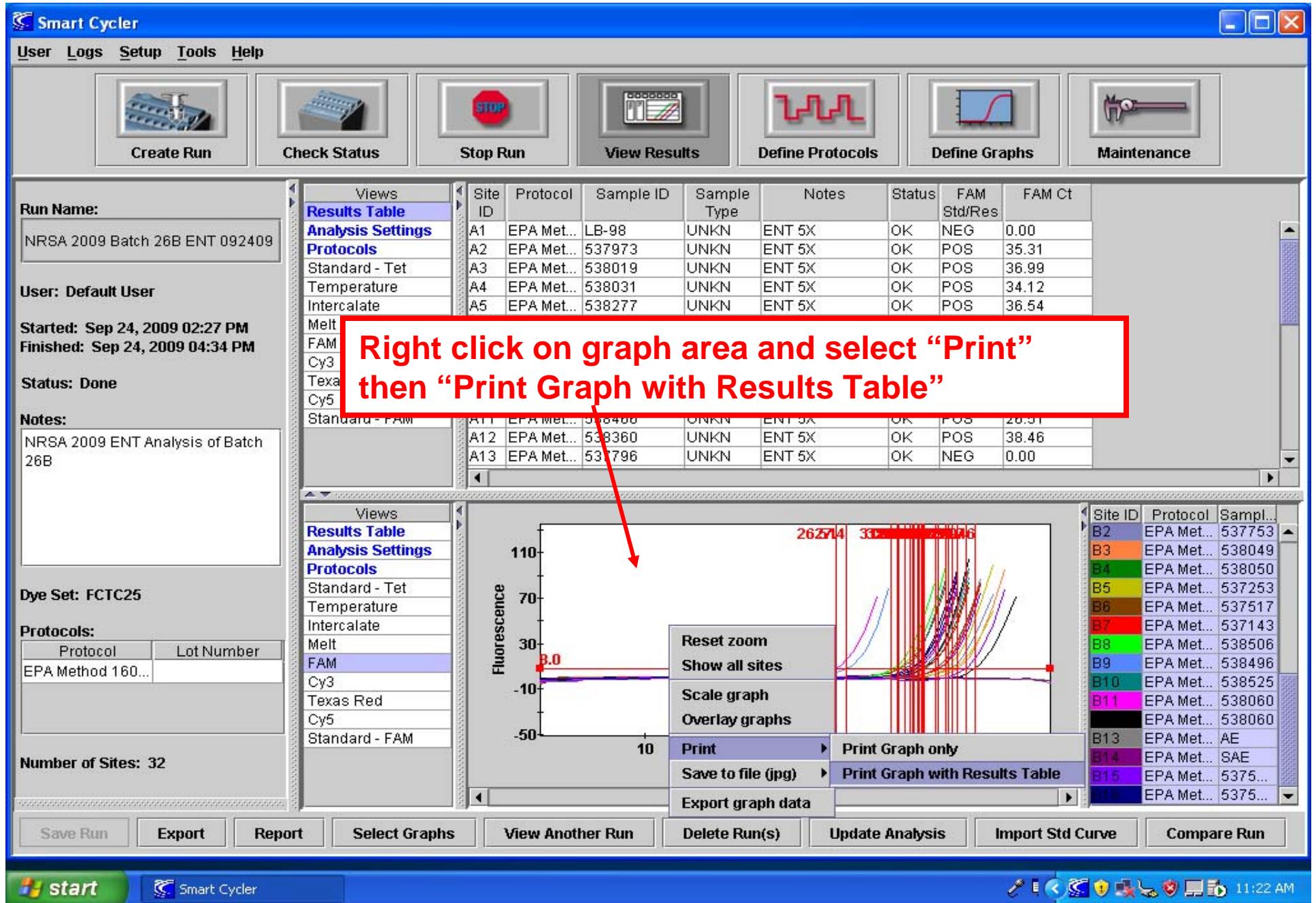


Check the Export Results Table and Analysis Settings box
then click “Export”



Choose the appropriate file folder to save the Raw Data to and click SAVE. NOTE: All raw data will export in the form of a CSV file. Analysts will later save file as an “XLS” file.

How to Print Run Data



Smart Cycler

User Logs Setup Tools Help

Create Run

Run Name: NRSA 2009 Batch 26B ENT 092409

User: Default User

Started: Sep 24, 2009 02:27 PM
Finished: Sep 24, 2009 04:34 PM

Status: Done

Notes: NRSA 2009 ENT Analysis of Batch 26B

Dye Set: FCTC25

This sheet will pop up. Click "Print" to print file

Print Preview

Valued Smart Cycler Customer 2009/10/2 11:25 AM

Optics Graph

Run Name : NRSA 2009 Batch 26B ENT 092409
User Name : Default User
Run Date : Sep 24, 2009 02:27 PM

Fluorescence

26274 331 346

8.0

110
70
30
-10
-50

10 20 30 40

Cycles

Site Legend

Site ID	Sample ID	FAM Ct	Protocol	Data Type	Line Type
A1	LB-98	0.00	EPA Method 1606 STOP	Primary Curve	—
A2	537973	35.31	EPA Method 1606 STOP	Channel	Symbol
A3	538019	36.99	EPA Method 1606 STOP	FAM	None

1 of 3 First Previous Next Last Print Close

11:25 AM

Smart Cycler



User Logs Setup Tools Help



Create Run



Check Status



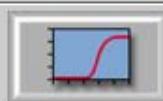
Stop Run



View Results



Define Protocols



Define Graphs



Maintenance

Run Name:

NRSA 2009 Batch 26B ENT 092409

User: Default User

Started: Sep 24, 2009 02:27 PM

Finished: Sep 24, 2009 04:34 PM

Status: Done

Notes:

NRSA 2009 ENT Analysis
26B

- Views
- Results Table**
- Analysis Settings
- Protocols
- Standard - Tet
- Temperature
- Intercalate
- Melt
- FAM
- Cy3
- Texas Red
- Cy5

Site ID	Protocol	Sample ID	Sample Type	Notes	Status	FAM Std/Res	FAM Ct
A1	EPA Met...	LB-98	UNKN	ENT 5X	OK	NEG	0.00
A2	EPA Met...	537973	UNKN	ENT 5X	OK	POS	35.31
A3	EPA Met...	538019	UNKN	ENT 5X	OK	POS	36.99
A4	EPA Met...	538031	UNKN	ENT 5X	OK	POS	34.12
A5	EPA Met...	538277	UNKN	ENT 5X	OK	POS	36.54
A6	EPA Met...	520186	UNKN	ENT 5X	OK	POS	31.96
A7	EPA Met...	520187	UNKN	ENT 5X	OK	POS	34.05
A8	EPA Met...	538579	UNKN	ENT 5X	OK	POS	31.30
A9	EPA Met...	538561	UNKN	ENT 5X	OK	POS	27.40
A10	EPA Met...	538583	UNKN	ENT 5X	OK	POS	32.48
						POS	26.51
						POS	38.46
						NEG	0.00

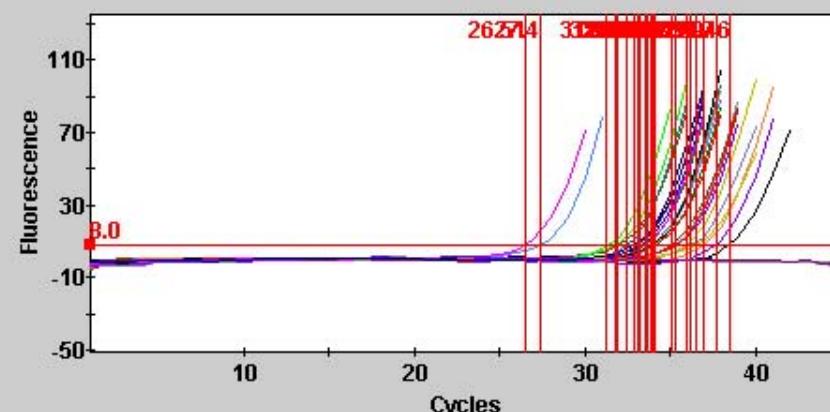
To print the generated report, click on "Report"

Dye Set: FCTC25

Protocols:

Protocol	Lot Number
EPA Method 160...	

Number of Sites: 32



Site ID	Protocol	Sampl...
B2	EPA Met...	537753
B3	EPA Met...	538049
B4	EPA Met...	538050
B5	EPA Met...	537253
B6	EPA Met...	537517
B7	EPA Met...	537143
B8	EPA Met...	538506
B9	EPA Met...	538496
B10	EPA Met...	538525
B11	EPA Met...	538060
B12	EPA Met...	538060
B13	EPA Met...	AE
B14	EPA Met...	SAE
B15	EPA Met...	5375...
B16	EPA Met...	5375...

Save Run

Export

Report

Select Graphs

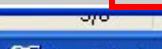
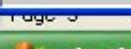
View Another Run

Delete Run(s)

Update Analysis

Import Std Curve

Compare Run



Doc1 - Microsoft Word



11:26 AM

Smart Cycler

Print Preview

Valued Smart Cycler Customer

2009/10/2 11:26 AM

Run Report

Run Information

Run Name: NRSA 2009 Batch 26B ENT 092409
User Name: Default User
Run Status: Done
Dye Set: FCTC25
Notes: NRSA 2009 ENT Analysis of Batch 26B
Analysis Settings:

Ch #	Dye Name	Usage	Bkgnd Sub	Bkgnd Min Cycle	Bkgnd Max Cycle	Curve Analysis	Thresh Setting
1	FAM	Assay	ON	5	40	Primary Curve	Manual

Manual Thresh Fluor Units	Auto Thresh #SD's	Auto Min Cycle	Auto Max Cycle	Valid Min Cycle	Valid Max Cycle	Boxcar Avg Cycles	Target
8.0	NA	5	10	3	60	0	

Protocol(s):
Name : EPA Method 1606 STOP
Lot Number: None
Stage 1: Hold 50.0°C for 120 seconds
Stage 2: Hold 95.0°C for 600 seconds
Stage 3: 2-Temperature Cycle repeat for 45 times.
95.0°C for 15 seconds
60.0°C for 120 seconds with Optics ON
Advance to Next Stage 2 Cycles after Ch 1

1 of 3 First Previous Next Last Print Close

Cycles

Number of Sites: 32

Save Run Export Report Select Graphs View Another Run Delete Run(s) Update Analysis Import Std Curve Compare Run

start Smart Cycler Doc1 - Microsoft Word

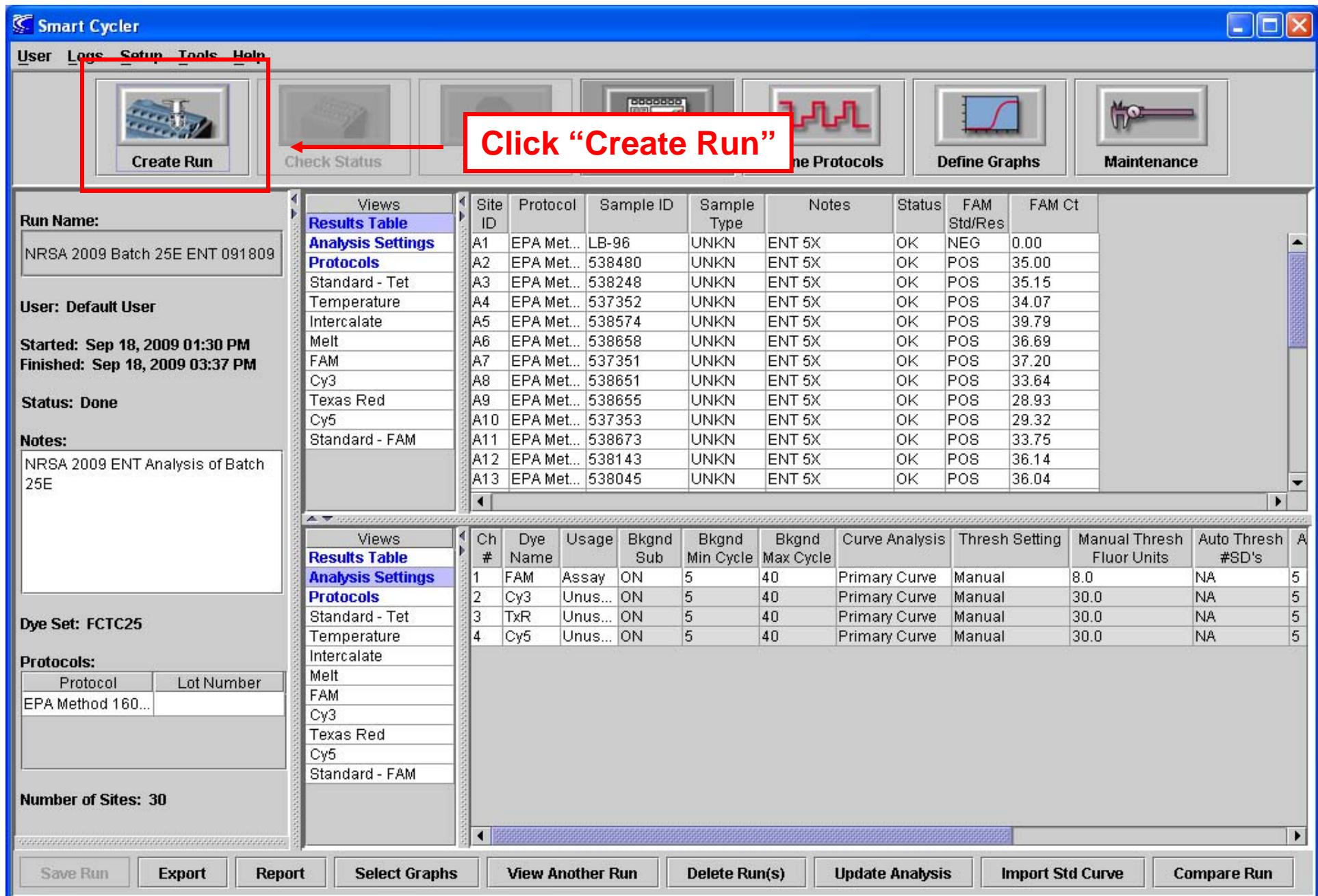
11:26 AM

Maintenance

1 Ct

Site ID Protocol Sampl...
B2 EPA Met... 537753
B3 EPA Met... 538049
B4 EPA Met... 538050
B5 EPA Met... 537253
B6 EPA Met... 537517
B7 EPA Met... 537143
B8 EPA Met... 538506
B9 EPA Met... 538496
B10 EPA Met... 538525
B11 EPA Met... 538060
B12 EPA Met... 538060
B13 EPA Met... AE
B14 EPA Met... SAE
B15 EPA Met... 5375...
B16 EPA Met... 5375...

How to Set Up a New Run



Smart Cycler



User Logs Setup Tools Help



Create Run



Check Status



Stop Run



View Results



Define Protocols



Define Graphs



Maintenance

Run Name:

Notes:

Dye Set: FCTC25

Protocols:

Protocol	Lot Number
----------	------------

Graphs:

- Standard - Tet
- Temperature
- Intercalate
- Melt
- FAM
- Cy3

Site ID	Protocol	Sample ID	Sample Type	Notes	FAM Std Conc	Cy3 Std Conc	TxR Std Conc	Cy5 Std Conc
---------	----------	-----------	-------------	-------	--------------	--------------	--------------	--------------

Add/Remove Sites

Ch #	Dye Name	Usage	Bkgnd Sub	Bkgnd Min Cycle	Bkgnd Max Cycle	Curve Analysis	Thresh Setting	Manual Thresh Fluor Units	Auto Thresh #SD's	Auto Min Cycle	Auto I Cyc
1	FAM	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
2	Cy3	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
3	TxR	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
4	Cy5	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10

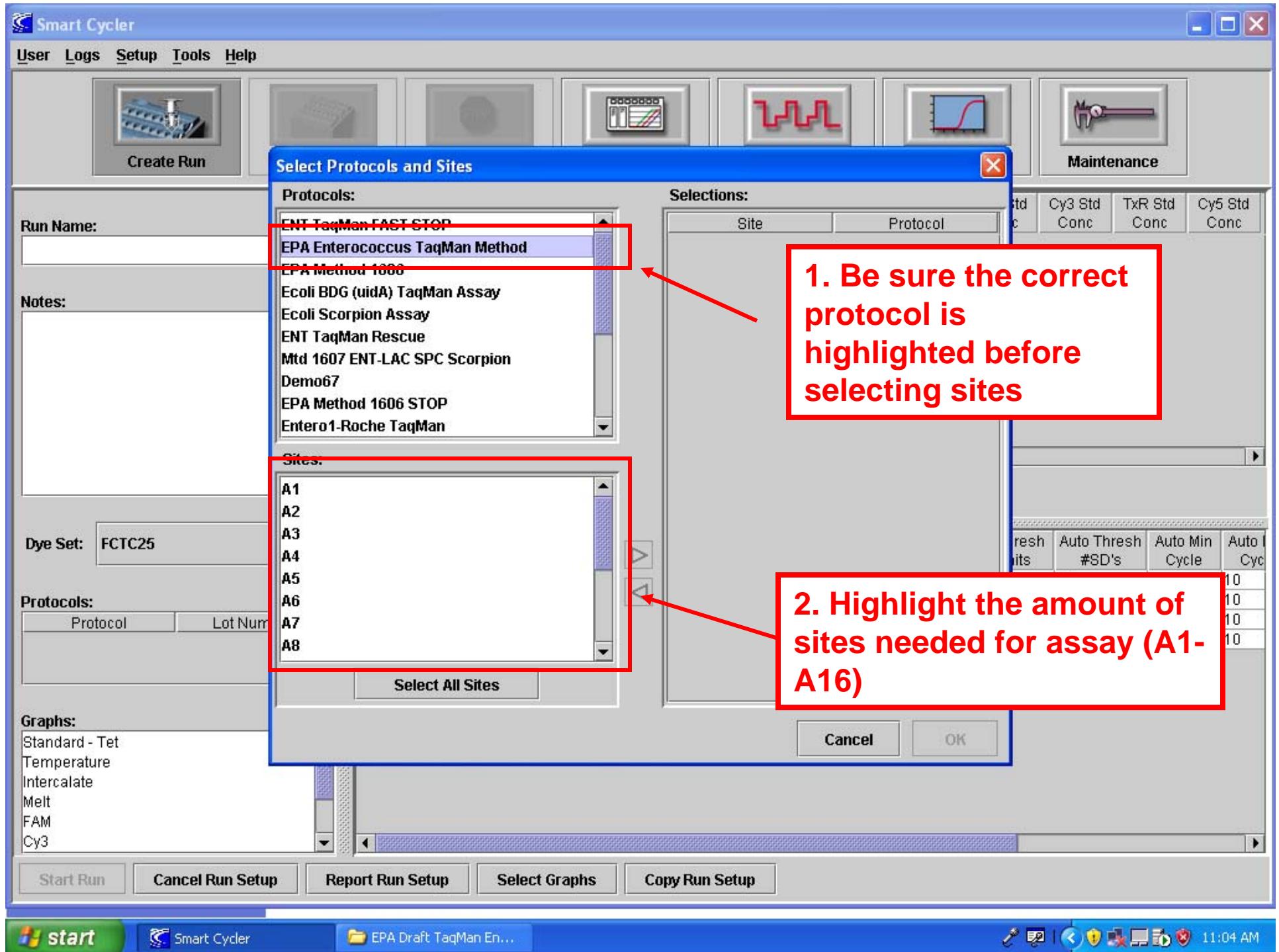
Start Run

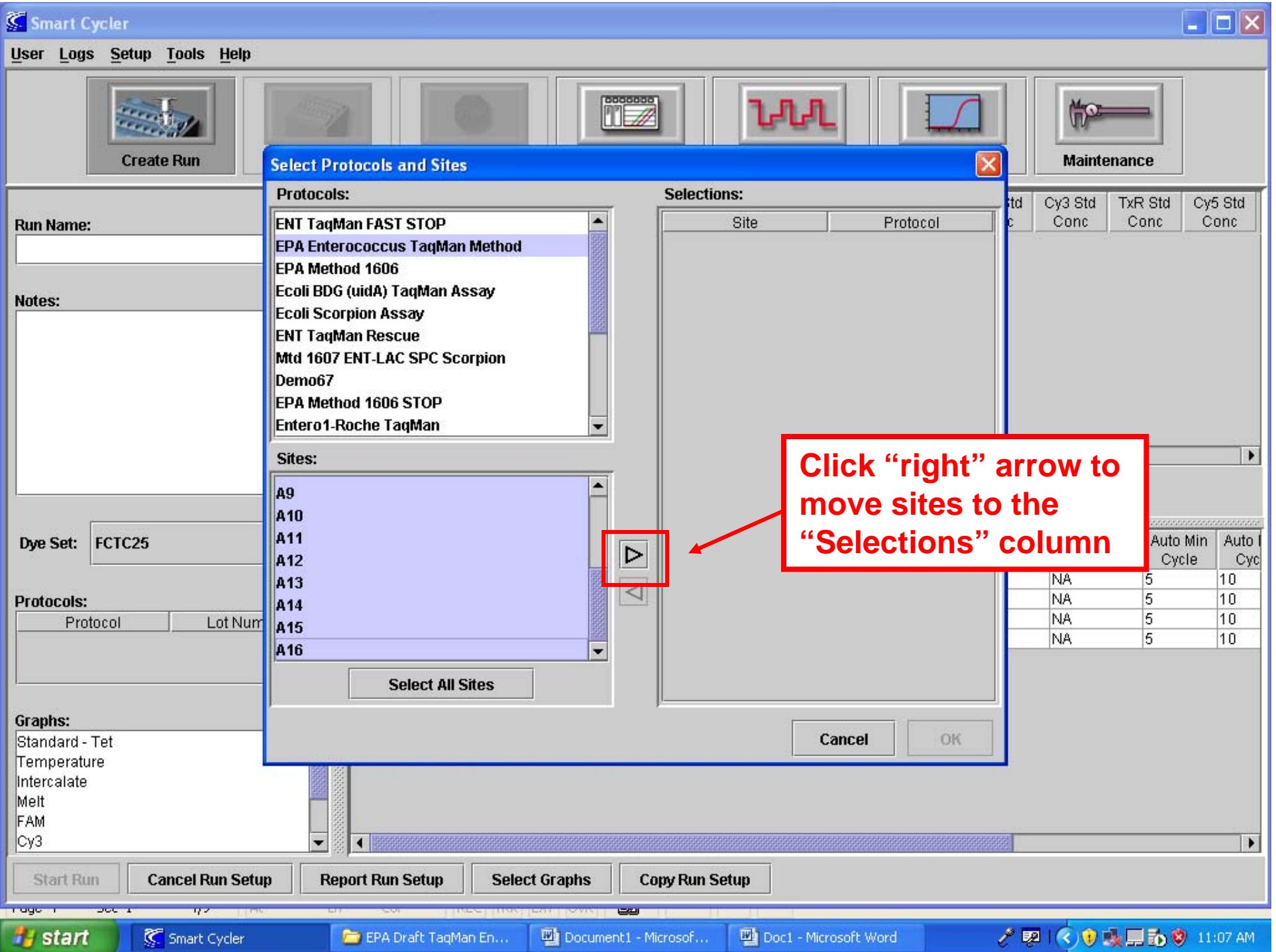
Cancel Run Setup

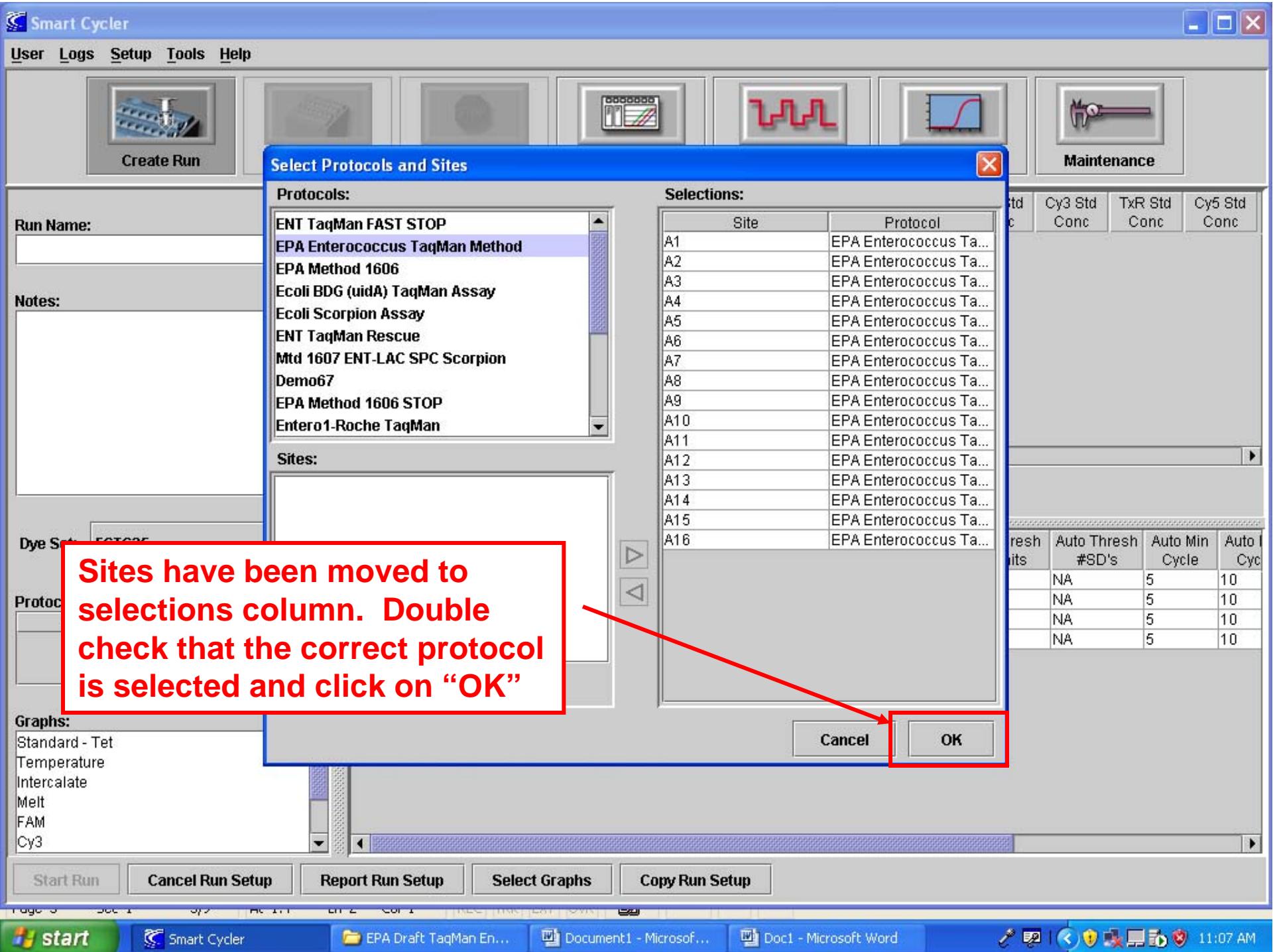
Report Run Setup

Select Graphs

Copy Run Setup









Create Run



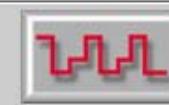
Check Status



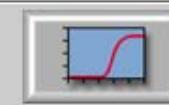
Stop Run



View Results



Define Protocols



Define Graphs



Maintenance

Run Name:

Notes:

Dye Set: FCTC25

Protocols:

Protocol Lot Number

EPA Enterococcus T...

Graphs:

Standard - Tet

Temperature

Intercalate

Melt

FAM

Cy3

Site ID	Protocol	Sample ID	Sample Type	Notes	FAM Std Conc	Cy3 Std Conc	TxR Std Conc	Cy5 Std Conc
A1	EPA Enterococcus ...		UNKN					
A2	EPA Enterococcus ...		UNKN					
A3	EPA Enterococcus ...		UNKN					
A4	EPA Enterococcus ...		UNKN					
A5	EPA Enterococcus ...		UNKN					
A6	EPA Enterococcus ...		UNKN					
A7	EPA Enterococcus ...		UNKN					
A8	EPA Enterococcus ...		UNKN					
A9	EPA Enterococcus ...		UNKN					
A10	EPA Enterococcus ...		UNKN					
A11	EPA Enterococcus ...		UNKN					

Add/Remove Sites

Ch #	Dye Name	Usage	Bkgnd Sub	Bkgnd Min Cycle	Bkgnd Max Cycle	Curve Analysis	Thresh Setting	Manual Thresh Fluor Units	Auto Thresh #SD's	Auto Min Cycle	Auto Cycles
1	FAM	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
2	Cy3	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
3	TxR	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
4	Cy5	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10

The Sample IDs and run name can be entered at this point

Start Run

Cancel Run Setup

Report Run Setup

Select Graphs

Copy Run Setup



User Logs Setup Tools Help



Create Run



Check Status



Stop Run



View Results



Define Protocols



Define Graphs



Maintenance

Run Name:

NRSA 2009 Batch 1 SKETA 092209

Notes:

NRSA 2009 SKETA Analysis of Batch 1

Dye Set: FCTC25

Protocols:

Protocol	Lot Number
----------	------------

EPA Enterococcus qP...

Graphs:

Standard - Tet
Temperature
Intercalate
Melt
FAM
Cy3

Site ID	Protocol	Sample ID	Sample Type	Notes	FAM Std Conc	Cy3 Std Conc	TxR Std Conc	Cy5 Std Conc
A1	EPA Enterococcus q...	LB-1	UNKN	SKETA 5X				
A2	EPA Enterococcus q...	1	UNKN	SKETA 5X				
A3	EPA Enterococcus q...	2	UNKN	SKETA 5X				
A4	EPA Enterococcus q...	3	UNKN	SKETA 5X				
A5	EPA Enterococcus q...	4	UNKN	SKETA 5X				
A6	EPA Enterococcus q...	5	UNKN	SKETA 5X				
A7	EPA Enterococcus q...	6	UNKN	SKETA 5X				
A8	EPA Enterococcus q...	7	UNKN	SKETA 5X				
A9	EPA Enterococcus q...	8	UNKN	SKETA 5X				
A10	EPA Enterococcus q...	9	UNKN	SKETA 5X				
A11	EPA Enterococcus q...	10	UNKN	SKETA 5X				

Add/Remove Sites

Ch #	Dye Name	Usage	Bkgnd Sub	Bkgnd Min Cycle	Bkgnd Max Cycle	Curve Analysis	Thresh Setting	Manual Thresh Fluor Units	Auto Thresh #SD's	Auto Min Cycle	Auto I Cyc
1	FAM	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
2	Cy3	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
3	TxR	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10
4	Cy5	Assay	ON	5	40	Primary Curve	Manual	30.0	NA	5	10

Under the “Usage” column, select “Unused” from the drop down menu for Cy3, TxR and Cy5. Leave the “Assay” on the FAM line for this particular protocol

Start Run

Cancel Run Setup

Report Run Setup

Select Graphs

Copy Run Setup



Create Run



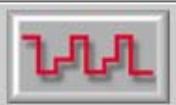
Check Status



Stop Run



View Results



Define Protocols



Define Graphs



Maintenance

Run Name:

NRSA 2009 Batch 1 SKETA 092209

Notes:

NRSA 2009 SKETA analysis of Batch 1

Dye Set:

FCTC25

Protocols:

Protocol	Lot Number
EPA Enterococcus T...	

Graphs:

Standard - Tet
Temperature
Intercalate
Melt
FAM
Cy3

Start Run

Cancel Run Setup

Report Run Setup

Select Graphs

Copy Run Setup

Site ID	Protocol	Sample ID	Sample Type	Notes	FAM Std Conc	Cy3 Std Conc	TxR Std Conc	Cy5 Std Conc
A1	EPA Enterococcus ...	LB-1	UNKN	SKETA 5X				
A2	EPA Enterococcus ...	1	UNKN	SKETA 5X				
A3	EPA Enterococcus ...	2	UNKN	SKETA 5X				
A4	EPA Enterococcus ...	3	UNKN	SKETA 5X				
A5	EPA Enterococcus ...	4	UNKN	SKETA 5X				
A6	EPA Enterococcus ...	5	UNKN	SKETA 5X				
A7								
A8								
A9								
A10								
A11								

1. Set the Manual Threshold
Fluorescence Units to 8.0 on the
FAM line

2. When smart cycler tubes have been put in to
appropriate sites, click "Start Run" - a red light
should appear on sites that are in use