



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

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Accepted for publication
May 3, 2017.

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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

Supported in part by the Kinderkankerfonds vzw (T.L.), the Research Foundation Flanders (Odysseus; P.V.V.), and Bloodwise (formerly Leukemia and Lymphoma Research, UK; C.S. and C.J.H.).

Disclosures: A.B.-S., K.d.G., I.Z., J.S., and S.S. are employed by MRC-Holland, the company developing the commercially available multiplex ligation-dependent probe amplification products used in this study. J.S. is a 100% shareholder of MRC-Holland.

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 (≥ 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 intra-chromosomal amplification of chromosome 21 (*iAMP21*), and flanking probes for the pseudoautosomal region 1 (Figure 1⁸ 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

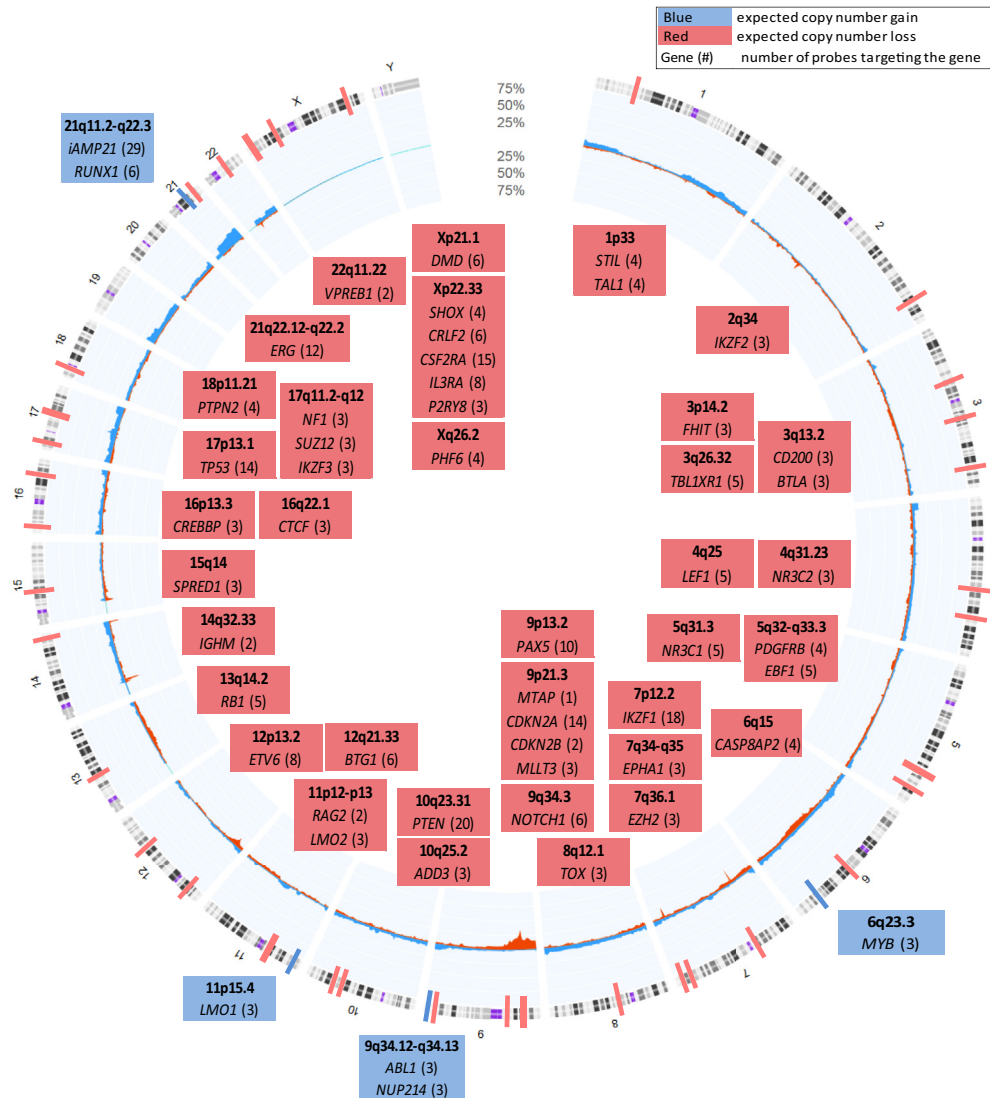


Figure 1 Target genes included in the D007 ALL digitalMLPA probe mix. Circos plot visualizing all target genes included in the D007-X2-0816 ALL digitalMLPA probe mix. The Circos plot was made using the Progenetix database (arraymap data)⁸ at <http://www.progenetix.org>, using subset “Leukemias: immature acute lymphoblastic leukemias,” which at the moment of generation of the figure consisted of 1067 samples from 40 publications. Numbers in parentheses refer to the number of probes included for the respective gene. In blue are the target genes with an expected copy number gain, in red the target genes with an expected copy number loss. Chromosome bands are indicated for each of the genes/regions. In the middle line of the Circos plot, the expected percentages of CNAs in immature ALL, according to the Progenetix database, are indicated (gains in blue and losses in red).

conclusions being based on single-probe alterations. Single-probe alterations should always be confirmed by a different method.

DigitalMLPA Experiments

DigitalMLPA was developed on the basis of the well-established MLPA method,⁵ but uses Illumina next-generation sequencing platforms for amplicon quantification (Figure 2). Briefly, 40 ng (in a total volume of 4 μ L) of each DNA sample was mixed with 2 μ L of a unique barcode solution (MRC-Holland), followed by DNA denaturation at 98°C for 10 minutes. After denaturation, a mixture of 1.25 μ L digitalMLPA probe mix (MRC-Holland) and 1.25 μ L digitalMLPA buffer (MRC-Holland)

was added to each sample, and reactions were incubated overnight at 60°C to ensure hybridization of the probes to the target DNA. Probes were ligated by incubating the reactions with 32 μ L of a ligase mastermix containing the ligase-65 enzyme (MRC-Holland) and buffers (MRC-Holland) at 48°C for 30 minutes, followed by heat inactivation of the ligase-65 enzyme at 98°C for 5 minutes and an additional incubation at 65°C for 20 minutes to reduce background. PCR amplification of the ligated probes was performed on a calibrated Biometra thermocycler (Biometra GmbH, Göttingen, Germany). The PCR-amplified products were then loaded onto an Illumina MiSeq sequencer (Illumina, San Diego, CA) for quantification using the MiSeq Reagent Kit version 3 (150 cycles; Illumina).

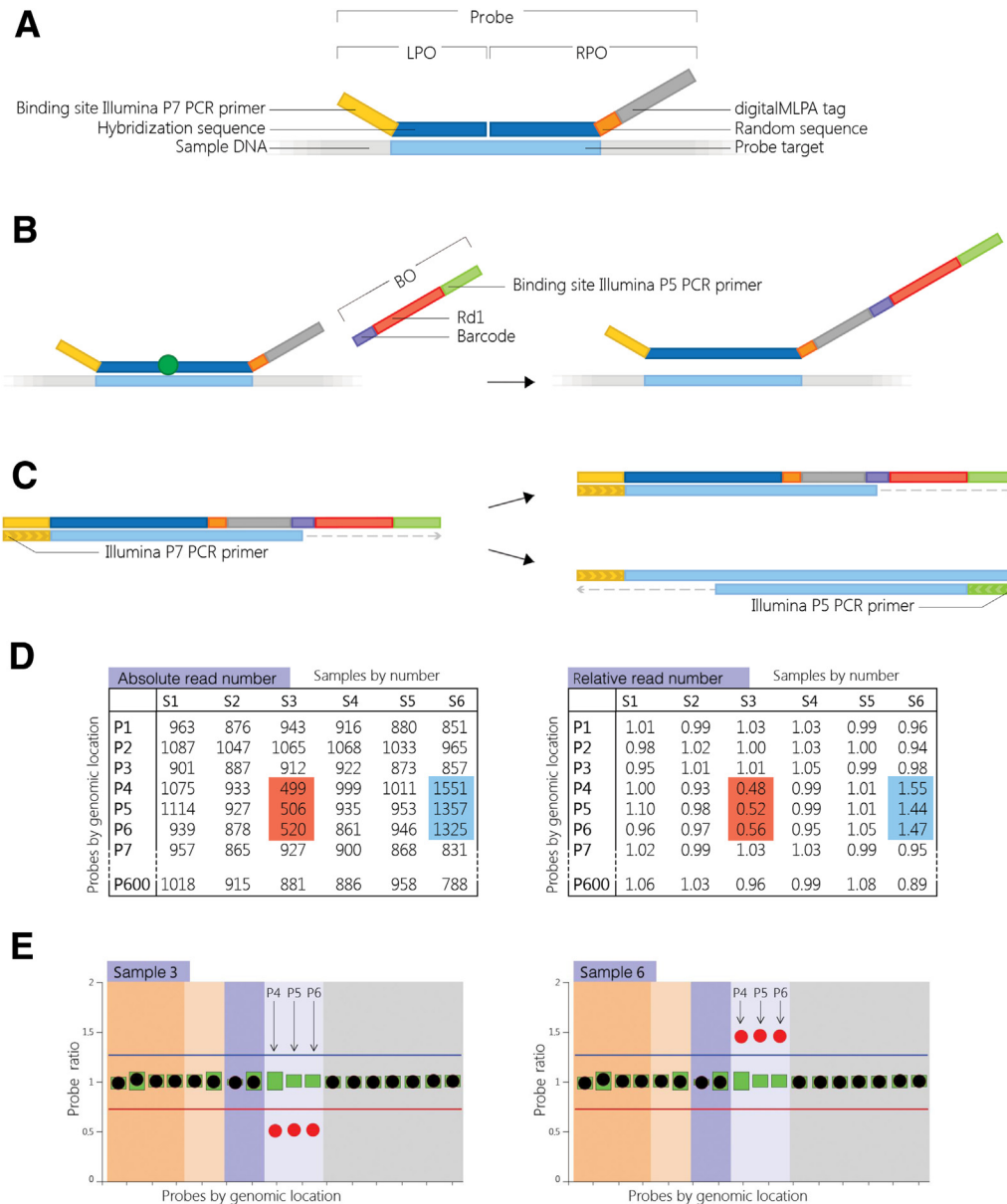


Figure 2 DigitalMLPA procedure. In digitalMLPA, sequencing is used solely to determine absolute read numbers of the various probe amplicons, not for sequence analysis of the sample DNA. As with conventional MLPA, reference DNA samples, preferably treated and extracted the same way as the DNA samples to be tested, are required to determine the relative copy number of each of the probes. **A:** After denaturation of the target DNA, the left (LPO) and right probe oligo (RPO) are hybridized to their target sequence overnight (at least 16 hours). **B:** The next day, the two oligonucleotides are enzymatically ligated and a barcode oligo (BO) is incorporated into the amplicon for sample identification. The Rd1 sequence, as part of the BO, is the Illumina tag required for quantification by Illumina sequencers (Illumina, San Diego, CA). **C and D:** After PCR (**C**), amplicons are quantified using Illumina sequencers (**D**). **E:** Absolute read counts are compared with reference samples to obtain probe ratios.

Quality Tests and Validation on Positive Cell Line Samples

All probes included in the digitalMLPA ALL probe mix were extensively quality tested for stability and variability on DNA from healthy individuals, according to internal guidelines and protocols of MRC-Holland. General probe performance was tested on Promega Human Genomic male DNA (Promega Benelux, Leiden, the Netherlands). Variability of the probes was tested on DNA of 48 healthy individuals; probes with an

SD of >0.08 were excluded from the probe mix and/or replaced with a newly designed probe. Sensitivity of probes to various experimental conditions, mimicking possible pipetting mistakes, deviating thermocycler temperatures, and giving variation in sample DNA quantity and quality, was also tested. Nonspecific probe oligo interactions were screened in reactions without DNA added (Tris-EDTA alone), and probes causing formation of nonspecific products likely to consume reads from the correctly ligated probes were removed or replaced. Lastly, the digitalMLPA ALL

probe mix was tested on commercial cell lines (Coriell Biorepositories, Camden, NJ; and European Collection of Authenticated Cell Cultures, Salisbury, UK) and research samples obtained from collaborating universities (Erasmus University Rotterdam, Rotterdam, the Netherlands; Leiden University, Leiden, the Netherlands; Utrecht University, Utrecht, the Netherlands; and University of Bonn, Bonn, Germany) harboring different well-characterized CNAs, including intragenic deletions, whole-gene deletions, and whole chromosome gains and losses.

Validation of the DigitalMLPA ALL Assay on ALL Patient Samples

DNA samples from patients with confirmed ALL were obtained from three different research laboratories (Ghent University, Ghent, Belgium; University of Helsinki, Helsinki, Finland; and Newcastle University, Newcastle upon Tyne, UK) and tested single blinded (A.B. and S.S.) at MRC-Holland. DNA was obtained from bone marrow or peripheral blood primary samples and extracted using the DNeasy Blood and Tissue Kit (Qiagen Benelux, Venlo, the Netherlands). The percentage of ALL blast cells in each sample was estimated to be at least 70%. digitalMLPA experiments were performed using 40 ng of sample DNA. Reference samples included in each experiment were triplicates of Promega male DNA. A total of 67 ALL patient samples were analyzed in this study: 9 T-ALL and 27 B-ALL samples from Ghent University, 1 T-ALL and 10 B-ALL samples from the University of Helsinki, and 20 B-ALL samples from Newcastle University. Concordance of digitalMLPA data with previously performed aCGH (Ghent University: Agilent180K custom-designed oligonucleotide array platform⁹; University of Helsinki: Agilent 244k CGH microarrays^{10,11}) and/or SNP arrays [University of Helsinki: Affymetrix Genome-Wide Human 6.0 SNP arrays^{10,11}; Newcastle University: SNP 6.0 (Affymetrix, Santa Clara, CA)], performed by AROS Applied Biotechnology (Aarhus, Denmark),^{12,13} was checked independently by each of the research institutes. Samples from Ghent University and the University of Helsinki were analyzed using the D007-X1-0316 ALL digitalMLPA probe mix. After analyzing the results of these patient samples, the product was updated to include several additional target genes having potential prognostic value in ALL (*FHIT*, *EPHA1*, *TOX*, *MTAP*, *ADD3*, *SPRED1*, *CREBBP*, *CTCF*, and *DMD*), and probes showing high variability (SD, >0.10) or formation of nonspecific amplification products were removed. Samples from Newcastle University were analyzed with the updated D007-X2-0816 ALL digitalMLPA probe mix.

Validation of the DigitalMLPA ALL Assay Using Standard MLPA Assays

To compare the results of this new digitalMLPA assay with those of the well-known and extensively tested standard

MLPA assays, patient samples from Ghent University were tested with MLPA assay P335-B2-0614 ALL-IKZF1, which includes probes for the pseudoautosomal region 1, *EBF1*, *IKZF1*, *CDKN2A/B*, *PAX5*, *ETV6*, *BTG1*, and *RBI*. Samples from the University of Helsinki were tested with P335-A4-0111, and samples from Newcastle University were tested with P335-A3 (N01, N02, N04-N06, N08, N09, and N12-N18), P335-A4 (N03 and N20), or P335-B1 (N07). Samples N10, N11, and N19 were not tested with the P335 standard MLPA assay. In addition, several samples with suspected *iAMP21* (N04-N08 and N10-N13) were tested with MLPA assay P327-B1 *iAMP21-ERG* containing probes for the region 21q11.2-21q22.3, including *RUNX1* and *ERG*. All probes included in these two standard MLPA probe mixes have equivalents in the digitalMLPA D007 ALL assay, but with different ligation sites, and could therefore be used for independent validation and verification of the findings observed with the digitalMLPA assay.

Dilution Series to Determine Detection Limits of Subclonal CNAs

A dilution series of three B-ALL patient samples (Ghent University) was analyzed to determine the detection limit for subclonal CNAs. The patient samples were diluted with corresponding remission material, to mimic the following percentages of subclonal populations: 50%, 30%, 25%, 20%, 15%, and 10%. Experiments using the digitalMLPA D007 ALL probe mix (X2-0816) were performed single blinded (A.B. and S.S.) at MRC-Holland using 40 ng of sample DNA. The read ratios of the undiluted patient samples (100%) were used as a reference to match the read counts in the diluted samples to a patient sample and a dilution percentage. Correct matching of the dilution percentages and patient samples was confirmed by Ghent University. The detection limit for subclonal CNAs was determined as follows: we used a value of 0.12, two times the average SD in DNA from healthy individuals across all probes included in the probe mix, to determine the limits of normal copy number. In other words, any read ratio outside of 0.88 to 1.12 was regarded as a potential deletion/gain, respectively. When two consecutive probes had ratios outside of this range, this was regarded as a true CNA call in this dilution series.

Dilution Series with Low Amounts of Input DNA for the DigitalMLPA Assay

To demonstrate that this digitalMLPA assay also produces robust results when the amount of input DNA is low, we performed experiments on serial dilutions of input DNA. The following amounts of input DNA per experiment were tested: 40 (standard), 30, 20, 15, 10, and 5 ng. Triplicates of 40 ng of Promega male DNA were used as a reference. The experiments were performed on three research samples from

the collection of MRC-Holland, of which one is a cell line (sample 1) and two are B-ALL patient samples.

Data Analysis of DigitalMLPA

For analysis of MiSeq data, FASTQ files were exported. To analyze the FASTQ files, software was designed at MRC-Holland that assigned each read in the FASTQ file to a complete digitalMLPA probe. Each read consisted of multiple parts: barcode, constant region, right-hybridizing sequence, and left-hybridizing sequence. Each part was analyzed using the Levenshtein distance algorithm¹⁴ that determines the number of insertions, deletions, and substitutions needed to change one sequence into another sequence. The first 10 nucleotides of the read contain the barcode sequence and were used to assign the read to a DNA sample. The second part of the read is a constant sequence of 26 nucleotides that was used to determine whether the read was a digitalMLPA product. All other, non-MLPA reads obtained from other experiments combined in the same (MiSeq) run, were ignored. After this constant region, the right-hybridizing sequence of the probe was used to find the corresponding probe. Using the Levenshtein distance algorithm, the last part of the read was then used to verify that the correct left-hybridizing sequence, and hence the correct combination of two ligated probe oligonucleotides, was present in the read. This allowed for detection/filtering of reads that are due to unanticipated interactions between any of the oligonucleotides present in each reaction. If the read could not be assigned to a specific probe included in the probe mix, the read was classified as unidentified. For further data analysis, read ratios of the probes are determined as in standard MLPA experiments, but using read counts instead of peak height. Read counts per probe were first compared with the median value of all reference probes in each sample. In a second step, the normalized values of each individual probe were compared with the median value of the corresponding probe in the reference samples; these were the final read ratios. A read ratio of 1.0 indicated normal copy number ($n = 2$) for autosomal gene regions, a read ratio of 0.5 indicated a heterozygous deletion, and a read ratio of 1.5 indicated a gain of one copy of the target sequence in a sample with 100% tumor cell percentage consisting of only one major clone. For X and Y chromosomal regions (except for genes located in the pseudoautosomal region 1 present on both X and Y chromosomes and, therefore, behaving as autosomal gene regions), a copy number of one was used as the normal copy number as the reference DNA used was male. When analyzing X and Y chromosomal regions, the sex of the patient was taken into account when interpreting the results. In females, the read ratio of X chromosome probes was divided by 2. A read ratio of 1.0 of an X-chromosomal region in a female would indicate a heterozygous deletion, whereas in males this indicates a normal copy number.

Results

Quality Testing on Healthy DNA Samples and Validation on Positive Cell Lines

In all digitalMLPA experiments, MiSeq runs generated approximately 600 single reads of at least 100 nucleotides in length for each probe in the reference DNA samples. Ten of a total of 642 probes were replaced on the basis of low read count, variation in normal healthy DNA samples, or formation of nonspecific amplification products. All probes included in this digitalMLPA probe mix showed minimal variability (SD, <0.08), had a relative read ratio of 0.80 to 1.20 as compared with the average read number across all autosomal probes, and did not form any harmful nonspecific amplification products. The mean SD across all target and reference probes on 48 healthy samples derived from blood was 0.06. Validation of the digitalMLPA ALL assay on positive cell lines harboring well-identified copy number changes showed accurate detection of all previously reported whole gene deletions (*TP53*, *RBI*, *CDKN2A*, *CDKN2B*, *NOTCH1*, and *PTEN*) or gains (*CRLF2*), gain of whole chromosomes (trisomies 13, 15, 18, 21, 22, and X), gain (3q) or deletion (18p, 3p telomere, 14q, and 18q) of chromosome arms, and intragenic deletions (*TP53* exons 10 to 11, *NFI* exon 26, and *PTEN* exons 1 to 2) in these cell lines (Supplemental Table S3).

Validation on ALL Patient Samples

Two samples were excluded from the analyses because of poor sample quality (H07 and G23; also failed on aCGH), as evidenced by high variability in MLPA read ratios (no clear baseline could be established). This could be because of the DNA extraction procedure or presence of impurities (eg, salt) in the extracted DNA. In three samples, no CNAs were detected by digitalMLPA (G18, H03, and H05), which was confirmed for all three samples by aCGH and standard MLPA (P335 ALL-IKZF1).

In the remaining 62 patient samples, a broad range of CNAs were detected, ranging from whole chromosome gains/losses, whole gene gains/deletions (both heterozygous and homozygous), intragenic deletions (both heterozygous and homozygous), and fusion genes to single-exon deletions (Supplemental Table S4). As expected, different CNA profiles were observed in the B- and T-ALL patient samples included in this pilot study. For example, *iAMP21* and copy number changes of *CD200/BTLA*, *IGHM*, *VPREB1*, and *CASP8A2* were only observed in B-ALL patient samples, whereas copy number changes of *STIL-TALI*, *LMO1*, *LMO2*, *NFI*, *SUZ12*, and *PTPN2* were exclusively observed in T-ALL patient samples. In addition, a larger number of target genes showed CNAs in both T- and B-ALL patient samples, such as *CDKN2A/B*, *LEF1*, *MYB*, *MLLT3*, and *PTEN*, genes in the pseudoautosomal region 1, *NR3C1*, and *NR3C2*.

Table 1 Concordance between DigitalMLPA and Other Methods

Method	Ghent University (Ghent, Belgium) (<i>n</i> = 35)		University of Helsinki (Helsinki, Finland) (<i>n</i> = 10)			Newcastle University (Newcastle upon Tyne, UK) (<i>n</i> = 20)		
	D007-X1-0316		D007-X1-0316			D007-X2-0816		
DigitalMLPA version	D007-X1-0316		D007-X1-0316			D007-X2-0816		
Other method	aCGH	P335	aCGH	SNPa	P335	SNPa	P335	P327
Total no. of CNAs	220	76	32	54	32	148	53	47
Counts confirmed	216	73	12	25	30	145	47	47
Detected by other method but missed by digitalMLPA (false negative)	1	3	1	12	0	0	1	0
Detected by digitalMLPA but missed by other method (false positive)	3	0	19	17	2	3	5	0

Samples of Ghent University (*n* = 35) and University of Helsinki (*n* = 10) were tested using D007-X1-0316; samples of Newcastle University (*n* = 20) were tested using D007-X2-0816. Results of the digitalMLPA assay were compared with aCGH, SNPa, and/or standard MLPA assays P335 ALL-IKZF1 and P327 iAMP21-ERG, when available. Total numbers of CNAs as observed by digitalMLPA, the number of counts confirmed by each individual method, and the counts missed by each method are included.

aCGH, array comparative genomic hybridization; CNA, copy number alteration; MLPA, multiplex ligation-dependent probe amplification; SNPa, single-nucleotide polymorphism array.

All detected CNAs were independently confirmed by other methods (aCGH and SNP arrays) and by data obtained with standard MLPA assay P335 and/or P327, when applicable. The concordance between digitalMLPA data and each of the other methods is shown in Table 1. Overall diagnostic sensitivity and specificity of the digitalMLPA assay (Table 2) were calculated based solely on data obtained from the samples from Ghent University (*n* = 35) and Newcastle University (15 of 20); as for the samples from the University of Helsinki (*n* = 10), many regions included in the digitalMLPA assay were not covered on the SNP array and array CGH platforms. For Newcastle University samples N15-N17 and N19-N20, no array CGH or SNP array analyses were performed; these samples (*n* = 5) were also omitted from the calculations. As we could not conclusively determine the number of false-positive or false-negative calls for the above mentioned samples (all 10 samples from the University of Helsinki and

five samples from Newcastle University), we excluded these particular samples from the sensitivity and specificity calculations. As can be concluded from Tables 1 and 2, the D007 digitalMLPA assay performs well in comparison with other methods and shows both high diagnostic sensitivity (98.9%) and specificity (97.8%). False-positive or false-negative findings detected in the samples used for the sensitivity and specificity calculations, observed using digitalMLPA but not using aCGH or SNP array (Supplemental Table S4) or vice versa, were never reported for the same probes/regions in multiple samples. False-positive CNAs detected by digitalMLPA, but not confirmed by aCGH or SNP array, included *VPREB1* deletion in sample G05, *RAG2* exon 1 deletion in sample N06, and *PTEN* exon 4 deletion in sample N07.

DigitalMLPA Identifies Known and Novel Genetic Aberrations in ALL

The digitalMLPA assay correctly detected the presence of intrachromosomal fusion genes (via copy number change), including *STIL-TAL1* (heterozygous deletion of *TAL1* exon 1 and *STIL* exons 2 to 12) (Figure 3A), *NUP214-ABL1* (Figure 3B), and *EBF1-PDGFRB* (Figure 3C). The presence of these fusion genes in the respective patient samples was confirmed by other methods. Of note, digitalMLPA will not detect inversions, balanced translocations, or copy number changes that lie outside (or only partially inside) the sequence detected by an MLPA probe.

Somatic loss of the entire Y chromosome was detected in one ALL sample, which was confirmed by cytogenetic analysis. One case harbored a heterozygous deletion of *CD200* and a homozygous deletion of *BTLA*. In addition to whole gene deletions, multiple intragenic deletions (both heterozygous and homozygous) were observed in different patient samples, including *PAX5* (exons 2 to 6), *IKZF1* (exons 1 to 3, 1 to 7, and 4 to 7), *RUNX1* (exons 2 to 4, 4 to

Table 2 Overall Diagnostic Sensitivity and Specificity of the D007 DigitalMLPA Assay

Variable	Value	
Total counts*	544	
Counts confirmed by other method	481	
Detected by other method but missed by digitalMLPA (false negative)	5	Sensitivity: 0.99
Detected by digitalMLPA but missed by other method (false positive)	11	Specificity: 0.98

The overall diagnostic sensitivity and specificity of the D007 digitalMLPA assay have been calculated using the samples from Ghent University (Ghent, Belgium) (*n* = 35) and Newcastle University (Newcastle upon Tyne, UK) (*n* = 15; samples N15 to N17 and N19 to N20 were excluded as no single-nucleotide polymorphism array or array comparative genomic hybridization data were available for these samples). All counts have been added up to calculate diagnostic sensitivity and specificity.

*Samples from Ghent University and Newcastle University.

MLPA, multiplex ligation-dependent probe amplification.

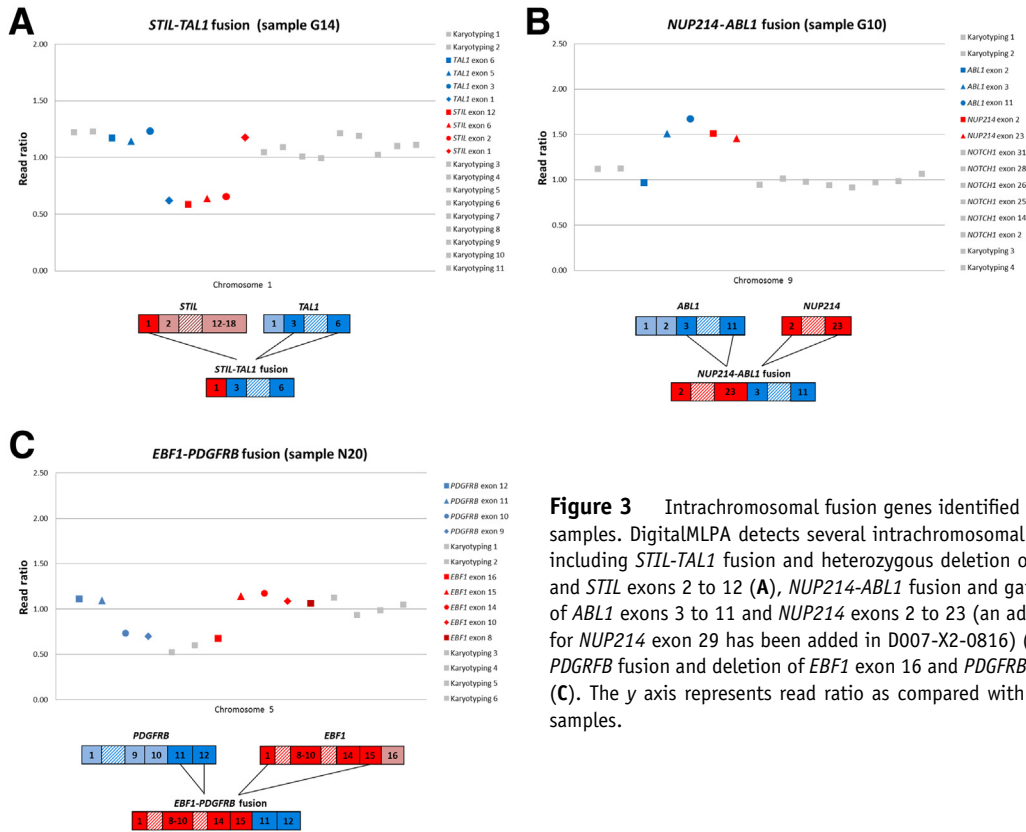


Figure 3 Intrachromosomal fusion genes identified in ALL patient samples. DigitalMLPA detects several intrachromosomal fusion genes, including *STIL-TAL1* fusion and heterozygous deletion of *TAL1* exon 1 and *STIL* exons 2 to 12 (A), *NUP214-ABL1* fusion and gain of one copy of *ABL1* exons 3 to 11 and *NUP214* exons 2 to 23 (an additional probe for *NUP214* exon 29 has been added in D007-X2-0816) (B), and *EBF1-PDGFRB* fusion and deletion of *EBF1* exon 16 and *PDGFRB* exons 9 to 10 (C). The y axis represents read ratio as compared with the reference samples.

6, and 8 to 9), *RBI* (exons 19 to 26), *CDKN2A* (exon 1 plus upstream probes, exons 2 to 4, and exons 2 to 5), *PHF6* (exons 1 to 3), *LEF1* (exons 3 to 4 and 4 to 12), *PTEN* (exons 1 to 5, 2 to 3, and 2 to 5), *MLL3* (exons 2 to 5), *ETV6* (exons 1 to 2, 2 to 5, and 6 to 8), and *ERG* (exons 5 to 12 and 5 to 9) (Figure 4A). Although most samples with *NR3C1* CNAs showed heterozygous deletions, one case harbored a gain of *NR3C1* exons 5 to 8 and a simultaneous heterozygous deletion of exons 1 and 2 (Figure 4B). Most deletions of *VPREB1* were heterozygous deletions of the whole gene, but in four cases, a heterozygous deletion was observed at the start of exon 2; the probe at the end of exon 2 showed a homozygous deletion. We also identified several cases with single-exon deletions, including heterozygous deletions of *BTG1* exon 2 (plus downstream probes), *MLL3* exon 1, 2, or 7, *RUNX1* exon 4, *NR3C2* exon 2, *FHIT* exon 4, *P2RY8* exon 2, *RAG2* exon 1, *PTEN* exon 4, *ETV6* exon 1 or 2, homozygous deletion of *IKZF1* exon 8 (Figure 4C), both heterozygous and homozygous deletion of *LEF1* exon 3 (Figure 4D) and *MLL3* exon 2. All intragenic deletions, including most reported single-exon deletions, were confirmed by aCGH and/or SNP arrays.

In addition to gene/exon deletions, gains were also detected. Among the patient samples tested, we identified one case displaying high hyperdiploidy (Figure 5A), which was correctly identified by digitalMLPA; by aCGH analysis, this case was misinterpreted as bearing multiple chromosomal losses. In two patient samples, a gain of *MYB* was

observed. Intragenic gains of *ETV6* exons 1 to 5 or 2 to 5 and *RUNX1* exons 4 to 9 (and downstream 21q probes) were also observed, as well as single-exon gains, such as *P2RY8* exon 1 and *PTEN* exon 5. iAMP21, currently defined as more than four copies of *RUNX1* on a single abnormal chromosome 21, as detected by FISH, was identified in 11 cases (Figure 5B), whereas gain of entire chromosome 21 was observed in six cases (Figure 5C).

Detection of Subclonal Copy Number Changes

Subclonal copy number changes were simulated using DNA from three B-ALL patient samples diluted to 50%, 30%, 25%, 20%, 15%, and 10% with corresponding remission material of the same patient. Using a cutoff value of 0.12 (as described in Materials and Methods), subclonal CNAs were reliably detected by the digitalMLPA assay if present in at least 20% to 30% of neoplastic cells (Table 3). One of the samples was obtained from a Down syndrome-associated ALL patient, explaining the gain of chromosome 21 with a read ratio of approximately 1.5 in all dilutions.

DigitalMLPA Using Minimal Amounts of Input DNA

Results from the experiments using low amounts of input DNA showed that digitalMLPA performs robustly on low amounts of DNA (some examples are included in

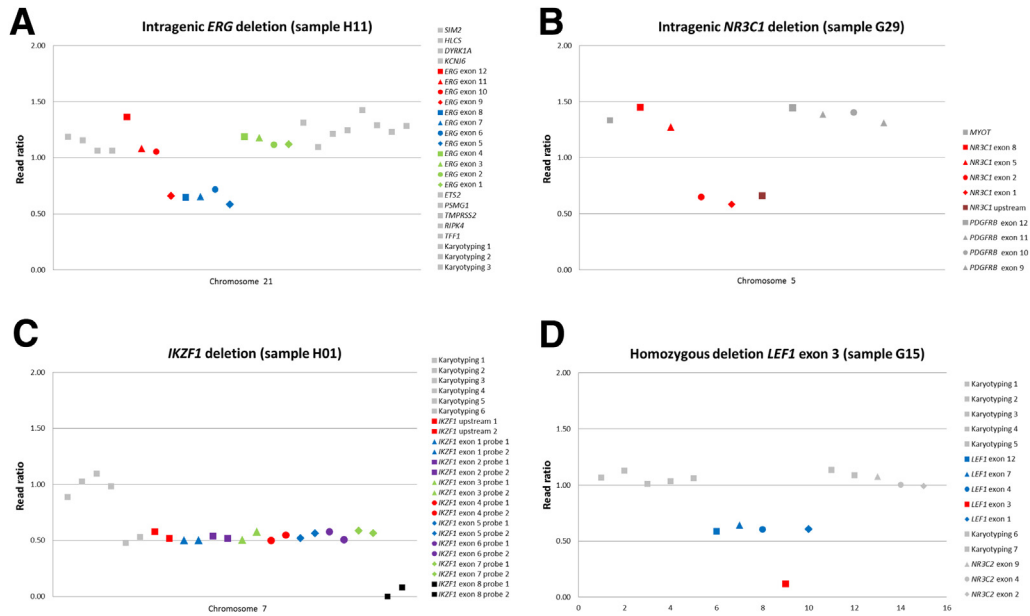


Figure 4 Intragenic and single-exon deletions identified in ALL patient samples. Several intragenic deletions are successfully identified in ALL patient samples, including deletion of *ERG* exons 5 to 9 (A), deletion of *NR3C1* exons 1 and 2 and upstream region on a background of gain of one allele of *NR3C1* exons 5 to 8 (B), homozygous deletion of *IKZF1* exon 8 (two probes are included for each exon of *IKZF1*) (C), and homozygous deletion of *LEF1* exon 3 (D). All copy number changes were confirmed by other methods. The y axis represents read ratio as compared with the reference samples.

Table 4). Read ratios for copy number gains and heterozygous and homozygous losses are stable, even when using only 5 ng of input DNA. Of note, when using <20 ng of input DNA, the number of unidentified reads (which

include incomplete probes and wrong combinations of right- and left-hybridizing sequences) increases markedly. Therefore, the use of ≥20 ng of input DNA for this digitalMLPA assay is recommended.

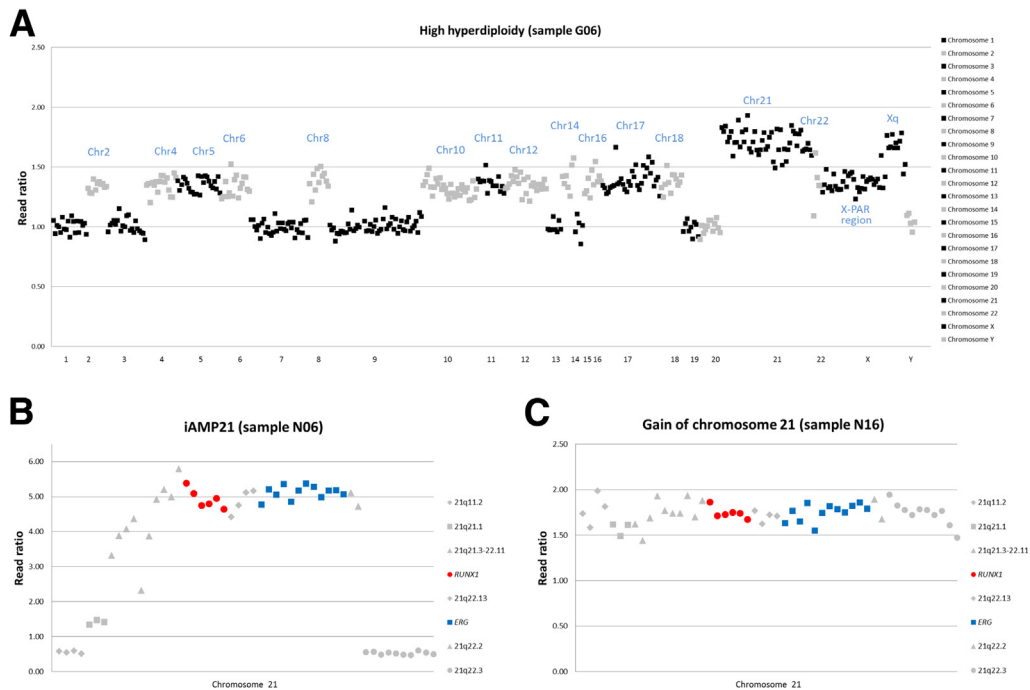


Figure 5 Gain of whole chromosomes or chromosomal regions in ALL patient samples. Gain of whole genes or chromosomal regions as identified by digitalMLPA. **A:** A case of high hyperdiploidy correctly identified by digitalMLPA showing the characteristic whole chromosome gains. **B:** Intrachromosomal amplification of chromosome 21 (iAMP21) showing the typical copy number profile along chromosome 21. **C:** Gain of entire chromosome 21. All copy number changes were confirmed by other methods. The y axis represents copy number as compared with the reference samples.

Table 3 Read Ratios of the Dilution Series

Sample	Gene/region	Aberration	Dilution (%)						
			100	50	30	25	20	15	10
G11	Chromosome 4	Gain	1.37	1.16	1.09	1.07	1.03	1.02	1.02
	Chromosome 6	Gain	1.36	1.12	1.09	1.03	1.00	1.03	1.02
	Chromosome 14	Gain	1.39	1.16	1.13	1.09	1.05	1.11	1.05
	Chromosome 17	Gain	1.36	1.16	1.13	1.08	1.08	1.07	1.06
	Chromosome 18	Gain	1.37	1.18	1.09	1.06	1.05	1.07	1.04
	Chromosome 21	Gain	1.80	1.36	1.21	1.17	1.14	1.10	1.08
	Chromosome X	Gain	1.86	1.38	1.24	1.16	1.14	1.16	1.08
	Pseudoautosomal region 1	Gain	1.36	1.17	1.12	1.06	1.05	1.06	1.06
G09	<i>CD200</i>	Deletion	0.55	0.75	0.86	0.89	0.89	0.88	0.89
	<i>BTLA</i>	Deletion	0.50	0.73	0.83	0.83	0.92	0.91	0.94
	<i>IKZF1</i> exons 1-7	Deletion	0.54	0.75	0.87	0.87	0.90	0.91	0.99
	8q Arm including <i>TOX</i>	Gain	1.45	1.22	1.13	1.12	1.08	1.05	1.05
	<i>MTAP</i>	Deletion	0.08	0.51	0.61	0.64	0.71	0.81	0.83
	<i>CDKN2A</i> exons 2-4	Deletion	0.09	0.50	0.63	0.67	0.72	0.77	0.85
	<i>CDKN2A</i> exon 1 + up	Deletion	0.51	0.70	0.80	0.77	0.91	0.84	0.93
	<i>CDKN2B</i> exon 2	Deletion	0.52	0.76	0.78	0.77	0.87	0.86	0.87
G01	20q13	Deletion	0.52	0.77	0.88	0.87	0.88	0.86	0.95
	<i>CD200</i>	Deletion	0.48	0.79	0.88	0.95	0.91	0.99	1.01
	<i>BTLA</i>	Deletion	0.00	0.63	0.75	0.82	0.83	0.92	0.98
	6q22.33-q27 including <i>MYB</i>	Gain	1.44	1.16	1.12	1.08	1.11	1.06	1.04
	9p22.2-p24.3	Deletion	0.51	0.74	0.85	0.86	0.88	0.92	0.94
	<i>MTAP</i>	Deletion	0.50	0.73	0.77	0.91	0.87	0.95	0.98
	<i>CDKN2A</i>	Deletion	0.02	0.59	0.77	0.78	0.83	0.87	0.87
	<i>CDKN2B</i> exon 2	Deletion	0.00	0.56	0.73	0.77	0.82	0.87	0.89
	<i>PAX5</i>	Deletion	0.50	0.82	0.88	0.92	0.95	0.98	1.01
	<i>RB1</i>	Deletion	0.49	0.78	0.94	0.93	0.95	0.97	0.97
	<i>IGHM</i>	Deletion	0.59	0.79	0.87	0.93	0.91	0.97	0.92
	Chromosome 21	Gain	1.47	1.48	1.51	1.5	1.49	1.52	1.49
<i>VPREB1</i>	Deletion	0.48	0.77	0.86	0.86	0.95	0.93	0.90	

Average read ratios across the gene or region of the dilution series of three lymphoblastic leukemia patient samples diluted with remission material of the same patient are listed. Dilutions tested were 50%, 30%, 25%, 20%, 15%, and 10% and matched to the correct patient sample using the undiluted patient sample (100%). Boldface are values <0.88 as these would be classified as a deletion, or >1.12 as these would be classified as a gain.

Discussion

The advent of high-resolution, microarray-based techniques, such as aCGH, has enabled the identification of multiple novel, often submicroscopic, DNA copy number alterations targeting key cellular pathways in ALL, several of which are clinically relevant and can be integrated into the risk classification of ALL patients.⁴ In diagnostic laboratories, conventional karyotyping is used to identify gross ploidy changes and FISH is used for detection of translocations. However, with these techniques, some submicroscopic CNAs may be missed. For example, the most common deletion of *IKZF1* (deletion 4 to 7, approximately 50 kb) is not detectable by bacterial artificial chromosome FISH because of the probe size limitation. Deletion of *CDKN2A* is underestimated by bacterial artificial chromosome FISH in 15% of ALL patients for the same reason.¹⁵ Next-generation sequencing methods are available to detect point mutations, fusion genes, and copy number changes, but these are labor intensive and costly. This digitalMLPA assay was

developed not aiming at replacing these techniques, but rather to accommodate an unmet need for accurate copy number detection of multiple genomic regions involved in ALL (eg, *IKZF1* and *ERG*) in a single experiment.

Our study demonstrates that digitalMLPA is a robust and reliable technique that can be used to genetically characterize ALL patients. The expected copy number changes were observed in all positive control cell lines, and most CNAs observed in patient samples were independently confirmed by other methods, including aCGH, SNP arrays, and standard MLPA assays.

Most CNAs observed in ALL patient samples were well-known, including intrachromosomal fusion genes, whole gene and intragenic deletions/gains, whole chromosome gains, and CNAs typically observed in either B- or T-ALL subtypes. Interestingly, we identified a sample harboring a heterozygous deletion of *CD200* and a homozygous deletion of *BTLA*, which has been previously described,⁹ although heterozygous deletion of both genes is more common. Various previously described intragenic deletions

Table 4 DigitalMLPA Using Low Amounts of Input DNA

Region	Research cell line sample 1						Research ALL sample 2						Research ALL sample 3					
	40 ng	30 ng	20 ng	15 ng	10 ng	5 ng	40 ng	30 ng	20 ng	15 ng	10 ng	5 ng	40 ng	30 ng	20 ng	15 ng	10 ng	5 ng
<i>RB1</i>																		
Exon 5	1.51	1.49	1.56	1.57	1.59	1.50	0.98	0.99	0.92	0.94	0.99	1.05	1.03	1.02	0.99	1.09	1.09	1.06
Exon 14	1.45	1.46	1.44	1.50	1.45	1.55	0.92	0.92	0.95	1.11	1.01	1.15	1.04	1.04	1.05	0.98	1.05	0.93
Exon 19	1.54	1.43	1.53	1.34	1.53	1.54	0.00	0.00	0.00	0.00	0.00	0.00	1.15	1.16	1.06	1.00	1.11	1.07
Exon 24	1.50	1.53	1.56	1.51	1.44	1.47	0.00	0.00	0.00	0.00	0.00	0.00	1.11	1.10	1.06	1.02	1.07	1.06
Exon 26	1.51	1.48	1.47	1.48	1.41	1.60	0.00	0.00	0.00	0.00	0.00	0.00	1.01	1.10	1.07	1.12	1.03	1.08
<i>CD200</i>																		
Exon 3	1.02	1.02	1.02	1.03	1.07	1.14	<u>0.53</u>	<u>0.52</u>	<u>0.53</u>	<u>0.52</u>	<u>0.48</u>	<u>0.51</u>	1.06	1.12	1.10	1.04	1.09	1.10
Exon 4	0.97	0.96	0.99	0.96	1.02	1.00	<u>0.49</u>	<u>0.46</u>	<u>0.45</u>	<u>0.52</u>	<u>0.45</u>	<u>0.50</u>	1.04	0.93	0.97	0.97	1.00	1.05
Exon 6	1.07	1.00	0.95	1.01	1.03	0.95	<u>0.50</u>	<u>0.46</u>	<u>0.49</u>	<u>0.47</u>	<u>0.47</u>	<u>0.57</u>	1.03	0.97	1.00	1.07	1.14	0.98
<i>BTLA</i>																		
Exon 5	1.02	0.96	1.00	0.99	0.94	0.98	<u>0.48</u>	<u>0.48</u>	<u>0.47</u>	<u>0.44</u>	<u>0.50</u>	<u>0.49</u>	1.03	0.97	1.01	0.95	1.01	0.94
Exon 2	0.98	0.93	1.09	1.00	1.07	0.87	<u>0.45</u>	<u>0.47</u>	<u>0.47</u>	<u>0.47</u>	<u>0.53</u>	<u>0.55</u>	1.00	1.00	1.04	0.89	0.92	0.93
Exon 1	1.00	0.99	1.11	1.17	1.03	0.94	<u>0.50</u>	<u>0.48</u>	<u>0.48</u>	<u>0.48</u>	<u>0.47</u>	<u>0.59</u>	0.97	1.06	0.97	0.97	1.08	0.95
<i>ETV6</i>																		
Exon 1	0.96	1.05	0.92	1.00	1.00	0.95	0.00	0.00	0.00	0.00	0.00	0.00	1.01	0.99	1.05	1.01	1.03	0.84
Exon 2	1.00	0.95	0.93	1.04	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.10	1.11	1.10	0.97	1.08	1.07
Exon 3	1.06	1.03	1.00	0.98	0.94	1.02	0.00	0.00	0.00	0.00	0.00	0.00	1.04	1.06	1.07	1.02	0.95	1.01
Exon 4	1.00	1.03	0.95	0.97	0.90	1.00	<u>0.50</u>	<u>0.51</u>	<u>0.51</u>	<u>0.50</u>	<u>0.50</u>	<u>0.45</u>	1.07	1.22	1.08	1.08	1.19	1.11
Exon 5	1.02	0.95	1.02	1.05	1.00	0.89	1.04	1.00	0.99	0.97	1.00	0.93	1.13	1.02	1.09	1.07	1.01	1.11
Exon 6	1.02	1.01	1.02	1.02	0.97	1.11	0.94	1.01	0.96	0.89	0.95	0.82	1.08	1.05	1.04	0.96	1.05	1.15
Exon 7	0.94	1.08	0.96	0.97	1.03	1.03	0.93	0.96	0.91	0.95	0.96	0.87	1.03	1.02	1.10	1.01	0.95	1.10
Exon 8	1.07	1.00	1.03	1.01	0.89	0.95	0.97	0.98	1.00	0.92	1.03	1.01	1.06	1.04	1.07	1.04	1.02	1.07
<i>IKZF1</i>																		
Exon 1, probe 1	0.93	0.90	0.85	0.87	0.95	0.90	0.92	0.92	0.90	0.94	0.87	0.86	1.06	1.07	1.05	0.91	1.06	1.06
Exon 1, probe 2	0.85	0.88	0.86	0.90	0.84	0.99	0.96	0.91	0.85	0.93	0.89	0.99	<u>0.64</u>	<u>0.65</u>	<u>0.62</u>	<u>0.66</u>	<u>0.68</u>	<u>0.68</u>
Exon 2, probe 1	0.89	0.84	0.92	0.89	0.98	0.88	0.88	0.95	0.88	0.87	0.98	0.93	<u>0.65</u>	<u>0.67</u>	<u>0.68</u>	<u>0.68</u>	<u>0.64</u>	<u>0.65</u>
Exon 2, probe 2	0.84	0.91	0.88	0.88	0.85	0.96	0.97	0.89	0.82	0.90	0.84	1.02	<u>0.67</u>	<u>0.66</u>	<u>0.68</u>	<u>0.62</u>	<u>0.76</u>	<u>0.68</u>
Exon 3, probe 1	0.93	0.97	0.91	0.92	0.94	1.01	0.95	0.92	0.89	0.89	0.93	0.82	<u>0.66</u>	<u>0.72</u>	<u>0.68</u>	<u>0.63</u>	<u>0.67</u>	<u>0.70</u>
Exon 3, probe 2	0.93	0.97	0.88	0.94	0.97	0.94	0.98	0.95	0.91	0.90	0.87	0.79	<u>0.71</u>	<u>0.75</u>	<u>0.77</u>	<u>0.66</u>	<u>0.63</u>	<u>0.77</u>
Exon 4, probe 1	0.90	0.93	0.90	0.88	0.99	0.94	<u>0.46</u>	<u>0.44</u>	<u>0.46</u>	<u>0.48</u>	<u>0.41</u>	<u>0.40</u>	0.29	0.31	0.29	0.34	0.34	0.30
Exon 4, probe 2	0.91	0.90	0.87	0.86	0.94	0.91	<u>0.41</u>	<u>0.45</u>	<u>0.48</u>	<u>0.44</u>	<u>0.41</u>	<u>0.43</u>	0.26	0.30	0.29	0.25	0.31	0.28
Exon 5, probe 1	0.91	0.89	0.99	0.94	0.97	0.91	<u>0.43</u>	<u>0.45</u>	<u>0.45</u>	<u>0.38</u>	<u>0.43</u>	<u>0.52</u>	0.30	0.31	0.33	0.29	0.31	0.25
Exon 5, probe 2	0.90	0.87	0.98	0.92	0.99	0.86	<u>0.46</u>	<u>0.44</u>	<u>0.47</u>	<u>0.50</u>	<u>0.43</u>	<u>0.45</u>	0.33	0.32	0.32	0.30	0.32	0.27
Exon 6, probe 1	0.91	0.91	0.93	0.83	0.96	0.86	<u>0.44</u>	<u>0.49</u>	<u>0.48</u>	<u>0.39</u>	<u>0.43</u>	<u>0.48</u>	0.32	0.32	0.31	0.24	0.26	0.21
Exon 6, probe 2	0.90	0.89	0.87	0.90	0.89	0.98	<u>0.45</u>	<u>0.47</u>	<u>0.45</u>	<u>0.44</u>	<u>0.45</u>	<u>0.41</u>	0.30	0.28	0.30	0.30	0.30	0.31
Exon 7, probe 1	0.89	0.94	0.97	0.89	0.91	0.87	<u>0.49</u>	<u>0.46</u>	<u>0.49</u>	<u>0.45</u>	<u>0.48</u>	<u>0.38</u>	0.29	0.31	0.32	0.23	0.27	0.26
Exon 7, probe 2	0.96	0.93	0.93	0.94	0.92	0.82	<u>0.45</u>	<u>0.45</u>	<u>0.48</u>	<u>0.45</u>	<u>0.46</u>	<u>0.42</u>	0.29	0.32	0.32	0.26	0.29	0.26
Exon 8, probe 1	0.88	0.94	0.87	1.00	0.89	0.86	0.86	1.00	0.88	0.87	0.88	0.91	1.02	0.98	1.02	1.02	0.94	1.13
Exon 8, probe 2	0.90	0.86	0.86	0.86	0.88	0.80	0.89	0.90	0.91	0.86	0.93	0.86	0.97	1.05	1.04	0.88	1.04	1.03
Unidentified reads	0.99	1.03	1.09	1.16	1.29	1.67	0.98	1.11	1.23	1.23	1.30	1.95	1.09	1.21	1.16	1.46	1.76	1.94

Displayed are read ratios from digitalMLPA experiments using varying amounts of input DNA. The dilution series tested included 40 (standard), 30, 20, 15, 10, and 5 ng of input DNA. Copy number gains are boldfaced, heterozygous losses are underlined italic, and homozygous losses are boldfaced italic. Unidentified reads include incomplete probes and wrong combinations of right- and left-hybridizing sequences.

ALL, lymphoblastic leukemia; MLPA, multiplex ligation-dependent probe amplification.

were detected using digitalMLPA, including *PAX5* exons 2 to 6,¹⁶ *IKZF1* exons 4 to 7,^{17–19} *RBI* exons 19 to 26,¹⁹ *ETV6* exons 6 to 8,^{20,21} and *BTG1*, harboring a breakpoint in exon 2.²² In addition to these well-known CNAs, several interesting intragenic deletions were observed, including an intragenic deletion of *ERG* exons 5 to 9. Previously described intragenic deletions of *ERG* encompass mostly deletions of exons 3 to 7, exons 3 to 8, or exons 3 to 9.^{23,24} We found no evidence in the literature describing intragenic deletions of *ERG* exons 5 to 9, as observed in one of

our patient samples. The clinical significance of this specific intragenic deletion is therefore unknown, but it would be expected to be equivalent to other intragenic deletions of *ERG*. Similarly, rare intragenic deletions of *PTEN* exons 1 to 5, exons 2 to 3, or exons 2 to 5 were observed. There are only a few previous reports of intragenic *PTEN* deletions (eg, exons 2 to 3 or exons 4 to 5).²⁵ There are no previous reports describing intragenic deletions of *RUNX1* exons 2 to 4 or *CDKN2A* exons 2 to 5, as were observed in our set of patient samples using digitalMLPA.

Several rare single-exon deletions were also detected, including a homozygous deletion of *IKZF1* exon 8. *IKZF1* exon 8 encodes two zinc fingers, which are required for homodimerization of *IKZF1* or heterodimerization with other transcription factors.²⁶ Deletions of *IKZF1* exon 8 are rare, but have been described in several studies and seem to be associated with a poor outcome.^{27,28} Another interesting case showed a homozygous deletion of *LEF1* exon 3, whereas the other exons of *LEF1* showed a heterozygous deletion. Deletions with distinct regions of heterozygous and homozygous deletions, suggesting that the two *LEF1* alleles were affected by independent genetic events, have been described previously.²⁹ Intragenic deletions of *VPREB1* have also been described in the literature, although most articles have not specified the exact breakpoints.³⁰ *VPREB1* deletions have been reported to be associated with decreased survival.³¹ Finally, we identified several novel single-exon deletions in *LEF1*, *FHIT*, and *ETV6*, gains of *ETV6* exons 1 to 5, or single-exon gains, such as *P2RY8* exon 1 or *PTEN* exon 5. It will be interesting to study the prevalence and clinical impact of these single-exon deletions in larger patient cohorts.

Another interesting result was one of high hyperdiploidy, which was misinterpreted as bearing multiple chromosomal losses on the basis of aCGH data. However, on the basis of the DNA index, it was concluded that this case should be regarded as high hyperdiploid in contrast to the hypodiploid state called by aCGH. As the absolute read numbers per sample are compared with a set of reference samples with normal copy number for all sequences included in this digitalMLPA assay, we were able to correctly identify a gain of 14 chromosomes in this high-hyperdiploid ALL patient sample. This case would likely have been identified correctly using conventional karyotyping or multilocus FISH. However, in cases with failed cytogenetics because of low numbers of dividing cells and concurrent problems with banding techniques, digital karyotyping with multiple probes covering all chromosome arms, as in this digital-MLPA assay, offers a reliable solution.

Both diagnostic sensitivity (98.9%) and specificity (97.8%) were high, as determined by comparing the digitalMLPA data to previously obtained aCGH data (Ghent University and Newcastle University). Aberrations not confirmed by aCGH and/or SNP array were mostly single-exon deletions (samples G02, N06, and N07) or subclonal deletions with relative read ratios >0.76 (samples G07 and N05). Several other subclonal alterations, however, were confirmed by aCGH and/or SNP array (samples G10 and N11). The false-positive results from digitalMLPA (*VPREB1*, *RAG2* exon 1, and *PTEN* exon 4), which were not confirmed by other methods, are unlikely to influence risk stratification. Single-exon deletions detected by digitalMLPA should always be confirmed by other methods.

To determine the detection limit for subclonal CNAs, we performed the ALL digitalMLPA assay on three B-ALL patient samples diluted with remission material from the

same patient. Results from this dilution series indicated that subclonal alterations could be reliably detected with this digitalMLPA assay if present in at least 20% to 30% of neoplastic cells, which is comparable to the detection limits observed with standard MLPA.^{7,32,33} Detection limits for subclonal alterations are dependent on the type of CNA present in the DNA sample. Homozygous deletions and high-level amplifications were easier to detect, even in highly diluted samples (down to 10% of neoplastic cells), whereas heterozygous deletions and one copy gains could be detected down to 25% to 30% of neoplastic cells. Inclusion of a higher number of digitalMLPA probes per target gene/region could possibly further lower the detection limit as statistically, subtle changes in read ratios of multiple adjacent probes could be more easily detected to make the call for a subclonal deletion. As the percentage of ALL blasts used for DNA analysis is usually $>70\%$, any CNA in the major clone will be accurately detected using digitalMLPA.

Advantages of digitalMLPA, as compared with other genetic methods, include a high dynamic range for copy number detection, a robust assay concerning impurities in the DNA sample as compared with standard MLPA, and the requirement of only small amounts (≥ 20 ng) of DNA. Usually, the amount of material available for DNA extraction in ALL patients is not an issue at diagnosis. However, use of small amounts of DNA allows laboratories to spare sufficient material for yet to be defined research experiments while maintaining appropriate clinical care. Alternatively, only low amounts of DNA may be available from valuable ALL patient-derived archived material (eg, from rare specimens within a research setting). With digitalMLPA, a large number of genomic loci of interest can be analyzed for copy number alterations in a single reaction with low hands-on time, and results are available within 36 hours. Because of the targeted approach, data analysis and result interpretation will be much easier as compared with array CGH or genome-wide sequencing platforms. DigitalMLPA can also be used for analysis of complex regions, such as the *PTEN* gene. In addition, as specific barcodes are used in the MLPA reactions, the PCR-amplified samples can be combined with any other sequencing reaction in one Illumina run. During data analysis, the MLPA reactions can also easily be separated from other sequencing data.

In conclusion, we have shown that digitalMLPA is a reliable technique to detect key CNAs in ALL. The digitalMLPA ALL probe mix is to be tested in independent laboratories and on a larger scale to assess its clinical and diagnostic utility. The results presented merit further consideration of digitalMLPA as a valuable alternative for genetic testing of newly diagnosed ALL patients.

Acknowledgments

We thank Saskia Quax for providing the images for [Figure 2](#); and Magali Meul for excellent technical assistance.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.jmoldx.2017.05.004>.

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