Benchmarks

Precise breakpoint localization of large genomic deletions using PacBio and Illumina next-generation sequencers

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Herein we present the applicability of single-molecule (PacBio RS) and second-generation sequencing technology (Illumina) to the characterization of large genomic deletions. By testing samples previously characterized using a Sanger approach, our methods determined that both next-generation sequencing platforms were able to identify the position of deletion breakpoints. Our results point out various advantages of next-generation sequencing platforms when characterizing genomic deletions; however, special attention must be dedicated to identical sequences flanking the breakpoints, such as poly(N) motifs.

The PacBio RS next-generation sequencing (NGS) technology (Pacific Biosciences, Menlo Park, CA, USA) has not only the potential to identify modified bases and thereby characterize methylation patterns (1,2), but it also provides previously unprecedented sequencing read lengths (>2kb), making it useful for quickly improving existing genome assemblies (3). In this study, we used the advantage of such long reads for the characterization of large deletions previously identified by multiplex ligation-dependent probe amplification (MLPA) and microarray analyses. Using traditional Sanger sequencing to characterize large deletions is time-consuming and work-intensive (4,5), increasing the need for effective breakpoint localization. Indeed, for Sanger sequencing a large fragment (2–10kb) containing the breakpoints has to be amplified by long-range PCR (LR-PCR) and subsequently

sequenced in order to identify exact breakpoint positions. Furthermore, since Sanger sequencing permits only ~600 bp to be sequenced using one primer, several sets of internal primers are required for large LR-PCR products.

In contrast, NGS may offer simplified sequencing in such cases. Herein, we tested this possibility by using not only the long reads of the PacBio platform, but also the short reads of a second-generation sequencing technology (HiSeq 2000, Illumina, San Diego, CA, USA). Illumina offers stable lengths of short reads (100 bp in this case) with errors most likely to be grouped at the ends of reads (6,7); the PacBio RS reads from this study had a mean length of 2459 bp and random distribution of errors affecting 10–15% of nucleotides. In addition, only a few dedicated computational techniques are available for the

characterization of large deletions by NGS (8), making data analysis a challenge.

The three DNA samples used in this study harbor previously characterized large hemizygous deletions. Deletions in sample 44 and sample 70 (of length 26,887 bp and 302,580 bp, respectively) affect the *FBN1* gene in patients with Marfan syndrome (4); a deletion in sample 53B has a size of 3,408,306 bp and comprises the entire *COL3A1* gene in a patient with Ehlers-Danlos syndrome vascular type (5). Accordingly, ~6.5–8.5 kb LR-PCR products were amplified using the Expand Long Template PCR System (Roche Diagnostics, Rotkreuz, Switzerland) as described previously (4,5) and purified by means of QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

SMRTbell libraries were prepared using the PacBio C2 chemistry (3–10 kb) DNA preparation kit (Part no. 001–540–726,

Method summary:

We present the applicability of single-molecule (PacBio RS) and second-generation sequencing technology (Illumina) to the characterization of large genomic deletions. Both next-generation sequencing platforms were able to identify the position of deletion breakpoints previously identified by Sanger sequencing.

Figure 1. Both ends of the 302,580-bp deletion on chromosome 15 in sample 70 displayed in the Integrative Genomics Viewer. Aligned reads are displayed as gray bars/arrows; letters indicate mismatched bases; purple vertical dashes indicate insertions; and black horizontal lines indicate deletions. The reads generated by PacBio RS (upper panel) and Illumina HiSeq 2000 (lower panel) were sorted by aligned position, base, and mapping quality, and compared with the results of Sanger sequencing (bottom). Sections of 22 reads are shown. The top tracks show a much clearer decrease in the PacBio reads, whereas the Illumina reads show a clearer increase in mismatches at the sites of breakpoints. Uppercase letters represent the sequence in the region of the start point of the deletion; lowercase letters represent the sequence in the region of the deletion end point. Due to identical sequences at the sites of breakpoints, the break and re-joining could have occurred at three positions, as indicated by open triangles. The dotted red line marks the most telomeric position of the possible breakpoints. Note that the total read counts (reads) and the percentage of reference bases (%) are given for the positions flanking the site where the coverage (gray bars) starts to lower. For more details, see Tables 1 and 2.

Pacific Biosciences) as well as 5 µg purified amplicons without fragmentation. Libraries were subsequently sequenced on the PacBio RS using one SMRT cell per sample and taking two movies of 45 min each. The reads have been mapped with the BLASR mapper (9), which is supplied in the SMRT Portal

software suite (Pacific Biosciences) and applies therefore as a standard mapper for PacBio reads. The same amplicons were sequenced on the HiSeq 2000 sequencer using Illumina's TruSeq DNA Sample Preparation v2 protocol with 1 µg input material and 100+100 bp pair-end reads. The reads were mapped using

the standard mapper, Bowtie (10). For both NGS platforms, the mappers have been used with default parameters. Respective sequences are available in the NCBI Sequence Read Archive (object ID: ERP002092).

For the PacBio data, the read coverage in the SMRT Portal software suite resulted in a clear drop of read depth in the deleted region (see Supplementary Figure S5), which was subsequently confirmed by zooming in on the breakpoint regions by means of the Integrative Genomics Viewer (IGV) (11) (Figure 1 and Tables 1 and 2 for sample 70; see Supplementary Figures S1–S2 as well as Supplementary Tables S1–S4 for samples 44 and 53B, respectively). Respective Illumina data displayed in IGV showed more gradually sinking patterns at the expected deletion ends; the site of breakpoints in these data was identified by an increase in mismatches (Figure 1, Tables 1 and 2, Supplementary Figures S1–S2, and Supplementary Tables S1–S4). This may be due to the fact that the mappers typically allow several mismatches, meaning that many of the short Illumina reads could be mapped over the breakpoints. In contrast, PacBio RS data show a number of reads spanning over the deletion, which have not been mapped by the SMRT Portal aligner to the standard reference due to the high number of mismatches. The read depth of both platforms is more than sufficient to find the breakpoint; tests using 1/2 or 1/3 of reads per sample also produced satisfactory results (data not shown).

An additional difficulty may be identical sequences on both sides of the deletion, a common phenomenon that has already been described for different genes (12–14).

Table 1. Read depth and percentage of the wild-type allele in the region flanking the breakpoint at the start site of the deletion in sample 70.

Location	Not deleted					Deleted				
Wild-type Sequence	A	C	C	C	C	C	C	A	T	T
PacBio RS	614 (100%)	531 (100%)	508 (100%)	492 (100%)	384 (99%)	103 (89%)	66 (88%)	66 (98%)	61 (95%)	29* (86%)
HiSeq 2000	12699 (100%)	11679 (100%)	9545 (100%)	8278 (100%)	7081 (100%)	5820 (1%)	4313 (48%)	3224 (100%)	1678 (99%)	1536** (98%)

*No mapped reads 243 bases after the most telomeric breakpoint (read depth = 0). **No mapped reads 117 bases after the most telomeric breakpoint (read depth = 0). Bold letters indicate identical bases at the sites of the breakpoint, which can be either up- or downstream of the breakpoint. The red dotted line indicates the most telomeric position of the three possible breakpoints. This is also the point where the read depth drops and the number of mismatches increases (see Figure 1).

Table 2. Read depth and percentage of the wild-type allele in the region flanking the breakpoint at the end of the deletion in sample 70.

Location	Deleted					Not deleted				
Wild-type Sequence	t	t	t	a	a	c	c	a	t	a
PacBio RS	231* (8%)	242 (10%)	251 (94%)	215 (95%)	315 (52%)	1316 (99%)	1359 (99%)	1411 (99%)	1465 (99%)	1547 (100%)
HiSeq 2000	5408** (100%)	8762 (87%)	10427 (83%)	13679 (92%)	14956 (62%)	16383 (100%)	17735 (96%)	18658 (100%)	19512 (100%)	20549 (100%)

*No mapped reads 210 bases before the most telomeric breakpoint (read depth = 0). **No mapped reads 16 bases before the most telomeric breakpoint (read depth = 0). Bold letters indicate identical bases at the sites of breakpoints, which can be either up- or downstream of the breakpoint. The red dotted line indicates the most telomeric position of the possible breakpoints. The most centromeric breakpoint, where the read depth drops and the number of mismatches increases, is indicated by a black bold line (see Figure 1).

PacBio										
	n=0	n=1	n=2	n=3	n=4	n=5	n=6	n=7	n=8	type II error
Flank 5	56	28	191	941	2132	348	37	8	0	3e-141
Flank 10	0	1	53	329	820	124	12	3	0	1e-46
Flank 20	0	0	5	56	127	19	2	1	0	5e-06
Illumina										
	n=0	n=1	n=2	n=3	n=4	n=5	n=6	n=7	n=8	type II error
Flank 5	2	3	0	29	68817	3	0	0	0	<2e-308
Flank 10	0	0	0	20	58769	0	0	0	0	<2e-308
Flank 20	0	0	0	13	43258	0	0	0	0	<2e-308

TCAGTACTTTAAACAGCCTA(C)nATAATCATCATGTTAGAGTC

Figure 2. Counts of exact matches for different lengths of a poly(C) motif (red) at the site of deletion breakpoints in sample 70 with 5-, 10-, and 20-nucleotide flanking sequences for both PacBio and Illumina reads. The count indicates that the true sequence includes 4 × C (n = 4) (see Figure 1). Corresponding type II errors were calculated using the R script provided in the Supplementary Materials.

In particular, this could be observed in all three deletions presented in this study (“CC” in samples 53B and 70 and “GC” in sample 44). In order to find the precise sequence of poly(N) motifs (tandemly repeated nucleotides) at the sites of break and rejoining, we have developed an AWK script to count matches at the sites of suspected deletion breakpoints (see Supplementary Materials). This counting was performed with perfect matches only, resulting in the data depicted in Figure 2 (sample 70) and Supplementary Figures S3 and S4 (samples 44 and 53B, respectively). When a single nucleotide (or pair, in the case of GC) has a fixed probability of being misinterpreted, it can be assumed without loss of generality that the distribution of the occurrences of specific motifs follows the Poisson distribution. The hypothesis that the maximum number of counts represents the appropriate motif has been tested. For PacBio RS reads in sample 70, the probabilities of wrongly accepting the null hypothesis are far below the 0.01 level of significance ($p = 1.5 \times 10^{-23}$, $p = 1.06 \times 10^{-46}$, and $p = 3.2 \times 10^{-141}$ in the cases of 20, 10, and 5 flanking bases, respectively) (Figure 2). In the case of Illumina, due to the high number of reads, error levels are so low that they go below that afforded by the small-number precision in the R language. For details on the calculations, see the R script in the Supplementary Materials. The script can be used on any FASTA or FASTQ data and checks the statistical power at a given significance level regardless of the platform.

As shown by this study, the determination of deletion breakpoints can be done with data obtained from both NGS platforms. However, whereas the long reads of PacBio RS showed a sharp decrease in read depth, Illumina short reads exhibited an increase in mismatches related to the position of

the breakpoints. Sample preparation costs are comparable for PacBio and Illumina platforms. However, sequencing using PacBio RS can be done within a working day, while Illumina’s system, even the smaller MiSeq version, requires more time. Both platforms are suitable for precise breakpoint localization, provide an alternative procedure for the characterization of large deletions, and require fewer resources and less time than traditional Sanger sequencing.

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Competing interests

The authors declare no competing interests.

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