

# qPCR Quantification Guide

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## Introduction

This document describes a qPCR method for quantifying 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.

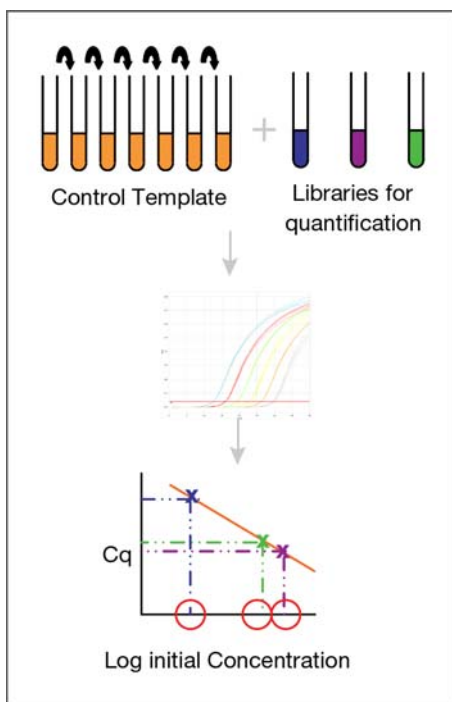
### Scope

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 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** qPCR Quantification Workflow



### NOTE

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). Illumina also recommends using a dedicated set of pipettes for qPCR to minimize contamination.



### NOTE

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
0.1N NaOH	General lab supplier
0.5% sodium hypochlorite (10% bleach)	General lab supplier
Control template (2 nM)	General lab supplier
Eco 48-well plates	Illumina, part # EC-200-1002
Eco Adhesive Seals	Illumina, part # EC-200-1003
Hybridization buffer	Illumina, part # 1000166
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
Pipettes (P2, P10, P200, P1000, and an 8 channel multichannel dispensing 18 µl)	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' CAAGCAGAAGACGGCATAACGA 3' HPLC purified	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, part # GD-1003-4001 2. Illumina, part # GD-1003-4010 3. Illumina, part # PE-2003-4001 4. Illumina, part # PE-2003-4005

**Table 2** Equipment Checklist

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, part # EC-100-1000 (110V) Illumina, part # EC-100-1001 (220V)
Genome Analyzer	Illumina, part # SY-301-1301
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 - Sample Preparation*).

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 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 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.

## Consumables

- ▶ 0.1% Tween 20 stored at room temperature (e.g., 50 ml water + 50  $\mu$ l 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  $\mu$ l of 0.1% Tween 20 to 2  $\mu$ l of the qPCR control template to make a 100-fold dilution.
- 2 Vortex the dilution to thoroughly mix the samples.
- 3 Add 100  $\mu$ l of 0.1% Tween 20 to 100  $\mu$ l of the diluted template to make a titration curve of six 2x serial dilutions to produce seven control template dilutions in the range of 20–0.3 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.

- 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.

### Consumables

- ▶ 0.1% Tween 20 stored at room temperature (e.g., 50 ml water + 50  $\mu$ l 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  $\mu$ l of 0.1% Tween 20 to 2  $\mu$ l of the unknown library template to make a 500-fold dilution for an approximate concentration of 20 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 2 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.

## Consumables

- ▶ KAPA SYBR FAST Master Mix Universal (2x)
- ▶ qPCR primer 1.1
- ▶ qPCR primer 2.1
- ▶ Water

## Procedure

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

Consumable	$\mu\text{l/well}$	$\mu\text{l/plate}$
KAPA SYBR FAST Master Mix Universal (2x)	10	550
qPCR Primer 1.1 (10 $\mu\text{M}$ )	0.2	11
qPCR Primer 2.1 (10 $\mu\text{M}$ )	0.2	11
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.

### 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 2  $\mu$ l 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:

**Figure 2** 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 2pM	Control 1pM	Control 0.5pM	Control 0.25pM	Control 0.125pM	Control 0.06pM	Control 0.03pM	NTC
E	Control 2pM	Control 1pM	Control 0.5pM	Control 0.25pM	Control 0.125pM	Control 0.06pM	Control 0.03pM	NTC
F	Control 2pM	Control 1pM	Control 0.5pM	Control 0.25pM	Control 0.125pM	Control 0.06pM	Control 0.03pM	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.

- 2 Add 18  $\mu$ l 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. Change the tips after each column.

- 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 following thermal profile:

Procedure	Temperature	Time
Hot start	95°C	3 minutes
X 40 {	95°C	3 seconds
	60°C	30 seconds

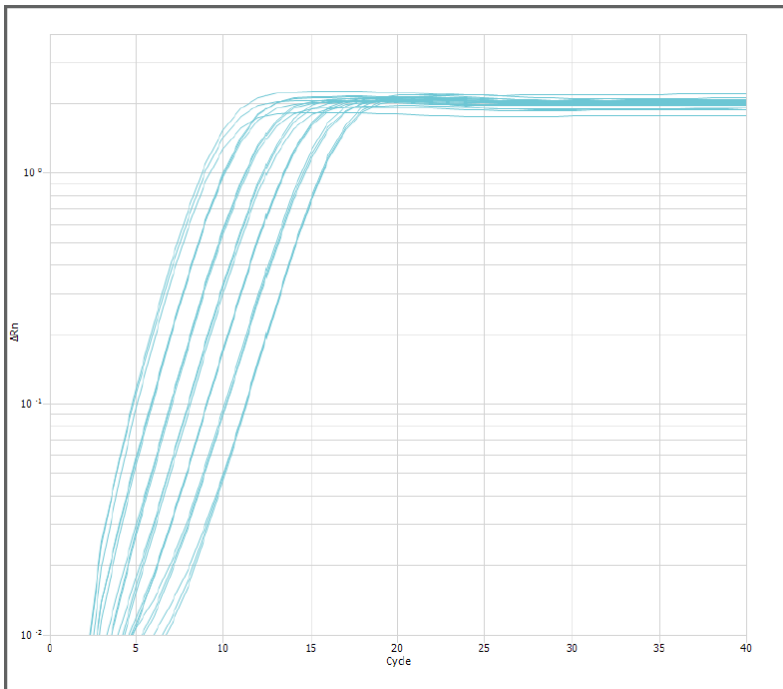
# Analyze Libraries

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

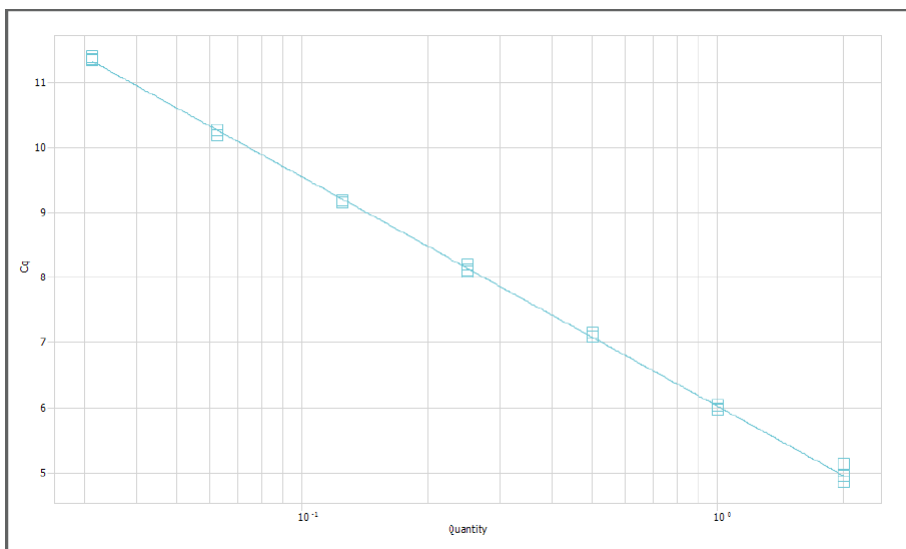
## Procedure

- 1 Check the NTC wells for any amplification. There should be no amplification, but data is acceptable if any amplification is >10 cycles after your last control template amplification.
- 2 Ensure that there is good amplification for the control template and remove any bad replicates.

**Figure 3** Control Template Amplification Example



- 3 Generate a standard curve from the control template by plotting the C<sub>q</sub> values against the log initial concentration.

**Figure 4** Standard Curve Example

- Slope = -3.527
  - Y-intercept = 6.023
  - Efficiency = 92.097%
  - $R^2 = 0.999$
- 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  $R^2 > 0.9$ . 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 any bad replicates.
  - 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 - Sample Preparation* on page 18.

## Appendix A - Cluster Count

A titration flowcell can be generated by preparing serial dilutions of template hyb from the control library and counting the number of clusters following the sequencing cycles.



### NOTE

The number of cycles required depends on the Pipeline used. Perform the same number of cycles as the Pipeline, plus 1 cycle, required for phasing.

Perform multiple cycles of sequencing to achieve an accurate cluster count, because the Pipeline analysis uses the cycles to identify individual clusters in a full length sequencing run.

Cluster counts from a first cycle report are not accurate, particularly at high cluster density, due to the different way in which the clusters are counted. A cluster titration for the control template should be linear up until the point at which the clusters become too dense to count accurately, based on current version of the Illumina Genome Analyzer (GA) being used. An example of a library titration is shown below.

### Consumables and Equipment

- ▶ Control library
- ▶ Sequencing reagents (enough for the required number of cycles)
- ▶ Single-Read or Paired-End Cluster Generation Kit
- ▶ GAII\_qPCR\_v#.xml

### Procedure

- 1 Prepare serial dilutions of the template hyb from 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.



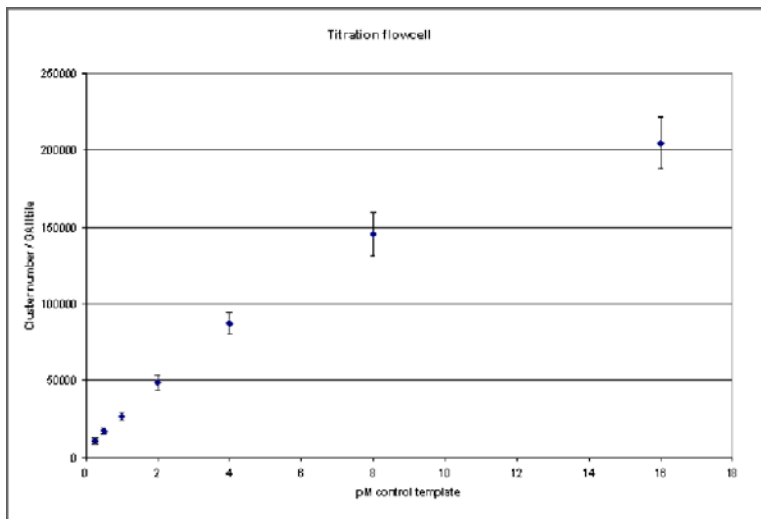
- Perform the sequencing cycles to count the clusters on the titration flowcell. Ensure that there is enough reagent for the run, with a minimum of the following per cycle. These can be left over reagents.

- 1 ml of incorporation mix
- 0.5 ml of cleavage mix
- 2.5 ml of scanning mix

For GAI, use the recipe GAI\_qPCR\_v#.xml. This recipe will scan 9 tiles/columns.

- There is no need to change any configuration file on the instrument. The offsets may not be recalculated and the default\_offsets.txt file used might be the one generated during the previous run analysis. To avoid any confusion, make sure that the run name states the name of the GA (e.g. 080908\_eas139\_0051\_FC30BKFAAX).
- Use the following settings for the Pipeline analysis: USE\_BASES YY. ANALYSIS sequence.
- Plot the cluster numbers displayed in the summary table against the initial concentration of control template.
- 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 16 pM, 8 pM, 4 pM, 2 pM, 1 pM, 0.5 pM, and 0.25 pM and clusters were counted through the Pipeline following five cycles of sequencing.

## Appendix B - Sample Preparation

Independently of DNA quantification, it is important to be able to achieve precise cluster numbers (i.e., a given library clustered at the same concentration should always give the same number of clusters). However, the same library clustered on separate occasions can give different cluster numbers, which can be attributed to the small pipetting volumes and large number of pipetting steps involved in the current cluster protocol. Illumina recommends a modified protocol for clustering to achieve more consistent cluster numbers when the same library will be used on multiple flow cells or lanes.

### Consumables and Equipment

- ▶ 0.1N NaOH
- ▶ Benchtop Microcentrifuge
- ▶ DNA Library
- ▶ Hybridization Buffer
- ▶ Pipettes (P2, P10, P200, P1000, and an 8 channel multichannel dispensing 18  $\mu$ l)



#### NOTE

To minimize errors in preparing 0.1N NaOH fresh each day, prepare it in large batches and aliquot it into 50 ml sealed tubes. These aliquots may be stored up to 6 months at 2° to 8°C and used in the protocol as needed. Once you open an aliquot, use it on the same day that it was opened. Discard any reagent that is left at the end of the day.

- ▶ QIAGEN EB Buffer + 0.1% Tween 20 (QIAGEN EB Tween)  
(e.g., 50 ml QIAGEN EB + 50  $\mu$ l Tween)
- ▶ Vortexer

### Procedure

- 1 Dilute the DNA library templates to 2 nM based on qPCR quantification in QIAGEN EB Tween using a minimum volume of 10  $\mu$ l using a P10 pipette.
- 2 Denature 10  $\mu$ l of 2 nM template DNA by adding 10  $\mu$ l 0.1N NaOH to generate 20  $\mu$ l of a 1 nM denatured template using a P10 pipette to make the dilutions.
- 3 Vortex the template solution.
- 4 Centrifuge the template solution to 280 xg for 1 minute.

- 5 Incubate for 5 minutes at room temperature to denature the template into single strands.
- 6 Add 980  $\mu\text{l}$  of pre-chilled hybridization buffer to the tube containing the denatured template solution, using a P1000 pipette, to generate a 20 pM template hyb solution.
- 7 Vortex the template hyb solution.

**NOTE**

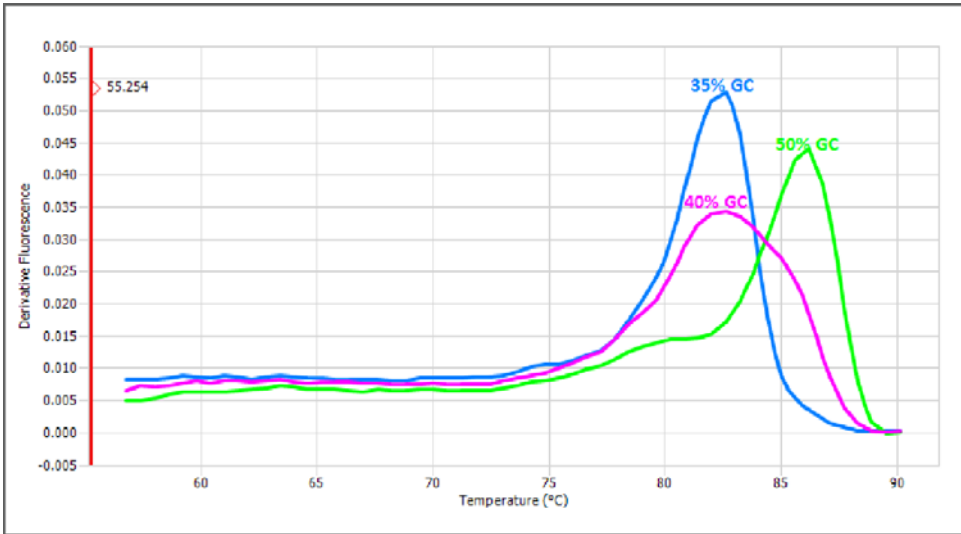
You may aliquot into smaller volumes to avoid multiple freeze/thaws. If so, the volumes should be large (e.g., 250  $\mu\text{l}$ ) to enable accurate pipetting for further dilutions.

- 8 The 20 pM template hyb solution can be diluted using large volume pipetting to the correct concentration for clustering, based on the qPCR control template titration flowcell.
- 9 Store any remaining 20 pM template hyb at  $-15^{\circ}$  to  $-25^{\circ}\text{C}$  for up to three weeks. Store the 20 pM template hyb solution in order to re-use of the same template hyb with the possibility of clustering at a different concentration in the future. When storing the 20 pM template hyb, the cluster process does not have to begin from the 2 nM stock and prevents any further cluster number variability. Since the libraries are quantified by qPCR before clustering it is assumed that a concentration above 20 pM is not required.

# 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	$\mu\text{l/well}$
KAPA SYBR FAST Master Mix Universal (2x)	10
qPCR Primer 1.1 (10 $\mu\text{M}$ )	0.2
qPCR Primer 2.1 (10 $\mu\text{M}$ )	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.



## Technical Assistance

For technical assistance, contact Illumina Customer Support.

**Table 3** Illumina General Contact Information

<b>Illumina Website</b>	<a href="http://www.illumina.com">http://www.illumina.com</a>
<b>Email</b>	<a href="mailto:techsupport@illumina.com">techsupport@illumina.com</a>

**Table 4** Illumina Customer Support Telephone Numbers

<b>Region</b>	<b>Contact Number</b>
North America toll-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 time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

## MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at <http://www.illumina.com/msds>.

## Product Documentation

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to <http://www.illumina.com/support/documentation.ilmn>. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF. To register for an iCom account, please visit <https://icom.illumina.com/Account/Register>.

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