

TECHNICAL MANUAL

ProNex[®] DNA QC Assay

for Use on the Applied Biosystems[®]
7500/7500 Fast Real-Time PCR Systems

Instructions for Use of Products
NG1002 and NG1003



ProNex[®] DNA QC Assay

for Use on the Applied Biosystems[®] 7500/7500 Fast Real-Time PCR Systems

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.....	1
2. Product Components and Storage Conditions	2
3. Materials Required	4
4. General Considerations	4
5. Protocol	5
5.A. Serial Dilution of the ProNex [®] DNA QC Assay gDNA Standard	5
5.B. ProNex [®] DNA QC Assay Reaction Setup	6
5.C. Run Setup and Thermal Cycling	8
6. Data Analysis	19
6.A. Using the ProNex [®] DNA QC Assay Analysis Software.....	19
6.B. Interpretation of Analyzed Data	20
7. Troubleshooting.....	22
8. Appendix.....	24
8.A. Composition of Buffer	24
8.B. References	24
8.C. Related Products	25

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 detect 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 Applied Biosystems[®] (ABI) 7500 or 7500 Fast Real-Time PCR System (Applied Biosystems[®] 7500 Software, Version 2.0.6 or greater) (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 ABI 7500/7500FAST	200 reactions	NG1002

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, ABI 7500/7500 Fast
- 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 ABI 7500/7500FAST	800 reactions	NG1003

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, ABI 7500/7500 Fast
- 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, ABI 7500/7500 Fast 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, ABI 7500/7500 Fast

The 20X ProNex[®] DNA QC Primer-Probe-IPC Mix, ABI 7500/7500 Fast includes all primers and probes, the IPC template and a passive reference dye.

- **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 TAMRA™ 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 PCR inhibitors than the other targets in the multiplex reaction.
- **Passive reference dye:** The CXR dye of the ProNex® DNA QC Assay is used as a passive reference and is included in each amplification reaction. Data from the other dye channels are normalized to the CXR signal. The CXR reference dye can be detected using the same filters and settings as those used for ROX™ dye.

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 used 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 Applied Biosystems® 7500 or 7500 Fast Real-Time PCR System (Applied Biosystems® 7500 Software, Version 2.0.6 or newer). Calibration with the ProNex® DNA QC Assay Calibration Kit (Cat.# NG1001) is required prior to DNA quantification.

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
- Applied Biosystems® 7500 or 7500 Fast Real-Time PCR System (Software Version 2.0.6 or newer must be used)
- vortex mixer
- microcentrifuge
- tubes to prepare the DNA standard dilution series
- MicroAmp® Optical 96-Well Reaction Plate (ABI Cat.# N8010560, 4316813, 4306737 or 4326659 for the Applied Biosystems® 7500 Real-Time PCR System); OR
- MicroAmp® Fast Optical 96-Well Reaction Plate, 0.1ml (ABI Cat.# 4346907, 4346906 or 4366932 for the Applied Biosystems® 7500 Fast Real-Time PCR System)
- MicroAmp® Optical Adhesive Film (ABI Cat.# 4311971)
- plate centrifuge
- MicroAmp™ 96-Well Support Base (ABI Cat.# 4379590)
- TE⁻⁴ buffer pH 8.0 (optional, see Section 8.A)

Note: 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 *Creating a Run Template* in Section 5.C).

Calibration of the Applied Biosystems® 7500 or 7500 Fast Real-Time PCR System

Before using the ProNex® DNA QC Assay on the Applied Biosystems® 7500 or 7500 Fast Real-Time PCR System, you must calibrate the instrument for FAM™, CAL Fluor® Gold 540, TAMRA™, Quasar® 670 and CXR dyes. See the *ProNex® DNA QC Assay Calibration Kit Technical Manual #TM515* for details.

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 2 μ l 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/ μ l) instead of an input amount in nanograms (concentration in ng/ μ l \times 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 accurately; 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/ μ l, 0.08ng/ μ l and 0.0032ng/ μ l.
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.



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

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 Nuclease-Free Water or TE⁻⁴ buffer (pH 8.0) 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, ABI 7500/7500 Fast 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, ABI 7500/7500 Fast 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, ABI 7500/7500 Fast 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 ^a	7µl	×		=	
2X ProNex® DNA QC Master Mix	10µl	×		=	
20X ProNex® DNA QC Primer-Probe-IPC Mix, ABI 7500/7500 Fast	1µl	×		=	
Total Volume	18µl				

^aThis volume of water assumes 2µl of template per 20µl reaction. If the volume of template is different, adjust the volume of water accordingly.

- Mix by vortexing for 10 seconds. Do not centrifuge after mixing.
- Add 18µl of reaction mix to the reaction wells of the MicroAmp® optical 96-well reaction 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 MicroAmp® optical adhesive film unnecessarily. Handle the plate by the edges, and avoid touching the top or bottom of the plate. Use a MicroAmp™ 96-Well Support Base to minimize contaminants on the bottom of the 96-well reaction 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 MicroAmp® optical adhesive film.
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 Applied Biosystems® 7500 or 7500 Fast Real-Time PCR System instrument using the Applied Biosystems® 7500 Software, Version 2.0.6 or newer.

Creating a Run 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, and run method and analysis settings.

1. Turn on the computer associated with the Applied Biosystems® 7500 Real-Time PCR System.
2. Turn on the Applied Biosystems® 7500 Real-Time PCR System.
3. Launch the Applied Biosystems® 7500 Software, Version 2.0.6 (or newer).
4. Before using the ProNex® DNA QC System, check that the instrument was calibrated using the ProNex® DNA QC Calibration Kit. See the *ProNex® DNA QC Calibration Kit Technical Manual #TM515* for details.
5. On the Home screen under Set-Up, click “Advanced Setup”. Alternatively, click “New Experiment”, and choose “Advanced Setup” from the drop-down menu.
6. In the Experiment Properties form (Figure 1), specify the following parameters:
 - Enter a name for the template in the “Experiment Name” field.
 - Select “7500 (96 Wells)” (or “7500 Fast (96 Wells)” if using a Fast instrument) for the instrument you are using to run the experiment.
 - Select “Quantitation-Standard Curve” for the type of experiment.
 - Select “TaqMan® Reagents” for the reagents you want to use to detect the target sequence.
 - Select “Standard (~2 hours to complete a run)”.

Note: If using an Applied Biosystems™ 7500 Fast instrument, “Fast (~40 minutes to complete a run)” can also be selected for the ramp speed during the instrument run.

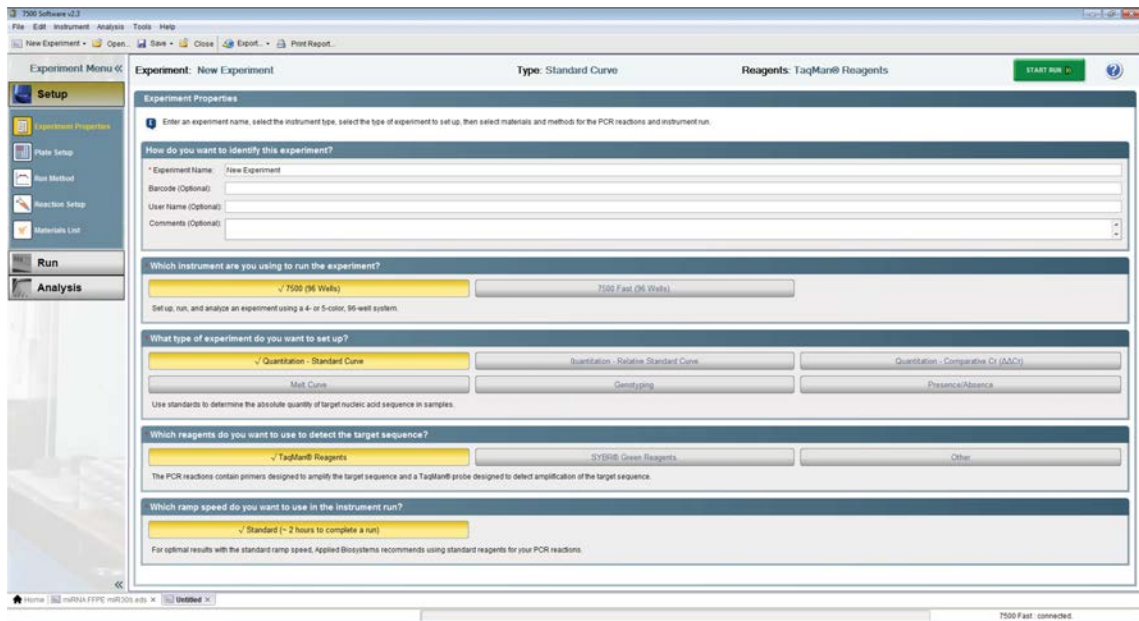


Figure 1. The Experiment Properties screen.

Creating a Run Template (continued)

- Select “Plate Setup” from the Setup menu in the left panel. Select the “Define Targets and Samples” tab. Click three times on “Add New Target” (Figure 2), and enter the following information to specify the targets:

Target Name	Reporter	Quencher
75bp	DNAQC_Q670	NFQ-MGB
150bp	DNAQC_FAM	NFQ-MGB
300bp	DNAQC_CFG540	NFQ-MGB
IPC	DNAQC_TMR	NFQ-MGB

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

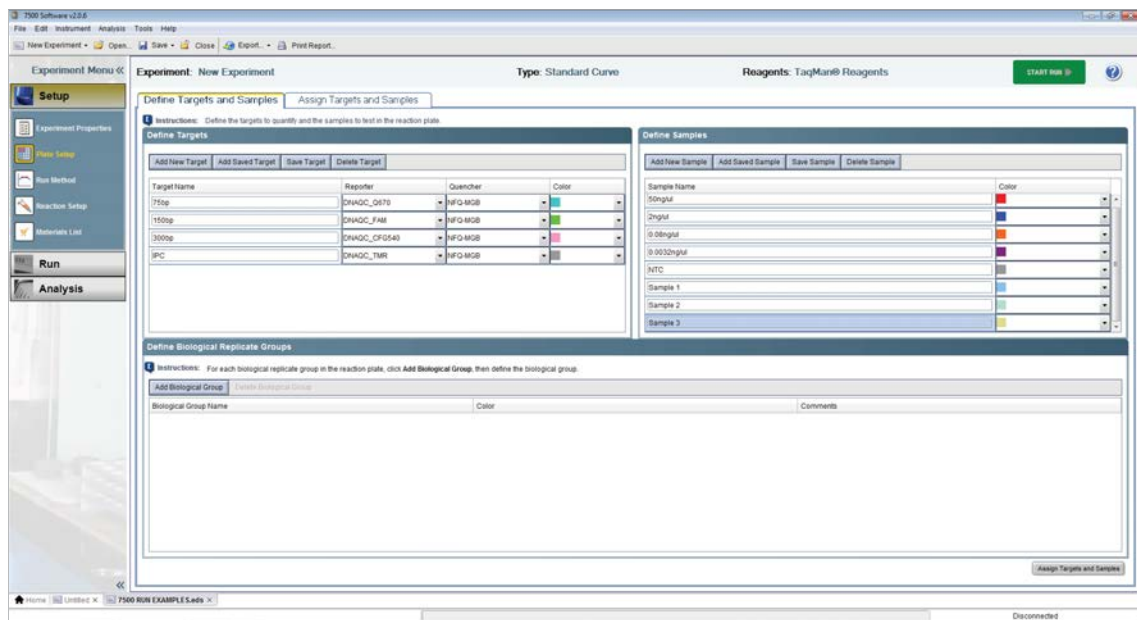


Figure 2. The Plate Setup screen.

- Under Plate Setup from the Setup menu in the left panel, select the “Define Targets and Samples” tab. Click “Add New Sample” (Figure 2), and enter a Sample Name. Repeat for each sample to be assayed.

- Select the “Assign Targets and Samples” tab (Figure 3), and highlight the wells in use on the View Plate Layout tab by dragging the pointer over the plate wells. Assign all four targets to the selected wells by selecting the boxes under “Assign”.

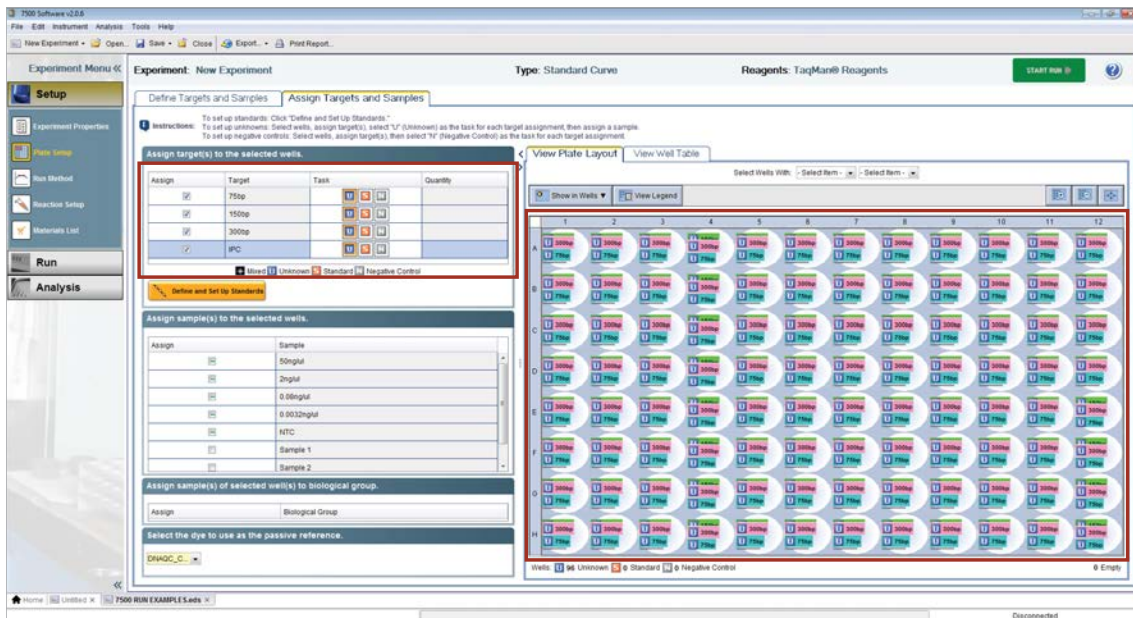


Figure 3. Assigning targets to wells.

Creating a Run Template (continued)

Optional: Steps 10-13 do not need to be completed if using ProNex® DNA QC Assay Analysis Software.

- Highlight the wells containing the DNA standards on the View Plate Layout tab, and select “S” as the Task for the 75bp, 150bp and 300bp targets (Figure 4).

Note: The Task for the IPC should be “U”.

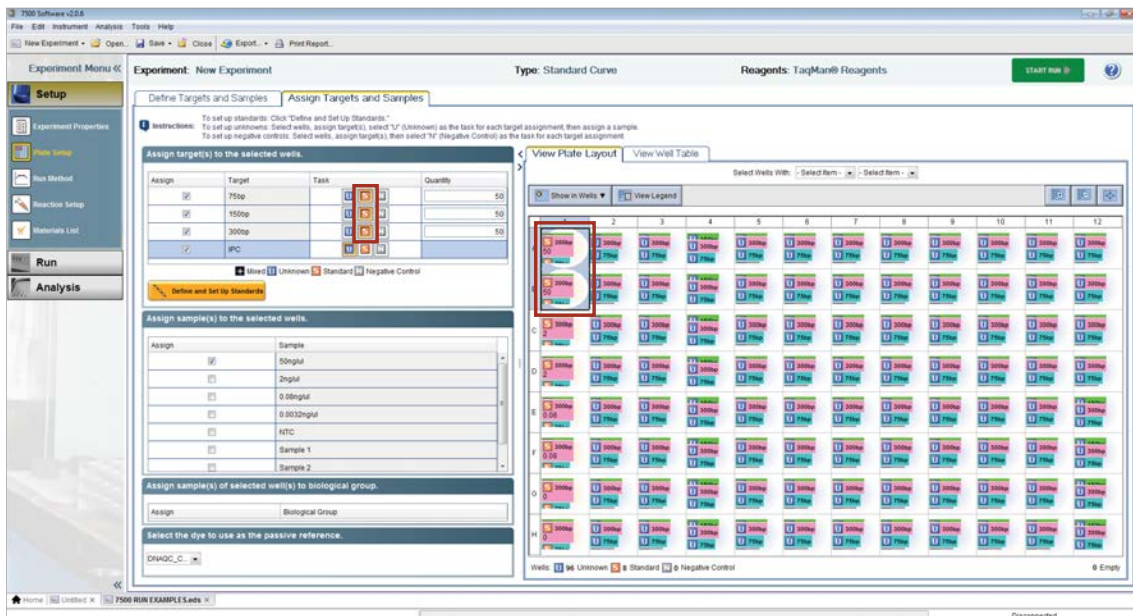


Figure 4. Assigning DNA concentrations to wells with DNA standards.

- Enter the concentration for each DNA standard in the Quantity field without the unit of measure (e.g., enter “50” for 50ng/μl).

Note: You can highlight multiple wells with DNA standards of the same DNA concentration at the same time, so that you need to enter values only once.

- Highlight the wells containing the NTCs on the View Plate Layout tab, and select “N” as the Task for the 75bp, 150bp and 300bp targets (Figure 5).

Note: The Task for the IPC should be “U”.

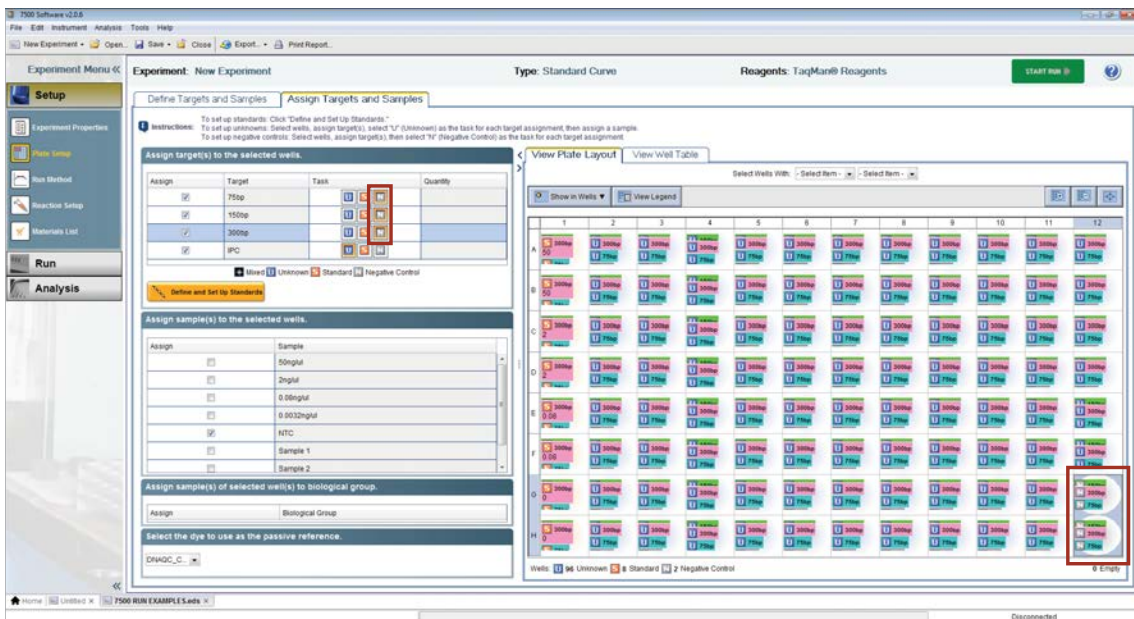


Figure 5. Assigning NTC Task to wells with NTC samples.

13. For each test sample, highlight the wells containing the Test Sample on the View Plate Layout tab, and assign the appropriate Sample Name.

Notes:

1. You can highlight replicates of the same Test Sample at the same time and assign a Sample Name.
2. The Task for the 75bp, 150bp, 300bp and IPC should be “U”.
3. Sample information can also be entered in the ProNex® DNA QC Assay Analysis Software.

Creating a Run Template (continued)

14. Select “DNAQC_CXR” from the drop-down menu titled “Select the dye to use as the passive reference” (Figure 6).

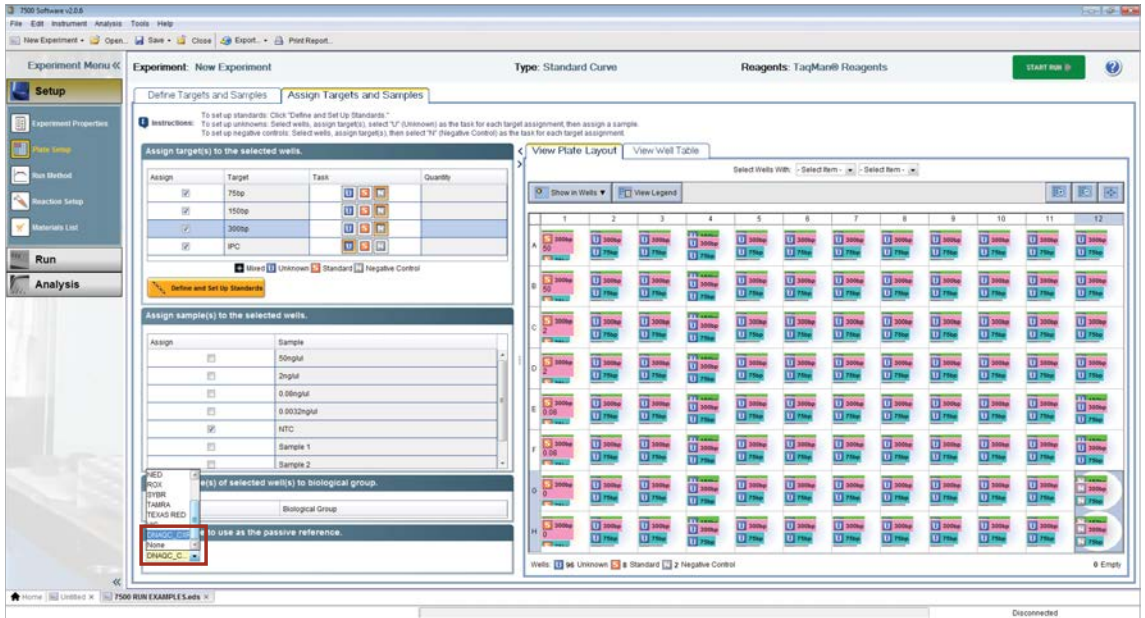


Figure 6. Assigning Sample(s) to selected wells.

15. From the Analysis menu at the top of the screen, select “Analysis Settings”.
16. On the C_T Settings tab (Figure 7) in the “Select a Target” panel, select the 150bp target.

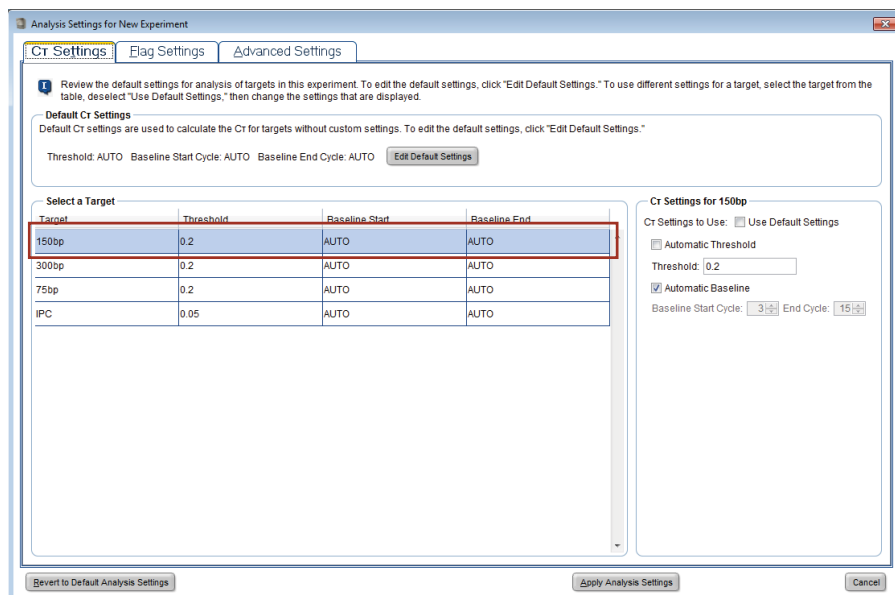


Figure 7. The Analysis Settings window.

17. In the C_T Settings for 150bp section, uncheck the “Use Default Settings” box and uncheck the “Automatic Threshold” box. Enter “0.2” for the threshold. Leave the “Automatic Baseline” box checked.
18. Repeat this process for each target listed in the “Select a Target” panel using the following threshold values:
 - 75bp: 0.2
 - 150bp: 0.2
 - 300bp: 0.2
 - IPC: 0.05

Note: ΔR_n is a relative fluorescent unit and is not absolute; it may differ between Applied Biosystems® 7500 or 7500 Fast Real-Time PCR Systems. Lamp Usage (hours) on an instrument can impact the relative fluorescence. The manual threshold values recommended in this technical manual were determined using maximum target ΔR_n values between 3.0–5.0. Manual thresholds may need to be increased or decreased according to the maximum ΔR_n values on individual instruments.

Creating a Run Template (continued)

19. Select “Apply Analysis Settings” (Figure 7). Select “Run Method” under Setup in the left panel. Modify the default run method as directed below:
 - a. Enter the total PCR volume (μl) in the Reaction Volume per Well field. If the reaction volume is different than $20\mu\text{l}$, enter the correct volume.
 - b. Select the first Holding Stage, and right-click to bring up a menu. Choose “Delete Selected” to delete this stage.
 - c. Change the second Holding Stage to 2 minutes at 98°C .
 - d. Enter “40” for “Number of Cycles” under Cycling Stage.
 - e. Change Step 1 to 98°C for 15 seconds and Step 2 to 60°C for 60 seconds
 - f. Ensure that the “Data Collection On” icon is active for Step 2 of the Cycling Stage.

Note: The “Data Collection On” icon is shown in the legend at the bottom of the screen in Figure 8.

The completed ProNex[®] DNA QC Assay run method is shown in Figure 8.

20. From the File menu at the top of the screen, select “Save as Template”, and choose a location to save the file. This action saves the instrument setup and thermal cycling program for future use. Use this *.edt file as a template to create the experiment document as described in the next section, *Starting a Thermal Cycling Run*.

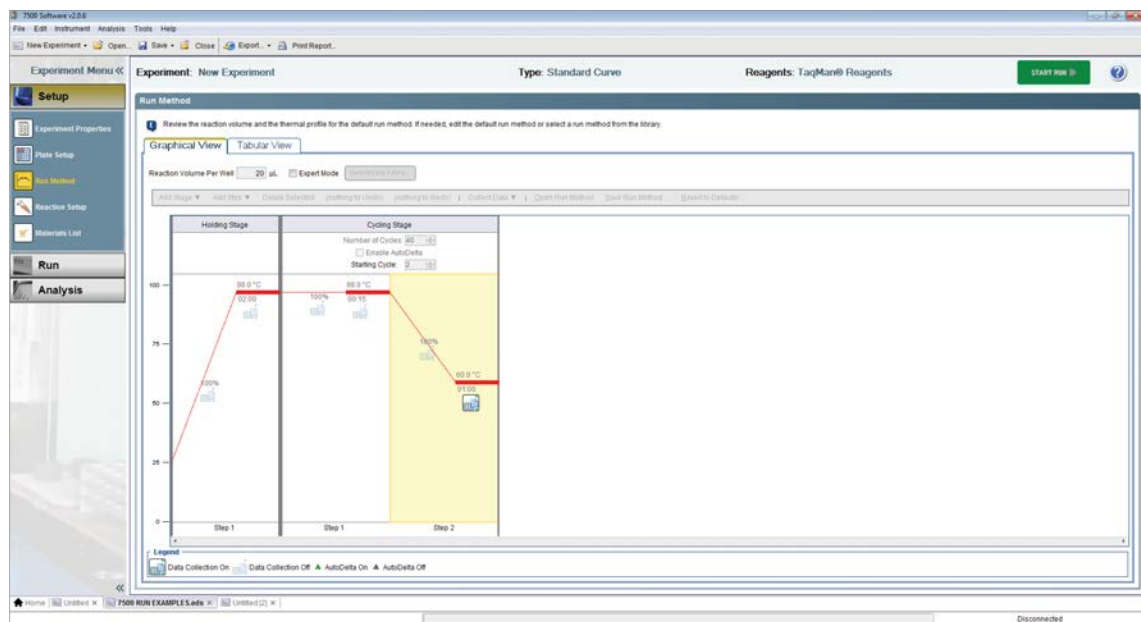


Figure 8. The completed ProNex[®] DNA QC run method.

Starting a Thermal Cycling Run

Prior to thermal cycling, create a run template (*.edt file) to store the dye information, target names and tasks, well locations and DNA concentrations for the DNA standards (optional), and the run method and analysis settings. Before using the ProNex® DNA QC Assay, ensure that the instrument is calibrated for the ProNex® DNA QC dyes.

1. Select “Open” from the File menu at the top of the screen. Navigate to the location of the .edt file, and click “Open”.
2. Save the .edt template as an .eds file by selecting “Save As...” from the File menu.
3. Deselect targets for unused wells.
4. **Optional:** Assign sample names and define standards.
 - a. To add sample names, select “Plate Setup” from the Setup menu, and select the “Define Targets and Samples” tab. Select “Add New Sample”, and enter the sample name in the section provided. Repeat for all samples.
 - b. Navigate to the Assign Targets and Samples tab, and highlight the wells that contain replicates of the same sample. Check the Assign box adjacent to the corresponding sample name in the section titled “Assign sample(s) to the selected wells”. Repeat until all samples are assigned.

Note: Sample names can be assigned either in the 7500 SDS software or the ProNex® DNA QC Assay Analysis Software Results. Any sample names entered in the SDS software will be imported with results in the ProNex® DNA QC Assay Analysis Software.

5. Confirm the Run Method.
6. Once samples are assigned properly, save the .eds file.
7. Press the tray door and place the plate in the open tray door. Ensure that the plate sits correctly in the plate holder, with well A1 in the top left corner. Push the tray door to close, and immediately press “Start Run”.

Amplification is complete in 83 minutes with standard cycling.

Optional: Evaluating Standard Curves Using the Applied Biosystems® 7500 Software

Standard curves can be evaluated in the Applied Biosystems® 7500 Software or the ProNex® DNA QC Assay Analysis Software Results. Any standard curves calculated in the SDS software will be independently calculated by the ProNex® DNA QC Assay Analysis Software.

1. Select “Analysis” in the left side panel, and ensure that all wells to be analyzed are highlighted on the View Plate that no targets are selected for unused wells.
2. Click “Analyze”.
3. To display standard curves, select “Standard Curve” from the Analysis menu in the left side panel. Display the standard curve for each target by selecting it from the Target drop-down menu (Figure 9).

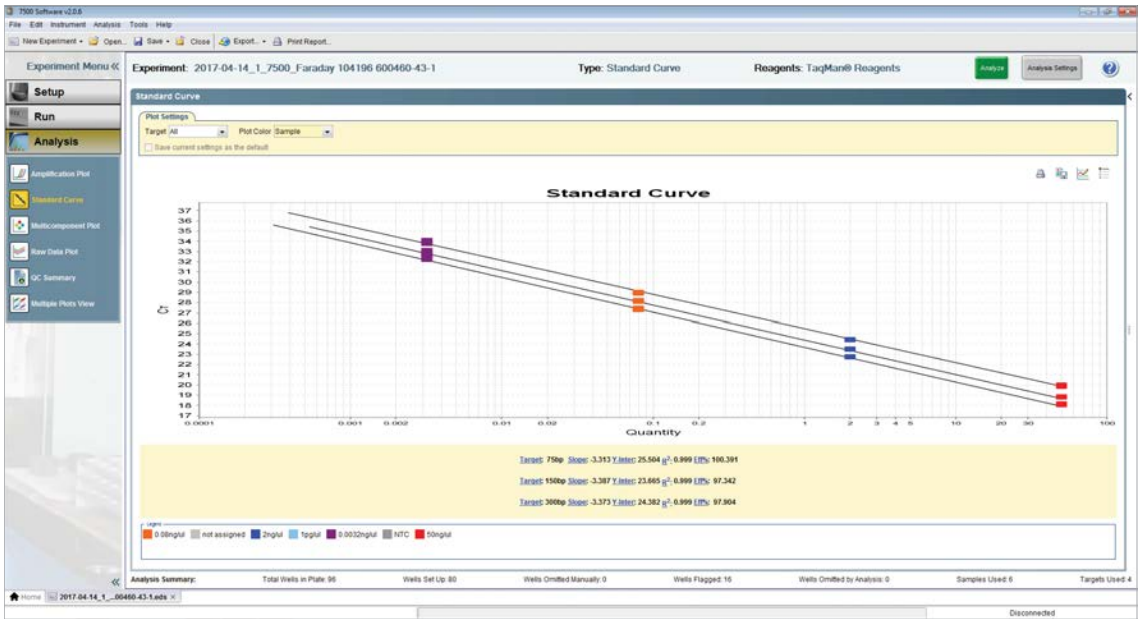


Figure 9. Displaying the standard curve for 75bp, 150bp and 300bp targets.

Exporting Analyzed Data from the Applied Biosystems® 7500 Software

1. Ensure that all wells with data for export are highlighted in the plate map. Select “Export” from the toolbar.
2. On the Export Properties tab of the Export Data window (Figure 10), select the following Export Properties:
 - a. Choose “Results” in the section titled “Select data to export”.
 - b. Choose “One File” from the drop-down menu titled “Select one file or separate files”.
 - c. Choose “.xls” as the File Type.
 - d. Specify the appropriate export file name.
 - e. Use the “Browse” button to select the file location.
 - f. Click “Start Export”.

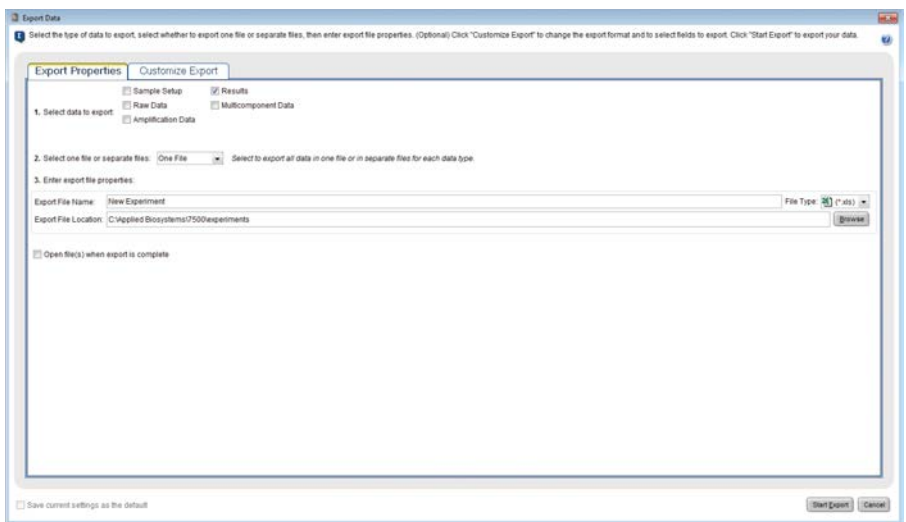


Figure 10. The Export Data window.

3. In the Export Completed window that appears, select “Close Export Tool”.

The results export file from the 7500 software will include the Well, Sample Name, Target Name, Task, Reporter/Quencher, CT, CT Mean and Standard Deviation, Quantity, Quantity Mean & Standard Deviation, as well as Threshold/Baselining information.

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 R²) 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

Standard Curves

The Applied Biosystems® 7500 Real-Time PCR System 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 $1\text{ ng}/\mu\text{L}$, 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 $>1.0\text{ pg}/\mu\text{L}$ per $2\mu\text{L}$ 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. E-mail: techserv@promega.com

Symptoms	Causes and Comments
ΔR_n of amplification plots (ΔR_n vs. Cycle) not normalized (greater than 1,000) with poor standard curves	No passive reference dye selected. Ensure that a passive reference is selected (e.g., DNAQC_CXR dye).
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	<p>Incorrect reaction setup.</p> <ul style="list-style-type: none"> • 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.
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	<p>Incorrect programming of the thermal cycler. Verify that the thermal cycler was programmed correctly. See <i>Creating a Run Template</i> in Section 5.</p> <p>Incorrect reaction setup.</p> <ul style="list-style-type: none"> • From the Experiment menu in the Applied Biosystems® 7500 software, select the “Analysis” section, and then select the “Multicomponent Plot” to determine if fluorescence was detected for any of the system dyes, including the passive reference dye. • If no fluorescence was detected, check to be sure that the 20X ProNex® DNA QC Primer-Probe-IPC Mix, ABI 7500/7500 Fast was added to the amplification reactions. <p>The 2X ProNex® DNA QC Master Mix may have lost activity, or the 20X ProNex® DNA QC Primer-Probe-IPC Mix, ABI 7500/7500 Fast was degraded. Minimize the number of freeze-thaw cycles. Store the 20X ProNex® DNA QC Primer-Probe-IPC Mix, ABI 7500/7500 Fast protected from light.</p>
Inconsistency between replicates of the same DNA sample	<p>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).</p> <p>DNA concentration was low. Differences in amplification results for replicate samples with low levels of DNA template can be due to stochastic effects.</p>
Quantity estimates for larger-sized targets (150bp and 300bp) are greater than the 75bp target	Incorrect dye assignment. Confirm that the correct custom dyes were assigned to each target.

Symptoms
Causes and Comments

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 1 μ l.

No passive reference dye selected. Ensure that the correct passive reference was selected (e.g., DNAQC_CXR dye).

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 Applied Biosystems® 7500 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 Applied Biosystems® 7500 Real-Time PCR System software. Verify that the analysis settings were set as described in *Creating a Run Template* in Section 5.C.

DNA detected in NTCs

DNA contamination occurred. Detection of >1.0 pg/ μ l per 2 μ l 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.



7. Troubleshooting (continued)

Symptoms	Causes and Comments
Noisy amplification plots with no distinct amplification curve and no IPC amplification plots detected	<p>Wells with no reaction mix were analyzed. Be sure that only wells containing amplification reactions are highlighted and analyzed. For empty wells, deselect the targets in the “Assign Targets and Samples” tab of the Setup Menu or use the “Omit” option in the View Plate Layout tab of the Analysis Menu.</p> <hr/> <p>Sample contamination occurred.</p> <ul style="list-style-type: none">• Avoid contamination of the Applied Biosystems® 7500 Real-Time PCR System by minimally handling plates and never running a reaction with a seal that is compromised or broken.• Decontaminate the sample block following the instructions in the 7500/7500 Fast Real-Time PCR Systems Maintenance Guide.
Sample with inhibitor not flagged by IPC	<p>Buffer composition interference. 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.</p> <hr/> <p>Samples with hematin can impact the passive reference dye (CXR) fluorescence, which can impact the effectiveness of the IPC to detect PCR inhibition. Use the Multicomponent Plot to monitor CXR fluorescence.</p>

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. Applied Biosystems® 7500/7500 Fast Real-Time PCR Systems Maintenance Guide. (2010) Part Number 4387777. Applied Biosystems, Foster City, 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 BioRad CFX96™	200 reactions	NG1004
	800 reactions	NG1005
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

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