GENOME TECHNOLOGY

Sample Prep Technical Guide

A TROUBLESHOOTING GUIDE: EXPERTS SHARE THEIR ADVICE ON EXTRACTING NUCLEIC ACIDS FROM FFPE TISSUES

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Q&A

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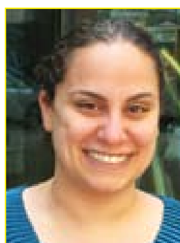
Letter from the editor



Nearly 90 percent of all solid tumor samples are fixed in formalin and embedded in paraffin — that's an estimated 1 billion FFPE samples stored throughout the world. The FFPE process tends to degrade the quality of nucleic acids, making it more difficult for genomic researchers to get any useful information out of the tissue, especially for older samples.

But researchers working on cancer and other diseases need to both store and use FFPE tissue samples — many cancer researchers are now attempting to use tissues that are decades old to better understand how cancer develops. Because of the need for these archival tissue samples, many researchers have developed methods to extract DNA and RNA with as little degradation or contamination as possible. Some use different techniques depending on what the tissue sample will be used for. Many companies have also developed kits to help researchers in the lab.

For this technical guide, we have asked pathologists, genomic researchers, and biomedical scientists from around the world to share the DNA and RNA extraction approaches they find most suited to their purposes when working with FFPE samples. Some of these methods are more complex than others, and some yield more DNA or RNA than others, but through their work, these researchers have found the methods that work best for them — from standard phenol and chloroform extractions, to incubation with proteinase K, to heat-induced retrieval, they're all included in this guide.

— Christie Rizk

Index of experts

Many thanks to our experts for taking the time to contribute to this technical guide, which would not be possible without them.

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Q1

What's the most efficient method for extracting DNA or RNA from FFPE samples?

DNA: Standard phenol/chloroform-extraction works fine for PCR-based methods and sequencing. Commercial kits are also available, focusing on FFPE material, e.g. QIAamp DNA Micro Kit from Qiagen.

RNA: Standard phenol/chloroform-extraction works fine for qPCR of mRNA. The RNeasy FFPE Extraction kit from Qiagen gives better results for miRNA.

Note: before DNA or RNA extraction is performed, the tissue has to be inspected by a pathologist and the areas of interest histologically confirmed.

— Karl-Friedrich Becker

DNA: We have found heat-induced retrieval at highly alkaline pH to be the best method, followed by Qiagen's QIAamp DNA Micro Kit.

RNA: I would use Ambion's RecoverAll kit or Qiagen's RNeasy FFPE kit.

— Ram Datar

We use mineral oil for the deparaffinization process for DNA and RNA extraction from FFPE tissues. By using mineral, oil we obtain better performance in the paraffin removal, and it results in less time consumed and is a non-toxic method. The extraction of DNA is followed up by using a Qiagen ATL buffer and proteinase K solution. The RNA extraction is performed by incubation of the samples for three hours at 56°C in a digestion buffer containing 5 percent SDS, sodium-citrate and proteinase K. Finally, we isolate the RNA following the indicated protocol by using TRIzol from Invitrogen.

— Angélica Figueroa

This will depend on the age and condition of the samples. There are also a number of commercial kits available and sometimes it is a matter of trying a few to see which will be best for a particular application. A key step is adequate incubation with proteinase K. We have had significant success using an overnight incubation step. Other measures include incubation at high temperatures, which also disrupts protein cross-links and removes chemical modifications induced by formalin fixation. We currently use the High Pure RNA Paraffin Kit from Roche.

— Vincent Gnanapragasam

Straight rehydration of the sample, followed by proteinase K digestion in Tris-hydrochloride/EDTA, followed by the usual phenol/chloro-

"Sometimes it is a matter of trying a few [commercial kits] to see which will be best."

— Vincent Gnanapragasam

form isolation for DNA, or TRIzol LS from Invitrogen for RNA, according to manufacturer's specification.

— Ronald Przygodzki

We perform a heat-based method to remove the paraffin from the samples, to avoid the headaches associated with the organics, and follow up with a Qiagen QIAamp genomic DNA isolation kit with a few modifications to the manufacturer's protocol.

The FFPE sections are placed in a 1.5 mL Eppendorf tube with 300 µl of tissue lysis buffer and heated at 75°C for five to 10 minutes to melt the paraffin. The samples are mixed partway through the procedure to ensure a good mixing of the sections with the buffer. The tube is centrifuged for one minute at 13K in a standard bench-top microfuge at room temperature, which allows the wax to form a soft layer on the top of the tissue suspension. This waxy layer is carefully removed with a pipette tip, making sure that no tissue is still attached to the wax. This heating and spinning process is repeated until there is no waxy layer remaining after centrifugation; this often takes three cycles. Our second modification involves the proteinase K digestion of the tissue. Formalin chemically cross-links protein to DNA, which can interfere with downstream enzymatic processes. We do a prolonged proteinase K digestion, for at least three days and up to a week at 55°C to 60°C at a final concentration proteinase K at 0.5 mg/ml. The samples are mixed periodically to check on tissue degradation, and more proteinase K may be added if needed. The last modification to the Qiagen QIAamp protocol is to elute the DNA in a lesser amount of TE buffer, using 50 µl to 100 µl of 70°C buffer instead of the recommended 200 µl and allowing at least five minutes at 70°C elution time.

— Janice Spence

We mostly extract DNA from small amounts of archived tissue to detect and genotype human papillomaviruses. For this purpose, the best results were seen with a combination of high heat treatment and a commercial kit for DNA purification. Typically, we incubate one 10-µm section with lysis buffer at 120°C followed by overnight digestion with proteinase K at 65°C. For further purification we use either Qiagen DNeasy or Chemagen's Chemagic MS1 for automated processing. Not only does this procedure eliminate the need for laborious deparaffination with xylene, quantity and quality of the resulting DNA was better than any of the traditional procedures we tried. Its simplicity allows processing of samples for a full 96-well plate with ease and the use of commercial kits warrants good consistency and standardization. The method also works well with stained tissues scraped from slides, although the required manual labor for that task limits throughput and efficiency.

— Martin Steinau

"The best results were seen with a combination of high heat treatment and a commercial kit."

— Martin Steinau

Q2

How does this approach affect the quality of the DNA or RNA?

Standard phenol/chloroform-extraction shows lower purity of the samples but this does not affect the results, at least in our hands.

— Karl-Friedrich Becker

DNA: The methods of HIR or QIAamp DNA Micro Kit both yield satisfactory results with DNA.

RNA: Both the RecoverAll kit and the Qiagen RNeasy kit yield satisfactory results with RNA.

— Ram Datar

We check the quality of DNA or RNA extracted samples by the determination of the concentration and 260/280 ratio in a Nanodrop spectrophotometer. We consider a sample to be good quality when ratios are between 1.8 and 1.99. However, in DNA samples there is higher risk of fragmentation when the size checked is too big (higher than 2 kb). We also use housekeeping genes, such as GAPDH and HPRT, as controls to estimate the quality of the extracted material. Our protocols have satisfactory quality for the experiments we perform in the laboratory.

— Angélica Figueroa

Overnight proteinase K incubation can affect RNA quality, but should not be a big problem for DNA quality. For mRNA and miRNA profiling, which is our main area of work, we have not found this to be a problem.

— Vincent Gnanapragasam

Column preparations of genomic DNA often generate DNA fragments of approximately 20 kb. Other approaches should be used if longer pieces are required.

The use of heat over organic solvents may prevent the elution of inhibitory products with the DNA, as many publications site the need to limit the use of FFPE DNA to 50 ng to 100 ng per PCR reaction, while we routinely use 250 ng to 400 ng per reaction when testing subsets of cells within the tissue.

The prolonged proteinase K treatment appears to greatly improve the ability to generate long PCR products from these DNA templates, as we routinely amplify over 1 kb products from FFPE DNA samples.

— Janice Spence

DNA quality from FFPE tissues will always be compromised, but this approach consistently yielded DNA in sufficient quality for our downstream applications. Mostly we target DNA regions 450 bp or smaller. Of roughly 6,500 processed samples from various places and labs, about 1 percent were insufficient for subsequent PCR amplification. We found that concentrations of PCR inhibitors are slightly higher in DNA extracts prepared without paraffin removal by xylene, but that disadvantage is well compensated by the higher yield overall.

— Martin Steinau

Q3

Does this approach usually provide enough DNA or RNA for your purposes?

Yes. Usually we perform mutation analyses or RT-qPCR reactions from the extracted nucleic acids. For these purposes we get plenty of nucleic acids from FFPE tissues.

— Karl-Friedrich Becker

DNA: Yes, adequate yield is obtained using both HIR and QIAamp DNA Micro Kit.

RNA: Yes, both Ambion's RecoverAll kit and Qiagen's RNeasy FFPE kit result in sufficient amounts of RNA, especially for RT-qPCR analyses.

— Ram Datar

Yes, we obtain enough sample to perform the experiments. We generally obtain 200 ng/μl of DNA and around 500 ng/μl of RNA.

— Angélica Figueroa

Our main application is from tumor microdissection of needle core biopsies of tissue. Even with this we are able to derive between 25 ng/μl to 30 ng/μl (about 60 μl total) of starting RNA/DNA. These are from four to six contiguous 5 μm thick slides (about 8,000 to 10,000 cells).

— Vincent Gnanapragasam

> "Being able to view the block before ordering the sections often proves useful."
>
> — Janice Spence

Techniques working around the 150-bp limit help us provide for the answers — if the question is a rearrangement, one works within the confines of the rearrangement, be it with amplification spanning the rearrangement or the RNA expression product.

— Ronald Przygodzki

One issue with FFPE samples is the actual proportion of tissue within each slice, so being able to view the block before ordering the sections often proves useful. Approximately 100 microns of section with over 35 percent tissue (4x25 microns) usually provides more than 10 micrograms of DNA, and it can be substantially more. DNA recovery is also dependent on the tissue samples, as some tissue types lack a nucleus, while necrotic tissues have often undergone DNA degradation.

— Janice Spence

Yes, although yields from one 10-μm section are certainly limited. Typically, our method yields 100 μl with DNA concentrations in the single digit nanogram range per microliter. This has been absolutely sufficient for a number of PCR based diagnostic assays with 10-μl input each.

— Martin Steinau

> "DNA quality from FFPE tissues will always be compromised."
>
> —Martin Steinau

Q4

How do you check the quality of the samples, and what level of degradation or contamination is acceptable?

We measure quantity and purity on a Nanodrop photometer. Degradation is not a problem because we only amplify short fragments.

— Karl-Friedrich Becker

DNA: We use spectrophotometry on a Nanodrop to determine 260/280 ratio of between 1.6 and 1.9, and agarose gel electrophoresis to ensure DNA fragment sizes spanning 0.3 kb to 2 kb.

RNA: We use analysis employing Agilent 2100 Bioanalyzer. The RNA Integrity Number (RIN) is typically between 5 and 7 from an FFPE isolate, which is adequate for RT-qPCR, particularly if the size of the target RNA is less than 0.5 kb.

— Ram Datar

We check the quality of DNA or RNA extracted samples by the determination of the concentration and 260/280 ratio in a Nanodrop spectrophotometer. We consider a sample to be of good quality when ratios are between 1.8 and 1.99. We also use housekeeping genes, such as GAPDH and HPRT, as controls to estimate the quality of the extracted material. We run agarose gels to analyze the level of degradation and contamination of the extracted RNA obtained. For the extracted DNA, we recommend a Bioanalyzer to rapidly and efficiently analyze DNA fragments amplified by PCR before sequencing.

— Angélica Figueroa

We accept the fact that our samples will be degraded and we use the Nanodrop to assess our material (primarily for quantification). In general, we need fragment lengths of between 100 bp and 300 bp. An important quality step for us is detection and expression of at least three housekeeping genes with acceptable Ct values — hence, keeping the design of the primers and amplicons small. I think the major step forward in using FFPE-derived material has been in the improvement of methods to accurately detect genes of interest despite degradation.

— Vincent Gnanapragasam

Samples are typically run in triplicate to see if the diagnostic findings are similar among all three, or at least two, of the tests per given case. One has to assume degradation among the samples — they never will be of the same quality as fresh or frozen samples.

— Ronald Przygodzki

The only quality-control check we perform is to quantify the samples using Nanodrop technology. The OD 260/230 ratio seems to predict which samples will fail some downstream processing, such as sequencing. We hypothesize this might be due to organic contamination.

— Janice Spence

Initially, we visualized DNA extracts by gel electrophoresis to see which conditions would produce the most intact DNA and high molecular weight. However, most important is the compatibility with downstream application. Therefore, we regularly check the DNA quality and quantity by real-time PCR targeting fragments with similar size to the relevant test applications.

— Martin Steinau

Q5

Do you find heat-induced retrieval to be an efficient extraction method? Why or why not?

So far we have not tested heat-induced retrieval methods for DNA/RNA extractions from FFPE tissues. We developed a method for protein extraction from FFPE tissues; here, heat-induced retrieval is essential.

— Karl-Friedrich Becker

DNA: It was demonstrated that the efficiency of DNA extraction from FFPE tissue sections is dependent on the temperature and the pH of the retrieval solution used, with higher temperature and higher pH producing better results. However, in contrast to enzymatic digestion, which results in near-complete dissolution of the tissue controllable by the incubation time of enzyme digestion, we found that integrity of the tissue sections remained significantly intact after heating in the Britton and Robinson buffer solution. Also, although heat-induced retrieval improved quality of DNA extracted from FFPE tissue sections, which was evident from successful PCR, the DNA yields were still lower than those obtained by the regular non-heating, enzymatic method. We tested some chemicals in combination with a heating protocol to determine an optimal method. A severely alkaline environment at high temperature (0.1M NaOH, pH 12.93 at 120°C) provides an effective retrieval strategy for maximal DNA extraction.

RNA: I prefer not to use heat-induced retrieval for RNA as both the quality and quantity obtained are often sub-optimal.

— Ram Datar

By using mineral oil for the deparaffinization process, we obtain an efficient extraction of the nucleic acids and we avoid the heat-induced retrieval as the high incubation temperature results in greater nucleic acid fragmentation.

— Angélica Figueroa

Heat induction is not performed *per se*, but the extraction with proteinase K is at the star value of 55°C, with heat inactivation for a few minutes at 95°C.

— Ronald Przygodzki

Clearly, from a number of evaluated approaches, heat-induced retrieval was the most effective method. It appeared that incubation at 120°C for limited time had no adverse effect on the DNA. Higher temperature was directly correlated to higher amplifiable DNA yield. It might be the most efficient method to reverse cross-linking induced during the tissue fixation. An additional indirect increase of efficiency results also from the associated improvements in workflow, such as the elimination of xylene washes and accidental removal of small tissue fragments during these pipetting steps.

— Martin Steinau

Q6

How does the intended use for the sample affect your decision on how to extract it?

Standard phenol/chloroform-extraction is not applicable for miRNA work, so we use the Qiagen kit for this approach.

— Karl-Friedrich Becker

DNA: FFPE DNA extracts from most samples are acceptable for PCR and mutation analyses, as long as the DNA is not excessively depurinated by extended formalin treatment.

RNA: Most FFPE RNA extracts are of acceptable quality for qualitative and quantitative RT-PCR (as long as the target amplicons are less than 300 nt) and microRNA analyses. If microarray analysis is the intended application, I would prefer to use RNA extract with RIN greater than 7.

— Ram Datar

In our case, we use RNA extraction from FFPE to perform RT-qPCR, and we use the DNA extraction for sequencing analysis. In our case, these methods described above are suitable for the protocol chosen in every specific case.

— Angélica Figueroa

> **"Some methods would require higher purity and paraffin removal."**
>
> **— Martin Steinau**

Optimized PCR and array platforms are making this question less of a problem. In general, our application is in PCR and the general extraction methods work well for this. We have now incorporated a pre-amplification step prior to PCR, which has also helped. We have not really altered our extraction methods for different uses.

— Vincent Gnanapragasam

No difference is perceived, given we are working with archival tissues.

— Ronald Przygodzki

The primary use of DNA in our lab is for PCR amplification, thus our reliance on the single protocol above.

— Janice Spence

We have not evaluated our method for other uses than PCR applications. It is possible that some methods would require higher purity and paraffin removal, but for our purposes the decision was clear: reduced labor, possible throughput and adaptability to automation favored a kit, plus heat treatment over any other method.

— Martin Steinau

> **"FFPE DNA extracts from most samples are acceptable for PCR and mutation analyses."**
>
> **—Ram Datar**

List of resources

Sometimes you need to know more. Here are some sources that may help.

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Keynote Speakers:

Richard K. Wilson, Ph.D., Professor of Genetics & Director, The Genome Institute, Washington University School of Medicine

Elizabeth A. Worthey, Ph.D., Assistant Professor, The Human and Molecular Genetics Center, Pediatrics, Bioinformatics, Medical College of Wisconsin

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