One Step Spin Column Solution for Optimized Purification of Nucleic Acids from Heavily Inhibited Clinical Samples for Improved Molecular Diagnostic Accuracy

Ana Barrionuevo, Zoë Hermsen, Erica Carrasco & Aaron Clausen
Zymo Research Corporation - 17062 Murphy Ave, Irvine, CA 92614

BACKGROUND

Melanoma is a highly aggressive skin cancer and the fifth most common cancer worldwide, accounting for 184,416 deaths between 1999 and 2020. While localized melanoma has a 95% survival rate, this rate drops to 30% once the cancer metastasizes to distant lymph nodes, making early detection critical. Molecular detection methods such as Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) are routinely used to identify melanoma-specific genetic markers, such as mutations in the BRAF gene. However, PCR performance can be severely compromised by the presence of PCR inhibitors or polyphenolics such as melanin in melanoma tissue, hematin in blood, and humic acid in feces. When co-purified with nucleic acids, these molecules disrupt enzymatic reactions and can cause false negatives in diagnostic assays. In this study, we evaluated the efficacy of Zymo Research's *OneStep* PCR Inhibitor Removal Kit in eliminating polyphenolic inhibitors from extracted DNA samples. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) melanoma tissue or HeLa cells spiked with melanin and treated with the *OneStep* kit. Post-treatment analyses using PCR and Sanger sequencing resulted in successful amplification and sequencing of the GAPDH and BRAF gene from melanin containing DNA. Our findings confirm that common polyphenolics significantly inhibit PCR amplification and that the *OneStep* PCR Inhibitor Removal Kit effectively removes these inhibitors, restoring assay sensitivity. This work underscores the importance of optimizing nucleic acid purification and amplification protocols for inhibitor-rich clinical samples. By improving the fidelity of molecular diagnostics in polyphenolic–rich samples, this study contributes to advancing accurate, early detection of melanoma.

MATERIALS & METHODS

- **PCR Inhibitor Solutions -** All PCR inhibitor stock solutions were prepared by dissolving the chemicals in water, except for melanin, which was diluted in 1 N NaOH and then brought to 600 ng/µl concentration using water. Stock solutions were diluted to various working concentrations in water for the experiments. For the PCR Inhibitor Cocktail, a mixture containing a final concentration of 0.61 µg/µl indigo, 5 µg/µl bile salts, and 0.133 µg/µl hematin was produced by diluting the chemicals in water.
- **Preparation of PCR Inhibitor Removal Column Containing Novel Slurry -** 600 µl of PCR Inhibitor Removal Slurry was placed inside a Zymo-Spin IV Spin-Column (Zymo Research Corporation, Irvine, CA). The liquid was removed from the slurry by centrifuging at 8,000 *x g* for 3 minutes, per the manufacturer's instructions. After removing the liquid, 100 µl of PCR Inhibitor Solution was passed through the resin by centrifuging the spin-column at 8,000 *x g* for 1 minute and the flow-through was collected for analysis.
- **Quantitative PCR of HeLa DNA -** DNA isolated from cultured HeLa cells using the *Quick*-DNA Miniprep Plus Kit (Zymo Research Corporation, Irvine, CA) was spiked with humic acid and the PCR Inhibitor Cocktail solution. Quantitative PCR was carried out using the Femto Human DNA Quantification Kit (Zymo Research Corporation, Irvine, CA) and CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA). Data was analyzed using the CFX Maestro Software (Bio-Rad, Hercules, CA).
- **FFPE DNA Extraction and Analysis -** Genomic DNA was extracted from melanoma FFPE blocks (THP-1500- B, AMSBIO) using the *Quick*-DNA FFPE Miniprep Kit (D3067, Zymo Research) following the manufacturer's protocol. DNA was eluted with 100 µl of EB buffer. The DNA was diluted to a 1 ng/µl stock concentration. 50 µl of stock was then treated with the *OneStep* PCR inhibitor removal column. The 100, 200, 300 and 400 bp fragments of the GAPDH gene and 107 bp fragment of the BRAF gene was amplified using quantitative PCR with ZymoTaq qPCR Premix, 20 ng of template for GAPDH gene, 50 ng of template for the BRAF gene, and 550 ng of melanin. Positive controls contained no melanin. Amplified fragments were analyzed in a 2% agarose gel ran at 120V for 45 min.
- **HeLa DNA Extraction and Analysis -** HeLa DNA stock was diluted to a final concentration of 0.86 ng/µl and melanin was spiked-in. Multiplex quantitative PCR for the GAPDH gene was carried out with ZymoTaq qPCR Premix and 2 ng of the template DNA stock. A second HeLa DNA stock was diluted to a final concentration of 8.6 ng/µl and melanin was spiked-in. Quantitative PCR was carried out using 2 ng of template for the GAPDH gene, 10 ng of template DNA for the BRAF gene, and 550 ng of melanin. Positive controls contained no melanin. Amplified fragments were analyzed in a 2% agarose gel ran at 120V for 45 min.

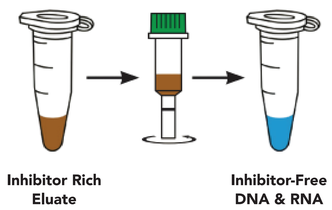


Figure 1. Rapid, one step PCR inhibitor removal system. Simply pass inhibitor-rich extracted nucleic acid sample through the column, which binds polyphenolics, to elute inhibitor-free DNA/RNA.

RESULTS

Novel PCR Inhibitor Removal Column Removes > 96% of Humic Acid, Urea, Bile Salts, Indigo, and Hematin.

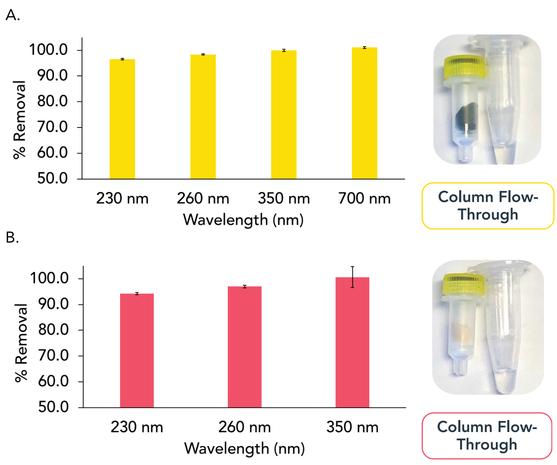


Figure 2. Evaluation of PCR inhibitor removal using common inhibitors and spectrophotometry. PCR inhibitor removal of PCR Inhibitor Cocktail (Figure 1A) and Humic Acid (Figure 1B). Absorbance was measured in the flow-through at the indicated wavelengths using a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, CA) and 1.5 µl of flowthrough. Percent removal was calculated by subtracting the absorbance of the flow-through from the absorbance of the stock PCR Inhibitor Solution then dividing the difference by the absorbance of the stock PCR Inhibitor Solution and multiplying by 100. Shown are n = 6, ± SDEV.

PCR Inhibitor Removal Column Treatment does not result in Significant DNA or RNA Loss.

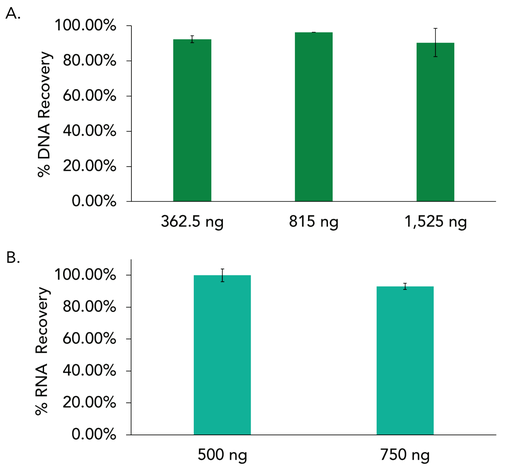


Figure 3. Evaluation of DNA & RNA recovery after PCR inhibitor removal treatment using spectrophotometry and fluorometry. Percent recovery of total DNA (Figure 3A) and RNA (Figure 3B) following inhibitor removal treatment at varying input amounts (ng). Nucleic acid concentration was measured using a Qubit Flex Fluorometer (ThermoFisher Scientific, Waltham, CA) for DNA and a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, CA) for RNA and multiplied by the volume recovered to determine nucleic acid input amount (ng). Shown are n = 2 for DNA, n = 10 for RNA ± SDEV.

PCR Amplification is Completely Restored after Passing Common Inhibitors through the PCR Inhibitor Removal Column.

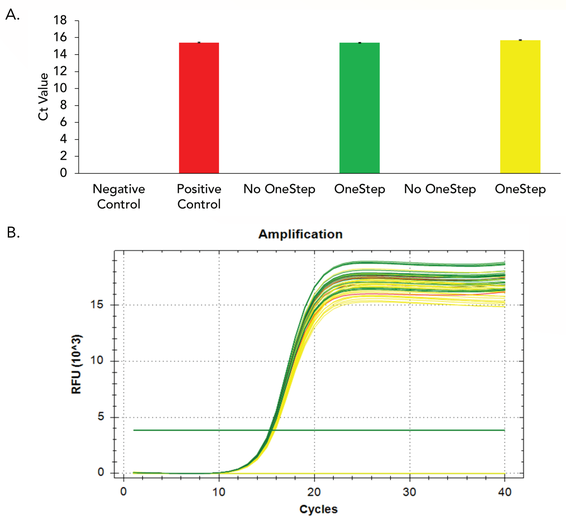


Figure 4. Quantitative PCR of HeLa DNA spiked into PCR Inhibitor Removal Column Flow-Through. 5 ng of HeLa DNA was added to a PCR reaction containing 3 µl of PCR Inhibitor Solution that was passed through the *OneStep* PCR Inhibitor Removal Column. Quantitative PCR was carried out using the Femto Human DNA Quantification Kit (Zymo Research). Untreated PCR inhibitor solutions were run in parallel for comparison. Shown are n = 6, ± SDEV.

qPCR Amplification of GAPDH Gene Fragments is Restored After Treatment.

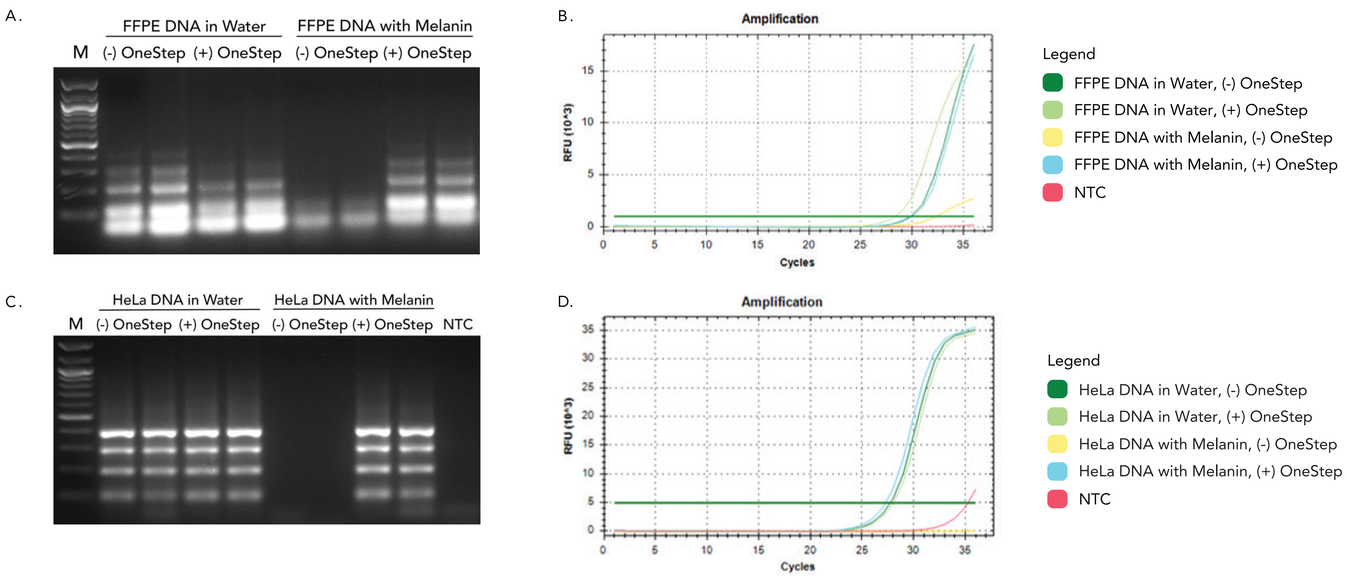


Figure 5. GAPDH Gene Multiplex Quantitative PCR. Multiplex quantitative PCR amplification using primers for 100, 200, 300 and 400 bp sized fragments of the GAPDH gene. Amplification was performed with either 20 ng of FFPE extracted DNA (B) or 2 ng of HeLa DNA as the template (D). The *OneStep* PCR inhibitor removal column was used to treat 50 µl of the template or 50 µl of melanin-spiked template prior to PCR amplification. The NTC contained only water. All PCR products, FFPE (A) and HeLa (C), were visualized on a 2% gel that ran at 120V for 45 minutes. M = 100 bp ladder (Zymo Research).

Successful qPCR Amplification & Sanger Sequencing of BRAF Gene After Treatment.

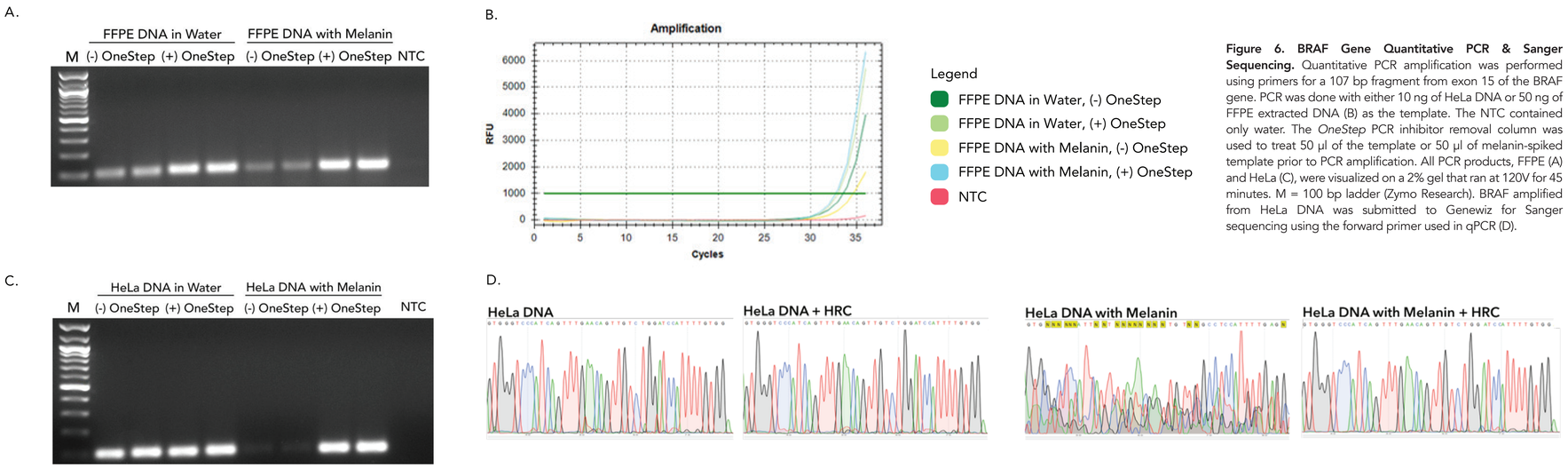


Figure 6. BRAF Gene Quantitative PCR & Sanger Sequencing. Quantitative PCR amplification was performed using primers for a 107 bp fragment from exon 15 of the BRAF gene. PCR was done with either 10 ng of HeLa DNA or 50 ng of FFPE extracted DNA (B) as the template. The NTC contained only water. The *OneStep* PCR inhibitor removal column was used to treat 50 µl of the template or 50 µl of melanin-spiked template prior to PCR amplification. All PCR products, FFPE (A) and HeLa (C), were visualized on a 2% gel that ran at 120V for 45 minutes. M = 100 bp ladder (Zymo Research). BRAF amplified from HeLa DNA was submitted to Genewiz for Sanger sequencing using the forward primer used in qPCR (D).

CONCLUSIONS

- The *OneStep* PCR inhibitor removal column proved to be very efficient at removing Humic Acid, Urea, Bile Salts, Indigo, and Hematin and restoring qPCR amplification with minimal loss of DNA or RNA.
- Quantitative PCR of HeLa and FFPE samples containing melanin resulted in little to no amplification for all four fragments from the GAPDH gene and the BRAF gene. Amplification was fully restored when samples were treated with the *OneStep* PCR inhibitor removal columns.
- Sanger sequencing of the HeLa DNA amplified BRAF gene was successful for all samples except the sample containing melanin which was then restored after *OneStep* treatment.

Contact: Ana Barrionuevo: abarrionuevo@zymoresearch.com