Guidelines for diagnostic next generation sequencing

17 October 2014

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This is the final draft version of a document on the diagnostic use of NGS that we wish to publish on behalf of EuroGentest.

The first version of this document was drafted by a small number of people. It was subjected to peer review by the participants to the Nijmegen meeting, November 21-22, 2013.
The document is ready for circulation and public consultation. Hence, it will be posted on the EuroGentest website for a few weeks. The procedure is in line with the process that other policy documents, generated by the European Society of Human Genetics, have to follow: the background document is posted and an invitation to comment is sent to the membership of the Society. Thereafter, a final version of the guidelines will be published in the European Journal for Human Genetics.

Of course, guidelines in a fast moving field can never be definitive, hence a system will be put in place to update them on a regular basis.

I wish to thank all the colleagues who have contributed to the development of the guidelines and the generation of the document. The members of the working group will be co-authors on the paper, the contribution of the other participants to the Nijmegen meeting will be acknowledged.

We believe that the document is timely, even though we have been slow in finalizing the editorial work. By posting it now, everybody who is interested in the guidelines or eagerly seeking advice will be able to consult the workgroup's viewpoints and recommendations.

Thanks for your interest! We hope that the guidelines will be of use, and that our work will contribute to the development of standard in the field of NGS.

Gert Matthijs
On behalf of the editorial group.

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Chapter 1: General introduction

1.1 Introduction

Next-generation sequencing (NGS) allows for the fast generation of thousands to millions of base pairs of DNA sequence of an individual patient. The relatively fast emergence and the great success of these technologies in research, hail a new era in genetic diagnostics. However, the new technologies bring challenges, both at the technical level and in terms of data management, as well as for the interpretation of the results. We believe that all these aspects warrant a consideration of what the precise role of NGS in diagnostics will be, today and tomorrow, before to even sets sail and acquire the machines and the skills. This is circular of course, as only the practice will tell us how well the tool performs.

Has NGS come of age? It is true that, technically, the available platforms aren't stable yet, in a sense that the technology and applications change constantly and rapidly. However, this should not prevent the implementation of NGS technology in diagnostics, since NGS offers a potential overall benefit for the patient. Thus, one can simply not wait or postpone the clinical use of NGS until the flawless massive parallel sequencing platform and the infallible test are available.

One thing that should prevent people from prematurely and untimely offering NGS diagnostics is bad quality. Insufficiently validated test do present a treat to patients, and their use in a clinical diagnostic setting is unacceptable.

Literature on the validation of diagnostic tests is available, and many genetic laboratories have gone through the phase of accreditation in genetic testing already (Berwouts et al. 2012). Thus, labs that have experience in evaluating and validating molecular tests should not be afraid of gearing up towards NGS. However, it is not possible to simply translate the rules for the validation of the classical laboratory tests to rules for NGS. Take the famous 'rule of 3', introduced to laboratory geneticists by Mattocks et al. (2010) for mutation scanning: to reliably cite a 99% sensitivity with a confidence of 95% one should have less than a single failure on 300 reference samples. Obviously, it is impossible to run 300 test samples or an equal number of runs prior to implementing a diagnostic NGS test. It would kill virtually all labs, while the clinical benefit of pushing the standards to such a scale would be small.

Hence, quality criteria have to be reinterpreted in view of this novel technology. We present a view on validation in this document from this perspective. It is an invitation for all experts involved in diagnostics and in quality assurance to jointly draw workable solutions. Practical solutions would be for the labs to collaboratively validate the platforms, pipelines and methods. Alternatively, the validation could be offered by independent organizations; however, it is unlikely for the latter to occur timely.

Nevertheless, there will always be costs associated with a thorough and acceptable validation, since validation is a requisite of the ISO norm 15189 for the accreditation of medical laboratories. The labs should not underestimate the efforts, neither should they try to pass under the bar or bend the rules. As a consequence of the costs, we anticipate that not all laboratories will be

offering the full scope, eventually. One way to prepare a service of laboratory for survival, is by thoughtful selection of the appropriate tests, and of the prime parameters that have to be considered for quality assurance. In parallel, the healthcare system should be made aware of the technical challenges, and be asked to adapt the reimbursement level of NGS tests accordingly.

STATEMENT 1.01: NGS should not be transferred to clinical practice without an acceptable validation of the tests according to the emerging guidelines .

If the NGS laboratory process is being outsourced, it is essential that the same quality criteria are achieved as for in-house sequencing. We recommend that the use be made of providers accredited by a recognized quality control body, and that a well-defined service agreement is drawn up to guarantee performance according to diagnostic accreditation standards (ISO 15189).

The guidelines presented here, basically deal with NGS testing in the context of rare and mostly monogenic diseases. The basics are also applicable to somatic testing in a context of cancer evaluation. However, the latter would involve additional quality parameters, like the threshold of variant detection, a feature which is generally not dealt with in the case of germ line variants. These parameters are not covered in the present document.

Similarly, the guidelines mainly focus on the targeted analysis of gene panels, either through specific capture assays, or by extracting data from exomes. Arguments in favor of such an approach have recently been comprehensively presented in literature, and will not be repeated here (Rehm 2013). In principle, whole genome sequencing (WGS) may - and shortly will -also be used to extract similar information. In that case, the guidelines would still apply but because WGS would also allow detecting other molecular features of disease, they would have to be extended accordingly. These extensions have not been addressed in this work.
The use of NGS for the determination of risk factors for multifactorial disease is currently not a clinically accepted practice. Hence, in these guidelines, we have not considered any features that may specifically apply to offering services for such risk factors.

STATEMENT 1.02: The laboratory has to make clear whether the test that is being offered may be used to exclude a diagnosis, or to confirm a diagnosis.

The distinction is significant, and warrants different settings and a different view on diagnostics. Similarly, if a laboratory offers somatic testing using NGS, the limits of the methods should be clearly indicated.

1.2 The generation of guidelines for diagnostic use

1.2.1 Scope

The massive parallel sequencing platforms are being used for different applications. We tend to distinguish the following NGS assays for diagnostics.

- Mutation scanning (for individual or small sets of genes). A typical example is the use of NGS platforms for amplicon based re-sequencing of the *BRCA1* and *BRCA2* genes, which has been described in several publications. Because this boils down to mutation scanning

in 2 genes that have been extensively characterized previously, and for which testing usually encompasses Sanger sequencing of the coding region (and flanking intronic sequences) plus deletion/duplication analysis, the NGS test should have at least the same sensitivity and specificity as the current diagnostic offer. The validation would largely occur as described by Mattocks et al. (2010), with several, additional features, to be taken from the specific instructions for quality assurance of NGS sequencing, as described in Chapter 4. Reporting would basically not be different from earlier reporting on *BRCA1* and *BRCA2* screening.

- Mutation screening by targeted capture or amplicon sequencing, for known genes. This is an extension of the previous, but with clearly novel features in terms of test design, comprehensiveness, limitations, sensitivity, specificity and possible adverse effects. The approach has been described in detail by Rehm in 2013. The present guidelines largely deal with this application.
- Exome sequencing shall actually be divided into 2 different applications. One is about targeted analysis for known genes, and the instructions are similar to the ones given for targeted mutation screening, except that aspects of unsolicited findings, and thus of informed consent, are to be dealt with more extensively. The other application is the use of the exome for the identification of novel genetic defects. In our view, this largely remains in the realm of research, especially if the genes in which mutations are identified, have not been previously associated with the particular disease; i.e. it is difficult to offer such a thorough analysis in diagnostics. An exception to that view is the use of exome sequencing in trios (patient and parents) for the identification of *de novo* defects.
- The so-called 'mendeliomes' combine the technical features of targeted assays with the side-effects of exomes, *in casu* the occurrence of secondary findings.
- Whole genome sequencing (WGS) will certainly come of age very soon. Laboratories that plan to offer WGS in a diagnostic context will have to deal with additional aspects, beyond the ones presented in the current guidelines. Still, the basics of NGS diagnostics will apply, including minimal technical achievements, diagnostic utility and informed consent issues.

There are technical limitations to the different platforms, like e.g. the accuracy with which the sequence is read, and subsequently assembled (Buermans and den Dunnen, 2014). Because the guidelines are meant to be generic, no attempt has been made to generate comprehensive lists of all possible platforms and their specific parameters.
There are also conceptual limitations to the different assays, like e.g. the fact that trinucleotide repeats cannot be detected by short read sequencing and mapping. It is difficult to provide an exhaustive list of these features; the laboratory geneticist shall have the necessary knowledge to identify them, and the laboratory shall consider them in the development of a diagnostic routing. It is important to guide the user of the test - i.e. the clinician who orders the analysis – of its limitations in view of the diagnostic request.

1.2.2 Methods

The different aspects of NGS and diagnostics were discussed during 3 workshops. The first took place in Leuven, February 25-26, 2013. The preliminary views were presented during the EuroGentest Scientific Meeting in Prague, March 7-8, 2013.
The second was an editorial workshop in Leuven, October 1-2, 2013, where the different people involved in writing the document, came together to discuss the layout of the document and prepare the first draft.
The first draft was finalized prior to the third meeting, in Nijmegen November 21-22, 2013. To the latter meeting, a larger group of stakeholders was invited. They were invited to comment on the draft, and on the statements presented therein. The comments were included in a new version, which was circulated among the editorial group, prior to publication on the EuroGentest website.

Right from the start, the aim was the write a document that would build on existing guidelines. At the beginning, several documents were available, while some appeared in the course of the procedure. The guidelines that were taken into consideration are listed below. Whenever information was taken from there or from the background therein, some specific reference has been given in the present document. The reader has to be aware that, indeed, the present guidelines try and compile what has been written before. Nevertheless, an attempt was made in each chapter to attribute – and acknowledge – the main features to the other guidelines. Whenever the current guidelines diverge from the view presented elsewhere, this is explicitly stated. Whatever is new to the current guidelines is emphasized as well.

The paper will be published eventually. The authors will be listed as follows:
Gert Matthijs, Erika Souche, Marielle Alders, Anniek Corveleyn, Sebastian Eck, Ilse Feenstra, Valérie Race, Erik Sistermans, Marc Sturm, Marjan Weiss, Helger Yntema, Egbert Bakker, Hans Scheffer and Peter Bauer.

List of other guidelines

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Other guidelines or related documents that have appeared towards the end of the consultation period:

- Aziz N, Zhao Q, Bry L, Driscoll DK, Funke B, Gibson JS, Grody WW, Hegde MR, Hoeltge GA, Leonard DG, Merker JD, Nagarajan R, Palicki LA, Robetorye RS, Schrijver I, Weck KE, Voelkerding KV. College of American Pathologists' Laboratory Standards for Next-Generation Sequencing Clinical Tests Arch Pathol Lab Med. (2014, in press) PMID: 25152313 (see http://www.archivesofpathology.org/doi/pdf/10.5858/arpa.2014-0250-CP, last accessed 9-9-2014)
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Kural D, Cariaso M, Lennon GG, Javed A, Agrawal S, Ng PC, Sandhu KS, Krishna S, Veeramachaneni V, Isakov O, Halperin E, Friedman E, Shomron N, Glusman G, Roach JC, Caballero J, Cox HC, Mauldin D, Ament SA, Rowen L, Richards DR, San Lucas FA, Gonzalez-Garay ML, Caskey CT, Bai Y, Huang Y, Fang F, Zhang Y, Wang Z, Barrera J, Garcia-Lobo JM, González-Lamuño D, Llorca J, Rodriguez MC, Varela I, Reese MG, De La Vega FM, Kiruluta E, Cargill M, Hart RK, Sorenson JM, Lyon GJ, Stevenson DA, Bray BE, Moore BM, Eilbeck K, Yandell M, Zhao H, Hou L, Chen X, Yan X, Chen M, Li C, Yang C, Gunel M, Li P, Kong Y, Alexander AC, Albertyn ZI, Boycott KM, Bulman DE, Gordon PM, Innes AM, Knoppers BM, Majewski J, Marshall CR, Parboosingh JS, Sawyer SL, Samuels ME, Schwartzentruber J, Kohane IS, Margulies DM. An international effort towards developing standards for best practices in analysis, interpretation and reporting of clinical genome sequencing results in the CLARITY Challenge. Genome Biol. 2014;15(3):R53. PMID: 24667040

1.2.3 Limitations

These guidelines do not deal with the evaluation of the pros and cons of disease targeted diagnostics by targeted captures assays versus exome sequencing. Nevertheless, it is the responsibility of the diagnostic laboratory to make such an evaluation, and list the arguments in a detailed validation plan, prior to implementing either one or the other.
By no means, these guidelines are comprehensive. The field of application is too broad to deal with all possible details.
Also, the current guidelines are the results of discussions in a relatively small group of experts. The group did not include representatives of all possible stakeholders. That would not allow one to move forward easily. Still, they are based on the knowledge and common sense of a group of people, involved in genetic diagnostics, who are keen to improve and harmonize the quality of NGS testing.
Nevertheless, the ambition is that these guidelines be adopted by the national accreditation bodies to complement the ISO 15189 norm, and thus facilitate both the installation – by the laboratory directors and co-workers – and the evaluation – by the experts and auditors from the accreditation bodies – of NGS diagnostic services.
As mentioned before, the guidelines do not address somatic testing. Interested colleagues are invited to extend the current guidelines in collaboration with practitioners in the field.

1.2.4 Contribution of EuroGentest

EuroGentest is a network, supported by the European Commission (FP7), to harmonize the process of genetic testing, from sampling to counseling, across Europe. The ultimate goal is to ensure that all aspects of genetic testing are of high quality thereby providing accurate and reliable results for the benefit of the patients (www.eurogentest.org).
The workshops were organized and sponsored by EuroGentest. None of the participants were paid for their work.

1.3 Highlights of the document

The contributors to the current guidelines acknowledge the work and ideas presented by other in the different guidelines on NGS that have appeared so far.
In the current document, the important issues are copied and discussed. Also, a few new insights have emerged during the preparations of the guidelines.

First, we believe that defining the 'diagnostic utility' of the NGS test is the laboratory's first duty, when preparing to offer diagnostic tests using NGS. This is not new to NGS, but the availability of a novel technology per se is not a sufficient argument to implement it.

Second, we hope that the proposal to rate the different NGS assays as type A, B or C depending on their quality and comprehensiveness, will be widely accepted, both by patients and clinicians, and by the health care system. This is the most important novel feature of this document.

Third, the quality parameters have to be standardized, and we propose the use of 3 specific percentages to report on the 'reportable range', which will allow comparing individual results.

Fourth, the laboratory has to adopt a policy for dealing with the additional features that are intrinsic to NGS testing, like secondary and unsolicited findings or the reporting of carrier status for recessive or X-linked diseases. It is beyond the scope of this document – and beyond the responsibilities of the individual laboratory to develop an institutional or national or even international viewpoint on these features. However, the laboratory has to consider these issues and publicize the policy that it adopts, and it has to adopt a policy before putting NGS into practice.

The same is true for issues like informed consent. In the document, they are addressed from the laboratories' standpoint. It is beyond the tasks of the laboratory directors to define what the content and the use of an informed consent should be. Hence, in this document it is put forward that it is not the laboratory's responsibility either to provide and collect it.

Fifth, we reiterate that the distinction between research and diagnostics has to be respected at all times, even if thanks to these novel technologies, the borders between them are blurred. We try to define ways in dealing with the transfer of research results to the medical records of the patients, and with the responsibilities of the diagnostic laboratory to husband tests results over time.

Finally, guidelines can never be permanent in a rapidly evolving field. Still, the essential aspects of quality and good laboratory and clinical practice, shall never change.

Contributions

Gert Matthijs

Chapter 2: Diagnostic/clinical utility

2.1 Introduction

Next-generation sequencing (NGS) is a valuable tool for diagnostic purposes. The benefit of implementing NGS in routine diagnostics is the introduction of testing many genes at once in a relatively short time and at relatively low costs, and thereby yielding more molecular diagnoses. This can be achieved by exome or genome sequencing or by targeted analysis of a selected set of genes.

When a targeted gene analysis approach is chosen, the selection of genes included in the panel should be done with care. The gene panel should only include genes that are known to be associated with the disease of interest.

This can either be done by not including genes not associated with the disease during enrichment, or by filtering them out during analysis using bioinformatics tools. The sensitivity of the diagnostic assay will depend on the quality of the assay. To allow the health care professionals and governing organizations to compare the diagnostic offer in the different laboratories, we propose to introduce a rating scheme for diagnostic NGS assays. We hope that this rating scheme will be further elaborated by different specialist committees and promoted at the national and international level.

2.2 Viewpoints and examples

2.2.1 Limitations of NGS and diagnostic yield

The limitations of NGS are dependent on the platform and on the enrichment methods (if any). PCR based enrichment is sensitive to allelic dropout caused by SNPs at the primer annealing site, but is less expensive and laborious, and can more easily be applied to small numbers of patients than capture based methods. The latter is probably less sensitive for allelic dropout but has problems with high GC content areas. It also allows the simultaneous enrichment of multiple patients in one reaction. Whole genome sequencing is least biased by allele dropout, but requires high sequencing capacity in return for a lower coverage, etc.

The platform that is chosen for sequencing will influence the sensitivity and error rate. For instance, pyrosequencing and pH based techniques have a problem detecting mutations in homopolymers. However, they can detect larger deletions or insertions than other platforms due to the longer read length that can be achieved. These and other factors will influence the choice of enrichment method and sequencing platform and determine which additional tests will be necessary to deliver high quality diagnostics. These are only a few examples of limitations and flaws of the different NGS approaches. We do not aim to provide an exhaustive list, nor do we want to discuss the pros and cons of the individual platforms.

STATEMENT 2.01: The aim and utility of the test or assay should be discussed at the beginning of the validation and a summary should be included in the validation report.

In general, the technical features of NGS are evolving rapidly, and it is expected that the limitations, in terms of detecting mutations, as compared to the current approaches, will disappear in the near future. Moreover, efforts are made to use NGS data for the detection of CNVs and exonic insertions and deletions. However, at each new step, a thorough validation is necessary. Since the laboratories acquire commercial sequencing platforms, they are generally

bound to use the performance criteria of the systems. On the other hand, the landscape is different and very variable as far as the software for sequence analysis and interpretation is concerned. Hence, the diagnostic laboratories will have to spend most of their efforts in optimizing and fixing the bio-informatics pipeline (see Chapter 4).

STATEMENT 2.02: When a laboratory is considering introducing NGS in diagnostics, it first has to consider the diagnostic yield.

This 'diagnostic yield' is defined as the chance that a disease causing variant is identified and molecular diagnosis can be made, calculated per patient cohort (Weiss, Van der Zwaag et al. 2012). It establishes the performance of NGS primarily from a clinical point of view. It is often not easy to determine the diagnostic yield, because it may be difficult to define the patient cohort for a given clinical entity or diagnostic request. Still, one could use literature, and compare to existing techniques (Neveling et al. 2013).
It is actually in view of the 'diagnostic yield' that the 'core gene list' and 'diagnostic routing' have to be developed (see below). Note that the diagnostic yield is not a lab quality parameter; for this we use sensitivity and specificity.

The diagnostic yield may be a good indicator to measure the efficiency of the test beyond its analytical aspects: it can also be used as a managerial tool, at the level of the laboratory or by the healthcare care system. For a disorder that is, in almost all cases, caused by a mutation in a single gene, testing a set of 10 or more genes with low, individual mutation detection rates is not beneficial from a clinical or healthcare point of view. For example, CFTR is the only gene known to cause cystic fibrosis (CF) and mutations in this gene are detected in over 98% of patients, even though mutations in handful of other genes are known to cause a CF like phenotype. Testing all patients with the clinical diagnosis of CF for a large number of genes using NGS will not necessarily yield a higher mutation detection rate (diagnostic yield) in patients, at least not at comparable costs. Testing other genes may be considered after mutations in CFTR have been excluded.
In contrast, for genetically and clinically heterogeneous diseases, where many different genes are known to be involved without a major contribution by a single gene, NGS analysis of large gene panels will substantially increase the diagnostic yield. For example, the number of genes known to cause cardiomyopathies has increased spectacularly over the past years. To date, over 50 genes are recognized as causal for either or both dilated cardiomyopathies (DCM) and hypertrophic cardiomyopathies (HCM). Sequencing all those genes in one test does increase the detection rate at considerably lower costs (Mook et al. 2013).
The decision whether or not to use an NGS approach should not only be based on the expected diagnostic yield and the benefit for the patient population, but also on financial grounds. It may thus depend on the number of patients being analyzed. Sequencing six genes using Sanger sequencing may be the method of choice when the test is only requested for few patients per year. Analyzing larger numbers of patients will favor the choice for NGS. In this light, it has been shown that NGS scanning of *BRCA1* and *BRCA2* will be profitable in most laboratories, eventually (Feliubadaló et al 2013). It is expected that in the near future this scale will tip more often towards NGS, as new technologies are emerging fast.

2.2.2 Core disease gene list

The first thing to do when developing a gene panel is to define the conditions for including a gene into a panel. Ideally, this is an issue that should be dealt with at the community level, in a multidisciplinary way. Several attempts to address the question of the core gene list are underway, for instance in the area of familial breast cancer testing. The aim is to compile the list of genes that constitute the diagnostic offer, minimally. There is an aspect of good medical practice linked to the development of these 'core disease gene lists'.
Genes with a lower contribution to the disease can be added, optionally.

STATEMENT 2.03: For diagnostic purpose, only genes with a known (i.e. published and confirmed) relationship between the aberrant genotype and the pathology, should be included in the analysis.

The second issue is to set the standards for coverage and sensitivity. In order to deliver high quality diagnostic NGS, it should be determined for which genes the analytical sensitivity should at least equal Sanger sensitivity. There is a strong opinion that for genes that are responsible for a significant proportion of the defects, the sensitivity should not be compromised by the transition from Sanger to NGS. Adding additional gene will of course increase the diagnostic yield, but this should not be at the expense of missing mutations that would previously have been detected.
As a result, it is a requisite to complete areas of low coverage in NGS for these genes by additional Sanger sequencing or by another approach (e.g. by combining amplicon based NGS with capture assays). However, a more pragmatic approach would also be acceptable. If the incremental detection rate of filling the gap would be virtually zero, the clinical relevance would be zero while the costs of testing would be increased.
For instance, if mutations have never been identified in a particular exon in hundreds to thousands of cases that were Sanger sequenced, and if this particular exon is badly covered by NGS, it would not make practical sense to add Sanger sequencing to fill the gap. In such a situation, it would suffice to provide evidence from literature or from a lab's own experience to argue that additional testing would be meaningless.
The incremental detection rate is thus the key determining factor in defining the core gene list and in dealing with the gaps. Hence, (inter)national efforts are necessary to determine what the incremental advantage would be of adding genes (and gene fragments) to the list. One might consider defining a core 1 and a core 2 list: core 1 meaning filing up with Sanger, while for core 2 the NGS coverage would suffice. The distinction will be an important factor in applying the scoring or rating system that will be presented in section 2.2.4.

In summary, the ideas about a core gene list are the following:
- the list must result in a 'substantial contribution' to the quality of life of a patient, and hence the genes must be chosen with care;
- a two-tier system would be acceptable, whereby some genes are scrutinized more in detail (in other words: with a more complete coverage) than others;
- the list must not inflict with the efficiency of a service, i.e. overzealous testing is not helpful;
- the use of core gene panels must lead to better diagnosis of the group of disorders, if not it lacks clinical utility.

STATEMENT 2.04: For the sake of comparison, to avoid irresponsible testing, for the benefit of the patients, 'core disease gene lists' should be established by the clinical and laboratory experts.

Consensus between labs about the core set promotes uniformity in testing between different laboratories. The statement also relates to the requirement of ISO15189 that the tests, which are being offered, have to be clinically relevant.

2.2.3 NGS versus other techniques: diagnostic routing

Some diagnostic tests warrant additional testing by other techniques than NGS (or Sanger) sequencing. Although NGS has the potential to detect CNVs, to date this is preferentially done by MLPA analysis (or other methodologies that reliably dose alleles). More importantly, repeat expansions including trinucleotide repeat expansions are not detectable with the NGS platforms, and the same would be true for deep intronic mutations or genomic rearrangements (like inversions), unless specific probes to detect the latter would be included in the NGS approach or WGS would be performed. Depending on the genes involved, a diagnostic test may consist of NGS sequencing plus additional testing. The comprehensive description of the diagnostic approach that is to be offered by the diagnostic laboratory for a specific disease or set of diseases is defined as the 'diagnostic routing' (Weiss, Van der Zwaag et al. 2013).
For instance, a test strategy may start by Sanger sequencing a single gene with high mutation rate, only to proceed with NGS panel if no mutation is found (Weiss, Van der Zwaag et al. 2013). This can be the choice in disease such as Marfan syndrome with one major gene (*FBN1*), and many minor genes. The rationale is well described in Weiss, Van der Zwaag et al. (2013). It is recommended that the laboratory procedures, including the genes tested, are recorded in a publicly available document describing this complete 'diagnostic routing'.

We provide a number of examples, with increasing complexity below. Note that this diagnostic routing may include different techniques like CGH array, MLPA, Sanger sequencing and NGS. Also note that these are just examples that depend on the current state of technology, and that they can vary and evolve depending on laboratory equipment and technological progress.

1. Clinical subgroups with a few genes with a high mutation detection rate

- Breast cancer. For this entity, the sensitivity of *BRCA1* and *BRCA2* testing should not be reduced as compared to Sanger plus MLPA/QFPCR. It is therefore necessary and cost efficient to analyze the *BRCA1* en *BRCA2* genes first (by NGS and/or Sanger sequencing plus deletion/duplication analysis). If negative it may be complemented by a more comprehensive gene testing by NGS, but the sensitivity of the available tests should not be compromised. If laboratories proceed to comprehensive testing, the detection rate for the original genes should not be compromised, to be in line with the requirements defined in section 2.2.2.

2. Strongly heterogeneous disorders

- Connective tissue disease. There are four (overlapping) clinical phenotypes described within the connective tissue diseases: (1) aortic or arterial aneurysm/dissection (such as Marfan syndrome, Ehlers–Danlos syndrome type IV, Loeys–Dietz syndrome, thoracic aneurysm and dissections); (2) Ehlers–Danlos syndrome; (3) osteogenesis imperfecta; and (4) lens luxation and/or Weill–Marchesani syndrome. For each of these clinical phenotypes, a different routing of genetic tests exists. In this routing the order of the different techniques and the genes which are

in the core lists are indicated. This diagnostic routing is described more extensively by Weiss, Van der Zwaag et al. (2013).

- Intellectual disability. Test Fragile X (trinucleotide repeat) and CGH array first (even though this may be obsolete soon, if NGS allows for the simultaneous evaluation of CNVs). After this, exome sequencing is probably the most cost effective choice (even this may eventually be replaced by whole genome sequencing, see e.g. Gilissen et al. 2014). It is advised to analyze the core list first, even if for this clinical entity this core list may contain more than 500 genes. If no (probably) pathogenic mutation is detected, the next step is filtering the exome data according to the suspected mode of inheritance (trio analysis -> de novo/recessive consanguineous/recessive not-consanguineous; more affected sibs -> recessive consanguineous/recessive not-consanguineous/dominant with mosaic parent). If no (probably) pathogenic mutation is detected, the further step may be to investigate the whole exome, whereby this last step would rather be performed in a research setting (see Chapter 6). For the 2nd and 3rd steps in the analysis informed consent may be necessary (see Chapter 3).
- Cardiomyopathy. To date over 50 genes are known or suggested to be involved in the etiology of cardiomyopathy. For most genes the evidence is solid and these genes should be included in the core gene list. If this list becomes too long there will be a trade-off between core list and diagnostic yield. Not completing by Sanger may still result in a higher diagnostic yield, but some mutations in the core genes will be missed that would have been found by Sanger sequencing. The discussion should be referred to expert groups. For some genes the evidence is still weak. Inclusion of those genes is optional and different quality parameters may apply for the analysis of this set. Similar considerations apply for deletion and duplication testing: MLPA for LMNA is included in the diagnostic routing (only) when the phenotype of the patients is suggestive of a LMNA defect (DCM and conduction defect).

3. Disorder with frequent deletions or duplications

- When deletions or duplications are a frequent cause of the disorder, these molecular defects should be excluded before continuing with NGS panels. Examples are hereditary spastic paraplegia, where deletions in *SPAST* are detected in 20% of the patients with the most common dominant form (SPG4), and Charcot Marie Tooth disease, where the *PMP22* (aka 17p11) duplication accounts for the majority of CMT1A cases.

4. Imprinting disorders

- Imprinting defects are not detectable with the (currently used) NGS approaches, and such disorders should thus not feature on the list of diseases tested by NGS.

2.2.4 A new rating scheme for diagnostic NGS

Laboratories will apply different (technical and diagnostic) standards for NGS tests, irrespective of guidelines. Indeed, there are too many variables still that cannot be fixed through prescriptive guidelines. Therefore, we propose a simple rating system for NGS diagnostics that will warrant fair scoring and easy comparison between what different labs are offering.

1. Type A test

This is the most complete analysis, as far as NGS is concerned. The lab warrants > 99% reliable reference or variant calls of the coding region and flanking intronic sequences, and fills all the gaps with Sanger sequencing (or another complementary sequencing analysis) and, depending on the platform used, performs extra analysis of e.g. the homopolymer stretches. This is the

highest level of exactitude a lab could offer for NGS at the current stage. In a type A test, all genes of the panel are comprehensively covered.

2. Type B test

The lab describes exactly which regions are sequenced at > 99 % reliable reference or variant calls, and fills some of the gaps with Sanger (or other) sequencing. This would be a respectable assay for confirming a diagnosis, but not for excluding it. In a type B test, the core genes would be comprehensively covered, in the way that was discussed earlier in section 2.2.2.

3. Type C test

The type C test solely relies on the quality of NGS sequencing, while no additional Sanger (or other) sequencing is offered. This would be the case, for instance, if gene panels are selected from exome sequencing, without any additional sequencing to complete the analysis. Therefore, the results of a type C test would often not fulfil the criteria for a core gene list. The lab would still be bound to specify what the reportable range would be, according to the instructions given in Chapter 4.

Adding MLPA and independent assays for repeat expansions may further increase the sensitivity of the test, but this aspect belongs to the 'diagnostic routing' rather than to the scoring system, presented here. The scoring system solely applies to the sequencing – by means of NGS or Sanger – of the region of interest, otherwise the scoring system would become too complicated or would require further (sub)classification. Admittedly, the scoring system will have to be updated when deletion and duplication analysis will be intrinsically covered by NGS, but the principles would remain the same.

STATEMENT 2.05: A simple rating system on the basis of coverage and diagnostic yield, should allow comparison of the diagnostic testing offer between laboratories.

In addition, it should allow people – patients, referring doctors, as well as private or public reimbursement agencies – to compare the tests and the prices.
We propose that the labs should mention this rating on their clinical reports and websites. For instance, a laboratory that uses a targeted capture assay for, say 10 or 20 genes, and warrants Sanger sequencing of all the genomic regions where reliable call cannot be obtained or guaranteed by NGS, would be allowed to publicise its test as a 'type A diagnostic NGS test'. As a result, most currently available tests are probably 'type B diagnostic NGS tests', except when no additional experiments are done to fill NGS sequencing gaps by Sanger (or other); in the latter case, the test would get the default 'type C diagnostic NGS test' rating. Note that, even for offering type C test, the (accredited) diagnostic lab is bound to calculate the quality parameters, mentioned in Chapter 4 and Chapter 5, and provide this information in the report.

A database for NGS panels is currently being compiled by EuroGentest, and will eventually be made available through Orphanet (J . Schmidke, M. Stuhrmann, personal communication). Such a database could adopt the above scoring system, to ease the comparison between the test offer of the different laboratories – or even make it a requisite for inclusion in the database.

In this way, the scoring system will become important for quality assurance as well. If professional, national or international organisations issue minimal test criteria for certain disease(s), a laboratory's offer would be evaluated against these criteria. This also implies that research laboratories that deliver "diagnostic results" have to adopt similar standards (see Chapter 5).

Eventually, the system could be completed with a utility score, which would focus on the clinical pertinence of a specific test. In this way, one could imagine that a two-dimensional frame would be generated, with the described 'technical' score on one axis, and a 'clinical' score on the other axis. Any particular test and disease combination could then be scored. It is a concept and has to be further developed.

2.3 Comparison to other guidelines

Several NGS strategies can be used for a diagnostic test: gene panel, whole exome or whole genome sequencing. Each of these techniques is described in Gargis et al. (2012) and Rehm et al. (2013). Rehm et al. (2013) propose to first perform a disease-targeted panel test. In such a test, only genes with sufficient scientific evidence for a causative role in the disease should be included and physicians must have the possibility to restrict analysis to a subpanel if genes with multiple overlapping phenotypes are included in the panel. Disease-targeted panels offer a higher analytical sensitivity and specificity than exome and genome sequencing and gaps can be easily completed by Sanger sequencing (or other techniques). In case of additional Sanger sequencing, the primers/assays should be designed in advance to allow for a decent turn-around time. The concept of core genes is also supported in these guidelines since it is strongly recommended to fully cover disease-genes with high yield. Ellard et al. (2014) also mention that regions that do not meet minimal read depth might be tested using other methods, unless a mutation is found.
In silico gene panels can also be selected from exome or genome data but according to Rehm et al. (2013) the coverage of specific genes should be described in the report to allow comparison with disease-targeted panel. The diagnostic routing described by Rehm et al. includes disease-targeted panels and exome or genome sequencing in case of negative results as well as supplementing assays to detect variants that cannot be detected by the test performed. The Australian guidelines also favor focusing on gene panels if it does not compromise the performance of the test.
The concepts of 'diagnostic yield', 'core genes' and 'diagnostic routing' have been comprehensively covered in the Dutch guidelines (Weiss, Van der Zwaag et al. 2013). Other guidelines, such as the Australian guidelines and Gargis et al. (2012), refer to clinical validity and clinical utility while Rehm et al. (2013) talk about predicted clinical sensitivity and Ellard et al. (2014) also use diagnostic yield. The list of genes included in a panel must be curated, regularly updated and made publicly available (Weiss, Van der Zwaag et al. 2013, Rehm et al. 2013).

Contributions

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Chapter 3: Informed consent and information to the patient and clinician

3.1 Introduction

In all forms of genetic testing, adequate genetic counseling and informed consent are critical (see e.g. Sequeiros et al. 2010). Informed consent is generally thought to have been given when the patient clearly understands the facts, implications and future consequences of a genetic test. In cases where an individual is considered unable to give informed consent (e.g. a child or patient with intellectual disability), another person (legal guardian) is authorized to give consent on his behalf.

The core principles of genetic testing also apply to diagnostic tests based on NGS, and patients should receive a pre-test oral counseling in which the different aspects of the genetic test are discussed. Since the implications of a genetic test based on NGS depend on the type of test that will be performed, the health care professional involved in the counseling should be well aware of the benefits and potential risks of the different tests. Although a written informed consent is legally not obligatory in most countries, it is advised for genetic tests that have a chance of unsolicited findings. In those countries where it is obliged, written consent will have to be adapted to NGS.

This chapter describes the implications of the different types of NGS tests for a patient, and provides tools for what needs to be discussed with a patient before starting a genetic test based on NGS. Clearly, the requirements for informed consent only vary if the clinical outcome of the result is different. For instance, if one moves to NGS only to replace Sanger sequencing without affecting the clinical sensitivity and without introducing a chance for secondary or unsolicited findings (as is the case for BRCA1 and BRCA2 testing in many laboratories), there is no need to adapt the practice of informed consent.

The chapter is also important in defining the role of the laboratory geneticists in the clinical setting: they cannot be held responsible for informing patients, but have a duty to inform the doctors and to help them to inform the patients correctly about the features and limitations of the diagnostic NGS test. The laboratory geneticists should also discuss – and, if needed, question – the usefulness of a test prescribed by a referring physician, for instance, propose a test for CAG-repeats versus a NGS panel for certain neurological diseases, or redirect the request from autosomal recessive to autosomal dominant genes on the basis of the family tree. In any case, the responsibilities for the informed consent lie with the referring clinician.

3.2 Viewpoints and examples

3.2.1 Implications of different NGS tests

The implications of a diagnostic test based on NGS depend on the procedures, platforms, filtering processes and data storage used in the laboratory. Since the referring physician is responsible for bridging between tests and patients, he should therefore be fully informed about the limitations and possible adverse effects of a genetic test. To start, one has to know whether targeted sequencing of a gene panel or exome (or even genome) sequencing will take place. In the latter case, it is important to know if the data analysis involves only known genes involved in a certain disease (gene panel) or if all variants in an exome or a genome are analysed. When a gene panel

is prescribed (either by targeted capture or by targeted analysis of an exome or genome), knowledge on the genes involved in the specific gene panel is required.

STATEMENT 3.01: The laboratory has to provide for each NGS test: the diseases it targets, the name of the genes tested, their reportable range, the analytical sensitivity and specificity, and, if any, the diseases not relevant to the clinical phenotype that could be caused by mutations in the tested genes.

The implications – or side effects, to put it frankly - of a test based on NGS are mainly based on the chance of unsolicited and secondary findings. While unsolicited findings are found in the genes linked to the tested disease, secondary findings are found in disease genes not implicated in the aetiology of the tested disease. Secondary findings are not an issue in the case of targeted sequencing but are particularly important in case of exome or genome sequencing. Since the results of a diagnostic test should be primarily directed towards answering the question related to the medical condition of a patient (see Chapter 6), it is advised to use a gene panel approach (either targeted capture or targeted analysis).

STATEMENT 3.02: The analysis pipeline of diagnostic laboratories should focus on the gene panel under investigation in order to avoid the chance of secondary findings, and be validated accordingly.

The chance of unsolicited findings in a gene panel is very low and is mainly dependent on the genes involved. Indeed some genes (and even some specific mutations) in a gene panel can be involved in diseases not related to the clinical phenotype: a gene panel for deafness may contain genes for Usher syndrome, a condition involving congenital hearing loss and later onset blindness; a gene panel for movement disorders may contain the ATM gene involved in ataxia-telangiectasia, but with specific mutations having a breast cancer susceptibility. Furthermore, one always has to be aware of the fact that heterozygous mutations in recessive conditions might be detected, thereby detecting disease carriers which might have consequences for reproduction. These two issues should be dealt with separately in the report of the NGS test (see Chapter 5).

STATEMENT 3.03: Laboratories should provide information on the chance of unsolicited findings.

Information on the risk of unsolicited findings might be specified by stating a risk for certain genes in the panel, as done in the examples given in the previous paragraph. However, this may not be straightforward: on one hand, the laboratory may not be capable of giving a comprehensive evaluation of the risk for the known genes (especially if the panel is large), while on the other hand, the risks are often not very clear and might even be unknown at the time the test is performed. The laboratories might have to provide a general statement about the fact that the results of a gene panel analysis might involve broader phenotypes than the disease initially tested for. Hence, it will also be related to the kind of test that is being offered.

In any case, the physician should consider – and check - a number of features before prescribing a NGS test:

1. Technical aspects, i.e. be aware that this is a comprehensive test versus a simple gene test, while the sensitivity may still be limited, depending on the disease;
2. The risk for unsolicited and secondary findings for the specific NGS test being offered;
3. The diagnostic indication, i.e. the appropriate test has to be prescribed (see Chapter 2);
4. The latter implicates the provision of extensive clinical information to the laboratory, knowing that this information is essential for the correct interpretation of the results and for the writing of an adequate report. In this context, it is noted that in some countries, the laboratory has a duty (e.g. in Germany) or a right (e.g. in Belgium) to refuse a genetic test, if it is not properly prescribed. This principle should be applied to NGS tests as well.

If the doctor is uncertain about any of the above, he or she should seek advice or refrain from prescribing the NGS test. The clinician must have a contact person responsible for NGS tests. The laboratory should announce whom to contact for further information.

Evidently, the quality with which the unsolicited and secondary findings is interpreted (in terms of pathogenic versus neutral versus 'unknown significance') should be the same as for the rest of the test.

3.2.2 Procedure for dissemination of unsolicited and secondary findings

Before implementing an NGS-based test, the clinical (genetic) centre needs to set up an 'unsolicited and secondary findings procedure' which has to be in accordance with the decisions of an ethical committee. It should be decided whether patients are offered opt-in, opt-out options to get additional information besides the initial diagnostic result. If these options are provided, the different outcomes should be classified based on the severity of a disease, the age of onset, mortality, existence of effective treatment, etc. Useful classification models have already been published (Berg et al. 2011, Bredenoord et al. 2011*)*, but the options that can be offered are highly dependent on local policies. The procedure should also specify whether unsolicited findings and carrier status are reported.

STATEMENT 3.04: If a clinical centre or a laboratory decides to offer patients the possibility to get carrier status for unrelated diseases and secondary findings, it should implement an opt-in, opt-out protocol and all the logistics need to be covered.

Unsolicited findings and carrier status on genes included in the tested gene panel should be reported in the main report. Secondary findings should be described in a separate report.
The availability of a multidisciplinary committee of experts or a local ethical board that can be assembled on an *ad hoc* basis to discuss the return of a debatable secondary finding to the referring physician is optional.
If no ethical board is available, e.g. in the case of a commercial laboratory offering NGS testing in a clinical context a board of experts should be consulted on a regular basis to discuss on how to deal with unsolicited finding and to determine whether the results are actionable or not. The board could consist of at least 3 experts with clinical experience, including board certified human geneticists and the clinician(s) of other specialities, directly involved in the care of the individual

case, should be consulted. The cases and the outcome of the discussions should be documented in a quality-managed form and signed by the board members.

3.2.3 Counselling for NGS diagnostics tests

Pre-test genetic counselling is necessary and should include a discussion on both expected results and the potential for unsolicited and secondary findings. Both unsolicited and secondary findings have to be defined and the policy of the laboratory on the dissemination of those findings should be outlined.

STATEMENT 3.05: The local policy about dissemination of unsolicited and secondary findings should be clear for the patient.

Information should be provided about the interpretation of results, especially the fact that this interpretation may alter with increasing knowledge. The concept of unsolicited and secondary findings needs to be discussed in the pre-test phase.
A written informed consent is recommendable, but not required unless several options for returning the results of unsolicited and secondary findings can be chosen.

STATEMENT 3.06: It is recommended to provide a written information leaflet or online available information for patients.

The consent must include a section on sharing anonymized variants in population and disease specific databases (see Chapter 6.2.5). This has to conform to privacy and security laws in respective countries. In clinical practice, contributing to these databases should be encouraged as it will ease variant interpretation and thus be beneficial to other patients.
If the *in silico* capture of a gene panel from an exome or a genome did not resolve the diagnosis, a second counselling should be done before the whole exome or genome is analysed. During this counselling, a new informed consent should be made.

3.3 Comparison to other guidelines

In their section on ethical and legal issues, the Australian guidelines state that a consultation between the referring clinician and the laboratory supervising the test is required. All guidelines insist on the fact that the clinician has to provide specific and adequate clinical information to facilitate interpretation of the analytical result.
Guidelines generally talk about incidental findings and do not make the distinction between unsolicited findings (found in the genes linked to the tested disease) and secondary findings (found in disease genes not implicated in the aetiology of the tested disease). However since they are usually described in the context of whole exome and whole genome sequencing, they must refer to secondary findings.
According to Australian guidelines, counselling should happen prior to genomic testing and discuss expected results as well as incidental findings. It should also specify that interpretation of results requires reference to population and disease specific databases and may alter with increasing knowledge. Patients should receive a written record of the policy used for incidental findings. Common examples of incidental findings include the detection of consanguinity and incest, the carrier status for autosomal recessive disorders, variants involving genes associated

with dominant or adult-onset conditions. Consent is required only if data generated in clinical setting is used for research purposes.
The laboratory must have a clear policy for disclosure of incidental findings and only report variants classified as pathogenic (Australian guidelines, Weiss, Van der Zwaag et al. 2013 and Rehm et al. 2013). This policy should conform to medical and ethical obligations. Rehm et al. also precise that it should be clear whether incidental findings are searched for and reported or whether only real incidental findings are reported. Reported findings must be confirmed and the laboratory must use criteria to decide which findings to report and how they can be requested.

Contributions

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Chapter 4: Validation

4.1 Introduction

All components of a diagnostics test must be validated prior to its use. For NGS-based diagnostic analyses, accuracy, analytical precision, analytical sensitivity, specificity, reportable range of test results, and reference range should be determined empirically and validated during validation (Gargis et al. 2012). These performance characteristics are assessed during platform, pipeline and/or test validation. Since platform, pipeline and test are highly interlinked, it is not straightforward to validate one independently of the other. This chapter describes the essential steps required for the development, optimization and validation of the diagnostics test and outlines when and how the performance characteristics can be assessed. The same rules should apply when data is generated within the lab's own facilities or when data is obtained through subcontracting.

4.1.1 Definitions

The platform does not only include the next generation sequencer but also DNA isolation, enrichment methods, library preparation, and data analysis.
Platform validation is the process of establishing that the massive parallel sequencing system can correctly read DNA sequence (Gargis et al. 2012). It should also evaluate how accurately each type of variant can be detected. To achieve that, performance specifications have to be determined for possible combinations of assay and analysis. Some variants will not be correctly identified using one or the other technology, but as far as they are not included in the test, it is not problematic.
Since the massive amount of data produced by NGS requires processing, the platform validation strongly depends on the pipeline validation. Analytical specificity and sensitivity should be inferred during pipeline evaluation and confirmed during validation.
The test validation in the context of NGS assays is the validation of the diagnostics test from end to end i.e. from the DNA sample to the reportable list of variants. Variant prioritization and interpretation are usually excluded from the test validation, because they require circumstantial evaluation of the clinical request, the literature, and the procedure may vary greatly, depending on the test. Nevertheless, the principle should be the same: an identical sample should lead to an identical clinical conclusion, if processed through the same pipeline by a different operator on a different day. The test validation includes and depends on the platform validation and the informatics pipeline validation. From a practical standpoint, we put forward that it should focus on the (genomic) regions under investigation. The test validation should prove the ability of the diagnostic test to detect variants in the regions defined during the development of the assay.

4.1.2 Analysis pipeline description

While all tools responsible for data acquisition and base-calling usually delivered with the sequencing platform might be looked at as the basis of every NGS dataset, choosing the right bioinformatics tools, software packages and even the appropriate hardware for the downstream data analysis has to be addressed by the responsible lab. Most vendors offer relatively mature software for the downstream data analysis, but many labs maintain their own data analysis

pipeline, based on FASTQ files generated by the vendor software. FASTQ files, an amalgamate of sequence reads and corresponding quality scores for each position in the reads, are the standardized sequence product of the common sequencing instruments.
Bioinformatics applications in diagnostics are very broad. For the sake of conciseness, we will not touch on somatic genetics with ultra-deep sequencing, differential sequencing and neither comment on arising technologies to interpret and diagnose genetic variation beyond small sequence changes, i.e. we will not address copy-number analysis and de-novo assembly. In this paragraph, recommendations for standard analyses in human germline diagnostics are put forth and standard workflows and applications are exemplified.

Generally, the analysis pipeline for NGS data consists of base calling and demultiplexing, mapping, annotating, and filtering steps. These steps are described in table 1 as well as several optional processing steps. During processing, three different files are produced. The FASTQ file contains the base calls of all the reads produced by the sequencer as well as the Phred quality score of each base. The BAM file (binary version of the Sequence Alignment/Map or SAM file) describes how the reads are mapped to the reference genome (position of mapping, mapping quality scores, number of matching and mismatching bases, etc.) and contains the reads sequence and quality scores. The Variant Call File or VCF file contains for each variant the chromosomal position, name and build of the reference genome, reference and alternative alleles, and various quality scores.

Table 1: Elements of a NGS bioinformatics pipeline

Processing step	Description	Tools and databases	Output
Base calling and demultiple-xing	Base calling and demultiplexing, are also referred as primary analysis.	vendor software of the sequencing platform	FASTQ file(s)
Primer trimming	In amplicon sequencing primers have to be trimmed from the reads	[CutAdapt (Martin et al. 2011)], [BWA (Li & Durbin, 2009)] (soft clipping while mapping)	FASTQ files or BAM file (if soft clipping by a mapper such as BWA)
Adapter trimming (optional)	Sequencing adapters may be trimmed from the read ends for those reads where the insert size is smaller than the read length. If not trimmed, sequenced adapters may interfere with mapping and variant calling, leading to false-positive or false-negative variant.	[Trimmomatic (Bolger et al. 2014)], [SeqPrep (https://github.com/jstjohn/SeqPrep)], [CutAdapt (Martin et al. 2011)], [BWA (Li & Durbin, 2009)] (soft clipping while mapping)	FASTQ files or BAM file (if soft clipping by a mapper such as BWA)

Low-quality trimming (optional)	Low quality bases may also interfere with mapping and variant calling and can be trimmed from the end (and begin) of reads.	[Trimmomatic (Bolger et al. 2014)], [SeqPrep (https://github.com/jstjohn/SeqPrep)], [CutAdapt (Martin et al. 2011)], [BWA (Li & Durbin, 2009)] (soft clipping while mapping)	FASTQ files or BAM file (if soft clipping by a mapper such as BWA)
Mapping	In the read mapping step, paired-end/ single-end reads are mapped to the reference genome allowing for base changes and indels. Mapping should always be performed against the full reference genome even when a small gene panel is sequenced. .	[BWA (Li & Durbin, 2009)], [Novalign (http://www.novocraft.com/main/index.php)], [Stampy (Lunter & Goodson 2011)], [SOAP2 (Li et al. 2009)], [LifeScope – for color space reads (http://www.lifetechnologies.com)], [Bowtie (Langmead & Salzberg 2012)]	BAM file
Duplicate removal (optional)	In shotgun sequencing few duplicates are expected since the DNA is randomly sheared. However, duplicates can occur during PCR and as an artifact of imaging. In amplicon sequencing, duplicates are expected and should not be removed.	[Picard MarkDuplicates (http://broadinstitute.github.io/picard)]	BAM file
Indel realign-ment (optional)	The presence of indels in the sequenced samples often leads to multiple single base mismatches around these sites, especially if they reside close to the start or end of reads. These artifacts may show up as false-positive variants during subsequent analysis. Local re-alignment algorithms identify such positions and try to minimize the amount of mismatching bases by performing a local re-alignment of the indel spanning reads, increasing the accuracy of the calls while minimizing false positives.	[GATK RealignerTargetCreator & IndelRealigner (DePristo et al. 2011)] and [SRMA (Homer & Nelson 2010)]	BAM file

Quality score recalibration (optional)	After mapping to the reference genome, the base quality score of the reads can be recalibrated to better match the probability of false base calls and to spread the quality scores wider over the valid range. In most algorithms, false base calls are distinguished from real variants by performing a simple base calling or using databases of known polymorphisms, e.g. [dbSNP].	[GATK BaseRecalibrator & PrintReads (DePristo et al. 2011)], [ReQON (Cabanski et al. 2012)]	BAM file
Variant calling	Variant calling consists of detecting and genotyping differences to the reference genome (base changes and small indels).	[samtools (Li et al. 2009)], [GATK UnifiedGenotyper (DePristo et al. 2011)], [GATK HaplotypeCaller (DePristo et al. 2011)] and [Platypus (Rimmer et al. 2014)]	VCF file
Annotation	Variant interpretation requires detailed annotation. Very basic annotations are gene name, region (exonic, splicing, intronic, intergenic, etc.) and coding change information. Additionally, minor allele frequency for known polymorphisms, pathogenicity and conservation scores and clinical databases can be used.	[Annovar (Wang et al. 2010)], [SNPeff (Cingolani et al. 2012)], [Cartagenia Bench Lab NGS (http://www.cartagenia.com/products/bench-lab-ngs/)] [dbSNP (Sherry et al. 2001)], [1000 Genomes (The 1000 Genomes Project Consortium 2012)], [ESP 6500 (https://esp.gs.washington.edu/drupal/)] [SIFT (Kumar et al. 2009)], [PhyloP (Cooper et al. 2005)], [MutationTaster (Schwarz et al. 2010)] [COSMIC (Forbes et	CSV, TSV, TXT, excel files or databases

		al. 2008)], [OMIM (http://omim.org/)], [ClinVar (Landrum et al. 2014)], [HGMD (Stenson et al. 2014)]	
Filtering	To find disease related variants in large variant lists, rigorous filtering is needed. Typical variant filters exclude low quality variants, intronic/intergenic variants, synonymous SNPs or known polymorphisms with low frequencies in the population. However, this kind of filtering selects both for deleterious and false-positive variant calls. To remove the false-positives, filtering according to variant frequencies of an *in-house* database, containing all the processed samples of a lab, is often applied. Because an *in-house* database accumulates false-positive variants that are specific for the used sequencing platform, sequencer and analysis pipeline, it can be used to identify and remove these false-positives.	[SnpSift (Cingolani et al. 2012)], [Cartagenia Bench Lab NGS (http://www.cartagenia.com/products/bench-lab-ngs/)]	CSV, TSV, TXT, excel files or databases

4.1.3 Quality parameters

In diagnostic setting, only good quality samples must be analysed. It is thus essential to define criteria to characterize high quality targeted gene panels, exomes or genomes.

The quality of a sample can/should be evaluated at three levels:

- **Technical target**; limiting the quality assessment to the technical target is a fair quality assessment allowing the technical evaluation of the capture procedure. For exome sequencing, it is kit dependent: the target defined by the kit should be used.
- **Clinical target – Region Of Interest (ROI)**; the clinical target has to be considered in order to define the reportable range and design the diagnostic test (see chapter 2 and following section). Since it is not necessarily included in the technical target the quality assessment of a sample cannot rely solely on the clinical target.
- **List of transcripts**; the kits used for exome or gene panel capture, the definition of clinical targets and the sequencing technologies may differ from one center to the other. In order to allow comparisons of quality across genetic centers, a quality criteria could be calculated according to a list of transcripts such as all coding transcripts from RefSeq.

Although the target plays an important role while measuring the quality of a sample, quality does not depend on target only, it is a combination of many parameters. The amount of data produced, the proportion of clusters assigned to each sample (when multiplexing), the proportion of PCR

duplicates and the coverage also have to be taken into account. In the same way, coverage alone is not enough especially if raw coverage is considered. Quality criteria should be based on informative coverage instead of raw coverage (Weiss, Van der Zwaag et al. 2013). Genes with pseudogenes or repetitive elements may show high raw coverage but low informative coverage (if all reads mapped with bad quality are discarded).

The proportion of the target that can be reliably genotyped, i.e. for which enough informative coverage is obtained to accurately call a genotype, provides a succinct quality measure that can be applied to the three targets previously defined. If all steps of the sample preparation have succeeded, this number should be high and reproducible. However if one step failed, the proportion of target reliably genotyped should be lower. Indeed the presence of lots of PCR duplicates due to a failed library preparation, for example, would decrease the overall coverage and reduce the number of sites reliably genotyped. A low amount of data would also result in low informative coverage and consequently reduce the number of sites reliably genotyped.

STATEMENT 4.01: All NGS quality metrics used in diagnostics procedures should be accurately described.

Especially the details of the calculation of a metric should be well-documented to make the interpretation of the metric clear. To facilitate automated handling of Quality Control (QC) values, quality metrics should be defined and documented in a uniform terminology and standardized file formats should be used. For example, the qcML project (Walzer et al. 2014) maintains a generic XML file format for storing QC data and an ontology of QC terms for proteomics and genomics.

4.1.4 Monitoring and sample tracking

NGS technology requires the monitoring of run specific features such as the number of samples pooled, the proportion of clusters assigned to each sample and the base quality score by position. Every sequencing run has to be monitored whether or not the instrument specifications are met. Moreover, there should be a definition of minimal requirements for important quality measures (i.e. base quality, read length, etc. depending on platform characteristics).
Analysis/sample specific features such as informative coverage, uniformity of coverage, strand bias, GC bias, mapping quality, proportion of reads mapped, proportion of duplicated reads, proportion of target covered at minimum coverage depth, proportion of target not covered, mean coverage, calling accuracy, number of variants and transition/transversion ratio also have to be monitored. Some of the QC measures that should be routinely monitored for all samples are described in more details in **Appendix 1** (QC metrics tracking for samples).

STATEMENT 4.02: The diagnostic laboratory has to implement a structured database for relevant quality measures for (i) the platform, (ii) all assays, (iii) all samples processed.

Monitoring data should not be reported but used as continuous validation.
It is important to keep track of exceptions such as the number of times that a sample has been sequenced to reach the defined quality criteria and the correction of eventual sample swaps. A sample tracking method should be used since NGS workflows are very complex and comprise

multiple processing steps both in the lab and during the computational analysis. For example, common SNPs could be included as enrichment targets and genotyped by independent methods (i.e. Sequenom or qPCR genotyping; see **Appendix 2**). Samples that have been swapped and for which the swap cannot be explained should not be considered for the diagnostic report.

STATEMENT 4.03: Aspects of sample tracking and the installation of bar-coding to identify samples, should be dealt with during the evaluation of the assay, and included in the platform validation.

The proportion of un-mapped reads and un-assigned MIDs should also be tracked as it can help identifying grossly deviant samples/analyses (due to contamination during the workflow). Finally, comparisons and monitoring between different assays should be achieved by generic enrichment contents. Indeed, quality control regions can be added to all panels/exome enrichments in addition to the SNPs for sample identification. Calculating the number of aberrant base calls (non-wild type calls), invalid base calls (denoted as base 'N') and sporadic indels in those regions would help identifying deviant samples. Moreover benchmarking these parameters allows for a direct comparison of different versions of a diagnostic test as well as for inter-test comparisons. Different sequencing platforms, enrichment methods, etc could be compared and these regions would allow for proficiency testing. Of course, the variants called in quality control regions have to be excluded from the quality metrics calculations.
We propose to use three large exons on different chromosomes that do not contain many known polymorphisms, especially indels (Table 2). The use of three regions instead of one region provides a backup in case of large deletions or enrichment problems. Exons are used since they are already contained in exome enrichments and, thus, have to be added as custom content to panels only.

Table 2: Quality control regions

chromosome	start (hg19)	end (hg19)
chr1	152057442	152060019
chr9	5919683	5923309
chr18	19995536	19997774

4.1.5 Comment on the a priori chance of finding a variant

Imagine that there is a chance of 99% of detecting a heterozygous variant at 20X. This will affect the detection rate for disease mutations differently, according to the different approaches but also depending on the inheritance pattern of the disease (for simplicity reasons, we assume that less than 20X coverage has a chance of 0% of detecting a heterozygous variant, which is not completely true)

In the case of recessive disorders:
For whole exome sequencing,
if 75% of the exome is covered at 20X,

- 2 compound heterozygous variants in 1 gene will be found in only 55.1% of the cases;
- in 38.3% of the cases, only one variant will be found;
- in 6.6% of the cases both variants will be missed.

if 86% of the exome is covered at 20X,
- 2 compound heterozygous variants in 1 gene will be found in 72.5% of the cases;
- in 25.3% of the cases, only one variant will be found;
- in 2.2% of the cases both variants will be missed.

In a target panel, if 96% of the target is covered at 20X,
- In 90.3% of the cases, both variants are detected
- In 9.5% of the cases, only one variant is found
- In 0.2% of the cases, both variants are missed.

In the case of dominant disorders:
For whole exome sequencing,
if 75% of the exome is covered at 20X,
- 1 heterozygous variant in 1 gene will be found in only 74.2% of the cases;
- in 25.8% of the cases, the variant will be missed.

If 86% of the exome is covered at 20X,
- 1 heterozygous variant in 1 gene will be found in only 85.1% of the cases;
- in 14.9% of the cases, the variant will be missed.

In a target panel, if 96% of the target is covered at 20X,
- 1 heterozygous variant in 1 gene will be found in only 95.0% of the cases;
- in 5.0% of the cases, the variant will be missed.

4.2 Viewpoints and examples

4.2.1 Platform validation

During platform validation, the laboratory has to make sure that all its devices and reagents satisfy the manufacturers requirements. The limitations of each technology must be identified and taken into account during data analysis and test development.

STATEMENT 4.04: Accuracy and precision should be part of the general platform validation, and the work does not have to be repeated for individual methods or tests.

Accuracy can be established by determining the discrepancy between a measured value and the true value, i.e. for NGS the most up-to-date reference sequence. Adequate coverage needed is dependent on the type of variation present in the sequence and its copy number. This parameter and thresholds for allelic read percentage therefore should be determined empirically and validated during test validation. Less coverage is needed to accurately detect homozygous or hemizygous SNPs than heterozygous SNPs.
Precision refers to the agreement between replicate measurements of the same material. An adequate number of samples (minimum 3) should be analysed to establish precision by assessing reproducibility (between-run precision) and repeatability (within-run precision) during test validation. Repeatability can be established by preparing and sequencing the same samples multiple times (minimum 3) under the same conditions and evaluating the concordance of variant detection and performance. Reproducibility assesses the consistency of results from the same sample under different conditions such as between different runs, different sample preparations, by different technicians, and using different instruments. A concordance between 95 and 98% would be satisfactory (Rehm *et al*. 2013).

Reference range is defined by Gargis et al. (2012) as "the range of test values expected for a designated population of persons." For NGS: "the normal variation of sequence within the population that the assay is designed to detect." In other words, any variant detected that is not known as normal should be considered as potentially pathogenic, and may require additional investigation, e.g. by using an automated prioritization tool to establish the clinical significance. This distinction between a normal and disease-associated variant obviously is not always well defined. Also cataloging known normal and disease-associated variants in databases will be invaluable (see chapter 5).

4.2.2 Analysis pipeline validation

Evidently every sequencing technology harbors its strengths and weaknesses. The bioinformatics tools must reflect these characteristics. For example, variants within homopolymer regions should be carefully looked at in pyrosequencing and semiconductor sequencing, while dual-color sequencing by hybridization warrants specific color spacing procedures.

STATEMENT 4.05: The bioinformatics pipeline must be tailored for the technical platform used.

During pipeline validation the diagnostic specifications must be measured by assessing analytical sensitivity and specificity. Several methods can be used to do so:

- the comparison of genotypes called from the diagnostics test with SNP array genotypes; however such a comparison might be biased since dbSNP variants included in most SNP arrays are usually used to train and enhance the genotyping algorithms;
- a blind comparison of genotypes called from the diagnostics test with Sanger confirmed variants, the drawback of this method being the low number of variants usually available;
- the comparison of genotypes called using two different NGS technologies;
- the analysis of an artificial datasets in which true variants and errors are know;
- the resequencing and/or analysis of well characterized publically available DNA samples such as 1000g DNA samples available via Coriell repositories while the corresponding sequencing datasets are accessible at www.1000genomes.org.

The availability of very well characterized samples is the ideal situation and approaches are made towards a **"platinum" data set** [GenomeInABottle (http://genomeinabottle.org/)]. The latter project provides open data access for an exhaustively sequenced three generation family for which DNA samples can be ordered via the Coriell repository. Consensus variant lists from sequencing data for three different technical platforms which have been fully validated by cross-checks or additional methods is available. DNA samples of these individuals can be used for platform and bioinformatic pipeline validation. In accordance with validation procedures set forth for Sanger sequencing validation (Mattocks et al. 2010), we suggest to validate about 300 variants per platform in order to specify the sensitivity and specificity of the system.

STATEMENT 4.06: Analytical sensitivity and analytical specificity must be established separately for each type of variant during pipeline validation.

Obviously, the same rules apply to commercial software and proprietary or public software used or developed by the lab.
Usually, updating the content of capture probes, selector probes or amplicons will not greatly affect these characteristics but the bioinformatics pipeline interdepends on the chemistry and the chosen enrichment. Therefore, any changes in chemistry, enrichment protocols or the bioinformatics analysis platform will warrant re-validation. Usually, the number of samples to use when repeating the analysis for revalidation should correspond to the number of samples of a normal test (e.g. 6 exomes on 2 lanes of HiSeq2500).

In general, the laboratories are encouraged to perform proficiency testing once the test has been validated, and participate in external quality assessment schemes as soon as they will be available. This is a requirement of the ISO 15189 norm for the accreditation of medical laboratories, but also effective in monitoring performance in the laboratories. In this context, laboratories are also invited to share well-characterized samples and data files to collaboratively improve and standardize practice for diagnostics.

STATEMENT 4.07: The diagnostic laboratory has to validate all parts of the bioinformatic pipeline (public domain tools or commercial software packages) with standard data sets whenever relevant changes (new releases) are implemented.

An *in-house* database containing all relevant variants provides an important tool in order to identify platform-specific artifacts, keep track of validation results, and provide an exchange proxy for locus-specific databases and meta-analyses. Typically, this database should allow for further annotations (for example false-positives, published mutations, segregating variants, etc.) which greatly streamlines the diagnostic process.

Care should be taken to choose a cut-off (i.e. variant frequency in the 'normal' population) for the (automated) classification of variants. The cut-off will differ depending on the expected inheritance pattern (dominant, recessive, X-linked) and the database that is being used as a reference.

STATEMENT 4.08: The diagnostic laboratory has to implement/use a structured database for all relevant variants with current annotations.

Storing NGS raw data is challenging because of the volume of the data. No standards exist for the extent of data storage. In general, a minimal data set that allows repetition of the diagnostic analysis should be stored. Currently, the consensus is that the FASTQ files have to be stored. Generally, data storage should stick to the standard open file formats FASTQ, BAM and VCF which should also be used for data exchange with other laboratories. If the BAM file is stored, it must be possible to generate the original FASTQ files from it, i.e. it should contain the unmapped reads and if the reads have been trimmed, the FASTQ files have to be stored as well. The stored VCF file should contain all good quality variants prior to filtering according to allele frequency, position in the genome, etc. If the VCF files are stored, it is advantageous to use a genome VCF (gVCF) file (including information on covered positions) so that variant frequencies can be reliably computed from them. Proprietary vendor file formats should be avoided because they

might become difficult to read once the vendor discontinues the use of the file format. The use of check-sums in order to guarantee integrity of the data is encouraged.
When storing the analysis results, full log files have to be stored in addition to the analysis results. The log files should be as complete as possible, making the whole pipeline from FASTQ data to the diagnostic report reproducible. The log files should contain all tools and databases used along with the tool and database version/timestamp and the parameters. Pipelines, tools and databases should be archived. It is recommended to use a version control system.

STATEMENT 4.09: The diagnostic laboratory has to take steps for long-term storage of all relevant datasets.

As a steady companion of NGS technology, a variety of bioinformatics tools has been put forth and tested for data analysis, data tracking and quality management. Albeit tremendous progress towards fast, accurate, and reliable algorithms and pipelines, many research tools are often poorly documented and tested. This will be the case for future tools as well, as the technological progress has outpaced traditional software development by far. A major drawback, at least a major challenge is still the correct genotyping of small and large indels and mosaic genotypes since all current tools struggle with complexities in mapping and variant calling of these types of variants. With the advent of whole genome sequencing and long-phased haplotype sequencing, part of these diagnostic weaknesses might be overcome by investing even more resources in accurate diagnostic NGS pipelines.

4.2.3 Test validation

A diagnostics test should be carefully developed and optimized prior to validation. Importantly, the 'regions of interest' (ROI) or clinical target, i.e. all coding regions plus the conserved splice sites (Ellard *et al*. 2012), have to be defined prior to launching the assay. When describing the clinical target, the name and version of the transcript used must be stated. The clinical target must be defined according to the best practices guidelines for genes and diseases available at the European level such as the gene cards (Dierking et al. 2013), the gene dossiers (http://ukgtn.nhs.uk/find-a-test/gene-dossiers/) or the EMQN best practice documents (http://www.emqn.org/emqn/Best+Practice). As the list of causative genes evolves constantly, the clinical target must be regularly updated.
Some areas of the clinical target may not be sequenced reliably and should therefore be excluded from the reportable range. Clinically relevant regions not included in the reportable range (due to technical reasons) should be genotyped by another technique such as Sanger sequencing (see Chapter 2 on diagnostic routing).
Mutation types that can be detected as well as the prevalence of such mutations in the tested disorders have to be taken into account when developing the test (see Chapter 2).

STATEMENT 4.10: The reportable range, i.e. the portion of the 'regions of interest' (ROI) for which reliable calls can be generated, has to be defined during test development and should be available to the clinician (either in the report, or communicated digitally).

An exome sequencing assay with the aim to achieve a high diagnostic yield does not require additional analysis to achieve high coverage in all genomic regions covered, but needs clear

communication to the clinician that the test cannot be used to exclude a particular clinical diagnosis (also cf. reportable range).

STATEMENT 4.11: The requirements for 'reportable range' depend on the aim of the assay.

During the test optimization, the number of samples that can be pooled, the cost and turn-around-time of the diagnostics test should be determined. It is also essential to ensure that the next generation sequencing data satisfies the quality criteria (based on technical and clinical targets) described in the previous section. All samples that do not fulfill these quality criteria should not be considered for routine reporting.

The performance of the diagnostics test must be evaluated in terms of accuracy, analytical sensitivity, analytical specificity and precision. Accuracy correlates with informative coverage; it depends on base quality, mapping quality, duplicated reads (PCR duplicates), GC content, strand bias, presence of repetitive sequences and existence of pseudogenes. Since it is sequence and context dependent, accuracy will vary across the genome/exome and should be determined at the test level, i.e. for each ROI. Analytical sensitivity depends on informative coverage and reportable range.
Finally the limitations of the diagnostics test should be clearly stated and listed in the report (see Chapter 5). They usually include the presence of repetitive sequences, pseudogenes, homologous regions, GC content, allele drop out and the fact that some type of variants, such as transversions and inversions, cannot be detected and/or are disregarded for the diagnostic test (e.g. if people do not extract CNV information from exome data, but could technically do so).

At the time being, it is advisable to confirm all reported variants to make sure that no sample swap occurred as well as to validate the informatics pipeline. However such a confirmation might no longer be required in a near future if the technology has been widely validated. Indeed one could define regions/variants for which genotyping is always reliable and only confirm variants detected outside of these regions.

STATEMENT 4.12: Whenever major changes are made to the test, quality parameters have to be checked, and samples will have to be re-run. The laboratory should define beforehand what kind of samples and what number of cases will be assayed whenever the method is updated or upgraded.

For instance, the test should be revalidated if a new genome build is used, software tools are updated, the gene panel is modified (for targeted re-sequencing), instrumentation and/or reagents are changed.
Laboratories are encouraged to take part of proficiency testing once their test has been validated.

4.3 Comparison to other guidelines

This chapter is the most covered in all guidelines published so far and all guidelines agree on some points such as having a sample tracking protocol in place, implementing and monitoring quality control measures, keeping track of exceptions, documenting and versioning the software

and pipeline used for analysis, confirming reported variants, etc... However, available guidelines also differ in some points outlined below.
Test development and optimization were described only by Rehm et al. (2013) and Gargis et al. (2012) although these two steps are essential and should be performed prior to the test validation. The Australian guidelines provide an extensive description of the wet lab process as well as the organization of the laboratory.
In their guidelines, Gargis et al. (2012) carefully defined accuracy, precision, reportable range, analytical sensitivity and analytical specificity. Following guidelines often refer to their definition. All guidelines state that these performance parameters have to be inferred but do not always specify that they should be inferred at the platform, informatics pipeline and test levels. There is a general agreement that precision can be assessed by sequencing samples in at least 3 different runs (Ellard at al. 2014, Gargis et al. 2012, Rehm et al.2013). A concordance of 95-98% should be aimed at (Rehm et al. 2013).
Although all guidelines mention coverage and state that the accuracy of variant detection depends on the depth of coverage, only Weiss, Van der Zwaag et al. (2013) define informative coverage in opposition to raw coverage. In their definition they only exclude duplicate reads but mention that other filtering criteria such as uniqueness of mapping, mapping quality, position of the base in the read, number of individual start sites represented by the reads could be used. Base quality scores can also be used. Gargis et al. (2012) also mention that only good quality reads should be used to assess depth. Criteria to decide when to call a variant are generally not given, except by Weiss, Van der Zwaag et al. who require a coverage of 30X and at least 20% of the reads containing the variant.
Target is often referred to, especially for quality assessment, but no distinction is made between technical and clinical target although both are primordial for establishing the quality of a sample and diagnostic test. We have emphasized this in the sections above. The concept of region of interest (referred to as clinical target in this document) is outlined by Ellard et al. (2014) as coding regions and conserved splice sites.
Many guidelines suggest the comparison of SNP arrays genotypes to genotypes inferred from NGS sequencing to assess pipeline and test performance (Gargis et al. 2012, Rehm et al. 2013, Weiss, Van der Zwaag et al. 2013). However, according to Rehm et al. this strategy should be used only for whole genome sequencing since most of the variants genotyped in SNP arrays are not on exome target. Gargis et al. would exclude this method, for the same reason, but only for disease-targeted panels (not for whole exome sequencing). A concordance of 95-98% should be aimed at (Rehm et al. 2013). The fact that the use of variants from dbSNP might bias the comparison as explained above is not mentioned in any guidelines. According to the Australian guidelines, reference materials containing variants, small indels and larger structural variants, homopolymers, repetitive sequences and sequences homologous to target should be used during validation and ongoing monitoring. Weiss, Van der Zwaag et al. (2013) and Rehm et al. (2013) suggest the use of samples with known Sanger-confirmed variants even though a large number of such samples would then be required. Indeed, according to Ellard et al. (2014) concordant results for at least 60 unique variants are necessary to have an error rate for heterozygote/homozygote variant lower than 5% with a confidence interval of 95%. Rehm et al.(2013) specify that the reference samples used for test validation must be renewable and may not contain pathogenic variants. Well characterized cell lines could be used with the inconvenient that they are not stable (Rehm et al. 2013, Gargis et al. 2012). Simulated electronic

files could also be used (Rehm et al. 2013, Gargis et al. 2013). For Rehm et al.(2013), it is essential to define a good quality exome (for example a mean target coverage of 100X with 90-95% of the bases covered at 10X if proband alone is sequenced or a mean target coverage of 70X if a trio is sequenced) and a good quality genome (mean coverage of 30X). Rehm et al. (2013) also propose to prioritize sensitivity over specificity when variants are confirmed and prioritize specificity for incidental findings.
Besides the standard quality measures, Gargis et al. (2012) suggest and discuss several strategies for quality control: the inclusion of a characterized external control with disease associated sequence variation in each run, reference materials, non-human synthetic control DNA, control sequence intrinsic to the sample and not on targeted regions such as highly conserved house-keeping genes or mitochondrial DNA.
Various storage strategies are proposed. According to the Australian guidelines, the laboratory should keep a copy (or at least be able to reprint) of the informed consent and the original report for at least 100 years. All files should be kept until a clinical report is issued and FASTQ, BAM and/or VCF files should be stored in the longer term. The data storage policy must comply with regulatory and legislative requirements. Ellard et al. (2014) propose to store the output file with variant annotation as well as a log of informatics processing. Gargis et al. (2012) mention that no rule are available so far but that if the VCF file is kept, FASTQ or BAM files should be stored as long as possible (at least till the next proficiency testing). Weiss, Van der Zwaag et al. (2013) suggest to store VCF files and statistics on vertical and horizontal coverage for an unlimited time and FASTQ or BAM files for one year. Rehm et al. (2013) state that a file that would allow regeneration of primary results should be stored for two years while VCF files and reports should be kept as long as possible. The policy on which files are kept and for how long should be clear and in accordance with local, state and federal requirements.
Proficiency testing and alternate assessment are mentioned and seen as necessary in all guidelines. They are discussed in details by Gargis et al. (2012), who propose to perform one proficiency test and one alternate assessment, each of two samples, each year. Proficiency testing can be done on reference materials, such as HapMap of 1000 Genome Project samples, synthetic DNA reference materials or FASTQ files.
Gargis et al. (2012) and Rehm et al. (2013) propose to repeat the validation when a new build of the reference genome is available, changes such as instrumentation, reagents, software updates and modification of gene panel. This revalidation can be modular but the number of samples that should be used is not specified.
Outsourcing a part of NGS test does not prevent the standards defined by the guidelines to be met (Australian guidelines, Weiss, Van der Zwaag et al. 2013) or can only be performed by certified laboratories (Ellard et al. 2014).
The Australian guidelines also provide a chapter on the required IT infrastructure.

Contributions

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Comparison to other guidelines written by Erika Souche

Appendix 1: QC metrics tracking for samples

Tracking QC metrics throughout the whole analysis pipeline is essential to ensure that each final report is based on diagnostics-grade read data. We will summarize the most important, but by far not all, QC metrics in the following table:

Quality metrics based on raw reads (FASTQ) or mapped reads (BAM)	
Parameter	**Comment**
median base quality by cycle	Base quality typically decreases towards the end of the reads. As a rule of thumb, the quality score should not fall below 20 (Phred quality score).
percentage duplicate reads	The percentage of duplicate reads is an indicator of the library complexity.
percentage trimmed bases (if applicable)	The percentage of trimmed bases during adapter trimming.
percentage of mapped reads	The percentage of reads that could be mapped to the reference genome.
percentage of reads on target region	The percentage of reads that could be mapped to the technical target region.
average depth on target region	The average sequencing depth on the technical and clinical target regions.
percentage of target region with depth 20 or more	The percentage of the technical and clinical target regions sequenced with an informative depth greater than or equal to 20 (or any other informative depth considered to be the minimum for diagnostics).
Quality metrics based on variants (VCF)	
Parameter	**Comment**
total number of variants	The total number of variants in the technical and clinical target regions should be similar for samples which were processed with the same panel/enrichment.
percentage of variants known polymorphisms	Most detected variants (> 90%) of each sample should be known polymorphisms.
percentage of variants indels	The percentage of indels with respect to the total number of variants.
percentage of variants homozygous	The percentage of homozygous variants with respect to the total number of variants.
percentage of nonsense variants	The percentage of nonsense variants with respect to the total number of variants.
transition/transversion ratio	The ratio of transitions/transversions

Appendix 2: SNPs for sample identification

In order to make samples traceable through the whole analysis workflow, we propose to include a number of common SNPs in all panels/exome enrichments. By comparing the genotypes determined in the NGS analysis to genotypes obtained by another assay such as PCR genotyping upon sample entry, sample swaps can be easily detected. We propose to include SNPs from different chromosomes, to mitigate the risk of missing genotypes due to larger deletions or enrichment problems.

E.g. the following SNPs are already used in diagnostic laboratory:

chromosome	position (hg19)	reference	Variant	dbSNP id	MAF
chr1	78578177	T	C	rs6666954	0.4524
chr2	147596973	A	G	rs4411641	0.4808
chr3	60898434	T	C	rs11130795	0.4533
chr4	185999543	G	A	rs6841061	0.4382
chr5	57617403	G	C	rs37535	0.4304
chr6	131148863	A	T	rs9388856	0.4483
chr8	107236280	G	T	rs1393978	0.4038
chr9	90062823	A	G	rs12682834	0.3892
chr11	13102924	G	A	rs2583136	0.4968
chr12	68195095	C	G	rs10748087	0.4881
chr13	79766188	A	G	rs2988039	0.4799
chr16	81816733	C	T	rs8045964	0.3846
chr20	14167283	A	G	rs6074704	0.4918
chr20	48301146	G	A	rs6512586	0.4318

Chapter 5: Reporting

5.1 Introduction

Genetic laboratories typically do better than reporting genotypes as +/+ or +/-. There is a good practice of reporting and interpreting results of a genetic analysis. This practice is being assayed through peer evaluation for laboratories that participate in external quality assessment (EQA) schemes. In the context of NGS, however, the amount of information and the level of detail that can be reported, is very significant. Still, a report has to be succinct, clear and interpretable by the non-expert, but at the same time, it has to contain sufficient data for the expert to infer what has been tested, and what not, and with which technology. In view of the rapid progress in the field, and the multitude of possible combinations of platforms, kits and software tools, versioning of methods and bioinformatics pipelines is of the utmost importance.

We list the information that should minimally be included in the report, and propose a model for reporting NGS results. By addressing the issue of 'unclassified variants' (UVs) or 'variants of unknown significance (VUS) in a rather conservative way, we want to protect laboratories – and patients – from overzealous interpretation of genetic variants in a diagnostic context. In this case, as well as in dealing with 'unsolicited findings', it is important for the laboratory to define and write down its policy beforehand. In relation to the 'duty to re-contact', we define two situations that have to be clearly distinguished.

5.2 Viewpoints and examples

5.2.1 Minimal content of a report

Reports of NGS results should follow the general principles of clinical genetic reporting (Claustres et al. 2013) and be in line with international diagnostic standards ISO 15189, and with professional guidelines like those issued by the Clinical Molecular Genetics Society(CMGS) in the UK (Treacy and Robinson, 2013), by the Human Genetics Society of Australasia) (https://www.hgsa.org.au/hgsanews/guidelines-for-implementation-of-massively-parallel-sequencing; 2013), and by the Swiss Society of Medical Genetics (http://www.sgmg.ch/user_files/images/SGMG_Reporting_Guidelines.pdf; 2003)). It is essential that results are reported in a clear and consistent manner, since laboratory reports may be read by both experts and non-experts. Therefore, the use of a phenotype checklist attached to the initial request form could be considered to maximize the quality of a report provided to the clinician.

In general, it is essential to use the mutation nomenclature according to Human Genome Variation Society (HGVS; http://www.hgvs.org/mutnomen/) and to include genome build and reference sequence used for gene, transcript and variant description. The HGNC approved gene symbol should be used at least once, for reference.

In addition, it is strongly recommended to include genomic coordinates in order to ensure uniform bioinformatics analysis and consistent documentation of identified variants. Exon annotation of the identified variants is not required since version updates of the reference sequences occur frequently.

To fulfill administrative, clinical and technical requirements, a patient report should contain patient and sample identification, restatement of the clinical question, specification of genetic tests used, results, interpretation, and a final conclusion.

STATEMENT 5.01: The report of an NGS assay should summarize the patient's identification and diagnosis, a brief description of the test, a summary of results, and the major findings on one page.

The one-page report thus list all the essential data about the test. In terms of the results, this includes all class 4 and class 5 variants, evidently. Whether or not class 3 variants are reported, will depend on local practice (see section 5.2.2).
The rationale for offering a one page summary is that the clinician will probably only scan the summary, and not look at all the information. Hence, the clinically significant conclusions and the relevant test and test quality data should feature on the first page.

The full report has to be much more elaborate, and contain much more details. We propose to work with supplements (or annexes) appended to the summary report, in which important test characteristics and details are described in addition to brief, clinical diagnostic report. Each page supplement should of course carry the patient identifier, a page number and the date, and unequivocally linked to the corresponding report.
One supplement is dedicated to test characteristics and bio-informatics details of targeted capture or exome sequencing. Exome sequencing in diagnostics is often initially restricted to the analysis of a disease-associated set of genes based on the patient's clinical indications. Therefore, it is required to include a complete gene list which is diagnostically targeted in the capture assay as well as in the exome. This gene list should be selected by a team of experts, according to the criteria given in chapter 2. The validation of the assay should warrant that the listed genes are tested at high quality, as explained in chapters 2 and 4.

Furthermore, a succinct but complete description of technical issues like the target enrichment approach, the NGS platform, and the data analysis pipeline used are required in the report. Versioning is very important in this respect, and a requisite of the report.
NGS testing meets new or other limitations in its performance and analysis compared with Sanger sequencing. It is therefore essential to include in the report disclaimers related to the test performance and the analytical limitations. For example, a thorough examination of all coding exons may not always be feasible due to lack of coverage. The test might also miss specific variant types (such as CNVs, repetitive DNA, deep intronic mutations,…). Also, the report should describe the pipeline-related test limitations, such as the possibility of incorrect template mapping due to pseudogenes and unreliable calling of large deletions/insertions. An indication on how the NGS test differs from previous tests – i.e. how it compares to earlier testing (possibly already applied to the same patient) – should also be given. What is the major change, what is the benefit of the new test? This could feature on the information sheet (annex 1) or on the website or in brochures provided by the laboratory.

It is essential that reports mention whether variants reported to be pathogenic were confirmed by another independent method. There are two main reasons to confirm variants with a second independent method: (a) remaining uncertainty about the quality of the variant calling and (b)

potential samples swaps. Sample swaps can also be excluded by an independent tracking system. We refer to chapter 4 for practical instructions.
All test characteristics and bio-informatics details could also be part of a test description on a dedicated website. One can refer to this website in the patient report, but then again, versioning is important.
A second supplement would be specific for each patient and include some quality issues as well as test performance data. It is essential to report (analytical) performance related to the minimum threshold that is guaranteed for the test. It is strongly recommended to report the performance related to the clinical target which is used for analysis in a given sample (see 4.1.3). The minimum threshold should be evidence based and must have been established during the test validation process. It is recommended to include the total number of variants observed in the analyzed gene panel in this specific sample; this can be used as a monitoring quality parameter of the whole pipeline. In addition, it is required that the report states whether some regions were not well covered and not complemented by another technique in a given sample. Laboratories must be able to show detailed information about the regions that were not successfully sequenced or analysed . Laboratories make opt to make this information available either in the report or by other means (i.e. on a secure website).
It might be useful to mention which gaps were filled by Sanger (or other means) that are not attainable using NGS. It is recommended to provide the estimated diagnostic yield of the test, if possible.

A third supplement would include the variants retained after analysis of the processed data in a clear and adequately structured format. It is essential to include the inheritance analysis model (autosomal dominant, recessive, X-linked, de novo, …) applied to the sequencing data and variant files. When summarizing the variant findings, it is recommended to include the gene name, zygosity, cDNA nomenclature, protein nomenclature, genomic position.

STATEMENT 5.02: A local policy, in line with international recommendations, for reporting genomic variants should be established and documented by the laboratory prior to providing analysis of this type.

Criteria for classifying variants can be found in the best practice guidelines. A brief discussion on the classification of variants from a diagnostic standpoint is given in section 5.2.2. In general, it is recommended not to report likely benign or benign variants (class 1 and class 2 variants according to Plon et. al. 2008) but instead to report only clearly causal variants or very strong candidate variants that suggest/predict functional impairment and warrant further testing in the family. Thus, it is a requisite to report all pathogenic and likely pathogenic variants (class 5 and class 4). When multiple variants of potential clinical significance are identified, it is recommended to discuss the likely relevance of each variant to the patient's phenotype and prioritize variants accordingly. When analyzing a large set of disease-related genes, the number of unknown variants (UVs) will become high. The choice to report UVs in a patient report is a local policy, but again, it has to be described beforehand. It is strongly advised to only limit such reports to UVs found in genes relevant to the primary indication for testing. It is acceptable to report the UVs in a separate 'supplementary data file' without confirmation by a second method as long as this is clearly stated in the clinical report.

Laboratories are also strongly encouraged to deposit well-curated data from clinical sequencing into national and international databases (see section 6.2.5).

Of course, laboratories are free to apply different layouts for the presentation of the results and supplements, but the report should include all the parameters mentioned above, and the accessibility of these parameters, as well as of the results in the patient's report, should be guaranteed.

5.2.2 Variants classification

In essence, the practice to report NGS variants should not differ from the custom to report variants found with the Sanger sequencing approach but the policy on the decision making process should be clearly documented. Criteria for classifying variants are available in the practice guidelines for the 'Interpretation and Reporting of Unclassified Variants (UVs) in Clinical Molecular Genetics' (Bell et al. 2007) and in different other recent publications. There is a growing consensus concerning the classification of genetic variants according to five categories. It is recommend the use of a variant classification into these 5 levels, namely: pathogenic (5), likely pathogenic (4), unclassified UVs (3), likely benign (2) and benign (1) variants. During the discussions on this topic, it has become clear that from a clinical standpoint, three categories could suffice: pathogenic variants (i.e. mutations that require clinical 'action'), unclassified variants and benign variants (i.e. polymorphisms). However, we argue that in the laboratory, the use of 5 classes should be maintained. Clearly, the distinction between class 5 and class 4, and between class 1 and class 2, resides in the amount of evidence – and thus certainty about the classification – that is available about the individual variant. Hence, for class 5 and class 1, there should be no concern about the nature of the variant, whereas for class 4 and class 2, a community activity is needed to collect and share the available information, with the aim to definitely classify the variants into class 5 and class 1 respectively. Evidently, this applies a fortiori for class 3, where further research and data sharing are necessary to better classify the variants .

STATEMENT 5.03: Data on UVs or VUS has to be collected, with the aim to eventually classify these variants definitively.

As stated above, the report should mention whether the proposed variants were confirmed or not by an independent method. It is recommended to report as 'pathogenic mutation' (class 5) only published mutations in genes which are clearly associated with the clinical request. It is rational to assign a 'likely pathogenic' status (class 4) to nonsense/frame shift/splice mutations in genes which are clearly associated with the disease. The challenge is to unmistakably classify missense mutations. Indeed, it is recognized that bioinformatic programs give inconsistent results and that mutation database contain mistakes (false mutation/false UVs). Several parameters could help in the interpretation but will always give a subjective interpretation. This demonstrates the crucial role of a multidisciplinary team where physicians, molecular geneticists and research experts confer and collaborate to prove the pathogenicity of a missense variant. The laboratories need also to recall the purpose of the test (exclusion of a diagnosis versus confirmation of a diagnosis) during the classification of variants.

The challenge with the NGS technology is the potentially extremely high number of variants. Because of this considerable amount of data, criteria most likely need to be adapted to find an appropriate strategy. Several parameters like the inheritance (de novo, autosomal dominant, recessive or X), the penetrance, the provenance of the data (trio analyses, core disease genes panel or large set of disease related genes panel) need to be integrated in this classification strategy. We also strongly encourage the development of analysis pipelines that can include multiple functional studies and phenotype data to improve the interpretation of variants.

To support data sharing and variants interpretation, we strongly encourage the use and/or creation of national or international database where diagnostically relevant data are collected. Several initiatives have been taken in this respect.

5.2.3 Unsolicited and secondary findings

A specific aspect of NGS strategies is the possibility of detecting unsolicited and secondary findings. In this document, we do not intend to rehearse the discussion about such findings, we only wish to point out that the laboratories should deal with the issue before engaging in NGS diagnostics. Even though the use of gene panels (see chapter 2) minimizes the chance of detecting such results, it is essential that laboratories have a clearly defined protocol for addressing unsolicited and secondary findings (see chapter 3).

As discussed in chapter 3, unsolicited findings and carrier states on genes included in the tested gene panel should be in the main report. The protocol should further define, prior to the result being available, (i) which secondary findings will systematically be searched for and reported in an additional separate data file or will be available on request; (ii) if unsolicited and secondary findings will be routinely confirmed by independent methods.
Commonly encountered examples of unsolicited and secondary findings detected during testing include: detection of carrier status for autosomal recessive disorders; detection of variants involving genes associated with dominant, adult-onset conditions; detection of variants related to cancer; detection of variants involved in pharmacogenetics.

STATEMENT 5.04: Laboratories should have a clearly defined protocol for addressing unsolicited and secondary findings, prior to launching the test.

Recent publications address this issue and discuss procedures how to report on unsolicited and secondary findings (Berg et al., 2013; Christenhusz et al., 2013; McGuire et al., 2013; van El et al., 2013). Uncertainty associated with reporting unsolicited and secondary findings is usually best managed with input from a medical genetic specialist. Clinicians may give patients the option of not receiving certain results (see chapter 3, informed consent).

5.2.4 Duty to re-contact

A diagnostic request is a contract at a certain point in time. The contract is finished once the lab has delivered a report.
The number of genes included in a gene panel will never be stable in time: as research evolves, more genes will become known for the heterogeneous diseases. Hence, a laboratory will only be able to offer what is known, and validated, at a given point in time. Even though novel

information about the disease may be hidden in the (raw) dataset, it is not possible to reiterate the question, i.e. reanalyse the patient's data again and again, in a diagnostic setting.

STATEMENT 5.05: The laboratory is not expected to re-analyse old data systematically and report novel findings, not even when the core disease genes panel changes.

The patient is responsible to recontact the physician. The lab cannot be made responsible to reinvestigate all the raw data nor to (re)classify all the variants that may have been detected before.

However, situations do occur when a variant changes from one class to another. Most often, it would concern a reclassification of a class 3 variant. However, it could also happen to other variants: a class 5 or class 4 variant may eventually be found to be non-pathogenic (or at least not causally related to that particular disease), or a class 1 or class 2 variant may be found to be pathogenic (or at least contribute to the phenotype). Class 3 variants would either be transferred to class 4 or 5, or class 1 or 2.All these changes would alter the conclusions of the diagnostic results and would have a significant impact on the clinical management of the patient. If at a particular moment, it is decided – by the lab or by the community of experts in the disease - to change a variant from class to another, the lab is responsible for reanalyzing the available data, to re-issue a report on the basis of the novel evidence, and also to re-contact the other patients, analyzed before, that are possibly affected by the new status of the variant. Again, this is not different from what people would do with data obtained by Sanger sequencing and other methods.
Such a situation can only be managed efficiently if the laboratory has installed a system that effectively links patients and variants, and allows for the retrieval of the affected cases when variants are re-classified.

STATEMENT 5.06: To be able to manage disease variants, the laboratory has to set up a local variant database for the different diseases for which testing is offered on a clinical basis.

Evidently, it is a daunting task to keep track of all variant reclassifications. Hence, well-curated (private or public) databases are needed to aid the diagnostic laboratories in this task.

5.3 Comparison to other guidelines

According to Ellard et al. (2014), the diagnostic report should follow the general principles of ACGS reporting best practice guidelines. The report should contain the test characteristics, the regions sequenced and analyzed (successfully or not), the type of variants detected and uniformity and average depth of coverage (Gargis et al. 2012, Weiss, Van der Zwaag et al. 2013, Rehm et al. 2013). If the assay includes core genes, the name of this genes must figure in the report with their status of core genes (Weiss, Van der Zwaag et al. 2013). For Ellard et al. (2014) and Rehm et al. (2013), reports of negative results must include the expected diagnostic yield as well as the genes and regions analyzed, the analytical sensitivity, the spectrum of detectable mutations and the limitations of the assay. The Australian guidelines state that the test

limitations should always be reported. Similarly, the conclusions of the CLARITY challenge state that it is critical to provide regions where coverage is insufficient (Brownstein et al. 2014).
Mutations must be described according to Human Genome Variation Society including information on genome build, reference sequence used for variant description, genomic coordinates. Weiss, Van der Zwaag et al. (2013) advise not to include the exon number while Rehm et al. (2013) advise its inclusion. Rehm et al. (2013) proposed that zygosity should also be reported.
Variants should be consistently categorized according to their clinical significance and this classification should be evidence-based. Filtering strategies must be outlined in the report (Weiss, Van der Zwaag et al. 2013, Rehm et al. 2013). Benign variants (common, well-known polymorphisms) should not be reported for Australian guidelines while the decision is left to the laboratory by Rehm et al. (2013). References for previously reported mutations should be included in the report (Ellard et al. 2014, Australian guidelines, Rehm et al. 2013).
For Ellard et al. (2014) UVs must be reported, according to ACGS best practice guidelines, in a separate technical report without Sanger confirmation. The Australian guidelines recommend to set up a protocol to address UVs and report them clearly and consistently. Weiss, Van der Zwaag et al. (2013) restrict the report of UVs to core disease genes whether or not they have been confirmed. Rehm et al. (2013) recommend the reporting of UVs in genes relevant to patient's indication.
Australian guidelines recommend laboratories to systematically review variant interpretations and have a formal process for evaluating new evidence, re-interpreting, re-contacting and contributing to patient reviews. The report must contain information on data storage and protocols for re-analysis and call-back. According to Rehm et al. (2013) laboratories should provide clear policies on the reanalysis of data and whether additional charges may apply. Physician should inquire whether status of UVs and likely pathogenic variants has changed.

Important publications on the classification of variants have appeared recently, including a publication specifically on the interpretation of *de novo* variants (e.g. Kircher et al. 2013, McArthur et al. 2014, Samocha et al. 2014). For some diseases, the diagnostic and research communities have gathered additional information and fine-tuned the classification accordingly (e.g. Hofman et al. 2013, Thompson et al. 2014). It is noteworthy these recent, influential publications have also raised the bar for the interpretation of genetic variants in a research setting.

Contributions

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Comparison to other guidelines written by Erika Souche

Chapter 6: Distinction between research and diagnostics

6.1 Introduction

Genome wide approaches such as exome and genome sequencing are routinely used in research to discover new candidate genes responsible for (rare) diseases. However these approaches may also reveal causative mutations in known genes that were not tested for beforehand. With the increasing possibilities of genome wide testing in diagnostics and research, the line between diagnostics and research is blurred.
This chapter describes what can be done with diagnostic patient data and for what type of analyses a specific (additional) research consent is needed.

6.2 Viewpoints and examples

6.2.1 Definitions of diagnostics and research

A diagnostic test is any kind of medical test performed to aid in the diagnosis or detection of disease. In genetics, this means that the genetic material of an individual is either searched for likely pathogenic variants that can explain the phenotype of a patient, or searched to show that a certain individual is not at risk of developing the disease that runs in the family. The diagnostic test used might be very specific, e.g. sequencing a certain gene or even a certain exon of a gene, but might also be less specific, e.g. genome wide copy number variant (CNV) detection to elucidate the cause of intellectual disability. Diagnostic testing is performed in specialized laboratories which produce reliable results conform to the requirements for quality and competence particular to medical laboratories (ISO15189 or comparable).

STATEMENT 6.01: A diagnostic test is any test directed towards answering the question related to the medical condition of a patient.

Research is usually aimed at the discovery and interpretation of new facts. Examples of genetic research are the elucidation of the genetic cause of a disease, to learn more about the pathogenesis of a genetic condition, or to unravel the function of specific genes. In general, a group of patients with the same genetic disease is needed to find the cause of the disease. Moreover, valuable research can only be performed when it is started with a project plan involving a hypothesis, a time schedule, and preferably preliminary data.

STATEMENT 6.02: A research test is hypothesis-driven and the outcome may have limited clinical relevance for a patient enrolled in the project.

6.2.2 The differentiation between diagnostics and research

The above mentioned definitions of diagnostics and research seem clear at first sight, but with the implementation of NGS in genetic testing, the line between diagnostics and research gets blurred. Everyone in the genetic field will accept that NGS can be done diagnostically when a gene panel is sequenced. Analysis of a gene panel after exome/genome sequencing is merely the same, but when it comes to analyzing the data of the rest of the exome/genome (when no mutations have been identified in the gene panel), people have different opinions on whether this can be done in a diagnostic setting. However, the analysis of whole exome/genome data can be performed in order to get a diagnosis in one particular patient/family (e.g. by looking at *de*

novo mutations in a trio, looking at homozygous mutations in consanguineous families, or by looking at genes involved in a certain pathway). In these cases, the results of the diagnostic test might not always lead to a direct diagnosis, but can be a starting point for further research (like segregation analysis in the family, functional analyses etc).

STATEMENT 6.03: The results of a diagnostic test can be hypothesis-generating.

6.2.3 What type of NGS can be done in a diagnostics laboratory?

If we keep in mind that a diagnostic test can be done as long as the result of the test can give a diagnosis for this particular patient/family, it is clear that the parallel testing of several genes involved in a heterogeneous disease (either by targeted sequencing or by targeted analysis of exomic/genomic data) can be offered in a diagnostic setting (Neveling et al. 2013). On the other hand, the search for a new disease gene using the exomic or genomic data from several patients with the same phenotype is a clear example of genetics research. One could argue that the analysis of exome orgenome data for the identification of a genetic defect in a particular patient/family (e.g. *de novo* analysis in a case-parent trio with intellectual disability) would also belong to research, but since the test is aimed at getting a diagnosis in this particular patient, it can be practiced in a diagnostic setting. It has been shown that analyzing exome data for *de novo* mutations has a high diagnostic yield (de Ligt et al. 2012, Rauch et al. 2012). Furthermore, it has been widely accepted that genome wide CNV detection with array CGH can be performed in a diagnostic lab; it has never been stated that laboratories only should look at known pathogenic CNVs. Hence, the use of exome or genome in a diagnostic setting is acceptable, if the objective is diagnostics indeed. Nevertheless, the identification of a novel gene related to disease is not within the realm of a diagnostic lab.

STATEMENT 6.04: Diagnostics tests that have the primary aim to search for a diagnosis in a single patient should be performed in an accredited laboratory.

Diagnostic laboratories should have a quality management system in place, and should aim at accreditation. The issue is not different for classical genetics versus NGS, but the burden of validating an NGS test and the newness of the platforms and applications should not be used to postpone or decline accreditation for NGS. Currently, most NGS tests are laboratory developed tests (LDT, which distinguished them from e.g. CE marked kits). This does not exempt them from quality assurance or accreditation, on the contrary. Both the IVD Directive and the ISO 15189 norm deal with them equally.

6.2.4 A duty to confirm research results in a diagnostic setting

When participating to a research project, patients and families must be aware that such a project may lead to a diagnosis. In this case, only clinically relevant results should be transferred to the patient's medical record and a protocol has to be defined within the research institute and clinic, for this transfer. This has become a major concern for both diagnostic laboratories and clinicians. Indeed samples from patients with specific phenotypes are increasingly and easily submitted to exome/genome sequencing in research studies, whereby the primary aim is not the research per se, but the resolution of an individual case or family – with the expectation, of course, that the results will be sufficiently interesting to warrant further publication. The clinicians, involved in such studies, either return the results instantly to the patients, or, as has happened in many

studies before, forget to return the results, so the family does not even get aware of the fact that a genetic cause of the disease was found.

STATEMENT 6.05: Research results have to be confirmed in an accredited laboratory before being transferred to the referring clinician and patient.

The argument is not about returning the results, but about making sure that certitude about the results is warranted before returning them to the patient. All conclusions relevant to the clinical file should be confirmed in an accredited lab, on an independent sample and communicated to the patient. There is no need to repeat the NGS analysis, as this would be overtly overshooting, but the pathogenic mutation that has been retained after a thorough interpretation of the results in a research context, has to be retested using Sanger sequencing (or the appropriate technology, in case the causal mutation is not detectable by Sanger, e.g. an exonic deletion). The diagnostic laboratory has to report on the analysis in a clinical report, stating why this specific analysis has been done and referring to the research data and research group. It may include a disclaimer of the sort of "The original result was obtained in a research context. The conclusions in this report are based on the assumption that this mutation is indeed the cause of the disease in this family. The latter has not been independently evaluated by the diagnostic laboratory."'. This practice is valid, even if it would incur costs for the testing (and thus possibly also for the patient).
For good clinical practice, it is advised to stick to the diagnostic needs of the patient, and not submit a sample to a research project until the diagnostic tools have been exhausted.

6.2.5 Share mutations and variants in international databases

Increasing the number of genes tested obviously leads to an increased number of variants that must be interpreted and classified. Although databases of variant frequencies provided by, among others, the Exome Sequencing Project (ESP; https://esp.gs.washington.edu/drupal/) or the 1000 Genomes Project (The 1000 Genomes Project Consortium, 2012), help distinguishing causative mutations from common variants, they may lack population specific variant frequencies. Most laboratories set up a database of variant frequencies of all locally sequenced and/or analyzed samples (ideally healthy parents) in order to ease variant interpretation. Such a database does not contain any sensitive information since only the frequencies of the variants (and sometimes the genotype counts) in the screened populations are reported. It could thus be shared across laboratories but this is unfortunately not often the case.

STATEMENT 6.06: The frequency of all variants detected in healthy individuals sequenced in a diagnostics and/or research setting should be shared.

If variant databases of healthy individuals help excluding variants, databases of pathogenic variants allow the identification of causative mutations and are thus equally, if not even more, important. Such databases include LOVD (Fokkema et al. 2011), HGMD (Stenson et al. 2014), MutDB (Singh et al. 2008), etc. Ideally, all variants detected in disease linked genes should be submitted to databases and linked to the clinical data of the patient. The criteria and arguments used for variant classification should also be described.

STATEMENT 6.07: All reported variants should be submitted to national and/or international databases.

The software for data management and for reporting genetic results should provide a mechanism to (automatically) contribute diagnostically validated results to international databases, to encourage participation in the collection of variant information. It is important to get this message of sharing data across.

6.3 Comparison to other guidelines

The distinction between diagnostic and research is barely mentioned in guidelines published so far. According to the Australian guidelines, diagnostics is based on evidence from peer-reviewed sources and genes with weak evidence should be used for research only. For Rehm et al. (2013), gene discovery was historically limited to research laboratories but can now also be done in clinical laboratories. However the follow up must be done in association with research laboratories.

Ellard et al.(2014) state that when transferring results from research to diagnostic, it is necessary to collect a new sample for result confirmation.

The Australian guidelines encourage laboratories to establish an internal database of genomic findings to allow the identification of common variants specific to patient population and recurrent false positives. Such a database should comply with regulatory and legislative requirements. Genomic data such as population frequencies and referenced clinical relevance of each variant should also be submitted to public databases. Ideally, both phenotypes and genotypes should be shared but this has to comply with privacy concerns. Ellard et al. (2014) propose to first share reported variants in public databases such as Diagnostic Mutation Database (DMuDB) but to aim at sharing all variants (including polymorphisms) and associated phenotype from every patient. Weiss, Van der Zwaag et al. (2013) suggest to use the existing databases to submit variants and encourages the development of national and international databases of reported variants for diagnostic laboratories only with traceable origin of submission. Rehm et al. (2013) recommend to deposit data to public databases such as ClinVar.

Contributions

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Comparison to other guidelines written by Erika Souche

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