ARTISAN PCR

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ARTISAN PCR: rapid identification of full-length immunoglobulin rearrangements without primer binding bias

B cells recognize specific antigens by their membrane-bound B-cell receptor (BCR). Functional BCR genes are assembled in pre-B cells by recombination of the variable (V), diversity (D) and joining (J) genes [V(D)J recombination]. When B cells participate in germinal centre reactions, non-templated point mutations are introduced into BCR genes by somatic hypermutation (SHM) (Rajewsky, 1996). V(D)J recombination and SHM create virtually unlimited BCR repertoires.

Many research applications require identification of full-length BCR sequences expressed in biological samples. BCR are commonly identified by multiplex polymerase chain reaction (PCR) with upstream primers that anneal to conserved stretches in variable or leader gene segments. BIOMED-2 is the current European standard protocol to assess BCR clonality as diagnostic criterion for lymphoma (van Dongen *et al*, 2003). However, a single point mutation in a primer binding site can obscure a dominant clonal BCR (Wu *et al*, 2009), whereas reliable identification of functional BCR genes is essential to avoid erroneous conclusions about the BCR (Koning *et al*, 2014). Therefore, several investigators have emphasized the need for an unbiased method of BCR amplification (Warren *et al*, 2013; Georgiou *et al*, 2014; Koning *et al*, 2014). We here describe ARTISAN PCR (*A*nchoring *R*everse *T*ranscription of *I*mmunoglobulin *S*equences and *A*mplification by *N*ested PCR) as a truly unbiased method with excellent applicability.

Polyadenylated mRNA (Dynabeads mRNA DIRECT Kit, Invitrogen, Grand Island, NY, USA) was directly isolated from thawed suspensions of cryopreserved cells, or from 10 μm sections of fresh frozen material that was homogenized immediately upon addition of lysis buffer. Isolation of B cells by magnetic beads prior to mRNA isolation was found to be dispensable. Reverse primers (Table SI) were designed to anneal to a consensus sequence from all functional alleles of μ, γ, α, κ, and λ Ig constant regions included in the ImMunoGeneTics (IMGT) database (Lefranc *et al*, 1999). Messenger RNA was mixed with reverse transcription primers and the SA.rt anchor oligonucleotide (Table SII). Synthesis of cDNA was performed with a reverse transcriptase that adds a non-templated 3′ polycytosine terminus (Zhu *et al*, 2001). Annealing of the SA.rt oligonucleotide to this polycytosine stretch creates a template for extension of the cDNA with the complementary SA.rt sequence in the same reaction. Subsequent PCR amplification of anchored cDNA was performed with anchor-specific primer SA.pcr in combination with a nested primer that anneals to one of the constant regions of interest (Cμ.pcr, Cγ.pcr, Cα.pcr, Cκ.pcr, or Cλ.pcr; Table SI and Table SII).

The efficacy of ARTISAN PCR was compared with a BIOMED-2-based multiplex PCR kit (IGH FR1, IGK tube A and IGL Clonality Assays, Invivoscribe, San Diego, CA, USA).

For massive parallel sequencing, column-purified ARTISAN amplicons were barcoded by PCR (Table SII). Samples were sequenced on the PacBio RS system (Pacific Biosciences, Menlo Park, CA, USA). After filtering for a minimum of eight passes (SMRT Portal software, Pacific Biosciences) and correction of indels in polyhomologous stretches (Koren *et al*, 2012), sequence reads were analysed with Geneious 6.1.6 software and IMGT HighV-QUEST tools (Alamyar *et al*, 2012).

PacBio sequencing of ARTISAN PCR amplicons identified full-length functional V(D)J sequences in all 39 Epstein–Barr virus-transformed lymphoblastoid cell lines (EBV LCL) tested (Table SIII). 10^3 cells sufficed as starting material. Each PacBio SMRT cell yielded $>25 \times 10^3$ reads with at least eight passes. Of 52 VDJ sequences, 50 were functional and 2 non-productive. Thirty-two EBV LCL were monoclonal and were six biclonal; one EBV LCL harboured six different B-cell clones. The sequence error rate as calculated for clonal EBV LCL sequences was $0{\cdot}126 \times 10^{-3}$ per bp without significant differences between μ, γ, α, κ, and λ amplicons. The multiplex protocol failed to amplify seven VDJ, one VJ-κ and nine VJ-λ, indicating success rates of 87%, 95%, and 78%, respectively, compared to ARTISAN PCR. Complete BCR heterodimers were obtained by the multiplex protocol for only 37 of the 50 functional EBV LCL clones identified by ARTISAN PCR (74%). Dilution experiments with B-cell lines demonstrated proportional BCR sequence representation according to cellular input (Fig 1A).

*A*nchoring *R*everse *T*ranscription of *I*mmunoglobulin *S*equences and *A*mplification by *N*ested PCR identified complete functional BCR sequences from cryopreserved primary cells in all 24 cases of various B cell (pre)malignancies (3 samples each from Burkitt lymphoma, mantle cell lymphoma, marginal zone lymphoma, follicular lymphoma, multiple myeloma, Waldenström macroglobulinaemia, chronic lymphocytic leukaemia and monoclonal B-cell lymphocytosis; Table SIV). Multiplex RT-PCR failed to identify four VDJ and eight VJ sequences, and identified the complete BCR heterodimer in only 13 cases (54%).

Finally, ARTISAN PCR was applied to 17 fresh frozen excisional biopsies archived between 1988 and 1991 and labelled

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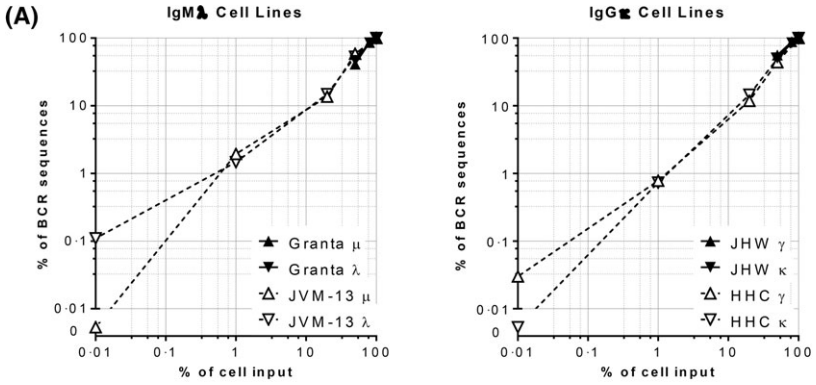

	Input				Output			
	Granta-519	JVM-13	JYW	HHC	Number of PacBio Reads			
	IgMλ	IgMλ	IgGκ	IgGκ	μ	λ	γ	κ
1	5×10^5	5×10^5	5×10^5	5×10^5	139	158	255	312
2	8×10^5	2×10^5	8×10^5	2×10^5	161	236	516	248
3	$9{\cdot}9 \times 10^5$	1×10^4	$9{\cdot}9 \times 10^5$	1×10^4	1337	2721	3708	5226
4	$9{\cdot}999 \times 10^5$	1×10^2	$9{\cdot}999 \times 10^5$	1×10^2	1825	2800	3116	2109

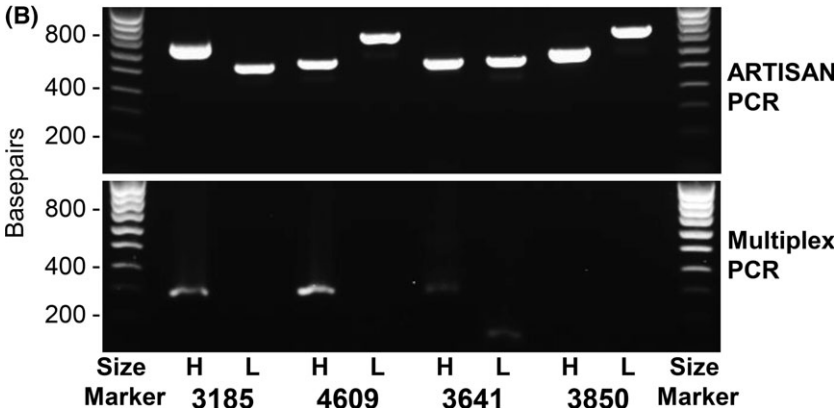

Fig 1. *A*nchoring *R*everse *T*ranscription of *I*mmunoglobulin *S*equences and *A*mplification by *N*ested (ARTISAN) PCR in combination with PacBio sequencing for identification of full-length B-cell receptor (BCR) transcripts. (A) Top panels: Number of PacBio BCR sequence reads per isotype/light chain in a dilution experiment of B-cell lines. Graphs display percentages of heavy and light chains reads of the respective cell line as a function of cellular input. Bottom panel: Absolute cell numbers in four analysed samples and number of reads obtained per ARTISAN library. (B) Gel electrophoresis of ARTISAN and multiplex PCR products from fresh frozen tissue in four selected cases. H: Ig heavy chain. L: Ig light chain.

with diagnoses consistent with diffuse large B-cell lymphoma. For 15 biopsies, dominant clonal sequences were identified for the heavy as well as the light BCR chain. In two cases, neither the heavy nor the light chain amplicons included clonal sequences. Pathological revision confirmed lymphoma infiltration in the 15 samples with clonal BCR sequences but demonstrated lack of lymphoma infiltration in both non-clonal biopsies. The multiplex approach failed to amplify three VDJ and three VJ clonal rearrangements, yielding complete BCR heterodimers for 10/15 (67%) cases only (Fig 1B, Table SV).

Across all experiments, functional clonal BCR sequences found by multiplex reverse transcription (RT)-PCR were also identified by ARTISAN PCR. Conversely, ARTISAN PCR identified specific causes for each multiplex RT-PCR failure: mismatches in primer binding sites ($n = 25$), lack of a primer for certain *IGLV* alleles ($n = 9$), and one truncation of the *IGHV3-33*01* F gene (Fig 2A). Failure of multiplex RT-PCR correlated with SHM mutation frequency. When only *IGV* alleles for which a primer was designed in BIOMED-2 were considered, multiplex RT-PCR failed for 36% of sequences with <90% identity to germline (Fig 2B). When considering all *IGV* alleles, the failure rate rose to 42% of sequences with high SHM load (Fig 2C).

*A*nchoring *R*everse *T*ranscription of *I*mmunoglobulin *S*equences and *A*mplification by *N*ested PCR, combined with a massive parallel sequencing technology that covers entire V(D)J sequences in a single read, e.g. the PacBio platform, permits unbiased comprehensive analyses of BCR repertoires in healthy, reactive, or neoplastic conditions. ARTISAN PCR has low error and apparently complete success rates but requires intact RNA as starting material for the protocol described here. In routine diagnostics performed on formalin-fixed tissue, amplification of DNA with BIOMED-2 primers has the advantage of feasibility but is limited by a substantial failure rate for hypermutated BCR sequences.

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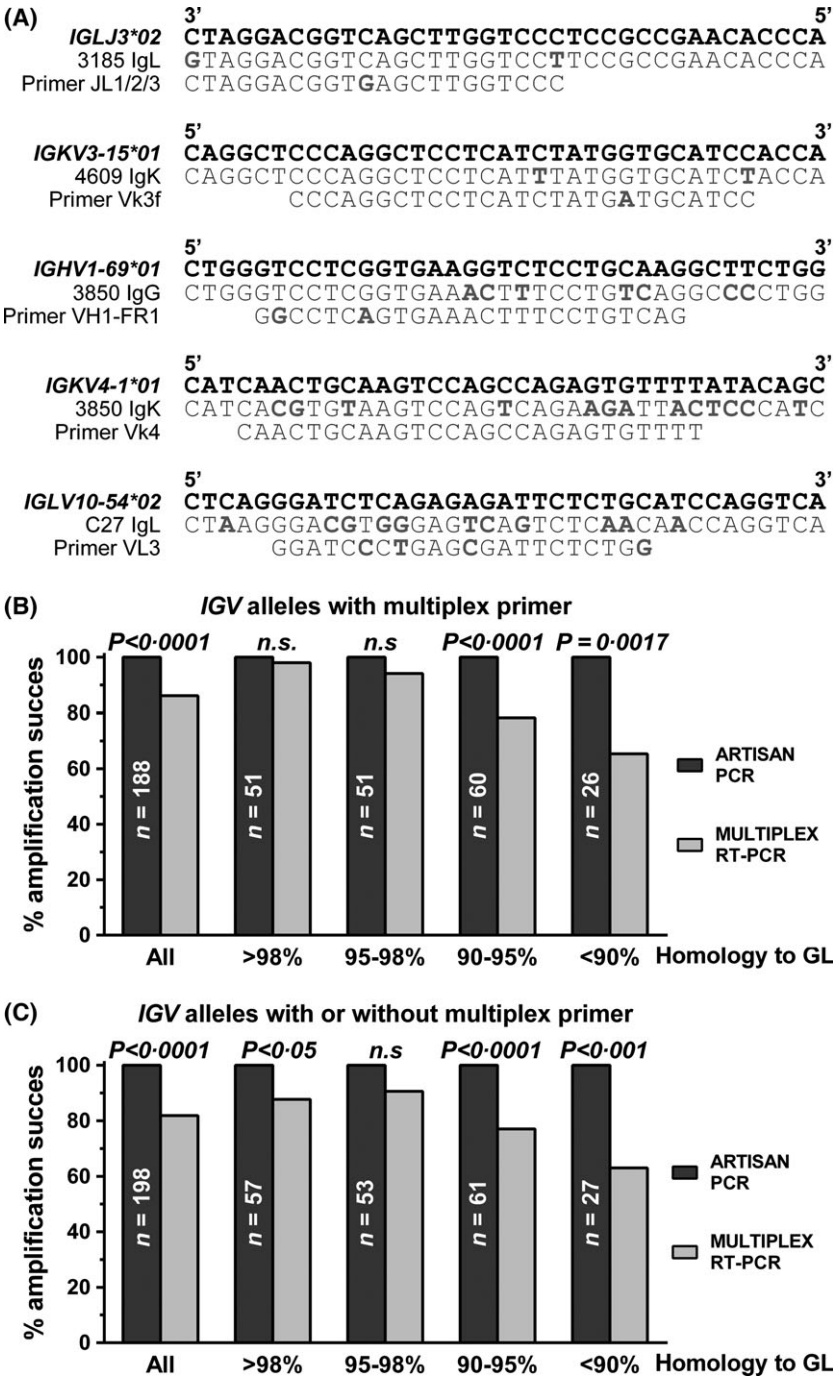

Fig 2. Impairment of multiplex polymerase chain reaction (PCR) through somatic hypermutation of B-cell receptor (BCR) sequences as deduced from ARTISAN PCR results. Success rates of ARTISAN PCR and multiplex RT-PCR were compared by Fisher's exact test (Prism 5·02, GraphPad Software, La Jolla, CA). (A) Exemplary reasons for failure of multiplex RT-PCR due to somatic hypermutation. Sequence alignments of germ-line *IGV/IGJ* genes, clonal BCR sequences obtained from cell lines or lymphoma samples as identified by ARTISAN PCR, and corresponding multiplex primers. Sequence identities to GL are indicated in grey, differences in red. Multiplex PCR failure occurs due to mismatches at the 3′ primer terminus (cases 3185, 4609), or multiple mismatches throughout the primer sequence (case 3850). In cell line C27 amplification failure is due to lack of a primer targeting the expressed *IGLV* allele in the multiplex RT-PCR primer set. (B) Comparison between ARTISAN PCR and multiplex RT-PCR for *IGV* alleles that are targeted by the multiplex primer mix. (C) Comparison between ARTISAN PCR and multiplex RT-PCR for all identified clonal VDJ and VJ sequences. GL, Germline; RT-PCR, reverse transcription polymerase chain reaction; ARTISAN PCR, *A*nchoring *R*everse *T*ranscription of *I*mmunoglobulin *S*equences and *A*mplification by *N*ested polymerase chain reaction.

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Authorship

H.V. and M.A.N. designed the study; P.M.K. and C.A.M.v.B. collected and archived biopsy material; M.K. and V.B. performed the experiments; M.K., S.K. and S.A.J.v.d.Z. analysed the data; H.P.J.B. and M.G. advised on methodology; M.K., H.V. and M.A.N. wrote the manuscript.

Conflict of interest

The authors have no conflicts of interest to report.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI: Oligonucleotide primers.

Table SII: ARTISAN PCR and Barcoding Reactions.

Table SIII: Overview of the sequences obtained from the cell lines.

Table SIV: Overview of the sequences obtained from the cell suspension biopsies.

Table SV: Overview of the sequences obtained from the fresh frozen solid tissue biopsies.

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Factor VIII inhibitor eradication with bortezomib in acquired haemophilia A

Acquired Haemophilia A is a rare autoimmune disease resulting in a potentially life threatening haemorrhagic diathesis. As the pathogenesis involves production of inhibitory antibodies directed against Factor VIII (FVIII), management includes immunosuppression to eradicate the inhibitory antibody and haemostatic support for haemorrhagic sequelae. Here, we report for the first time, a case of successful FVIII inhibitor eradication with the proteasome inhibitor bortezomib in a patient with relapsed, refractory acquired haemophilia A complicated by recurrent limb-threatening bleeding episodes.

A 45-year-old male, with a past history of schizophrenia, presented with a large iliopsoas bleed (Fig 1A) in conjunction with a FVIII assay of 7% and inhibitor titre of 2·6 Bethesda units/ml (Fig 1B). This occurred in the context of a past history of acquired haemophilia diagnosed in 2005, which was treated successfully with rituximab. Haemostatic support was initiated with recombinant activated Factor VIIa (rFVIIa) (90 μg/kg) 2-hourly. Rituximab (375 mg/m^2 weekly for 4 doses) was commenced immediately for inhibitor eradication. Corticosteroid was not initially administered given its potential to exacerbate his mental state.