Consultations in Molecular Diagnostics

Capillary Electrophoresis Artifact Due to Eosin

Implications for the Interpretation of Molecular Diagnostic Assays

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Capillary electrophoresis (CE) is a commonly used tool in the analysis of fluorescently labeled PCR amplification products. We have identified a CE artifact caused by the tissue stain eosin that can complicate the interpretation of CE data. The artifact was detected during routine analysis of a DNA sample isolated from a formalin-fixed, paraffin-embedded tissue sample considered histologically suspicious for a B-cell neoplasm. A standard clinical PCR and CE assay for immunoglobulin heavy chain (*IGH*) gene rearrangement revealed a weak polyclonal population of rearranged *IGH* genes and a 71 base peak suspicious for *IGH* clonality. The spectral properties of the 71 base peak were unusual in that although the dominant fluorescence of the peak was blue, it also fluoresced in green and yellow (blue>green>yellow), raising the suspicion that the peak might represent an artifact. CE analysis of the genomic DNA sample without PCR amplification demonstrated the presence of the 71 base peak, suggesting that the artifact was caused by a contaminant within the DNA sample itself. We demonstrate that eosin, which was used to stain the formalin-fixed tissue during processing, yields a discrete 71 base peak of similar morphology to the contaminant peak on CE analysis. The data suggest that eosin in the fixed tissue was not completely eliminated during nucleic acid extraction, resulting in the artifact peak. We discuss the implications of this potentially common contaminant on the interpretation of CE data and demonstrate that artifacts caused by eosin can be avoided by using more stringent DNA purification steps. Histological dyes may fluoresce, and artifacts from them should be

considered when primary peaks contain additional underlying peaks of other colors. (J Mol Diagn 2005, 7:143–148)

Products of PCR amplification have traditionally been detected using agarose gel electrophoresis or polyacrylamide gel electrophoresis (PAGE) with visualization using either a DNA intercalating agent such as ethidium bromide or radionucleotide labeling. More recent trends in DNA detection include a variety of fluorescent detection platforms such as real-time PCR, fluorescent gel electrophoresis, and capillary electrophoresis (CE). CE has become a popular tool for PCR product analysis due to its relatively low PCR product requirements (high detection sensitivity), highly accurate sizing capability (resolution of 1 base or less), and an automated format that requires minimal user intervention. In CE analysis, PCR product detection and sizing is typically accomplished by labeling PCR primers with fluorescent molecules, which are incorporated into PCR products during thermal cycling. Labeled products are separated by high-voltage electrophoresis in small-bore capillaries filled with a size selective matrix. The PCR products are detected semi-quantitatively during migration by laser-induced fluorescence (LIF) and a charge-coupled device (CCD) camera. Sizing accuracy is ensured by the addition of an internal size standard to each sample.

Because of the advantages of CE, many clinical molecular diagnostic labs now use this technology rather than PAGE or agarose gel electrophoresis for PCR product detection. Examples of clinical molecular diagnostic assays that have been developed for CE platforms include bone marrow engraftment analysis by amplification of microsatellites and gene rearrangement analysis by amplification of the immunoglobulin heavy chain (*IGH*)

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and T-cell receptor (*TCR*) gene loci.¹⁻⁵ Despite the many advantages provided by CE, artifacts or anomalies can occur using this technical approach that have the potential to affect interpretation of the resultant data. The presence of bubbles in the electrophoresis polymer is a relatively frequent and well-described finding that often results in the generation of a peak or peaks in the electropherogram with simultaneous fluorescence in multiple color channels. Crystal deposits in the polymer as well as other contaminants in the electrophoresis system can also generate artifact peaks. So-called "pull-up peaks" occur when signals from one color (ex. blue) are interpreted by the software as a different color (ex. green). This artifact is due to the overlapping emission spectrum of fluorescent dyes and usually occurs when the fluorescent signals exceed the dynamic range of the instrument (off-scale data). This particular type of artifact peak is most commonly a problem when performing multiplex PCR using multiple primers labeled with different-colored fluorophores, especially when the resulting PCR products overlap in size. Recently, the phenomenon of dye-associated fluorescence peaks in CE was described.⁶ These peaks occur at constant positions that are characteristic of the specific dye. Fortunately, the morphology of many of these different artifact peaks is dissimilar from that generated by labeled PCR products, enabling their identification if the interpreter is familiar with the artifact. For reliable interpretation of CE data it is essential that all "non-specific" or artifact peaks be identified as such and ideally eliminated.

Here we report an artifact in CE analysis resulting from eosin staining of paraffin-embedded tissue. Eosin is commonly used to stain tissue before paraffin-embedding so that the histology technologist does not inadvertently section through and discard the entire sample, a potential problem especially with small biopsy samples. We discuss a case in which this artifact could have resulted in the incorrect interpretation of CE data for *IGH* gene rearrangement analysis (B-cell clonality). Our findings underscore the need for careful interpretation criteria for all data generated by CE.

Materials and Methods

Tissue Acquisition and IRB Approval

The tissue sample in question consisted of a formalin-fixed, paraffin-embedded skin biopsy, processed by standard histological techniques. Following the initial dehydration steps of processing, the tissue was stained in a 1:77 (vol:vol) dilution of 0.5% eosin-Y (Richard Allan Scientific, Kalamazoo, MI) in absolute ethanol added to the processing bath, to enable identification of small tissue fragments during sectioning. The paraffin-embedded tissue was submitted for clinical molecular diagnostic testing. Exemption from Institutional Review Board (IRB) review for case presentation was obtained from the Johns Hopkins University School of Medicine IRB.

DNA Isolation and PCR

Several 10- μ m sections of the paraffin-embedded sample were cut and placed in an Eppendorf tube. Paraffin was removed using multiple xylene washes, followed by ethanol washes. The tissue was then digested with Proteinase K and Tween-20 (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.2 mg/ml ProK, 0.5% Tween-20) overnight at 37°C. DNA was purified from the lysate using the Qiagen QIAmp DNA Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

IGH PCR and Capillary Electrophoresis

PCR reactions consisted of: 1X PCR buffer with 1.5 mmol/L MgCl₂ (Applied Biosystems, ABI, Foster City, CA), 100 μ mol/L each dNTP (ABI), 400 nmol/L each primer, 1% PCR-grade formamide, and 0.5 U *Taq* Gold DNA polymerase (ABI). Primers FR3a 5'ACACGGC(C/T)(G/C)TGTTACTGTG3' and VLJH, 5'FAM-GTGACCAGGGT(A/G/C/T)CCTTGGCCCCAG3' were synthesized by Oligos Etc. (Wilsonville, OR).^{7,8} Replicate PCR analyses of the sample were performed using 25, 35, and 50 ng of input DNA. Reactions were heated to 95°C for 10 minutes, followed by 35 cycles of 95°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute, and followed by 72°C for 10 minutes. Products generated from the PCR reactions were prepared for CE analysis according to the manufacturer's instructions (ABI). In brief, 1 μ l of PCR product was added to 9 μ l of hi-di-formamide with TAMRA size standard, heated to 95°C for 2 minutes and ice quenched. Samples were subjected to electrophoresis under standard conditions using the ABI 310 Genetic Analyzer and POP4 polymer (ABI). PCR products were detected and sized using the GeneScan software (ABI). Using this assay, the possible size ranges of PCR products generated from rearranged *IGH* genes are 50 to 150 bases with the majority of the products typically limited to 60 to 110 bases. POP4 polymer was also used on the ABI 3100 sequencer.

Case Description

The patient is an 83-year-old female who presented with a suspicious cutaneous lesion on her left cheek. A biopsy of the lesion was performed. Histological sections of the biopsy showed a moderately dense, atypical lymphoid infiltrate composed of mature B-lymphocytes of small to intermediate size. Although there were some histological features suggestive of a reactive process, the findings were considered suspicious for a lymphoproliferative disorder. The paraffin-embedded skin biopsy sample was submitted to the molecular diagnostics laboratory for *IGH* gene rearrangement analysis. DNA was isolated from the fixed tissue and the *IGH* gene locus was PCR amplified and the resultant products analyzed by CE.

Results and Discussion

CE analysis of rearranged *IGH* gene PCR products generated from the sample revealed a dominant peak at 71

Figure 1. A–B and D–E: Examples of capillary electrophoresis electropherograms. *x* axis is size in bases, *y* axis is fluorescence intensity. For all colors, the color of the fluorescence is depicted by the color of the tracing, except for yellow fluorescence, depicted by a **black tracing**. *IGH* PCR amplification products are in blue. Red peaks are internal size standards. **A:** The original CE results from the *IGH* gene rearrangement assay of the reported case. Note the peak at 71 bases that is predominantly blue (**blue arrow**), but also fluoresces to a lesser extent in green (**green arrow**) and yellow (**black arrow**). **B:** CE analysis of the genomic DNA sample without PCR amplification. Both the major peak at 71 bases and the minor peak at 54 bases are consistent with the peaks seen in (**A**). **C:** The paraffin-embedded tissue from which DNA was isolated and analyzed. Note the pink color due to the tissue stain eosin. **D:** Capillary electrophoresis electropherogram of eosin diluted 1:1,000,000 in H₂O. The peak at 71 bases fluoresces predominantly blue, but also fluoresces to a lesser extent in green, and even less in yellow. Also note the minor peak at 54 bases fluorescing in blue and green. These findings are consistent with those generated by CE analysis of the original genomic DNA sample without PCR amplification (**B**). **E:** CE analysis of the repeat *IGH* gene rearrangement PCR after additional purification of the DNA sample. The pattern is consistent with polyclonality with no evidence of the 71 base contaminant peak.

bases superimposed on a background of polyclonal *IGH* gene amplicons (Figure 1A). This pattern was consistent in all three PCR replicates (at different input DNA concentrations) and was highly suspicious for a monoclonal B-cell population present in the context of a background polyclonal B-cell population. On closer examination of the data it was noted that while the 71 base peak showed predominantly blue fluorescence, it also fluoresced to a lesser extent in green, and very slightly in yellow (displayed in black by the computer software). The presence of multiple fluorescent colors superimposed on a single peak of a single size raised suspicion that the peak could represent an artifact rather than a monoclonal *IGH* gene rearrangement. Interestingly, the morphology of the peak was not typical of a bubble or other contaminant in the capillary, which in addition to fluorescence in multiple

colors, usually results in a peak with a very narrow width. The peak was also not consistent with a “pull-up” peak artifact especially since the data were not off-scale. Importantly, the positive, negative, and no-template controls on the run showed no evidence of the 71 base peak, nor did any of the other patient samples on the run. These findings suggested that the artifact was the result of a contaminant in the sample itself rather than in the reagents used in the analysis.

To identify the etiology of the contaminant peak, we performed CE analysis on the genomic DNA sample itself without PCR amplification. The genomic DNA sample was diluted into water to a concentration corresponding to that of a PCR reaction and then analyzed by CE without prior PCR amplification. The genomic DNA sample revealed evidence of a dominant peak migrating at 71

bases fluorescing in blue, green, and yellow, and a minor peak migrating at 54 bases fluorescing in blue and green (Figure 1B). Both peaks are of comparable fluorescence and morphology to that seen in the post-PCR CE analysis of the same sample (Figure 1A). The data confirmed the suspicion that the contaminant was present within the DNA sample itself.

To identify the source of the contaminant within the genomic DNA sample we retrieved the formalin-fixed, paraffin-embedded block from which the DNA sample had been isolated. On inspection of the block, it was noted that the tissue sample within the block was pink in color (Figure 1C). The pink color was found to result from the use of the tissue stain eosin after tissue dehydration to aid in the identification of small tissue samples during histological sectioning. We obtained a sample of the eosin used in this processing step, diluted it 1:1000,000 in H₂O, and injected the sample on the ABI 310. The result, shown in Figure 1D, demonstrated a dominant peak migrating at 71 bases that fluoresced in blue > green > yellow and a minor peak migrating at 54 bases and fluorescing in blue > green. This pattern is essentially identical to that identified in the original suspicious PCR product and the genomic DNA sample. This provided compelling evidence that the source of the artifact peak was eosin in the genomic DNA sample that had not been completely eliminated during nucleic acid isolation. Interestingly, others have described the use of eosin as a fluorescent molecule for CE analysis for quantification of anions in water and also as a tracer molecule to detect groundwater migration.^{9,10} These investigators' descriptions of the physical properties of eosin, its excitation at wavelength 488 nm and emission at 520 nm, are consistent with our findings. It is important to note that the spectral pattern observed in CE is, in part, a reflection of the fluorescence dye matrix used for analysis. Our clinical *IGH* assay uses the ABI310 dye matrix C (standardized for the use of the fluorescent dyes 6-FAM, TET, HEX, and Tamra) which yielded an eosin spectral pattern of blue>green>yellow at consistent relative intensities. Analysis of eosin fluorescence using alternate dye matrixes (ABI310 dye matrix F (5-FAM, JOE, NED, ROX), ABI3100 dye matrix D (6-FAM, HEX, NED, ROX), or F (5-FAM, JOE, NED, ROX)) slightly alters the observed fluorescent pattern. For each matrix, however, blue remained the dominant color post-analysis, with green and yellow varying in relative intensities (data not shown). The relative color ratios in the raw spectrum also varied somewhat depending on what wavelength ranges were selected for collection (data not shown).

Because the eosin artifact can create interpretation difficulties, we sought to develop a protocol to eliminate the presence of eosin in genomic DNA extracts before PCR/CE analysis of the samples. Our standard protocol for isolation of DNA from paraffin-embedded samples calls for DNA to be purified after tissue lysis using the Qiagen QIAmp DNA Mini Kit. During this procedure, the DNA is bound to a silica-based column and washed twice with proprietary buffers (once with wash buffer AW1 and once with wash buffer AW2) before nucleic acid elution. We tested whether additional wash steps would

remove contaminating eosin. Aliquots of lysates from the tissue sample were processed identically except that the number of wash steps was varied. Each sample was then assayed identically by our standard *IGH*/CE assay. Without the washing steps, the PCR reaction apparently failed (no *IGH* or β -globin PCR products were detected) and only the eosin peak was identified on CE analysis. Interestingly, other reports have previously described the inhibitory effects of other histological stains on PCR amplification.^{11,12} When one wash step with each buffer was used for each sample (standard protocol), the *IGH* PCR/CE result was similar to the original result with some polyclonal amplification and an eosin peak at 71 bases (consistent with Figure 1A). When two washes with each wash buffer were used, the eosin peak was absent and CE analysis of the resultant *IGH* PCR products revealed only a polyclonal distribution of *IGH* gene amplification products (Figure 1E). Thus, by simply adding additional wash steps to the genomic DNA isolation procedure (a relatively rapid and inexpensive approach), we were able to eliminate the eosin artifact. The resulting data without the eosin artifact is consistent with a polyclonal B-cell population, not suspicious for monoclonality, which has obviously different implications for patient management compared to the original impression of the data. Our protocol for nucleic acid isolation from paraffin-embedded tissue now includes the additional purification steps to help avoid eosin contaminants. In addition, because we anticipate that eosin may not be the only fluorescent contaminant in paraffin-embedded tissue (other possibilities include ink from marked margins, etc), it is also now standard protocol in our laboratory for the technologist isolating DNA from paraffin-embedded tissues to note any coloration or markings on the sample on the sample sheet so that the information is available to the laboratory directors when signing out cases.

Paraffin-embedded tissue has become a routine source of DNA for many clinical molecular analyses despite limitations in the quality and quantity of DNA that can be extracted. The practice of using eosin during processing tissue sample processing is relatively common, but we wanted to determine the approximate frequency of its use. We performed an informal survey on a pathologist's assistant listserver (<http://groups.yahoo.com/group/Pathassistants>). The majority of respondents use eosin for staining small tissue samples. Some labs stain all small or pale-colored tissue with eosin before processing (such as during the grossing step or during formalin-fixation); while some labs add eosin to one of the alcohol steps in the processor, in which case all tissue on the processor is stained. A few labs responded that they use alternative dyes, such as mercurochrome or toluidine blue.

We also retrospectively analyzed data in our lab from other molecular diagnostics assays that employ paraffin-embedded tissue as starting material for genomic DNA extraction. These assays include *T-cell receptor (TCR)* gene rearrangement, microsatellite instability (MSI), and loss of heterozygosity (LOH) of chromosome arms 1p and 19q (1p19qLOH), which are routine clinical assays in our laboratory.^{5,13,14} Some of the patient data generated

from these assays revealed evidence of eosin peaks with characteristics similar to those seen in the *IGH* assay. For these assays, however, the eosin peak did not create an interpretation dilemma for two reasons. First, none of the loci analyzed by these specific assays generate PCR products in the 71 base size region. Second, because the latter two assays analyze microsatellite loci (mono- and di-nucleotide repeats), specific peaks must have appropriate stutter peaks associated with them to be interpreted as valid PCR amplicons.^{13,14} The absence of a stutter peak associated with the eosin artifact indicated that the 71 base peak was not likely to be the result of amplification of a microsatellite locus. Thus, although the 71 base eosin peak is present in these assays, the peak is functionally superfluous to assay interpretation. This artifact, however, could also be a significant problem in *TCR* assays, or any other clonality assay where a single prominent peak indicates clonality. Whether or not eosin is likely to present a problem is dependent on the specific fluorochrome used in the assay and whether the products for a particular assay migrate in the same region. Since some *TCR* assays use primers that generate products in this range, we also urge caution in *TCR* assay interpretation.

Eosin is a very common dye used not only for detection of embedded tissue, but almost universally in conjunction with hematoxylin (H&E) in histological tissue staining. H&E staining is the most common histological stain used in diagnostic surgical pathology. Although not always necessary, H&E (or eosin alone) staining can be helpful for molecular analysis of tissues by facilitating the targeted microdissection of specific tissues. Microdissection allows a more homogenous DNA sample to be isolated from the cell population of interest and generally improves testing accuracy and sensitivity. We have previously detected artifact peaks in CE data resulting from the PCR amplification of DNA isolated from microdissected, H&E-stained tissue (unpublished data). Additional experiments revealed that both hematoxylin as well as a commonly used hematoxylin additive, phloxine B, each individually resulted in fluorescent artifacts migrating at approximately 130 to 140 and 160 to 170 bases, respectively.

The use of CE in molecular diagnostic labs continues to expand, primarily due to the advantages in DNA sizing and throughput that the technology provides. Recent College of American Pathologists (CAP) Molecular Oncology surveys suggest that the use of capillary electrophoresis in clinical diagnostic labs for detection of *IGH* gene rearrangement has almost tripled from 2001 to 2003.^{15,16} The MO-A and MOP-A surveys in 2001 indicate that approximately 10% of responding laboratories used CE for their *IGH* gene rearrangement analysis, while the same surveys performed in 2003 indicate that approximately 30% of these labs used CE for analysis.^{15,16} The advantages of CE analysis have opened exciting new avenues for the molecular analysis of tissue, but as shown here, also warrant caution in data interpretation. Importantly, CE may not be the only technology for molecular analyses affected by histological stains including eosin. Any platform measuring fluorescence as an end-

point, including real-time quantitative PCR and chip-based gene expression analysis could potentially be affected by the fluorescent nature of eosin and other histological stains. In this regard, analysis using alternative platforms such as PAGE might help in sorting out this type of problem since it seems likely that the small molecule eosin would migrate at a position other than 71 bases under these substantially different electrophoretic conditions. Fortunately, as demonstrated here, at least the CE artifact generated by eosin can be avoided by adding a few simple steps during nucleic acid purification. Despite precautions, artifacts occur during CE that demand careful interpretation by well-qualified individuals, trained in the interpretation of clinical CE molecular diagnostic assays. Histological dye artifact should be considered when a peak of one dominant color contains subordinate peaks of other colors.

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