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BRIEF COMMUNICATION

Evaluation of a new flow cytometry crossmatch procedure for simultaneous detection of cytotoxicity and antibody binding

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Abstract

In this study we have evaluated an alternative 96-well format flow cytometry based (FCtox) method which enable simultaneous detection of cytotoxicity and human leukocyte antigen (HLA) antibody binding. Comparable results were obtained in side-by-side comparisons with conventional complement-dependent cytotoxicity (CDC) and flow cytometric crossmatch (FCXM) in terms of sensitivity and specificity. There was 91 and 93% agreement between results obtained by FCtox and CDC for T and B cells, respectively. In addition, comparable results were obtained with FCtox IgG and FCXM IgG for both T and B cells. Furthermore, compared with a recently developed and highly sensitive Luminex based C1q assay we obtained close to 90% method agreement with the FCtox assay. Our alternative cytotoxicity and IgG binding assay which exhibit low intra-and inter-assay variation will improve the workflow and speed up the pre-transplant testing and also allow continuous monitoring of assay performance and proper quality assurance.

Numerous reports published over the last 40 years have demonstrated that cytotoxic donor-specific human leukocyte antigen (HLA) antibodies (DSA) can mediate acute and chronic kidney graft rejections (1). Although the mechanism of graft destruction is not entirely clear, the HLA antibodies are believed to target endothelial cells of the transplanted organ (2). The use of crossmatch assays such as the CDC assay (3), FCXM assay (4) and solid phase techniques using HLA antigen coated beads for detection of DSA antibodies (5) are valuable tools for selection of suitable patient–donor pairs. The determination of cytotoxic antibodies with the CDC assay was developed already in 1964 (6). At least in Europe, CDC assays are still an important part of clinical decision making. Intriguingly, apart from a few modifications introduced to overcome some of its weaknesses, such as insensitivity and nonspecific responses, it has basically remained unaltered since its introduction (7, 8). Since 1983, a flow cytometric assay based on the determination of antibody binding to HLA class I and/or class II expressing lymphocytes have been used in clinical transplantation (Tx) laboratories (4). This assay enabled detection of both complement-fixing and noncomplement-fixing antibodies and due to its high sensitivity, even low avidity HLA antibodies, previously unrecognized by CDC, could be identified (9). Since the introduction of flow cytometry in Tx laboratories, there has been a growing interest in developing an assay for detection of cytotoxic antibodies using flow cytometry (10–13). In recent years, the flow cytometry based (FCtox) approach has been further refined, which has enabled simultaneous detection of both antibody binding and cytotoxicity (14–16). Taken together, all of these studies show that the FCtox assessment of antibody and cytotoxicity in one single assay is sensitive and objective.

In this report, we present a FCtox assay for detection of both antibody and cytotoxicity specifically designed for routine application. The performance of the assay was compared with conventional CDC and FCXM as well as with the presence of single antigen bead binding IgG antibodies and C1q fixing antibodies, as determined with the recently developed complement C1q assay (17, 18).

Immuno-magnetic bead pre-enriched T (CD3+; Figure 1A) and B cells (CD19+; Figure 1B) were used as target cells and purity-checked at the time of crossmatch. The purity for T and B cells were 98% ± 2.9 ($n = 37$) and 94% ± 5.3 ($n = 37$), respectively. The crossmatch assays were performed in multi-well plate (96-well) which enabled processing of multiple samples simultaneously. The time required for completing all steps in the protocol was approximately 3.5 h. A forward/side scatter (FSC/SSC) gate was set on live and dead (low FSC) lymphocytes to exclude cell debris (Figure 1C, left panels). The percentage of dead [7-aminoactinomycin D (7-AAD+)] cells (Figure 1C, middle panels) and level of IgG antibody binding (Figure 1C, right panels) on gated T and B cells were displayed on one-dimensional histograms. Generally, no or only minor regating of the FSC/SSC gate or the positive marker set on dead cells in the 7-AAD histogram was required between different experiments. Note that 7-AAD positive cells show reduced cell size (low FSC) (Figure 1C, DSA + sera). The robustness of the FCtox assay was determined by intra- and inter-assay crossmatches with HLA antibody negative and positive sera at different dilutions (Table 1).

In the first round of FCtox assay validation we analyzed antibody binding (IgG) and cytotoxicity (7-AAD) using sera with or without donor-specific IgG antibodies (DSA). The target cells were isolated from 14 ($n = 14$) different HLA-typed donors and crossmatched against a panel of 21 ($n = 21$) different HLA antibody negative and positive sera. In total 208 cell−serum combinations (i.e. crossmatches) were performed. For T cell targets, 129 and 79 crossmatches were performed with DSA+ and DSA− sera, respectively. For B cells targets, 148 and 60 crossmatches were performed with DSA+ and DSA− sera, respectively (Table 2). The majority of crossmatches (>95%) using DSA+ sera resulted in IgG binding and a fraction (>50%) of these sera were also able to induce cytotoxicity. A small proportion of crossmatches using DSA+ sera did not generate positive T FCtox IgG data. All of these sera had relatively low levels of DSA antibodies [mean fluorescence intensity (MFI) < 3000, data not shown]. A similar frequency of DSA+/B FCtox IgG− was observed and a fraction (15%) of the crossmatches using DSA− sera resulted in B FCtox IgG+.

In a second round of experiments the FCtox assay was performed in parallel with the conventional CDC and FCXM assays (Appendix S1, Supporting Information). The donor cells were from 9 ($n = 9$) different HLA-typed individuals and crossmatched against a panel of 29 ($n = 29$) different HLA antibody negative ($n = 12$) and positive sera ($n = 17$). In total 53 ($n = 53$) cell−serum crossmatches were performed. There was >90% agreement between results obtained by the FCtox 7-AAD and CDC assay as shown in Table 3. Similar assay correlation was observed for FCtox IgG and FCXM. Detailed analysis of the sera used in crossmatches with FCtox−/CDC+ outcome revealed that in the majority (5/7, 71%) of cases no DSA IgG, IgM and/or C1q fixing antibodies could be detected (data not shown). Furthermore, in both of the FCtox+/CDC− crossmatches C1q fixing antibodies (MFI: 5750 and 16976) were detected. In T FCtox IgG−/T FCXM IgG+ crossmatches ($n = 4$) DSA IgG were detected in two of the sera. In two of three (2/3) B FCtox IgG+/B FCXM IgG− crossmatches DSA IgG antibodies (MFI: 1497, 3058) were detected.

In order to evaluate the cytotoxicity parameter of FCtox further we performed crossmatches using sera with and without DSA C1q fixing antibodies (Table 4). Seventy seven percent of the T FCtox 7-AAD and 80% of the T CDC crossmatches were found to be positive when sera containing donor-specific C1q fixing antibodies were used. For B cells, even larger frequencies (~90%) of crossmatches were positive. The data obtained from C1q assay correlated well ($P < 0.0001$) with crossmatch outcome for both FCtox and CDC. No significant difference ($P > 0.05$) was observed between FCtox and CDC in their ability to detect sera with C1q fixing antibodies. A number of crossmatches with C1q+ sera did not result in lysis using FCtox or CDC assay. In the majority of C1q+/T FCtox 7-AAD− crossmatches (9/15, 60%) the sera had C1q MFI values below 3000 (Range: 1273 to 22,848). Notably, lack of lysis was observed for both FCtox and CDC even in sera with relatively high C1q MFI values. The C1q MFI values for B FCtox 7-AAD− assay were between 1273 and 19160. The MFI values for C1q fixing antibodies in T CDC− were from 1684 to 7658 and in B CDC− assays from 1848 to 16976.

Donor-specific complement-fixing antibodies are a well documented contraindication for Tx and it is therefore important to determine their existence prior to Tx. Although Tx centers worldwide to some extent use different criteria in their selection of suitable patient−donor pairs, the unifying cellular technique for many laboratories is the CDC assay. Many Tx centers also perform HLA antibody binding assay by flow cytometry (19). We perform CDC and FCXM on all patient−donor samples for both living and diseased donors as a part of the immunological work-up. The criteria to proceed with Tx are that both CDC (T and B cells) and FCXM (T and B cells) are negative. Using CDC together with FCXM in the patient−donor evaluation has clearly minimized the risk of early antibody mediated graft rejection (20). As a consequence of increased work-load in our laboratory there are higher demands on the laboratory staff to master several techniques under time-restrains. In order to circumvent that (and other issues discussed below) we here aimed at developing a FCtox assay which would enable us to perform both IgG binding and cytotoxicity using a single assay.

In this study we show that the outcome from FCtox crossmatches were generally in good agreement with the expected outcome based on current available methodologies such as CDC and FCXM. Occasionally although, disparate results between conventional methods and FCtox were obtained. Importantly, the majority of the discrepancies observed here may be explained by false positive CDC, as the majority of the

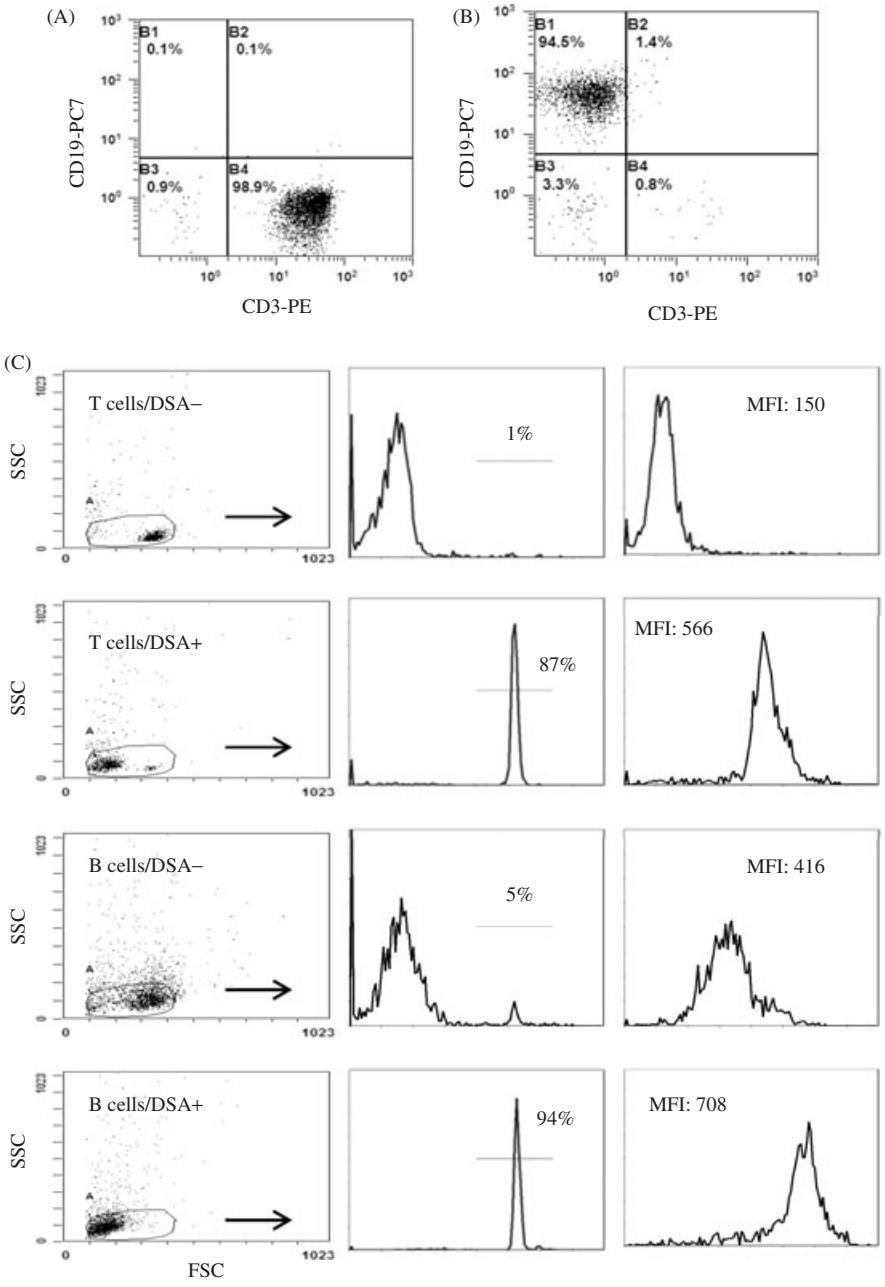

Figure 1 Representative plots from a flow cytometric cytotoxic crossmatch assay with immuno-magnetic bead (EasySep® Human cell Enrichment Kits #19051 and 19054; Stem Cell Technologies, Vancouver, BC, Canada) sorted T cells (A) and B cells (B) against donor-specific antibody negative (DSA−) and DSA+ serum (C). T and B cells were incubated with serum for 30 min at room temperature (22°C). Then after washing steps, incubated for 30 min at room temperature with 20 µl of standard rabbit complement (CL3111; Cedarlane Laboratories Ltd, Ontario, Canada). Wash steps were then repeated and goat anti-human IgG F(ab'2) FITC (109-096-098; Jackson ImmunoResearch Europe Ltd., Suffolk, UK) was then added for 20 min at +4°C. After washing steps 7-aminoactinomycin D (7-AAD) solution (A07704; Beckman Coulter, Miami, FL) was added. Percentage of lysis above background for each sample (Δ%) was calculated as follows: percentage 7-AAD+ cells of sample − percentage 7-AAD+ cells of negative control serum. The IgG channel shift [Δ mean fluorescence intensity (ΔMFI)] was calculated as follows: median fluorescence intensity (MFI) of sample − median (MFI) of negative control serum. Cell debris were excluded by applying a forward and side scatter gate on live and dead lymphocytes as shown in the left hand forward/side scatter (FSC/SSC) dot plot panels. The percentage of dead cells (7-AAD+) and the MFI of IgG antibody binding for T cells and B cells are shown in the mid and right hand histograms, respectively.

sera lacked C1q fixing antibodies and/or IgG or IgM antibodies. In addition, all of the CDC+ crossmatches with negative FCtox results had weak reactivity (score 2) which supports the FCtox and LMX data. Twenty-three percent of T cell crossmatches were found to be FCtox 7-AAD−/LMX-C1q+. This is somewhat expected because the LMX C1q assay is highly sensitive and not all C1q+ antibodies may result in cell lysis or can be detected with a less sensitive assay such as FCtox (or CDC). It is important to point out that *in vitro* cytoxicity negative sera with low-titer DSA may induce recall B-cell memory response *in vivo* which could lead to rejection. Future validation will be needed in order to determine whether the C1q assay reliably can predict which antibodies will or will not fix antibodies.

In contrast to all previously described FC protocols using anti-CD3 and CD19 mAb staining to discriminate between T and B cells, we here use a fast immuno-magnetic isolation negative selection procedure to enrich T and B cells with high purity, without the use of any affinity columns. The advantage with this approach is that no beads are attached to the target cells and that no other (nontarget) cells are present during the crossmatch assay. Furthermore, the negative selection strategy also enables FCtox purity-check of enriched target cells; an important step towards an improved quality control

Table 1 Intra- and inter-assay variation of the FCtox assay[a]

	T cells		B cells	
Intra-assay[b]	Negative control serum	Positive control serum	Negative control serum	Positive control serum
7-AAD + (%)	2.4 ± 0.7	67.2 ± 2.2	4.2 ± 0.9	50.9 ± 5.8
IgG (MFI)	112 ± 6	491 ± 6	388 ± 10	564 ± 10

	T cells				B cells			
Inter-assay[c]	1/8[d]	1/16	1/32	1/64	1/8	1/16	1/32	1/64
7-AAD + (Δ%)[e]	80 ± 4	65 ± 9	29 ± 11	5 ± 3	73 ± 5	62 ± 9	36 ± 10	16 ± 6
IgG (ΔMFI)[f]	409 ± 6	374 ± 10	356 ± 5	317 ± 7	193 ± 34	159 ± 30	141 ± 24	123 ± 27

7-AAD, 7-aminoactinomycin D; MFI, mean fluorescence intensity.
[a]HLA antibody positive control sera consisted of pooled sera from highly immunized patients with HLA class I and/or class II antibodies (>80% panel reactive antibodies; PRA).
[b]Cells isolated from one donor. Mean ± SD (replicates $n = 8$). Representative data from one of totally four donors.
[c]Cells isolated from cryopreserved PBMC from one donor.
[d]HLA Ab + serum at indicated dilutions.
[e]Frequency of 7-AAD+ cells minus background (negative serum). Mean ± SD ($n = 4$)
[f]Fluorescence intensity of IgG expression minus background. Mean ± SD ($n = 3$)

Table 2 FCtox crossmatches against sera with or without donor-specific antibodies

	T FCtox 7-AAD[a]		T FCtox IgG[a]	
DSA IgG	Positive	Negative	Positive	Negative
Positive[b]	76/129 (59%)	53/129 (41%)	124/129 (96%)	5/129 (4%)
Negative	0/79 (0%)	79/79 (100%)	3/79 (4%)	76/79 (96%)
	B FCtox 7-AAD		B FCtox IgG	
DSA IgG	Positive	Negative	Positive	Negative
Positive[c]	111/148 (75%)	37/148 (25%)	140/148 (95%)	8/148 (5%)
Negative	2/60 (3%)	58/60 (97%)	9/60 (15%)	51/60 (85%)

7-AAD, 7-aminoactinomycin; DSA, donor-specific antibody; FCtox, flow cytometry based method.
[a]The frequencies of T and B cell FCtox positive and negative crossmatches against DSA IgG positive and negative sera. All of the test sera were from patients undergoing evaluation for kidney transplantation. Negative control sera were obtained from healthy blood donors (ABO negative males). The HLA-type (A, -B, -DRβ1 and -DQβ1) of donor cells were molecularly typed on genomic DNA using either polymerase chain reaction (PCR)-sequence-specific oligonucleotides (SSOs) (LABType®; One Lambda Inc., Canoga Park, CA) or PCR-sequence-specific primers (SSPs) (Olerup SSP®; Olerup SSP AB, Stockholm, Sweden) as described by the manufacturers.
[b]HLA class I specific antibodies as determined by Luminex single antigen bead (SAB) based HLA antibody IgG (LMX-IgG) assay.
[c]HLA class I and/or class II specific antibodies determined as above.

of the assay. Immuno-magnetic bead based sorting of cells also has the advantage of being a well-known procedure for the laboratory staff, as it is used in the conventional CDC protocol. To perform CDC our laboratory currently uses positive selection of target cells (T and B cells) using magnetic beads that are noncompatible with FCtox purity-check. In addition, the relatively large beads attached to cells occasionally disturb the visual read-out in the CDC. Another reason for us to choose pre-sorted T and B cells [instead of bulk peripheral blood mononuclear cells (PBMC)] for the FCtox protocol, was that we noted that the CD19 surface expression on B cells was either masked or highly reduced in crossmatches where high level of lysis was observed (M. Alheim, unpublished observation). This clearly hampered proper gating and reliable interpretation of B cell crossmatch data. The reason is not known but warrants further investigations. Using pre-sorted target cells, as described here minimizes the number of markers (i.e. fluorochromes) required to generate T and B cell crossmatch data. Thus, the laboratory staff do not need to master multicolor flow cytometry as basically no compensation is required using the reagents described in this study.

Most studies published on FC-based cytotoxicity have used either prolonged complement incubation times (45 min–120 min) and/or addition of AHG or pretreatment of donor cells with pronase (14–16). However, we are reluctant to use additional steps to enhance sensitivity as a higher degree of result inconsistencies has been reported for AHG enhanced CDC as compared with direct CDC (19). A fraction (15%) of the crossmatches performed in this study using DSA− sera resulted in B FCtox IgG+ outcome. Importantly, similar frequencies of 'false' positive B cell crossmatches have been observed with conventional FCXM (M. Alheim, unpublished observation). It is a well recognized problem that B cells crossmatch is prone to false-positive reactions (21–23). Pre-treatment with proteolytic enzyme pronase may improve the accuracy of B cell crossmatch (24). Saw et al. used pronase to eliminate Fc receptors and thereby reduced nonspecific binding in the cytotoxic flow crossmatch assay (15). However, recent reports suggest that pronase treatment may contribute to increased sensitivity for lysis

Table 3 Side-by-side comparison of crossmatch results for FCtox, CDC and FCXM[a]

	T FCtox 7-AAD[b]			T FCtox IgG[c]	
T CDC[b]	Positive	Negative	T FCXM IgG[c]	Positive	Negative
Positive	20/53 (38%)	4/53 (7%)	Positive	37/53 (70%)	4/53 (7%)
Negative	1/53 (2%)	28/53 (53%)	Negative	0/53 (0%)	12/53 (23%)
	B FCtox 7-AAD[b]			B FCtox IgG[c]	
B CDC[b]	Positive	Negative	B FCXM IgG[c]	Positive	Negative
Positive	29/53 (55%)	3/53 (5%)	Positive	37/53 (70%)	0/53 (0%)
Negative	1/53 (2%)	20/53 (38%)	Negative	3/53 (5%)	13/53 (25%)

7-AAD, 7-aminoactinomycin; FCtox, flow cytometry based method; FCXM, flow cytometry lymphocyte crossmatch.
[a]Complement-dependent cytotoxicity (CDC) assay and FCXM was performed according to standard procedures.
[b]Crossmatch outcome for CDC *vs* FCtox 7-AAD.
[c]Crossmatch outcome for FCXM IgG *vs* FCtox IgG.

Table 4 FCtox and CDC crossmatches against sera with or without C1q fixing donor-specific antibodies

	T FCtox 7-AAD[a]		T CDC[a]	
DSA C1q	Positive	Negative	Positive	Negative
Positive[b]	51/66 (77%)	15/66 (23%)	20/25 (80%)	5/25 (20%)
Negative	3/61 (5%)	58/61 (95%)	3/24 (13%)	21/24 (87%)
	B FCtox 7-AAD[a]		B CDC[a]	
DSA C1q	Positive	Negative	Positive	Negative
Positive[c]	80/90 (89%)	10/90 (11%)	29/32 (91%)	3/32 (9%)
Negative	7/39 (18%)	32/39 (82%)	4/17 (24%)	13/17 (76%)

7-AAD, 7-aminoactinomycin; CDC, complement-dependent cytotoxicity; DSA, donor-specific antibody, FCtox, flow cytometry based method.
[a]The frequencies of positive and negative FCtox 7-AAD and CDC assays against serum with or without DSA C1q fixing antibodies. The C1q (LMX-C1q) assay was performed according to the instructions of the manufacturer (LABscreen®, One Lambda Inc., Canoga Park, CA).
[b]HLA class I specific antibodies.
[c]HLA class I and/or II specific antibodies.

(25, 26). In addition, pronase treatment can affect the level of HLA expression on the surface of T and B cells, which could lead to incorrect crossmatch data (27). Other disadvantages are that the enzymatic digestion affects the cell viability and adds at least 30 min to the protocol.

In our selection process to find an appropriate patient for Tx of an organ from a diseased donor, we initially perform a T and B cell CDC crossmatch (>3 h to result) and thereafter select five CDC negative patients for additional FCXM analysis (>2 h). Thus the total time needed to complete both type of crossmatches is at least 5 h. To perform two separate assays either in parallel or sequentially, requires several operators or leads to an extended time to obtain the results. The time aspect is very important for crossmatching prior to diseased donor organ Tx, where cold ischemic time is a major concern. A great advantage with the FCtox described here is that both cytotoxicity and IgG binding data is obtained within 3.5 h.

Another important aspect is the choice of cytotoxicity assay read-out. CDC is based on visual inspection and an estimation of the frequencies of lyzed cells. This approach is obviously more subjective than flow cytometry. Our experiments presented here show that the FCtox assay has low intra-and inter-assay variation. Ongoing experiments show that the FCtox assay has low inter-instrument and inter-operator variability (M. Alheim, unpublished observation). Importantly, the latter type of variation is occasionally observed for the CDC and this variability should be minimized using a more objective FCtox assay. Furthermore, CDC as it is designed today, with manual scoring hampers the introduction of automation. Our laboratory perform other cellular assays such as immunophenotyping (CD4/CD8 ratio) and have successfully automated the data management and transfer of the results directly into our laboratory information system (LIS) via commercially available softwares. With this automated approach we avoid several manual steps in our data analysis and are able to process a large number of samples in a short time. This strategy could easily be applied for flow cytometry crossmatching using the herein described FCtox assay. In addition, the use of FCtox will clearly simplify the quality assurance and monitoring of day-to-day validations of assay performance. However, further retrospective and prospective studies are required to fully evaluate and validate its clinical usefulness in a routine laboratory setting. It could be valuable to accurately estimate the incidence of graft rejection in donor–recipient pairs evaluated with CDC, FCXM and FCtox and C1q assay.

Conflict of Interests

The authors have declared no conflicting interests.

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Supporting Information

The following supporting information is available for this article:

Appendix S1. Material and methods.