TECHNICAL MANUAL

ProNex® DNA QC Assay

for Use on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

Instructions for Use of Products
NG1004 and NG1005

9/17
TM513

ProNex® DNA QC Assay

for Use on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Bulletin.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The ProNex® DNA QC Assay evaluates the quantity and quality of genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) samples or other potentially degraded DNA sources. It is a human-specific, multiplexed probe-based quantitative polymerase chain reaction (qPCR) assay that may also be used to evaluate the ratio of circulating cell-free DNA (ccfDNA) to higher molecular weight genomic DNA in plasma samples. The multiplex assay detects 75bp, 150bp and 300bp human genomic DNA sequences, and it includes an internal positive control (IPC) to test for false-negative results that may occur in the presence of PCR inhibitors. This technical manual describes the ProNex® DNA QC Assay and provides instructions for use with the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (1).

Due to the inherent variability of FFPE and ccfDNA quality, knowing the quantity of DNA is not in itself reliably predictive of downstream assay success. The ProNex® DNA QC Assay allows researchers to determine the amount of amplifiable DNA in a sample. The ProNex® DNA QC Assay may be used to evaluate if a sample is suitable for downstream analysis using techniques such as next-generation sequencing (NGS) or droplet digital PCR (ddPCR).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ProNex® DNA QC Assay Bio-Rad CFX96™	**200 reactions**	**NG1004**

For Research Use Only. Not for use in diagnostic procedures. Includes:

- 2 × 1.1ml 2X ProNex® DNA QC Master Mix
- 1 × 220µl 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™
- 1 × 150µl ProNex® DNA QC gDNA Standard
- 2 × 1.5ml ProNex® DNA QC Dilution Buffer
- 2 × 1.25ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
ProNex® DNA QC Assay Bio-Rad CFX96™	**800 reactions**	**NG1005**

For Research Use Only. Not for use in diagnostic procedures. Includes:

- 8 × 1.1ml 2X ProNex® DNA QC Master Mix
- 4 × 220µl 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™
- 4 × 150µl ProNex® DNA QC gDNA Standard
- 8 × 1.5ml ProNex® DNA QC Dilution Buffer
- 8 × 1.25ml Nuclease-Free Water

Storage conditions: Store the ProNex® DNA QC Assay at –30°C to –10°C in a non-frost-free freezer. Store the ProNex® DNA QC gDNA Standard at 4°C overnight prior to the first use; do not refreeze. For short-term storage (less than 1 week), store the other components at 2–10°C. Minimize the number of freeze-thaw cycles. The 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ is light-sensitive and must be stored in the dark.

Dilutions of the ProNex® DNA QC gDNA Standard in ProNex® DNA QC Dilution Buffer can be stored at 4°C for up to 1 week.

20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™

The 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ includes all primers, probes and IPC template.

- **Primers and probe for the 75bp human genomic target:** The Quasar® 670 dye-labeled probe of the ProNex® DNA QC Assay detects a multicopy human DNA target. The primers are used to amplify a 75bp amplicon. Data from this target quantify the total amount of human DNA in a sample. The 75bp target is the shortest amplicon, most robust to inhibitors and least likely to be affected by degradation events.
- **Primers and probe for the 150bp human genomic target:** The FAM™ dye-labeled probe of the ProNex® DNA QC Assay detects a multicopy human DNA target. The primers are used to amplify a 150bp amplicon. Data from this target also quantify the amount of human DNA in a sample, but due to its greater length, the amplicon is more susceptible to degradation and/or the presence of inhibitors compared to the 75bp target. The 150bp target is the closest in size to ccfDNA. Some ccfDNA may fall below this size; therefore, some users may prefer to choose the 75bp target for this purpose.

- **Primers and probe for the 300bp human genomic target:** The CAL Fluor® Gold 540 dye-labeled probe of the ProNex® DNA QC Assay detects a multicopy human DNA target. The primers are used to amplify a 300bp amplicon. Data from this target also quantify the amount of human DNA in a sample, but due to its greater length, the amplicon is more susceptible to degradation and the presence of inhibitors compared to the 75bp and 150bp targets. The 300bp target can be used to detect genomic DNA contamination in ccfDNA samples.
- **Primers, probe and template for the IPC:** The Quasar® 705 dye-labeled probe of the ProNex® DNA QC Assay detects the IPC, a novel DNA template that is included in every amplification reaction. The primers produce an amplified 435bp product. Amplification performance of the IPC is used to detect PCR inhibitors in the sample. This is the longest target in the ProNex® DNA QC Assay, making the IPC more susceptible to inhibitors than the other targets in the multiplex reaction.

2X ProNex® DNA QC Master Mix

The 2X ProNex® DNA QC Master Mix is optimized for the four-color multiplex reaction and uses GoTaq® Hot-Start Polymerase. The reaction should be set up at room temperature and is amenable to automation. Amplification is complete in 83 minutes.

ProNex® DNA QC gDNA Standard

The ProNex® DNA QC gDNA Standard is supplied with the ProNex® DNA QC Assay. It consists of pooled human genomic DNA supplied at 50ng/µl. Serial dilutions of this DNA standard are amplified in the same plate as the unknown samples, and the results are used to generate a standard curve for the 75bp, 150bp and 300bp targets. The standard curve can be used to determine the DNA concentration for each unknown sample.

ProNex® DNA QC Dilution Buffer

The ProNex® DNA QC Dilution Buffer is the diluent for serial dilution of the ProNex® DNA QC gDNA Standard to create the standard curve.

Instrumentation

The ProNex® DNA QC Assay is designed and optimized for use with the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. Due to differences in available dye channels, the ProNex® DNA QC Assay can only be used on the CFX96 Touch™ system. The CFX384 Touch™ system does not include channel 5 (Quasar® 705/Cy5.5® dyes).

ProNex® DNA QC Assay Analysis Software

The ProNex® DNA QC Assay Analysis Software is available for download at:
www.promega.com/resources/software-firmware/pronex-dna-qc-assay/

It will assist with data analysis and data review of DNA standards and unknown samples generated using the ProNex® DNA QC Assay. The *ProNex® DNA QC Assay Analysis Software Technical Manual* #TM512 is also available for download.

3. Materials Required

Materials to Be Supplied by the User

- sterile, aerosol-resistant pipette tips
- CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Cat.# 1855195)
- vortex mixer
- microcentrifuge
- tubes to prepare the DNA standard dilution series
- Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear (Bio-Rad Cat.# HSP9601)
- Microseal® 'B' PCR Plate Sealing Film, adhesive, optical (Bio-Rad Cat.# MSB1001)
- plate centrifuge
- TE^{-4} buffer pH 8.0 (optional, see Section 8.A)

Notes:

1. Use of the Bio-Rad Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/white (Bio-Rad Cat.# HSP9655) results in increased fluorescent signal and requires using different baseline threshold values than those provided in this technical manual.

2. For support of other instruments with similar fluorescent dye capabilities, contact Promega Technical Services for more information. Email: **techserv@promega.com**.

4. General Considerations

The ProNex® DNA QC Assay is extremely sensitive; take precautions to minimize human DNA contamination. We recommend storing the ProNex® DNA QC Assay reagents separately from DNA samples.

- Use clean, designated work areas and separate pipettes for pre- and post-amplification steps to minimize the potential for cross-contamination between DNA samples and to prevent carryover of nucleic acid from one run to the next.
- Wear a lab coat and protective eyewear. Wear gloves and change them often.
- Prevent contamination by using aerosol-resistant pipette tips. Always include a no-template control (NTC) reaction to detect contamination. We recommend performing NTC reactions in duplicate.
- Use low-retention or siliconized microtubes when preparing DNA standard dilutions or diluting unknown samples.
- Do not unseal reaction plates after amplification is complete. Unsealing the plates increases the risk of contaminating subsequent reactions with amplified product.
- Always check to ensure that the instrument software's analysis settings are correct (see *Applying Thresholds Using the CFX Manager™ Software* in Section 5.C).
- The CFX96 Touch™ systems are factory-calibrated with commonly used fluorophores. No custom dye calibration is required to use the ProNex® DNA QC Assay on the CFX96™ system.

5. Protocol

When using the ProNex® DNA QC Assay for the first time, we recommend completing the steps in Section 5.C prior to assembling the reactions. Once you are familiar with the instrument setup, you can program the instrument after setting up the reaction.

The recommended protocol for the ProNex® DNA QC Assay uses a four-point standard curve. We recommend performing a minimum of duplicate amplification reactions with each dilution of the DNA standard and using a dedicated set of pipettes to increase run-to-run consistency. Use the same pipettes to dispense the DNA standard and unknown samples to minimize variability. Do not use other DNA (e.g., DNA isolated from a cell line) as the DNA standard.

This protocol uses 2µl of template DNA per reaction. The template volume can be increased as long as the final reaction volume remains constant. If the template DNA volume and final reaction volume are the same for both the DNA standards and unknown DNA samples, the DNA standards can be considered a concentration (in ng/µl) instead of an input amount in nanograms (concentration in ng/µl × volume). The ProNex® DNA QC Assay Analysis Software can accommodate different template volumes for standards and unknowns; however, all standards must be of the same volume and all unknowns must be of the same volume. In analysis, the ProNex® DNA QC Assay Analysis Software will apply a dilution factor to the results in order to correct for volume differences between standards and unknowns.

We recommend performing at least duplicate amplifications of each DNA standard and each unknown DNA sample. Performing replicate analysis and averaging the quantification results can reduce variability.

When diluting the ProNex® DNA QC gDNA Standard or unknown DNA samples, use the ProNex® DNA QC Dilution Buffer or TE^{-4} buffer (pH 8.0); do not use water as a diluent. Serial dilutions of the ProNex® DNA QC gDNA Standard prepared with ProNex® DNA QC Dilution Buffer can be stored for up to 1 week at 4°C. Serial dilutions prepared with TE^{-4} buffer (pH 8.0) should be prepared fresh for each experiment.

Note: Change gloves often, especially after handling high-concentration DNA.

5.A. Serial Dilution of the ProNex® DNA QC Assay gDNA Standard

Prepare serial 25-fold dilutions of the ProNex® DNA QC gDNA Standard, and then amplify these dilutions to create four-point standard curves to determine the concentration of 75bp, 150bp and 300bp targets in the unknown DNA samples. Accurate serial dilution of the ProNex® DNA QC gDNA Standard is essential to quantify unknown DNA samples correctly; carefully mix and pipet each DNA standard dilution.

1. Ensure that the ProNex® DNA QC gDNA Standard was stored at 4°C overnight before first use. If necessary, thaw the ProNex® DNA QC Dilution Buffer completely. Mix the ProNex® DNA QC gDNA Standard by vortexing the tube three times at high speed for 10 seconds each.

   Note: After the initial thaw, store the ProNex® DNA QC gDNA Standard and ProNex® DNA QC Dilution Buffer at 4°C.

2. Label three tubes with the following concentrations: 2ng/µl, 0.08ng/µl and 0.0032ng/µl.

3. Dilute the ProNex® DNA QC gDNA Standard as indicated in Table 1. Mix each dilution by vortexing for 10 seconds prior to removing an aliquot for the next dilution. Change pipette tips between dilutions.

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Table 1. ProNex® DNA QC gDNA Standard Dilutions.

DNA Concentration	Volume of ProNex® DNA QC gDNA Standard	Volume of ProNex® DNA QC Dilution Buffer
50ng/µl	Use undiluted ProNex® DNA QC gDNA Standard	0µl
2ng/µl	4µl of undiluted ProNex® DNA QC gDNA Standard	96µl
0.08ng/µl	4µl of 2ng/µL dilution	96µl
0.0032ng/µl	4µl of 0.08ng/µL dilution	96µl

5.B. ProNex® DNA QC Assay Reaction Setup

Include an NTC reaction for each set of reactions. Add 2µl of TE^{-4} buffer (pH 8.0) or Nuclease-Free Water to these reactions instead of template DNA. No amplification product (undetermined C_q or a DNA concentration <1.0pg/µl per 2µl input volume) should be detected in the NTC reaction; a higher DNA concentration indicates the presence of contaminating DNA.

Note: The ProNex® DNA QC Assay is extremely sensitive. The NTC reaction may yield amplification products in the subpicogram range. We recommend performing duplicate amplifications of the NTC reaction.

Sample DNA may be diluted in either the ProNex® DNA QC Dilution Buffer or TE^{-4} buffer (pH 8.0).

1. Thaw the 2X ProNex® DNA QC Master Mix, 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ and Nuclease-Free Water completely at room temperature.
2. Mix the 2X ProNex® DNA QC Master Mix and 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ by vortexing for 10 seconds.

   Note: Do not centrifuge after mixing as this may cause the primers and probes to be concentrated at the bottom of the tube.
3. Determine the number of reactions to be set up, including the NTC reactions. Increase this number by 10–15% to compensate for pipetting error and reagent loss on the sides of pipette tips. While this approach requires using a small amount of extra reagent, it ensures that enough reaction mix is prepared for all amplification reactions. Amplification of the unknown DNA samples and DNA standards using the same reaction mix is critical.
4. Prepare sufficient reaction mix by combining the volumes of Nuclease-Free Water, 2X ProNex® DNA QC Master Mix and 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ calculated in Table 2. Add reagents in the order listed in Table 2.

Table 2. Setup of ProNex® DNA QC Standards, Unknown Samples and NTC Reaction

Component	Volume per Reaction		Number of Reactions		Final Volume
Nuclease-Free Water[1]	7µl	×		=	
2X ProNex® DNA QC Master Mix	10µl	×		=	
20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™	1µl	×		=	
Total Volume	18µl				

[1]This volume of water assumes 2µl of template per 20µl reaction. If the volume of template is different, adjust the volume of water accordingly.

5. Mix by vortexing for 10 seconds. Do not centrifuge after mixing.

6. Add 18µl of reaction mix to the reaction wells of the Hard-Shell® 96-well PCR Plate. See Table 3 for an example of a 96-well plate that shows locations of the DNA standards (yellow), NTC reactions (blue) and unknown samples (UNKN).

   Note: Wear gloves at all times when handling the plate, and take care to avoid touching the plate wells and Microseal® 'B' adhesive seals unnecessarily. Handle the plate by the edges, and avoid touching the bottom of the plate.

Table 3. Example of a 96-Well Plate Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
B	50 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
C	2 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
D	2 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
E	0.08 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
F	0.08 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN
G	0.0032 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	NTC
H	0.0032 (ng/µl)	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	UNKN	NTC

5.B. ProNex® DNA QC Assay Reaction Setup (continued)

7. Add 2µl of Nuclease-Free Water or ProNex® DNA QC Dilution Buffer to the NTC reactions.
8. Add 2µl of the ProNex® DNA QC gDNA Standards prepared in Section 5.A or unknown DNA samples to the appropriate wells.
9. Seal the plate with a Microseal® 'B' adhesive seal. Handle the plate by the edges, and avoid touching the top of the plate.
10. Centrifuge the plate briefly to collect the contents of each well at the bottom. The plate is ready for thermal cycling. Protect the plate from extended exposure to light or elevated temperatures prior to thermal cycling.

5.C. Run Setup and Thermal Cycling

The following instructions are for the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System using the Bio-Rad CFX Manager™ Software.

Creating a Protocol Template

A template file (.edt file) can be used to store the dye information, target names and tasks, well locations and DNA concentrations for the DNA standards, as well as run method and analysis settings.

1. Turn on the computer associated with the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System.
2. Turn on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System.
3. Launch the Bio-Rad CFX Manager™ Software.
4. From the Start-up Wizard or Run Menu select the "User Defined Run..." option.
5. On the Protocol Tab, select "Create New...".
   a. Enter in the cycling information to match the image shown in Figure 1:
      - Step 1: 98°C for 2 minutes
      - Step 2: 98°C for 15 seconds
      - Step 3: 60°C for 1 minute
      - Highlight Step 3 (60°C for 1 minute) and confirm that the camera icon is displayed. If not, press the "Add Plate Read to Step" button on the left to add the camera.
      - To run the protocol for a total of 40 cycles, enter "39" in the GOTO option. The red arrow should pass through steps 2 and 3.
   b. Enter the total PCR volume (µl) in the Sample Volume field. If the running volume is different than the default 20µl, enter the correct volume.

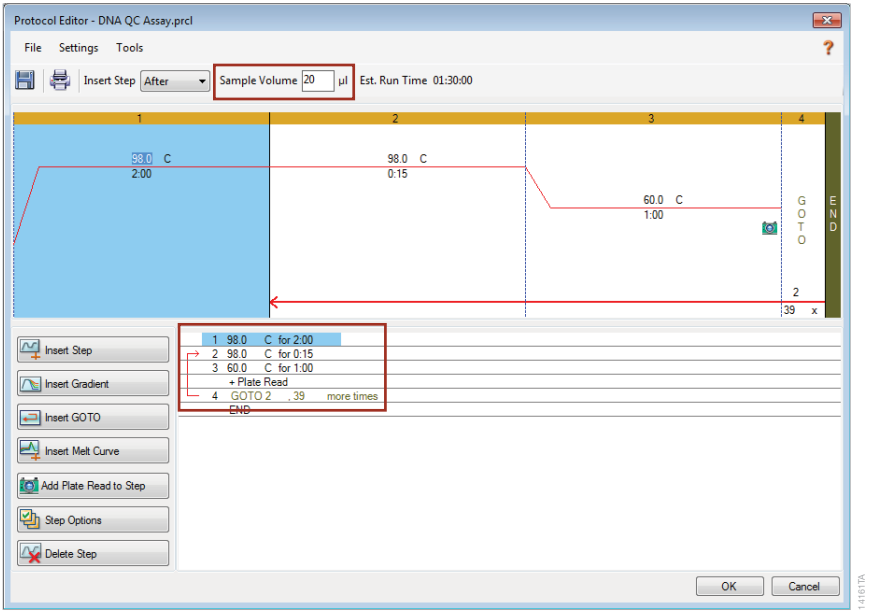

Figure 1. The Protocol Editor—New screen.

6. Save the Protocol File as “DNA QC Assay”.
7. Click “OK” to exit the Protocol Editor.
8. Select “Next >>” to move to the Plate Screen.

Creating a Plate Template

1. In the Express Load drop-down menu, select “QuickPlate_96 wells_All Channels.pltd”.
2. Click “Edit Selected…”. The Plate Editor menu appears (Figure 2).

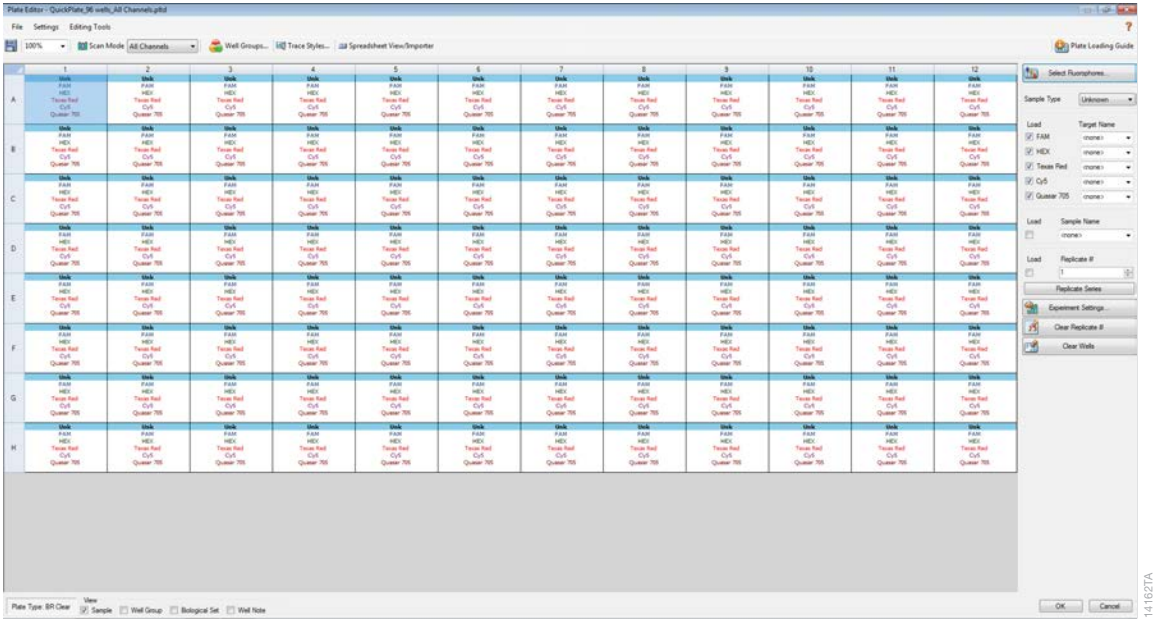

Figure 2. The Plate Editor screen.

3. Highlight all 96 wells of the plate, and click “Select Fluorophores…” to bring up the fluorophore options (Figure 3).
   - For Channel 1, select FAM.
   - For Channel 2, select Cal Gold 540.
   - For Channel 3, deselect all dyes.
   - For Channel 4, select Quasar 670.
   - For Channel 5, select Quasar 705.

   At this time, you can choose the default display color for each fluorophore.
4. Click “OK” to save your selections.

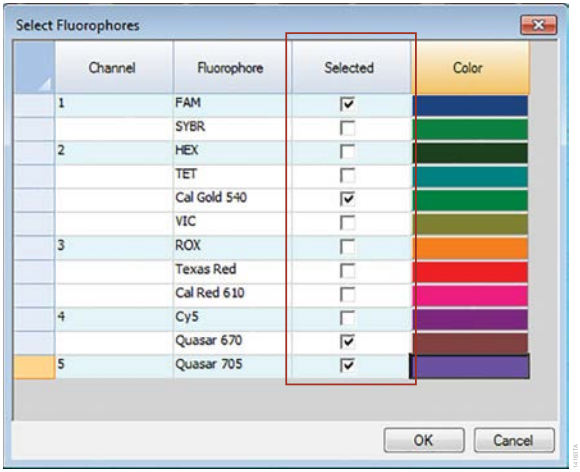

Figure 3. The Select Fluorophores screen.

5. Highlight all 96 wells of the plate, and check "Load" for FAM, Cal Gold 540, Quasar 670 and Quasar 705.

6. Highlight all 96 wells of the plate, type the names as listed below and press the Enter key (Figure 4).

   - FAM: 150bp
   - Cal Gold 540: 300bp
   - Quasar 670: 75bp
   - Quasar 705: IPC

   Note: Target name identifiers are necessary for the ProNex® DNA QC Assay Analysis Software to recognize these targets.

Creating a Plate Template (continued)

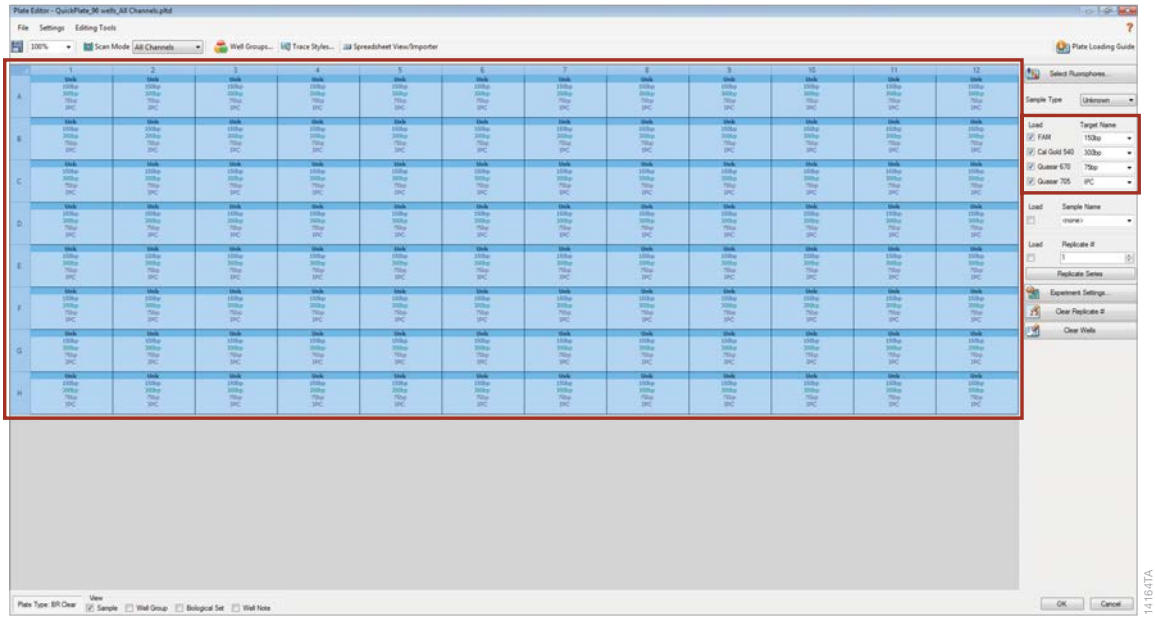

Figure 4. The Plate Editor screen—Assigned Targets.

7. Name and save the Plate file, e.g., "QuickPlate_96 wells_DNAQC.pltd". This Plate file can be used as a template for future ProNex® DNA QC Assay experiments.
8. Select "Next >>" to move to the Start Run screen.

Starting a Thermal Cycling Run

There are three options to start a new user-defined run.

1. Use the instructions in the previous sections, *Creating a Protocol Template* and *Creating a Plate Template*.
2. If you have previously saved the Protocol File (.prcl) and Plate File (.pltd) templates for the ProNex® DNA QC Assay, choose the Startup Wizard/Run Setup tab and use the "Select Existing..." options at each screen instead of creating new files.
3. Use the "Repeat Run" option in the Startup Wizard. This option will load the Protocol file (.prcl) and Plate File (.pltd) from a previous run, and display the Start Run screen.

On the Start Run screen:

1. Click "Open Lid" to open the lid of the CFX96 Touch™ system and load the qPCR plate.
2. Click "Close Lid" to close the lid of the CFX96 Touch™ system.
3. The "Start Run" button will now be active. Click it and name/save the run.

Amplification is complete in 83 minutes.

Applying Thresholds Using the CFX Manager™ Software

Once cycling is complete the run file will save/open as a PCR Data File (*.pcrd) that can be analyzed. For consistent analysis, you must set manual thresholds.

1. On the Quantification Tab, set manual thresholds for each dye. This must be done individually for each dye channel.
    a. Uncheck the CalGold 540, Quasar 670 and Quasar 705 boxes under the Amplification Chart (Figure 5). Keep FAM checked.

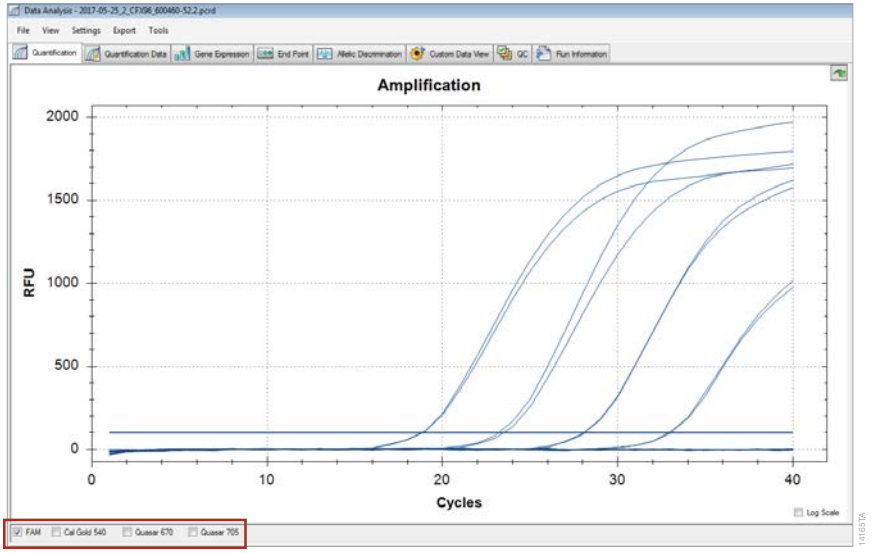

Figure 5. The Amplification plot on the Quantification tab in the DNA Analysis window.

Applying Thresholds Using the CFX Manager™ Software (continued)

b. Under the Settings menu, select "Baseline Threshold..." (Figure 6).

c. Change the Single Threshold from Auto Calculated to User Defined. Enter the value of 100 as the threshold. Click "OK".

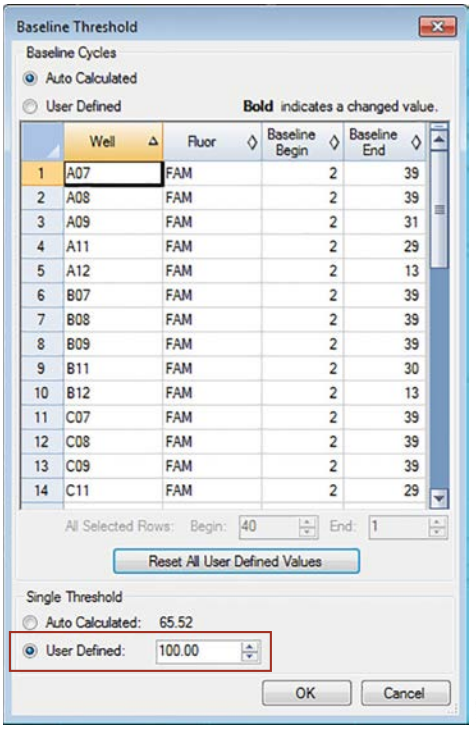

Figure 6. The Baseline Threshold window (from the amplification plot on the Quantification Tab) in the DNA Analysis window.

2. Repeat this process for each target using the following threshold values:

   - FAM: 100
   - Cal Gold 540: 100
   - Quasar 670: 100
   - Quasar 705: 50

   Note: RFU is a relative fluorescent unit and is not absolute; it may differ between Bio-Rad CFX96 Touch™ Real-Time PCR Detection Systems. The manual threshold values recommended in this technical manual were determined using maximum target RFUs between 1500–2500. Manual thresholds may need to be increased or decreased according to the maxium RFUs on individual instruments.

Optional: Entering and Evaluating Standard Curves Using the CFX Manager™ Software

Standard curves can be evaluated in the CFX Manager™ Software or the ProNex® DNA QC Assay Analysis Software Results. Any standard curves calculated in the CFX Manager™ software will be independently calculated by the ProNex® DNA QC Assay Analysis Software.

Entering Sample Information

You can enter sample information in the Plate Editor step before (.pltd file) or after (Plate Setup >> View/Edit Plate…) thermal cycling. Sample information can also be entered in the ProNex® DNA QC Assay Analysis Software.

1. Highlight the wells containing the DNA standards and use the Sample Type drop-down menu to change the well assignment from Unknown to Standard. "Unk" should change to "Std" for the selected wells (Figure 7).

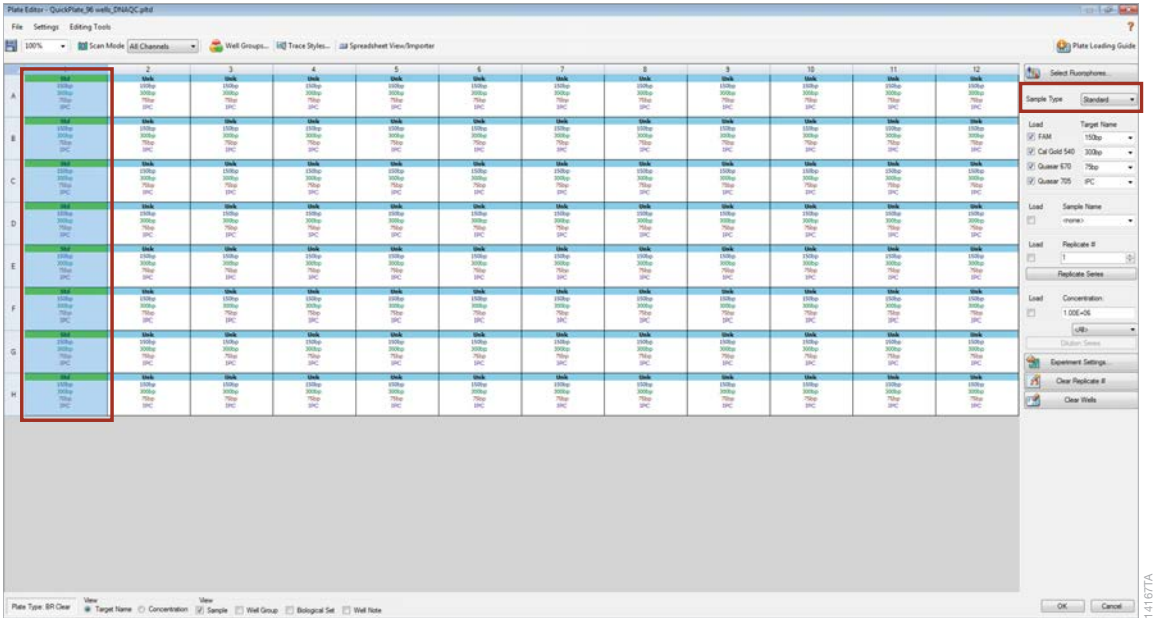

Figure 7. Assigning Standards.

Optional: Entering and Evaluating Standard Curves Using the CFX Manager™ Software (continued)

2. Enter the Sample Name and Concentration for each DNA standard in the labeled fields (Figure 8). The Concentration field is numeric only (e.g., enter "50" for 50ng/µl).
   - Multiple wells with DNA standards at the same DNA concentration can be highlighted at the same time, so that you only need to enter values once.
   - Always press the Enter key after entering the sample name or concentration for the selected wells.
   - The units for standards can be chosen under the Settings/Units. "Copy Number" is the default; ng/µl and pg/µl are not included options, so you can use the copy number setting.

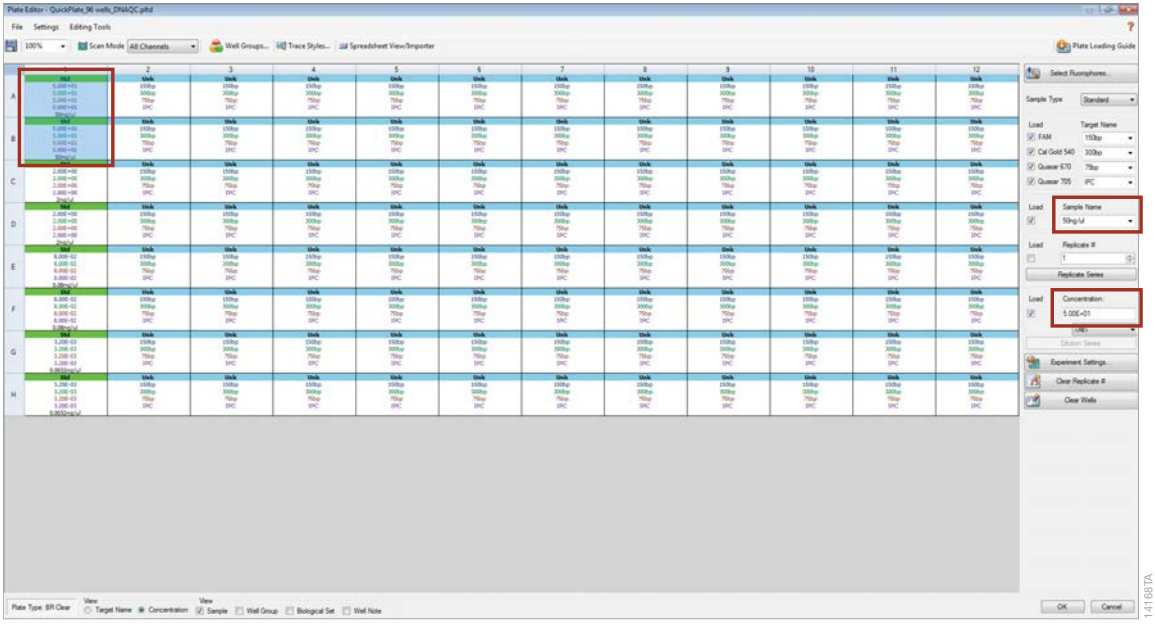

Figure 8. Assigning DNA concentrations to wells with DNA standards.

3. Highlight the wells containing the NTCs and use the Sample Type drop-down menu to change the well assignment from “Unknown” to “NTC” (Figure 9).

4. Enter the Sample Name for each NTC in the Sample Name field. Press the Enter key.

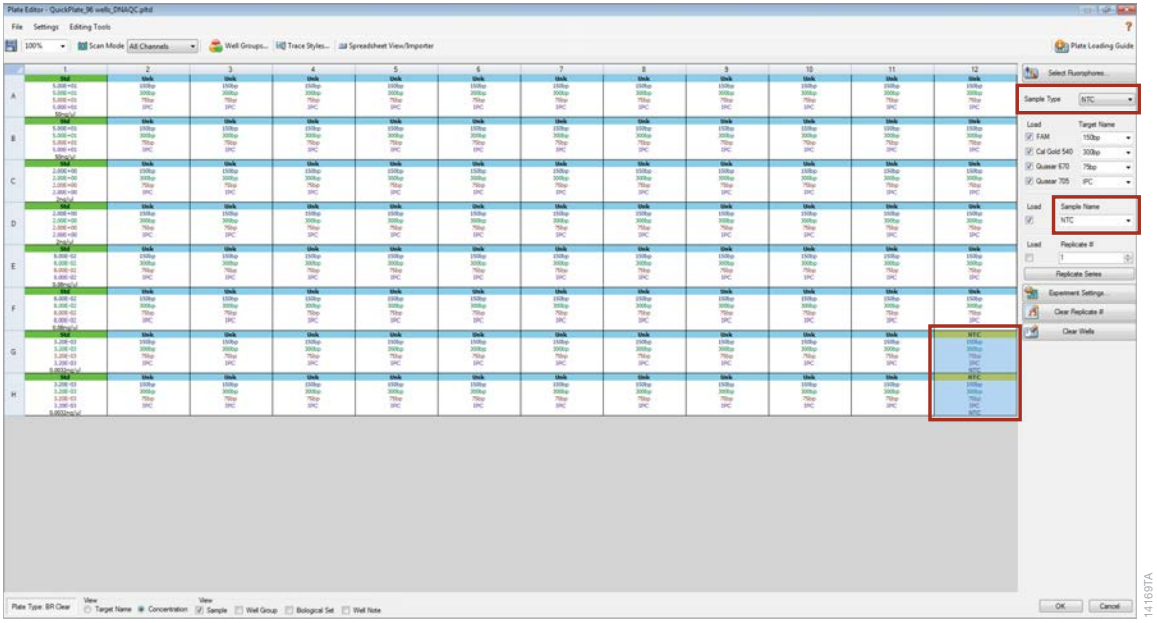

Figure 9. Assigning NTC Task to wells with NTC samples.

Optional: Entering and Evaluating Standard Curves Using the CFX Manager™ Software (continued)

5. For each test sample, highlight the wells containing the Test Sample, and assign the appropriate Sample Name. The Sample Type should be "Unknown" (Figure 10).
   - You can highlight replicates of the same Test Sample at the same time and assign a Sample Name.

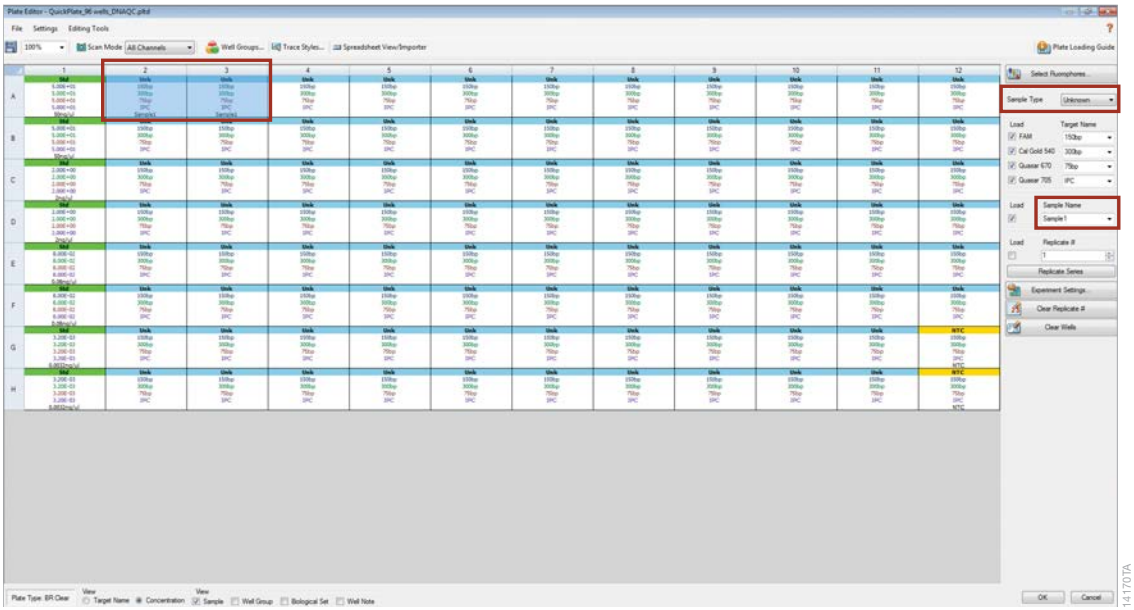

Figure 10. Assigning Sample(s) to selected wells.

6. For any empty wells, highlight the wells and use the Clear Wells button to remove all assigned information from them (Figure 11). If all wells on the plate contain samples, skip this step.

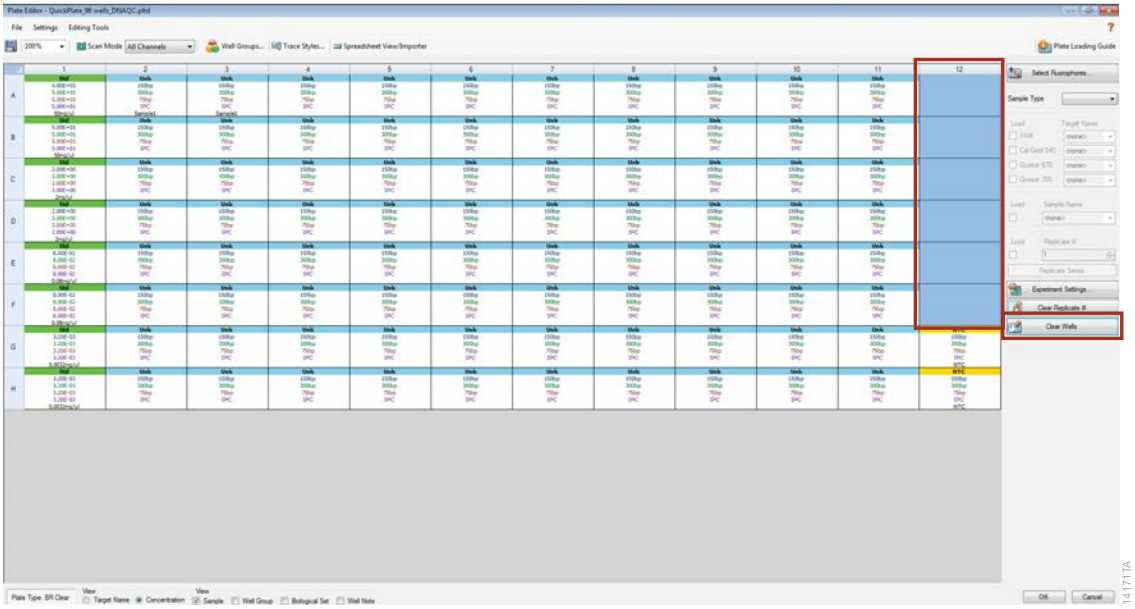

Figure 11. Using Clear Wells to remove information from selected wells.

7. If you entered sample information **before** thermal cycling, name and save the Plate file, e.g., "QuickPlate_96 wells_DNAQC Plate1.pltd." Click "OK" to exit the Plate Editor.
   - The Preview should reflect changes made in the Plate Editor window.
   - This Plate file can be used as a template for future experiments.
8. If you entered sample information **after** thermal cycling, click "OK" to save changes within the run file (.pcrd) only.

Optional: Entering and Evaluating Standard Curves Using the CFX Manager™ Software (continued)

After plate editing is complete, the Run Setup screen should resemble the one shown in Figure 12.

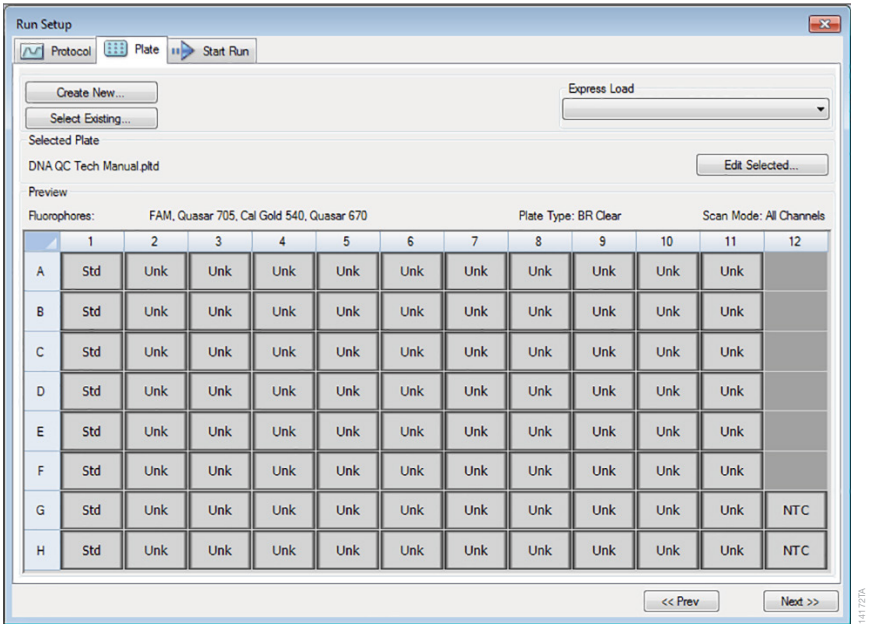

Figure 12. The Run Setup screen after plate editing.

Evaluating Standard Curves in the CFX Manager™ Software

1. On the Quantification tab, check the FAM, CalGold 540 and Quasar 670 boxes under the Amplification chart. Deselect "Quasar 705".
2. Highlight only the Standards and NTCs in the well selector map (Figure 13). These samples will now be displayed on the Standard Curve chart.
3. Under the Standard Curve plots, the PCR Efficiency, slope, R^2 and y-intercept are displayed for each Target/Dye.

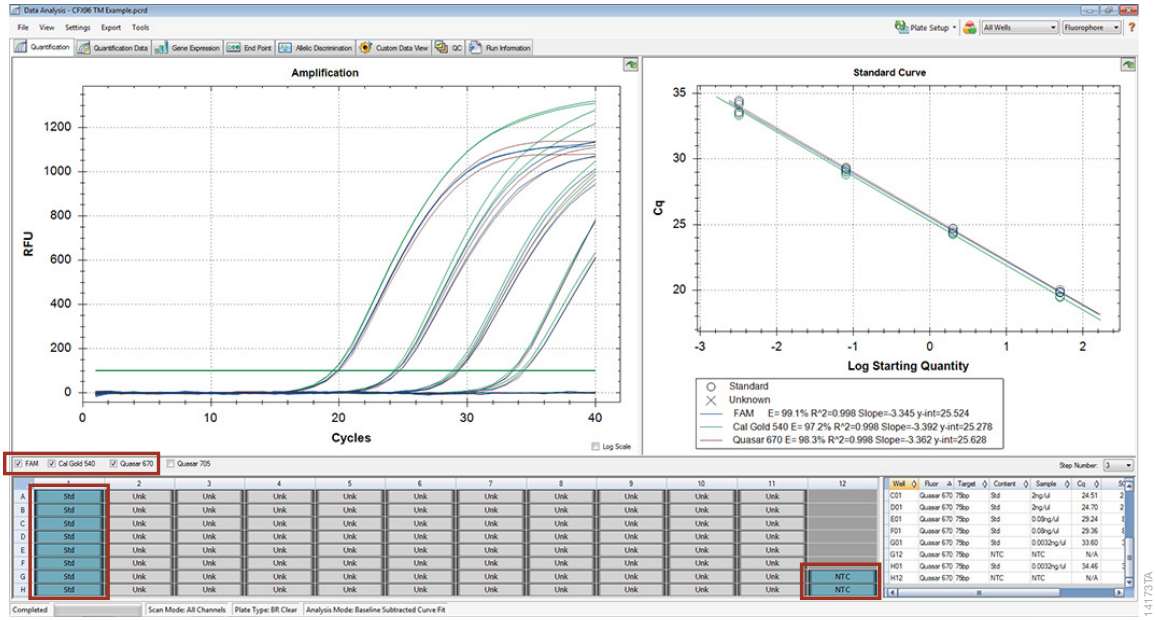

Figure 13. The entire Quantification tab in the DNA Analysis window.

Exporting Analyzed Data from the CFX Manager™ Software

1. Ensure that all Wells and Fluorophores with data for export are highlighted in the plate map.
2. Under the Export Menu, select "Custom Export…" and choose the following Export Properties (Figure 14):
   a. Export Format: Choose Excel 2007 from the drop-down menu.
   b. Check the "Include Run Information Header" box.
   c. Under Sample Description, check the following:
      - Well
      - Fluorophore
      - Target Name
      - Content
      - Sample Name
   d. Under Quantification, check the following:
      - C_q
      - Starting Quantity

   All selected Export Columns will be displayed on the right side of the Custom Export menu.
3. Click "Export". Choose the save location and file name, and click "Save".

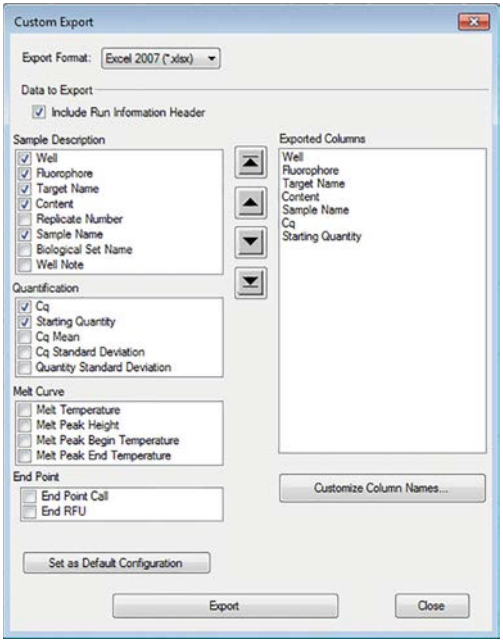

Figure 14. The Custom Export window.

6. Data Analysis

6.A. Using the ProNex® DNA QC Assay Analysis Software

See the *ProNex® DNA QC Assay Analysis Software Technical Manual* #TM512.

- The ProNex® DNA QC Assay Analysis Software will calculate the standard curves for the 75bp, 150bp and 300bp targets and compare the results (slope and R^2) to user-defined settings.
- For each unknown sample, a sample quantity for each target (75bp, 150bp and 300bp), an IPC Shift and the quantity ratios will be calculated.
- The ProNex® DNA QC Assay Analysis Software will compare the IPC Shift and ratios for each unknown sample to user-defined settings.

6.B. Interpretation of Analyzed Data

In addition to the concentrations of DNA from each sample for each target (75bp, 150bp and 300bp), the ProNex® DNA QC Assay yields information that can be used to evaluate amplification performance, detect PCR inhibitors in amplification reactions, evaluate the degree of DNA degradation and/or indicate genomic DNA contamination in ccfDNA samples.

Note: Quantity ratios may not be reliable in samples with low DNA concentrations (e.g., less than 1pg/µl) due to stochastic effects.

Standard Curves

The Bio-Rad CFX Manager™ Software and ProNex® DNA QC Assay Analysis Software perform a linear regression to the standard dilution series data and calculate the equation for the line of best fit (the standard curve). The equation is in the form of $y = mx + b$, where x = log concentration and $y = C_q$. The R^2 value is a measure of the fit of the data points to the regressed line. The slope (m) is an indication of the PCR efficiency. A slope of −3.3 indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). The Y-intercept (b) is defined as the y value (C_q) when x (log concentration) equals 0. Therefore, b corresponds to the C_q value for a sample with a concentration of 1ng/µl, since $\log_{10}(1) = 0$.

In general, the standard curve for each target (75bp, 150bp and 300bp) should have an average slope (m) in the range of −3.1 to −3.6 and an R^2 value >0.990. See Section 7 for standard curves that fall out of these ranges.

Note: C_q value (quantification cycle) is used rather than C_T (cycle threshold) per Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (2).

No-Template Controls

No amplification product should be detected in the NTC samples of the 75bp, 150bp and 300bp targets.

Detection of >1.0pg/µl per 2µl input volume DNA in the no-template controls can indicate the presence of contaminating DNA.

Internal Positive Control

Amplification performance for the IPC can be used to evaluate overall performance of the amplification reaction or detect PCR inhibitors in the DNA sample. PCR inhibitors in samples can mimic degradation by underestimating sample quantity, preferentially in larger PCR targets.

- If a sample yields no detectable amplification for the 75bp, 150bp and 300bp targets but has an IPC amplification curve that crossed the amplification threshold without an IPC shift greater than that specified in the Settings of the ProNex® DNA QC Assay Analysis Software, then insufficient DNA template was added to the ProNex® DNA QC amplification reaction.
- If a sample has an IPC shift greater than the value specified in the Settings of the ProNex® DNA QC Assay Analysis Software, or if the C_q value for the IPC is undetermined (regardless of detectable amplification for the 75bp, 150bp and 300bp quantification targets), then a PCR inhibitor may be present in the amplification reaction.
- The IPC is the longest target in the ProNex® DNA QC Assay and therefore is more susceptible to inhibition relative to the shorter 75bp, 150bp and 300bp targets.

Unknown Samples

The ProNex® DNA QC Assay Analysis Software uses the quantity for each target (75bp, 150bp and 300bp) and calculates ratios for each unknown sample ([75bp]/[150bp], ([75bp]/[300bp] and ([150bp]/[300bp]). It allows you to customize both threshold values and sample assessment messages based on individual needs.

Note: For both Degradation and Genomic DNA Contamination applications, individual laboratories should determine acceptable threshold values relevant to their downstream application success. The diversity of downstream applications, sample types and purifications, and potential PCR inhibitors does not allow for specific threshold value recommendations. Default threshold settings in the ProNex® DNA QC Assay Analysis Software are values intended to be customized by the user. You should validate the correlation between success in downstream applications and one (or more) target ratios.

Degradation

The [75bp]/[300bp] and [75bp]/[150bp] ratios can be used to evaluate whether a DNA sample is degraded.

- If a sample has a low [75bp/300bp] and/or [75bp/150bp] ratio, then the DNA in the sample is likely not degraded. Genomic DNA samples have ratios near 1.
- If a sample has an increased [75bp/300bp] and/or [75bp/150bp] ratio, then the DNA in the sample is likely degraded. In general, the higher the ratio, the more degraded the sample.
- Evaluating both the [75bp]/[300bp] and [75bp]/[150bp] ratios can indicate the degree of degradation in a sample, and establish what size amplicon is appropriate for downstream analysis with the sample.

Genomic DNA Contamination

The [75bp]/[300bp] and [150bp]/[300bp] ratio can be used to evaluate whether a ccfDNA sample has genomic DNA contamination.

- If a sample has a low [75bp]/[300bp] and/or [150bp]/[300bp] ratio, then the ccfDNA in the sample could have genomic DNA contamination. Genomic DNA samples have ratios near 1.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**.

Symptoms	Causes and Comments
Flat amplification curves and no C_q values detected for any of the targets, including the IPC, for a subset of samples in the 96-well plate	Incorrect reaction setup. • Verify that reaction mix was added to the failed amplification reactions. • Examine the volume of liquid in each well of the reaction plate to verify that evaporation did not occur during cycling. Ensure that all wells were adequately sealed to prevent evaporation during thermal cycling.
	Amplification inhibitor present in the DNA template. Dilute the template DNA and quantify using 2µl of the diluted template.
Flat amplification curves and no C_q values detected for any of the targets, including the IPC, for all samples in the 96-well plate	Incorrect programming of the thermal cycler. Verify that the thermal cycler was programmed correctly. See *Creating a Protocol Template* in Section 5.C.
	Incorrect reaction setup. If no fluorescence was detected, check to be sure that the 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ was added to the amplification reactions.
	The 2X ProNex® DNA QC Master Mix may have lost activity, or the 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ was degraded. Minimize the number of freeze-thaw cycles. Store the 20X ProNex® DNA QC Primer-Probe-IPC Mix, Bio-Rad CFX96™ protected from light.
DNA detected in NTCs	DNA contamination occurred. Detection of >1.0pg/µl per 2µl input volume DNA in the NTCs can indicate the presence of contaminating DNA. Refer to Section 4 for guidelines to prevent DNA contamination.
	Non-specific background signal observed. Occasionally instrument/baseline noise is observed and can cross the threshold, resulting in a C_q. These baseline noise samples lack an amplification curve and exhibit low jagged signal. Manual thresholds can be increased and data re-analyzed above this noise to eliminate C_q values for these samples.
Noisy amplification plots with no distinct amplification curve and no IPC amplification plots detected	Sample contamination occurred. • Avoid contamination of the CFX96 Touch™ system by minimally handling plates and never running a reaction with a seal that is compromised or broken. • Clean the block and inner lid periodically to prevent the buildup of dust or fluorescent solution. See the CFX96 Touch™ instruction manual for additional details.
Sample with inhibitor not flagged by IPC	Buffer composition intereference. Buffers with both PCR inhibitors and alcohols can result in PCR inhibition not being detected by the IPC, due to the early shift of the IPC by the alcohol. Dilute the template DNA and quantify using 2µl of the diluted template.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Quantity estimates for larger-sized targets (150bp and 300bp) are greater than the 75bp target	Incorrect dye assignment. Confirm that the correct dyes were assigned to each target.
Nonlinear standard curve (R^2 values less than 0.98) or slope outside the specified range (−3.1 to −3.6)	Inconsistent sampling. • Store the DNA standard at 4°C overnight prior to use to improve sampling consistency. • Be sure that the ProNex® DNA QC gDNA Standard was thawed completely and well mixed prior to use. • Be sure that each dilution of the ProNex® DNA QC gDNA Standard was well mixed before removing an aliquot for the next serial dilution. Use the same pipette to dispense each aliquot. Change pipette tips between each dilution step. Change pipette tips between each addition of DNA standard to the 96-well plate. • Calibrate pipettes to minimize variability when pipetting. • Amplify each DNA standard in duplicate to minimize the effects of variation on the standard curve. • An error was made during dilution of the ProNex® DNA QC gDNA Standard. Verify all calculations, and repeat the dilution. • Avoid pipetting volumes less than 1µl.
	Sample loss during cycling. Examine the volume of liquid in each well of the reaction plate to verify that evaporation did not occur during cycling. Ensure that all wells were adequately sealed to prevent evaporation during thermal cycling.
	Incorrect DNA concentrations. • Verify that all DNA standard concentrations were entered correctly into the CFX Manager™ Real-Time PCR System software. • Verify that all DNA standard concentrations were entered correctly into the ProNex® DNA QC Analysis Software.
	Incorrect analysis settings in the Bio-Rad CFX Manager™ software. Verify that the analysis settings were set as described in *Applying Thresholds using the CFX Manager™ Software* in Section 5.C.
Inconsistency between replicates of the same DNA sample	Sample loss due to evaporation. Examine the volume of liquid in each well of the reaction plate of the same DNA sample to verify that evaporation did not occur during cycling. Evaporation of the reaction mix affects amplification of the human and IPC targets (late C_q values).
	DNA concentration was low. Differences in amplification results for replicate samples with low levels of DNA template can be due to stochastic effects.

For support of other instruments with similar fluorescent dye capabilities, contact Promega Technical Services for more information. Email: **techserv@promega.com.**

8. Appendix

8.A. Composition of Buffer

TE-4 Buffer

10mM Tris
100µM EDTA

8.B. References

1. CFX96 Touch™, CFX96 Touch™ Deep Well, CFX Connect™, and CFX384 Touch™ Real-Time PCR Detection Systems Instruction Manual. (2013) 10021337 Rev E. Bio-Rad Laboratories, Hercules, CA.
2. Bustin, S.A. *et al.* (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–22.

8.C. Related Products

Product	Size	Cat.#
ProNex® DNA QC Assay Calibration Kit, 7500	1 kit	NG1001
ProNex® DNA QC Assay ABI 7500/7500FAST	200 reactions	NG1002
	800 reactions	NG1003
ProNex® DNA QC Assay Software		7002422
ReliaPrep™ FFPE gDNA Miniprep System	10 reactions	A2351
	100 reactions	A2352
Maxwell® RSC DNA FFPE Kit	48 preps	AS1450
Maxwell® RSC ccfDNA Plasma Kit	48 preps	AS1480
GoTaq® qPCR Master Mix	5ml	A6001
	25ml	A6002
GoTaq® Probe qPCR Master Mix	2ml	A6101
	10ml	A6102