invitrogen

Invitrogen™ Collibri™ Library Quantification Kit

USER GUIDE

for qPCR-based quantification of libraries prepared for Illumina™ NGS platforms

Catalog Numbers A38524100, A38524500

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Revision A.0

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Revision history: MAN0017585

Revision	Date	Description
A.0	17 May 2018	New user guide.

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1. Product information

Product description

Invitrogen™ Collibri™ Library Quantification Kits (Cat. Nos. A38524100, A38524500) contain reagents that are optimized for qPCR-based quantification of next-generation sequencing (NGS) libraries that are prepared for Illumina™ platforms.

Collibri™ Quantification Master Mix contains all the reagents that are required to amplify from Illumina™ adapter sequences: DNA polymerase, reaction buffer, dNTPs, ROX passive reference dye, and primers that target the Illumina™ adapter sequences—only the template needs to be added. Together with six pre-diluted DNA standards (a 10-fold dilution series of a 387-bp template) and Collibri™ Library Dilution Buffer, the kit is ideally suited for accurate quantification of NGS libraries between 150–1300 bp in length.

Collibri™ Library Quantification Kit is compatible with any qPCR instrument, regardless of its ROX requirement due to the universal concentration of ROX passive reference dye in the Master Mix.

The Master Mix contains the Platinum™ II *Taq* DNA Polymerase, which is an engineered *Taq* DNA polymerase complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures. Polymerase activity is restored after the initial denaturation step at 95°C, providing an automatic "hot start". This offers increased sensitivity, specificity, and yield, while allowing reaction assembly at room temperature. Like standard *Taq* DNA polymerase, Platinum™ II *Taq* DNA Polymerase has both 5' to 3' polymerase and 5' to 3' exonuclease activities, but lacks the 3' to 5' exonuclease activity.

Colored components of the kit provide visual control of the proper workflow progress – when the blue Collibri™ Quantification Master Mix is mixed with the yellow Collibri™ DNA Standards and the yellow Collibri™ Library Dilution Buffer, the reaction mix becomes green. This allows the tracking of pipetting steps during reaction setup (see Figure 1).

Colors of the kit components have no influence on product performance and library quantification results.

Figure 1 When the blue Collibri™ Quantification Master Mix is mixed with the yellow Collibri™ DNA Standards and the yellow Collibri™ Library Dilution Buffer, the reaction mix becomes green, which allows visual tracking of the pipetting steps during reaction setup.

Kit contents and storage

Sufficient amount of reagents are supplied in the Collibri™ Library Quantification Kit for the qPCR-based quantification of 100 (Cat. No. A38524100) or 500 (Cat. No. A38524500) reactions.

Component	Amount		Storage[1]
	100 reactions (Cat. No. A38524100)	500 reactions (Cat. No. A38524500)	
Collibri™ Quantification Master Mix	1.6 mL	8 mL	–25°C to –15°C Protect from light
Collibri™ Library Dilution Buffer	20 mL	5 × 20 mL	–25°C to –15°C
Collibri™ DNA Standard 1 (20 pM)	100 µL	100 µL	
Collibri™ DNA Standard 2 (2 pM)	100 µL	100 µL	
Collibri™ DNA Standard 3 (0.2 pM)	100 µL	100 µL	
Collibri™ DNA Standard 4 (0.02 pM)	100 µL	100 µL	
Collibri™ DNA Standard 5 (0.002 pM)	100 µL	100 µL	
Collibri™ DNA Standard 6 (0.0002 pM)	100 µL	100 µL	

[1] All components of the kit can be stored at 2–8°C for up to 1 month. Protect the Collibri™ Quantification Master Mix from light. All components of the kit are stable for 20 freeze/thaws cycles.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Real-time PCR instrument (see "Instrument compatibility" on page 4 for a list of Applied Biosystems™ real-time PCR instruments)[1]	Various
PCR plates appropriate for your instrument, for example: MicroAmp™ Fast Optical 96-well reaction plate with barcode, 0.1-mL	4346906
Optical sealer for the PCR plate, for example: MicroAmp™ Optical Adhesive Film	4360954
0.5-mL non-stick microfuge tubes	AM12350
1.5-mL non-stick microfuge tubes	AM12450
Microcentrifuge	MLS
Vortex mixer	MLS
Pipettors: 10-µL, 20-µL, 200-µL, 1000-µL	MLS
Barrier (filter) pipettor tips	MLS

[1] Also compatible with instruments from other suppliers.

Instrument compatibility

The Collibri™ Library Quantification Kit can be used with any real-time qPCR instrument regardless of its ROX passive reference dye requirement, including the following Applied Biosystems™ instruments:

- ViiA™ 7
- QuantStudio™ 3
- QuantStudio™ 5
- QuantStudio™ 6
- QuantStudio™ 7
- QuantStudio™ 12 K
- StepOne™
- StepOnePlus™
- 7500 Fast
- 7900 HT
- 7900 HT Fast

Workflow

The workflow is illustrated in Figure 2. The sample library is diluted in Collibri™ Library Dilution Buffer to a concentration that falls within the range of the Collibri™ DNA Standards. Following qPCR of the DNA standards and the diluted sample library, a standard curve is generated, from which the concentration of the sample library is calculated.

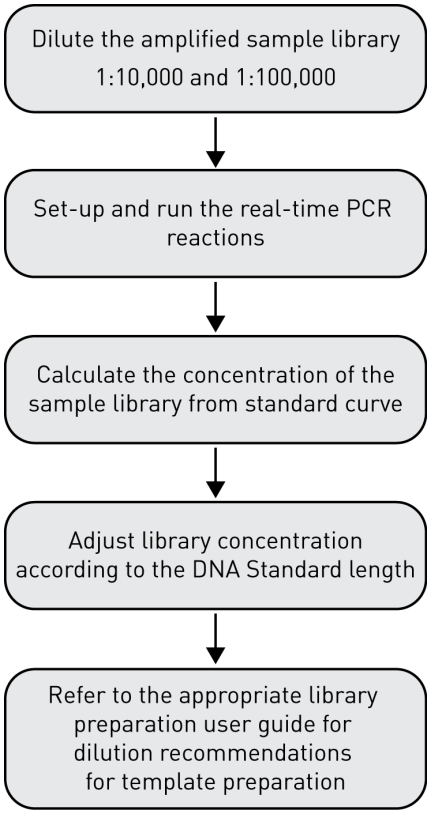

Figure 2 Collibri™ Library Quantification workflow

Note: Colored components of the kit provide visual control of the proper workflow progress – reaction mix changes color as the correct reaction component is added.

2. Methods

Important procedural guidelines

Because qPCR is a very sensitive technique and the dynamic range of the Collibri™ Library Quantification assay extends to very low template copy numbers, the reliability of results is highly dependent on accurate liquid handling. Therefore, it is essential that you perform the library dilutions and dispense the samples with special care. To achieve the highest accuracy, we recommend the following:

- Ensure that all kit components are thawed on ice and mixed well before use.
- Equilibrate the Collibri™ Library Dilution Buffer to room temperature before use.
- Maintain a sterile environment when handling sample libraries and the Collibri™ DNA Standards to avoid contamination between each other and from DNases.
- Take precautions to avoid cross-contamination by using aerosol-resistant barrier tips.
- Always use a new pipette tip to avoid cross-contamination. Improper handling of samples and reagent affects the accuracy of quantification.
- Do not use multi-channel pipettes for this assay. Even small differences in volume can result in data variation and misinterpretation of the results.
- Always dispense the DNA Standards from the lowest to the highest concentration (i.e., from DNA Standard 6 to DNA Standard 1) and use a fresh pipette tip for each DNA Standard.
- We strongly recommend that you include a no-template control (NTC) in each assay to detect any contamination that is introduced during reaction setup.
- Ideally, NTC samples should show no amplification. However, due to contamination of amplicons, libraries, or standards during setup, NTC samples can show amplification. As long as the Cq value from the NTC sample is 3 cycles from the average Cq value for DNA Standard 6, NTC will have no effect on library quantity estimation.
- Set up all samples, including no-template controls (NTC), in triplicate to increase accuracy.

Dilute the libraries

Guidelines for library dilution

- Equilibrate the Collibri™ Library Dilution Buffer to room temperature before use.
- Mix Collibri™ Library Dilution Buffer gently. Extensive shaking causes foaming.
- Prepare fresh library dilutions for each qPCR-based quantification assay.
- To reduce DNA adsorption to plastic tubes, use low binding microfuge tubes for library dilution (such as the 1.5-mL Non-Stick RNase-Free Microfuge Tubes, Cat. No. AM12450).
- Keep diluted libraries on ice to prevent degradation.
- Dilute the libraries 1:10,000 and 1:100,000. You can use other dilutions as well, but at least one dilution point has to fall within the range of the Collibri™ DNA Standards (20–0.0002 pM) to interpret the data.
- For accurate quantification, run two library dilution points (1:10,000 and 1:100,000).
- Check the Bioanalyzer™ data for the presence of primer or adapter dimers. They will be amplified during the quantification reaction and skew the results.

Dilute the libraries

1. Prepare an initial 1:100 dilution of each library sample:
   a. Add 2 µL of library to 198 µL of Collibri™ Dilution Buffer.
   b. Vortex the dilution to thoroughly mix the samples, then briefly centrifuge to collect all the droplets at the bottom of the tube.
2. Prepare the 1:10,000 dilution:
   a. Add 2 µL of the 1:100 dilution (from step 1) to 198 µL of Collibri™ Dilution Buffer.
   b. Vortex the dilution to thoroughly mix the samples, then briefly centrifuge to collect all the droplets at the bottom of the tube.
3. Prepare the 1:100,000 dilution:
   a. Add 10 µL of the 1:10,000 dilution (from step 2) to 90 µL of Collibri™ Dilution Buffer.
   b. Vortex the dilution to thoroughly mix the samples, then briefly centrifuge to collect all the droplets at the bottom of the tube.
4. Use 1:10,000 and 1:100,000 dilutions (from steps 2 and 3) for qPCR setup (page 7).

Set up and run qPCR reactions

Before you begin

- Collibri™ Quantification Master Mix is ready-to-use and does not require any preparation steps. It is compatible with any qPCR instrument, no matter its requirements for ROX passive reference dye.
- Calculate the total number of reactions that include the six DNA standards, two dilutions of each tested library, and no template controls (NTC). We strongly recommend that you run each reaction in triplicate.
- The number of repeats can be reduced to two, but this can also decrease the reliability of the data.
- Figure 3 shows the recommended plate layout. Note that the Collibri™ DNA Standards and NTC require 21 wells of the plate, leaving 75 wells for the libraries. This allows the quantification of 12 libraries with 2 dilutions in triplicate.
- Total qPCR reaction volume in each well of the 96-well plate is 20 µL. Do not decrease the reaction volume, because evaporation and pipetting errors can lead to inaccurate results.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	NTC	Lib 1 1:100,000	Lib 1 1:100,000	Lib 1 1:100,000	Lib 5 1:100,000	Lib 5 1:100,000	Lib 5 1:100,000	Lib 9 1:100,000	Lib 9 1:100,000	Lib 9 1:100,000
B	Std 6 0.0002 pM	Std 6 0.0002 pM	Std 6 0.0002 pM	Lib 1 1:10,000	Lib 1 1:10,000	Lib 1 1:10,000	Lib 5 1:10,000	Lib 5 1:10,000	Lib 5 1:10,000	Lib 9 1:10,000	Lib 9 1:10,000	Lib 9 1:10,000
C	Std 5 0.002 pM	Std 5 0.002 pM	Std 5 0.002 pM	Lib 2 1:100,000	Lib 2 1:100,000	Lib 2 1:100,000	Lib 6 1:100,000	Lib 6 1:100,000	Lib 6 1:100,000	Lib 10 1:100,000	Lib 10 1:100,000	Lib 10 1:100,000
D	Std 4 0.02 pM	Std 4 0.02 pM	Std 4 0.02 pM	Lib 2 1:10,000	Lib 2 1:10,000	Lib 2 1:10,000	Lib 6 1:10,000	Lib 6 1:10,000	Lib 6 1:10,000	Lib 10 1:10,000	Lib 10 1:10,000	Lib 10 1:10,000
E	Std 3 0.2 pM	Std 3 0.2 pM	Std 3 0.2 pM	Lib 3 1:100,000	Lib 3 1:100,000	Lib 3 1:100,000	Lib 7 1:100,000	Lib 7 1:100,000	Lib 7 1:100,000	Lib 11 1:100,000	Lib 11 1:100,000	Lib 11 1:100,000
F	Std 2 2 pM	Std 2 2 pM	Std 2 2 pM	Lib 3 1:10,000	Lib 3 1:10,000	Lib 3 1:10,000	Lib 7 1:10,000	Lib 7 1:10,000	Lib 7 1:10,000	Lib 11 1:10,000	Lib 11 1:10,000	Lib 11 1:10,000
G	Std 1 20 pM	Std 1 20 pM	Std 1 20 pM	Lib 4 1:100,000	Lib 4 1:100,000	Lib 4 1:100,000	Lib 8 1:100,000	Lib 8 1:100,000	Lib 8 1:100,000	Lib 12 1:100,000	Lib 12 1:100,000	Lib 12 1:100,000
H				Lib 4 1:10,000	Lib 4 1:10,000	Lib 4 1:10,000	Lib 8 1:10,000	Lib 8 1:10,000	Lib 8 1:10,000	Lib 12 1:10,000	Lib 12 1:10,000	Lib 12 1:10,000

Figure 3 Recommended plate layout for qPCR reaction

Set up the qPCR reaction

1. For each reaction, pipette 16 µL of Collibri™ Quantification Master Mix (blue) into a well of the 96-well PCR plate.

   IMPORTANT! The Collibri™ Quantification Master Mix contains SYBR Green I dye, which is light sensitive. Prolonged exposure of the master mix to light can cause a decrease in the fluorescent signal and changes in Ct values.

2. Add 4 µL of each DNA standard in triplicates. We recommend that you start from the lowest concentration DNA Standard 6 (0.0002 pM).
3. Add 4 µL of Collibri™ Library Dilution Buffer to wells designated for NTC samples.
4. Add 4 µL of each diluted library in triplicate to the designated wells. Note that reducing the number of replicates increases the risk of obtaining data that is not reliable, which will require you to reassay the libraries.

   Note: When the blue colored Collibri™ Quantification Master Mix is mixed with the yellow colored Collibri™ DNA Standards or libraries diluted in the yellow Collibri™ Library Dilution buffer, the reaction mix becomes green.

5. Seal the plate, then centrifuge it briefly to collect the contents at the bottom of the wells and to eliminate air bubbles.

Run the qPCR reaction

1. Place the plate in the real-time qPCR instrument, then run the assay using the following cycling parameters:

PCR cycles	Step	Temperature	Time
1 cycle	Initial denaturation	95°C	2 minutes
35 cycles	Denature	95°C	30 seconds
	Anneal/Extend	60°C	45 seconds

   Ensure that the data collection is included during the annealing/extension step through the SYBR Green or SYBR/FAM channel.

2. *(Optional)* Include melt curve analysis using default settings.

   Note: Melt curve analysis step is not required in the Collibri™ Library Quantification Kit protocol. The melt curve analysis can be used to identify adapter-dimers that are left after library preparation steps and can give a hint that library concentration may be overestimated. Libraries prepared by different methods show a wide variety of melt curve shapes, but this does not affect library quality parameters such as cluster density and sequencing quality.

Analyze the data

1. Annotate the DNA Standards and their concentrations in the instrument software:

Name	Concentration
DNA Standard 1	20 pM
DNA Standard 2	2 pM
DNA Standard 3	0.2 pM
DNA Standard 4	0.02 pM
DNA Standard 5	0.002 pM
DNA Standard 6	0.0002 pM

2. Use the instrument software to generate the standard curve.
3. Review the data and exclude any obvious outliers from the analysis.

> **Note:** Do not omit more than 1 data point from a single standard curve point and more than 2 data points from the standard curve for the analysis. If the data set contains many outliers, results are unlikely to be reliable.

4. Ensure that the standard curve meets the following criteria:
   a. Average Cq value between the DNA standards is between 3.1–3.6.
   b. Calculated reaction efficiency is between 90–110%.
   c. $R^2 \geq 0.99$

> **Note:** If the standard curve does not meet these criteria, calculated library concentration is not reliable and the experiment needs to be repeated.

5. Exclude library samples that fall outside of the dynamic range of the assay (i.e., that return an average Cq value lower than that of Standard 1 or higher than that of Standard 6).

> **Note:** If both library dilutions amplify before DNA Standard 1, data cannot be interpreted and the experiment needs to be repeated with an appropriate dilution.

6. ΔCq between two library dilution points should be in the range 3.1–3.6. You can exclude one data point from the three repeats, if it falls out of the range.
7. Using the standard curve, convert the average Cq value for each library data point to average concentration in pM.
8. Multiply the library concentration by the dilution factor for each dilution, then average the concentrations from the two dilution points.

> **Note:** Deviation between the two dilutions should not exceed 10%. If the results deviate more than 10%, they should be interpreted with caution.

9. Size adjustment is necessary to determine final library concentration. To calculate the size adjustment factor, divide the length of the DNA standard (387 bp) by the average library fragment length (in bp).
10. To calculate the final library concentration, multiply the calculated library concentration with the size adjustment factor:

$$\frac{\text{DNA Standard length (387 bp)}}{\text{Average library fragment length (bp)}} \times \text{Library conc.} = \text{Final library concentration}$$

11. Continue with library preparation for next-generation sequencing.

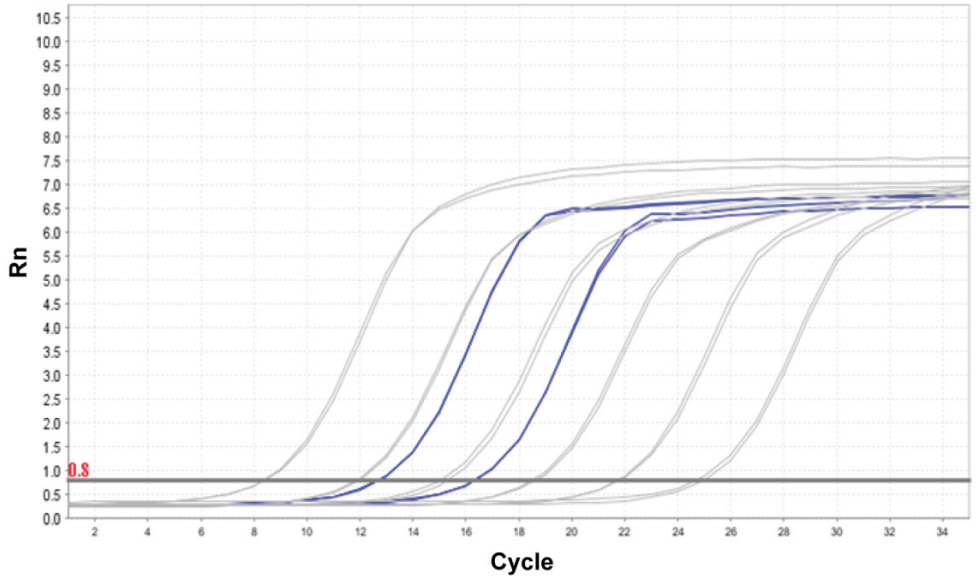

Figure 4 Example amplification plot.

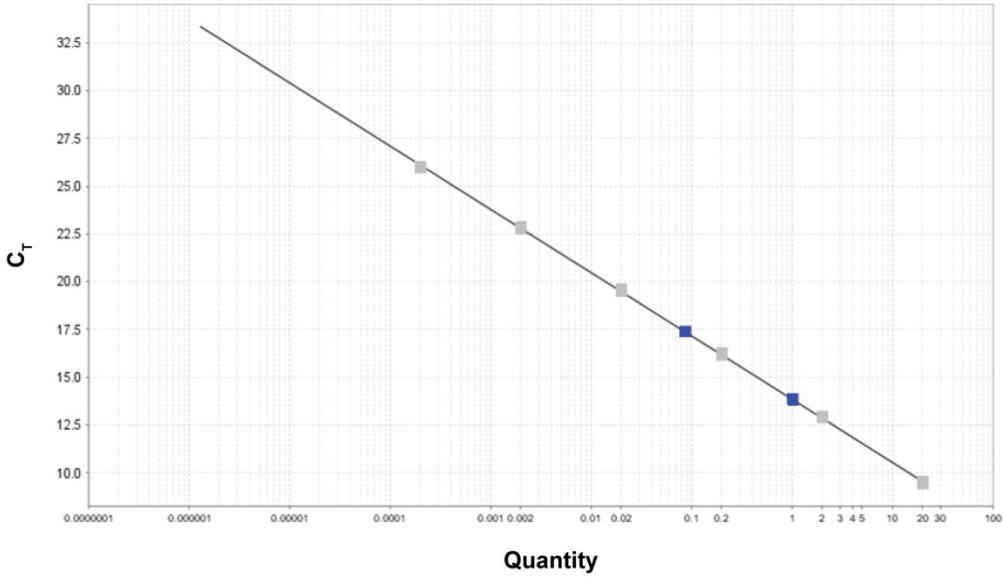

Figure 5 Example of standard curve and NGS library amplification (two dilutions) with the Collibri™ Library Quantification Kit.

Appendix A: Troubleshooting

Observation	Possible cause	Recommended action
DNA Standard curve falls outside the efficiency range of 90–110%	Threshold is set incorrectly.	Adjust threshold manually in the exponential region of amplification curves.
	qPCR conditions are suboptimal.	Verify that all steps have been followed and the correct reagents, dilutions, volumes, and cycling parameters have been used.
	Pipetting errors.	Ensure proper pipetting techniques. Avoid using multichannel pipettors.
	Bubbles in the reaction mix.	Briefly centrifuge the reaction plate before running the reactions in the thermal cycler.
	Collibri™ Quantification Kit was stored incorrectly.	Verify that the kit was stored according to the storage conditions provided in this user guide.
qPCR curves are different from each other	Pipetting errors.	Ensure proper pipetting techniques. Avoid using multichannel pipettors.
	Evaporation of liquids due to improper seal.	Ensure that the reaction plate is sealed properly before running the reactions
	Bubbles in the reaction mix.	Briefly centrifuge the reaction plate before running the reactions in the thermal cycler.
	Incorrect instrument set up for passive reference dye.	Ensure that the ROX passive reference dye is selected.
NTC amplification is within 3 cycles of the Collibri™ DNA Standard 6	Collibri™ Quantification Master Mix or Collibri™ Library Dilution Buffer is contaminated.	Use a new bottle of Collibri™ Library Dilution Buffer (or nuclease-free water) for library dilution. If the NTC remains within 3 cycles from the Collibri™ DNA standard 6, use a new Collibri™ Library Quantification Kit.
Poor reproducibility between sample replicates	Pipetting errors and/or improper liquid handling.	Ensure proper pipetting techniques. Avoid using multichannel pipettors.
	Evaporation of liquids due to improper seal.	Ensure that the reaction plate is sealed properly before running the reactions
Library dilution points fall outside of DNA Standard range	Incorrect library dilution.	Library dilution points that fall outside of DNA Standard range cannot be used for quantity estimation. Adjust library dilution factors according to obtained results. Usually, 1:10,000 and 1:100,000 dilutions fall in the DNA Standard range; however, there can be exceptions.
Concentrations from different library dilutions differ >10%	Improper library dilution.	Repeat library dilution. Do not use multichannel pipettes. Use Collibri™ Library Dilution Buffer instead of water.

Observation	Possible cause	Recommended action
DNA Standards amplify and meet required criteria for analysis, but libraries do not amplify	Libraries do not have correct adapter sequences for amplification primers to anneal.	Verify that the libraries contain Illumina™ adaptors with P5 and P7 sequences.
	Libraries are degraded.	Check the bioanalyzer data to ensure that the library is not degraded.
	Improper library dilution.	Repeat library dilution. Do not use multichannel pipettes. Use Collibri™ Library Dilution Buffer instead of water.
Non-uniform fluorescence intensity	Contamination of the thermal cycler.	Decontaminate the thermal cycler. Maintain a sterile environment and follow proper sterile technique when handling the reagents and equipment.
	Poor calibration of the thermal cycler.	Calibrate the thermal cycler.
	Collibri™ Quantification Master Mix was exposed to light for too long.	Repeat the experiment. If the fluorescence intensity is decreased, use a new Collibri™ Quantification Master Mix.
Excessive foaming in the Collibri™ Library Dilution Buffer	Bottle of Collibri™ Library Dilution Buffer was shaken too strongly.	Collibri™ Library Dilution Buffer contains materials that can foam if shaken vigorously. Mix the buffer gently.

Appendix B: Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organisation (WHO), *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

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  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.