Generating Sequencing Libraries from FFPE Samples

Guidelines for extraction and qualification of DNA from FFPE samples for optimal use with TruSight® Tumor 26 library preparation for next-generation sequencing.

Introduction

Formalin-fixed, paraffin-embedded (FFPE) human tissues are a valuable source of material for molecular analyses and clinical studies. Various processes and protocols now exist for the extraction and purification of nucleic acids from FFPE samples. However, the assays used to evaluate DNA and RNA have evolved from simple monoplex PCR to higher plexity products. Therefore, the quality and amount of nucleic acid extracted from FFPE material has become more critical to the success of these assays.

This white paper provides a review of FFPE DNA extraction and qualifications methods as they apply to higher plexity assays in next-generation sequencing analysis. In particular, it will focus on the use of FFPE samples as starting material for library preparation with the TruSight Tumor 26 panel for sequencing on the Illumina MiSeq® desktop sequencer.

DNA Requirements for TruSight Tumor 26

TruSight Tumor 26 can be used to generate sequencing libraries that are highly multiplexed at both the target and sample level. The high level of assay complexity is enabled by combining an oligo extension-ligation process with universal PCR. Both of these reactions require an intact DNA template of sufficient quality. The formalin fixation and paraffin embedding process impacts DNA quality by fragmenting, cross-linking, and otherwise introducing damage through various chemical modifications. Optimizing the DNA extraction method from FFPE samples and assessing the quality of the resulting DNA produces high library success rates with TruSight Tumor 26.

Table 1: Amount of DNA Required for TruSight Tumor 26 Varies Based on Input Sample Quality

Sample ID	FF DNA Conc. (ng/µl)	FF Input (ng)	FFPE DNA Conc. (ng/µl)	FFPE ΔCq	Dilution Factor	Amplifiable DNA Input (ng)
1	15.3	38	23.6	4.74	--	37
2	45.8	29	43.8	3.54	--	86
3	23.6	30	25.2	2.09	--	235
4	23.9	60	22.2	3.96	--	64
5	23.3	29	32.8	2.00	--	250
6	24.8	31	34.8	2.36	--	195
7	107	33	24.7	4.18	--	55
8	41.5	26	23.5	3.23	--	107
9	25.0	31	24.0	3.45	--	92
10	24.4	31	21.4	2.85	--	139
11	36.3	45	49.1	2.38	--	192
12	28.5	36	29.0	1.57	2	168
13	54.1	68	50.7	1.00	2	250
14	32.4	20	24.4	2.46	--	182
15	47.4	30	27.5	1.92	2	132
16	22.7	28	22.4	1.03	2	245
17	89.3	112	16.6	2.45	--	183
18	23.7	30	34.1	3.12	--	115

DNA derived from FFPE tissues is degraded and damaged reducing amplify ability. Here 18 matched fresh frozen and FFPE tissues were evaluated for DNA quality. Fresh frozen DNA was found to be 100% amplifiable by comparison to (ΔCq) non-FFPE reference DNA (QCP). The ΔCq was used to determine the appropriate dilution factor and DNA input into the library preparation assay.

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Qualification of DNA Extracted from FFPE Samples

The TruSight Tumor 26 extension-ligation process relies upon the ability of a DNA polymerase to read across a single-stranded template. Therefore, a quantitative PCR approach was taken to qualify the performance of DNA extracted from FFPE tissue. By comparing the ability of FFPE DNA to be amplified relative to that of a non-FFPE reference gDNA, a ΔCq value can be calculated for each sample. This value can be used to predict its performance with TruSight Tumor 26. A non-FFPE reference gDNA Quality Control Template (QCT) is provided with the Quality Control Primers (QCP). It is important to note that the amplifiable mass (ng) of FFPE DNA capable of generating library product will often be only a fraction of the total amount extracted from FFPE tissues. The exact amount of FFPE DNA input will vary according to the quality of the extracted DNA (Table 1).

To make sure that the ΔCq value accurately reflects the ability of a sample to be amplified, the QCT must be used carefully and consistently to maintain a reliable baseline Cq value. Aliquot 5 µl of the stock QCT into PCR tube strips for long-term storage to improve pipetting accuracy and template performance. Then, each time the assay is run, 5 µl of QCT from a single aliquot is added to 495 µl of DEPC water to make the 1:100 dilution needed as input for qPCR. This can then be run according to the following protocol:

1. Vortex the diluted QCT and spin briefly at 280 × g.
2. Dilute 1.5 µl of each FFPE gDNA with 148.5 µl DEPC water.
3. Aliquot 10 µl of DEPC water for use as the No Template Control (NTC).
4. Prepare the qPCR premix using the following volumes per replicate:

2X qPCR Master Mix*	5.0 µl
0.5 µM QCP**	1.0 µl
DEPC water	2.0 µl
Total volume per well	**8.0 µl**

* Volume provided reflects use of Bio-Rad iQ SYBR Green Supermix.

** The QCP primers are prepared as mixed 5 µM stock, then diluted 1:10 to generate a 0.5 µM working stock.

To calculate the total volume to premix, 8 µl is multiplied by (# of samples + QCT + NTC)† 3 replicates each + 10% overfill. The final volume can be transferred to a trough or PCR tube strips.

5. Use a multichannel pipette to dispense 8 µl of premix into each well to be used in the qPCR plate (data was generated with the Bio-Rad CFX384 Real-Time System with clear 384-well plates).
6. Pipette 2 µl NTC into each of 3 wells, 2 µl QCT into each of 3 wells, and 2 µl FFPE DNA into each of 3 wells per sample. After each dispense, pipet up and down 3X to ensure transfer.
7. Seal the plate and spin briefly at 280 × g.

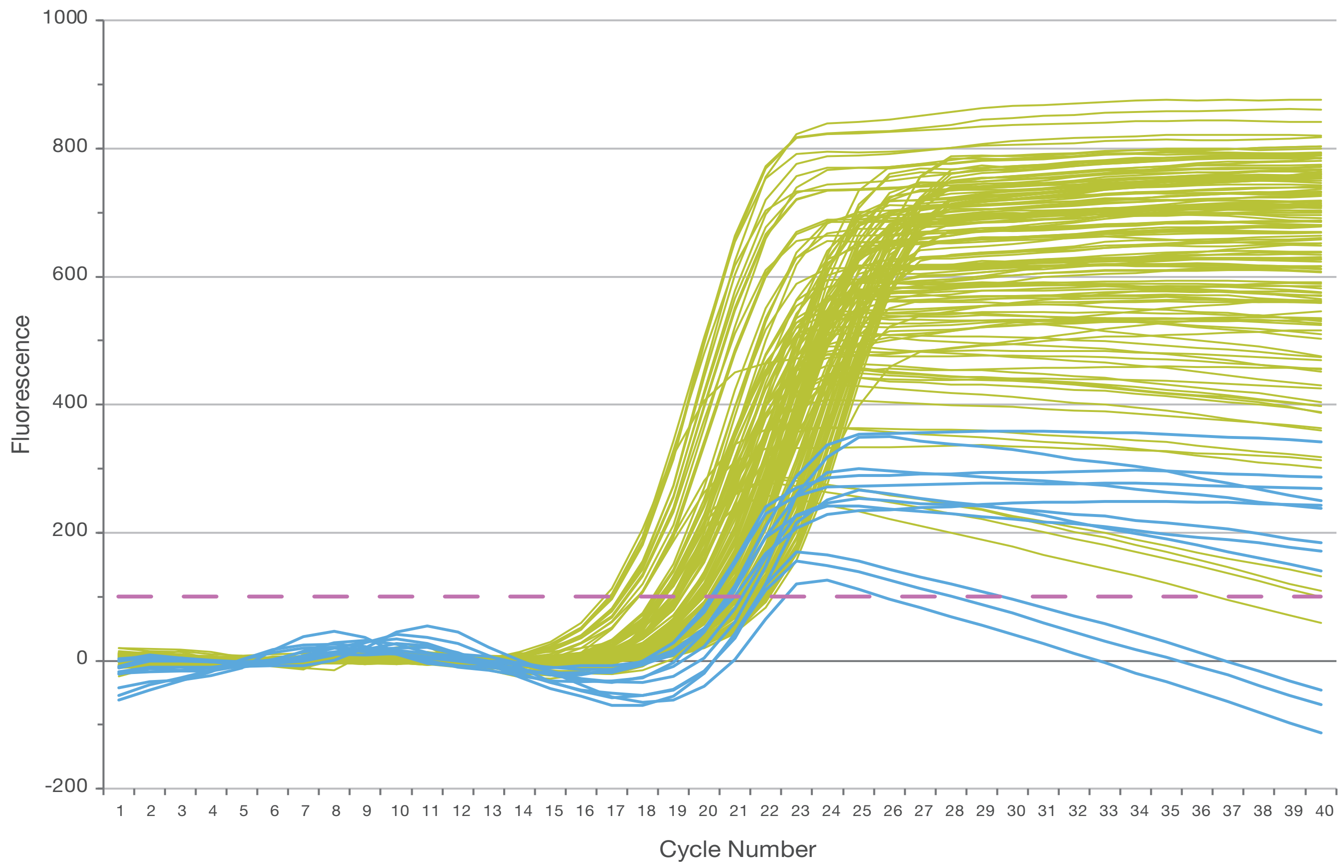

Figure 1: Qualification of DNA Samples by PCR—DNA samples described in Table 1 were qualified for use in creating libraries for next-generation sequencing using real-time PCR following parameters stated in the text. The graph shows qPCR amplification curves generated by these samples. Purple: Cq threshold. Blue: DNA samples with abnormal amplification profiles that did not yield libraries suitable for sequencing. Green: DNA samples with profiles passing qPCR qualification that can be used to prepare sequencing libraries.

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Sample	FFPE			Fresh Frozen			FFPE			Fresh Frozen		
Sample	1	2	3	1	2	3	4	5	6	4	5	6
ΔCq	4.74	3.54	2.09	0.46	-1.53	-0.32	3.96	2.00	2.36	0.66	-0.07	-0.52

Sample	FFPE			Fresh Frozen			FFPE			Fresh Frozen		
Sample	7	8	9	7	8	9	10	11	12	10	11	12
ΔCq	4.18	3.23	3.45	-3.11	-1.36	-0.44	2.85	2.38	1.57	-0.28	-0.86	-0.52

Sample	FFPE			Fresh Frozen			FFPE			Fresh Frozen		
Sample	13	14	15	13	14	15	16	17	18	16	17	18
ΔCq	1.00	2.46	1.92	-0.78	-1.04	-1.68	1.03	2.45	3.12	-0.78	-0.70	-0.34

Figure 2: Analysis of Prepared TruSight Tumor 26 Libraries—Samples described in Table 1 were qualified and normalized based on qPCR data shown in Figure 1. Libraries were then prepared with TruSight 26 for use in next-generation sequencing. These libraries were run on agarose gels (above, flanked by 100 bp DNA ladder) to check for uniform library preparation.

8. Make sure that the seal is free of any liquid or dust, place plate in the qPCR machine in the proper orientation, and run the following thermal profile:

Temperature	Time
50°C	2 minutes
95°C	10 minutes
Cycle 40 times as follows:	
95°C	30 seconds
60°C	30 seconds
72°C*	30 seconds

† Confirm that the instrument captures images after this step.

Before measuring the Ct value, or Cq value as noted in the MIQE guidelines, the Ct threshold should be set to avoid inaccurate measurements due to background (100 RFU with the Bio-Rad CFX384 Real-Time System). After the Cq value has been obtained, data quality can be determined by confirming that amplification of NTC occurs at least 10 cycles after QCT. Any replicate Cq values that diverge by more than 1 Cq from the remaining replicates should be discarded due to pipetting or other error. Finally, any replicates exhibiting an abnormal amplification curve (Figure 1, shown in blue) should be excluded due to PCR inhibitors or other tissue factors that impair PCR performance.

When complete, subtract the average Cq for the QCT from the average Cq for each sample to yield the ΔCq values. The QCT average Cq in these studies ranged from 17.4 to 17.7 across 3 lots of QCT. Samples with ΔCq values ≤ 4 consistently yielded libraries of sufficient quality and quantity to provide reliable sequencing data. Performance of samples with ΔCq > 4 was less reliable. Also, samples with ΔCq values ≤ 1.5 were found to perform with more uniformity when diluted two-fold for every cycle below 1.5. Table 1 summarizes the amplifiable ng of DNA input used to generate libraries for sequencing; Figure 2 shows the uniform concentration of the library products.

Evaluation of FFPE DNA Extraction Methods

Having a method for qualifying the performance of DNA extracted from FFPE tissue enables evaluation of the DNA extraction methods themselves. As described above, those methods yielding the highest amount of amplifiable DNA will be most successful with TruSight Tumor 26. Other aspects to consider when choosing a DNA extraction and qualification method include ease of use, versatility, and robustness. Also important is how well it integrates into the overall process workflow, and whether it is amenable to automation for reduced user error and higher throughput. For these reasons, the QIAGEN Supplementary Protocol: Purification of genomic DNA

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from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution was run on the QIAcube to extract all FFPE DNA. Optimization of the QIAGEN protocol for use with TruSight Tumor 26 included multiple steps. DNA was extracted from eight 5 µm FFPE tissue sections treated with 320 µl of QIAGEN Deparaffinization Solution and digested with 40 µl Proteinase K in a thermal mixer overnight at 1,000 rpm. Elution volume was decreased to 30 µl to maximize the final DNA concentration.

Summary

Following these guidelines for extracting and qualifying FFPE DNA samples before generation of sequencing libraries, we see 90% of evaluated samples passing QC. Of these samples, all have produced successful libraries (Figure 2). It should be noted, however, that some types of samples such as melanomas, may contain PCR inhibitors that impair performance and lower the success rate for these particular samples. Prolonged fixation times may also affect ability of a sample to be amplified, again emphasizing the importance of initial qualification of FFPE DNA samples by qPCR.

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