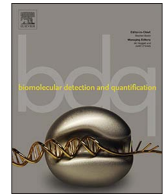
Biomolecular Detection and Quantification 15 (2018) 6–12

Contents lists available at ScienceDirect

Biomolecular Detection and Quantification

journal homepage: www.elsevier.com/locate/bdq



Research paper

An optimized targeted Next-Generation Sequencing approach for sensitive detection of single nucleotide variants

S. Stasik[a,c,1], C. Schuster[b,1], C. Ortlepp[b], U. Platzbecker[a], M. Bornhäuser[a,c], J. Schetelig[a], G. Ehninger[a], G. Folprecht[a,1], C. Thiede[a,*,1]

[a] *Universitätsklinikum Carl Gustav Carus, Medizinische Klinik und Poliklinik I, Dresden, Germany*
[b] *AgenDix GmbH, Dresden, Germany*
[c] *National Center for Tumor Diseases (NCT), Heidelberg, Partner Site Dresden, Germany*

ARTICLE INFO

Handled by Jim Huggett

Keywords:
Cancer
Minimal residual disease
Next-Generation sequencing
Low-level single nucleotide variants
Detection
Quantification

ABSTRACT

Monitoring of minimal residual disease (MRD) has become an important clinical aspect for early relapse detection during follow-up care after cancer treatment. Still, the sensitive detection of single base pair point mutations via Next-Generation Sequencing (NGS) is hampered mainly due to high substitution error rates. We evaluated the use of NGS for the detection of low-level variants on an Ion Torrent PGM system. As a model case we used the c.1849G > T (p.Val617Phe) mutation of the *JAK2*-gene. Several reaction parameters (e.g. choice of DNA-polymerase) were evaluated and a comprehensive analysis of substitution errors was performed. Using optimized conditions, we reliably detected *JAK2* c.1849G > T VAFs in the range of 0.01–0.0015% which, in combination with results obtained from clinical data, validated the feasibility of NGS-based MRD detection. Particularly, PCR-induced transitions (mainly G > A and C > T) were the major source of error, which could be significantly reduced by the application of proofreading enzymes. The integration of NGS results for several common point mutations in various oncogenes (i.e. *IDH1* and *2*, c-*KIT*, *DNMT3A*, *NRAS*, *KRAS*, *BRAF*) revealed that the prevalent transition vs. transversion bias (3.57:1) has an impact on site-specific detection limits of low-level mutations. These results may help to select suitable markers for MRD detection and to identify individual cut-offs for detection and quantification.

1. Introduction

The implementation of Next-Generation Sequencing (NGS) technologies into clinical diagnostics and research is a promising approach for the optimization of diagnosis and treatment of cancer. Malignant diseases are characterized by a stepwise and clonal accumulation of DNA sequence alterations that affect various cell signaling pathways, ultimately causing tumor initiation and progression [1]. Numerous genes are involved, among which several are affected in a variety of neoplasms (e.g. *KRAS*, *TP53*), whereas others are involved only in selected tumor entities (e.g. *IDH1*, *IDH2* in CNS tumors and leukemia) or even in specific diseases (e.g. *NPM1* mutations in myeloid leukemias) [2–5].

Detailed knowledge on the cancer genome and somatic hotspot mutations is crucial to improve risk assessment, to identify target lesions for specific treatment and to predict the response to therapy [6]. In addition to prognosis, molecular profiling is suitable to understand clonal evolution in cancer and to integrate adequate clinical biomarkers for the determination of minimal residual disease (MRD) status during long-term follow-up [7,8].

Based on a continuous decrease in costs and increase in throughput during the past years, NGS increasingly becomes an application for routine clinical testing [9]. While Sanger sequencing is largely restricted to a limited range of well-described mutations at specific genomic loci, the throughput of NGS enables novel diagnostic procedures using highly multiplexed mutational profiling across a large number of genes for virtually all types of DNA alterations [9,10] In addition to genome wide analyses, targeted NGS using amplicon resequencing allows the detection of point mutations with much higher sensitivity compared to Sanger sequencing. Accordingly, by increasing the number of reads per amplicon up to several 100000, it is theoretically possible to detect subclonal mutations at ultra-deep frequencies [11–13].

However, still there is paucity in the transfer and validation of NGS-

* Corresponding author: Universitätsklinikum Carl Gustav Carus, Medizinische Klinik und Poliklinik I, Fetscherstraße 74, 01307 Dresden, Germany.
E-mail address: christian.thiede@uniklinikum-dresden.de (C. Thiede).

[1] Equally contributing authors.

https://doi.org/10.1016/j.bdq.2017.12.001
Received 25 August 2017; Received in revised form 27 November 2017; Accepted 18 December 2017
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based MRD detection for routine clinical services, as sensitivity in massively parallel sequencing is typically limited due to high per-base substitution error rates (e.g. as a result of errors induced by DNA-polymerase) and by the capacity of NGS to process larger amounts of nucleic acids (template DNA input), typically needed for MRD detection [14]. Moreover, the specificity for low-frequency variants might vary across different target regions (e.g. various genes; high/low GC content sequences), enrichment technologies (e.g. hybridization capture and PCR amplicon based), tumor specimens and biopsy types, respectively [15,16].

In order to meet clinical standards and to distinguish true variants from sequencing errors, NGS has to be accurate and robust [17]. Several solutions have been described, e.g. the use of complex barcoding strategies, which enable the separation of true single nucleotide variants (SNVs) from errors [11–14]. Other procedures focused on minimizing false-positive variant calls at the respective genomic positions [18,19].

To further improve NGS based detection of SNVs we used an Ion Torrent PGM semiconductor system, which is well suited for targeted sequencing in clinical settings due to a comparably short running time and high accuracy for SNP calling [20]. As a model for optimization, we used the c.1849G > T mutation of the *JAK2*-gene leading to p.Val617Phe as a prototypical single base pair point mutation. In order to come up with an optimized procedure for the sensitive and quantitative application of NGS-based minimal residual disease detection, we carefully tested several reaction parameters to determine potential sources of error (e.g. the choice of an adequate proofreading polymerase, the amount of genomic template DNA, the number of PCR cycles etc.). We evaluated the reproducibility of our protocol on several other common target oncogenes (*IDH1* and *2*, c-*KIT*, *DNMT3A*, *NRAS*, *KRAS*, *BRAF*) which are potentially relevant as clinical biomarkers. In order to better understand the principal mechanisms behind the observed differences, we performed a more detailed analysis of substitution errors and could clearly show that transition errors, especially artefactual G > A substitutions, are the most common alteration. These results may lead to a better selection of suitable markers for MRD detection.

2. Material and methods

2.1. Samples and DNA extraction

Genomic DNA from the HEL cell line (DSMZ No. ACC 11, DSMZ, Braunschweig, Germany) (*JAK2* – p.Val617Phe positive) [21] or *JAK2* mutant patient samples was diluted in genomic DNA from healthy donors to obtain c.1849G > T variant allele frequencies (VAFs) of 0.001–10%. Control DNA was derived from healthy individuals (< 50 years of age) and served to assess per-base substitution error rates. Unless otherwise specified, DNA was extracted using DNeasy blood mini kit (Qiagen, Hilden, Germany) and quantified with a Qubit 2.0 fluorometer (Life Technologies Life Technologies, Grand Island, NY, USA). All patient samples were obtained with written informed consent of the patients, all studies involving human primary material were performed after approval of the local ethical board of the University Hospital Dresden.

2.2. Primer design and PCR amplification

PCR to detect the c.1849G > T mutation of the *JAK2*-gene was performed on genomic DNA with various concentrations of template DNA input (50 ng and 250 ng) as well as different PCR cycle numbers (35 and 40 cycles) using proofreading and non-proofreading polymerases from different vendors (Table 1). Fusion PCR primer for the preparation of amplicon libraries were designed (Primer Premier 6; Premier Biosoft, Palo Alto, CA, USA) according to the manufacturer's recommendations (Fusion Method; Life Technologies), illustrated in Supplementary Fig. S1. Briefly, the Fusion PCR method uses oligonucleotides containing the Ion A (5′-CCATCTCATCCCTGCGTGTC TCCGACTCAG-3′) and truncated P1 (trP1) (5′-CCTCTCTATGGGCAGT CGGTGAT-3′) adapters linked to a gene specific part to generate amplicons with the required motifs for parallel sequencing during the PCR. For unidirectional sequencing only one forward (with A adapter (or trP1 adapter)) and one reverse primer (with trP1 adapter (or A adapter)) were used for PCR amplification. Primer sequences for *JAK2* (p.Val617Phe) were 5′-GAAGCAGCAAGTATGATGAGCAAGC-3′ (Forward) and 5′-CTGAGAAAGGCATTAGAAAGCCTGTAGT-3′ (Reverse), amplifying a 182 bp fragment. PCR primer sequences and specific PCR conditions of all other target regions (*IDH1* and *2*, c-*KIT*, *DNMT3A*, *NRAS*, *KRAS*, *BRAF*) are listed in Supplemental Table S1. All PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).

2.3. Library preparation, sequencing and data analysis

The PCR reactions were purified using a two-round purification process with Agencourt AMPure XP Reagent (Beckman Coulter, Krefeld, Germany) and eluted in 30–50 µl ddH_2O. The barcoded PCR products were quantified with a Qubit 2.0 fluorometer (Life Technologies) using the Qubit dsDNA HS Assay (Life Technologies) and sequenced uni-directionally on an Ion Torrent PGM semiconductor-based device (Life Technologies), according to manufacturer's protocols with 18 MOhm water and argon gas to drive fluidics. Briefly, the pooled library was clonally amplified on Ion Sphere™ Particles (ISPs) in an emulsion PCR using the Ion PGM Hi-Q OT2 Kit (Life Technologies). Enrichment of positive Ion Spheres (ISPs) was achieved using DynaBeadsMyOne streptavidin C1 beads (Life Technologies). Quantification of recovered particles was performed using a Qubit 2.0 fluorometer (Life Technologies) and an Ion Sphere quality control kit (Life Technologies). PGM sequencing (Ion PGM Hi-Q Sequencing Kit; Life Technologies) was done using different semiconductor chips, with output of 6×10^5 reads (314 chip), 3×10^6 reads (316 chip) and 6×10^6 reads (318 chip). Raw read mapping was done using Torrent Suite Software version 3.2 or higher based on the TMAP (Torrent Mapping Alignment Program) Smith-Waterman alignment algorithm using default settings with alignment to the hg19 human reference genome from the UCSC Genome Browser (http://genome.ucsc.edu/). For data analysis, adapter trimming and alignment QC we used the Torrent Variant Caller (TVC, v.4.0) plugin with default settings (somatic low stringency), providing optimized pre-set parameters for low-frequency variants assessment with minimal false negative calls.

3. Results

3.1. Application of NGS for the sensitive and quantitative detection of JAK2 (p.Val617Phe)

While there was a small but non-significant difference in base-calling error for the simultaneous increase of input DNA and reduction of PCR cycles (Fig. 1a), the application of high accuracy proofreading polymerases (Phusion HSII and Q5 High Fidelity) significantly (~5fold) reduced median per-base substitution error rates and consequently increased NGS sensitivity towards the identification of the *JAK2* c.1849G > T (p.Val617Phe) variant at 0.1% VAF (Fig. 1e-f). For all tested polymerases, PCR induced transitions (mainly G > A and C > T) were the major source of error, with an average transition bias vs transversion bias ratio of 3.57:1 (Fig. 1 b). Most significantly, Q5 High Fidelity polymerase reduced both transition and transversion bias, mainly for T > C (25fold), T > A and G > C (11fold each) substitution errors (compared to Platinum*Taq*). Virtually no difference between non-/proofreading enzymes was observed for A > G (1.35fold), C > G (1.25fold) and T > G (0.95fold) errors. Comparing sensitivity at c.1849G > T (*JAK2*) (Fig. 1c), false-positive G > T background was significantly reduced when the Q5 High Fidelity polymerase was used

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