



Critical points for an accurate human genome analysis

DOI:

[10.1002/humu.23238](https://doi.org/10.1002/humu.23238)

Document Version

Final published version

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

White, S. J., Laros, J. F. J., Bakker, E., Cambon-Thomsen, A., Eden, M., Leonard, S., Lochmüller, H., Matthijs, G., Mattocks, C., Patton, S., Payne, K., Scheffer, H., Souche, E., Thomassen, E., Thompson, R., Traeger-Synodinos, J., van Vooren, S., Janssen, B., & den Dunnen, J. T. (2017). Critical points for an accurate human genome analysis. *Human Mutation*. <https://doi.org/10.1002/humu.23238>

Published in:

Human Mutation

Citing this paper

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Critical Points for an Accurate Human Genome Analysis

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/humu.23238](https://doi.org/10.1002/humu.23238).

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The 3Gb-TEST project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 602269.

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Abstract

Next-generation sequencing is radically changing how DNA diagnostic laboratories operate. What started as a single-gene profession is now developing into gene panel sequencing and whole exome and genome sequencing (WES/WGS) analyses. With further advances in sequencing technology and concomitant price reductions, whole genome sequencing (WGS) will soon become the standard and be routinely offered.

Here we focus on the critical steps involved in performing WGS, with a particular emphasis on points where WGS differs from WES, the important variables that should be taken into account, and the quality control measures that can be taken to monitor the process.

The points discussed here, combined with recent publications on guidelines for reporting variants, will facilitate the routine implementation of WGS into a diagnostic setting.

Key words: Whole genome sequencing, Whole exome sequencing, Genetic variation, Bioinformatics, Next generation sequencing

Introduction

Next-generation sequencing (NGS) is being rapidly implemented into the diagnostic setting. Due to the cost of whole genome sequencing (WGS) the first tests were limited to selected regions of the genome, and later either gene panels or whole exome sequencing (WES). These approaches have clearly demonstrated the value of this type of analysis, simplifying genetic screening and identifying a number of new disease genes. They have also given the first indications of the complicating factors that are part of genomic screening, such as unsolicited findings.

There have been several publications suggesting guidelines for reporting NGS-based findings (Green, et al., 2013; Hehir-Kwa, et al., 2015; Rehm, et al., 2013; Weiss, et al., 2013), and a comprehensive report regarding the implementation of exome sequencing in the clinic has also been published (Matthijs, et al., 2015). Many of the points addressed by (Matthijs, et al., 2015) are also applicable to whole genome sequencing, but the greater size and complexity of the human genome mean that there are additional issues to be considered (Table 1). Using (Matthijs, et al., 2015) as a baseline, we here focus on the different technical aspects involved in sequencing a complete human genome; from DNA sample source and preparation to variant calling and description (Table 2), but we are also fully aware of the other clinical, ethical and professional dimensions of such a technology (Julia, et al., 2016). A key consideration, addressed elsewhere, is the need for robust processes of health technology assessment (Payne, et al., 2017, in press).

Sample source

The first step in sequencing a genome is to obtain a DNA sample. There are many possible sources, each of which can influence the findings. For routine clinical purposes a venous blood sample is usually obtained, with genomic DNA isolated from nucleated cells such as lymphocytes. Depending on the isolation method used, such samples may also contain cell-free DNA, usually from dead cells (and would therefore be expected to have the same DNA as found in the nucleated cells). If the sample is obtained during pregnancy, there will be fetal DNA (actually derived from the placenta) present in the blood. Indeed, a recent report showed that it was possible to sequence the fetal genome solely by analysing circulating cell-free DNA from the mother (Kitzman, et al., 2012).

Likewise, if the person providing the sample has a cancer, then cells/DNA from a tumour may have been shed into the circulation. The proportion of different cell types within a blood sample can vary considerably (Whitney, et al., 2003) and it is important to note that B and T lymphocytes will have undergone somatic rearrangements at their respective cell receptor genes. Genomic DNA isolated from these sources may appear to contain deletions at these loci, if compared to DNA from non-lymphocytic cells.

When analysing a tumour, the issue of sample purity is a critical issue, as the biopsy may contain varying amounts of contaminating non-tumour tissue. In addition, it is well recognised that tumours are heterogenous, and may contain clonal subtumours within the sample (Marusyk and Polyak, 2010).

It is also possible for non-human DNA to be present in a sample. Low invasive methods for obtaining DNA such as buccal swabs or spit kits are often used (Freeman, et al., 1997). However, food material may contaminate DNA derived from a buccal swab or saliva sample. Additionally, bacteria such as *Streptococcus parasanguinis* are commonly found in the oral cavity, and may be a significant contributor to a DNA sample isolated from this source (Mahfuz, et al., 2013). Potential contaminations in a specific genomic DNA sample may derive from infections of the individual (viral, bacterial, etc.) or any cell culturing when performed. This is not necessarily an issue for WES, as the enrichment step will enrich for human sequences and therefore automatically reduce the level of contamination, but most WGS protocols will sequence all DNA present in a sample, irrespective of source.

Although cultured cells are an easy source of DNA, it is known that culturing can lead to genomic instability (Adey, et al., 2013; Maitra, et al., 2005; Narva, et al., 2010). In addition, depending on the stringency of the culture conditions, contaminants might be present like mycoplasma, yeast, etc. It is also possible that the cell line in question becomes contaminated with another cell line, or even overgrown by a different cell line completely. There have been numerous reports demonstrating that a significant proportion of cell lines are not in fact what they are thought to be (American Type Culture Collection Standards Development Organization Workgroup, 2010; Capes-Davis, et al., 2010; Chatterjee, 2007), and many journals now require evidence that cell lines used in a report are correct. It has now become a service of a number of biobanks to verify and authenticate cell lines used in research projects, prior to publication submission.

Another potential issue is the replication state of the cells. If DNA is isolated from proliferating cells, then DNA from early replicating regions will be over-represented in comparison with sequence from late replicating regions. This is especially important when looking at possible copy number variants or mosaic changes (Koren, et al., 2014).

Reports have shown it is possible to sequence the genome of a single cell (Navin, et al., 2011; Wang and Navin, 2015) and even DNA and RNA from the same cell (Dey, et al., 2015). Such sequences will never be complete, however, as part of the genome will be lost during preparation and sequencing. It is also important to note that every cell has its “own” genome, even when isolated from the same tissue within a single individual, due to *de novo* variation introduced during growth, derived from DNA replication and/or DNA damage repair. WGS analysis of individual cells has shown significant variation, ranging from large CNVs (Cai, et al., 2015; Knouse, et al., 2016) to LINE1 retrotranspositions (Evrony, et al., 2015).

DNA isolation

Ideally, clean and high molecular weight DNA will be obtained, and this will usually be the case from freshly obtained samples. Older DNA samples or those isolated from archived material may well be degraded or contaminated. DNA degradation is rarely random, meaning that genomic regions will be unequally represented. This will be reflected in non-

uniform coverage of specific regions (e.g. chromosome ends, AT/GC rich regions, etc.), and may preclude sequence analysis with long read instruments. In fact the data obtained can be used to monitor the extent of degradation, when present.

Sequencing Platforms

Although there are now a number of different NGS platforms available (reviewed in (Reuter, et al., 2015)), only a few are amenable for routine human WGS. These can be loosely divided into short read (<1kb read length) and long read (>1 kb) sequencers. The current market leaders for short read sequencers are the Illumina systems. Illumina sequencing chemistry is based on sequencing-by-synthesis, using nucleotides linked to fluorescently labelled terminators (Bentley, et al., 2008). Paired end sequencing allows both ends of the molecule to be read, theoretically doubling the amount of sequence produced and potentially allowing the identification of structural variants through discordant mapping of the two ends.

Of the sequencers that can routinely produce reads >1kb in length, the Pacific Biosciences RSII is the most commonly used for human WGS. Sequencing takes place in individual wells on a Single Molecule, Real-time (SMRT) cell. Using this approach it is possible to produce sequence reads >50kb in length, although with a much lower output and higher error rate than the Illumina. The error rate can be compensated for with sufficient read depth, as the errors are unbiased. This allows for regions not amenable to sequencing with Illumina chemistry e.g. homopolymer stretches or loci with high or low GC content, to be sequenced.

Several human genomes sequenced with this technology have been described (Chaisson, et al., 2015a; Zook, et al., 2016).

Another example of a long read sequencer is the Oxford Nanopore system. This technology reads the nucleotides in a DNA strand by measuring the change in electrical current as the DNA molecule passes through a pore in a membrane. Advantages over other sequencing platforms are the low startup cost, portability, and speed from sample to data. Primarily used for smaller genomes (Loman, et al., 2015; Quick, et al., 2016) the first human genome completely sequenced with using this technology was announced at the end of 2016.

Sample preparation

The method of DNA isolation used determines whether specific contaminants may be present or not. When blood is centrifuged cells can be separated from cell-free DNA and other methods may be used to reduce the amount of DNA derived from viruses or bacterial contaminants. Sample preparation mostly involves steps that depend on the sequencing platform to be used, but generally involves some form of DNA fragmentation followed by the attachment of linker sequences to facilitate the sequencing reaction. Again, fragmentation is not random, and depends on different factors including GC content, genomic location, etc. Currently popular methods using enzymatic fragmentation (transposases) are known to demonstrate some bias due to e.g. GC content (Lan, et al., 2015; Marine, et al., 2011). In addition, the size of the DNA fragments generated will influence possibilities for haplotype construction.

A PCR step is often used during library preparation, particularly when only small amounts of starting DNA are available. However, this step will also introduce biases. A study looking at the effect of GC content found that there was reduced abundance of DNA fragments with extreme GC% (<10% and >60%) (Aird, et al., 2011). This could be ameliorated to a degree, by optimising the PCR conditions with regards to temperature and DNA polymerase. Another study also showed that DNA polymerase choice can be critical in reducing bias (Dabney and Meyer, 2012).

It is also possible to prepare a genomic DNA sample for sequencing using an amplification-free protocol. In one such approach, adapters containing the sequences necessary for attachment to the Illumina flow cell are ligated onto the fragmented DNA (Kozarewa, et al., 2009). Amplification during cluster generation on the flow cell enriches for sequences containing the correct adapters. This amplification step is inherent to the Illumina sequencing system, and it is only single molecule sequencing platforms like Pacific Biosciences (Eid, et al., 2009) and Oxford Nanopore (Clarke, et al., 2009) that do not include some type of amplification. As every amplification step introduces biases, amplification should be restricted to a minimum (Aird, et al., 2011; Kozarewa, et al., 2009; van Dijk, et al., 2014).

Read alignment

The two main variables in read alignment are the choice of aligner and the choice of reference genome. Many different algorithms have been developed for aligning short read

sequences (reviewed in (Ye, et al., 2015)), with Bowtie/Bowtie2 (Langmead and Salzberg, 2012; Langmead, et al., 2009) and BWA (Li and Durbin, 2009) amongst the most popular.

The reference sequence used for mapping has significant consequences, especially with regards to repeat regions, unassigned sequences (unplaced contigs), and different haplotypes (present as haplotype chromosomes or alternative alleles). Before a transition is made to a new human genome build (e.g. hg19 to hg38) existing data should be analysed relative to the old and new reference, and all differences should be understood.

Metrics of all sorts and from all stages in the pipeline can and should be stored in a QC database. The aim of such a database is to gather information about the distribution of these metrics, in order to automatically find outliers. If, for example, the GC content of all fastq files is stored, it can be noted that the GC content on average will be 40%, with a very small deviation (less than 1%). Any sample that has a GC content that significantly deviates from this distribution should be set aside for further scrutiny. Likewise, the Transition/Transversion ratio (Tr/Ti) can be used as a quality control for single nucleotide polymorphisms (SNPs), which is typically higher for exons compared to introns, and higher for synonymous SNPs compared to non-synonymous SNPs (Wang, et al., 2015).

There are several important stages in the pipeline where QC metrics are gathered. In general one should try to capture a distribution of a metric rather than one value. For example, do not store the average insert size of the library, but rather the distribution of insert sizes (using 100 bp bins).

Short read sequencers, e.g. Illumina HiSeq, produce sequence reads that are not sufficiently long to be unambiguously mapped to certain repetitive sequences in the genome, especially when they contain expanded (disease-associated) alleles. This has consequences for the analysis of e.g. CNVs/SVs in these regions, and for trinucleotide repeat disorders. Not only are repeat expansions like CGA and CGG extremely sensitive to PCR-bias, when there is insufficient unique flanking sequence present, reads will not be mapped with high confidence and it is unlikely that variants will be called reliably.

Given the difficulties observed with read mapping, and the associated consequences for accurate variant calling, it might be advantageous to make a reference genome that has been optimised for diagnostic purposes. This diagnostic reference genome should be modified at all sites where read mapping is not optimal for reliable variant detection. Such regions include those underlying trinucleotide repeat expansion disorders, where the ideal reference genome would contain an extended repeat, ensuring the mapping of all reads and thereby accurate repeat-length scoring. Similarly, repeated segments of genes such as *DRD4* (48bp unit) and *PRNP* (48bp unit), can be extended to improve variant detection and allele sizing. Larger duplicated sequences can be reduced to a single copy, ensuring that variants will not be missed because they are randomly distributed over the different copies. Linking variants based on this clinical reference genome to the standard reference genome build (e.g. hg38) can be achieved using a simple genomic coordinate translation table.

For quality purposes a number of specific genomic regions should be selected and used to generate quality metrics, as well as for determining coverage and the ability to detect

variants. These can be based on specific criteria, e.g. high (around the promoter of *EGFR* (Obradovic, et al., 2013)) and low (around exon 2 of *DMD* (White, et al., 2002)) GC content, repeats (variable number of tandem repeat (VNTR) polymorphisms within the *DRD4* gene (Van Tol, et al., 1992) or the *MUC1* gene (Kirby, et al., 2013)) and include sequences that are/are not included in exome analysis (exonic and intronic/intergenic regions respectively). Loci not expected to be covered using the applicable sequencing approach should also be included.

Small sequence variants (SNPs/Indels/STRs)

Many different algorithms have been described for calling variants in NGS data (reviewed in (Nielsen, et al., 2011)). Comparative studies have shown that no single algorithm can detect all variants, that there is <100% overlap between algorithms, and that indels are especially difficult to detect (Cornish and Guda, 2015; Liu, et al., 2013). Analysis of short tandem repeats (Press, et al., 2014) are complicated by the high mutation rate due to polymerase slippage. It has been shown that non-PCR amplification is better for STR analysis when compared to an amplification-based protocol, with a 9-fold reduced error rate (Fungtammasan, et al., 2015).

Structural Variation

There are many different types of structural variation (SV), including deletions, duplications and amplifications (collectively known as copy number variation, or CNV), insertions, transpositions, and translocations (Alkan, et al., 2011). There have been several approaches described for CNV analysis of WES data (Fromer, et al., 2012; Wu, et al., 2012) but all suffer from highly variable coverage derived from several steps in the process (incl. GC-percentage, PCR-bias, protocol changes, capture probes, capture efficiency, etc.). To address the variability in coverage, tools that are designed to do CNV calling on WES data either use reference sets or call CNVs in a population. An example of such a tool is cn.MOPS (Klambauer, et al., 2012), which aims to explain the coverage distribution per position within a set of samples by a mixture of Poisson distributions. If more than one distribution is needed to explain the observed variation, a (common) copy number variation is detected. This approach works well if the copy number variation is relatively common in the input dataset. Another well known tool,XHMM (Miyatake, et al., 2015), uses a combination of principal component analysis normalisation and a hidden markov model to detect CNVs. A relatively large number of samples (at least 50) should be used to reliably call CNVs within this set. Finally, WISECONDOR, a tool originally developed for non-invasive prenatal testing (NIPT) (Straver, et al., 2014) has successfully been used to detect CNVs in WES data. This tool detects violations of correlated coverage of bins within one sample. A reference set is used to establish the correlation, the CNV detection itself is done per sample. For all of the approaches described above, either a batch of samples, or a reference set is needed to cope with the high variability of coverage within a WES data set. Additionally, there is usually no possibility of determining the exact breakpoint, meaning that the exact nature of

duplications will usually be unclear and other types of SV, e.g. inversions will not be detected. Using WGS data, especially when using amplification-free protocols, gives a more uniform coverage, making SV detection more reliable and sensitive. In addition, WGS samples should contain the unique breakpoint sequences, instrumental for resolving the identity and exact borders of the SV.

In contrast to SNPs, a relatively low level of sequence coverage is sufficient to detect many types of SV. Aneuploidies can be detected in cell-free DNA as part of NIPT (Brady, et al., 2015), with <10 million reads (<1x average coverage) being sufficient for whole chromosome aneuploidies (Chiu, et al., 2008; Fan, et al., 2008). Multiallelic CNVs (mCNV) are specific genomic regions that can be present in a range of different copy numbers, and are difficult to accurately genotype (Cantsilieris and White, 2012). A report by Handsaker et al. (Handsaker, et al., 2015) modified a previously published read depth approach (Handsaker, et al., 2011) to identify and genotype >1000 mCNV loci in samples from the 1000 genome project.

Unmapped Reads (Dustbin analysis)

Reads that do not initially map to the reference may still be informative. This may be due to a read containing multiple variants, spanning a deletion/duplication/inversion breakpoint, insertion of non-reference sequence (viral, repeat), or representing a sequence not present in the reference genome. Different analytical approaches need to be applied for each of these possibilities. Not all reads are mapped perfectly or with only a small number of

mismatches. Different types of unmapped reads include: (i) split reads, (ii) discordant reads, (iii) unmapped mates, (iv) soft clipped reads. Split reads are direct evidence of translocations, but are very hard to find as they require rather clean breakpoints on both ends of the structural variation event. Pindel (Ye, et al., 2009) was designed for this specific use case. It uses one of the reads in a read pair as an anchor. Fragments of the other read are then located in the vicinity of the anchor read. This method is able to detect deletions, inversions, translocations and, to a certain extent, novel insertions. A related technique named BreakDancer (Chen, et al., 2009) was developed to exploit the presence of discordant reads. Especially in combination with mate pair sequencing, this technique is suitable for breakpoint detection of larger structural variation events, even in the absence of clean breakpoints.

Specific algorithms have been developed for identifying inversion breakpoints in PacBio sequence data, by reanalysing reads that did not initially align (Chaisson, et al., 2015a).

Unmapped mates and soft clipped reads are indirect evidence of large structural variations that may be translocations, but also large insertions, tandem duplications, etc. Although analysing these reads by themselves is not sufficient to draw any conclusions about the nature of the structural variation, it does indicate that there may be an aberration. Also, in combination with CNV calling it may exclude or support certain types of events.

Some read pairs cannot be mapped to the reference genome at all. There are several possible reasons for this, e.g. the reference sequence contains too many copies of this sequence, or the reference sequence does not contain the sequence of interest. The first

case is easy to detect from the alignment file, however there are multiple potential causes for the latter case:

- The sequence is sample specific, or the population that this sample comes from significantly differs from the reference.
- The sequence comes from a different organism (contamination, an infection, etc.).

To classify these read pairs, it is possible to use a BLAST-like approach to see if there is any significant enrichment for known pathogens. In practice, high throughput tools like Kraken (Wood and Salzberg, 2014) or Centrifuge (Kim, et al., 2016) are used for rapid classification of unmapped reads. Both tools use an index based strategy (in the case of Kraken based on *k*-mers, in the case of Centrifuge based on Burrows-Wheeler transform and the Ferragina-Manzini index). Both techniques have successfully been used for general metagenomics projects, but also for pre-filtering of WES and WGS data. This may be necessary when the sample was obtained via a buccal swab, especially when looking for *de novo* variants. Any reads that are classified as human can be assembled into a contig. This contig may be aligned again to the reference sequence, as this occasionally reveals a previously undetected structural variant, or it can be set aside for further analysis.

De novo assembly

There are several issues with assembling a genome through alignment to a reference. The reference genome will contain gaps, due to difficulties in assembling complex regions such

as repeat structures. In addition, homology between repeats means that unambiguous alignment will not always be possible. Routine sequence analysis will not necessarily identify on which allele a specific variant is located (Snyder, et al., 2015). Whether variants are in *cis* (on the same allele) or in *trans* (on different alleles on the two chromosomes) can have important clinical consequences. Two deleterious variants in a gene associated with a recessive condition may have no effect if they are in *cis*, but are likely to be disease-causing if in *trans* (“compound heterozygosity”).

Most studies attempting to associate copy number variations of a specific locus use the sum of the different alleles, rather than measuring each allele separately (White, 2015). Although different haplotypes representing the range of copy numbers have been generated for several loci e.g. the amylase locus (Carpenter, et al., 2015), this information is not routinely available in typically used reference genomes. For WGS data, one option to obviate many of these problems is to perform a *de novo* assembly (Chaisson, et al., 2015b). Although this is more complex and computationally intensive, it will produce a more complete genome. If short read technology is used, a combination of paired end and mate pair libraries of different sizes is costly, but advantageous, and it will still not be sufficient to completely assemble a genome. A study used the PacBio system to sequence previously uncharacterised regions of the human genome (Chaisson, et al., 2015a). Mapping long reads to the ends of gaps and assembling from these points allowed the generation of >1 Mb of previously unmapped sequence.

Independent of what sequencing technology will predominate in the future, it can be anticipated that *de novo* assembly will eventually be the standard approach for genome sequencing.

Variant reporting

Ultimately, whether a mapping or a *de novo* assembly approach is used, the sequence will be compared to a reference to detect and call variants. The HGVS recommendations for the description of sequence variants are widely accepted standards for how each type of variant should be reported. It should be noted however that the standard output from NGS is not HGVS, but typically a VCF file, a semi-standard. The problem is that in VCF the same variant can be reported in different ways. When in HGVS a one nucleotide deletion is reported as g.12345678del, in VCF it may appear as position:12345677 refGC sampleG, or position:12345678 refCT sampleT. In addition, NGS software calls deletions on the 5' site of repeated sequences while the HGVS recommends the 3' rule. Needless to say this causes errors when tools or users annotate these variants and perform database searches using previous reports, and they do not realize that one variant may be reported using different formats. Although tools are available to cope with this problem (e.g. Mutalyzer (Wildeman, et al., 2008) and the Description Extractor (Vis, et al., 2015) they are not yet frequently used. Even when such tools do not, as stated, give correct HGVS descriptions the errors made are consistent and the same description is generated each time.

Each variant may be described in more than one manner e.g. with reference to a genomic position, as well as within a reference sequence for a specific gene. Although it may be possible to predict the effect of a coding variant on the mRNA or protein, or a non-coding variant on mRNA splicing, it should be stated whether this is a prediction or if there is supporting experimental evidence.

Suggestions for what is required in a clinical WGS report have been published (McLaughlin, et al., 2014). Especially when the purpose of the sequence analysis is to provide a genetic diagnosis, it is important to specify which regions have not been covered sufficiently to report any sequence variants (Brownstein, et al., 2014). This should include (i) regions known to not be covered using the technology implemented for sequencing e.g. repetitive regions, extreme GC percentages and (ii) regions that did not achieve sufficient coverage to allow variants to be called.

It may be that the WGS is being performed for non-clinical reasons e.g. out of general interest or to identify “nice-to-know” variants. In such a case it is essential that there are clear guidelines regarding what types of genes/variants will be analysed, as well as what counselling (if any) will be provided when reporting the findings.

Variant analysis of WGS data.

Even if the entire genome is sequenced, it may be that only a subset of loci is screened for variants. Largely due to biases introduced during the enrichment step, WGS provides more

consistent coverage of the exonic sequences than WES for the same depth of sequence (Meynert, et al., 2014). This can be compensated for by increasing the WES sequence depth, but this increases the cost of the assay further.

A study by Gilissen et al. used WGS to screen 50 intellectual disability (ID) cases where no causative variant had previously been identified with microarray CNV analysis and whole exome sequence (WES) analysis (Gilissen, et al., 2014). A comparison of variants identified by WGS and/or WES (only at loci covered by the exome capture kit) yielded >10x more variants identified by WGS only, when compared to those identified by WES only. For both approaches the differences were primarily due to differences in sequence coverage, with WGS giving a more uniform coverage. The discordance between WES and WGS was also described in Belkadi et al. (Belkadi, et al., 2015), who also showed that the false positive rate was higher in WES compared to WGS. Interestingly, the accuracy of indel calling was similar for both, demonstrating the inherent difficulty in calling indel variants.

A report by Sun et al., (Sun, et al., 2015) focussing only on 500 genes previously implicated in ID, found that WES detected all variants identified by WGS in nine samples, and that 99% of the 500 genes were covered to a sufficient depth. These findings demonstrate that the effective efficiency of WES vs WGS depends on the purpose of the analysis. However, as sequencing becomes cheaper it will eventually be more cost-effective, from a laboratory perspective, for WGS to be the default option for genetic analysis, irrespective of how much of the genome will subsequently be analysed.

A large study reported by Taylor et al used WGS to analyse 156 individual cases (Taylor, et al., 2015). The proportion of cases for which a causative variant could be identified varied according to condition, with the highest success rate (8/14) observed for trios (index and both parents). For Mendelian disorders a disease-causing variant was identified in 23/68 cases, with an overall study result of 33/156.

Assigning non-coding variants to a specific disease faces several challenges. In some cases, large *de novo* deletions or duplications upstream or downstream of a given gene can be linked to a condition with high certainty, e.g. *SOX9* with disorders of sex development, campomelic dysplasia, and / or craniofacial disorders (Gordon, et al., 2009; Kleinjan and van Heyningen, 2005). In most cases, however, it is not always immediately clear which gene is affected by a given non-coding variant.

The study of (Taylor et al. 2015) was only able to link non-coding variants to a condition in two cases. In one, a SNV in the 5' UTR of the *EPO* gene, identified in two unrelated families, was the only rare exonic variant in an identical by descent, 8Mb interval. The role of *EPO* in red blood cell development makes it a compelling candidate for the erythrocytosis seen in these families.

The other was a complex rearrangement near the *SOX3* gene, consisting of a deletion combined with an insertion of part of chromosome 2, identified in a case with X-linked hypoparathyroidism. *SOX3* is known to be involved in parathyroid development, providing a link between the affected locus and the condition. This example also highlights a strength of WGS, as the insertion would not have been detected with exome or microarray analysis.

Several consortia, such as the Encyclopedia of DNA Elements (ENCODE) project (Consortium, 2012), and Roadmap Epigenomics Mapping Consortium (Roadmap Epigenomics, et al., 2015), have generated genomewide data sets from multiple cell types that allow the identification of genomic regions that have regulatory potential. Even with this information, it is still challenging to predict the effect of a SNV. Different approaches have been described for using these data to predict functional effects (Boyle, et al., 2012; Lee, et al., 2015), however in the vast majority of cases it is necessary to combine these predictions with other biological information to make a link with disease.

Conclusions

Although routine WGS, as a laboratory activity, is rapidly becoming feasible from a financial viewpoint, there is currently no single sequencing technology able to generate complete, fully haplotype resolved, human genomes. The most accurate genome assemblies to date have used a combination of short and long range sequencers, along with other genomic assembly technologies such as optical mapping (Hastie, et al., 2013). Initiatives such as Genome In A Bottle are assembling high quality, haplotype aware, diploid reference genomes. These genomes can serve as superior references for mapping studies, and the approaches used can be applied more broadly for further genome-based studies. Ultimately, however, it will require the development of a technology that can sequence single DNA molecules, hundreds of kilobases in length, before complete genomes can routinely be generated, as well as considerable efforts to achieve the best benefit for patients regarding

their informed choices and relevant medical service. As a common endeavour, taking the time to share data, clinical findings and experiences with the implementation and use of different policies for these issues, whenever it is possible and appropriate to do so, is necessary. Cooperation between both doctor- patient and between research teams will be the key to achieving the successful use of this powerful tool, which offers many more challenges than the technical ones we have outlined here.

Acknowledgements

We thank all the people that contributed (Speakers/Participants) in the many 3Gb-TEST organised meetings/events listed at the consortium website: 3Gb-TEST.eu/meetings/.

Disclosure of Conflicts of Interest

Stefan Van Vooren is employed by Agilent Technologies. Bart Janssen is employed by GenomeScan B.V.

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Table 1. Critical Differences between WES and WGS

	WES	WGS
Sample source	Enrichment step likely to reduce impact of contaminating DNA	All DNA in sample can be sequenced, appropriate care should be taken
Library prep	Enrichment step will introduce biases	No enrichment leads to more even coverage
Platform	Short reads sufficient	Longer reads preferable
Variant detection	Amplification/enrichment step will lead to unequal genomic representation; CNV calling relies on read depth	More even genomic coverage; structural variation analysis may allow breakpoint detection
Sequence alignment	Compare to reference genome	Can perform de novo assembly; allows for more accurate genome construction
	Focus on reads aligning to exome	Whole genome analysis, increased complexity due to repeats etc

Variant reporting	Focus on coding/splice site variation	More complex variation, genome-wide
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Table 2. A summary of points to consider when performing whole genome sequencing

Aspect	Points
DNA Sample	<p data-bbox="491 427 584 461"><i>Source</i></p> <ul data-bbox="544 533 1485 1272" style="list-style-type: none"> <li data-bbox="544 533 1485 645">• Blood - circulating DNA from e.g. tumour or fetus (pregnant female) can be isolated along with cellular DNA <li data-bbox="544 689 1394 723">• Buccal swab - can be contaminated with non-human material <li data-bbox="544 768 1310 801">• Cell line - Genetic variation introduced during culturing <li data-bbox="544 846 1485 1037">• Mosaicism - lymphocytes undergo rearrangements; neurons affected by LINE1 retrotranspositions; new variants will be introduced with each cell division due to replication errors <li data-bbox="544 1081 1485 1272">• Cell cycle - Late replicating DNA will be under-represented compared to early replicating DNA, can influence copy number analysis <p data-bbox="491 1317 608 1350"><i>Isolation</i></p> <ul data-bbox="544 1422 1485 1771" style="list-style-type: none"> <li data-bbox="544 1422 1485 1534">• DNA quality / integrity - Isolation with e.g. phenol can influence downstream enzymatic reactions <li data-bbox="544 1579 1485 1771">• Severely fragmented DNA is unsuitable for long read sequencing; sites of degradation will be non-random, significantly affecting coverage <p data-bbox="491 1816 754 1850"><i>Sample preparation</i></p>

	<ul style="list-style-type: none">• Enzymatic or mechanical fragmentation will be non-random and introduce different biases• To reduce biases, especially GC% extremes, PCR amplification during sample preparation should be minimized (ideally avoided)
Sequencing	<p><i>Type of sequencer</i></p> <ul style="list-style-type: none">• Short read sequencers generate many reads, but the read length can make it difficult to unambiguously align reads, especially in repetitive sequences• Long read sequencers e.g. PacBio, generate relatively fewer reads, but the longer reads are easier to align and assemble
Alignment/Assembly	<p><i>Quality control</i></p> <ul style="list-style-type: none">• Important to check for duplicate reads, coverage vs. GC%, completeness of coverage etc. <p><i>Alignment</i></p> <ul style="list-style-type: none">• Choice of aligner, as well as specific conditions chosen during alignment, will impact final results• Reference genome (build, presence / absence of different haplotypes) will affect the completeness and accuracy of alignment.• Some loci cannot be unambiguously mapped, especially with short read sequencing

	<ul style="list-style-type: none">• Unmapped reads can be due to genetic variation incompatible with standard genomic alignment, e.g. certain types of structural variation• Aligning to a reference will never lead to a complete genome <p><i>De novo assembly</i></p> <ul style="list-style-type: none">• Is more accurate, but also more time consuming and complex• With current sequencing technology it is not possible to completely assemble a human genome
Variant Detection/Reporting	<p><i>Variant calling</i></p> <ul style="list-style-type: none">• No single algorithm can reliably detect all variant types, e.g. multiallelic copy number variation; specific types require a focused analysis approach• Sequencing platform used and sample preparation method will determine which types of variation can/can not be reliably called <p><i>Variant reporting</i></p> <ul style="list-style-type: none">• Variants should be reported using standardised nomenclature• Unless experimentally confirmed, it should be clear that changes at RNA or protein level are predictions only• VCF files can report the same variant in different ways, and care

	should be taken when comparing files from different sources and/or when querying databases
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