Sequencing Library qPCR Quantification Guide

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Introduction

This document describes a qPCR method for quantifying sequencing by synthesis (SBS) libraries generated using the Illumina® sample preparation protocols and Eco™ Real-Time PCR System. qPCR is a method of quantifying DNA based on PCR. qPCR tracks target concentration as a function of PCR cycle number in order to derive a quantitative estimate of the initial template concentration in a sample. As with conventional PCR, it uses a polymerase, dNTPs, and two primers designed to match sequences within a template.

For the purposes of this protocol, the primers match sequences within the adapters flanking an Illumina sequencing library. qPCR is, therefore, an ideal method for measuring libraries in advance of generating clusters, because it will only measure templates that have both adaptor sequences on either end which will subsequently form clusters on a flowcell. In addition, qPCR is a very sensitive method of measuring DNA and therefore dilute libraries with concentrations below the threshold of detection of conventional spectrophotometric methods are amenable to quantification by qPCR.

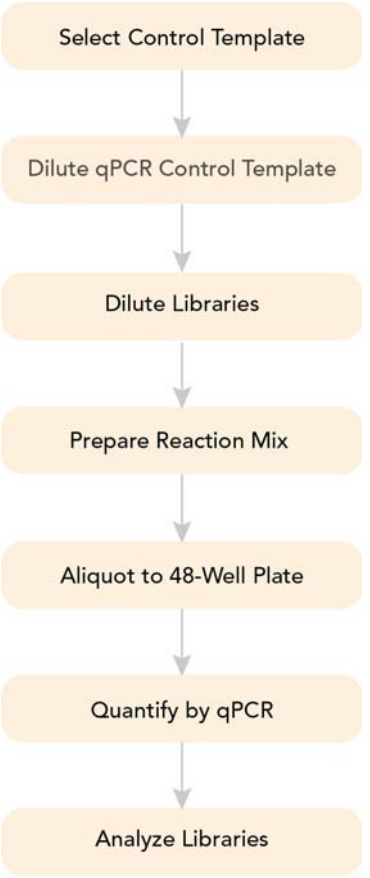
Scope

This document describes a protocol designed for the Eco Real-Time System. However, there are several different qPCR instruments available for running experiments and analyzing data.You will need to adapt this protocol when using a qPCR platform other than Eco. For additional information regarding Eco, reference the Illumina *Eco System User Guide*.

Quantification Workflow

The following figure illustrates the Sequencing Library qPCR Quantification workflow. Dilute the control template and the libraries for quantification to the pM range and run qPCR. From the qPCR results, calculate the concentration of the quantified libraries and dilute them to a standard concentration (e.g., 2 nM).

Figure 1 Sequencing Library qPCR Quantification Workflow



Best Practices

When preparing libraries for sequencing, you should always adhere to good molecular biology practices.

- During qPCR setup, it is important to avoid DNA cross-contamination. Clean the set up area, including all equipment to be used, thoroughly with 0.5% sodium hypochlorite (10% bleach).
- Wear gloves and use sterile technique at all times.
- Use a dedicated set of pipettes for qPCR to minimize contamination.
- The accuracy of qPCR is highly dependent on accurate pipetting and thorough mixing of solutions. Take extra care to avoid pipetting errors during qPCR set up and when preparing templates for clustering.

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding.

Table 1 User-Supplied Consumables

Consumable	Supplier
0.1% Tween 20	General lab supplier
0.2 ml eight-tube strip	General lab supplier
0.5% sodium hypochlorite (10% bleach)	General lab supplier
2 N NaOH	General lab supplier
Control template (2 nM)	General lab supplier
Eco 48-well plates	Illumina, catalog # EC-200-1002
Eco Adhesive Seals	Illumina, catalog # EC-200-1003
Hybridization buffer	Illumina, catalog #0801-1001
KAPA SYBR FAST Master Mix Universal 2X qPCR Master Mix (2 x 5 ml =10 ml)	Kapa Biosystems, part #KK4602
Libraries to be quantified diluted to approximately 10 nM	General lab supplier
Nuclease-free water	General lab supplier
QIAGEN EB 250 ml elution buffer	QIAGEN, part # 19086
qPCR primer 1.1: 5' AATGATACGGCGACCACCGAGAT 3' HPLC purified	General lab supplier
qPCR primer 2.1: 5' CAAGCAGAAGACGGCATACGA 3' HPLC purified	General lab supplier
Tris-Cl 10 mM, pH 8.5	General lab supplier

Table 1 User-Supplied Consumables (Continued)

Consumable	Supplier
One or more of the following kits in order to correspond to the number of libraries to be quantified: 1. Single-Read Cluster Generation Kit (1 flow cell) 2. Single-Read Cluster Generation Kit (10 flow cells) 3. Paired-End Cluster Generation Kit (1 flow cell) 4. Paired-End Cluster Generation Kit (5 flow cells)	 1. Illumina, catalog # GD-1003-4001 2. Illumina, catalog # GD-1003-4010 3. Illumina, catalog # PE-2003-4001 4. Illumina, catalog # PE-2003-4005

Table 2 User-Supplied Equipment

Equipment	Suggested Supplier
Benchtop centrifuge with swing out rotor	Sorvall Legend RT
Benchtop microcentrifuge	General lab supplier
Eco Real-Time PCR System (110V) or Eco Real-Time PCR System (220V)	Illumina, catalog # EC-100-1000 (110V) Illumina, catalog# EC-100-1001 (220V)
Vortexer	General lab supplier

Select Control Template

Before starting qPCR, select the control template against which the libraries for quantification can be measured. Any library prepared for sequencing on the Illumina platform can be used as a control for qPCR and you can tailor a control template to suit your specific needs. The control template should be as similar as possible to the libraries for quantification, in terms of template size, GC content and library type (e.g., genomic, ChIP-Seq, etc.). It is also important that a sufficient quantity of the control template is available, as specified in this protocol, for use in multiple qPCR reactions.

In order to correlate library concentration with cluster number, it is recommended to generate a titration flowcell for the control template (see *Appendix A - Cluster Count* and *Appendix B - Preparing DNA Template*).

The GC content of a given library is not always known and this can be a problem for matching the library to an appropriate control template for sequencing library qPCR quantification. However, it is possible to estimate the GC content of Illumina libraries relative to other Illumina libraries of the same template size by performing a limited number of cycles of qPCR followed by a dissociation curve. The higher the GC content of the library, the higher the melting temperature of the PCR product (see *Appendix C - Library GC Content*). Once the GC content of a library is known, an appropriate control template can be selected for sequencing library qPCR quantification.

Dilute qPCR Control Template

Use the appropriate control library for the libraries you wish to quantify.

NOTE
Illumina recommends using a control library that gives a good range of cluster numbers when clustered between 2–20 pM.

User-Supplied Consumables

- 0.1% Tween 20 stored at room temperature (e.g., 50 ml water + 50 µl Tween 20)
- qPCR control template (2 nM)

NOTE
Store the qPCR 2 nM library template in small aliquots to avoid multiple freeze and thaw cycles.

Procedure

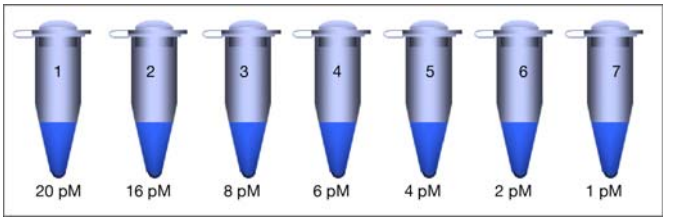
1 Add 198 µl of 0.1% Tween 20 to 2 µl of the qPCR control template to make a 100-fold dilution.

2 Vortex the dilution to thoroughly mix the samples.

3 Add 100 µl of 0.1% Tween 20 to 100 µl of the diluted template to make a titration curve of six 2x serial dilutions to produce seven control template dilutions in the range of 20–1 pM.

NOTE
It is important to make fresh dilutions of the qPCR control template immediately before qPCR because DNA does not store well at low concentrations.

Figure 2 Serial Dilutions



4 Vortex the dilution to thoroughly mix the samples.

5 Repeat steps 1–4 to produce three independent serial dilutions of the control template.

NOTE

Control dilutions are diluted a further 10X into the final SYBR mix, so the final concentration in the qPCR is 2–0.03 pM.

Dilute Libraries

This process dilutes the libraries for quantification to the same range as the control template for qPCR.

User-Supplied Consumables

- 0.1% Tween 20 stored at room temperature (e.g., 50 ml water + 50 µl Tween 20)
- Libraries for quantification diluted to approximately 2 nM in QIAGEN EB Buffer

NOTE
It is important to make fresh dilutions of the qPCR unknown library template before qPCR as the DNA does not store well at low concentrations.

Procedure

1. Add 998 µl of 0.1% Tween 20 to 2 µl of the unknown library template to make a 500-fold dilution for an approximate concentration of 4 pM.
2. Vortex the dilution to thoroughly mix the samples.
3. Repeat steps 1–2 to produce three independent dilutions of the library template. Triplicate results for qPCR are important for subsequent analysis.

NOTE
Unknown sample dilutions are diluted a further 10X into the final SYBR mix so the final concentration in the qPCR is approximately 0.4 pM.

Prepare Reaction Mix

It is important to make a master mix to minimize pipetting errors. This process makes enough master mix to fill a 48-well plate.

User-Supplied Consumables

- KAPA SYBR FAST Master Mix Universal (2x)
- qPCR Primer 1.1
- qPCR Primer 2.1
- Nuclease-free Water

Procedure

1 Prepare the SYBR master mix reaction mix as follows. The master mix contains extra volume to accomodate up to 55 wells:

Consumable	µl/well	µl/plate
KAPA SYBR FAST Master Mix Universal (2x)	10	550
qPCR Primer 1.1 (10 µM)	0.2	11
qPCR Primer 2.1 (10 µM)	0.2	11
Nuclease-free Water	7.6	418
Total Volume	**18**	**990**

2 Mix gently but thoroughly.

3 Place the reaction mix on ice and protect it from light until use.

Aliquot to 48-Well Plate

This process aliquots the control template dilutions, unknown library dilutions, and master mix. It is important to pipette as accurately as possible, because small variations in volumes will greatly affect the qPCR results.

User-Supplied Consumables

- 48-well plates
- Control template dilutions (see *Dilute qPCR Control Template*)
- Eco adhesive seals
- Libraries for quantification dilutions (see *Dilute Libraries*)
- Reaction Mix (see *Prepare Reaction Mix*)

Procedure

1 Add 18 µl of the master mix to each well of the 48-well plate using a multichannel pipette. Take care to pipette accurately into the wells as variations in volume will affect the assay.

2 Add 2 µl of the control template dilutions, the unknown library dilutions, or water to each well of a 48-well plate. Take care to pipette accurately into the wells as small variations will affect the assay. For example, the 48-well plate can be filled as follows:

Table 3 48-Well Plate Format

	1	2	3	4	5	6	7	8
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
B	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
C	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
D	Control 20 pM	Control 16 pM	Control 8pM	Control 6 pM	Control 4 pM	Control 2 pM	Control 1 pM	NTC
E	Control 20 pM	Control 16 pM	Control 8pM	Control 6 pM	Control 4 pM	Control 2 pM	Control 1 pM	NTC
F	Control 20 pM	Control 16 pM	Control 8pM	Control 6 pM	Control 4 pM	Control 2 pM	Control 1 pM	NTC

Wells in rows D–F contain the control template dilutions and the no template control (NTC) in triplicate. Wells in rows A–C contain the sample dilutions in triplicate.

NOTE

The Eco software provides a dilution template and default cycling conditions.

3 Seal the plate using an Eco adhesive seal, taking care to avoid cross contamination and to avoid smudging the surface of the lids.

4 Place the 48-well plate on the plate adapter and centrifuge the plate to 250 xg for 1 minute.

Quantify by qPCR

This process quantifies the libraries by qPCR.

Procedure

1 Place the 48-well plate on the qPCR machine in the correct orientation, then close the lid.

2 Use the thermal profile provided in the **Eco** software, by selecting the **SBS Library Quantification.ecot** template from the **Templates** tab. The thermal profile is as follows and can be viewed on the **Setup | Thermal Profile** tab:

Procedure	Temperature	Time
Hot start	95°C	10 minutes
X 40 {	95°C	10 seconds
	60°C	30 seconds

3 View the **Setup | Plate Layout** tab to verify that the layout matches your template.

4 Select **Start Run**.

Analyze Libraries

This final step in the qPCR process analyzes the quantified libraries.

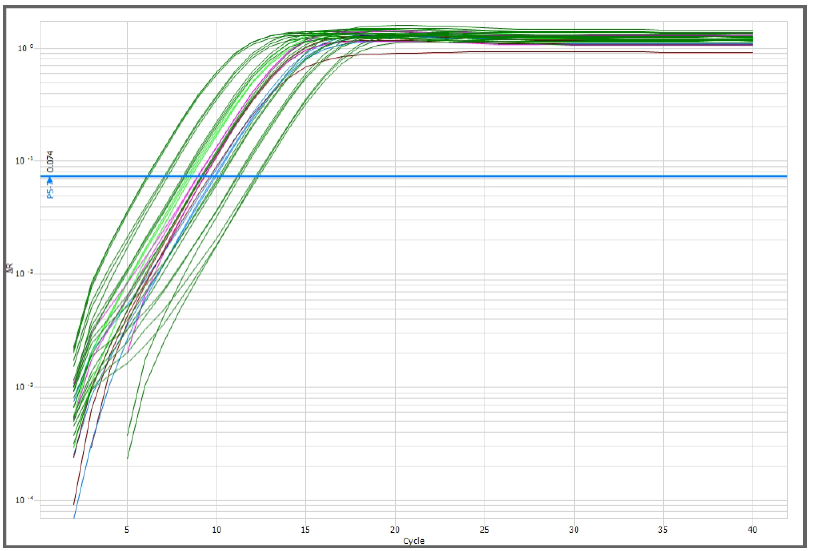
Procedure

1 Check the NTC wells for any amplification. There should be no amplification.

2 Ensure that there is good amplification for the control template and remove outliers from a replicate group that are > 0.5 Cq.

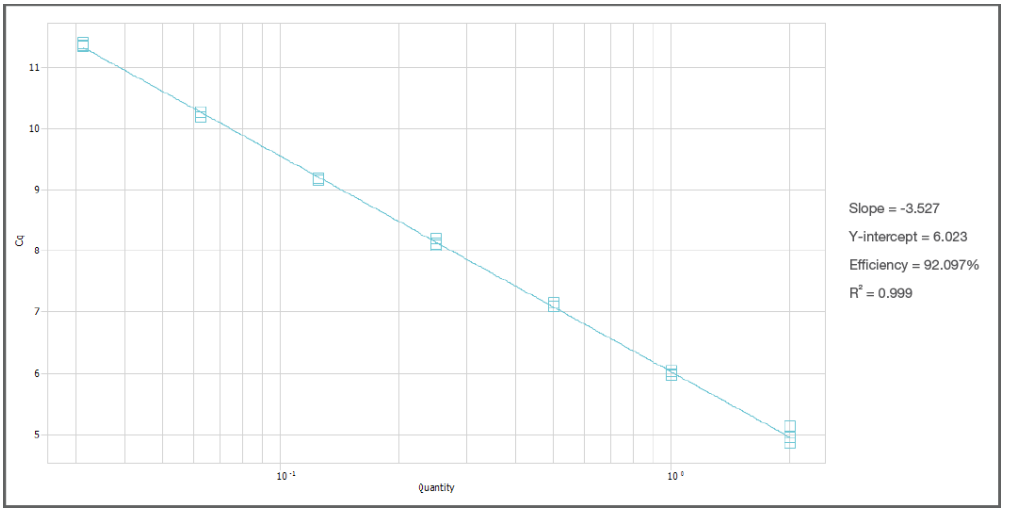
CAUTION
Four or more outliers per plate indicate technical errors.

Figure 3 Control Template Amplification Example



3 Generate a standard curve from the control template by plotting the Cq values against the log initial concentration.

Figure 4 Standard Curve Example



4 Ensure that the efficiency of amplification of the control template is 90–110% (a slope of -3.6 to -3.1) and that the R^2 >0.99. If not, reassess the datapoints used to calculate the standard curve.

5 Lock the threshold fluorescence based on the standard curve.

6 Ensure that there is good amplification for the unknown library templates and remove outliers from a replicate group that are > 0.5 Cq.

CAUTION
Four or more outliers per plate indicate technical errors.

7 Calculate the initial concentration of your unknown library templates based on the standard curve generated from the control template dilutions.

NOTE
Remember to factor in the 5000-fold dilution of unknown sample.

8 Dilute the quantified library to a standard concentration for clustering. A suggested protocol for preparing sample DNA for cluster generation is given in *Appendix B - Preparing DNA Template* on page 20.

Appendix A - Cluster Count

A titration flowcell can be generated by preparing serial dilutions. Perform five cycles of sequencing to obtain accurate cluster counts with RTA. Cluster counts from a first cycle report are not accurate due improved algorithms present in RTA. A cluster titration for the control template should be linear up until the point at which the clusters become too dense to count accurately. An example of a library titration is shown below.

User-Supplied Consumables

- Control library
- Sequencing reagents (enough for the required number of cycles)
- Single-Read or Paired-End Cluster Generation Kit

Procedure

1 Prepare eight serial dilutions of the control library and cluster on a flowcell.

NOTE
The number of clusters required from the libraries to be quantified by qPCR should fall within the range of the titration flowcell.

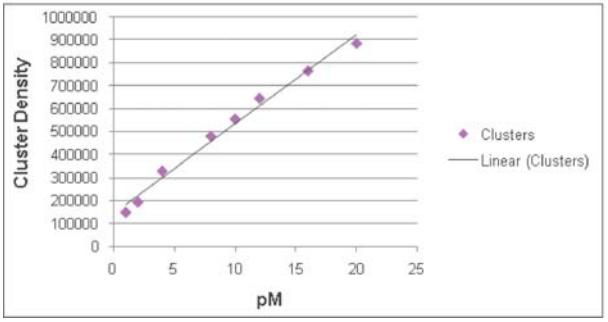
2 Perform six sequencing cycles to count the clusters on the titration flowcell. Ensure that there is enough reagent for the run, with a minimum of the following per cycle. These can be left over reagents.

3 Obtain cluster counts from the summary file.

4 Plot the cluster numbers displayed in the summary table against the initial concentration of control template.

5 Calculate the pM concentration required for the desired number of clusters using the equation of the line.

Figure 5 Library Titration Example



In the above figure, an *E. coli* control library with a template size of 300 bp was clustered on four flowcells at 20 pM, 16 pM, 8 pM, 6 pM, 4 pM, 2 pM, and 1 pM and clusters were counted through RTA following six cycles of sequencing.

Appendix B - Preparing DNA Template

This section explains how to prepare your DNA template for cluster generation.

There are two steps involved in preparing the DNA template:

- Denature with 2 N NaOH
- Dilute DNA into hybridization buffer.

User-Supplied Consumables

- 2 N NaOH
- Hybridization Buffer
- Tris-Cl 10 mM, pH 8.5
- 0.2 ml eight-tube strip

Denature DNA Template

Use the following instructions to denature the template DNA with 2 N NaOH to a final DNA concentration of 1 nM. This is suitable for performing the hybridization step at a DNA concentration up to 8 pM.

If you require a higher DNA concentration, see *Denaturing High Concentrations of DNA* on page 21 for suggested adjustments.

CAUTION

Excess NaOH concentrations (greater than 800 µM) in diluted samples inhibits the formation of clusters, an effect which occurs if you add more than 8 µl of NaOH denatured DNA sample to 1 ml of hybridization buffer.

1. Combine template DNA, Tris-Cl, pH 8.5, and 2 N NaOH in the following volumes:
   - 10 nM Template DNA (2 µl)
   - Tris-Cl 10 mM, pH 8.5 (17 µl)
   - 2 N NaOH (1 µl)

   The total volume should be 20 µl (template final concentration 1 nM).
2. Vortex briefly to mix the template solution.
3. Pulse centrifuge the solution.
4. Incubate for five minutes at room temperature to denature the template into single strands.

5 Place the denatured DNA template on ice until you are ready to proceed to final dilution.

Denaturing High Concentrations of DNA

Use the following table only if you require a DNA concentration higher than 8 pM; otherwise, follow the protocol *Denature DNA Template* on page 20.

Table 4 Adjustments to the Protocol for High Final DNA Concentrations

Desired Final DNA Concentration in 1 ml	Template DNA (10 nM)	Tris-Cl 10 mM, pH 8.5	2 N NaOH	Concentration of Denatured Template DNA
8–12 pM	3 µl	16 µl	1 µl	1.5 nM
12–16 pM	4 µl	15 µl	1 µl	2.0 nM
16–20 pM	5 µl	14 µl	1 µl	2.5 nM
20–24 pM	6 µl	13 µl	1 µl	3.0 nM

Dilute Denatured DNA

Use the following instructions to dilute the denatured DNA with pre-chilled xxxHT1 to a total volume of 1,000 µl. Illumina recommends that you perform a titration of your DNA template to determine a good density of clusters.

1 Remove the Hybridization Buffer from -15° to -25°C storage and thaw at 2° to 8°C overnight or in a beaker of room temperature deionized water.

2 To reach the desired final concentration for the hybridization step, dilute denatured DNA as follows:

Required Final Concentration	5 pM	6 pM	7 pM	8 pM
1 nM denatured DNA	5 µl	6 µl	7 µl	8 µl
Pre-chilled HT1	995 µl	994 µl	993 µl	992 µl

3 Invert several times to mix the template solution.

4 Pulse centrifuge the solution.

5 Label the tubes of an eight-tube strip 1–8.

6 Dispense 120 µl of the control library into tube 4 of the eight-tube strip. This places the control sample in lane 4 on the flow cell.

7 Add 120 µl of diluted, denatured sample DNA template into the remaining tubes of an eight-tube strip. Take careful note of which template goes into which tube.

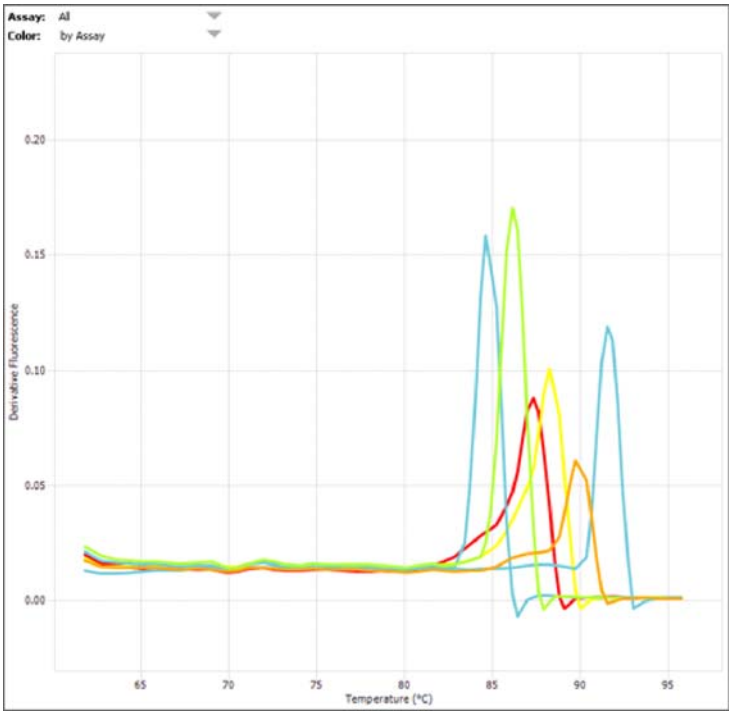
8 Record each sample position and concentration on the lab tracking form.

9 Set aside on ice until you are ready to load it onto the instrument.

Appendix C - Library GC Content

The GC content of a library is not always known, but it is possible to estimate the GC content of Illumina libraries relative to other Illumina libraries of the same size by performing a dissociation curve on the qPCR instrument. The higher the GC content of the library, the higher the melting temperature. Therefore, libraries of the same template length can be directly compared and GC content of unknown libraries estimated relative to libraries of known GC content.

Figure 6 Dissociation Curves Example Generated for 300 bp Illumina Libraries



Consumables and Equipment

- 48-well plates
- Benchtop centrifuge with swing out rotor
- Eco adhesive seals
- Eco Real-Time PCR System
- Illumina libraries of known GC content (~10 nM)

- Illumina libraries of unknown GC content (~10 nM)
- KAPA SYBR FAST Master Mix Universal 2x

Procedure

1 Prepare the following reaction mix for each library (including controls) in a 48-well plate/reaction.

Consumable	µl/well
KAPA SYBR FAST Master Mix Universal (2x)	10
qPCR Primer 1.1 (10 µM)	0.2
qPCR Primer 2.1 (10 µM)	0.2
Water	7.6
Illumina library (approx 10 nM)	2
Total Volume	**20**

2 Put the optical strip lids on the wells and briefly centrifuge the 48-well plate to 250 xg for 1 minute.

3 Place the 48-well plate on the qPCR machine in the correct orientation, then close the lid.

4 Use the following thermal profile:

Procedure	Temperature	Time
Hot start	95°C	3 minutes
X 10 {	95°C	3 seconds
	60°C	30 seconds

5 At the end of the thermal profile ramp slowly from 60° to 95°C to generate a dissociation curve.

Notes

Notes

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 5 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 6 Illumina Customer Support Telephone Numbers

Region	Contact Number
North AmericaToll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom Toll-free	0800.917.0041
Germany Toll-free	0800.180.8994
Netherlands Toll-free	0800.0223859
France Toll-free	0800.911850
Other European Timezones	+44.1799.534000
Other Regions and Locations	1.858.202.ILMN (1.858.202.4566)

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