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A novel digital PCR-based method to quantify (switched) B cells reveals the extent of allelic involvement in different recombination processes in the IGH locus

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ABSTRACT

B cells fulfill an important role in the adaptive immunity. Upon activation and immunoglobulin (IG) class switching, these cells function in the humoral immunity compartment as plasma cells. For clinical applications, it can be important to quantify (switched) B cells accurately in a variety of body fluids and tissues of benign, inflammatory and malignant origin. For decades, flow cytometry and immunohistochemistry (IHC) have been the preferred methods for quantification. Although these methods are widely used, both depend on the accessibility of B cell epitopes and therefore require intact (fixed) cells. Whenever samples are low in quantity and/or quality, accurate quantification can be difficult. By shifting the focus from epitopes to DNA markers, quantification of B cells remains achievable. During differentiation and maturation, B cells are subjected to programmed genetic recombination processes like VDJ rearrangements and class switch recombination (CSR), which result in deletion of specific sequences of the IGH locus. These cell type-specific DNA "scars" (loss of sequences) in IG genes can be exploited as B cell markers in digital PCR (dPCR) based quantification methods. Here, we describe a novel, specific and sensitive digital PCR-based method to quantify mature and switched B cells in DNA specimens of benign and (copy number unstable) malignant origin. We compared this novel way of B cell quantitation with flow cytometric and immunohistochemical methods. Through cross-validation with flow cytometric sorted B cell subpopulations, we gained quantitative insights into allelic involvement in different recombination processes in the IGH locus. Our newly developed method is accurate and independent of the cellular context, offering new possibilities for quantification, even for (limited) small samples like liquid biopsies.

1. Introduction

B cells play an important role in the adaptive humoral immunity. In terms of diagnostics, prognostics, monitoring of disease progression or minimal residual disease, prediction to therapy response and monitoring treatment efficacy, accurate quantification of (infiltrating) B cells in benign, inflammatory and malignant tissues and body fluids is of great importance in a variety of clinical applications (Itoh, 2000; Costa, 2016; Trouvin, 2015; Talmadge, 2011; Fridman, 2011; Castaneda, 2016; Schatton, 2014; Linnebacher and Maletzki, 2012; Shen et al., 2018; Schwartz et al., 2016; Theurich, 2016; Helmink, 2020; Amaria, 2018).

Established quantification methods to determine B cell content in body fluids or solid tissues are flow cytometry and immunohistochemistry (IHC). These methods are very accurate through the use of cell-specific antibodies. Presence and accessibility of marker epitopes are mainly related to the specimen's condition and applied preparation method, with fresh or processed material mostly meeting the required criteria for accurate quantification (Wood, 2013; Walker, 2006). Whenever sample quantity and/or quality is too low (loss of cellular context e.g. due to degradation), quantification of B cells can be

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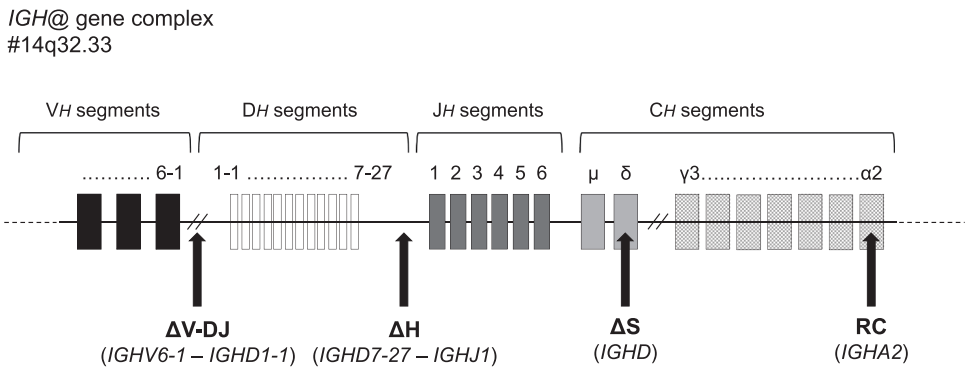

Fig. 1. Schematic depiction of the *IGH@* gene complex (14q32.33). Arrows indicate the regions targeted by dPCR relative to the different genomic features of the gene complex. ΔH is a mature B cell marker that becomes deleted due to the first VDJ rearrangement step (i.e. D-J joining) in the bone marrow during early B cell development. This marker is located between the *IGHD7–27* and *IGHJ1* genes. ΔV-DJ is located between the *IGHV6–1* and *IGHD1–1* genes and is removed by a second subsequent VDJ rearrangement step (i.e. V-DJ joining). ΔS is a switched B cell marker that becomes deleted by class switch recombination (CSR) in germinal centers during isotype switching of activated B cells. This marker is located in the constant gene *IGHD*. The regional corrector (RC) assay targets the most downstream constant gene *IGHA2* and is not deleted by neither VDJ rearrangements nor CSR.

impeded. Alternatively, the method of quantification may be shifted to cell-specific DNA markers. Nevertheless, whereas a broad variety of transcriptionally and translationally expressed cell-type specific molecules has been identified, genomic DNA is essentially identical between different somatic cells within an individual, thereby barely providing cell-type specific DNA markers. However, during B cell differentiation and maturation, the IGH locus is subjected to programmed genetic recombination processes like VDJ recombination and class switch recombination (CSR)(Ollila and Vihinen, 2005). As a consequence, B cells have an altered genome as compared with other cells and specific sequences can be used as cell-type specific markers.

Conventional (multiplex) PCR, combined with deep sequencing techniques, can be applied to determine B cell content at the genomic level. These approaches typically require a pre-amplification step, thereby limiting possibilities for absolute quantification of target molecules. Moreover, these approaches generally target the whole repertoire of IG genes and thereby supply additional information about gene usage (van Dongen, 2003; Evans, 2007; Carlson, 2013). Consequently, a simple B cell quantification results into a complex, expensive and time-consuming procedure. To avoid this, we took advantage of the genetic dissimilarity between B cells and cells of other origin by measuring loss of specific sequences of the IGH locus. Thus, instead of counting a whole repertoire of rearranged IG genes, we designed an indirect counting approach based on the same rationale as previously published by our group for the quantification of T cells (Zoutman, 2017; Zoutman et al., 2019; Nell, 2022; Nell, 2021).

Here we introduce two novel and generic digital PCR (dPCR) assays to quantify mature B cells and switched B cells specifically in DNA specimens of benign and malignant origin. This quantification method was based on measuring two specific markers (ΔH and ΔS) of the IGH locus (14q32.33), which are lost due to different recombination processes (Fig. 1). Marker ΔH is located between the *IGHD7–27* and *IGHJ1* genes and becomes deleted due to the first VDJ rearrangement step (i.e. D-J joining) in the bone marrow during early B cell development (Ollila and Vihinen, 2005). Therefore, it is expected that loss of this marker will be observed in all peripheral mature B cells (both unswitched and switched). Marker ΔS is located in the constant region encoding C delta (*IGHD*01/02)* that becomes deleted by CSR in germinal centers during isotype switching of activated B cells (Liu, 1996; Lefranc, 2001). Therefore, it is expected that loss of this marker will be observed in peripheral switched B cells (i.e. plasma cells and memory B cells) specifically. Conversely, unswitched B cells have not undergone CSR and ΔS must still be present in these cells.

Although it is known that parts of the IGH locus are deleted by these recombination processes, exact quantitative knowledge about ubiquitous loss is lacking and the extent of allelic involvement remains open for debate (Ollila and Vihinen, 2005; Outters, 2015; Vettermann and Schlissel, 2010; Alves-Pereira, 2014; Pichugin, 2017). However, by utilizing the absolute quantitative capacity of dPCR and through cross-validation against flow cytometric sorted B cell subpopulations, we could effectively elucidate these uncertainties.

Specificity of ΔH and ΔS assays was evaluated in a variety of samples and accuracy and dynamic range were assessed by using standard curve data from serial diluted B cell DNA. Moreover, we validated applicability of these assays by determining extents of B cell infiltration in heterogeneous (and copy number unstable) uveal melanoma specimens.

2. Materials and Methods

2.1. Cell Isolation and Sorting

Heparinized blood was drawn from healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. All donors gave their written informed consent.

For standard curve preparations, freshly isolated PBMC samples from three donors were pooled and enriched for B cells up to 90% purity by depletion of non-B cells, using the B Cell Isolation Kit II, Human, according to the protocol supplied by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). We also used L-363 cells, a leukemic IgG plasma cell line and passage 5 cultured fibroblasts from a healthy donor. These cells were cultured from donor skin, as obtained by mammary reduction surgery (Janson, 2013).

A variety of 6 unswitched and 6 switched B cell subpopulations were obtained from 5 healthy donors by fluorescence-activated cell sorting

Table 1
Applied gating strategies for flow cytometric sorting of unswitched and switched B cell subpopulations.

Donor	Unswitched B cells		Switched B cells		
	IgD+ CD27-	IgM+ IgD-	IgA+	IgG+	CD27 + IgD- CD19 + ($CD24^{hi}$ and $CD38^{hi}$ excluded)
1	**X**	**X**	**X**	**X**	
2	**X**	**X**	**X**	**X**	
3	**X**	**X**			
4					**X**
5					**X**

Table 2
Detailed primer and probe information of dPCR assays used to quantify (switched) B cells.

Assay	Description	Gene	Genomic feature	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Probe (5′ to 3′)	Label
ΔH	**B cell marker**	*IGH@*	*IGHD7–27 - IGHJ1 (intergenic)*	AGGGTTTTGGCTGAGCTG	TGGTTTTTGTAGAGCTGCCA	ACCACTGTGCTAACTGGGGACACAGTG	FAM
ΔS	**Switched B cell marker**	*IGH@*	*IGHD*	CTTTCTGCTCTCTGGTAGCC	GGCTGTCTTTCAGGTGAAGT	GGGCGTCCTGCTCTTCTGGGGC	HEX
ΔV-DJ	**V-DJ joining marker**	*IGH@*	*IGHV6–1 - IGHD1–1* (intergenic)	AGCATGTGCCACTTACAACA	TGAGATGAGAACCCAGCCAT	AGCAGAACGAACAGACTCCCCCGC	FAM
REF	**Reference**	*DNM3*	Exon 14	CTAAACACCTCTGCTGATTTCTGC	CCGCCTTTCATGATGCCAATG	TGAGCCACCCCTTGCGAATCACCT	FAM and HEX
REF	**Reference**	*TTC5*	PrimePCR ddPCR CNV Assay dHsaCP2506733 (Bio-Rad Laboratories, Hercules, CA, USA)				HEX
RC	**Regional corrector**	*IGH@*	*IGHA2*	AGCCTCGACAGCACCCC	GGTCACACTGAGTGGCTCCT	CGTGGTCGTCGCATGCC	FAM

(FACS) using a BD FACSAria II cell sorter and BD FACSDiva Software version 8.0.1 (BD Biosciences, San Jose, CA, USA) following diverse gating strategies: Sorted unswitched B cells were either $IgD^{+}CD27^{-}$ or $IgM^{+}IgD^{-}$ and switched memory B cells were either IgA^{+}, IgG^{+} or $CD27^{+}IgD^{-}CD19^{+}$ ($CD24^{hi}$ and $CD38^{hi}$ excluded) (Table 1).

2.2. Uveal Melanoma samples

Snap-frozen and Formalin Fixed Paraffin Embedded (FFPE) primary uveal melanoma samples, obtained via enucleation, were available from the Department of Ophthalmology, Leiden University Medical Center (LUMC). All patient samples were collected with informed consent and this study was approved by the LUMC Biobank Committee and Medisch Ethische Toetsingscommissie under no. B14.003/DH/sh and B20.026.

2.3. IHC analysis

4 μm Sections of FFPE tissue were mounted on adhesive glass slides. Deparaffinization was executed according to the BenchMark Ultra protocol. Antigen retrieval was performed by CC1 antigen retrieval solution (Ventana Medical Systems, Oro Valley, USA). Specimens were incubated with PAX-5 antibodies (ref: M7307; clone: Dak-PAX5, DAKO, Glostrup, Denmark), detection was performed with the ultraView Universal DAB Detection Kit and amplification with the Amplification Kit (Ventana Medical Systems). Next, all specimens were counterstained with hematoxylin II (Ventana Medical Systems) and coverslipped. Each slide contained a positive control and stainings were performed on the VENTANA BenchMark ULTRA (Ventana Medical Systems).

Stained slides were scanned by a Panoramic Midi Scanner (version 11, 3DHISTECH, Budapest, Hungary). Sections were analyzed using the QuantCenter software (version 2.1, 3DHISTECH). In short, Cutting- and staining artefacts were excluded from scoring by annotating the rest of the tissue. Then one has to train the system by selecting a few representative example regions/cells. In this way the system learns which cells to count. After selecting these areas one can preview the training session to check if the correct cells are directed to the proper assigned label. If not correct, the training session can be adjusted. After correct training of the system quantification of the annotation occurs. Since the samples presented large differences in pigmentation, this procedure had to be repeated for each section. The percentage of B cells was calculated by dividing the PAX-5 nuclei (red) by the total amount of nuclei (i.e. PAX-5 + hematoxylin stained nuclei (blue)) multiplied by 100.

2.4. DNA Isolation

Cultured, enriched and sorted cell samples were subjected to DNA isolation using either the QIAamp DNA Blood Mini or Micro kit depending on the amount of input material, according to the protocol supplied by the manufacturer (Qiagen, Hilden, Germany). DNA from snap-frozen primary uveal melanoma samples was isolated from 25 × 25 μm sections using the QIAamp DNA Mini Kit (Qiagen).

2.5. Standard curve preparation

To evaluate the accuracy and dynamic range of the developed dPCR assays, a standard curve was prepared with L-363 and enriched B cell DNA. These samples were individually serially diluted in a background of non-rearranged *IGH@* DNA, originating from fibroblasts. Combined, the prepared standard curve contained 16 dilution points consisting of 0.7%, 0.8%, 1.4%, 1.6%, 2.8%, 3.1%, 5.6%, 6.3%, 11.3%, 12.5%, 22.5%, 25%, 45%, 50%, 90% and 100% (w/w) B cell DNA.

2.6. Digital PCR (dPCR)

Digital PCR (dPCR) represents the third generation of PCR and provides new possibilities for molecular quantification. dPCR enables sensitive, precise and reproducible absolute quantification of single nucleic acid molecules by combining sample partitioning (limiting dilution) with Poisson statistical data analysis(Vogelstein and Kinzler, 1999). When partitioning samples, admixed nucleic acid molecules and PCR solution are separated into thousands of partitions (droplets) prior to amplification. This limiting dilution typically results in droplets containing only one or zero amounts of target molecules. After amplification, presence of target DNA is measured by detecting signals from used fluorescently labeled probes, resulting in droplets scoring '1′ or '0′; similar to a digital readout.

To quantify mature B cells and switched B cells, dPCR assays were developed targeting ΔH and ΔS loci (*IGH@* at 14q32.33) respectively (Fig. 1). *DNM3* (1q24.3) and *TTC5* (14q11.2) were used as stable genomic reference genes in our cohort of samples. Duplex experiments were carried out for measuring ΔH or ΔS and normalization against one of the mentioned reference genes.

In the event of a copy number alteration (CNA) involving the IGH locus, it is crucial to implement a regional corrector (RC) assay to recognize and correct mathematically for such a gain or loss. This methodology was previously published by our group and now extended for the quantification of B cells(Nell, 2022; Nell, 2021). B cell markers ΔH and ΔS, a RC targeting *IGHA2* and reference *DNM3* or *TTC5* were measured using a 2 × 2 multiplex dPCR experimental setup.

Initially, in accordance to the rationale for quantification of T cells, (switched) B cell fractions were calculated as published previously (Zoutman, 2017; Zoutman et al., 2019; Nell, 2022; Nell, 2021). Applicability of this approach is firstly dependent on the degree of ubiquitous loss of marker ΔH in mature B cells and ΔS in switched B cells, and secondly, on the number of involved alleles (i.e. mono- or biallelic loss).

All dPCR experiments were performed in 22 μL using 10–50 ng of

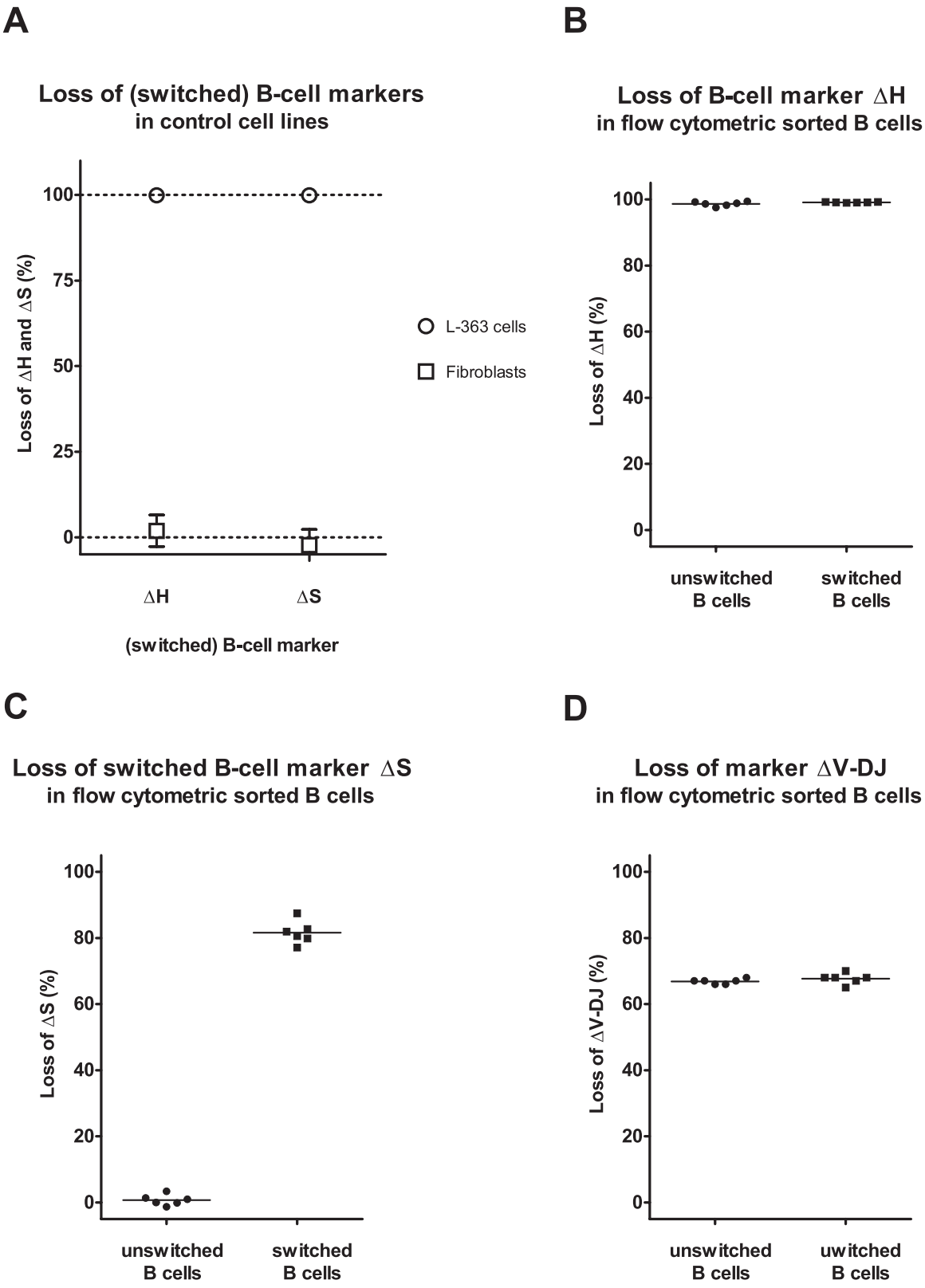

Fig. 2. Quantification of B cell DNA markers with dPCR in cell lines and in cells sorted by flow cytometry. A. Loss of mature B cell marker ΔH and switched B cell marker ΔS in leukemic IgG plasma cell line L-363 (circles) and retention of these markers in fibroblasts (squares). B. Loss of mature B cell marker ΔH in unswitched (left) and switched (right) B cell subpopulations. C. Retention of switched B cell marker ΔS in unswitched B cell subpopulations (left) and loss in switched B cell subpopulations (right). D. Loss of V-DJ joining marker ΔV-DJ in unswitched (left) and switched (right) B cell subpopulations.

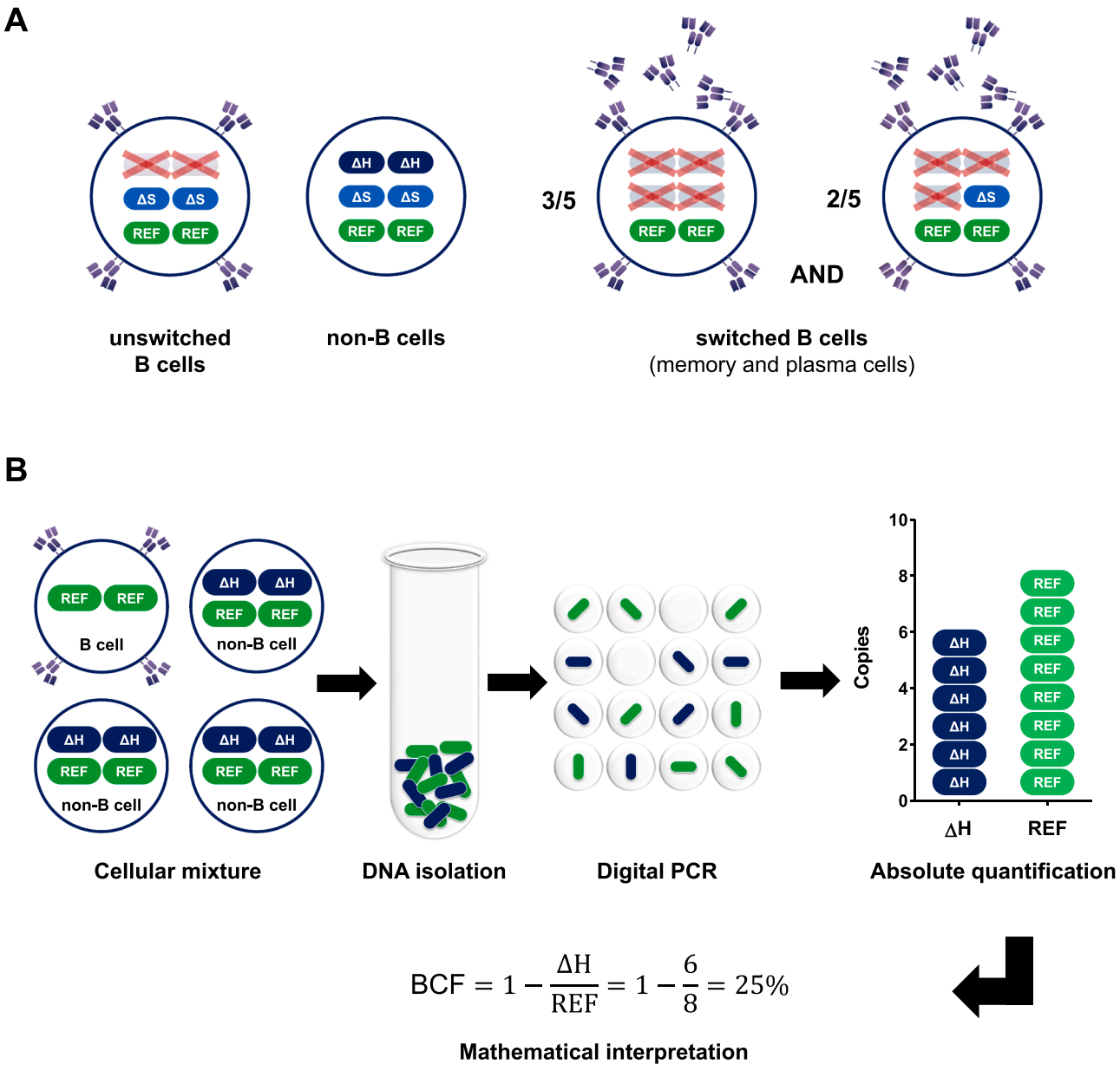

Fig. 3. Cell specific quantification of mature B cells and switched B cells by measuring loss of IGH loci using dPCR. A. B cells and cells of other origin (non-B cells) differ genetically because of VDJ rearrangements and CSR of the *IGH@* gene complex (14q32.33). Relative to non-B cells, unswitched and switched B cells are lacking mature B cell marker ΔH (dark blue) biallelically. In addition, switched B cells specifically lack the switched B cell marker ΔS (light blue) biallelically in 3/5 and monoallelically in 2/5 of the cells. B. Workflow example of mature B cell quantification by measuring loss of ΔH. A DNA specimen, containing genomes from B cells (25%) and non-B cells (75%), is partitioned into up to twenty thousands droplets before PCR amplification of ΔH with FAM labeled hydrolysis probes and a reference gene (REF; HEX labeled). After amplification, positive and negative droplets are counted to calculate the absolute B cell fraction (BCF) of the analyzed sample. C. Similar workflow example, here for the quantification of switched B cells. In a DNA specimen, containing genomes from switched B cells (62.5%) and non-B cells (37.5%), marker ΔS is quantified by dPCR with FAM labeled hydrolysis probes and the reference gene (REF) with HEX labeled probes. After amplification, droplets are counted to calculate the absolute switched B cell fraction (SBCF) of the analyzed sample.

DNA, ddPCR Supermix for probes (Bio-Rad Laboratories, Hercules, CA, USA), FAM- and HEX-labeled hydrolysis probes, forward and reverse primers (Table 2), and DNA restriction digestion enzyme *Hae*III (New England Biolabs, Ipswich, MA, USA). All components were added directly to the dPCR reaction solution, according to the protocols we described (Zoutman, 2017; Zoutman et al., 2019; Nell, 2022; Nell, 2021). Droplets were generated using an AutoDG System (Bio-Rad) and PCR amplification was performed in a T100 Thermal Cycler (Bio-Rad). Cycle parameters were as follows: enzyme activation for 10 min at 95°C; denaturation for 30 s at 94°C; annealing and extension for 1 min at 60°C for 40 cycles; enzyme deactivation for 10 min at 98°C; cool step for 30 min at 4°C followed by an infinite cooling at 12°C. The ramp rate for all cycles was 2°C/second. Droplets were measured by a QX200 Droplet Reader (Bio-Rad) and analyzed using Quantasoft software (version 1.7.4; Bio-Rad) and Roodcom WebAnalysis (version 1.9.4, available via https://webanalysis.roodcom.nl).

2.7. Pan-cancer analysis of CNAs involving the IGH locus

The pan-cancer analysis of CNAs involving the IGH locus was carried

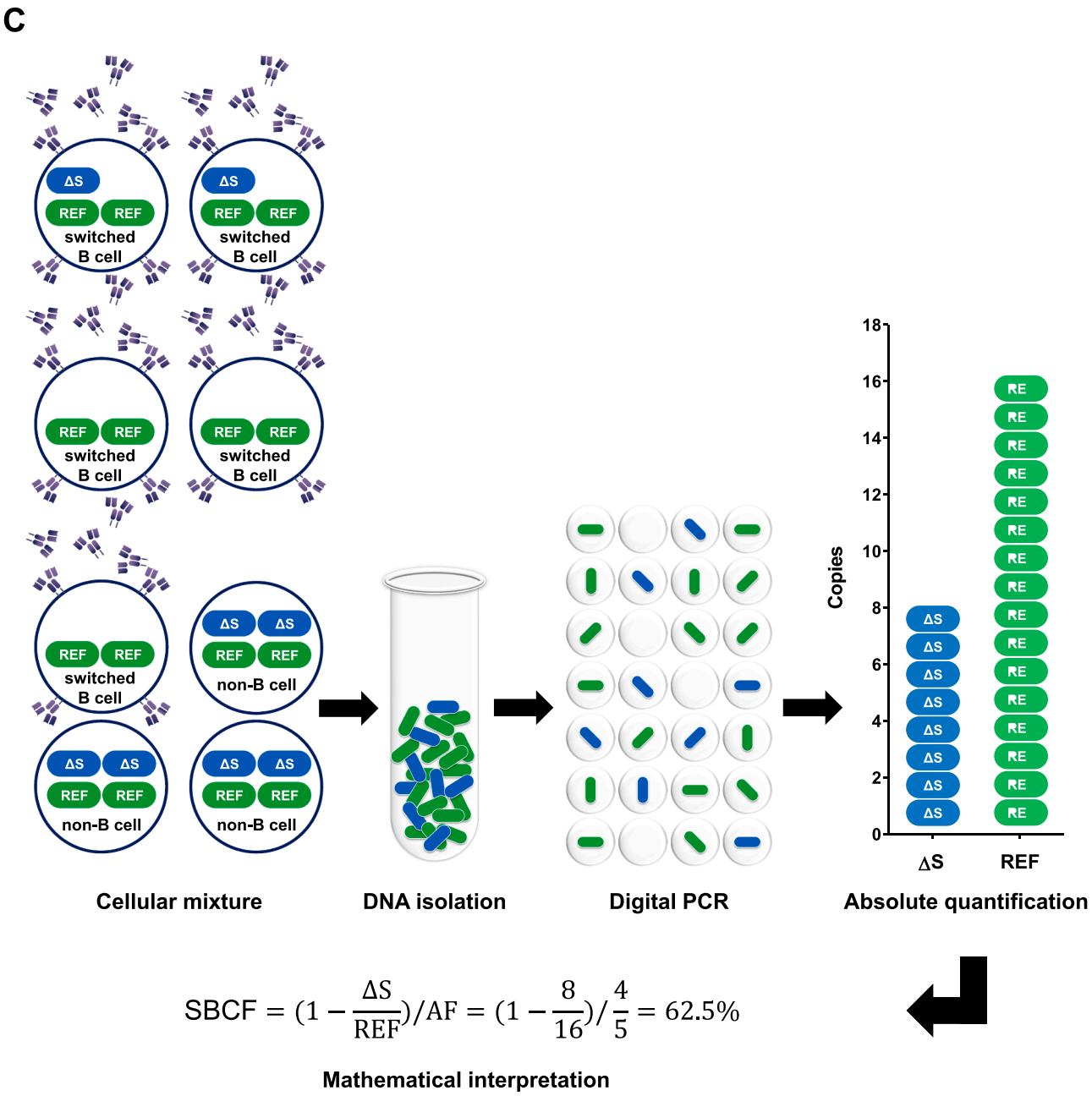

Fig. 3. (*continued*).

out using the 'Copy Number' and 'Merged Sample Quality Annotations' data acquired via https://gdc.cancer.gov/about-data/publications/pancanatlas. For each tumor specimen, the segment mean copy number values for the IGH locus were assessed and thresholded by a noise cutoff of 0.3: Values smaller than − 0.3 were marked as 'loss' and values larger than 0.3 as 'gain'. Next, the proportion of cases per tumor type with a loss, gain or no CNA was calculated. The pan-cancer statistics were based on the mean of these frequencies. The Lymphoid Neoplasm Diffuse Large B cell Lymphoma (DLBCL) dataset was excluded from our analysis as in these malignancy (precursors of) B cells form the population of tumor cells. The complete analysis was carried out using R (version 4.0.3), RStudio (version 1.4.1103) and R package data.table (version 1.13.6). All custom scripts are available via https://github.com/rjnell/b-cell-quantification.

2.8. Statistical Analysis

Statistical analysis was performed by using GraphPad Prism version 8.0.1 (GraphPad Software, La Jolla, CA). To evaluate dynamic range and accuracy of ΔH and ΔS assays, linear regression and standard Pearson *r* correlation test were performed on standard curve data.

3. Results

3.1. ΔH is a Mature B Cell DNA Marker That is Biallelically Lost in B Cells

To evaluate the specificity of marker ΔH we measured its loss in a variety of samples. Initially, we analyzed DNA specimens from L-363 and fibroblast cell cultures which served as pure sources of B cells and non-B cells respectively. We observed no loss of ΔH in fibroblasts, however, in L-363 cells this marker was undetectable, suggesting a

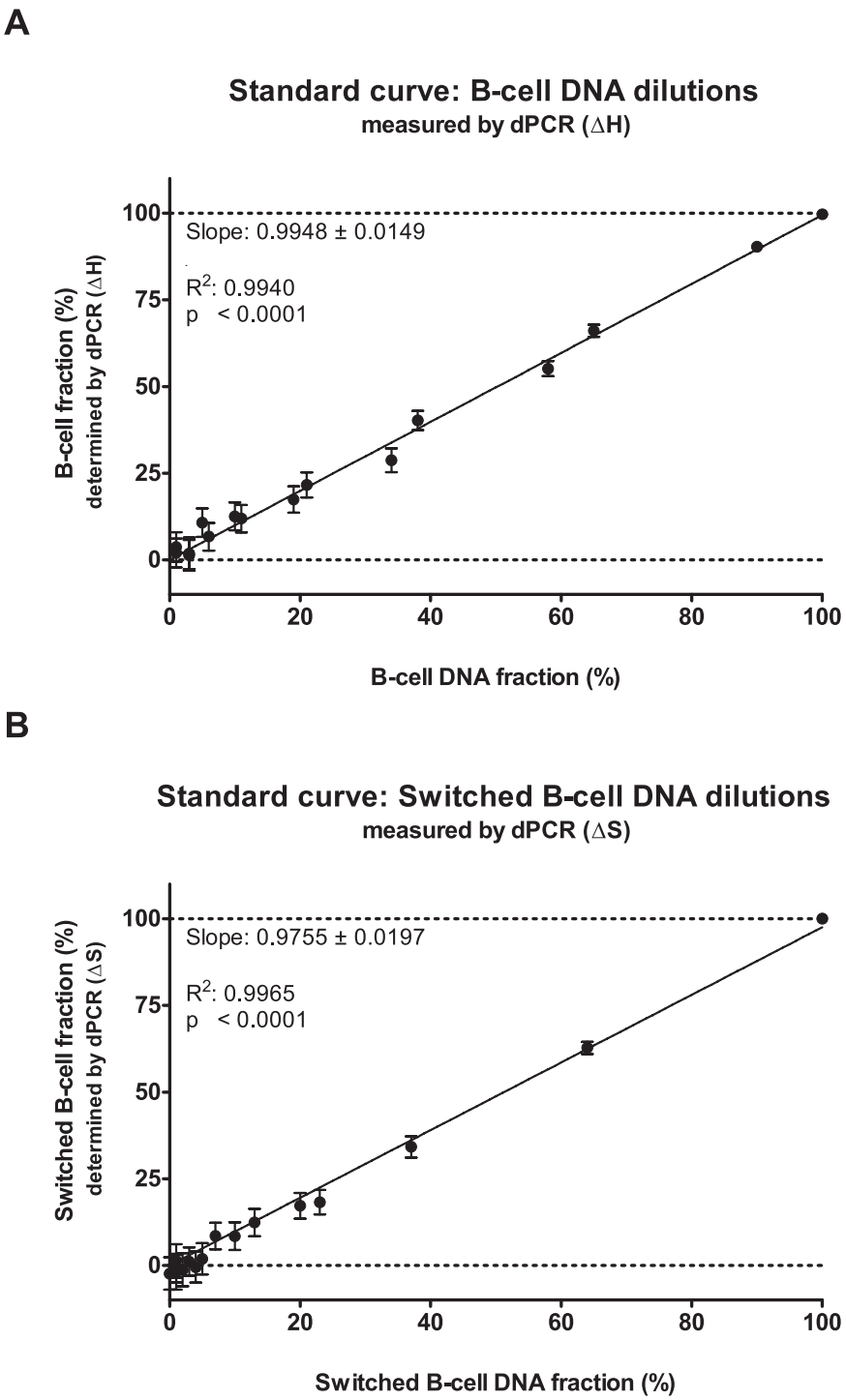

Fig. 4. Validation of the ΔH and the ΔS markers in dPCR experiments with in vitro dilutions of (switched) B cell DNA. B cell DNA from enriched B cell samples from healthy donors and L-363 cells were serial diluted in fibroblast DNA. Scatter plots with linear regression for measuring serial diluted (switched) B cell DNA were generated. The graphs illustrate the relation between fractional input of mature B cell (A) and switched B cell (B) DNA (x-axis) and the cellular fraction as determined by dPCR (y-axis). Error bars indicate 95% confidence intervals, as calculated by QuantaSoft according to Poisson distribution on scored droplets.

biallelic loss (Fig. 2A).

To confirm this finding in benign, polyclonal and uncultured B cells, we analyzed a variety of 12 sorted mature B cell samples as obtained by FACS (Table 1). Based on the previously measured biallelic loss of ΔH in L-363 cells, we quantified the B cell purity in DNA specimens of the sorted samples. In 11 out of 12 samples the purity was > 99% and in the remaining sample it was > 98% (Fig. 2B).

These results demonstrate effectively executed cell sorting of B cell subpopulations and, moreover, verified that marker ΔH is specifically, ubiquitously and biallelically lost in mature (unswitched and switched) B cells (Fig. 3A). Hence, it is allowed to calculate a B cell fraction according to the same arithmetic approach as previously published by our group for the quantification of T cells (Zoutman, 2017; Zoutman et al., 2019). This implies that the *classic model* can be applied to calculate a B cell fraction (BCF) in a copy number stable sample(Nell, 2022; Nell, 2021). This model requires measured concentrations of the mature B cell marker ([ΔH]) and a genomically stable reference target ([REF]), according to the following formula.

Classic model:

$$BCF = 1 - \frac{[\Delta H]}{[REF]}$$

An exemplary workflow for the quantification of B cells is given in Fig. 3B.

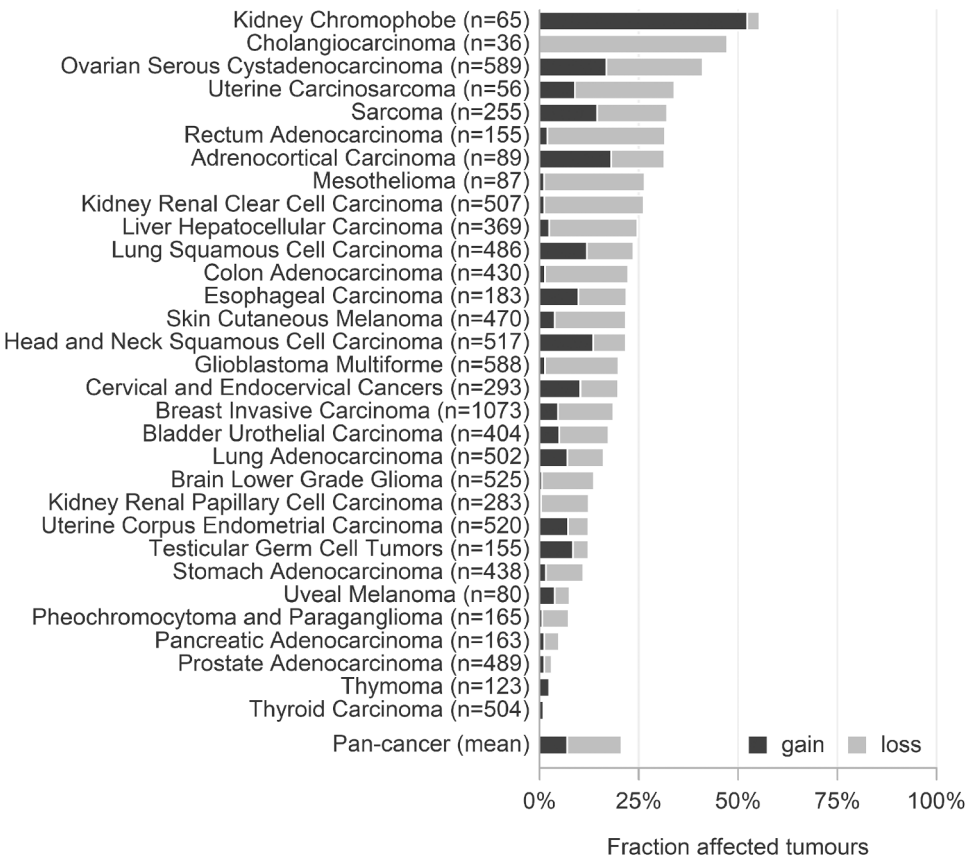

Fig. 5. Pan-cancer analysis results of copy number alteration (CNA) prevalence involving the IGH locus. Bars represent the percentage of gains (black) and losses (grey) of IGH found in a variety of malignancies. Numbers (n) represent the amount of individual cases included per tumor type.

3.2. ΔS is a Switched B cell DNA Marker That is Often Biallelically Lost in Switched B Cells

To evaluate the specificity of marker ΔS, we used the same sample selection as in **paragraph 3.1**. When analyzing cell cultures, we observed no loss in fibroblasts. But in L-363 cells marker ΔS was undetectable, thereby indicating biallelic loss (Fig. 2A). However, since L-363 cells are derived from leukemic IgG plasma cells and are clonally expanded, observed biallelic loss may not be a generic event in benign switched B cells accordingly.

To investigate to what extent biallelic loss occurs in benign, polyclonal and uncultured B cells, we analyzed the same set of sorted B cell samples (Table 1). In unswitched B cells, indeed, we observed no loss in any of the samples (Fig. 2C). In combination with the ubiquitous biallelic loss of ΔH, we are sure that these cells have not undergone CSR and consequently are true unswitched B cells as well. In contrast, marker ΔS was specifically lost in switched B cells, however, not in a pure mono- or biallelic manner. We observed that ΔS was consistently lost in 4/5 of the measured alleles, ranging from 77.1% to 87.4% (Fig. 2C). Since switched B cells underwent CSR, ΔS must be deleted at least monoallelically and, consequently, it was expected that it would be absent in 50% of the analyzed alleles. Obviously, it was lost more often, thereby indicating a common biallelic deletion as well. Presuming that CSR essentially must have taken place with at least one allele, we can extrapolate that in 3/5 of switched B cells ΔS is biallelically lost and in 2/5 monoallelically (Fig. 3A).

Since ΔS was not lost in either a pure mono- or biallelic fashion, it is not allowed to calculate a switched B cell fraction following the same arithmetic approach as described in **paragraph 3.1** for mature B cells. In order to make switched B cell quantification still feasible, we needed to correct for this imbalance. By adding an allelic-factor (AF) to the formula, we were still able to enumerate these cells accurately. This AF is determined by the fractional observed loss of ΔS of the measured alleles from sorted switched B cell samples.

A slightly modified *classic model* can be applied to calculate a switched B cell fraction (SBCF) in a copy number stable sample (see **paragraph 4.1** for the mathematical derivation). This model requires measured concentrations of the switched B cell marker ([ΔS]) and a genomically stable reference target ([REF]). Including the AF of 4/5, the formula will be as follows:

Classic model:

$$\mathrm{SBCF} = \frac{1 - \frac{[\Delta S]}{[REF]}}{4/5}$$

An exemplary workflow for the quantification of switched B cells is given in Fig. 3C.

3.3. Biallelic involvement in V-DJ joining is less ubiquitous than in D-J joining

To gain more detailed insights into allelic involvement in different stages of VDJ rearrangements, we developed an additional assay. We called this assay ΔV-DJ and it targets a sequence between the *IGHV6–1* and *IGHD1–1* genes (Fig. 1). Where marker ΔH is deleted during the first step of recombination (i.e. D-J joining), ΔV-DJ is removed by a second subsequent event (i.e. V-DJ joining) (Ollila and Vihinen, 2005).

We measured loss of ΔV-DJ in the same set of 12 sorted B cell samples (Table 1). Since mature B cells underwent VDJ rearrangements, ΔV-DJ must be deleted at least monoallelically and, consequently, it was expected that it would be absent in 50% of the analyzed alleles. However, we observed that it was lost in 2/3 (on average 67%) of the measured alleles. This loss was consistent in both unswitched and

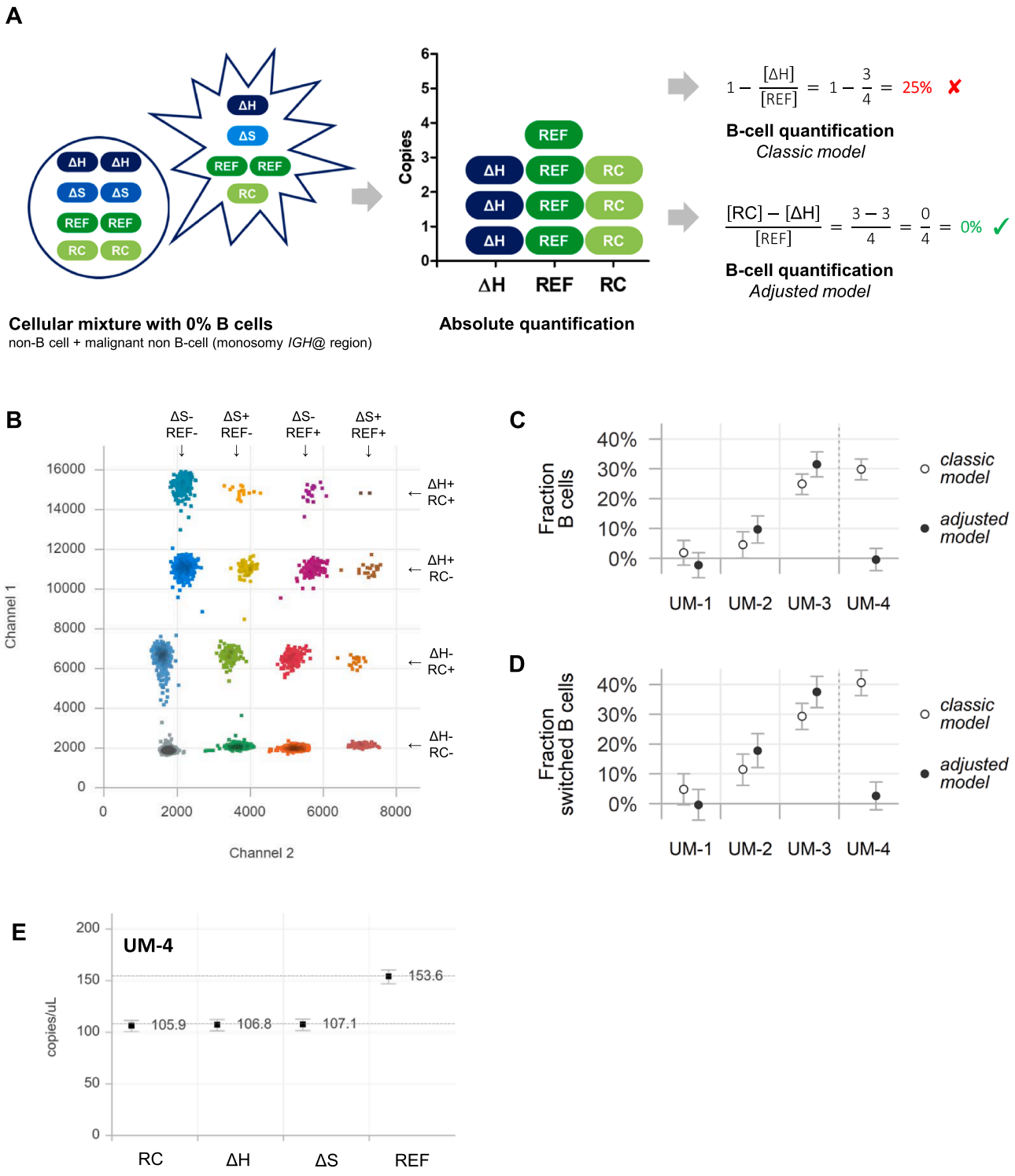

Fig. 6. Absolute quantification of mature B cells and switched B cells with dPCR in copy number stable and unstable samples by using a regional corrector (RC). A. Exemplary workflow of a B cell quantification following the *classic* and *adjusted model* in a DNA specimen of malignant origin. A sample without infiltrating B cells contains exclusively genomes from healthy stromal cells (50%) and tumor cells with monosomy of the IGH locus (50%). After quantification of mature B cell marker ΔH, a stable reference (REF, dark green) and the RC, (light green), the fraction of B cells can accurately be calculated by using the *adjusted model* solely. B. 2D-plot example of a 2 × 2 multiplex dPCR experiment performed on a UM sample. Here, ΔH, ΔS, RC and REF measurements were combined into a single dPCR experiment. By using Roodcom WebAnalysis, 16 distinct clusters were identified and used to calculate the concentrations of the individual targets as indicated by the arrows. C. Quantified B cell fractions of UM samples as determined by dPCR following the *classic* (open circles) and *adjusted model* (closed circles). Tumor cells in UM-4 harbor monosomy of chromosomal arm 14q (including the IGH locus). Because of this, the actual B cell fraction can only be determined by using the adjusted model (similar to the situation as described in "A"). D. Same situation as shown in "C", now displayed for the quantification of switched B cell fractions. E. Measured concentrations of all targets in sample UM-4 (harboring monosomy of IGH). Due to loss of markers ΔH and ΔS in the tumor cells, an accurate (switched) B cell fraction calculation is impeded. However, since the RC was equally lost, a correct quantification is still feasible by using the *adjusted model*.

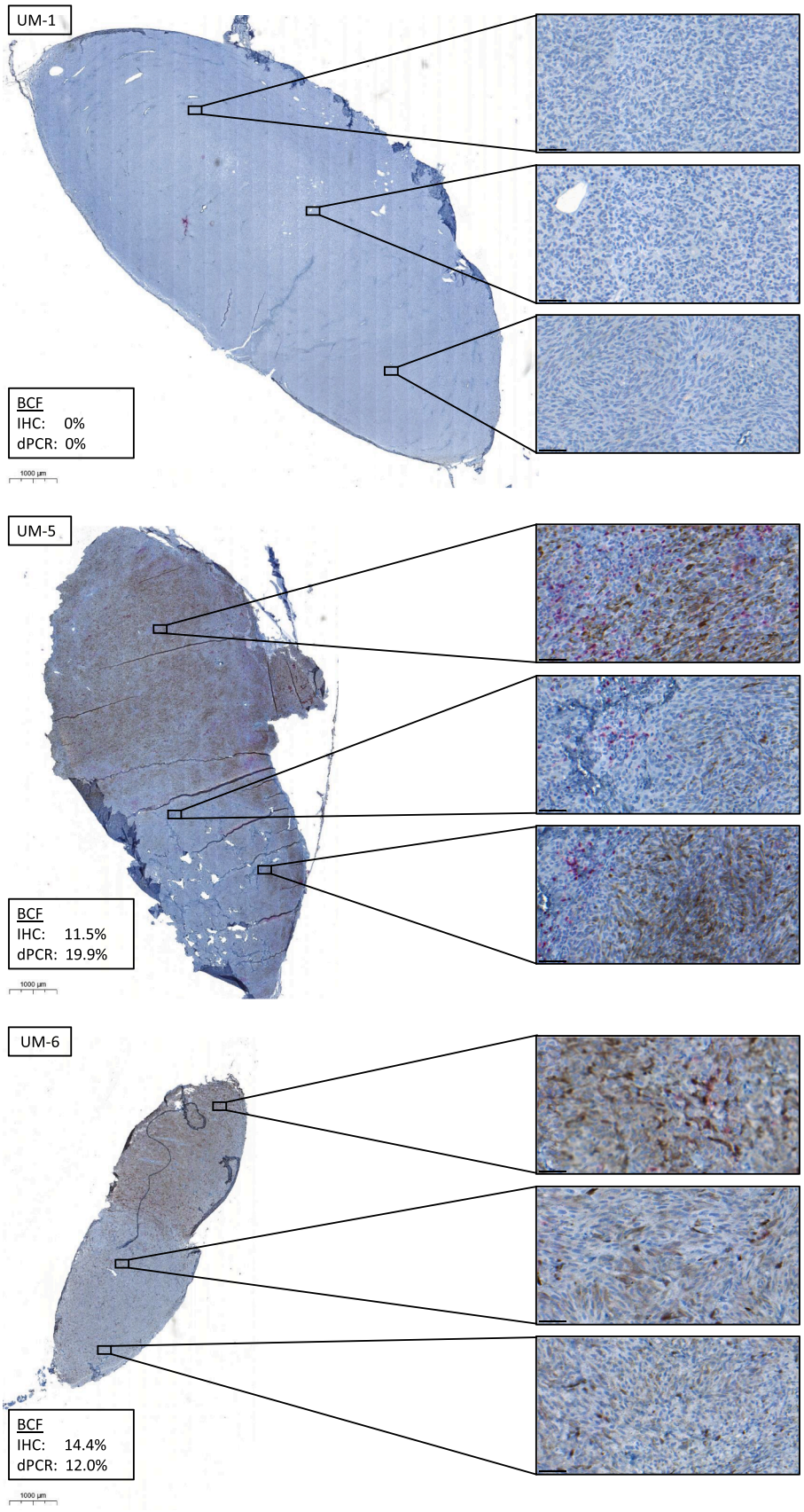

Fig. 7. IHC-based quantification of B cells in sections of 3 different Formalin Fixed Paraffin Embedded UM samples with varying extends of infiltration. All cell nuclei were stained with hematoxylin (blue) and B cell nuclei were specifically stained with PAX-5 (red). Double stained B cell nuclei appear purple. Overview pictures (1x) of UM cross sections and a representative selection of 40x magnified pictures (scale bars represent 50 µm) show a spatial heterogeneity of (clusters of) B cell infiltration and presence of pigmentation (brown). B cell fractions were determined by calculating the fractional abundance of PAX-5 stained nuclei among all nuclei (IHC) and compared with the B cell fractions as determined by dPCR.

switched B cells (Fig. 2D). Presuming that V-DJ joining essentially must have taken place with at least one allele, we could extrapolate that in approximately 1/3 of mature B cells this region is biallelically lost and in 2/3 monoallelically.

3.4. Accuracy and Dynamic Range of ΔH and ΔS dPCR are High

After demonstrating cell specificity of mature B cell marker ΔH and switched B cell marker ΔS, we evaluated the accuracy and dynamic range of dPCR based quantification by using a standard curve. This curve contained 16 dilution points ranging from 0.7% to 100% (w/w) B cell DNA.

Linear regression revealed a high relationship between DNA input and the fraction of B cells as determined by dPCR. Regarding quantification of mature B cells, the slope for ΔH dPCR was significantly ($P < 0.0001$) close to a linear regression of 1 (i.e. 0.9948), the coefficient of determination (R^2) was 0.9940 (Fig. 4A). The slope for quantification of switched B cells by ΔS dPCR was similar: 0.9755 ($P < 0.0001$) and corresponding R^2 was 0.9965 (Fig. 4B). The dynamic range of quantification guarantees high accuracy for detecting low to absolute concentrations of (switched) B cell DNA, as indicated by the small 95%-confidence intervals for both assays.

3.5. Use of Multiplex dPCR in Uveal Melanoma Samples Validates Quantification of (Switched) B cells in Heterogeneous and Copy Number Unstable Specimens

Our previous results validated that the ΔH and ΔS assays can be used to accurately determine (switched) B cell fractions in DNA samples. Importantly, however, the mathematical rationale behind these calculations requires that the chosen target (ΔH or ΔS) and reference are not involved in any CNA and would follow our predefined *classic model*. This assumption is met in most benign conditions, as usually none of the cells harbors unexpected CNAs. In contrast, copy number instability is a frequent characteristic of malignant cells(Taylor, 2018), challenging the validity of our *classic model* in specimens where (healthy) B cells are mixed with cells from a cancerous origin.

While an unstable reference can be replaced (as demonstrated earlier, practically any disomic locus may suffice) (Zoutman et al., 2019), ΔH and ΔS are unique markers only found as part of the IGH locus. As a consequence, a CNA of this locus in admixed cancer cells cannot be resolved by switching markers. A loss or gain would be erroneously attributed to the presence or absence of (switched) B cells, leading to an over- or underestimation of these cells as demonstrated previously for T cells(Nell, 2022; Nell, 2021).

To evaluate the prevalence of CNAs affecting marker ΔH and ΔS in cancer, we analyzed genomic profiles of 10,599 cases spanning 31 tumor types that are part of the TCGA pan-cancer dataset (Taylor, 2018; Weinstein, 2013). Alterations affecting the IGH locus were identified in ~23% of the tumors, with losses and gains of both markers in ~14% and ~9% of the cases, respectively (Fig. 5). Consequently, use of the *classic model* would lead to incorrect (switched) B cell fractions in ~1/4 of the tumor specimens.

For our DNA-based T cell quantification, we recently introduced the *adjusted model*, an extended form of the *classic model* which allows for the detection and normalization of CNAs affecting the T cell marker locus(Nell, 2022; Nell, 2021). Similarly, this concept can be applied on our B cell quantification model as well. The *adjusted model* is based on the incorporation of an extra target, a so-called regional corrector (RC), into the quantitative analysis. This target must be located in close proximity of marker ΔH and ΔS (ideally within the IGH locus), but should be present on both alleles in B cells. Consequently, when a CNA in non-B cells encompasses the IGH locus, the copy number of the RC will be equally altered. Next, quantification of the RC allows for a mathematical correction of the disturbed B cell quantification. An exemplary workflow of the *adjusted model* is presented in Fig. 6.A.

For our application, the most suitable RC is *IGHA2 * 01/02/03*, the most downstream C gene of IGH locus (Fig. 1). Relative to marker ΔH and ΔS, *IGHA2* is the most proximally located target which is not deleted by neither VDJ rearrangements nor CSR in B cells (Pan-Hammarström et al., 2007). To combine ΔH, ΔS, RC and REF measurements in a single dPCR experiment, we developed a 2 × 2 multiplex experimental setup (Fig. 6B), according to previously described methodology (Nell, 2022; Nell, 2021; Whale et al., 2016).

Whenever a B cell fraction (BCF) has to be calculated in a copy number unstable sample (such as a malignancy), besides the measured [ΔH] and [REF], also the concentration of regional corrector *IGHA2* ([RC]) should be taken into account according to the formula below.

Adjusted model:

$$BCF = \frac{[RC] - [\Delta H]}{[REF]}$$

Whenever a switched B cell fraction (SBCF) needs to be calculated in a copy number unstable sample, [ΔS], [REF] and [RC] should be taken into account, including the AF of 4/5 (see **paragraph 4.2** for a detailed explanation and mathematical derivation).

Adjusted model:

$$SBCF = \frac{\frac{[RC]-[\Delta S]}{[REF]}}{4/5}$$

To validate the applicability of the *adjusted model* for a dPCR-based (switched) B cell quantification in cancer, we selected four primary uveal melanoma samples (UM-1 to UM-4) with various degrees of B cell infiltration to be analyzed using our multiplex experimental setup. We assured that the chosen reference is copy number stable in the four tumors and used the *classic* and *adjusted model* to determine B cell and switched B cell fractions.

Uveal melanomas are characterized by low levels of aneuploidy (Furney, 2013) and, based on our pan-cancer analysis, only ~7% of the uveal melanomas contains a CNA comprising the IGH locus (Fig. 5). In line with this, no gains or losses were present in most samples (UM-1, 2 and 3) and the calculated (switched) B cell fractions did not differ significantly between the *classic* and *adjusted model* (Figs. 6C and 6D). In contrast, UM-4 was previously characterized by a loss of the entire chromosome arm 14q in the cancer cell population (data not shown), similar to the situation proposed in Fig. 6A. This CNA resulted in monoallelic ΔH and ΔS loss and, consequently, in an incorrect, overestimated (switched) B cell fraction when following the *classic model* (Figs. 6C and 6D). However, as the RC was equally lost, lower abundance of ΔH and ΔS did not originate from B cells and the *adjusted model* was able to correct for this CNA in admixed cancer cells (Fig. 6E).

3.6. Evaluation of dPCR- and IHC-Based Quantification of B cells

After demonstrating cell specificity and quantitative accuracy of our assays in benign and (copy number unstable) malignant samples, we compared dPCR- with IHC-based quantification of B cells in UM specimens. To avoid arbitrary enumeration in situ we applied an automated approach by using quantitative image analysis software. B cell fractions were determined by calculating the fractional abundance of PAX-5 stained (red) nuclei among all hematoxylin stained (blue) nuclei; Double stained B cell nuclei appear purple (Fig. 7). Despite clear spatial heterogeneity of (clusters of) B cell infiltration and difficulties in quantification due to pigmentation, obtained fractions correlated well with the abundances as determined by dPCR; Low to moderate infiltration followed the B cell fraction as calculated by ΔH dPCR.

4. Calculations

4.1. Classic Model for Switched B cell Fraction Calculation with Allelic-Factor

Previously we introduced the *classic* and *adjusted model* to quantify T cells in DNA specimens (Nell, 2022; Nell, 2021). In **paragraph 3.1** and **3.5** we showed that these approaches can be used to quantify B cell fractions as well. However, in order to quantify a switched B cell fraction (SBCF) accurately we are dependent on a allelic-factor (AF) (**paragraph 3.2** and **3.5**), which will alter the original mathematical derivation.

In the slightly modified *classic model* two DNA markers are quantified: the switched B cell marker ΔS and a copy-number stable, independent genomic reference REF. Square brackets refer to measured concentration of the respective DNA targets, as obtained by dPCR. This model is based on the following principles: ΔS is present on 2•(1-AF) alleles derived from switched B cells. For example, with AF set to 4/5, 1–4/5 = 20% of the ΔS alleles is still present in switched B cells, which is on average 2•20%= 0.4 allele per switched B cell. ΔS is present on 2 alleles derived from all other cells (Fig. 3A):

$$[\Delta S] = 2\bullet(1-AF)\bullet SBCF + 2\bullet(1-SBCF)$$

REF is present on 2 alleles derived from switched B cells, and present on 2 alleles derived from all other cells:

$$[REF] = 2\bullet SBCF + 2\bullet(1-SBCF)$$

$$= 2$$

The ratio $\frac{[\Delta S]}{[REF]}$ can then be rewritten as follows:

$$\frac{[\Delta S]}{[REF]} = \frac{2\bullet(1-AF)\bullet SBCF + 2\bullet(1-SBCF)}{2}$$

$$= (1-AF)\bullet SBCF + (1-SBCF)$$

$$= SBCF - AF\bullet SBCF + 1 - SBCF$$

$$= 1 - AF\bullet SBCF$$

Which results in the formula to calculate the switched B cell fraction:

$$AF\bullet SBCF = 1 - \frac{[\Delta S]}{[REF]}$$

$$SBCF = \frac{1 - \frac{[\Delta S]}{[REF]}}{AF}$$

4.2. Adjusted model for Switched B cell Fraction calculation with Allelic-Factor

In the modified *adjusted model* three DNA markers are quantified: switched B cell marker ΔS, regional corrector $RC_{\Delta S}$ and a copy-number stable, independent genomic reference REF. Square brackets refer to measured concentration of the respective DNA targets, as obtained by dPCR. It is based on the following principles: ΔS is present on 2•(1-AF) alleles derived from switched B cells (Fig. 3A), and present on 2 +A alleles derived from other, possibly malignant cells (e.g. as in the illustrated situation of monosomy in Fig. 6A):

$$[\Delta S] = 2\bullet(1-AF)\bullet SBCF + (2+A)\bullet(1-SBCF)$$

As an example, when all other cells have gained one copy of ΔS: 2 +A= 2 + 1 = 3. When all other cells have lost one copy of ΔS: 2 +A= 2–1 = 1.

$RC_{\Delta S}$ is present on 2 alleles derived from switched B cells and present on 2 +A alleles derived from other, possibly malignant cells:

$$[RC_{\Delta S}] = 2\bullet SBCF + (2+A)\bullet(1-SBCF)$$

The ratio $[RC_{\Delta S}] - [\Delta S]$ can then be rewritten as follows:

$$[RC_{\Delta S}] - [\Delta S] = (2\bullet SBCF + (2+A)\bullet(1-SBCF)) - (2\bullet(1-AF)\bullet SBCF + (2+A)\bullet(1-SBCF))$$

$$= 2\bullet SBCF + (2+A)\bullet(1-SBCF) - 2\bullet(1-AF)\bullet SBCF - (2+A)\bullet(1-SBCF)$$

$$= 2\bullet SBCF - 2\bullet(1-AF)\bullet SBCF$$

REF is present on 2 alleles derived from B cells, and present on 2 alleles derived from all other cells:

$$[REF] = 2\bullet SBCF + 2\bullet(1-SBCF)$$

$$= 2$$

The ratio $\frac{[RC_{\Delta S}]-[\Delta S]}{[REF]}$ can then be rewritten as follows:

$$\frac{[RC_{\Delta S}] - [\Delta S]}{[REF]} = \frac{2\bullet SBCF - 2\bullet(1-AF)\bullet SBCF}{2}$$

$$= SBCF - (1-AF)\bullet SBCF$$

$$= SBCF - SBCF + AF\bullet SBCF$$

$$= AF\bullet SBCF$$

Which results in the formula to calculate the switched B cell fraction according to the *adjusted model*:

$$AF\bullet SBCF = \frac{[RC_{\Delta S}] - [\Delta S]}{[REF]}$$

$$SBCF = \frac{\frac{[RC_{\Delta S}]-[\Delta S]}{[REF]}}{AF}$$

5. Discussion

In contrast to the highly variable numbers of expressed surface molecules, all somatic nucleated cells contain genomic DNA in equal (diploid) amounts, sex chromosomes excluded. Hence, presence or absence of genomic DNA correlates directly to the number of originating cells in a digital-like fashion. Whereas epitopes may vary in expression levels between individual cells, cell types or subpopulations and can be influenced under physiological conditions (e.g. aging), genomic DNA typically remains in a diploid conformation (Ginaldi, 2001). Consequently, DNA markers have a high quantitative potential, especially when epitope-based approaches are not feasible.

In this manuscript we introduced a novel dPCR based method to quantify (switched) B cells specifically and accurately in DNA specimens. dPCR enables sensitive, precise and reproducible absolute quantification of nucleic acids by combining sample partitioning (limiting dilution) with Poisson statistical data analysis (Vogelstein and Kinzler, 1999). This dPCR approach is less devious, less time-consuming and cheaper as compared to multiplex PCR and deep sequencing techniques wherein the full repertoire of recombined IG genes is amplified and quantified. Moreover, an absolute quantification of mature and switched B cells, as performed by dPCR, is less biased because of its digital design. Other methods, like flow cytometry, IHC and conventional (multiplex) PCR combined with deep sequencing techniques are more vulnerable to bias in quantification due to sample processing steps (e.g. pre-amplification), dependency on standards and replicates, but also arbitrary aspects like instrument settings and even personal factors (Wood, 2013; Walker, 2006; Vogelstein and Kinzler, 1999; Robins, 2013; Bustin, 2009). Importantly, the sample requirements are much lower compared to cell-based methods, as no intact cells and preserved epitopes are needed. Instead, reliable quantification can be obtained

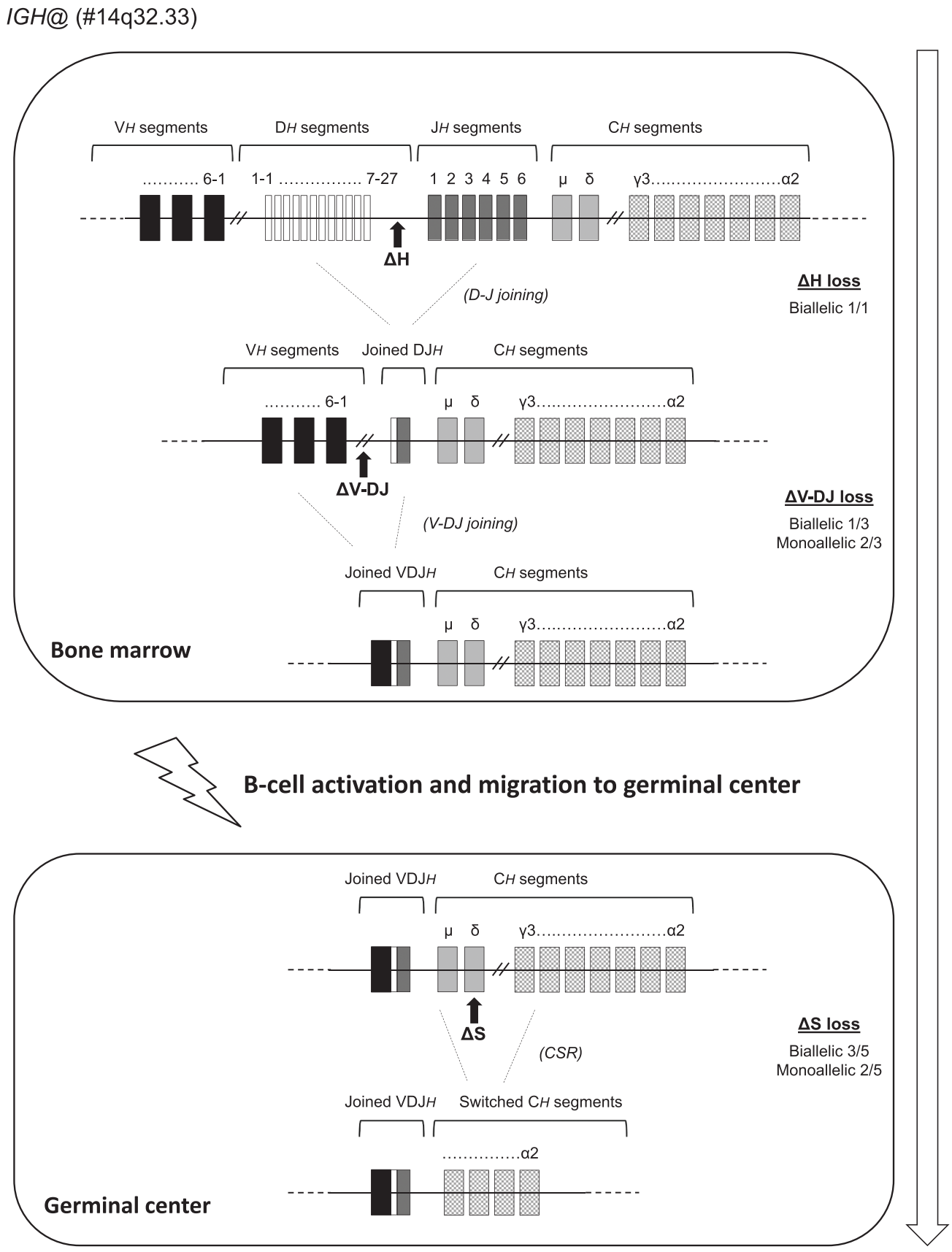

Fig. 8. Quantitative verified model describing the allelic involvement in different recombination processes in the IGH locus as retrospectively measured by dPCR in flow cytometric sorted B cell subpopulations. By measuring ΔH loss we concluded that VDJ rearrangements initially start (i.e. D-J joining) biallelically during early B cell development in the bone marrow. This biallelic recombination is continued by V-DJ joining in 1/3 of the B cells as measured by ΔV-DJ loss. By measuring ΔS loss we concluded that CSR initially starts biallelically in 3/5 of the switched B cells during isotype switching in germinal centers.

from a few nanograms of DNA, thus offering new possibilities for quantification in (limited) small samples like liquid biopsies. Besides the opportunity to quantify lymphocytes, dPCR also allows enhanced mechanistic studies, for instance to investigate V(D)J recombinations more accurately and into detail(Hsieh et al., 2020).

The methodology discussed here was based on our previously published rationale for the quantification of T cells in copy number stable and unstable samples (Zoutman, 2017; Zoutman et al., 2019; Nell, 2022; Nell, 2021). Here we took advantage of the genetic dissimilarity between (switched) B cells and cells of other origin by measuring loss in the ΔH and ΔS regions of the IGH locus. These "scars" (loss of sequences) can be regarded as cell-specific markers for mature B cells and switched B cells (i.e. plasma cells and memory B cells) respectively.

Since ΔH is biallelically and ubiquitously lost in mature B cells, we could apply the same workflow and arithmetic approach to quantify these cells as previously introduced for the quantification of T cells (Zoutman, 2017; Zoutman et al., 2019; Nell, 2022; Nell, 2021). In contrast, this was not possible for ΔS, since this marker is not lost in a pure mono- or biallelic fashion in switched B cells. However, by adding an allelic-factor (AF) to the formula, we could effectively correct for this biological imbalance and accurate quantification remains feasible. Related to this, accuracy of quantification is influenced by this AF and is, unavoidably, dependent on the degree of consistency throughout individuals. However, in our cohort, inter-sample fluctuations of ΔS loss were small, ranging from 77.1% to 87.4% of the alleles. Nonetheless, in response to abundant antigens, clonally expanded switched B cells (with either a mono- or biallelic loss of ΔS) potentially may not reflect the AF as identified in a normal repertoire of switched B cells(Paijens, 2020).

The ΔS dPCR assay was designed to quantify all classically switched B cells (i.e. switched to *IGHG3*, *IGHG1*, *IGHA1*, *IGHG2*, *IGHG4*, *IGHE* and *IGHA2*). We targeted a sequence in close proximity of the first switch-cassette (S-region) downstream of *IGHD*, since this is genetically speaking 'the line of demarcation' for classic CSR. Classic CSR to *IGHD* is genetically impossible, since a S-region between *IGHM* and *IGHD* is lacking. However, non-classic CSR to *IGHD* occurs, but it is a very rare and enigmatic event and seems to be restricted to a few B-cell subsets in specific lymphoid areas. It is described for some, tonsil located, CD38 + B cells. These cells were switched to IgD expression after an AID-mediated deletion of *IGHM*(Rouaud, 2014). It is also described for some rare B cell malignancies(van der Burg, 2002). Given the rarity of CSR to *IGHD*, it will not influence the accuracy of quantification of peripheral switched B cells by using ΔS.

To verify whether CSR truly has taken place in our analyzed switched B cells, one can choose to detect switch (excision) circles(von Schwedler et al., 1990). Although switch circles can be used, it can never be utilized as a direct one-on-one marker as our ΔS marker does. Sequential CSR can occur and, moreover, switch circles are diluted as a result of cell division(Zhang et al., 1994; Cameron, 2003). For this reason we focused on IgG/IgA/IgE-expressing switched B cells as obtained by FACS.

Accurate IHC-based quantification remains challenging due to a variety of parameters. Although we applied an automated quantification method, we were confronted with interfering pigmentation. Furthermore, regional differences in (clusters of) B cell infiltration made it difficult to calculate a representative B cell fraction. In contrast, while tissue context was lost in the matched DNA specimens, spatial heterogeneity is leveled out and represented a larger part of the tumor (25×25 μM versus 1×4 μM). In this respect, IHC can be regarded as a semi-quantitative method as compared to the absolute quantitative capacity of dPCR. However, only IHC can provide valuable information on, for example, localization and infiltration patterns. For this reason, using both methods will supply mutually additional information.

Special care should be taken when using our B cell markers in analyzing DNA samples from malignancies of a lymphoproliferative origin, such as lymphomas and leukemias. The maturation stage during onset of the malignancy, the clonality and genetic stability of (pre-)B cell malignancies may have different consequences on the presence of ΔH and ΔS. For example, whereas mature B cell proliferations have undergone VDJ-rearrangements and have generally lost ΔH biallelically, in undifferentiated/immature B cell proliferations the IGH locus might be incompletely rearranged. As a result, ΔH may be mono- or biallelically present in (malignant) B cells, not following the *classic* or *adjusted model*.

Regarding allelic involvement during different stages of VDJ rearrangements and CSR, a variety of theoretical, stochastic and experimental models are available(Ollila and Vihinen, 2005; Outters, 2015; Vettermann and Schlissel, 2010; Alves-Pereira, 2014; Pichugin, 2017). Despite this, exact (numerical) knowledge about allelic involvement and ubiquitous loss of IGH loci is lacking. However, by accurate quantification of ΔH, ΔS and ΔV-DJ loss in flow cytometric sorted B cell subpopulations, we could now make numerical statements retrospectively. These findings are summarized in our novel, quantitative verified, model as presented in Fig. 8. By measuring ΔH loss, we concluded that VDJ rearrangements initially start (i.e. D-J joining) biallelically. However, by measuring ΔV-DJ loss, we demonstrated that this biallelic recombination is less frequently continued (i.e. V-DJ joining), namely in only 1/3 of B cells. Concerning CSR, it is generally accepted that recombination only occurs on the active allele. However, circumstantial evidence suggests involvement of the non-productive allele as well (Pichugin, 2017). By dPCR we could demonstrate that ΔS was biallelically lost in 3/5 of switched B cells. This observation shows that CSR would at least start often on both the active and inactive allele. To gain more detailed knowledge regarding abortive CSR in the non-productive allele, one can perform in vitro experiments in which IgM+IgD+ B cells are induced to switch to IgG, IgA and IgE expression(Kracker and Radbruch, 2004). Currently, in collaboration with another partner, we are investigating this.

6. Conclusion

We designed and validated an accurate, specific, sensitive and relatively fast and cheap method to quantify mature and switched B cells in DNA specimens of benign or (copy number unstable) malignant origin. By combining our assays with the absolute quantitative capacity of dPCR and through cross-validation against flow cytometric sorted B cell subpopulations, we gained quantitative insights into allelic involvement in different recombination processes in the IGH locus. Together with our previously developed and validated dPCR assays for the quantification of T cells, these mature B cell and switched B cell assays enlarge the array to enumerate a variety of lymphocytes in (small) bulk DNA specimens in a meaningful way (Zoutman, 2017; Zoutman et al., 2019; Nell, 2022; Nell, 2021).

Declaration of Competing Interest

WHZ, RJN an PAV are inventors on patent application WO/2021/071358 which describes the methodology in this manuscript.

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