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ENELI OITMAA

Development of arrayed primer extension microarray assays for molecular diagnostic applications

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Development of arrayed primer extension microarray assays for molecular diagnostic applications

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Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu

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II Gardner P, **Oitmaa E**, Messner A, Hoefsloot L, Metspalu A, Schrijver I. Simultaneous multigene mutation detection in patients with sensorineural hearing loss through a novel diagnostic microarray: a new approach for newborn screening follow-up. Pediatrics. 2006 Sep;118(3):985–94.

III Pereiro I, Hoskins BE, Marshall JD, Collin GB, Naggert JK, Piñeiro-Gallego T, **Oitmaa E**, Katsanis N, Valverde D, Beales PL. Arrayed primer extension technology simplifies mutation detection in Bardet-Biedl and Alström syndrome. Eur J Hum Genet. 2011 Apr;19(4):485–8.

IV **Oitmaa E**, Peters M, Vaidla K, Andreson R, Mägi R, Slavin G, Velthut A, Tõnisson N, Reimand T, Remm M, Schneider M, Õunap K, Salumets A, Metspalu A. Molecular diagnosis of Down syndrome using quantitative APEX-2 microarrays. Prenat Diagn. 2010 Dec;30(12–13):1170–7.

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My contributions to the original articles are as follows:

Papers I, II, III: Participated in study design, performed the experiments, analyzed APEX assay results, participated in the preparation and writing of the paper.

Paper IV: Designed the study, performed the experiments, analyzed APEX-2 assay results, prepared and wrote the paper.

LIST OF ABBREVIATIONS

ACs	AmnioCytes
aCGH	Array Comparative Genomic Hybridization
ACMG	American College of Medical Genetics
ACOG	American College of Obstetricians and Gynecologists
ADO	Allele DropOut
ALMS	ALströM Syndrome
APEX	Arrayed Primer EXtension
ARMS	Amplification Refractory Mutation System
ASA	Allele-Specific Amplification
ASO	Allele-Specific Oligonucleotide hybridization
ASPE	Allele-Specific Primer Extension
BBS	Bardet-Biedl Syndrome
CBAVD	Congenital Bilateral Absence of the Vas Deference
CF	Cystic Fibrosis
CFTR	CF Transmembrane Conductance Regulator
chr21	Chromosome 21
CNV	Copy Number Variation
CVS	Chorionic Villi Sampling
ddNTP	DiDeoxyriboNucleoside TriPhosphate
DHPLC	Denaturing High-Performance Liquid Chromatography
dNTP	DeoxyriboNucleoside TriPhosphate
DS	Down Syndrome
DSCR	Down Syndrome Critical Region
FN	False Negative
FP	False Positive
FRET	Fluorescence Resonance Energy Transfer
gDNA	Genomic DNA
HHL	Hereditary Hearing Loss
hME	Homogeneous MassEXTEND assay
HT	High Throughput
iFISH	Interphase Fluorescence In Situ Hybridization
indel	INsertion/DELetion
MALDI-TOF MS	Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry
MIP	Molecular Inversion Probe
MLPA	Multiplex Ligation-dependent Probe Amplification
NGS	Next Generation Sequencing
NIPD	NonInvasive Prenatal Diagnosis
NS-HHL	Non-Syndromic HHL
OLA	Oligonucleotide Ligation assay
PLP	PadLock Probe
QF-PCR	Quantitative Fluorescence PCR

RAD	Rapid Aneuploidy Detection
RFLP	Restriction Fragment Length Polymorphism
S-HHL	Syndromic HHL
sAP	Shrimp Alkaline Phosphatase
SBE	Single Base Extension
SNHL	SensoriNeural Hearing Loss
SNP	Single Nucleotide Polymorphism
SSCP	Single-Strand Conformation Polymorphism
T21	Trisomy of chromosome 21
TDI	Template-directed Dye- terminator Incorporation
TIRF	Total Internal Reflection Fluorescence
Tm	Melting Temperature
TN	True Negative
TP	True Positive
TUH	Tartu University Hospital
UNG	Uracil N-Glycosylase

INTRODUCTION

With the application of revolutionary technological advances, such as the PCR technique (Saiki *et al.*, 1985, Mullis and Faloona, 1987) and automated Sanger sequencing (Sanger *et al.*, 1977, Madabhushi, 1998), new insights into the genetic causes of inherited diseases are continuously being made. Such breakthroughs have led to an explosion of new molecular technologies that have the potential to serve as platforms for diagnostic tests in routine clinical practice. The growing list of molecular tests can be utilized for diagnosing genetic disorders, carrier testing, prenatal testing, presymptomatic testing for late-onset disorders, infectious disease testing, and testing of pharmacogenetic biomarkers in clinical oncology, among others.

The first widely used genetic tests for Mendelian diseases, such as cystic fibrosis, were based on restriction fragment length polymorphism / polymerase chain reaction (Friedman *et al.*, 1991, Raskin *et al.*, 1992). Such tests are still popular today due to their simplicity, straightforwardness, low set-up costs, and easy handling. If more extensive testing is needed, then mutation detection methods amenable to automation and multiplexing are preferred. Recent advances in genetic research and, especially, the introduction of next-generation sequencing technology and high-density genome-wide genotyping arrays have accelerated the discovery of new genes and disease-associated mutations. However, integration of these powerful technologies into everyday clinical practice remains challenging because these methods generate huge amounts of data with unclear clinical significance.

Use of molecular methods amenable for the targeted analysis of causative alterations would allow a more straightforward interpretation of the test results and subsequent diagnosis of disease. For instance, DNA microarray-based technologies allow the targeted detection of common disease-causing genetic alterations. These very useful tools enable the rapid and simultaneous analysis of numerous mutations at different scales in a single assay. Several DNA microarrays have been successfully introduced in routine clinical practice, including the xTAG® (Luminex Inc.), AmpliChip (Roche/Affymetrix), and arrayed primer extension (APEX) assays (Asper Biotech) for mutation detection and the GeneChip® array (Affymetrix) for detection of DNA copy number changes.

The aim of the current thesis was to develop and validate the suitability of DNA microarrays based on APEX (Kurg *et al.*, 2000) and APEX-2 assays (Krjutskov *et al.*, 2008) for the molecular diagnosis of monogenic and oligogenic hereditary disorders and for the rapid detection of chromosomal aneuploidies in uncultured fetal cells.

1. REVIEW OF LITERATURE

1.1. Genetic testing strategies

Genetic disorders are caused by different types of mutations that alter the DNA sequence in a single or multiple genes up to the chromosome level. Point mutations, which affect a single nucleotide pair, are the most common mutation type. Mutations involving deletions and insertions of nucleotide pairs or larger chromosome segments (copy number variations, **CNVs**) are another source of genetic disease.

Genetic testing techniques for identifying mutations can be broadly divided into two groups. The first group involves scanning methods used for screening both unknown and known sequence alterations. The second group involves targeted methods applied for screening only known mutations. Although a wide variety of technologies have been developed for identifying sequence variations, there is still no standard or preferred technique(s) for the routine testing of inherited diseases. All available methods have advantages as well as limitations and require specific skills and experience to perform and interpret (Dequeker *et al.*, 2009).

1.1.1. Mutation scanning methods

Mutation scanning methods utilize various techniques and are based on the detection of the altered mobility of DNA strands in gel electrophoresis or other high-resolution matrices. Examples of these methods include single-strand conformation polymorphism (**SSCP**) analysis (Orita *et al.*, 1989, Kakavas *et al.*, 2006), denaturing gradient gel electrophoresis (Myers *et al.*, 1985, de Cid *et al.*, 2010), and denaturing high-performance liquid chromatography (**DHPLC**) (Liu *et al.*, 1998, D'Apice *et al.*, 2004, Zainal *et al.*, 2012). High-resolution melt analysis is another popular scanning method that involves fluorescence monitoring of the melting profiles of PCR products in solution (Ririe *et al.*, 1997, Li *et al.*, 2011, Gisler *et al.*, 2012). These scanning methods are sensitive, specific, and cost-effective for the screening of known and unknown mutations or single-nucleotide polymorphisms **(SNPs)** (Dequeker *et al.*, 2009). The main benefit of their implementation in routine clinical diagnostics is the reduced need for full-gene sequencing of patient samples to discover the presence of sequence alterations.

1.1.2 DNA sequencing

Sequence alterations identified by the above-mentioned methods are usually confirmed with Sanger sequencing (or dideoxy sequencing) technology (Sanger *et al.*, 1977, Madabhushi, 1998). Sanger sequencing has been considered the "gold standard" method over the past three decades for direct DNA sequencing.

It has been used extensively as a first-line mutation detection method for identifying both known and unknown sequence variations. However, although Sanger technology provides highly accurate sequence quality and long read lengths, its high cost is prohibitive for routine diagnostic testing.

To overcome these limitations, recent efforts have been directed towards the development of high-throughput **(HT)** sequencing technologies, also referred to as next-generation sequencing **(NGS)**, to achieve major advances in cost and throughput without loss of sensitivity. Genetic testing strategies incorporating NGS platforms allow the simultaneous study of many genes and the detection of a large variety of mutation types, which will increase the detection rates for genetic testing, substantially reduce the cost, and improve turnaround time of testing.

Currently, the top-ranking NGS platforms are based on either sequencing-by-synthesis, including cyclic-reversible termination (Illumina/Solexa, Illumina, Inc.) (Zeitz *et al.*, 2013), ion semiconductor sequencing (Ion Torrent system, Life Technologies) (Costa *et al.*, 2013), and pyrosequencing (454, Roche Applied Science) (Neveling *et al.*, 2012), or sequencing-by-ligation, including the support oligonucleotide ligation detection system (SOLiD, Life Technologies) (de Ligt *et al.*, 2012) and the Complete Genomics Analysis platform (CGA™, Complete Genomics) (Drmanac *et al.*, 2010). The technical details and performance of NGS technologies have been extensively reviewed in numerous publications (Pettersson *et al.*, 2009, Metzker, 2010, Liu *et al.*, 2012). The sequencing landscape is dominated by Illumina sequencing platforms. Complete Genomics (recently obtained by BGI) is no longer accepting orders, and the future of the SOLiD platform is not clear after the recent acquisition of Life Technologies by Thermo Fisher Scientific.

1.1.3. Scanning molecular methods for CNV detection

Whereas the above techniques (except NGS) enable detection of point mutations and small insertions / deletions **(indels)**, semi-quantitative PCR methods, such as semi-quantitative fluorescent multiplex PCR (Niel *et al.*, 2004, Pallares-Ruiz *et al.*, 2010), quantitative fluorescence PCR (**QF-PCR**) (Mansfield, 1993, Mann *et al.*, 2012), multiplex ligation-dependent probe amplification (**MLPA**) (Schouten *et al.*, 2002, Van Opstal *et al.*, 2009, Cui *et al.*, 2013), and array comparative genomic hybridization (**aCGH**) (Pinkel *et al.*, 1998, Lee *et al.*, 2012, Magbanua *et al.*, 2013), allow accurate detection of both micro- and macro-rearrangements in genes and chromosomes, which remain undetected with conventional cytogenetic analysis. These methods are able to process many samples simultaneously, allowing faster turnaround times compared to conventional karyotyping (the standard method used for detecting chromosomal alterations). The major drawbacks of these techniques are that balanced rearrangements remain undiscovered and their high sensitivity to DNA quality or reaction conditions.

1.1.4. Targeted mutation detection methods

Genetic testing for inherited diseases mainly relies on direct gene analysis, which incorporates prior knowledge of disease-causing alterations with targeted mutation detection methods. These methods include, for example, allele-specific amplification (**ASA**), ligation-based assays, allele-specific oligonucleotide (**ASO**) hybridization, enzymatic cleavage techniques, single base extension (**SBE**) or mini-sequencing, and multiple variations of these technologies. Targeted mutation methods could be made more cost effective through their automation, either *via* the HT parallel analysis of a large number of samples for a limited number of mutations or *vice versa*, depending on the technology used.

1.1.4.1. Allele-specific amplification

ASA techniques are based on the preferential amplification of rare (mutant) alleles using matched / mismatched primers and a polymerase that lacks 3'–5' exonuclease activity. There are various modifications of the ASA approach, such as the amplification refractory mutation system (**ARMS**) (Newton *et al.*, 1989), bi-directional PCR allele-specific amplification (Liu *et al.*, 1997), and the competitive allele-specific TaqMan® PCR™ technology (Life Technologies) (Richter *et al.*, 2013).

Although ASA techniques include a diverse group of methods, they are all relatively rapid, sensitive, inexpensive, and simple to perform. They are amenable to automation and low-level multiplexing, low-to-medium throughput, and do not require restriction endonuclease cleavage sites. Their main limitation is the degree of specificity of mismatched primers, which correlates with the mismatch type. Purine / purine and pyrimidine / pyrimidine mismatches are more refractory to extension by Taq polymerase than purine / pyrimidine mismatches (Newton *et al.*, 1989). ASA techniques can be used to detect known point mutations (Hassan *et al.*, 2013, Richter *et al.*, 2013), indels (Hashemi *et al.*, 2012), and polymorphisms (Poe *et al.*, 2012).

1.1.4.2. Ligation-based assays

Ligation-based techniques utilize the specificity of ligases to achieve allelic discrimination. When two juxtaposed oligonucleotides hybridize perfectly to a single-stranded target DNA, the DNA ligase joins them together to form a single oligonucleotide. Ligation-based methods such as the oligonucleotide ligation assay (**OLA**) (Landegren *et al.*, 1988) and padlock probe (**PLP**) method (Nilsson *et al.*, 1994) use allele-specific probes with their 3′ ends at the variable sites because DNA ligases are more selective to mismatches at the 3′ end of strands. In contrast to OLA (Figure 1A) and PLP, the molecular inversion probe (**MIP**) technique (Figure 1B) employs a modified padlock

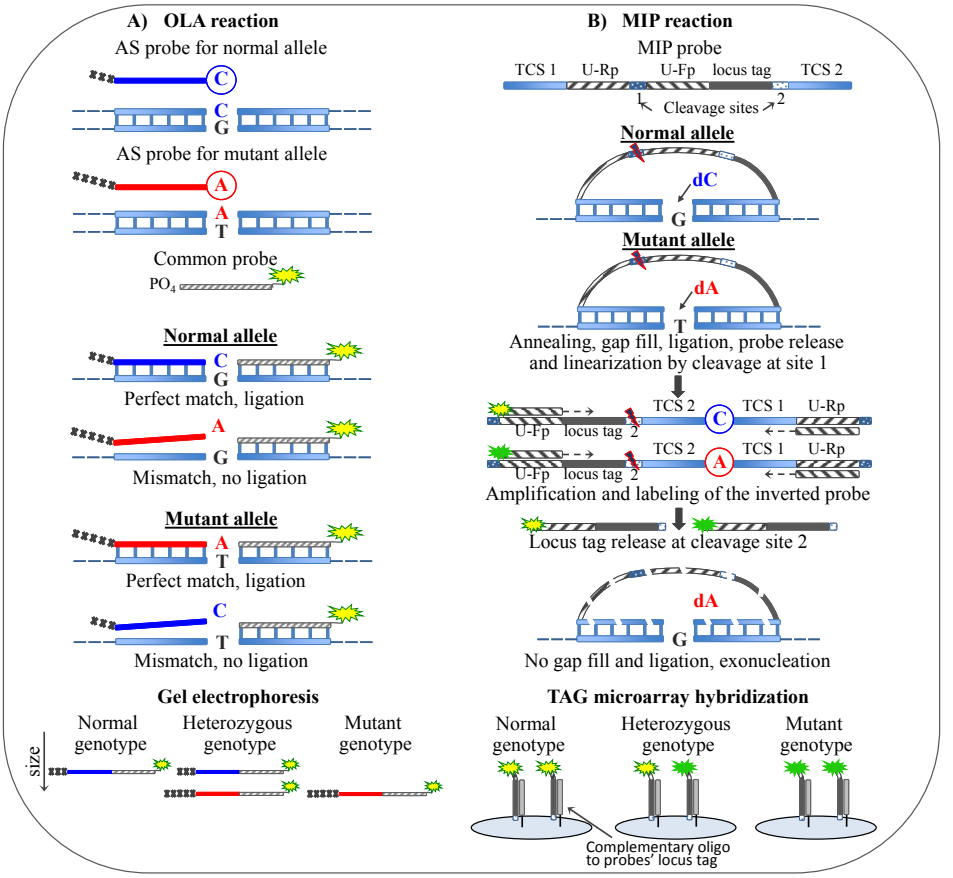

Figure 1. Schematic representation of ligation-based approaches. (A) Oligonucleotide ligation assay (OLA) utilizes the one-tube, two-step multiplex PCR amplification of genomic DNA (gDNA) and a subsequent OLA reaction with two mobility-modifying 5'-tailed allele-specific (AS) probes per variation site and 5'-phosphorylated common probes carrying a 3' fluorescent tag. Only the perfectly target-matched AS probe will be ligated to the common probe by the DNA ligase. This step is followed by electrophoretic separation of the ligation product (by Abbott Molecular). **(B)** Molecular inversion probe (MIP) assay utilizes hybridization of the pool of MIP probes to gDNA. SBE is used to fill the gap with complementary dNTP (deoxyribonucleoside triphosphate) at the variation site. The assay is performed in four separate tubes, one per dNTP. Probe ends are ligated by ligase, resulting in a circularized probe. Uncircularized probes and gDNA are destroyed by exonucleases. The probe is digested at cleavage site 1, resulting in an inverted probe that is amplified with universal primers (U-Fp and U-Rp) and labeled. This step is followed by locus tag release and identification of each amplicon through unique locus tags, which are complementary to probes on the TAG-microarray (by Affymetrix Inc.).

Abbreviations: TCS 1 and TCS 2, target-complementary segments.

probe without the polymorphic 3' end, allowing detection of both alleles of each SNP. A SBE fills the gap at the polymorphic site prior to ligation (Hardenbol *et al.*, 2003).

Ligation-based assays can be used with different assay formats and detection platforms, allowing fully automated workflow, semi-automated interpretation of the results, and medium-to-high throughput (Hardenbol *et al.*, 2005, Bruse *et al.*, 2008, Schwartz *et al.*, 2009, Su *et al.*, 2010, Ke *et al.*, 2011). OLA and MIP technologies have been successfully commercialized by Abbott Molecular and Affymetrix Inc., respectively. Their limitations include highly variable capturing efficiencies with different probes (Deng *et al.*, 2009), high probe costs, and complex protocols with multiple steps. The OLA, PLP, and MIP assays can be applied to the analysis of point mutations, small indels, and CNVs (Schiffman *et al.*, 2009, Schwartz *et al.*, 2009, Wang *et al.*, 2009, Su *et al.*, 2010, Chandler *et al.*, 2012).

1.1.4.3. Allele-specific oligonucleotide hybridization

Hybridization approaches exploit differences in the thermal stabilities of perfectly matched compared to mismatched oligonucleotides, to target the DNA for achieving allelic discrimination (Wallace *et al.*, 1981). The reverse ASO hybridization method allows the simultaneous analysis of multiple sequence variations in a single hybridization reaction (Saiki *et al.*, 1989). The simplest reverse ASO hybridization assays include line-probe assays (*e.g.*, InnoLiPA, Linear Array), which were developed for genotyping virus strains (De Stefani *et al.*, 2013) and detecting mutations in genes of medical interest (Giuliani *et al.*, 2010).

Because the reverse ASO hybridization approach does not require any enzymatic reactions to discriminate alleles (Figure 2A), it is easily adapted to HT platforms using microarrays. The GeneChip® array from Affymetrix Inc. is commonly used for genome-wide association studies (Lee *et al.*, 2013) and copy number analysis (Sapkota *et al.*, 2013). The main drawback for ASO hybridization approaches is the need for extensive optimization of the assay conditions, to ensure that hybridization occurs only between the perfectly complementary probe and the target DNA.

The TaqMan® genotyping assay (Life Technologies) combines the ASO hybridization approach and the 5' nuclease assay (Figure 2B) for the amplification and detection of specific alleles in homogeneous solution by real-time PCR technology (Livak, 1999, Kutyavin *et al.*, 2000). The major advantage of this assay is the discrimination of alleles in a single reaction without any post-PCR processing, an advantage that is unavailable with most other genotyping techniques. It is a widely used strategy for SNP analysis (Ney *et al.*, 2013) and mutation detection (Miyagawa *et al.*, 2012).

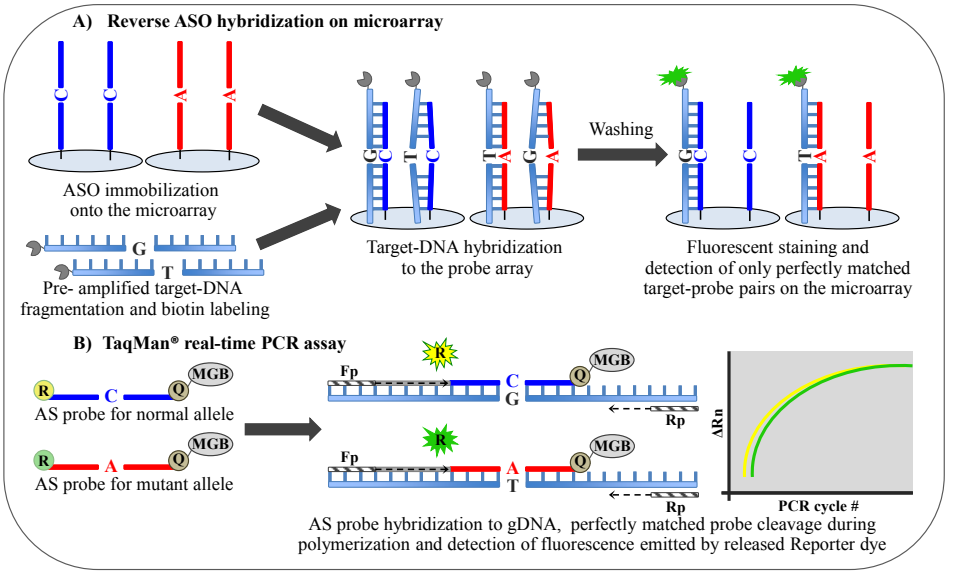

Figure 2. Hybridization-based approaches for genotyping of DNA variations. (A) Reverse allele-specific oligonucleotide (ASO) hybridization on a microarray platform. **(B)** TaqMan® real-time PCR assay utilizes two allele-specific (AS) fluorescence resonance energy transfer (FRET) probes labeled with reporter (R) and quencher (Q) dyes at both ends and a minor grove binder (MGB) at the 3' end to increase binding stability. Degradation of the probe releases an allele-specific 5' dye label (R), thus relieving the Q effect and allowing detection of the fluorescence emitted by the R dye. Fluorescence is detected and measured in a real-time PCR machine.

1.1.4.4. Enzymatic cleavage techniques

This group of techniques relies on the ability of certain classes of enzymes to cleave DNA by recognizing specific sequences or structures. Historically, the first widely used method for mutation detection was restriction fragment length polymorphism (**RFLP**), which is based on the selectivity of restriction endonucleases for short and specific DNA sequence sites that could be created or disrupted by a mutation (Kan and Dozy, 1978). Although RFLP is simple and straightforward, it is limited by the availability of informative restriction sites. This disadvantage can be overcome, to some extent, by creating restriction sites using a partially mismatched PCR primer (Kumar and Dunn, 1989, Tsai *et al.*, 2011). RFLP-based assays are suitable for detecting single nucleotide substitutions and indels in genetic and infectious disease applications (Pandey *et al.*, 2012, Tauseef *et al.*, 2012).

Invasive cleavage assays (Invader® and InvaderPlus®, Hologic Inc., formerly Third Wave Technologies) utilize structure-specific cleavage by a flap endo-nuclease (Lyamichev *et al.*, 1999). Coupling the original invasive cleavage assay with the serial invasive signal amplification reaction approach greatly

improved signal amplification (Figure 3), eliminating the need for PCR amplification of the target DNA and an additional signal amplification step (Hall *et al.*, 2000, Usami *et al.*, 2012). The Invader® assay can be performed in several assay formats, including homogenous assay, microfluidic card, or solid-phase capture of probes to microspheres. It is suitable for automated, HT analysis of single nucleotide substitutions, indels, and CNVs associated with genetic and infectious diseases (Rao *et al.*, 2003, Tadokoro *et al.*, 2006, Hosono *et al.*, 2008, Naoki *et al.*, 2011).

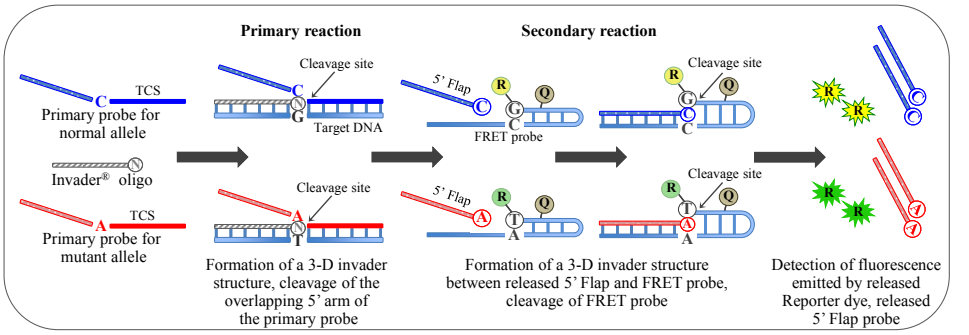

Figure 3. Invader® assay utilizing the serial invasive signal amplification reaction. In the primary reaction, a three-dimensional (3D) invader structure is formed by annealing the invader oligonucleotide and a perfectly matched primary probe to the target PCR product, generating a 5' overhang structure of primary probe (5' Flap) at the mutation position. The flap endonuclease (FEN) recognizes this 3D structure and cleaves 5' Flap from the primary probe after its first paired base. If the primary probe does not match, then cleavage of the primary probe will not occur. Signal amplification occurs in the second simultaneous FEN reaction. Each released 5' Flap cycles on and off the FRET probes to form a 3D invader structure. The second cleavage reaction releases fluorescent dye, resulting in a measurable signal.

Abbreviation: TCS, target-complementary segment.

1.1.4.5. Single base extension or minisequencing reaction

SBE reaction approaches rely on the fidelity of the DNA polymerase to add only complementary single bases to the 3' end of an oligonucleotide annealed to the target DNA molecule immediately upstream of the nucleotide to be detected (Figure 4A). Characteristics of the extended primer for either the wild-type (WT) or mutant allele allow discrimination between the homozygous and heterozygous genotypes (Sokolov, 1990, Syvanen *et al.*, 1990, Kuppuswamy *et al.*, 1991). In the SBE reaction, discrimination between sequence variants is based on the high accuracy of the nucleotide incorporation reaction catalyzed by the DNA polymerase, rather than differences in thermal stability between mismatched and perfectly matched hybrids formed with ASO probes (Syvanen, 1999). This difference leads to the higher flexibility and specificity of the SBE

assay compared to the ASO approach. The SBE reaction allows detection of single nucleotide substitutions, insertions and deletions, and homopolymeric and repetitive sequence stretches shorter than the primer itself.

Because of its easy set-up, quick and robust performance, and suitability for automation and HT analysis, the SBE reaction principle has been employed in various assay formats and applications. SBE assays can be broadly divided into three categories, according to the assay platform: **i)** solution-phase homogenous assays, wherein genotyping is performed completely in solution; **ii)** semi-solid-phase methods, wherein primer extension is performed in solution and the detection step on a solid support; and **iii)** solid-phase methods, wherein both primer extension and detection are performed on a solid support. Most SBE assays use four consecutive steps: **i)** PCR amplification of the template, **ii)** removal of residual deoxyribonucleoside triphosphates (**dNTPs**) and PCR primers, **iii)** primer extension of a common primer (SBE-primer), and **iv)** signal detection. Initially, radioactive nucleotides were used to determine the extended primer; however, these have been replaced with myriad nucleotide-labeling strategies, such as fluorescent tags (Kurg *et al.*, 2000), mass tags (Fei *et al.*, 1998), and haptens (Nikiforov *et al.*, 1994), allowing detection of multiple mutations / SNPs in parallel.

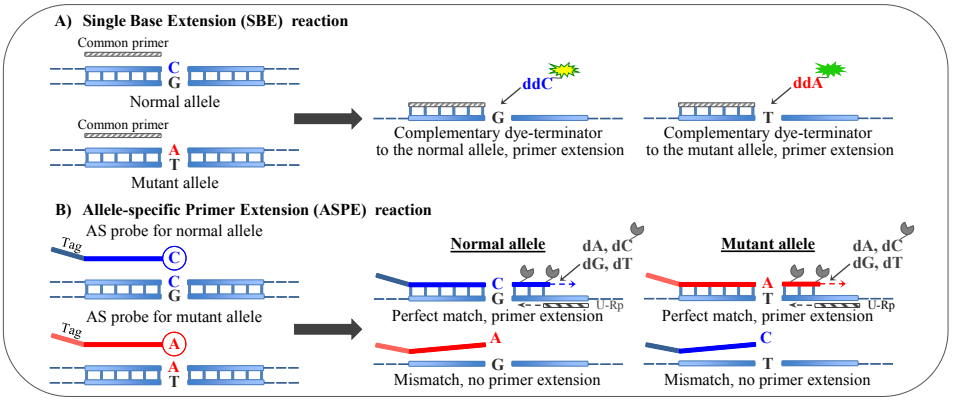

Figure 4. Primer extension approaches. (A) SBE reaction utilizes annealing of the common primer one base upstream of the mutation site, followed by its extension by complementary dideoxyribonucleoside triphosphates (ddNTPs) (*e.g.*, dye-labeled ddNTPs) and detection of the extension product with a corresponding platform. **(B)** ASPE reaction utilizes two AS primers to anneal with their 3′ ends at the mutation site and a common reverse primer (U-Rp) for the PCR reaction (*e.g.*, xTAG® Technology, Luminex). The reaction only occurs when the AS primer is perfectly annealed to the mutation site at its 3′ end. Resulting biotinylated PCR products are hybridized to a fluid microbead-based array and analyzed by flow cytometry (not shown).

Allele-specific primer extension **(ASPE)** assays, such as xTAG® Technology (formerly known as TAG-IT™ and multiplexed target-specific primer extension) (Bortolin *et al.*, 2004) utilize two identical allele-specific primers except for a mismatch at their 3′ end. Primer extension occurs only if the nucleotide at their 3′ end binds with perfect complementarity to the variation site, allowing for allelic discrimination (Figure 4B).

1.1.4.5.1 Solution-phase homogeneous SBE assays

Solution-phase homogenous SBE assays include the template-directed dye-terminator incorporation **(TDI)** (Chen and Kwok, 1997) and TDI with fluorescence polarization detection (Chen *et al.*, 1999). These techniques employ a microtiter plate format for simple and rapid analysis of target sites in solution-phase, without the need for product separation or specially designed and fabricated solid supports. The TDI assay with fluorescence polarization was commercialized by Perkin-Elmer Inc., allowing fully automated HT screening (>50,000 genotypes per day), and has been implemented for SNP genotyping studies (Freeman *et al.*, 2002).

1.1.4.5.2 Semi-solid-phase methods

To highlight the technological diversity of semi-solid-phase primer extension approaches, in this section, some popular methods utilizing different detection platforms are briefly described.

Gel-electrophoresis. Initial SBE approaches utilized cyclic primer extension of genomic DNA **(gDNA)** or PCR product in four (or two, bi-allelic) identical reaction mixtures, each containing SBE primer, one of four (or two) [α^{32}-P] dNTPs, and Taq-polymerase. Radiolabeled SBE products were electrophoresed in polyacrylamide gel and exposed to X-ray film (Sokolov, 1990, Kuppuswamy *et al.*, 1991).

Krook et al. introduced the multiplex single nucleotide primer extension approach, which utilizes the cyclic primer extension of pre-amplified DNA samples. SBE primers of different lengths are multiplexed in one tube, followed by size separation by gel electrophoresis (Krook *et al.*, 1992). This principle is employed in the SNaPshot® Multiplex System (Life Technologies), which allows multiplexing up to 10 polymorphic sites in a single reaction and throughput of up to 42,240 genotypes per day. The SNaPshot® Multiplex System has been used for various applications, including the mutation analysis of genetic diseases (Wang *et al.*, 2003, Zafra-Ceres *et al.*, 2012) and genotyping of SNPs (Esteves *et al.*, 2013).

Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry (**MALDI-TOF MS**). The **PinPoint assay** is one of the simplest approaches among SBE-based methods. The cyclic SBE reaction is performed in one tube and involves chemically unmodified dideoxyribonucleoside

triphosphates (**ddNTPs**) and differently sized SBE primers. Extension products are detected by MALDI-TOF MS (Haff and Smirnov, 1997, Liao *et al.*, 2005). Limitations of this approach include its low spectral peak resolution (A/T heterozygotes) and contamination by residual reagents (*e.g.*, salt, detergent, and glycerol), leading to degraded spectral quality and reduced accuracy of mass assignment. These limitations can be overcome by using a "mass tuning" strategy (*e.g.*, optimizing the primer sequence and length, using a higher voltage ion source, including sugar additives for MALDI matrices, etc.), adding an extra desalting step, or using mass-tagged ddNTPs to increase the mass difference between alleles (Fei *et al.*, 1998, Liao *et al.*, 2005).

To overcome the insufficient allele separation problem, mass-tagged ddNTPs instead of natural ddNTPs have been used in the iPLEX® assay (Sequenom, Inc.), allowing unambiguous allele identification (Meyer *et al.*, 2009). The iPLEX® assay allows multiplexing up to 24 loci per reaction with 384 samples in parallel (Sequenom, Inc.). Both the PinPoint and iPLEX® assays have been used for mutation analysis of genetic diseases (Dymerska *et al.*, 2010, Farkas *et al.*, 2010), large-scale SNP genotyping for association studies (Meyer *et al.*, 2009, Hou *et al.*, 2013), and CNV studies (Trewick *et al.*, 2011).

Another popular MALDI TOF MS-based primer extension assay is the homogeneous MassEXTEND **(hME)** assay (Sequenom, Inc.), which was developed as an improvement of the primer oligo base extension assay (Braun *et al.*, 1997). The hME assay utilizes cyclic multibase primer extension: primers are extended by one to four appropriate bases through the polymorphic site before the reaction is terminated by a ddNTP, and allele-specific products of different masses are generated (Storm *et al.*, 2003). The hME assay is routinely performed with up to 12plex SNPs per reaction in 384 plates, but higher multiplexing levels can be obtained by careful design of the extension primers. The hME assay has been implemented in various SNP association studies (Li *et al.*, 2012) and mutation analyses of genetic diseases (Li *et al.*, 2007).

TAG-array assay. Primer extension assays, such as xTAG® Technology (Luminex Diagnostics), utilize solid-phase detection of liquid-phase ASPE products in the Tag-array format. Use of Tag-arrays allows separation of multiplexed extension products carrying unique sequence tags (TAGs) by hybridization to a generic microarray containing complementary TAG-oligo-nucleotides (anti-TAGs). The universal anti-TAGS immobilized to the generic microarray are unrelated to the particular set of markers and could be used to genotype any group of markers. These easily generated generic Tag-arrays enable a high degree of multiplexing with high accuracy (99%). They provide opportunities for efficient large-scale genetic studies, allowing parallel geno-typing of up to tens of thousands of markers. The main disadvantages include the larger number of processing steps required from PCR to genotype determination and the standardizing of the SBE reaction because all tag-probes may not work reliably under the standard conditions (Bortolin *et al.*, 2004, Rohlfs *et al.*, 2011).

1.1.4.5.3 Solid-phase SBE assays

Performing the isothermal SBE reaction on a solid phase is a simple and rapid approach for achieving HT genotyping / mutation analyses of multiple variation sites under the same reaction conditions. Immobilization of one of the reaction components on a solid support that further serves as the separation method decreases the number of sample manipulation steps and allows the full automation of the entire sample analysis process. Solid-phase SBE assays involve many methodological modifications and detection platforms, from gel electrophoresis to DNA microarrays on activated glass or silica slides (Nikiforov *et al.*, 1994, Pastinen *et al.*, 1996, Shumaker *et al.*, 1996, Kurg *et al.*, 2000, Shapero *et al.*, 2001, Steemers *et al.*, 2006, Kisaki *et al.*, 2010). These assays include, for instance, the solid-phase mini-sequencing method (Syvanen *et al.*, 1990, Pastinen *et al.*, 1996), whole-genome genotyping SBE assay (Infinium® Assay, Illumina Inc.) (Steemers *et al.*, 2006) and arrayed primer extension (**APEX**) assay (Shumaker *et al.*, 1996, Kurg *et al.*, 2000).

The solid-phase mini-sequencing method has been used in multiple studies to analyze point mutations, insertions and deletions for the diagnosis of inherited genetic disorders (Jalanko *et al.*, 1992, Syvanen *et al.*, 1992), carrier screening of recessive diseases (Roomets *et al.*, 2012), and quantitative analysis of mRNAs (Ikonen *et al.*, 1992). The bases for the different modifications of this method include, for instance: i) the principle of primer design (similar or different length), ii) immobilization on solid-phase, iii) use of single- or double-stranded PCR product in the SBE reaction, iv) different sets of labeled or unlabeled dNTPs and ddNTPs, which define the number of different mini-sequencing reactions per DNA sample, and v) the signal detection assay / device (Syvanen *et al.*, 1992, Nyren *et al.*, 1993, Pastinen *et al.*, 1996, Pecheniuk *et al.*, 1997, Shapero *et al.*, 2001). Currently, the popular solid-phase SBE technologies utilize a microarray format.

The HT Infinium® Assay is a modification of the whole-genome genotyping SBE assay (Steemers *et al.*, 2006). This assay can genotype at scales ranging from 10,000 up to millions of SNPs per sample. Infinium® assays are suitable for whole-genome genotyping applications (Green *et al.*, 2012, Jasmine *et al.*, 2012). The assay utilizes the whole-genome amplification of DNA, followed by template fragmentation and capture on silica beads assembled on planar silica slides or fiber optic bundles (Beadchip format, Illumina Inc.) by hybridization to immobilized SNP-specific primers. Primers are hybridized adjacent to the SNP and extended with a single hapten-labeled ddNTP corresponding to the SNP allele. Dual-color signal detection of incorporated ddNTPs is obtained by adding fluorescently labeled antibodies in several steps to amplify the signals (www.illumina.com).

The medium-to-high throughput APEX assay is another widely used solid-phase SBE microarray technology. It is applicable for the simultaneous analysis of tens to hundreds of mutations / SNPs at one locus or multiple loci in a rapid and cost-effective way (Shumaker *et al.*, 1996, Kurg *et al.*, 2000, Dawson *et al.*,

2002). The method is specific, reproducible, and technically robust for detection of single nucleotide substitutions, short homopolymers or repeats, insertions and deletions. It has a very simple and flexible array design (*i.e.*, easy addition and removal of oligonucleotides) without the need for extensive optimization (Cremers *et al.*, 2007, Rodriguez-Paris *et al.*, 2010).

The APEX assay employs solid-phase SBE reaction conditions with primers covalently attached to an appropriately activated solid support (*e.g.*, epoxy- or amino-activated glass surface) *via* an aminolinker at the 5'-end of the primer. As a result of the spatial separation of the immobilized primers on the microarray, local complexity of the primers is greatly reduced, which eliminates artifactual primer-primer interactions and improves specificity. The APEX primer is designed to anneal with its 3' end adjacent to the variation site to be identified. Most APEX primers have an average of 25 to 35 bases, depending on the sequence context (*e.g.*, GC content) (Shumaker *et al.*, 1996, Kurg *et al.*, 2000, Schanne *et al.*, 2008, Duskova *et al.*, 2011).

In solution, the amplified PCR product is subjected to fragmentation and purification to remove residual dNTPs and primers, followed by hybridization to the immobilized APEX primers. The subsequent SBE reaction utilizing DNA polymerase catalyzes the incorporation of four dye-labeled ddNTPs complementary to the target DNA molecule (Figure 5A). The direct fluorescence readout of the extended product for genotype determination is acquired by a four-color confocal laser scanner (Schanne *et al.*, 2008) or laser imager using a total internal reflection fluorescence **(TIRF)** excitation mechanism combined with a charge-coupled device for HT image acquisition (Kurg *et al.*, 2000, Tonisson *et al.*, 2002, Duskova *et al.*, 2011).

The advanced APEX-2 assay (Figure 5B) allows the simultaneous two-step amplification of hundreds of variations in a single tube. Each APEX-2 primer contains a locus-specific region at the 3' end for hybridization adjacent to the variation to be identified and subjected to locus-specific primer extension and amplification of the extended product (first phase). A universal sequence tail at the 5' end of the primer is identical to a single universal primer used for second phase of amplification of extension products with universal sequence tails only.

Major advantages of the APEX-2 assay include its use of only two oligonucleotides per variation site and its remarkably reduced genome complexity, due to the amplification of only the primer extension products. These advantages lead to increased multiplexing capability, improved quality, and reduced cost and time for analysis. The simplified genotyping procedure is accomplished quickly because all of the amplified PCR products are short (100–200 bp) and no further fragmentation step is necessary. Only small amounts of DNA are needed for sample analysis (Krjutskov *et al.*, 2008). The required quantity of gDNA (0.3 ng) per variation site is less than that required by other multiplex PCR-based genotyping methods, such as SNaPshot® (1 ng) (Geppert and Roewer, 2012), and comparable to the amount required by iPLEX (0.2–0.4 ng) (Dymerska *et al.*, 2010).

In general, the quality of results from an APEX/APEX-2 reaction depends on the primer performance. Redesigning the microarray by replacing or rejecting unreliably performing oligonucleotides can solve most problems concerning genotype calling or allele discrimination. If template-independent primer extension due to intra- or intermolecular secondary primer structures is detected for one strand, then the other strand of the DNA template can be used for analysis because SBE primers are designed for both strands of the DNA template. Similarly, if primer extension is prohibited by secondary structures of the primer complementary to one strand, then the correct genotype could be obtained from the opposite strand (Tonisson *et al.*, 2002, Schanne *et al.*, 2008).

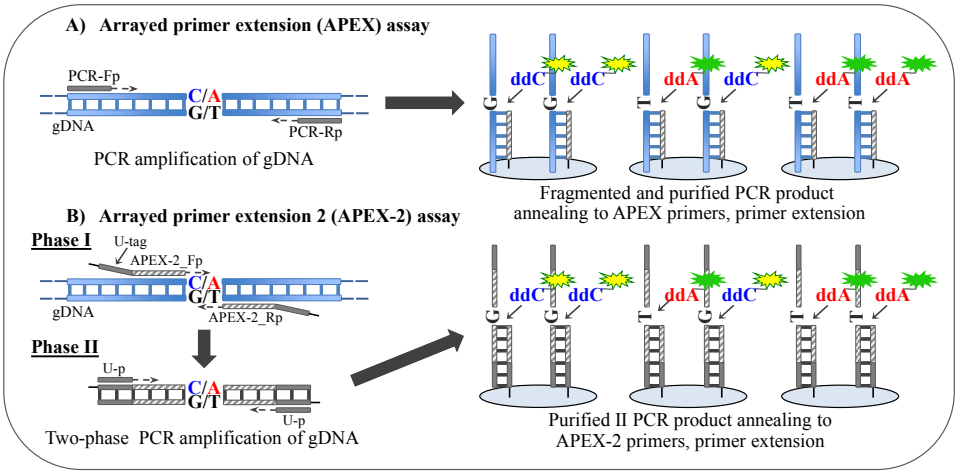

Figure 5. APEX assays. (A) APEX microarray assay utilizes the annealing of fragmented and purified PCR products to APEX primers immobilized on a glass slide. The subsequent primer extension step utilizes DNA polymerase and four dye-labeled ddNTPs to extend the APEX primer according to the complementary sequence at the mutation site. Fluorescence is detected. **(B)** APEX-2 assay involves the two-phase PCR amplification of gDNA. During phase I, the complexity of gDNA is reduced by amplification of the mutation site with the same APEX-2 primer set immobilized on the glass slide. APEX-2 primers include a tag sequence (U-tag) at the 5' end identical to the sequence of the universal primer (U-p) and a complementary sequence to bases before the mutation site at the 3' end. A single U-p is used for amplification of the phase I PCR product with U-tag sequences only. Subsequently, phase II PCR products are purified. Primer extension is performed as described above.

APEX assays have been developed and used for molecular diagnostics and carrier screening of various single-gene Mendelian disorders, such as autosomal recessive Stargardt disease (Jaakson *et al.*, 2003) and Wilson disease (Gojova *et al.*, 2008), and Mendelian disorders with several genes responsible for a similar phenotype, including β-thalassemia (Kurg *et al.*, 2000, Gemignani *et al.*, 2002), Usher syndrome (Cremers *et al.*, 2007, Vozzi *et al.*, 2011), familial hyper-cholesterolemia (Duskova *et al.*, 2011), and others. APEX assays have also

been utilized for genotypic resistance testing in HIV (Schanne *et al.*, 2008), identification of pathogenic fungi (Campa *et al.*, 2008), and SNP genotyping in a linkage disequilibrium study (Dawson *et al.*, 2002). The APEX-2 assay has been used for association studies of nonsyndromic cleft lip with or without cleft palate (Nikopensius *et al.*, 2011) and forensic testing (Krjutskov *et al.*, 2009).

1.2. Genetic background and testing of cystic fibrosis

Cystic fibrosis (**CF**) (MIM #219700) is the most common life-threatening autosomal recessively inherited disease among Caucasians, with a prevalence of 1 in 2500 and a carrier frequency of 1 in 25. The prevalence of CF is highly variable between different ethnic populations of Caucasians, ranging from 1 in 25,000 in Finland to up to 1 in 1353 in Ireland. The estimated incidence of CF in Estonia is approximately 1 in 7500 live births. Among other racial groups, such as Africans and Asians, the estimates of the prevalence of CF is very low, presenting as 1 in 17,000 and 1 in 350,000 (in Japan), respectively (Hamosh *et al.*, 1998, Kahre, 2004, Farrell *et al.*, 2007, Rohlfs *et al.*, 2011).

CF is caused by mutations in the *CF Transmembrane Conductance Regulator* gene (***CFTR****;* MIM #602421) (Riordan *et al.*, 1989, Rommens *et al.*, 1989, Zielenski *et al.*, 1991), which encodes the CFTR protein that functions as a chloride channel and regulator of other ion channels and transporters. CFTR is expressed at the apical membrane of epithelial cells in various organs (Anderson *et al.*, 1991, Toczylowska-Maminska and Dolowy, 2012). Mutations in *CFTR* can affect the CFTR protein quantitatively and/or qualitatively (Zielenski, 2000, Geborek and Hjelte, 2011).

CF clinical manifestations vary markedly with regard to disease severity and rate of progression in the organs involved. Most affected individuals have classical CF, with chronic obstructive pulmonary disease, elevated chloride ion concentration in sweat (>60 mmol/L), exocrine pancreatic insufficiency, specific gastrointestinal or nutritional abnormalities, salt loss syndromes, and infertility. According to a consensus panel, the diagnosis of CF should be based on the presence of one or more clinical features (*e.g.*, family history, positive sweat test result on two occasions) and detection of causative mutation in the *CFTR* gene (Moskowitz *et al.*, 2008).

Approximately 2% of CF patients have “non-classical” or “atypical” CF, with normal (<30 mmol/L) or borderline sweat chloride values (30–60 mmol/L) and multi-organ or single-organ involvement (*e.g.*, exocrine pancreatic sufficiency and milder lung disease). Most of these clinically atypical cases are confidently diagnosed with help of sweat test and/or genetic test (De Boeck *et al.*, 2006, Farrell *et al.*, 2008). Various conditions with single-organ involvement resulting from CFTR dysfunction that does not fulfill the diagnostic criteria for CF, such as disseminated bronchiectasis, acute recurrent or chronic pancreatitis, nasal polyps, congenital bilateral absence of the *vas deferens* (**CBAVD**) etc., are designated as CFTR-related disorders (Bombieri *et al.*, 2011).

1.2.1. Spectrum of CFTR mutations worldwide

The spectrum and frequency of *CFTR* mutations are highly variable among different ethnic / geographic populations (Bobadilla *et al.*, 2002). The *CFTR* mutation spectrum has been well characterized in most European populations (or populations with European descent), but the mutations are less well defined in minorities and other racial groups (Schrijver, 2011). Among the various *CFTR* mutations (numbering based on Genebank accession NM_000492.3), the most common mutation is p.Phe508del (c.1521–1523delCTT), accounting for about 70% of all mutant CFTR alleles. More than 90% of CF patients are homozygous or compound heterozygous for p.Phe508del (Stanke *et al.*, 2008). Frequency of p.Phe508del among Caucasians varies from 100% of disease alleles in Faroe Islands in Denmark (Schwartz *et al.*, 1995) to 18.8% in Turkey (Onay *et al.*, 1998). In Estonia, the major mutation is p.Phe508del, accounting for 51.7% of all CF chromosomes (Teder *et al.*, 2000).

Ten to twenty less-common mutations represent over 0.1% of all CF alleles worldwide (Bobadilla *et al.*, 2002). Some mutations have striking heterogeneity in allelic frequency, depending on the ethnic / religious / racial background of the individual. For instance, c.54–5940_273+10250del21kb (legacy name CFTRdele2,3) is relatively common (6.4–1% of all CF chromosomes) in Slavic populations (Dork *et al.*, 2000), p.Trp1282X (W1282X) is the most common (48%) in Ashkenazi Jews (Kerem *et al.*, 1995), p.Met1101Lys (M1101K) is the most common (69%) in the Hutterite population (Zielenski *et al.*, 1993), and c.2988+1G>A (3120+1G>A) is the most common mutation in the South African black population (46%) and the second most common mutation among African Americans (9–12%), but rare among Caucasians (Goldman *et al.*, 2001, Palomaki *et al.*, 2004).

1.2.2. CF carrier screening panels

CFTR gene studies are some of the most frequently performed genetic analyses worldwide. Analyses are carried out for carrier screening, molecular diagnosis, and prenatal diagnosis of CF, among other reasons (Dequeker *et al.*, 2009). When implementing a comprehensive CF carrier screening program or diagnostic test in an ethnic or ethnically mixed population, researchers and clinicians must consider the frequency of ethnic-specific alleles.

In the United States, the CF carrier screening program follows guidelines published by the American College of Medical Genetics **(ACMG)** and the American College of Obstetricians and Gynecologists **(ACOG)**. Initially, CF carrier screening was recommended only for high-risk Northern European and Ashkenazi Jewish populations. The revised standard CF screening panel includes 23 mutations prevalent in these two populations, with a threshold of ≥0.1% frequency in the general US population (Watson *et al.*, 2004). Strom *et al.* estimated the detection rate of this 23-mutation panel for a pan-ethnic US

population. The panel provided high estimated detection rates for Ashkenazi Jews (88%) and Caucasians with Northern European descent (86%), but was less effective for Hispanic Caucasians (69%), African Americans (63%), and Asians (43%) (Strom *et al.*, 2011). Rohlfs *et al*. extended the 23-mutation panel to 98 mutations with ethnicity-limited mutations. They reported that non-ACMG / ACOG mutations accounted for 22.7%, 26.9%, and 37.0% of CF alleles identified in African Americans, Hispanics, and Asians, respectively (Rohlfs *et al.*, 2011).

The European CF Society organized the Consensus Conference in 2009 to establish a consensus document on CF carrier screening programs. They recommended that carrier screening and diagnostic test panels include mutations with a frequency above 0.5–1% in the CF population. Results in tests with approximately 30 to 40 of the most frequent CF-causing mutations achieved mutation detection rates ranging from 70% to 90%, depending on the North-to-Southeast gradient of the p.Phe508del allele (Dequeker *et al.*, 2009). Castellani *et al*. summarized the consensus achieved and presented standards for efficacious, safe, and ethical practice of CF carrier screening programs. Such programs have had difficulties accounting for the different ethnicities of participants and determining an accurate ethnic-specific panel because of the mixture of ethnic groups and inaccurate self-reporting of ethnicity (Castellani *et al.*, 2010).

1.2.3. *CFTR* mutation testing strategies in diagnostic laboratories

Over 1900 mutations in the *CFTR* gene have been listed in the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca) and a broad range of technologies are available to clinical laboratories for *CFTR* mutation testing. Many studies have compared the most popular methods and commercial platforms used for CF diagnostics or carrier screening (Krafft and Lichy, 2005, Eshaque and Dixon, 2006, Johnson *et al.*, 2007, Dequeker *et al.*, 2009). Commercial assays cover the most common *CFTR* mutations, including, but not limited to, the panel of 23 *CFTR* mutations recommended by ACMG / ACOG. According to the US Centers for Disease Control and Prevention Newborn Screening Quality Assurance Program, the most popular methods have been the Invader® technology from Hologic Inc. (30%), xTAG® Technology from Luminex Diagnostics (18.6%), and ARMS assay from Gen-Probe Inc. (9.3%) (Earley *et al.*, 2011). Laboratories that participated in the CF External Quality Assessment Scheme 2012 most often used reverse ASO hybridization assays from Innogenetics (31%), OLA assays from Abbott Molecular (27%), and ARMS assays from Gen-Probe Inc. (17%) (CF European Network 2012, authorized by: Dequeker and Girodon).

Many other technologies have been developed and implemented for CF testing in clinical practice. One group includes scanning techniques, including SSCP (Sachdeva *et al.*, 2012), denaturing gradient gel electrophoresis (de Cid *et al.*, 2010), DHPLC (D'Apice *et al.*, 2004), high-resolution melt analysis (Krenkova *et al.*, 2009) and MLPA (Schrijver *et al.*, 2008). The second group contains direct mutation detection methods, beginning with RFLP (Raskin *et al.*, 1992) and Sanger sequencing (Lucarelli *et al.*, 2006) and including assays that allow HT analysis, such as the multiplex allele-specific diagnostic assay (Shuber *et al.*, 1997), the CF Portrait™ system (Strom *et al.*, 2004), a pyro-sequencing-based assay (Bickmann *et al.*, 2009), and the hME assay (Farkas *et al.*, 2010).

1.3. Molecular etiology and genetic testing of hereditary hearing loss

Hearing loss is an etiologically heterogeneous trait with many genetic and environmental causes. It is a common birth defect, with an estimated prevalence of one to two per 1000 newborns, and affects an estimated 70 million people worldwide (Morton and Nance, 2006, Eisen and Ryugo, 2007). Genetic changes account for more than 50% of cases with congenital deafness (Friedman and Griffith, 2003). Hereditary hearing loss **(HHL)** can be classified according to the absence (nonsyndromic; **NS-**) or presence (syndromic; **S-**) of additional clinical manifestations (Van Camp *et al.*, 1997, Schrijver, 2004).

Non-syndromic HHL (**NS-HHL**) most often involves sensorineural deficits in auditory processing. This type of HHL is also called sensorineural hearing loss (**SNHL**) and accounts over 70% of all HHL cases (Schrijver, 2004). Syndromic HHL (**S-HHL**) accounts for up to 30% of all HHL cases. More than 400 forms of S-HHL have been characterized. According to the relative frequency among persons with hearing loss, the most common S-HHL forms are Pendred syndrome (4–10%), Usher syndrome (4–6%), Waardenburg syndrome (1–4%), Branchio-oto-renal syndrome (1%), and Alport syndrome (1%) (Morton and Nance, 2006).

1.3.1. Genetic heterogeneity of NS-HHL

The genetic basis of NS-HHL is very complex. Based on the mode of inheritance, HHL can be subdivided into autosomal recessive (DFNB, 75–80% of HHL cases), autosomal dominant (DFNA, 10–20%), X-linked (DFN, ~1%), and mitochondrial groups (0% up to 20% in some populations) (ACMG, 2002, Finsterer and Fellinger, 2005). As a general rule, DFNB usually has pre-lingual onset, whereas DFNA has post-lingual onset and progressive manifestation (Schrijver, 2004).

In most cases, HHL is the result of a single gene harboring mutations, but mutations in two independent genes may be involved in some cases (Del Castillo *et al.*, 2003). Different mutations in the same gene (*e.g.*, in *GJB2*) can cause recessive (DFNB1; MIM #121011) or dominant NS-HHL (DFNA3A; MIM #601544) (Denoyelle *et al.*, 1998). Furthermore, mutations in *SLC26A4* (PDS; MIM #274600) may cause an S-SNHL form (*i.e.*, Pendred syndrome) as well as NS-SNHL (DFNB4; MIM #600791) (Cirello *et al.*, 2012). According to the Hereditary Hearing Loss Homepage (http://hereditaryhearingloss.org/, updated in June, 2013), over 70 loci and 41 genes have been identified for DNFB, over 50 loci and 25 genes for DFNA, and five loci and three genes for DFN.

1.3.2. Molecular genetic epidemiology of HHL

Genetic epidemiological studies have revealed that the overall prevalence and causes of deafness are variable among populations and at different times. Such variability is due to the founder effect, assortative mating, and relaxed selection against deafness (Arnos *et al.*, 2008). Information obtained from such studies could be used for the improvement of genetic diagnosis of hearing loss.

GJB2 (Genebank accession NM_004004.5) was the first gene identified as responsible for autosomal recessive NS-HHL (Kelsell *et al.*, 1997). *GJB2* mutations account for up to 30% to 60% of autosomal recessive NS-SNHL cases and approximately 20% of pre-lingual deafness cases (Kenneson *et al.*, 2002). The frequency of *GJB2* mutations is sufficiently high in most populations to make mutation analysis of this gene clinically useful.

The most frequent *GJB2* mutation is c.35delG, representing about 70% of all *GJB2* mutations in the Caucasian population, with relative frequencies ranging from 28% to 94% depending on the population (Gasparini *et al.*, 2000, Snoeckx *et al.*, 2005, Primignani *et al.*, 2009). The highest c.35delG carrier frequency (1 in 22) among European countries was found in Estonia (Gasparini *et al.*, 2000). The most common *GJB2* mutations in Asian, Ashkenazi Jew, Indian, and African populations are c.235delC, c.167delT, p.Y24X, and p.R143W, respectively (Brownstein and Avraham, 2009, Joseph and Rasool, 2009, Yang *et al.*, 2010, Kabahuma *et al.*, 2011, Qu *et al.*, 2012). The *GJB6* (Genebank accession NM_006783.4) truncating mutation del(GJB6-D13S1830) was an accompanying mutation in 6.9–71% of affected *GJB2* heterozygotes in different populations. The del(GJB6-D13S1830) allele is the most frequent allele in Spain, France, the United Kingdom, Israel, and Brazil, accounting for 5.9–9.7% of all the DFNB1 alleles (Del Castillo *et al.*, 2003).

More than 10 different mutations in *GJB3* have been found in patients with deafness from Chinese, Brazilian, Austrian, and Spanish populations. However, the pathogenicity of most of these sequence alterations is controversial. *GJB3* mutations may be an infrequent cause of NS-HHL, and the relevance of *GJB3* mutations in different populations needs to be proven in further studies (Xia *et*

al., 1998, Lopez-Bigas *et al.*, 2000, Mhatre *et al.*, 2003, Alexandrino *et al.*, 2004, Frei *et al.*, 2004).

The homoplasmic m.1555A>G mutation in the 12S rRNA gene (Genebank accession NC_012920.1) was identified as the main cause of aminoglycoside-induced and nonsyndromic deafness in families of different ethnic backgrounds (Prezant *et al.*, 1993, Matthijs *et al.*, 1996, Estivill *et al.*, 1998, Li *et al.*, 2004). Similarly, the m.7511T>C mutation in the mitochondrial $tRNA^{Ser(UCN)}$ gene (Genebank accession NC_012920.1) was associated with nonsyndromic deafness in different ethnic groups (Sue *et al.*, 1999, Chapiro *et al.*, 2002, Ishikawa *et al.*, 2002). Bizhanova *et al*. reviewed more than 150 mutations in the *SLC26A4* gene (Genebank accession NM_000441.1) reported in patients with Pendred syndrome. Prevalence rates of recurring mutations varied among different ethnic groups. For instance, p.L236P, p.T416P, and c.1001+1G>A occurred frequently in European populations, whereas p.H723R accounted for 53% of the mutant alleles in Japanese patients and 40% of Korean patients with Pendred syndrome (Bizhanova and Kopp, 2010).

1.3.3. Strategy for molecular testing of hereditary SNHL

Nearly 100 genes are involved in HHL. Although genetic testing is available for some of these genes (GeneTests™, www.ncbi.nlm.nih.gov/sites/GeneTests/), screening genes individually is a time- and cost-consuming approach. Therefore, an effective genetic testing strategy focusing on the most common mutations that are likely to be encountered in the clinical setting is needed.

Currently, hereditary SNHL is frequently screened by complete Sanger sequencing of the coding region and flanking exon / intron boundaries of the subjected gene. The most common genetic testing of NS-HHL utilizes Sanger sequencing of the small *GJB2* gene (only one coding exon) and direct mutation testing (MLPA and PCR analysis) for the recurrent deletion mutation del(GJB6-D13S1830) in *GJB6* (according to EMQN External Quality Assessment Scheme for Hereditary Deafness (DFNB1) 2011, authorized by Simon Patton). Test sensitivity is correlated with estimated accounts of *GJB2* mutations (30–60%) for autosomal recessive NS-SNHL (Kenneson *et al.*, 2002). The implementation of medium-to-high throughput platforms, such as the Invader® (Usami *et al.*, 2012) or APEX assay (Rodriguez-Paris *et al.*, 2010), would improve test sensitivity by allowing the simultaneous detection of numerous mutations of deafness genes. In particular, NGS technologies could offer new capabilities for the rapid and cost-effective screening of all deafness genes (Hilgert *et al.*, 2009, Park *et al.*, 2013).

1.4. Genetic background and testing of ciliopathy diseases

Ciliopathies are an emerging group of clinically distinct but overlapping genetic disorders, largely caused by ciliary dysfunction (Badano *et al.*, 2006). According to the Ciliopathy Alliance, over 20 ciliopathies have been identified to date (http://www.ciliopathyalliance.org). Although each ciliopathy is rare, combined they represent a clinical continuum with an estimated frequency exceeding 1 in 1000 live births (Zaghloul and Katsanis, 2009).

Autosomal recessive multisystem ciliopathies discussed in detail here include Bardet-Biedl syndrome (**BBS**; MIM #209900) (Bardet, 1920, Biedl, 1922, Beales *et al.*, 1999) and Alström syndrome (**ALMS**; MIM #203800) (Alstrom *et al.*, 1959). BBS has an estimated prevalence of 1:140,000 to 1:160,000 in North America and Europe (Sowjanya *et al.*, 2011). ALMS has a prevalence of less than 1.4 in 1,000,000 worldwide (to date, approximately 700 cases have been identified) (Pineiro-Gallego *et al.*, 2012). These diseases display variable age of onset, rate of progression, and severity of phenotypic features, even within siblings (Marshall *et al.*, 2005, Mahamid *et al.*, 2012). The clinical similarity of BBS and ALMS (Table 1) in the late-onset of some features, such as renal disease in ALMS, often leads to delayed diagnosis and misdiagnosis (Pineiro-Gallego *et al.*, 2012).

Table 1. Clinical manifestations of BBS (Beales *et al.*, 1999, Zaghloul and Katsanis, 2009) **and ALMS** (Marshall *et al.*, 2005, Marshall *et al.*, 2007)

Clinical manifestations	BBS	ALMS
Rod-cone dystrophy (retinitis pigmentosa)	+++	+++
Obesity	+++	+++
Renal dysplasia	++	++
Mental retardation / developmental delay	+++	++
Hypogonadism in males	+++	++
Postaxial polydactyly	+++	–
SNHL	+	+++
Heart defects	+	++
Type 2 diabetes mellitus	+	+++
Hepatic dysfunction	+	+++

+++ common; ++ intermediate frequency, + uncommon, – not reported

1.4.1. Genetic heterogeneity of BBS and ALMS

ALMS is caused by mutations in Alström syndrome 1 gene (*ALMS1*; MIM #606844; chromosome 2p13) (Collin *et al.*, 2002, Hearn *et al.*, 2002). Marshall *et al*. reviewed 109 different mutations in *ALMS1*, and found that exons 8, 10, and 16 represented mutational hotspots. Mutations in exon 16 accounted for 36% of the total mutational load in ALMS (Marshall *et al.*, 2011).

The genetically heterogeneous BBS is caused by mutations in 16 different loci (*BBS1* to *BBS16*), without a clear genotype-to-phenotype correlation (Redin *et al.*, 2012). The most frequently involved BBS loci are *BBS1*, *BBS10*, and *BBS2*, involving 18–23%, 20–22% and 8–13% of BBS families, respectively. Known BBS genes account for only 50–75% of the total mutation load in BBS patients (depending on patient ancestry), suggesting that additional BBS genes remain to be identified, or mutations linked to BBS may be underestimated if only patients with strictly defined BBS phenotypes are included. The presence and potential effect of triallelism or oligogenism in BBS have been widely discussed but remain controversial (Katsanis, 2004, Zaghloul and Katsanis, 2009, Redin *et al.*, 2012).

Most BBS proteins can be divided into two groups. The first group includes proteins encoded by *BBS1* (MIM #209901), *BBS2* (MIM #606151), *BBS4* (MIM #600374), *BBS5* (MIM #603650), *BBS7* (MIM #607590), *BBS8* (also TTC8; MIM #608132), and *BBS9* (also PTHB1; MIM #607968) that form a core protein complex called the BBSome. In cooperation with the non-BBSome protein GTPase ARL6 encoded by *BBS3 (ARL6*; MIM #608845), the BBSome promotes ciliogenesis. The BBS phenotype may be caused by defects in vesicular transport to the cilium (Nachury *et al.*, 2007).

The second group includes the chaperonin-like proteins encoded by *BBS6*, *BBS10* (MIM #610148), and *BBS12* (MIM #61683) that may be involved in the regulation of BBSome assembly (Seo *et al.*, 2010). Mutations in these genes account for 36.5% of the mutational load in BBS (Billingsley *et al.*, 2010). In their analysis of a large multiethnic cohort, Deveault *et al*. concluded that although several common mutations, such as p.M390R in *BBS1*, p.C91LfsX5 in *BBS10*, and p.Y24X in *BBS2*, were involved in BBS, most mutations (66.7%) were private (Deveault *et al.*, 2011).

In addition to the overlapping phenotypes of BBS and ALMS, substantial genetic overlaps have been described. For instance, Aliferis *et al*. revealed that the rate of ALMS1 mutations among patients diagnosed to have BBS was 4.2% (Aliferis *et al.*, 2012).

1.4.2. Molecular genetic epidemiology of BBS and ALMS

BBS occurs with variable frequency among populations, with prevalence rates of 1:160,000 in Europe to 1:13,500 in the Arab population of Kuwait because of a high rate of consanguinity (Sowjanya *et al.*, 2011). The prevalence of BBS in

Newfoundland is approximately 1:18,000, likely due to founder effect, consanguinity, and large sibship size. A particularly high prevalence of families (40%) with mutations in the *BBS6* gene has been recorded in this population, in contrast to 4% of families of North American / European descent (Moore *et al.*, 2005).

In contrast to white populations of European descent, *BBS1* is not the major BBS locus in Arab populations, accounting for only 5% and 8% of families of Tunisian and Saudi Arabian descent, respectively (Beales *et al.*, 2003, Mykytyn *et al.*, 2003, Smaoui *et al.*, 2006). Beales *et al*. demonstrated that *BBS4* is most prevalent among Turkish and Pakistan populations (Beales *et al.*, 2001). Stoetzel *et al*. detected pathogenic mutations of *BBS12* mostly in Caucasians of European descent, but also in Gypsies, Chinese, and Middle Eastern or Arabic populations (Stoetzel *et al.*, 2007). Unlike these results, Billingsley *et al*. found *BBS12* mutations only in individuals of non-European descent. Additionally, they revealed that mutations in *BBS6* and *BBS10* were represented in different populations (Billingsley *et al.*, 2010).

Marshall *et al*. analyzed a multiethnic cohort of ALMS patients, reporting that the most common *ALMS1* mutant allele, c.10775delC, accounted for 12% of mutated alleles across different populations. They also described numerous ethnic-specific mutations (Marshall *et al.*, 2007). Similarly, several other studies have been reported high prevalence rates of ethnic-specific mutations due to the founder effect and/or consanguinity in certain ethnic groups (Minton *et al.*, 2006, Aldahmesh *et al.*, 2009).

1.4.3. Diagnostic molecular testing for BBS and ALMS

The extensive non-allelic genetic and clinical heterogeneity of BBS and ALMS has been a problem for molecular diagnostic and genetic counseling applications. Researchers have discovered 16 known BBS genes and 14 other genes (including *ALMS1*) involved in ciliopathies that share overlapping clinical features with BBS (Redin *et al.*, 2012). Although the most widely used molecular testing strategy for BBS and ALMS involves Sanger sequencing of hotspot genes / gene regions or all coding exons (Marshall *et al.*, 2005, Billingsley *et al.*, 2011, Aliferis *et al.*, 2012), this approach is very time-consuming and expensive. Use of screening methods such as restriction enzyme digestion, DHPLC, and SSCP for the most frequently recurring alleles or hotspot genes would reduce the need for Sanger sequencing, but these approaches are still labor-intensive and time-consuming (Hichri *et al.*, 2005, Imhoff *et al.*, 2011). A cost-effective alternative to Sanger sequencing is the implementation of genotyping microarrays (*i.e.*, APEX microarray) for the initial screening of known (*e.g.*, common) mutations for BBS and ALMS (Pineiro-Gallego *et al.*, 2012). Alternative NGS-based strategies, including whole genome / exome sequencing or targeted sequencing, have been implemented for the cost-effective simultaneous screening of many genes associated with BBS and ALMS (Janssen *et al.*, 2011, Redin *et al.*, 2012).

1.5. Molecular diagnosis of Down syndrome

Down syndrome (**DS**, MIM #190685) as a distinct entity was described by Dr. John Langdon Haydon Down in 1866 (Down, 1866). DS is the most frequent chromosomal aneuploidy, occurring in approximately 1 in 700 live births. Its frequency depends on maternal age and the structure and utilization of prenatal screening and pregnancy termination in a population (Cocchi *et al.*, 2010). The highly pleiotropic phenotypes of DS (Table 2) are most commonly caused by trisomy of the entire chromosome 21 (**T21**) (Lejeune *et al.*, 1959) or, in rare cases, by duplication of the relatively small region at the distal part of chromosome 21 (21q21–21q22.3), called the DS Critical Region (**DSCR**) (Ronan *et al.*, 2007, Korbel *et al.*, 2009). According to a review by Bornstein *et al*., the full T21 is estimated to account for up to 92–95% of all DS pregnancies and is mainly the result of a maternal meiotic nondisjunction event (Bornstein *et al.*, 2009). About 2–4% and 1–4% of all DS cases are caused by mosaicism of T21 (mosaic-T21) and Robertsonian translocations, respectively (Dreux *et al.*, 2008, Zhu *et al.*, 2012).

DS can be considered as a multigene disorder, involving several relevant genes in almost the entirety of the 21q region (except the most centromeric region) rather than whole chromosome 21 (**chr21**) (Gardiner and Davisson, 2000). Published studies suggest that the phenotype of DS is mainly determined by only a few genes, with a major phenotypic effect accomplished by altered gene dosage (*i.e.*, production of increased amounts of gene products rather than alteration of the genes or gene products) (Arron *et al.*, 2006, Korbel *et al.*, 2009, Roy *et al.*, 2012). Contributions of potentially causative mutations in genes located on chr21 or other chromosomes should still be evaluated for distinct DS phenotypes (Ackerman *et al.*, 2012).

1.5.1. Molecular diagnostic methods for rapid aneuploidy detection of DS

Conventional cytogenetic analysis of metaphase chromosomes by Giemsa staining techniques is the gold standard for laboratory confirmation of DS (Dolan, 2011). Although full karyotype analysis is effective to reveal trisomies, monosomies, mosaicism, and rearrangements involving large chromosomal segments, rearrangements of <5 Mb usually remain undetected. The main limitation of conventional cytogenetic analysis involves the need for cell culture, which makes the analysis too time-consuming (up to 22 days), labor-intensive, and costly to fulfill requirements for rapid aneuploidy detection (**RAD**) (Gekas *et al.*, 2011).

Table 2. Major clinical phenotypes of DS in chronologic order of appearance (Antonarakis and Epstein, 2006)

Appearance	Clinical phenotype
At birth	Dysmorphic features (82% have upslanting palpebral fissures, 50% have folded or dysplastic ears, brachycephaly, flat nasal bridge, wide gap between the first and second toes, *etc*.); congenital heart disease; Hirschprung disease; imperforate anus; hypotonia; transient myeloproliferative disorder
Infancy and childhood	Growth retardation and obesity; developmental and mental retardation; decreased sensitivity to pain; leukemia; thyroid dysfunction; immune defects and/or infection
Adulthood	Decrease in cognitive function; Alzheimer disease; male sterility; reduced longevity

Development of reliable, fast, and less-expensive molecular methods, like interphase fluorescence *in situ* hybridization **(iFISH)** (Klinger *et al.*, 1992, Witters *et al.*, 2002, Leclercq *et al.*, 2008), QF-PCR (Mann *et al.*, 2001, Mann *et al.*, 2004, Cirigliano *et al.*, 2009, Hills *et al.*, 2010), and MLPA (Slater *et al.*, 2003, Kooper *et al.*, 2008, Van Opstal *et al.*, 2009), for detection of common aneuploidies (trisomies 21, 18, and 13 and sex chromosome abnormalities) in uncultured fetal cells has allowed the increased use of RAD in routine prenatal clinical practice. One benefit of the implementation of RAD techniques is the reduced reporting time of 1 to 2 days, which allows patients to make an informed decision about pregnancy management substantially earlier.

Currently, iFISH is the most common rapid screening method for aneuploidies in a clinical diagnostic setting. However, compared to QF-PCR and MLPA, iFISH is labor-intensive, more expensive, not suitable for automation, and, therefore, not easily applicable to a large number of samples (Witters *et al.*, 2002, Leclercq *et al.*, 2008). In contrast to QF-PCR, iFISH and MLPA do not detect the presence of maternal cells in samples from female fetuses (Mann *et al.*, 2004). All of these RAD techniques detect mosaicism to some extent, but detection levels in iFISH depend on the number of analyzed cells (25–50 cells used in routine). Low-grade mosaicism is likely to be missed (Hulten *et al.*, 2003). QF-PCR and MLPA have been reported to detect mosaicism as low as 20% (Mann *et al.*, 2004, Kooper *et al.*, 2008). Despite the ongoing debate about the use of these RAD techniques as stand-alone tests (Leung *et al.*, 2008, de Jong *et al.*, 2011, Papoulidis *et al.*, 2012), this strategy has been already implemented in an increasing number of diagnostic centers (Cirigliano *et al.*, 2009, Gekas *et al.*, 2011, Hamilton and Waters, 2012).

An alternative molecular diagnostic tool for RAD in prenatal diagnosis is aCGH, which allows simultaneous high-resolution analysis of multiple genomic loci, unlike iFISH, MLPA or QF-PCR (Fiorentino *et al.*, 2011, Lee *et al.*, 2012). The main disadvantages of aCGH include its more complicated and time-

consuming (3–5 days) protocol compared to MLPA or QF-PCR. It costs significantly more than conventional karyotype analysis or MLPA and QF-PCR. Although experience with diagnostic aCGH in pediatric patients is extensive (Hochstenbach *et al.*, 2009), experience in clinical prenatal diagnosis and suitability as a first-line diagnostic tool in place of traditional karyotype analysis are still under extensive debate (Bui *et al.*, 2011, Fiorentino *et al.*, 2011, Lee *et al.*, 2012).

1.5.2. Noninvasive prenatal diagnosis of Down syndrome

Noninvasive prenatal diagnosis **(NIPD)** has been an ultimate goal in the prenatal diagnosis of DS. The discovery of the presence of cell-free fetal nucleic acids in maternal plasma opened up new possibilities for early NIPD of DS (Lo *et al.*, 1997). NIPD of DS from the maternal circulation would offer a non-invasive, early, safe, and easy sampling approach compared to amniocentesis and chorionic villi sampling **(CVS)**, which increase the risk of miscarriage (Lo, 2012).

Detection of fetal T21 and other chromosomal aneuploidies from maternal plasma is very challenging. In particular, measurement of the fetal chromosome dosage should be performed in a background of high maternal DNA in maternal plasma, which only contains a small amount of fetal DNA (≤10% in the first trimester) (Lo *et al.*, 1998, Lun *et al.*, 2008). Currently, there is no consensus on the best strategy to detect DS based on cell-free fetal nucleic acid from the maternal circulation, although various approaches have been described. Some have focused on determining the chromosome dosage from the quantification of the allelic ratio of SNPs by analyzing fetal RNA of placental origin (Lo *et al.*, 2007) or fetal DNA (Dhallan *et al.*, 2007). Other approaches, such as digital PCR (Lo *et al.*, 2007) and NGS (Chiu *et al.*, 2011, Liao *et al.*, 2012), have determined the chromosome dose by counting single plasma DNA molecules.

Although NGS is currently expensive and time-consuming, the potential of using NGS as a first-tier screening test in clinical settings has been revealed in several large-scale validity studies (Chiu *et al.*, 2011, Palomaki *et al.*, 2011, Bianchi *et al.*, 2012). Consequently, NGS of maternal plasma DNA for the NIPD of fetal chromosomal aneuploidies has already been introduced as a clinical service in the USA and in some regions of Asia (Lo, 2012).

2. AIMS OF THE PRESENT STUDY

The aims of this thesis were to develop and evaluate the suitability of APEX and APEX-2 microarray-based assays for the diagnosis and carrier screening of rare genetic diseases and for rapid molecular diagnosis of DS from uncultured fetal cells. In particular, to develop and validate:

I. a novel APEX-based microarray for the detection of *CFTR* mutations in ethnically diverse populations (Paper I);

II. a novel APEX-based microarray for the simultaneous detection of mutations in multiple genes of SNHL patients (Paper II);

III. a novel APEX microarray-based BBS-ALMS assay for the molecular analysis of BBS and ALMS patients (Paper III);

IV. a modified APEX-2 assay for the rapid prenatal diagnosis of DS from uncultured fetal cells (Paper IV).

3. MATERIAL AND METHODS

3.1. Selection of DNA sequence variants

Selection of CF mutations (Paper I)

We selected 204 mutations / variations across the *CFTR* gene from the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca) and the literature (Zielenski *et al.*, 1991, Highsmith *et al.*, 1994, Mercier *et al.*, 1994, Wong *et al.*, 2001). Selected mutations represented the most commonly reported mutations across racial / ethnic groups. The full set of mutations is listed in Table 1 of Paper I. The mutation nomenclature for *CFTR* mutations was changed according to the guidelines of the Human Genome Variation Society (http://www.hgvs.org). The previous nomenclature is described as "legacy name" (Cystic Fibrosis Mutation Database). The mutations originated from 26 exons (except legacy exons 22 and 23) and 15 introns (legacy introns 2, 3, 4, 5, 6b, 8, 10, 11, 12, 14b, 16, 17a, 17b, 19, and 20).

Selection of SNHL mutations (Paper II)

We selected 198 mutations / sequence variants from multiple sources, including: (i) the Connexin Deafness homepage (http://davinci.crg.es/deafness/), (ii) mitochondrial mutation literature (Fischel-Ghodsian, 2003) and Mitomap database (http://www. mitomap.org/), (iii) Hereditary Hearing Loss homepage (http://hereditaryhearingloss.org), and (iv) Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/). The full set of mutations / variations is listed in Table 1 of Paper II.

Mutation panel of BBS-ALMS assay (Paper III)

We selected 253 mutations / sequence variants from *BBS1-7*, *BBS9*, *BBS10*, *BBS12*, and *ALMS1*. The full set of mutations / variations is listed in Supplementary Table 1 of Paper III.

SNP panel of T21 APEX-2 assay (Paper IV)

The set of SNPs from the 21(q21.1q22.2) region (DSCR) was selected. The selected 143 SNPs had an average heterozygosity of 0.47 for populations of European ancestry (http://hapmap.ncbi.nlm.nih.gov/). SNPs and primer sequences were based on NCBI dbSNP build 127 (Sherry *et al.*, 2001) and NCBI human genome build 36 (Sayers *et al.*, 2009), respectively. The full set of 90 validated SNPs (out of 143 SNPs) is listed in Supplementary Table 1 of Paper IV.

3.2. Design of the oligonucleotide microarrays

APEX primer design of CF, SNHL, and BBS-ALMS arrays (Papers I–III)

For each DNA sequence variant included in the panels, at least two unique oligonucleotide primers (APEX primers) were designed (sense and antisense). The average length of APEX primers was 25 nucleotides, but the primer length depended on the sequence context. In the case of AT-rich regions, the primer length was increased to raise the melting temperature **(Tm)** of the primer to be close to the annealing temperature of the APEX reaction. The Tm of the primer was calculated using the Primer3 software (Rozen and Skaletsky, 2000).

Longer APEX primers result in stronger signals, mainly due to increased duplex stability (Kurg *et al.*, 2000). However, the possibility for stable secondary structures (*e.g.*, hairpins or primer dimers) is also increased, which may affect primer binding to the template and result in signal loss or false signals due to self-extension (Tonisson *et al.*, 2002). Several computational programs, including NetPrimer (PREMIER Biosoft) and ProbeDesigner (Biodata, Estonia), were used to predict and estimate the stability of secondary structures. When necessary, a deliberate mismatch or modified nucleotide was introduced into the primer sequence, to avoid a strong secondary structure. A mismatch was only introduced when stable secondary structures were created in the middle to 5' end of APEX primer; they were not applicable for stable secondary structures in the 3' end. Mismatches in the 3' end result in failed primer extension and subsequent genotype calling.

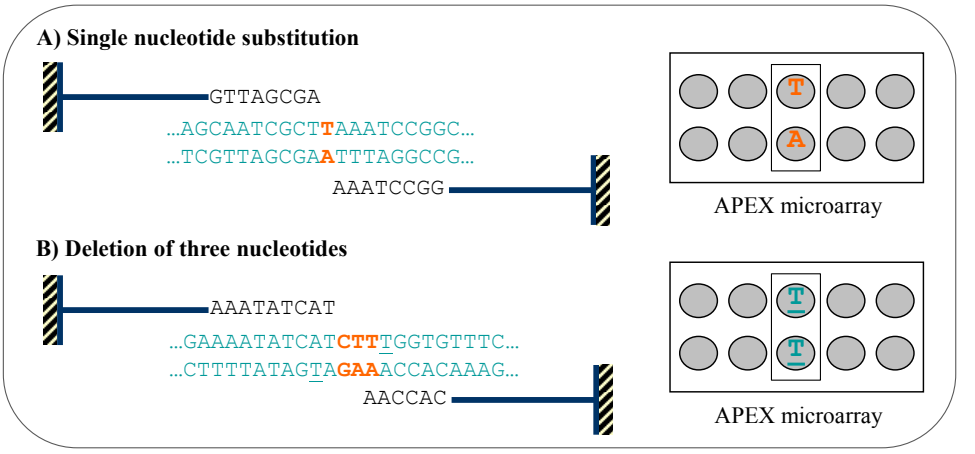

Figure 6. APEX primer design for sense and antisense directions. (A) Detection of single nucleotide substitution (T). APEX primer is designed –1 nucleotide before variation site. **(B)** Detection of three nucleotide deletion (CTT). Sense strand APEX primer ends –1 position before the first deleted base. In the case of deletion, the primer incorporates the next base (T) after the three deleted nucleotides. On the antisense strand, the first deleted nucleotide is A. The next nucleotide after deletion is also A, which makes allele discrimination impossible. Therefore, the antisense strand APEX primer is designed to end +1 base inside the deletion. In the case of deletion, the primer incorporates +2 bases after the last deleted nucleotide (modified after image of Asper Biotech Ltd).

Most APEX primers were designed to anneal –1 base upstream of the nucleotide to be identified (Figure 6A). In several cases for indel detection, when the downstream nucleotide(s) after the indel was identical to the first base of the indel, the primer 3' end was positioned further into the indel until discrimination of the nucleotide change was achieved (Figure 6B). All APEX primers included six-carbon amino linkers at their 5' end to enable covalent binding of the primer to the amino-activated glass slide.

Design of T21 APEX-2 array (Paper IV)

The same set of APEX-2 primers was utilized for multiplex PCR amplification of the DNA template and the subsequent APEX reaction on the glass slide as illustrated in Figure 5B. Each APEX-2 primer consisted of the complementary region for a genomic sequence flanking either side of the SNP at the 3′ end. The 5′ end of APEX-2 primer included the identical sequence of a universal primer and a six-carbon amino linker, allowing PCR amplification (in phase II) of only the primer extension products generated in PCR phase I, and immobilization of the APEX-2 primer to the amino-activated glass slide, respectively (Krjutskov *et al.*, 2008).

The complementary region of the T21 APEX-2 primers ranged from 19 to 41 bases, depending on Tm (55–62 °C), which was calculated using Primer3 software (Rozen and Skaletsky, 2000). Primer specificity was verified using the GenomeTester 1.3 software that predicts self-pairings or dimers and the number of binding sites in the human genome. Only primers that were predicted to produce a single product were used. SNPmasker software was used to calculate the product length and the presence of the closest SNP (Andreson *et al.*, 2006).

Preparation of the microarray slides (Papers I–IV)

The microarray slides used for printing APEX / APEX-2 primers have a dimension of 24 × 60 × 0.1 mm and are coated with 3-aminopropyltrimethoxysilane plus 1,4-phenylenediisothiocyanate (Asper Biotech Ltd). The primers were diluted to 50 μM in 100 mM of carbonate buffer (pH 9.0) and printed onto the activated surface with BioRad VersArray (BioRad Laboratories, Hercules, CA). The slides were subsequently blocked with 1% NH_4OH solution and stored at 4 °C until needed. Before the APEX / APEX-2 reactions, the slides were washed with 95 °C distilled water and 100 mM of NaOH to reduce background fluorescence and to prevent rehybridization of unbound oligonucleotides to the slide (Tonisson *et al.*, 2002).

3.3. Study subjects

DNA samples for CF chip development (Paper I)

To validate the APEX primers spotted on glass slides, synthetic oligomers and/or gDNA samples with CF-specific mutations were used. The gDNA samples for the CF assay were derived from the Molecular Diagnostics Centre of the United Laboratories of Tartu University Hospital (**TUH**), Estonia; the Molecular Pathology Laboratory at Stanford Hospitals and Clinics, USA; and Charles University, Institute of Biology and Medical Genetics, Department of Molecular Genetics, CF Center, Czech Republic.

In the case of the CF APEX assay, 51 patients or cell-line DNA samples with known *CFTR* mutations (Table 2 in Paper I) were used. Because gDNA samples for all mutations were not available, 136 synthetic template DNAs for both the sense and antisense directions were designed. Each synthetic template was approximately 50 bases in length (optimized for Tm), with a poly(T) tract at the 5' end to minimize the possibility of self-extensions and/or self-annealing (Table 4 in Publication I).

DNA samples for SNHL chip development (Paper II)

Synthetic oligomers or gDNA samples with SNHL-specific mutations were used. The gDNA samples for the SNHL assay were obtained from the Molecular Pathology Laboratory at Stanford Hospitals and Clinics, USA and the Molecular Diagnostics Centre of United Laboratories of TUH, Estonia. For validation of the SNHL APEX assay, 22 gDNA samples with 39 sequence variants were used. Because patient samples could not be obtained for most of the mutations present on the SNHL APEX array, approximately 45-mer synthetic oligomers were designed for each mutation site.

Study subjects of BBS-ALMS assay (Paper III)

The study group included 340 patients (205 BBS and 135 ALMS) with a confirmed or suspected diagnosis of BBS or ALMS. DNA samples were derived from at least 24 different countries.

Study subjects of T21 APEX-2 assay (Paper IV)

The gDNA samples were extracted from the blood lymphocytes of 16 DS patients, which were obtained from 108 amniotic fluid samples and 26 CVS samples. Amniotic fluid and CVS samples were collected from pregnant women at 16 to 17 and 11 to 12 gestational weeks, respectively. Blood samples from DS patients were received from the Department of Genetics of the United Laboratories of TUH and the University of Ulm, Germany. Fetal cells were collected during routine amniocentesis or CVS and underwent conventional karyotyping in the Department of Genetics of the United Laboratories of TUH,

and in Nova Vita Clinic, Estonia. The gDNA was extracted from peripheral blood cells using a salting-out protocol (Aljanabi and Martinez, 1997). The gDNA from amniotic cells and CVS samples was extracted using a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations.

3.4. Template preparation

Template preparation for CF, SNHL, and BBS-ALMS assays (Papers I–III)

Template preparation for the APEX reaction involved following steps: **i)** PCR amplification of gDNA with PCR mixtures that included 20% substitution of dUTPs for dTTPs, enabling later fragmentation of PCR products with thermolabile uracil *N*-glycosylase (**UNG**), **ii)** spin column-based PCR product concentration and purification of residual dNTPs and primer left over after PCR, and **iii)** PCR product purification from residual dNTPs with shrimp alkaline phosphatase (**sAP**) and fragmentation with UNG.

Template fragmentation is essential in APEX assays. It reduces the effects of secondary structures and the Tm of target duplexes, among other advantages. Shorter template strands have greater mobility, which increases the effective concentration. The method of introducing a 20% fraction of dUTP in the PCR reaction and UNG treatment is preferred over other fragmentation techniques (*e.g.*, DNase I, mechanical shearing), due to its better reproducibility in APEX assays (Kurg *et al.*, 2000). Another key issue in template preparation for a successful APEX reaction is the removal of all residual dNTPs after PCR. Residual dNTPs will interfere with the ability to obtain accurate data, because DNA polymerase will incorporate them into the 3' end of the APEX primer during primer extension.

Template preparation for T21 APEX-2 assay (Paper IV)

Template preparation for the T21 APEX-2 assay involved: **i)** a one-tube two-phase multiplex PCR amplification of gDNA (Figure 5B), and **ii)** exonuclease I/sAP treatment of PCR II products. The protocol did not include UNG fragmentation. The obtained PCR products were short (~100 bp) and suitable for APEX reaction without further fragmentation.

3.5. APEX reaction and signal detection

APEX reaction and signal detection (Papers I–IV)

The isothermal APEX reaction on glass slides was performed similarly in all experiments. Briefly, heat-denatured, double-stranded template was added to a reaction mix containing four dye-labeled ddNTPs and DNA polymerase. The

mixture was pipetted onto a pre-warmed (58 °C) glass slide with immobilized APEX primers. Annealing of the single-stranded template to 5' immobilized primers and subsequent DNA polymerase-assisted incorporation of dye-labeled ddNTPs on a complementary basis to the 3' ends of primers took place at 58 °C for 20 min (or 10 min in SNHL APEX assay). APEX reaction products were visualized with the Genorama® QuattroImager™ microarray imaging system, which combines an automated TIRF-based excitation (Figure 7) mechanism with a charge-coupled device to record the emitted fluorescence (Kurg *et al.*, 2000).

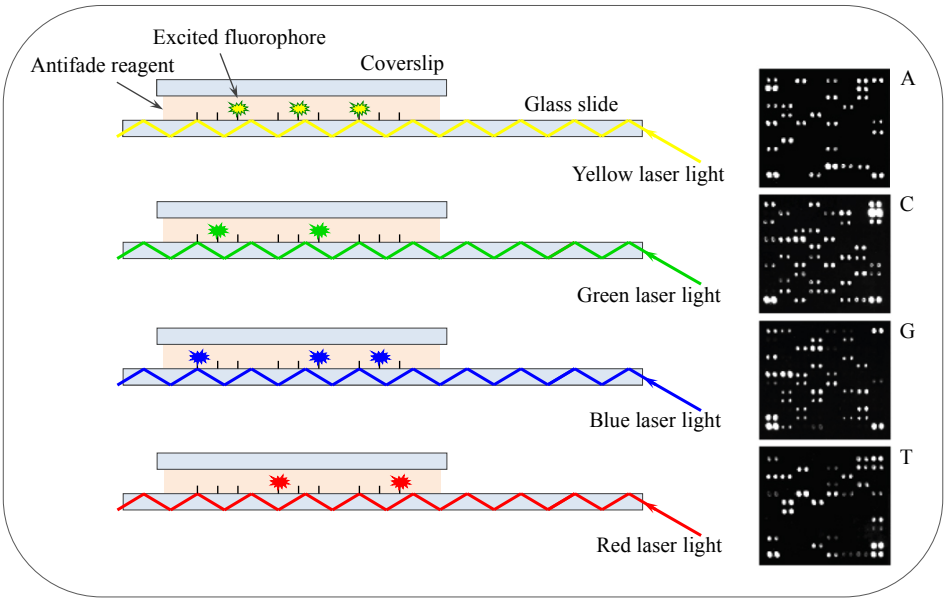

Figure 7. **Signal detection with the Genorama® QuattroImager™ imaging system.** Fluorescence on the surface of a DNA chip is recorded as follows. The chip is excited by the spreading of the laser beam in the glass slide, which acts like a wave guide, through total internal reflection (TIRF). One image is captured for each of the dye-labeled ddNTPs, which are excited by laser with the corresponding wavelength (modified after image of Asper Biotech Ltd).

3.6. Data analysis of APEX / APEX-2 assay

For each mutation / variation site, APEX primers for the sense and antisense strands were spotted in duplicate onto the APEX array. This approach reduced the possibility of false-positive signal interpretation due to nonspecific reactions (dust particles etc.) and simplified the distinction between homo- and heterozygosity (Figure 8). The genotypes for each position on captured array images were identified using Genorama Genotyping Software.

Data analysis of CF, SNHL, and BBS-ALMS assay (Papers I–III)

In principle, the mutation analysis of the APEX microarray involves the qualitative evaluation of data (*i.e.*, the obtained sequence is compared with a reference sequence). The analysis of the same array position for DNA samples in parallel in one database allows the researcher to obtain an overview of the performance of each APEX primer through a sample set of results included in the database and enables automated genotype calling *via* cluster analysis.

Quality parameters for the CF, SNHL, and BBS-ALMS assays were obtained as follows: **i)** Assay specificity = true negative (TN) / [TN + false positive (FP)]; **ii)** Assay sensitivity = true positive (TP) / [TP + false negative (FN)]; **iii)** Reproducibility of the SNHL assay = (total number of genotyped mutations − number of wrong mutation genotypes) / total number of genotyped mutations; **iv)** Call rate of the BBS-ALMS assay = (number of correctly genotyped mutations / total number of genotyped mutations).

Data analysis of T21 APEX-2 assay (Paper IV)

Genotyping data from the T21 APEX-2 assay were analyzed with a modified PicDB Image Database analysis program of the Genorama Genotyping Software. The Density-Based Spatial Clustering of Applications with Noise algorithm was used for cluster analysis of samples (Ester *et al.*, 1996). A two-tailed *t*-test was performed for allelic fractions of heterozygous SNPs after APEX analysis to calculate *P*-values for the determined clusters of each SNP. At least six control DNA samples with known ploidy, three euploid and three T21, were used to annotate each DNA sample examined. $P < 0.05$ was considered statistically significant.

Genotype data quality was assessed by the following parameters (all converted to %): **i)** Assay call rate = (number of correctly genotyped SNPs / total number of genotyped SNPs); **ii)** Assay specificity = TN disomic DNA samples / (TN disomic DNA samples + FP trisomic DNA samples); **iii)** Assay sensitivity = TP trisomic DNA samples / (TP trisomic DNA samples + FN disomic DNA samples); **iv)** SNP specificity = TN disomic SNP genotypes / (TN disomic SNP genotypes + FP trisomic SNP genotypes); **v)** SNP sensitivity = TP trisomic SNP genotypes / (TP trisomic SNP genotypes + FN disomic SNP genotypes); **vi)** Reproducibility = (total number of genotyped SNPs − number of wrong SNP genotypes) / total number of genotyped SNPs.

4. RESULTS AND DISCUSSION

4.1. Detection of *CFTR* mutations by CF APEX assay (Paper I)

To date, over 1900 *CFTR* sequence variants in the *CFTR* gene have been described in the Cystic Fibrosis Mutation Database. The CF screening panel recommended by ACMG/ACOG includes the 23 most common mutations in the high-risk populations of Northern European and Ashkenazi Jewish descent. Only some mutations prevalent in other races or ethnicities are included in the CF panel because they do not fulfill inclusion criteria for the general population threshold (*i.e.*, mutations with 0.1% frequency in general U.S. population) (Watson *et al.*, 2004).

We selected 204 of the most common mutations / variations (Table 1 in Paper I) across the *CFTR* gene (Genebank accession NM_000492.3) for a carrier screening and diagnostic panel to increase CF carrier and disease detection rates among different racial / ethnic groups. Mutations originated from 26 exons and 15 introns. Alterations included single nucleotide substitutions, small indels, a large 21-kb deletion (removal of exons 2 and 3), and small repeats, like c.1210-12T[5_9] (legacy name IVS8-5T/7T/9T) important in CBAVD.

The CF APEX assay was validated using 51 patient gDNA samples (Table 2 in Paper I) and 136 synthetic templates (Table 4 in Paper I). This approach allowed validation of the APEX reaction, but could not assess optimization of the PCR steps, because synthetic templates were not amplified. Therefore, four mutation sites were tested using both gDNA samples and synthetic templates, and concordant results were obtained. To discover assay reproducibility, each site was tested from 3 to 20 times with identical results under our test conditions. According to the reported results of this pilot study, the CF APEX assay allowed reliable and reproducible detection of the WT versus mutation sequence at each array position on the microarray. The assay sensitivity was 100% (*i.e.*, no FNs were observed) and specificity was 100% (*i.e.*, no FPs were present).

The developed assay was suitable both for screening of CF carriers and for confirming the diagnosis of CF patients. Representative results are presented in Figure 8 from three patient samples, one heterozygous, one homozygous, and one normal for the common mutation p.Phe508del, located in exon 11 (legacy exon 10). One of the most technically difficult sequence variations to identify reliably was the c.1210-12T[5_9] polymorphism in intron 9 (legacy intron 8), of which the 5T variant is particularly important in CBAVD. Accurate and reliable differentiation of the poly(T) length required three sets of allele-specific APEX primers.

One important concern is the genotyping quality control of microarrays for mutations, which are very rare in the general population. Ideally, a positive

control (DNA sample with the same mutation) for each mutation should be included with each run. This control would demonstrate that all components of the assay, including APEX primers, are working properly. However, in the case of microarrays that include primers for hundreds or thousands of different and rare mutations, this control is not possible. To perform the assay in a clinical diagnostic setting, there are two options: several positive controls can be pooled and run in parallel with test samples and/or external quality assessment schemes (if available) can be used. The negative control (*i.e.*, no template) slide is mainly used to monitor the array for nonspecific reactions with the APEX primer. This control could be included in each run or not, depending on the quality control scheme established.

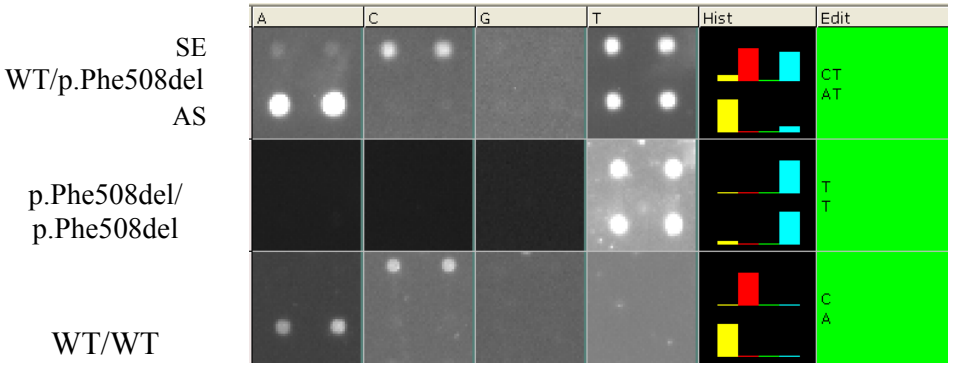

Figure 8. APEX analysis at mutation site p.Phe508del in *CFTR* gene (NM_000492.3). Samples of three individuals, one each with WT/p.Phe508del, p.Phe508del/p.Phe508del, and WT/WT, respectively, are presented. For each DNA sample, sense (SE) strand (upper row) and antisense (AS) strand (lower row) signals are presented. For a normal allele, a signal for the SE primer is expected in the C channel and for the AS primer in the A channel (C/A). For a mutant allele, a signal for the SE primer is expected in the T channel and for the AS primer in the T channel (T/T). In the first row, cells from left to right: Fluorescent image in channels A, C, G, and T, respectively; Hist represents relative signal intensities; Edit, detected signal.

Due to the extended pan-ethnic mutation panel on the CFTR array, this CF test had a higher mutation detection capability for CF diagnosis and carrier screening with different ethnicities, including Caucasians, non-Caucasians, and individuals of mixed ethnicity, than assays currently available on-site in diagnostic laboratories. Compared to, for example, the X-Tag®CF assay (Luminex Diagnostics), which detects up to 71 mutations, our array enabled us to detect more than double the number of mutations.

Although 204 mutations were included in the CF APEX test to improve first-line CFTR mutation screening, many common mutations that may contribute to CF in Hispanics, African Americans, Asians, etc., were not included in the panel and remained undetected. Once new information about causative CFTR

mutations common in some ethnic populations is obtained, these mutations can be included in the CF APEX test. This approach will improve the early diagnosis of CF, leading to appropriate genetic counseling and treatment in all ethnic groups.

An increased incidence of CFTR mutations has been found in diseases other than classical CF, including CBAVD, chronic pancreatitis, disseminated bronchiectasis, and allergic pulmonary aspergillosis (Bombieri *et al.*, 2011). In these cases, the CF APEX test can serve as the first-tier research tool to screen for mutations, coupled with other more comprehensive but resource-intensive technologies if the results are negative. When relevant disease-contributive *CFTR* mutations are identified, these can be added to the CF APEX array for routine analysis.

In conclusion, the results demonstrated the feasibility of the APEX approach for CF mutation detection. The CF APEX assay is specific, reproducible, and technically robust. In general, it could be accomplished in 4 to 5 hours, as illustrated in Figure 9.

Figure 9. **Estimated sample turn-around time of CF APEX assay.** Template preparation step includes PCR amplification of gDNA, spin-column purification, and sAP / UNG treatment for removal of residual dNTPs and fragmentation of PCR product.

Because the template preparation and APEX reaction steps can be performed in parallel, the rate-limiting factor in sample analysis is the number of detection devices per laboratory. At 6 to 7 minutes of analysis time per array, the throughput in 8 hours would be approximately 60 to 70 samples per imager unit.

4.2. Detection of SNHL mutations by SNHL APEX assay (Paper II)

Genetic etiology of SNHL is highly complex, due to the involvement of multiple genes and a diverse pattern of inheritance. SNHL can be inherited in an autosomal dominant, or recessive, X-linked, or mitochondrial manner. In addition to the variable prevalence of SNHL among different populations and ethnicities, the frequency and distribution of the mutation spectrum are also highly variable.

To compensate for the lack of a multigene diagnostic for SNHL, our goal was to create an SNHL APEX assay that provides a molecular diagnosis for patients affected with SNHL and allows carrier detection of recessive SNHL. We selected 198 mutations / sequence variants (Table 1 in Paper II) in six nuclear and two mitochondrial genes involved in the etiology of SNHL and diagnostically practical in terms of the number of mutations included. The mutation panel included single nucleotide substitutions and indels, including a 309-kb deletion affecting *GJB6*.

The SNHL APEX assay contained commonly reported mutations in the most frequently involved genes underlying NS-SNHL and S-SNHL among population groups. For instance, the c.35delG mutation is the most commonly identified mutation in the *GJB2* gene (Genebank accession NM_004004.5) (Gasparini *et al.*, 2000). This mutation causes a deletion of a one guanine from a poly(G) tract at positions 30–35, leading to a frame shift. Figure 10 demonstrates representative APEX results of several individuals / patients for the c.35delG mutation.

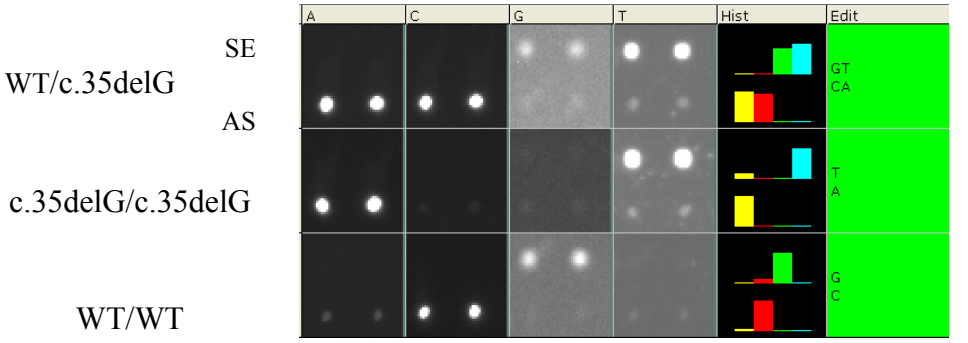

Figure 10. APEX analysis of deletion NM_004004.5:c.35delG in the *GJB2* gene. Samples of three individuals, one each with WT/c.35delG, c.35delG/c.35delG, and WT/WT, are presented. For each DNA sample, sense (SE) strand (upper row) and antisense (AS) strand (lower row) signals are presented. For a normal allele, a signal for the SE primer is expected in the G channel and for the AS primer in the C channel (G/C). For a mutant allele, a signal for the SE primer is expected in the T channel and for the AS primer in the A channel (T/A).

Although Usher syndrome is common in S-SNHL, mutations for Usher syndrome detection were not included, because they are involved in a separate APEX microarray (Cremers *et al.*, 2007). Several mutations / variations of uncertain or evolving clinical significance were included in this panel: for instance, p.V27I, p.M34T, p.V37I, and p.E114G in *GJB2*, NM_206885.1:c.-53-2A>G (legacy name IVS2-2A>G) in *SLC26A5* (Tang *et al.*, 2005), and p.L11F and p.V24A in *GJA1* (Genebank accession NM_000165.3) (Paznekas *et al.*, 2003). The advantage of the APEX assay is manifested by the easy removal or

replacement of DNA sequence variations with uncertain clinical significance as our knowledge of the implicated genes and mutations evolves.

The SNHL APEX assay was validated in a pilot study by using 22 patient DNA samples with 39 sequence variants (18 in *GJB2*, 1 in *GJB6*, and 18 in *SLC26A4*) and synthetic oligomers for each mutation site. The sequence of synthetic templates consisted of the reference sequence segments flanking the mutation site. In validation with the gDNA samples, we detected one unexpected homozygous result, instead of the expected heterozygosity for mutation NM_004004.5:c.-23+1G>A (legacy name IVS1+1G>A) in *SNHL-9* (Table 2 in Publication II). The homozygous result was reproduced twice with the same PCR product on APEX analysis, but re-amplification and subsequent APEX analysis demonstrated the expected heterozygosity. We concluded that there was allele dropout (**ADO**) in the original PCR amplification.

Considering the ADO event, the specificity was 97% (32/32+1). However, as ADO is a very rare event in PCR and is unrelated to validation of the microarray position or condition of the APEX reaction itself, the true calculated specificity was considered to be 100% for the APEX reaction. The sensitivity of the assay was 100%, and no FNs were observed. The reproducibility of the APEX reaction was determined by re-testing a heterozygous c.35delG sample in eight replicons. The results revealed 100% reproducibility of the APEX reactions under optimized reaction conditions.

The results of the pilot study revealed the accuracy, reproducibility, and technical robustness of the SNHL APEX assay for detecting multiple mutations in various genes essential for the comprehensive molecular diagnosis of SNHL. The sample turn-around time of the SNHL APEX assay was approximately 6 hours, including a hands-on time of approximately 1 hour and 20 min. In most laboratories, the SNHL testing strategy involves testing only for SNHL mutations in *GJB2* by Sanger sequencing, which is expensive, labor-intensive, and suitable mainly for the sequential analysis of one or a few genes. In contrast, the APEX platform is more resource-effective for monitoring mutations in numerous genes simultaneously, and could be easily updated for emerging clinical needs.

One reason to consider molecular diagnostic genetic testing in both children and adults with hearing loss includes the ability to determine the etiology of nonsyndromic forms of hearing loss that lack other clinical features early in life (Schrijver, 2004). The SNHL APEX assay-based testing is ordered by pediatricians, otolaryngologists, and geneticists. It can be integrated into more standardized follow-up after newborn hearing screening, where clinically indicated. Patients will benefit from accurate genetic counseling and, in some cases, treatment, including early intervention with hearing aids or cochlear implants, which will improve the quality of life for hearing-impaired individuals (Yoshinaga-Itano *et al.*, 1998). Additionally, the use of large diagnostic platforms, like the APEX array, for testing many cases provides extensive information about mutation frequencies and distributions in distinct populations, as

well as genotype-phenotype correlations, permitting more accurate assessments of the clinical significance of individual mutations or their combinations.

4.3. Mutation detection by BBS-ALMS APEX assay (Paper III)

BBS and ALMS are multisystem genetic disorders showing highly variable phenotypes and considerable phenotypic overlap that may lead to misdiagnosis between the two disorders (Aliferis *et al.*, 2012). To date, the genetic heterogeneity of BBS involves 16 genes and ALMS involves one gene, making mutational analysis rather complicated (Redin *et al.*, 2012). From the BBS-ALMS APEX assay, 253 mutations / sequence variants in 10 BBS and one ALMS gene (Supplementary Table 1 in Paper III) were selected for molecular diagnostic testing and carrier screening of common mutations present in different populations. The selected panel of BBS/ALMS mutations included the most common disease alleles among different populations described in the literature (Zaghloul and Katsanis, 2009, Marshall *et al.*, 2011). A set of intragenic SNPs was also included. These SNPs could be used in a linkage study to a particular locus and may lead to the identification of novel BBS and ALMS1 mutations (this analysis was not performed herein). The BBS-ALMS APEX assay included single nucleotide substitutions and indels. An example of the primer design and captured images of the APEX reaction results are shown in Supplementary Data in Paper III.

After the development phase, we obtained an assay call rate of 99.5%, sensitivity of 99.4%, and specificity of 100%. The validated array was used to screen 205 BBS and 135 ALMS patient samples (340 in total) with a confirmed or suspected diagnosis of BBS or ALMS. At least one likely causative mutation was identified in 35% (119/340) of families (Figure 1 in Paper III). In most cases (53 BBS + 16 ALMS families), homozygous or compound heterozygous mutations were discovered, and no further analysis was required (Tables 1 and 2 in Paper III). The mutation detection rate of the assay was higher in European than non-European patients (BBS: 47 *vs.* 3%; ALMS: 19 *vs.* 5%). A summary of polymorphisms that were detected is shown in Supplementary Table 2 in Paper III.

More mutations were detected in BBS (40.5%; 83/205) than in ALMS (26.7%; 36/135) families, mainly due to the two "common" mutations in *BBS1* (p.M390R) and *BBS10* (p.C91fsX95), identified in 71.1% (59/83) of BBS families. The most common contributors to BBS syndrome were the *BBS1* and *BBS10* genes, involved in 84.1% (69/82) of all BBS families with a BBS mutation detected. Consistent with findings in other studies, the most frequent disease alleles in north European origin BBS families were p.M390R, identified in all *BBS1* families, and p.C91fsX95 (Zaghloul and Katsanis, 2009), identified in 50% (10/20) of *BBS10* families (Table 1, Paper III).

Although the most frequently reported mutation in *ALMS1* was c.10775delC (p.T3592fs) (Marshall *et al.*, 2007), the most frequently observed *ALMS1* mutation in this study was c.11316_11319delAGAG (p.R3772fs), identified in three families (Table 2 in Paper III). The BBS-ALMS1 assay detected two mutations in 25.8% (53/205) of BBS and 11.9% (16/135) of ALMS families. Cases with one heterozygous mutation identified should be screened for the same gene in full length.

In these series, we determined two BBS cases with oligogenic inheritance, carrying three BBS mutations, whereas the third heterozygous allele was a nonsense mutation (p.R275X in *BBS2* and p.Q355X in *BBS9*) that should affect the function of the protein (Table 1 in Paper III). Digenic triallelism has previously been reported in BBS. Most often, the third heterozygous mutation (at a second BBS locus) behaves as a modifier allele (Badano *et al.*, 2003) rather than being necessary for the phenotypic manifestation of BBS (Katsanis *et al.*, 2001). To date, no evidence of digenic triallelism has been seen in patients with ALMS.

Although we continue to update of BBS-ALMS assay on a regular basis, the major limitation of the array is incomplete coverage of all genes and all published mutations related to BBS and ALMS. Alternative technologies, like NGS, could be used for the identification of deleterious mutations in this group of patients. However, at this time, NGS platforms are more resource-consuming compared to the genotyping array to be a first-pass screening option. In conclusion, the BBS-ALMS assay is an efficient first-pass screening tool for molecular diagnosis of BBS and ALMS and has beneficial outcomes for resolving phenocopy issues between these syndromes.

4.4. Molecular detection of fetal Down syndrome by quantitative T21 APEX-2 assay (Paper IV)

In most cases, DS is caused by an extra chr21, but complete or partial clinical DS features may also be associated with the duplication of a few or one gene in the DSCR (Rachidi and Lopes, 2007, Korbel *et al.*, 2009). These small chromosomal aberrations are not revealed by conventional karyotype analysis or metaphase FISH. Our goal was to develop an APEX-2 assay-based SNP genotyping method as a proof of principle for the rapid determination of both whole-chromosome duplication and gene-/exon-level duplications that cause DS. In addition to the previously described qualitative analysis of the APEX assay for mutation analysis, the T21 APEX-2 assay included quantitative data analysis for DNA sample annotation. Copy number analysis of chr21 by the APEX-2 assay relied on a comparison of signal intensities of the allelic fractions of heterozygous SNPs after the APEX reaction, as illustrated in Figure 11 (Figure 1 in Paper IV).

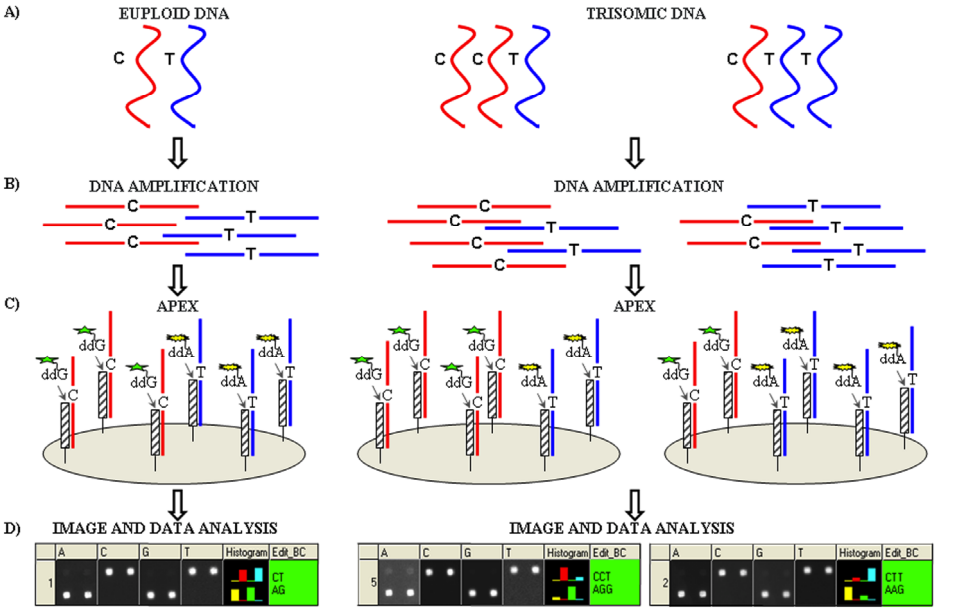

Figure 11. Principle of the T21 APEX assay. Trisomic samples are discriminated from euploid samples by comparing signal intensities of allelic fractions of heterozygous SNPs obtained after the APEX reaction. (**A**) Trisomic DNA has two copies of one allele of a heterozygous SNP, compared to euploid DNA with one copy of both alleles. (**B**) One-tube multiplex PCR amplification. (**C**) APEX reactions are performed on a glass slide with covalently attached SNP-specific primers for both DNA strands. Four different terminator nucleotides, each tagged with an individual fluorophore, are used. To simplify the illustration, only one strand is shown. (**D**) Imaging and analysis of genotyping data of DNA samples. Left to right: fluorescent image in channels A, C, G, and T; histograms represent relative signal intensities; Edit BC, base call; in sections A, C, G, T; Histogram & Edit BC, upper row represents signals of sense strand primer, lower row antisense strand primer (Figure 1 in Paper IV).

We expected that double-copy alleles from trisomic DNA samples would have different mean allelic fraction values compared to the euploid DNA samples; more than three genotype clusters should be present. Examples of the data analysis of two SNPs are shown in Figure 12 (Figure 2 in Paper IV). Figures 12A and 12B illustrate a reliable discrimination between T21 and euploid DNA samples on both DNA strands. DNA samples with the duplicated A or T allele are clearly clustered apart from the DNA samples with a normal allele ratio (for sense strand, $P = 0.000242$ and $P = 0.006$, respectively; for antisense strand, $P = 0.007$ and $P = 0.0000124$, respectively). In contrast, Figure 12C shows genotyping data in which no distinct clusters were formed for the allelic fractions of the duplicated C or T allele, apart from DNA samples with a normal allele ratio using the sense strand ($P = 0.085$ and $P = 0.2$, respectively). Reliable

discrimination of the duplicated G or A allele was possible using the antisense strand ($P = 0.0000250$ and $P = 0.00120$, respectively) (Figure 12D).

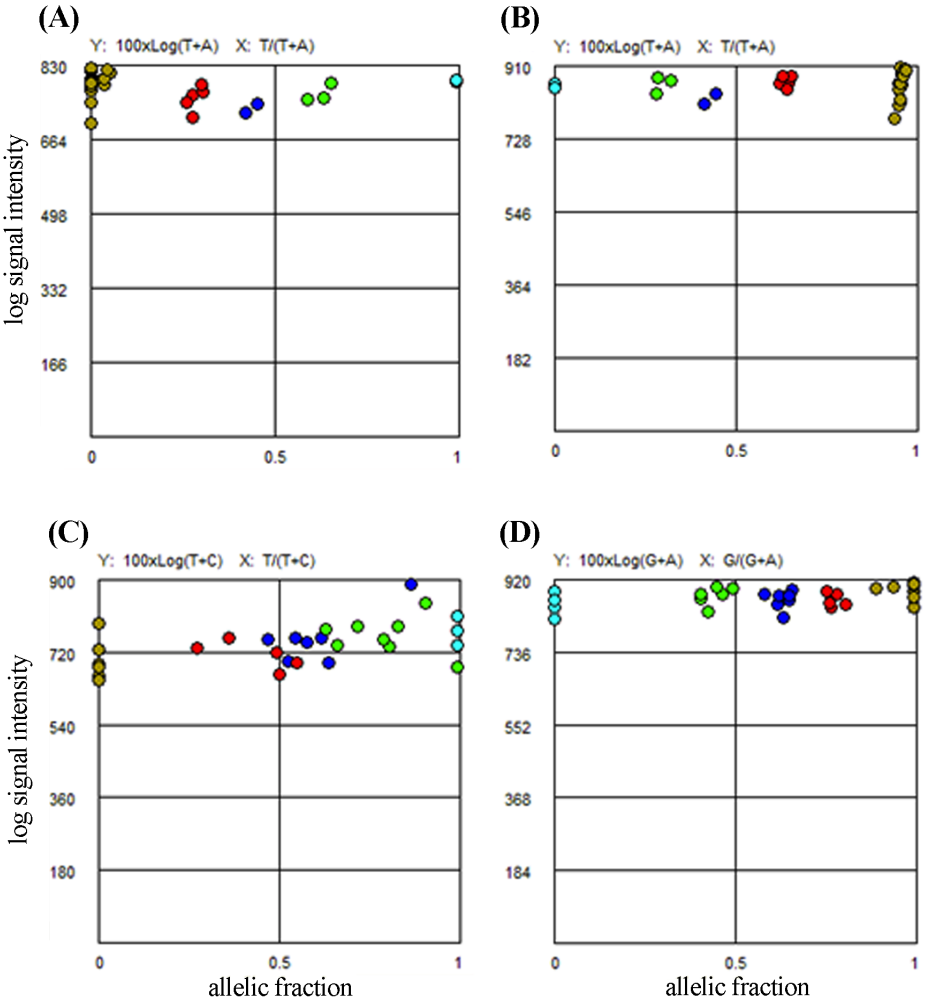

Figure 12. Log plots of allelic fraction. DNA samples homozygous for a given SNP (light brown and blue) are expected to be at zero or one on *x*-axis, with DNA samples heterozygous for a given SNP between them. **(A)** Sense strand for rs2000419 (A/T). T21 DNA samples are highlighted with red or green for A or T allele duplication, respectively. **(B)** Antisense strand for rs2000419 (T/A). T21 DNA samples are highlighted with red or green for T or A allele duplication, respectively. **(C)** Sense strand for rs2833844 (C/T). T21 DNA samples are highlighted with red or green for C or T allele duplication, respectively. **(D)** Antisense strand for rs2833844 (G/A). T21 DNA samples are highlighted with red or green for G or A allele duplication, respectively. Dark blue, euploid DNA samples (Figure 2 in Paper IV).

The T21 APEX-2 assay was validated through several steps, and quality parameters were obtained (Table 3, modification of Table 1 in Paper IV). We selected SNPs from the 21(q21.1q22.2) region (DSCR) and performed an initial assay validation on euploid DNA samples. Reliably performing oligos for 90 SNPs of 143 SNPs that passed *in silico* quality control were revealed. Oligos for 53 SNPs (37%) were excluded from subsequent analysis because of low call rate, low reproducibility, or false signals. The 90 validated SNPs (listed in Supplementary Table 1 in Paper IV) were further used to discriminate between 11 T21 patients and 9 normal individuals, genotyped in three to four replicates (70 experiments in total). As shown in Figure 13A (Figure 3A in Paper IV), the T21 and normal DNA samples were separately distributed from each other. Data from 58 experiments were used to calculate the call rate for each SNP (range 97–100%), (Supplementary Table S1 in Paper IV).

Table 3. Genotyping quality parameters for the T21 assay (modification of Table 1 in Paper IV)

Parameter	Assay validation	Blind study	CULT AC	UNCULT AC	CULT CVS	UNCULT CVS
Assay call rate (%)[a]	99.2	98.9	99.8	99.9	99.8	99.9
Assay specificity (%)	100	100	100	100	100	100
Assay sensitivity (%)	100	100	100	100	100	NA
Reproducibility (%)[b]	99.3	NA	NA	NA	NA	NA
Specificity of SNPs (%)	98.7	98.6	100	98.6	99.2	100
Sensitivity of SNPs (%)	98.4	96.9	98.8	99.1	98.3	NA

Abbreviations: NA, not applicable; CULT, cultured; UNCULT, uncultured; ACs, amniocytes; CVS, chorionic villus sample.

[a] Assay call rate: 6252/6300 counts in assay validation step; 3738/3780 counts in blind study; in evaluation step, 5209/5220 counts with cultured ACs; 4498/4500 counts with uncultured ACs; 1616/1620 counts with cultured CVSs; 809/810 counts with uncultured CVSs.

[b] Reproducibility of the assay was determined by genotyping five DNA samples as eight replicates and three DNA samples as seven replicates (61 experiments in total).

Subsequently, a blinded study using 16 T21 patients and 11 normal individuals for T21 was performed. Of the T21 patients, 10 DNA samples were duplicates. Of the normal samples, five were duplicates. Of the 42 DNA samples, 26 DNA samples were correctly classified as T21 and 16 DNA samples were classified as euploid (Figure 13B).

The threshold for annotating DNA samples as euploid or trisomic for T21 was determined by a validation assay. Results were confirmed *via* a blinded study of the clinical samples. According to this data set, T21 was confirmed when ≥80% of the heterozygous SNPs had an altered allele ratio compared to the normal controls. The DNA sample was annotated as euploid for chr21 when ≤20% of the heterozygous SNPs had an altered allele ratio. The analysis of the

APEX results revealed that the failure to detect the expected allele ratio of a SNP and incorrect determination of heterozygous SNPs might be caused by amplification bias of the analyzed SNP and/or low DNA quality. Hence, if the APEX primers for each SNP designed for both DNA strands could be analyzed autonomously, the number of incorrectly assigned SNPs would be narrowed down. Re-analysis of DNA samples should be performed if borderline results are obtained.

Finally, the T21 assay was used to analyze cultured and uncultured amniocyte (**AC**)/CVS samples. Of the 58 cultured AC samples, 55 DNA samples were annotated correctly as normal for chr21, and three were classified as T21. From the uncultured AC samples, 49/50 DNA samples were correctly determined to have two copies of chr21, and one DNA sample was correctly determined as T21. All uncultured CVS samples appeared to be normal for chr21, and 5/17 cultured CVS samples were annotated as trisomic for chr21 (Figure 13C).

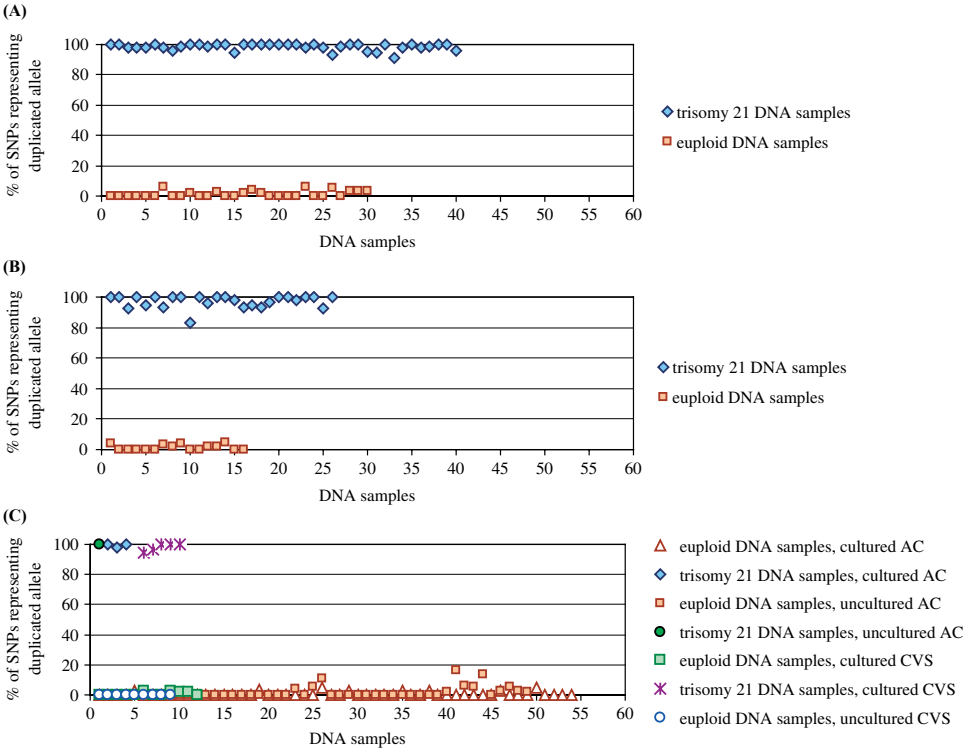

Figure 13. APEX-2–based analysis of 90 SNPs from euploid and trisomy 21 samples. Genotyping results are shown from (**A**) the validation step, (**B**) blinded study, and (**C**) cultured and uncultured fetal cells (Figure 3 in Paper IV).

To confirm the robustness of the APEX-2 assay concerning DNA quality, five DNAs extracted from cultured CVS samples fixed in 3:1 methanol-acetic acid (Carnoy's fixative) were used. Three of these samples were correctly annotated as T21 and two as normal for chr21. All of obtained results were confirmed by conventional cytogenetic analysis.

The presence of maternal cell contamination in fetal samples may be a potential cause for prenatal misdiagnosis. We compared fetal genotypes derived from 50 uncultured AC and 5 CVS samples with their mother's genotypes. No misleading maternal cell contamination was detected with the T21 APEX-2 assay. In contrast, maternal cell contamination could not be detected with MLPA, which is a popular technology for RAD (Kooper *et al.*, 2008). Another commonly used stand-alone RAD test is QF-PCR assay that allows the detection of maternal cell contamination and female triploidies. However, the set of markers used in that method can occasionally be uninformative (Cirigliano *et al.*, 2009).

In principle, the APEX-2 assay could potentially be used to detect triploidy when SNPs from other chromosomes are included on the array, because we estimate allele ratios of heterozygous SNPs in each locus autonomously. The problem with the uninformative marker set is minimized by the large number of SNPs included in the T21 array. The current panel of 90 highly heterozygous SNPs enabled the assessment of T21 in the general population, but a more specific selection of SNPs in ethnic minority groups and ethnic groups with high consanguinity may need to be added. For instance, two DNA samples from highly inbred families were excluded from the T21 APEX-2 assay validation, because only one SNP out of 90 was heterozygous.

The highly flexible microarray format of the APEX-2 assay is amenable for the addition of new loci without extensive optimization. It could be updated to analyze other common autosomal aneuploidies, such as trisomies 13 and 18, sex chromosome aneuploidies, and different gene mutations. For instance, along with the elevated risk of fetal T21, increased nuchal translucency has been associated with a wide range of gene mutations in diverse syndromes of chromosomally normal fetuses (Souka *et al.*, 1998). Introducing these causative loci to the APEX-2 assay would enable the abnormal ultrasound finding to be associated with either aneuploidy or gene mutations in a single test.

Data obtained from our proof of principle experiments demonstrated that the T21 APEX-2 assay is suitable for RAD because it is fast and reliably discriminates between T21 and nontrisomic DNA samples derived from cultured and uncultured fetal cells. The advantages of the T21 APEX-2 assay include its easy handling and robust template preparation compared to, for example, the aCGH platform. Additionally, the APEX-2 assay is not highly sensitive to input DNA quality, quantity, or extraction method, unlike MLPA (Kooper *et al.*, 2008) and aCGH (Fiorentino *et al.*, 2011). High-quality results were achieved from as little as 60 ng of gDNA extracted from uncultured fetal cells derived from 2 mL of amniotic fluid. The amount of starting material needed for analysis was

comparable to that for QF-PCR and MLPA, but much less compared to aCGH (without whole-genome amplification) (Bi *et al.*, 2008).

The developed T21 APEX-2 assay would be advantageous as a rapid pre-test to reduce parental anxiety during genetic counseling before finalizing karyotyping. The main advantages of the APEX-2–based assay for prenatal chromosomal aneuploidy detection include: **i)** short sample turnaround time (30 hours), **ii)** no need for fetal cell culturing, **iii)** simultaneous analysis of chromosomal and gene mutation loci, **iv)** possibility of multiplexing for hundreds of loci, **v)** cost-effectiveness, and **vi)** a flexible and easily customized set-up.

CONCLUSIONS

The developed APEX and APEX-2 microarray-based assays are suitable tools for the comprehensive screening of mutations / variations and CNVs in clinical practice. Main advantages of the APEX/APEX-2 assay include its flexibility and single-step hybridization of the template, followed by primer extension, which make the assay relatively fast, simple, and robust with fewer components, pipetting steps, and manipulations in the workflow. These advantages shorten the hands-on time and reduce operator-dependent errors and variability.

The main outcomes of the current thesis are:

1. A novel APEX-based genotyping microarray for the detection of 204 *CFTR* mutations was designed and validated. The significantly extended pan-ethnic mutation panel includes frequent CF mutations detected in both Caucasian and non-Caucasian populations. The CF APEX assay has been implemented in the molecular diagnosis of disease and routine CF mutation carrier screening in Caucasians and is especially beneficial in non-Caucasians and individuals of mixed ethnicity (Schrijver *et al.*, 2005).
2. Development of a novel diagnostic APEX microarray for simultaneous multigene mutation detection in SNHL patients. The designed and validated panel includes 198 mutations in eight genes underlying mostly NS-SNHL. The integration of the comprehensive SNHL APEX assay into newborn hearing screening follow-up allows the detection of genetic causes of deafness in children (and adults), improving medical management for patients and genetic counseling for families (Gardner *et al.*, 2006).
3. Molecular analysis of BBS and ALMS patients with a *BBS-ALMS1* mutation array. Development of the BBS-ALMS assay included the design and validation of 253 sequence variants in 10 BBS genes and one *ALMS1* gene. The assay detected at least one mutation in 40.5% of BBS families and 26.7% of ALMS families. Two BBS families segregating three BBS alleles were found, supporting oligogenicity or modifier roles for additional mutations. In contrast, more than two mutations were not found in any ALMS family. The study results confirm the value of the BBS-ALMS assay as an efficient and cost-effective first-pass diagnostic screening test (Pereiro *et al.*, 2011).
4. Proof-of-principle demonstration of the use of the modified APEX-2 assay as a new and reliable method for the rapid prenatal diagnosis of DS from uncultured fetal cells (AC / CVS). The developed T21 APEX-2 assay could be used as a rapid pre-test to reduce parental anxiety during genetic counseling before final karyotyping (Oitmaa *et al.*, 2010).

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WEB RESOURCES

Ciliopathy Alliance homepage: http://www.ciliopathyalliance.org
Connexin Deafness homepage: http://davinci.crg.es/deafness/
Cystic Fibrosis Mutation Database: http://www.genet.sickkids.on.ca/
Hereditary Hearing Loss homepage: http://hereditaryhearingloss.org
Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/
Human Genome Variation Society: http://www.hgvs.org
Illumina Inc. homepage: http://www.illumina.com
International HapMap Project: http://hapmap.ncbi.nlm.nih.gov/
Mitomap database: http://www. mitomap.org

SUMMARY IN ESTONIAN

Praimerekstensiooni metoodil põhinevate DNA kiipide väljatöötamine ja kasutamine molekulaardiagnostikas

Polümeraasi ahelreaktsiooni ja DNA didesoksü (Sangeri) meetodil sekveneerimise kasutuselevõtmine 1980. aastatel võimaldas ka pärilike haiguste molekulaarse diagnostika kiiret arengut. Järgnevate aastate jooksul on nende meetodite põhjal välja arendatud mitmeid erinevaid tehnoloogilisi lahendusi, mida oleks võimalik rakendada rutiinses kliinilises praktikas väga erinevate pärilike haiguste diagnostikaks ja sõeluuringuks.

Käesolev töö keskendus APEX / APEX-2 kiibitehnoloogial põhineva nelja erineva molekulaardiagnostilise testi väljatöötamisele. Antud töö tulemused võib kokkuvõtvalt sõnastada järgmiselt:

1) Arendasime välja APEX meetodil põhineva tsüstilise fibroosi (CF) testi, mis võimaldab teha nii molekulaardiagnostikat kui ka mutatsioonikandluse määramist. Selleks valisime välja 204 erinevates populatsioonides sagedasemat haigus-seoselist mutatsiooni tsüstilise fibroosi transmembraanse regulaatori geenist. Arendustöö tulemusena saime 100% sensitiivsuse ja 100% spetsiifilisusega CF APEX testi, mis võrreldes teiste kasutusel olevate paneelidega võimaldab kuluefektiivselt määrata üle kahe korra rohkem CF seoselisi mutatsioone nii kaukasoididel kui ka mitte-kaukasoidsete populatsioonide ja segunenud etnilise taustaga indiviididel (Schrijver *et al.*, 2005).
2) Arendasime välja APEX meetodil põhineva geenitesti peamiselt mittesündroomse sensorineuraalse kuulmislanguse (SNHL) patsientide diagnoosi molekulaarseks kinnitamiseks ja retsessiivse SNHL kandluse määramiseks. Selleks valisime välja 198 järjestuse varianti põhiliselt mittesündroomse SNHL fenotüübi väljakujunemisega seotud 8 geenist. Valideerimise tulemusena saime 100% tundlikkuse ja 100% spetsiifilisusega SNHL APEX testi. Selle testi kasutamine võimaldab kuulmislanguse geneetilisi põhjuseid tuvastada nii vastsündinute kuulmise sõeluuringu nn. järelkontrollis kui ka kuulmispuudega täiskasvanutel ja seeläbi parandada nii patsientide ravi kui nende perekondade geneetilist nõustamist (Gardner *et al.*, 2006).
3) Disainisime ja valideerisime APEX kiibi, mis võimaldab mutatsioonianalüüsi BBS ja ALMS patsientidel. Selleks valisime 253 DNA järjestuse varianti *ALMS1* geenist ja 10 erinevast *BBS* geenist. Antud uuringugrupi patsientide hulgas tuvastasime 40.5% BBS perekondadest ja 26.7% ALMS perekondadest vähemalt ühe mutatsiooni. Lisaks leidsime kahes BBS perekonnas 3 haigus-seoselise alleeli pärandumisjuhtu, mis kinnitab haiguse oligogeensust või vähemalt modifitseeriva mutatsiooni osalust haiguse väljakujunemisel. ALMS perekondades me üle kahe mutatioonialleeli ei leidnud. Selle uuringu tulemuste põhjal võib öelda, et BBS-ALMS test on piisavalt tõhus ja kuluefektiivne, et tema kasutamine esmase testina diagnostilises skriiningus oleks õigustatud (Pereiro *et al.*, 2011).

4) Töötasime välja APEX-2 tehnoloogial põhineva molekulaardiagnostiline testi Downi sündroomi kiireks sünnieelseks tuvastamiseks kultiveerimata looterakkudest. Valideerimise tulemusena saime kiire ja usaldusväärse, 100% spetsiifilisuse ja 100% tundlikkusega testi Downi sündroomi määramiseks. Kuna see meetodika võimaldab loote aneuploidiat määrata kultiveerimata looterakkudest on analüüsiks kuluv aeg (kuni 30 tundi) väga lühike võrreldes standardse karüotüpiseerimise ajakuluga (2–3 nädalat). Antud testi kasutamine geneetilise nõustamise skeemis võimaldaks kiiret loote eeluuringut Downi sündroomi suhtes ja seega lühendaks oluliselt vanematel pingerohket vastuste ootamise aega (Oitmaa *et al.*, 2010).

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