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Technical comment

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A sensitive protocol for *FOXP3* epigenetic analysis in scarce human samples

$CD4^+CD25^{high}$ regulatory T (Treg) cells are key players in the maintenance of peripheral immune tolerance [1]. Stable expression of the FOXP3 transcription factor is essential for Treg cells' ability to suppress the immune responses of conventional T (Tconv) cells [2]. FOXP3 stability in murine Treg cells has been linked to *FOXP3* locus demethylation at the CNS2 [3, 4], also called the Treg-specific demethylated region (TSDR) [5]. In contrast to FOXP3 expression [6, 7], complete demethylation of the TSDR [3], in addition to other genomic regions [8], is a true hallmark of human and mouse Treg cells, and its role in the early Treg lineage specification is currently under intense scrutiny in the mouse [3, 8, 9]. Unfortunately, the investigation of TSDR demethylation to define whether $FOXP3^+$ T cells are bona fide Treg cells in translational research settings has so far been inaccessible due to limitations in clinical sample amount. Indeed, methods currently available to inspect the TSDR at single-CpG resolution [5] lack sensitivity due to bottlenecks at the amplification stage (Fig. 1A). Other methods originally aimed at counting Treg cells in unsegregated populations [10] can be adapted to study the regulatory lineage commitment of sorted cells. However, because these methods separately amplify methylated and unmethylated TSDRs, the sample amount requirement is a function of the degree of TSDR methylation, growing exponentially at the two ends of the range, where one of the two species becomes limiting.

In this work, we report an inexpensive, single-CpG resolution, PCR-based protocol with very low requirements on sample amount and robust to aldehyde-based fixation, features making it of immediate relevance for sample-limited research settings. Our optimized protocol enhances the sensitivity of existing techniques [5] while retaining specificity. We introduced nested PCR, touchdown preamplification, primer tailing, and a two-step sequencing cycle (detailed protocol available as Supporting Information). The nested PCR targets 15 commonly investigated CpGs sites [5, 10], thereby allowing for direct comparison of results with previous literature. However, we found that measurements of the first CpG dinucleotide are typically noisier due to proximity to the sequencing primer, and we recommend excluding this first CpG dinucleotide, unless several technical replicates are performed. The touchdown preamplification step was introduced not to compromise specificity with the enhanced sensitivity. Moreover, we added 5′ tails to the inner primers in order to (i) increase their length, which allows raising the annealing temperature, thereby minimizing spurious amplification; (ii) introduce C and G nucleotides, which are rarer in amplicons from bisulphite-converted templates, thereby increasing DNA complexity and, consequently, the specificity of primer annealing; (iii) extend the amplicon at the 5′ end, which allows to shift the sequencing primer upstream, thereby improving base resolution at the 5′ end; and (iv) allow the sequencing primer to anneal only to the inner-PCR product. Finally, the two-step sequencing cycle yields a better signal balance when compared with the standard three-step denaturation/annealing/extension cycle. Electropherograms were analyzed with ESME to quantitatively determine the methylated/unmethylated CpG ratio [11]. We favored Sanger sequencing for its widespread availability, but pyrosequencing is also an attractive option.

Our method was able to successfully generate TSDR amplicons from much lower amounts of template than single-round PCR techniques (Fig. 1A). The TSDR methylation profiles of $CD25^{low/-}$ Tconv cells and $CD25^{high}CD127^{low/-}$ Treg cells generated by our protocol are in line with published data [5, 8–10], demonstrating its accuracy (i.e. proximity to the true value) in measuring highly methylated (Tconv cells), highly demethylated (male Treg cells), and partially demethylated (female Treg cells, due to *FOXP3* methylation on the inactive X chromosome) samples (Fig. 1B). Accuracy was linearly preserved across the whole range of TSDR methylation, indicating no amplification bias of either methylated or unmethylated TSDR templates (Fig. 1C). In addition, to carefully characterize the lower limit of detection, we calculated the percentage error (a normalized measure of the difference between observed and expected value, i.e. an estimate of accuracy) and the coefficient of variation (a normalized measure of data dispersion around the mean, i.e. an estimate of precision) of technical replicates across decreasing amounts of template gDNA. We were able to amplify down to 1.25 ng bisulphite-converted gDNA with 100% success rates

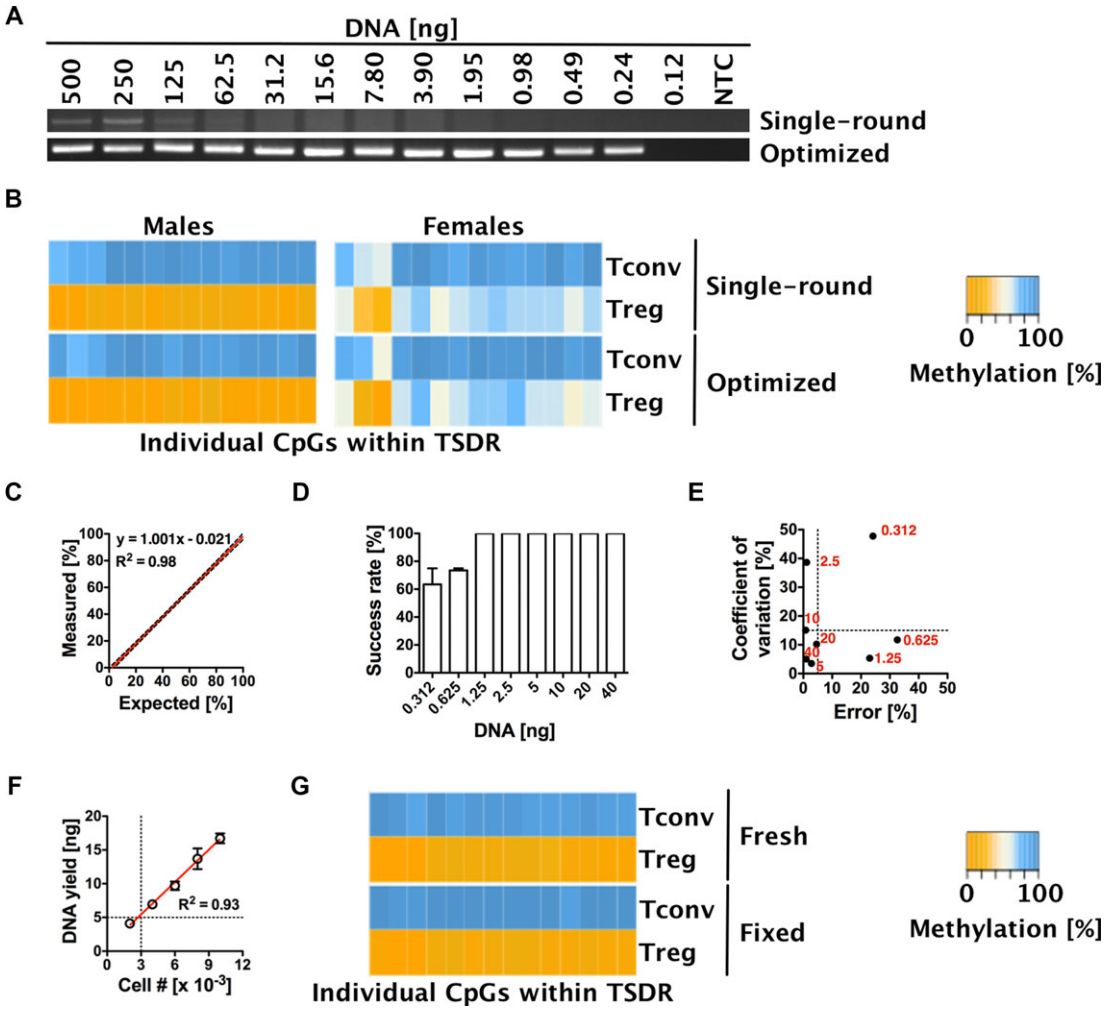

Figure 1. Technical characterization of the TSDR methylation analysis protocol. (A) Amplification performance of decreasing amounts of bisulphite-converted gDNA from $CD4^+$ T cells using a single-round PCR protocol [5] or our optimized protocol. NTC, no template control. (B) Male and female Tconv cells and Treg cells were sorted as $CD4^+CD25^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$. The degree of methylation for each CpG site was color-coded according to the legend. Two representative healthy donors out of eight analyzed are shown. (C) pUC57 plasmids carrying sequences corresponding to bisulfite-converted methylated and unmethylated TSDR were mixed at known ratios to generate increasingly methylated templates. Each reaction received 2 500 plasmid copies. The methylation degree of four replicates per point was fitted to a linear model, which is displayed with 95% confidence intervals (gray area delimited by dotted lines). (D) The amplification success rate and (E) percentage error and coefficient of variation of four technical replicates amplified from male Tconv and Treg-cell samples mixed at 1:1 ratio are shown. Each point indicates the average of 14 CpGs, with the amount of bisulfite-converted gDNA per amplification (in ng) indicated in red. Dotted lines mark the thresholds of acceptance. (F) The yield of bisulphite-converted gDNA from varying cell numbers, FACS-sorted in lysis buffer, was fitted to a linear model. (G) Male Tconv cells and Treg cells were sorted as $CD4^+CD25^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$ (fresh), or $CD4^+CD25^-$ and $CD4^+CD25^+FOXP3^+$ (fixed). The degree of methylation for each CpG site was color-coded according to the legend. Data shown are representative of at least three independent experiments. In D and F, error bars represent the SD.

(Fig. 1D). The percentage error remained below 5% for gDNA input down to 2.5 ng, and the coefficient of variation remained within 15% down to 5 ng (Fig. 1E). Based on these data, we are confident that our optimized protocol allows a gDNA input as low as 5 ng. As few as 3 000 Treg cells were sufficient to obtain this amount of bisulphite-converted gDNA (Fig. 1F). Below this threshold, stochastic sampling would degrade accuracy and precision. Finally, our protocol can be performed on cells that have previously undergone intracellular staining, as we obtained similar results when using either fresh ($CD25^{high}CD127^{low/-}$) or paraformaldehyde-fixed ($CD25^+FOXP3^+$) Treg cells (Fig. 1G).

We showed the feasibility of our technique on Treg cells isolated from limiting samples of thymus and blood of thymectomized infants. Differently from circulating $CD4^+$ T cells, a significant fraction of $CD4^+$ thymocytes was positive for FOXP3 while remaining negative for CD25 (Fig. 2A). As such, thymic samples were sorted as either total $CD3^+CD4^+CD8^-FOXP3^+$ or $CD3^+CD4^+CD8^-CD25^+CD127^-FOXP3^+$ cells. Our method was able to successfully measure the TSDR methylation profile of all sorted populations (Fig. 2B), demonstrating its value for follow-up studies

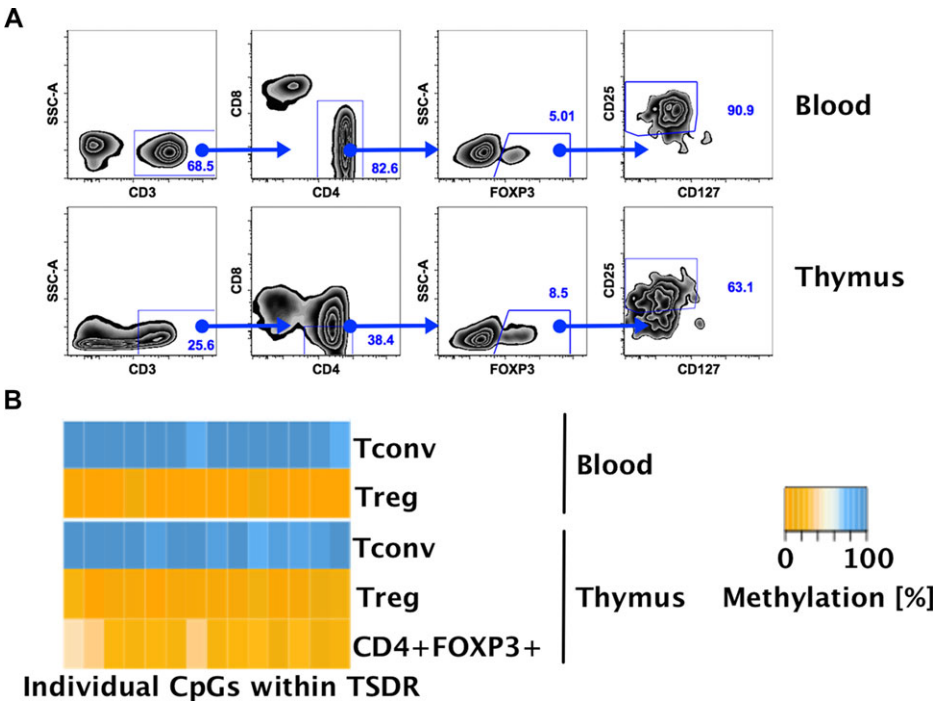

Figure 2. TSDR methylation of Treg cells isolated from pediatric samples. (A) CD25, CD127, and FOXP3 staining within $CD3^+CD4^+CD8^-$ T cells from peripheral blood and thymus. (B) Fixed cells from blood and thymus were sorted as $CD3^+CD4^+CD8^-CD25^-$ (Tconv), $CD3^+CD4^+CD8^-CD25^+CD127^-FOXP3^+$ (Treg) or $CD3^+CD4^+CD8^-FOXP3^+$ ($CD4^+FOXP3^+$). The degree of methylation of each CpG site was color-coded according to the legend. Amplifications were performed using 15–20 ng bisulphite-converted gDNA, and two to three replicates were run per sample. A representative male infant out of five is shown throughout the figure.

aimed at characterizing the human Treg lineage specification in the thymus.

In conclusion, we have optimized and validated an inexpensive, accessible, single-CpG resolution tool for *FOXP3* methylation analysis that is immediately applicable in translational research settings previously uncharted due to inaccessible requirements, which will help to shed new light on the development and stability of the Treg compartment in health and disease.

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Abbreviations: **Tconv cell**: conventional T cell · **TSDR**: Treg-specific demethylated region

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The detailed *Materials and methods* for Technical comments are available online in the Supporting information

