Customer feedback on products

<table>
<thead>
<tr>
<th>Product Name</th>
<th>KAPA Hyper Prep Kit (KK8500, KK8502)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>KAPA BIOSYSTEMS</td>
</tr>
<tr>
<td>Application</td>
<td>Exome sequencing from small input-volume of FFPE genome DNA (50ng) using SureSelect XT (Agilent Technologies, Inc.)</td>
</tr>
</tbody>
</table>

All data here are available by courtesy of Dr. Hidewaki Nakagawa and Dr. Kazuhiro Maejima, Laboratory for Genome Sequencing Analysis, RIKEN Center for Integrative Medical Sciences, Japan. We are deeply grateful for their cooperation.

**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.

In the study reported here, WES (Whole-exome sequencing) analysis of FFPE tissue samples were conducted by combining the SureSelect XT target enrichment system (Agilent Technologies) with KAPA Hyper Prep Kit, and very promising improvement was obtained.

**Workflow of WES library preparation using KAPA Hyper Prep Kit and SureSelect XT target enrichment system (Agilent Technologies, Inc.)**

1. Extraction of genomic DNA (Pretreatment, including deparaffinization of FFPE samples, and DNA extract were conducted using QIAamp DNA FFPE Tissue Kit)  
   Size distribution check with Agilent TapeStation or Bioanalyzer

2. Fragmentation of genome DNA

3. End-repair, dA-tailing

4. Adapter-ligation

5. Purification with AMPureXP

6. Library amplification (pre-capture)

7. Purification with AMPureXP

8. Size distribution check and validation of concentration with Agilent TapeStation or Bioanalyzer

9. Concentration of pre-capture PCR product

10. SureSelect Library Capture

11. Library amplification (post-capture)

12. Purification with AMPureXP

13. Size check and concentration validation with Agilent TapeStation or Bioanalyzer

14. Library pool

15. Next-generation sequence (illumina HiSeq2500)
Production of Pre-capture PCR product from FFPE genomic DNA using KAPA Hyper Prep Kit and SureSelect Adapter

1. Preparation of FFPE genomic DNA
   Measurement of concentration by Picogreen
   Quality evaluation of FFPE DNA by qPCR
   Preparation of 50 ng to 200 ng of FFPE genomic DNA to make 50 µL (using 1x Low TE to increase the volume)

2. Covaris fragmentation
   Fragmentation of DNA using Covaris according to the protocol for SureSelect XT Kit
   Cycle setting: 3 cycles (60sec x 3 cycles; Total 180sec)

   Example: Setting for Covaris S2
   - Duty Cycle: 10%
   - Intensity: 5
   - Cycles per Burst: 200
   - Time: 60sec, 3 cycles
   - Setting mode: Frequency sweeping
   - Temperature: 4°C ~ 7°C

3. End Repair & A-Tailing
   End repair and A-tailing according to the protocol for KAPA Hyper Prep Kit

   Reaction composition
   - Fragmented DNA: 50 µL
   - End Repair & A-Tailing Buffer: 7 µL
   - End Repair & A-Tailing Enzyme: 3 µL
   - Total Volume per well: 60 µL

4. Adapter Ligation
   Adapter ligation according to the protocol for KAPA Hyper Prep Kit

   Reaction composition
   - End Repair & A-Tailing reaction Product: 60 µL
   - Nuclease-Free Water (*): 5 µL
   - Ligation Buffer: 30 µL
   - DNA Ligase: 10 µL
   - SureSelect Adapter Oligo Mix (*): 5 µL
   - Total Volume per well: 110 µL

   * Adapter may be undiluted, diluted or increased depending on the quality of FFPE gDNA. When adapter is increased, Nuclease-free water should be decreased.

5. Post-Ligation Clean up
   Cleanup according to the protocol for KAPA Hyper Prep Kit
   For elution, 20 µL of nuclear-free water should be used.

6. Library Amplification (PreCapture PCR)
   PCR conducted after changing the primer volume to 2.5 µL and the annealing temperature to 65°C

   Reaction composition
   - Adapter-Ligated Library: 20 µL
   - 2x KAPA Hi-Fi HotStart Ready Mix: 25 µL
   - SureSelect Primer: 2.5 µL
   - SureSelect ILM Indexing PreCapture PCR Reverse Primer: 2.5 µL
   - Total Volume per well: 50 µL

   Thermal Cycler settings
   - 20°C: 15min
   - 65°C: 30min
   - 4°C: ∞
   - 1 cycle
   - The HotTop of the thermal cycle should be set to OFF.

   Thermal Cycler settings
   - 20°C: 15min
   - 65°C: 30min
   - 4°C: ∞
   - 1 cycle
   - The HotTop of the thermal cycle should be set to OFF.

   * PCR cycle should be optimized according to the initial volume of DNA. SureSelect hybridization requires 750 ng of yield.

7. Post-Amplification Clean up
   Cleanup of PCR product according to the protocol for KAPA Hyper Prep Kit
   Elution conducted with 30 µL of nuclear-free water

8. Evaluation by TapeStation or Bioanalyzer
   Evaluation of pre-capture PCR products by TapeStation D1000 or Bioanalyzer DNA1000

9. Concentration of pre-capture PCR products
   Concentration of 750 ng of pre-capture PCR product (recommended: 750 ng; minimum: 500 ng) using SpeedVac DNA 120 (Thermo Scientific)
   Dissolution with 3.4 µL of nuclear-free water

10. From hybridization to preparation of capture library
    Start with hybridization according to the protocol for SureSelect XT Kit using Agilent SureSelect XT Human All Exon V5 and hybridization reagent

    Process after 10 should be operated according to the protocol for Agilent SureSelect XT kit.
Evaluation of SureSelect XT library

Capture library reagent : SureSelect XT Human All Exon V5

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>DNA quality</th>
<th>Input DNA [ng]</th>
<th>PCR Cycles</th>
<th>Adapter dilution</th>
<th>Pre-capture Yield [ng]</th>
<th>comment</th>
<th>Hybridization amount [ng]</th>
<th>Post-capture Yield [ng]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFPE7_Cancer</td>
<td>Good</td>
<td>50</td>
<td>14</td>
<td>No</td>
<td>1179</td>
<td>2nd peak around 400-1000bp</td>
<td>750</td>
<td>264</td>
</tr>
<tr>
<td>FFPE5_Cancer</td>
<td>Good</td>
<td>50</td>
<td>14</td>
<td>No</td>
<td>1083</td>
<td>2nd peak around 400-1000bp</td>
<td>500</td>
<td>402</td>
</tr>
<tr>
<td>FFPE2_Cancer</td>
<td>Medium</td>
<td>50</td>
<td>14</td>
<td>No</td>
<td>1340</td>
<td>2nd peak around 400-1000bp</td>
<td>500</td>
<td>157</td>
</tr>
<tr>
<td>FFPE3_Cancer</td>
<td>Medium</td>
<td>50</td>
<td>14</td>
<td>No</td>
<td>1694</td>
<td>2nd peak around 400-1000bp</td>
<td>500</td>
<td>122</td>
</tr>
<tr>
<td>FFPE4_Cancer</td>
<td>Medium</td>
<td>50</td>
<td>14</td>
<td>No</td>
<td>1231</td>
<td>2nd peak around 400-1000bp</td>
<td>750</td>
<td>460</td>
</tr>
<tr>
<td>FFPE9_Cancer</td>
<td>Poor</td>
<td>50</td>
<td>14</td>
<td>No</td>
<td>1019</td>
<td></td>
<td>500</td>
<td>114</td>
</tr>
<tr>
<td>FFPE9_Cancer</td>
<td>Poor</td>
<td>200</td>
<td>12</td>
<td>No</td>
<td>634</td>
<td></td>
<td>634</td>
<td>187</td>
</tr>
</tbody>
</table>

* Use original qPCR method for FFPE DNA quality evaluation, not published.

50 ng DNA of FFPE5 (Good quality)

PreCapture PCR Product

Adapter No Dilution Yield: 1083ng

PCR x14

A shoulder is shown.

SureSelect Exome V5 (500~750ng)

Exome Library

Sequencing PCR-duplication

6.6%

Sufficient volume of adapter decreased duplication.

Adapter x15 Dilution Yield: 1850ng

No shoulder is shown.

Reduced volume of adapter increased duplication.

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50 ng DNA of FFPE9 (Poor quality)

PreCapture PCR Product

Adapter No Dilution Yield: 1019ng

PCR x14

SureSelect Exome V5 (500~750ng)

Exome Library

Sequencing PCR-duplication

44.4%

Low quality of DNA increased duplication

Adapter x15 Dilution Yield: 280ng

51.2%

200 ng DNA of FFPE9

Adapter No Dilution Yield: 634ng

PCR x12

23.3%

Even with low-quality DNA, higher input DNA volume and sufficient adapter volume improved duplication.
Results of NGS (interim result)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>DNA quality</th>
<th>Input DNA [ng]</th>
<th>Initial fastq</th>
<th>Mapped</th>
<th>mapping rate</th>
<th>duplication rate</th>
<th>on target rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFPE7_Cancer</td>
<td>Good</td>
<td>50</td>
<td>67,960,522</td>
<td>62,814,748</td>
<td>92.4%</td>
<td>8.2%</td>
<td>66.8%</td>
</tr>
<tr>
<td>FFPE5_Cancer</td>
<td>Good</td>
<td>50</td>
<td>70,034,926</td>
<td>67,040,360</td>
<td>95.7%</td>
<td>6.6%</td>
<td>66.0%</td>
</tr>
<tr>
<td>FFPE2_Cancer</td>
<td>Medium</td>
<td>50</td>
<td>72,517,124</td>
<td>67,819,596</td>
<td>93.5%</td>
<td>14.0%</td>
<td>67.9%</td>
</tr>
<tr>
<td>FFPE3_Cancer</td>
<td>Medium</td>
<td>50</td>
<td>63,595,416</td>
<td>59,910,508</td>
<td>94.2%</td>
<td>15.8%</td>
<td>69.1%</td>
</tr>
<tr>
<td>FFPE4_Cancer</td>
<td>Medium</td>
<td>50</td>
<td>63,394,208</td>
<td>60,942,730</td>
<td>96.1%</td>
<td>7.2%</td>
<td>65.0%</td>
</tr>
<tr>
<td>FFPE9_Cancer</td>
<td>Poor</td>
<td>50</td>
<td>109,083,544</td>
<td>91,226,884</td>
<td>83.6%</td>
<td>44.4%</td>
<td>57.2%</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>200</td>
<td>69,105,486</td>
<td>63,668,336</td>
<td>92.1%</td>
<td>23.4%</td>
<td>66.6%</td>
</tr>
</tbody>
</table>

* Use original qPCR method for FFPE DNA quality evaluation, not published.

**Conclusion**

- Exome sequencing is possible from 50 ng of FFPE DNA (biopsy samples).
- Performance may vary depending on the “quality” of FFPE evaluated by qPCR.
- Using extra volume of adapter may increase the yield and may be able to control the duplication.
- Existence of a “hump” during the library production may associate with the ratio of PCR duplication and other factors which decide feasibility of FFPE DNA sequencing.

**<Customer’s comments>**

NGS analysis of FFPE samples is required for analyses of rare disorders (tumors), and for utilizing of a large amount of clinical data. For the development of the technique to clinical sequencing, methods suitable for NGS analysis of minute amounts of DNA taken from FFPE, such as biopsy samples taken in the past, must be established. We had hesitated to use DNA taken from FFPE samples because they tend to lead to a significantly high level of PCR duplication (more than 50% in some cases), and their data tend to be of low quality. The efficiency of KAPA Hyper Prep Kit in library construction was surprisingly high. There is no doubt that KAPA Hyper Prep Kit will make a great impact on the methods of clinical sequencing using FFPE. In the clinical sequencing, cancer tissue samples for example, are often available only in the form of biopsy FFPE. In such cases, KAPA Hyper Prep Kit is a very promising tool because it allows efficient construction of NGS library even from a several slices of biopsy FFPE (50–100 ng).

**<Comment from Nippon Genetics Co., Ltd. >**

We would like to show our deep gratitude to Dr. Nakagawa and Dr. Maejima for their performance to obtain good results in a short time.

In general, construction of NGS libraries from a minute amount of DNA samples, such as FFPE samples, tends to form dimer due to the extra amount of adaptor. Therefore, an appropriate amount of adaptor must be examined for the template. In addition, obtaining enough volume of library for sequencing is difficult, and as a result, the duplication rate will be high in the analysis of sequence data, causing a problem of insufficient levels of coverage and sequence depth.

With large improvement of reagents and protocol, KAPA Hyper Prep Kit has achieved very high efficiency for adapter ligation. In Dr. Nakagawa and Maejima’s study, this superior performance should have contributed to the effective construction of the library without dilution of DNA even with FFPE-derived genome DNA, thus low and favorable duplication rates.

**KAPA Hyper Prep Kit**

Library preparation kit for illumina

- **End repair and A-tailing buffer**
- **DNA Ligase**
- **HiFi HotStart Ready Mix**
- **Library amplification primer mix**
- **Notes: Adapter is not included. Magnet beads “AMPure XP” are not included**

**Components**
- End repair and A-tailing buffer
- End repair and A-tailing enzyme
- Ligation buffer

**Storage conditions**
- Kits can be stored for up to 12 months at -20°C.

**CatNo** | **Kit size** | **Component**
---|---|---
KK8500 | Hyper Prep Kit | 8 reaction
KK8502 | Hyper Prep Kit | 24 reaction
KK8504 | Hyper Prep Kit | 96 reaction