NIPPON Genetics Co.,Ltd.

Customer feedback on products

Product Name : KAPA HTP Library Preparation Kit (KK8234)
Manufacturer : KAPA BIOSYSTEMS
Application : Sequence analysis of small input-volume formalin-fixed paraffin-embedded (FFPE) tissue samples in whole-exome sequencing (WES) using the SureSelect target enrichment system

Introduction

Next-generation sequence analysis of genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples is expected to play a very important role as an application, especially in the field of clinical research. However, it is also a well- known fact that this type of analysis is very difficult to perform due to such causes as:

- Degradation of DNA (e.g., fragmentation and/or substitution of cytosine to uracil due to hydrolysis)
- Protein-DNA cross-linking through formaldehyde
- Low efficiency of DNA extraction (only small amounts of DNA available)

In general, it is difficult to prepare an enough volume of library for sequence analyses when using genomic DNA from FFPE. This will result in higher duplication rates and thus insufficient coverage and lower sequence depth.

As a solution, KAPA BIOSYSTEMS Library Preparation Kit was used combined with a SureSelect target enrichment system (Agilent Technologies, Inc.) and the application resulted in a very promising improvement in Whole-exome sequencing (WES) analyses of FFPE tissue samples as shown in the example below.

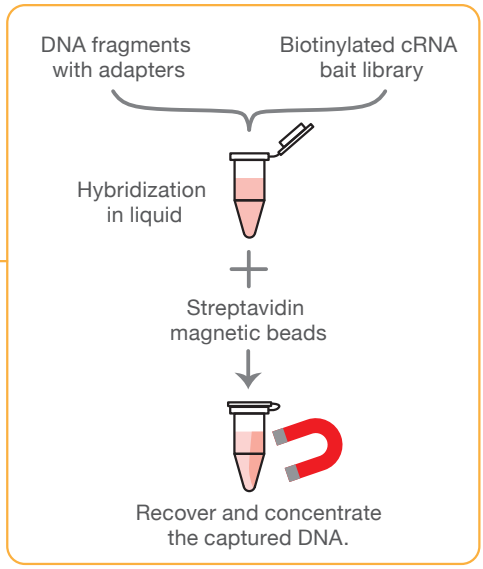
The following data were provided by the courtesy of Dr. Kota Ouchi of the Dr.Aburatani Lab for the Systems Biology of Cancer Regulatory Genomics Translational Research, the Research Center for Advanced Science and Technology, the University of Tokyo, Japan.

Workflow of the WES library preparation using KAPA BIOSYSTEMS Library Preparation Kits and SureSelect target enrichment system (Agilent Technologies, Inc.)

Extraction of genomic DNA — ①
↓
Size distribution check with Agilent Bioanalyzer
↓
Fragmentation of genomic DNA — ②
↓
End-repair、dA-tailing、Adapter-ligation — KAPA BIOSYSTEMS KAPA Library Preparation Kit
↓
Purification with AMPureXP
↓
Library amplification (pre-capture) — KAPA BIOSYSTEMS KAPA Library Preparation Kit
↓
Purification with AMPureXP
↓
Size distribution check and validation of concentration with Agilent Bioanalyzer
↓
SureSelect Library Capture
↓
Library amplification (post-capture) — KAPA BIOSYSTEMS KAPA Library Preparation Kit
↓
Purification with AMPureXP
↓
Size check and concentration validation with Agilent Bioanalyzer
↓
Library pool
↓
Next-generation sequence (illumine Hiseq2500)

① Deparaffinization of the FFPE samples and other pretreatments were all conducted using the QIAamp DNA FFPE tissue Kit (QIAGEN).

② Fragmentation by Covaris was performed principally under the conditions recommended in the SureSelect protocol.
Note: Please refer to Supplemental Information on Protocol Changes (1).



Notes
- Detailed information on sample type, volume of first sample, library preparation kit used, and DNA capture kit is described in the order of the Experiment 1 and experiment 2.
- Please refer to the Supplemental Information on Protocol Changes for the changes made on the protocol.

Experiment 1

<Method>

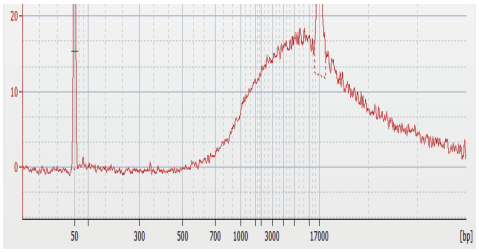
In hepatocellular carcinoma (HCC) tissue samples (Cases A and B), 1 µg each of DNA was taken from frozen specimens and FFPE samples using the conventional method, while 50 ng of DNA was taken from FFPE samples using KAPA Library Preparation Kit (KK8201) and prepared for library. The data taken from WES were compared.

	Sample	Starting DNA	Library Preparation Kit	PrePCR cycle	Capture kit	On-bead PCR
Frozen-SS	Frozen specimens	1ug	Conventional method (SureSelect)	6cycle	SureSelect Human All Exon v4	No
FFPE-SS	FFPE	1ug	Conventional method (SureSelect)	8cycle	SureSelect Human All Exon v4	No
FFPE-KP	FFPE	50ng	KAPA Library Preparation Kit*	10cycle	SureSelect Human All Exon v5	No

*SureSelect adapter oligo and PCR primer were used.

Size distribution of ordinary genomic DNA

Control

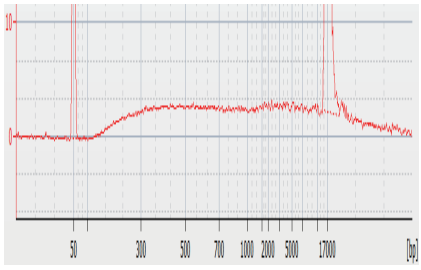


Peak is observed at around 10 kbp

Note: Bioanalyzer is used in this experiment for comparison with degraded DNA. In general, bioanalyzer is not recommended for measurement of intact genomic DNAs.

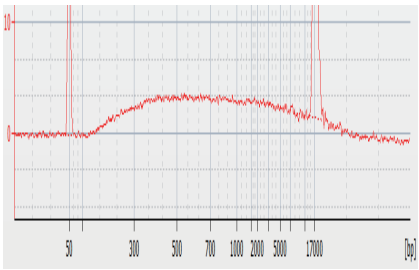
Size distribution of genomic DNA extracted from FFPE samples

Case A



Peak by degradation is observed between 300 bp and 1,000 bp.
Embedding period was 4.5 years.

Case B



Peak by degradation is observed between 400 bp and 1,000 bp.
Embedding period was 1 year.

<Results>

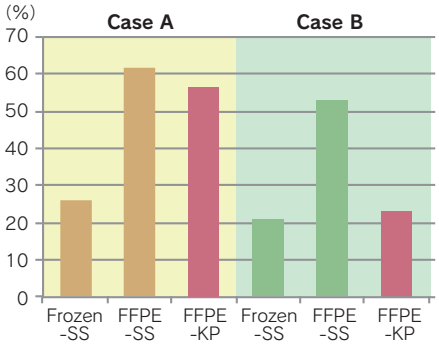
Sequence data

		trimmed count	mapped count	mapped %	Duplicate %	covered roi %
Case A	Frozen-SS	157,315,593	153,914,267	97.8379	26.1	99.908
	FFPE-SS	59,933,963	53,505,094	89.2734	61.6	99.7247
	FFPE-KP	52,987,579	45,382,310	85.65	56.4	99.9055
Case B	Frozen-SS	160,978,185	157,644,530	97.9291	21	99.9245
	FFPE-SS	93,019,529	86,910,573	93.4326	52.7	99.8339
	FFPE-KP	77,074,404	71,978,395	93.99	23	99.8904

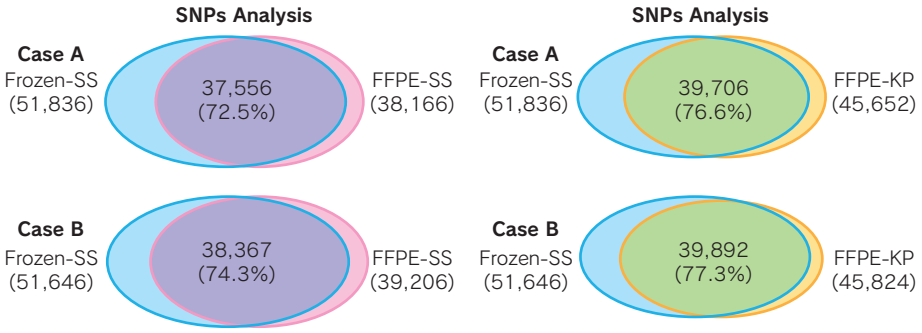
Results from SNP analysis

		Detected SNPs	SNPs common with frozen samples	Rate of SNPs detected with each condition to the SNPs detected from frozen samples (%)
Case A	Frozen-SS	51,836		
	FFPE-SS	38,116	37,556	72.5
	FFPE-KP	45,652	39,706	76.6
Case B	Frozen-SS	51,646		
	FFPE-SS	39,206	38,367	74.3
	FFPE-KP	45,824	39,892	77.3

Comparison of duplication rates (%)



Comparison of results from SNP analysis



Even when the amount of starting DNA was small (50 ng) for KAPA Library Preparation Kit, the rates of duplication were improved and detection sensitivities for SNP were equivalent to the case where 1 µg of DNA extracted from FFPE with the conventional method was used. This suggests that use of the KAPA BIOSYSTEMS Library Preparation Kit will improve the duplication rates and enables sequencing from a minute amount of specimens.

Experiment 2

<Method>

Using 500 ng* each of genomic DNA from FFPE tissue samples of large intestine cancer (CLC) (Case 1 to 12), a library was prepared with KAPA HTP Library Preparation Kit (KK8234) set with the “With-beads” purification protocol and the protocol for SureSelect on-bead PCR method. The data obtained with WES were verified. (* 500 ng of samples were used to ensure library diversity.)

The embedding period for the FFPE tissue samples used in this experiment is 3 years or longer, indicating that the DNA used in this experiment had the same or higher level of degradation compared with the DNA used in the Experiment 1 (data not shown).

Sample	Starting DNA	Library Preparation Kit	PrePCR cycle	Capture kit	On-bead PCR method
FFPE	500ng	KAPA HTP Library Preparation Kit*	8-10cycle	SureSelect Human All Exon v5	Yes

* SureSelect adapter oligo and PCR primer were used.

<Results>

Sequence data

	Total tags	Mapped tags	Nonduplicate tags	Duplicate (%)	Target tags	On target %	Mean Library Size	Mean Depth	More Than ×20%
Case 1	184,766,058	171,778,197	129,085,721	25.21	87,526,498	76.9	155.4	127.8	96.5
Case 2	204,840,798	188,542,887	149,394,760	21.59	83,300,400	70.4	159.2	123.2	96.2
Case 3	114,490,282	110,015,026	55,922,981	49.44	33,981,113	74.49	161.2	43.1	88.4
Case 4	152,532,920	144,823,494	89,132,452	38.87	51,815,419	74.97	151.1	70.9	92.1
Case 5	178,781,216	170,379,240	153,875,995	10.15	116,573,202	83.05	174.4	156.9	97.2
Case 6	168,582,548	157,733,747	127,413,273	19.9	76,895,366	81.67	148.7	104.9	94.4
Case 8	186,252,298	176,849,882	157,869,391	11.28	115,975,702	83.5	168.2	163.2	96.6
Case 9	196,300,966	186,665,363	171,852,539	8.43	128,258,976	82.11	183.3	171.5	97.7
Case 11	178,567,150	167,004,784	120,801,998	27.99	86,365,830	79.99	170.7	124.2	96
Case12	233,082,164	215,238,751	159,250,534	26.55	100,484,330	73.58	172	137.4	97.4

Duplication rates were significantly improved in all cases, and as a result sufficient depths were successfully ensured.
When the SureSelect Human All Exon V4 was used along with the protocol before adopting the on-bead PCR method, as well as KAPA Library Preparation Kit was not used, the mean depth of 20 to 60 was obtained (data not shown).

KAPABIOSYSTEMS

KAPA HTP Library Preparation Kit

The kit includes Illumina sequencer library preparation and 4 kinds of enzymes (End-repair, dA-tailing, Adapter-ligation, and Library-amplification) and an improved protocol for AMPureXP purification after each enzymatic reaction.
The original “With-beads” protocol allows End-repair, dA-tailing, and Adapter-ligation in one tube and thus improves DNA recovery rate (PEG/NaCl SPRI Solution needed for the “With-beads” protocol is included in the kit).

Notes:
- Adaptor and primer are not included in the kits.

Supplemental Information on Protocol Changes

① Covaris fragmentation of genomic DNA

For the genomic DNA from FFPE, the peak of the extracted DNAs was checked with the bioanalyzer (Agilent Technologies) and fine tuning was applied to the duty cycle of Covaris as needed.
The cycle number for FFPE was reduced from 6 (as suggested in the standard protocol for frozen specimens) to 2–4.

② Volume of Adapter Oligo added at the Adapter Ligation step of the KAPA BIOSYSTEMS Library Preparation Kit

In the conventional protocol for SureSelect, 10 µl of Agilent Adapter Oligo Mix is used for 3µg of starting DNA. When the volume of start DNA is to be reduced, diluted Oligo Mix is used (e.g., 10 µl of diluted (3 x) Oligo Mix is used for 1µg of start DNA).
With KAPA BIOSYSTEMS Library Preparation kit, the reaction conditions are prepared using, for example, 5µl of 3 x diluted oligo mix (i.e., 10µl of 6 x dilution) for 500 ng of start DNA, or 5µl of 30 x diluted oligo mix (i.e., 10µl of 60 x dilution) for 500 ng of start DNA.

Adapter Ligation reaction conditions for KAPA BIOSYSTEMS Library Preparation Kit

Ligation Master Mix	45 µl	
Agilent Adapter Oligo Mix*	5 µl	* Diluted according to the volume of the start DNA.
Total volume per well/tube	50 µl	

③ Library Amplification reaction composition for KAPA BIOSYSTEMS Library Preparation Kit (pre-capture amplification)

1.25 µl each of Agilent SureSelect Primer and SureSelect ILM Indexing Pre Capture PCR Reverse primer was used as described in the SureSelect protocol.
The elution volume of the library DNA was increased to 22.5 µl from 20 µl in the previous step of elution to adjust the final total volume to 50 µl.
The peak of the extracted DNA from FFPE was examined with the bioanalyzer (Agilent Technologies) and fine tuning was applied to the duty cycle as needed (8–10 cycle).

Library DNA	22.5 µl
KAPA Library Amplification Master Mix(2x)	25 µl
Agilent SureSelect Primer	1.25 µl
SureSelect ILM Indexing Pre Capture PCR Reverse primer	1.25 µl
Total volume per well/tube	50 µl

④ Library Amplification reaction composition for KAPA BIOSYSTEMS Library Preparation Kit (post-capture amplification)

1 µl each of SureSelect Indexing Post Capture Forward PCR Primer and PCR Primer Index was used as described.
The primer and index were added to KAPA Library Amplification Master Mix. The final composition was as listed below as the Library Amplification Master Mix is originally designed for 2 x dilution.

Library DNA*	23 µl	
KAPA Library Amplification Master Mix(2x)	25 µl	*For the on-bead PCR method, 23 µl of Nuclease Free Water was added to a tube containing beads absorbed with Library DNA.
Indexing Post Capture Forward PCR Primer	1 µl	
PCR Primer Index(1～96)	1 µl	
Total volume per well/tube	50 µl	

<Customer's comments>

In recent years, large-scale parallel sequencing using fresh frozen samples has been generating a large number of findings in genetic mutation occurring in the cancer cells. At the same time, the restriction regarding frozen specimens, such as the limited number of facilities and the difficulty to ensure enough numbers of specimens, has become an urgent issue.
On the other hand, the formalin-fixed paraffin-embedding (FFPE) is a most common and standardized treatment method used for pathological diagnoses of cancer, and therefore many medical institutions already own FFPE specimens. If FFPE specimens can be used for parallel sequencing, genetic information with detailed clinical information can become readily available from the massive volume of samples stored throughout the world. However, genomic sequencing with FFPE specimens has been known to be very difficult due to the chemical modification and degradation of DNA caused by formalin fixation.
When we conducted whole-exome sequencing (WES) with the conventional method using DNA extracted from FFPE tissues, we faced with a problem of significantly high duplication rates (60–80%). Higher duplication rates often prevent us from obtaining enough coverage and depth, making the analysis difficult or impossible.
To solve this problem, we used KAPA HTP Library Preparation Kit in conjunction with the on-bead protocol for Agilent SureSelect Human All Exon to successfully improve the duplication rates (approx. 8–50), and therefore the coverage and depth. This examination demonstrated that WES can be successfully performed even when only a minute volume of specimens is available. This is a very important improvement for studies using FFPE specimens, for which obtaining stable yields of DNA is difficult.

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