

Quantification and Quality Assessment of Human DNA Samples

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

Introduction

For next-generation sequencing (NGS) 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. Included in this kit are 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. Please 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 TechnicalSupport@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'- CCTGAGGTCAGGAGTTTCGAG-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)

Protocol

Assemble 20 µl reactions for each Alu-qPCR. A standard curve can be generated from serial dilutions (11 ng, 1.1 ng, 0.11 ng, 0.011 ng, 0.0011 ng) of human genomic DNA and no template control. A melting curve can also be performed to ensure that only one peak was amplified for all samples.

Reagent	Volume (1 Reaction)
iTaq Universal SYBR Green Supermix	10 µl
Alu115 or Alu247 primers	2 µl
DNA	X µl
Low EDTA TE	Adjust total volume to 20 µl
Total	20 µl

Thermocycler Conditions

95 °C for 3 minutes, lid heating ON	
95 °C for 5 seconds, lid heating ON	
62 °C for 30 seconds, lid heating ON	35 cycles
Read SYBR	

Data Analysis

To determine the DNA quantity of each sample, generate a standard curve from the C_t values of your serial dilution samples (11 ng, 1.1 ng, 0.11 ng, 0.011 ng, 0.0011 ng) to determine the y-intercept and slope. The formula to determine concentration is:

$$\text{ng}/\mu\text{l} = 10^{\text{(average sample } C_t - \text{y-intercept)} / \text{slope}}$$

The concentration for the Alu115 amplicon can be used to determine the total quantity of usable DNA.

The concentrations for both Alu247 and Alu115 amplicons can be used to determine the DNA Integrity Score:

$$\text{DNA Integrity Score} = (\text{ng}/\mu\text{l of Alu247}) / (\text{ng}/\mu\text{l of Alu115})$$

The DNA Integrity Score is intended to be used as an indicator of probability of successful library construction. Specific recommendations concerning library construction and sequencing metrics are difficult to define in terms of a DNA Integrity Score due to the diversity of sample types and protocols that can be used with this assay. Please consider genome complexity and sample quality when choosing input DNA quantity. Although libraries may be successfully prepared from ultra-low inputs, reduced representation of genome complexity may occur.



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