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Direktor: Prof. Dr. med. Wolfgang Hiddemann

# Detection of *DNMT3A* R882 Mutations in Patients with Acute Myeloid Leukaemia

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## Abbreviations

| | |
|---|---|
| ASB-PCR | Allele-specific PCR with a blocking reagent |
| *ADD* | Ataxia telangiectasia mutated — DNA methyltransferase 3A — DNA methyltransferase 3-like |
| *BCR-ABL* | Breakpoint cluster region Abelson |
| bp | Base pair |
| CBFB | Core-binding factor, beta subunit |
| *CEBPA* | CCAAT/enhancer-binding protein α |
| CGI | CpG Island |
| CI | Confidence interval |
| *cKIT* | Proto-oncogene receptor tyrosine kinase |
| CML | Chronic myeloid leukaemia |
| (CN-)AML | (Cytogenetically normal) acute myeloid leukaemia |
| CR | Complete remission |
| $C_t$ | Cycle threshold |
| dHPLC | Denaturing high performance liquid chromatography |
| DMSO | Dimethyl sulfoxide |
| *DNMT* | DNA methyltransferase |
| EFS | Event free survival |
| FCS | Foetal calf serum |
| *FLT3* | FMS-like tyrosine kinase receptor-3 |
| FISH | Fluorescence In-Situ Hybridisation |
| HRM | High Resolution Melting Analysis |
| HSCT | Haematopoietic stem cell transplantation |
| *IDH1* | Isocitrate dehydrogenase 1 |
| MDS | Myelodysplastic syndrome |
| MGB | Minor groove binder |
| MGUS | Monoclonal gammopathy of unkown significance |
| MLL | Myeloid/lymphoid or mixed lineage |

| | |
|---|---|
| | leukaemia |
| MPN | Myeloproliferative neoplasm |
| MRD | Minimal residual disease |
| *MYD88* | Myeloid differentiation primary response gene 88 |
| NGS | Next-generation sequencing |
| *NPM* | Nucleophosmin |
| *NRAS* | Neuroblastoma Ras proto oncogene |
| OS | Overall survival |
| *p53* | Tumour protein p53 |
| PBS | Phosphate buffered saline |
| (q)PCR | (Quantitative) polymerase chain reaction |
| PHD | Polybromo homology |
| *PML-RARA* | PML-Retinoic Acid Receptor α mutations |
| PNA | Peptide nucleic acid |
| R | Arginine |
| RAEB | Refractory anaemia with excess blasts |
| *RB1* | Retinoblastoma protein |
| RCMD | Refractory cytopenia with multilineage dysplasia |
| RPMI-1640 | Roswell Park Memorial Institute-1640 Medium |
| *RUNX1* | Runt-related transcription factor 1 |
| *WT1* | Wilms tumour 1 |
| WT | Wild type |

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# I. Introduction

## 1.1 Acute myeloid leukaemia:

Acute myeloid leukaemia is a heterogeneous malignancy with clonal proliferation of haematopoietic precursor cells infiltrating in blood, bone marrow and other tissues. It represents the most common form of acute leukaemia in adults and occurs increasingly frequently with age. The median onset of AML is 65 years resulting in an incidence of 16.8 per 100,000 for individuals older than 65 years and 1.8 in those younger than 65 years (Howlader N, 2013; Hoffbrand AV & Moss PAH, 2011). More than 18,000 cases of AML are diagnosed annually in Europe with a five year relative survival rate of 19% (Visser O, et al. 2012).

Within the process of haemopoiesis, leukaemic transformation is considered to take place at the stage of the haematopoietic stem cell or early progenitor cell that has already started to differentiate. These immature cells are assumed to gain specific survival advantages by at least two mechanisms: Alteration of genes that affect proliferation known as class 1 genes, e. g. internal tandem duplications or tyrosine kinase domain mutations of the *FMS*-like tyrosine kinase 3 (*FLT3*-ITD or -TKD mutations), mutations of the neuroblastoma RAS viral oncogene (*NRAS* mutations), mutations in the Janus Kinase 2 (*JAK2*), *cKIT* mutations etc. and modification of those that affect differentiation classically named class 2 genes, e. g. mutations in the nucleophosmin gene (*NPM1* mutations), PML-Retinoic Acid Receptor α mutations (*PML-RARA*), CCAAT/enhancer-binding protein α (*CEBPA*-mutations), Runt-related transcription factor 1 (*RUNX1* mutations), as illustrated in Figure 1. However, recently it has been shown that this theory needs to be expanded as not all of the patients with AML carry mutations in class 1 or 2 genes (Shen Y et al., 2011; Shih A et al., 2012; Thiede C, 2012). Whole genome and exome sequencing studies have revealed that somatic mutations in epigenetic regulators are a frequent occurrence in AML and contribute to malignant transformation (Ding L et al. 2012; Schoofs T & Mueller-Tidow C, 2011). One of the most common mutations is located in the de novo methyltransferase 3A (*DNMT3A*) gene regulating DNA methylation.

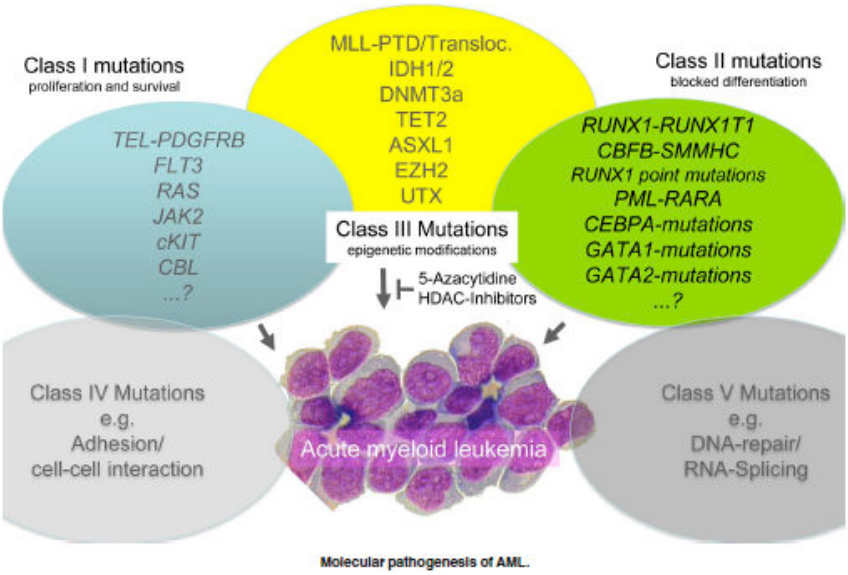


Figure 1: Mutations contributing to the pathogenesis of AML (Reproduced from Thiede C, 2012)

## 1.2 Prognostic marker in AML:

Conventionally, AML cases were classified into three risk groups (favourable, intermediate and adverse) by morphologic features, flow cytometric examination and cytogenetic fluorescence in situ hybridisation (FISH) analysis but recently recurrent genetic alterations have been taken into consideration and were incorporated into the WHO classification of acute myeloid leukaemia (Vardiman JW et al., 2009; Godley LA, 2012).

In general, patients with AML with a favourable risk classification receive conventional chemotherapy whereas patients with an unfavourable risk classification are chosen for allogeneic haematopoietic stem cell transplantation (HSCT) (Martelli MP et al., 2013). However, the prognostic group encompassing most of the adult AML patients (40-50% of all AML cases) is cytogenetically normal AML (CN-AML), part of the intermediate risk group (Lin TL & Smith BD, 2011). Patients out of this risk group are difficult to assign to different treatment options because of the high variance of molecular subtypes and their highly differing treatment outcomes.

The prognostic importance of further risk sub classifications of the large and heterogeneic diverse CN-AML group by mutational analysis was shown by many studies examining the outcome of genes that are frequently associated with leukaemia. It was shown that genetic

predictors, among them *DNMT3A* mutations, indicated improved risk stratification (Renneville A et al., 2014; Shivarov V et al., 2013, Patel JP et al., 2012; Shen Y et al., 2011).

### 1.3 De novo methyltransferase 3A:

DNA methylation status as well as covalent histone modifications, chromatin remodelling functions and interfering RNA are epigenetic regulating mechanisms (Schoofs T & Mueller-Tidow C, 2011) and have an important impact on carcinogenesis (Munoz P et al., 2012). In particular aberrant changes in DNA methylation patterns are a hallmark of cancer cells (Yamashita Y et al., 2010). Recently, it has also been reported that DNA methylation has crucial influence on key transcription factors, like WT1 and RUNX1, during the development of myeloid progenitor cells (Rönnerblad M et al., 2014; Qu Y et al., 2014). Three different types of DNA methyltransferase enzymes have been classified: DNA methyltransferases (DNMTs) 3A and B which both mediate de novo methylation while in contrast DNMT1 mainly maintains methylation (Ribeiro AFT et al., 2012, Cagnetta A et al., 2014). *DNMT3A* has been shown to be repeatedly mutated in myeloid malignancies (Yan XJ et al., 2011; Ley TJ et al., 2010). This de novo methyltransferase 3A catalyses the addition of a methyl group donated from the cofactor S-Adenosyl methionine to the 5'-position of cytosine residues at CpG dinucleotide residues (known as CpG islands) in DNA (figure 2) which are located mainly in the promoter region of genes.

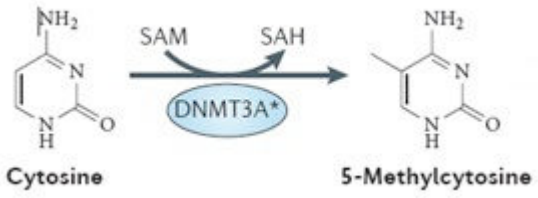


Figure 2: Enzymatic function of DNMT3A (adapted from: Shih AH et al., 2012)

However, increased methylation of CpG islands in the promoter region is somehow correlated with reduced expression of the downstream gene and represents a regulatory mechanism of gene expression (Ley TJ, Ding L & Walter M, 2010; Munoz P et al., 2012). The affected genes involve tumour suppressor genes and their inactivation is considered to partially initiate leukaemogenesis (Agrawal S et al., 2007).

In contrast, the global methylation status of AML cells outside CGI islands is mostly hypomethylated which might lead to increased genomic instability and contribute to the malignant process (Eriksson A et al., 2015; The Cancer Genome Atlas Research Network, 2013).

## 1.4 DNMT3A mutations:

Frequency and structure:

*DNMT3A* mutations occur in approximately 22% of adult AML patients and are especially common in cases with intermediate risk normal cytogenetic AML (up to 36%) (Ley TJ et al., 2010; Thiede C, 2012). The majority of the *DNMT3A* mutations (60-83%) affect codon R882 which is allocated in exon 23 in the catalytic domain of the *DNMT3A* gene (Ley TJ et al., 2010; Yamashita et al., 2011; Renneville A et al., 2012; Marcucci G et al., 2012; Ibrahem L et al., 2015). Figure 3 illustrates the structure of the *DNMT3A* gene: The elements of the regulatory region, namely the tetrapeptide PWWP domain and the polybromo homology (PHD) domain; and the methyltransferase (MTase) component which represents the catalytic region of the enzyme.

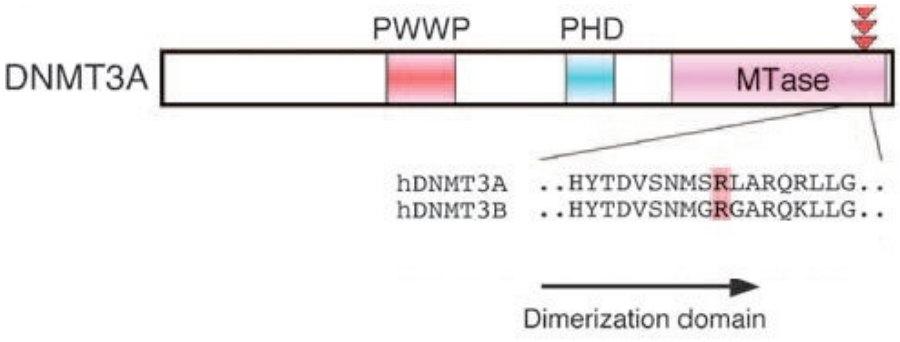


Figure 3: Structure of the *DNMT3A* gene, PWWP = proline-tryptophan-tryptophan-proline domain, PHD = polybromo homology domain, MTase = methyltransferase domain (Adapted from: Yamashita Y et al, 2010)

Characterisation of R882 mutations:

The majority of mutations at the R882 hotspot (within the catalytic domain of the *DNMT3A* gene) are heterozygous and caused by different missense base sequences: Notably, the R882H mutation, a G-to-A transition producing histidine substitution instead of arginine; the

R882C mutation, a C-to-T transition leading to cysteine substitution and the R882S mutation, a C-to-T transversion resulting in serine substitution. They occur with different frequencies: 62-65% R882H, 24-36% R882C and 2-3% R882S of all R882 mutations (Ley TJ et al., 2010; Brewin JN et al., 2012; Gaidzik et al., 2013).

Effects of the mutation:

Most of the somatic *DNMT3A* mutations prevent the formation of active homotetramers and heterotetramers between DNMT3A and DNMT3L which serves as a coactivator of the functionally active enzyme (Eriksson A et al., 2015; Shih A et al., 2012).
The R882 mutation results in a decreased catalytic activity (50-80%) compared to wild type and a loss in DNA binding affinity (Yamashita Y et al., 2010; Russler-Germain DA et al., 2014).
Interestingly, the frequent R882H mutation does not appear to alter the methyltransferase activity when analysed as an *in vitro* mix of WT and R882H mutated enzyme. Yet, the coexpression of both proteins in mammalian cells results in loss of enzymatic function indicating that the R882H mutation causes haploinsufficiency in the sense of a dominant-negative effect on the WT protein (Stewart H, et al., 2015; Genovese G et al., 2014; Russler-Germain DA et al., 2014).
These findings are deduced from *in vitro* experiments and have recently been further analysed in *in vivo* studies: Loss of *DNMT3A* function in murine haematopoietic stem cells (HSC) impairs their differentiation and interferes with normal haematopoiesis which leads to the development of several lymphoid and myeloid malignancies. Those *DNMT3A*-knock-out mice have an aberrant methylome pattern and underline the preleukaemic function of the mutation (Mayle A, et al., 2015).
Furthermore, the methylation pattern in humans with CN-AML and *DNMT3A* mutations is different from those without the mutation: recent data reveals significant hypomethylation of the homeobox genes affecting embryogenesis as well as hematopoietic development and leukemogenesis (Qu Y et al., 2014).

*DNMT3A* mutations in clonal evolution of AML:

The impact of the *DNMT3A* mutation on the clonal evolution and pathogenesis of AML was examined by a comparison of the karyotypes of M3-AML with the fusion gene *PML-RARA*

as the known initiating event to M1-AML cases with normal cytogenetics and exomes of haematopoietic stem cells from healthy donors. Because of the pattern of *DNMT3A* mutations and co-occurring mutations like *NPM1, IDH1, FLT3* and others *DNMT3A* mutations were thought to be initiating events in AML in cases with normal cytogenetics rather than random or background mutations in the hematopoietic stem cell and therefore contribute to AML pathogenesis as a "key initiating mutation" (Welch JS et al., 2012).

Further research groups provide evidence that *DNMT3A* mutations contribute to clonal expansion of haematopoietic stem or progenitor cells as a result of impaired differentiation and represent an initiating event and respectively act as an oncogene during the development of AML (Genovese G et al., 2014; Roy DM et al., 2014).

Moreover, *DNMT3A* mutations are considered as potential driver mutations because they seem to arise mutually exclusive to transcription-factor fusions that were previously acknowledged to advance leukaemia (Kihara R et al., 2014). The influence of the analysed mutation on clonal expansion is even more pronounced in relation to different age groups resulting in increasing percentages of leukaemia-associated genetic alterations in patients above 70 years of age (Xie M et al., 2014; Jaiswal S et al., 2014).

Recent data confirms the role of *DNMT3A* as a driver mutation in the specific case of clonal haematopoiesis and development in AML in patients with aplastic anaemia (Yoshizato T et al., 2015).

Clinical characterisation of patients with *DNMT3A* mutations:

*DNMT3A* mutations are mostly seen in AML subtype M4 and M5 (after the French-American British morphological classification). Moreover, patients with *DNMT3A* mutations present with a higher peripheral white blood count at time of first diagnosis and also often harbour *NPM1, IDH1/2, FLT3*-ITD and TKD mutations but not *CEBPA* and *NRAS* mutations compared to patients with AML without *DNMT3A* mutations. The *DNMT3A* mutation is recurrently observed in patients older aged 50 and over rather than in younger patients (Kihara R et al., 2014). Importantly, *DNMT3A* mutations are associated with decreased overall and relapse free survival especially in patients of younger age (below 60 years) with cytogenetically normal AML (Thiede C, 2012; Ribeiro AFT et al., 2012; Renneville A et al., 2012; Ibrahem L et al., 2015). Furthermore, the mutation presents as an independent adverse prognostic factor in high-risk genotypes as defined by criteria of the European Leukaemia Net (Shivarov V et al., 2013).

Treatment options:

Aberrant DNA methylation is also the pharmacological target of inhibitors of DNA methyltransferases. The hypomethylating agent decitabine is already licensed as a first line therapy for the treatment of older AML patients who seem too frail to sustain standard induction chemotherapy (DiNardo CD et al., 2014; Ramos F et al., 2014). Elderly AML patients with a *DNMT3A* mutation have a significantly better response rate after treatment with decitabine than those without the mutation (Metzeler KH et al., 2012). However, there is also contradictory data showing the high-complexity between epigenetic mutations and hypomethylating drugs as well as the need for further studies (DiNardo CD et al., 2014).
In terms of maintenance therapy a phase 1 b study demonstrated that lenalidomide which is beneficial in high-risk myelodysplastic syndrome in combination with azacitidine might control *DNMT3A* positive residual disease and therefore protect patients from clinical relapse (Wei A et al., 2015).
Another approach for induction therapy of *DNMT3A* carriers is the comparison of the treatment with idarubicin versus daunorubicin. This study conducted by LaRochelle O et al. (2011) indicates that patients with *DNMT3A* exon 23 mutations have better median disease free and overall survival rates than those without the mutation when treated with idarubicin. Both patient groups have similar results when treated with daunorubicin (LaRochelle O et al., 2011).
Furthermore, patients with *DNMT3A* mutations seem to have a better response and improved overall survival after induction chemotherapy with high-dose daunorubicin (cytarabin plus 90 mg daunorubicin/$m^2$) in comparison to standard dose (cytarabin plus 45 mg daunorubicin/$m^2$) than patients without the *DNMT3A* mutation (Patel JP et al., 2012).

### 1.5 *DNMT3A* mutations as prognostic marker in AML:

Patients with cytogenetically normal AML harbouring *DNMT3A* mutations have significantly shorter event-free (EFS) and overall survival (OS) with a 5 year EFS of 13% vs. 32% and an OS of 23% vs. 45% (accordingly a 2-year OS of 41% versus 68%) as compared to those without the *DNMT3A* mutation (Renneville A et. al, 2012; Renneville A et al., 2014). The EFS and OS were also significantly worse in the comparison of *DNMT3A* mutation versus *DNMT3A* wild type when all AML risk groups were taken into account; with a median OS of 11.9 months when carrying the *DNMT3A* mutation against 24.0 months when the mutation

was absent. These findings were confirmed even when codon R882 in exon 23 alone was analysed as it contains most of the mutations within the *DNMT3A* gene (Ribeiro AFT et al., 2012; Ostronoff F et al., 2013).

In univariate and multivariate analyses *DNMT3A* mutation status was defined as an independent adverse prognostic factor for overall survival in the majority of patient subgroups above all in CN-AML patients (Renneville A et. al., 2014; Hou HA et al., 2013; Ribeiro AFT et al., 2012; Thol F et al., 2011). Kihara R et al. showed that the original European Leukemia Net risk stratification system for CN-AML could be improved by assigning patients with the *DNMT3A* mutation rather to the intermediate risk I group than to the favourable risk group (Kihara R et al., 2014; Dohner et al., 2010). In a recent meta-analysis examining 6377 AML patients including 1161 *DNMT3A* mutation carriers the mutation appeared to predict an adverse OS for various patients risk groups as well as in the total non-classified population. Apart from the favourable risk subgroup and the non-R882-*DNMT3A* mutant subgroup all other allocated patients were clearly stratified by *DNMT3A* prognostic status (Tie R et al., 2014).

Several studies showed that patients with *DNMT3A* mutations have a significantly shorter overall and disease-free survival, this appeared not to be an effect of a decreased rate of complete remission (CR), but reflected an increase in disease recurrence as CR rates were shown to be similar between *DNMT3A* mutant and *DNMT3A* wild type patients (Thiede C, 2012). In addition to that, AML patients with *DNMT3A* mutation suffered from higher relapse rates (Hou H et al., 2012).

### 1.6 Minimal residual disease detection:

Although 50-80 % of adult AML patients reach complete remission after treatment with chemotherapy, the great majority of these patients will finally sustain disease relapse resulting in long-term survival rates of 30-40% in younger patients and 20% in elderly patients (Buccisano F et al., 2012). The main cause of relapse is minimal residual disease (MRD). This term describes residual leukaemic cells that remain in patients' blood and bone marrow after chemotherapy or stem cell transplantation and cannot be detected by conventional morphological methods. Measurement of MRD provides crucial prognostic information as it allows further subdivision of the large intermediate risk AML group into MRD-positive and MRD-negative (above and below of a certain threshold as defined by the methodology applied) (Paietta E, 2012). This has important clinical implications for the

assessment of response after chemotherapy and possible postremission procedures to evaluate the patient's individual risk of relapse (Buccisano F et al., 2012). Moreover, MRD levels could also be used as a relative end point to compare treatment efficiencies at any point during AML treatment (Paietta E, 2012). One of the main limitations of MRD detection is the lack of consistency of gene mutations during disease progression.

However, *DNMT3A* mutations appear to remain stable during AML disease evolution as several studies of sequential AML samples reveal. In these studies, AML patients with initially positive *DNMT3A* mutation status lacked the *DNMT3A* mutation at remission but regained the same mutation at disease relapse (Hou H-A et al., 2012; Pløn et al., 2014). Additionally, *DNMT3A* mutations seem to persist (or remain stable) from diagnosis to relapse and might induce other mutations, namely the *FLT3*-ITD mutation (Wakita S et al., 2012). In accordance with these observations, all patients in whom *DNMT3A* mutations were absent at initial diagnosis maintained this status at relapse or had mutations at low allele frequencies.

These results indicate that the *DNMT3A* mutation could be applied as an effective biomarker for minimal residual disease (Hou H-A et al., 2012; Wakita S et al., 2012; Krönke et al., 2013; Pløn et al., 2014; Mancini M et al., 2015).

### 1.7 Methods to detect *DNMT3A*:

Up to now, the following techniques to detect *DNMT3A* mutations are available: direct Sanger sequencing of PCR amplified genomic or complementary DNA (Hou H-A et al., 2012), denaturing high-performance liquid chromatography (dHPLC or WAVE$^{TM}$ system) (Ribeiro AFT et al., 2012), high resolution melting curve (HRM) analysis (Singh RR et al., 2012) and next-generation sequencing (Brewin et al., 2012; Shivarov V et al., 2014; Gorniak P et al., 2015). Although next-generation sequencing procedures have undergone many improvements they are still complicated and time consuming techniques that are neither available in average hospital laboratories nor suitable for large sample numbers and therefore do not represent an ideal method to test for *DNMT3A* mutations (Yamashita Y et al., 2010, Berenstein R et al., 2014). Direct Sanger sequencing was considered as “gold standard” for detection of somatic mutations yet it is certainly limited due to its relatively low sensitivity of 10-20% mutant allele burden (Shivarov V et al., 2014; Gorniak P et al., 2015).

High resolution melting curve analysis was reported to have a sensitivity of 6.7% for exon 23 *DNMT3A* mutations (Singh RR et al., 2012) to about 2.5% (Hayette S et al., 2012) but has

limitations as the accuracy of the assay is highly dependent on the resolution of the machine used (Wittwer CT, 2009).

Hitherto, although its high prognostic value, as has been demonstrated in 1.5, no simple screening test for detection of the *DNMT3A* mutation has been established yet. A fast determination of *DNMT3A* status could provide the attending physician with valuable information about potential treatment strategies and predict an individual outcome for the affected patient.
Furthermore, the current *DNMT3A* MRD tests do not supply results that are accurate enough to monitor patients post treatment on residual leukaemic cells harbouring the *DNMT3A* mutation.

## 1.8 Quantitative polymerase chain reaction:

Quantitative polymerase chain reaction (qPCR) has been established as a reliable test for MRD detection for patients carrying genetic alterations with high sensitivities (depending on the target structure) (DiNardo CD & Luger SM, 2012).

Quantitative PCR is based on the principle that detection of the PCR product is measured while the reaction still proceeds. It is therefore also called real-time PCR. qPCR has been developed from traditional PCR which can be divided up into 3 sections (figure 4):

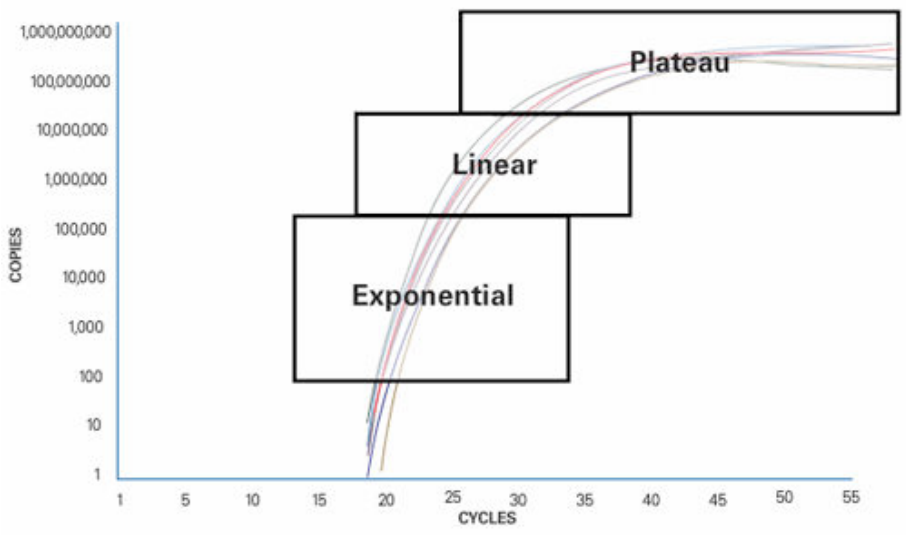


Figure 4: PCR phases

1.: Exponential: During the exponential phase the PCR product doubles every cycle if the reaction has 100% efficiency because the reagents are available in abundance and the reaction's kinetics optimal for amplification.

2.: Linear: The reaction slows down due to depletion of the reagents and the product is not duplicated at each cycle any more.

3.: Plateau: The PCR reaction has finished, each experimental sample at its own plateau following the reaction's kinetics. In traditional PCR the product is measured during this phase.

While using qPCR the product is measured during the exponential phase because the amplification rate is stable enough to give precise results for quantification of a target structure. In order to detect the product a fluorescent probe that is complementary to one section of the amplicon is added to the reaction. This oligonucleotide probe (in the present sample similar the original trademark Taqman©) contains a fluorescent reporter at the 5' end and a quencher at the 3' end which suppresses the fluorescent group as long as the probe is intact. Due to its intrinsic exonuclease ability the DNA polymerase synthesizing the new fragment cleaves the quencher from the probe (Figure 5) allowing absorbance of the fluorescence by the qPCR machine. Therefore, the amount of fluorescence increases directly proportionally to the accumulation of new DNA strands (Dale J, 2012).

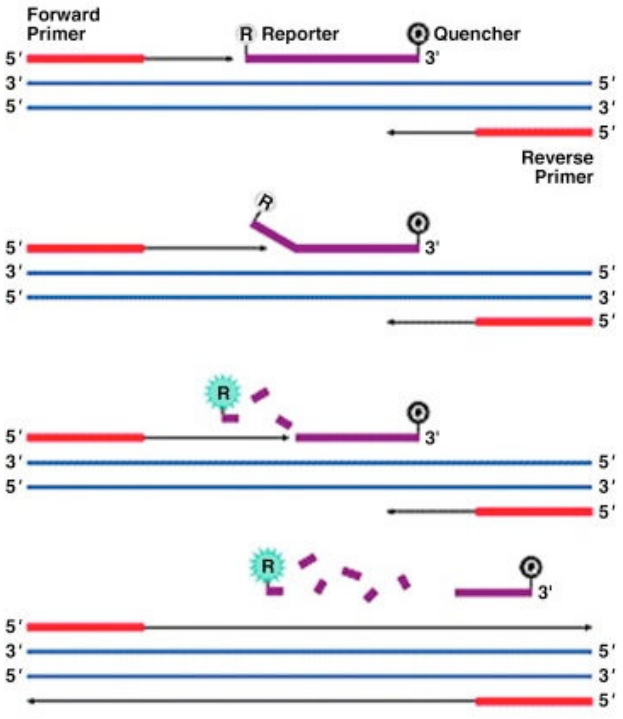


Figure 5: Taqman© style qPCR detection (Reproduced from Wang SQ et al., 2010)

To enable quantification of a target structure, like in this case that of a specific mutation, the following terms are of great importance: The cycle threshold ($C_t$) which describes the PCR cycle number during which the fluorescence of the reporter dye reaches a level higher than the background called the threshold line. The threshold line is usually automatically allocated by the qPCR machine.

## II. Aims

On the basis of the reasons argued in the introduction, our aim was to establish two assays for detection of *DNMT3A* mutations:

Firstly, we sought to develop a rapid screening test using differential restriction digestion of PCR amplified DNA products for reliably detecting the presence of an R882 mutation of the *DNMT3A* gene and determining the most common types of the R882 mutations within a few hours of bone marrow aspiration.

Secondly, we aimed to establish a method for detecting and quantifying low copy numbers of *DNMT3A* R882 mutations for the purpose of monitoring minimal residual disease in AML patients.

Both methods were designed to use the OCI/AML-3 cell line which harbours a heterozygous *DNMT3A* R882C mutation (Tiacci E et al.,2012; Brewin JN et al., 2012) as a positive control. Furthermore, after verification of results for the OCI/AML-3 DNA we intended to analyse AML patients' samples.

# III. Material and methods

## 3.1 Material:

### 3.1.1 Oligonucleotides:

**PCR**:

Forward Primer:

5'-GTGATCTGAGTGCCGGGTTG-3'……Sigma-Aldrich Company Ltd., Dorset, UK

Reverse Primer:

5'-TCTCTCCATCCTCATGTTCTTG-3'……Sigma-Aldrich Company Ltd., Dorset, UK

**qPCR:**

Forward qPCR primer “DNMT3A green”: 5’-ACAGAAGATTCGGCAGAACTAAG-3’

Reverse qPCR primer “DNMT3A green”: 5’-CGGAGGACCAGCAGCCA-3’

Forward qPCR primer “DNMT3A yellow”: 5’-CCAGGGTATTTGGTTTCCCAGT-3’

Reverse qPCR primer “DNMT3A yellow”: 5’-ATGACTGGCACGCTCCATG-3’

Forward qPCR primer “DNMT3A blue”: 5’-CACCTCTTCGCTCCGCTG-3’

Reverse qPCR primer “DNMT3A blue”: 5’-TTTTTGTTTGTTTGTTTAACTTTGTGT-3’

All qPCR primer pairs were purchased from PrimerDesign Ltd., Southampton, UK.

### 3.1.2 Enzymes:

Ampli Taq Gold Polymerase……..Life Technologies Ltd., Paisley, UK

FastDigest AciI (SsiI)……...Fisher Scientific Ltd., Loughborough, UK

FastDigest AluI…….Fisher Scientific Ltd., Loughborough, UK

### 3.1.3 Cell lines:

OCI/AML-3 cell line……Leibniz Institute DSMZ, Braunschweig, Germany

KG-1 cell line…….American Tissue Culture Collection, Teddington, UK

Cell line characteristics: Following information was taken out of the Guide to Leukemia-Lymphoma Cell Lines (Drexler HG, 2010).

| | OCI/AML-3 | KG-1 |
|---|---|---|
| Age and sex | 57-year-old male | 59-year-old male |
| Sample type | peripheral blood | bone marrow |
| Doubling time | 35-40 hours | 40-50 hours |
| Immunology | CD3-, CD4+, CD13+ CD14-, CD15+, CD19-, CD 33-, CD34+, CD68+, HLA-DR- | CD3-, CD4-, CD13+, CD14-, CD15+, CD19-, CD33+, CD34+, HLA-DR+ |
| Cytogenetics | hyperdiploid karyotype - 48(45-50)<2n>X/XY, +1, +5, +8, der(1)t(1;18)(p11;q11), i(5p), del(13)(q13q21), dup(17)(q21q25) - sideline with r(Y)x1-2 | hypodiploid with 4.5% polyploidy; 45(42-47)<2n> X/XY, -4, +8, +8, -12, -17, -20, +2mar, der(5;17)(q10;q10) del(5)(q?11q?13), dup(7)(q12q33), del(7)(q22q35), i(8q)x2, der(8)t(6;8)(p11;q22), der(8)t(8;12)(p11;q13), der(11)t(1;11)(q13-21;p11-p13),der(16)t(?12;16)(?p13;q13/21) |
| Gene alterations | *NPM1* mutation, hemizygous for *RB1,* *DNMT3A* R882C mutation (Tiacci et al., 2012) | *NRAS* mutation, $P15^{INK4B}$ methylation, $P16^{INK4A}$ methylation, *P53* mutation, *RB1* rearrangement |

Table 1: Characteristics of the Cell lines used as positive and negative controls

### 3.1.4 Reagents and kits:

10,000X Ethidiumbromide……VWR International Ltd., Lutterworth, UK

100 bp PCR DNA ladder……..Fisher Scientific Ltd., Loughborough, UK

10X Tris Acetate-EDTA buffer……...Sigma-Aldrich Company Ltd., Dorset, UK

6X Gel Loading Dye…..Glycerol, EDTA, Bromophenol Blue, Xylene Cyanol, distilled water

Ampli Taq Gold PCR Master Mix…….Life Technologies Ltd., Paisley, UK

Custom designed real-time PCR assay……PrimerDesign Ltd., Southampton, UK

DNeasy blood and tissue kit……..Qiagen, Crawley, UK

Foetal calf serum……..Biosera, Ringmer, UK

Genetic Analysis Grade Agarose……..Fisher Scientific Ltd., Loughborough, UK

Histopaque 1077……..Sigma-Aldrich Company Ltd., Dorset, UK

L-glutamine (100mM)……Life Technologies Ltd., Paisley, UK

Nuclease-free water……..Fisher Scientific Ltd., Loughborough, UK

Penicillin (100 IU/ml)……Life Technologies Ltd., Paisley, UK

Phosphate buffered saline……Sigma-Aldrich Company Ltd., Dorset, UK

Precision 2X qPCR Mastermix……...PrimerDesign Ltd., Southampton, UK

QIAquick PCR Purification Kit……Qiagen, Crawley, UK

QuantiTect Reverse Transcription Kit……...Qiagen, Crawley, UK

RPMI 1640…….Life Technologies Ltd., Paisley, UK

Streptomycin (100mM)……..Life Technologies Ltd., Paisley, UK

### 3.1.5 Equipment:

Gene Amp PCR System 9700……Applied Biosystems, Carlsbad, USA

Power Pack 200……..Bio-Rad Laboratories Ltd., Hemel Hempstead, UK

Nanodrop 1000 Spectrophotometer……...Thermo Scientific, Loughborough, UK

Syngene Bio Imaging Gene Flash…….Synoptics Ltd., Cambridge, UK

Stratagene Mx 3005P qPCR System…….Agilent Technologies, Cheshire, UK

### 3.1.6 Software:

Stratagene Mx3005P Software version 4.10……..Agilent Technologies, Cheshire, UK

Nanodrop 1000 Software version 3.7.0…….Thermo Scientific, Loughborough, UK

Minitab Statistical Software version 16……..Minitab Ltd., Coventry, UK

### 3.1.7 Databases:

Tissue bank of the Medical Research Building Brighton and Sussex Medical School, UK

Database of the National Center for Biotechnology Information NCBI, Bethesda, USA

## 3.2 Methods:

### 3.2.1 Cell culture:

The leukaemic cell lines OCI/AML-3 and KG-1 were cultured in a medium containing RPMI 1640 supplemented with 10% foetal calf serum, 100mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were maintained at 37° with 5% $CO_2$ in a humidified incubator and passaged at the ratio of 1:3 every three days (Stewart HJ et al., 2011).

### 3.2.2 Preparation of patients' samples:

The bone marrow aspirate was diluted at the ratio 1:1 with PBS, then pipetted onto Histopaque 1077 and centrifuged for 20 minutes at 450 x g. Next, the white cell layer was removed and placed into a 15 ml tube and washed with 10 ml PBS. The sample was centrifuged for 15 minutes at 250 x g. After removing the supernatant, the cell pellet was resuspended in 1ml PBS. Unless the cells were used immediately after the preparation, they were diluted 1:1 with cell freezing medium containing 20%v/v DMSO, 40%v/v FCS in Iscove's modified Dulbecco's medium, frozen and stored in liquid nitrogen (Stewart et al., 2011). All patients agreed by written informed consent to analysis of their bone marrow or peripheral blood samples and the Brighton East Research Ethics Commission gave permission to perform the research.

### 3.2.3 DNA extraction:

DNA extraction of OCI/AML-3 and KG-1 cells as well as patients' blood mononuclear cells was performed using the QIAGEN DNeasy Blood & Tissue kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

The concentration and purity of the extracted DNA was analysed by spectrophotometry using Nanodrop 1000.

### 3.2.4 Serial dilutions:

The OCI/AML-3 DNA concentration and KG-1 DNA concentration were both adjusted to 84 ng/µl. An initial dilution consisting of 100 µl OCI/AML-3 and 100 µl KG-1 DNA was set up. 50 µl of this assay was taken out and mixed in a fresh Eppendorf tube with 50 µl of KG-1 DNA. This step was repeated 8 times. Finally, 10 different dilutions were completed (Table 2).

| Dilution No. | Serial dilutions: % OCI/AML-3 and KG1 | Serial dilutions: % mutation strand |
|---|---|---|
| 1 | 100 % OCI | 50 % |
| 2 | 50 % OCI + 50 % KG1 | 25 % |
| 3 | 25 % OCI + 75 % KG1 | 12.5 % |
| 4 | 12.5 % OCI + 87.5 % KG1 | 6.25 % |
| 5 | 6.25 % OCI +93.75 % KG1 | 3.125 % |
| 6 | 3.125 % OCI + 96.875 % KG1 | 1.5625 % |
| 7 | 1.5625 % OCI + 98.4375 % KG1 | 0.78125 % |
| 8 | 0.78125 % OCI + 99.21875 % KG1 | 0.390625 % |
| 9 | 0.390625 % OCI + 99.60938 % KG1 | 0.1953125 % |
| 10 | 0.1953125 % OCI + 99.80469 % KG1 | 0.0976562 % |

Table 2: Serial dilutions of OCI/AML-3 in KG-1 DNA

### 3.2.5 Polymerase chain reaction:

Polymerase chain reaction was performed to amplify the relevant region of the *DNMT3A* gene including codon R882. A PCR assay of 50 µl in total contained the following elements: 25 µl Ampli Taq Gold Master mix, 20 – 22 µl RNAse and DNAse free water, 1 – 3 µl (100 – 200 ng) genomic DNA, 1 µl forward primer and 1 µl reverse primer. The reaction settings were as described: Denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds for 35 cycles (Brewin JN et al., 2013).

### 3.2.6 PCR product purification:

The PCR product was purified using the QIAquick® PCR Purification Kit according to the manufacturer's instructions except for the last step which was elution of DNA in 50 µl nuclease-free water instead of buffer.

### 3.2.7 Restriction enzyme digestion:

The purified PCR product was digested by two different rapid-digestion restriction enzymes: AciI and AluI. AciI specifically recognises the 4 base-pair sequence 5'-CCGC-'3 (Polisson and Morgan, 1990), cleaving between the two cytosine residues on the forward strand and between the guanine and cytosine on the reverse strand (Figure 6).


Figure 6: AciI restriction site

AluI recognises the nucleotide sequence 5'-AGCT-3', respectively 3'-TCGA-5' and cuts between the guanine and cytosine residue resulting in blunt ends both sides of the digested fragment (Figure 7).


Figure 7: AluI restriction site

Both AciI and AluI were advanced restriction enzymes for rapid DNA digestion enabling to completely digest 1µg of lambda DNA within 5 minutes. According to the manufacturer, no degradation of 1µg of lambda DNA as a result of nuclease contamination or star activity (reduced enzyme specificity due to prolonged incubation) after prolonged incubation for 4 hours was detected (Product Information, Thermo Scientific).

Both enzyme assays were set up using to the following protocol:

PCR product: 10µl

RNAse free water: 7µl

10X FastDigest buffer: 2µl

FastDigest enzyme: 1µl

Each sample was mixed thoroughly, spun down by centrifugation for 30 seconds in a bench top centrifuge and incubated at 37°C in a water bath for 60 minutes.

### 3.2.8 Gel electrophoresis:

After the restriction enzyme digestion the samples were run out on a 1.5% agarose gel and stained with 10mg/ml ethidium bromide to confirm complete digestion. The gel was then looked at under a UV transilluminator allowing the analysis of size and molecular weight of the digested DNA fragments.

### 3.2.9 Quantitative polymerase chain reaction:

The digested samples were diluted serially in nuclease-free water to a final concentration of 1:1000. Quantitative PCR was performed with following primer pairs:

TTCTGCTGGGTTGTGAAACTCTAGGCAGTGATGACCTTACTACCTTTAAGGTCACAGAAACC
AGCACAGTGCCTGGCACATGGTTGGTGATCTGAGTGCCGGGTTGTTTATAAAGGACAGAAGA
TTCGGCAGAACTAAGCAGGCGTCAGAGGAGTTGGTGGGTGTGAGTGCCCCTGTCCCTGCACT
TCGGGTGGCTGCTGGTCCTCCGGGTCCTGCTGTGTGGTTAGACGGCTTCCGGGCAGCCTGGT
CTGGCCAGCACTCACCCTGCCCTCTCTGCCTTTTCTCCCCCAGGGTATTTGGTTTCCCAGTC
CACTATACTGACGTCTCCAACATGAGCCGCTTGGCGAGGCAGAGACTGCTGGGCCGGTCATG
GAGCGTGCCAGTCATCCGCCACCTCTTCGCTCCGCTGAAGGAGTATTTTGCGTGTGTGTAAG
GGACATGGGGGCAAACTGAGGTAGCGACACAAAGTTAAACAAACAAACAAAAAACACAAAAC
ATAATAAAACACCAAGAACATGAGGATGGAGAGAAGTATCAGCACCCAGAAGAGAAAAAGGA
ATTTAAAACAAAAACCACAGAGGCGGAAATACCGGAGGGCTTTGCCTTGCGAAAAGGGTTGG
ACATCATCTC

CCGC = AciI site (destroyed by both R882H and R882C mutations)
CCGC = further AciI sites
GTGATCTGAGTGCCGGGTTG = forward primer sequence
TCTCTCCATCCTCATGTTCTT = reverse primer sequence
CCAGGGTATTTGGTTTCCCAGT = forward qPCR primer "DNMT3A R882 codon"
CATGGAGCGTGCCAGTCAT = reverse qPCR primer "DNMT3A R882 codon"
CACCTCTTCGCTCCGCTG = forward qPCR primer "DNMT3A digestion control"
ACACAAAGTTAAACAAACAAACAAAAA =reverse qPCR primer "DNMT3A digestion control"
ACAGAAGATTCGGCAGAACTAAG = forward qPCR primer "DNMT3A loading control"
TGGCTGCTGGTCCTCCG = reverse qPCR primer "DNMT3A loading control"

Figure 8: Real-time PCR primer pair sequence

Figure 8 shows the DNA sequence of the *DNMT3A* gene up- and downstream from codon R882 flanked by the outer standard PCR primer pair. Within this amplicon 3 qPCR primer pairs were placed to produce three different fragments with and without an AciI restriction site (a 4 base pair sequence CCGC as it occurred at the R882 hotspot). The first qPCR primer pair served as an internal DNA loading control because it amplified up a fragment without an AciI restriction site. The second primer pair amplified the region containing the AciI restriction site which matched the R882 hotspot. This restriction site was destroyed if a *DNMT3A* R882 mutation occurred. The third primer pair generated an amplicon containing a further AciI restriction site that was used as a relative digestion control.
The thermal profile of each qPCR run was as follows: Enzyme activation for 10 minutes at 95°C, than denaturation for 15 seconds at 95°C and data collection for 60 seconds at 60°C. The last two steps were repeated for 50 times.

Additionally, every run included DNA from OCI/AML-3 cell line which harbours a heterozygous *DNMT3A* R882C mutation as a positive control and KG-1 as a negative control.

### 3.2.10 Calculations:

Based on this, the mutational proportion of a sample could be calculated: The loading control fragment which was not cut by AciI was used to assess the amount of DNA present in each sample. The relative abundance of the R882 codon-containing fragment was then used to quantify the amount of mutant present in each sample (equation 1).

$$M = \frac{(\gamma g / y - c)}{m} \qquad (1)$$

$M$ = proportion of mutants present in the sample

$\gamma$ = correction factor to adjust for differential primer binding after mock-digestion (see also 4.2.4)

$g$ = abundance of loading control fragments

$y$ = abundance of mutant fragments

$m$ = gradient of the calibration curve

$c$ = y-intercept of the calibration curve

The correction factor $\gamma$ was calculated from mock-digestion experiments (see also 4. 2. 3. 1) and represented what the amount of sample should be multiplied by to equal the amount of loading control for an AciI-digested sample as the primer binding efficiency was altered after digestion. $g$ and $y$ were calculated by equation number 2 and 3 accounting for the amplification efficiencies of each primer pair as measured in a separate experiment (4. 2. 2):

$$g = 1.8533^{\text{Ct of loading control amplicon}} \quad (2)$$

$$y = 1.8538^{\text{Ct of R882 containing amplicon}} \quad (3)$$

m and c in equation number 1 were given by the slope of the calibration curve given by the equation of a line (equation no. 4) (for further information on the calibration curve see 4. 2. 4):

$$y = mx + c \quad (4)$$

with m = 0.5596 and c = 0.0328.

Furthermore, the cutting efficiency of the restriction enzyme AciI was calculated by relating the relative abundance of the loading control amplicon to the digestion control amplicon and carried out as an internal quality control for each sample tested (equation number 5).

$$C = \frac{1.7897^{CtDC}}{1.8533^{CtLC}} \quad (5)$$

$C$ = Relative Cutting Efficiency

$C_t$ DC = $C_t$ of digestion control amplicon

$C_t$ LC = $C_t$ of loading control amplicon

### 3.2.11 Sanger sequencing:

To confirm the generated results externally all samples were sent to Eurofins MWG Operon, Ebersberg, Germany and sequenced using the company's value read tube service. Therefore, 15 µl of purified PCR product per sample was analysed by Sanger sequencing.

When the results were available, the sequence was compared to human reference DNA by using the Basic Local Alignment Search Tool provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

# IV. Results

## 4.1 Differential restriction enzyme assay:

### 4.1.1 Restriction enzyme digestion pattern:

The *DNMT3A* wild type PCR product contained an AciI recognition site (CCGC) at codon R882 and two other sites further downstream as illustrated in Figure 9. Therefore, AciI digestion and subsequent agarose gel electrophoresis showed one major fragment of 252bp length and three smaller fragments measuring 125bp, 51bp and 16bp which served as a control for complete digestion.

GCCTGGCACATGGTTGGTGATCTGAGTGCCGGGTTGTTTATAAAGGACAGAAGATTCGGCAG
AACTAAGCAGGCGTCAGAGGAGTTGGTGGGTGTGAGTGCCCCTGTCCCTGCACTTCGGGTGG
CTGCTGGTCCTCCGGGTCCTGCTGTGTGGTTAGACGGCTTCCGGGCAGCCTGGTCTGGCCAG
CACTCACCCTGCCCTCTCTGCCTTTTCTCCCCCAGGGTATTTGGTTTCCCAGTCCACTATAC
TGACGTCTCCAACATGAGCCGCTTGGCGAGGCAGAGACTGCTGGGCCGGTCATGGAGCGTGC
CAGTCATCCGCCACCTCTTCGCTCCGCTGAAGGAGTATTTTGCGTGTGTGTAAGGGACATGG
GGGCAAACTGAGGTAGCGACACAAAGTTAAACAAACAAACAAAAAACACAAAACATAATAAA
ACACCAAGAACATGAGGATGGAGAGAAGTATCAGCACCCAGAAGAGAAAAAGGAAT

CCGC = AciI site/codon R882
CCGC = AciI site/downstream cutting controls
AGCT = AluI site
GTGATCTGAGTGCCGGGTTG = forward primer sequence
TCTCTCCATCCTCATGTTCTT = reverse primer sequence

Figure 9: *DNMT3A* sequence flanking the R882 codon

The *DNMT3A* R882H, R882C and R882S codon mutations altered the CCGC sequence at position R882, which subsequently couldn't be cut by AciI anymore and therefore lead to an aberrant band pattern on the agarose gel: An additional band at 303 bp representing the mutated allele that lost the AciI recognition site was visible next to the band at 252 bp of the non-mutated allele. The band of the mutant allele was 51 bp longer because the fragment was amplified up to the AciI recognition site further downstream in the gene. If the *DNMT3A* mutation was homo- or hemizygous the bands of 252 and 51 bp wouldn't be present.

In the case of the R882C and R882S mutation an AluI site (AGCT) was generated resulting in bands of 250 bp and 253 bp lengths respectively. *DNMT3A* wild type PCR product was not cut by AluI and displayed a 444 bp band on the gel (table 3).

| | DNA sequence | Amino acid sequence (position 880-884) | Fragments after AciI digest (in bp) | Fragments after AluI digest (in bp) |
|---|---|---|---|---|
| Wild type | ATGAG**CCGC**TTGG | M S **R** L A | 252 + 125 + 51 + 16 | 444 (not cut by AluI) |
| R882H mutation | ATGAG**CCAC**TTGG | M S **H** L A | 303 + 252 + 125 + 51 + 16 | 444 (not cut by AluI) |
| R882C mutation | ATG**AGCT**GGTTGG | M S **C** L A | 303 + 252 + 125 + 51 + 16 | 444 + 250 + 194 |
| R882S mutation | ATGAGC**AGCT**TGG | M S **S** L A | 444 (not cut by AciI) | 444 + 253 + 191 |

Table 3 (adapted from Brewin JN et al., 2012) (All mutations were considered as heterozygous).

### 4.1.2 Restriction enzyme sensitivity:

To determine the sensitivity of the restriction enzyme AciI heterozygous mutant R882C DNA from the OCI/AML-3 cell line was serially diluted with wild type DNA from the KG-1 cell line in the following ratios: 50%, 25%, 12.5%, 5% and 0.5%. Following this, PCR amplification was performed as described in 3. 2. 4 using all dilutions and pure wild type DNA. In subsequent gel electrophoresis the presence of the additional 302 bp band in the samples 50%, 25%, 12.5% and 5% proved that AciI could detect the mutation at sensitivity of at least 5% mutant to wild type copy number (figure 10) (Brewin JN et al., 2012).

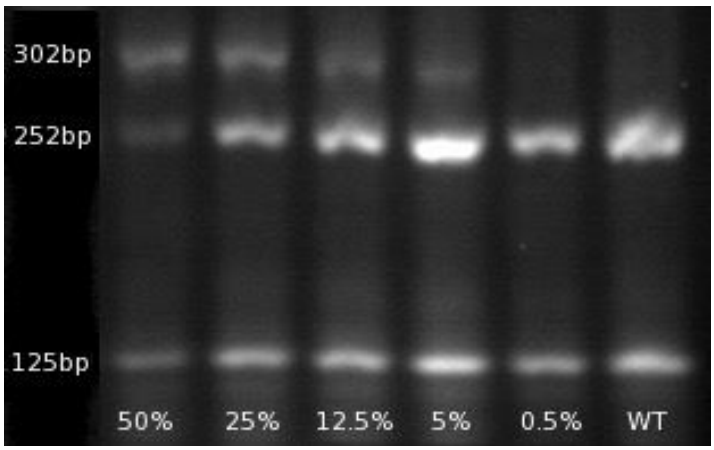


Figure 10: AciI sensitivity for *DNMT3A* R882C mutated DNA (Reproduced from Brewin JN et al., 2012)

### 4.1.3 Cell lines OCI/AML-3 and KG-1:

OCI/AML-3 R882C mutated PCR product and KG-1 wild type PCR product were used as a positive and negative control respectively and run aside the unknown samples in each experiment (figure 11).

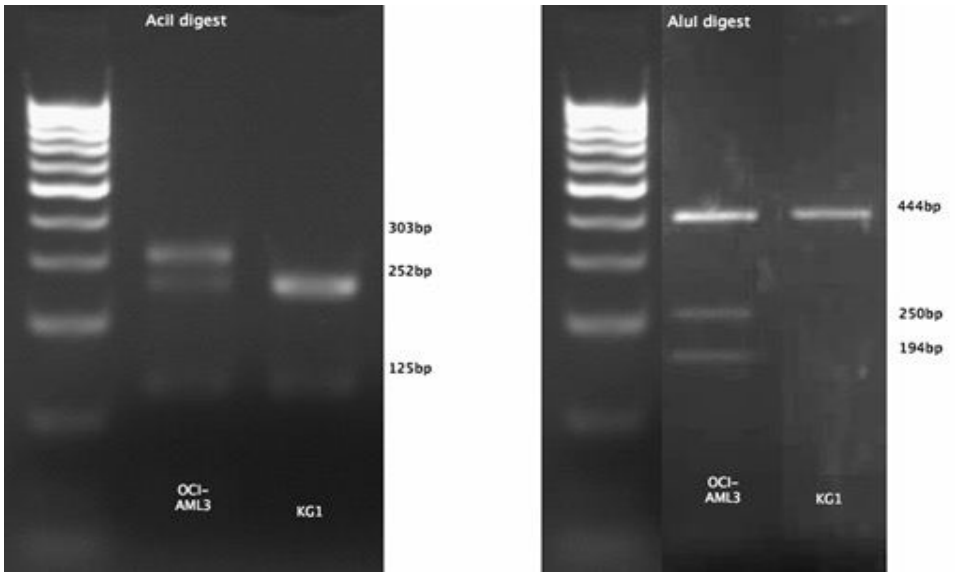


Figure 11: Band pattern of AciI and AluI digestion of OCI/AML-3 and KG-1 cell line DNA (Reproduced from Brewin JN et al., 2012).

### 4.1.4 Patients' samples:

42 AML patients' samples were screened in total (table 6 in chapter 4.4.1.). 39 of those were found to be *DNMT3A* negative, 2 *DNMT3A* R882H positive and 1 R882C positive. Figure 12 illustrates one exemplary negative sample (WT) and the R882H and R882C mutated samples out of the AML patients' samples pool. The AciI digest resulted in complete digestion of the wild type and displayed 252 bp and 125 bp, the bands of 125 bp and 51 bp are not visible on figure 12. Both the R882C and R882H mutation lead to incomplete digestion with an additional band at 303 bp (figure 12). AluI digestion allowed differentiation between the R882C and R882H mutation and confirmed the presence of the R882C mutation demonstrated by two bands at 250 bp and 194 bp. Furthermore, 6 samples from patients suffering from other haematological diseases (mostly myelodysplastic and myeloproliferative syndromes) were tested. The presence of the *DNMT3A* mutation was excluded in all those 6 haematological non-AML samples.

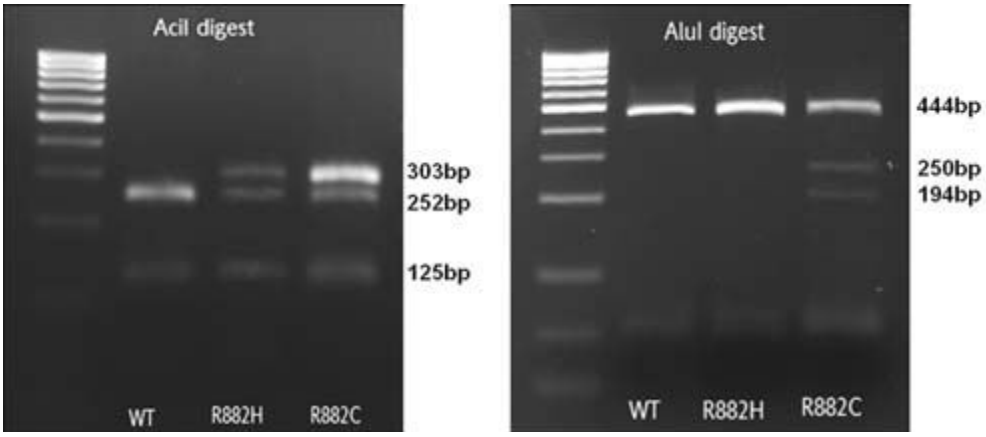


Figure 12: Differential restriction enzyme digestion of PCR product of *DNMT3A* wild type (WT), R882H and R882C *DNMT3A* mutated DNA (Reproduced from Brewin JN et al., 2012)

## 4.2 Quantification of the mutation:

### 4.2.1 Principle of relative quantification:

Quantity of the *DNMT3A* R882 mutation was measured relative to a calibrator which was a sample of known mutational proportion; in this case the heterozygous OCI/AML-3 PCR product. After verification of effective primer binding and amplification efficiencies a dilution series of the calibrator was performed to produce a calibration curve with the purpose

of evaluating the detection threshold for the R882 mutation. The patients' samples to be analysed were then calculated by correlating the abundances of three independent amplicons flanking the R882 codon with the slope of the calibration curve (3. 2. 10).

### 4.2.2 Amplification efficiencies of qPCR primer pairs:

To test the amplification efficiency of each double dye (Taqman© style) qPCR primer pair a 6-log serial dilution of OCI/AML-3 PCR product in nuclease-free water was made at the ratios of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ and amplified using all three primer pairs.

Based on this, $C_t$ values of the dilutions $10^{-1}$ - $10^{-6}$ were used for measurement of the primer amplification efficiency using the $C_t$ slope method (Schmittgen & Livak, 2008). Therefore, the obtained $C_t$ values were plotted against the logarithm of the corresponding PCR product dilution (figure 13). Amplification efficiencies were then calculated from the slope of these graphs according to the following equation (with $E_x$ representing the efficiency):

$$E_x = 10^{(-1/\text{slope})} - 1 \qquad (6)$$

For the loading control primer pair the slope of the line was – 3.7321 which lead to following calculations:

$$E_x = 10^{(-1/-3.7321)} - 1 \qquad (7)$$

$$= 0.8533 \text{ or } 85.33\%$$

This was equally done for the primer pair containing the R882 codon resulting in an efficiency of 85.38% and for the digestion control primer pair concluding in 78.97% efficiency. As the correlation coefficient ($R^2$) of each graph was above 0.99, $C_t$ values were precisely predicting the logarithmic concentration of PCR product. Due to experimental error one outlier was ignored in the digestion control primer graph so that data points of only 5 log dilutions are demonstrated.

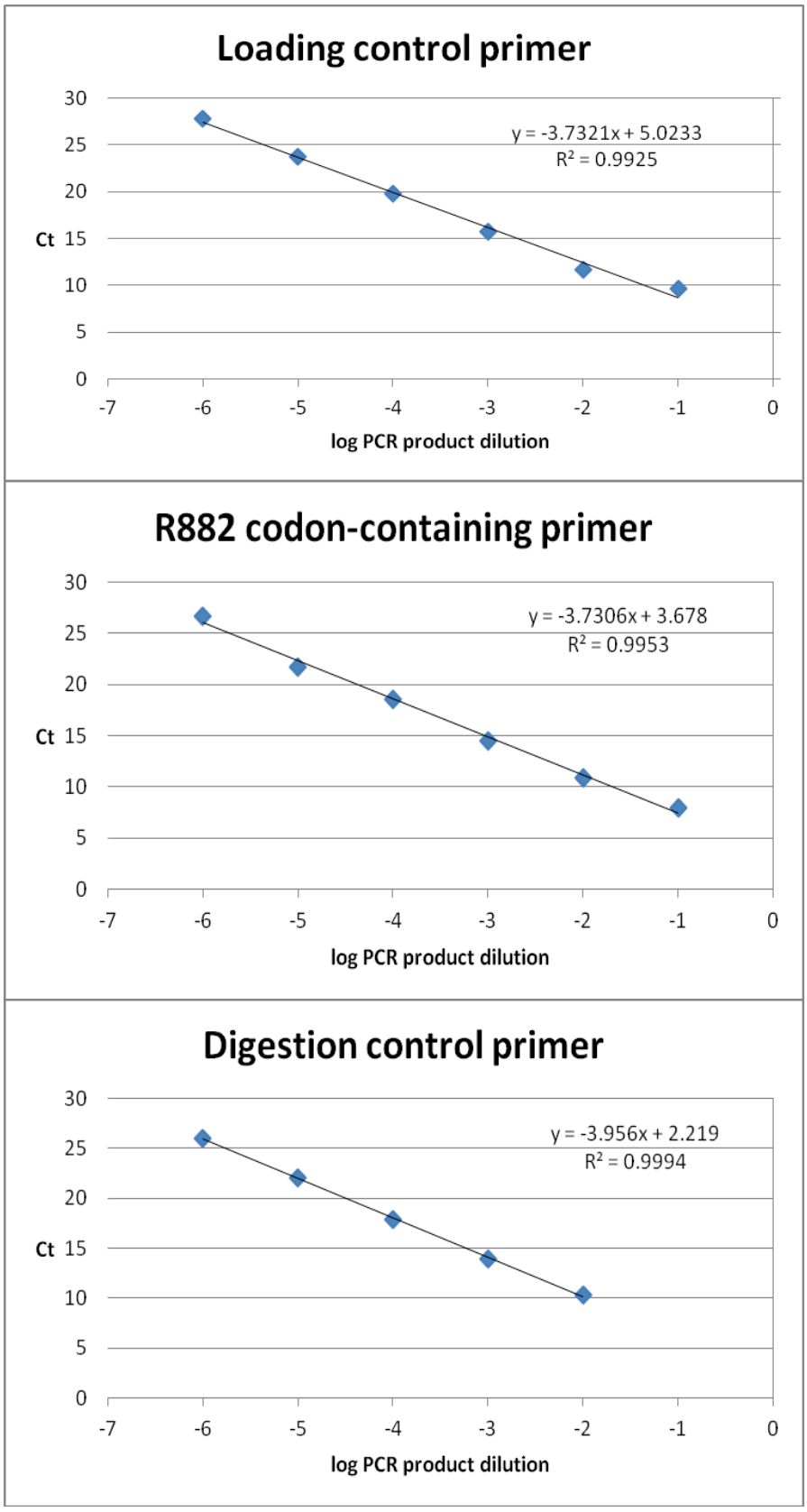


Figure 13: qPCR primer pair amplification efficiencies

#### 4.2.3.1 Primer binding efficiencies of digested PCR product:

The primer binding qualities and effects on $C_t$ values after AciI digestion of the PCR product were tested. Therefore, KG-1 wild type PCR product was digested according to the protocol (3. 2. 7.) and in a second assay mock-digested (all enzyme buffers added, only difference: no enzyme added). These two assays were then amplified by qPCR as described in 3. 2. 10 and following $C_t$ values were measured:

| KG-1, Mock-Digestion | Loading control | R882 codon amplicon | Digestion control | % Mutation | % Relative Cutting |
|---|---|---|---|---|---|
| $C_t$ Replicate 1 | 17.01 | 16.95 | 17.99 | - | - |
| $C_t$ Replicate 2 | 16.76 | 16.93 | 17.75 | - | - |
| $C_t$ Replicate 3 | 17 | 17 | 17.89 | - | - |
| $C_t$ Replicate 4 | 16.81 | 16.94 | 17.77 | - | - |
| $C_t$ Replicate 5 | 16.48 | 16.94 | 17.68 | - | - |
| Mean | 16.81 | 16.95 | 17.82 | - | - |
| KG-1, AciI-Digestion | Loading control | R882 amplicon | Digestion control | % Mutation | % Relative Cutting |
| $C_t$ Replicate 1 | 18.15 | 23.63 | 22.46 | 0.11 | 95.11 |
| $C_t$ Replicate 2 | 17.82 | 23.62 | 22.57 | -0.60 | 97.93 |
| $C_t$ Replicate 3 | 17.67 | 23.56 | 22.53 | -0.77 | 98.51 |
| $C_t$ Replicate 4 | 17.55 | 23.44 | 22.57 | -0.77 | 99.28 |
| $C_t$ Replicate 5 | 17.66 | 23.41 | 22.62 | -0.50 | 99.00 |
| Mean | 17.77 | 23.53 | 22.55 | -0.51 | 97.96 |
| Standard deviation of the mean | | | | 0.16 | 0.75 |

Table 4: $C_t$ values of qPCR primer binding efficiency experiments

For the mock-digested samples the $C_t$ values of the loading control and the R882-containing amplicon were very similar with a difference of only 0.14 of a $C_t$ value. The digestion control's $C_t$ value was higher which was probably due to the slightly lower amplification efficiency of that primer pair. For the AciI digested KG-1 PCR product the values for the loading control amplicon, the R882 amplicon and the digestion control amplicon were expected to be the same because KG-1 does not contain a DNMT3A mutation and therefore should be completely cut by the restriction enzyme. The fact that the loading control amplicon and the latter ones had different $C_t$ values was incorporated into the mutation calculations as correction factor $\gamma$ (3.2.10).

#### 4.2.3.2 Measuring the relative cutting efficiency:

The relative digestion efficiency was measured by comparing the $C_t$ of the digestion control amplicon of AciI-digested *DNMT3A* KG-1 against the $C_t$ of the digestion control amplicon of the mock-digested KG-1 sample (table 4). As the patient samples were very limited it was not possible to run a mock-digestion control for each sample. However, the mean of the $C_t$ values for the mock-digested digestion control and the mean of the $C_t$ values for AciI-digested loading control on KG-1 PCR product (table 4) were very much alike (difference of 0.05 $C_t$ values), therefore it was considered as valid to estimate the cutting efficiency on the difference in $C_t$s of the digestion control and the loading control for each AciI digested patient sample.

### 4.2.4 Calibration curve:

The calibration curve was based on a qPCR experiment generating multiple amplicons of a serial dilution series of OCI/AML-3 DNA in wild type/KG-1 DNA at the following ratios:

| Dilution No. | Serial dilutions % OCI/AML-3 | Serial dilutions % mutation strand |
|---|---|---|
| 1 | 100 % OCI | 50 % |
| 2 | 50 % OCI + 50 % KG-1 | 25 % |
| 3 | 25 % OCI + 75 % KG-1 | 12.5 % |
| 4 | 12.5 % OCI + 87.5 % KG-1 | 6.25 % |
| 5 | 6.25 % OCI +93.75 % KG-1 | 3.125 % |
| 6 | 3.125 % OCI + 96.875 % KG-1 | 1.5625 % |
| 7 | 1.5625 % OCI + 98.4375 % KG-1 | 0.78125 % |
| 8 | 0.78125 % OCI + 99.21875 % KG-1 | 0.390625 % |
| 9 | 0% OCI + 100 % KG-1 | 0 % |

Table 5: Dilution series of OCI/AML-3 in KG-1 DNA

Dilutions No. 1-9 using the OCI/AML-3 cell line were shown to have the mutational load detailed above. Due to its heterozygosity the percentage of the DNA strand containing the mutation is half of the amount of OCI/AML-3 DNA in each dilution. Figure 14 demonstrates how the mathematical quantification of *DNMT3A* R882 mutation based on experimental $C_t$ values was consistent with the actual percentage of the mutation created by the dilution series.

The y axis represents the experimentally detected/calculated proportion of mutants whereas the x axis symbolises the proportion of mutant cells in the dilution samples. In other words, the graph depicts the relationship of the detected proportion and the actual proportion of mutant percentage in the samples and contains errors bars of 2% which equalises to 1% mutant DNA.

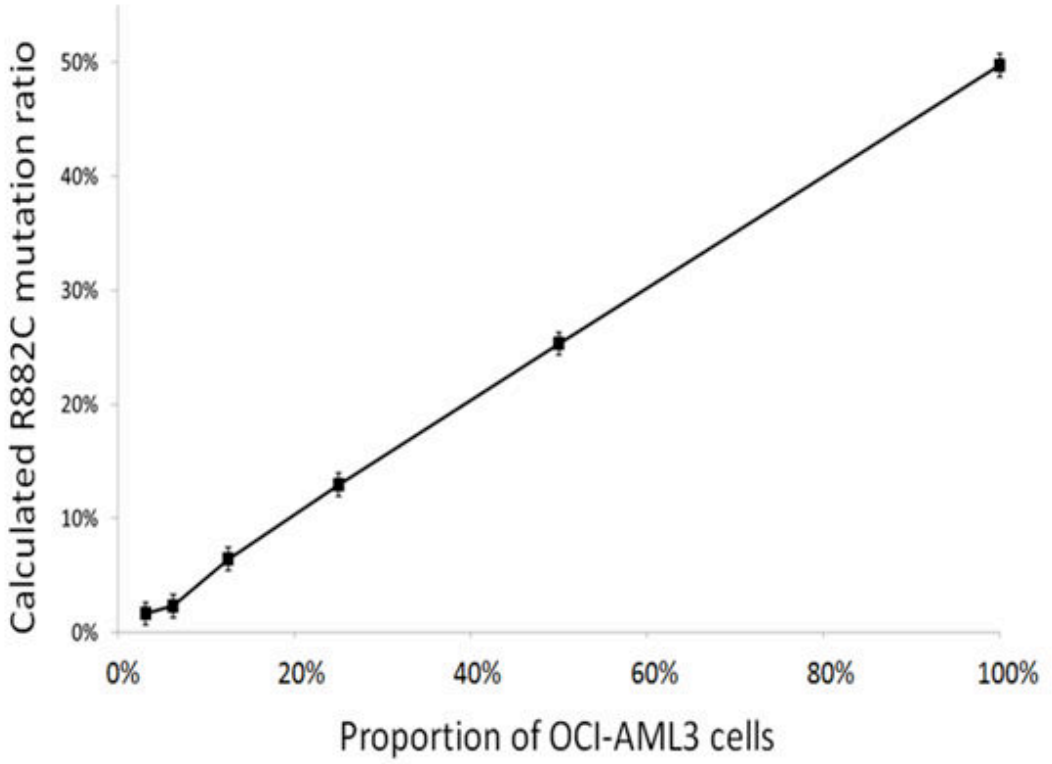


Figure 14: Calibration curve (Reproduced from Bisling KE et al., 2013)

**4.2.5 Sensitivity of the MRD detection assay:**

The sensitivity of the MRD detection assay was determined by the calibration curve using the OCI/AML-3 dilutions as mentioned above. The lowest concentration of mutant in wild type DNA that could still be amplified and detected was 0.78% of OCI/AML-3 R882C mutant DNA in KG-1 wild type DNA. Further dilutions containing 0.39% and 0.19% of OCI/AML-3 DNA could not be reliably amplified and detected.

Therefore, the sensitivity of the assay was declared as less than 1% of total cells respectively 0.5% of allele frequency (assuming heterozygosity among the samples carrying the mutation).

### 4.2.6 Precision of qPCR replicates:

A minimum of two technical qPCR replicates was performed for each AML sample. To test the precision of these replicates mean $C_t$ values were calculated and plotted against the minimal and maximal replicates (Figure 15). In guidelines on MRD analysis Van der Velden and colleagues (2003) suggest that the variation in $C_t$ values with a mean of below 36 should not vary more than 1.5. In this project most of the qPCR replicates are within the appropriate range as demonstrated in figure 15.

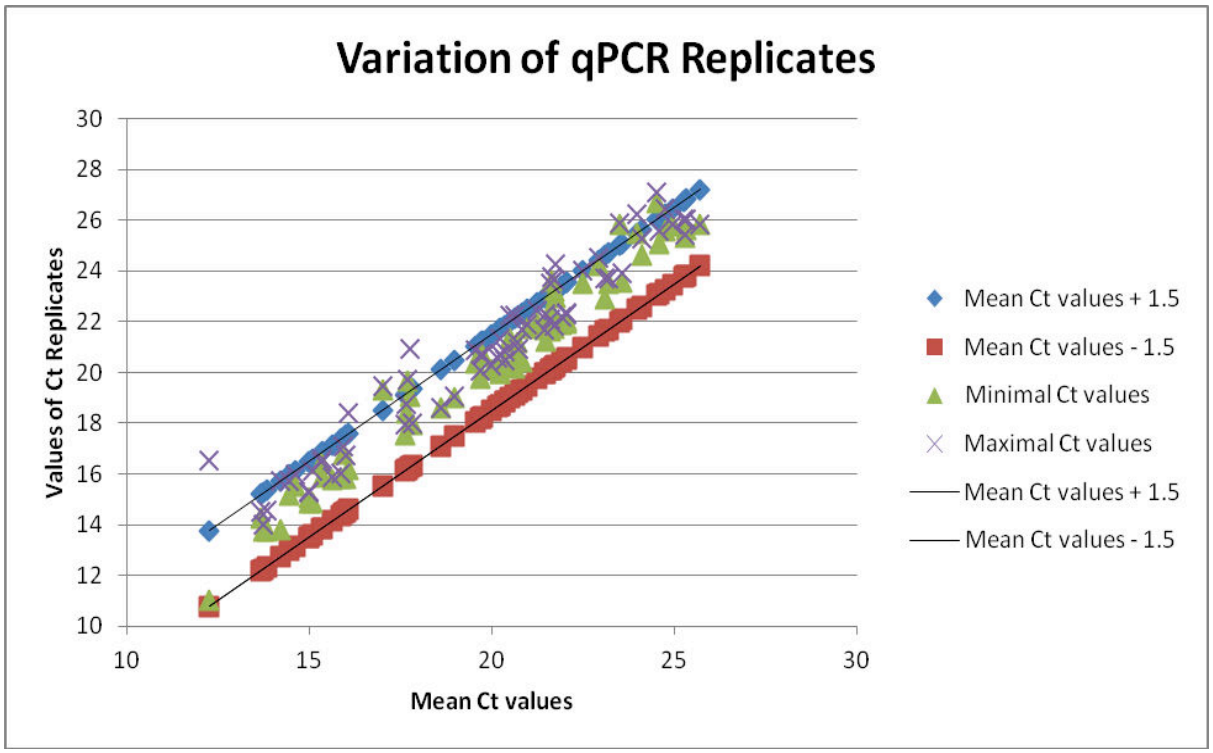


Figure 15: Experimental variation range of the qPCR replicates

### 4.2.7 Reproducibility:

All AML samples were repeated once in a separate qPCR. The results of the mutation calculation based on the first qPCR and second qPCR values were analysed by paired Wilcoxon test. No statistically significant difference was found between both measurements ($p$=0.186, n=22). This was confirmed by using paired t-test on the same data set to test whether there was a significant difference between both observations (95% CI -2.94, 1.006; $p$=0.388). However, the analysis by paired t-test confirmed that the results were reliably reproducible.

### 4.2.8 Patients' samples:

19 patients who had been previously screened by the rapid digestion assay were additionally analysed by the quantification method/MRD assay. 3 patients out of this pool relapsed and were retested so that in total 22 AML samples were tested. 2 patients harboured the *DNMT3A* R882H mutation and were quantified as 97.6 ± 1.9% and 71.1 ± 3.3% of total cells (or 48.8 ± 0.95% and 35.55 ± 1.65% mutant allele frequency respectively). These amounts represent the mean of separate calculations (listed in 3. 2. 10) based on precise $C_t$ values as detected by the qPCR machine of each technical replicate and the corresponding standard deviation. The remaining 20 samples were negative for the *DNMT3A* R882 mutation. OCI/AML-3 and KG-1 PCR product were used as positive and negative controls in each experimental run to standardise the reaction and following calculations. The following scheme (figure 16) demonstrates the qPCR graphs of the loading control amplicon (labelled as LC), the R882 codon containing amplicon (labelled as R882) and the digestion control amplicon (labelled as DC) for a typical negative patient sample, the R882H positive patient sample and OCI/AML-3 as positive and KG-1 negative control for one experimental run. The x axis represents the qPCR cycle number and the y axis shows the level of fluorescence detected.

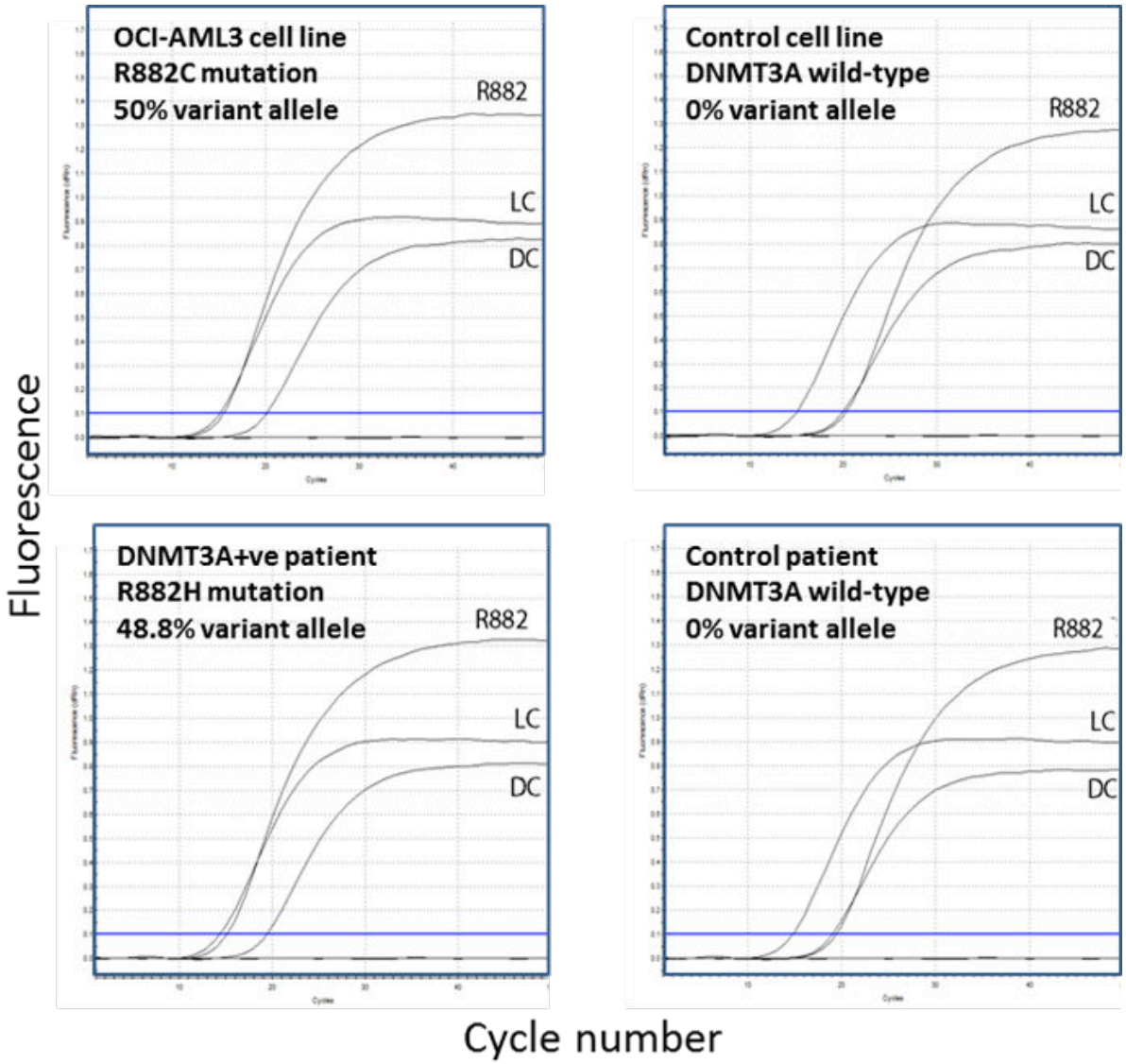


Figure 16: qPCR curves for three primers pairs for the mutated OCI/AML-3 and control KG-1 cell line (top) and for patients' samples (below) (Reproduced from Bisling KE et al., 2013)

## 4.3 Sequencing:

The mutational status of all analysed patients was confirmed by Sanger sequencing. The detected mutations (two R882H mutations, one R882C mutation) were consistent with the sequencing results. Furthermore, all negative samples were confirmed to lack the *DNMT3A* R882 mutation.

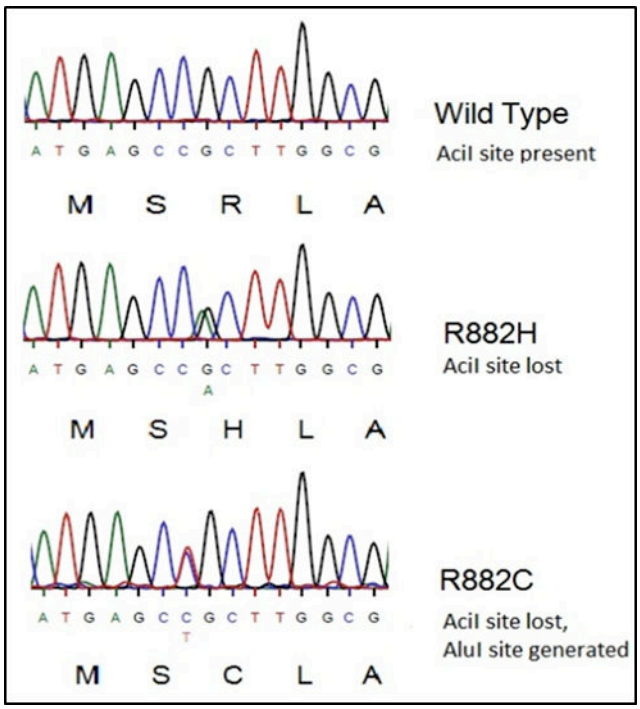


Figure 17: Sequencing results (Reproduced from Bisling KE et al., 2013)

Figure 17 displays the base and amino acid sequence of a typical wild type patient sample, one of the two patient samples harbouring the R882H mutation and the patient sample carrying the R882C mutation. The R882H mutation was caused by a G-to-A transition leading to a histidine substitution at codon position 882 in exon 23 which caused loss of an AciI restriction site. The R882C mutation was generated by a C-to-T transition causing cysteine substitution which resulted in loss of the AciI site but generation of an AluI restriction site (Brewin JN et al., 2013).

## 4.4 Clinical characteristics of the patients:

### 4.4.1 Clinical details of all patients' samples:

42 AML and 6 MPN/MDS samples were screened using the rapid screening test. 19 AML samples were further analysed with the MRD detection assay. Table 6 and 7 summarise the clinical characteristics including age, sex, percentage of bone marrow blasts, the sample's origin, type of AML, karyotype and the allocated risk status of the AML and MPN/MDS samples.

| Variable | **AML samples** (N=42) |
|---|---|
| Age at time of bone marrow biopsy – yrs. | 64.2 ± 11.2, 1 N/A |
| Male sex – no. (%) | 21 (53%), 2 N/A |
| Bone marrow blasts– (%) | 41.8 ± 30.6, 12 N/A |
| Origin of sample – BM/PB | 35: BM, 6: PB, 1 N/A |
| Type of AML – no. (%): | 1 N/A |
| - De Novo AML | 27 (64%) |
| - Secondary AML | 10 (24%) |
| - Relapsed AML | 4 (10%) |
| Normal karyotype – no. (%) | 23 (55%) |
| Risk status – no. (%): | |
| - Favourable | 1 (2.4%) |
| - Intermediate | 27 (64.3%) |
| - Adverse | 4 (9.5%) |
| - Indeterminate | 10 (23.8%) |

Table 6: Clinical characteristics of the 42 AML samples. N/A means information not available, ± values are means plus/minus standard deviation of the mean; BM = Bone marrow, PB = Peripheral blood, risk status was stratified according to the Cancer and Leukemia Group B (CALGB 8461) (Byrd JC et al., 2002)

**Other malignant diseases:**

The following non-AML bone marrow samples were screened because the *DNMT3A* mutation was not only identified in patients with *DNMT3A* but also in cases with myelodysplastic syndromes and myeloproliferative neoplasms (Stegelmann F et al., 2011; Walter MJ et al., 2011).

| | Age (years) | Sex | Disease | Further details |
|---|---|---|---|---|
| BSMS 14 | 47 | N/A | Polycythaemia | *JAK 2* pos. |
| BSMS 28 | 63 | M | Monoclonal gammopathy of undetermined significance | |
| BSMS 53 | 73 | F | Chronic myeloid leukaemia | *BCR-ABL* |
| BSMS 66 | 64 | F | Refractory anaemia with excess blasts type 2 | Blasts: 12% |
| BSMS 88 | 68 | M | Refractory cytopenia with multilineage dysplasia | *JAK 2* pos. |
| BSMS 90 | 76 | M | Refractory anaemia with excess blasts | Blasts: 22% |

Table 7: Diagnostic and cytogenetic details of the 6 haematologic non-AML samples that were tested for the *DNMT3A* mutation

Furthermore, all AML patient samples were analysed regarding any clinical differences between the ones harbouring the *DNTM3A* R882 mutation and those classified as wild type for *DNMT3A* (table 8).

**Comparison of *DNMT3A* R882 mutation versus *DNMT3A* R882 wild type:**

| Variable | *DNMT3A* R882 Wild type (N=39) | *DNMT3A* R882 Mutation (N=3) | *p Value* |
|---|---|---|---|
| Median age – in yrs. (range) | 66.00 (23-82) | 78.00 (76-80) [1] | *0.0736** |
| Male sex – no. (%) | 19 (50) [1] | 2 (100) [1] | *0.4885†* |
| Median bone marrow blast count – in % (range) | 30.8 (1-98) [11] | 58.0 (17-99) [1] | *0.3827** |
| Type of AML – no. (%): | [3] | | |
| - De Novo AML | 26 (66.67) | 2 (66.67) | *1†* |
| - Secondary AML | 6 (15.38) | 1 (33.33) | *0.43†* |
| - Relapsed AML | 4 (10.26) | 0 | *1†* |
| Normal karyotype – no. (%) | 21 (53.85) | 2 (66.67) | |
| Risk status – no. (%): | | | |
| - Favourable | 1 (2.6) | 0 | *1†* |
| - Intermediate | 24 (61.5) | 3 (100) | *0.5414†* |
| - Adverse | 4 (10.3) | 0 | *1†* |
| - Indeterminate | 10 (25.6) | 0 | *1†* |

Table 8: Numbers in [] brackets indicate how many patients didn't have appropriate values available and were omitted from statistical analysis.* *p* values were calculated with Mann Whitney U test for the comparison of *DNMT3A* wild type with *DNMT3A* R882 mutation. † *p* values were calculated using the Fisher exact test for the comparison between *DNMT3A* R882 wild type and *DNMT3A* R882 mutation.

No statistically significant differences were observed between the AML patients carrying the *DNMT3A* mutation and those with wild type *DNMT3A*. However, there was a slight tendency towards higher age at disease presentation, the percentage of male sex, the occurrence of a higher blast count and the probability of the intermediate risk group status of patients carrying the *DNMT3A* R882 mutation than those without the mutation.

### 4.4.2 Clinical outcome of the patients with *DNMT3A* R882 mutation:

The two patients harbouring the *DNMT3A* R882H mutation are referred to as BSMS 33 and BSMS 85: BSMS 33 was a 76-year-old male patient harbouring a deletion of the long arm of chromosome 5 (del(5)(q13q33)) which was identified at the time of initial diagnosis. Deletion of the long arm of chromosome 5 is a common monosomal karyotype in acute myeloid leukaemia and myeloprofilerative disease and is associated with an extremely unfavourable prognosis in AML patients (Breems DA, Van Putten WLJ, & Löwenberg B, 2013). He had a blast count of 99% at initial diagnosis and was positive for the *FLT3*-ITD mutation. The co-occurrence of the *NPM* mutation as well as the *CBFB* or *MLL* gene rearrangement was excluded. In this project, the *DNMT3A* R882H mutation within his genome was quantified as 71.1 ± 3.3% of total cells (or 35.55 ± 1.65% mutant allele frequency).

BSMS 85 was also a male patient, aged 80 years, suffering from secondary AML as a progression of MDS with normal cytogenetics. He was allocated to the intermediate risk group (risk group allocation following indications of the Cancer and Leukemia Group B (CALGB 8461), Byrd JC et al., 2002) and he did not have a *FLT3*-ITD or *NPM* mutation.

They both received 2 cycles of intensive chemotherapy consisting of Cytarabine as a continuous infusion for seven days plus Daunorubicin on days one until three. Both patients received morphological response to chemotherapy but an incomplete blast recovery and relapsed quickly post treatment. BSMS 33 survived for 7 months, BSMS 85 for 8 months after initial diagnosis.

Unfortunately, there is only very limited clinical information on the patient with the *DNMT3A* R882C mutation: He was a male patient with primary AML and normal cytogenetics who died quickly after diagnosis.

# V. Discussion

## 5.1. Synopsis of principal findings:

In this chapter the methodology and principal findings of both *DNMT3A* mutation detection assays will be argued and compared to other techniques. Briefly, genomic DNA was extracted from AML samples, PCR amplified and analysed by a rapid differential restriction enzyme digestion assay. By differentiating between two specific digestion enzyme patterns and confirmation through comparison with control cell line OCI/AML-3, three cases of *DNMT3A* R882 mutations could be identified. Two R882H and one R882C mutation were detected within the pool of 42 AML samples. In order to detect minimal residual disease with low copy numbers of *DNMT3A* mutations, the test was further expanded through quantitative PCR allowing quantification of the mutational proportion. A subset of 22 samples were further analysed using this highly sensitive test. The two R882H mutations could be confirmed and were calculated as 97.6% and 71.1% of total cells. However, *DNMT3A* R882 mutations were not present in any of the other 20 samples. The clinical details of the patients harbouring the *DNMT3A* mutation were examined and compared to the patients that didn't show the mutation but no statistically significant differences between both groups were recognised.

## 5.2. Evaluation of the strengths and limitations of the *DNMT3A* detection assays:

### 5.2.1 Differential restriction enzyme assay:

The following paragraph will highlight the strengths and weaknesses of the differential AciI/AluI restriction enzyme assay: The biggest advantage of this method is the rapid determination of presence or absence of the most frequent mutations, namely the R882H and R882C mutations at the R882 locus within the *DNMT3A* gene. Only two to three hours in total is enough time to process an AML bone marrow sample. Another major strength is that the sensitivity of the test is 5% of mutant to wild type copy number. In addition, the test uses basic techniques such as DNA purification, PCR, restriction enzyme digest and therefore equipment that is broadly available in most diagnostic laboratories. Furthermore, it is a cost effective test as it requires mostly mass-produced, relatively inexpensive standard kits, e.g. PCR Mastermix (Brewin JN et al., 2013).

On the other hand, a great disadvantage is the risk of potential incomplete digestion, hence the danger of falsely predicting the absence of the mutation. Normally, complete digestion should be confirmed by the two smaller fragments (16 bp and 51 bp) but it can be difficult to visualise smaller bands on agarose gels.

A further limitation is that the test doesn't allow a differentiation between R882C and R882S mutations as it is not possible to distinguish a band with a 3 bp difference on a standard agarose gel. However, as the subtypes of the *DNMT3A* R882 mutation are rather of academic interest than of clinical relevance, this disadvantage is acceptable.

Moreover, although each step of the method is fast and simple post PCR processing can make it fairly laborious.

Another disadvantage is that the AciI enzyme digests the DNA leaving cohesive ends which could re-ligate if over digestion occurs.

### 5.2.2 Choice of qPCR chemistry:

Several different types of qPCR detection are available: The most common qPCR chemistry methods are Taqman©, SYBR Green, Molecular Beacons and MGB (3′-Minor groove binder-DNA) probes (Forlenza M et al., 2012). We chose Taqman© style double-dye probes because they provide precise results while being very cost effective. The only qPCR probe that is lower in costs is SYBR green but this probe has the disadvantage of non-specific primer binding and false-positive signals as SYBR Green does attach to any type of double stranded DNA.

### 5.2.3 Relative quantification using internal amplicons:

In this project, relative quantification of the *DNMT3A* mutation in comparison to absolute quantification (measuring the absolute copy number of a specific pathogen) was considered as sufficiently precise because the knowledge of the exact number of gene copies was not deemed necessary for the clinical application of this potential MRD assay. Rather, the project's aim was to identify a certain percentage of cells in relation to a known reference. This reference is often a reference gene that is as similar to the target gene as possible. For this project an internal control instead of a separate reference gene was chosen because the mutational quantification was based on the difference in the amount of restriction enzyme digested amplicons. Therefore, these amplicons should be digested in exactly the same way

to prevent technical influence on quantification. The use of an internal control had also the practical advantage that only one sample (the *DNMT3A* sequence containing all three amplicons) was digested so that laboratorial work was shortened and the amount of restriction enzyme reduced.

### 5.2.4 Calculations:

#### 5.2.4.1 Mutational proportion:

The calculation of the mutation was based on a mathematical model for relative quantification in real-time PCR reactions by Pfaffl MW (2001) which estimates the relative expression ratio of a target gene in relation to a reference gene transcript by taking into account the PCR amplification efficiencies. For this project, this model was further refined by expressing the level of mutation in relation to a calibration curve, as usually done for absolute quantification, with the aim to obtain measurements as precise as possible. The benefit of this refined calculation method is that the results are corrected for differences in PCR efficiencies between target and control sample and that the threshold detection is defined very precisely through a calibration curve. An inconvenience of the refined efficiency correction method is the need for dilution series experiments of the target to create the standard curve. If several reference genes or internal controls are used this has a great impact on the laboratory time and effort. However, in the present case this disadvantage has less importance as only one internal control is used. Another option to estimate quantification is the comparative $C_t$ method ($2^{-\Delta\Delta}Ct$ method) which postulates several assumptions (Schmittgen TD & Livak KJ, 2008). One of them is that the PCR efficiency is 100% which is why this method wasn't appropriate for the present data setting.

#### 5.2.4.2 Relative cutting control:

To confirm complete restriction enzyme digestion without having the facility to run a separate mock-digestion assay in each experiment, the *relative* cutting control was examined by comparing $C_t$ values of the loading control and the digestion control amplicon of each AML bone marrow sample. The digestion experiments on KG-1 PCR product as described in 4.2.3.2 resulted in very similar $C_t$ values for the digestion control amplicon of the mock-digested KG-1 PCR product and the loading control amplicon of the AciI digested KG-1 PCR product. This lead to the conclusion that the mock-digested digestion control could be replaced by the loading control amplicon of the AciI digested sample which was generally

applied in all subsequent experiments. However, this observation needs to be further validated by testing the stability of this phenomenon across different cell lines as well as bone marrow samples. This disadvantage of the MRD detection assay which could easily be improved in future experiments by adding a mock-digestion sample of each AML sample or by further analysing the consistency of the above mentioned $C_t$ values.

### 5.2.5 qPCR primer binding and amplification efficiencies:

As literature on relative quantification of gene expression levels based on qPCR (Schmittgen TD & Livak KJ, 2008) suggested that primer amplification efficiencies of the target and control gene should be within 10% of each other, the primer efficiencies of the method established in this project were considered valid to estimate the proportion of the mutation, especially as the calculations were corrected for each primer amplification efficiency. However, the digestion control primer had a slightly lower efficiency of 79% which is downgrading the predictive value of the digestion control calculations. According to Schmittgen and Livak's protocol primer amplification efficiencies should be at least 80%, therefore an adjustment of that primer might have been useful to improve the precision of the estimation of the digestion control. As mock-digestion experiments showed, there were small differences in $C_t$ values due to potential qPCR inhibition by restriction enzyme buffers. These were corrected by a further correction factor obtained through comparisons of mock and enzyme digested PCR product, so that the calculations were as accurate as possible. In this model we assume that the altered primer binding qualities are similar when digested KG-1 PCR product is amplified as well as patients' samples or other cell lines. To prove this assumption further experiments are needed to confirm that the primer binding qualities are consistent across different cell lines and patients samples.

### 5.2.6 Calibration curve:

The use of a calibration curve offers great precision when measuring expression fold ratios or quantifying leukaemic cells (Forlenza M et al., 2012). In this project, the calibration curve was based on a dilution series with 9 different dilutions allowing the determination of the lowest possible detection threshold with great accuracy because the amount of mutant DNA was gradually decreased. On the other hand, the establishment of a calibration curve is laborious and requires very clear technical working as methodology is based on this curve.

### 5.2.7 Sensitivity and reproducibility:

The limit of detection of the minimal residual disease assay was between $10^{-3}$ and $10^{-4}$ as the *DNMT3A* mutation was still reliably detected in less than 1% of leukaemic cells or 0.5% allele frequency respectively. This level complies with the requirements stated by VJH Van der Velden et al. (2003) and E Paietta et al. (2012) for detection of recurrent gene mutations in acute myeloid leukaemia. One restraint is that the sensitivity was measured based on dilution experiments of the *DNMT3A* R882C mutation so that the level of minimal detection is formally only confirmed for the R882C type of the mutations at the R882 hotspot. However, as the R882H mutation is based on the same mechanism, there should be no objections against transferring this level of detection to the R882H type of mutation.

The reproducibility was tested by repeating the qPCR and subsequent mutation calculations leading to similar results based on statistical analysis by Wilcoxon test and paired t- test. A repetition of the experiments from the beginning including the initial PCR and especially the restriction enzyme digestion would have been more precise and would allow more realistic conclusions about the reproducibility of the assay. This should be taken into consideration in future experiments.

### 5.2.8 *DNMT3A* mutation status and clinical characteristics of the AML samples:

In this report, out of the AML sample pool containing 42 samples in total, two *DNMT3A* R882H mutations were detected and quantified as 97.7% and 71.1% respectively and one R882C mutation was detected but not further analysed due to depletion of the sample. Moreover, 20 other AML samples were screened using the quantification method and the absence of the *DNMT3A* R882 mutation was confirmed.

The prevalence of the *DNMT3A* mutation usually has a range from 15 to 25% (Thiede C, 2012). In this project, the prevalence of the *DNMT3A* R882 mutation was much lower, namely at the 7% level which is unusual and could be due to several different reasons, e.g. the small sample size, by chance or patient selection. As *DNMT3A* mutations most frequently arise in cytogenetically normal AML (CN-AML) with prevalence rates from 27.2% (Thol F et al., 2011) to 34.2 % (Marcucci G et al., 2012) we sought to analyse the mutational recurrence in patients with CN-AML in our sample pool. In this project, out of 23 patients suffering from AML with normal cytogenetics three were *DNMT3A* R882 mutant leading to a ratio of 13.04% in CN-AML. Potential explanations for this difference are mentioned above.

In most studies from Europe and the United States patients with AML harbouring the *DNMT3A* mutation were about 10 years older, of male sex, had a high blast count and typically presented with cytogenetically normal AML compared to those without the mutation (Tie R et al., 2014; Renneville A et al., 2014; Gaidzik VI et al., 2103; Thiede C, 2012). There are tendencies towards these characteristics in the present patient cohort as well: Both were male, of older age, had a considerably elevated blast count and one of them had cytogenetically normal AML. The other patient differed in the genetic profile as he had a deletion of the long arm of chromosome 5. Furthermore, one of the two was positive for the *FLT3*-ITD mutation which has been observed as a frequent co-occurrence of *DNMT3A* mutations (Abdel-Wahab O & Levine RL, 2013). However, no statistically significant differences were observed between patients with the *DNMT3A* mutation compared to those without the *DNMT3A* mutation. One possible reason for this is that only a small number of patients was analysed as only a limited number of patients were diagnosed with acute myeloid leukaemia during the study period in East Sussex. Another reason could be the fact that patients with AML were analysed at all points during their treatment, not only ones with primary AML but also those with relapsed disease (see table 8, 4.4.1.) which could potentially reduce the number of patients with *DNMT3A* mutations and mask their typical presentation.

An advantage of this patient cohort was that 3 patients had samples available at initial diagnosis and also at relapse. Analysis showed that these patients were *DNMT3A* mutation negative at initial diagnosis and remained so during the disease development. Unfortunately, no follow-up samples from *DNMT3A* mutation positive patients were available.

## 5.3 Analysis of the *DNMT3A* detection tests in comparison to others:

The following subchapter will discuss the strengths and weaknesses of the present study in comparison to other *DNMT3A* mutation detection methods as well as to similar MRD tests screening for other recurrent genetic mutations in acute myeloid leukaemia or other haematological diseases. The great majority of studies analysing *DNMT3A* mutations use sequencing technologies as the detection method by which the genetic alteration was first identified. The *DNMT3A* mutation was initially described by two independent research groups in 2010: Yamashita and colleagues primarily identified the *DNMT3A* mutation by carrying out large scale array-based genomic resequencing of AML samples (Yamashita Y et

al., 2010), whilst Ley et al. performed massively parallel deep sequencing of 281 AML patients (Ley et al., 2010). Shortly after this, Yan X-J et al. distinguished the mutation by using exome sequencing in acute monocytic leukemia (the M5 subtype of acute myeloid leukemia) (Yan X-J et al., 2010). Subsequent studies used direct Sanger sequencing of either all coding exons or only exons enriched for genetic alterations within the *DNMT3A* gene (Ostronoff F et al., 2013; Tiacci E et al., 2012; Renneville A et al., 2012; Patel JP et al., 2012).

However, the ongoing development of these sequencing technologies lacks practical applicability and availability in standard diagnostic laboratories and rather advances research purposes. Therefore, the differential restriction enzyme screening test provides an advantageous rapid and simple analysis for diagnostic laboratories with basic equipment and represents a valid method for smaller health care centres with less advanced technologies accessible (Brewin JN et al., 2013).

More recently a comparable endonuclease restriction test to our assay has been developed using another specific restriction enzyme with a sensitivity of 0.05% mutant allele frequency which is higher than our restriction enzyme test (Berenstein R et al., 2014). This might be due to insufficient optimisation in our laboratory, interaction of different buffers or the use of a more efficient enzyme.

Additionally, Mancini M and colleagues (2015) developed a restriction enzyme based RT-PCR assay to screen for *DNMT3A* R882 mutations. Compared to our differential restriction enzyme test their approach detects R882H, C, S and P alterations while we only confirmed our assay for the more common R882H and C mutations. Theoretically, our assay also has the ability to detect R882S mutations because this C-to-A transversion results in a serine substitution that generates an AluI restriction site, the second endonuclease used in the differential restriction enzyme sequence. Though, in our study population no R882S substitution was present so that we couldn't validate the detection of this rare substitution (Brewin JN et al., 2013).

Secondly, Mancini's research team confirmed the data by capillary electrophoresis while we verified our results by Sanger sequencing. In addition, they designed the forward RT-PCR primer to generate an additional restriction cut site thus providing an internal cutting control whereas the cutting efficiency in the present restriction enzyme test was validated visualising two smaller PCR fragments that are generated during complete digestion on an agarose gel.

However, those caveats are compensated for the high sensitivity of 5% mutant to wild type copy number of our differential restriction enzyme test compared to 10% (Mancini M et al., 2015).

The detection of minimal residual disease in acute myeloid leukaemia promises to be a powerful parameter for both post therapy monitoring of selected patients for appropriate post induction or consolidation therapy along with a potential control during ongoing initial treatment to check therapy response but it is still subject to many difficulties such as non consistent baseline levels between the different detection methods or the absence of longitudinal stability of gene mutations (Paietta E, 2012). In the following different paragraphs *DNMT3A* detection analyses are evaluated and compared to the instant MRD test:

High resolution melting (HRM) analysis is a technique that measures the melting point of double stranded DNA fragments after PCR amplification by measuring fluorescence dye levels and is often applied for detection of aberrant sequence variants (Pfaffl MW, 2010).
In a recent study showing that high *DNMT3B* (DNA methyltransferase B) levels predict a poor prognosis in acute myeloid leukemia *DNMT3A* mutations in exon 23 were detected by RT-PCR and HRM analysis. The method was established using a dilution series of an AML sample carrying the R882H mutation resulting in a sensitivity of 2.5% (Hayette S et al., 2012). This approach is relatively similar to the present work in terms of development of the detection threshold. However, our assay was able to reliably detect *DNMT3A* R882 mutations with a sensitivity of 1%. Nonetheless, the report by Hayette et al. (2012) examines for mutations positioned between codon V867 and R891 including codon R882. Admittedly, they include 24 codons into the mutational analysis while the present methodology focuses only on codon R882 where indeed around 60% of all *DNMT3A* mutations are found (Ley TJ et al., 2010; Yamashita et al., 2011; Renneville A et al., 2012). In conclusion, our assay offers a higher sensitivity with a focus on the most frequently altered codon as opposed to a lower sensitivity analysis of a larger sequence.

Further HRM tests include a study by Singh RR et al. (2012) who achieved lower sensitivities for the *DNMT3A* mutation (for exon 8 and 10 within the PWWP domain: 7.2%, and 6.8% of cells respectively, for exon 15 within the ADD (*ATM-DNMT3A-DNMT3L*) zinc finger domain 6.4% and finally for exon 18 and 23 within the catalytic domain 8.4% and 6.7% respectively. Again this work has the advantage of examining several loci. Another report observed the occurrence of *DNMT3A* mutations in Chinese patients and achieved HRM

analysis sensitivity for codon R882 of 2% which was shown to be much more sensitive than direct DNA sequencing with 10% sensitivity (Lin J et al., 2011).

One further, more up-to-date HRM assay by Gorniak P et al. (2015) analysed 67 AML samples, among those 12 containing the *DNMT3A* R882 mutation, with a detection limit of 10% mutant to wild type DNA. They defined the detection threshold by creating a dilution series of a plasmid encompassing the *DNMT3A* R882 codon in concentrations of 100%, 50%, 25%, 10%, 5%, 1% and 0%. However, our assay performs better in terms of sensitivity levels as the present test repeatedly generated results at a sensitivity level of 1% mutant to wild type, 0.5% mutant allele frequency respectively (Bisling KE et al., 2013).

In addition, denaturing high-performance liquid chromatography is widely used to detect gene mutations based on the physicochemical difference between homoduplexes (PCR amplicons whose DNA strands are completely complementary) and heteroduplexes (PCR amplicons with base changes in one DNA strand as a result of a heterozygous mutation) as measured by reversed-phase chromatography after partial denaturation (Kosaki K et al., 2005). This high-capacity and low-cost method has been used to identify *DNMT3A* mutations and allowed their detection with a sensitivity of 5% screening all coding exons of the *DNMT3A* gene in paediatric AML cases and adults younger than 60 years respectively (Liang D-C et al., 2013; Ribeiro AFT et al., 2012). In comparison to the present *DNMT3A* detection method the aforementioned assays are able to screen for *DNMT3A* mutation with a greater sensitivity than Sanger sequencing but not more sensitive than the hereby presented methodology.

More importantly, none of the above discussed methods has the capability to quantify the *DNMT3A* mutations in relation to the total number of leukaemic cells.
A mutation quantification test was recently developed to detect and monitor *BRAF*V600E mutations in hairy cell leukaemia with the purpose of diagnosis and follow-up of affected patients (Schnittger S et al., 2012). This assay is based on real-time PCR measurement of the expression changes between *BRAFV*600E mutant to wild type DNA and was established from dilution series of a plasmid containing the mutation. In further detail, the sensitivity reaches $10^{-4}$ – $10^{-5}$ which is at least 10 times more precise than the sensitivity for the *DNMT3A* mutation reached in our test ($10^{-2}$ – $10^{-3}$) and is probably due to higher qPCR efficiencies of 1.93 to 1.96 compared to 1.85 to 1.79 (as described in 4.2.2.). An additional strength of the afore-mentioned test is the external validation of variant patients as assessed by the newly

developed test by multiparameter flow cytometry (MFC) and deep-sequencing instead of verification by Sanger sequencing as performed in our study.

Lately, PCR technologies determining *DNMT3A* mutations have significantly improved: Mancini M et al., (2015) established a quantification test based on peptide nucleic acid (PNA) real-time quantitative PCR which uses artificially synthesised DNA elements that are characterised by neutral electrical qualities and therefore obtain stronger interactions between DNA strand and PNA probe. As exclusively wild type *DNMT3A* sequence is linked to the PNA probe PCR amplification cannot continue and a different fluorescent signal is registered for mutant and WT DNA sequence. As discussed in 5.2.3 our qPCR test uses an amplicon inside the *DNMT3A* gene as a loading control whereas Mancini and colleagues use *ABL1* gene amplification because their quantification methodology is independent of the endonuclease study design. In contrast to these differences, the herein presented detection method as well as Mancini and colleagues' work achieves similar sensitivity levels of about $10^{-4}$.
Moreover, another research group set up a bead-based bridged-nucleic acids (BNA) assay using oligonucleotide probes with improved hybridisation capability to DNA strands that are coupled with fluorescently labelled microbeads. This test features quantitative detection of *DNMT3A* R882C, H, R and S mutations with a sensitivity of 2.5% for the different mutant alleles and was validated on dilutions of mutant and wild type plasmid (Shivarov V et al., 2014; Biosynthesis User Information). Again, this test includes R882R and S mutations in the detection profile but fails to reach our sensitivity level of less than 1% of total cells.

One step further towards absolute quantification of the *DNMT3A* R882H mutation provides a study undertaking allele-specific PCR with a blocking reagent (ASB-PCR). In the experiments, a plasmid containing the R882H defect is used and enables us to differentiate 1% R882H positive DNA. Aside from a good sensitivity level of $10^{-3}$ the test offers exact knowledge of the *DNMT3A* R882H mutational load at different MRD disease stages of AML (Berenstein R et al., 2015).

Similarly, Xu L et al. (2013) developed conventional and real-time allele-specific PCR assays for detection and quantification of the somatic mutation *MYD88* L265P in patients with Waldenström's macroglobulinemia, IgM monoclonal gammopathy and other B-cell lymphoproliferative diseases. The conventional PCR test had a sensitivity of 0.1% of mutant

allele frequency while the real-time PCR assay could identify *MYD88* mutant alleles down to a sensitivity of 0.08%. The mutant allele amount was calculated by the $^{\Delta\Delta}C_t$ method using values for mutant and reference PCR amplicons (Xu L et al., 2013; Schmittgen TD & Livak KJ, 2008). Compared to the herewith described *DNMT3A* detection assays, the sensitivity levels of both the real-time and the conventional PCR detection approaches allow very precise detection of the corresponding mutation. The validation of results by Sanger sequencing was carried out similarly in both projects.

When analysing the MRD monitoring of *NPM1* mutations in acute myeloid leukaemia an exemplary study performed by the German-Austrian Acute Myeloid Leukemia Study Group (Krönke J et al., 2011) using RNA based real-time quantitative PCR showed maximum sensitivity levels of $10^{-5}$-$10^{-6}$ for the analysed mutation types. The advantages of this study are that the established method examines several types of the mutation as well as the high sensitivity and specificity.

In conclusion, our MRD detection assay has a higher sensitivity to screen for *DNMT3A* R882 mutations than most other available *DNMT3A* tests but on the other hand it only focuses on one mutational hotspot and doesn't analyse additional loci. The *DNMT3A* mutation seems to be more difficult to detect than the *MYD88*, *NPM1*, and *BRAFV*600E mutations in comparable real-time PCR tests in other haematological illnesses as the maximum sensitivities of most of these tests were better.

## 5.4 Conclusions about the significance of the study:

### 5.4.1 Significance as a clinical test:

The differential restriction enzyme test provides a practical and fast approach to detect the presence of *DNMT3A* R882 mutations and also offers some evidence about the subtype of R882 mutation. Due to the simplicity and rapidity of the assay it could help the clinician reach a timely decision on treatment options as patients with *DNMT3A* mutations might benefit from enhanced chemotherapy regimens (Brewin JN et al., 2012). Therefore, it is probably of greater benefit in a small laboratory with limited resources than a laboratory with access to deep sequencing techniques. Besides, tests based on conventional PCR need less DNA than whole genome- or Sanger sequencing so that this test represents an option for very small and precious samples sizes as well (Xu L et al., 2013).

The quantitative test provides a method for detection of *DNMT3A* R882 mutations which is more sensitive than Sanger sequencing as well as less time-consuming and cost intensive than deep- and whole-genome sequencing techniques. This assay provides information on prognosis, treatment decisions and minimal residual disease monitoring in AML patients.

The combination of both tests gives the opportunity to screen patients that are deemed as low-risk for the *DNMT3A* mutation with the basic restriction enzyme method and those that are more likely to carry the *DNMT3A* mutation with a more sensitive analysis using the quantification test.

As recent findings consider *DNMT3A* mutations to be a founding clone in AML evolution and remain in subclinical stages for a long time, management of minimal residual disease could be modified to screen not only post-therapy blasts but also pre-leukaemic stem cells (Shlush LI et al., 2014; Genovese G et al., 2014). However, further research on the impact of *DNMT3A* on the probability of developing AML is needed before MRD detection could be offered to individuals harbouring the *DNMT3A* mutation prior to outbreak of overt AML.

### 5.4.2 Indications about *DNMT3A* during clonal evolution in AML:

A recent study by Ding L et al. (2012) analysed the clonal evolution in relapsed acute myeloid leukaemia by whole-genome sequencing of primary tumour and relapsed genomes of eight AML patients. The comparison of relapse-specific versus primary tumour mutations evolved into the following models of clonal evolution during AML disease development: The first pattern was based on a founding clone in the primary tumour which obtained mutations and developed into the relapsed clone. The second pattern was that a subclone of the founding clone sustained initial therapy, acquired additional mutations and accumulated at relapse. *DNMT3A* mutations were found at high variant allele frequencies in two out of eight patients indicating that the mutation was part of all leukaemic cells in those samples and probably also present in the founding clone. Under the assumption that the mutations were heterozygous, Ding L and colleagues quantified the founding clone and subsequent minor clones as 93.72% (which is commensurate with the malignant cellular content of the sample), 53.12%, 29.04% and 5.10% at first diagnosis while the subclone comprising 5.10% developed into the major clone at relapse. As the development of AML is thought to be mainly the result of accumulation of multiple mutations in genes affecting differentiation, proliferation/cell death and epigenome regulators (Jan M & Majeti R, 2013), *DNMT3A* mutations would then randomly appear during the disease process and therefore derive as major or founding and minor clones. However, the present levels of the *DNMT3A* mutation in this project show that of the 22 patient samples 2 major clones (97.6% and 71.1%) but no minor clones were present. This clonal pattern suggests that *DNMT3A* R882 mutations are initiating events in AML and contribute to a founding clone. On these grounds, our data seems to confirm the work of Welch JS et al. (2012) who used whole-genome sequencing to analyse the genomes of M3-AML patients with *PML-RARA* translocation as a known initiating event versus those of normal karyotype M1-AML and the exomes of HSPC (haematological stem/progenitor cells) from healthy people. Their findings suggest that the majority of mutations observed in AML are haphazard occurrences in HSPCs that were accumulated in the background (increasingly more frequent with age) until only a few cooperating mutations suffice to create the founding clone. *DNMT3A* was shown to be significantly more often recurrently mutated in M1 AML cases and therefore deemed rather as an initiating than a cooperating event.

It was also reported that mutations in AML affecting epigenetic regulators like *DNMT3A* were mutually exclusive of the presence of transcription-factor fusions that are classically

characterised as class II mutations. This acts as indirect evidence supporting the hypothesis that *DNMT3A* is a driver mutation (Kihara R et al., 2014).

An analysis of clonal haematopoiesis in aplastic anaemia also corroborates this hypothesis, as *DNMT3A*-mutated clones seemed to increase in size over time and arise in AML (Yoshizato T et al., 2015).

## 5.5 Future research:

Most of the future work necessary to improve both *DNMT3A* detection tests is already mentioned in each section discussing the strengths and limitations of the assays (chapter 5.2.). In contrast to the evaluation of the advantages and disadvantages, the following paragraph will highlight two main principles that are required in respect of future research. Firstly, further studies would involve an increase in the analysed sample size so that more mutant samples can be distinguished and quantified. This is crucial and would considerably validate the tests because the chance of coincidental results is diminished and it is easier to deduce general rules from greater data sets.

Secondly, the examination of longitudinal samples and a related analysis of potential changes in *DNMT3A* mutation frequency would give an important example of how the quantification test can be applied as a minimal residual disease test.

Above that, despite the high prognostic impact of epigenetic mutations especially that of *DNMT3A*, single mutation detection tests require revision with regard to their clinical suitability. As AML is such a heterogeneous and complex disease critical review of single-reaction qPCR versus multiplexing of several gene loci in one experiment is essential.

Evidently, current next-generation sequencing approaches identifying *DNMT3A* mutations and other genetic lesions in AML are progressing and will become more broadly available and cost-efficient (Shen T et al., 2015).

At present, numerous different next-generation sequencing technologies are advancing: To name just a few examples, target-enrichment methods concentrate on specific tumorigenic regions thus reducing costs and material - post-analytic bioinformatics and DNA input respectively (Bolli N et al., 2015). Another technique represents microdroplet PCR deep

sequencing data allowing simultaneous amplification of several mutational objectives (Cheng DT et al., 2014).

Notably, Luthra R et al. (2014) already accomplished the sequencing of multiple genes (among those *DNMT3A*) within 72h turnaround time, diminished DNA input and with a high concordance between two NGS platforms as well as direct Sanger sequencing.

Nonetheless, further experiments addressing the clinical practicality and applicability of sequencing techniques of AML causing mutations will provide substantial and demanding tasks for the future.

## VI. Summary

Acute myeloid leukaemia is a molecularly heterogeneous clonal disorder that has traditionally been stratified in three risk groups to predict patients' outcome. Recent studies suggest that this traditional risk classification requires re-evaluation as numerous somatic genetic alterations in epigenetic regulators with effects on the clinical outcome of AML patients have been detected. Mutations in the gene de novo methyltransferase 3 A (*DNMT3A*) are one of the most frequent aberrant epigenetic changes in this malignancy comprising 15-25% of all AML cases and up to 36% in cytogenetically normal AML. As event-free and overall-free survival is significantly worse amongst patients harbouring the *DNMT3A* mutation compared to those with *DNMT3A* wild type AML, different detection methods have been developed. However, all tests published up to date have certain limitations regarding the level of sensitivity or practicability in a standard diagnostic laboratory. Therefore, we sought to develop firstly a rapid screening test in order to identify *DNMT3A* mutant patients prior to treatment and secondly to establish a highly sensitive quantification method to assay for *DNMT3A* mutant minimal residual disease to prevent relapse. Both assays focus on codon 882 within the *DNMT3A* gene because more than 60% of all *DNMT3A* mutations occur at this locus.

The screening test is based on analysis of an aberrant restriction enzyme digestion pattern of preliminarily PCR amplified fragments allowing the detection of the *DNMT3A* R882 mutation as well as the differentiation between the R882H, R882C and R882S mutation subtypes. The sensitivity of the restriction enzyme assay was confirmed by semi-quantitative PCR experiments of a dilution series of the *DNMT3A* R882C mutant cell line OCI/AML-3 in wild type cell line KG-1 leading to a test sensitivity of 5% mutant to wild type copy number.

The quantification method consists of three steps: First, genomic DNA from the OCI/AML-3 cell line, the KG-1 cell line or from patients' samples is isolated and the region encompassing the *DNMT3A* R882 codon is amplified by PCR. Then, this PCR product is digested by a restriction enzyme sensitive for the *DNMT3A* R882 mutation and the processed fragments are re-amplified by quantitative PCR producing three independent fragments within the *DNMT3A* gene. The quantification of the mutation is calculated as the relative ratio of the abundance of an upstream amplicon representing a loading control and a fragment encompassing the R882 codon. The third amplicon serves as a control for complete restriction enzyme digestion.

The sensitivity of the quantification assay was determined by calibration curve experiments with the lowest detectable level of 0.78% *DNMT3A* R882C mutant in wild type DNA leading to a test sensitivity of less than 1% of total cells or 0.5% of allele frequency. The reproducibility of the quantification method for each observation was verified by a separate experimental run confirming that there was no statistically relevant difference between the original and the repeated data set.

After reproducing reliable results on the cell lines, we analysed 42 AML samples and 6 other haematological samples (mostly MDS and MPN) using the differential restriction enzyme test and the quantification method. Out of the 42 AML samples, 2 *DNMT3A* R882H mutations and one R882C mutation were identified. The presence of the *DNMT3A* mutation was excluded in all other 39 AML samples and the 6 MDS/MPN samples. The two R882H mutations were quantified as 97.6 ± 1.9 % and 71.1 ± 3.3 % respectively. The mutational status of all patients' samples was validated by external Sanger sequencing.

Finally, the clinical outcome of the patients positive for the *DNMT3A* R882 mutation was compared to those with *DNMT3A* wild type but no statistically significant differences were assessed. However, we observed a moderate trend towards higher probability of male sex, an accumulation of a higher blast count and an increased affiliation to the intermediate risk group within the *DNMT3A* R882 mutant study group.

In consideration of the aims of this study, we successfully established a screening test for the presence of the *DNMT3A* R882 mutation with following advantages: The rapidity of the complete process with a duration of a few hours between a patient's bone marrow aspiration and determination of the mutation status as well as the benefit of the relatively low experimental cost and the use of basic laboratorial techniques. On top of that, we succeeded to reach a sensitivity of 5% mutant to wild type copy number. Although there were limitations regarding the differentiation between the subtypes of the *DNMT3A* R882 mutation, the test offers a facilitation of the risk classification and appropriate choice of treatment in situations with an urgent need of a decision based on prognostic risk factors.

In regard to the quantification method, we succeeded in setting up a highly sensitive detection and quantification test to be able to screen for minimal residual disease. While we focused on the mutational hotspot codon R882 other research groups developed tests using HRM curve analyses and dHPLC that examine up to 24 codons. Nevertheless, none of the afore

mentioned studies reached the sensitivity level of 1% mutant to wild type copy number at the R882 locus. Furthermore, our quantification technique is less time-consuming and more cost saving.

In conclusion, the differential restriction enzyme test provides the clinician with a practical approach to screen for the *DNMT3A* R882 mutation to evaluate the patient's prognosis while the quantification method could serve as a reliable parameter to verify post treatment remission as well as to investigate for minimal residual disease to prevent AML relapse.

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## VIII. List of illustrations

## IX. Zusammenfassung

Akute myeloische Leukämie (AML) ist eine heterogene Erkrankungsgruppe klonaler Neoplasien, die traditionell in drei Risikogruppen eingeteilt wird. Neuere Studienergebnisse verdeutlichen die Notwendigkeit einer Re-Evaluation dieser Einteilung, da insbesondere Mutationen in epigenetischen Regulatoren und deren klinischer Einfluss bisher nicht berücksichtigt werden. Eine der häufigsten epigenetischen Veränderungen bei akuter myeloischer Leukämie ist eine Mutation im Gen der De Novo Methyltransferase 3A (*DNMT3A*), die bei 15-25% sämtlicher Fälle von AML und bei bis zu 36% der AML Erkrankungen mit unauffälliger Zytogenetik vorkommt. Aufgrund der Verkürzung der rezidivfreien Lebensspanne sowie der Gesamtüberlebenszeit der Patienten, die diese Mutation aufweisen, wurden unterschiedliche Detektionsmethoden entwickelt. Dabei bestehen bislang jedoch deutliche Einschränkungen, beispielsweise durch niedrige Sensitivitätsraten oder eingeschränkte Praktikabilität in diagnostischen Laboren mit standardisierter Ausrüstung.

Darauf basierend gab es folgende Zielsetzung für diese Studie: Erstens die Entwicklung eines Screeningtests, der die Präsenz der *DNMT3A* Mutation vor Behandlungsbeginn überprüft, sowie zweitens eine hochsensitive Quantifikationsmethode, mithilfe derer minimale residuale *DNMT3A* Mutationen, beispielsweise zum Zeitpunkt der Remission, identifiziert werden können, um Rezidive frühzeitig zu erkennen. Bei beiden Ansätzen liegt der Fokus auf dem Codon 882, da mehr als 60% der *DNMT3A* Mutationen die Aminosäure Arginin in dieser Position alterieren.
Der Screening Test baut auf einer Analyse von zuvor durch PCR amplifizierten DNA Fragmenten und deren voneinander abweichenden Restriktionsenzymmustern auf, wodurch sowohl die Identifikation von *DNMT3A* R882 Mutationen als auch die Unterscheidung zwischen R882H, R882C und R882S Subtypen ermöglicht wird. Die Sensitivität des Tests wurde durch Verdünnungsreihen der *DNMT3A* positiven Zellreihe OCI/AML-3 und der für dieses Gen als Wildtyp vorliegenden Zelllinie KG-1 bestimmt und lag bei 5%.

Zur Quantifizierung der Mutation werden drei Schritte benötigt: Zuerst wird die genomische DNA einer Zelllinie oder einer Patientenprobe isoliert und die Zielregion, die das R882 Codon beinhaltet, mittels PCR amplifiziert. Dann wird das PCR Produkt durch Restriktionsenzyme geteilt. Die dabei entstehenden drei unabhängigen Amplikons werden

wiederum durch quantitative PCR vervielfältigt. Die Quantifizierung erfolgt durch den relativen Anteil des durch die Mutation alterierten R882 Amplikons in Bezug zu einer „Loading Control“, die im gleichen Gen liegt, aber durch das Restriktionsenzym nicht geschnitten wird. Das dritte Amplikon dient als Kontrolle für die Effizienz des Restriktionsenzyms („Digestion Control“) und enthält eine weitere Zielstruktur des Enzyms. Die Sensitivität der Quantifikationsmethode wurde durch Kalibrierungsexperimente bestimmt, welche die niedrigste detektierbare Rate von *DNMT3A* mutierten Allelen zu Wildtyp Allelen von 0,78% zeigte. Die Reliabilität der Quantifikationsmethode wurde durch separate Wiederholungen der Experimente und statistischen Vergleich der erhobenen Daten verifiziert. Nachdem die Experimente basierend auf den Zelllinien reliabel reproduziert werden konnten, wurden 42 AML und 6 weitere hämatologische Proben (vorrangig aus dem myelodysplastischen und myeloproliferativen Formenkreis) mit beiden Ansätzen untersucht. Unter den 42 AML Proben lagen 2 *DNMT3A* R882H Mutationen und eine R882C Mutation vor. Bei den übrigen 39 AML Fällen und den 6 weiteren Proben konnte das Vorkommen der *DNMT3A* Mutation, auch in geringer Anzahl, ausgeschlossen werden. Die zwei R882H positiven Befunde wurden als jeweils 97.6 ± 1.9 % und 71.1 ± 3.3 % quantifiziert. Der Mutationsstatus sämtlicher Proben wurde durch Sanger Sequencing validiert.

In der Folge wurden die klinischen Charakteristika der *DNMT3A* Träger analysiert. Dabei wurden unter den *DNMT3A* Trägern vermehrt männliche Probanden gefunden, eine höhere Anzahl von Blasten in der Knochenmarkbiopsie sowie ein vermehrtes Auftreten einer Eingruppierung in der „intermediate risk“ Gruppe beobachtet; dies allerdings ohne statistische Signifikanz.

Durch die erfolgreiche Entwicklung des Screeningtests konnten mehrere Vorteile erarbeitet werden: Die schnelle Erfassung der Prognose eines Patienten mit lediglich 4-5 Stunden Zeitbedarf zwischen Blutentnahme bzw. Knochenmarkbiopsie und der Diagnose des *DNMT3A* Genstatus, wodurch eine frühzeitige Risikostratifizierung eines Patienten und eine stärker individualisierte Wahl des Therapieregimes ermöglicht wird. Dazu kommt der relativ geringe Kostenaufwand sowie der Gebrauch von weit verbreiteten und standardisierten Labortechniken.

Mit der Quantifizierungsmethode wurde ein hoch sensitiver Ansatz zur Detektion von minimalen residualen Leukämiezellen etabliert, der eine reliable Möglichkeit zur Früherkennung der häufigsten Ursache für Rezidive bietet.

# Eidesstattliche Versicherung

Bisling, Kathrin

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema
Detection of DNMT3A R882 Mutations in Patients with Acute Myeloid Leukaemia

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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München, den 06.09.2016

Ort, Datum

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