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Analytical Validation and Field Feasibility of a MIQE-Compliant Immune-Transcript Assay (CD3E, CD4, CD8A, IL10) from Dried Blood Spots for Integrated Classification of Pediatric Iron-Deficiency Anemia

MOMENA SAMI AHMED MOHAMMED
University of Karary, Faculty of Medical Laboratory Sciences
Prof. NADIA MADANI MOHAMED AHMED
University of Karary, Faculty of Medical Laboratory Sciences
Dr. TARIG A. M. HAMID
University of Karary, Faculty of Medical Laboratory Sciences
Dr. SHIREEN SHEREEN MAHY ALDIN ABDALLAH
University of Karary, Faculty of Medical Laboratory Sciences
Dr. MUTAZ MOHAMED IBRAHIM ALI[1]
Basil Biomedical, Egypt

Abstract

Background: *Iron-deficiency anemia (IDA) remains the leading pediatric hematologic disorder worldwide, compounded by infection-driven inflammation that confounds traditional iron biomarkers. Translational diagnostics increasingly seek multiplexed yet field-deployable molecular assays capable of retaining analytical rigor outside reference laboratories.*

Methods: *A cross-sectional study (n = 100; age 6 months–12 years) evaluated a four-gene immune-transcript panel (CD3E, CD4, CD8A, IL10) quantified by MIQE-compliant RT-qPCR from paired EDTA blood and dried blood spots (DBS). Validation parameters included primer efficiency (90–110 %), melt-curve specificity, reference-gene stability (geNorm $V_{2/3} \leq 0.15$; NormFinder ≤ 0.30), and replicate precision (CV ≤ 5 %). Diagnostic analyses compared mild (Hb ≥ 10 g/dL) vs moderate/severe (Hb < 10 g/dL) IDA and explored associations with BRINDA-adjusted ferritin, Ret-He, and hepcidin.*

Results: *Mean RNA yield was higher from EDTA (4.2 µg) than DBS (3.1 µg; p = 0.002), while purity and Ct precision were equivalent. All primers achieved 95–103 % efficiency with single melt peaks. Reference genes GAPDH and HPRT1 were stable (M = 0.56). CD3E expression declined with anemia severity (p = 0.04) and correlated with ferritin (r = 0.29, p = 0.004); IL10 rose as Hb decreased (r = –0.22, p = 0.03). The Combined Immune Score (CIS = mean z-CD3E – z-IL10) yielded AUC 0.79 (95 % CI 0.70–0.88) with 10-fold cross-validated AUC 0.78 (SD 0.05). Expression ratios were matrix-invariant.*

Conclusions: *A disciplined RT-qPCR workflow applied to DBS quantifies biologically meaningful immune axes in pediatric IDA. CD3E and IL10 serve as reproducible reporters of T-cell tone and counter-regulation. The CIS adds orthogonal diagnostic context to ferritin, Ret-He, and hepcidin and is operationally compatible with humanitarian field pipelines.*

Keywords: iron-deficiency anemia; child; BRINDA; Ret-He; hepcidin; RT-qPCR; MIQE; DBS; CD3E; IL10

[1] corresponding author. Email: moatasa@hotmail.com

1. INTRODUCTION

Iron-deficiency anemia (IDA) remains the most prevalent nutritional disorder of childhood, affecting nearly one in three children worldwide (1, 2). In malaria- and infection-endemic settings, inflammation alters canonical iron markers, complicating interpretation and leading to both under- and over-treatment. WHO's 2024 guideline on hemoglobin thresholds emphasizes context-specific biomarker interpretation and reinforces the need for affordable yet reliable diagnostic strategies (1). Ferritin, the standard indicator of iron stores, is an acute-phase reactant that rises in infection. BRINDA algorithms correct ferritin for inflammation using regression adjustment against CRP and AGP (3-5), yet even adjusted ferritin reflects storage rather than functional iron supply. Reticulocyte hemoglobin equivalent (Ret-He) and hepcidin add functional and regulatory insight (6-10), but neither captures the immune-metabolic processes that modulate erythropoiesis during chronic inflammation. Immune–hematopoietic crosstalk tightly links iron metabolism and cellular immunity. Iron scarcity impairs T-cell proliferation, while cytokines such as IL-6 and IL-10 induce hepcidin and suppress erythropoietin signaling (15-17). CD3E, encoding the CD3ε chain of the T-cell receptor complex, reflects overall T-cell activity and metabolic tone, whereas IL10 marks counter-regulation and iron-restrictive anti-inflammatory pathways. Measuring these transcripts could reveal immune signatures that explain variance in IDA severity not captured by biochemical markers. DBS sampling offers minimally invasive collection, room-temperature stability, and transport simplicity (18, 19). Yet RNA degradation and amplification variability must be controlled. By adhering to MIQE standards (11, 12) efficiency testing, melt-curve verification, reference-gene validation field-collected DBS can produce quantitatively credible qPCR data. Therefor this study was conducted to validate a four-gene immune-transcript assay (CD3E, CD4, CD8A, IL10) for quantitative accuracy, reference-gene stability, and diagnostic utility in DBS samples, and to evaluate its integration with BRINDA-adjusted ferritin, Ret-He, and hepcidin for pediatric IDA classification.

2. METHODS

Cross-sectional diagnostic-validation study conducted between March 2024 and February 2025 among 100 children (6 months–12 years) attending outpatient clinics in [Institution]. Participants were stratified as mild (Hb ≥ 10 g/dL) or moderate/severe (Hb < 10 g/dL) IDA. Exclusion criteria included acute infection, hemoglobinopathies, chronic inflammatory disease, or recent iron therapy. Paired EDTA (0.5–1 mL) and DBS (50–100 µL on Whatman 903) samples were collected. DBS were air-dried 3–4 h, sealed with desiccant, stored ≤ 14 days at room temperature, then –20 °C. EDTA samples were stored 2–8 °C and processed within 48 h. RNA was isolated (QIAamp RNA Blood Mini for EDTA; Zymo Quick-RNA Microprep for DBS) with on-column DNase. Yield and purity measured spectrophotometrically (A_{260}/A_{280} 1.95–2.10). Samples passing ΔCt (no-RT ≥ 10) and replicate SD ≤ 0.3 proceeded to analysis. Reference-gene Ct values served as integrity proxies. SYBR Green chemistry (QuantStudio 5). Primers (Table 1) met efficiency (90–110 %) and single-peak criteria. Normalization to geometric mean of GAPDH and HPRT1 validated by geNorm and NormFinder (13, 14). Relative

quantification via $2^{-\Delta\Delta Ct}$. Means ± SD, t-tests or Mann–Whitney as appropriate. Pearson and partial (age/sex-adjusted) correlations for transcripts vs biochemical indices. Logistic regression for moderate/severe IDA. ROC AUC for CIS (mean z-CD3E – z-IL10), with 10-fold cross-validation. $\alpha = 0.05$.

3. RESULTS:

Analytical validation

Gene	Efficiency %	R^2	Amplicon (bp)	Peak pattern
CD3E	97.4	0.992	112	Single
CD4	98.1	0.993	104	Single
CD8A	99.0	0.991	120	Single
IL10	95.3	0.994	98	Single
GAPDH	101.2	0.997	101	Single
HPRT1	100.4	0.996	94	Single

Reference-gene stability was confirmed (geNorm M = 0.56; $V_{2/3}$ = 0.11; NormFinder 0.23). Intra- and inter-assay CVs were 3.8 % and 4.5 %, respectively.

RNA yield and purity

Metric	EDTA	DBS	p
Yield (µg)	4.2 ± 1.1	3.1 ± 1.0	0.002
$A_{260/280}$	1.98 ± 0.03	1.97 ± 0.04	0.45
QC pass rate (%)	96	94	0.62

Yield loss from DBS was counterbalanced by comparable purity and Ct precision. All NTC and no-RT controls were negative, indicating absence of contamination.

Results:

Children with moderate/severe IDA displayed lower CD3E ($\Delta\Delta Ct$ mean –0.62 ± 0.20, p = 0.04) and higher IL10 (+0.55 ± 0.19, p = 0.04) expression relative to mild cases, while CD4 and CD8A showed downward but non-significant trends. CD3E positively correlated with ferritin (r = 0.29, p = 0.004) and weakly with Ret-He (r = 0.21, p = 0.06); IL10 correlated negatively with Hb (r = –0.22, p = 0.03). The CD3E–IL10 relationship was inverse (r = –0.25, p = 0.02), suggesting reciprocal T-cell and counter-regulatory dynamics.Linear models showed CD3E as a positive predictor of hemoglobin (β = 0.31, p = 0.01), IL10 as a negative predictor (β = –0.25, p = 0.03). The CIS combined index achieved AUC 0.79 (95 % CI 0.70–0.88), with cross-validated AUC 0.78 ± 0.05 and balanced sensitivity (72 %) and specificity (74 %). Results were consistent across DBS and EDTA (p interaction > 0.2). Subgroup analysis showed age modestly modified signal strength (CIS–Hb r = 0.33 in <5 years vs 0.21 ≥5 years). Sex had no effect. Hepcidin was negatively correlated with CD3E (r = –0.26, p = 0.02) and positively with IL10 (r = 0.27, p = 0.02), supporting the immune-iron regulatory link. DBS storage duration (≤ 14 days) had no impact on Ct values (p > 0.5), validating short-term ambient stability.

4. DISCUSSION:

The assay met MIQE criteria throughout, including efficiency within 95–105 % and linearity $R^2 \geq 0.99$. DBS yielded slightly less RNA but retained purity and Ct precision

comparable to EDTA, demonstrating that quantitative accuracy is preserved with field-ready collection.

Dual reference-gene normalization (GAPDH + HPRT1) minimized matrix bias and ensured inter-plate stability. Intra/inter-assay CVs under 5 % affirmed technical reproducibility, satisfying MIQE and allowing inter-laboratory transferability. The inverse relationship between CD3E and IL10 captures a biologically coherent immune-iron interaction: as iron restriction intensifies, T-cell transcriptional activity wanes while anti-inflammatory signaling rises. This recapitulates experimental evidence that iron deficiency dampens T-cell effector function and that IL-10–driven tolerance sustains hepcidin-mediated iron sequestration. By integrating CIS with conventional indices (ferritin, Ret-He, hepcidin), clinicians gain context on whether anemia reflects simple deficiency or inflammatory lockdown…rather than purely nutritional deficiency. This distinction matters in pediatric practice, where inflammatory anemia and iron deficiency frequently overlap. The CIS (CD3E–IL10 composite) functioned as an interpretable metric capturing this immunohematologic balance, achieving cross-validated AUC 0.78, which is notable given biological heterogeneity and field sample variability. The strong equivalence between EDTA and DBS matrices confirms that field-collected DBS samples, if stored properly, can retain qPCR-grade RNA integrity for up to two weeks. This expands feasibility for humanitarian and low-resource deployments where phlebotomy, cold chain, or immediate centrifugation are not viable. The short workflow—from finger-prick DBS to qPCR quantification—enables decentralized screening and aligns with WHO's recommendation for affordable diagnostic decentralization. Beyond anemia, the analytical framework exemplifies how MIQE-disciplined molecular quantification can bridge clinical hematology and immune diagnostics. Because CD3E and IL10 reflect distinct regulatory nodes—activation and tolerance—they could be incorporated into multiplex panels for monitoring vaccine responses, infection recovery, or nutritional immunology interventions. The study's demonstration that dual reference-gene normalization and melt-curve verification suffice for DBS ensures replicability even in small laboratories. While cross-sectional, the findings justify longitudinal validation to evaluate whether CIS predicts hemoglobin recovery or iron therapy responsiveness. Sample size limited power for stratified subgroup analysis (e.g., malaria exposure, under-5 vs school-age). Protein-level correlation (flow cytometry or ELISA) and single-cell transcriptomics could further clarify cellular contributors. Technological adaptation to isothermal platforms (e.g., RT-LAMP with lateral flow) could enable true point-of-care deployment in rural clinics.

5. CONCLUSION:

A four-gene immune-transcript assay (CD3E, CD4, CD8A, IL10) met MIQE standards and maintained analytical reliability when applied to dried blood spots. CD3E and IL10 provided stable, biologically relevant axes reflecting T-cell competence and counter-regulation. The resulting Combined Immune Score improved classification of IDA severity and remained consistent across sample matrices. This compact, reproducible workflow bridges laboratory precision and field practicality, offering a scalable diagnostic tool for anemia surveillance in low-resource settings.

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