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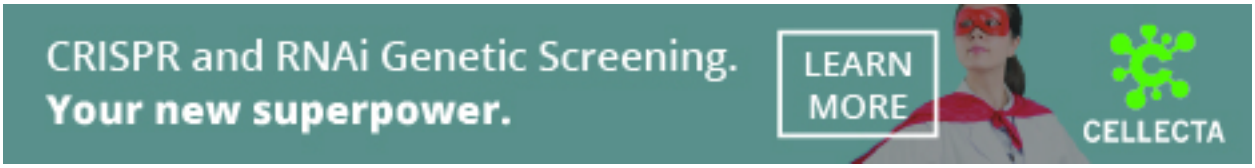

Sample Preparation and PCR Amplification from Paraffin-embedded Tissues

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The exquisite sensitivity of PCR has afforded molecular studies of fixed paraffin-embedded tissue (PET) specimens, which comprise most archival clinical material. Combined with subsequent hybridization methods or DNA sequencing, PCR has provided the sensitive and specific detection of infectious agents and host genetic alterations. This technology provides the tools for retrospective molecular studies of human disease processes. The presence of human papillomaviruses (HPV)[1,2] and herpesviruses[3] was investigated in early PCR-based studies of PET specimens. Other studies probed cancer-associated mutations, such as in the *ras* gene.[4,5] PCR-based microsatellite analysis has greatly increased our ability to test primary human tumor DNA for areas of allelic loss and genomic instability from carefully microdissected PET specimens.[6,7]

The use of PET specimens has been essential in the identification of etiologic agents associated with rare syndromes such as Whipple's disease.[8] In the case of the Whipple's agent, *Tropheryma whippelii*, a new microbial evolutionary relationship was also established. In a recent study, both genetic (HLA DR-DQ haplotypes) and infectious agent (HPV) information was obtained from PET specimens in a cervical cancer collection.[9] Using PCR-based methods, associations between the risk of HPV-16-related cervical cancer and particular HLA DR-DQ haplotypes were demonstrated with this archival tumor series. Archival PET specimens will certainly continue to provide molecular epidemiologists with invaluable clinical material.

The preparation of PET specimens for use in PCR amplification is theoretically very simple. The paraffin must be dissolved from the tissue slice, and the dried tissue must then be treated to liberate DNA.[10] Complete DNA purification is possible but often unnecessary. Such extensive purification procedures must be weighed against the increasing risk of sample contamination with each manipulation. (This is of extreme importance when PET specimens are being analyzed for the presence of an infectious agent.)

The success of any PCR-based study of fixed, paraffin-embedded material depends on several factors, including (1) the fixative used in the tissue processing, (2) the duration of the fixation, (3) the age of the paraffin block, and (4) the length of the DNA fragment to be amplified. Below, we review the results of studies conducted to evaluate the suitability of PET specimens as subsequent PCR targets. We also provide updated protocols[10] and methodologic considerations for retrospective PCR-based studies.

EFFECTS OF FIXATION

Although PCR DNA amplification is a powerful tool for retrospective studies, not all preservation or fixation methods render DNA that is suitable for subsequent amplification.[10–14] Previously, we reported extensive analyses of the effects of commonly used fixation methods on the efficiency of subsequent PCR amplification.[12,13] In those studies (see Table 1), the effect of fixation was measured by the ability of the DNA in a treated tissue to act as a template for the amplification of DNA fragments of increasing lengths. The effect of each fixation method tested is clearly reflected by the maximum product length obtained from each treated tissue. Of the fixatives tested, those most successful in subsequent PCR amplifications are fixed in ethanol, acetone, or OmniFix, followed by 10% buffered neutral formalin (BNF). Another group of fixatives including Zamboni's, Clarke's, paraformaldehyde, formalin/alcohol/acetic acid, and methacarn compromise amplification efficiency. Tissues fixed in highly acidic solutions (Carnoy's, Zenker's, or Bouin's) are seriously compromised for amplification and were not considered desirable.[13,14]

Closely associated with the effects of type of fixative used in sample processing is the length of time a sample is maintained in a fixative. Our previous studies indicate (Table 1) that after 24 hr of tissue fixation, the ability to

TABLE 1 Effect of Fixation on Subsequent PCR

Fixative	Maximum length product after 24-hr fixation (bp)	Largest fragment generated (bp) and duration of fixation	
Acetone	1327	1327	8 days
Alcoholic formalin	989	989	24 hr
Bouin's	0	110	1 hr
10% BNF	1327	1327	24 hr
Carnoy's	268	989	4 hr
Clark's	989	1327	4 hr
Ethanol	1327	1327	30 days
Formalin/alcohol/acetic acid	989	1327	1 hr
Methacarn	989	1327	1 hr
Paraformaldehyde	989	1327	1 hr
OmniFix	1327	1327	72 hr
		989	30 days
Zamboni's	989	1327	4 hr
Zenker's	110	268	4 hr

Data are summarized from previous reports.[12,13]

amplify large PCR products decreases with all fixatives tested except ethanol, acetone, and OmniFix. The nonacidic fixatives afford the subsequent amplification of fragments 536 bp or greater in length. Although many clinical laboratories routinely fix tissues for 24 hr or less, some tissues may be treated for up to several days, thereby reducing the amplifiable fragment size. For example, biopsies are often placed in buffered formalin and shipped to reference laboratories for embedding and analysis. A more extreme situation exists when tissue sampling occurs in remote regions, thus requiring fixation and storage for extended periods of time prior to analysis. Consequently, the length of time a tissue is immersed in a fixative can be as critical as the type of fixative used. Both factors should be taken into consideration when planning either a retrospective or prospective study using PET specimens. When the DNA is compromised, an amplification strategy utilizing smaller PCR products (<200 bp) is necessary.

EFFECTS OF SPECIMEN AGE

The approach used to assess the effects of fixation on subsequent DNA amplification was also applied to test the effects of specimen age. An unpublished study conducted at the University of New Mexico included 240 PET samples representing all invasive cervical carcinomas that were diagnosed at the University of New Mexico Hospital over a 20-year period. These specimens were tested for their ability to generate three sizes (268, 536, and 989 bp) of PCR fragments when used as template. All samples had been fixed in 10% BNF, and each specimen age point included at least 20 specimens. The results (Fig. 1) showed that after 16 years, 90% of the samples were suitable for the amplification of the 268-bp β-globin fragment. Successful amplification of this fragment decreased to 45% for 20-year-old specimens. As the size of the amplified fragment was increased from 268 to 536 bp, a significant effect of specimen age was detected. Only 60% of the reactions using 5-year-old specimens were successful in the amplification of a 536-bp fragment. The most dramatic effect of specimen age was seen when the amplified fragment size was increased to 989 bp. Here, a linear decrease in the number of successful amplifications was observed such that by 5 years there was no appropriate template DNA generated for the amplification of the 989-bp fragment. Clearly, in the case of samples older than 5 years, the smaller the fragment, the greater the likelihood of successful amplification.

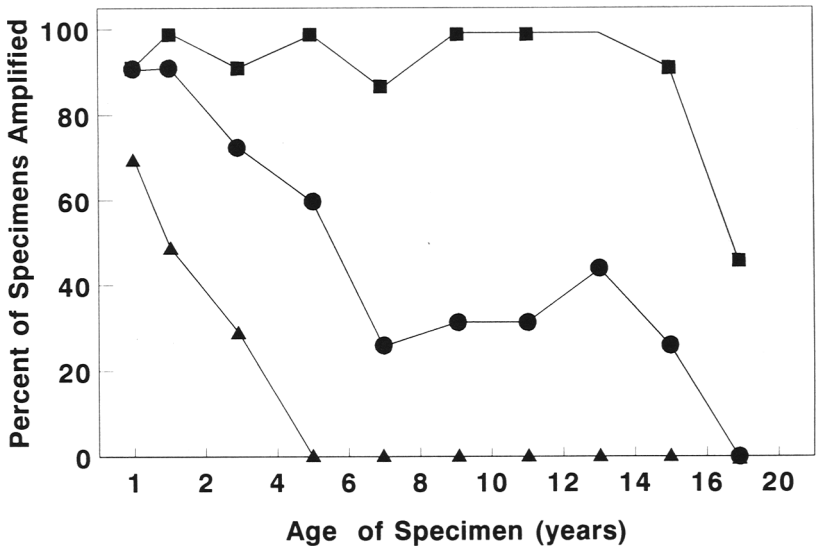

FIGURE 1. Effect of specimen age on maximum size of amplification product. Tissue blocks used in this study were 1–20 years old. Invasive cervical cancer biopsies that had been fixed in 10% BNF and paraffin-embedded were deparaffinized, subjected to proteinase K digestion, and amplified with various β-globin primer pairs. (■) 268-bp β-globin; (●) 536-bp β-globin; (▲) 989-bp β-globin. Each time point represents at least 20 PET specimens from the specimen age group.

Because of the many variables involved in PET fixation and processing and the variable efficiencies of primer pairs, it is recommended that comprehensive pilot studies be conducted to assess the DNA quality of any collection of PET specimens. Results of these studies would help to determine the optimal amplification product lengths that could be obtained from the specimens chosen.

MATERIALS AND METHODS

Reagents

Tissue sections
10% bleach solution (freshly diluted)
Octane, xylene, or AmeriClear (Baxter Scientific)
100% ethanol
HPLC-grade acetone (optional)
Proteinase K (20 mg/ml stock solution)
Digestion buffer: 50 mM Tris-HCl (pH 8.5); 1 mM EDTA; 1% Laureth 12 ["Macol LA-12," PPG Company (formerly Mazer Chemicals), Gurnee, IL] or 0.5% Tween 20 or 1% Laureth-10 (Sigma)

Materials

Two-inch sterile, gauze pads (Johnson & Johnson, Thomas Scientific 2904-C12)
1.5-ml microcentrifuge tubes, flat top and tight fit (Sarstedt 3207, 3210)
Cap-Locks (Inner-Mountain Scientific C-3270-1)
Sterile disposable plastic pipettes

*Dry heat blocks at 55°C and 95°C
*Microcentrifuge (PCR-product and plasmid clean)
*PCR-product clean test tube racks
*Vortex mixer Rotating or rocking platform shaker
Quartz sand (Sigma S-9887)
*These items must be dedicated for clean, pre-PCR use.

Preparation of Tissue Sections

A fundamental safeguard to prevent PCR product contamination is to process the samples in an area physically separated from post-PCR sample analysis. The sectioning of paraffin blocks requires considerable effort to prevent sample-to-sample contamination. Rigorous cleaning of both the microtome, microtome blade, and any equipment used in the sectioning area must be completed between each paraffin block. This is accomplished by squirting freshly diluted 10% bleach[15,16] onto gauze squares and carefully cleaning the microtome. The bleach wash should be followed by an ethanol rinse to prevent corrosion of the microtome. The blade must be removed and carefully wiped clean of any debris with another clean, bleach-soaked gauze. Any tissue remaining on the blade may easily contaminate the next sample. Disposable blades provide the greatest protection from block-to-block contamination. Finally, it is essential to change gloves between the cleaning of the microtome and the sectioning of each new block.

Once the microtome has been cleaned thoroughly, the first section is taken to expose a PCR "clean" surface. Replicate sections (5–20 μm) can be cut from each block, and a single section placed in a sterile, 1.5-ml microcentrifuge tube. The thickness of the section depends on the size of the tissue. For a small biopsy (2–3 mm), 10- to 20-μm sections may be required, whereas larger tissues (5×5 mm) can be sectioned at 5 μm. Although multiple sections can be placed in a single tube, fewer thick sections are more practical for processing.

If the target tissue is localized within a limited portion of the block, such as the area of tumor invasion, it is critically important to prepare adjacent, flanking "first and last" sections (5 μm) for hematoxylin and eosin (H&E) staining. First, it will ensure, in the case of a negative result, that the tissue of interest was present. Second, it will allow confirmation of the original histologic diagnosis. In some cases, the tissue may be microdissected within the block or on a mounted section. This is common, for example, when identifying tumor-specific mutations.[5] Because such manipulations are vulnerable to minute amounts of contamination, this is not recommended for infectious disease studies.

Deparaffinizing Sections

1. Centrifuge the tissue section to the bottom of the 1.5-ml tube (~5 sec).
2. Open the tube by holding a clean gauze square over the cap and gently prying off the top. Never "pop" or "flip" the tube open with your thumb or touch the inside of the cap; this can cause sample-to-sample contamination.
3. Add 1 ml of octane (or AmeriClear, or xylene) and gently vortex to loosen the paraffin from the bottom of the tube. Most paraffins dissolve quickly (2–5 min), but others may require gentle vortexing or continued mixing on rotating platform shaker at room temperature (up to 30 min) to be dissolved. Deparaffinized tissue is opaque and "fluffy" in appearance, whereas undissolved paraffin is solid white and rigid.
4. Pellet the tissue and any remaining paraffin by centrifugation for 2–5 min at full speed in a microcentrifuge.
5. Carefully remove the solvent with a single-use, fine-tipped glass pipette. Do not disturb the tissue as it is easily dislodged. Do not remove any tissue while pipetting. If this occurs, expel it into the tube and repeat the centrifugation. (If Pasteur pipettes are used, each must be cotton-plugged to prevent contamination of the pipette bulb and thus other samples.)
6. Repeat steps 3–5 if any paraffin remains.
7. Carefully add 0.5 ml of 100% ethanol to the tube and mix well.
8. Centrifuge for 2–5 min, and carefully remove the ethanol.

9. A drop (10–30 μl) of HPLC-grade acetone can be added to speed the evaporation of the ethanol. To dry the tissue, the open tubes should be carefully placed in a sand-filled, 55°C heat block. **Note:** Never use speed-vacs or vacuum bottles, where sample-to-sample contamination readily occurs, to dry the tissue pellets. The open tubes are vulnerable to contamination at this point. Therefore, do not allow contact between individual tubes and eliminate air flow around the tubes. PCR product or plasmid-containing tubes should never be used in this area and, specifically, this heat block. The sand in the block should be changed frequently to reduce the possibility of contamination.

10. The appearance of the tissue changes from opaque when wet to solid white when dry. Handle the tubes carefully, as static electricity can cause the dry tissue to pop out of the tube.

Proteinase K Digestion

1. Suspend the dry pellet in fresh digestion buffer (typically 100 μl). The required volume varies with the amount of tissue present after deparaffinization. For example, a 3×5-mm× 5-μm tissue pellet should be digested in ~250 μl of buffer. Smaller pellets, 2×3 mm×10–20 μm, should be digested in 50 μl of buffer. In general, the dried tissue should occupy ~25–30% of the volume of digestion buffer when the buffer is first added to the tube.

The tissue must be completely immersed in the digestion buffer. If necessary, vortex and briefly centrifuge tubes before incubation.

2. Incubate at 55°C for 3 hr (small biopsies) to overnight (larger pieces of tissue). Intermittent mixing or continuous rocking of the tubes may help with larger pieces of tissues. Very large specimens may require a longer incubation (up to 48 hr) and additional proteinase K.

3. Just prior to proteinase K inactivation, briefly centrifuge the tubes to ensure that all liquid is at the bottom of the tube. Place tubes in a 95°C heat block for exactly 10 min. Prolonged heating can damage the DNA, and heating for less than 8 min may not fully inactivate the proteinase K. Additional time is required for volumes >0.5 ml. **Note:** For this incubation, cap locks are usually necessary to prevent caps from popping open. Alternatively, some brands of microcentrifuge tubes (Sarstedt, Costar) can accommodate this high-temperature step and do not require cap locks when heat-inactivating volumes <0.6 ml.

4. Just prior to amplification, pellet any remaining debris by microcentrifugation for ~10 sec. Amplify 1 and 10 μl of the crude DNA preparation.

5. It is recommended that the samples be amplified promptly after digestion. However, the digested tissue can be stored for 1 month at −20°C or below. Some targets, such as multiple-copy genomic sequences, are amplifiable after several months.

Alternative Methods of Crude DNA Extraction

Recently, several reports have introduced simplified methods for PET sample preparation and subsequent amplification. Although these procedures may be more rapid than those outlined in this chapter, they may not be suitable in all situations. Methods such as sonication[17,18] and boiling[19] do not extract longer DNA fragments as efficiently as proteinase K digestion,[20,21] and may not liberate adequate copies of target DNA. This can be particularly problematic if the desired target is present in low copy numbers or if the concentration of background DNA is particularly high. Furthermore, these methods may promote the preferential amplification of small DNA fragments. These issues must be addressed and determined experimentally with

pilot studies using several samples of the PET collection. Ultimately, the decision must be based on the requirements of the individual study.

Preparation from Cytology Slides

While amplification of DNA extracted from cells fixed on microscope slides has not been widely investigated, some researchers have utilized these materials.[22,23] In this procedure, the coverslip is removed by immersion in xylene for up to 2 days. The H&E stains are removed by successive incubations in ethanol solutions with increasing concentrations of water. Complete removal of the stains is required for successful amplification. Cells are removed from the slide by the addition of a proteinase K digestion buffer and carefully scraping the cells into a 1.5-ml microcentrifuge tube. Samples are digested at 50°C for 60 hr. After digestion, the proteinase K is heat-inactivated and samples are amplified directly. In some cases, further DNA purification (phenol/chloroform extraction, EtOH precipitation) may be required.

General Considerations for Preventing Sample Contamination

Be conscious of cross-contamination and use techniques that reduce the occurrence. Never work directly over open vials. Be extremely careful opening any tubes and always centrifuge tubes before opening. Use gauze squares to cover the lid when opening tubes to prevent aerosols. Gently pry or pull open tubes; never pop or flip caps open with your thumb.

Do not create aerosols, particularly when pipetting solvents.

Wear clean, dedicated lab coats and change gloves frequently.

Appropriate Controls for Sample Processing and Amplification

Because of the numerous manipulation steps in this protocol, negative controls must be included to monitor and identify sample-to-sample contamination. With microbial DNA detection, a negative control specimen should be a PET that does not contain the sequences being amplified, but that does contain internal control sequences (such as β-globin). For example, we use BNF-fixed, paraffin-embedded appendix tissue as a negative control for our HPV studies. A negative control PET section should be the first sample processed and then interspersed (after every tenth sample), starting with the microtome sectioning. Controls must be carried through all phases of sample processing and amplification. Positive controls are also useful but should only be included as the final sample, to reduce the possibility of contaminating other samples. These PET controls are included in addition to amplification controls such as no addition, and positive and negative purified DNA samples. These types of controls should serve as standards for all investigators to adopt.

Recommendations for the Assessment of Sample DNA Quality

As noted above, a preliminary study to assess the quality of the specimen DNA should be made when using archival materials. In addition, the use of a primer pair as an internal or coamplification control is critical when testing for infectious agents. Without this marker of DNA suitability (product size and quantity), the lack of detection of the infectious agent in a particular specimen is not informative. Therefore, we recommend the use of a single primer pair (of the appropriate size) as a coamplification control for routine sample analysis. As mentioned previously, these primers should amplify a fragment of greater length than the PCR fragment to be obtained from the target of interest.

Listed in Table 2 are four oligonucleotide primer pairs[12] located in the

TABLE 2 Sequences of Human β-globin Amplification Primers

Primer	Sequence	Primer pair	Predicted product size (bp)
PC03	5′-ACACAACTGTGTTCACTAGC-3′	PC03/PC04	110
PC04	CAACTTCATCCACGTTCACC	GH20/PC04	268
GH20	GAAGAGCCAAGGACAGGTAC	RS42/KM29	536
KM29	GGTTGGCCAATCTACTCCCAGG	RS80/RS40	989
RS40	ATTTTCCCACCCTTAGGCTG	KM29/RS80	1327
RS42	GCTCACTCAGTGTGGCAAAG		
RS80	TGGTAGCTGGATTGTAGCTG		

The predicted DNA fragment sizes for each of the five primer pairs are listed.

human β-globin gene that can be used to assess the quality of the DNA sample to produce amplification fragments of 268, 536, 989, and 1327 bp. Amplification parameters are as follows: Aliquots (1 and 10 μl) of prepared samples are amplified separately with each of the four primer pairs. Each 100-μl reaction should contain 1 or 10 μl of sample DNA; 100 nM of each primer; 200 μM of each dNTP (dATP, dGTP, dCTP, dTTP); 2.5 units of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer, Norwalk, CT); 50 mM KCl, 4 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.5). A 100-μl mineral oil overlay is added to prevent evaporation during thermal cycling (not required when using a Perkin-Elmer TC9600). Cycling parameters are 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C for 40 cycles (or the number of cycles you plan to use in your assay), followed by an additional 5 min at 72°C (Perkin-Elmer, DNA Thermal Cycler).

Aliquots of amplified DNA can be resolved on either 7% (wt/vol) polyacrylamide gels, or 1% agarose gels, stained with ethidium bromide, and photographed under UV light. The resulting profiles will give an indication of how efficiently increasing sizes of DNA fragments can be amplified. A control DNA fragment at least 100 bp longer than the fragment of interest should be readily amplified. If it is not, then either a scheme generating a smaller PCR product must be sought or alternative specimens found for study.

Troubleshooting

No amplification? There can be many explanations:

1. Too much or too little crude DNA in amplification. Amplify a range of sample volumes, usually 1 to 20 μl. If the digestion is too concentrated, a dilution of the digested sample may be required. If dilution is frequently required, an increase in the digestion volume is indicated. Similarly, if volumes >20 μl are routinely required, a reduction in digestion volume is indicated. In some cases, use of higher volumes may inhibit PCR (owing to inhibitors); the DNA must then be concentrated by further purification.
2. DNA may be too degraded. This may be the result of several factors, including the fixation process and age of the PET sample. To determine the maximum "amplifiable" fragment length, follow the recommendations above. In addition, determining the average size of the sample DNA directly, by agarose gel electrophoresis, is also informative.
3. Incomplete heat inactivation of proteinase K may result in digestion of the *Taq* polymerase. Repeat the heat inactivation. Spin tubes prior to inactivation making sure all liquid is in the bottom of the tube. Check temperature of heat source to be sure the sample temperature reaches 95°C.
4. Cycling intervals are insufficient. The cycling parameters for PET specimens may require modification to accommodate the fragmented genomic DNA, particularly when amplifying DNA fragments >400 bp. An increase in

time (e.g., from 1 min to 2 min) may be useful during the 72°C extension. In addition, an increase in the number of cycles (e.g., from 30 to 40) is also recommended to accommodate inefficient amplification during early cycles.

5. Too much nonspecific priming. A "hot start" may remedy this.[24] The use of either Ampliwax Gems (Perkin-Elmer, Norwich, CT) or the delayed addition of *Taq* may greatly reduce nonspecific bands while increasing the amount of specific product generated. This can, in some cases, also be very helpful in increasing sensitivity.

6. PCR inhibitors in the sample. We have observed that further DNA purification is helpful in some cases. Proteinase K-digested material can subsequently be subjected to phenol/chloroform extraction and ethanol precipitation of the DNA. It is possible that the addition of this step may also function to better liberate the DNA from the highly cross-linked protein matrix obtained after fixation.

Complete DNA purification should only be implemented when absolutely necessary, as these additional manipulations provide increased opportunities for contamination. (Some laboratories doing genetic studies routinely prepare purified DNA from PET specimens to obtain sufficient material for multiple PCR analyses. In these cases, contamination is much less of an issue than in infectious disease work.)

DISCUSSION

The use of paraffin-embedded tissues in PCR-based studies has resulted in many exciting new insights in the areas of cancer research, genetic and infectious disease, and molecular epidemiology. However, this tool has some limitations owing to the intrinsic properties of PET specimens. As discussed in this paper, tissue fixation and the age of embedded tissue are important factors affecting the size of target DNA that can be amplified successfully. Although each paraffin-embedded tissue will have individual intrinsic properties, a general rating can be made regarding the quality of DNA derived from PET specimens from a particular time period and institution. We have found considerable variability in the quality of DNA derived from different institutions using BNF. This variability may be attributed to modification of fixation procedures and/or the quality or the age of the chemicals used in the fixation process. We stress that pilot studies to examine the quality of the extracted DNA are essential.

Many researchers gather DNA sequence information from PCR products derived from PET specimens, although extensive studies have not addressed the accuracy of sequence information from PET specimens. Fortunately, most studies use sufficient amounts of input DNA such that artifacts are unlikely to affect results. However, in experiments where minute amounts of target are available for PCR, some concerns about the effects of PET DNA damage are warranted. We have shown that many fixatives, particularly those containing acid, cause a significant decrease in the length of genomic DNA that can be amplified. Acids may hydrolyze glycosidic bonds, thus generating abasic sites in DNA. Randall and colleagues[25] extensively studied the kinetics of nucleotide insertion opposite abasic sites in DNA using *Drosophila* DNA polymerase-α and found that the specificity of nucleotide insertion was 6–11 times greater for A over G and 20–50 times greater for A over C and T. If *Taq* polymerase has its own preferences, this would have implications for the analysis of point mutations from low-copy number targets. Furthermore, observations made from studying ancient or highly degraded DNA demonstrated that *Taq* polymerase can "jump" to another template during PCR when the polymerase encounters strand scission or abasic sites.[26] Such jumping can generate artifactual hybrids, for example, between alleles or microbial genomes, when amplifying from few copies of input target.

DNA amplification methods have allowed archival PET specimens to become routinely useful clinical investigative material for molecular genetic studies. Because retrospective studies can often provide the most cost-effective and expedient approaches to epidemiologic questions, PCR- based analyses of PETs have contributed to our recent progress in many areas of biomedical research.

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