The use of new technology to improve genetic testing

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Chapter 1

1. General introduction

The identification of the molecular genetic basis is crucial for the definitive diagnosis of individuals with congenital malformations and inherited diseases and for the risk evaluation in relatives. Currently, molecular genetic diagnosis depends on the recognition of distinctive clinical features (syndrome), on linking the syndrome to a known underlying defect, and on the availability of a laboratory that offers the diagnostic test for the particular gene(s) of interest (1-2).

In the majority of cases, one sequences the gene(s) of interest to look for genetic variation that can explain the clinical phenotype. Various techniques are available for targeted sequencing, for example, conventional Sanger sequencing (3) and Next Generation Sequencing (NGS) (4-8). Sanger sequencing is the gold standard for diagnostic analysis of single candidate genes. Most diagnostic DNA laboratories worldwide use this method for the identification of disease causing mutations. Although DNA testing by Sanger sequencing is useful for most Mendelian diseases, its use is still hampered by limited throughput and high cost. However, one can enhance the speed and reduce the cost by using any one of several pre-sequencing screening methods for DNA fragment analysis (9-12). One can then sequence only those fragments that contain the variants. We have described the various pre-sequencing methods in **Chapter 2**. Among these, High Resolution Melting Curve Analysis (HR-MCA) offers a cost efficient, fast and convenient method for assessing the presence of variants in a diagnostic setting (13).

However, when there are too many samples and /or too many possible candidate genes to be tested in patients with genetically heterogeneous disorders, this combined technology (HR-MCA followed by Sanger sequencing) becomes time consuming, labour intensive and inefficient. For these diseases, such as, cardiomyopathies (14), retinitis pigmentosa (15), deafness (16), Noonan syndrome (17), one can use NGS to search for gene mutations. NGS circumvents the bottleneck

by interrogating all candidate genes or genomic regions of interest simultaneously (4-7). It is useful for sequencing DNA on a massive-scale, even an entire human genome (8, 18-21). The problem is that Whole Genome Sequencing (WGS) is very time consuming for many applications and is likely to remain prohibitively expensive for some time. However, a number of methods are now available to select targeted regions for sequencing in a more cost and time efficient manner (22). Such methods, described collectively as genome capture or genome enrichment technologies, include PCR- based methods (long range PCR or multiplexed short PCR) (23, 24), capture by hybridization (on–array and in–solution) (25-29), and capture by circularization (30).

The aim of the work reported in this thesis was to optimize, test and apply different new molecular techniques, which include HR-MCA, targeted sequencing and exome enrichment followed by NGS. The purpose of this was to facilitate the detection of disease causing mutations in several disorders with suspected Mendelian inheritance, to speed up the identification of disease genes and to provide a systematic tool for classifying previously intractable genetic diseases.

We review a number of the above-mentioned new techniques below. We also present several technical approaches for detecting candidate pathogenic variants in different Mendelian disorders.

1.1 High Resolution – Melting Curve Analysis (HR-MCA)

The introduction of the Sanger sequencing method over 30 years ago marked a milestone in the history of genetic analysis. It quickly became indispensable for basic biological research and in various applied fields, such as biotechnology, diagnosis and forensic biology. The key principle of this methodology is to utilize dideoxynucleotide triphosphates as DNA chain terminators and to use specifically labelled nucleotides to read a DNA template during DNA synthesis (3).

A series of technical modifications and innovations have slowly improved the accuracy and efficiency of the Sanger method and have finally led to the development of automated Sanger sequencing (31-34). The automated Sanger method became the method of choice for DNA sequencing and has dominated the field for almost two decades. During this time, the capacity of

the Sanger method has become so enhanced that it can read up-to 1000 base pairs per sequencing reaction (34).

Sanger sequencing of single candidate genes for a given disease is useful for diagnosis if minimal locus heterogeneity and distinctive clinical symptoms exist. However, the high cost of sequencing and the rarity of many conditions still hampers the application of this method to many Mendelian disorders. The number of clinical situations where results of testing for DNA mutations has implications for the management and treatment of patients is increasing. The already overburdened laboratories with serious financial constraints have to deal with the growing demand for rapid turnaround time. Therefore, one needs cost-effective techniques that are simple to perform, and which show high sensitivity and specificity. HR-MCA has been attracting more and more attention among analytical nucleic acid techniques in recent years. It is a simple, powerful and robust post-PCR analysis method for scanning of variants and for genotyping (13).

HR-MCA is based on a melting (dissociation) curve and does not require sample processing after PCR. This technology was made possible by the recent advances in real-time PCR instrumentation (that allows for highly controlled temperature transitions and data acquisition) and by the availability of improved double-stranded DNA (dsDNA)–binding saturation dyes and hardware and software to monitor and analyze the melting. These advances allow accurate detection of sequence variations based on melting analysis. The advantage here is that one does not need to use labelled probes and not all amplicons need to be sequenced (35-37). The fluorescent binding dyes, known as intercalating dyes, do not inhibit PCR. They have a high fluorescence when bound to dsDNA and low fluorescence in the unbound state (13). HR-MCA is done after PCR amplification of the region of interest in the presence of a dsDNA dye. The amplicons are warmed up and the fluorescence data is collected at 55–95°C at a temperature transition rate of 0.1 °C/s and 200 data points/°C (Idaho Technology Inc., Salt Lake City, UT). Specific PCR amplification of the intended targets is critical, requires careful design of primers, the correct length of the PCR product and optimal number of cycles.

At the beginning of the HR-MCA, there is a high level of fluorescence in the sample, but as the sample is heated up and the dsDNA melts into single strands, the dye is released resulting in a

change in fluorescence. The fluorescence reduces as the number of double stranded DNA fragments decrease (35, 36, 38). A camera in the machine monitors this process and the machine plots the data in a graph known as a melting curve that represents the level of fluorescence and the temperature (Idaho Technology Inc., Salt Lake City, UT). The fluorescence signal is plotted against the temperature and the fluorescence data that is generated is assessed, based on the shape of the melting curve or on the melting temperature (Tm) (38, 39). The highest rate of decrease of the fluorescence signal occurs at the melting temperature (Tm) of the DNA amplicon. The Tm is the temperature at which 50% of the DNA sample is double stranded and the other 50% is single stranded. In general, the Tm is higher when DNA fragments are long and/or have a high GC content (40). The fluorescence data collected during the HR-MCA ranges from pre-melt (initial fluorescence) to post-melt (final fluorescence) signals. The raw data is first normalized by selecting the pre-melt and post-melt regions for each primer set, onto a 0 to 100% scale (Figure 1A) before plotting the HR MCA curves. The temperature is then shifted in order to reduce the well-to-well variations. The amplicons with heterozygous variants can then be separated from the wild type and this is visible in the distinct shapes of the melting curves (Figure 1B) (38-40).

To visualize normalized data, difference plots can be generated (Figure 1 C) by subtracting the curves of the samples that are analysed from a reference curve. The machine automatically clusters samples with similar melting curves/genotypes into groups. So the distinct shape of the melting curve, the derivative plot, and/or the difference plot can be used for amplicon analysis (Idaho Technology Inc., Salt Lake City, UT). Amplicons that amplify poorly will have low fluorescence and should not be analyzed further.

The melting temperature of an amplicon at which the double DNA strands separate is predictable and depends on the sequence of the DNA bases. This allows one to compare different samples, which should give the same shaped melting curve, for the same amplicon. However, if one of the selected samples has variant in the amplified DNA sequence, it will alter the melting curve profile. In diploid organisms, which have two alleles, there are three possibilities for a given variant: both alleles contain the variant (homozygote variant), either allele has the variant (heterozygote), neither allele contains a variant (homozygous wild type).

One can distinguish between homozygous samples by a shift in the Tm, and between heterozygous samples by changes in the shape of the melting curve (13, 41, 42). However, not all homozygotes can be readily distinguished; homozygous or hemizygous variants can easily be missed due to subtle differences between some variants. The standard solution to overcome this problem is to mix patient DNA with wild-type DNA in order to generate heteroduplexes.

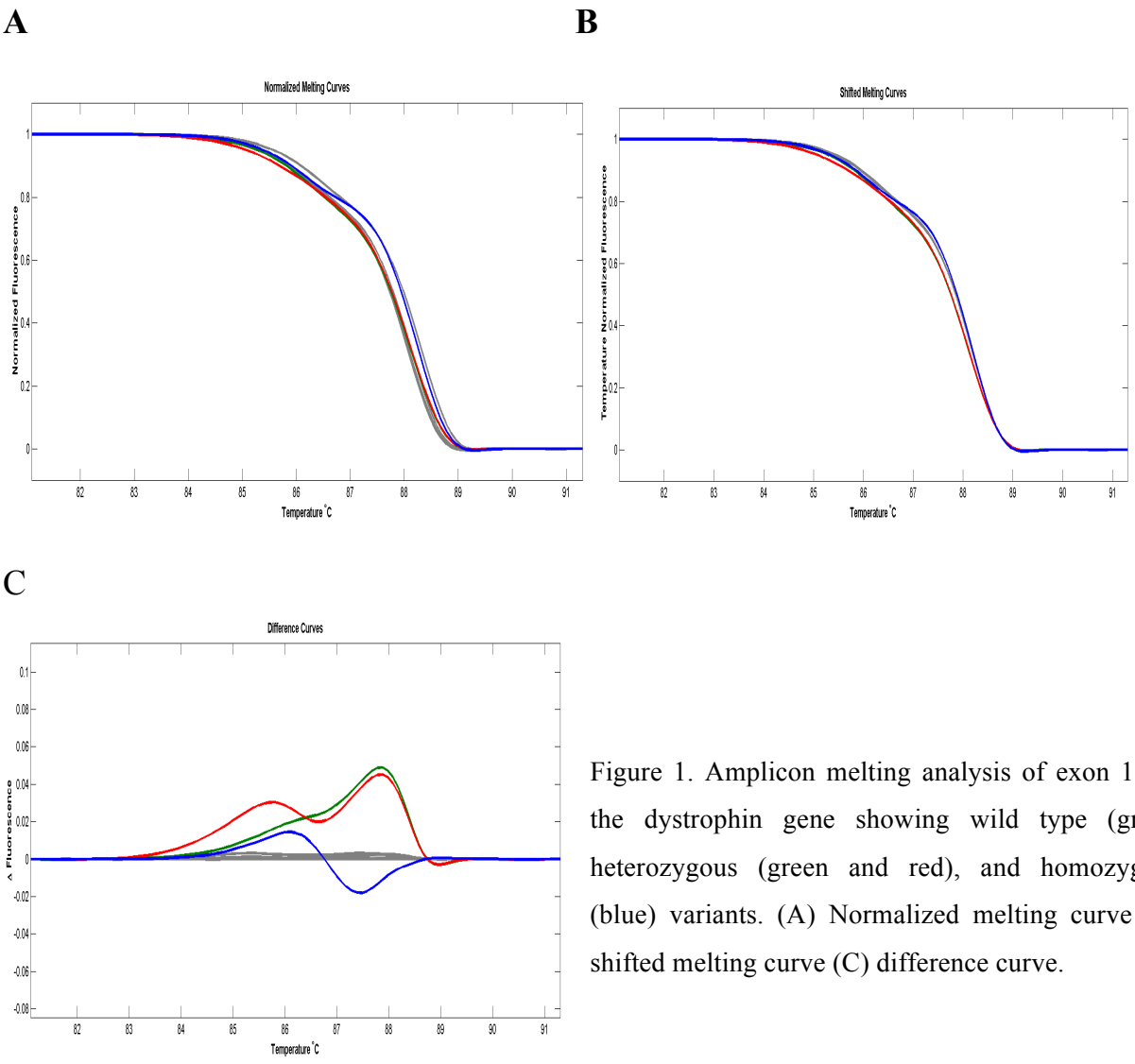

Figure 1. Amplicon melting analysis of exon 17 of the dystrophin gene showing wild type (gray), heterozygous (green and red), and homozygous (blue) variants. (A) Normalized melting curve (B) shifted melting curve (C) difference curve.

The melting profile of a PCR product depends on its GC content, its length and its sequence (39). Short PCR products normally melt in a single transition while longer PCR products often melt in multiple transitions corresponding to melting domains of different stability.

The HR-MCA method is simple and flexible, has minimal requirement for optimization and has superb specificity and sensitivity. For this reason it is used by a wide range of disciplines for a variety of applications such as DNA methylation analysis (43-45), genetic mapping (46), species identification (47), HLA compatibility typing (48), genotyping and mutation detection; the last of these is discussed in detail in the following section.

1. 1.1 Genotyping and mutation detection

It is important to determine differences in the genetic make-up (genotyping) for the study of genes and gene variants associated with disease. HR-MCA was first used as a genotyping technique (13). However, in most cases, the shape of an HR-MCA curve in itself is not sufficient to type a specific variant (40); moreover, the type of base change, the presence of a homozygous and/or a non-pathogenic variant (common variants) may complicate the interpretation of the melting profiles. Either adding an unlabeled probe (49, 50) or sequencing the fragment can solve the problem. Unlabeled probes are around 30 base pairs in length and are blocked at their 3´-end to prevent extension. An excess amount of the strand complementary to the probe is produced by asymmetric PCR (1:5 or 1:10 ratio). Unlabeled probes can be designed to match either the variant or wild type sequence. Data can be viewed by using the derivative plot: if two melting regions are visible, the allele that is complementary to the probe will show a single peak at the highest temperature, whereas other alleles will produce a peak at lower temperatures. Typically, heterozygotes will display two peaks representing the two alleles (39, 51).

Hundreds of variants in many genes that are associated with genetic diseases, with autosomal recessive, autosomal dominant or X-linked inheritance, have been examined; more than 60 different genes have been analyzed using HR-MCA (35, 39, 51-61).

In **Chapter 2** of this thesis, we show the successful application of HR-MCA as a pre-sequencing screening method. We have optimized and validated the HR-MCA method and used it in combination with dsDNA dye LCGreen Plus to scan all coding exons and the exon/intron junctions and to genotype frequently found variants in the largest known human gene to date, the DMD gene. Mutations in the DMD gene can cause Duchenne and Becker muscular dystrophy (DMD). We found that amplicons up to 600 base pairs and more can be used for HR-MCA but the technique is more sensitive when shorter fragments, that result in melting profiles with no

more than two melting domains are tested. In addition, we found that HR-MCA is capable of distinguishing amplicons that differ by a single base pair. Therefore, we can use it to detect single nucleotide substitutions and small deletions and duplications. HR-MCA is a highly reliable and a quick method for mutation scanning and genotyping. It is now ready for routine diagnostic use on patients with Duchenne or Becker muscular dystrophy and on female carriers.

1.2 Next generation sequencing (NGS)

The ability to read the sequence of bases that comprise polynucleotide molecules such as DNA has had an enormous impact on biological and medical research. Sanger sequencing is the gold standard sequencing technology since 1977 (3). It has led to a number of monumental accomplishments, including the completion of the human genome sequence (62). However, the limited throughput of Sanger sequencing makes it expensive, laborious and time consuming, and therefore unsuitable for large-scale sequencing projects. The advent of Next-Generation Sequencing (NGS) technologies in 2005 has changed the paradigm of DNA sequencing and has opened fascinating new opportunities in biomedicine (4-6). NGS technologies have made it possible to process hundreds of thousands to millions of DNA templates in parallel. This results in a high throughput (gigabase) scale and low cost per base (6, 7). The cost of DNA sequencing keeps reducing due to rapid innovations in sequencing technology. The inexpensive production of huge amounts of sequence data is the main advantage of NGS over the Sanger sequencing method.

NGS uses a number of different technologies that have appeared since 2005. In several NGS methods, fragmented genomic DNA ligated to universal adaptors amplifies into PCR colonies. Each colony has many copies of the same fragment, and some NGS methods can sequence all of them in parallel, whereas other NGS methods read single DNA sequences. Older NGS technologies read sequences from one end while newer platforms allow for paired-end sequences (4-8). Once the NGS produces a sequence, the sequenced data is mapped to a reference genome, such as the human reference genome, which provides the basis for all subsequent steps of data analysis. Several NGS platforms are now available on the market and among them, the Roche/ 454, Illumina (Genome Analyzer/ Hiseq), and the Life Technologies SOLiD System are the

commercially dominant ones. Table 1 shows a summary and a comparison between these three different platforms.

Table 1. A summary and a comparison between three NGS platforms.

Companies	**Roche GS FLX**	**Illumina-Solexa**	**Life Technologies**
Company homepage	http://www.454.com	www.illumina.com	http://www.appliedbiosystems.com
Platforms	GS FLX Titanium, GS 20	HiSeq 2000, Genome Analyzer II (GA II), Solexa platform	ABI SOLiD, SOLiD 4
Sample requirements	1 µg for shotgun library, 5 µg for paired end	<1 µg for single or paired-end	<2 µg for shotgun library, 5–20 µg for paired end,
duraton of library prep/feature generation (days)	3–4	2	2–4
Method of feature generation	Bead-based/emulsion PCR	Isothermal bridge PCR amplification on flow cell surface	Bead-based/emulsion PCR
Chemistry	Pyrosequencing (sequencing-by-synthesis with pyrophosphate)	Reversible Dye Terminators	Sequencing by ligation
Reads per run	1 million	up to 3 billion	1.2 to 1.4 billion
Raw accuracy	99.99%	98%	99.99%
Read length	700 bases	50 to 250 bp bases	50+35 or 50+50 bp
Sequencing run time	10-24 hours	1 to 10 days (based on the sequencer)	6 days
References	63-67	68-70	66, 67, 71

In a relatively short time, NGS technologies have revolutionized the research on the human genome. They have been applied to genomic sequences, the trancriptome (RNA seq) and chromatin immunoprecipitation in combination with DNA microarray (ChIP- chip) or sequencing (ChIP-seq) (72-98).

In the following sections, we address the important features and applications of NGS for whole-genome and targeted re-sequencing.

1.2.1 Whole-genome and targeted re-sequencing

The most common use of NGS platforms has been re-sequencing (99). The introduction of these technologies has made it possible for some laboratories to sequence an entire human genome.

Several completely sequenced human genomes have been published so far, for example: human genome sequences of the well known James D. Watson and of an African, a Chinese and two Korean individuals (18, 19, 100- 102). Sequencing whole human genomes will lead to a deeper understanding of the full spectrum of genetic variation. It will also throw some light on the role of genetic variation in phenotypic variation and disease susceptibility. However, the cost and capacity required for whole genome sequencing (WGS) is still significant. Routine sequencing of large numbers of whole human genomes is not yet feasible. Nevertheless, we expect that it will become routine in the near future. For the time being, for time and cost effectiveness, we have to select and enrich genomic regions of interest before sequencing. Moreover, the resulting data from target-enrichment methods is significantly less cumbersome to analyze. Thus, for some projects, the sequencing of large numbers of samples after targeted enrichment provides more answers to biological questions than the sequencing of the whole genomes of fewer individuals.

Several methods to target specific areas in the genome prior to NGS have been developed (86). In general, there are three categories: PCR based methods (103- 105), capture-by-circularization (106-108) and capture-by-hybridization (25, 27, 28, 109).

1.2.1.1 PCR

For over 20 years, PCR has been the most widely used pre-sequencing technique for sample preparation, as it is compatible with Sanger sequencing (3). PCR is also potentially well suited for NGS platforms, but to make full use of the high throughput, a large number of amplicons or samples must be pooled and sequenced together. However, multiplex PCR is difficult to perform since simultaneous use of many primer pairs can lead to differential amplification, formation of primer dimmers and to high rates of mispriming events (110, 111). Some amplicons may even fail to amplify. In addition, the length of amplicons that can be generated by long range PCR is limited (111, 112).

In our experience, working with very long PCR fragments tends to be laborious, time consuming and expensive. This is because each individual PCR has to be optimized, with a maximum length of 11 kilobases, in order to make amplification as efficient as possible. Theoretically, you can design primers for long range PCR for all desired targets. However, in practice, not all reactions will yield a PCR product. This can also be a problem when amplifying fragments with a low

DNA integrity. Furthermore, the presence of SNPs in the primer annealing regions may lead to amplification of only one allele. These problems can be overcome by redesigning and optimizing the primer, and using a combination of short and long range PCR. After PCR amplification, the concentration of uniplex PCR products should be normalized to avoid sequencing one dominant PCR product from one sample or amplicon above all others. Only then, can one pool the PCR products before sequencing. The RainStorm method circumvents many of the problems of the standard PCR–based approach. RainDance Technologies have developed this method (http://www.raindancetechnologies.com), which involves the use of emulsion PCR (70).

1.2.1.2 Capture-by-circularization

Molecular Inversion Probe (MIP) belongs to the category of molecular techniques that capture sequences by circularization. MIPs were initially developed for multiplex target detection and SNP genotyping (113, 114). In this technique, single-stranded oligonucleotide molecule (probe) consists of two target complementary arms separated by a linker region (one or two sequence tags and two amplification primers common to all MIPs). This assay is performed in three steps: hybridization of probes to the target sequences, circularization of bounded MIPs by ligase and amplification using common primers. The main advantages of capture-by-circularization with MIPs are reproducibility and high specificity with high levels of multiplexing (at least 300,000 independent targets). It can be applied directly to genomic DNA, which needs low amounts of starting material. Moreover, MIP amplification products can be directly sequenced by NGS (22, 107, 108).

1.2.1.3 Capture-by-hybridization (Hybridize capture)

Capture-by-hybridization can selectively target any specific area in the genome, such as genes of interest, linkage regions, whole chromosomes (X-exome) and all exons (exome sequencing) (22, 115). This approach relies on the hybridization of fragmented genomic DNA libraries to a complex mixture of capture probes. The capture probes may potentially be in solution (28) or fixed to a solid matrix such as a microbead or a glass surface (on array) (25, 26). This method has clear advantages over other methods that are based on the extension or ligation by an enzyme. These include the possibility of greater degree of multiplexing and potentially higher tolerance for polymorphisms that overlap with the present capture probes (22).

In the on-array capture methodology, total genomic DNA is fragmented and applied to the probes; non-bound fragments are removed by washing after hybridization. The hybridized DNA fragments are then eluted and enriched for sequencing (25) (Figure 2). Roche/NimbleGen was the first to adapt this technique. They provide different types of microarrays with different capture size varying from 5 Mb to 34 Mb on a single array, with different numbers of probes with lengths ranging from 60 to 90 basepairs. Agilent also offers commercial kits implementing this technology.

In our hands, on-array target enrichment of large targeted regions showed several advantages over PCR methods: it is quicker, cheaper and less laborious. However, there are also disadvantages: working with large numbers of samples is not feasible, because arrays that are hybridized at the same time must also be eluted together and a single person cannot perform more than 20 arrays per day. Working with arrays requires expensive equipment such as a hybridization station. In addition, irrespective of whether the on-array capture experiment is for 100 kb or an entire exome, a large amount of input DNA is needed (10–20 μg) to start a library preparation.

In **Chapter 3**, we describe custom high-density microarrays (NimbleGen) to enrich exons and intron-exon junctions of 112 distinct genes potentially involved in mental retardation and congenital malformation, which were sequenced on the Illumina analyzer (Solexa). Our results show that this methodology offers a versatile tool for successfully selecting sequences of interest from the total human genome. In addition, we have discussed a number of advantages and disadvantages characteristics of this methodology. To overcome many of the disadvantages, Agilent, NimbleGen, and other companies have developed an in-solution-based target enrichment. In-solution capture is similar to the on-array capture method, but the probes in this technology are biotinylated (DNA or RNA bait) and not attached to a solid support.

Many different methods have been described for targeted hybrid capture but only a few have been extended to capture the whole human exome (exome sequencing) (http://www.illumina.com/products/truseq_exome_enrichment_kit.ilmn, http://www.genomics.agilent.com/,http://www.nimblegen.com/exomev3launcheq/)

1.2.1.3.1 Exome sequencing (ES)

Elucidation of the genetic basis of rare and common human diseases is the main goal of genetics and molecular biology. To date, causal variants for about 3,000 different Mendelian disorders have been identified (Online Mendelian Inheritance in Man OMIM, http://www.ncbi.nlm.nih.gov/omim) (116, 117).

In the past two decades, linkage studies followed by positional cloning and homozygosity mapping have been very successful in identifying causal mutations for Mendelian disorders (118, 119) due to a perfect segregation pattern of the causal variant with the disorder. However, these traditional methods are not suitable for studying all Mendelian disorders. Several factors limit the power of these methods such as, availability of patients for investigation, small number of cases or families (extremely rare Mendelian disorders), sporadic cases where variants have arisen de novo during meiosis, reduced penetrance and locus heterogeneity (117).

Exome sequencing encompasses 1% of the genome and includes 180,000 exons from more than 20,000 genes. It has become the main tool for studying the genetic causes of Mendelian disorders and of sporadic cases in which traditional methods have failed (120-123). Even when the traditional methods are expected to succeed, ES provides a tool for accelerating the discovery of disease genes (124). **In Chapter 5**, we have shown that we were able to rapidly identify a missense mutation and a splice site mutation in *TPP1*, which causes the autosomal recessive spinocerebellar ataxia type 7 (SCAR7), by ES of only one patient.

ES starts with random shearing of genomic DNA and flanking the library fragments by adaptors. Next, the library is enriched for sequences corresponding to exons either by in-solution or on–array hybridization capture. After that, the fragments are hybridized to the probes in the presence of blocking oligonucleotides. The recovered hybridized fragments are then enriched by PCR amplification, and this is followed by NGS (Agilent sureselect/ Roche/Nimblegen) (Figure 2). For sample indexing, barcodes can be introduced during the initial library construction or during post-PCR capture amplification (28). After NGS, data mapping and analysis of candidate variants are performed. At the Human and Clinical Genetics Center in Leiden, we have built a data analysis pipeline, which automatically maps the data to the reference genome, retrieves

sequence variants, checks their presence in known databases (e.g. dbSNP, 1000 genome) and predicts the potential consequences at the level of protein translation.

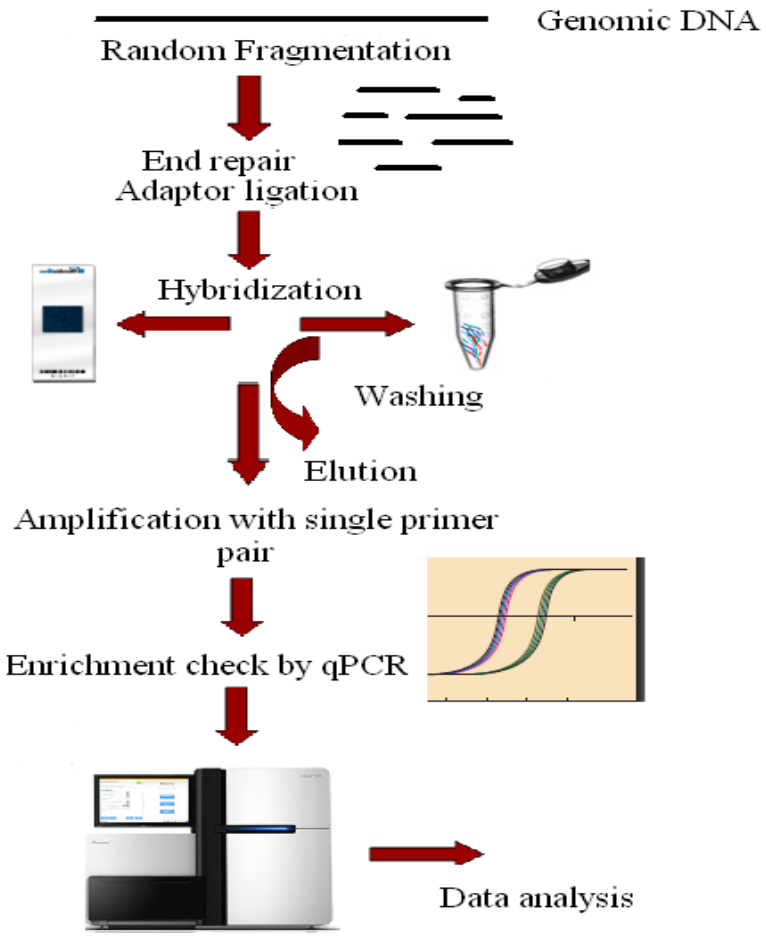

Figure 2. On- array and in-solution hybrid capture protocol.

At least three vendors (Agilent, Illumina and Nimblegen) offer in-solution whole exome capture kits. There are technical differences between them: for instance, Agilent uses RNA probes while Illumina and Nimblegen use DNA probes. These kits also differ in the definition of the exome (fraction of the genome targeted). We found the performance of exome kits from Agilent and Nimblegen (34 Mb) to be largely equivalent (see Table 2); each is scalable in 96-well plates by using a thermal cycler with no need for special equipment.

Table 2. The performance of whole exome kits from Agilent and Nimblegen (34 Mb).

	Agilent Sure Select	NimbleGen
# bases covered by baits (target base)	33,149,893	31,389,337
# bases covered by reads	32,878,642	31,233,008
% target base covered by reads	99.18%	99.50%
Average (base coverage) for the target	30.32x	29.97x
# consecutive-bait regions (CBR)	150,742	164,191
% CBR not covered by reads	1.18%	0.61%
% CBR max(base_coverage) < 5x	1.60%	1.09%
% CBR avg(base_coverage) >= 10x	86.09%	85.63%
% CBR avg(base_coverage) >= 20x	60.53%	60.74%
% CBR min(base_coverage) >= 20x	19.29%	20.96%

Despite the fundamental limitation of the current ES technology, which does not cover non-coding regions, it is a powerful strategy for discovering genes that underlie Mendelian disorders. This is for two reasons: First, large fractions of rare protein-altering variants, which are predicted to be deleterious, are located in exons (22); second, the price of ES is lower than that of sequencing an entire human genome.

Many proof-of-concept studies using ES to identify new disease genes for Mendelian disorders (125, 126) have been carried out in the past two years. There are an increasing number of successful studies that have found pathogenic variants of different diseases. More than 100 genes have been identified by ES in several Mendelian disorders with dominant, recessive and X-linked inheritance (Table 3), and this number is expected to increase.

Table 3. Overview of Mendelian disease genes identified by NGS, based on Rabbani *et al. (*2012).

Disorder	Inheritance	Gene identified	Reference
Miller syndrome	AR	DHODH	127
Autoimmune lymphoproliferative syndrome	AR	FADD	128
Nonsyndromic hearing loss	AR	GPSM2	124
Combined hypolipidemia	AR	ANGPTL3	129
Perrault syndrome	AR	HSD17B4	130
Complex I deficiency	AR	ACAD9	131
Hyperphosphatasia mental retardation syndrome	AR	PIGV	132
Sensenbrenner syndrome	AR	WDR35	133
Cerebral cortical malformations	AR	WDR62	134
3MC syndrome	AR	MASP1	135
Kabuki syndrome	AD	MLL2	121
Schinzel–Giedion syndrome	AD	SETBP1	136
Spinocerebellar ataxia	AD	TGM6	137
Terminal osseous dysplasia	XLD	FLNA	138
Nonsyndromic mental retardation	AR	TECR	139
Retinitis pigmentosa	AR	DHDDS	140
Osteogenesisimperfecta	AR	SERPINF1	141
Skeletal dysplasia	AR	POP1	142
Combined malonic and methylmalonicaciduria	AR	ACSF3	143
Amelogenesis	AR	FAM20A	144
Chondrodysplasia and abnormal joint development	AR	IMPAD1	145
Progeroid syndrome	AR	BANF1	146
Infantile mitochondrial cardiomyopathy	AR	AARS2	147
Heterotaxy	AR	SHROOM3	148
Mosaic variegated aneuploidy syndrome	AR	CEP57	149
Hypomyelinatingleukoencephalopathy	AR	POLR3A, POLR3B	150
Spastic ataxia-neuropathy syndrome	AR	AFG3L2	151
Dilated cardiomyopathy	AR	GATAD1	152
Gonadal dysgenesis	AR	PSMC3IP	153
Autosomal recessive progressive external ophthalmoplegia	AR	RRM2B	154
Knobloch syndrome	AR	ADAMTS18	155
Spinocerebellar ataxia with psychomotor retardation	AR	SYT14	156
Adams–Oliver syndrome	AR	DOCK6	157
Steroid-resistant nephrotic syndrome	AR	MYO1E, NEIL1	158
Complex bilateral occipital cortical gyration abnormalities	AR	LAMC3	159
Intellectual disability	AR	AP4S1, AP4B1, AP4E1	160

Hypertrophic cardiomyopathy	AR	MRPL3	161
Retinitis pigmentosa	AR	MAK	162
3M syndrome	AR	CCDC8	163
Seckel syndrome	AR	CEP152	164
ADK deficiency	AR	ADK	165
Nephronophthisis-like nephropathy	AR	WDR19	166
Psuedo–Sjögren–Larsson syndrome	AR	ELOVL4	167
Idiopathic infantile hypercalcemia	AR	CYP24A1	168
EMARDD	AR	MEGF10	169
Gray platelet syndrome	AR	NBEAL2	170
Immunodeficiency, centromeric instability and facial anomalies	AR	ZBTB24	171
Leber congenital amaurosis	AR	KCNJ13	172
Hereditary spastic paraparesis	AR	KIF1A	173
Ohdo syndrome	AD	KAT6B	174
Paroxysmal kinesigenicdyskinesias	AD	PRRT2	175
Hajdu–Cheney syndrome	AD	NOTCH2	176
Bohring–Opitz syndrome	AD	ASXL1	177
Hereditary diffuse leukoencephalopathy with spheroids	AD	CSF1R	178
Spondyloepimetaphyseal dysplasia	AD	KIF22	179
Adult neuronal ceroid-lipofuscinosis	AD	DNAJC5	180
KBG syndrome	AD	ANKRD11	181
Dendritic cell, monocyte, B and NK lymphoid deficiency	AD	GATA-2	182
Late-onset Parkinson's disease	AD	VPS35	183
Sensory neuropathy with dementia and hearing loss	AD	DNMT1	184
Dilated cardiomyopathy	AD	BAG3	185
High myopia	AD	ZNF644	186
Autosomal dominant retinitis pigmentosa	AD	RPE65	187
Charcot–Marie–Tooth disease	AD	DYNC1H1	188
Hereditary hypotrichosis simplex	AD	RPL21	189
Geleophysic and acromicric dysplasia	AD	FBN1	190
Myhre syndrome	AD	SMAD4	191
Leucoencephalopathy	XLR	MCT8	192
Split hand and foot malformation	AR	DLX5	193
Global eye developmental defects	AR	ATOH7	194
Primary hypertrophic osteoarthropathy	AR	SLCO2A1	195
Bartsocas–Papas Syndrome	AR	RIPK4	196
Familial aplastic anemia	AR	MPL	197
Peeling skin syndrome	AR	CHST8	198
Sengers syndrome	AR	AGK	199
Hypertension	AR/AD	KLHL3, CUL3	200
Weaver syndrome	AD	EZH2	201
Genitopatellar syndrome	AD	KAT6B	202

Hypothyroidism	AD	THRA	203
Floating–Harbor syndrome	AD	SRCAP	204
Hereditary spastic paraplegia type 12	AD	RTN2	205
Microcephaly associated with lymphedema	AD	KIF11	206
Congenital disorders of glycosylation (CDG)	AR	DDOST	207
Congenital mirror movements	AD	RAD51	208
Mandibulofacialdysostosis with microcephaly	AD	EFTUD2	209
Limb-girdle muscular dystrophy	AD	DNAJB6	210
Congenital stationary night blindness	AR	GPR179	211
Autosomal recessive primary microcephaly	AR	CEP135	212
Aplastic anemia and myelodysplasia	AD	SRP72	213
Acrodysostosis	AD	PDE4D	214
Olmsted syndrome	AD	TRPV3	215
Familial diarrhea	AR	GUCY2C	216
Nager syndrome	AD	SF3B4	217
Infantile cerebellar retinal degeneration	AR	ACO2	218
Coffin–Siris syndrome	AD	ARID1B	219
Joubert syndrome	AR	C5ORF42	220
Cerebroretinal microcephaly with calcifications and cysts	AR	CTC1	221
Kohlschutter–Tonz Syndrome	AR	ROGDI	222
UV-sensitive syndrome	AR	UVSSA	223
Pulmonary arterial hypertension	AD	CAV1	224

In **Chapters 4-7,** we have shown several examples of successful application of ES for detecting pathogenic mutations in various diseases. Moreover, ES has been used for detecting somatic mutations in tumours (225). The strength of ES in both research and as a diagnostic tool is becoming increasingly evident. It is used to find pathogenic mutations and to confirm the clinical diagnosis. ES can also be used as a genetic screening method to determine the carrier status of an individual, with respect to mutations that cause a particular autosomal recessive disorder (125, 126, 226). The diagnostic application of ES has been demonstrated by several examples (126, 225). However, a number of technical factors are still challenging for which ES requires further optimization and standardization. It is likely that ES, at least for the time being, will coexist with other NGS-based strategies, namely the targeted NGS and WGS in molecular diagnostics. Although, the total cost of WGS is much higher for the present, it is expected to become much more affordable soon (227). Obviously, one approach does not fit all different diagnostic applications and one needs to select the best approach based on available resources. For example,

a targeted NGS or ES approach is suitable for detecting mutations in disorders with genetic heterogeneity. Similarly, the diagnosis of X-linked disorders would require an NGS approach targeting the genes located on the X chromosome. We have used X-exome sequencing in two affected individuals from two unrelated families to detect the pathogenic mutation (c.5217G>A) in *FLNA* causing the X-linked Terminal Osseous Dysplasia (TOD) (**Chapter 4**). For other diseases such as mental retardation and congenital malformations, which are often due to copy number variations (CNV) or small mutations, the first step is to apply ES. Failure of ES to identify the causative genetic defect would suggest a possible extragenic location of the pathogenic mutation. WGS could then be used to detect deep intronic mutations or variants in remote regulatory elements. The advantage of WGS is that it does not require 30× or more coverage; so, sequencing of paired-end or mate-pair libraries with sufficient coverage across the genome is enough to identify CNVs, small mutations and chromosomal rearrangements. Recently, a cryptic fusion oncogene in acute promyelocytic leukemia was identified by WGS (228).

Definitive genetic diagnosis for a particular Mendelian disease cannot be established based on only one newly identified variant; for that, additional cases are required. However, it may be difficult to find additional cases in rare disorders to validate the potential candidate mutation. In that case, one has to perform functional studies of the putative pathological variant to confirm the pathogenicity. This method should be considered when the mutated gene has a key role in a well-defined molecular pathology of a certain disease. Indeed, the discovery of pathogenic variants and candidate genes responsible for different Mendelian disorders will help in understanding the function of the gene and the related biological pathways underlying health and disease. For example, we discovered that pathogenic variants in the *SMCHD1* (Structural Maintenance of Chromosomes Flexible Hinge Domain containing ***1***) gene responsible for Facioscapulohumeral Dystrophy type 2 (FSHD2) act as epigenetic modifiers of the D4Z4. This has provided new insights into the possible role of *SMCHD1* mutations in modifying the epigenetic repression of other genomic regions (**Chapter 7**).

In summary, the widespread availability of NGS technologies and the ever evolving field of gene sequencing is changing the approach to detecting genes, which cause Mendelian diseases as well as those responsible for complex traits. In particular, ES is expediting the detection of genes for

Mendelian diseases and the detection of mutations because of a rapid and straightforward laboratory workflow. Thousands of variants are identified per individual genome by NGS but only one or two of these variants may explain the Mendelian disease. Therefore, the interpretation of variants has become the new challenge. The number of variants that are identified in ES varies depending on the exome kit used, the NGS platform and the algorithms used for data mapping, and variant calling; this number can be huge. The following are, therefore, crucial for the identification of disease genes: prioritization of the variants, use of suitable bioinformatics tools and automated variants annotation algorithms, and the characterization of the functional impact of variants. It is expected that in due course standards and guidelines for ES or WGS will be established. The new technologies are therefore likely to become the most commonly used tools for the detection of genes for Mendelian diseases as well as for other diseases in the coming years.

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