TECHNICAL MANUAL

ProNex® NGS Library Quant Kit

Instructions for Use of Product
NG1201

Revised 5/20
TM521

Promega

ProNex® NGS Library Quant Kit

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 Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

Promega

1. Description

The ProNex® NGS Library Quant Kit[a] contains reagents to determine the concentration of next generation sequencing (NGS) libraries compatible with Illumina platforms using qPCR. Data generated using the ProNex® NGS Library Quant Kit can be used to normalize the DNA concentration for NGS libraries to the desired concentration.

ProNex® NGS Library Quant 10X Primer Mix

The ProNex® NGS Library Quant Primer Mix includes primers that target the P5 and P7 Illumina adapter sequences for quantification of Illumina libraries (1).

ProNex® NGS Library Quant 2X Master Mix

The ProNex® NGS Library Quant 2X Master Mix formulation includes BRYT Green® dye, a passive reference dye, a hot start polymerase, $MgCl_2$, dNTPs and a proprietary reaction buffer. The BRYT Green® dye is detected using the same filters and settings as the SYBR® Green I dye. The passive reference is a low level of the carboxy-X-rhodamine (CXR) reference dye identical to ROX™ dye. Data from the other dye channels are normalized to the signal from the passive reference.

ProNex® NGS Quant DNA Standard

A standard curve is required to determine the DNA concentration of the sample libraries. The ProNex® NGS Quant DNA Standard is supplied with the ProNex® NGS Library Quant Kit. Serial dilutions of this DNA standard are amplified in the same plate as the sample libraries, and the results are used to generate a standard curve. The standard curve is used to determine the DNA concentration of the sample libraries.

ProNex® NGS Library Dilution Buffer

The ProNex® NGS Library Dilution Buffer is supplied as the diluent for serial dilution of the ProNex® NGS Quant DNA Standard and the sample libraries. The dilution buffer is also the recommended input for the no-template controls.

Instrumentation

The ProNex® NGS Library Quant Kit can be used with any real-time instrument capable of detecting SYBR® Green I or FAM™ dye. The BRYT Green® dye has spectral properties similar to those of SYBR® Green I: excitation at 493nm and emission at 530nm. The CXR reference dye has the same spectral properties as ROX™ dye: excitation at 580nm and emission at 602nm. Instrument optical settings established for SYBR® Green I and ROX™ dyes should be used with the ProNex® NGS Library Quant Kit.

The concentration of CXR passive reference dye in the ProNex® NGS Library Quant 2X Master Mix is low. Some instruments require higher concentrations of passive reference dye. Check the user guide for your instrumentation to determine whether a higher concentration of CXR is needed. A tube of CXR Reference Dye can be purchased separately for instruments that require a higher level of reference dye. If you are using an instrument that requires additional CXR, supplement the ProNex® NGS Library Quant 2X Master Mix with 0.2µl of CXR Reference Dye per 20µl reaction.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ProNex® NGS Library Quant Kit	**500 reactions**	**NG1201**

For Research Use Only. Not for use in Diagnostic Procedures. The ProNex® NGS Library Quant Kit includes sufficient reagents for 500 reactions at 20µl per reaction. Components are shipped on dry ice. Includes:

- 5 × 1ml ProNex® NGS Library Quant 2X Master Mix
- 1 × 1ml ProNex® NGS Library Quant 10X Primer Mix
- 1 × 200µl ProNex® NGS Quant DNA Standard (200pM)
- 2 × 1,250µl Nuclease-Free Water
- 2 × 150ml ProNex® NGS Library Dilution Buffer

Storage Conditions: Store the ProNex® NGS Library Quant Kit at –30°C to –10°C in a nonfrost-free freezer protected from light. For immediate use, components may be stored at 2–10°C, protected from light, for up to 3 months. Dilutions of the ProNex® NGS Quant DNA Standard in ProNex® NGS Library Dilution Buffer can be stored at 4°C for up to 3 months.

3. General Considerations

The BRYT Green® dye in the ProNex® NGS Library Quant 2X Master Mix has spectral properties similar to those of SYBR® Green I dye: excitation at 493nm and emission at 530nm. Instrument optical settings established for SYBR® Green I assays should be used with the ProNex® NGS Library Quant Kit. The ProNex® NGS Library Quant Kit can be used with any real-time instrument capable of detecting SYBR® Green I or FAM™ dye. Additional testing in your laboratory may be required to show instrument compatibility. The CXR reference dye has the same spectral properties as ROX™ dye: excitation at 580nm and emission at 602nm. Use the instrument settings for ROX™ dye for reactions containing ProNex® NGS Library Quant 2X Master Mix.

The dynamic range of this assay is 20pM to 0.02pM. Sample libraries must be diluted to fall in the dynamic range. If the concentration of a diluted library falls out of this range, the library should be retested with a dilution that falls in the dynamic range of the assay.

We recommend performing triplicate 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 the sample libraries to minimize variability. Do not use any other DNA as the DNA standard.

The ProNex® NGS Library Dilution Buffer may take several hours to thaw at room temperature. Alternatively, thaw the ProNex® NGS Library Dilution Buffer at 2—10°C overnight. Store the ProNex® NGS Library Dilution Buffer at 2—10°C after thawing.

The ProNex® NGS Library Quant Kit is extremely sensitive; take precautions to minimize cross contamination. We recommend storing the ProNex® NGS Library Quant Kit reagents separately from DNA samples. We also recommend using clean designated work areas and separate pipettes for pre- and post-amplification steps to minimize the potential for cross-contamination between DNA samples and prevent carryover of nucleic acid from one run to the next. Wear a lab coat and protective eye wear. 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 triplicate.

Do not unseal reaction plates after amplification is complete. Unsealing the plates increases the risk of contaminating subsequent reactions with amplified product.

4. Reaction Plate Setup

Materials to Be Supplied by the User

- sterile, aerosol-resistant pipette tips
- low-retention or siliconized microcentrifuge tubes
- optical multiwell reaction plates and adhesive film covers
- real-time thermal cycler
- **optional:** CXR Reference Dye (Cat.# C5411) (**Note:** Additional CXR reference dye is needed for some instrumentation. Check the user guide for your instrument to determine whether additional CXR is required.)

4.A. Serial Dilution of the ProNex® NGS Quant DNA Standard

Vortex the ProNex® NGS Quant DNA Standard thoroughly before each use. We recommend long-term storage at 2–10°C. Perform serial tenfold dilutions of the ProNex® NGS Quant DNA Standard, and then amplify these dilutions to create four-point standard curves to determine the concentration of targets in the unknown DNA samples. Accurate serial dilution of the ProNex® NGS Quant DNA Standard is essential to accurately quantify unknown DNA samples; carefully mix and pipet each DNA standard dilution. The ProNex® NGS Library Dilution Buffer should be used as the diluent for the ProNex® NGS Quant DNA Standard. Serial dilutions of the ProNex® NGS Quant DNA Standard prepared with ProNex® NGS Library Dilution Buffer can be stored for up to 3 months at 2–10°C.

The four-point serial dilutions prepared in this section include DNA standards in the range of 20pM to 0.02pM. When diluting the ProNex® NGS Quant DNA Standard, use the ProNex® NGS Library Dilution Buffer; do not use water as a diluent. Use low-retention or siliconized tubes to prepare the serial dilutions.

1. If not already thawed, thaw the ProNex® NGS Library Dilution Buffer completely. Vortex the ProNex® NGS Quant DNA Standard three times at high speed for 10 seconds each time.

   Note: After the initial thaw, store the ProNex® NGS Quant DNA Standard and ProNex® NGS Library Dilution Buffer at 2–10°C.

2. Label four tubes with the following concentrations: 20pM, 2pM, 0.2pM and 0.02pM.

3. Prepare serial dilutions of the ProNex® NGS Quant DNA Standard as indicated in Table 1. Vortex each dilution for 10 seconds before removing an aliquot for the next dilution. Change pipette tips between dilutions.

Table 1. Serial Dilution of the ProNex® NGS Quant DNA Standard (tenfold dilutions).

DNA Concentration	Volume of ProNex® NGS Library Quant DNA Standard	Volume of ProNex® NGS Library Dilution Buffer
20pM	Use 5µl of the ProNex® NGS Quant DNA Standard (200pM)	45µl
2pM	5µl of 20pM dilution	45µl
0.2pM	5µl of 2pM dilution	45µl
0.02pM	5µl of 0.2pM dilution	45µl

Notes: [1]We recommend performing amplification reactions of each DNA standard in triplicate. [2]Change gloves after handling the high-copy number DNA standard. [3]Serial dilutions of the ProNex® NGS Quant DNA Standard prepared with the ProNex® NGS Library Dilution buffer can be stored for up to 3 months at 4°C.

4.B. Dilute DNA Libraries

1. Dilute the sample libraries to concentrations within the dynamic range of the assay (20pM to 0.02pM).

   Note: If microcentrifuge tubes will be used for the serial dilution of the sample libraries, use low-retention or siliconized tubes.

2. Pipette a minimum volume of 2µl of the undiluted library and an appropriate volume of ProNex® NGS Library Dilution Buffer to prepare the dilution.

   Note: Libraries may require dilutions that fall in the range of 1:1,000 to 1:100,000. Serial dilutions of the libraries may be used to determine a dilution factor that falls in the dynamic range of the assay.

   For example:

   a. Dilute sample libraries 1:100 by carefully adding 2µl of undiluted library to 198µl of ProNex® NGS Library Dilution Buffer.

   b. Mix the dilution thoroughly by gently pipetting 10 times using a 200µl single-channel or multichannel pipette set to 198µl.

   c. Dilute the sample library 1:10,000 by carefully adding 2µl of the 1:100 dilution (from Step 2.b) to 198µl of ProNex® Library Dilution Buffer.

4.C. Reaction Setup

Include no-template control reactions for each set of reactions. Add 4µl of ProNex® NGS Library Dilution Buffer to these reactions instead of template DNA.

1. Thaw the ProNex® NGS Library Quant 2X Master Mix, ProNex® NGS Library Quant 10X Primer Mix, and Water, Amplification Grade, completely at room temperature.

2. Vortex the ProNex® NGS Library Quant 2X Master Mix and ProNex® NGS Library Quant 10X Primer Mix for 10 seconds to mix.

4.C. Reaction Setup (continued)

3. Determine the number of reactions to be set up, including NTC reactions. Increase this number by 10–15% to compensate for pipetting error and reagent loss on 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. Use Table 2 to calculate the volume of each component required to prepare sufficient reaction mix for the number of reactions determined in Step 3.

Table 2. Preparation of Reaction Mix for NGS Library Quantification Using the ProNex® NGS Library Quant Kit.

Component	Volume Per Reaction	Number of Reactions	Final Volume
Water, Amplification Grade	4µl		
ProNex® NGS Library Quant 2X Master Mix	10µl		
ProNex® NGS Library Quant 10X Primer Mix	2µl		
Total Volume	16µl		

Note: A separate tube of CXR Reference dye should be purchased for instruments that require a higher level of reference dye than in the ProNex® NGS Library Quant 2X Master Mix. If you are using an instrument that requires additional CXR, supplement the ProNex® NGS Library Quant 2X Master Mix with 0.2µl of CXR Reference Dye per 20µl reaction.

5. Prepare the reaction mix by combining the volumes of ProNex® NGS Library Quant 2X Master Mix, ProNex® NGS Library Quant 10X Primer Mix, and Water, Amplification Grade, calculated in Step 4.
6. Vortex for 10 seconds to mix.
7. Add 16µl of reaction mix per well to the reaction wells of an optical multiwell reaction plate.

   Note: Wear gloves at all times when handling the plate, and take care to avoid touching the plate wells and optical adhesive film unnecessarily. Handle the plate by the edges, and avoid touching the bottom of the plate.
8. Add 4µl of the of the ProNex® NGS Library Quant DNA Standards prepared in Section 4.A or the diluted NGS libraries prepared in Section 4.B for a final reaction volume of 20µl.
9. Add 4µl of the ProNex® NGS Library Dilution Buffer to the NTC reactions for a final reaction volume of 20µl.
10. Seal the plate with optical adhesive film.

    Note: Ensure that all wells are adequately sealed to prevent evaporation during thermal cycling.
11. Centrifuge the plate briefly to collect contents of each well at the bottom. The plate is ready for thermal cycling. Protect the plate from extended light exposure or elevated temperatures before thermal cycling.

12. Program the real-time thermal cycler using the following cycling parameters:

    95°C for 2 minutes, then:

    30 cycles of

    95°C for 15 seconds,

    60°C for 15 seconds,

    72°C for 45 seconds (perform fluorescence data collection during this step)

    Note: Select the SYBR® Green I dye for target detection. Select the ROX® dye for the passive reference.

13. Place the plate in the instrument, and run the program.

14. When the program is complete, remove the plate from the instrument.

5. Data Analysis

1. Review standard curve.

2. In general, using the default settings, we see slopes for the standard curves in the range of −3.10 to −3.70. The R^2 values for all standard curves should be > 0.990.

6. Determine Library Concentration

1. To determine the library concentration, calculate the average concentration of the sample replicates and multiply by the dilution factor used in Section 4.B.

2. Use the following equation to calculate the size-adjusted correction factor for the library.

$$\text{Correction factor for library size} = \frac{\text{350bp (Size of ProNex® NGS Quant DNA Standard)}}{\text{(Average length (bp) of library fragment)}}$$

3. Multiply the library concentration calculated in Step 1 by the correction factor calculated in Step 2 to calculate the size-adjusted concentration.

   Note: This will give the concentration in pM. Convert to nM for the library concentration to be compatible with Illumina sequencing manuals.

Use the size adjusted concentration of the original library to normalize the library concentration. The optimal loading concentration may vary for different sample types or library construction methods. Refer to the library preparation kit and Illumina instrument manuals for guidance on loading concentrations and library pooling.

7. Troubleshooting

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

Symptoms	Causes and Comments
Flat amplification curves and no C_q values detected for a subset of samples in the in the multiwell reaction plate	Verify that the 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.
	Verify that the thermal cycler was programmed correctly. See Section 4.
	Verify that ProNex® NGS Library Quant 10X Primer Mix and/or DNA libraries were added to the amplification reactions.
	The ProNex® NGS Library Quant 2X Master Mix lost activity or was degraded. Minimize the number of freeze-thaw cycles. Store the ProNex® NGS Quant 2X Master Mix protected from light.
DNA standards amplify and produce a linear standard curve with late C_q values or no C_q values for the DNA libraries	The library dilution is significantly outside the dynamic range of the assay. Retest the library with corrected dilution.
	Adapter sequences were not ligated to the DNA libraries. Prepare the DNA libraries again.
Non-linear standard curve (R^2 values <0.98) and/or slope outside of the specified range	Be sure that the DNA standard is completely thawed and mixed well before use.
	Be sure that each dilution of the DNA standard is well mixed before removing an aliquot for the next serial dilution. Use the same pipette for each aliquot. Change the pipette tip between each addition of DNA standard to the multiwell reaction plate.
	Calibrate the pipettes to minimize variability when pipetting.
	Amplify the standard curve in triplicate to minimize the effects of variation.
	An error was made during dilution of the DNA standard. Verify all calculations and repeat the dilution of the DNA standard. Avoid pipetting volumes less than 2µl.

Symptoms	Causes and Comments
Non-linear standard curve (R^2 values <0.98) and/or slope outside of the specified range	Incorrect concentration values were entered into the instrument software. Verify that all of the DNA standard concentrations were entered correctly.
	Ensure that the correct passive reference was selected (ROX™).
	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.
	Review the instrument settings and adjust as necessary.
	Repeat the DNA standard dilutions if the serially diluted standards were stored longer than recommended.
	Check that low-retention or siliconized tubes were used to prepare the serial dilutions of the DNA standard.
Inconsistency between replicates of the same DNA sample	Examine the volume of liquid in each well of the reaction plate to verify that evaporation did not occur during cycling. Evaporation of the reaction mix can affect C_q values.
	Check that library dilution falls into the dynamic range of the standard curve (20pM to 0.02pm). Retest the library with the corrected dilution.
DNA detected in no-template controls (NTC)	An amplification curve that crosses the amplification threshold could indicate the presence of contaminating DNA.
Noisy amplification plots with no distinct amplification curve	Wells with no reaction mix were analyzed. Be sure that only wells that contain amplification reactions are selected and analyzed in Section 4.
Irregular amplification curve for the 20pM standard	Instrument software subtracted early amplification as background. Review the baseline and threshold values and manually adjust as necessary.

8. Appendix

8.A. Reference

1. *Illumina Adapter Sequences* (2017) Illumina, Inc. [Internet: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-03.pdf Accessed 11/8/2017]

Promega

8.B. Related Products

Product	Size	Cat.#
ProNex® DNA QC Assay ABI 7500/7500 FAST	200 reactions	NG1002
	800 reactions	NG1003
ProNex® DNA QC Assay BioRad CFX 96™	200 reactions	NG1004
	800 reactions	NG1005
ProNex® Size-Selective Purification System	10ml	NG2001
	125ml	NG2002
	500ml	NG2003
CXR Reference Dye	100µl	C5411
2800M Control DNA (10ng/µl)*	25µl	DD7101
2800M Control DNA (0.25ng/µl)*	500µl	DD7251

*Not For Medical Diagnostic Use.

Nucleic Acid Extraction

Product	Size	Cat.#
Maxwell® RSC Instrument	1 each	AS4500
Maxwell® RSC ccfDNA Plasma Kit	48 preps	AS1480
Maxwell® RSC Blood DNA Kit	48 preps	AS1400
ReliaPrep™ FFPE Total RNA Miniprep System	10 reactions	Z1001
	100 reactions	Z1002

9. Summary of Changes

The following changes were made to the 5/20 revision of this Technical Manual:

1. In Section 2, the volume of ProNex® NGS Quant DNA Standard was changed from 25µl to 200µl.
2. Disclaimers were updated to remove an expired statement.

[a]U.S. Pat. Nos. 8,598,198 and 9,206,474 and other patents and patents pending.

BRYT Green and ProNex are registered trademarks of Promega Corporation.

MiSeq is a registered trademark of Illumina, Inc. SYBR is a registered trademark of Life Technologies. FAM and ROX are trademarks of Applera Corporation.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.