PCR-free library construction

Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
NEB Paired-End Sample Prep Premix Kit – End Repair	NEB	E6875B-GSC		✓
NEB Paired-End Sample Prep Premix Kit – A Tail	NEB	E6876B-GSC		✓
NEB Paired-End Sample Prep Premix Kit – Ligation	NEB	E6877B-GSC		✓
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
dNTPs, 10 mM each	Invitrogen	46-0519		✓
Ice bucket	Fisher	11-676-36		✓
Covaris LE220 with WCS and Chiller	Covaris	LE220	✓	
DNA *AWAY*	Molecular BioProducts	21-236-28		✓
Gilson P2 pipetman	Mandel	GF-44801	✓	
Gilson P10 pipetman	Mandel	GF-44802	✓	
Gilson P20 pipetman	Mandel	GF23600	✓	
Gilson P200 pipetman	Mandel	GF-23601	✓	
Gilson P1000 pipetman	Mandel	GF-23602	✓	
Diamond Filter tips DFL10	Mandel Scientific	GF -F171203		✓
Diamond Filter tips DF30	Mandel Scientific	GF-F171303		✓
Diamond Filter tips DF200	Mandel Scientific	GF-F171503		✓
Diamond Filter tips DF1000	Mandel Scientific	GF-F171703		✓
Galaxy mini-centrifuge	VWR	37000-700	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
Black ink permanent marker pen	VWR	52877-310		✓
Clear Tape Sealer	Qiagen	19570		✓
Aluminum Foils seals	VWR	60941-126		✓
Aluminum foil tape, 3"x 60 yds	Scotch/3M	34000740		✓
Eppendorf BenchTop Refrigerated Centrifuge 5810R	Eppendorf	5810 R	✓	
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10”X15”	Fisher	01-826-4		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	CommercialAlcohols	00023878		✓
IKA Works Vortexer	Agilent	MS2S9-5065-4428	✓	
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
Peltier Thermal Cycler	MJ Research	PTC-225	✓	
Power Supply, LVC2kW, 48VDCV	Tyco Electronics	RM200HA100	✓	
Plate, 96-Well reservoirs, diamond-bottom, Low-Profile	Seahorse	EK2036		✓
AB1000 96-well 200µl PCR plate	Fisher	AB1000		✓
MagMax express 96 Deep Well plates (EtOH and waste)	Applied Biosystems	4388476		✓
Storage Plate, 96-well, 1.2 mL, square well, U-bottomed	ABgene	AB1127		✓
Microlab NIMBUS	NIMBUS	Hamilton	✓	
Eppendorf Benchtop Centrifuge	Eppendorf	5810 R	✓	
70% Ethanol	In house	N/A	N/A	N/A
Qiagen Buffer EB – 250 mL	Qiagen	19086		✓
UltraPure Distilled Water	Invitrogen	10977-023		✓
Nuclease Free 2.0 mL eppendorf tube	Ambion	12400		✓
5 mL Screw Cap tubes	Ultident	SCT-5ML-S		✓
TruSeq Indexed Adapters	IDT	NA		
TruSeq Universal Primers	IDT	NA		
Alpaqua Magnum FLX	Alpaqua Engineering	A000400	✓	
Ampure XP Beads, 450mL	Agencourt	A63882		✓
PCR Clean DX (ALINE beads)	ALINE Biosciences	C-1003-450		✓
NIMBUS P50 tips 50uL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235831		✓

NIMBUS P300 tips 300uL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235832		✓
Tween 20, 10%, for easy pipetting, 1L	Bio-Rad	161-0781		✓
ALPS 50V Microplate Heat Sealer	Thermo Scientific	AB-1443	✓	
EZPierce 20uM Thermal foil	ThermoFisher	AB1720		✓

1. Shearing & QC

1.1. Transfer gDNA, to Covaris LE220 vessels

1.2. Shearing was performed using Covaris LE220 as per the following parameters:

Duty	PIP	Cycles/Burst	Treatment Time	Labware: 96 microTUBE Plate
20	450	200	2x 45sec (spin in between)	P/N 520078

1.3. QC: Agilent HS DNA Assay

2. End Repair

Solution	1 rxn (µL)
End-Repaired gDNA	60
NEB Adenylation Premix	40
Reaction volume	**100**

2.1. Transfer 40 µL of NEB End Repair Premix into wells of a destination plate.

2.2. Transfer 60 µL of sheared & repaired DNA to the brew, mix using 80% volume, 10X.

2.3. Tetrad Program **LIBCOR > ER-** 20^0C for 30 minutes: hold 4°C

2.4. Safe stopping point if stored at -20^oC.

3. Upper/Lower Size Selection

3.1. Ethanol and Magnetic beads must be incubated at room temperature for at least 30 minutes before use.

Upper Cut

DNA volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Supernatant Volume
100	50	120	15	7	150

Lower Cut

Supernatant Volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Ethanol Air Dry Time (mins)	EB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)

Supernatant Volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Ethanol Air Dry Time (mins)	EB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
150	25	140	15	7	5	32	3	2	30

3.2. Note: This is a safe stopping point. Do not proceed to adenylation unless you have adequate time to perform ligation reaction as well.

4. A-Tailing

Solution	1 rxn (µL)
End-Repair + BC DNA	30
NEB Adenylation Premix	20
Reaction volume	**50**

4.1. Transfer 20 µL of NEB Adenylation Premix to 30 µL of size selected and repaired/phosphorylated DNA.

4.2. Tetrad Program: LIBCOR>ATAIL-37ºC for 30 minutes; 70ºC for 5 minutes; 4ºC for 5 minutes; 4ºC hold.

4.3. Proceed directly to in-tandem ligation (**do not bead clean after Adenylation**). Store on ice while preparing Ligation premix and adapters.

5. Adapter Ligation

Solution	1 rxn (µL)
Adenylated template	**50**
NEB Ultra Premix 2X	21
TruSeq Adapter, 6.25uM	4
Reaction volume	**75**

5.1. Transfer Template to single use adapter plate containing 4uL of TruSeq adapter per well.

5.2. Transfer 21µL of ligation brew to 54 µL of template plus index adapter.

5.3. Reset pipette to 80% total volume, mix 10X.

5.4. Select tetrad program: LIBCOR>LIGATION- 20^0C for 15 minutes: hold 4°C

5.5. Set a timer for 15 minutes. Quick spin plate and store on ice immediately after the 15 minute ligation.

6. Double Bead Clean post Ligation (1:1)

Bead clean #1

DNA volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	2x 70% EtOH Wash Vol (µL)	Ethanol Air Dry Time (mins)	EB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
75	75	120	15	7	150	5	52	3	2	50

Bead clean #2

DNA volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	2x 70% EtOH Wash Vol (µL)	Ethanol Air Dry Time (mins)	EB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
50	50	80	15	7	150	5	20	3	2	19

6.1. The ligated template can be stored at -20^{o}C after the first or second bead clean up step.

7. qPCR.

Libraries were quantified using the KAPA Library Quantification kit.

Supplementary Figure Legends

Supplementary Figure 1. Majority of SSARs involve non-contiguous sequences that map within 500 bases from each other. SSARs were identified by searching for reads whose alignments to the reference genome were broken into two or more disparate segments with at least two segments aligning 1) on opposite strands and 2) within a pre-defined maximum distance of each other. The SSAR artifacts were identified as the subset of these multiple strand split reads whose segments align within the indicated gaps in bp of each other (Y-axis). From here, it can be observed that a distance threshold of 500 bp allows the detection of ~80% of SSARs.

Supplementary Figure 2. Levels of SSAR and unpaired reads are largely non-overlapping. SSAR levels are shown as percentages of total number of all aligned reads, total number of properly paired (PP) reads and total number of improperly paired reads.

Supplementary Figures 3-7. Effect of extraction protocols on FFPE library quality. Data from indicated metrics were plotted for libraries that were derived from fresh-frozen DNA (FF), for libraries that were prepared from nucleic acid that was extracted using the FormaPure protocol (FFPE F) and the Allprep/HighPure protocol (FFPE A-H).

Supplementary Figures 8. Differences between the the FormaPure (FFPE F) and the Allprep/HighPure (FFPE A-H) extraction protocols. Note that the FormaPure protocol includes longer incubations at higher temperature and is magnetic bead-based (as opposed to the column-based A-H protocol).

Supplementary Figure 9. Measures to improve FFPE library quality and their effects on the levels of paired-end reads, chimeric reads and base error rates. Mouse FFPE scrolls were used as starting materials. 100 ng TNA that was extracted using the FormaPure protocol was used (F). 100 ng DNA that was extracted using the Qiagen/HiPure protocol was used with (A-H). Additional input conditions included FormaPure extracted DNA that was stringently size selected (F+ Stringent SS) using 0.7:1 (beads to sheared nucleic acid ratio) as opposed to standard size selection with 1:1 beads to sheared nucleic acid as well as FormaPure extracted nucleic acid that was treated with S1 nuclease (F+S1). Error bars=Standard deviations.

Supplementary Figure 10. Effects of S1 nuclease treatment on the levels of duplicate rates. . 100 and/or 300 ng TNA extracted using the FormaPure protocol was used with (F+S1) or without (F) S1 nuclease treatment. 100 ng DNA extracted using the Qiagen/HiPure protocol was used with (A-H+S1) or without (A-H-S1) S1 nuclease treatment. N=5 (FFPE samples from 5 patients). Of note, these samples were not patient-matched between the extraction protocols (A-H and F). Error bars=Standard deviations.

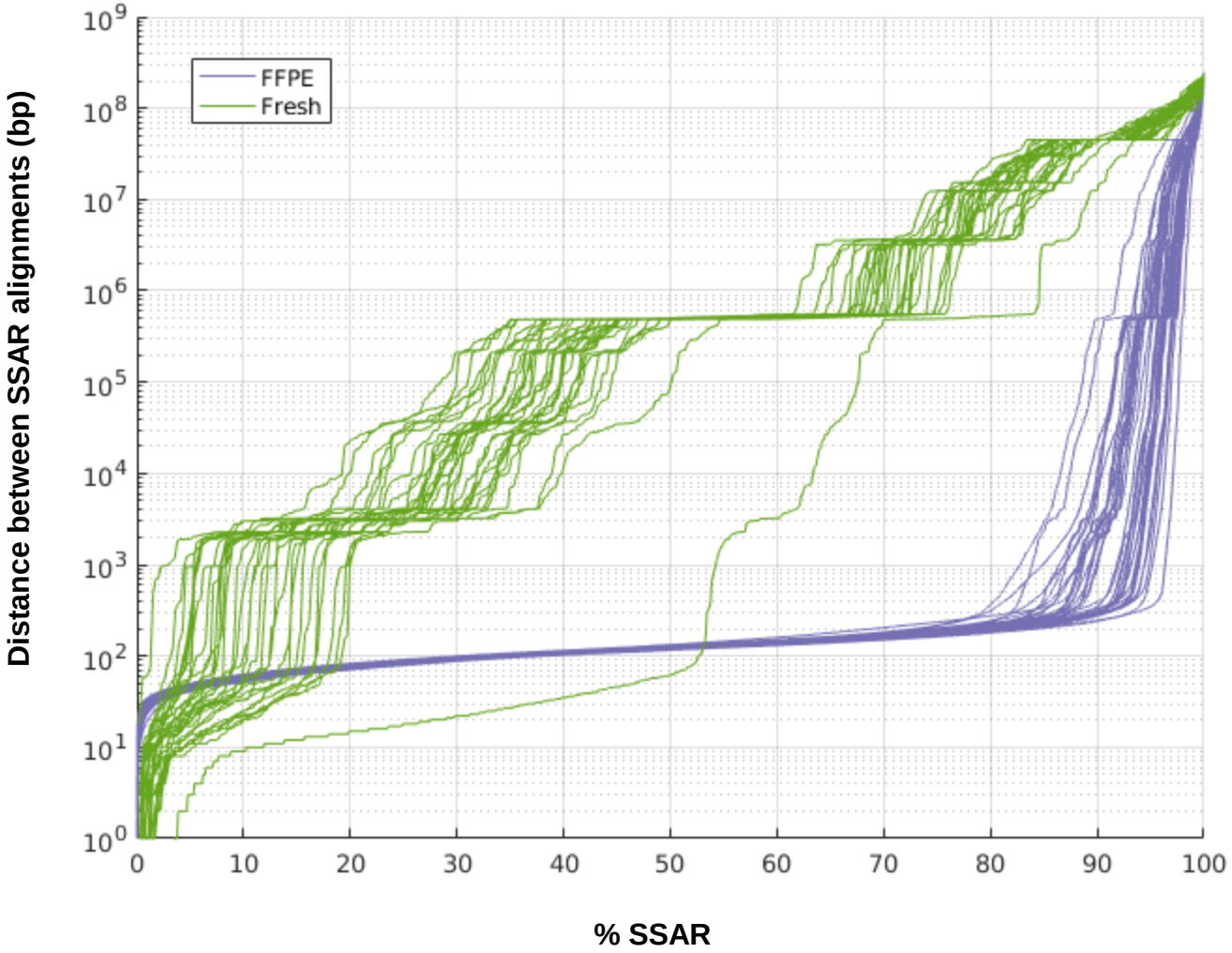
Supplementary Figure 11. Effects of S1 nuclease treatment on deep sequencing metrics. Various metrics are shown for libraries that were prepared from fresh normal and tumor samples, and matching FormaPure FFPE samples with (F+S1) or without (F-S1) S1 nuclease treatment.

Supplementary Figure 12. Comparisons of single nucleotide variant allelic frequencies (VAFs). Libraries that are being compared are from fresh-frozen (FF) tumor samples, and matching FormaPure FFPE tumor samples with (F+S1) or without (F-S1) S1 nuclease treatment. Single nucleotide VAFs shown with a QSS score ≥ 15 (left panel) and QSS score ≥ 35 (right panel).

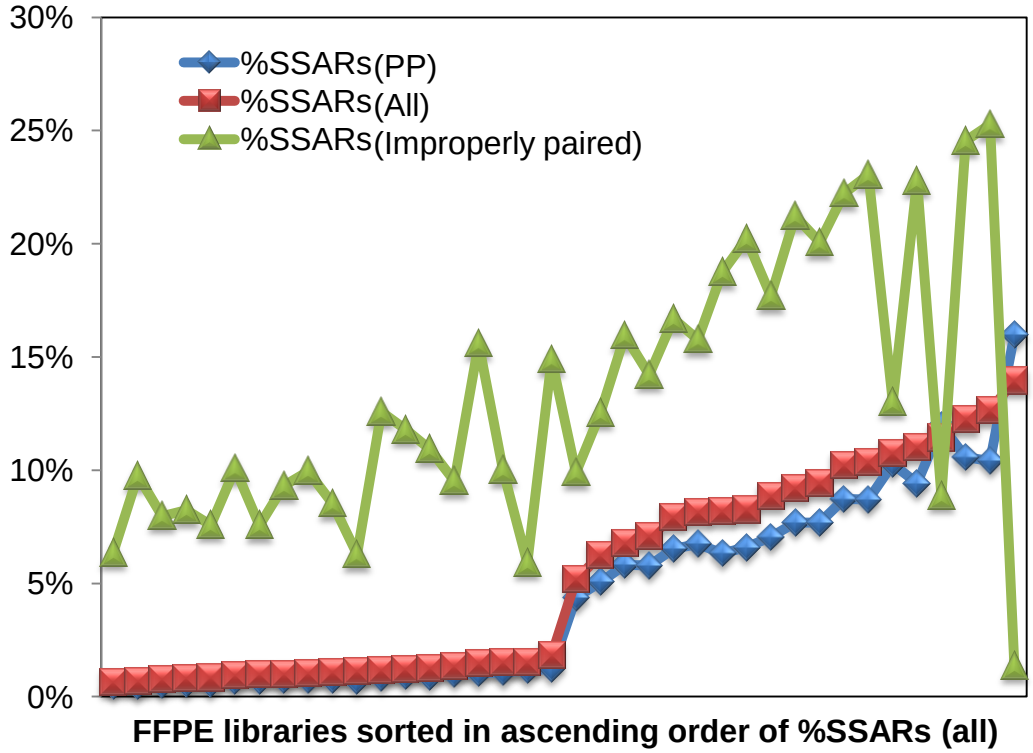
Supplementary Figure 13. Effects of S1 nuclease treatment on FFPE-associated single base INDELs. Libraries were prepared from fresh normal and tumor samples from the same patient, and matching FormaPure FFPE samples with (F+S1) and or without (F-S1) S1 nuclease treatment. For each of the three latter libraries, INDELs were identified relative to the library from the normal blood sample and a Venn diagram representing the resulting data is shown.

Supplementary Figure 14-16. Effects of S1 nuclease treatment on FFPE-associated copy number variants (CNVs). Libraries were prepared from fresh normal and tumor samples from the same patient, and matching FormaPure FFPE samples with (F+S1) and or without (F-S1) S1 nuclease treatment. For each of the three latter libraries, CNVs were identified relative to the library from the normal blood sample and a Venn diagram representing the resulting data is shown. CNV segments in a bin size of 200 reads were calculated in the tumor samples relative to the normal blood sample and the resulting profiles are shown for all chromosomes in Supplementary Figure 14 (Fresh-frozen), Supplementary Figure 15 (FFPE -S1), and Supplementary Figure 16 (FFPE +S1).

Supplementary Figure 17. Comparision of S1 nuclease treatment of FormaPure extracted nucleic acid with DNA extracted Qiagen/HiPure protocol (Q-H). Data from F+S1 was compared to a range of data obtained for libraries that were prepared from DNA that was extracted using the Qiagen/HiPure protocol (Q-H) for the indicated metrics.

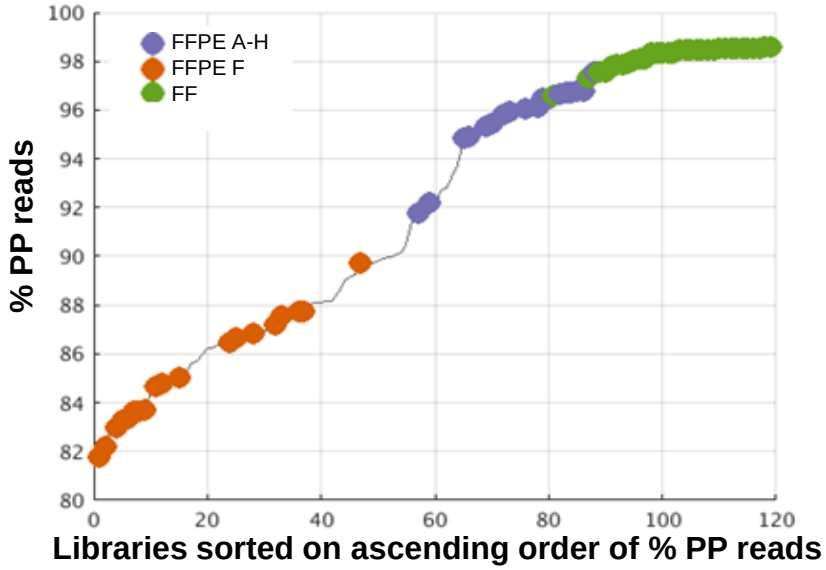


Supplementary Figure 1

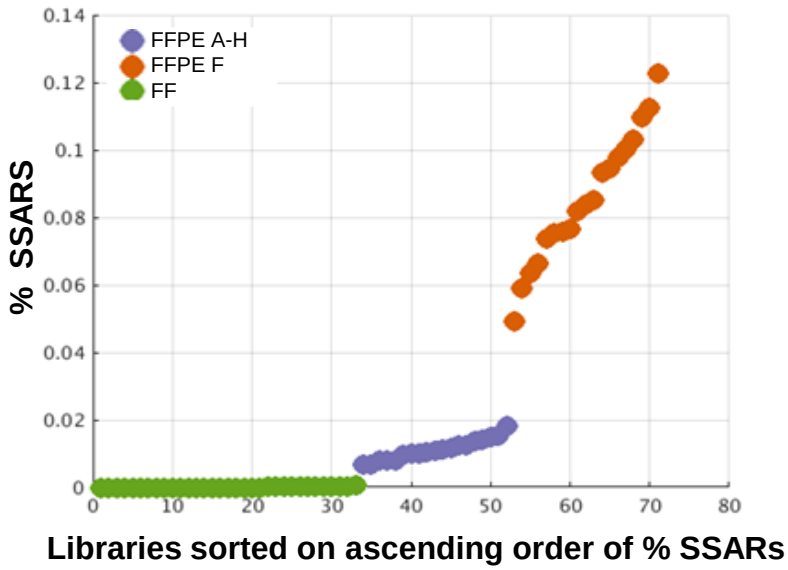


Supplementary Figure 2

A.

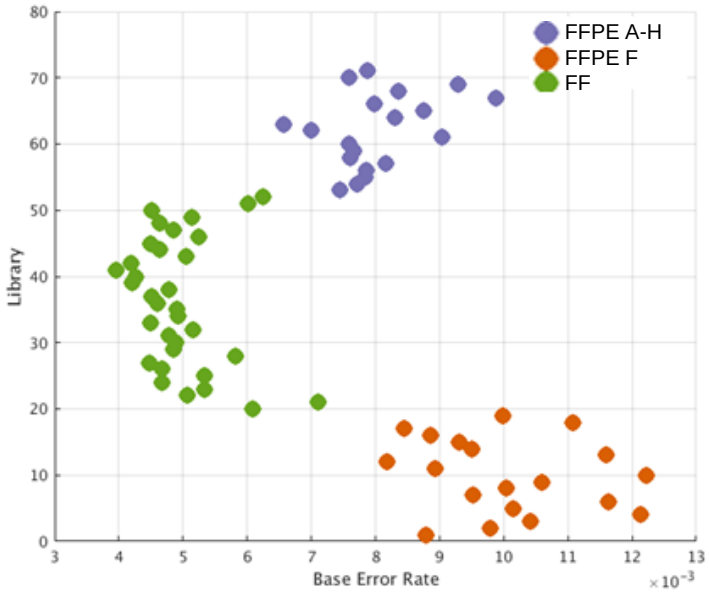


B.

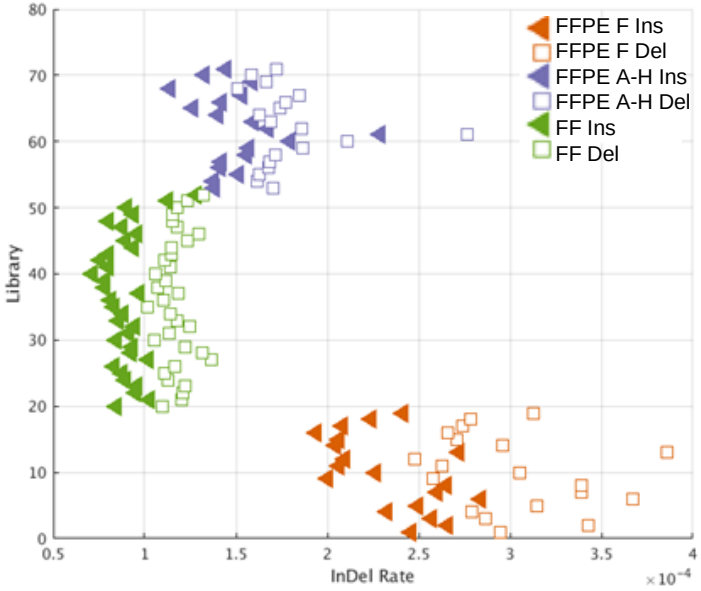


Supplementary Figure 3

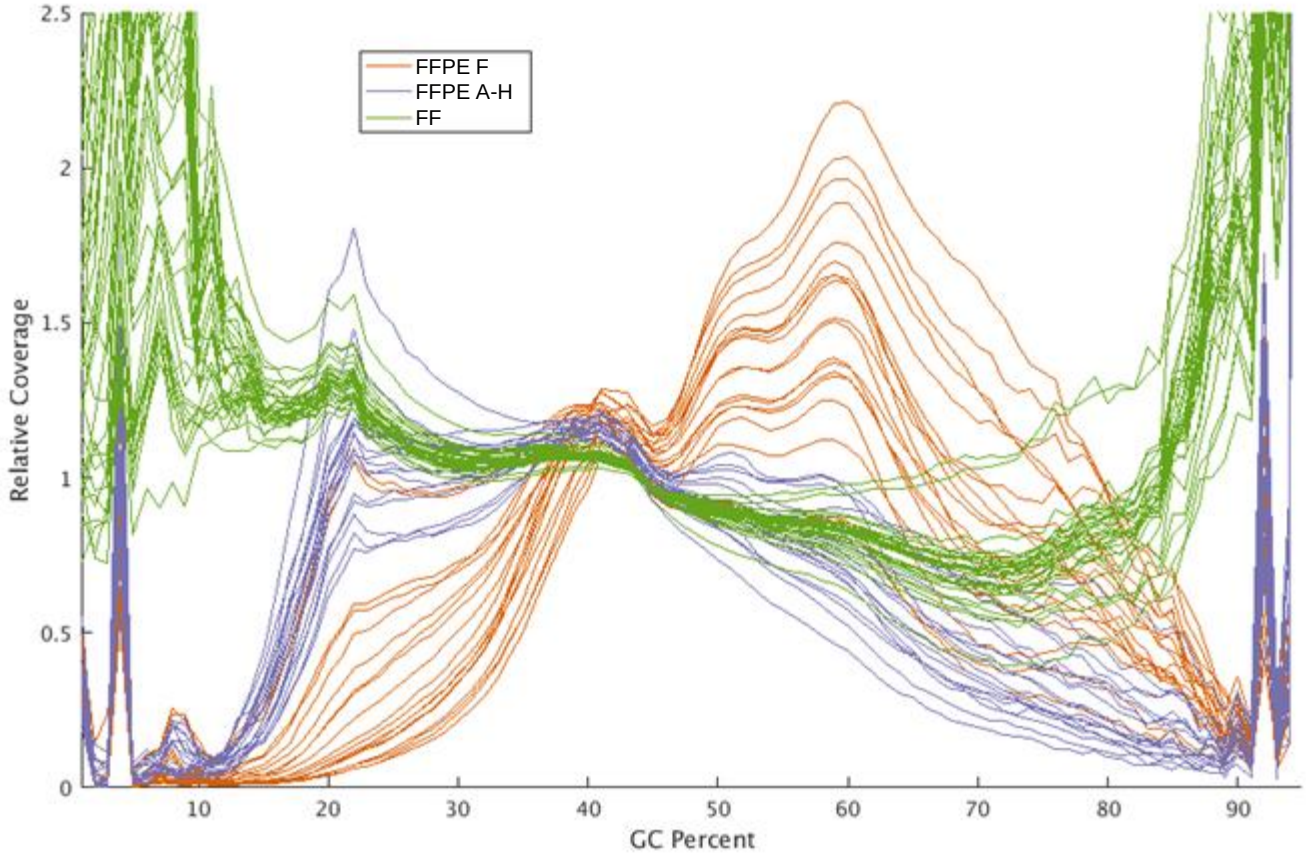
A.



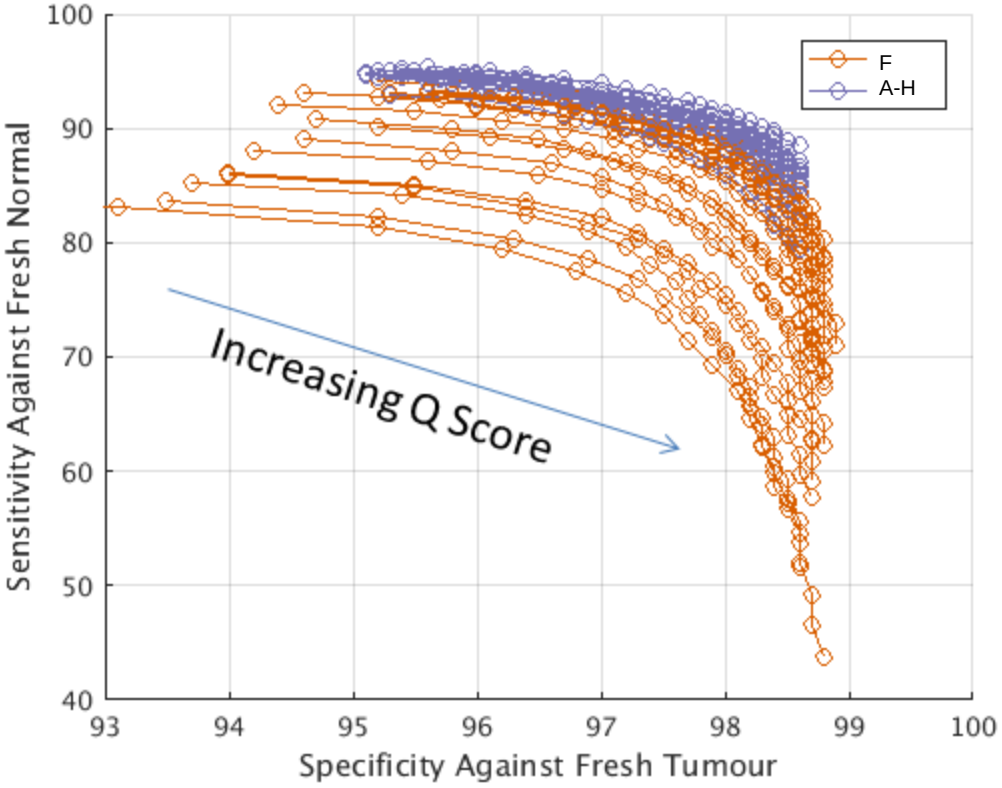
B.



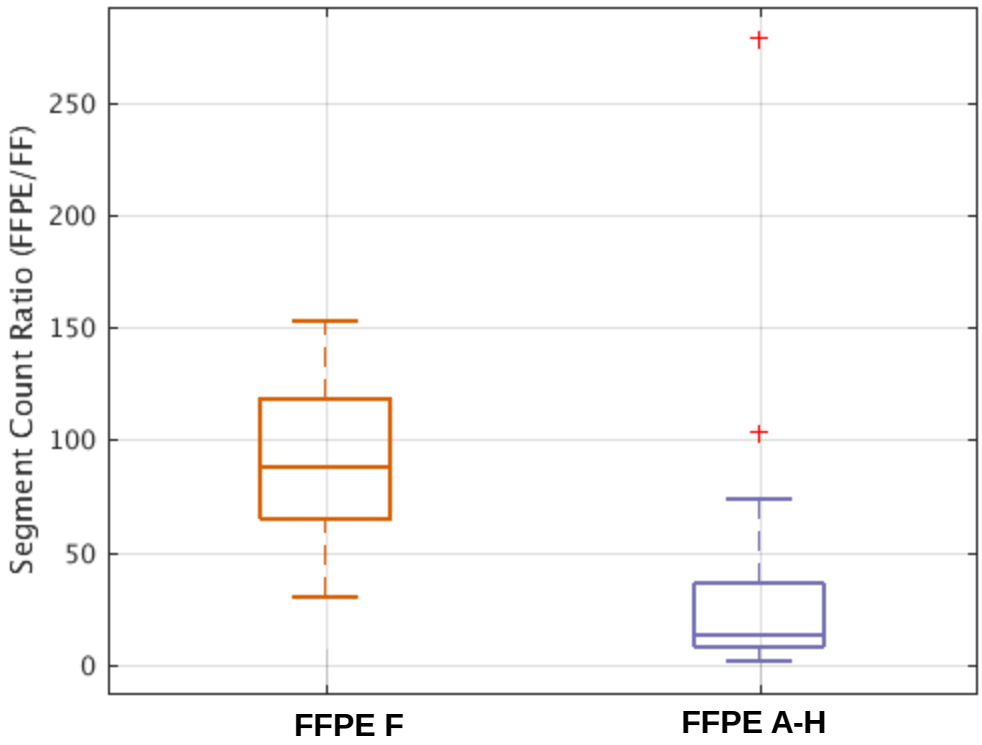
Supplementary Figure 4



Supplementary Figure 5



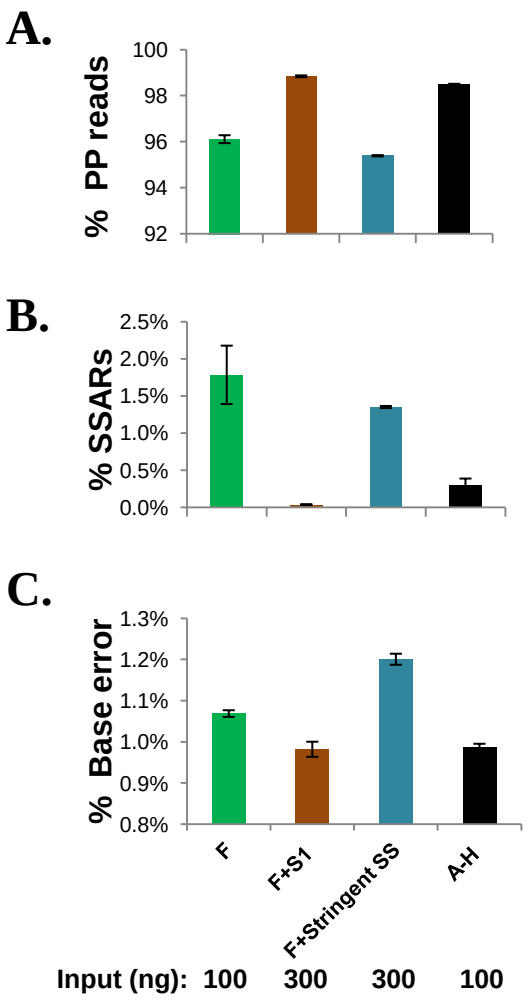
Supplementary Figure 6



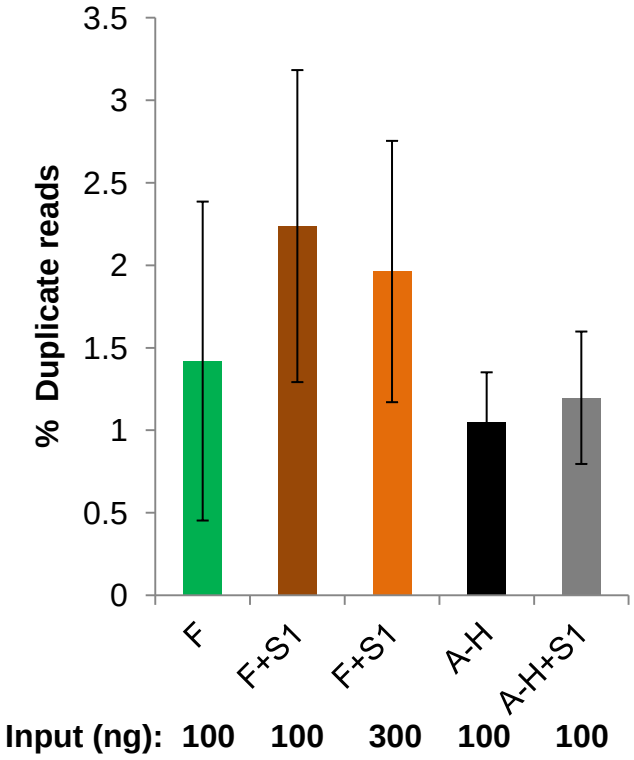
Supplementary Figure 7

Step	A-H	Modified-FormaPure
Deparaffinization	Heptane; Methanol	Heat (2hr@70) ^{O}C
Lysis buffer	HiPure + SDS	Agencourt
Proteinase K In respective lysis/special buffer	45 min (RNA&DNA) +60min (DNA) @56^{O}C	Overnight(RNA&DNA) @55^{O}C
Reverse crosslink In respective lysis/special buffer	2hr@90^{O}C	2hr@90^{O}C
Nucleic acid purification	Column	Magnetic beads
Retention of <75 bp DNA fragments	No	Yes
RNA and DNA partitioning?	Yes	No

Supplementary Figure 8



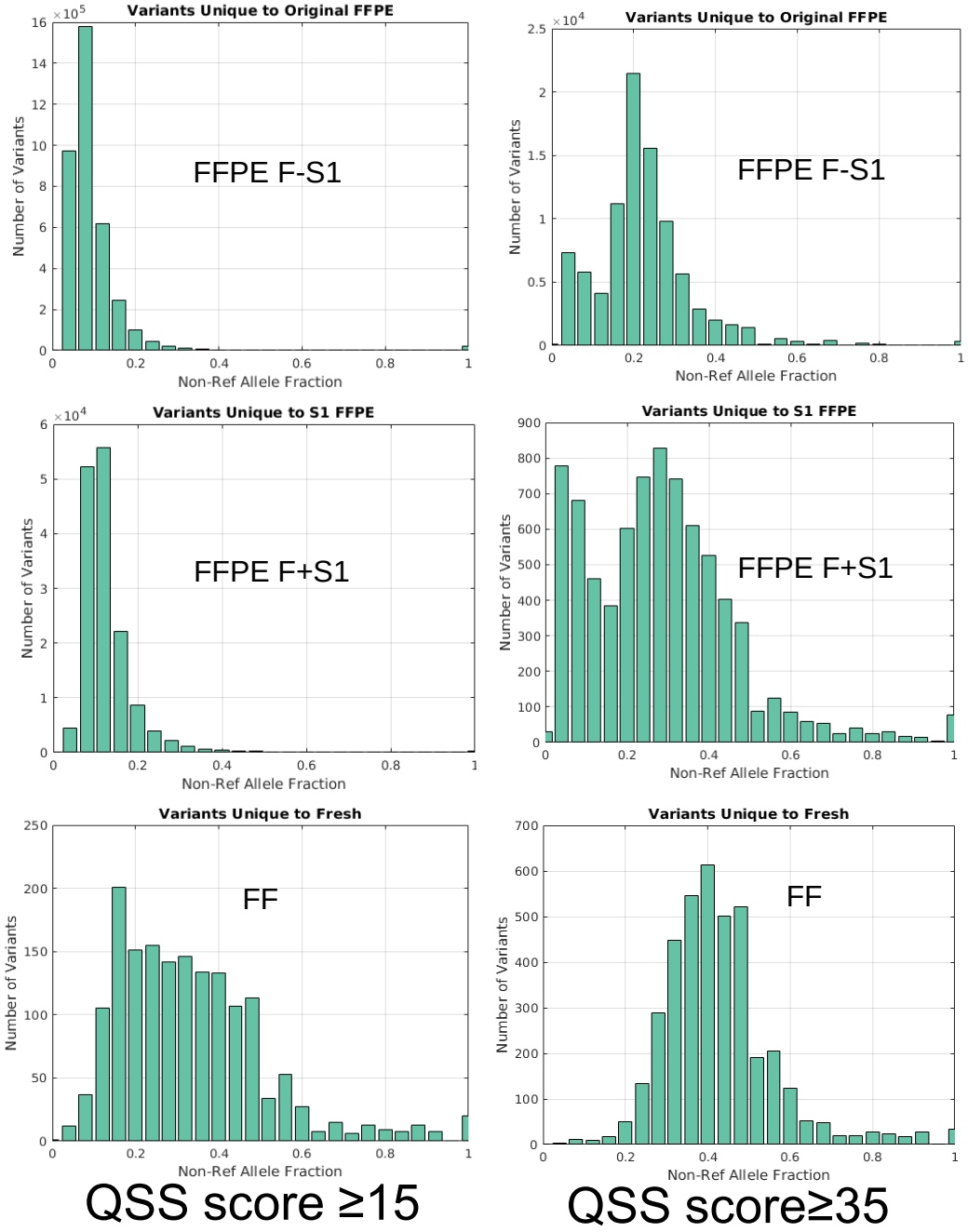
Supplementary Figure 9



Supplementary Figure 10

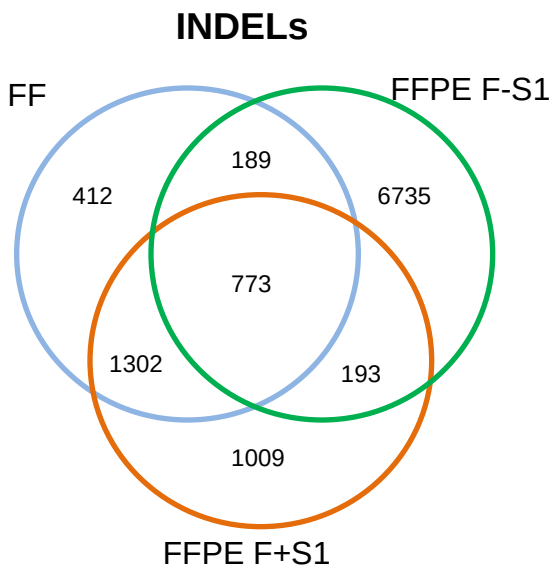
	Fresh Tumour	Fresh normal	FFPE F	FFPE F+S1
Sequenced on:	HiSeq 2500	HiSeq 2500	HiSeq X	HiSeq X
# Reads	613405720	1243367554	943013536	977846050
% chastity failed	8.458	9.67	0	0
Coverage of genome	95.54	48.19	54.83	33.37
%aligned	99.668	99.622	99.854	99.891
% PP reads	98.39	98.287	89.683	98.019
% uniquely aligned	94.884	94.732	86.683	93.147
Mean Insert Size	418	438	157	181
X coverage estimate	23.612928	48.198601	31.505802	33.374752
read length	125	125	157	150
general error rate	0.008	0.0081	0.0132	0.0059
mapped reads with insertion percentage	1.25	1.22	2.2	1.27
mapped reads with deletion percentage	1.51	1.47	2.71	1.51
% stand-split artifact reads	0.	0	6.4	0.5

Supplementary Figure 11

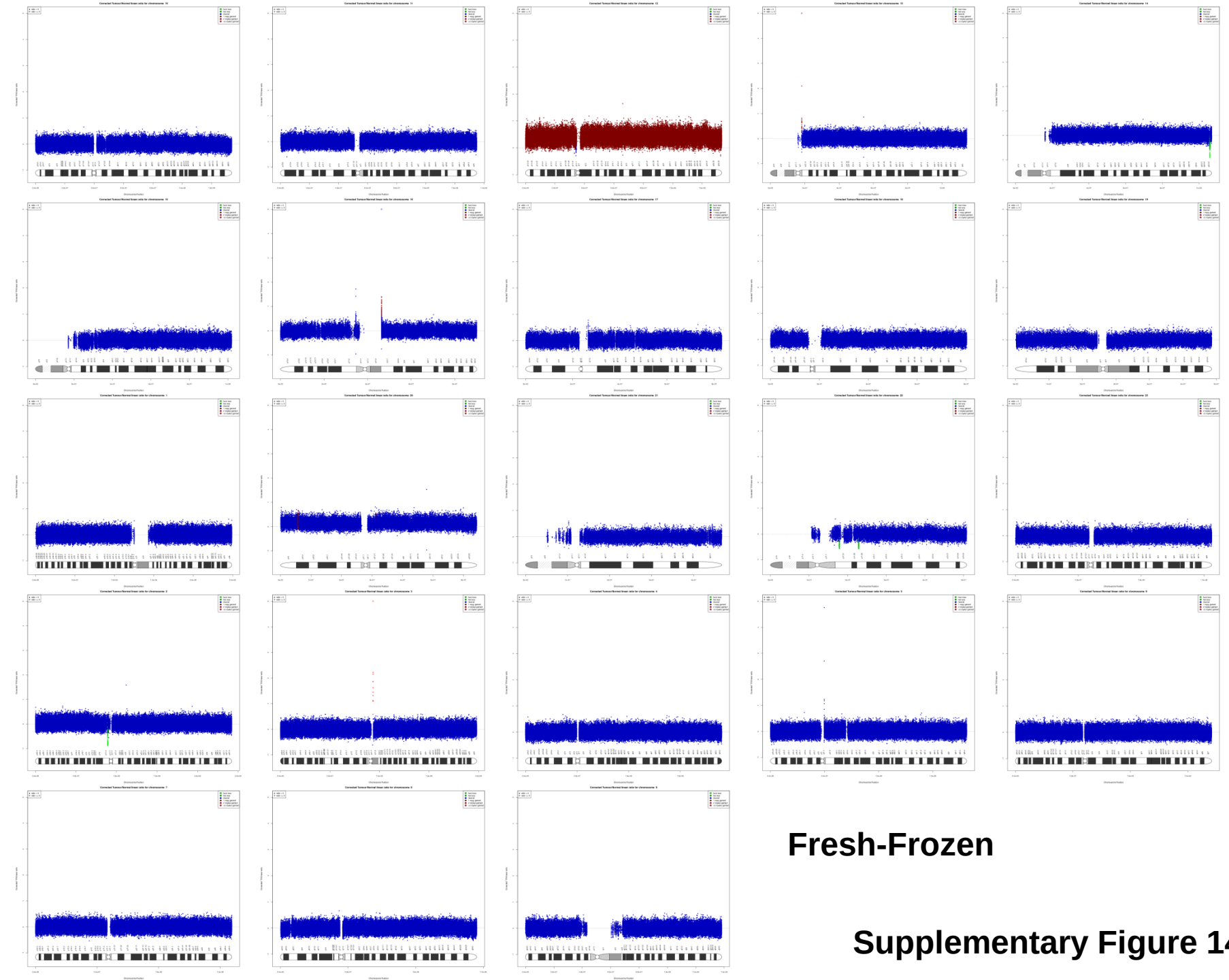


QSS score ≥15 QSS score≥35

Supplementary Figure 12

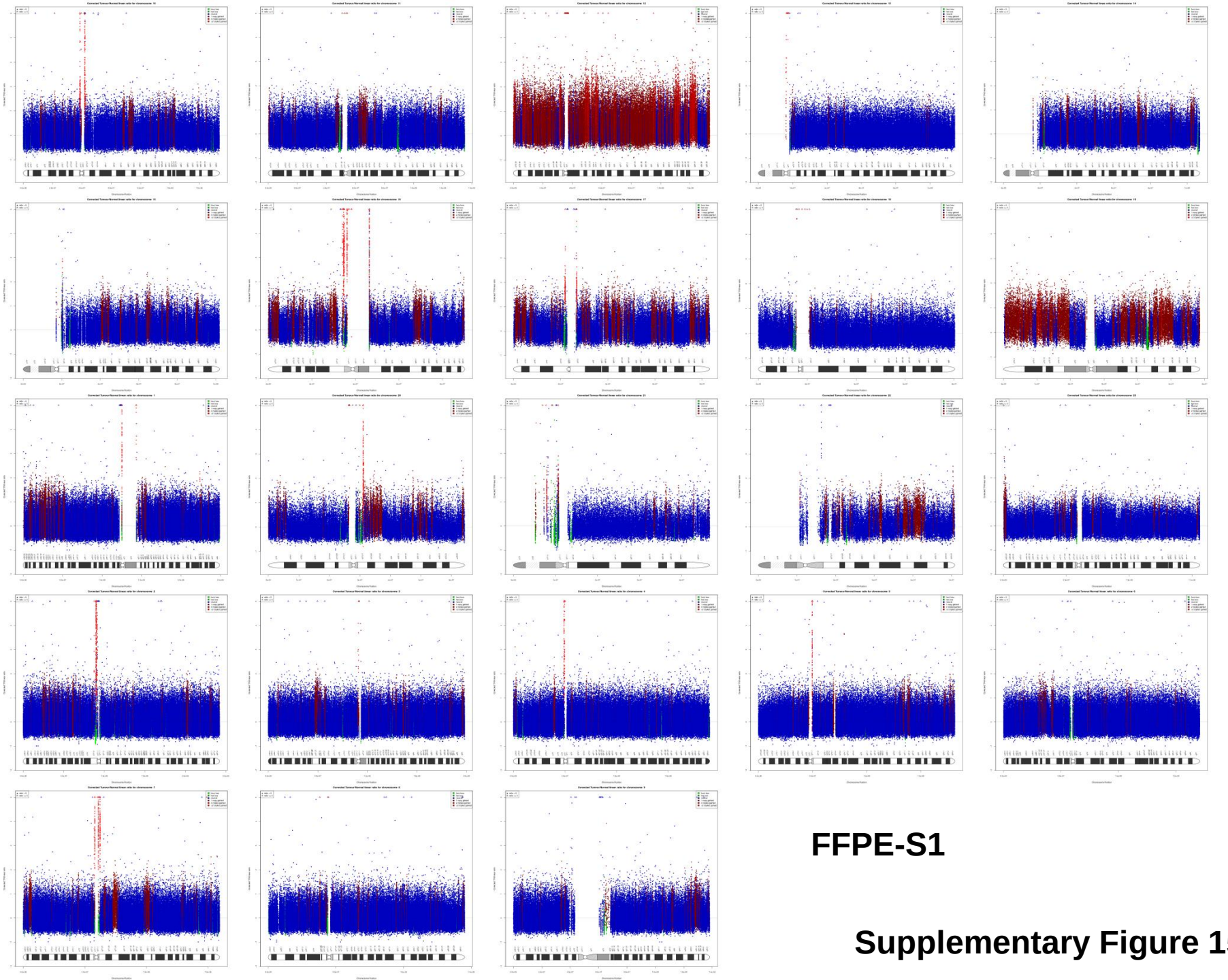


Supplementary Figure 13



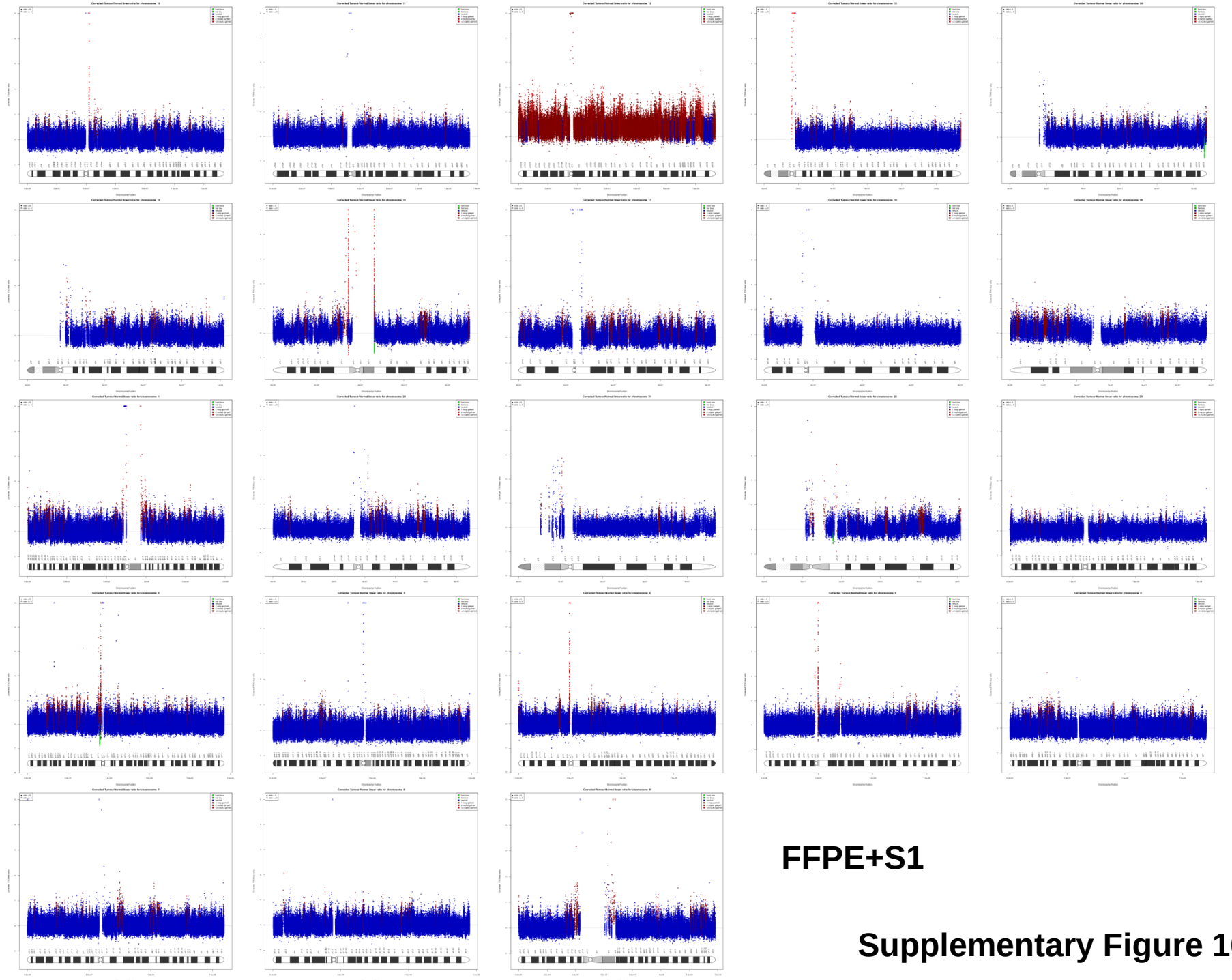
Fresh-Frozen

Supplementary Figure 14



FFPE-S1

Supplementary Figure 15



FFPE+S1

Supplementary Figure 16

	FFPE A-H Range (n=15)	FFPE F+S1 nuclease
# Reads	1652- 2005 million	978 million
Coverage of genome	74.1-90.0	33.37
Mean insert (bp)	200-260	181
% PP reads	92-98	98
% SSAR	0.7-2	0.5
GC bias	S1 slightly better at low GC	
% General Base Error Rate	0.7-1.0	0.6
CNV # segments (#FFPE/#Fresh)	10-40	35.8
% Somatic SNVs unique to FFPE	88-99.9	87.9
% Somatic Indels unique to FFPE	24-60	36.7

Supplementary Figure 17