ZYMO RESEARCH

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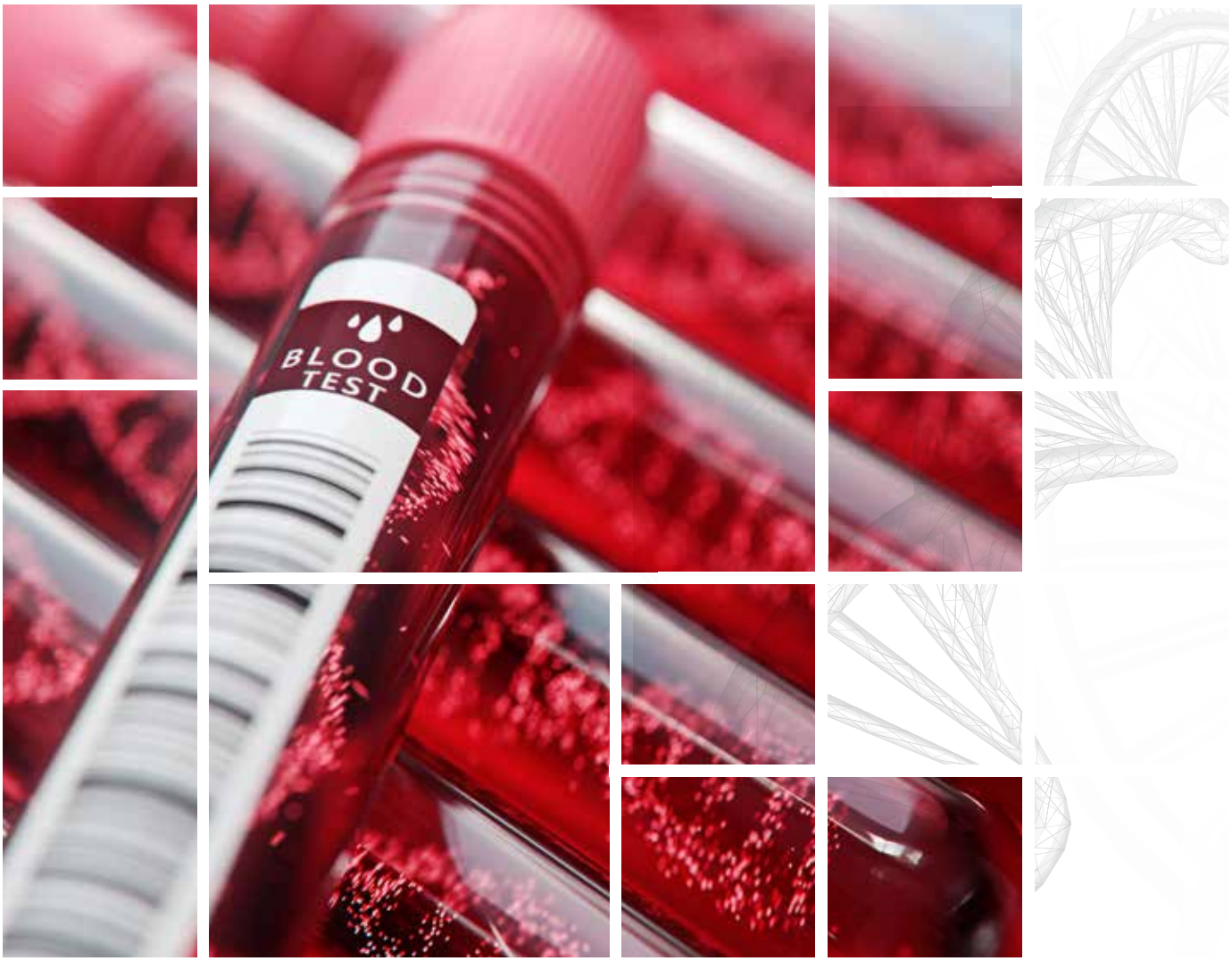
Case Study: Validation Methods for the Implementation of an Automated Process for DNA Extraction



Introduction

Presented here is a case study to show the implementation of an automated process for genomic DNA extraction of human and mouse blood samples for downstream DNA methylation analysis. The laboratory had a number of technical and operational requirements. The Zymo Research Automation team designed and established a suitable workflow with the following criteria:

Technical Criteria:

- Extraction and Purification of Genomic DNA from Whole Blood
  - o Extraction and purification of > 800ng genomic DNA from an input volume of 133uL of whole blood (Human and Mouse) for downstream bisulfite conversion reaction
- Bisulfite Conversion of Genomic DNA for downstream targeted bisulfite sequencing
  - o Purified, bisulfite-converted, genomic DNA (200ng input) was assay-ready for direct downstream targeted Next-Generation Sequencing (NGS) library preparation

Operational Criteria:

- Instrument: Thermo Scientific™ KingFisher™ Flex Purification System, with 96-Deep Well Magnetic Head
- Purification and Bisulfite conversion of 4x Batches of 96 whole blood samples performed per operational day; customer requirement of 2x Batches of 96 per day allowing for a 100% increase flexibility to the operational pipeline
- Quality of resulting sample was not operator dependent, nor sample species dependent

  Several validation experiments were conducted to ensure the automation process was optimized to reliably meet the established needs and requirements. The experiments will be described in detail in the next section and are outlined below:
  - o Consistency and Reproducibility of Yield and Purity
  - o Compatibility with Bisulfite Conversion and Library Preparation
  - o Scalability of Extraction Chemistry
  - o Cross Contamination on Instrument

Materials and Methods

The Thermo Scientific™ KingFisher™ Flex Purification System, with 96-Deep Well Magnetic Head in combination with BindIt 4.0 Software was used to perform genomic DNA extraction using the Zymo Research Quick-DNA MagBead Plus kit[1]. The KingFisher™ Flex is equipped with a 96-well magnetic head for high throughput processing. The Quick-DNA MagBead Plus kit has a robust buffer system that is compatible with a wide array of sample types including blood, saliva, cells, tissues, etc.

After sample and reagent preparation, the KingFisher™ Flex can process up to 96 samples in a single run in under 45 minutes using the optimized script provided and developed by Zymo Research. This meets the established processing requirements. The optimized script was then used for all the following validation experiments.

<u>Consistency and Reproducibility of Yield and Purity</u>

Multiple human and mouse blood samples were collected in DNA/RNA Shield Blood Collection Tubes[2]. Low conforming samples were specifically chosen to ensure the extraction workflow was robust to handle the wide variability typically seen in the patient samples processed by this laboratory. All samples were processed using Quick-DNA MagBead Plus kit on the KingFisher™ Flex and then analyzed by UV-vis spectrometer (Thermo Scientific NanoDrop™ 2000). This experiment was performed to ensure the workflow on the instrument can reliably meet the established technical specifications of the extraction chemistry and the customer's needs.

Seven human blood samples and eight mouse blood samples were collected. Each sample was proteinase K treated at 5% v/v and incubated overnight at room temperature. Overnight incubation allowed for the laboratory to batch prepare samples for extraction the following day to streamline the entire workflow. DNA/RNA

Shield provides nucleic acid stability through this incubation allowing for the sample preparation to be performed independently of the extraction.

Aliquots of 400 µl prepared samples were processed using the Quick-DNA MagBead Plus kit on the KingFisher™ Flex. All human samples were run in duplicates. The results showed consistent recovery and purity. All human blood samples yielded over 800 ng DNA with an average of 1453.1 ± 397 ng and A260/A280 and A260/A230 absorbance ratios over 1.7 (Figure 1). All mouse blood samples yielded over 800 ng DNA with an average of 1571.9 ± 534 ng and A260/A280 and A260/A230 ratios over 1.8 (Figure 2). The automated workflow for genomic DNA extraction meets the established technical and processing throughput requirements.

Figure 1A

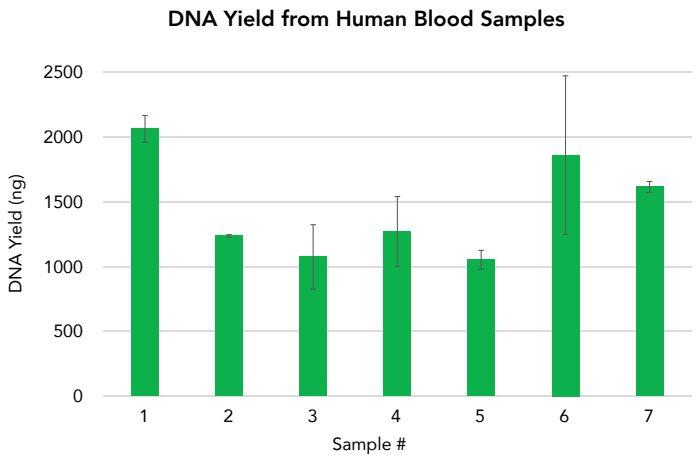


Figure 1B

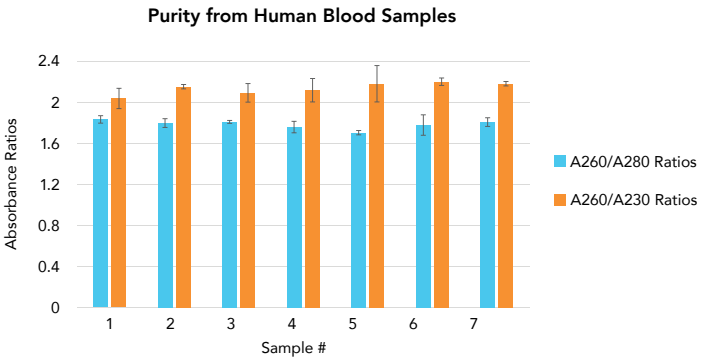


Figure 1: 7 unique human blood samples were processed on the KingFisher™ Flex and analyzed using the NanoDrop™ 2000. (A) Total DNA yields from all samples were above 900 ng. (B) Purity absorbance ratios (A260/A230 and A260/A280) were all above 1.7.

Figure 2A

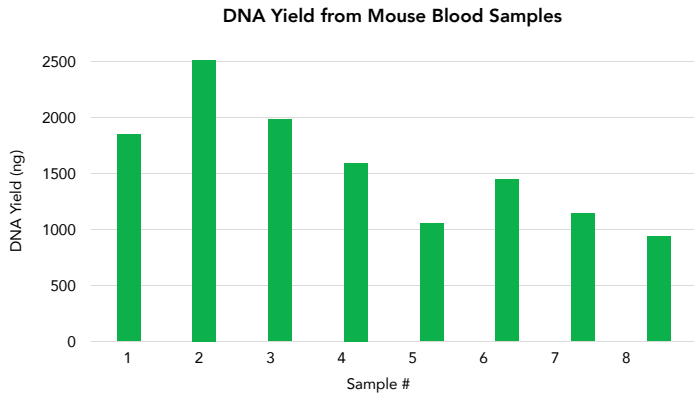


Figure 2B

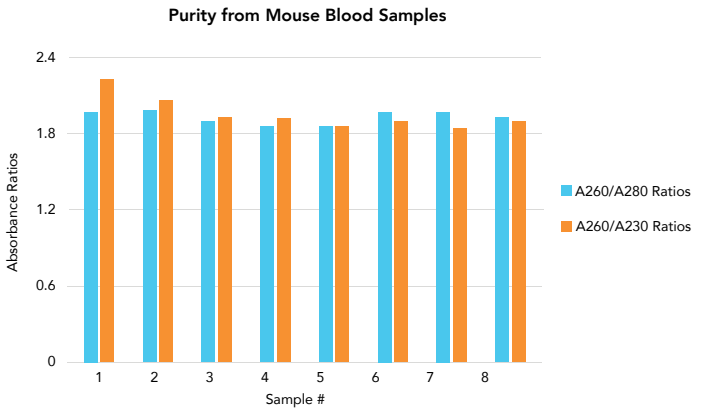


Figure 2: 8 unique mouse blood samples were processed on the KingFisher™ Flex and analyzed using the NanoDrop™ 2000. (A) Total DNA yields from all samples were above 900 ng. (B) Purity absorbance ratios (A260/A230 and A260/A280) were all above 1.8.

To confirm that the automated extraction workflow also had inter run consistency and reproducibility, additional extractions of mouse blood sample #1 were performed. A total of 14 technical replicates were processed in 4 extraction runs on the KingFisher™ Flex by multiple operators on different days.

The average DNA yield of these replicates is 1754.9, 95% CI [1666.374 – 1843.426]. All A260/A230 and A260/A280 absorbance ratios were above 1.8 (Figure 3).

Figure 3A

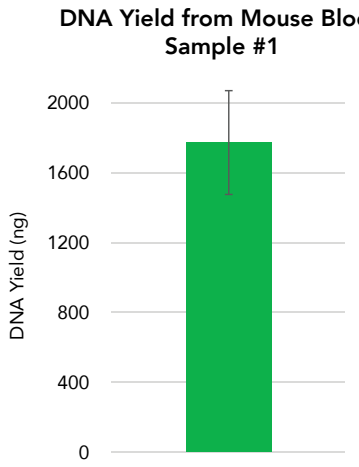


Figure 3B

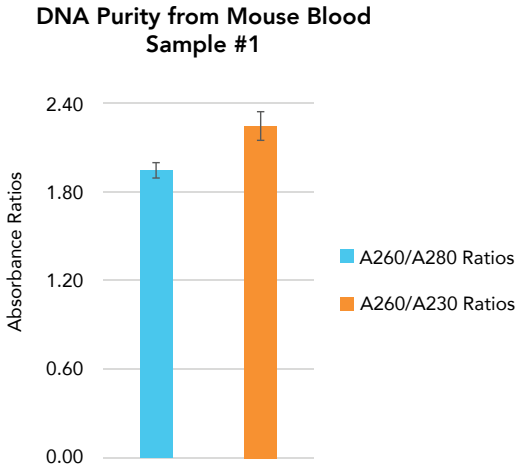


Figure 3: 14 technical replicates of the same mouse blood sample were processed on the KingFisher™ Flex over multiple runs, days, and operators. Yield and purity were determined using the NanoDrop™ 2000. (A) Average total DNA yield was 1754.9 ± 169 ng. (B) Purity absorbance ratios (A260/A230 and A260/A280) for all replicates were all above 1.8.

Compatibility with Bisulfite Conversion and Library Preparation

For the successful implementation of the automated extraction workflow, the extracted DNA must also be compatible with the laboratory's downstream application.

One mouse blood sample and two human blood samples were selected for further analysis. 200 ng DNA from these extracted mouse and human blood samples were normalized and underwent bisulfite conversion utilizing the EZ-96 DNA Methylation-Lightning MagPrep[3] kit before library preparation and targeted sequencing. The mouse blood sample was performed in duplicate and the human blood samples in quadruplets.

All DNA samples successfully produced libraries after bisulfite conversion. Prepared libraries were confirmed and analyzed by Agilent 2200 Tapestation® System before sequencing on the Illumina HiSeq 1500 instrument for targeted methylation analysis. (Figure 4) All libraries passed the laboratory's quality control metrics.

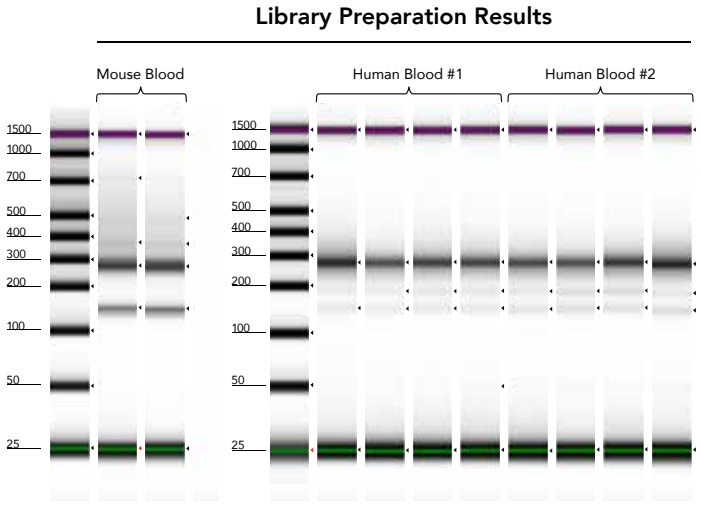


Figure 4: Target bisulfite sequencing libraries prepared with DNA extracted from human and mouse blood samples using the established automated workflow were analyzed by Agilent 2200 Tapestation® HSD1000.

Scalability of Extraction Chemistry

Due to the wide variety in the laboratory's expected samples, a validation experiment was performed to confirm that the DNA recovery performed optimally even with exceptionally low biomass samples.

As a control, a well characterized HeLa cell pellet was resuspended in DNA/RNA Shield. A dilution series (1:10) was prepared using DNA/RNA Shield ranging from 1 x 10^6 cells to 1 cell equivalent. Each sample was prepared and processed using the automated workflow process methods described above on the KingFisher™ Flex.

The extracted DNA was quantified using the Femto Human DNA Quantification Kit[4] targeting the Line-1 region (approximately 500,000

copies/genome) on the Bio-Rad™ CFX Connect Real-Time PCR Detection System. The dilution series showed a linear recovery of DNA with an R^2=0.9942 (Figure 5).

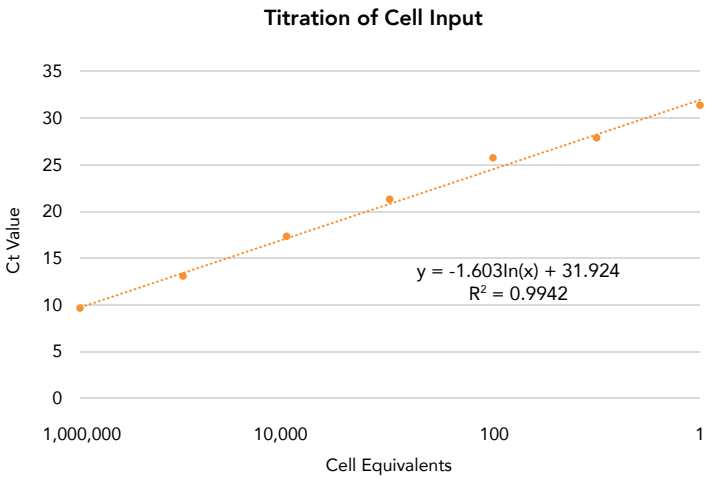


Figure 5: Plot showing the linear recovery (R^2 = 0.9942) of DNA extracted from HeLa cells using the automated workflow in dilution series (1:10 dilutions, n = 2).

Cross Contamination on Instrument

This laboratory will be processing unique samples for every prep. It is essential that no cross contamination occurs on the KingFisher™ Flex.

75 ng of a synthesized alien DNA sequence was spiked in a checkerboard pattern to columns 1-6 of the 96-deep-well plate (n=24). Negative controls (no DNA) added to alternating wells (n=24). The samples were then processed using the same automated extraction workflow as described above. The resulting eluates were analyzed by qPCR using ZymoTaq qPCR Premix[5] with target specific primers on the Bio-Rad™ CFX Connect Real-Time PCR Detection System.

The qPCR analysis showed an average Ct value of 12.24 ± 0.6 for samples spiked with DNA and no amplification for all negative control samples (Figure 6). The automated extraction process does not show any cross contamination.

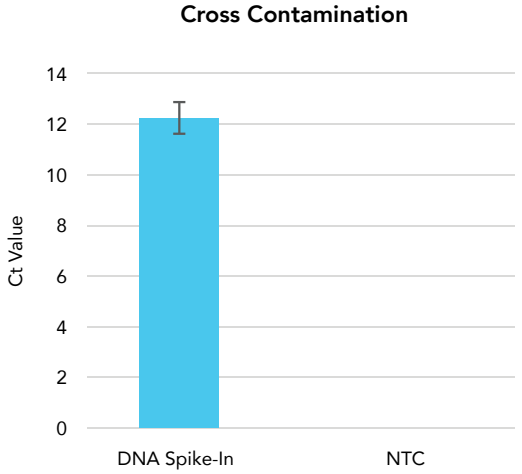


Figure 6: Synthesized alien DNA sequence in DNA/RNA Shield was spiked into every other well across columns 1-6 of a 96-deep-well plate. Negative controls were added to alternating wells. All samples were processed using the automated extraction workflow on the KingFisher™ Flex and eluates were quantified using ZymoTaq qPCR Premix with target specific primers. No amplification was found in NTC samples.

Conclusion

Given the laboratories requirements and circumstances, the automation workflow utilizing the KingFisher™ Flex has been successfully optimized. The process was validated for genomic DNA extraction from mouse and human blood samples collected in DNA/RNA Shield for methylation analysis. The automated workflow has been shown to be robust, consistent, and reproducible with no cross contamination.

Footnotes:
[1] D4081 Quick-DNA Magbead Plus Kit, 1x96 Preps
[2] R1150 DNA/RNA Shield Blood Collection Tube, 50 pack
[3] D5046 EZ-96 DNA Methylation-Lightning MagPrep, 4 x 96 Rxns.
[4] E2005 Femto Human DNA Quantification Kit
[5] E2055 ZymoTaq qPCR Premix, 200 Rxns