INPUT DNA QUANTIFICATION ASSAY

For Use with Accel-NGS® DNA Library Kits and Accel-Amplicon™ Panels

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Introduction

For next-generation sequencing library preparation, input quantification by spectrophotometric-based (NanoDrop®) or fluorometric-based (Qubit®) methods may not provide an accurate assessment of the usable DNA within the sample. Quantification by spectrophotometric-based methods commonly overestimates DNA concentration and is limited to relatively high concentration samples. Quantification by fluorometric-based methods provides accurate DNA concentrations for samples with high quality DNA (e.g., whole blood, fresh frozen samples, cultured cells), but performs poorly with low quality samples and cannot distinguish between circulating, cell-free DNA (cfDNA) and high molecular weight cellular gDNA. Therefore, for low quality samples [e.g., formalin-fixed, paraffin-embedded (FFPE) samples] and cfDNA samples, we recommend quantification by a qPCR-method, using both short and long amplicons to accurately determine the concentration and quality of sample DNA [Simbolo M. et al. PLoS ONE (2013) 8(6): e62692].

Alu sequences (highly abundant in the human genome) can be used for the sensitive quantification of human genomic DNA. Swift Biosciences' kits include primers that can be used to amplify two differently sized amplicons: short (115 bp; Alu115) and long (247 bp; Alu247) amplicons from genomic Alu repeats. Following input analysis, the appropriate amount of sample DNA can be used as input for NGS library preparation with an Accel-NGS Library Kit and Accel-Amplicon Panel.

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

As shown here with 10 FFPE samples of varying quality, NanoDrop non-specifically measures all double-stranded DNA (dsDNA), single-stranded DNA, and contaminants within the sample. Qubit quantifies dsDNA content, however it cannot assess DNA damage. Whereas the qPCR assay specifically measures DNA quantity and provides a metric for damage. For sample types with more consistent high quality DNA including whole blood, fresh-frozen samples, and cultured cells, quantification by Qubit is a reliable indicator of amplifiable content.

FFPE Samples

The QIAamp® DNA FFPE Tissue Kit (Qiagen Cat. No. 56404) is recommended for DNA extraction from FFPE tissue sections. Be sure to elute the sample in water.

DNA extracted from FFPE samples can exhibit varying degrees of DNA damage, and the adverse consequences of DNA damage will be more pronounced for amplification of the longer (Alu247) amplicon. Therefore, with FFPE samples, Alu115-qPCR results accurately detect 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.

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. Fix	3.6	0.5
Kidney + 24 hr. Fix	2.4	0.6
Kidney + 48 hr. Fix	1.0	0.4

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 had either been fresh-frozen, or fixed for 6, 24, or 48 hours before being paraffin-embedded.

Circulating cfDNA Samples

Cell-Free DNA BCT® tubes (Streck Cat. No. 218961) and the QIAamp Circulating Nucleic Acid Kit (Qiagen Cat. No. 55114) are recommended for sample collection and cfDNA extraction with the Accel-NGS 2S DNA Library Kits. However, carrier RNA in this kit (and other extraction kits) will be detected by NanoDrop or Qubit, resulting in inaccurate quantification. Quantification by qPCR

[as presented by T.B. Hao in the British Journal of Cancer (2014) 111, 1482–1489] was used during validation of the Accel-NGS 2S DNA Library Kit's cfDNA protocol and is recommended to determine the concentration and integrity index of the input cfDNA.

As cfDNA exhibits a narrow size distribution around 165 bp, Alu115-qPCR results accurately detect the total quantity of cfDNA and high molecular weight cellular gDNA. Alu247-qPCR results indicate only the presence of high molecular weight cellular gDNA contamination. Therefore, the Alu247/Alu115 ratio illustrates the DNA Integrity Score of the sample.

Based on results from Hao et al., expected DNA Integrity Scores are as follows:

Sample	Median DNA Integrity Score
Normal cfDNA	0.38 (0.29-0.49)
Primary colorectal cancer patient cfDNA	0.62 (0.51-0.65)
gDNA	1.0

If you have questions related to FFPE or cfDNA sample quality, please contact Swift Biosciences' Technical Support at TechSupport@swiftbiosci.com or tel: 734.330.2568.

Before You Start

Contents

Contains enough reagents for the preparation of 48 reactions in duplicate, as well as standards.

Reagents		48 Reactions	Sequence
Amplicon Primers	Alu115	290 µl	Forward: 5'- CCTGAGGTCAGGAGTTCGAG-3' Reverse: 5'-CCCGAGTAGCTGGGATTACA-3'
	Alu247	290 µl	Forward: 5'-GTGGCTCACGCCTGTAATC-3' Reverse: 5'-CAGGCTGGAGTGCAGTGG-3'

Required Materials Not Supplied

- iTaq™ Universal SYBR® Green Supermix (Bio-Rad Cat. No. 172-5120)
- Standard human genomic DNA (Promega Cat. No. G3041)
- Microcentrifuge
- Programmable thermocycler operating within manufacturer's specifications (Bio-Rad CFX96)
- 0.2 mL PCR tubes or 96-well plate
- Aerosol-resistant tips and pipette ranges from 1-1000 µl
- Nuclease-free water (molecular biology-grade)

Alu Assay

1. Prepare a standard curve using serial dilutions of human genomic DNA of known quantities (11 ng, 1.1 ng, 0.11 ng, 0.011 ng, 0.0011 ng) for each 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 so as to increase the likelihood it will fall within the standards and, therefore, the dynamic range of the assay. 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 in a 1.5 mL tube by adding reagents in the order listed below. We suggest the use of iTaq Universal SYBR Green Supermix (Bio-Rad 172-5120).

Reagents	**1 rxn**
iTaq Universal SYBR Green Supermix	10 µl
Alu115 or Alu247 primers	2 µl
DNA	X µl
Low EDTA TE	up to 20 µl
Total Volume	**20 µl**

4. Place in the thermocycler and run the Alu Primer PCR Quantification program as described below.

Alu Primer qPCR Quantification Thermocycler Program	95 ˚C for 3 minutes, lid heating ON	
	95 ˚C for 5 seconds, lid heating ON 62 ˚C for 30 seconds, lid heating ON	(x35 cycles)

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.

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 of 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 in FFPE or cfDNA samples, respectively. The DNA Integrity Score is intended to be used as an indicator of probability of successful library construction. Due to 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. Use your best judgement.

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

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