qPCR Quantification Protocol Guide

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Topics

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Introduction

This document describes a qPCR method for quantifying libraries generated using the Illumina® sample preparation protocols. 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. Moreover, qPCR is a very sensitive method of measuring DNA and thus dilute libraries with concentrations below the threshold of detection of conventional spectrophotometric methods are amenable to quantification by qPCR.

Scope

There are several different qPCR instruments available from instrument vendors, each with its own proprietary software for running experiments and analyzing data. It is beyond the scope of this document to describe protocols for all instruments and analysis packages. Instead, this document describes a protocol designed around a Stratagene Mx3000P qPCR machine and Stratagene MxPro software. You will need to adapt this protocol to your specific qPCR platform.

Quantification Workflow

Figure 1 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).

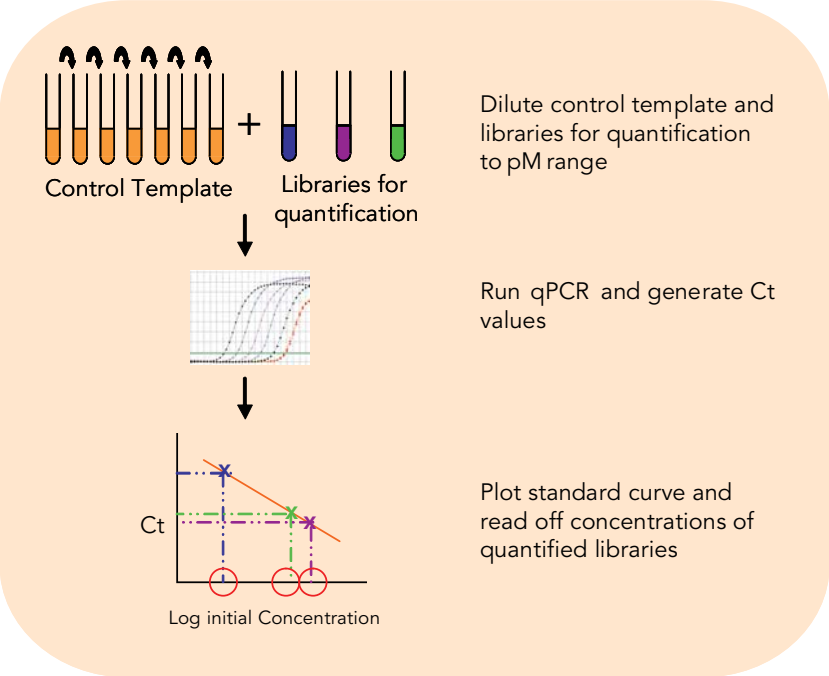

Figure 1 *qPCR Quantification Workflow*

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.

User-Supplied 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
0.1N NaOH	General lab supplier
0.5% sodium hypochlorite (10% bleach)	General lab supplier
0.1% Tween 20	General lab supplier
96-well plates	Stratagene, part # 410088
Control template (10 nM)	General lab supplier
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
Optical strip caps	Stratagene, part # 401425
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' CAAGCAGAAGACGGCATACGA 3' HPLC purified	General lab supplier
One or more of the following kits in order to correspond to the number of libraries to be quantified: **a.** Single-Read Cluster Generation Kit (1 flow cell) **b.** Single-Read Cluster Generation Kit (10 flow cells) **c.** Paired-End Cluster Generation Kit (1 flow cell) **d.** Paired-End Cluster Generation Kit (5 flow cells)	 **a.** Illumina, part # GD-1003-4001 **b.** Illumina, part # GD-1003-4010 **c.** Illumina, part # PE-2003-4001 **d.** 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
Genome Analyzer	Illumina, part # SY-301-1301
qPCR machine	Stratagene Mx3000P
Vortexer	General lab supplier

Select Control Template

Before starting qPCR, select the control template against which the libraries for quantification can be measured.

In principle, any library prepared for sequencing on the Illumina platform can be used as a control for qPCR and you may wish to tailor a control template to suit your specific needs. The control template should be as similar as possible in terms of template size, GC content and library type (e.g., genomic, CHIP-seq, etc.) to the libraries for quantification. It is also important to note that the control template needs to be available in sufficient quantity, as specified in this protocol, for it to be used 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 - Determine Cluster Numbers for Control Library* and *Appendix B - Sample Preparation for Cluster Generation*).

The GC content of a given library is not always known and this poses 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 - Determine Relative GC Content of Library*). 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.

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

Consumables

- qPCR control template (10 nM)

Store the qPCR 10 nM library template in small aliquots to avoid multiple freeze and thaw cycles.

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

Procedure

1. Add 198 µl of the 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 the 0.1% Tween 20 to 100 µl of the diluted template to make a titration curve of six 2x serial dilutions. This will give 7 control template dilutions in the range of 100–1.6 pM.

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

4. Vortex the dilution to thoroughly mix the samples.
5. Repeat steps 1 through 4 to produce 3 independent serial dilutions of the control template.

Control dilutions are diluted a further 10X into the final SYBR mix, so the final concentration in the qPCR is 10–0.16 pM.

Dilute Libraries

The libraries for quantification need to be diluted to the same range as the control template for qPCR.

Consumables

- Libraries for quantification diluted to approximately 10 nM in QIAGEN EB Buffer

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.

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

Procedure

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

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. The method here makes enough master mix to fill a 96-well plate, but it is possible to make less master mix if you do not wish to use all 96 wells.

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:

Consumable	µl/well	µl/plate (master mix for 110)
KAPA SYBR FAST Master Mix Universal (2x)	10	1100
qPCR Primer 1.1 (10 µM)	0.2	22
qPCR Primer 2.1 (10 µM)	0.2	22
Water	7.6	836
	18	1980

2. Mix gently but thoroughly.
3. Place the reaction mix on ice and protect it from light until use.

Aliquot to 96-Well Plate

The next step in the process is to aliquot the control template dilutions, unknown library dilutions, and master mix. It is important during this step to pipette as accurately as possible, because small variations in volumes will greatly affect the qPCR results.

Consumables

- Control template dilutions (see *Dilute qPCR Control Template*)
- Libraries for quantification dilutions (see *Dilute Libraries*)
- Reaction Mix (see *Prepare Reaction Mix*)
- 96-well plates
- Optical strip caps

Procedure

1. Add 2 µl of the control template dilutions, the unknown library dilutions, or water to each well in a 96-well plate. Take care to pipette accurately into the wells as small variations will affect the assay. For example, the 96-well plate can be filled as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control 100pM	Control 100pM	Control 100pM	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17
B	Control 50pM	Control 50pM	Control 50pM	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
C	Control 25pM	Control 25pM	Control 25pM	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
D	Control 12.5pM	Control 12.5pM	Control 12.5pM	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
E	Control 6.25pM	Control 6.25pM	Control 6.25pM	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
F	Control 3.13pM	Control 3.13pM	Control 3.13pM	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
G	Control 1.56pM	Control 1.56pM	Control 1.56pM	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
H	NTC	NTC	NTC	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24

Figure 2 Well Plate Format

Wells in columns 1–3 contain the control template dilutions and the no template control (NTC) in triplicate. Wells in columns 4–12 contain the sample dilutions in triplicate.

2. Dispense 18 µl of the master mix into each well of the 96-well plates using a multichannel pipette. Take care to pipette accurately into the wells as variations in volume will affect the assay. Change tips for each new column.
3. Place the optical strip lids on the wells, taking care to avoid cross contamination and to avoid smudging the surface of the lids.
4. Centrifuge the 96-well plate to 280 xg for 1 minute.

Quantify by qPCR

Quantify the libraries by qPCR.

Procedure

1. Place the 96-well plate in the qPCR machine in the correct orientation and clean the optical lids with lens tissue to remove any dust before closing the qPCR machine 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

The final step in the qPCR procedure is to analyze 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).

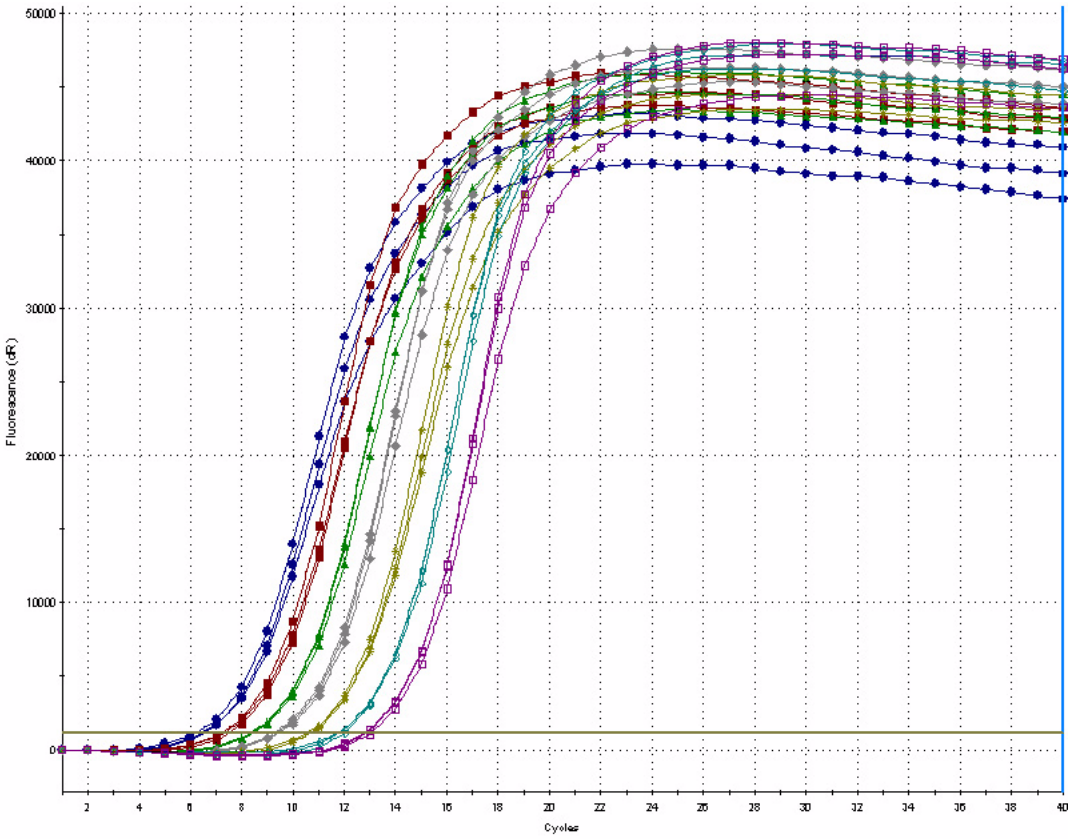

Figure 3 Example of Control Template Amplification

3. Generate a standard curve from the control template by plotting the Ct values against the log initial concentration (Figure 4).

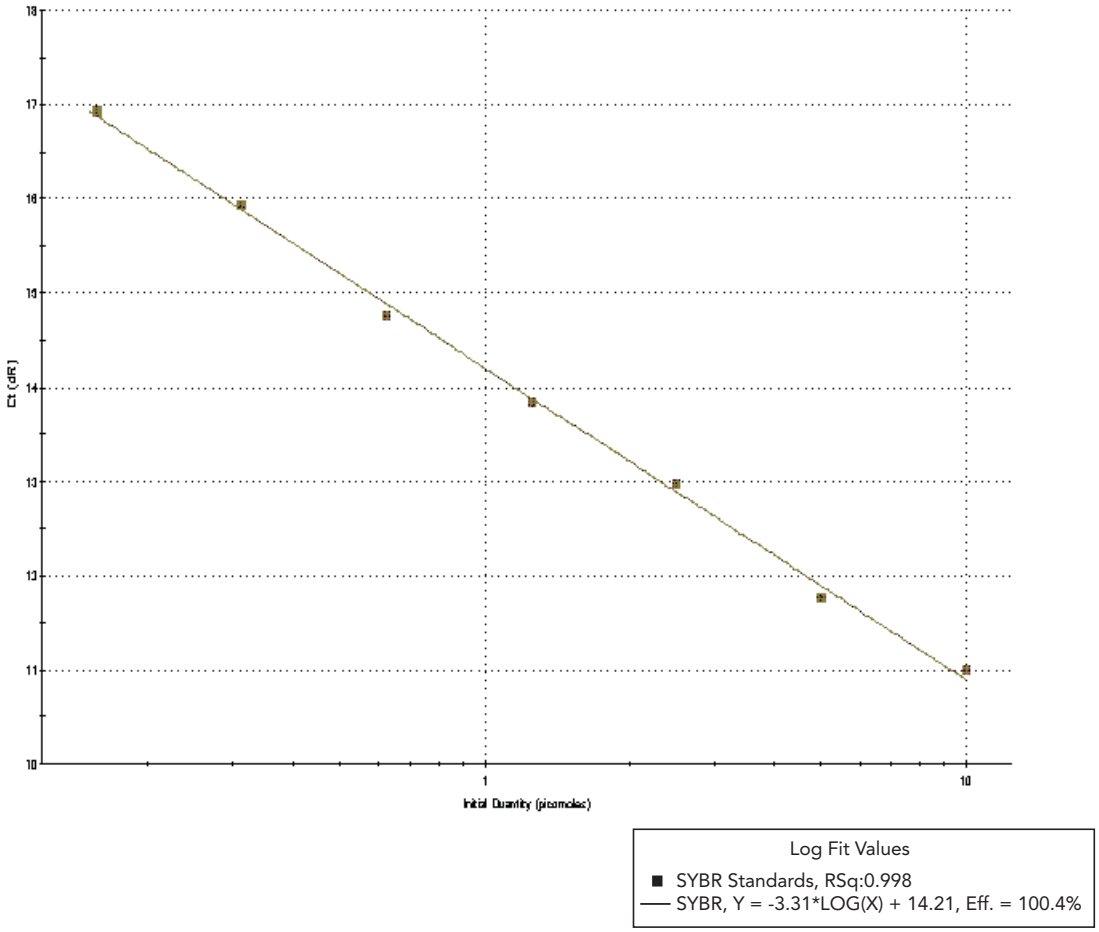

Figure 4 Example of Standard Curve

4. Ensure that the efficiency of amplification of the control template is 90–110% (a slope of -3.58 to -3.10) and that the R2 >0.9. If not, reassess the datapoints you are using 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 for Cluster Generation* on page 17.

Appendix A - Determine Cluster Numbers for Control Library

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.

It is necessary to perform multiple cycles of sequencing to achieve an accurate cluster count, since 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 in Figure 5.

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.

2. 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 GAII, use the recipe GAII_qPCR_v#.xml. This recipe will scan 9 tiles/columns.
3. 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_FC30BKFAAXX).

4. Use the following settings for the Pipeline analysis:
   USE_BASES YY. ANALYSIS sequence.
5. Plot the cluster numbers displayed in the summary table against the initial concentration of control template.
6. Calculate the pM concentration required for the desired number of clusters using the equation of the line.

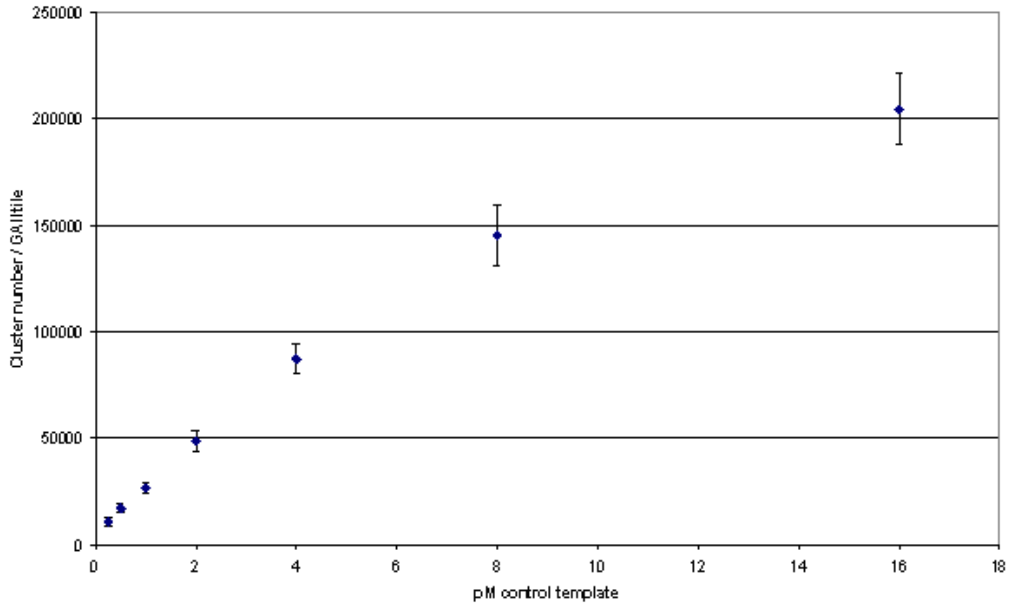

Figure 5 Example of Library Titration

In Figure 5, 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 for Cluster Generation

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, it is often observed that the same library clustered on separate occasions can give different cluster numbers and this 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

- DNA Library
- QIAGEN EB Buffer + 0.1% Tween 20 (QIAGEN EB Tween)
  (e.g., 50 ml QIAGEN EB + 50 µl Tween)
- 0.1N NaOH
- Hybridization Buffer
- Vortexer
- Benchtop Microcentrifuge
- Pipettes
  (P2, P10, P200, P1000, and an 8 channel multichannel dispensing 18 µl)

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.

Procedure

1. Dilute the DNA library templates to 2 nM based on qPCR quantification in QIAGEN EB Tween using a minimum volume of 10 µl using a P10 pipette.
2. Denature 10 µl of 2 nM template DNA by adding 10 µl 0.1N NaOH to generate 20 µl 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 µl of pre-chilled hybridization buffer using a P1000 pipette to the tube containing the denatured template solution to generate a 20 pM template hyb solution.

7. Vortex the template hyb.

You may aliquot into smaller volumes to avoid multiple freeze/thaws. If so, the volumes should be large (e.g., 250 µl) to enable accurate pipetting for further dilutions.

The 20 pM template hyb solution can be diluted using large volume pipetting to the correct concentration for clustering, as judged from the qPCR control template titration flowcell. Any remaining 20 pM template hyb can be stored at -15° to -25°C for up to 3 weeks.

Storage of the 20 pM template hyb solution enables you to re-use the same template hyb with the possibility of clustering at a different concentration at a later time. By storing the 20 pM template hyb you do not have to begin the cluster process again from the 2 nM stock, thus preventing any further cluster number variability. Since the libraries are quantified by qPCR before clustering it is assumed that a concentration above 20 pM would not be required.

Appendix C - Determine Relative GC Content of Library

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 then 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).

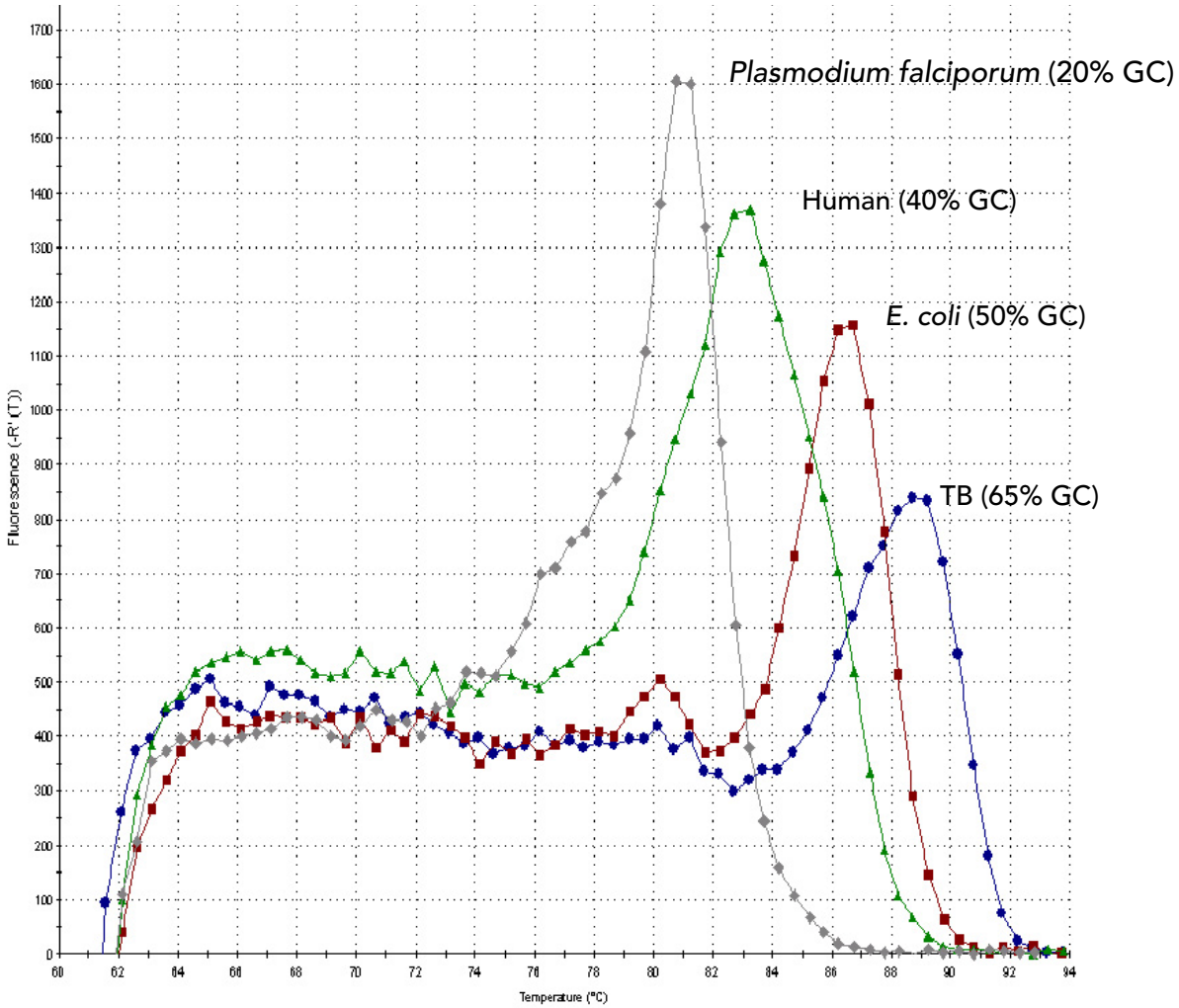

Figure 6 *Example of Dissociation Curves Generated for 300 bp Illumina Libraries*

Consumables and Equipment

- Illumina libraries of known GC content (~10 nM)
- Illumina libraries of unknown GC content (~10 nM)
- KAPA SYBR FAST Master Mix Universal 2x
- qPCR machine
- 96-well plates
- Optical strip caps
- Benchtop centrifuge with swing out rotor

Procedure

1. Prepare the following reaction mix for each library (including controls) in a 96-well plate/reaction

Consumable	µl/reaction
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
	20

2. Put the optical strip lids on the wells and briefly spin the 96-well plate down in a bench top centrifuge to 280 xg for 1 minute.
3. Place the 96-well plate in the qPCR machine in the correct orientation and clean the optical lids with lens tissue to remove any dust before closing the qPCR machine 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.

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