Application Note

Identifying rare mutations in Diamond-Blackfan anemia using target enrichment and next-generation sequencing

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Introduction

Diamond-Blackfan anemia (DBA) is a rare congenital stem cell disorder associated with monoallelic inactivating mutations in the ribosomal protein (RP) genes. It leads to bone marrow failure syndrome by causing defects in erythroid progenitor and precursor cell development (1).

Loss of function mutations in 10 of the c. 80 RP genes have been definitively associated with DBA. *RPS19* is mutated in up to 25% of DBA cases, and 13 other RP genes are mutated in a further 25–35%. The molecular basis of the remaining 40–50% of cases is unknown. Since such cases may harbor mutations in one or more of the remaining RP genes and such mutations may occur at very low frequencies, genetic screening using conventional Sanger sequencing on a per-exon/per-gene basis is challenging. Therefore, we developed a methodology based on custom target enrichment technology combined with high-throughput sequencing. To ensure high quality libraries, rapid quality control using the QIAxcel® Advanced System was used at several steps of the library preparation process. We chose the QIAxcel for capillary electrophoresis because it uses ready- to-run gel cartridges and has very short, automated runs that are suitable for high-throughput analysis. Next-generation sequencing was performed using MiSeq®, an Illumina® platform, to screen all 80 RP genes. This proved to be a powerful approach for finding rare mutations in a large set of genes.

Materials and methods

Target enrichment

SureSelect® XP (Agilent®, US) was used for the target enrichment, which employed a custom designed RNA bait hybridization solution to capture the target genes, including the intronic regions and 500 bp of the flanking untranslated region. The regions of interest were collated from the Ribosomal Protein Gene Database (**http://ribosome.med.miyazaki-u.ac.jp**) and uploaded to the Agilent eArray design facility.

Library preparation and sequencing

Library preparation is a complex process with multiple steps. Therefore it is important to assess the sample quality after several steps to ensure the appropriate quality of the final libraries. DNA was purified from peripheral blood leukocytes using QIAamp® DNA Mini Kit on the QIAcube®. A 3 µl sample of gDNA was sheared using a Covaris® e220 sonication platform, and the fragment size was determined via capillary electrophoresis using the QIAxcel Advanced System. Samples were diluted 1 µl to 10 µl with DNA dilution buffer and run on the instrument with the appropriate screening kit. The AM320 method was used in combination with the 15 bp/5 kb alignment marker and a 100 bp – 2.5 kb DNA size marker.

Additional quality control was done after amplification of the ligated libraries and after amplification of the capture libraries. All three steps were analyzed with the QIAxcel DNA Screening Kit using method AM320. The analysis protocol parameters were: baseline filter 160 sec, minimum distance 5 sec, and threshold 10 S/N. The run, reference marker table (RMT), and analysis parameters were defined as a standard protocol that was saved with ScreenGel® software and used for all of the runs.

The capture hybridization was carried out at 65°C for 48 h. Libraries were quantified for pooling using qPCR. Sequencing was performed on an Illumina MiSeq using 150 bp paired-end reads and multiplexed into two runs of 10 samples each. Putative mutations were validated with Sanger sequencing in a CPA-accredited laboratory using an Applied Biosystems® 3500xL Genetic Analyzer.

Results

Per the SureSelect Target Enrichment protocol for Illumina Multiplex sequencing, quality control was performed after shearing of the genomic DNA (Figure 1), amplification of the adapter-ligated libraries (Figure 2), and after amplification of the captured libraries (Figure 3).

The QIAxcel Advanced System allows complete analysis of 12 samples, including reporting, in 8 min. The results are presented as a gel-like image, electropherogram, and result table with different requested parameters (in this case, size and concentration of libraries).

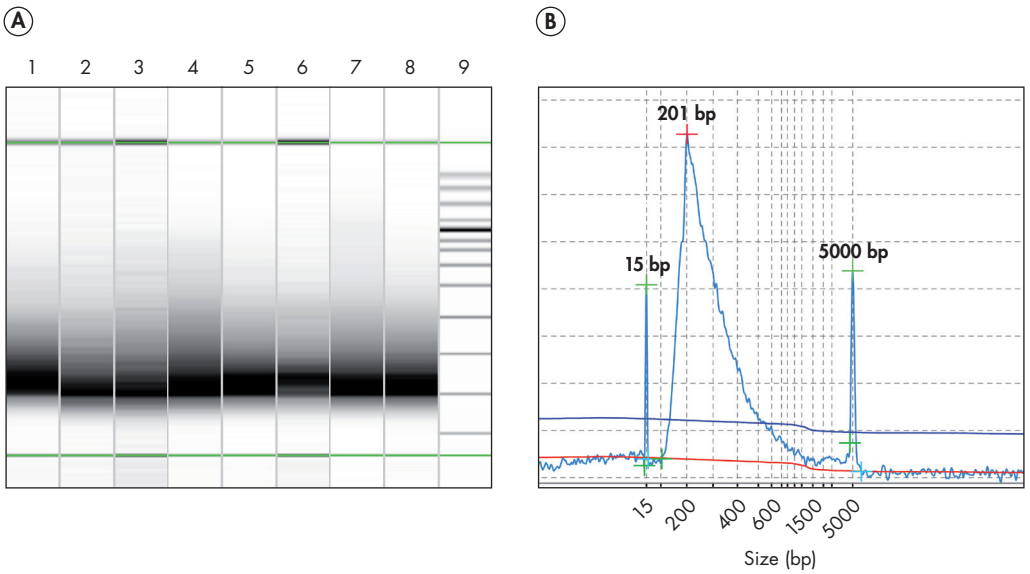

Figure 1. Analysis of sheared genomic DNA. **A** Lanes 1–8: Genomic DNA sheared using a Covaris e220 sonication platform was analyzed using the QIAxcel DNA Screening Kit and method AM320. QX Alignment Marker 15 bp/5 kb (marked in green) was run simultaneously with all of the samples. Lane 9: The QX DNA Size Marker 100 bp – 2.5 kb was used for precise size and concentration assessments. The image shows a smear in the range of 50 – 350 bp. **B** The electropherogram shows the distribution. The medium peak size is 200 bp.

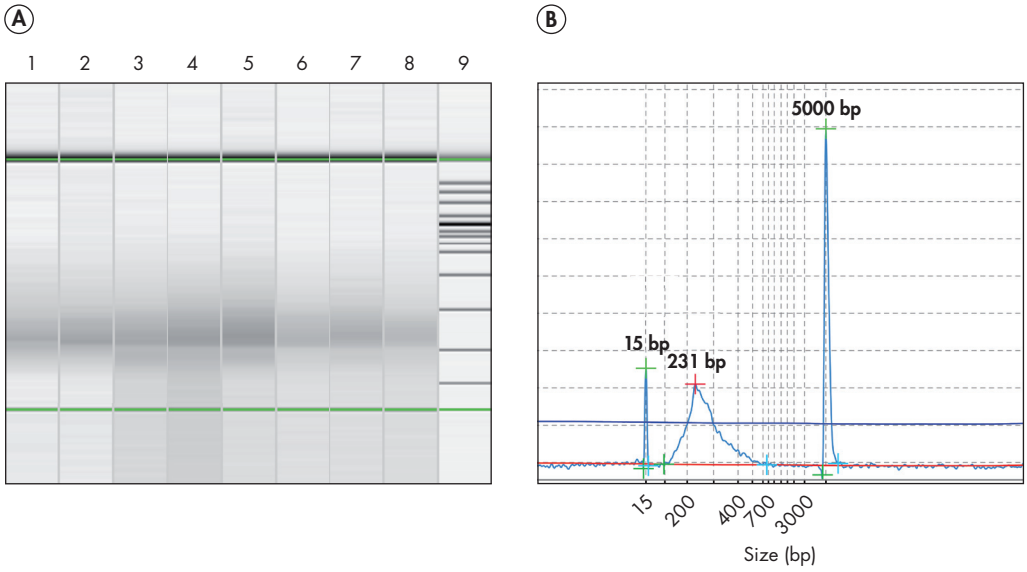

Figure 2. Analysis of amplified, adapter-ligated libraries. **A** Lanes 1–8: The adapter-ligated libraries were analyzed using the QIAxcel DNA Screening Kit and method AM320. Lane 9: QX DNA size marker 100 bp – 2.5 kb. **B** The electropherogram shows a single peak with a median size of 231 bp. The baseline filter is marked with a red line and the threshold with a blue line. As the shortest and longest DNA fragments, the QX Alignment Marker 15 bp/5 kb fragments are visible as sharp peaks at the beginning and the end of the run.

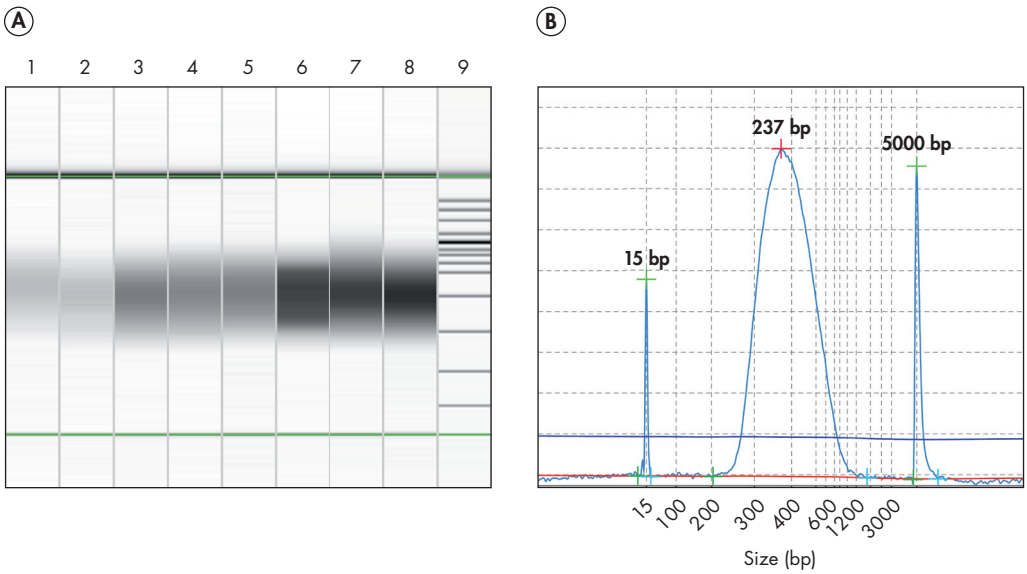

Figure 3. Analysis of amplified capture DNA. **A** Lanes 1–8: The amplified capture DNA was analyzed using the QIAxcel DNA Screening Kit and method AM320. Lane 9: QX DNA size marker 100 bp – 2.5 kb. **B** The electropherogram shows a single peak with a median size of 237 bp.

Using this method, we identified and validated known and novel inactivating mutations in 88% of individuals with DBA. Target enrichment combined with high-throughput sequencing is a robust methodology for the genetic detection of DBA and one that shows considerable improvement over existing methods.

Conclusions

Target-gene enrichment followed by multiplexing and high-throughput sequencing is a powerful approach that allows rare mutations to be found in large sets of genes. Advantages over conventional approaches include the capacity for rapid, accurate, and cost-effective screening of all 80 ribosomal protein genes and the ease of identification of DBA-associated mutations. The method could also prove useful when screening for rare mutations in other genetically complex diseases. The QIAxcel Advanced System allowed reliable and cost-effective library quality control through the whole library preparation procedure. Thus, the sample quality was continuously monitored, securing the good quality of libraries and the high quality of the final sequencing data.

References

1. Gerrard, G., et al. (2013) Target enrichment and high-throughput sequencing of 80 ribosomal protein genes to identify mutations associated with Diamond-Blackfan anaemia. Brit. J. Haematology **162(4)**, 530

Ordering Information

Product	Contents	Cat. no.
QIAxcel Advanced	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel software, and 1-year warranty on parts and labor	9001941
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929002
QIAxcel DNA Screening Kit (2400)	QIAxcel DNA Screening Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929004
QX Alignment Marker, 15 bp/5 kb (1.5 ml)	Alignment marker with 15 bp and 5 kb fragments	929524
QX DNA Size Marker, 100 bp – 2.5 kb (50 µl)	DNA size marker with fragments of 100, 200, 300, 400, 500, 600, 700, 800, 1000, 1200, 1500, 2000, and 2500 bp, and a concentration of 100 ng/µl	929559

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