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Digital Multiplex Ligation-Dependent Probe Amplification for Detection of Key Copy Number Alterations in T- and B-Cell Lymphoblastic Leukemia

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Recurrent and clonal genetic alterations are characteristic of different subtypes of T- and B-cell lymphoblastic leukemia (ALL), and several subtypes are strong independent predictors of clinical outcome. A next-generation sequencing—based multiplex ligation-dependent probe amplification variant (digitalMLPA) has been developed enabling simultaneous detection of copy number alterations (CNAs) of up to 1000 target sequences. This novel digitalMLPA assay was designed and optimized to detect CNAs of 56 key target genes and regions in ALL. A set of digital karyotyping probes has been included for the detection of gross ploidy changes, to determine the extent of CNAs, while also serving as reference probes for data normalization. Sixty-seven ALL patient samples (including B- and T-cell ALL), previously characterized for genetic aberrations by standard MLPA, array comparative genomic hybridization, and/or single-nucleotide polymorphism array, were analyzed single blinded using digitalMLPA. The digitalMLPA assay reliably identified whole chromosome losses and gains (including high hyperdiploidy), whole gene deletions or gains, intrachromosomal amplification of chromosome 21, fusion genes, and intragenic deletions, which were confirmed by other methods. Furthermore, subclonal alterations were reliably detected if present in at least 20% to 30% of neoplastic cells. The diagnostic sensitivity of the digitalMLPA assay was 98.9%, and the specificity was 97.8%. These results merit further consideration of digitalMLPA as a valuable alternative for genetic work-up of newly diagnosed ALL patients. *(J Mol Diagn 2017, 19: 659—672; http://dx.doi.org/10.1016/j.jmoldx.2017.05.004)*

T- and B-cell lymphoblastic leukemia (T- and B-ALL, respectively) is the most common childhood cancer and shows profound heterogeneity at the clinical and genetic level. Classification of ALL is based on immunophenotype, taking into account the lymphocyte lineage (B cell, T cell, or mixed lineage). However, within these groups, genetic subtypes exist, with great differences in clinical outcome

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and treatment response.[1–4] Genetic changes in ALL include copy number alterations (CNAs) of genes involved in lymphocyte lineage differentiation and cell cycle control, gene rearrangements, fusion genes, and whole chromosomal losses or gains. A combination of technologies, such as fluorescence *in situ* hybridization (FISH), immunophenotyping, array comparative genomic hybridization (aCGH), and single-nucleotide polymorphism (SNP) arrays has made it possible to detect these key genetic abnormalities and thereby distinguish the different subtypes of ALL. However, these techniques and associated data analysis are costly and labor intensive.

Multiplex ligation-dependent probe amplification (MLPA)[5] has become one of the standard methods for the detection of common CNAs, such as *IKZF1* deletions associated with poor outcome in B-ALL.[6] MLPA has been shown to perform with high concordance to FISH, chromogenic *in situ* hybridization, and quantitative PCR.[6,7] However, standard MLPA assays are restricted to a maximum of 60 MLPA probes and require a minimum of 50 ng of good quality DNA. As some of the genetic subtypes in ALL are complex, and may encompass multiple chromosomal locations, there is need for an improved approach to simultaneously perform copy number analysis of multiple regions with high sensitivity and specificity. To address this need, a next-generation sequencing–based MLPA variant (digitalMLPA) has been developed with the potential to include up to 1000 probes in a single reaction. In addition, minimal amounts of DNA are required ($\geq$20 ng) for robust performance of the assay. In the digitalMLPA assay described herein, 642 probes were included, targeting the following: i) all genes/regions included in five standard MLPA assays (MRC-Holland, Amsterdam, the Netherlands) used routinely on diagnostic ALL patient samples: P202 IKZF1 (IKAROS), P327 iAMP21-ERG, P329 CRLF2-CSF2RA-IL3RA, P335 ALL-IKZF1, and P383 T-ALL; ii) additional target genes of potential prognostic and/or therapeutic relevance in ALL, including *NOTCH1*, *CD200/BTLA*, *VPREB1*, *TBL1XR1*, *EBF1-PDGFRB*, *IGHM*, *NR3C1/2*, *CREBBP*, *CTCF*, *ADD3*, *EPHA1*, *FHIT*, *SPRED1*, *DMD*, and *TOX*; and iii) a set of 208 digital karyotyping probes for detection of ploidy changes (hyperdiploidy or hypodiploidy), to determine the extent of copy number changes, and to be used as reference probes for data normalization.

After extensive validation of the novel digitalMLPA probes on normal samples and positive cell lines harboring known CNAs, a validation study was performed on 67 ALL patient samples previously characterized using other methods (aCGH and/or SNP arrays and standard MLPA assays). Samples were analyzed single blinded (A.B. and S.S.). The sensitivity and specificity of the assay were calculated by comparing the digitalMLPA data to the data previously obtained by other methods. Finally, the detection limits for subclonal CNAs were determined.

Materials and Methods

Development of DigitalMLPA Probe Mix for ALL

The digitalMLPA D007 ALL probe mix was designed to contain probes for genes and chromosomal regions known or proposed to have significant diagnostic or prognostic roles in ALL, selected by extensive literature review and suggestions from experts in the field of ALL research. In the literature review, we focused on recurrent reports of CNAs in individual genes and possible clinical relevance in terms of prognostic and/or diagnostic use for ALL, when available. Both B-ALL– and T-ALL–associated CNAs were included in this digitalMLPA D007 ALL probe mix (X2-0816) up to a total of 306 target-specific probes for 56 target genes (at least three probes per gene when possible), 27 different genes on chromosome 21 (including *RUNX1*) to detect intrachromosomal amplification of chromosome 21 (iAMP21), and flanking probes for the pseudoautosomal region 1 (Figure 1[8] and Supplemental Table S1). The set of 208 digital karyotyping probes covers all chromosome arms with three to six probes, with at least one probe on each of the following locations: close to the centromere, middle of the chromosome arm, and close to the telomere (Supplemental Table S2). Most target probes were designed to locate in the coding sequence of the target genes. Inclusion of probes for specific exons within each target gene was based on breakpoint information and those exons most frequently affected by intragenic deletions. A set of 128 internal quality control probes was also included to determine reaction quality, to determine amount of input DNA, and for troubleshooting purposes. These include a set of 39 pairs of SNP probes for sample identification and detection of sample contamination.

Probe Design for the DigitalMLPA D007 ALL Assay

Probes were designed based on University of California, Santa Cruz, Human Genome build GRCh37/hg19. Probes consist of two parts, the right- and left-hybridizing sequences, which are ligated only when bound adjacent to each other on their target DNA. Ligation sites of most probes are located within exons, as existing sequence information of exons is more reliable than it is for intronic sequences. SNPs at and around the ligation site can affect the probe ligation and were, therefore, checked using the dbSNP146 database to avoid frequent and validated SNPs. SNPs can also reduce a probe signal by destabilizing probe-sample DNA binding. The length and melting temperature of each probe oligonucleotide were therefore chosen in such a way as to ensure stable binding to the target DNA even in the presence of known SNPs. As each submission of sequence data provides more information about possible SNPs in the human genome, there is always a possibility of an SNP being present at the ligation site, which might influence probe binding and ligation and might thereby cause a false-negative result. Multiple probes were included for each target gene/region to prevent