qPCR quantification of Onso™ libraries

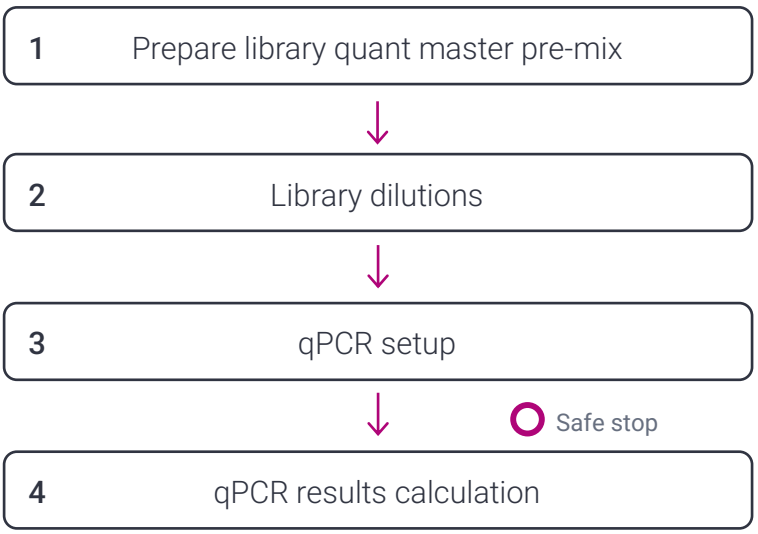
Procedure & checklist

Before you begin

This procedure describes the workflow for the qPCR quantification of Onso libraries constructed for sequencing on PacBio® short-read sequencing systems.

Overview	
Samples per Onso library quant kit	Varies, up to 500 reactions
Workflow time	2 hours

Workflow



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Required materials and equipment

Materials	
Onso™ library quant kit	PacBio® 102-431-800
Library quant primer mix	PacBio® 102-553-100
Library quant standard 1	PacBio® 102-553-300
Library quant standard 2	PacBio® 102-553-400
Library quant standard 3	PacBio® 102-553-500
Library quant standard 4	PacBio® 102-553-600
Library quant standard 5	PacBio® 102-553-700
Library quant standard 6	PacBio® 102-553-800
Library quant master mix	PacBio® 102-647-800
Library dilution buffer	PacBio® 102-647-900
ROX (high)	PacBio® 102-552-900
ROX (low)	PacBio® 102-553-000
qPCR Instrument	Any MLS (major lab supplier)
0.2 mL 8-tube strips and/or 96-well qPCR plate	Any MLS
Nuclease-free, 1.5 mL DNA low-bind tubes	Any MLS
Microcentrifuge	Any MLS
Vortex mixer (or bioshaker)	Any MLS
Single-channel or 8-channel pipettes	Any MLS
Optical seals	Any MLS
Nuclease-free water, molecular biology grade	Any MLS

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General best practices

Library input

Quantification using this procedure is compatible for Onso libraries generated using the following Procedures & checklists:

- "Preparing Onso libraries from genomic DNA for short-read sequencing"
- "Preparing Onso libraries from fragmented DNA for short-read sequencing"
- "Generating hybrid capture libraries for sequencing on the Onso system".

Onso compatible libraries generated using the conversion kit are not compatible with the kit and workflow described here. For those libraries, refer to "Preparing Onso compatible libraries from existing P5/P7 libraries for short-read sequencing", specifically section 3 titled "Library conversion QC and quant guidance".

Reagent and sample handling

qPCR is sensitive to small amounts of DNA contamination. Use proper sterile technique to achieve accurate quantitation results.

It is recommended that users run replicate reactions for each standard and library sample, ideally in triplicate. This increases the accuracy of the quantitation and allows the exclusion of outlier reactions due to instrument, plate, or pipetting errors.

Room temperature is defined as any temperature in the range of **18-23°C** for this protocol.

Thaw reagents at room temperature. Ensure all reagents are completely thawed. If a precipitate is seen, pipette up and down several times to break it up, and quickly vortex to mix.

Quick spin all reagents in a microcentrifuge to collect liquid at the bottom of the tube prior to use.

Keep all temperature-sensitive reagents listed in the table below on wet ice, or cold blocks if wet ice is unavailable.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
Prepare library quant master mix	Light Blue	Library quant primer mix
	Light Green	Library quant master mix
	Amber, Red	ROX (Low)
	Amber, Green	ROX (High)
Library dilutions	n/a	10X dilution buffer
Set up qPCR reactions	Red	Library quant DNA standard 1
	Orange	Library quant DNA standard 2
	Yellow	Library quant DNA standard 3
	Green	Library quant DNA standard 4
	Blue	Library quant DNA standard 5
	Purple	Library quant DNA standard 6

Recommended ROX input for indicated instruments used with Onso library quant kit can be found below:

qPCR Instrument	ROX
Bio-Rad iQ 5, CFX96, CFX384, Opticon **Roche Lightcycler** **Qiagen Rotor-Gene** **Eppendorf Mastercycler** **Cepheid SmartCycler**	Not Recommended
Applied Biosystems 7500, QuantStudio, ViiA7 **Agilent Mx**	Low ROX
Applied Biosystems 700, 7300, 7700, 7900HT, StepOne, StepOnePlus	High ROX

For qPCR instruments not included in the table above, consult your qPCR instrument user manual to determine the appropriate ROX solution to be used.

qPCR Program

Program thermocycler(s) prior to beginning the protocol for the first time.

Step	Time	Temperature	Cycles
1	1 min	95°C	1
2	15 sec 45 sec	95°C 63°C	35

Set the plate read to occur at the end of the 63°C extension in step 2.

If ROX is included in the master mix, verify that ROX is selected in the appropriate normalization settings.

Use only the “SYBR Green” or “SYBR/FAM” channel of the qPCR instrument. For qPCR instruments capable of reading at multiple wavelengths, taking additional readings at other wavelengths unnecessarily increases the instrument run time.

Workflow steps

1. Prepare library quant master pre-mix

Note that 8.0 mL of Library quant master mix working solution prepared here, without ROX, supports exactly 500 reactions (each requiring 16 µL of master mix solution).

✔	Step	Instructions
	1.1	Add **500 µL** of **library quant primer mix** to **7.5 mL** of new **library quant master mix.**
	1.2	If necessary for the qPCR instrument, add either **100 µL** of **ROX (Low)** or **ROX (High)**.
	1.3	Vortex well and spin down.
	1.4	Aliquot, if desired. Mark bottle or aliquoted tubes of master mix with the date of first use, and record that the primer mix was added for future reference. Store at **-20°C** (stable for at least 30 freeze/thaw cycles).

2. Library dilutions

Dilute each sample and positive control to a concentration within the range of the standards.

It is recommended that users run replicate reactions for each standard and library sample, ideally in triplicate. This increases the accuracy of the quantitation and allows the exclusion of outlier reactions due to instrument, plate, or pipetting errors.

✔	Step	Instructions
	2.1	Identify the quantity of 1X library dilution buffer required to prepare all library dilutions, positive control(s), and negative control according to the table below. Adjust the final volume of library dilution buffer required to include a 10% overage. ⚠ Library quant DNA standards do not require dilution prior to use.

Library Prep Input Amount	Dilution Factor	Volume of 1x Library Dilution Buffer Required (µL)	
		Samples & Positive Controls	Negative Controls
≤25 ng	1:100	198	12
25 - 200 ng	1:2,000	236	12
200 - 1000 ng	1:10,000	396	12

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	2.2	Prepare an appropriate volume (according to step 2.1) of **1X dilution buffer** by diluting **10X dilution buffer** with nuclease-free water.
	2.3	Dilute DNA libraries and positive control(s) by the appropriate dilution factor by following **one** of steps 2.4-2.6
	2.4	For libraries were prepared starting with **low input amounts (≤ 25 ng):** • Add 2 µl library sample to 198 µl of 1X dilution buffer to create a 1:100 final dilution. • Mix for 2 minutes at 2200 rpm. • Quick spin using a microcentrifuge.

	2.5	For libraries were prepared starting with **intermediate input amounts (25-200 ng)** or moderate PCR amplification: • Add 2 µl library sample to 38 µl of 1X dilution buffer to create a 1:20 dilution. • Mix for 2 minutes at 2000 rpm. • Quick spin using a microcentrifuge. • Add 2 µl 1:20 dilution to 198 µl of 1X dilution buffer to create a 1:2000 final dilution. • Mix for 2 minutes at 2200 rpm. • Quick spin using a microcentrifuge.
	2.6	For libraries that were prepared starting with **high input amounts (200-1000 ng)** or preparatory PCR amplification: • Add 2 µl library sample to 198 µl of 1X dilution buffer to create a 1:100 dilution. • Mix for 2 minutes at 2200 rpm. • Quick spin using a microcentrifuge. • Add 2 µl of 1:100 dilution to 198 µl of 1X dilution buffer to create a 1:10,000 final dilution. • Mix for 2 minutes at 2200 rpm. • Quick spin using a microcentrifuge.
	2.7	Use remaining 1X dilution buffer as a negative (no template) control for the qPCR reaction.

3. qPCR setup

✔	Step	Instructions
	3.1	Add **16 µl** of **Library quant master mix** to each plate well.
	3.2	Add **4 uL** of diluted library, positive control, negative control, or **library quant DNA standards 1-6** to the user designated plate wells. Note well locations and contents for future use.
	3.3	Securely seal qPCR plate with optical film. If hand sealing the reaction plate, avoid wells along the edges of the plate, if possible, as these wells are more prone to seal failures during qPCR runs, leading to evaporation of the reaction and outlier traces.
	3.4	Mix at **2200 rpm** for **1 minute**.
	3.5	Spin down in plate centrifuge. Check to ensure there are no bubbles.
	3.6	Load the plate and run the qPCR program.

4. qPCR results calculation

✔	Step	Instructions
		Generation of Standard Curve
	4.1	Use the qPCR instrument to label the standard concentrations as follows:

Sample	Concentration (pM)
Library quant DNA standard 1	100
Library quant DNA standard 2	10
Library quant DNA standard 3	1
Library quant DNA standard 4	0.1
Library quant DNA standard 5	0.01
Library quant DNA standard 6	0.001

	4.2	Once the appropriate wells are set as "Standard", confirm that the efficiency for the library quant DNA standards (derived from the slope of the linear fit of the DNA standard curve plotted on a log-scale x-axis) is 90–110%. The coefficient of determination (R^2) for the linear fit of the standards should be ≥ 0.99. qPCR efficiency and R^2 can be calculated with the assistance of the NEBioCalculator here.
		Calculation of Sample Concentrations
	4.3	Obtain the concentration (pM) of each diluted library sample from the qPCR thermal cycler using the standard curve generated by DNA standards 1–6. Note: If the Cq value of the samples fall outside of the range of the standard curve (i.e lower than DNA standard 1 or greater than DNA standard 6), qPCR must be re-attempted with a different dilution factor.
	4.4	Calculate the average concentration of the triplicate library dilutions and multiply by the dilution factor to determine the concentration of the undiluted library stock. Note: If one of the replicates of a diluted library sample is an outlier (> 0.5 Cq from the other replicates), then the data from this outlier may be excluded from the data analysis.
	4.5	Calculate the size adjusted concentration of the library using the size of the standard fragment (304 bp) and the average size of the amplified library: $Size\ adjusted\ concentration\ (pM) = undiluted\ library\ stock\ concentration\ (pM) \times \left(\frac{304\ bp}{average\ library\ size\ (bp)}\right)$

The NEBioCalculator (found here) is recommended for any additional calculations and analysis of qPCR data.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release	01	AUG2023