Video Article

Simple and Rapid Method to Obtain High-quality Tumor DNA from Clinical-pathological Specimens Using Touch Imprint Cytology

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Abstract

It is critical to determine the mutational status in cancer before administration and treatment of specific molecular targeted drugs for cancer patients. In the clinical setting, formalin-fixed paraffin-embedded (FFPE) tissues are widely used for genetic testing. However, FFPE DNA is generally damaged and fragmented during the fixation process with formalin. Therefore, FFPE DNA is sometimes not adequate for genetic testing because of low quality and quantity of DNA. Here we present a method of touch imprint cytology (TIC) to obtain genomic DNA from cancer cells, which can be observed under a microscope. Cell morphology and cancer cell numbers can be evaluated using TIC specimens. Furthermore, the extraction of genomic DNA from TIC samples can be completed within two days. The total amount and quality of TIC DNA obtained using this method was higher than that of FFPE DNA. This rapid and simple method allows researchers to obtain high-quality DNA for genetic testing (*e.g.*, next generation sequencing analysis, digital PCR, and quantitative real time PCR) and to shorten the turnaround time for reporting results.

Video Link

The video component of this article can be found at https://www.jove.com/video/56943/

Introduction

Next generation sequencing technology has provided researchers significant advancements in analyzing genome information in genetic variations, Mendelian disease, hereditary predisposition, and cancer [1,2,3]. The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) have pursued the identification of genetic alterations in several types of common cancers[4]. Hundreds of essential cancer driver genes have been successfully identified, and some of these molecules are being targeted for drug development[1,5,6].

In the clinical setting, FFPE specimens are commonly used for pathological diagnosis and molecular testing for various diseases, including cancer. However, during the fixation process with formalin, DNA-protein or DNA-DNA cross-linking occurs and DNA fragmentation is induced. Thus, FFPE DNA samples are not always suitable for genetic analysis because of low quality and quantity of DNA[7,8,9]. Additionally, it takes several days to prepare FFPE specimens, and technical skill is necessary to accurately prepare the sections. Therefore, it is desirable to develop a simple and rapid method for obtaining high-quality intact DNA.

Cytology is an alternative method for pathological diagnosis. Cytological sample preparation is a simpler, less expensive, and more rapid approach compared with FFPE preparation[10]. The TIC technique has been performed on sentinel lymph nodes and marginal tissues from breast cancer patients for intraoperative rapid diagnosis for some years[11,12]. However, there are few reports that have examined whether high-quality genomic DNA can be extracted from TIC specimens and used for subsequent genetic analysis. Cytological specimens are commonly stained with Papanicolaou (Pap) or Giemsa staining, and we previously reported that the amount and quality of DNA extracted from TIC specimens (especially Giemsa-stained samples) are superior to samples obtained from FFPE tissues[13]. Compared with Pap staining, Giemsa staining has an advantage in requiring less staining procedures. In Pap staining, after the samples have been fixed and stained, they must be mounted with mounting medium (*e.g.*, Malinol) for distinguishing sample contents, such as tumor cells, normal cells, and inflammatory cells under a microscope. If the Pap specimen is prepared without the mounting step, it is almost impossible to observe the cells under a microscope because the specimen is dried. In comparison, Giemsa staining can be observed in the dried state, therefore, the mounting step is not necessary for quick cellular evaluation. For microdissection, Giemsa staining is more suitable because it requires dry specimens.

In this report, we introduce a simple and rapid method for preparing TIC specimens with Giemsa staining and demonstrate that TIC is a better source for DNA compared with FFPE specimens.

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Protocol

1. TIC Preparation for Quick Microscopic Assessment Using Normal Glass Slides

1. Perform the TIC preparation as soon as possible after clinical pathological tissue materials are available. If TIC specimens cannot immediately be prepared, keep the tissue materials covered with saline moistened sterile-gauze and store in the refrigerator to prevent drying of tissues.
2. Prepare 5 mm^3 tissue material such as solid tumors (*e.g.*, liver, lung, and breast tissues) clinically obtained by surgery or endoscopy.
    1. Gently wipe the tissue with sterile-gauze coated with physiological saline and remove blood, if the tissue surface has a lot of blood.
    2. For microscopic specimens such as biopsy material, keep the sample moistened with sterile-gauze soaked in physiological saline.
3. Cut and trim the normal tissue with a trimming knife and expose the surface of the tumor lesion, if the tumor masses are not visible grossly.
4. Touch the tumor surface of the resected specimens onto a normal glass slide several times with gloved hands. Visually confirm the touched area is over 80% of the normal glass slide.
5. Lightly press the normal glass slide against a polyethylene naphthalate (PEN) membrane slide and rub gently 2 - 3 times with gloved hands. Visually confirm the cells are transferred from the normal glass slide to the PEN membrane slide.
6. Air-dry both the glass and PEN membrane slides for 5 min at room temperature.
7. Stain the normal glass slide for direct cytological examination. Dip the glass slides with fixative solution for 5 s, and then stain with Giemsa staining solution for 15 s.
8. Assess and screen the tumor contents and cellularity on the normal glass slide entirely with a microscope for quick assessment. Evaluate tumor cells based on several criteria; nuclear enlargement, abnormal karyotype, abundant chromatin, unequal distribution of cells, ratio of nuclear component/cytoplasmic component, cell size, and cell polarity.

2. Preparation of the PEN Membrane Slide Film for Genetic Testing

1. If the sample shows tumor cellularity over 60% by quick microscopic assessment (step 1.8), cut the tumor-touched film of the PEN membrane slides for DNA extraction with a knife and gloved hands. Transfer the cut film to a sterile microcentrifuge tube with a pincette and gloved hands.
2. If the tumor cellularity was determined as low (less than 60% of the tumor contents) by quick microscopic assessment (step 1.8), use laser capture microdissection and obtain tumor samples.
    1. Perform Giemsa staining to assess the tumor cells using standard protocols.
    2. Cut the film of the PEM membrane slide by appropriate laser capture microdissection.
    3. Transfer the cut film to a sterile microcentrifuge tube with a pincette and gloved hands.
    4. Store the film-containing microcentrifuge tube at 4 °C until DNA extraction (the protocol can be paused here).

3. DNA Extraction

1. Perform DNA extraction from the TIC or FFPE tissue samples using a FFPE DNA extraction kit according to the manufacturer's instructions with minor modifications. An equivalent kit is available for the FFPE DNA extraction step.
2. Add 180 µL of tissue lysis buffer (pH = 8.3) to the film-containing microcentrifuge tube with a manual 200-µL pipette. Add 20 µL of proteinase K with a manual 20-µL pipette and mix by vortexing with a vortex mixer at maximum speed (approximately 2,500 rpm) for 5 s.
3. Incubate the samples at 56 °C overnight with an air incubator.
4. Incubate the FFPE and TIC samples at 90 °C in a heat block for 1 h and 10 min, respectively. Briefly spin down the microcentrifuge tube at 1,500 x g for 5 s at room temperature with a mini centrifuge.
5. Add 200 µL of lysis buffer to the sample with a 200-µL pipette and mix thoroughly by vortexing at maximum speed for 5 s.
6. Add 200 µL of ethanol (96 - 100%) with a 200-µL pipette and mix thoroughly by vortexing at maximum speed for 5 s. Briefly spin down the microcentrifuge tube at 1,500 x g for 5 s at room temperature with a mini centrifuge.
7. Carefully transfer the entire lysate to the spin column with a 1,000-µL pipette and centrifuge at 6,000 x g for 1 min at 25 °C.
8. Place the spin column in a clean 2-mL collection tube with gloved hands, and discard the collection tube containing the flow-through into a plastic disposal box.
9. Add 500 µL of wash buffer to the spin column with a 1,000-µL pipette and centrifuge at 6,000 x g for 1 min at 25 °C.
10. Place the spin column in a clean 2-mL collection tube with gloved hands, and discard the collection tube containing the flow-through into a plastic disposal box.
11. Add 500 µL of wash buffer to the spin column with a 1,000-µL pipette and centrifuge at 6,000 x g for 1 min at 25 °C.
12. Discard the collection tube containing the flow-through into a plastic disposal box. Place the spin column in a clean 1.5-mL microcentrifuge tube with gloved hands and centrifuge at 20,000 x g for 3 min at 25 °C to dry the membrane.
13. Place the spin column in a DNA-low binding tube with gloved hands.
    1. Add 40 - 50 µL of elution buffer to the center of the membrane with a 100-µL pipette.
    2. Incubate at room temperature for 5 min and centrifuge at 20,000 x g for 1 min at 25 °C.
    3. Store the DNA samples at -20 °C until the next step (the protocol can be paused here).

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4. Estimation of DNA Quality by Quantitative Real Time PCR

1. Prepare the master mix in a sterile microcentrifuge tube with a pipette, as follows: 10 µL of 2x Real-Time PCR Master Mix, 1 µL of 20x RNase P Primer-Probe Mix (amplicon size: 87 bp), and 8 µL of sterile nuclease-free water.
2. Prepare the second master mix in one sterile microcentrifuge tube as follows: 10 µL of 2x Real-Time PCR Master Mix, 1 µL of 20x RNase P Primer-Probe Mix (amplicon size: 268 bp), and 8 µL of sterile nuclease-free water.
3. Perform serial dilutions of human control genomic DNA (supplied in the kit) 4 times for a five-point standard curve and determine the absolute DNA concentrations[13].
4. Add 19 µL of the two prepared master mixes (prepared in step 4.1 and 4.2) into separate wells of an optical 96-well reaction plate with a 20-µL pipette.
5. Add 1 µL of FFPE DNA or TIC DNA to separate wells containing the reaction mix with a 2-µL pipette. Add 1 µL of nuclease-free water into a separate well containing the reaction mix for the no template control.
6. Hold the non-adhesive side of an optical adhesive film and peel back the protective backing from the center of the film. Gently drag the applicator over the film and seal the film over the 96-well plate.
7. Gently mix the 96-well plate using a 96-well plate mixer for 10 s at room temperature at 2,000 rpm. Centrifuge the plate briefly at 1,000 x g for 3 min at room temperature.
8. Power on the real-time PCR instrument and insert the 96-well plate. Run the PCR reactions using the following protocol: 95 °C for 20 s, followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s. Use "standard curve" and "fast mode."
9. Assess DNA fragmentation with the ratio of DNA (relative quantification; RQ) obtained for the long amplicon (268 bp) to the short amplicon (87 bp). RQ is the mean value of the long amplicon/the mean value of the short amplicon[13].

5. Preparation of the Next Generation Sequencing Library

1. Prepare the sequencing library for next generation sequencing according to the manufacturer's instructions.
2. Prepare the multiplex PCR master mix in a sterile microcentrifuge tube per sample as follows: 4 µL of 5x Multiplex PCR reaction solution, 4 µL of 5x primer pool, ≤6 µL TIC or FFPE DNA (1 - 100 ng), and add nuclease-free water up to 20 µL.
    1. Add the multiplex PCR master mix to a PCR tube and mix gently by tapping the tube.
    2. Briefly spin down the microcentrifuge tube at 1,500 x g for 5 s at room temperature with a mini centrifuge.
3. Run the PCR reactions using the following protocol: 99 °C for 2 min, followed by 20 cycles of 99 °C for 15 s and 60 °C for 4 min, and holding step 10 °C. Briefly spin down the PCR tube with a mini centrifuge at 1,500 x g for 5 s at room temperature.
   NOTE: Determine the number of cycles based on the number of primer pairs.
4. Open the lid of the PCR tube, and add 2 µL of the restriction enzyme with a 2-µL pipette. Close the lid of PCR tube and mix gently by tapping the PCR tube. Briefly spin down the PCR tube with a mini centrifuge at 1,500 x g for 5 s at room temperature.
5. Run the PCR reactions using the following protocol: 50 °C for 10 min, 55 °C for 10 min, 60 °C for 20 min, and holding step 10 °C. Briefly spin down the PCR tube with a mini centrifuge at 1,500 x g for 5 s at room temperature.
6. Add the adaptor ligation master mix into the each well containing the digested PCR amplicons with a pipette as follows: 4 µL of adaptor ligation solution, 0.5 µL of barcode, 0.5 µL of adaptor, 2 µL of nuclease-free water, and 2 µL of DNA ligase. Close the lid of PCR tube and mix gently by tapping. Briefly spin down the PCR tube with a mini centrifuge at 1,500 x g for 5 s at room temperature.
7. Run the PCR reactions using the following protocol: 22 °C for 30 min, 68 °C for 5 min, 72 °C for 5 min, and holding step 10 °C.
8. Purify the sequencing library with magnetic beads according to the manufacturer's instructions.
9. Transfer the adaptor-ligated library solution into the 1.5-mL DNA low-binding tube. Add 45 µL of magnetic beads into the DNA low-binding tube for the 1st purification. Mix gently by tapping the tube and incubate for 5 min at room temperature.
10. Place the DNA-low binding tube in a magnetic rack, then incubate for 2 min at room temperature until the solution is clear. Carefully discard the supernatant with a 200-µL pipette without disturbing the magnetic beads.
11. Add 150 µL of freshly prepared 70% ethanol with a 200-µL pipette, then move the tube side-to-side of the magnet to wash the beads. Carefully discard the supernatant without disturbing the magnetic beads.
12. Repeat step 5.11 for a second wash.
13. Briefly spin down the tube with mini centrifuge at 1,500 x g for 5 s at room temperature. Place the DNA low-binding tube in a magnetic rack, and carefully discard the ethanol droplets with a 10-µL pipette.
14. Add 50 µL of Low TE into the DNA low-binding tube containing the magnetic beads pellet to disperse the beads. Incubate for 2 min at room temperature.
15. Place the DNA low-binding tube in a magnetic rack, and incubate at room temperature for 2 min until the solution is clear.
16. Transfer the 50 µL of supernatant into the new DNA low-binding tube and add 75 µL of magnetic beads with a 100-µL pipette for the 2nd purification. Mix gently by tapping the tube and incubate for 5 min at room temperature.
17. Place the DNA low-binding tube in a magnetic rack, then incubate for 2 min at room temperature until the solution is clear. Carefully discard the supernatant with a 200-µL pipette without disturbing the magnetic beads.
18. Add 150 µL of freshly prepared 70% ethanol with a 200-µL pipette, then move the tube side-to-side of the magnet to wash the beads. Carefully discard the supernatant without disturbing the magnetic beads.
19. Repeat step 5.18 for a second wash.
20. Briefly spin down the tube with a mini centrifuge at 1,500 x g for 5 s at room temperature. Place the DNA low-binding tube in a magnetic rack, and carefully discard the ethanol droplets with a 10-µL pipette.
21. Add 50 µL of low TE into the DNA low-binding tube containing the magnetic beads pellet to disperse the beads. Incubate for 2 min at room temperature.
22. Place the DNA low-binding tube in a magnetic rack, and incubate at room temperature for 2 min until the solution is clear.
23. Transfer the 45 µL of supernatant containing purified library into the new DNA low-binding tube with a 100-µL pipette.

6. Quantify the Library Concentration by Quantitative Real Time PCR

1. Determine the concentration of each library according to the manufacturer's instructions[13].
    1. Prepare a 20-fold dilution solution as follows: mix 2 µL of purified library and 38 µL of nuclease-free water in a DNA low-binding tube with a 2-µL and 100-µL pipette.
    2. Store the undiluted libraries at -20 °C until step 7.3.

2. Prepare a 200-fold dilution solution as follows: mix 5 µL of the 20-fold diluted purified library (prepared in step 6.1) and 45 µL of nuclease-free water in a DNA low-binding tube.
3. Prepare a 2,000-fold dilution solution as follows: mix 5 µL of the 200-fold diluted purified library (prepared in step 6.2) and 45 µL of nuclease-free water in a DNA low-binding tube.
4. Prepare the reaction master mix as follows: mix 10 µL of 2x master mix solution and 1 µL of 20x primer-probe assay solution in sterile microcentrifuge tube with a pipette, then mix by tapping the tube. Add 11 µL of the reaction master mix into the wells of an optical 96-well reaction.
5. Add 9 µL of the 2,000-fold diluted library, 9 µL of each standard control, or 9 µL of nuclease-free water to each well with a 10-µL pipette.
6. Hold the non-adhesive side of optical adhesive film and peel back the protective backing from the center of the film.
    1. Gently drag the applicator over the film and seal the film over the 96-well plate.
    2. Gently mix the 96-well plate using a 96-well plate mixer for 10 s at room temperature.
    3. Centrifuge the plate briefly at 1,000 x g for 3 min at room temperature.

7. Power on the real-time PCR instrument and insert the 96-well plate. Run the PCR reactions using the following protocol: 50 °C for 2 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Use "standard curve" and "fast mode."
8. Calculate the undiluted library concentration by multiplying the concentration determined with qPCR by 2,000.

7. Next Generation Sequencing

1. Plan the run condition and set the run parameter within the software.
    1. Click [Plan tab] and [Template], and select the appropriate run method.
    2. Select the application and technique type, and click [Next].
    3. Select the instrument, sample preparation kit (optional), library kit type, template kit, sequencing kit, base calibration mode, chip type, control sequence (optional), and barcode set, and click [Next].
    4. Select plugins and click [Next].
    5. Select project and click [Next].
    6. Select default reference and BED files of the targeted region.
    7. Type the sample name, select the barcode, and click [Plan Run].

2. Perform template preparation and chip loading in an automated instrument according to the manufacturer's instructions. Thaw the reagent cartridge at room temperature for 45 min before use.
3. Dilute the undiluted library with nuclease-free water according to the library concentration calculated in step 6.8 and make 20 pM libraries.
    1. Prepare a pooled library for sequencing and store on ice.
    2. Add 25 µL of the pooled library with a 100 µL pipette to the bottom of the sample tube. Use the pooled library within 48 h.

4. Power on and open the cover of the automated instrument.
    1. Place the sequencing chip, chip adaptor, enrichment cartridge, tip cartridge, PCR plate, PCR frame seal, recovery tube, solution cartridge, and reagent cartridge to the appropriate position of the automated instrument.
    2. Touch the [Set up Run] and [Step by step] on the screen.
    3. Close the cover and touch [Start check] on the screen.
    4. After the deck scan process, touch [Next] on the screen.
    5. Check the display contents (kit type, chip type, chip ID, sample ID, plans), set the time, and touch [OK] on the screen.

5. After finishing the chip loading:
    1. Touch [Next] on the screen and open the cover.
    2. Unloaded the sequencing chip from chip adaptor with gloved hands.
    3. Place the chip into the chip container, rap with parafilm and store at 4 °C until the sequencing reaction.
    4. Removed the enrichment cartridge, PCR plate, PCR frame seal, recovery tube, solution cartridge, and reagent cartridge from the appropriate position of the automated instrument with gloved hands.
    5. Transfer an empty-tip cartridge to the waste tip position of the automated instrument with gloved hands.
    6. Touch [Next] and close the cover.
    7. Touch [Start] and clean the automated instrument by ultraviolet rays for 4 min.

6. Dissolve a sodium chlorite tablet in 1,000 mL of ultrapure water and filter solution with a 0.22-µm filter flow filter unit.
    1. Power on the sequencing instrument.
    2. Touch [Clean] and [Next] on the screen of the sequencing instrument.
    3. Clean the sequencing instrument with 250 mL of filter-sterilized sodium chlorite solution and subsequently 250 mL of ultrapure water.

7. Touch [Initialize] and select the appropriate sequencing kit on the screen.
    1. Install a gray shipper at the appropriate location with gloved hands.
    2. Initialize the sequencing instrument with the wash solution (provided in the kit), the pH adjustment solution (containing 350 µL of 100 mM sodium hydroxide), and the pH standard solution (provided in the kit).

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8. Add 20 µL of dATP, dGTP, dCTP, and dTTP nucleotides (provided in the kit) in 50-mL tubes (provided in kit) with a 100-µL pipette.
    1. Install a gray shipper at the appropriate location with gloved hands.
    2. Load the 50-mL tube and screw onto the sequencing instrument.
    3. Touch the [Next] on the screen to start the initialization step, which takes approximately 25 min.

9. After completing the initialization step:
    1. Touch [Run] on the screen and select the appropriate library preparation instrument.
    2. Scan the two-dimensional barcode of the chip.
    3. Insert the sequencing chip on the appropriate position.
    4. Close the chip clamp and instrument door.
    5. Touch [Chip check], [Next], and [OK] on the screen to start the sequencing run.

10. After the sequencing reaction, transfer the data and perform data-analyzing pipeline on the sequencing server[13,17].

Representative Results

Figure 1 shows the entire process from preparing TIC specimens to DNA extraction. Notably, the procedure takes only two days to obtain genomic DNA from TIC samples. We evaluated any effects of tumor storage before the slide processing. We found that tumor cells were attached onto the glass slide when tissue specimens were immediately touched onto the slide, and when tissues were kept in saline moistened sterile-gauze for 1 h (**Figure 2**). However, when tissues were kept at room temperature, the tumor cells were not well attached to the slide. Prepared slides could be stored at 4 °C for three months. Therefore, it is important not to allow the specimens to dry.

We examined the utility of our method and compare it with specimens acquired using FFPE. TIC and FFPE samples were prepared from 14 tumor specimens, and we confirmed that tumor contents and purity could be assessed from the TIC specimens. After we performed Giemsa staining, the number of tumor cells and the tumor morphology could be assessed by microscopy. We were thus able to routinely evaluate the tumor cells and subsequently perform a DNA quality check.

The DNA quantity and quality were estimated by quantitative real time PCR[13,14,15]. We determined the absolute DNA quantities and the RQ value, which is an indicator of the degradation level of genomic DNA. The results showed that a higher DNA yield was achieved using the TIC specimens compared with the FFPE specimens (**Table 1**). In addition, the RQ values of the TIC DNA were significantly higher compared with that of the FFPE DNA (**Figure 3**, $p = 2.3 \times 10^{-8}$, two-tailed Student's *t* tests). We also assessed the RQ values of the TIC and FFPE DNA extracted from different tumor types and found that the TIC DNA was higher in quality compared to the FFPE DNA (**Figure 3**). These results indicated that the TIC DNA was less fragmented than the FFPE DNA.

We next assessed whether the TIC DNA could be used for next generation sequencing analysis. FFPE and TIC DNA were prepared from primary colorectal cancer and metastatic liver cancer obtained from a patient (**Figure 4A**). PCR amplification and library preparation were conducted using the Cancer Hotspot Panel and subsequently targeted sequencing was performed. As a result, *APC* Q1367* was identified in both FFPE and TIC DNA extracted from Site 1 (**Table 2**). Furthermore, *APC* S1356*, *KRAS* G12D, and *TP53* M237I were detected in both FFPE and TIC DNA extracted from Site 2, 3, and 4 (**Table 2**). These results suggested that the identical somatic mutations were identified in paired FFPE and TIC DNA samples prepared from the same tumor site (**Figure 4B** and **Table 2**). Notably, the same somatic mutations were detected between primary colorectal cancer (Site 2, not Site 1) and two metastatic liver cancer samples, suggesting that the tumor clones from Site 2 metastasize to liver (**Figure 4B** and **Table 2**). Together these findings suggest that the TIC DNA is high-quality and is suitable for a wide range of genetic testing including next generation sequencing.

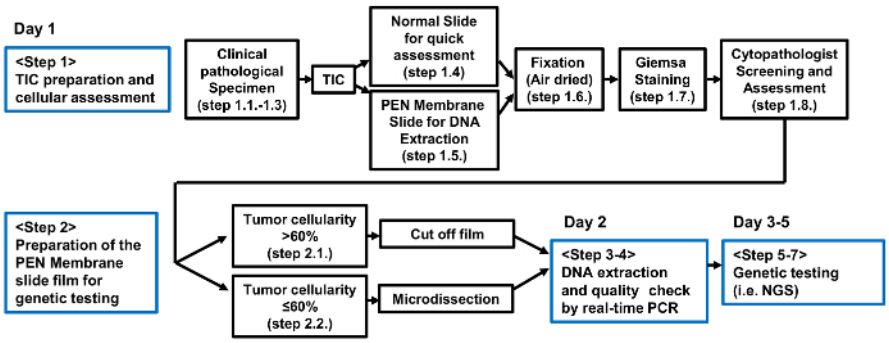

Figure 1.Schematic for obtaining DNA from a TIC sample preparation. The tumor tissue is touched on the slide glass to prepare the TIC sample. After assessment of the tumor morphology and contents under a microscope, the tumor DNA can be extracted and used for genetic testing. By using TIC, the tumor DNA can be obtained from a clinical pathological specimen within two days. Please click here to view a larger version of this figure.

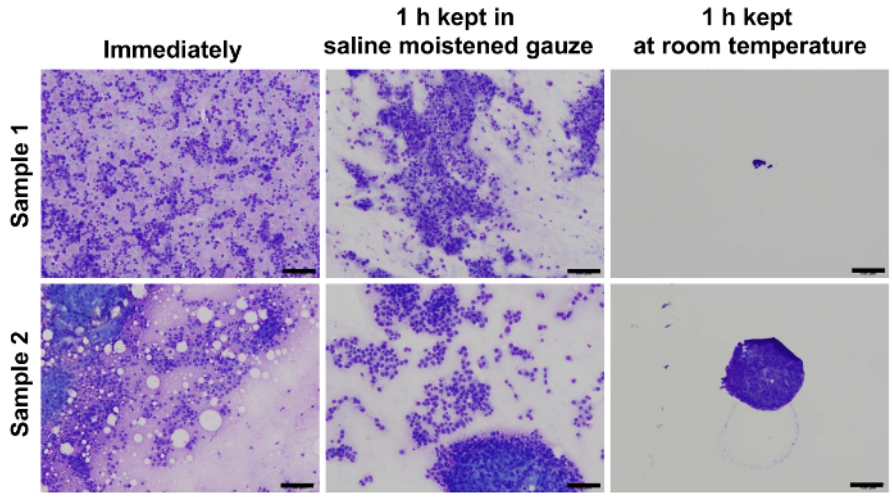

Figure 2. Preparation of TIC samples. Resected tumor specimens were immediately touched onto a normal glass slide (left panel), kept in saline moistened sterile-gauze for 1 h (middle), and kept at room temperature for 1 h (right). Sample #1 was hepatocellular carcinoma and sample #2 was breast cancer. Microscopic pictures were captured using a digital camera mounted to a microscope. Scale bar: 100 µm. Please click here to view a larger version of this figure.

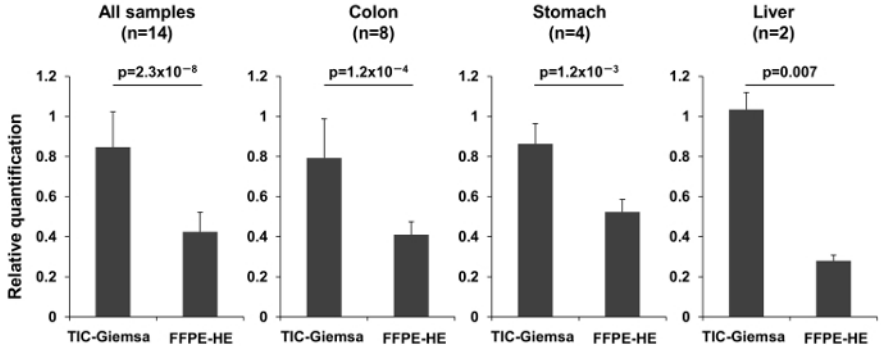

Figure 3. DNA quality check estimated by quantitative real-time PCR analysis. TIC and FFPE DNA were extracted from 14 tumor tissues from colorectal (n = 8), stomach (n = 4), and metastatic liver cancer (n = 2). Comparison of the relative quantification scores between TIC-Giemsa and FFPE-HE samples. RQ values of TIC DNA were significantly higher than that of FFPE DNA. Statistical analysis between the two groups was performed and *p*-values were calculated by unpaired two-tailed Student's *t* test using Excel. Please click here to view a larger version of this figure.

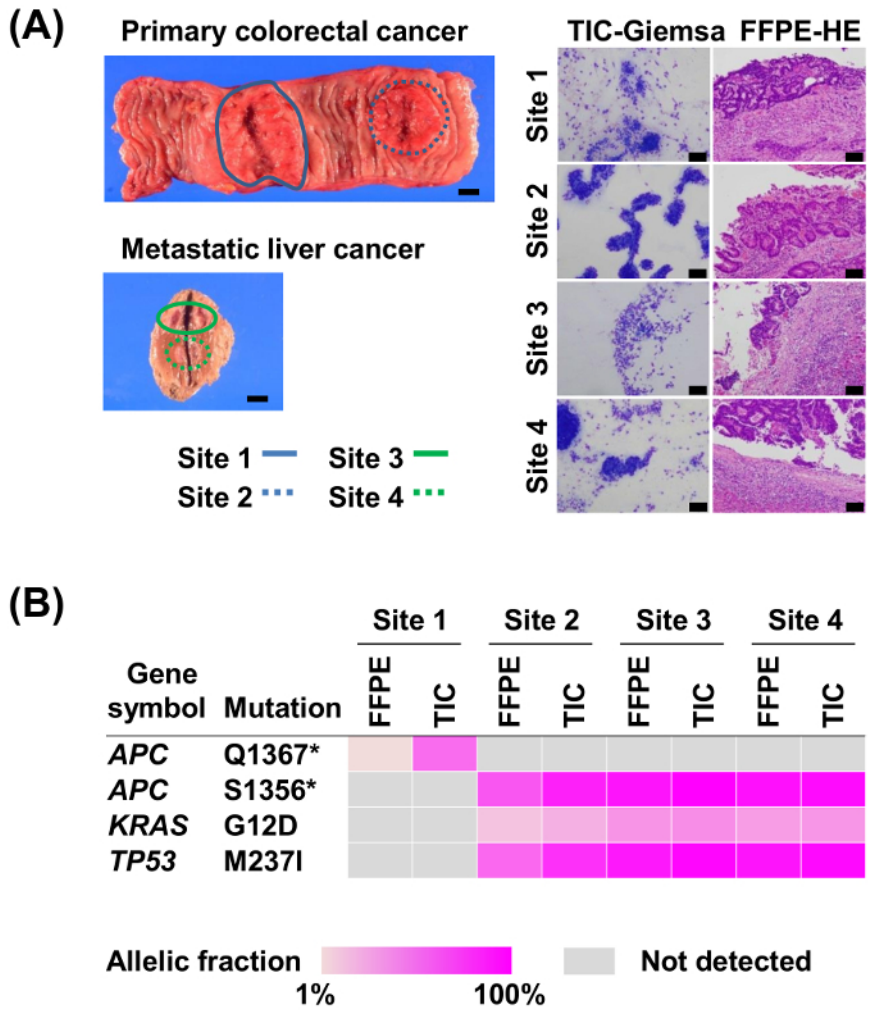

Figure 4. Next generation sequencing analysis data using TIC and FFPE DNA. (**A**) Macroscopic and microscopic images. Representative images of TIC-Giemsa and FFPE-HE staining from the colorectal cancer (site 1 and 2) and metastatic liver carcinoma samples (site 3 and 4). Scale bar in macroscopic images: 1 cm, Scale bar in microscopic images: 100 µm. (**B**) Heat map showing the distribution of somatic mutations for each tumor site (n = 8). Identical mutations were detected among paired TIC and FFPE DNA samples. Values of allelic fractions are indicated in graduation color scale from 1% (light pink) to 100% (pink). Gray columns showed no identified mutation. Please click here to view a larger version of this figure.

		TIC-Giemsa (n=14)					FFPE-HE (n=14)			
		Total DNA (ng)					Total DNA (ng)			
Sample	**Tumor site**	**Short**	**Long**		**RQ**		**Short**	**Long**		**RQ**
Site1	Colon	1495	1247		0.83		939	349		0.37
Site2	Colon	991	1057		1.07		556	204		0.37
Site3	Liver	467	511		1.09		130	39		0.3
Site4	Liver	2172	2115		0.97		488	127		0.26
Site5	Colon	749	598		0.8		529	205		0.39
Site6	Stomach	330	286		0.86		211	98		0.46
Site7	Stomach	636	499		0.78		154	84		0.55
Site8	Stomach	27	27		1.01		135	81		0.6
Site9	Colon	1986	1611		0.81		476	163		0.34
Site10	Colon	280	218		0.78		209	83		0.39
Site11	Colon	1546	575		0.37		366	159		0.43
Site12	Stomach	1501	1200		0.8		274	132		0.48
Site13	Colon	1556	1404		0.9		326	179		0.55
Site14	Colon	1565	1210		0.77		680	295		0.43
	Mean±SD	**1093±682**	**897±600**		**0.85±0.18**		**391±253**	**157±86**		**0.42±0.10**

Table 1. DNA quality data. Paired TIC (n = 14) and FFPE specimens (n = 14) were prepared from colon, liver, and stomach cancers. TIC samples were stained with Giemsa, and FFPE samples were stained with hematoxylin and eosin (HE). DNA samples were extracted and quantified by quantitative real-time PCR with two primer pairs amplifying RNaseP locus (long amplicon (268 bp) and short amplicon (87 bp)). RQ values were calculated as follow: the mean value of long amplicon divided bythe mean value of short amplicon. SD, standard deviation; RQ, relative quantitation

Sample Name	Location	Preparation	Gene symbol	Mutation	Position	Reference	Variant	Coding	Coverage	Allelic fraction
Primary colorectal cancer	Site 1	FFPE	*APC*	Q1367*	chr5:112175390	C	T	c.4099C>T	1999	45%
Primary colorectal cancer	Site 1	TIC	*APC*	Q1367*	chr5:112175390	C	T	c.4099C>T	1011	72%
Primary colorectal cancer	Site 2	FFPE	*APC*	S1356*	chr5:112175358	C	A	c.4067C>A	1968	77%
Primary colorectal cancer	Site 2	FFPE	*KRAS*	G12D	chr12:25398284	C	T	c.35G>A	1993	52%
Primary colorectal cancer	Site 2	FFPE	*TP53*	M237I	chr17:7577570	C	A	c.711G>T	1991	73%
Primary colorectal cancer	Site 2	TIC	*APC*	S1356*	chr5:112175358	C	A	c.4067C>A	1967	89%
Primary colorectal cancer	Site 2	TIC	*KRAS*	G12D	chr12:25398284	C	T	c.35G>A	1994	56%
Primary colorectal cancer	Site 2	TIC	*TP53*	M237I	chr17:7577570	C	A	c.711G>T	1995	86%
Metastatic liver cancer	Site 3	FFPE	*APC*	S1356*	chr5:112175358	C	A	c.4067C>A	1974	92%
Metastatic liver cancer	Site 3	FFPE	*KRAS*	G12D	chr12:25398284	C	T	c.35G>A	1995	62%
Metastatic liver cancer	Site 3	FFPE	*TP53*	M237I	chr17:7577570	C	A	c.711G>T	1296	91%
Metastatic liver cancer	Site 3	TIC	*APC*	S1356*	chr5:112175358	C	A	c.4067C>A	1964	97%
Metastatic liver cancer	Site 3	TIC	*KRAS*	G12D	chr12:25398284	C	T	c.35G>A	1993	64%
Metastatic liver cancer	Site 3	TIC	*TP53*	M237I	chr17:7577570	C	A	c.711G>T	1998	95%
Metastatic liver cancer	Site 4	FFPE	*APC*	S1356*	chr5:112175358	C	A	c.4067C>A	1965	93%
Metastatic liver cancer	Site 4	FFPE	*KRAS*	G12D	chr12:25398284	C	T	c.35G>A	1992	60%
Metastatic liver cancer	Site 4	FFPE	*TP53*	M237I	chr17:7577570	C	A	c.711G>T	1993	92%
Metastatic liver cancer	Site 4	TIC	*APC*	S1356*	chr5:112175358	C	A	c.4067C>A	1960	94%
Metastatic liver cancer	Site 4	TIC	*KRAS*	G12D	chr12:25398284	C	T	c.35G>A	1992	62%

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Metastatic liver cancer	Site 4	TIC	*TP53*	M237I	chr17:7577570	C	A	c.711G>T	1995	95%

Table 2. Comparison of mutations in paired FFPE and TIC DNA detected by targeted sequencing analysis. Paired TIC and FFPE samples were prepared from 2 primary colorectal cancers (Site 1 and 2) and 2 metastatic liver cancers (Site 3 and 4). Targeted sequencing was performed with these DNA samples and somatic mutations were identified. Mutation profiles were identical between paired TIC and FFPE DNA samples.

Discussion

In this study, we presented an alternative method for obtaining tumor DNA from clinical pathological specimens using TIC. TIC preparation is very simple and needs less time compared with FFPE methods, without the requirement for special instruments[10]. All procedures from the TIC preparation to DNA extraction can be completed within two days (**Figure 1**). This method thus shortens the turnaround time for performing genetic testing. Notably, this provides a significant advantage in shortening the number of days required for molecular analysis. This short turnaround time allows us to immediately offer an appropriate therapy for progressive cancer patients who require molecularly targeted drugs. This method thus provides benefits for analyses of genetic alterations in tumors and administration of molecularly targeted drugs for cancer therapy.

There are some key points for the success of using TIC DNA for genetic analysis. Tumor tissues may be necrotic because of treatment such as chemotherapy or other treatments. Thus, caution is needed in preparing TIC specimens to avoid sampling from necrotic sections in the tumor as much as possible. Further, initial microscopic assessment is very important for subsequent procedures. In addition, preventing the drying of tumor tissues is required for successful TIC sample preparation. If the tumor tissues are dried, this results in fewer attached cells on the glass slide. It will also be difficult to observe the tumor cellularity and morphology because drying leads to degeneration of tumor tissues.

There are some potential benefits to using TIC DNA. First, we can check the tumor cellularity during the first assessment with microscopy. If normal cells, such as lymphocytes and stromal cells, were abundant in the tissue samples, the contamination of these normal cells would prevent the ability to detect somatic mutations in the tumor cells. Quick microscopic assessment of the samples may help the evaluation of the tumor cellularity and estimation of whether adequate tumor DNA samples were obtained from the TIC samples before DNA extraction. Second, our results showed that the TIC DNA is both high in quality and quantity. FFPE DNA has been used for next generation sequencing analysis including targeted sequencing, exome sequencing, and whole genome sequencing[16,17,18,19,20]. Archival FFPE DNA is also routinely used for genetic analysis[21,22], however FFPE DNA can be fragmented during formalin fixation. This can result in problems in PCR amplification or DNA extraction, causing a lack of sequence coverage, uniformity at target regions, and increase risk of sequence error[23,24]. In contrast, TIC DNA is not fragmented, which may be due to the alcohol fixation that has less of an influence on the nucleic acid. As TIC DNA is not fragmented like the FFPE DNA, these samples will be more suitable for next generation sequencing analysis, real-time PCR, and digital PCR. Indeed, we previously showed that TIC DNA from a tumor specimen can be used in next-generation sequencing analysis, a method called "TIC-seq", and the results could precisely capture the tumor somatic mutations[13]. Third, this technique is applicable for a wide range of materials. In the current report, we used surgical specimens. In addition to surgical tissues, metastatic lymph node, bronchial or endoscopic biopsy can be used for preparing TIC DNA.

In conclusion, we present a simple and rapid method for preparing high-quality tumor DNA using TIC samples. This method will expand new possibilities in the field of genetic analysis and help promote precision medicine in the clinical setting.

Disclosures

The authors have nothing to disclose.

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