APPLICATION NOTE

Ion AmpliSeq Direct FFPE DNA Kit

A simplified DNA extraction method for Sanger sequencing of FFPE samples

In this application note we show:

- The Ion AmpliSeq™ Direct FFPE DNA Kit minimizes the need for complicated extraction of DNA from archived samples for Sanger sequencing
- Sanger sequencing of DNA extracted with the Ion AmpliSeq Direct FFPE DNA Kit performs equivalently to purified DNA from the same samples
- Sanger sequencing of directly extracted DNA coupled with analysis by Minor Variant Finder Software facilitates detection of minor alleles in preserved samples, and data is concordant with next-generation sequencing data

Introduction

Preserving tissue samples by fixation in paraformaldehyde and embedding in paraffin blocks is a standard way to preserve and archive biological materials. The nucleic acids present in these formalin-fixed, paraffin-embedded (FFPE) samples provide a snapshot of the state of the genome and transcriptome in cells, yielding important information for retrospective studies. Thus, methods have been developed for extracting DNA and RNA from such archived samples. However, the methods developed for extracting these nucleic acids are often time-consuming and involve multiple manipulative steps, each of which could result in sample mix-up or loss. Moreover, the reagents required for removing the paraffin and extracting the nucleic acids are noxious and toxic, incurring further expenses for handling and disposing of them properly. Thus, a simple method for extracting DNA and RNA from FFPE samples could greatly facilitate retrospective studies.

We have developed a simple and fast kit for extracting nucleic acids from tissue sections preserved on slides. Briefly, the protocol involves adding a reagent to the tissue section and scraping the tissue off the slide into a tube. The mixture is then incubated with a second reagent for 15 minutes, followed by a high-temperature denaturation step for 10 minutes. After incubation, the extracted material is ready for analysis.

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The Ion AmpliSeq Direct FFPE DNA Kit was initially developed to streamline the input for generating Ion AmpliSeq™ libraries and sequencing on Ion Torrent™ next-generation sequencing (NGS) systems. In this application note, we show that DNA extracted with the Ion AmpliSeq Direct FFPE DNA Kit can also be used for Sanger sequencing on Applied Biosystems™ capillary electrophoresis (CE) platforms (Figure 1). To illustrate the utility of this workflow, we show how DNA extracted with the Ion AmpliSeq Direct FFPE DNA Kit can be used to detect single nucleotide variants (SNVs) in tumor tissues. We developed Minor Variant Finder Software, a user-friendly desktop software package specifically for the detection and reporting of SNVs in Sanger sequencing traces. Minor Variant Finder Software can detect variants at a frequency as low as 5% from as little as 1 ng of DNA extracted from FFPE samples. Together, these tools provide clinical researchers a rapid and powerful method for obtaining sequence information from archived samples.

Protocol

The protocol for direct DNA extraction and Sanger sequencing is summarized below. For further details, please see the user guide for the Ion AmpliSeq Direct FFPE DNA Kit (Pub. No. MAN0014881), product bulletin for the Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit (Pub. No. CO13911), and protocol summary for the Applied Biosystems™ BigDye™ XTerminator Purification Kit (Pub. No. 107PR04-01).

Direct DNA extraction with the Ion AmpliSeq Direct FFPE DNA Kit

1. Using a single 20 µL pipette tip for each sample:
    a. Pipet 10 µL of Transfer Solution onto the region of interest of the FFPE tissue section mounted on a slide (Figure 2).
    b. Using the same 20 µL pipette tip, spread the Transfer Solution to ensure complete coverage of the region of interest, then scrape and break up the tissue with the pipette tip. The tissue should be a slurry of fine particles in the Transfer Solution.
2. Pipet the slurry from the slide into a 0.2 mL tube.
3. Pipet the slurry up and down at least 5 times.
4. If needed, use the same tip to repeat steps 1–3, transferring as much of the region of interest as possible into the tube.

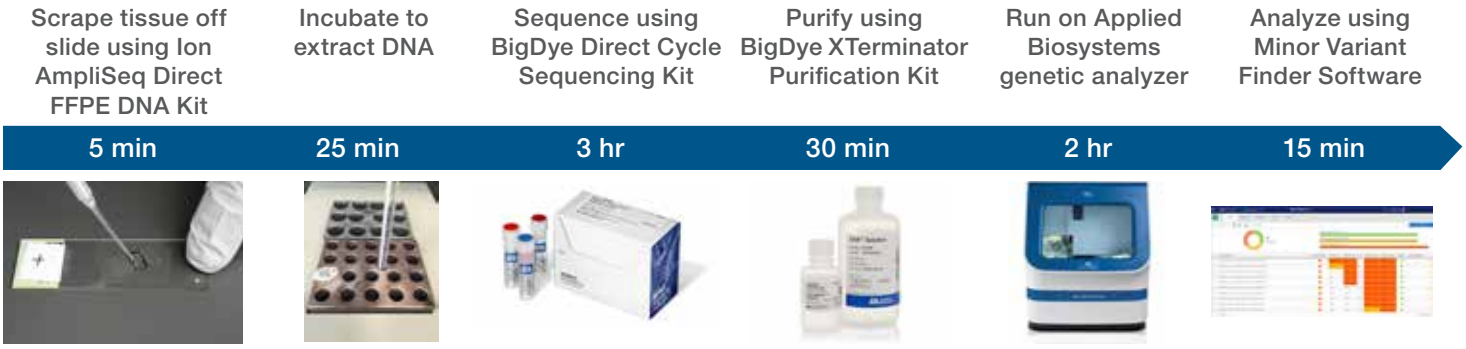

Figure 1. Workflow for direct DNA extraction and Sanger sequencing from FFPE samples. In this simplified protocol, Sanger sequencing information can be obtained from FFPE samples in less than 7 hours total, with less than 1 hour of hands-on time. A simplified workflow helps reduce the chance of sample mix-up or loss.

Figure 2. Example of FFPE sample removal from slides. (A) A starting sample of human liver preserved as an FFPE section on a slide is shown. **(B)** Transfer Solution is added and the tissue is scraped off the slide. In some cases, this is facilitated by using a 20 µL pipette tip as a squeegee. **(C)** The slide with paraffin left behind is shown after transfer of the scraped material to a new tube.

Note: The final volume of slurry may vary, but no volumetric adjustment during steps 5–8 is required.

5. Add 21 µL of Direct Reagent to the tube.
6. Set a pipette to 30 µL, then mix the Direct Reagent and slurry by pipetting up and down 10 times.
7. Incubate samples at 65°C for 15 minutes, then 95°C for 10 minutes. Please note that the extra incubation step at 95°C for 10 minutes is not in the protocol for Ion AmpliSeq library preparation.
8. Centrifuge briefly to collect contents at the bottom of the tube. The bottom phase contains the extracted DNA and can be used for sequencing.

Sanger sequencing with BigDye Direct and BigDye XTerminator kits

1. Combine 1.5 µL M13-tailed sequencing primers (0.8 µM each primer), 5 µL BigDye™ Direct PCR Master Mix, and 1–3.5 µL extracted DNA. Bring total volume to 10 µL with water. Amplify on a thermal cycler using the thermal cycling profile provided in the BigDye Direct Cycle Sequencing Kit user guide (Pub. No. 4458016). If Minor Variant Finder Software is being used, set up duplicate reactions for sequencing the forward and reverse strands. A DNA extract from a tissue section that appears histologically normal can be used as a control sample.
2. Add 2 µL BigDye™ Direct Sequencing Master Mix and 1 µL of either forward or reverse M13 sequencing primer. Run the reactions in a thermal cycler using the sequencing profile provided in the BigDye Direct Cycle Sequencing Kit user guide.
3. For each reaction, combine 45 µL SAM™ Solution with 10 µL XTerminator™ Solution as indicated in the BigDye XTerminator Purification Kit user guide (Pub. No. 4374408). Mix well and add 55 µL of the slurry to each sequencing reaction, making sure the slurry is well suspended between addition steps.
4. Place plate on a vortexer and shake for 30 minutes.
5. Centrifuge plate at 1,000 x *g* for 1 minute. Run samples on an Applied Biosystems™ CE instrument using the appropriate run module.
6. Analyze .ab1 files using Minor Variant Finder Software.

Results

To demonstrate the direct DNA extraction and Sanger sequencing workflow, we obtained tumor samples from various tissue types with different oncogenic alleles and frequencies. Each sample was a 7 µm section from FFPE tissue mounted on a glass microscope slide. The efficiency of direct DNA extraction was determined by measuring the yield of DNA obtained from these samples using the Invitrogen™ Qubit™ 3 Fluorometer. In general, the yield and concentration of DNA in the extract was adequate for downstream analysis by Sanger sequencing (Table 1). At a recommended DNA input of at least 1 ng for sequencing using the BigDye Direct Cycle Sequencing Kit, each sample provided sufficient DNA for many Sanger sequencing reactions.

Table 1. DNA concentration and yield obtained from each 7 µm mounted section that was extracted using the Ion AmpliSeq Direct FFPE DNA Kit.

Sample name	Tissue type	Concentration	Yield
Control	Normal liver	3.2 ng/µL	48 ng
1188466b	Uterus	26.4 ng/µL	396 ng
1188945b	Brain	6.0 ng/µL	89 ng
1189051b	Lung	21.6 ng/µL	324 ng
35466214	Pancreas	10.8 ng/µL	162 ng
370	Melanoma	19.5 ng/µL	293 ng

The suitability of direct DNA extraction for Sanger sequencing was determined by comparing Sanger sequencing reactions performed with directly extracted DNA and column-purified DNA from the same FFPE block. For each of the tissue sections, we sequenced 10 ng of DNA obtained by column purification and 10–20 ng (1–2 µL) of DNA obtained by direct extraction. Sequencing primers were chosen using the Primer Designer™ Tool (**thermofisher.com/primerdesigner**). For optimal flexibility, the database of the Primer Designer Tool includes sequencing primer combinations for different-size amplicons of the same locus. For these experiments, the sequencing primers chosen were selected for the shortest amplicons that would facilitate sequencing of degraded DNA from FFPE samples.

In each case, the sequencing quality metrics were similar between directly extracted DNA and column-purified DNA from the same FFPE sample, indicating that components present in the extract did not interfere with the Sanger sequencing reactions (Figure 3). Furthermore, the sequencing traces were also indistinguishable (example shown in Figure 4). The level of background sequence was low, the same somatic variant SNP was detectable, and no novel SNP differences were observed between the reactions. Therefore, DNA directly extracted from FFPE samples can provide quality Sanger sequencing data with a streamlined workflow.

To determine the efficiency of using directly extracted DNA to examine allele frequencies, we analyzed the Sanger sequencing traces using Minor Variant Finder Software and compared the results to SNP variant frequencies determined by NGS. DNA was purified from a single tissue section using a column-based protocol and sequenced by NGS using the Ion AmpliSeq™ Cancer Hotspot Panel v2. DNA from a different section of the same tumor sample was extracted using the same column-based protocol and sequenced in the forward and reverse directions by Sanger sequencing. Finally, DNA from a third section of the same tumor sample was extracted using the Ion AmpliSeq Direct FFPE DNA Kit and sequenced in the forward and reverse directions by Sanger sequencing. After Sanger sequencing, allele frequencies of the samples were determined using Minor Variant Finder Software.

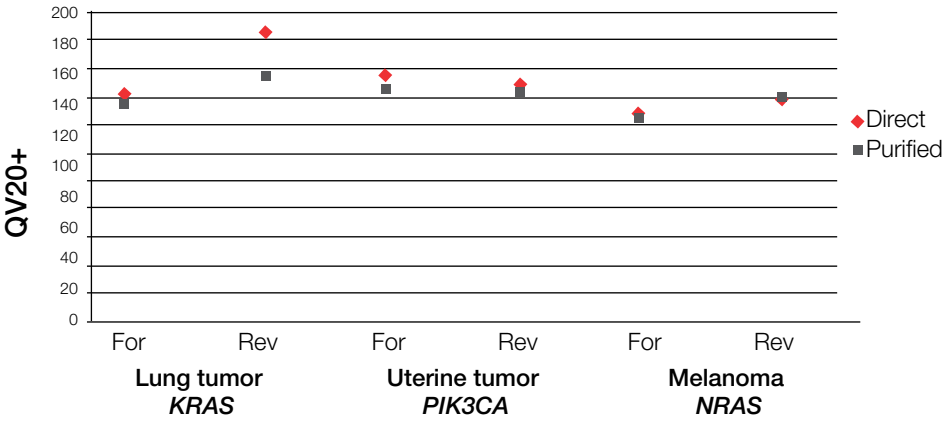

Figure 3. Comparison of CE quality scores of Sanger sequencing traces. QC metrics obtained by sequencing the indicated samples were similar between the directly extracted DNA and column-purified DNA from a different section of the same block. QV20+ is the total number of bases in the trace that have quality values of at least 20.

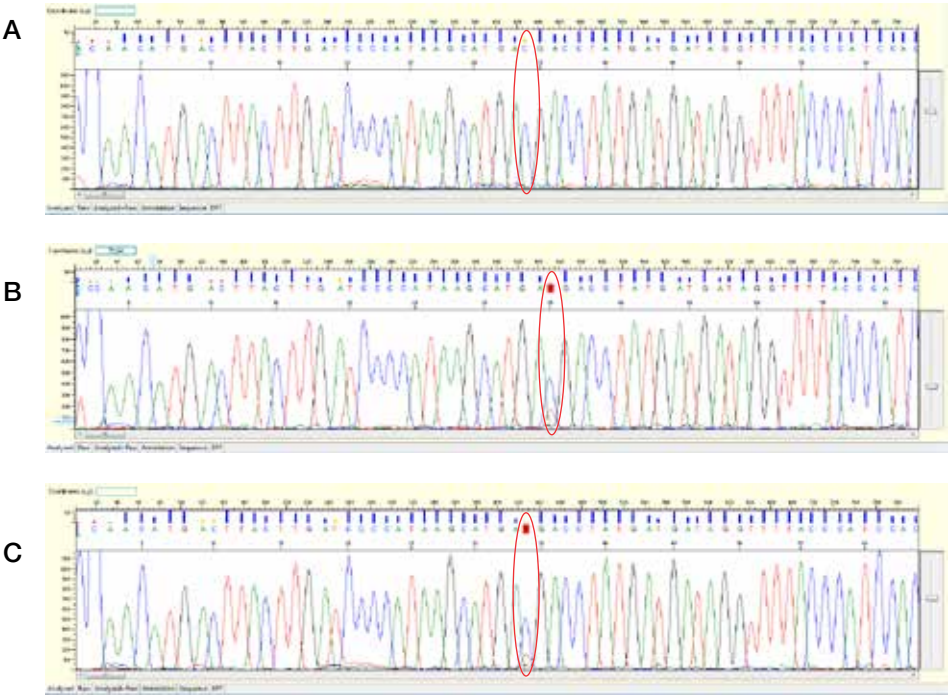

Figure 4. Comparison of sequencing traces. (A) Sequence of the *IDH1* locus from control-purified genomic DNA from a normal sample. **(B)** Sequence of the *IDH1* locus from column-purified DNA from an FFPE brain tumor sample. Note the SNP variant observable in this sample (circled). **(C)** Sequence of the *IDH1* locus from DNA directly extracted from the same brain tumor sample. The quality of the sequencing trace was indistinguishable from the trace obtained with purified DNA. The same SNP variant (circled) was detected with both methods.

Overall, the variant frequencies obtained with the different methods correlated well (Table 2). For example, the melanoma sample, with a very high fraction of an *NRAS* p.Q61L allele determined by NGS, was present at very high frequencies in both the purified and directly extracted DNA samples. In addition, the uterine sample with a low-frequency *PIK23CA* p.E545K (6.8%) allele was also accurately called by Sanger sequencing in the two sample preparation methods (average of 3.9% and 3.8% in purified and directly extracted DNA, respectively). One sample (lung *KRAS* p.G12A) gave more variable allele frequency results with the three methods: 70.6% with NGS, 54.1% with column-purified DNA, and about 20% with directly extracted DNA. Since these samples came from different sections of the same tumor, the different allele frequencies could reflect somatic heterogeneity of the tumor, which is frequently seen in late-stage tumor samples [1]. Nevertheless, these results indicate that DNA directly extracted using the Ion AmpliSeq Direct FFPE DNA Kit and sequenced by Sanger sequencing can produce allele frequency data that are consistent with that observed from purified DNA of the same samples.

Conclusions

We have developed a kit that streamlines the extraction of genomic DNA from FFPE samples without the use of toxic reagents. The concentration of DNA recovered meets or exceeds that obtained using other kits, and the amount of DNA recovered can be sufficient for many sequencing reactions. When used as a template for Sanger sequencing, DNA directly extracted from FFPE samples can provide high-quality results that are comparable to those obtained with traditional column-based purified DNA. Finally, the traces obtained by Sanger sequencing of extracted DNA can be used with Minor Variant Finder Software to determine allele frequencies of somatic variants in tumor samples that are similar to frequencies obtained by NGS workflows. The simplicity of the workflow described in this application note provides a mechanism for clinical researchers to quickly obtain sequence information from archived samples.

Reference

1. Ryu D, Joung JG, Kim NK et al. (2016) Deciphering intratumor heterogeneity using cancer genome analysis. *Hum Genet* 135:635–642.

Table 2. Comparison of allele frequency results between methods.

				Sanger allele frequency			
Sample name	**Tissue type**	**Allele**	**NGS allele frequency**	**Purfied DNA, forward reaction**	**Purified DNA, reverse reaction**	**Direct extract, forward reaction**	**Direct extract, reverse reaction**
1188466b	Uterus	*PIK3CA* p.E545K	6.8%	3.9%	4.0%	4.7%	3.1%
1188945b	Brain	*IDH1* p.R132H	25.0%	27.3%	22.5%	24.1%	21.4%
1189051b	Lung	*KRAS* p.G12A	70.6%	56.6%	51.7%	15.0%	25.0%
35466214	Pancreas	*KRAS* p.G12A	11.9%	Not done	Not done	23.4%	14.5%
370	Melanoma	*NRAS* p.Q61L	91.2%	>80%	>80%	>80%	>80%

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