An approach for analyzing genetic heterogeneity in retrospective tumor samples using laser capture microdissection, real-time PCR, and next-generation sequencing

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- A workflow is presented here for obtaining high-quality genomic information for cancer research from a small amount of preserved tissue using laser capture microdissection (LCM).

- We provide a simple method to analyze the quality of the extracted DNA by real-time PCR. This method is used as a time-saving step to both quantify the extracted DNA and determine if the DNA can be sequenced.

- High-quality sequence information was obtained from the extracted DNA using a targeted sequencing approach on the Ion PGM™ System. We anticipate that the speed and flexibility of this workflow will make it a useful tool for the cancer genomics laboratory to identify disease-causing mutations, even from difficult sample types.
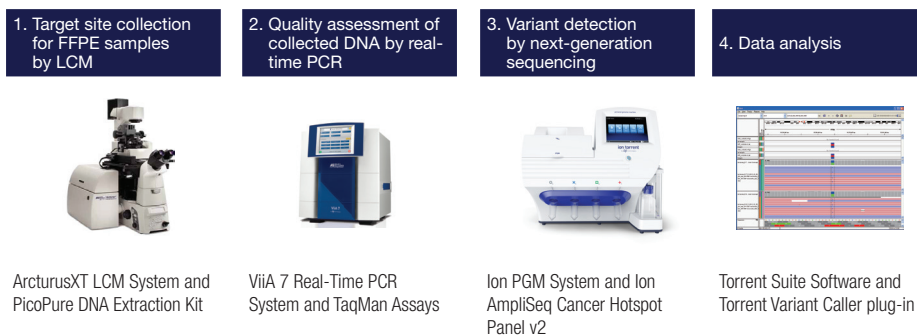
1. Target site collection for FFPE samples by LCM	2. Quality assessment of collected DNA by real-time PCR	3. Variant detection by next-generation sequencing	4. Data analysis
ArcturusXT LCM System and PicoPure DNA Extraction Kit	ViiA 7 Real-Time PCR System and TaqMan Assays	Ion PGM System and Ion AmpliSeq Cancer Hotspot Panel v2	Torrent Suite Software and Torrent Variant Caller plug-in

Figure 1. A workflow for next-generation sequencing starting from FFPE tissue samples for cancer research.

Introduction

Understanding the genetic makeup of tumor specimens is critical for appreciating their underlying biology. DNA sequencing is an ideal method to obtain a complete snapshot of the genotypes in a tumor to identify potential disease-causing mutations. Dramatic progress in the accuracy, cost, and speed of next-generation sequencing (NGS) technology with the Ion PGM System enables these studies to be immediately possible. However, several factors should be considered, from sample preparation to sequencing, in order to successfully integrate this workflow into the cancer genomics laboratory.

Thermo Fisher
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The onset and proliferation of tumors is the result of an accumulation of genomic aberrations, changing the genetic composition of the tissue from homogeneous to a complex and diverse population. Since most tumors are composed of cells with different genomic compositions, it can be difficult to obtain accurate analyses by observing "average" genomes extracted in bulk from such tumors. Ideally, genomic DNA that is extracted from the cancerous subpopulation, or from single cells in the tumor, should be analyzed. However, cell numbers are often limiting, which can result in only small amounts of DNA available for downstream analyses. Furthermore, DNA extracted from formalin-fixed, paraffin-embedded (FFPE) samples is often at least partially degraded. Therefore, a workflow utilizing NGS that is capable of analyzing lower-quality DNA from preserved tumor samples is highly desirable.

In this application note, we provide a specimen-to-sequence workflow resulting in high-quality genomic information from small amounts of cells (Figure 1). Briefly, tumor and non-tumor cells were collected from FFPE tissue sections using the Applied Biosystems™ ArcturusXT™ LCM System. Genomic DNA was then isolated from the cells using the Applied Biosystems™ PicoPure™ DNA Extraction Kit. The quality of the extracted genomic DNA was analyzed using the Applied Biosystems™ ViiA™ 7 Real-Time PCR System and custom-designed Applied Biosystems™ TaqMan™ Gene Expression Assays. Specific loci were then amplified with the Ion AmpliSeq™ Cancer Hotspot Panel v2 and sequenced on the Ion PGM System. These studies were performed under the guidance of Professor Masao Omata, President of Yamanashi Prefectural Hospital Organization (Professor Emeritus, Faculty of Medicine, University of Tokyo), and were designed to facilitate research in understanding the genomic changes observed in tumor cells.

Laser capture microdissection of tissue samples

The ArcturusXT LCM System was used to isolate cells from tissue samples in this study. This system combines an infrared (IR) capture laser and an ultraviolet (UV) cutting laser in one microdissection platform. The IR laser enables capture under gentle conditions, preserving biomolecule integrity, which is optimal for the microdissection of single cells or a small number of cells. The UV laser performs microdissection with unprecedented speed and accuracy and is suitable for difficult and dense tissue structures. This dual-laser system allows simple and secure microdissection of individual cells, groups of cells, and large regions of tissue from a single sample.

FFPE sections (10 μm) from retrospective colon carcinoid cases were mounted onto polyethylene naphthalate (PEN) membrane glass slides (see ordering information) and stained with hematoxylin and eosin (H&E). The H&E-stained slides were then loaded onto the ArcturusXT LCM System, and tumor and non-tumor regions were identified. Tumor and non-tumor tissues were each excised from the FFPE sample (Figure 2) using both the IR and UV microdissection lasers, and collected on individual Applied Biosystems™ CapSure™ Macro LCM Caps. CapSure LCM caps retain the microdissected tissue for subsequent DNA extraction. The average microdissected area was 2.5 mm^2 for the tumor samples and 2.2 mm^2 for the non-tumor samples.
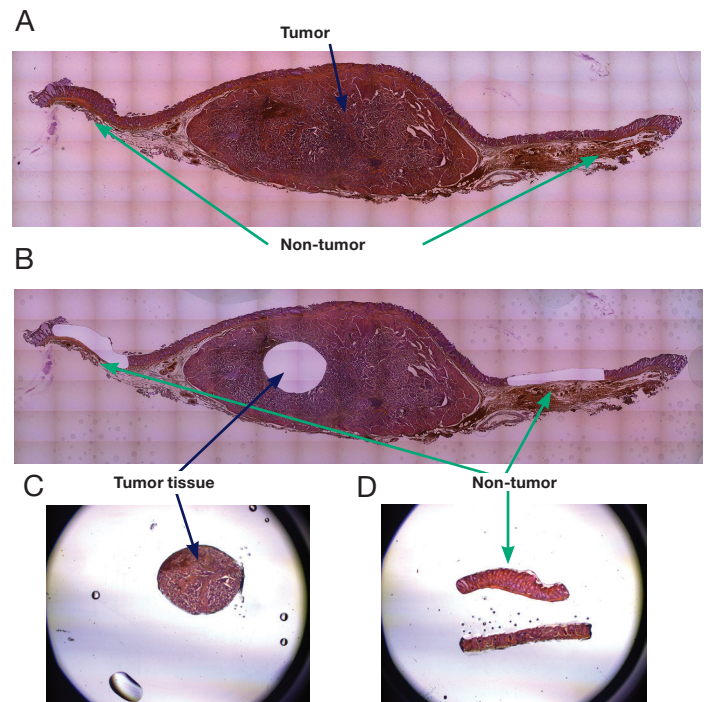

Figure 2. Laser capture microdissection of a colon carcinoid tissue sample. The tissue section is shown (A) before and (B) after microdissection. Both tumor and non-tumor regions of the sample were excised. The microdissected areas of (C) tumor and (D) non-tumor tissue are shown on their respective CapSure LCM cap sample holder.

DNA extraction

Genomic DNA (gDNA) was extracted from LCM caps containing the microdissected tissue, using the PicoPure DNA Extraction Kit. This kit provides a fast and easy extraction process, with no organic extractions or spin columns required. The extraction solution (50 µL, composed of Proteinase K and reconstitution buffer provided in the kit) was added to a 0.5 mL microcentrifuge tube. The LCM cap with captured cells was coupled with the tube and inverted so that the lysis solution would have contact with the LCM cells. To extract the gDNA, the samples were incubated for 16 hours at 65°C and then centrifuged to collect the lysate into the microcentrifuge tube. The LCM cap was detached from the tube, and the lysate was then heated to 95°C to inactivate the Proteinase K.

Quality analysis of gDNA

The extracted gDNA may be degraded in some cases, especially when working with FFPE tissue samples. This degradation can be evaluated by a number of methods, but analysis can be difficult or impractical with limited amounts of DNA. Due to its high sensitivity and specificity, real-time PCR is well suited for this application. For this study, a workflow was developed to evaluate DNA degradation levels utilizing the ViiA 7 Real-Time PCR System and TaqMan Gene Expression Assays.

The Applied Biosystems™ TaqMan™ RNase P Detection Reagents Kit was used to generate a short amplicon (87 bp) for the RNase P gene (one copy of which is present in human gDNA). In addition, a custom TaqMan Assay was designed to generate a relatively long amplicon (256 bp). Since a longer amplicon is generally more difficult to amplify during PCR, it can serve as a model for the amplification of challenging samples such as FFPE tissue. Longer amplicons accumulate at a lower rate than smaller amplicons during PCR,

Table 1. Amplification parameters for quality assessment of tumor, non-tumor, and human RNase P control DNA samples.

Parameter	Used for workflow
Instrument	ViiA 7 Real-Time PCR System
Reaction mode	Standard mode
Master mix	TaqMan Gene Expression Master Mix
Primer and probe final concentrations	900 nM primer, 250 nM probe
Reaction volume	20 µL/well (DNA sample added at 1 µL/well)
Replicates	n = 3
Standard curve	Human control gDNA (10 ng/µL, included with TaqMan RNase P Detection Reagents Kit) serially diluted 4 times for a 5-point calibration curve

particularly when analyzing challenging samples, because there is a lower amount of unfragmented template available for amplification in degraded DNA samples. Therefore, higher C_t values are expected for longer amplicons when PCR is performed with degraded DNA samples. By comparing the quantification of the short and long amplicons, researchers can evaluate the extent of DNA degradation and decide whether it is worthwhile to proceed to NGS. Table 1 outlines the amplification parameters used for this assay.

Figure 3 shows the resulting amplification curves generated from the samples tested. Since the human control DNA was of high quality and not compromised, similar amplification profiles were observed for both the short and long amplicons. However, a shift to higher C_t values was observed for the long amplicon for both the tumor region and the non-tumor region of the FFPE sample sections, which suggests that the DNA had been degraded.
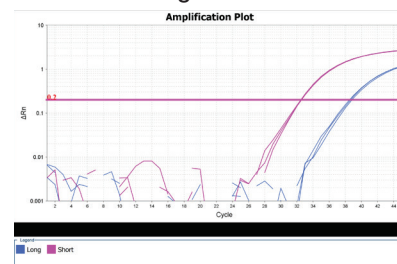These data show that although the DNA recovered from
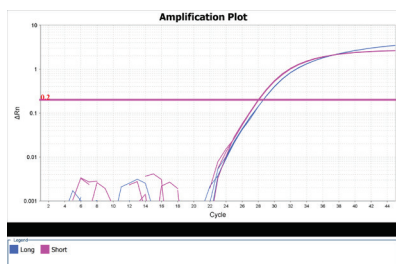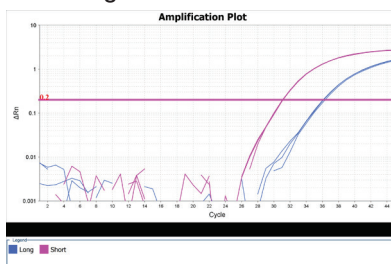
Figure 3. Quality evaluation of extracted nucleic acid by qPCR. (A) Human control DNA (included with TaqMan RNase P Detection Reagents Kit). Since this control DNA is of high quality and is not compromised, similar amplification curves for both the short and long amplicons are expected and observed. DNA from (B) tumor region and (C) non-tumor region of the tissue sample. PCR products accumulated later for the long amplicons in each sample, reflecting a lower quantity of complete template available for amplification.

Table 2. DNA quantitation by real-time PCR of the tumor and non-tumor regions of the sample.

Sample	Short amplicon (87 bp)		Long amplicon (256 bp)	
	Quantity (ng/μL)	SD	Quantity (ng/μL)	SD
Tumor region	1.309	0.023	0.151	0.012
Non-tumor region	0.553	0.018	0.033	0.003

FFPE samples are degraded to some extent, they are amplifiable and interpretable, so moving forward in the workflow to the sequencing steps is warranted.

A standard curve based on the amplification of the control DNA sample was generated, and the concentration of the DNA extracted from the tumor and non-tumor samples was determined from these data (Table 2). As expected, the quantity of DNA detected was lower when the long amplicon was used for analysis, for both the tumor and non-tumor sample regions. These values can be used to calculate relative quantification (RQ), which is an indicator of the degradation level of genomic DNA (Figure 4). For the long amplicon, the RQ was 0.064 for the tumor region and 0.034 for the non-tumor region, relative to human control DNA. A lower RQ value indicates that there is less intact DNA available for downstream applications, which is an important factor to consider when proceeding to NGS.

Target selection and library preparation prior to NGS

For this study, specific loci corresponding to known variants in cancer genes were amplified in a single multiplex assay, prior to sequence analysis, using the Ion AmpliSeq Cancer Hotspot Panel v2. This panel consists of 207 primer pairs interrogating 50 oncogenes and tumor suppressor genes, such as *KRAS*, *BRAF*, *EGFR*, and *TP53*, and covers approximately 2,800 "hotspot" mutations. Since the average length of the amplicons generated with this panel is only 154 bp, the panel can be successfully used with degraded gDNA preparations, such as those obtained from FFPE samples. In addition, the Ion AmpliSeq Cancer Hotspot Panel v2 can analyze all target loci with as little as 10 ng of gDNA, and the workflow can be completed in one day, from DNA preparation to annotated mutations. Due to sample availability limitations in this study, only 5 ng of gDNA was used for both the tumor and non-tumor samples, and satisfactory results were observed. To compensate for the
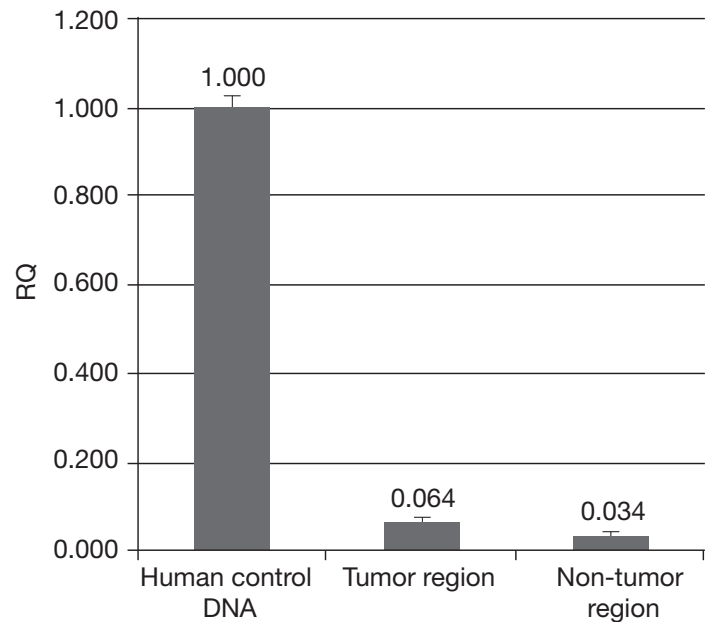

Figure 4. Relative quantification (RQ) of samples. The ratio of DNA obtained for the long amplicon to the short amplicon was calculated (i.e., 0.151/1.309 = 0.115 for the tumor region of the sample). This value was then normalized to the long/short ratio obtained for the control gDNA sample (1.79, data not shown). Results are plotted for both the tumor region (0.115/1.79 = 0.064) and non-tumor region of the sample.

lower amount of gDNA, 4 additional cycles were added to the Ion AmpliSeq panel protocol. A complete list of loci analyzed by the Ion AmpliSeq Cancer Hotspot Panel v2 can be seen at **thermofisher.com/ampliseq**.

Next-generation sequencing

After multiplex amplification of the target loci, the Ion PGM System was used for DNA sequencing. Unlike other NGS platforms, the Ion PGM System utilizes semiconductor technology to detect hydrogen ions that are naturally released during DNA synthesis, which eliminates the need for fluorescent dye chemistries and system optics. Library and template preparation steps are automated using the Ion Torrent™ AB Library Builder™ and Ion OneTouch™ systems to further simplify the NGS workflow. Sequencing analysis data are shown in Figure 5. Satisfactory sequence data were obtained for both the tumor and non-tumor samples despite the suboptimal quantity of starting material, with coverage of 100x for 99% or more of the targeted regions and also with high sequence accuracy.

Data were analyzed using Torrent Suite™ Software and

Ion Reporter™ Software. With Torrent Suite Software, the quality and accuracy of the sequencing run can be reviewed, in addition to performing further analyses such as variant calling. Torrent Suite Software contains the Torrent Variant Caller plug-in, which has been optimized to detect single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) from Ion Torrent™ sequencing data. An example of data analysis using the Torrent Variant Caller is shown in Figure 6. Although not performed as part of this study, mutations can be validated using TaqMan Assays (see sidebar, "TaqMan Mutation Detection Assays").

A

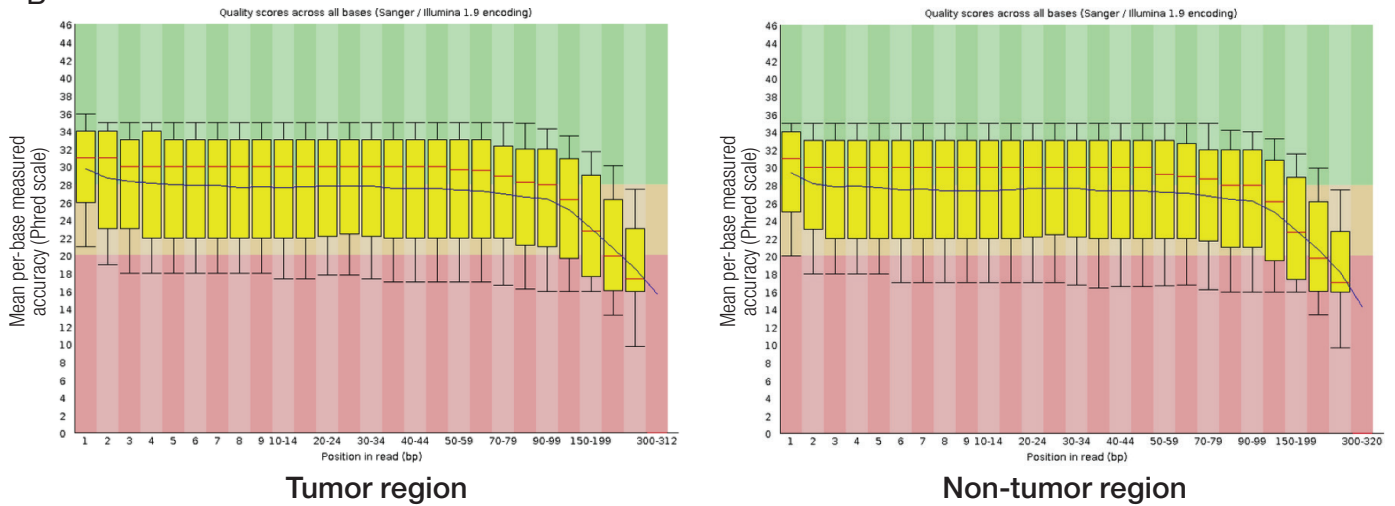|	20x coverage	100x coverage	Reads on-target	Depth	Number of reads (thousands)
Tumor region	100%	99.17%	94.41%	1,551	386
Non-tumor region	100%	99.61%	88.69%	3,939	1,048

B

Tumor region

Non-tumor region

Figure 5. Sequencing results with the Ion PGM System. (A) Summary of results obtained for the tumor and non-tumor regions of the sample. (B) Phred score of the respective reads. These data are plotted as a function of base position at an individual read level. The quality of called bases is shown on a Phred scale, where a score of 20 represents 99% sequence accuracy and a score of 30 denotes 99.9% sequence accuracy.
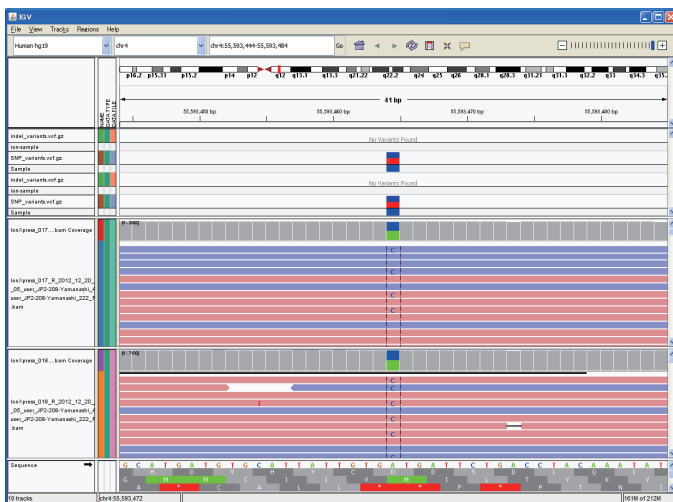

Figure 6. An example of data analysis with Torrent Variant Caller software. Torrent Suite Software allows you to customize your analysis at the end of each run through the use of different plug-ins. The Torrent Variant Caller plug-in is used for this workflow to detect SNPs and indels from Ion Torrent sequencing data. The software calls SNP and indel variants across a reference or within a targeted subset of that reference. Plug-ins can be set to run automatically on every analysis, and also can be run manually on a completed analysis.

TaqMan Mutation Detection Assays

Candidate mutations identified by next-generation sequencing can be validated by real-time PCR. Applied Biosystems™ TaqMan™ Mutation Detection Assays detect somatic mutations in genes that are associated with cancer. These assays use Applied Biosystems™ castPCR™ (competitive allele-specific TaqMan PCR) technology. The assays are highly specific and sensitive, detecting as low as 0.1% mutant alleles in a background of wild type DNA. Our current assay set supports 46 genes covering 778 mutations, and more assays are planned in the future. Figure 7 illustrates the TaqMan Mutation Detection Assay technology.

A
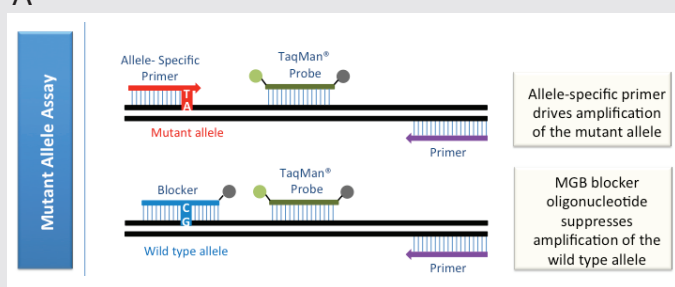
B

ABL1	CSF1R	FGFR3	KDR	NRAS	SMARCB1
AKT1	CTNNB1	FLT3	KIT	PDGFRA	SMO
ALK	EGFR	GNAS	KRAS	PIK3CA	SRC
APC	ERBB2	HNF1A	MET	PTEN	STK11
ATM	ERBB4	HRAS	MLH1	PTPN11	TP53
BRAF	FBXW7	IDH1	MPL	RB1	VHL
CDH1	FGFR1	JAK2	NOTCH1	RET	
CDKN2A	FGFR2	JAK3	NPM1	SMAD4	

Figure 7. The principle behind TaqMan Mutation Detection Assays. (A) TaqMan Mutation Detection Assays contain mutant allele assays, which specifically detect one or more mutant alleles. An allele-specific primer drives amplification of the mutant allele, while a blocker oligonucleotide suppresses amplification of the wild type allele. **(B)** Currently supported genes for the TaqMan Mutation Detection Assays.

Discussion

In this application note, we describe a specimen-to-sequence solution for analysis of DNA collected from FFPE samples of retrospective carcinoid cases. Since both tumor and non-tumor cells were isolated by LCM and analyzed in parallel through all steps of the workflow, researchers can have increased confidence that their results more accurately reflect the actual genomic changes present in cancer cells.

Genomic DNA isolated from FFPE samples is often degraded and present in low quantities. We therefore implemented a quality control step that evaluates the degree of degradation prior to NGS analysis. In addition, evaluating the RQ value, which compares sample DNA quantity obtained by real-time PCR relative to control DNA quantity, enables researchers to quantify the DNA samples prior to NGS. Although the recommended input DNA amount for NGS using the Ion AmpliSeq Cancer Hotspot Panel v2 is 10 ng, results observed in this study were satisfactory with just 5 ng of input DNA per reaction for both the tumor and the non-tumor samples. Evaluating sample DNA degradation level and DNA quantity before proceeding to NGS is recommended to help save time and resources.

The Ion PGM System enables a wide range of applications, such as targeted sequencing, exome sequencing, and de novo genome sequencing. A targeted sequencing approach was used here to perform multiplex PCR from genomic regions that are frequently mutated in human cancer genes. High-quality sequence reads were obtained for both tumor and non-tumor cells, even though the samples were obtained from FFPE tissue. The workflow, from targeted DNA amplification through sequencing, can be completed in a single day. Based on the speed of this workflow and quality of results obtained, further studies are currently underway that we hope will help clarify the underlying genetic changes that occur in cancer cells.

Professor Masao Omata

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Professor Emeritus,
University of Tokyo

Professor Masao Omata has been engaged in medical research on gastrointestinal and liver diseases for 43 years. Since 1981, his research has focused on the molecular biology of the hepatitis B virus (HBV). He has conducted basic and clinical studies on the emergence of liver cancer associated with hepatitis B and C, as well as gastric cancer associated with *Helicobacter pylori*. Research supports the hypothesis that tumorigenesis of these cancers cannot initiate in the absence of these pathogens [1,2].

Comprehensive studies of gastric and liver cancer cells show that many types of genetic alterations are observed in cells 30 to 40 years after initial exposure to the pathogens. It seems unlikely that a single gene or pathway is responsible for this pathology. Although it may be possible to significantly reduce gastric and liver cancers by eliminating these pathogens, it has been difficult to fully understand the pathology and develop treatments for the cancers they cause.

In-depth, large-scale genomic studies of lung and colon cancers have enabled clinical researchers to identify a number of previously unknown driver mutations associated with these tumors. Targeted therapies for lung and colon tumors are now being developed based on this research. Dr. Omata and other cancer researchers remain optimistic that deep sequencing studies will result in similar breakthroughs for gastric and liver cancers.

References

1. Arzumanyan A, Reis HM, Feitelson MA (2013) Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. *Nat Rev Cancer* 13:123–135.

2. Bhandari A, Crowe SE (2012) *Helicobacter pylori* in gastric malignancies. *Curr Gastroenterol Rep* 14:489–496.

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Ordering information

Product	Cat. No.
Laser capture microdissection	
PEN Membrane Glass Slides	LCM0522
CapSure Macro LCM Caps	LCM0211
Arcturus PicoPure DNA Extraction Kit	KIT0103
ArcturusXT Laser Capture Microdissection Instrument	thermofisher.com/arcturus
Real-time PCR	
TaqMan RNase P Detection Reagents	4316831
TaqMan Gene Expression Master Mix	4369016
ViiA 7 Real-Time PCR system	thermofisher.com/viia7
Next-generation sequencing	
Ion AmpliSeq Cancer Hotspot Panel v2	4475346
Ion AmpliSeq Library Kit 2.0 (96 LV)	4480441
Ion PGM Template OT2 200 Kit	4480974
Ion PGM Sequencing 200 Kit v2	4482006
Ion 316 Chip Kit v2	4483188
Ion Xpress Barcode Adapters 1-96 Kit	4474517
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