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Whole Exome Sequencing of FFPE Samples and Recommendations

Description

This data summary discusses whole exome sequencing of FFPE samples performed by Otogenetics in reference to published studies. The purpose of this summary is to illustrate (I) the yield of gDNA from a given size of FFPE block; (II) the QC standards employed by Otogenetics for FFPE samples and the correlation of sequencing quality with the initial sample QC quality, (III) the percentage of FFPE samples processed with satisfactory exome sequencing data based on the initial sample QC. In addition, (IV) the correlation between outcome of exome sequencing and the age of FFPE samples is cited from extensive studies in the literature. Finally, (V) key considerations for FFPE sample exome sequencing are recommended.

Summary of Conclusions and Recommendations (Based on data compiled from Otogenetics and in cited references)

Conclusions:

- A single slice of FFPE tissues at 10um x 4 mm x 8 mm can yield sufficient amount of gDNA for whole exome sequencing. However, 4 slices or amount of FFPE tissues are recommended.
- Initial QC of gDNAs from FFPE tissues can classify the gDNA into three grades: Grade 1 of size range to high MW >10Kb, Grade 2 of size range from 250 bp to <10Kb, and Grade 3 of size range at low MW ~250 bp.
- The age of the FFPE tissues does not need to be known. The above grade classification of gDNAs based on size distribution can be used to predict the whole exome sequencing of FFPE gDNA. gDNA from >20years tend to have a lower molecular weight distribution than the gDNA from <10 years.
- FFPE gDNAs of Grade 1 are expected to achieve a similar sequencing quality as the gDNA from fresh tissues. Sequencing data size may need to be increased to achieve equivalent coverage.
- 83% of FFPE gDNAs of Grade 2 are expected to achieve a similar sequencing quality as the gDNA from fresh tissues with increased sequencing data size. Up to 17% of the samples of Grade 2 may fail at library preparation.
- 75% of FFPE gDNAs of Grade 3 are expected to achieve a similar sequencing quality as the gDNA from fresh tissues. Approximately 50% more sequencing data is needed. Up to 25% of the samples of Grade 3 may fail at library preparation.

Recommendations:

- proceed with FFPE gDNAs of Grade 1-3, make an assessment at library preparation step
- assign up to 50% more sequencing data allowance for FFPE samples that have succeeded at library preparation
- a higher cost of whole exome sequencing for FFPE tissue gDNAs than fresh tissue gDNAs will be charged, due to need for larger data sizes

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(I) gDNA yield from FFPE tissues

Description:

Otogenetics performed gDNA extraction from 1, 4, 8, 12 slices of FFPE tissue.

The size of each slice: 4 mm x 8 mm x 10 μm.

The yields of gDNA are listed below:

Slice number	Gel position	Conc. (ng/ul)	$OD_{260/280}$	Volume (ul)	Total amount (ug)
1	Lane1	158.2	1.92	25	3.9
4	Lane2	186.9	1.92	30	5.6
8	Lane3	172.4	1.91	55	9.4
12	Lane4	222.8	1.92	110	24.5

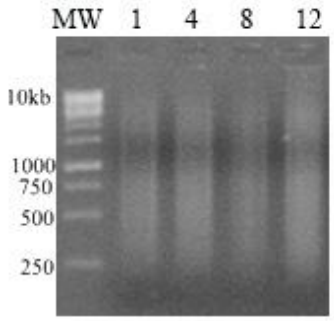

Fig. 1. gDNA yield and quality from FFPE slices.

Conclusions and recommendations:

- A single slice of FFPE tissue at the size of 4 mm x 8 mm x 10 μm is sufficient to generate >3 μg of gDNA, which is enough for exome sequencing. Recommend to submit an equivalent of 4-8 slices at this size.
- gDNA from FFPE tissues commonly appear as smears on the gel. The gDNA from this particular FFPE tissue ranges from 250 bp to <10Kb in size.

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(II) QC standards for FFPE gDNA samples and the correlation between initial QC and exome sequencing data

(a) QC of gDNA from FFPE tissues

Description:

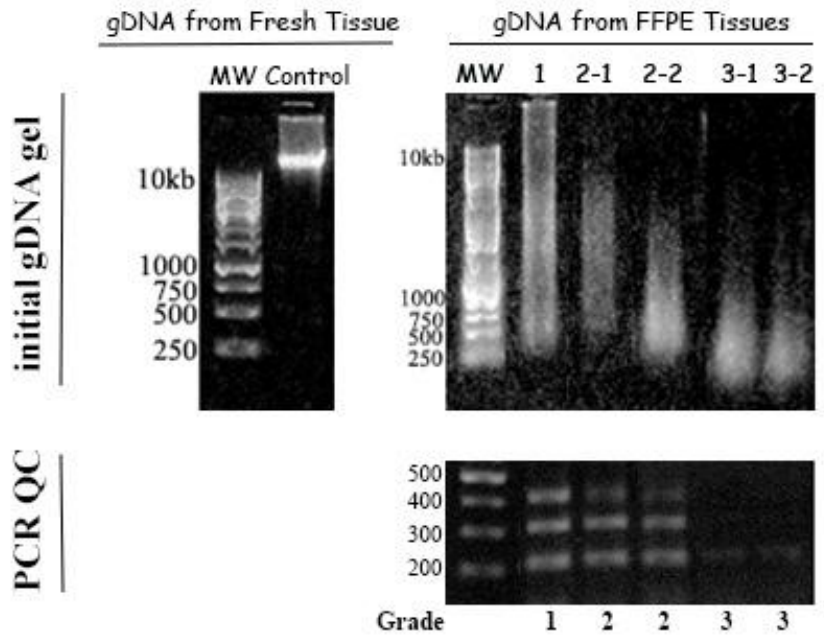
Otogenetics uses gel electrophoresis and spectrophotometer assays to assess the quality of initial gDNA. gDNA from fresh tissues appears as a large molecular weight band above 10Kb on the gel (Fig. 2).

gDNA from FFPE samples have various appearances on the gel (Fig. 2), depending on how fixation was done, how gDNA extraction was performed, and how well gDNAs were stored after purification. The best ones appear as smears across from low to high molecular weight species, some appear as medium-size smears, and some as low molecular weight smears only.

In addition to gel electrophoresis and spectrophotometer assays, Otogenetics also employs a PCR assay that examines the degree of degradation/cross-linking in gDNA from FFPE tissues. For FFPE gDNA of the best quality, PCR products of 400bp, 300bp, 200 bp are expected. For FFPE gDNA of the medium quality, PCR products of 300 bp and 200 bp are expected. For FFPE gDNA with only low molecular weight smears, PCR products of the shortest amplicon, 200 bp, may be only fragments. Figure 2 illustrates the FFPE gDNA of various initial quality.

QC results illustration:

A



B

	Sample#	sample type	Adjusted Duplicates (%)
	Normal tissue	Normal gDNA	9.15%
good quality FFPE (Grade 1)	FFPE #1	FFPE gDNA	18.56%
medium quality FFPE (Grade 2)	FFPE #2-1	FFPE gDNA	25.44%
	FFPE #2-2	FFPE gDNA	25.26%
low quality FFPE (Grade 3)	FFPE #3-1	FFPE gDNA	42.04%
	FFPE #3-2	FFPE gDNA	51.77%

Fig. 2. (**A**) QC of gDNA from FFPE tissues and Grades 1, 2, and 3 based on original smear appearance and PCR QC. (**B**) Exome sequencing data from higher grade FFPE gDNA is of lower duplication rates, which is a key quality measure for exome sequencing.

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(III) Correlation of exome sequencing outcome with initial QC

Description:

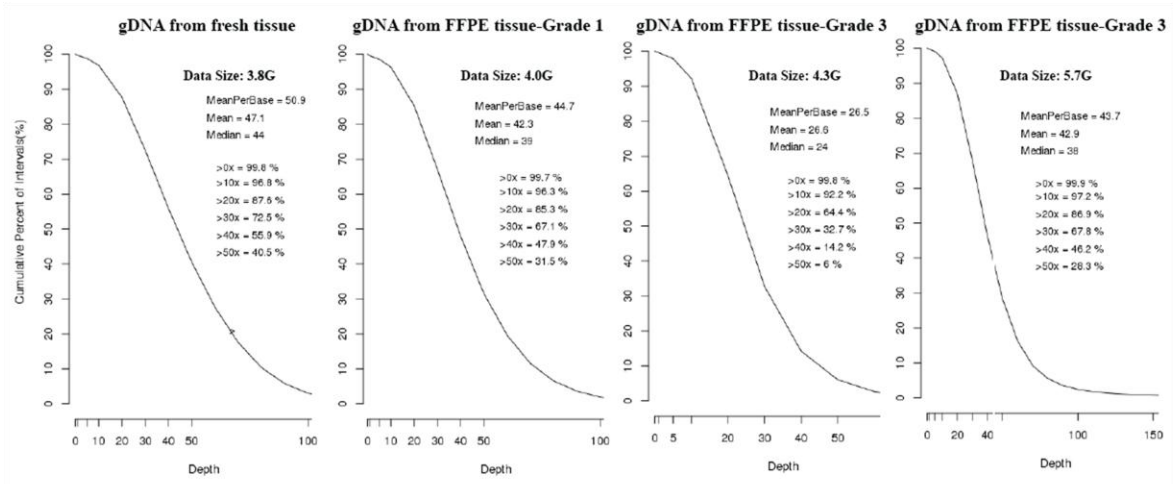
The outcome of exome sequencing quality was assessed based on (a) duplication rates and (b) across target coverage. In addition, (c) the percentages of samples which achieved satisfactory whole exome sequencing for gDNA with different grades were calculated as a prediction factor for whole exome sequencing of FFPE samples. Sequencing data quality itself (Q30) is not affected by the quality of gDNAs from FFPE tissues.

(a) Duplication rates increase for lower quality FFPE samples.

The duplication rate correlated with the quality of the initial samples, as shown by the duplication rate (Fig. 2B). As the grade of gDNA decreased, the duplication rate increased (Fig. 2B).

(b) Similar across target coverage can be achieved by increasing data size for lower grade gDNAs from FFPE tissues.

The percentage of targets covered at 10x, 20x, and 30x under a given data size is indicative of the across target coverage. As shown in Fig. 3, the across target coverage is lower for lower graded gDNA when compared to higher graded gDNA at the same data size. Interestingly, however, the across target coverage for lower graded gDNA samples could be compensated by a large data size. Therefore, gDNAs at lower grades could achieve the same and excellent across target coverage with larger data size which does incur a higher sequencing cost.



Sample	Data Size	% of Targets >10x coverage	% of Targets >20x coverage	% of Targets >30x coverage	%Data Size
Fresh	3.8 G	96.8	87.6	72.5	100%
FFPE G1	4.0 G	96.3	85.3	67.1	105%
FFPE G3	4.3 G	92.2	64.4	32.7	113%
FFPE G3	5.7 G	97.2	86.9	67.8	150%

Fig. 3. Additional data can make up for the lower grade FFPE gDNA. The top panel shows the across target coverage of a gDNA from fresh tissue (intact and control gDNA), from a high grade FFPE gDNA (grade 1), from a low grade FFPE gDNA (grade 3) at the same data size, and from a low grade FFPE gDNA (grade 3) at 1 1/2 times data size. This figure indicates that with additional data (~50% more data), the lower grade FFPE gDNA could achieve the same and satisfactory across target coverage.

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(c) The percentage of samples completed with satisfactory whole exome sequencing correlated with the QC grade of the initial FFPE gDNAs.

As shown in Fig 4, among FFPE gDNA samples submitted to Otogenetics for whole exome sequencing, most of them were classified as Grade 2. For Grade 1 FFPE gDNAs, all of the samples achieved satisfactory sequencing results. For Grade 2 FFPE gDNAs, clients decided to go forward with 83% of the samples while the remaining 17% of the samples were opted out for processing either after the initial QC report or after failure of high quality library preparation. For Grade 3 FFPE gDNAs, clients decided to go forward with 75% of the samples while the remaining 25% of the samples were opted out for processing either after the initial QC or after library preparation (failure in library preparation). Therefore, 0% of the Grade 1 FFPE gDNA failed at library preparation, up to 17% of the Grade 2 FFPE gDNAs may fail at library preparation, and up to 25% of the Grade 3 FFPE gDNAs may fail at the library preparation. Samples of all grades passing library preparation almost generally achieve satisfactory whole exome sequencing, albeit the percentage of samples with higher duplication rates increases for lower grade FFPE gDNA (Fig. 4).

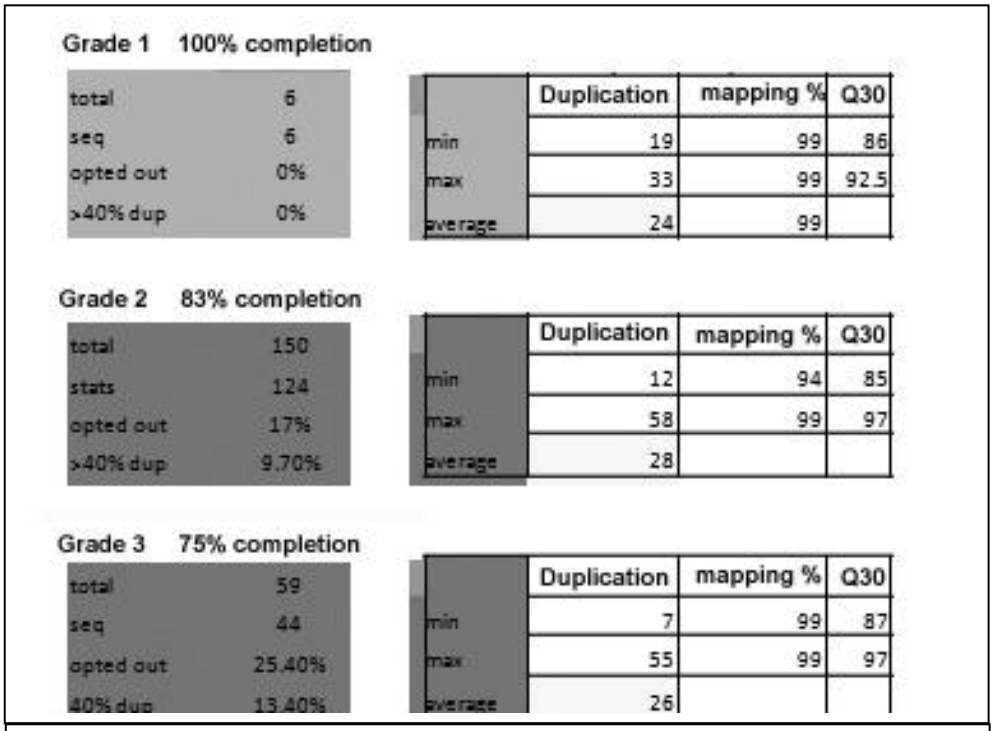

Fig. 4. Correlation of the percentage of successful whole exome sequencing of FFPE gDNAs with the initial grade classification of gDNAs.

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(IV) Age of the FFPE tissues does not influence significantly the whole exome sequencing if given larger data size compensation.

There are several publications summarizing from extensive studies on the correlation of the age of FFPE tissues and the quality of whole exome sequencing. As shown in Fig. 5, the age of the FFPE tissues correlates with the size distribution of the gDNA and resulting mean inset size. This reduced insert size correlates with fragment size in the Grade classification established experimentally by Otogenetics. Furthermore, both the duplication rate and across target coverage of gDNAs from these studies are consistent with Otogenetics' result compiled from FFPE gDNA whole exome sequencing, or (1) the lower graded gDNAs from older FFPE tissues (>20years) showed a higher duplication rate than the higher graded gDNA from FFPE tissues of < 10 years and (2) the across target coverage of lower graded gDNA could be compensated by a larger data size (Fig. 5).

FFPE Pilot – Sequencing Metrics

Experiment	FFPE Samples - ≥20years (n=8)	FFPE Samples - ≤10 years (n=12)	HapMap Control (n=1)
Year of Sample	1995-1996	2002-2005	2015
Sequencing Data Output			
Raw GB	9.3792	8.5336	8.2456
Mean Target Coverage	74	79	92
% On Target @ 10x	96.73	97.24	98.21
% Zero Bases	0.65	0.62	0.69
Mean Insert Size	173	213	223
% Dups	36.27	21.55	7.42
data needed for across target coverage	41% more data	18% more data	

Modified from Marosy et al., JHU, ASHG 2015

Fig. 5. Age of FFPE tissues correlates with the duplication rate and data size requirement observed by Otogenetics' grade classifications.

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(V) Conclusions and Recommendations for FFPE gDNA whole exome sequencing.

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References:

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