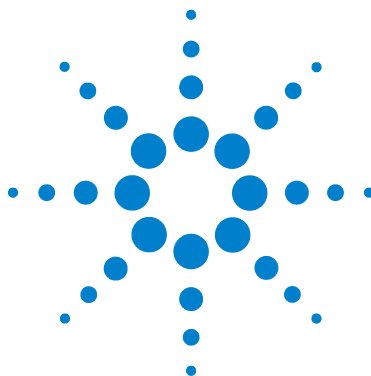




QPCR NGS Library Quantification Kit (illumina GA)

Part Number G4880A

Protocol

Version A, April 2010

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Procedures.**



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In this Guide...

This document describes how to use the QPCR NGS Library Quantification Kit (illumina GA) for quantifying the concentration of a DNA sequencing library.

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

2 Procedures

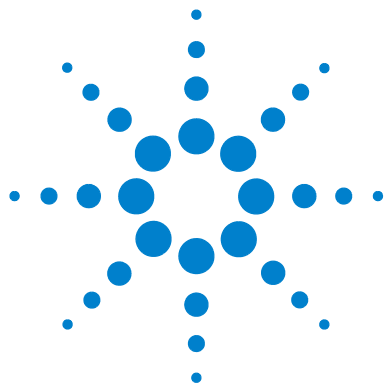
This chapter contains instructions on quantifying the concentration of a DNA library using the QPCR NGS library quantification kit.

3 Troubleshooting

This chapter contains additional information on improving quantification results.

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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.



Materials Provided

Stratagene Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix, Catalog #600882 (400 QPCR reactions)	
2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix	2 × 2 ml
Reference Dye, 1 mM	100 µl
Agilent QPCR Library Quantification Kit, Catalog #5190-2379	
100 pM illumina DNA Standard (288-bp target)	20 µl
200× Dilution Buffer	0.5 ml
50× illumina GA Primer Mix	200 µl
10× GC Additive	1 ml

Storage Conditions

Store all components at -20°C upon receipt.

Once the 200× Dilution Buffer has been thawed, it can be stored at 4°C. The solution may appear opaque upon thawing. This appearance does not affect the performance of the buffer. If desired, you may briefly warm the 200× Dilution Buffer to 37°C before use to clarify the solution.

Once the 2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix has been thawed, it can be stored at 4°C for up to one month or returned to -20°C for long term storage.

The QPCR Master Mix and Reference Dye are light sensitive; protect these solutions from light whenever possible.

Required Equipment, Supplies and Reagents

Additional Equipment, Supplies and Reagents Required
Nuclease-free PCR-grade water
Real-time PCR instrument
96-well QPCR plates or tubes

The QPCR NGS Library Quantification Kit (illumina GA) is included in Agilent's SureSelect Target Enrichment protocols for indexing applications on the illumina Genome Analyzer.

Overview

When preparing a DNA library for next generation sequencing (NGS) on the illumina Genome Analyzer system, accurate quantification of the molarity of the DNA library is required to ensure high-quality reads and efficient data generation. DNA concentrations that are too high can lead to mixed signals and data that are impossible to resolve. Conversely, concentrations that are too low can reduce sequencing coverage and read-length.

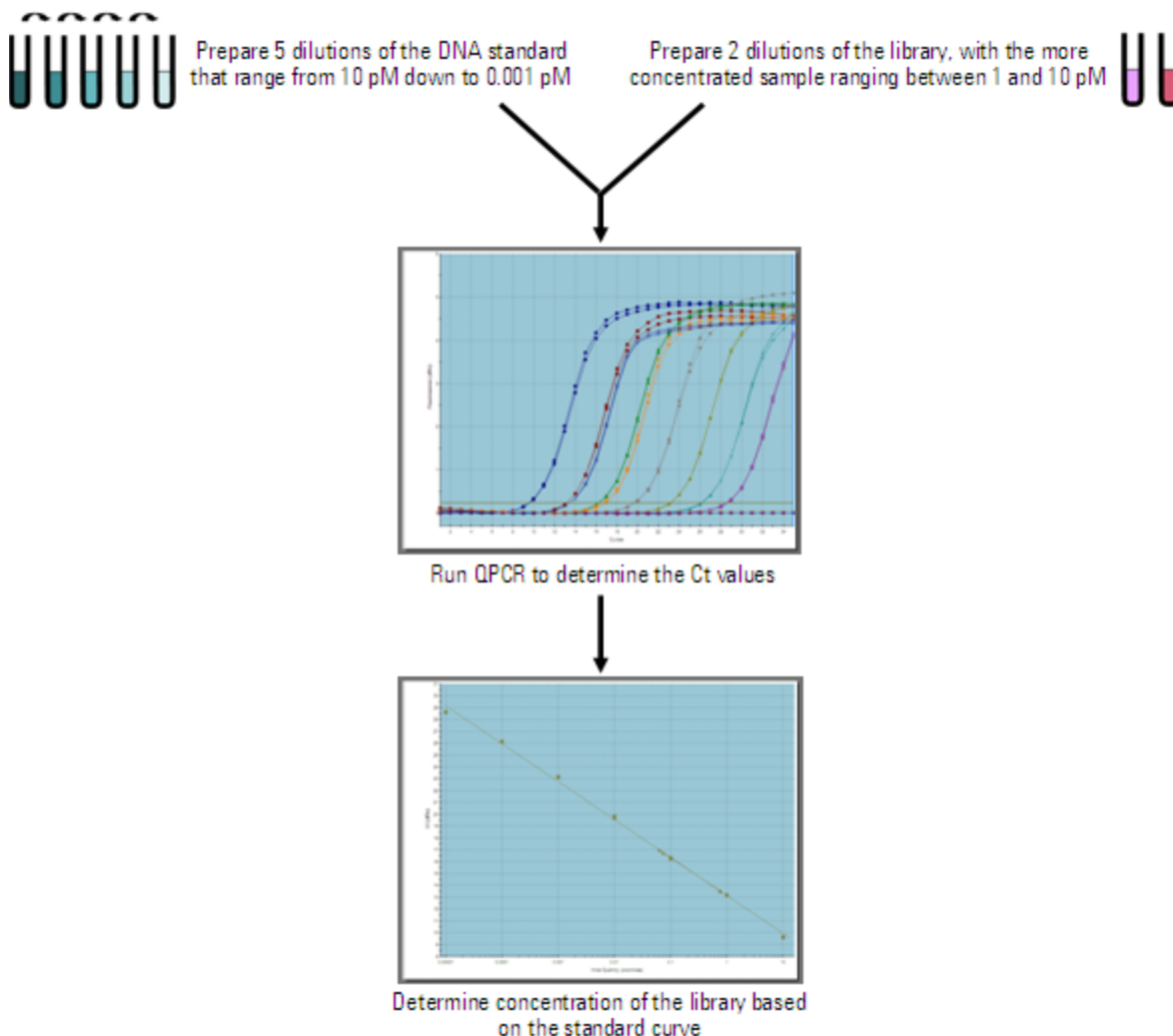
QPCR is a highly sensitive approach for quantifying a DNA NGS library. It uses a minimal amount of material compared to other quantification approaches, and it can be automated for high-throughput applications.

The QPCR NGS Library Quantification Kit provides a fast, reliable method for determining the concentration of an DNA library using QPCR. The kit is compatible with libraries designed for the illumina GA Sequencing System, and up to 21 libraries can be quantified in a single QPCR run. The kit includes the Stratagene Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix. This 2× master mix contains a mutant *Taq* DNA polymerase, dNTPs, and the double-stranded DNA-binding dye SYBR Green I for detection. A passive reference dye (an optional reaction component) is provided in a separate tube.

The kit also includes a DNA standard of known concentration that is used to generate a standard curve to which the library samples are compared. The DNA standard consists of a 288-bp linear DNA target that includes the same adapter sequences used for illumina GA libraries. The primers needed to amplify the DNA standard and the library are also included with the kit. An overview of the quantification protocol is shown in [Figure 1](#).

The QPCR NGS library quantification kit is recommended for use with Agilent's SureSelect™ kits for the illumina GA system. Information on the SureSelect platform can be found at www.agilent.com/genomics/nextgen.

Figure 1 Overview of the library quantification protocol



Recommended Plate Setup for QPCR

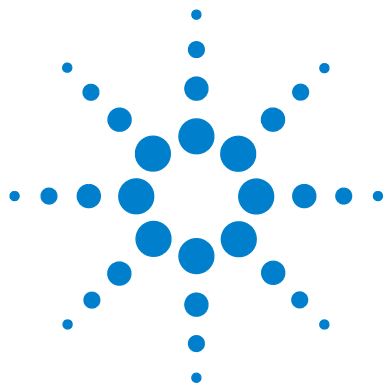
To ensure accurate quantification while maintaining high-throughput capability, we propose the following scheme for the QPCR reactions:

- Include five 10-fold serial dilutions of the DNA standard (in duplicate) with each QPCR run. With five dilutions, the range of concentrations for the standard curve covers 10 pM down to 0.001 pM, a range of 10,000 fold.
- Include two 10-fold serial dilutions of the DNA library (in duplicate), with the molarity of the more concentrated dilution estimated to be between 1 and 10 pM. The rough concentration of the library can be determined by running a sample on a gel or from the OD₂₆₀ reading on a spectrophotometer, or it can be estimated based on data gathered during library preparation.
- Include a no-template control (NTC) reaction in duplicate.

Using the above guidelines, 21 libraries can be quantified on a single 96-well plate (and a total of 84 libraries can be quantified with each library quantification kit). See [Figure 2](#) for an example of a plate setup that uses these recommendations.

Figure 2 Recommended plate setup for QPCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 pM Standard	10 pM Standard	Library 2 Dilution 1	Library 2 Dilution 1	Library 6 Dilution 1	Library 6 Dilution 1	Library 10 Dilution 1	Library 10 Dilution 1	Library 14 Dilution 1	Library 14 Dilution 1	Library 18 Dilution 1	Library 18 Dilution 1
B	1 pM Standard	1 pM Standard	Library 2 Dilution 2	Library 2 Dilution 2	Library 6 Dilution 2	Library 6 Dilution 2	Library 10 Dilution 2	Library 10 Dilution 2	Library 14 Dilution 2	Library 14 Dilution 2	Library 18 Dilution 2	Library 18 Dilution 2
C	0.1 pM Standard	0.1 pM Standard	Library 3 Dilution 1	Library 3 Dilution 1	Library 7 Dilution 1	Library 7 Dilution 1	Library 11 Dilution 1	Library 11 Dilution 1	Library 15 Dilution 1	Library 15 Dilution 1	Library 19 Dilution 1	Library 19 Dilution 1
D	0.01 pM Standard	0.01 pM Standard	Library 3 Dilution 2	Library 3 Dilution 2	Library 7 Dilution 2	Library 7 Dilution 2	Library 11 Dilution 2	Library 11 Dilution 2	Library 15 Dilution 2	Library 15 Dilution 2	Library 19 Dilution 2	Library 19 Dilution 2
E	0.001 pM Standard	0.001 pM Standard	Library 4 Dilution 1	Library 4 Dilution 1	Library 8 Dilution 1	Library 8 Dilution 1	Library 12 Dilution 1	Library 12 Dilution 1	Library 16 Dilution 1	Library 16 Dilution 1	Library 20 Dilution 1	Library 20 Dilution 1
F	NTC	NTC	Library 4 Dilution 2	Library 4 Dilution 2	Library 8 Dilution 2	Library 8 Dilution 2	Library 12 Dilution 2	Library 12 Dilution 2	Library 16 Dilution 2	Library 16 Dilution 2	Library 20 Dilution 2	Library 20 Dilution 2
G	Library 1 Dilution 1	Library 1 Dilution 1	Library 5 Dilution 1	Library 5 Dilution 1	Library 9 Dilution 1	Library 9 Dilution 1	Library 13 Dilution 1	Library 13 Dilution 1	Library 17 Dilution 1	Library 17 Dilution 1	Library 21 Dilution 1	Library 21 Dilution 1
H	Library 1 Dilution 2	Library 1 Dilution 2	Library 5 Dilution 2	Library 5 Dilution 2	Library 9 Dilution 2	Library 9 Dilution 2	Library 13 Dilution 2	Library 13 Dilution 2	Library 17 Dilution 2	Library 17 Dilution 2	Library 21 Dilution 2	Library 21 Dilution 2



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- Analyze the Data 17
 - To Determine the Concentration of the Library 17

This chapter contains instructions on quantifying the concentration of a DNA library using the QPCR NGS library quantification kit.



Perform QPCR

To Prepare the Reagents

Dilution Buffer

Dilute the 200× dilution buffer to 1× using PCR-grade water. You will need 100 µl of 1× dilution buffer to prepare 5 serial dilutions of the DNA standard.

Note that the 200× dilution buffer may appear opaque. This appearance does not affect the performance of the buffer. If desired, you may briefly warm the 200× dilution buffer to 37°C before diluting it to 1× with water.

DNA Standard

Using 1× dilution buffer, prepare dilutions of the 100 pM DNA standard. We recommend preparing five 10-fold serial dilutions of the DNA standard (from 10 pM down to 0.001 pM) and running the diluted samples in duplicate during QPCR. Prepare each dilution by mixing 2 µl of DNA standard with 18 µl of 1× dilution buffer. The diluted samples can be stored at 4°C for up to a day.

Reference Dye

The 1 mM stock of Reference Dye needs to be diluted using PCR-grade water prior to adding it to the QPCR reactions.

- If using the Stratagene Mx3000P or Mx3005P QPCR system or the ABI 7500 Real-Time PCR system, dilute the Reference Dye to 2 µM. This equates to a 1:500 dilution of the stock.
- If using the ABI StepOnePlus or 7900HT Real-Time PCR system, dilute the Reference Dye to 20 µM using PCR-grade water. This equates to a 1:50 dilution of the stock.

Keep all solutions containing the reference dye protected from light. The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the same day for setting up additional assays.

DNA Library

We recommend preparing two 10-fold serial dilutions of the DNA library to be run in duplicate during QPCR. Using 1× dilution buffer, dilute a sample of the library to obtain a DNA concentration somewhere between 1 and 10 pM. ***Make note of the dilution factor used to prepare this sample.*** Then, dilute this sample 10-fold to obtain a second sample.

NOTE

For libraries with an average DNA size of 300 bp, a 1–10 pM range equates to approximately 2 to 0.2 ng/ml.

To Set Up the QPCR Reactions

- 1 Thaw the tube containing the 2× Brilliant III Ultra-Fast SYBR Green QPCR Master Mix and keep the tube on ice while setting up the reactions. *SYBR Green is light sensitive; solutions containing the master mix should be protected from light whenever possible.*
- 2 Prepare the reactions by combining the components in [Table 1](#) in order. Prepare a single reagent mixture for all reactions using multiples of the volumes listed below.

Table 1 PCR Reagent Mixture

Component	Volume
dH ₂ O, sterile	5.3 µl
2× Brilliant III Master Mix	10 µl
10× GC Additive (optional)*	2 µl
50× illumina GA Primer Mix	0.4 µl
Diluted Reference Dye	0.3 µl
Total volume	18 µl

*The GC-Additive is required for amplification of GC-rich DNA libraries. If any of the libraries being quantified has a GC content over 60%, or if you are unsure of the GC content, include the additive in the reagent mixture. If leaving out the additive, adjust the volume of water accordingly.

- 3 Vortex the reagent mixture briefly, then distribute 18 µl to each individual well of a 96-well QPCR plate.

- 4 Add 2 μ l of DNA sample to each well to bring the final reaction volume to 20 μ l. If using the plate setup in [Figure 2](#), you will have 10 wells containing a DNA standard sample and 82 wells containing a library DNA sample. For the no-template control reactions, add 2 μ l of 1 $\times$ Dilution Buffer in place of the DNA.

After adding each sample, gently mix the reaction by pipetting the contents of the well up and down.

To Run the QPCR Protocol

- 1 In the QPCR system software, assign well information as needed to generate a standard curve from the wells containing the serially diluted DNA standard.

The concentration of the DNA standard in each reaction is one-tenth the concentration of the diluted sample that was added to that reaction. When assigning template quantities to the wells in the QPCR system software, we recommend assigning the concentration of the DNA standard present in the diluted sample (i.e. 10, 1, 0.1, 0.01 and 0.001 pM) rather than the final concentration in the reaction. This way, the 10-fold dilution that occurs when DNA is added to the reactions does not need to be considered.

- 2 Place the plate in the QPCR instrument and run the PCR program shown in [Table 2](#). Set the instrument to report and detect fluorescence at each cycle during the 60°C annealing/extension step. Note that a melt/dissociation step is not necessary.

Table 2 PCR Cycling Protocol

Number of Cycles	Temperature	Duration
1	95°C	3 minutes
30	95°C	10–20 seconds*
	60°C	30 seconds

*A denaturation time of 20 seconds is recommended if any of the libraries being tested has a high GC content. A 10-second denaturation is suitable for amplification of libraries with <60% GC.

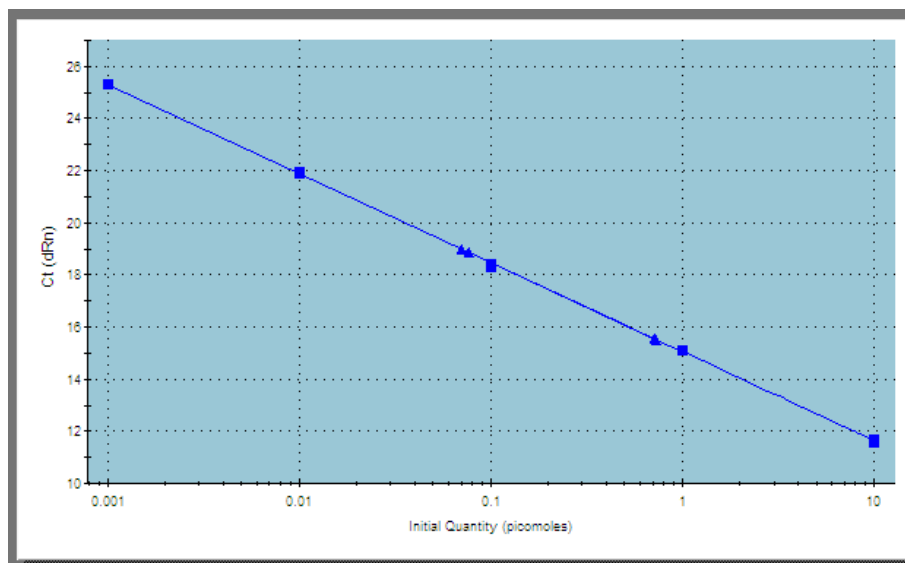
NOTE

This PCR cycling protocol has been optimized on the Stratagene Mx3000P/Mx3005P QPCR systems. Some optimization of cycling parameters may be required when using another instrument. A protocol is sufficient for library quantification if the standard curve yields an R-squared value >0.98 and an amplification efficiency of 90–110%.

Analyze the Data

To Determine the Concentration of the Library

- 1 In the QPCR software, view the standard curve generated from the serially diluted DNA standard. The standard curve is a plot of DNA concentration versus Ct. An example is shown below.



- 2 Verify that the standard curve has an R-squared value >0.98 and an amplification efficiency between 90% and 110%.

If the standard curve does not meet these specifications, the library quantification may not be accurate. See the [“Troubleshooting Guide”](#) on page 20 for suggestions on obtaining a high-quality standard curve with the DNA standard.

- 3 For each library dilution tested, use the standard curve to find the concentration of DNA based on the Ct.

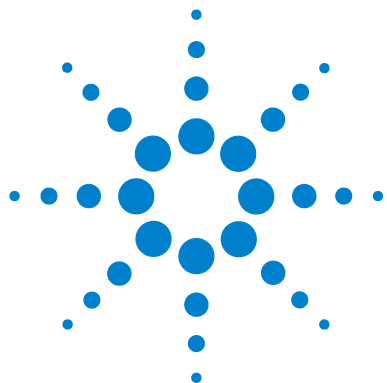
When calculating concentrations, be sure to multiply by the dilution factor used to generate that particular sample.

- 4 Calculate the average concentration of the library:
 - If both library dilutions have Ct values within the range of the standard curve, use the average of the two concentrations.
 - If only one of the library dilutions has a Ct value in the range of the standard curve, use the concentration value obtained from that one Ct and disregard the Ct from the other dilution.
- 5 If the average target length for the library is significantly different from that of the 288-bp DNA standard (either >310 bp or <260 bp), adjust the concentration measured in [step 4](#) using the formula below. (*The average target length for a library can be estimated on a gel or on the Agilent 2100 Bioanalyzer.*)

$$\text{Adjusted Concentration} = \text{Measured Concentration} \times (288 \text{ bp} / \text{average length of library})$$

2 Procedures

To Determine the Concentration of the Library



3 Troubleshooting

Troubleshooting Guide [20](#)

This chapter contains additional information on improving quantification results.



Troubleshooting Guide

If the R-squared value of the standard curve is <0.98 , or the amplification efficiency is not between 90% and 110%

- ✓ Repeat the QPCR using fresh dilutions of the DNA standard. Be sure to use 1× dilution buffer as the diluent (not water) and use pipettes that have been properly calibrated. Do not use diluted samples of the DNA standard that have been stored for longer than a day.
- ✓ Repeat the QPCR using a freshly-thawed aliquot of the 2× QPCR master mix. Multiple freeze-thaws, excessive exposure to light, and long-term storage at temperatures higher than -20°C can all impact the performance of the master mix.
- ✓ The cycling parameters provided in the protocol may need to be optimized for your QPCR instrument. Try optimizing the annealing temperature and extension time to improve the quality of the standard curve.

If none of the Ct values obtained from the library dilutions are within the range covered by the standard curve

- ✓ If all Ct values were too high, repeat the QPCR with library dilutions that are more concentrated.
- ✓ If all Ct values were too low, repeat the QPCR with library dilutions that are less concentrated.

If the concentrations obtained from the 2 dilutions are significantly different

- ✓ A more careful assessment of the amplification efficiency of the library may be necessary. Try running a standard curve (in duplicate or triplicate) using 4 or more serial dilutions of the library DNA (this can be done by defining a second standard curve on the plate in the QPCR instrumentation software). If the efficiency obtained from this standard curve is not between 90% and 110%, consider repurifying the library.

If replicate reactions of a library sample yield significantly different Ct values

- ✓ If one of the replicate reactions failed to amplify, the data from the failed replicate can be discarded. To calculate the concentration, use only the data from the replicate that amplified.
- ✓ If both replicates amplified, but they yielded significantly different Ct values, repeat the QPCR.

In This Book

This document describes how to use the QPCR NGS Library Quantification Kit (illumina GA) for quantifying the concentration of a DNA sequencing library.

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