INPUT DNA QUANTIFICATION ASSAY

For use with:

- xGen™ Input DNA Quantification Primers
- xGen DNA Library Preparation Kits
- xGen Amplicon Panels

Introduction

Next generation sequencing (NGS) library preparation require DNA quantification to be accurate. The methods commonly used for DNA quantification are spectrophotometry or fluorometry. However, these methods may inaccurately determine DNA concentration due to their requirements for samples to have a high DNA input and a high DNA quality. Spectrophotometric-based methods require a high DNA input, which can overestimate DNA concentration. In contrast, fluorometric-based methods can provide accurate concentrations for samples with high-quality DNA derived from whole blood, fresh frozen samples, and cultured cells while inaccurately determining concentrations for formalin-fixed, paraffin-embedded (FFPE) samples or cell-free DNA (cfDNA) due to low-quality and low-input [1]. Additionally, fluorometric-based methods cannot distinguish between cfDNA and high molecular weight cellular genomic DNA (gDNA). To overcome these limitations, the xGen Input DNA Quantification Primers are designed to provide an accurate DNA quantification for all DNA samples, including those with low quality and low input.

Since Alu sequences are highly abundant in the human genome, these sequences have become useful in quantifying gDNA. By using the xGen Input DNA Quantification Primers, two differently sized amplicons, short and long versions derived from genomic Alu repeats, are used for performing DNA amplification. The short amplicon (Alu115) has a sequence length of 115 bp and the long amplicon (Alu247) length is 247. After successfully amplifying the DNA, quantify the DNA before using DNA as input for NGS library preparation. Use xGen DNA Library Preparation Kits and xGen Amplicon Panels for constructing NGS libraries.

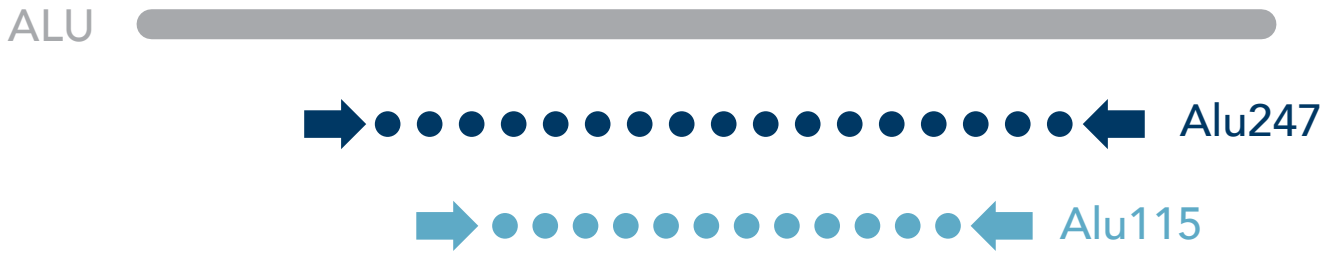

Figure 1. Overview of xGen Input DNA Quant Primers.

Consumables—IDT

Item	Catalogue #
xGen Input DNA Quantification Primers	10009856

Consumables—other suppliers

Item	Supplier	Catalogue #
QIAamp® DNA FFPE Tissue Kit	Qiagen	56404
Cell-Free DNA BCT® tubes	Streck	218961
QIAamp Circulating Nucleic Acid Kit	Qiagen	55114
iTaq™ Universal SYBR® Green Supermix	Bio-Rad	1725120
Standard human genomic DNA	Promega	G3041
Microcentrifuge	Varies	Varies
Programmable thermal cycler	Bio-Rad	CFX96
0.2 mL PCR tubes or 96-well plate	Varies	Varies
Aerosol-resistant tips and pipette ranges from 1–1000 µl	Varies	Varies
Nuclease-free water (molecular biology-grade)	Varies	Varies

Table 1. Comparison of the quantification methods using NanoDrop® (Thermo Fisher Scientific), Qubit™ (Thermo Fisher Scientific), and Alu115-qPCR.

Sample	Nanodrop (ng/µL)	Qubit (ng/µL)	Alu115-qPCR (ng/µL)
FFPE 1	7.1	2.3	1.5
FFPE 2	26.2	11.4	7.3
FFPE 3	25.2	11.5	10.2
FFPE 4	35.4	15.0	14.7
FFPE 5	59.9	32.4	20.7
FFPE 6	43.0	23.0	16.8
FFPE 7	67.6	35.6	27.4
FFPE 8	76.6	42.2	17.5
FFPE 9	14.1	5.9	3.4
FFPE 10	246.0	84.0	5.8

Table 1. Ten (*N*=10) FFPE samples were measured using a spectrometric-based method (NanoDrop), a fluorometric-based method (Qubit), and xGen DNA Input Quantification Primers (Alu115) coupled with qPCR. NanoDrop nonspecifically measures all double-stranded DNA (dsDNA), single-stranded DNA, and contaminants within the sample. Qubit quantifies dsDNA and does not assess DNA damage. The xGen DNA Input Quantification Primers with qPCR assay specifically measures DNA quantity and provides a metric for DNA damage.

FFPE SAMPLES

To perform a DNA extraction for FFPE samples, we recommend using the QIAamp® DNA FFPE Tissue Kit. However, be sure to elute the sample in water before extraction.

Note: DNA extraction from FFPE samples can exhibit a varying degree of DNA damage, and the adverse consequences of DNA damage can affect the amplification of the longer (Alu247) amplicon.

Note: For FFPE samples, the Alu115-qPCR results accurately identify the total quantity of usable DNA, and the Alu247/Alu115 ratio illustrates the DNA Integrity Score of the sample. High-quality DNA is expected to have a DNA Integrity Score of 1.0, while lower quality DNA will have a score between 1.0 and 0.

Table 2. High-quality and degraded samples scores using xGen primers.

Sample	Alu115-qPCR (ng/µL)	DNA Integrity Score (Alu247/Alu115)
HD701	14.4	0.9
HD-C751	1.2	<0.1
Fresh frozen kidney	8.6	0.8
Kidney + 6 hr fixation	3.6	0.5
Kidney + 24 hr fixation	2.4	0.6
Kidney + 24 hr fixation	1.0	0.4

Table 2. Alu115-qPCR concentration values and Alu247/Alu115 DNA Integrity Scores are shown here for two Horizon Discovery standards. HD701 is not a formalin-compromised sample; HD-C751 is a formalin compromised version of the same DNA present in HD701. Concentrations and scores are also shown for DNA extracted from the same normal kidney sample, which has been either fresh-frozen or fixed for 6, 24, or 48 hours before being paraffin-embedded.

Circulating cfDNA Samples

For sample collection of circulating cfDNA, the Cell-Free DNA BCT® tubes and the QIAamp Circulating Nucleic Acid Kit are recommended. Note that the carrier RNA found in this kit (and other extraction kits) will be identified by NanoDrop or Qubit, which can result in inaccurate quantification. To better determine the concentration and integrity index of the input cfDNA, we recommend using qPCR [2].

cfDNA fragments range from 120 to 222 base pairs, peaking around 167 bp, so the results generated using Alu115-qPCR accurately identifies the total quantity of cfDNA and high molecular weight cellular gDNA. The results generated using Alu247-qPCR indicate only the presence of high molecular weight cellular gDNA contamination. Therefore, the Alu247/Alu115 ratio illustrates the DNA Integrity Score of the sample.

Before You Start

xGen Input DNA Quantification Primers, Contents

The volumes listed in the reaction tables in this section contain enough reagents for the preparation of 48 reactions, in duplicate.

Alu Assay

1. Prepare a standard curve of serial standard dilutions using gDNA ranging from 0.0022–2.2 ng/µL, then combine 5 µl of each standard with the Alu primer pair, in duplicate.
2. Prepare to run each sample and a no template control, in duplicate, for sample quantification. Determine the volume of sample DNA to load to increase the likelihood it will fall within the standards—and to determine the dynamic range of the assay.

Tip: For limiting samples, a minimum of 1 µl is required. If your DNA is more concentrated than the highest standard, dilute it to fall between the standards.

3. Prepare the qPCR reaction by adding reagents in the order listed (see Table 3). We suggest the use of iTaq Universal SYBR Green Supermix.

Table 3. Quantitative PCR master mix for Alu quantification for 1 reaction.

Component	Volume (µL)
iTaq Universal SYBR Green Supermix	10 µL
Alu115 or Alu247 primers	2 µL
DNA	1–8 µL
Low EDTA TE	Up to 20 µL
Total volume	20

4. Place samples in the thermal cycler and run the Alu Primer PCR Quantification program (see Table 4).

Table 4. Quantitative PCR thermal cycler conditions for Alu quantification.

Step	Volume (µL)	Temperature (°C)	Time (sec)
Denaturation	1	95	180
Amplification	35	95	5
		62	30
Hold	—	4	∞

Note: Lid should be heated to between 100–105°C during denaturation and amplification steps.

5. Plot Ct values (y-axis) vs. DNA quantity of the serial dilutions (x-axis) on a log scale to produce the standard curve. Identify the slope and the y-intercept. Solve using the following formula to determine the sample DNA concentration. Consider any sample dilutions or lower input DNA volumes.

   ng/µl = 10 ^ (Ct – y-intercept) / slope

6. The concentration for the Alu115 amplicon can be used to determine the total quantity of usable DNA in ng/µl. Verify that the calculated concentration of your sample is between the DNA standards of the assay.
7. Use a ratio of the Alu247 and Alu115 amplicons to calculate a DNA Integrity Score. High quality DNA is expected to have a DNA Integrity Score around 1.0, while lower quality DNA will have a score between 0.1 and 1.0 due to either damage, or high molecular weight DNA contamination for FFPE or cfDNA samples. The cfDNA, though not intrinsically damaged, is expected to have an integrity score around 0.3. The DNA Integrity Score is intended to be used as an indicator for the probability of a successful library construction. Because of the diversity of sample types and protocols, specific recommendations concerning library construction and sequencing metrics are difficult to define in terms of the DNA Integrity Score.

   DNA Integrity Score = (ng/µl of Alu247) / (ng/µl of Alu115)

REFERENCES

1. Simbolo M, Gottardi M, Corbo V, et al. **DNA qualification workflow for next generation sequencing of histopathological samples**. PLoS One. 2013;8(6):e62692. Published 2013 Jun 6. doi:10.1371/journal.pone.0062692

2. **Hao TB, Shi W, Shen XJ, et al. Circulating cell-free DNA in serum as a biomarker for diagnosis and prognostic prediction of colorectal cancer. Br J Cancer**. 2014;111(8):1482-1489. doi:10.1038/bjc.2014.470

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