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Heteroduplex analysis of VDJ amplified segments from rearranged IgH genes for clonality assessments in B-cell non-Hodgkin's lymphoma. A comparison between different strategies

MARCOS GONZÁLEZ, DAVID GONZÁLEZ, RICARDO LÓPEZ-PÉREZ, RAMÓN GARCÍA-SANZ, M. CARMEN CHILLÓN, ANA BALANZATEGUI, M. VICTORIA MATEOS, IRENE ALAEJOS, ANTON W. LANGERAK,* ALBERTO ORFÃO, JACQUES J.M. VAN DONGEN,* JESÚS F. SAN MIGUEL

*Hematology Service, University Hospital of Salamanca; Centro de Investigación del Cancer (CIC), Universidad de Salamanca, Spain; *Department of Immunology, University Hospital, Erasmus University, Rotterdam, The Netherlands*

ABSTRACT

Background and Objective. **The main difficulty of PCR-based clonality studies for B-cell lymphoproliferative disorders (B-LPD) is discrimination between monoclonal and polyclonal PCR products, especially when there is a high background of polyclonal B cells in the tumor sample. Actually, PCR-based methods for clonality assessment require additional analysis of the PCR products in order to discern between monoclonal and polyclonal samples. Heteroduplex analysis represents an attractive approach since it is easy to perform and avoids the use of radioactive substrates or expensive equipment.**

Design and Methods. **We studied the sensitivity and specificity of heteroduplex PCR analysis for monoclonal detection in samples from 90 B-cell non Hodgkin's lymphoma (B-NHL) patients and in 28 individuals without neoplastic B-cell disorders (negative controls). Furthermore, in 42 B-NHL and in the same 28 negative controls, we compared heteroduplex analysis vs the classical PCR technique. We also compared ethidium bromide (EtBr) vs. silver nitrate ($AgNO_3$) staining as well as agarose vs. polyacrylamide gel electrophoresis (PAGE).**

Results. **Using two pair consensus primers sited at VH (FR3 and FR2) and at JH, 91% of B-NHL samples displayed monoclonal products after heteroduplex PCR analysis using PAGE and $AgNO_3$ staining. Moreover, no polyclonal sample showed a monoclonal PCR product. By contrast, false positive results were obtained when using agarose (5/28) and PAGE without heteroduplex analysis: 2/28 and 8/28 with EtBr and $AgNO_3$ staining, respectively. In addition, false negative results only appeared with EtBr staining: 13/42 in agarose, 4/42 in PAGE without heteroduplex analysis and 7/42 in PAGE after heteroduplex analysis.**

Interpretation and Conclusions. **We conclude that $AgNO_3$ stained PAGE after heteroduplex analysis is the most suitable strategy for detecting monoclonal rearrangements in B-NHL samples because it does not produce false-positive results and the risk of false-negative results is very low.**

Correspondence: M. González, Service of Hematology, University Hospital of Salamanca, Paseo de San Vicente, 58-182, Salamanca, 37007, Spain. Phone: international +34.23.291375 – Fax: international +34.23.294624 – E-mail: margondi@gugu.usal.es

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B-cell lymphoproliferative disorders (B-LPD) can be diagnosed easily by routine cytomorphologic examination and immunophenotyping.[1] However, there are some cases in which it is difficult to establish a definitive diagnosis, mainly due to problems in the immunologic assessment of the monoclonal nature of the disorder (e.g. in cases without surface Ig expression, presence of oligoclonality, poor quality of the sample or low number of tumor cells).[2] Molecular analysis of IgH genes can be an alternative to confirm or exclude the presence of monoclonality in these cases. Usually, this has been assessed by Southern blot (SB) analysis, which is a reliable but time-consuming and labor-intensive method.[3] Accordingly, new techniques based on PCR amplification of the junctional regions of IgH genes are being investigated as alternatives to SB analysis.[4-7]

Although PCR techniques can be sensitive and fast, there are two major drawbacks in clonality studies of IgH genes. Firstly, rearrangements involving particular VH gene segments might remain undetectable, mainly because of the existence of primer mismatches originated from somatic mutations which are frequent in mature B-LPD.[8-11] This produces an annealing failure of primers leading to a false-negative result. The second and more important problem is the risk of false-positive results due to the background generated by the amplification of rearranged IgH genes from polyclonal B lymphocytes. This *polyclonal background* pitfall also occurs in T-cell clonality studies based on T-cell receptor (TCR) gene rearrangement amplifications.[4] Several methodologic approaches have been investigated to solve these problems, including direct sequencing of

PCR products,[12] single-strand conformation polymorphism (SSCP),[13] denaturing gradient gel electrophoresis (DGGE),[14] temperature gradient gel electrophoresis (TGGE),[15] genescanning[12,16] and heteroduplex analysis.[17-21] Among these techniques (frequently labor intensive and time-consuming), heteroduplex analysis has been described as a simple, fast, and non-expensive method to asses monoclonality in T-cell lymphoproliferative disorders.[17-19] However, less experimental data has been reported for B-LPD,[20,21] and most of the data refer to precursor B-cell leukemias, while, to the best of our knowledge, no results have been published on B-cell non-Hodgkin's lymphoma (B-NHL). In heteroduplex analysis, PCR products are denatured at high temperatures and subsequently renatured to induce homo- or heteroduplex formation. Discrimination between PCR products derived from monoclonal and polyclonal lymphoid cell populations is based on the presence of homoduplexes (PCR products with identical junctional regions) or a smear of heteroduplexes (derived from PCR products with heterogeneous junctional regions), respectively.[17-21]

In this study, we evaluated heteroduplex analysis for clonality assessment in 90 patients with B-NHL, using 28 samples from patients with reactive disorders (n=18) and healthy volunteers (n=10) as negative controls. Moreover, in 42 B-NHL samples and in the 28 negative controls we compared heteroduplex analysis to classic PCR-based techniques, in which PCR products are not analyzed after amplification. Other technical variables included in different strategies, such as ethidium bromide (EtBr) or silver nitrate ($AgNO_3$) staining as well as agarose or polyacrylamide gel electrophoresis (PAGE), were been evaluated. In addition we also tested the sensitivity of the technique in order to explore its potential role in MRD detection.

Design and Methods

Cell samples

Fresh samples from lymph nodes, bone marrow or peripheral blood from 90 B-NHL patients were included in the analysis. All samples had more than 15% neoplastic cells. The number of tumor cells was counted by flow cytometric analysis based on κ/λ and B cell antigen expression.[2] The presence of clonal B cells was also assessed by detection of IgH clonal rearrangements by Southern blot (SB) analysis (IgHJ6 probe)[22] in order to confirm not only the B-cell clonality of the disease but also the presence of sufficient clonal cells in the sample. Histologic distribution of the patients according to the Working Formulation (WF) classification was as follows: group A, n=33; B, n=6; C, n=10; D, n=1; E, n=10; F, n=4; G, n=11; H, n=1; I, n=1; J, n=9 and 4 unclassifiable.

Lymph nodes from 18 patients with reactive disorders and peripheral blood (PB) samples from 10 healthy volunteers were used as negative controls. The number of B-cells in the samples was counted by flow cytometry according to the surface expression of the CD19/CD22 antigens. The polyclonality of the samples was confirmed by SB analysis together with the absence of Ig light chain restriction on the cell surface, and the absence of malignant B-cell lymphoproliferative disorders (clinical diagnosis and outcome).

DNA preparation and Southern blot analysis

High molecular weight DNA was isolated by standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation.[23] For Southern blot analysis, 10 µg of DNA were digested to completion with the Bgl II and Bam HI/Hind III restriction enzymes, size fractionated and blotted onto nylon membranes as previously described.[24] The blots were hybridized with IgHJ6 ^{32}P-labeled probes and autoradiographed 72 hours later.

PCR amplifications

Two PCR analyses were carried out on all samples in order to amplify the VDJ region of the heavy chain Ig genes (IgH) using 1 µg of genomic DNA. Two consensus primer pairs were used for these amplifications: FRIII^5–JHc^5 and FRII^{11}–JHc^5. PCRs were performed in a 100 µL final volume containing: 200 nM of oligonucleotide primers, 200 µM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$ and 2 U of Taq polymerase (Perkin Elmer, Norwalk, CT, USA) in PCR buffer (50 mM KCl, 10 mM Tris-HCl). Amplifications with the FRIII-JHc primer pair were performed in a Perkin Elmer Cetus 9600 thermal cycler with an initial denaturation over 5 minutes at 94ºC and 35 cycles consisting on 45" of denaturation at 94ºC, 45" of annealing at 55ºC and 45" of extension at 72ºC, with a final 10 minutes extension at 72ºC. When the FRII-JHc primer pair was used, temperature conditions were: denaturation at 94ºC for 60", annealing at 60ºC for 60" and extension at 72ºC for 60", with the same initial denaturation and final extension as for FRIII-JH.

In order to test the PCR products generated, after each amplification 20 µL of the final reaction were analyzed by electrophoresis in a 2% agarose gel (Nu Sieve, FMC, Rockland, ME, USA) in tris-borate electrophoresis buffer (TBE) and visualized by ethidium bromide (EtBr) staining under UV light. In all experiments, a negative control (sterile distilled water) and a positive control were used.

Heteroduplex analysis

For heteroduplex analysis, PCR products were denatured at 94ºC for 10 min. and subsequently cooled at 40ºC to induce duplex formation.[18] The hetero- and/or homoduplexes generated were immediately loaded on a 10% non-denaturing polyacrylamide gel in 0.5 x TBE buffer, run at room temperature, and visualized by silver nitrate staining (fixation for 6' in a 0.5% acetic acid, 10% ethanol solution,

staining for 15' in a 0.1% $AgNO_3$ solution and final development for 20' in a formaldehyde 0.15%, NaOH 1.5% solution). High DNA Mass-Ladder™ (Life Technologies, Inching, UK) was used as DNA size marker. The presence of one or two thin clear bands within the expected size (between 75-140 bp for FRIII and between 200-300 bp for FRII) indicated clonality, while polyclonal samples appeared as a rough smear in the gel. If no amplification was achieved, we tested the DNA quality of the sample by amplification of the ABL gene.

Comparison between different approaches

In order to compare all technical variables involved in the PCR product analysis, we compared different methodologic strategies in 42 of the B-NHL samples and in the 28 negative controls using the same PCR products (100 µL final volume PCR). After agarose gel electrophoresis we divided PCR products into two 40 µL aliquots and only one of them underwent heteroduplex analysis. Briefly, both aliquots were loaded in 10% non-denaturing polyacrylamide gels, which were visualized after EtBr and $AgNO_3$ staining. Thus, using this strategy we were allowed to compare agarose vs. polyacrylamide gel electrophoresis, EtBr vs. $AgNO_3$ staining and heteroduplex vs. no heteroduplex formation in the same PCR product.

Sensitivity studies

In order to determine the sensitivity of the technique, dilution experiments were performed. DNA from five samples which yielded monoclonal products (tumor load ranging from 50 to 80%) were serially diluted into two types of polyclonal DNAs: 1) DNA extracted from polyclonal lymph nodes with more than 30% polyclonal B-cells (DNA type I), which were considered to be samples with a high B-cell polyclonal background and, 2) DNA extracted from the polynuclear phase of Ficoll-hypaque separated cells from normal peripheral blood samples, with less than 5% of polyclonal B cells, which were considered to be samples with low B-cell polyclonal background (DNA type II). These two types of diluted samples subsequently underwent the same heteroduplex PCR analysis to test sensitivity.

Results

Polyclonal samples

None of the 28 negative controls produced monoclonal results by Southern blot analysis. In PCR clonality studies, agarose gel electrophoresis showed a polyclonal pattern assessed by two independent observers in 23 samples (82%). In the remaining five samples, a confusing result (strong smear difficult to distinguish from a real clonal band) was obtained probably because of the existence of a background originating from the amplification of the rearranged IgH genes from the polyclonal population. The percentage of B polyclonal cells in these five samples ranged between 22% and 59%. False positive results were still present in polyacrylamide gel electrophoresis (PAGE) before heteroduplex analysis, increasing to eight samples when silver staining was used. These conflicting results were clarified after heteroduplex analysis in PAGE, in which all 28 polyclonal samples showed a clearly distinguishable smear without any band complicating the interpretation (Table 1).

B-NHL samples

Southern blot analysis. After Southern blot analysis, all 90 lymphoma samples showed IgH gene rearrangements (49 monoallelic and 41 biallelic), confirming not only the existence of B-cell monoclonality, but also the presence of sufficient numbers of tumor cells in the samples.

Monoclonality detection with PCR/heteroduplex and $AgNO_3$ staining. Heteroduplex PCR analysis using the primer pairs for the VDJ_H junctional regions showed monoclonal results in 90% (81/90) and 77% (69/90) of cases when FRIII or FRII respectively were used (Table 2). Of the nine samples which were negative for FRIII-JH_C amplification, one was positive with the alternative primer pair FRII-JH_C increasing the positivity to 91% of cases (82 out of 90) (Table 2). The eight false-negative samples came from two patients with hairy cell leukemia, four with follicular lymphoma and two with Burkitt's lymphoma.

In some cases, alternative bands could be seen above in addition to the clonal band, corresponding to heteroduplexes, single strand PCR products and/or next-JH-amplification of the rearranged JH.[18]

Table 1. Polyclonal samples: false-positive or unclear clonality assay results comparing different strategies.

Diagnosis	*% BL*	*Agarose*	*PAGE-SN no HTX*	*PAGE-SN and HTX*
nPB (n=10)	*1-5*	*NP*	*NP*	*NP*
Lymph node #1	59	U	Polyclonal	Polyclonal
Lymph node #2	21	Polyclonal	Clonal	Polyclonal
Lymph node #3	39	Polyclonal	Clonal	Polyclonal
Lymph node #4	12	Polyclonal	Clonal	Polyclonal
Lymph node #5	50	Polyclonal	Polyclonal	Polyclonal
Lymph node #6	31	Polyclonal	Clonal	Polyclonal
Lymph node #7	36	U	Polyclonal	Polyclonal
Lymph node #8	46	Polyclonal	Polyclonal	Polyclonal
Lymph node #9	5	NP	NP	NP
Lymph node #10	39	Polyclonal	Polyclonal	Polyclonal
Lymph node #11	52	U	Clonal	Polyclonal
Lymph node #12	15	Polyclonal	Polyclonal	Polyclonal
Lymph node #13	15	Polyclonal	Polyclonal	Polyclonal
Lymph node #14	22	U	Clonal	Polyclonal
Lymph node #15	30	U	Clonal	Polyclonal
Lymph node #16	22	Polyclonal	Clonal	Polyclonal
Lymph node #17	27	Polyclonal	Polyclonal	Polyclonal
Lymph node #18	34	Polyclonal	Polyclonal	Polyclonal

BL: B lymphocytes; PAGE-SN: polyacrylamide gel electrophoresis and silver nitrate staining; HTX: heteroduplex analysis; nPB: normal peripheral blood; NP: no PCR product; U: unclear result.

Table 2. Numbers of clonal and no clonal results in B-NHL patients by amplifying VDJ with JH and both FRIII and FRII primer pairs after heteroduplex formation and silver nitrate stained PAGE.

	B-NHL (n=90)	
	Clonal	No clonal
FR III (n=90)	81 (90%)	9 (10%)
FR II (n=90)	69 (77%)	21 (23%)
(FR III + FR II) (n=90)	82 (91%)	8 (9%)

Table 3. Comparison between the different strategies.

	Agarose EtBr		*PAGE EtBr*		*PAGE $AgNO_3$*		*PAGE EtBr–HX*		*PAGE $AgNO_3$–HX*	
	+	–	+	–	+	–	+	–	+	–
Negative controls (n=28)	5	23	2	26	8	20	0	28	0	28
B-NHL (n=42)	29	13	38	4	42	0	35	7	42	0

(+): clonal result; (-): polyclonal result; EtBr: ethidium bromide staining; PAGE: polyacrylamide gel electrophoresis; $AgNO_3$: silver nitrate staining; HX: heteroduplex analysis.

Comparison between different methodologic strategies

In 28 negative controls and in 42 B-NHL samples, we compared heteroduplex analysis with classic PCR-based techniques, which do not analyze PCR products after amplification. Simultaneously, we compared agarose vs. PAGE and two different staining methods: EtBr and $AgNO_3$ (Table 3).

False-positive results. In the 28 negative controls, we found a confusing result in five cases after agarose gel electrophoresis. When PAGE and no heteroduplex analysis of the PCR product was conducted, two and eight cases gave rise to *monoclonal* bands after EtBr or AgNO3 staining, respectively. All these positive results disappeared when heteroduplex analysis was performed in the same PCR product (Figure 1, Table 3).

False-negative results. Comparing results from 42 B-NHL samples, we observed false-negative results in a variable number of cases in all strategies except when employing silver nitrate staining (Figure 2, Table 3)

Thus, heteroduplex analysis in PAGE and silver nitrate staining was the only strategy which did not produce either false-positive or false-negative results.

Sensitivity of heteroduplex analysis for clonality detection

Serial dilutions of five monoclonal DNA samples diluted in DNA type I (high B-cell polyclonal background), showed a sensitivity of at least 10^{-2} (identification of the monoclonal band when only 1% tumor cells was present in the sample). However, when dilution experiments were performed using dilutions of monoclonal DNA in DNA type II (low B-cell polyclonal background), the sensitivity of the method reached 10^{-3}.

Discussion

Southern blot analysis has long been the only reliable method for molecular clonality assessment in the screening of B-cell lymphoproliferative disorders (B-LPD).[3] However, over the last few years several PCR based methods have been tested as alternatives. The main difficulty of PCR-based clonality detection is the discrimination between monoclonal and polyclonal PCR products, especially when a high background of polyclonal B cells exists in the tumor sample. At present, PCR-based methods for clonality assessment require additional analysis of the PCR products in order to distinguish monoclonal from polyclonal samples.[12-18] Heteroduplex analysis has been described as a simple, fast method that does not require radioactive substrates or expensive equipment.[18] Some preliminary reports have shown the validity of this strategy in T-cell LPD,[17-18] but information on B-cell LPD is still scanty.[20-21] In this paper we report on the value of heteroduplex PCR analysis of junctional regions from rearranged IgH genes to detect monoclonality in 90 B-NHL samples. The specificity of the method was

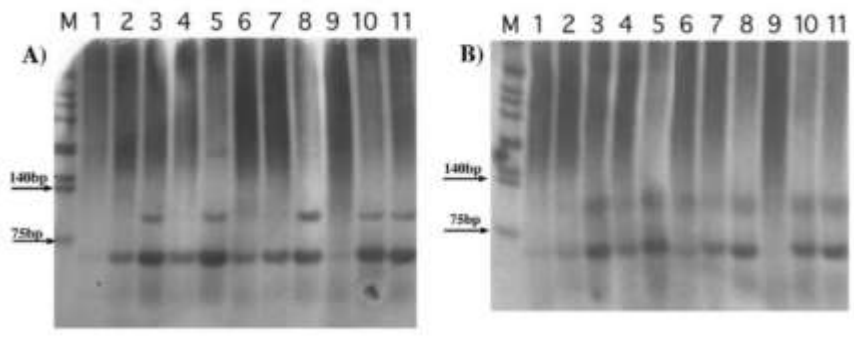

Figure 1. Polyclonal samples analyzed by PAGE and silver nitrate staining before (A) and after (B) heteroduplex analysis with CDRI-II primers. M: molecular weight marker; line 1: negative control; line 2-11: polyclonal samples. Before heteroduplex analysis some cases (lines 3, 5, 8, 10 and 11) gave rise to clonal bands, all disappearing after heteroduplex analysis.

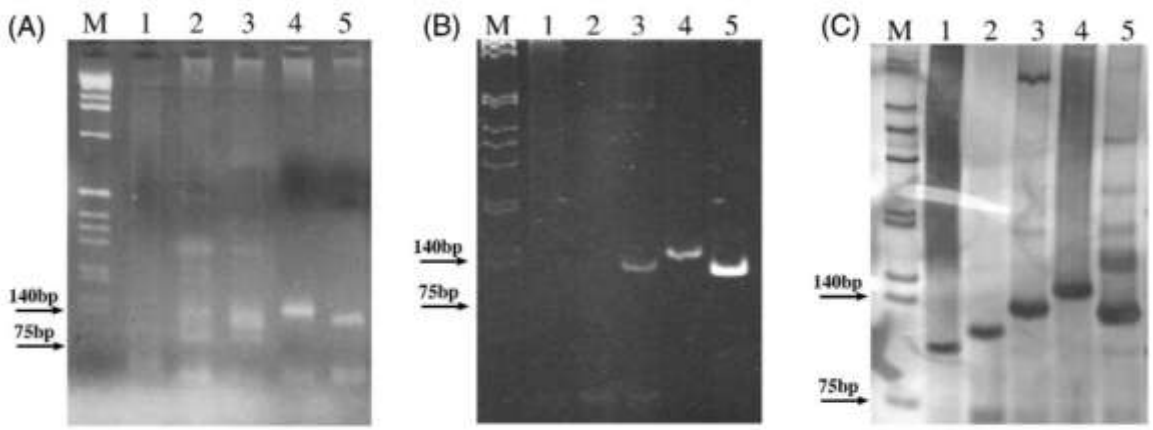

Figure 2. CDRIII–PCR product analysis in five of the positive controls. (M: molecular weight marker). A) Agarose gel electrophoresis with EtBr staining; clonal results were obtained only in cases 4 and 5, while polyclonal results were obtained in cases 2 and 3, and no product was obtained in case 1. B) PAGE with EtBr after heteroduplex analysis. Cases 3, 4 and 5 were clearly clonal, while in 1 and 2 no products were visualized. C) PAGE with AgNO3 staining after heteroduplex analysis. All cases appeared clearly as clonal bands.

assessed by analysis of 28 negative controls. All these latter samples showed a polyclonal distribution at heteroduplex analysis, although they had given rise to confusing bands (pseudo-clonal bands) in agarose (five samples) and PAGE before heteroduplex analysis (two and eight samples with EtBr and $AgNO_3$, respectively), probably because of a high number of reactive B-lymphocytes in these samples. Accordingly, heteroduplex analysis will solve the problem of false-positive results inherent to the PCR strategy for diagnosis of monoclonal B-cell proliferation.

An additional problem of PCR of VDJ junctional regions of the IgH genes when consensus primers are used is the lack of amplification, ranging from 15% to 30% of cases depending on the different strategies used for clonality assays.[4-7] Possible reasons for this PCR negativity are numerous, but probably the most frequent limitation is the existence of primer mismatches within the FR3 and FR2 regions originating from somatic mutations, which are very frequent in mature B-LPD, such as follicular and MALT lymphomas.[8-11] Nevertheless, in the present study, only 9% of B-NHL cases did not amplify the VDJ region using both FR3 and FR2 consensus primers. This high clonality detection rate is probably the result of two technical modifications: i) heteroduplex analysis of PCR products allows a reduction in the stringency of PCR conditions, which increases the efficiency of the highly degenerated primers that we used; ii) most studies on PCR-based clonality detection techniques employed agarose or PAGE stained with EtBr but in our experience, silver nitrate staining significantly enhances the sensitivity of the method to a degree ranging from 30% (13/42 false-negative results in agarose) to 17% (7/42 false-negative results in PAGE with EtBr staining). Using VH family specific primers the rate of clonality detection should be increased.[4,6-11]

Analysis of serial dilutions of tumor DNA in control DNA revealed sensitivities of 1% when a high *background* of polyclonal B-cells was present in the dilution-used samples. This low sensitivity is adequate for initial diagnosis, but it is certainly not sufficient for detection of minimal residual disease (MRD).[25,26] The sensitivity did, however, increase to 10^{-3} when the *background* of polyclonal B-cells was lower. This second situation is the most likely to be found in patients in whom MRD studies may be required (i.e. after intensive chemotherapy treatment, a very low number of polyclonal B cells would be expected). Nevertheless, heteroduplex analysis has a lower detection level than that achieved using clone-specific markers, which are highly desirable for monitoring MRD.[25]

In summary, the heteroduplex PCR procedure described here, including silver nitrate staining, for analysis of IgH rearranged genes is a simple, rapid, and non-expensive alternative to Southern blot analysis for the assessment of clonality in B-cell non-Hodgkin's lymphoma.

Contributions and Acknowledgments

MG and DG were the principal investigators, designed the study, performed the literature revision and wrote the paper. MG, DG, and RG-S were responsible for the data interpretation, direct supervision and day-to day contact with the participants. RL-P, MCC, AB, MVM and IA helped to collect/interpret the data and standardize the techniques. Preliminary results were produced at AWL and JJvD's laboratory and their suggestions contributed greatly to solving technical problems. AO and JFSM contributed to the writing of the paper and gave final approval of the version to be published.

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Disclosures

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