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(54) **Title:** METHOD FOR EPIGENETIC IMMUNE CELL DETECTION AND COUNTING IN HUMAN BLOOD SAMPLES FOR IMMUNODIAGNOSTICS AND NEWBORN SCREENING

(57) **Abstract:** The present invention relates to improved methods for epigenetic blood and immune cell detection and counting, and respective uses and kits.

Method for epigenetic immune cell detection and counting in human blood samples for immunodiagnostics and newborn screening

The present invention relates to methods for epigenetic blood and immune cell detection and counting in human blood samples, in particular for immunodiagnostics and newborn screening, and respective uses and kits.

Background of the invention

Quantitative abnormalities of lymphoid and myeloid immune cell subsets are indicative for several human diseases and therefore constitute important parameters for diagnosis and patient monitoring. Currently, immune cell quantification is mostly performed by flow cytometry (FCM), which provides flexibility with respect to the analyzed cell types and accuracy (1). However, although hematology analyzers used in diagnostic laboratories are highly developed and sample logistics are extensively adapted, FCM suffers from intrinsic limitations. FCM-based cell counting requires fresh, anti-coagulated or well-preserved blood samples with intact leukocytes. Even with fresh samples, it is advisable to work quickly since time-to-analysis can influence the results with cell deterioration beginning in the initial hours after blood draw. Time-to-analysis influences results due to cell deterioration within few hours after blood collection. Standardization remains a challenge due to biological, technical and operational variations (2-5) and standardized protocols remain to be established, especially for samples with low numbers of certain cell populations, e.g. in immunodeficiencies (6, 7). A critical challenge is that FCM-based cell counting requires intact leukocytes, but fresh or well-preserved blood is not available for all medical applications.

The most critical challenge, however, is that not all medical applications warrant availability of fresh or well-conserved blood samples and flow cytometry cannot be applied in these cases.

Therapeutic decisions for HIV-infected patients depend on $CD4^+$ T cell counting. At frequencies below 500 $CD4^+$ T cells/µl blood, antiretroviral therapy is recommended and becomes imperative below 200 cells/µl. In resource-poor regions, appropriate cell counting is

hampered when blood collection and measurement cannot be performed in close succession. Therefore, treatment is initiated solely based on HIV-related clinical symptoms, which can result in suboptimal outcomes (8, 9). Furthermore, FCM is not applicable in newborn screening for severe, but treatable inborn defects, routinely performed on dried blood spots (DBS). Primary immunodeficiencies (PID) constitute such inborn disease group and are considered or are already part of screening programs (10). Typically, genetic defects lead to quantitative deficiencies of specific leukocyte subpopulations. Severe combined immunodeficiencies (SCID) represent such PID and are clinically characterized by the absence of T or B cells. Detection of SCID in newborns is currently based on quantitative PCR-assisted T cell receptor (TREC) and immunoglobulin kappa-deleting recombination excision circles (KREC) analyses (11). These methods reliably detect the lack of recent thymic T cell and bone marrow B cell emigrants, the predominant T and B cell subtypes present in neonatal blood. However, TREC/KREC analysis fails to detect other specific lymphocyte subsets defective in severe PID, such as Natural Killer (NK) cells or neutrophils. Despite this limitation, TREC newborn screening is effective and shows improved disease outcome due to earlier diagnosis (12). TREC analysis in newborn analysis is exclusively used for initial screening. Differential diagnosis and patient monitoring prior to and upon the curative hematopoietic stem cell transplantation requires change of technology and is performed by flow cytometry.

To overcome current technological and diagnostic limitations and to broaden applicability of immune monitoring, the inventors established DNA (un-) methylation-based, quantitative assessment of immune cells (epigenetic qPCR). This technique provides relative and absolute immune cell counts applicable to fresh, frozen or paper-spotted, dried blood. Signals are digital, i.e., indicating either one positive or negative value per cell rather than arbitrarily defined thresholds for “positiveness” as in FCM. It can be performed in an automated, operator-independent manner and reduces susceptibility to reagent variability, such as antibodies.

In a first aspect of the present invention, the above object is solved by a method for an improved methylation assay for identifying blood immune cells, comprising the steps of

a) providing a sample of human blood, in particular from a newborn, comprising genomic DNA of blood immune cells;

b) treating said genomic DNA of said immune cells with bisulfite to convert unmethylated cytosines into uracil;
c) amplifying of said treated genomic DNA using suitable primer pairs to produce amplicons, and
d) identifying said blood immune cells based on analyzing said amplicons as amplified,
wherein said amplification and analysis comprises amplification and/or qPCR using primers and probes selected from at least one of the sets of SEQ ID NOs. 1 to 12 for CD4, SEQ ID NOs. 13 to 20 for CD8beta, SEQ ID NOs. 21 to 28 for LRP5, SEQ ID NOs. 29 to 36 for MVD, SEQ ID NOs. 37 to 44 for LCN2, and SEQ ID NOs. 45 to 56 for CD3gamma/delta, and
wherein a demethylation of at least one CpG position in said amplicon is indicative for at least one blood immune cell selected from $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, $CD14^+$ monocytes, $CD56^+$ NK cells, and $CD19^+$ B cells.

Preferred is a method according to the present invention, further comprising an analysis of an amplicon for CD3 epsilon, as disclosed, for example, in US 2012-0107810.

Preferred is the method according to the present invention, further comprising an additional FCM of said blood immune cells to be identified.

Epigenetic immune cell counting provides a robust platform, capable of diagnosing immune defects, and optionally and conveniently complementing flow cytometry and T cell receptor excision circles analysis, nevertheless, without their respective limitations.

The present invention furthermore relates to the accurate quantification of methylation data as obtained using the above assay. This involves several components and considerations:
1. An internal standard, e.g. in silico converted plasmids.
2. A (e.g.) GAPDH normalizer in contrast to the methylated variant of a specific gene.
3. Thus, a comparison of all demethylated copies by the obligatory demethylated GAPDH with the specific (but present in the same number of copies) demethylated gene according to the quantification with 1.
4. Nevertheless, the above does not allow a truly "absolute" quantification, since the in silico converted standard does not correspond to the biological sample (which is converted only in the reaction vial.

5. Solving the problem at 4. based on adding and measuring a so-called GNoMs (Genomic Normaliser of Methylation), here, all original sequences are equimolarly included into a plasmid and then submitted to the overall process (bisulfite treatment and purification). Since they are present 1:1 a standard can be identified after the quantification using the standards in 1 showing the difference between in silico and in situ methylation. Using this factor, the methylation value of the measurements can be corrected, which improves the result considerably.
6. Using a defined amount of a nucleic acid (plasmid) with a standard gene having inverted CG bases, furthermore, any loss of material during the process can be accounted for, which further improves the method.
7. Reliable and specific assay components designed for clinical practice and needs.

Cell-type specific DNA methylation markers (13-15) amplified in qPCR potentially allow for immune cell detection and quantification even in samples of limited quantity and quality. The rationale for the identification of cell-type specific epigenetic markers has been described before (14, 16-18). Alternative methods for DNA methylation-based immune cell quantification include the analysis of individual CpG-sites on a genome-wide scale relying on microarray analysis (19). Such method allows estimation of leukocyte subpopulations based on calculated beta values (methylation intensities). The inventors assumed that locus-specific individualized epigenetic qPCR is highly specific and sensitive and thus well-suited for diagnostic approaches.

For epigenetic qPCR, genomic DNA is treated with bisulfite. Unmethylated CpG dinucleotides are converted to TpGs, whereas methylated CpGs remain unaltered. Thus, bisulfite conversion translates epigenetic marks into sequence information, allowing discrimination and quantification of both variants. Epigenetic qPCR is non-susceptible to loss of cell integrity since DNA is a stable substrate. It can be performed on fresh-frozen blood, DBS or other specimens without particular demands on preservation state. In addition, PCR components are synthetically produced and standardization is easy to achieve. Nevertheless, immune cell counting via epigenetic qPCR has not yet been demonstrated, due to absence of well-defined specific biomarkers and a lack of definitive and absolute quantification (20).

The inventors studied immune cell-type specific epigenetic qPCR for quantification of leukocyte populations in human blood. For total $CD3^+$, $CD4^+$ and $CD8^+$ cytotoxic T cells (21,

22), regulatory elements in the genes coding for the cell type determining proteins were analyzed regarding their methylation status. Epigenetic markers for neutrophils, B and NK cells were identified from genome-wide discovery and profiling of resulting candidate genes. Determination of absolute cell numbers (i.e., cells/µl blood) constitutes the gold standard, e.g. for counting of $CD4^+$ T cells in HIV patients. The inventors tested definitive and absolute counting of immune cells based on their cell-type specific epigenetic signals in healthy donors as well as a cohort of HIV patients and analyzed their equivalence to FCM. For DBS, where the blood volume is difficult to define, copies of unmethylated immune cell-type specific marker genes were related to copies of a universal denominator (GAPDH). Moreover, the diagnostic potential of epigenetic qPCR was demonstrated by identifying PID cases in a cohort of clinically inconspicious newborns using DBS.

In a preferred embodiment of the method(s) according to the present invention, the method is integrated, and further comprises an analysis and a first normalization using a demethylation standard gene selected from a gene expressed in all cells to be identified, such as, for example, a housekeeping gene, such as, for example, GAPDH and beta-actin, preferably using primers and probes selected from SEQ ID NOs. 57 to 61 for the gene for said GAPDH.

In yet further preferred embodiment of the method(s) according to the present invention, the method is integrated, and further comprises a second normalization using an *in silico* bisulfite-converted recombinant nucleic acid comprising a sequence inversing all CpG dinucleotides to GpC of said at least one demethylation standard gene (GAP[GC] construct), preferably using primers and probes selected from SEQ ID NOs. 62 to 64 for said GAP[GC] construct.

In yet further preferred embodiment of the method(s) according to the present invention, the method is integrated, and further comprises a third normalization using a calibrator plasmid comprising one copy of each amplicon sequence in its unconverted genomic (i.e., unmethylated) state.

In yet further preferred embodiment of the method(s) according to the present invention, the method further comprises a quantification of said blood immune cells as identified.

In a preferred embodiment thereof, a method according to the present invention comprises

a) providing a defined volume of a sample of human blood, in particular from a newborn, comprising (e.g. diploid) genomic DNA of blood cells to be quantitated;
b) providing an in silico bisulfite-converted recombinant nucleic acid comprising a demethylation standard gene, a sequence inversing all CpG dinucleotides to GpC of said demethylation standard gene, and a blood cell specific gene;
c) providing a recombinant nucleic acid comprising the demethylated genomic sequence of said demethylation standard gene of b), a sequence inversing all CpG dinucleotides to GpC of said demethylation standard gene, and said blood cell specific gene of b);
d) providing a recombinant nucleic acid comprising the sequence inversing all CpG dinucleotides to GpC of said at least one demethylation standard gene of b);
e) adding a defined amount of said recombinant nucleic acid of d) to said sample of a) ("spiking");
f) treating said (e.g. diploid) genomic DNA of the cells to be quantitated of a) and said recombinant nucleic acids of c) and d) with bisulfite to convert unmethylated cytosines into uracil;
g) amplifying of said nucleic acid molecules of a), b), c), and f) using suitable primer pairs to produce amplicons; and
h) identifying the blood immune cells based on analyzing said amplicons,
wherein said amplification and analysis comprises amplification and/or qPCR using primers and probes selected from at least one of the sets of SEQ ID NOs. 1 to 12 for CD4, SEQ ID NOs. 13 to 20 for CD8beta, SEQ ID NOs. 21 to 28 for LRP5, SEQ ID NOs. 29 to 36 for MVD, SEQ ID NOs. 37 to 44 for LCN2, and SEQ ID NOs. 45 to 56 for CD3gamma/delta, and
wherein a demethylation of at least one CpG position in said amplicon is indicative for at least one blood immune cell selected from $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, $CD14^+$ monocytes, $CD56^+$ NK cells, and $CD19^+$ B cells.

Optionally, a quantification step for said blood immune cells is included, as described herein.

Preferred is the method according to the present invention, further comprising an analysis of an amplicon for CD3 epsilon as above.

Preferred is the method according to the present invention, further comprising an additional FCM of said blood immune cells to be identified.

Preferably, said demethylation standard gene is selected from a gene expressed in all cells to be detected, such as, for example, a housekeeping gene, such as, for example, GAPDH and beta-actin.

In one aspect of the method according to the present invention, more than one blood cell specific gene is analyzed, e.g. a panel of 1, 2, 3, 4, 5 or 6 blood cell specific genes is generated as needed or desired, optionally together with more than one demethylation standard gene as described herein

Preferably, the nucleic acids are plasmids, e.g. linearized plasmids, such as bacterial plasmids, e.g. pUC, a yeast artificial chromosome (YAC), human artificial chromosome (HAC), PI-derived artificial chromosome (PAC), a bacterial artificial chromosome (BAC), and/or a PCR-product

In an aspect of the method, the amplification is normalized using a first *in silico* bisulfite converted nucleic acid (plasmid), comprising a demethylation standard gene (e.g. GAPDH), an "artificial sequence“ (the sequence inversing all CpG dinucleotides to GpC), as well as a blood cell specific gene (a “specific gene”, e.g. CD4). All three elements are equally present (equimolar) on said nucleic acid, and are *in silico* bisulfite converted. Therefore, the normalization curve and the corresponding calibration curves can be directly compared with the sample, and the relative cell count can be determined from the ratio of blood cell specific gene to demethylation standard gene. Nevertheless, the nucleic acid does not correspond to the “real” sequence, since each C is replaced by a T. A serial dilution and determination of each concentration with all genes as mentioned generated the calibration curve for the assay.

In order to improve the accuracy of the approach, a second nucleic acid (plasmid) is used comprising the demethylation standard gene (e.g. GAPDH), the "artificial sequence“ (the sequence inversing all CpG dinucleotides to GpC), and the blood cell specific gene (a “specific gene”). Nevertheless, these sequences are NOT *in silico* bisulfite converted, and correspond to the genomic sequences (in as far as the have a genomic counterpart, see below) – and thus can only be used for measuring the amplification (e.g. qPCR) efficiency.

The reason for the second standard is two-fold. A) For a definitive quantification a standard is required that is identical as in the biological sample to be analyzed (this is also a regulatory requirement). In the first nucleic acid, nevertheless, a double stranded AT-rich sequence is compared with a single-stranded U-rich sequence. Only the "true" bisulfite conversion of the double stranded nucleic acid allows for this definitive comparison. Then, the quotient of bisulfite conversion of blood cell specific gene to demethylation standard gene, normalized using the first nucleic acid, gives a factor of the efficiency. The same holds true for a quotient based on the division of the bisulfite conversion of the sequence inversing all CpG dinucleotides to GpC by the bisulfite conversion of the demethylation standard gene.

Preferably, the "artificial sequence" (the sequence inversing all CpG dinucleotides to GpC) is a random sequence comprising C and CpG sequences (for bisulfite conversion) that does not occur in the human genome. In one embodiment, the artificial sequence is the exact sequence of the part of GAPDH that is amplified (amplicon) wherein the CpG sequences are inverted into GpC sequences. The "artificial sequence" is found on all three nucleic acids as described above, namely on the first one (*in silico* bisulfite converted), the second one (for bisulfite conversion), and - as the only analyzed sequence - on the third nucleic acid (*in silico* bisulfite converted).

The third nucleic acid is given in a defined amount into a defined amount of blood, in particular from a newborn, and is then analyzed (e.g. purification, bisulfite treatment, second purification, desulfonation, specific amplification). Then, a normalization is performed against the first nucleic acid (how many copies were measured and given into the reaction), the efficiency is determined using a comparison with the second nucleic acid, and the (residual) copy number is determined using the third nucleic acid. Any losses are compared with a loss of genomic DNA that was subjected to the same procedure. The overall process allows for a precise definitive and absolute quantification of said DNA, and through this the cells in a blood sample, such as, for example, whole blood.

In one embodiment, the invention relates to an artificial sequence that is the exact sequence of the part of GAPDH that is amplified (amplicon) wherein the CpG sequences are inverted into GpC sequences as a tool when performing the method(s) of the present invention.

The composition of the cellular immune system holds valuable diagnostic information for various diseases. The standard technology for quantitative immune cell monitoring is flow cytometry. However, this method is limited to blood samples in which cell-integrity is retained. In clinical routine, this effectively restricts analysis to fresh blood samples as analytical substrate.

In order to widen the margin of use of diagnostic immune monitoring, the inventors implemented epigenetic qPCR systems for quantification of the major leukocyte populations. Upon determining immune cell type specific methylation marks, whole blood from 25 healthy donors, 97 HIV patients and 325 Guthrie cards from newborns including 25 cards from patients with primary immunodeficiencies (PID), including but not limiting to XLA, SCID, SCN, were analyzed. Methodological concordance between flow cytometric and epigenetic data for B-, NK-, total T cells, T helper cells, neutrophils, and cytotoxic T cells was determined and the ability of this new technique to identify quantitative immune cell deficiencies was tested.

Data show that quantification via epigenetic qPCR assays and flow cytometry perform equivalently in healthy subjects and HIV patients according to Bland-Altman testing. Epigenetic quantification is applicable for relative and absolute frequencies of leukocyte subsets in fresh and frozen blood samples. In contrast to flow cytometry, immune cell analysis of Guthrie cards accurately identifies cases PID in newborns. Epigenetic quantification of immune cell populations performs with high equivalence to standard flow cytometry offering a wider range of possible applications, including analysis of dried blood spots possibly laying a path to blood counting of patients in remote areas or from newborns.

Thus, preferred is the method according to the present invention, wherein said blood sample is selected from peripheral, capillary or venous blood samples or subfractions thereof, such as, for example, peripheral blood monocytes, blood clots, and dried blood spots. Also preferred is the method according to the present invention, comprising the step of diagnosing primary immunodeficiencies (PID) in a human, in particular a newborn, based on said quantification, wherein said sample preferably is a dried sample, like a Guthrie card (see also further below), or DBS (dried blood spots).

Preferred is a method according to the present invention, further comprising the step of concluding on the immune status of a human based on at least one quantification of said at last one immune cell type.

Preferred is the method according to the present invention, wherein said recombinant nucleic acid molecule is selected from a plasmid, a yeast artificial chromosome (YAC), human artificial chromosome (HAC), PI-derived artificial chromosome (PAC), a bacterial artificial chromosome (BAC), and a PCR-product.

Preferred is the method according to the present invention, wherein said demethylation standard gene is selected from a gene expressed in all cells to be detected, such as, for example, a housekeeping gene, such as, for example, GAPDH and beta-actin.

Preferred is the method according to the present invention, wherein said blood cell specific gene is selected from: A gene known or found to be expressed in all blood cells to be detected, CD4, CD8beta, and/or alpha, low-density lipoprotein receptor-related protein 5 (LRP5), mevalonate pyrophosphate decarboxylase (MVD), lipocalin-2 (LCN2), and the CD3gamma/delta region (gene).

Another aspect of the invention relates to a diagnostic kit, comprising materials for performing the method according to the present invention, optionally with instructions for use. Preferred materials are the nucleic acid molecules, and/or a bisulphite reagent. Preferred materials are selected from primers and probes selected from any one of SEQ ID NOs. 1 to 12 for the gene for CD4, SEQ ID NOs. 13 to 20 for the gene for CD8beta, SEQ ID NOs. 21 to 28 for the gene for LRP5, SEQ ID NOs. 29 to 36 for the gene for MVD, SEQ ID NOs. 37 to 44 for the gene for LCN2, SEQ ID NOs. 45 to 56 for the CD3gamma/delta genetic region, SEQ ID NOs. 57 to 61 for the gene for GAPDH, and SEQ ID NOs. 62 to 64 for the GAP[GC] construct.

Another aspect of the invention relates to the use of the kit according to the invention for performing a method according to the invention.

Yet another aspect of the invention then relates to a primer or probe selected from any one of SEQ ID NOs. 1 to 64, and an amplicon as amplified by a primer pair selected from SEQ ID

NOs. 1 and 2; 3 and 4; 5 and 6; 7 and 8; 10 and 11; 13 and 14; 15 and 16; 18 and 19; 21 and 22; 23 and 24; 26 and 27; 29 and 30; 1 and 32; 34 and 35; 37 and 38; 39 and 40; 42 and 43; 45 and 46; 47 and 48; 49 and 50; 51 and 52; 54 and 55; 57 and 58; 59 and 60; and 62 and 63.

The present invention also encompasses a method for treating an immune-related disease in a human, in particular a newborn, patient in need thereof, comprising performing a method as described herein, and providing a treatment for said immune-related disease based on the results of said method. One additional embodiment comprises an immune cell monitoring, and immune-related diseases include, for example, PIDs, other immunodeficiencies or cancer.

Current immune cell monitoring requires fresh or well-conserved blood hampering diagnostics in medical fields where such substrates are unavailable. Here, the inventors describe immune cell-type specific epigenetic qPCR which allows determination of immune cell counts from unobservantly conserved, paper-spotted dried blood or fresh samples. General feasibility of epigenetic qPCR has been shown previously using "Treg specific de-methylated region (TSDR)" in T regulatory cells (13). Upon identification of specific epigenetic markers for a number of diagnostically relevant immune cell populations, the inventors demonstrate performance equivalence of the according epigenetic qPCR with the gold standard technologies (FCM, TREC/KREC analysis) of immune cell analytics. For this, the quantification of immune cells in fresh-frozen blood and/or DBS from healthy controls, a cohort of primary (PID) or acquired (HIV) immunodeficiencies, and a cohort of newborns with or without inborn immune deficiencies was analyzed.

Ideal DNA-methylation markers for cell type identification are discriminative between target (near 0% methylation) and all control cells (near 100% methylation). In addition to analysis of T cell associated genes CD3G/D, CD4, and CD8B, loci in the genes MVD, LRP5 and LCN2 were found to be unmethylated only in NK cells, B cells and neutrophils, respectively. MVD is a component of the mevalonate pathway (33), and is expressed in testis, duodenum and colon. LRP5 is involved in bone generation (34). LCN2 is an extracellular transport protein and a major protein of the human tear fluid (35). Reason and function of specific absence of methylation in these regions remains to be analyzed but does not affect its use for cell quantification in peripheral blood. All markers were validated by bisulfite sequencing and the discriminatory CpG-dinucleotides were selected for qPCR development and characterized on artificially generated methylated and unmethylated DNA. Quantitative amplification of target

DNA was achieved without detecting background from non-target templates. qPCR assay performance was robust with low variation as shown by small intra and inter assay CVs in fresh, frozen, or dried blood.

For simultaneous quantification of different cell types in biological samples, the inventors designed a calibrator plasmid containing the unmethylated genomic sequences of GAPDH as reference quantifier and the cell type-specific markers. Whereas GAPDH was previously described as instable gene expression normalizer (36) and to contain segmental duplications (37), the GAPDH locus selected here is stably diploid and always unmethylated. Therefore, through adjusting the quantification of biological samples with the *in silico* bisulfite-converted standard and by the calibrator, assay specific technical inefficiencies can be corrected and allows definitive quantification (20) of the respective loci relative to unmethylated GAPDH, i.e., all nucleated cells. As such, epigenetic qPCR displays a direct proportional relation to cell types as determined by FCM. The remaining observed biases between the methods (38) may result from the biological and technical disparity between nucleic acid-based and antibody-based methods. Homogeneous error distribution and precision were comparable to data from previously performed method comparisons among different antibody-based methods (39). Together, these data suggest that epigenetic qPCR, both from liquid and dried blood substrates, performs equivalent to FCM for the relative quantification of immune cells.

With respect to clinical applications, relative cell quantification is accepted by WHO in HIV-treatment guidelines, but in medical practice treatment decisions depend on cell counting per volume (40, 41). For epigenetic qPCR, this poses a problem since DNA recovery is not quantitative and the relationship between DNA amount and blood volume is not fixed. For that reason, the inventors' experimental setup included the spiking of a defined concentration of artificial GAP[GC] into blood providing for an approximated inference to the original DNA content in a defined blood volume upon subsequent qPCR analysis. Whereas different efficiencies of genomic and plasmid DNA have been described (42), such differences are more reduced after bisulfite treatment and resulting genomic DNA fragmentation. When applied to healthy donors and a HIV cohort, the data showed high correlation and biases and limits of agreements similar to the data described for relative quantification method comparison. Accordingly, the inventors concluded that immune cell counting per microliter can be performed by epigenetic qPCR equivalent to FCM.

At present, neonatal screenings are always performed from DBS. Since FCM is not applicable to this substrate, TREC/KREC analysis is used for PID screening. Introducing epigenetic qPCR in such screening would therefore require equivalence testing to TREC/KREC. Due to different parameters tested, i.e., DNA excision circles vs. genomic DNA, method comparison is not feasible. Instead, the inventors estimated the specificity and sensitivity of TREC/KREC from (43). Epigenetic qPCR reliably identified newborns suffering from different types of PID with similar sensitivity and specificity when using the 99% confidence regions. It only failed to identify one newborn PID patient with maternal cell engraftment, i.e., a patient, where the absence of T and B cells is masked by maternal cells. Unlike the analysis of excision circles, epigenetic analysis is not limited to the main lymphocyte subsets. Such problems may be addressed by expanding the epigenetic qPCR portfolio to markers for memory T or B cells, which are absent in newborns without engraftment. When detected in newborn, such markers may allow detection of engraftment and thereby indicate the absence of a healthy inherent immune system.

Further quantitative defects of other immune cell populations occur in neutrophils and highly specialized Tregs. The inventors' data indicate that identification of such patients based on epigenetic qPCR for neutrophils and Tregs is possible early after birth, allowing for early diagnosis of SCN, which constitutes potentially life-threatening PIDs (43, 44).). The importance to detect and treat these severe immune disorders has been exemplified before (46).

Because of the scarceness of patients, conducting comprehensive studies of rare genetic diseases poses major challenges. Here, this limitation most affected the analysis of only six SCN patients, yet the set of SCID patients with different genetic backgrounds is well comparable to previously published studies (47). The limited set of data provided in this invention only proves technical feasibility but does not yet allow translation into newborn screening. Despite strict limitations of this invention, the inventors' data indicate that epigenetic qPCR may provide an option in medical screening procedures.

Taken together, the invention shows that epigenetic qPCR provides precise and accurate means for immune cell detection and monitoring, and it underscores that epigenetic qPCR

may assist or even replace current immunodiagnostics in particular for unobservantly preserved blood or DBS.

The present invention will now be described and explained further in the following examples and figures, nevertheless, without being limited thereto. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

Figure 1 shows the bisulfite sequencing derived DNA methylation of cell specific marker genes in purified immune cells. Immune cell types are arranged in columns with amplicons (AMP) and the associated gene names in rows. Different gene loci are separated by red lines, Amplicons within the same locus by black lines. Each individual row represents a single CpG site. Methylation rates are color-coded from light gray (0%) to dark gray (100%).

Figure 2 shows a comparison of immune cell quantification by FCM and epigenetic qPCR. Immune cells from 25 blood samples of independent donors were measured with flow cytometry (y-axis) and epigenetic qPCR (x-axis). In A) relative immune cell counts are shown as percent of total leukocytes. In B) absolute immune cell counts are shown as cell number per µl whole blood. The regression line is depicted in red as computed from all data points, the black line indicates the bisectrix.

Figure 3 shows the method comparison of T cell subsets in a HIV cohort. Relative counts of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in % of total nucleated cells determined by (A) FCM and epigenetic qPCR in liquid whole blood, (B) FCM as in A) and epigenetic qPCR from DBS, and (C) comparison of epigenetic qPCR from liquid blood and DBS. On the lefthand side, data are presented as scatterplots. The regression line is depicted in red as computed from all data points, the black line indicates the bisectrix. On the righthand side Bland-Altman plots show average cell counting of the respective analyses (x-axis) plotted over their relative difference (y-axis). Red lines reflect limits of agreement. Central grey lines illustrate the systematic bias. The respective 95% CIs are shown as dotted lines. Upper panel: Total $CD3^+$ T cells; Middle panel: $CD4^+$ T cells; bottom right: $CD8^+$ T cells.

Figure 4 shows the epigenetic qPCR of neonatal DBS. Copies from cell-type specific qPCRs (y-axis) plotted against GAPDH copies (x-axis). (A) unmethylated CD3G/D, B) MVD and C) LRP5. DBS from healthy neonates (n=250, grey circles) estimate reference ranges for each

assay as defined by 99% confidence region (red ellipse) and 99.9% confidence region (blue). 24 DBS from PID-diagnosed newborns are shown as colored circles, each referencing disease characteristics shown in Table 2.

Figure 5 shows the epigenetic qPCR on DBS from newborns with SCN. DBS from healthy controls (grey+box) and newborns with confirmed SCN were subjected to epigenetic qPCR for quantification of $CD15^+$ neutrophils. Healthy cohorts are represented in the boxplot and results from diseased patients are depicted in light gray.

Figure 6 shows a schematic overview over the different quantification approaches for epigenetic cell counting. In A) locus-specific relative percentage quantification is illustrated. qPCRs allow counting of copy numbers as based on the calculation of serially diluted in silico converted plasmids by a linear interpolation (f-1) of the amplification results (f). Relative percentage methylation at the genomic locus is calculated by the interpolated copy number of originally unmethylated copies at this locus divided by all copies at this locus, i.e., the methylated and unmethylated ones. Conversion in the biological sample perturbs the integrity of the genomic DNA, whereas the plasmid represents the amplification product and not the substrate. The resulting difference in amplification efficiency is given by an unknown "conversion factor, (CF)". It is considered negligible when comparing amplification of two highly homologous sequences with few methylation-status dependent SNPs. In (B) the universally unmethylated GAPDH locus (representing the total number of genomic DNA copies) is used as denominator to determine the ratio of any cell-type specifically unmethylated locus. Here, CF leads to substantial shifts between the different qPCR assays. In C) a calibrator plasmid containing equimolar genomic target sequences is used to compensate for conversion efficiencies at the different genomic loci introducing the efficiency factor (EF). D) For counting absolute numbers of cells in a defined volume of blood, a known copy number of plasmid containing a synthetic, not natural DNA sequence (GAP-GC) is supplemented. Interpolating the starting amount of GAP-GC allows monitoring of DNA preparation, conversion and qPCR providing a good estimator for process efficacy.

Figure 7 shows a correlation analysis of absolute quantification for T cell subsets in a HIV cohort. Absolute immune cell counts (as expressed in cells per µl blood) were measured from blood samples of 97 HIV^+ patients by means of flow cytometry and epigenetic qPCR analysis. The graph at the left side scatter plots three T cell populations as measured by epigenetic

qPCR analysis (x-axis) and flow cytometry (y-axis). The lines in black and red represent the bisectrix and the regression line, respectively. Pearson correlation coefficient r=0.955 ($p<0.0001$). The graphs on the right side display a Bland-Altman plot with the mean cell count (cells per µl) averaged between each epigenetic and cytometric measurement on the x-axis plotted over their (relative) difference (y-axis). Red lines reflect the limits of concordance and the central grey line illustrates the systematic bias. Above and below each of these lines, the respective 95% confidence intervals are shown as dotted grey lines. Upper panel: Total $CD3^+$ T cells; middle panel: $CD8^+$ T cells; bottom right: $CD4^+$ T cells.

Figure 8 shows epigenetic immune cell quantification on DBS from newborns. Dried blood spots on Guthrie cards were subjected to epigenetic qPCR analysis for the quantification of unmethylated CD4 (A; specific for $CD4^+$ T cells) and CD8B (B, specific for $CD8B^+$ T cells) gene copies. Calculated values from the immune cell specific assays (y-axis) were scatter plotted over parallel measured GAPDH copies (x-axis). Reference samples from healthy neonates (n=250, grey dots) were measured and used to estimate normal ranges for each assay as defined by red (99% confidence region) and azure (99.9% confidence region) ellipses, respectively. 24 samples from newborns each with a diagnosed PID (classification as indicated in the legend box at the lower right) are shown as red, blue, green and black circles, each associated with an identifier referencing disease characteristics according to table 2.

Table 1. Cell-type specific epigenetic qPCR systems

Cell type specificity	Target gene of qPCR assay	Amplification system	Quantification mode	Plasmid based controls			Analyzed immune cell populations					
				TpG-variant	CpG-variant	Calibrator	$CD4^+$ T cells	$CD8^+$ T cells	$CD19^+$ B cells	$CD56^+$ NK cells	$CD15^+$ Granulocytes*	$CD14^+$ Monocytes
$CD4^+$ T cells	CD4	TpG-system [#TpG]		30100	0	6443	4795	244	50	58	61	57
		CpG-system [#CpG]		0	29650		8	2300	7990	5100	5335	3600
			RD_{ls} [%]	**100**	**0**		99.8	*9.6*	0.6	1.1	1.1	1.6
			RD_u [%]				53.4	2.7	0.6	0.6	0.7	0.6
			EF			*0.53*						
			DD_u [%]				91.4	6.1	0.6	1.1	1.3	1.4
$CD8B^+$ T Cells	CD8B	TpG-system [#TpG]		29850	0	10457	622	5845	51	36	37	19
		CpG-system [#CpG]		0	27150		6400	608	11100	7375	7985	5720
			RD_{ls} [%]	**100**	**0**		8.9	90.6	0.5	0.5	0.5	0.3
			RD_u [%]				6.9	65.1	0.6	0.4	0.4	0.2
			EF			*0.87*						
			DD_u [%]				7.3	90.6	0.4	0.4	0.5	0.3
$CD19^+$ B cells	LRP5	TpG-system [#TpG]		30550	0	8723	2	2	9970	24	1	5
		CpG-system [#CpG]		0	31500		4760	3205	1125	5105	5790	3655
			RD_{ls} [%]	**100**	**0**		0.0	0.1	89.9	0.5	0.0	0.1
			RD_u [%]				0.0	0.0	111.0	0.3	0.0	0.1
			EF			*0.72*						
			DD_u [%]				0.0	0.0	91.7	0.3	0.0	0.1
$CD56^+$ NK cells	MVD	TpG-system [#TpG]		27750	0	12400	150	169	170	10550	172	95
		CpG-system [#CpG]		0	25750		9585	6850	16450	494	11200	7220
			RD_{ls} [%]	**100**	**0**		1.5	2.4	1.0	95.5	1.5	1.3
			RD_u [%]				1.7	1.9	1.9	117.5	1.9	1.1
			EF			*1.03*						
			DD_u [%]				1.5	2.2	1.1	101.2	1.9	1.2
$CD15^+$ Neutrophils	LCN2	TpG-system [#TpG]		29100	0	4520	14	17	95	9	3230	65
		CpG-system [#CpG]		0	31400		3485	2420	5170	3620	30	2110
			RD_{ls} [%]	**100**	**0**		0.4	0.7	1.8	0.2	99.1	3.0
			RD_u [%]				0.1	0.2	0.6	0.1	36.0	0.8
			EF			*0.38*						
			DD_u [%]				0.4	0.6	1.7	0.2	94.7	2.2
$CD3^+$ T cells	CD3 D/G	TpG-system [#TpG]		33350	0	14133	12050	8320	37	59	28.8	23
		CpG-system [#CpG]		0	29450		4	1	13800	9505	9125.0	6810
			RD_{ls} [%]	**100**	**0**		100.0	100.0	0.3	0.6	0.3	0.3
			RD_u [%]				122.8	112.1	0.2	0.6	0.3	0.3
			EF			*1.17*						
			DD_u [%]				104.4	95.2	0.2	0.5	0.2	0.3
Leukocytes	GAPDH	TpG-system [#TpG]				12050	9815	7425	15100	10110	8980	7655

Table 1. RD_{ls}: Relative unmethylation (locus specific) in %; RD_u: Relative unmethylation (universal) in %; EF: Efficiency factor; DD_u: Definitive unmethylation (universal) in %

Table 2. Genetic defects and diagnostic classification by TREC/KREC and epigenetic qPCR for PID patients.

Disease Description				TREC/KREC Newborn Screening			Epigenetic qPCR Analysis			
Identifier	Classification	Gene Defect	Loss of Function Type	TREC[1] Positive [yes/no]	KREC[2] Positive [yes/no]	Screening Classification	(CD3 G/D, GAPDH)[3] Conspicious [yes/no]	(MVD, GAPDH)[3] Conspicious [yes/no]	(LRP5, GAPDH)[3] Conspicious [yes/no]	Screening Classification
1	SCID	*ADA*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
2	SCID	*ADA*	amorph	no	yes	correctly identified	yes	yes	yes	correctly identified
3	DO-SCID[4]	*ADA*	hypomorph	no	yes	correctly identified	no	yes	yes	correctly identified
4	DO-SCID[4]	*ADA*	hypomorph	no	yes	correctly identified	yes	yes	yes	correctly identified
5	SCID	*AK2*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
6	SCID	*AK2*	amorph	yes	yes	correctly identified	yes	yes	no	correctly identified
7	SCID	*Artemis*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
8	SCID	*CD3D*	amorph	yes	no	correctly identified	yes	yes	no	correctly identified
9	SCID w ME[5]	*IL2RG*	amoprh	yes	no	correctly identified	no	no	no	not identified
10	SCID	*IL2RG*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
11	SCID	*IL7RA*	amorph	yes	no	correctly identified	yes	no	no	correctly identified
12	SCID	*IL7RA*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
13	SCID	*IL7RA*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
14	DO-SCID[4]	*JAK3*	hypomorph	no	no	not identified	yes	yes	yes	correctly identified
15	SCID	*PNP*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
16	SCID	*PNP*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
17	SCID	*RAG1*	hypomorph	yes	yes	correctly identified	yes	yes	no	correctly identified
18	SCID	*RAG1*	amorph	yes	yes	correctly identified	no	yes	yes	correctly identified
19	SCID	*RAG2*	amorph	yes	yes	correctly identified	yes	no	yes	correctly identified
20	XLA	*BTK*	amorph	no	yes	correctly identified	yes	no	yes	correctly identified
21	XLA	*BTK*	amorph	no	yes	correctly identified	no	no	yes	correctly identified
22	XLA	*BTK*	amorph	no	yes	correctly identified	no	no	yes	correctly identified
23	XLA	*BTK*	amorph	no	yes	correctly identified	yes	yes	yes	correctly identified
24	XLA	*BTK*	hypomorph	no	no	not identified	no	yes	yes	correctly identified

[1]TREC values ≤ 6 copies per dot were considered positive; [2]KREC values ≤ 4 copies per dot were considered positive; [3]Values outside the joint 99% reference range were considered conspicious, see Fig. 5; [4] Delayed onset SCID; [5] SCID with maternal engraftment

Table 3: Selected candidate regions for neutrophils, NK and B cells from genome-wide discovery on Illumina's Infinium methylation-specific array. DNA methylation marker candidates for NK cells, B lymphocytes and neutrophils identified from a genome-wide discovery experiment using Illumina's 450k Infinium human methylation bead chip array are shown. Bisulfite converted gnomic DNA from purified immune cell populations (as specified in the table's header, each from two independent healthy donors A and B) was used for a genome-wide DNA methylation analysis applying Illumina's 450k Infinium human methylation bead chip array. The assay interrogates 450,000 methylation sites quantitatively across the genome at single-nucleotide resolution. From measured signal intensities, beta values were calculated representing the degree of methylation at a locus. The table shows calculated beta values of 6 loci (as indicated by CpG site ID acc. to Illumina 450k micro array) that are associated with genes MVD, LRP5 or LCN2.

CpG site ID (acc. to Illumina 450k micro array)	Associated gene	Tested immune cell populations											
		$CD4^+$ T cells		$CD8^+$ T cells	NK cells			B cells		Monocytes		Neutrophil granulocytes	
		CD3+ CD4+ CD45RA+ CCR7+		CD3+ CD8+ CD45RA+ CCR7+	CD56+CD16−/dim			CD19+		CD14++		CD15++ CD16++	
		A	B	A	A	B	C	A	B	A	B	A	B
cg05355684	MVD	0.90	0.92	0.91	**0.10**	**0.08**	**0.08**	0.92	0.92	0.92	0.89	0.90	0.91
cg27467516	MVD	0.79	0.84	0.85	**0.52**	**0.51**	**0.48**	0.82	0.86	0.84	0.84	0.82	0.82
cg13738327	LRP5	0.97	0.96	0.97	0.97	0.97	0.97	**0.02**	**0.04**	0.97	0.98	0.98	0.98
cg00464927	LRP5	0.99	0.99	0.97	0.95	0.96	0.97	**0.04**	**NA**	0.97	0.97	0.98	0.99
cg14615559	LCN2	0.73	0.89	0.85	0.84	0.85	0.88	0.83	0.88	0.71	0.66	**0.04**	**0.04**
cg22438810	LCN2	0.70	0.87	0.86	0.74	0.75	0.79	0.74	0.79	0.80	0.82	**0.17**	**0.19**

Table 4: Data from method comparison analysis. Pearson coefficients, bias and precision from the comparison of epigenetic qPCR and flow cytometry performed on blood samples from 97 HIV+ patients (liquid; spotted and dried on Guthrie cards, DBS) are presented.

		Analysis of $CD3^+$ T cells	Analysis of $CD4^+$ T cells	Analysis of $CD8^+$ T cells
Scatter plot analysis	**Pearson coefficient r (p-Value)**			
	FCM vs. epigen. qPCR (blood)	0.977 (p<0.001)	0.957 (p<0.001)	0.966 (p<0.001)
	FCM vs. epigen. qPCR (DBS)	0.953 (p<0.001)	0.742 (p<0.001)	0.924 (p<0.001)
	Epigen. qPCR (blood) vs. epigen. qPCR (DBS)	0.954 (p<0.001)	0.792 (p<0.001)	0.951 (p<0.001)
Bland-Altman analyis	**Bias [%] (95% C.I.)**			
	FCM vs. epigen. qPCR (blood)	4.32 (3.48, 5.16)	-6.59 (-8.48, -4.70)	10.27 (8.95, 11.59)
	FCM vs. epigen. qPCR (DBS)	-0.16 (-1.43, 1.10)	-42.58 (-46.07, -39.08)	-1.15 (-3.03, 0.73)
	Epigen. qPCR (blood) vs. epigen. qPCR (DBS)	-4.45 (-5.70, -3.21)	-35.83 (-38.69, -32.98)	-11.00 (-12.58, -9.42)
	Precision [%] (95% C.I.)			
	FCM vs. epigen. qPCR (blood)	8.12 (6.94, 9.31)	17.63 (14.96, 20.31)	12.73 (10.86, 14.60)
	FCM vs. epigen. qPCR (DBS)	12.43 (10.64, 14.22)	32.98 (28.04, 37.93)	18.32 (15.66, 20.98)
	Epigen. qPCR (blood) vs. epigen. qPCR (DBS)	12.05 (10.29, 13.80)	27.07 (23.04, 31.11)	15.21 (12.98, 17.44)

Table 5: Stability testing of DBS. T cell subpopulations measured by epigenetic qPCR analysis from blood, spotted, dried on Guthrie cards and stored for various times and at different temperatures.

Storage condition		$CD3^+$ T cells Mean [%]	Standard Deviation [%]	$CD4^+$ T cells Mean [%]	Standard Deviation [%]	$CD8^+$ T cells Mean [%]	Standard Deviation [%]
1 day	4°C	20.94	0.62	18.85	0.36	5.73	0.51
	Room temperature	21.19	1.07	17.87	2.39	5.22	0.13
	37°C	24.36	1.64	22.21	4.29	5.94	0.64
1 week	4°C	27.46	3.88	21.11	1.28	7.14	2.24
	Room temperature	24.21	0.50	23.87	2.50	7.39	0.75
	37°C	24.41	2.32	21.42	0.86	7.14	0.26
6 weeks	4°C	21.56	3.38	22.78	5.26	6.19	0.80
	Room temperature	24.09	3.44	19.76	6.24	7.62	2.54
	37°C	22.91	1.42	21.34	1.23	5.98	0.39

Table 6: Epigenetic qPCR from DBS spotted with diluted blood. The three main T cell subpopulations were measured by epigenetic qPCR in different concentrations from a dilution series of EDTA-blood samples.

		$CD3^+$ T cells Mean [%]	Standard Deviation [%]	$CD4^+$ T cells Mean [%]	Standard Deviation [%]	$CD8^+$ T cells Mean [%]	Standard Deviation [%]
Donor A	undiluted	22.46	0.00	15.20	0.00	8.43	0.00
	1:3 dilution	19.89	-11.42	12.01	-20.97	5.84	-30.80
	1:9 dilution	19.49	-13.22	16.45	8.21	7.26	-13.97
	1:27 dilution	15.90	-29.21	NA	NA	NA	NA
Donor B	undiluted	19.39	0.00	20.92	0.00	5.70	0.00
	1:3 dilution	17.27	-10.96	17.60	-15.88	4.03	-29.31
	1:9 dilution	18.64	-3.90	21.60	3.22	5.39	-5.46
	1:27 dilution	15.88	-18.08	23.10	10.41	NA	NA
Donor C	undiluted	12.40	0.00	10.86	0.00	4.09	0.00
	1:3 dilution	12.45	0.45	11.18	2.89	3.29	-19.59
	1:9 dilution	8.61	-30.57	10.45	-3.76	5.50	34.33
	1:27 dilution	23.11	86.42	NA	NA	NA	NA

Table 7A/B: Oligonucleotides used for bisulfite sequencing and qPCR and their respective concentrations as used in the reactions.

Table 7A

Gene	ENSEMBL-ID	**Oligonucleotides for bisulfite sequencing**			
		Amp	Sequence (5' - 3')	SEQ ID No.	Conc. [µM]
CD4	ENSG00000010610	1255			
		Fw.	GGTTTAGGAGGGGTTGTATATT	1	0.5
		Rev.	GGTTTAGGAGGGGTTGTATATT	2	0.5
CD4	ENSG00000010610	2000			
		Fw.	GGGTTAGAGTTTAGGGTTGTT	3	0.5
		Rev.	ACTATCCCCAATATCCTCTACTT	4	0.5
CD4	ENSG00000010610	2001			
		Fw.	TCTAAAATATACAAAACTAACCCAAT	5	0.5
		Rev.	GTGTTAGATAGAGTTTGGGGGT	6	0.5
CD8B	ENSG00000172116	2007			
		Fw.	AATGTTTTATTTGGGGGTTTAT	13	0.5
		Rev.	CCTACTACTCCTTCAATTCTCAA	14	0.5
LRP5	ENSG00000162337	2249			
		Fw.	ATTTTTGTGTGATTTTAGGGTT	21	0.5
		Rev.	ATATCCAAATATCCTACCCTCC	22	0.5
MVD	ENSG00000167508	2674			
		Fw.	AACCCCTAATTTCCTTCTTACT	29	0.5
		Rev.	GGTGTGGGTTTGAGTTTATTT	30	0.5
LCN2	ENSG00000148346	1730			
		Fw.	GATTAGGTTTGAGGTGGAGTT	37	0.5
		Rev.	TATCCCTACCAAAAATACAACA	38	0.5
CD3G/D	ENSG00000160654 ENSG	1405			
		Fw.	GATTTTTAGATGTTTGGGGTT	45	0.5
		Rev.	TTATTCCACCTATTACCTTCCA	46	0.5

	00000167286				
CD3G/D	ENSG 00000160654 ENSG 00000167286	1406 Fw. Rev.	 TTTAGGTTGTGTGTAAATGTGG ATAAACCTCACTCCCATCAATA	 47 48	 0.5 0.5
CD3G/D	ENSG 00000160654 ENSG 00000167286	1408 Fw. Rev.	 AGGATGAGGATAGTTAGGTTTTT AATCCCTCCTAAATTCATTACC	 49 50	 0.5 0.5
GAPDH	ENSG 00000111640	1570 Fw. Rev.	 AAACCCACTTCTTTAATTTACC TGGGGGTAGGGTAGTTG	 57 58	 0.5 0.5
GAP [GC]	-				

Table 7B

Oligonucleotides for qPCR analysis

Gene	Assay variant	Sequence (5' - 3')	SEQ ID No.	Conc. [µM]
CD4	TpG Fw. Rev. Probe	 CCCTACTCTTATAATAAACATTTTTATCAA GAAATTATTTTTTGAGTGTTTTTAATG TGATTTTGAGGGTGGTGGTTATTTTG	 7 8 9	 4.5 3 0.25
CD4	CpG Fw. Rev. Probe	 CCCTACTCTTATAATAAACATTTTTATC GGAAATTATTTTTCGAGTGTTTTTAACG ATTTTGAGGGCGGCGGTTATTTT	 10 11 12	 1.5 1.5 0.25
CD8B	TpG Fw. Rev. Probe	 GTGGTTAAGAAATTAATAGGAAAAAGAATG CTTCCCCACCACAATACAACA TGTTTGTGAGGTATTTAGTTGATGGGAGTTT	 15 16 17	 1.5 1.5 0.125
CD8B	CpG Fw. Rev. Probe	 GGTTAAGAAATTAATAGGAAAAAGAAC CCCCATATTACTTCCCCG CGTTTGTGAGGTATTTAGTCGACGGGAG	 18 19 20	 1.5 1.5 0.125
LRP5	TpG Fw. Rev. Probe	 AATATTACAACCATACACCCAACAA AAGTGATAGAATTTTATGTTTTTTTTATG TTAGTTGAGGTGAGGTGTTTTGTTAGT	 23 24 25	 1.5 1.5 0.25
LRP5	CpG Fw. Rev. Probe	 ATTAATATTACGACCGTACGC CGATAGAATTTTACGTTTTTTTTAC ACGAAACGCCTCGCCTCGA	 26 27 28	 1.5 1.5 0.25
MVD	TpG Fw. Rev. Probe	 GGTTTTGTGGTATTTTTATAGAGTAGT CCATATACACCCTCCTCAA CCCTAAACCACCTCTTCCCCTACAC	 31 32 33	 1.5 1.5 0.125
MVD	CpG			

	Fw.	TTTTGTGGTATTTTTATAGAGTAGC	34	1.5
	Rev.	CCATATACGCCCTCCTCG	35	1.5
	Probe	AAACCGCCTCTTCCCCTACG	36	0.25
LCN2	TpG			
	Fw.	ACCAAAAATACAACACTTCAA	39	1.5
	Rev.	GGTAATTGTTAGTAATTTTTGTG	40	1.5
	Probe	CACTCTCCCCATCCCTCTATC	41	0.15
LCN2	CpG			
	Fw.	TACCAAAAATACAACACTCCG	42	1.5
	Rev.	AGGTAATTGTTAGTAATTTTTACG	43	1.5
	Probe	CTCACTCTCCCCGTCCCTCTATC	44	0.15
CD3G/D	TpG			
	Fw.	CCTAAACACTACCACATCTCAA	51	1.5
	Rev.	AGAAATTTAGTTGTTATGGTTTGT	52	1.5
	Probe	AAAAAACCATCAACCCCATAACACAAA	53	0.25
CD3G/D	CpG			
	Fw.	CTAAACACTACCACATCTCGA	54	1.5
	Rev.	AAATTTAGTTGTTACGGTTTGC	55	1.5
	Probe	CCGTCGACCCCATAACGC	56	0.25
GAPDH	TpG			
	Fw.	GGTTTTTGGTATTGTAGGTTTT	59	1.5
	Rev.	CCAATTACAACATAACAACCA	60	1.5
	Probe	TGTTTGGATGTTGTGTTTGTGGTAGAGTG	61	0.25
GAP [GC]	TpG			
	Fw.	GGTTTTGTGTATGTTAGGTTTG	62	0.75
	Rev.	CCACATTACAACATAAACACAC	63	0.75
	Probe	TGTTGTGATGTTGGTTTTGGTGTAGAGGT	64	0.125

Examples

Study Design - The research objective was to determine if epigenetic qPCR can complement current methods for diagnostic immune cell counting. To test this, the inventors identified and evaluated cell-type specifically unmethylated DNA loci, for relevant immune cells including $CD15^+$ neutrophils, $CD19^+$ B, $CD56^+$ NK, $CD3^+$, $CD4^+$ and $CD8^+$ T cells and Tregs. Epigenetic qPCR was developed and standardized using established normalization parameter. Critical steps for this normalization were to provide for comparable measurement for all cell-specific qPCRs by adjusting for qPCR efficiency between different genomic loci and different bisulfite conversion effects of different regions as well as normalization for DNA purification efficiency for absolute quantification of cells per blood volume. Both, relative and absolute quantification was applied to evaluate whole blood from 25 healthy donors, 97 HIV patients, as well as dried spots from 250 dried blood spots from healthy newborn and 24 newborns cards from newborn patients with primary immunodeficiencies. Results of epigenetic qPCR were verified for equivalence to standard FCM and furthermore tested in applications with

current diagnostic undersupply in immune cell counting, in particular primary and acquired immune deficiencies. Patient material was provided from German and Californian hospitals and blinded prior to data analysis.

Dried blood spots - Three 3.2 mm DBS punches of genetically confirmed IPEX, SCID, SCN and XLA patients, from 250 randomly selected anonymous newborns and from capillary blood of one patient with confirmed IPEX were obtained. The sequencing and genetic confirmation of the included PID patients was performed in compliance with the practitioner toolkit of the Clinical Sequencing Exploratory Research (CSER) Consortium. Written parental consent was obtained for all participants. The study was approved by the Medical Association Chamber of Saxony ethics committee or institutional review board at University of Freiburg, Germany.

Peripheral whole blood - EDTA-anticoagulated peripheral blood was collected from 25 healthy subjects and 97 HIV^+ patients under treatment at Leipzig University with ethical consent. Samples were subjected to epigenetic qPCR and to standard FCM (48). Information was blinded to experimenters.

DNA preparation and bisulfite conversion - For purified cells, genomic DNA was isolated and bisulfite treated using DNeasy tissue and EpiTect Fast Bisulfite conversion kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For EDTA-blood, 20 µl substrate was supplemented with 16 µl lysis buffer, 3 µl proteinase K (30 mg/mL) and GAP[GC] plasmid (final concentration 20,000 copies/µl) and lysed for 10 minutes at 56°C. For conversion, EpiTect Fast Bisulfite Conversion Kit was used. 3x3.2 mm DBS punches were added to 68.75 µl lysis buffer, 10.75µl proteinase K (30 mg/mL), 20,000 copies/µl GAP[GC] plasmid (final concentration) and lysed for 60 minutes at 56°C. Conversion was performed for 45 min at 80°C adding 180 µl ammonium bisulfite (68%-72%, pH 4.8-5.3, Chemos AG, Munich, Germany) and 60 µl tetrahydrofuryl alcohol (Sigma-Aldrich). For purification "My Silane Genomic DNA kit" (Invitrogen, Carlsbad, CA) was used following manufacturer's instructions. Bisulfite conversion rates were analyzed previously and are provided in the manufacturer's manual with values above 98% (49). Efficiency of conversion was routinely checked by bisulfite sequencing showing rates above 98%. As process control, the genomic calibrator included conversion controls in each individual qPCR. BioPerl was used for *in silico* bisulfite conversion of sequences (50).

Epigenetic qPCR - Thermal cycling was done as follows: 1 x 95°C for 10 or 35 min followed by 50 x 95°C for 15 sec, and 61°C for 1 min in 5 µl (DBS) or 10 µl (EDTA-blood) using Roche LightCycler 480 Probes Master. For calculation of cell numbers from autosomal genes, a 2:1 allel-to-cell ratio was assumed. For RD_{ls} [%], TpG-copies were divided by TpG- + CpG-copies. For RD_u [%], the quotient of TpG copies (of the respective immune cell type) and GAPDH copies was calculated. For DD_u [%], RD_u were corrected by EF compensating for performance differences between different qPCRs. For assay-specific EF, the inventors used a plasmid-based calibrator harboring the genomic target region of all qPCRs, including GAPDH (universal denominator) and an artificial GAP[GC] region. The calibrator was subjected to bisulfite conversion followed by qPCR. EF was calculated by dividing measured TpG copies by parallelly measured GAPDH copies. EFs were derived from approximately 25 experiments. 95% CI were 0.90-1.19 (CD3G/D), 0.47-0.63 (CD4), 0.75-1.00 (CD8B), 0.58-0.77 (LRP5), 0.89-1.18 (MVD) and 0.38-0.48 (LCN2). For absolute quantification, an artificial GAPDH sequence inversing all CpG dinucleotides to GpC (GAP[GC])and its corresponding epigenetic qPCR were designed without cross reactivity with endogenous GAPDH. EF for GAP[GC] was 0.87 with an 95% CI of 0.75-1.00.

Combined TREC/KREC newborn screening assay - TREC/KREC screening was applied as described previously (51). Briefly, DNA from one 3.2-mm punch of the original DBS was extracted in a 96-well format, and quantitative triplex real-time qPCR for TREC, KREC, and ß-actin (ACTB) was performed using a ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). TREC and KREC copy numbers were determined per 3.2-mm punch. ACTB was used to verify suitable DNA amounts per DBS and not for normalizing TREC/KREC copies.

Plasmids - Sequences, corresponding to methylated or unmethylated, bisulfite-converted genomic regions, were designed *in silico* and inserted into plasmid pUC57 (Genscript Inc., Hongkong, China) and used for assay establishment and as qPCR quantification standard. Standard plasmids harbor all assay target sequences equimolarly. Plasmids were spectrophotometrically quantified, linearized by ScaI and serially diluted in 10 ng/µl of λ-phage DNA (New England Biolabs) to obtain 31250, 6250, 1250, 250, 50 or 30 copies in the final reaction. Calibrator plasmid harbors all assay target sequences equimolarly in genomic

unconverted, unmethylated version. Artificial spike-in plasmid carries unconverted GAPDH with inverted CpG dinucleotides (GAP[GC]).

Oligonucleotides - Oligonucleotides (Metabion AG, Munich, Germany) are described in Table 7.

Flow cytometry - For leukocyte purification, peripheral blood from healthy adult donors was fractionated by FCM into $CD15^+$, $CD14^+$, $CD56^+$ NK, $CD19^+$ B, $CD4^+$ and $CD8^+$ T cells with cell purities >97% and viability >99% as described previously (13). For analytical cell quantification, absolute $CD45^+$ leukocyte counts were determined by a MACSQuant cytometer (Milteny Biotec, Bergisch Gladbach, Germany). Frequencies and absolute counts of $CD15^+$ neutrophils, $CD19^+$ B, $CD56^+$ NK, $CD3^+$, $CD4^+$ and $CD8^+$ T cells and $FOXP3^+$ Tregs were calculated as previously described (13, 48).

Statistical analysis - CP (crossing point) of triplicate measurements was computed by second-derivative maximum applying LC480 software (Roche, Mannheim, Germany) to yield copy numbers (plasmid units) by interpolating amplification (f) from calibration curves generated with serial dilutions of plasmid-based standards. Sample sizes for method comparison were chosen as 100 to provide 95% CI for limits of agreement at +/- 0.34x the underlying standard deviation. Estimation of reference ranges demands a healthy population of at least 120 individuals for the nonparametric estimation of the 95% CI. The number of healthy cases was increased until exhaustion of available samples to accommodate for multidimensionality and estimation of extreme quantiles. Henze-Zinkler test was used to check for multivariate normality. Method comparison between flow cytometric and qPCR-based measuring technique was done as follows: Bivariate data from the two methods were illustrated in a scatterplot. Linear regression was performed testing a) for a slope different from 1 and b) an intercept different from 0. Bland-Altman plots were inspected analyzing bias and precision statistics (29). Acceptable precision was regarded as average deviation from the bias in percent. The limit of quantification for qPCR assays defined by the inter assay CV (0.2) was used as precision criterion and acceptable limits of agreement of 0.4. Wilcoxon-Rank-Sum Test was used to for median differences. The estimated bias, precision statistic and respective 95% CI are reported. For correlation, Pearson product-moment correlations were used. All p-values are two-sided. Statistics software R 3.3.0 was employed.

Cell type-specific bisulfite-conversion - Methylation-dependent conversion of CpG-dinucleotides was analyzed by bisulfite sequencing *(24)* aiming at marker identification for immune cell populations from human peripheral blood. Candidate loci were selected from literature or discovered using Illumina's 450k array-based assay. The inventors' data showed distinctive absence of methylation at individual CpG positions for $CD56^+$ NK cells, $CD19^+$ B cells and neutrophils (target cell types), whereas the same CpGs were methylated in control cell types (Table 3). Based on these findings, amplicons (AMP) were designed for denser CpG methylation analysis in the identified regions. As a likely candidate marker for $CD4^+$ T cells, the inventors designed three AMPs for bisulfite sequence analysis covering regulatory elements within the 5' region of the first intron (AMPs 1255, 2000 and 2001) in the CD4 gene (21, 22). Unmethylated CpG-sites are detected as TpG residues after bisulfite-conversion and amplification occurs exclusively in target cells, i.e., $CD4^+$ T lymphocytes. The same CpGs were inert to bisulfite-conversion in control cell types, including $CD56^+$ NK cells, $CD8^+$ T lymphocytes, $CD14^+$ monocytes, $CD19^+$ B lymphocytes and $CD15^+$ granulocytes (Figure 1). Next, the inventors investigated the CD8B gene as a potential epigenetic marker for $CD8^+$ T cells (21, 22) by designing amplicons targeting regulatory elements within its third intron (AMP2007). Here, bisulfite-mediated conversion of CpGs was observed exclusively in $CD8^+$ T cells, while those CpGs were inert to conversion in control cells. According epigenetic marks were identified for B cells, NK cells and neutrophils in the genes coding for low-density lipoprotein receptor-related protein 5 (LRP5, AMP2249), mevalonate decarboxylase (MVD, AMP2674) and lipocalin 2 (LCN2, AMP1730), respectively. Each AMP was uniquely unmethylated in the target cell type and fully methylated in the corresponding control leukocyte populations (Figure 1). DNA methylation of the intergenic CD3G and CD3D region (AMPs 1405, 1406 and 1408), constituting a marker for $CD3^+$ T cells, and the methylation profile of GAPDH (AMP1570) were published previously (15).

Locus-specific relative qPCR measurements - To target differentially methylated CpG positions described above, discriminating qPCR assay systems were designed. These were characterized on synthetic template DNA cloned into plasmids. Templates correspond to the bisulfite modified genomic DNA, i.e., replacing all cytosines (C) with thymidines (T). For the TpG template (mimicking unmethylated CpGs), a universal plasmid carrying targets for all assays in an equimolar stoichiometry was designed. A universal CpG-plasmid (mimicking methylated CpGs) was generated accordingly. Exclusive amplification of the desired DNA sequence without cross-reactivity with mutually antithetic templates was demonstrated for all

qPCRs (Table 1). Assay specificity was tested on immune cell populations, which were purified as described in the Materials and Methods section. For target cells, high copy numbers were observed in their respective TpG-specific system, and low copy numbers were measured in the corresponding CpG-system. Conversely, for control cells low and high copies were found in the TpG- and CpG-systems, respectively. The original copy number of the target gene was determined by relating qPCR signals from the according amplification (f') to amplification of serially diluted standard plasmids (f), each with a defined concentration of the *in silico*-converted unmethylated version. Relative determination of locus-specifically unmethylated DNA (RD_{ls}) ranged from 89.9 to 100% in target cell types and from 0 to 3% in controls (Table 1). Exceptions were observed for $CD4^+$ T cells, showing 8.9% RD_{ls} at the CD8B locus and vice versa (i.e., 9.6% CD4 RD_{ls} in $CD8^+$ T cells), possibly due to mutual and residual cell contaminations.

Universal and definitive quantification - Amplification efficiency and estimated copy numbers vary for each locus-specific qPCR system (25). Therefore, an invariably unmethylated regulatory region of the GAPDH (26) gene was used as a universal denominator to determine each cell-type locus relative to all nucleated cells. This system was applied to purified $CD3^+$, $CD4^+$ and $CD8^+$ T cells, neutrophils, $CD14^+$ monocytes, $CD56^+$ NK and $CD19^+$ B cells. Quantification differs when using methylated and unmethylated amplification data at specific epigenetic loci (RD_{ls}) compared with quantification of the unmethylated cell type-specific locus and the universally unmethylated GAPDH as the denominator (RD_u) (Table 1).

Since *in silico*-converted double-stranded, GC-rich plasmids do not fully represent *de facto* bisulfite-converted, single-stranded GC-depleted DNA (27, 28), a "calibrator plasmid" was adopted harboring one copy of all assay targets in their unconverted genomic (i.e., unmethylated) state. This calibrator is bisulfite-converted in parallel to samples. When copy numbers from this calibrator are obtained by standard plasmid interpolation, systematic amplification differences between the assays were detected and translated into an efficiency factor (EF), adjusting for biases between cell-type specific assays and GAPDH. Cell type-specific EFs were measured in approximately 25 experiments ranging between 0.53 (95% confidence interval (CI) = 0.42, 0.61) for CD4 and 1.17 (95% CI = 0.95, 1.31) for CD3D/G (see *"Epigenetic qPCR"* in Material and Method Section). Calculated EFs provide universal definitive determination of unmethylated DNA (DD_u) for each assay (Table 1). Using this

approach, the inventors applied epigenetic qPCR for universal and definitive quantification of immune cells from biological samples. The concepts of immune cell quantification used in this work are illustrated in Figure 6.

Method comparison of FCM and epigenetic qPCR - To allow absolute cell quantification comparable to FCM (i.e., cell/µl) the inventors introduced a "spike-in plasmid" harboring an artificial GAPDH-derived sequence, created by inversing all CpG dinucleotides to GpC (GAP[GC]) and an according epigenetic qPCR. For absolute immune cell counting, this plasmid was added to blood samples in a defined concentration. The *in silico* bisulfite-converted, artificial GAP[GC] sequence was included in the quantification standard and the unconverted sequence into the calibrator plasmid.

To assess the overall performance of the epigenetic cell counting, markers for B cells, NK cells, $CD3^+$, $CD4^+$, $CD8^+$ T cells, $FOXP3^+$ Tregs and neutrophils were analyzed in blood samples from 25 adult healthy donors in comparison with FCM. Data from both methods were scatter plotted either as relative (Figure 2A) or absolute cell counts (Figure 2B), each yielding Pearson coefficients (r) of above 0.95 ($p<0.0001$).

To test the individual epigenetic markers in a clinically relevant setting, the inventors used blood from 97 HIV^+ subjects and quantified $CD3^+$, $CD4^+$ and $CD8^+$ T cell counts by standard FCM and epigenetic qPCR. For the latter, EDTA-blood or DBS served as substrates. Method comparisons were conducted for all three approaches. For comparison of FCM to epigenetic qPCR in liquid blood, correlation analyses yielded Pearson r coefficients from 0.96 to 0.98 ($p<0.001$) for relative quantification (Figure 3A). Leukocyte numbers per microliter blood as determined by FCM and epigenetic qPCR were highly correlated (Pearson $r=0.8$; $p<0.001$) (Figure 7). Comparative analyses from DBS and FCM (Figure 3B) yielded Pearson r between 0.7 and 0.95 ($p<0.001$). Epigenetic measurements from liquid blood and DBS yielded Pearson r between 0.8 and 0.95 ($p<0.001$) (Figure 3C).

Bland-Altman analyses ((29) shown in Figure 3A-C, (right panels) determines systematic biases and precision for all methods and markers which are listed in Table 6. Biological read-outs of FCM and epigenetic counting from either substrate correlated well for the tested cell types. Minor biases (4.3-; -6.6; 10.3 for CD3, CD4, CD8, respectively) and high precision (all < 20%) were detected between FCM and epigenetic qPCR of liquid blood whereas a

pronounced variation (>20%) was observed between DBS and both other methods for $CD4^+$ T cells. To investigate the influence of substrate instability in DBS, different storage times and conditions and sample dilutions, mimicking unobservantly collected DBS, were monitored. Data showed no sign of degradation at different storage conditions with change of variation (CV) below 15% for all temperatures and time points (Table 5). CV was below 30% down to blood dilutions of 1:9 (Table 6). As previously analyzed by others, genomic DNA is a stable analyte and can be extracted from year-long stored DBS (30-32).

Epigenetic qPCR in neonatal screening samples - Epigenetic qPCR was applied in a case/control study consisting of original neonatal screening cards (i.e., DBS) from 24 PID patients and 250 randomly selected newborns, measuring total T, B and NK cells (Figure 4). PID cases included SCID patients with different gene defects and X-linked agammaglobulinemia (XLA) associated with BTK-mutation (Table 2). Reference ranges were established using the joint distribution of all leukocytes (GAPDH-specific qPCR) and specific immune cell types. Copy numbers were log-transformed and used to estimate a bivariate normal distribution, whose confidence regions (99% and 99.9% curves) defined reference ranges for newborns. As each of the three panels is tuned to 99% or 99.9% confidence regions, Bonferroni correction guarantees family-wise-error-rates below 3% or 0.3% yielding final confidence of 99.7 or 97%, respectively.

For $CD3^+$ T cell- and GAPDH-measurements, 13 out of 16 samples from SCID patients were outside the 99.9 % confidence region, SCID15 was found outside the 99%, but inside the 99.9 % region and SCID9 and 18 presented as non-suspicious. However, SCID15 and 18 were outside the 99.9% confidence region for NK cell and GAPDH measurements. Moreover, for B cell and GAPDH measurements, SCID18 was found outside the 99.9% confidence region and SCID15 was found outside the 99% region (Figure 4). Hence, 15 out of 16 SCID patients were unambiguously identified as non-normal by epigenetic testing based on their newborn cards. SCID9 presented with maternal lymphocyte engraftment, as confirmed by FCM and chromosomal analysis, did however not show a quantitative impairment of cell counts and is classified as normal. Epigenetic qPCR for $CD4^+$, $CD8^+$ T cells and GAPDH confirmed these findings without adding diagnostic information to $CD3^+$ T cell analysis in SCID samples. DBS of delayed-onset SCID (DO-SCID) associated with hypomorphic JAK3 or ADA mutations were also analyzed. The JAK3 deficient delayed-onset patient (DO-SCID14) showed reduced CD3, NK, B and CD4, CD8 values (Figure 4) outside the 99.9% confidence

region. ADA-associated DO-SCID4 was outside the 99.9% confidence region for NK and B cells and outside the 99% region for CD3, whereas DO-SCID3 was outside the 99.9% confidence region in B cells and the 99% confidence region in NK but normal for $CD3^+$ T cells. Overall, all three DO-SCID samples were identified based on epigenetic analysis. DBS from four out of five patients with XLA showed B cell counts outside of the 99.9% confidence region. Hypomorphic XLA24 was outside the 99% confidence region in B and NK cells. NK and T cell counts were at the borders of reference ranges for other XLA samples (XLA23 in NK and XLA20, XLA23 in Tcells). Altogether the XLA phenotype was in accordance with B cell deficiency. Comparison with TREC/KREC values showed that epigenetic quantification detected all but one patient, whereas TREC/KREC failed to detect two of five cases with delayed-onset or hypomorphic genetic background. However, maternal engraftment masks detection via epigenetic counting, whereas TREC analysis is not affected (Table 2). Screening classification is based on the three tests for conspicuous cell counts attains a sensitivity of 0.958 and a specificity of 0.984 using the 99% confidence regions. For the 99.9% confidence regions, sensitivity falls to 0.9167, while the specificity reaches 1.

IPEX and severe congenital neutropenias (SCN) are other forms of severe PID with no currently available newborn screening. Given their severe early-onset and morbidity, patients would benefit from neonatal diagnosis. In juvenile IPEX patients peripheral Tregs are uniquely increased (23), when compared to healthy age-matched donors and disease controls.

Applying epigenetic qPCR for neutrophils, neonatal patients with SCN were detected by a significant reduction ($p = 4.4x10e-6$) of neutrophils (Figure 5A). The median percentage of neutrophils was at 55% in the control cards (n=26) and at 17% in neutropenic patients (n=6).

Tested was a DBS each from a newborn and a two-year old IPEX patient by epigenetic qPCR of Treg and $CD3^+$ T cells (Figure 5B). The ratio of Treg/$CD3^+$ T cells of IPEX patients is conspicuously increased compared to the non-affected healthy donors.

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Claims

1. A method for an improved methylation assay for identifying blood immune cells, comprising the steps of

a) providing a sample of human blood, in particular from a newborn, comprising genomic DNA of blood immune cells;

b) treating said genomic DNA of said immune cells with bisulfite to convert unmethylated cytosines into uracil;

c) amplifying of said treated genomic DNA using suitable primer pairs to produce amplicons, and

d) identifying said blood immune cells based on analyzing said amplicons as amplified,

wherein said amplification and analysis comprises amplification and/or qPCR using primers and probes selected from at least one of the sets of SEQ ID NOs. 1 to 12 for CD4, SEQ ID NOs. 13 to 20 for CD8beta, SEQ ID NOs. 21 to 28 for LRP5, SEQ ID NOs. 29 to 36 for MVD, SEQ ID NOs. 37 to 44 for LCN2, and SEQ ID NOs. 45 to 56 for CD3gamma/delta, and

wherein a demethylation of at least one CpG position in said amplicon is indicative for at least one blood immune cell selected from $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, $CD14^+$ monocytes, $CD56^+$ NK cells, and $CD19^+$ B cells.

2. The method according to claim 1, further comprising an analysis of the methylation status of an amplicon for CD3 epsilon.

3. The method according to claim 1 or 2, further comprising an analysis and a first normalization using a demethylation standard gene selected from a gene expressed in all cells to be identified, such as, for example, a housekeeping gene, such as, for example, GAPDH and beta-actin, preferably using primers and probes selected from SEQ ID NOs. 57 to 61 for the gene for said GAPDH.

4. The method according to any one of claims 1 to 3, further comprising a second normalization using an *in silico* bisulfite-converted recombinant nucleic acid comprising a sequence inversing all CpG dinucleotides to GpC of said at least one demethylation standard

gene (GAP[GC] construct), preferably using primers and probes selected from SEQ ID NOs. 62 to 64 for said GAP[GC] construct.

5. The method according to any one of claims 1 to 4, further comprising a third normalization using a calibrator plasmid comprising one copy of each amplicon sequence in its unconverted genomic (i.e., unmethylated) state.

6. The method according to any one of claims 1 to 5, wherein said analysis further comprises a quantification of said blood immune cells as identified.

7. The method according to any one of claims 1 to 6, wherein said method further comprises an additional FCM of said blood immune cells to be identified.

8. The method according to any one of claims 1 to 7, wherein more than one blood cell specific gene is analyzed, e.g. a panel of 1, 2, 3, 4, 5 or 6 blood cell specific genes, optionally together with more than one demethylation standard gene.

9. The method according to any one of claims 1 to 9, wherein said blood sample is selected from peripheral, capillary or venous blood samples or subtractions thereof, such as, for example, peripheral blood monocytes, blood clots, and dried blood spots.

10. The method according to any of claims 3 to 10, further comprising the step of concluding on the immune status of a human based on at least one quantification of said at last one immune cell type.

11. A diagnostic kit, comprising materials for performing the method according to any of claims 1 to 10, optionally with instructions for use.

12. The kit according to claim 11, wherein said materials are selected from primers and probes selected from any one of SEQ ID NOs. 1 to 12 for the gene for CD4, SEQ ID NOs. 13 to 20 for the gene for CD8beta, SEQ ID NOs. 21 to 28 for the gene for LRP5, SEQ ID NOs. 29 to 36 for the gene for MVD, SEQ ID NOs. 37 to 44 for the gene for LCN2, SEQ ID NOs. 45 to 56 for the CD3gamma/delta genetic region, SEQ ID NOs. 57 to 61 for the gene for GAPDH, and SEQ ID NOs. 62 to 64 for the GAP[GC] construct.

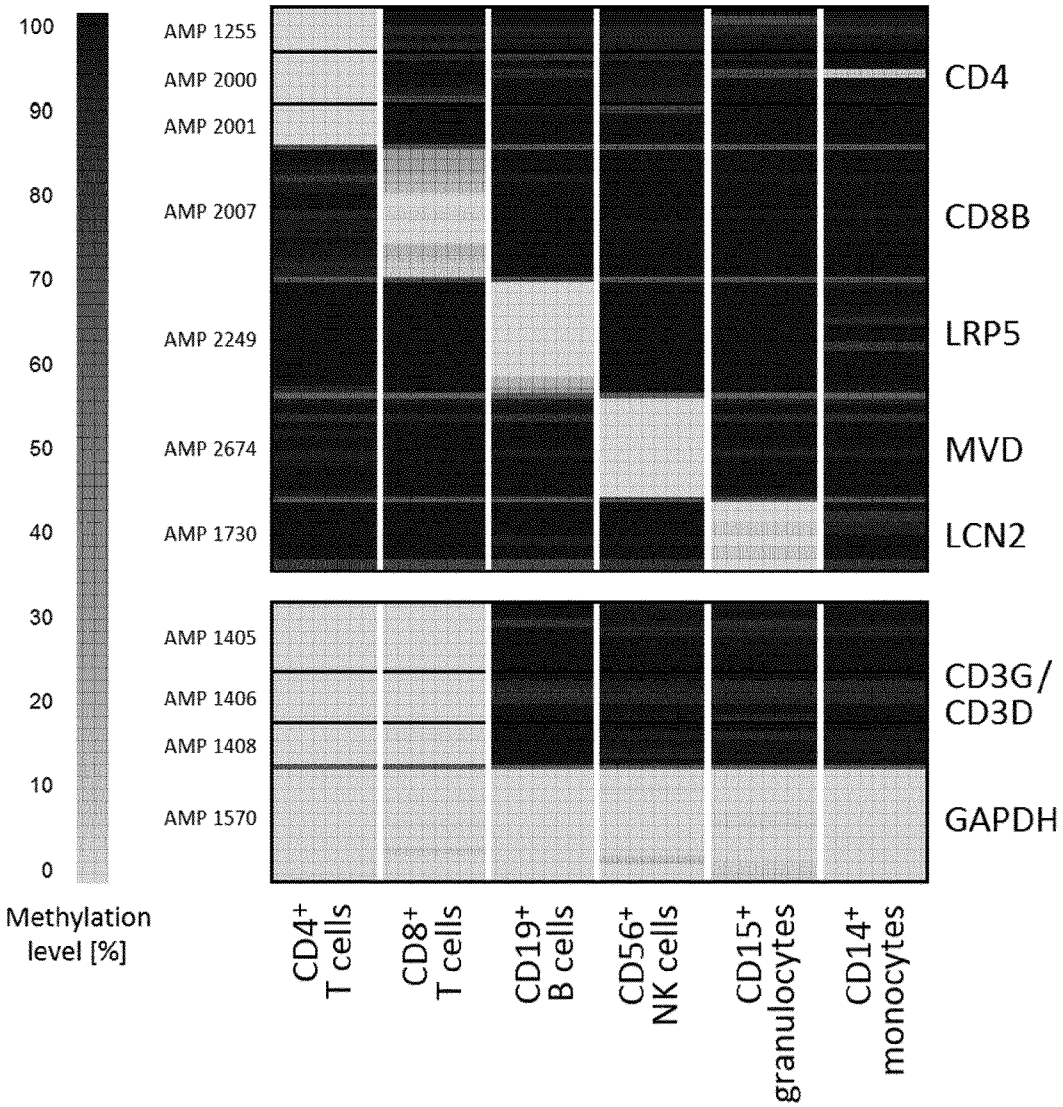
13. Use of the kit according to claim 11 or 12 for performing a method according to any of claims 1 to 10.

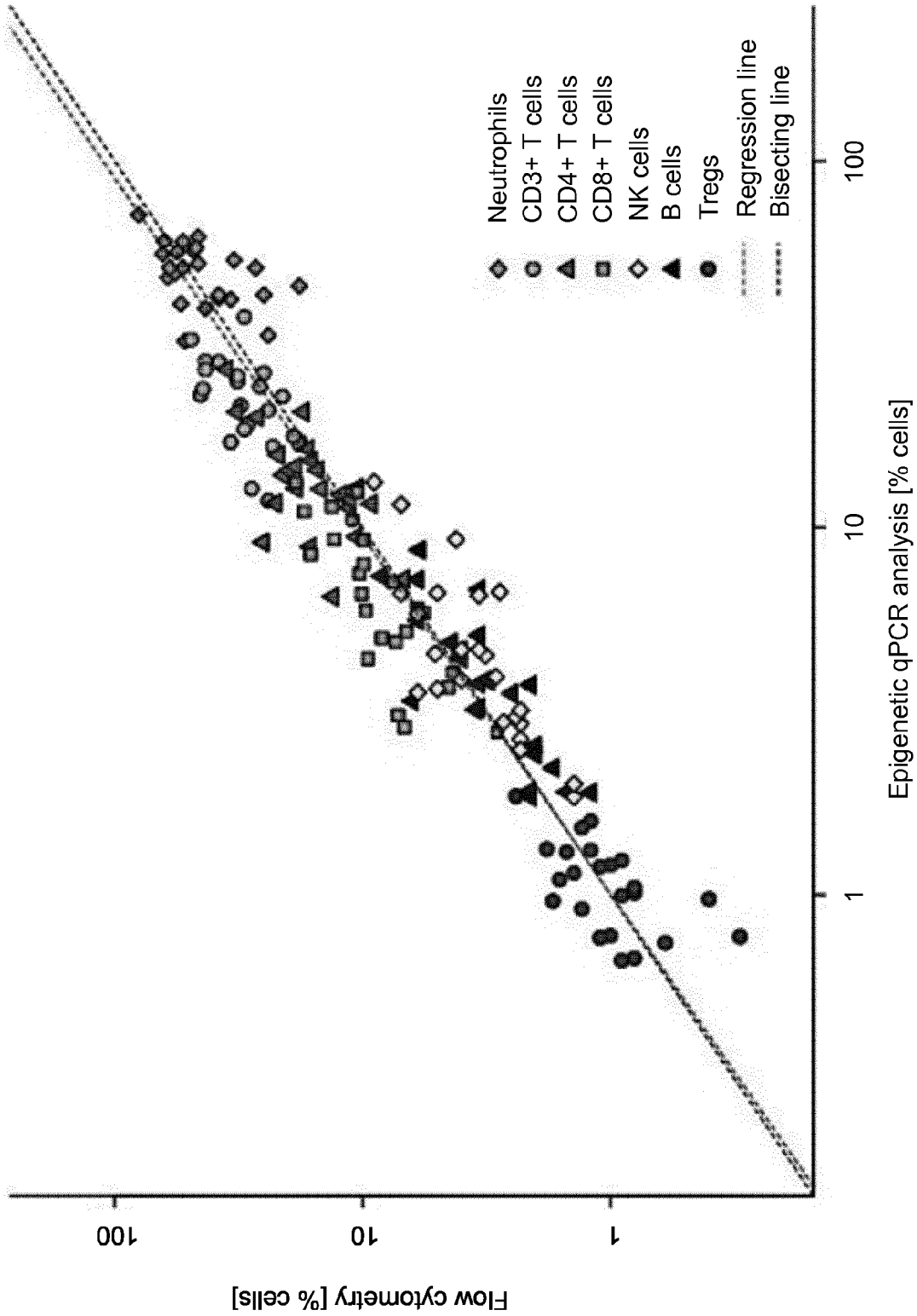
14. A primer or probe selected from any one of SEQ ID NOs. 1 to 64, and an amplicon as amplified by a primer pair selected from SEQ ID NOs. 1 and 2; 3 and 4; 5 and 6; 7 and 8; 10 and 11; 13 and 14; 15 and 16; 18 and 19; 21 and 22; 23 and 24; 26 and 27; 29 and 30; 1 and 32; 34 and 35; 37 and 38; 39 and 40; 42 and 43; 45 and 46; 47 and 48; 49 and 50; 51 and 52; 54 and 55; 57 and 58; 59 and 60; and 62 and 63.

Epiontis GmbH
E31941WO

FIGURES

Figure 1

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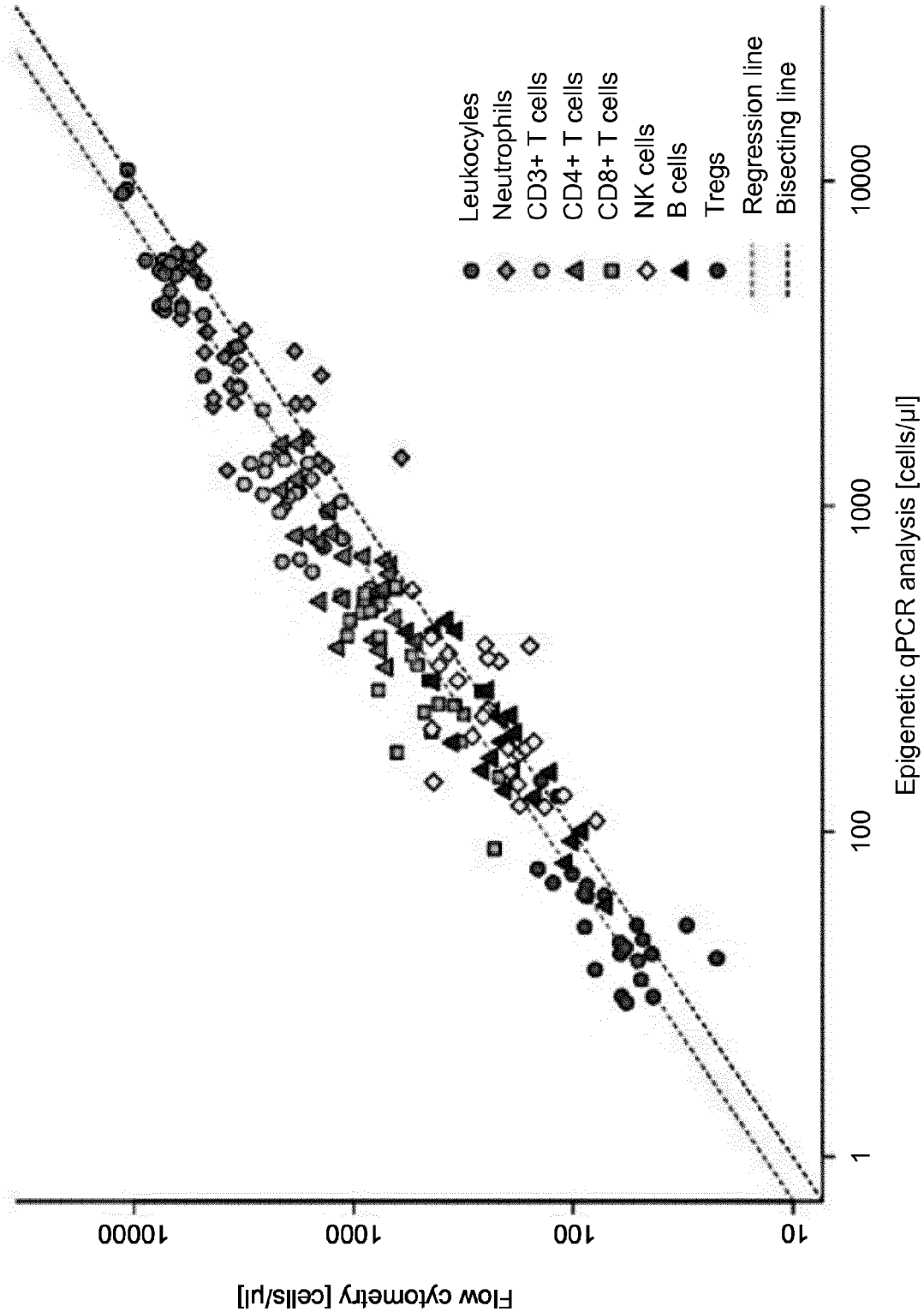
Flow cytometry [% cells]
1
10
100
Epigenetic qPCR analysis [% cells]
1
10
100
Neutrophils
CD3+ T cells
CD4+ T cells
CD8+ T cells
NK cells
B cells
Tregs
Regression line
Bisecting line

Figure 2



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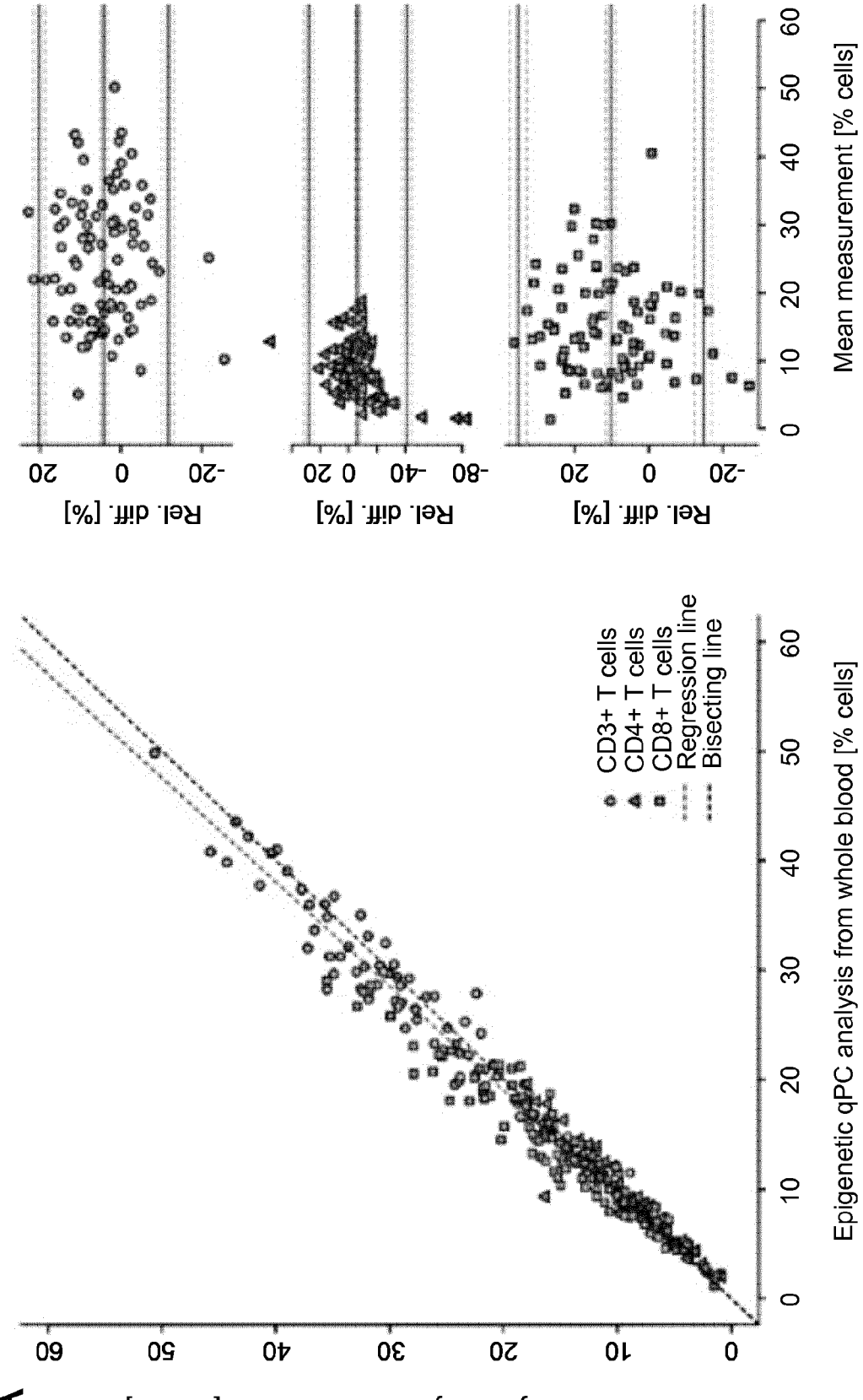
A
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Epigenetic qPC analysis from whole blood [% cells]
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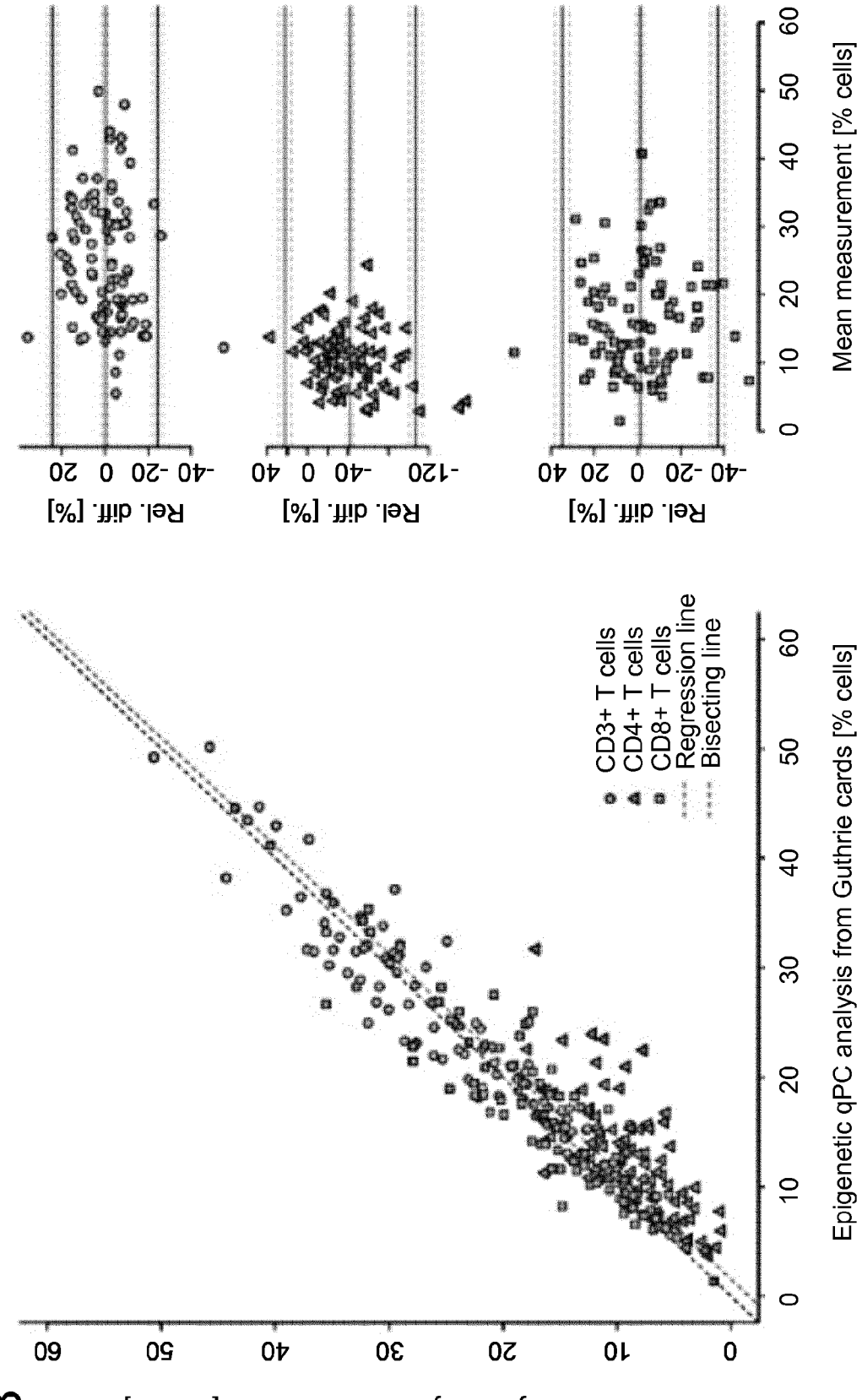
CD4+ T cells
CD8+ T cells
Regression line
Bisecting line
Rel. diff. [%]
Mean measurement [% cells]

Figure 3



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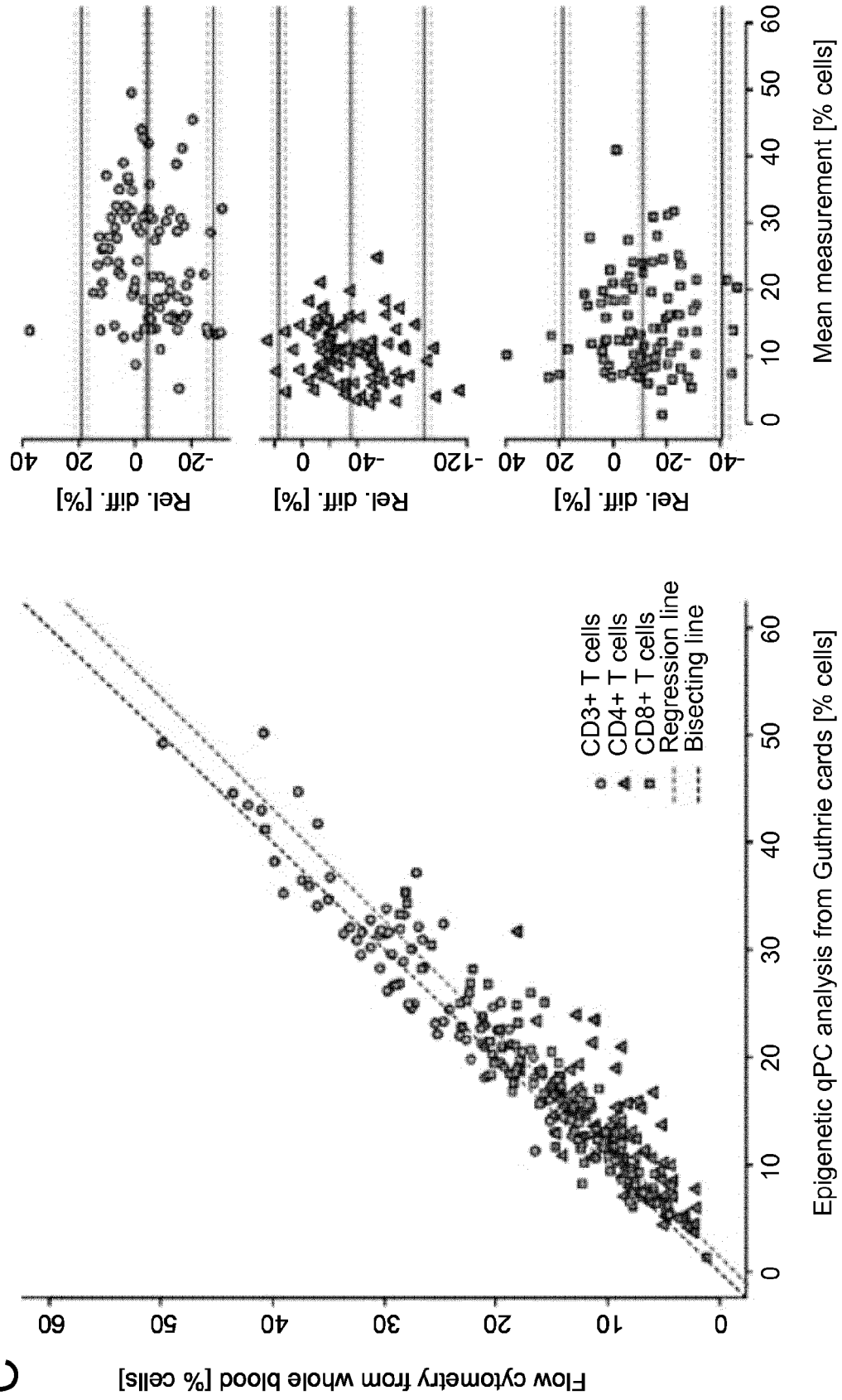

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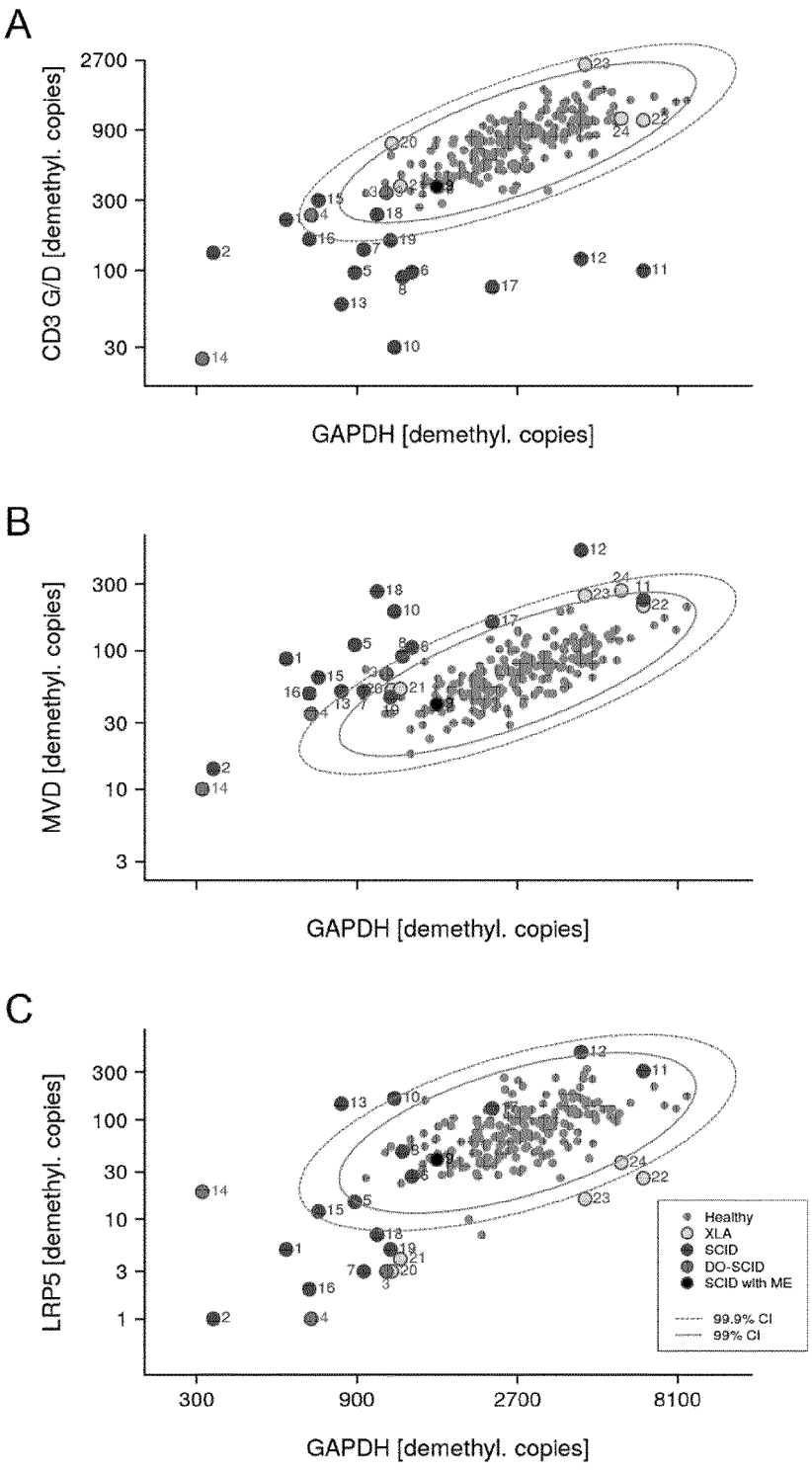
Figure 4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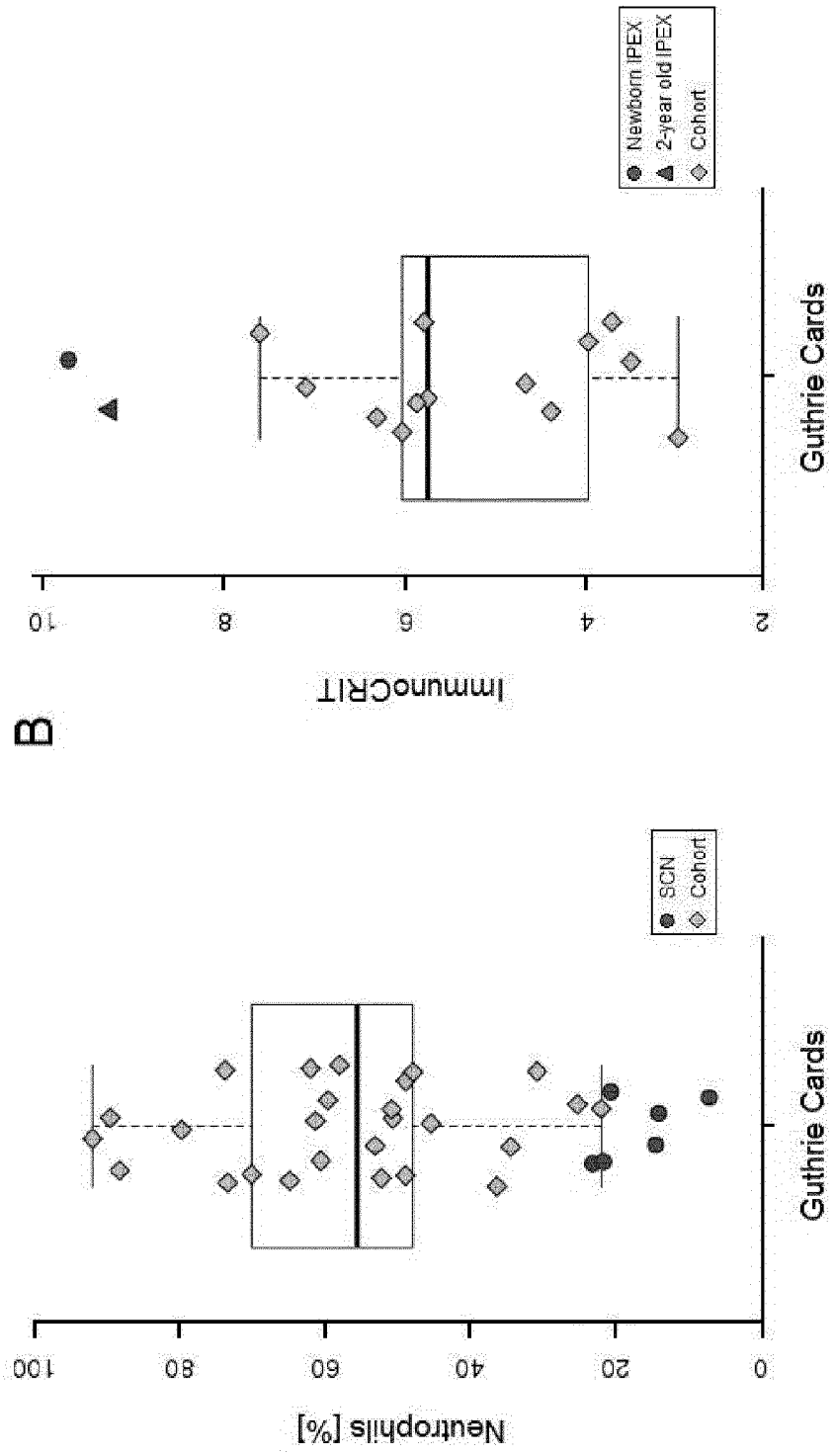
900
300
100
30
GAPDH [demethyl. copies]
B
MVD [demethyl. copies]
300
100
30
10
3
GAPDH [demethyl. copies]
C
LRP5 [demethyl. copies]
300
100
30
10
3
1
300
900
2700
8100
GAPDH [demethyl. copies]
Healthy
XLA
SCID
DO-SCID
SCID with ME
99.9% CI
99% CI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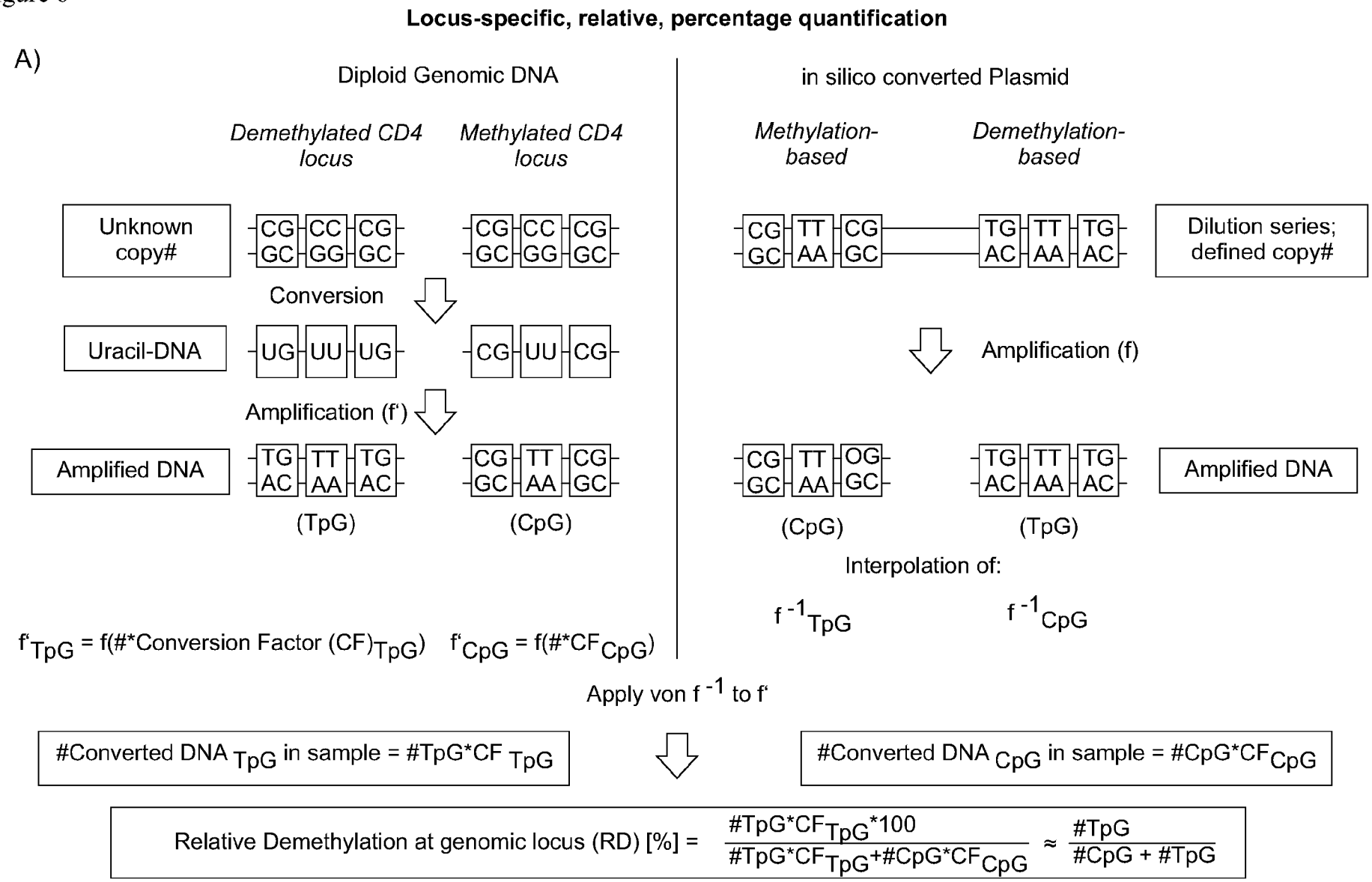
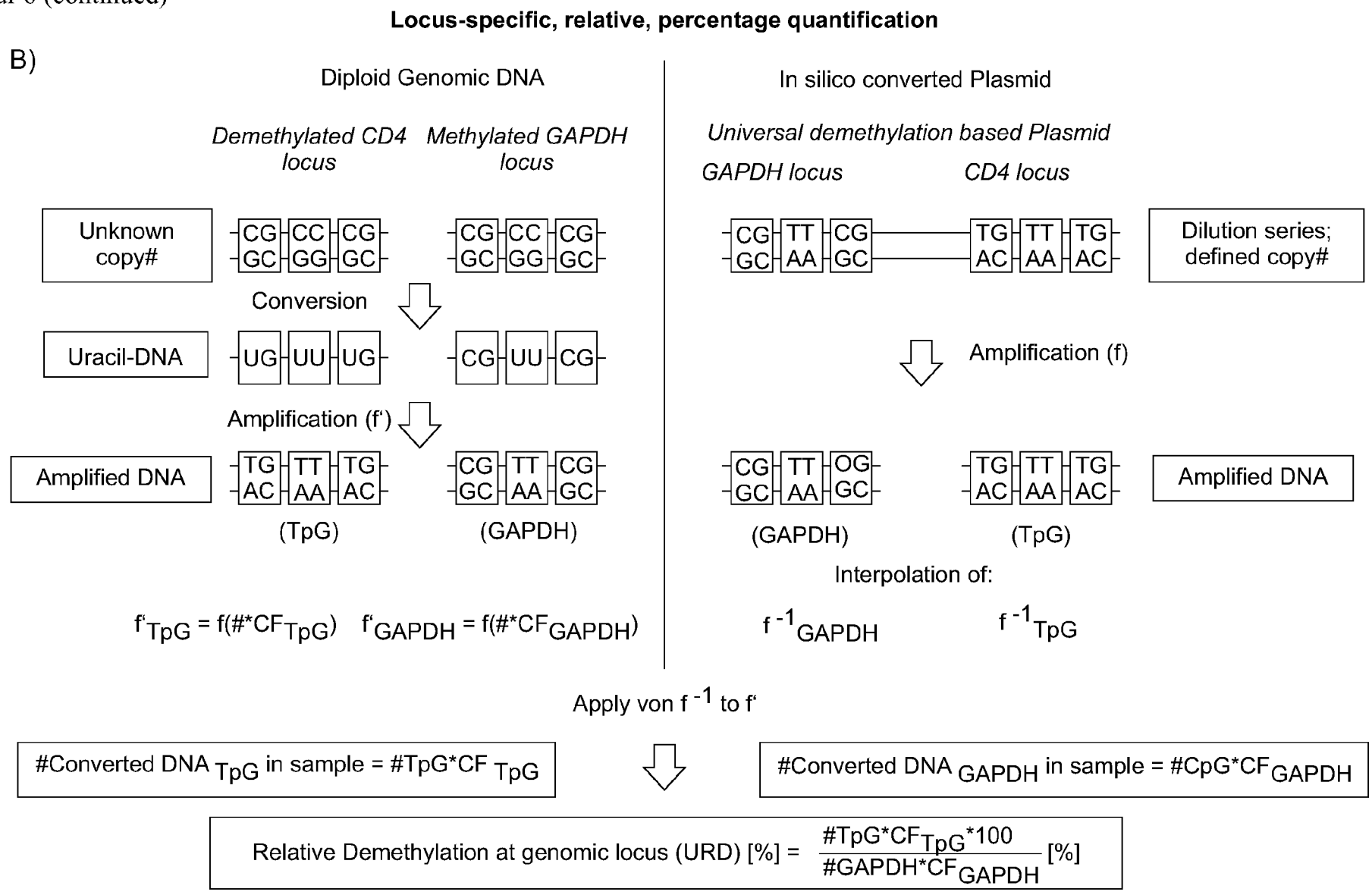
Neutrophils [%]
0
20
40
60
80
100
Guthrie Cards
SCN
Cohort
B
ImmunoCRIT
2
4
6
8
10
Guthrie Cards
Newborn IPEX
2-year old IPEX
Cohort

Figure 5

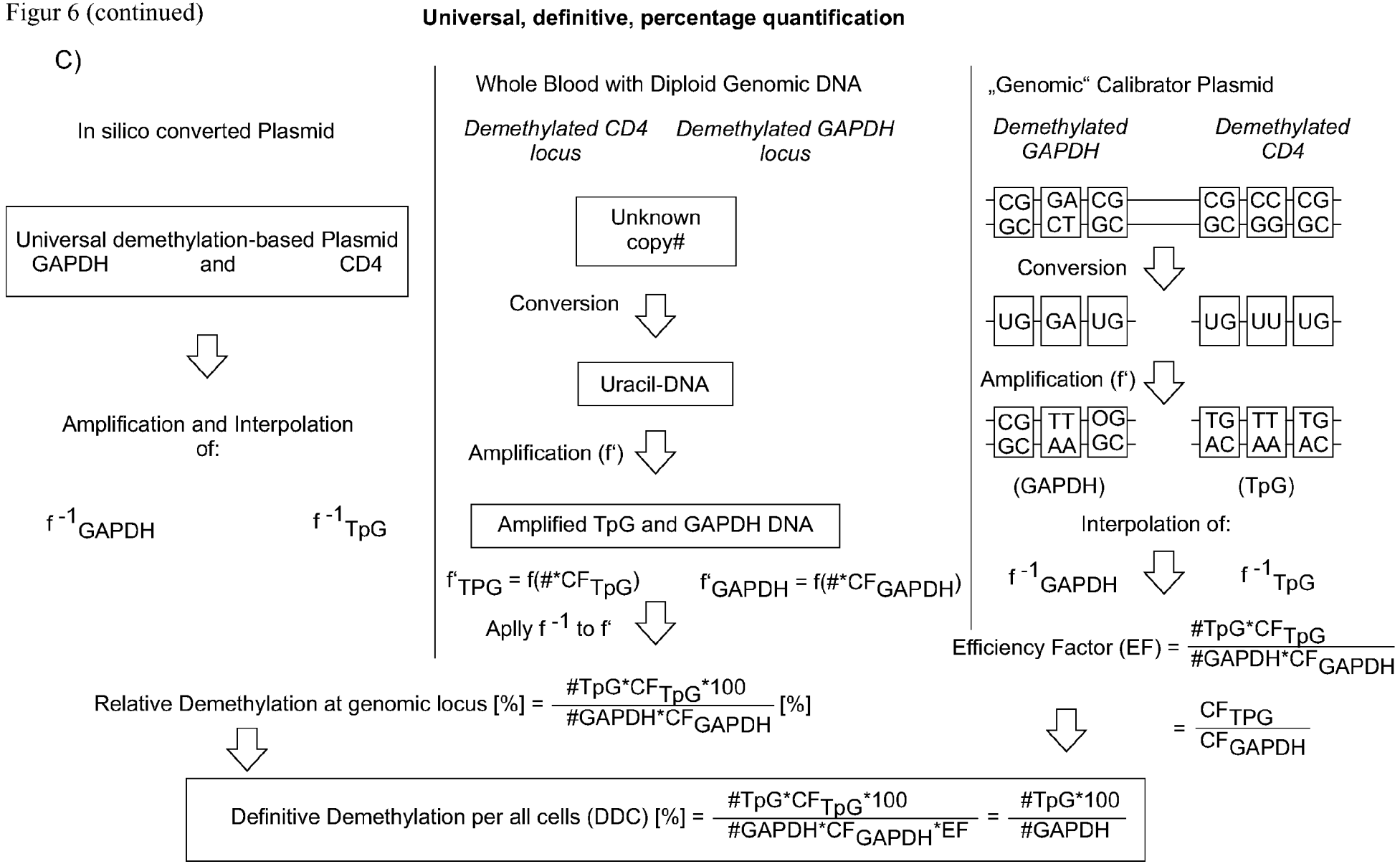
Figure 6



Figur 6 (continued)



Figur 6 (continued)



Figur 6 (continued)

D)

Universal, definitive, absolute quantification

In silico converted Plasmid

Universal demethylation-based Plasmid
GAPDH and GAP-GC

Amplifikation und Interpolation von:

f^{-1}_{GAPDH} $f^{-1}_{GAP\text{-}GC}$

Defined volume (V) of Whole Blood with „Spiker“ Plasmid

„Spiker“ Plasmid with defined #GAP-GC *Demethylated genomic GAPDH locus*

Genomic DNA with Plasmid spike

Conversion

Uracil-DNA

Amplification (f‘)

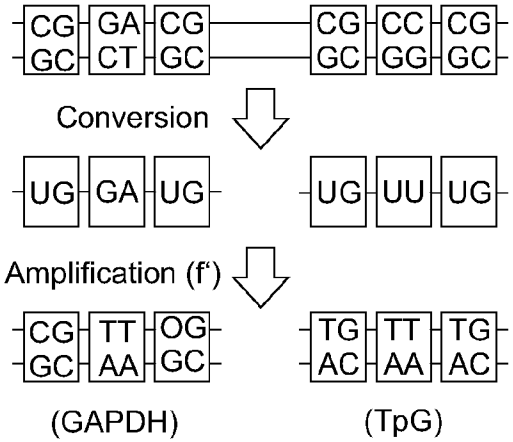
Amplified GAP-GC and GAPDH DNA

$f'_{GAP\text{-}GC} = f(\#*CF_{GAP\text{-}GC})$

Apply f^{-1} to f‘ $f'_{GAPDH} = f(\#*CF_{GAPDH})$

„Genomic“ Calibrator Plasmid with „GAP-GC“

Demethylated GAPDH *Artifical GAP-GC sequence*



Interpolation of:

f^{-1}_{TpG} $f^{-1}_{GAP\text{-}GC}$

$$\text{Leukocyte count (LC) [per µl blood]} = \frac{\#GAPDH*CF_{GAPDH}}{\#GAP\text{-}GC*CF_{GAP\text{-}GC}} * \frac{1}{EF} * \frac{\#GAP\text{-}GC}{V} = \frac{\#GAPDH}{2V}$$

$$EF = \frac{CF_{GAP\text{-}GC}}{CF_{GAPDH}}$$

Absolute copy number of immune cell type [per µl] = LC * DDC

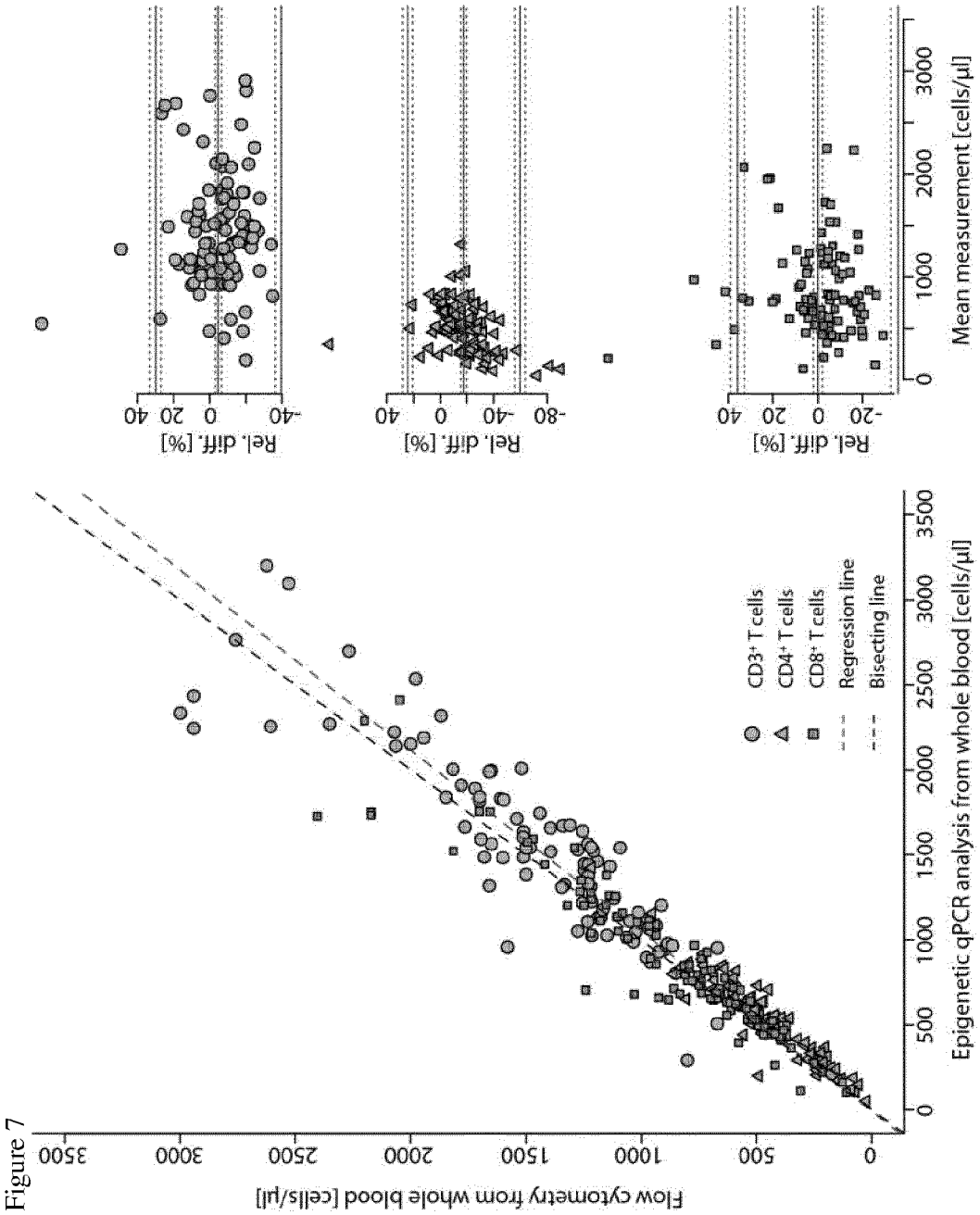
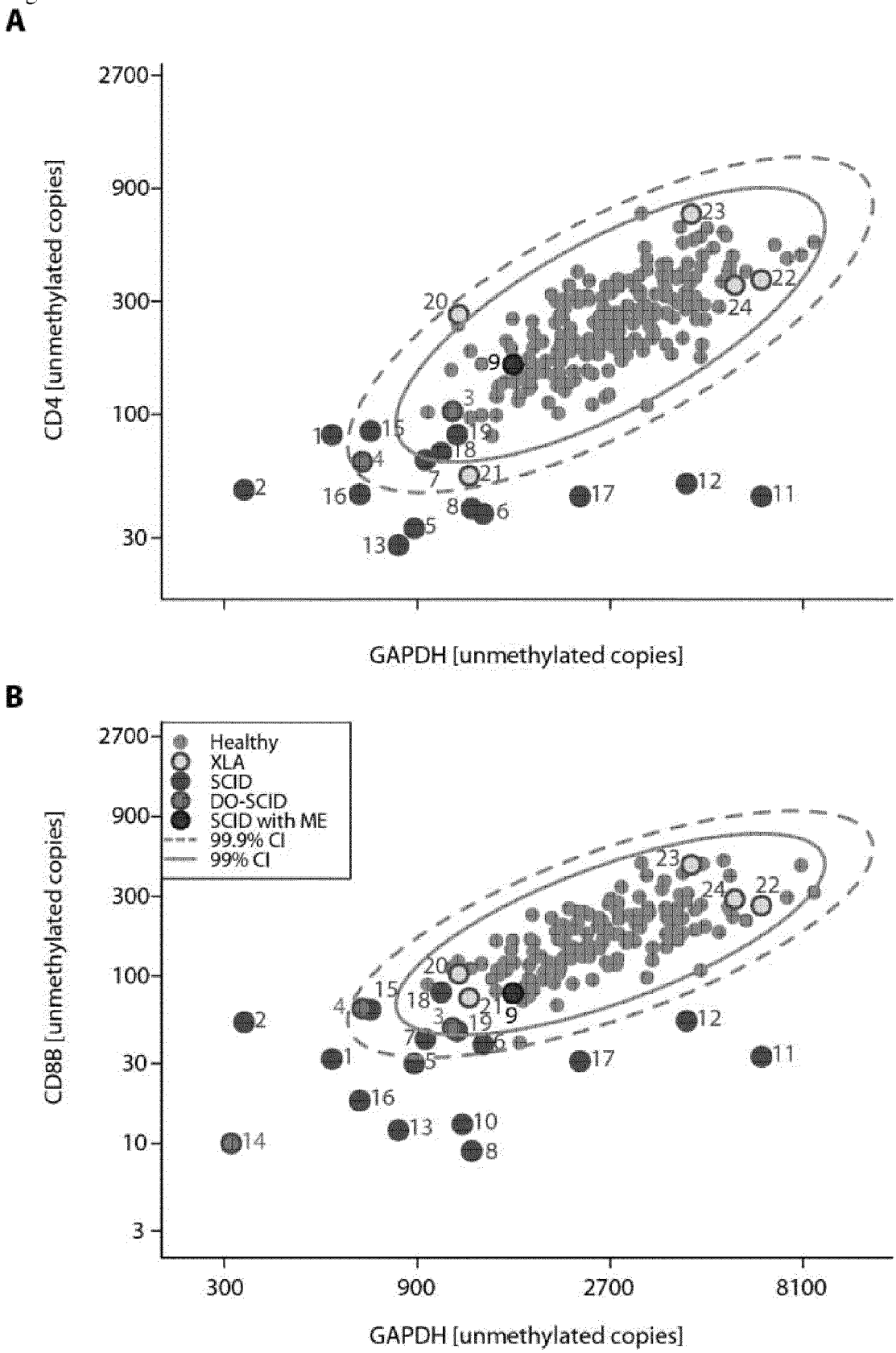

Flow cytometry from whole blood [cells/µl]
Epigenetic qPCR analysis from whole blood [cells/µl]
CD3+ T cells
CD4+ T cells
CD8+ T cells
Regression line
Bisecting line
Rel. diff. [%]
Mean measurement [cells/µl]

Figure 7

Figure 8


A
2700
900
300
100
30
CD4 [unmethylated copies]
GAPDH [unmethylated copies]
B
Healthy
XLA
SCID
DO-SCID
SCID with ME
99.9% CI
99% CI
2700
900
300
100
30
10
3
CD8B [unmethylated copies]
300
900
2700
8100
GAPDH [unmethylated copies]

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/067876

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6881 C12Q1/6851 C12Q1/686
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/135454 A1 (EPIONTIS GMBH [DE]) 19 September 2013 (2013-09-19) the whole document, in particular the claims	1-14
X	WO 2014/080017 A1 (EPIONTIS GMBH [DE]) 30 May 2014 (2014-05-30) the whole document, in particular the claims	1-14
X	WO 2017/050916 A1 (EPIONTIS GMBH [DE]) 30 March 2017 (2017-03-30) the whole document, in particular the claims	1-14

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 2019	13/09/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bassias, Ioannis

1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/067876

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/050882 A1 (EPIONTIS GMBH [DE]) 30 March 2017 (2017-03-30) the whole document, in particular the claims	1-14
X	WO 2014/170497 A2 (EPIONTIS GMBH [DE]) 23 October 2014 (2014-10-23) the whole document, in particular the claims	1-14
X	WO 2012/098215 A1 (UNIV BRUXELLES [BE]; FUKS FRANCOIS [BE] ET AL.) 26 July 2012 (2012-07-26) the whole document, in particular the claims	11-14
X	EP 2 199 411 A1 (EPIONTIS GMBH [DE]) 23 June 2010 (2010-06-23) the whole document, in particular the claims	1-10
X	US 2017/233807 A1 (OLEK SVEN [DE] ET AL) 17 August 2017 (2017-08-17) the whole document, in particular the claims	1-10
X,P	UDO BARON ET AL: "Epigenetic immune cell counting in human blood samples for immunodiagnostics", SCIENCE TRANSLATIONAL MEDICINE, vol. 10, no. 452, 1 August 2018 (2018-08-01), page eaan3508, XP055617272, US ISSN: 1946-6234, DOI: 10.1126/scitranslmed.aan3508 the whole document	1-14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/067876

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		PL 2788510 T3	31-07-2017
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/067876

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 2010069499 A2	24-06-2010
US 2017233807 A1	17-08-2017	US 2017233807 A1	17-08-2017
		US 2019241957 A1	08-08-2019