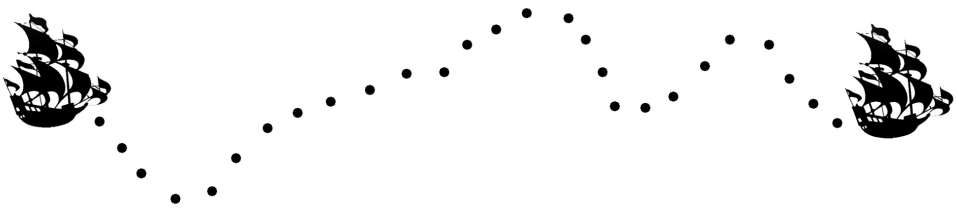
Chapter 1

General Introduction

Parts of this chapter have been published previously and have been adapted in a modified form.

High-Resolution Analysis of Genomic Copy Number Changes.
Hermsen M, Coffa J, Ylstra B, Meijer G, Morreau H, van Eijk R, Oosting J and van Wezel T. (2010) High-Resolution Analysis of Genomic Copy Number Changes, in Genomics: Essential Methods (eds M. Starkey and R. Elaswarapu), John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9780470711675.ch1

Genotyping and LOH Analysis on Archival Tissue using SNP Arrays.
van Eijk R, Middeldorp A, Lips EH, van Puijenbroek M, Morreau H, Oosting J and van Wezel T. (2010) Genotyping and LOH Analysis on Archival Tissue using SNP Arrays, in Genomics: Essential Methods (eds M. Starkey and R. Elaswarapu), John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9780470711675.ch3



Pathology: a historical perspective

The history of pathology began 3500 years ago with the documentation of disease by the Egyptians. Throughout the centuries, individuals in the Greek (Hippocrates and Aristotle, 4th century BC), Roman (Celsus, 1st century AD, and Galen, 2nd century AD), medieval Byzantine (Aetius, 6th century AD) and Arab (Avicenna and Avenzovar, 11th century AD) empires contributed to the medical field. [1] It can be claimed that anatomical pathology, or pathology as a separate medical specialty, began with the work of the Florentine physician Antonio Benivieni (1443-1502) [1,2]. Benivieni described autopsies and case histories and his work was published titled *De Abditis Morborum Causis* (The Hidden Causes of Disease). Some of his autopsy protocols are similar to those currently in use [2]. The first modern book of anatomy is mostly attributed to Andreas Vesalius (1514-1564). He published his *De humani corporis fabrica* (The Fabric of the Human Body) in 1543 (Nutton 2012). In 1554 Jean Francois Fernel (1497-1558) introduced the term "Pathology" in his *Medicina [3].*

The work of these pioneers was continued by others, including Giovanni Batista Morgagni (1682-1771) who started correlating signs and symptoms with findings at dissection, John Hunter (1728-1793) considered founder of scientific surgery, Mathew Baillie (1761-1823) by introducing the systematic study of pathology and Marie Francois Xavier Bichat (1771-1802), who contributed to the founding of histology [1,4–7]. Unfortunately, all this work did not contribute much to the health of the individual patient. Many pathological observations were made post-mortem, and patient treatment did not significantly improve for centuries [8]. Pathology was inseparable from other medical specialties, and individuals often had both pathological and clinical skills [1].

New spectacular developments in pathology arose in the mid-nineteenth century, largely because of the introduction and implementation of novel medical technologies; for the first time, it became common practice to apply pathological findings in patient care. Together with these changes, pathology developed as an independent medical profession. Thomas Hodgkin (1798-1866), later known for the eponymous disease, was one of the first to recognize that the "*microscope might lead to useful discoveries in the future*" [1]. Indeed, the microscope changed pathology by making it possible to histologically examine tissue on the cellular level. Since Rudolf Virchow published his text entitled *Cellular Pathology* in 1858, the basic understanding of cancer has greatly changed from an organ-based disease to a cell-based disease [9,10]. During this time period, along with anatomical pathology, "surgical pathology" was introduced in 1819 [10]. Other technological advances further enhanced the ability to pathologically examine tissue. In 1863, the introduction of the natural dye hematoxylin, derived from the logwood tree (*Haematoxylum campechianum*), led to the first successful description of the hematoxylin staining technique that is utilized today [11]. Beginning in 1826 synthetic aniline dyes were developed and contributed to the development of numerous histochemical stains [12]. The introduction of

the freezing microtome in the 1870s, paraffin wax embedding (1869, Edward Klebs) and tissue fixation with formaldehyde (1893, Ferdinand Blum) began a new area for the “modern” pathologist. It became possible to pre- or intra-operatively contribute to the diagnosis and treatment of a patient [10]. In the 1870s, Carl Ruge, a German gynecologist, microscopically diagnosed cervical and uterine cancer and may have been one of the first international consultants to interpret material from other countries [13]. In the 1890s, frozen sections were examined during breast cancer surgery. In this early period of surgical pathology, misdiagnosis and technological issues contributed to debate on the usefulness of these technologies, which lasted until additional technological advances were introduced in the pre-World War II era [10]. In 1941, Albert Coons and colleagues labeled an antibody with a fluorescent dye and used it to identify an antigen in tissue sections: Immunohistochemistry (IHC) was born. Since that time, tumor diagnosis has relied primarily on histopathological and immunohistological features [14,15].

The dawn of another era that significantly influenced pathology was at the horizon, “Molecular Pathology”. Although the cellular nature of tumors was described in the early nineteenth century, it was not until 1890 that David Paul von Hansemann (1858-1920), a German pathologist and a coworker of Rudolf Virchow, introduced the term “*anaplasia*” and proposed that normal cells are converted to tumor cells when they acquire chromosomal abnormalities[16]. At the same time, Theodor Boveri (1862-1915), who did not focus his studies on cancer, applied his observations of dividing sea urchin eggs and their abnormalities to what he perceived to be the genetic basis of malignancy. In 1914, he formulated 20 specific hypotheses regarding cancer biology in his book *zur Frage der Entstehung maligner Tumoren*; almost all of these hypotheses have been verified by studying cancer chromosomes in the 100 years after his publication [16,17]. These discoveries would not have been possible without yet another breakthrough that revolutionized pathology.

This was the description of the DNA double helix in 1953 by Watson and Crick [18]. This was the starting point of many new developments in molecular technologies which yielded many new insights into the molecular pathogenesis of cancer. These insights have had a large impact on cancer diagnosis, prognosis and therapeutics [19]. Fluorescence in situ hybridization (FISH) was first described in 1969 [20] and was applied in clinical diagnostics to detect HER2 amplification in breast cancer in 1992 [21]. The application of Southern blotting [22] and comparable techniques, such as pulsed-field gel electrophoresis [23], allowed researchers to identify molecular variations more rapidly. The construction of molecular probe collections and the discovery of restriction fragment length polymorphisms (RFLPs) enabled the mapping of the human genome [24] and the positioning of many genes [25–27] (figure 1). The study of the molecular basis of disease was further facilitated by the development of the polymerase chain reaction in the early 1980s at Cetus Corporation in California. Kary Mullis

was awarded the Nobel Prize for chemistry in 1993 for his contributions [28]. The introduction of fluorescent PCR, in which the increase in fluorescence per cycle can be monitored, made PCR the method of choice for gene expression studies and direct mutation analysis (Deepak et al., 2007).
Sanger sequencing was described in 1977 [29], and the combination of this method with PCR led to the detection of point mutations, polymorphisms and other small DNA rearrangements [30]. Sanger sequencing was most likely the first molecular methodology suitable for high-throughput, fully automated data acquisition and commercialization [31] and led to the first complete sequencing of the human genome [32,33]. With the introduction of massive parallel sequencing or "next-generation" sequencing strategies, DNA sequencing costs were dramatically reduced. The 1000 Genomes Project was consequently launched in 2008 [34–36], and reliable sequencing and analysis of complete cancer genomes became possible [37].

3110 *Nucleic Acids Research, Vol. 18, No. 10*

An additional MspI RFLP at the human hepatic lipase (HL) gene locus

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Source/Description: λHL2 contains a cDNA corresponding to the human hepatic lipase mRNA from position −8 to the poly A tail (1).

Polymorphism: In addition to the previously reported 1.0/1.2 kb MspI RFLP (1) MspI detects an additional RFLP with two allelic fragments of 4.8 and 5.8 kb.

Frequency: Studied in 61 Caucasians: 5.8 kb allele = 0.95
4.8 kb allele = 0.05

Not Polymorphic For: See (1).

Chromosomal Localisation: Human hepatic lipase is assigned to chromosome 15q15-q22 (1).

Mendelian Inheritance: Co-dominant inheritance demonstrated in four 2-generation families with 30 members.

Probe Availability: Request for probe to LC at the above address.

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Reference: 1) S. Datta *et al.* (1988) *J. Biol. Chem.* **263**, 1107−1110.

Figure 1. An additional MspI RFLP at the human hepatic lipase (HL) gene locus.

These technological advances over the years have and will continue to have a large impact on cancer research. Molecular pathology in combination with molecular tumor diagnostics has become standard hospital practice for genetic and genomic testing for clinically relevant discoveries in cancer [38,39].
The discovery of activating mutations in *BRAF* in several cancer sub-types in 2002 and the report of activating mutations in *EGFR* in lung cancer [19] in 2004 led to the development of high-throughput molecular screening methodologies. Personalized medicine has become an important strategy for oncologists, with the consequent need to test small or limited amounts of material and deliver the test results to the clinic as quickly as possible [40,41]. This all contributed to unprecedented beneficial outcomes for oncology patients. One of the most imaginative examples is the “Lazarus” effect, the concept that patients almost literally rise from the dead, on lung cancer patients with a specific EGFR mutation, and therefore a poor prognosis, that are subsequently treated with tyrosine kinase inhibitors [42].

Pathology: a local perspective
Leiden University, founded in 1575, and the Leiden University Hospital have a long history of developing and implementing novel ideas and technologies in anatomical and clinical pathology. In 1593, one of the first anatomical theaters in Europe was established [43]. A reconstruction of this theater can be visited in the Boerhaave museum in Leiden. Nicolaes Tulp (1593-1674), later portrayed by Rembrandt in “The Anatomy Lesson of Dr Nicolaes Tulp”, studied medicine in Leiden and, after moving to Amsterdam, contributed to medicine with his work *Observationes Medicae*. In it, he described in detail more than 200 cases of disease and death [44].

In the same period, Franciscus de le Boë Sylvius (1614-1672) came to Leiden as a physician and anatomist. At his instigation, the first University Chemical laboratory in Europe was founded in 1669 [45]. One of his students was Theodor Kerckring (1638-1693), who published the *Spicilegium anatomicum*, an anatomical atlas of clinical observations, medical curiosities, autopsy discoveries and general anatomical information. He used a microscope to investigate the folds in the small intestine [46].

Herman Boerhaave (1668-1738) made an important contribution to pathology by publishing autopsy reports of patients with a documented recent medical history [1]. Bernhard Siegfried Albinus (1697-1770), one of Boerhaave’s students, became one of the most famous anatomy teachers in Europe. In his work, *Tabulae sceleti et musculorum corporis human*, Albinus and his coworker, the artist and engraver Jan Wandelaar (1690-1759), employed a novel technique to increase the scientific accuracy of the anatomical illustrations. It was based on the artists’ traditional drawing-frame, which contained a grid to achieve systematic control over the rendering from a precisely established viewpoint [47].

Figure 2. Leiden Professors of Pathology in the 19th and 20th century.

A
S.S. Rosenstein (1832-1906)
Special Collections, Leiden University library

B
T.H. MacGillavry (1835-1921)
From the LUMC Department of Pathology Collection

C
D.E. Siegenbeek van Heukelom (1850-1900)

D
N. P. Tendeloo (1864-1945)

E
G.O.E. Lignac (1891-1954)

F
Th. G. van Rijssel (1917-1994)

G
A. Schaberg (1918-1999)

H
Ph.J. Hoedemaeker (1937-2007)

Gerard Conrad Bernard Suringar (1802-1874) contributed to this early part of the "Leiden history of medicine" by publishing 18 articles on it in the Nederlandsch Tijdschrift voor Geneeskunde between 1860 and 1870. Allard Calcoen, in his thesis *Onder Studenten* [48] describes the role of Leiden pathologists contributing to new directions in pathology in the second half of the 19th century.

Samuel Siegmund Rosenstein (1832-1906, figure 2), a prominent student of Rudolf Virchow became, after a period in Groningen, a professor at Leiden in 1873. He was one of the first clinicians in the Netherlands to recognize the importance of micro-organisms as a cause of infectious disease, and he was the first to describe and demonstrate the presence of tuberculosis cells in patients with kidney tuberculosis [48]. In 1899, he reminisced about the significant progress in the clinical treatment of patients, especially in the second half of the 19th century. Medicine moved from a time when theoretical and practical medicine were distinct to a period of close collaboration between physiological and pathological anatomy and the clinic, which he described as a "threefold alliance". As examples of the technical advances that contributed to this alliance, he described the application of the ophthalmoscope, the laryngeal mirror and the thermometer and laboratory developments in electric equipment, microscopy, the microtome and staining methods. Significant breakthroughs in surgery, bacteriology and pharmacology also contributed to improved patient care [49].

Other pioneers in this "new directions in medicine" included Theodorus Henricus MacGillavry (1835-1921, figure 2) and Daniel Eliza Siegenbeek van Heukelom (1850-1900, figure 2). Macgillavry, once characterized as "a man who can think microscopic", employed light microscopy techniques to study human leukemia [50]. When he arrived at Leiden, he remarked that the University was flourishing; however, he was unable to find a space that, in his opinion, could rightly be named a "Pathological Laboratory" unless "pathologically would be translated as inadequate and laboratory by booth." He sometimes performed pathological experiments in his house. His efforts contributed to the construction of a new laboratory that opened in 1885: The Boerhaave Pathologic Anatomic Laboratory located at Steenstraat 1A in Leiden (Figure 3). Siegenbeek van Heukelom, another skilled microscopist, garnered the most fame in the area of medicina forensis. In his capacity as 'police doctor', he observed organic changes in individuals who died post-operatively or collapsed after receiving chloroform anesthesia and consequentially contributed to the reduced use of chloroform as an anesthetic [48].
Nicolaas Philip Tendeloo (1864-1945) elevated the level of study in general pathology and pathological anatomy in the Netherlands, and a new laboratory for general pathology, anatomy and forensic medicine was designed and built based on his ideas and opened in 1925 at Wassenaarseweg 62/70 (Figure 3) [51,52]. This laboratory was used until 1994, when the department of pathology moved to its current location in the LUMC building at the Albinusdreef (Cicero 1994, vol 9, p16).

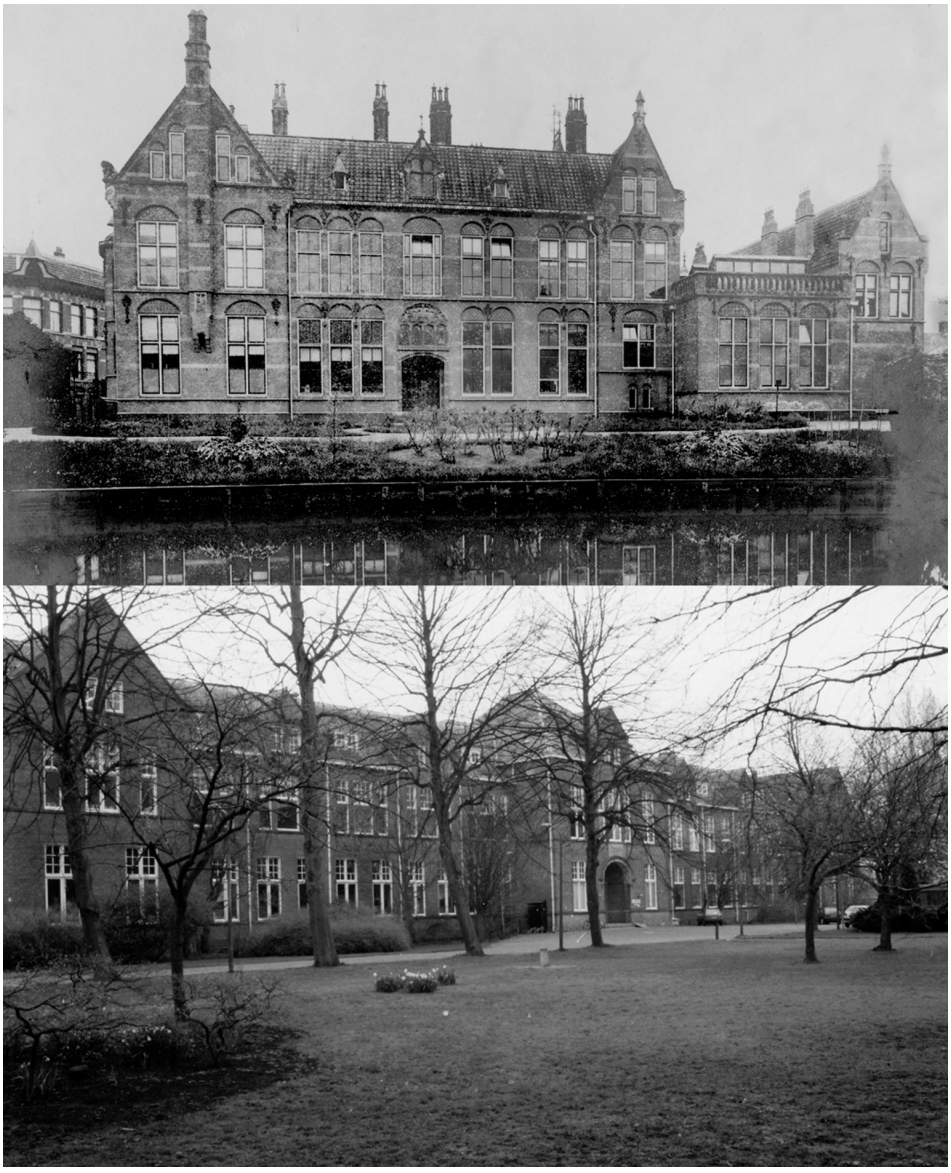

Figure 3.
Upper: The Boerhaave Pathologic Anatomic Laboratory at Steenstraat 1A, Leiden. In use from 1885-1925. Photo by amanuensis A. Mulder 1915.
Lower: Pathology Laboratory at Wassenaarseweg 62, Leiden. In use from 1925-1994. Photo by K.G. van der Ham, ca. 1993.

"*Quo vadis?*" was the intriguing title of an article published in 1952 in the "Nederlandse tijdschrift voor Geneeskunde" by George Otto Emile Lignac (1891-1954, figure 2) [53]. After reviewing the history of pathology, he predicted the biological importance of ribonucleotides and deoxyribonucleotides and concluded by stating: "Van de cellulaire tot de moleculairer pathologie, zal men zeggen. Inderdaad, deze weg moet onvermijdelijk worden begaan", which means:
"From cellular to molecular pathology, man will say: Indeed, this road must inevitably be chosen". And indeed, that road was taken.
An early reference to the use of molecular technology in the department is found in the 1958-1959 Pathology Annual Report. It states that Aart Schaberg (1918-1999, figure 2) initiated research to image chromosomes in malignant tumors (Verslag over de cursus 1958-1959, p4-§G3
As early as 1954, the year that Prof. Lignac tragically died in an airplane crash, Piet van Duijn (1921-2007) published a "new method" for the "combined staining of DNA and a number of polysaccharides" [54]. Theo van Rijssel (1917-1994, figure 2) succeeded Lignac in 1956, and he united diagnostics, educational and research. Several new technologies were introduced [55]. In the early 1960s Piet van Duijn expanded his interest to quantitative cytochemistry of DNA in the nucleus of different cell types and at different stages of cell division. He and his coworkers contributed much to the automation of cytochemical and cytogenetic analysis and the introduction of FISH (fluorescent in situ hybridization) in cytogenetic diagnosis. (van Duyn, 1960) (http://www.knaw.nl, accessed January, 2013).
In the same period and in collaboration with the Department of Histo and Cytochemistry, Sebastiaan Ploem developed an epi-illuminator known as the Ploem-opak, which has become an indispensable element in fluorescence microscopy (Cicero 2005, vol 12, p9).
The creation in 1971 of the "Foundation Pathological Anatomical National Automated Archive" (PALGA) was important for pathology in the Netherlands. Philippus Jacobus Hoedemaeker (1937-2007, figure 2) was one of the founders. Because of this system, the national cervical cancer screening study successfully began. Since then, the PALGA database has become a valuable resource for PA departments in the Netherlands to quickly and efficiently diagnose and determine the best treatment for cancer (http://www.knaw.nl, accessed January, 2013).
In the 1970s and 1980s, Cees Cornelisse, Dirk Ruiter, Philip Kluin and Gert-Jan Fleuren (head of the department 1993-2012), supported by their coworkers, further developed the molecular research in the department. DNA imaging technology and flow cytometry were used for the analysis of cervical and ovarian cancer [56–58]. From the early 1990s, in situ hybridization and chromosome and cosmic libraries were made available and used for the interpretation of chromosomal rearrangements [59–62]. PCR was introduced [63] and used to detect the loss of heterozygosity in fresh and archival tissue [64,65]. Polymorphic microsatellite markers were used to type flow cytometric sorted cells [66,67] and to identify potentially mixed-up samples [67].

Because of its clinical relevance, molecular diagnostic testing was first used in 1992 when clonality testing was performed using Southern blotting on 32 T-cell lymphomas (Jaarverslag Laboratorium voor Pathologie 1992, p28§F). Under the supervision of Hans Morreau, a preliminary list for molecular pathological indications was prepared in 1997, and in 1998 a quality control system for molecular testing was implemented in the laboratory (Jaarverslag Laboratorium voor Pathologie 1997, p32§E). In 1996, the first automated DNA sequencer, a gel-based ABI Prism® 377, was implemented in the laboratory, enabling new molecular testing techniques that focused on loss of heterozygosity (LOH) detection and microsatellite instability testing in colorectal cancer.
Since 1992, the number of molecular diagnostic consultations has increased to over 3000 in 2010. Over the years, there has been a constant demand for the development, validation and implementation of new and often high-throughput molecular technologies. In addition, new technological developments have been applied in the laboratory for molecular research. In 2003, the tissue microarray technique, which was developed by Sauter and Kallionemi [68], was introduced in Leiden [69], followed by microarray-based gene expression technologies [70]. Microarrays based on single nucleotide polymorphisms (SNPs) were used to detect the genome-wide loss of heterozygosity and other chromosomal aberrations [71,72], and since 2009, the first steps toward high-throughput sequencing have been made.

Pathological workflow
Tumor tissue becomes available for pathological examination at various disease stages through pre-operative testing by fine needle aspiration, tissue biopsy and the surgical treatment of patients with cancer. After delivering the crude material to the Department of Pathology, representative tissue samples are taken for further processing, including formalin-fixation and paraffin-embedding. In Dutch academic centers such as the LUMC, pathology departments maintain a systematic archive with millions of FFPE tissue blocks that have been collected and stored over the years. The oldest series of accessible paraffin blocks in the LUMC Department of Pathology dates back to 1946, and we estimate that a total of over 2.2 million blocks have been archived over the last 65 years.
To examine the material, pathological tissue sections are cut from the FFPE blocks with a microtome, stained with hematoxylin and eosin and delivered to the pathologist for initial examination and diagnosis.
The pathologist can further refine his diagnosis by making use of a selection of immunostaining, microscopy and molecular analysis techniques. After a molecular request is made, nucleic acids are extracted, the requested molecular analysis is performed and the results are reported and integrated in the pathological reports, which are communicated to the clinic. The remaining material is stored in the archives. Patient material can be subsequently used for scientific research and analysis according to medical ethical guidelines described in the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (http://www.federa.org, accessed January, 2013) as well as

local medical ethical guidelines. According to these guidelines, all human material used in research studies should be anonymized.
All clinical pathology processes are defined in protocols and standard operating procedures. The process of molecular analysis can be defined in a four-part workflow:

1: *Molecular pathological consultation*
2: *Pre-analysis technology*
3: *Molecular testing*
4: *Data acquisition, analysis and storage*

These four parts are schematically illustrated in figure 4. The majority of molecular pathological consultations can be handled using high-throughput processes, while customized solutions are available for less frequent tests. The workflow is not static but is influenced by technological and biological demands, developments and improvements. New developments should be validated and implemented to maintain high standards in patient care.

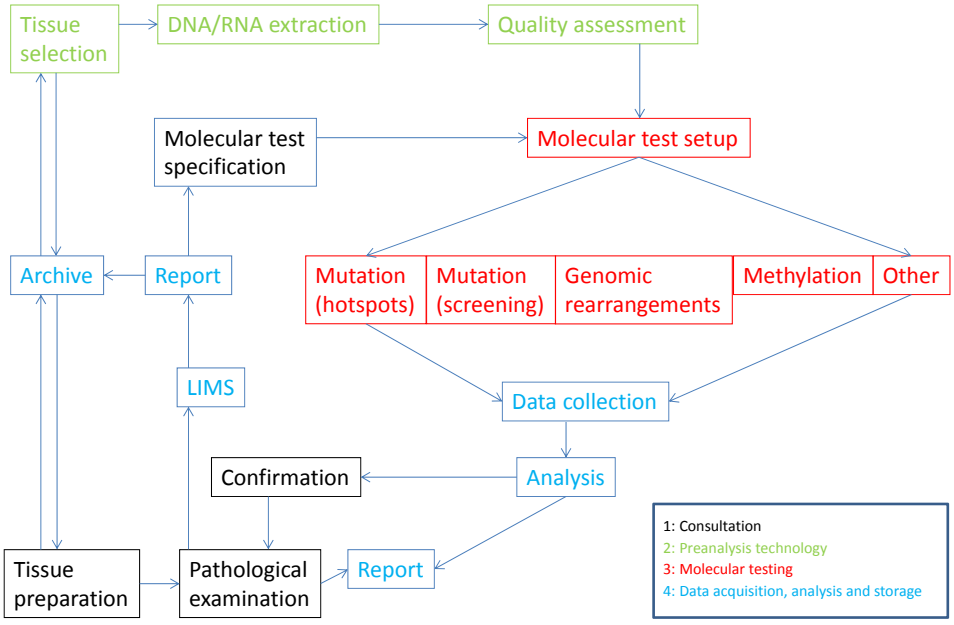

Figure 4. Diagram of the molecular analysis workflow.

Molecular analysis workflow

1: Molecular pathological consultation

The pathologist first decides which molecular pathological test to perform. Consultations for mutation hotspot analysis or extended mutation screening are frequent. Other consultations can be requested to detect genomic rearrangements and multi-gene or methylation-specific events. After a brief introduction to tumor genesis and general molecular principles, the different types of consultations for the detection of somatic mutations, multiple gene events, genomic rearrangements and methylation-specific events will be discussed.

Tumorigenesis

In essence, cancer is a genetic disease. Although certain cancers have specific unique characteristics, the development of human tumors is characterized by hallmarks that have been postulated and refined over recent decades. Mutations that affect oncogenes, tumor suppressor genes and tumor stability genes have been discovered, and these genes play an important role in tumor formation and progression [73–75].

Oncogenes and activated proto-oncogenes are characterized by a dominant gain of function and can have different origins. The first confirmed oncogene, Src, was discovered in 1976 by Bishop and Varmus, who received a Nobel Prize in 1989 for their work. Src encodes a tyrosine kinase, and mutations in Src lead to the malignant progression of cancer [76,77]. Src inhibitors have been developed and are utilized to treat cancer patients [78]. The Philadelphia Chromosome is another early example of an oncogene and became an early example of the use of chromosome analysis for cancer diagnostics. This chromosomal abnormality was first described in 1960 when Hungerford and Nowell detected a tiny aberration in the chromosomes of cultured blood cells taken from two patients with chronic myelogenous leukemia [79]. The Philadelphia Chromosome is created by the translocation of the sections of chromosomes 9 and 22 that include the Abl and Bcr genes, respectively. The Bcr-Abl fusion gene formed by this translocation codes for a constitutively active receptor tyrosine kinase that causes uncontrolled cell proliferation. Research efforts led to the development of imatinib mesylate (Gleevec), which was the first in a new class of genetically targeted agents, a major advance in cancer treatment [80]. *MYC* is another proto-oncogene and has been implicated in Burkitt's Lymphoma, named after Denis Parsons Burkitt, the surgeon who first described the disease in 1958 [81]. The MYC gene encodes a common transcription factor. In Burkitt's lymphoma, patients have a chromosomal translocation that moves an enhancer sequence near the *MYC* gene, resulting in increased expression of this transcription factor [82]

Tumor suppressor genes are divided in gatekeeper genes and caretaker genes, based on their function and generally follow the hypothesis that both alleles of the gene must be affected for cancer to develop. This two-hit hypothesis was

formulated by A.G. Knudson while studying retinoblastoma [83,84]. There are exceptions to the "Knudson" model for tumor suppressors; for example, "dominant-negative" mutations in the *TP53* gene produce a mutated p53 protein that inhibits the function of p53 produced by the wild-type allele [85]. Other exceptions to the Knudson model include tumor-suppressor genes that exhibit haploinsufficiency. In these cases, the level of one or multiple gene products is not sufficient for the cell to function normally. Haploinsufficiency of many genes, including *APC, ATM, BRCA1, BRCA2, TP53,* and *RB,* has been shown to contribute to tumorigenesis [86]. Gatekeeper genes such as *APC*, *RB* and *TP53* inhibit tumor growth or promote tumor death. Inactivation of a gatekeeper gene often leads to tissue specific types of cancer such a Retinoblastoma or Adenomatous polyposis coli [87,88]. Caretaker or stability genes are another class of tumor suppressor genes, that promotes tumorigenesis in a different, more indirect way, when mutated [75,88]. This class includes the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes responsible for repairing mistakes that occur during DNA replication or that are induced by mutagen exposure. Consequently, mutations in this class of genes increase the mutation rate of other genes. Similarly to tumor-suppressor genes, both alleles of stability genes typically must be inactivated to produce an effect [75]. Examples of genes in this class include *BRCA1, BRCA2,* which have been implicated in breast cancer and *MLH1, MSH2, PMS2, MSH6* and *MUTYH* in colon cancer. Many of these genes are currently being tested as molecular diagnostic markers.

Cancer cells can develop as a consequence of aberrations in these classes of genes. Eight different hallmarks of cancer cells have been postulated, and collectively they dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. Cancer cells do not act in isolation; rather, they subsist in a rich and heterogeneous microenvironment where the tumor stroma contributes to cancer initiation, growth and progression. All this should be considered in the molecular diagnosis and treatment of cancer [73,74,89].

Somatic mutations

Many examples are available to illustrate the need for somatic mutation analysis in patient care. For instance, mutations in the *KRAS* oncogene, codons 12, 13 and 61 are frequently found in many cancers. Activating mutations in *KRAS* codons 12 and 13 are associated with resistance to TKIs in non-small-cell lung carcinoma (NSCLC) [90,91] and are used to predict resistance to monoclonal antibody therapy in colorectal cancer (CRC) [92,93]. A specific *KRAS* c.34G>T transversion may indicate a failure in the base excision repair mechanism in colon cancer due to germline mutations in the *MUTYH* gene [94].

For *BRAF*, the V600E variant and the more rare V600K variant are found in the majority of cutaneous melanomas, and mutation-positive tumors can be treated with vemurafenib (PLX4032), which targets these molecules [95,96]. In papillary thyroid carcinoma (PTC), the V600E mutation appears to be refractory to radioactive iodine treatment, which consequently leads to a poor clinical prognosis [97,98]. However, initial studies have demonstrated that these thyroid cancers do not clinically react upon vemurafenib (PLX4032) treatment. Although reports indicate that the V600E alteration predicts resistance to monoclonal antibody therapy in colorectal cancer and NSCLC [90–93] , this knowledge has not been used clinically to date.
Mutations in the PIK3CA gene may play an important role in CRC but have not been associated with specific therapies and are still under study [99]. The *PIK3CA* hotspot mutations E542K, E545K, H1047R are also reported in NSCLC [90,91].
Hotspot mutations in *NRAS* and *HRAS* are present in specific types of benign and malignant thyroid cancers. *NRAS* mutations in codon 61 are reported to be involved in tumor progression and a more aggressive clinical behavior of the tumor [97,98,100].
Specific mutations in *GNA11* and *GNAQ* are found in uveal melanoma, which can be treated with MEK inhibitors [101].
Specific somatic heterozygous mutations in the isocitrate dehydrogenase genes *IDH1* and *IDH2* have been detected in the non-hereditary skeletal disorders Ollier disease and Maffucci syndrome and aid in the subclassification of these tumors [102]. Mutations in the *IDH1* gene are also found in malignant gliomas and have been used to further evaluate the disease [103]
A subset of NSCLC cancers may harbor an activating mutation in the *EGFR* kinase domain [104]. Deletions in exon 19 and the L858R variant in exon 21 are the most frequently found mutations, and tumors with these mutations are, in many cases, sensitive to tyrosine kinase inhibitors (TKIs). If the exon 19 and 21 hotspot mutations in NSCLC are not present, Sanger sequencing can be performed on exons 18-21 to identify rare variants that may predict a favorable response to TKI inhibitors [90,91].
The majority of gastrointestinal stromal tumors exhibit oncogenic activating mutations in the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT* tyrosine kinase) and platelet-derived factor receptor α (*PDGFRA*) [105–107]. In different types of melanomas, mutations in *KIT* exons 11, 13 and 17 are observed, and designer compounds, such as Imatinib, may offer an immediate therapeutic benefit for these patients [108].
Sanger sequencing of exons 5-8 in *TP53* can predict if the tumor is metastatic or if a secondary primary tumor has emerged [107,109]. Beta-catenin (*CTNNB1*) analysis is performed in desmoid fibromatosis to establish differential diagnosis and prognosis. The most frequently found mutations are in exon 3 and serve as potential molecular tools for disease management [110].

Multiple gene events and genomic rearrangements
Two major genetic mechanisms are frequently involved in CRC formation: the

chromosome instability (CIN) and microsatellite instability (MSI) pathways. Testing for CIN and MSI status can provide insight into the prognosis of the patient. In general, CIN+ and MSI+ cancers have worse and better prognoses, respectively [111–113].
The detection of 1p/19q chromosomal deletions has become essential for treatment decisions for cancer of the central nervous system. Oligodendrogliomas presenting with 1p/19q chromosomal deletions have favorable responses to chemotherapy and a substantially longer survival [103,114].
Other markers used in the evaluation of malignant gliomas are alterations in the epidermal growth factor receptor (EGFR) pathway and chromosomal deletions and amplifications in *CDKN2A* (p16), *EGFR*, *ERBB2* (HER2), *PTEN* and *TP53* [114,115].
Chromosomal rearrangements also play an important role in the formation of the thyroid cancer variant PTC. The rearranged during transfection (RET) gene is a tyrosine kinase receptor located on chromosome 10 and is often found to be mutated in PTC. Thus far, 13 different types of *RET*/PTC rearrangements have been identified [116]. *RET*/PTC chimeric proteins lead to constitutive activation of the tyrosine kinase domain and other downstream pathways. Compounds that have an inhibitory effect on the kinase activity of RET have been identified and tested in multiple clinical trials [97,98,100].
Gene fusions, which occur due to specific chromosomal translocations, are observed in many soft tissue tumors, such as Ewing sarcomas. These rearrangements help to improve the diagnosis of a wide variety of sarcomas in children and young adults [117]. The fusion of anaplastic lymphoma kinase (*ALK*) with echinoderm microtubule-associated protein-like 4 (*EML4*) is present in approximately 5% of all NSCLC cases and has become a clinical target for ALK inhibitors [118].
Recently, more and different *RET*, *ALK* and *ROS* fusions have been identified in lung adenocarcinomas through whole-genome and transcriptome sequencing [119], targeted next-generation sequencing [120] and integrated molecular- and histopathology-based screening [121] and will most likely be identified as relevant clinical targets.

Methylation specific events
The inactivation of tumor-suppressor genes by DNA methylation in the promoter region of the gene is associated with a loss of expression and plays an important role in gene silencing. These effects are well recognized in carcinogenesis and can have diagnostic, prognostic and predictive value [122].
Hypermethylation of the *MLH1* promoter can be used to subclassify sporadic colon cancer patients with a microsatellite instable (MSI) pattern from those with Lynch syndrome MSI tumors [111,123].
The methylation status of the O-6 methylguanine-DNA methyltransferase (*MGMT*) promoter is used to evaluate malignant gliomas [124]. Epigenetic silencing of *MGMT* augments sensitivity to temozolomide, which damages DNA by methylating the O-6 position of guanine, leading to cell death [114,115]

2: Pre-analysis technology

The application of DNA methodology in tumor genetics and genomics has been hampered by two major factors. First, because the patient material is mainly used for microscopic examination and has to be prepared for long-term storage, almost all available patient material is formalin-fixed and paraffin-embedded . The fixation and embedding procedures leave cellular structures mainly intact but damage nucleic acids. Consequently, nucleic acid isolation is a challenging task and often yields heavily degraded DNA for use in further analyses. Second, due to early diagnosis and the use of novel neo-adjuvant patient treatments, the number of cases for which only very small amounts of material are available is increasing [15].

In this part of the workflow, the type of available material and how it must be processed must be defined before DNA can be isolated. In a minority of cases, high-quality DNA can be isolated from freshly frozen tumor samples, but generally, FFPE or cytological tissue is the primary source available for processing. The pathologist decides if tissue cores can be taken or if the isolation should be performed on tissue slices. If tissue slices are available, whether the whole slice can be used or if micro-dissection is necessary, should be determined. After isolation, the quality and quantity of the isolated DNA should be assessed to determine if it is sufficient to perform the next steps in the process. In some cases, the DNA has to be diluted, concentrated or treated with a whole genome amplification step. Material preservation, micro-dissection, DNA isolation, whole genome amplification and DNA quantification will be discussed further.

Tissue preservation

Fresh or freshly frozen materials are the best sources for the extraction of nucleic acids and protein. However, for the microscopic analysis of tissue slices, FFPE material has been used for over a century in daily pathological practices [125]. Storage of FFPE tissues is inexpensive, and the embedded tissue can be kept almost indefinitely at room temperature. Therefore, laboratories with pathological archives have endless amounts of FFPE tissue samples [125,126].

Tissue fixation is commonly achieved by the addition of a 4% aqueous solution of buffered formaldehyde [127]. However, in some tissue types, additional or pretreatment steps must be performed [128,129]. Unfortunately, in this process of fixation and embedding, chemical crosslinking between RNA, DNA and protein occurs. Together with the addition of monomethylol groups to nucleotide base pairs, the quality of the nucleotides in the tissue is diminished. Only degraded and short fragments of the DNA and RNA remain for use in molecular analyses [130].

Microdissection versus macrodissection

For pathological examination of FFPE material, tissue sections are cut from tissue blocks. The sections are stained with hematoxylin, which stains the cell nuclei blue, and eosin, which stains the cytoplasm and other extracellular substances red or pink. There are different methods for further processing the material to make it suitable for molecular testing. For solid tumors, it is likely that

tumor cells are present in high concentrations, making it possible to use whole tissue sections or tissue cores for DNA extraction. However, in the presence of abundant stroma and other normal cells, the tumor cells may be obscured, making these samples more difficult for specific DNA or RNA analysis. To circumvent this complication, tumor cell enrichment strategies, such as cell sorting, laser capture microdissection or manual microdissection, can be performed. With these methods, the tumor epithelium is separated from the surrounding stroma and healthy tissue [131,132].
In laser capture microdissection (LCM), a transparent thermoplastic film or other coating is applied to the surface of a tissue section on a glass slide. A laser pulse then specifically activates the film above the cells of interest, and consequently, a strong focal adhesion permits the selective procurement of the targeted cells [133,134]. LCM can process very tiny amounts of pure tumor cells, but unfortunately, this method is time consuming and labor intensive. Therefore, in many routine settings, manual microdissection is performed with a scalpel blade. In this method, guided by an H&E-stained slide, tumor fields are scratched from deparaffinized, hematoxylin-stained copies of the original tissue slice. If very little material is present, which is often the case in cytological smears of the lung, microdissection is initiated by marking the tumor foci with a diamond needle on the back of a H&E- or Giemsa-stained slide. The cover slips are removed by soaking the slides in xylene. Finally, the tumor foci are collected with a scalpel blade [135] [136].

DNA isolation
The isolation procedure begins with the deparaffinization of the tissue, which is a time-consuming step in most protocols and is often performed by xylene incubation followed by ethanol washing steps [137,138]. Several methods have been described to isolate DNA after deparaffinization. The majority of the methods require manual isolation steps, although some (semi-) automated methods have been described. Column- or bead-based methods are most commonly used [139–141]. The quality and quantity of the DNA obtained with these different techniques is variable, and the final DNA yield can be low and of reduced quality. The type, quality or quantity of the input material contributes to the DNA yield, but the isolation method can also play an important role [142,143].

Whole genome amplification
To obtain sufficient DNA for further molecular testing, additional steps, such as whole genome amplification (WGA) or other pre-amplification steps, may be necessary [144,145]. WGA ideally generates a new whole genome sample of amplified DNA (wgaDNA) that is indistinguishable from the original sample but contains a higher DNA concentration [142]. There are two types of WGA: WGA based on PCR and WGA with non-PCR-based linear amplification [146]. The major disadvantage of all WGA methods is that ideal conditions do not exist. De novo mutations can be introduced, and parts of the genome can be preferentially under- or over-represented in the wgaDNA due to GC content or repetitive

sequences. The introduction of de novo point mutations should be considered, particularly when performing further DNA tests, and wgaDNA should not be used in single nucleotide hotspot mutation analysis [147]. Some WGA methods require high molecular weight DNA. An example is the strand displacement amplification method (SDA), which is based on rolling circle amplification [148]. Primer extension pre-amplification methods have been more successfully applied to FFPE tissue because these methods can better accommodate fragmented DNA [149,150].

DNA quantification

Different methods are available to measure the quality and quantity of the isolated or amplified DNA. One method is to perform a spectrophotometric measurement using a NanoDrop® instrument. In this approach, the ratio of the absorbances at 260 nm and 280 nm is used to assess the purity of the DNA. A ratio of ~1.8 is generally accepted as “pure” for DNA. Little material is needed, and the method can be performed rapidly. However, the measured DNA concentration may be an overestimate because all DNA fragments, even if partly degraded, give a fluorescent signal. Additionally, ionic strength and pH can influence the estimated DNA concentration [151]. Nanodrop analysis is mainly used to measure the concentration of amplified DNA. If heavily degraded DNA isolated from FFPE material is measured, the DNA concentration can be overestimated, leading to the failure of downstream applications. In this situation, more accurate approaches should be used. This can be achieved by making use of an intercalating dye such as PicoGreen®. This dye is essentially non-fluorescent and will only exhibit fluorescence after binding to double-stranded DNA. A high linearity is achieved, and dsDNA concentrations can be deducted by making use of a standard curve [152]. Therefore, PicoGreen-based assays are preferred for quantitative measurements of DNA extracted from FFPE material over spectrophotometric approaches in which no, or limited, information is gained on the quality of the DNA. An apparently highly concentrated sample may be composed of heavily degraded DNA and short DNA fragments, while a sample with an apparently low DNA concentration may be composed of longer and better quality DNA. Consequently, the best way to gain insight into the quantity and quality of the DNA may be to perform “quality” PCR. In this approach, amplicons located on different loci in the genome and of different lengths (for instance, 100, 200 and 400 base pairs) are generated and further analyzed on an agarose gel or by real-time PCR, making use of threshold cycles (Cq) and melting curve profiles. The longer the fragments generated, the higher the quality of the DNA, and this can be taken into account when making DNA dilutions for genetic testing [153,154]. Alternatively, capillary electrophoresis using LabChip instruments, such as the low-throughput Agilent 2100 Bioanalyzer and the high-throughput Caliper LabChip GX can be used to give both qualitative and quantitative information about the samples. The Life technologies Qubit® 2.0 Fluorometer, used in combination with Molecular Probes® dyes or methods, is another alternative; it makes use of fluorescent dyes specific for non-degraded nucleic acids.

3 and 4: Molecular testing, data collection, analysis and storage

In the third part of the workflow, molecular testing is performed. Mutations can be detected with closed-tube (real-time) PCR technology and different types of low-throughput sequencing. Copy number and chromosomal rearrangements can be detected with more complex technologies such as multiplex ligation-dependent probe amplification (MLPA), CGH and SNP arrays. High-throughput sequencing technology can be used to combine mutation screening with chromosomal rearrangement analysis. In the fourth and last part of the workflow, all of the acquired data are processed and analyzed using dedicated software or specialized analysis tools. The analyzed data are linked to the clinical and pathological information of the patient. After a careful process of analysis and quality evaluation in a diagnostic setting, the final results are reported to the clinic. In a research setting, the data can be further processed and analyzed to answer biological hypotheses. In this section, different methods for somatic mutation detection and prescreening, including hydrolysis probe assays and high resolution melting and sequencing, will be presented. Copy number variation assays with MLPA and SNP arrays will be discussed, and high-throughput next-generation sequencing will be introduced.

Somatic mutation detection and analysis

Over the years, many different techniques have been developed and used in research and diagnostics to detect somatic mutations. Most of these techniques are based on PCR. Hotspot mutation analysis can be performed with hydrolysis probe assays [136], Matrix-assisted laser desorption ionization Time of Flight (MaldiToF) [155], SNPshot [156] and pyrosequencing [157]. For mutation scanning, Sanger sequencing, although it's sensitivity is limited to 10-20% for somatic mutations, remained for long the gold standard [158]. To accelerate the process and reduce costs, many types of prescreening methods can be applied. An example of prescreening methodology is high-resolution melting analysis (HRM or HRMA) [142], which can be used in combination with COLD-PCR [159]. Other examples include denaturing high-performance liquid chromatography (DHPLC) [160], conformation-sensitive gel electrophoresis (CSGE) [161] and single-strand conformational polymorphism detection (SSCP) [162]. High-throughput next-generation sequencing (HT-NGS), massive parallel sequencing and third-generation sequencing are all different terms for the methodologies developed over the last decade. These techniques have greatly increased sequence throughput while decreasing costs [163,164]. The first generation of HT-NGS platforms delivered 100 Mb (Roche 454 Genome Sequencer) to 3 Gb (Illumina Solid Genome Analyzer) of sequence data per run [165]. Since the introduction of HT-NGS, sequencing chemistry and hardware has rapidly improved. New small bench-top sequencers have been developed with simple sample preparation protocols and the potential for faster data generation and analysis, making them suitable for implementation in molecular diagnostics [164].

Hydrolysis probe assays

Real-time quantitative PCR permits the sensitive, specific and reproducible quantitation of nucleic acids [166] and can be used in high-throughput, automated technologies with lower turnaround times [167]. Some of the various real-time PCR chemistries use the double-stranded DNA-intercalating agent SYBR® Green 1, while others use hydrolysis probes, dual hybridization probes, molecular beacons or scorpion probes [168]. To detect hotspot mutations, the hydrolysis probe method is frequently used. This method is often referred to as the "TaqMan" assay, but this is a brand name. Concerning the chemistry of this method, the Expert Review of Molecular Diagnostics states, "*In the real-time quantitative TaqMan® assay, a fluorogenic nonextendable 'TaqMan' probe is used. The probe has a fluorescent reporter dye attached to its 5´ end and a quencher dye at its 3´ terminus. If the target sequence is present, the fluorogenic probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of the Taq polymerase enzyme during the extension phase of the PCR. While the probe is intact, FRET occurs, and the fluorescence emission of the reporter dye is absorbed by the quenching dye. Cleavage of the probe by Taq polymerase during PCR separates the reporter and quencher dyes, thereby increasing the fluorescence from the former. Additionally, cleavage removes the probe from the target strand, allowing primer extension to continue to the end of template strand, thereby not interfering with the exponential accumulation of PCR product. Additional reporter dye molecules are cleaved from their respective probes with each cycle, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced*." [168]

Real-time PCR data acquisition is performed using the software provided with the real-time PCR equipment. These analysis platforms are often too basic for further data analysis, and additional dedicated software is required. For instance, to analyze expression data, Vandesompele developed the widely used tool "Genorm" [169]. The analysis of real-time SNP-type data is easier to perform. However, dedicated approaches must be used if multiple variations on the same locus are interrogated (this thesis). A very important development in real-time PCR analysis is the effort to come to a worldwide consensus on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. This developments led to the drafting of a list of guidelines, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) [170].

High Resolution Melting

HRM is a fast and simple alternative method for hydrolysis probe assays and mutation scanning in general [171]. This method is based on the principle that heating DNA results in the transition of the double-stranded DNA molecule into its two single strands. This process can be accurately monitored by measuring the fluorescence after the addition of a saturating DNA dye to the PCR reaction and after increasing time points and decreasing temperature units in an instrument with improved temperature precision [172]. A review by Erali et al. describes the main advantages of this method: *"Simultaneous genotyping with one or more*

unlabeled probes and mutation scanning of the entire amplicon can be performed at the same time in the same tube, vastly decreasing or eliminating the need for re-sequencing in genetic analysis." [173]
The analysis of HRM data depends on the instrument used and consists of one or two normalization steps. First, the fluorescence (Y) axes of HRM plots are normalized on a 0 to 100% scale. In the next, optional step, normalization to the temperature (X) axis can be applied to compensate for well-to-well temperature measurement variations between samples. Finally, the different genotypes can be identified by plotting the difference in fluorescence between the normalized melting curves. One melting curve is chosen as a reference, and the difference between each curve and the reference is plotted against temperature to yield a "fluorescence difference" plot. The original reference curve is a horizontal line at zero, and the different genotypes are clustered along different paths [171].

Sanger DNA sequencing
Sanger DNA sequencing has been one of the most widely used molecular techniques because it provides direct insight into the molecular composition of the material under investigation and can be easily automated. Sanger sequencing is based on the synthesis of a complementary copy of a single-stranded DNA template. To perform a sequencing reaction, a buffered mixture of DNA polymerase, a template-specific oligonucleotide, deoxynucleotides and fluorescently labeled dideoxynucleotides is added to the single-stranded DNA template. After cycling, DNA copies of various lengths are formed from the original template. The length of the products is determined by the position at which a fluorescent dideoxynucleotide is incorporated in the strand [31]. After capillary electrophoresis, the different length products are visualized and further analyzed with dedicated sequence analysis software. Mutation Surveyor, PolyPhred Sequencher and Sequence Pilot are commercial packages, but freeware for basic (Chromas, FinchTV) or more advanced sequence analysis (InSNP) can also be used [174]. It is important that the software can detect somatic mutations in cancer, which are often obscured as a consequence of tumor heterogeneity or the presence of excess normal DNA in the isolates. In addition, it is essential that information on mutations and variations in the human genome is communicated in a uniform way. In an effort to clarify the nomenclature, the Human Genome Variation Society (HGVS) has formulated guidelines and recommendations for gene variation nomenclature and variation databases [175–177]. Sequence variants in multiple genes per patient can be stored in a patient information system or database dedicated to the storage of gene variants, such as the Leiden Open-Source Variation Database (LOVD) [178,179].

Copy number variation detection and analysis
A number of methods can be used to detect copy number variation. A distinction can be made between methods that interrogate only one or a few loci and methods that can be applied for copy number variation analysis of the whole genome. Fluorescence in situ hybridization (FISH) and loss of heterozygosity (LOH) typing

with microsatellite markers are used to detect chromosomal imbalances in a single locus. To interrogate up to 50 different loci, multiplex ligation-dependent probe amplification (MLPA) can be performed. Genome-wide high-throughput methods, such as array-based comparative genome hybridization (array CGH) and single nucleotide polymorphism arrays (SNP arrays), have been applied in cancer research and diagnostics [72,180,181]. New developments in this field include the introduction of digital PCR for a single locus and high-throughput sequencing for whole genome-based copy number variation testing [182,183]. The use of MLPA and SNP array genomics will be discussed in more detail.

Multiplex ligation-dependent probe amplification

The MLPA technique was first described in 2002 [184] and has become a multiplex technique for determining the copy numbers of genomic DNA sequences and promoter methylation status, as well as for mRNA profiling [185]. MLPA is a PCR-based approach that is sufficiently sensitive, reproducible and sequence-specific and allows the relative quantification of up to 50 different targets simultaneously. MLPA is relatively easy to perform with standard laboratory equipment, a PCR instrument and a capillary sequencer. In MLPA, probes, not sample nucleic acids, are subject to amplification and quantification. Each locus is interrogated with two MLPA probes, which hybridize to adjacent sites of the target sequence. One probe is a short synthetic oligonucleotide, and the other is an M13-derived, long oligonucleotide. The short probe contains a target-specific sequence (21–30 nucleotides) and a 19-nucleotide sequence at the 5' end that is identical to the sequence of a labeled PCR primer. The long MLPA probe contains 24–43 nucleotides of target-specific sequence at the 5' phosphorylated end, a 36 nucleotide sequence that contains the complement of an unlabeled PCR primer at the 3' end, and a stuffer sequence of variable length in between. This variable-length fragment gives each complete probe the necessary size difference for detection and quantification using capillary gel electrophoresis [184]. When both probes are stably hybridized to adjacent sites of the target sequence, they are ligated by a specific ligase enzyme, permitting subsequent amplification. MLPA probes are identified after capillary separation by size using a selected size standard for the size calling procedure. The relative MLPA probe signals (fluorescent units) reflect the relative copy number of the target sequence. An indication of the DNA input in the MLPA reaction may be obtained by examining the dosage quotient (DQ) control fragments, fragments whose lengths always co-vary and are present in all MLPA kits. The signals of these fragments will be prominent if the amount of sample DNA is very low. By contrast, the fifth control band of 92 nucleotides is ligation-dependent and should have a signal similar to most of the other MLPA amplification products. Visual inspection of the peak pattern of a patient sample superimposed over a peak pattern of a reference run can be used to analyze a few samples [185].

The analysis of a larger series of samples, more complex diseases and MLPA runs performed with miscellaneous sample types and quality requires exportation of the peak signals and reliable normalization methods. Statistical methods

must be applied to identify probes that show aberrant copy numbers. Analysis overviews must be made available before the method can be applied in molecular diagnostics [186–188].

SNP arrays

Different methodologies of SNP typing and types of commercially available SNP arrays have been developed. Basically, two types of arrays exist: arrays with universal capture oligonucleotides or locus-specific arrays of oligonucleotides.

The SNP typing assays include methodologies such as allele-specific primer extension and whole genome sampling. Two different genotyping methods, molecular-inversion probe (MIP) genotyping and GoldenGate genotyping, are based on high-level multiplex PCR with universal primers in combination with universal arrays.

Molecular-inversion probe (MIP) genotyping arrays are commercially offered as Affymetrix OncoScan™ arrays. MIP genotyping utilizes a pool of locus-specific probes. The 5' and 3' ends of each circularizable probe anneal upstream and downstream of the SNP, respectively. The 1 bp gap is filled in a different reaction for each nucleotide. The probes are subsequently circularized using ligase to seal the remaining nick, and non-annealed and noncircular probes are removed by exonuclease treatment. Restriction digestion then releases the circularized probe, and the resulting template is PCR-amplified using common primers [189]. The reactions for each of the four nucleotides are labeled in different colors and pooled. Subsequently, the pool is hybridized to an array of universal-capture probes, and the four colors are read. With MIP arrays, the entire genome can be interrogated with more than 335,000 markers using 75 ng DNA isolated from FFPE tissue [190,191].

GoldenGate genotyping makes use of a multiplex mixture of probes for 96, 384, 768 or 1536 SNPs per array [192]. For each SNP, a combination of allele-specific and locus-specific primers are annealed to the SNP locus. These primers are tailed with common forward and reverse primers and a universal capture probe that is complementary to the locus-specific primer. The small gap between the allele and locus-specific probes is filled by allele-specific primer-extension and sealed with a ligase, resulting in an artificial allele-specific PCR template. This template is then PCR amplified using fluorescently labeled universal PCR primers. The resulting probe is hybridized to an array of universal-capture probes, and the array is scanned in a special reader, generating two fluorescent signals that represent the two different alleles of a SNP.

Locus-specific arrays of oligonucleotides, such as Affymetrix GeneChips, can detect over one million SNPs on a single chip. For instance, the Genome-Wide Human SNP Array 6.0 features 1.8 million genetic markers, including more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes to detect copy number variation. For each SNP, a set of locus-specific 25-mer oligonucleotides is present on the array. The sample is prepared according to the whole-genome sampling assay [193], a method in which the genomic complexity is reduced through restriction enzyme (RE) treatment of high-quality genomic

DNA and ligation of a common adaptor to the digested DNA. The subsequent single-primer PCR step reduces the genomic complexity through efficient size-selection in the PCR reaction. The product is then hybridized to a locus-specific array. The SNPs on the array are selected from the DNA that is represented after the complexity reduction PCR step [194].
Illumina Infinium arrays are locus-specific arrays with allele-specific capture probes. In this assay, whole-genome DNA is amplified and subsequently fragmented. The resulting probes are then denatured and hybridized to the array. An 'on the array'-allele-specific primer extension assay is followed by staining and read using standard immunohistochemical detection methods [195]. This type of array is available for genotyping, copy number variation (CNV) and cytogenetic analysis and consists of 300,000 to nearly 1.2 million markers.
After scanning the SNP arrays, the signal intensities must be converted into genotype calls. SNP calling software is available for each platform: BeadStudio for GoldenGate and Infinium, GTYPE and Genotyping Console for GeneChips, and GTGS for the MIP assay. All programs are essentially similar with three clusters automatically computed for each SNP: heterozygous AB and homozygous AA or BB. The clusters are based upon the allele-specific signal intensities. Genotyping errors and no-calls will hamper linkage and association studies, and reliable SNP calls are essential for these applications. Therefore, additional genotyping algorithms have been developed to improve the quality of the genotypes from SNP arrays. Examples of these methods are SNIPer [196] AccuTyping [197], SNPchip [198] and RLMM [199]. GTC software is available for the simultaneous analysis of SNPs, copy number polymorphisms (CNPs), rare copy number variations (CNVs) and cytogenetic aberrations (http://www.affymetrix.com/, accessed January, 2013). For sensitive analysis of copy number variation in tumors, BeadarraySNP, a Bioconductor package was introduced for the analysis of Illumina SNP array data. An algorithm, the lesser allele intensity ratio (LAIR), was developed to accurately determine allelic (im)balances. Further incorporation of the ploidy status of the tumor permits the identification of the allelic state of all chromosomal aberrations, including LOH, copy-neutral LOH, balanced amplifications and allelic imbalances. For the validation series, 300 k CytoSNP-12 (Illumina, USA) high-density SNP arrays were used [72].

High Throughput Next Generation Sequencing (HT-NGS)
With the current technological advances in next-generation sequencing, the simultaneous sequencing of hundreds of candidate genes up to the whole exome [34], the transcriptome [37,200], the epigenome [201] and the whole genome [202] has recently become feasible. HT-NGS technology provides the opportunity to identify previously unknown cancer-predisposing genes or somatic mutations in individual patients, families and tumors [203,204]. For instance, the identification of novel genes that predispose patients to colorectal cancer could be directly implemented in clinical practice. In clinical genetics centers, the knowledge of disease-related genetic mutations could be used for counseling and to advise the surveillance of these mutations in families. The identification of at-risk individuals

will result in timely and efficient customized surveillance through colonoscopy. Furthermore, these gene mutations may provide therapeutic leads to improve the treatment of cancer.

Commonly used second-generation HT-NGS platforms include the Roche 454 Genome sequencer, the Illumina Genome Analyzer and the Applied Biosystems SOLiD system [34]. These instruments are based on the massive parallel sequencing of spatially separated clonal amplicons [163]. They are mainly used to target thousands of genes in one or a few samples from which as much sequence as possible is retrieved, with the disadvantage of long runtimes of up to two weeks. In molecular tumor diagnostics, the demand will most likely be focused on the rapid sequencing of smaller subsets of genes in multiple samples with sufficient sequence depth to identify rare somatic variants in heterogeneous tumors. This demand can be met by using a combination of smaller, faster, benchtop sequencers in combination with the targeted sequencing of "DNA barcoded" samples. Instruments such as the 454 GS Junior (Roche), MiSeq (Illumina) and Ion Torrent PGM™ or Proton™ (Life Technologies) are currently available for this purpose and will likely have a decisive impact on diagnostics in the near future [205]. DNA barcoding can be achieved by adding unique tags to the ends of DNA fragments. These tags can be linked to the DNA during PCR or after the isolation of targeted sequences [34,206,207]. Targeted sequencing can be performed with customized panels, such as the Ion AmpliSeq™ Target Selection Technology, or dedicated "cancer panels", such as the Ion AmpliSeq™ Cancer Panel, which promises to assess hundreds of mutations in 10 ng of FFPE-DNA in a single day (http://www.lifetechnologies.com, accessed January, 2013). Another method that is likely suitable for this strategy was developed in Uppsala, Sweden and was first described in 2005 [208]. This method, the HaloPlex™ target enrichment system, was adapted by Agilent and is based on the digestion of DNA with different sets of restriction enzymes (http://www.agilent.com, accessed January, 2013). The targeted nucleic acid sequences are hybridized with oligonucleotide constructs called selectors. The selectors contain target-complementary end-sequences that are joined by a general linking sequence and that act as ligation templates to direct the circularization of target DNA fragments. Circularization only takes place if a ligation reaction has occurred, which makes the method theoretically very sensitive and specific. Only these circularized targets are then amplified in multiplex using one universal PCR primer pair that is specific for the general linking sequence in the selectors. By combining selector technology with high-throughput parallel sequencers, rapid resequencing can be accomplished from multiple genes and multiple specimens if DNA barcoding is applied [34,208]. A collaboration between Agilent Technologies and the LUMC Department of Pathology was initiated in 2011 to investigate the possibility of using this method for FFPE material; the performance of the method was determined by comparing DNA isolated from freshly frozen tumors with DNA from formalin-fixed and paraffin-embedded material isolated from the same tumor. The initial results are promising, even for heavily degraded DNA (Crobach and van Eijk et al., 2013 article in preparation).

Considerations in Molecular Pathology.
The role of the pathologist and his laboratory team has changed dramatically over time. After centuries of organ-based pathology that provided little benefit to patients, the field moved in the mid-nineteenth century to cellular-based clinical pathology with the potential to improve diagnosis and tumor classification, and, currently, the molecular pathologist plays an important role in 'personalized medicine'.

A pathologist has been described as the following: "a physician, concerned with human suffering and willing to make a considerable effort to decrease this suffering; a scientist with an inquiring mind, using advanced tools to study disease; an educator, sharing his knowledge, scientific inquiry methods and spirit with his students and other medical colleagues; and a leader in both pathology and medicine because he believes in quality assurance and the role of pathology in the overall advancement of medicine". Because of their multiple roles, pathologists continually build bridges between clinical medicine, surgery and basic science [209]. Microscopic tissue anatomy guides the initial classification of disease. Immunohistochemistry enables proteins to be visualized in tissue and facilitates the determination of the origin and nature of normal and aberrant cells. Developments in molecular biology improve the ability to examine the functional and genetic qualities of tissues, leading to better classification, diagnosis and treatment of disease [9].
Important aspects of molecular pathology must be further developed because the criteria and processes for implementing a molecular diagnostic test as the "standard-of-care" in a clinical setting have not been fully established. The following must be considered: resourcing appropriate patient material, assay development and supply, quality control, reporting and auditing, ethical and regulatory elements such as reimbursement and the role of the pharmaceutical industry [210]. With these considerations, it should be possible to develop a system that works locally to balance the increasing demands for higher quality specialist services [211].

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