

**SPECIAL ISSUE ARTICLE**

Ecoimmunology in the field: Measuring multiple dimensions of immune function with minimally invasive, field-adapted techniques

Aaron D. Blackwell¹ | Angela R. Garcia^{2,3}

¹Department of Anthropology,
Washington State University, Pullman,
Washington, USA

²Research Department, Phoenix
Children's Hospital, Phoenix,
Arizona, USA

³Department of Child Health, University
of Arizona College of Medicine, Phoenix,
Arizona, USA

Correspondence

Aaron D. Blackwell, Department of
Anthropology, Washington State
University, Pullman, WA, USA.
Email: aaron.blackwell@wsu.edu

Abstract

Objective: Immune function is multifaceted and characterizations based on single biomarkers may be uninformative or misleading, particularly when considered across ecological contexts. However, measuring the many facets of immunity in the field can be challenging, since many measures cannot be obtained on-site, necessitating sample preservation and transport. Here we assess state-of-the-art methods for measuring immunity, focusing on measures that require a minimal blood sample obtained from a finger prick, which can be: (1) dried on filter paper, (2) frozen in liquid nitrogen, or (3) stabilized with chemical reagents.

Results: We review immune measures that can be obtained from point-of-care devices or from immunoassays of dried blood spots (DBSs), field methods for flow cytometry, the use of RNA or DNA sequencing and quantification, and the application of immune activation assays under field conditions.

Conclusions: Stable protein products, such as immunoglobulins and C-reactive protein are reliably measured in DBSs. Because less stable proteins, such as cytokines, may be problematic to measure even in fresh blood, mRNA from stabilized blood may provide a cleaner measure of cytokine and broader immune-related gene expression. Gene methylation assays or mRNA sequencing also allow for the quantification of many other parameters, including the inference of leukocyte subsets, though with less accuracy than with flow cytometry. Combining these techniques provides an improvement over single-marker studies, allowing for a more nuanced understanding of how social and ecological variables are linked to immune measures and disease risk in diverse populations and settings.

1 | INTRODUCTION

The immune system is fundamental to understanding health. Immune function measures are useful for

assessing a broad range of characteristics, at both the population-level (i.e., infection prevalence (Blackwell et al., 2011); maintenance of immunity [Demonbreun et al., 2021]) and individual-level (i.e., informing clinical

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care [Cals et al., 2010]). Immune characterization is also central to studies investigating life history allocations (Blackwell et al., 2010; McDade et al., 2008; Urlacher et al., 2018), behavioral responses (Cepón-Robins et al., 2021), and interactive effects of infection on other disease risks (Schneider-Crease et al., 2021). However, capturing the complexity of the immune system and its function outside of a clinical setting presents challenges, primarily arising from limitations on sample collection, preservation, and transport (Blackwell et al., 2021). Blood is the primary tissue used to analyze immune function, but blood collection from venous samples typically requires a trained phlebotomist and participants comfortable providing venous blood. As an alternative, finger prick blood collection is easily accomplished with limited training (McDade et al., 2007). With practice, a finger prick can typically provide ~250 to 500 μ l of blood, especially with high-flow lancets (we prefer the blue BD Microtainer Contact-Activated Lancets #366594; Becton, Dickinson and Company). By using small samples and preservation methods such as drying, freezing, or mixing with stabilizers, sample transport from finger prick samples is simplified, relative to large venous draws or analyses with fresh blood. Finger prick blood can even be collected and mailed by participants themselves (e.g., Demonbreun et al., 2021).

The immune system is complex. It is composed of a wide array of cells, signaling molecules, and effector molecules that exist or act in coordination to prevent or limit infection. Consequently, there are a large number of parameters that studies might measure when seeking to quantify the immune system. For some studies, single measures may be sufficient. For example, C-reactive protein (CRP) levels are useful for capturing a snapshot of systemic inflammation and infection (Black et al., 2004; McDade et al., 2012). However, single measures tell us little about interactions between the immune and other systems, or more abstract constructs like immunocompetence, immunosenescence, and immunological strategies or trade-offs. Additionally, circulating levels may not always be reflective of immunological response to infection; measuring immune responses to stimuli—rather than levels at a single, arbitrary time point—may sometimes be more informative.

Here we evaluate current state-of-the-art methods for measuring immune function in “non-clinical” or field settings using blood collected from a minimally invasive finger prick. First, we provide a brief introduction to the components of immune function which might be measured, and discuss the theoretical and scientific contexts in which these measures might be useful. Next, we briefly discuss point-of-care devices which give immediate results in the field, and then focus the remaining bulk of this review on methods for collection and preservation of

finger-prick blood samples and their subsequent quantification for immune measures including immunoassays of dried blood spots (DBSs), flow cytometry, RNA sequencing, DNA quantification and sequencing, and direct, experimental measures of immune response. After reviewing these, we also briefly discuss prospects for measuring immune function in other biospecimens such as urine, saliva, and feces and discuss further considerations for sample collection and analysis.

2 | APPROACHES TO IMMUNE FUNCTION

2.1 | Aspects of immune function

The immune system is extraordinarily complex and a full accounting of its components and functioning is outside the scope of this review. (A number of excellent textbooks are available on this subject, such as [Parham, 2021]). However, we briefly summarize some major components, which are also listed in Table 1.

The immune system consists primarily of specialized white blood cells (leukocytes) and their secreted products, as well as some components secreted by other tissues for the purposes of signaling or immune defense. These components are typically transported by and measured in the blood but exist throughout the tissues of the body. Note, however, components in the blood and tissues actually represent the last line of defense against parasites and pathogens, as there are many other barriers to infection that include behavioral avoidance and response (the behavioral immune system), physical barriers (such as skin), and secreted immune components at points of entry into the body (the mucosal immune system).

Leukocytes can be broken down into six main subsets: neutrophils, lymphocytes, basophils, eosinophils, monocytes, and macrophages. One subset, lymphocytes, can further be categorized into three main subtypes (T cells, B cells, and natural killer cells), with several distinct classes to each of these cell types. Humoral immunity consists of immunoglobulins (antibodies) secreted by B cells, the main classes of which are IgG (the predominant type), IgM (produced in response to new infections), IgE (a response to macroparasites), and IgA (most commonly investigated in its secreted form, sIgA, as the immunoglobulin in saliva, milk, and other secretions). A wide range of signaling molecules called cytokines (e.g., interleukins, chemokines) are secreted by leukocytes and other cells in the body. Finally, effector molecules (e.g., nitric oxide), members of the complement cascade, and inflammatory proteins such as CRP are secreted by various tissues and participate in immune defense and regulation of other responses.

TABLE 1 Measures of immunity and their applications

Measure	Description	Proxy for	Short-term variation ^a	Long-term variation ^a
C-reactive protein	Acute phase reactant	Infection; chronic inflammation	High	Medium
Cytokines	Immune signaling molecules	Infection; chronic inflammation	High	Medium
Leukocytes	All white blood cells	Infection; immune investment	High	Medium
Helper T cells	Recognize antigen and activate killer T cells and B cells	Cell mediated adaptive immunity; extracellular defense	Low	Medium
Cytotoxic T cells	Trigger apoptosis in cells infected by viruses	Cell mediated adaptive immunity; intracellular defense	Low	Medium
Natural Killer cells	Trigger apoptosis in cells infected by viruses; may defend against parasites	Innate cell mediated immunity	Low	Medium
B cells	Produce antibody	Cellular and humoral immunity	Low	Medium
Naïve T cells, sjTREC	Pool of cells with randomly generated receptors, from which those recognizing novel threats will be selected	Thymic output; investment into defense against future threats; immunosenescence	Low/medium	High
Eosinophils/ Basophils	Respond to macroparasite infections and contribute to allergy	Endo or ectoparasite infection and responses; allergic responses	Medium	Medium
Neutrophils	Phagocytic cells that migrate to infection sites to attack microbes	Infection; immune investment	High	Medium
Monocytes	Phagocytic cells that migrate to infection sites	Chronic inflammation; low levels in blood may indicate migration into tissues to fight infections	High	Medium
Regulatory T cells	Moderate other immune responses	Immunological tolerance; regulation of autoimmunity and allergy	Low	High
IgG	Long-term antibodies to past infections	Humoral immunity; long term protection	Low	Low
IgE	Antibodies related to parasite defense and allergy	Humoral immunity; endoparasite exposure; allergy	Low	High
sIgA	Secreted antibody in saliva, milk, and other fluids	Secretory immunity	Medium	Medium
IgM	The first class of antibody produced after a new infection	Humoral immunity; recent infection	High	Medium
Gene expression	mRNA transcripts from particular genes; representative of production of the related gene product	Depends on the gene and analysis method. See Table 2.	Generally high but varies by gene	Generally medium but varies by gene
Gene methylation	Methylation of regulatory regions of genes, affecting mRNA expression levels	Depends on the gene and analysis method.	Generally low but varies by gene	Generally medium but varies by gene
Immune response assays	In vitro response to an antigen, mitogen, foreign erythrocyte, or microbe	Ability to respond to infection	Unknown	High

^aAssessment of short-term within individual lability (e.g., due to infection) versus long-term differences between individuals or populations. Actual differences for any study will depend on context, for example, the degree of variation in exposures, genetic diversity in the study population, comparison groups, and so forth. Selected reference for variation in these measures: (Blackwell et al., 2011, 2016; Braun et al., 2018; Campisi et al., 2012; Garcia, Trumble, et al., 2020; Lorenzi et al., 2008; Mcdade et al., 2012; Ou et al., 2011; Petrovsky & Harrison, 1998; Sbiera et al., 2011; Urlacher et al., 2018).

2.2 | Categorizing aspects of immune function

Given the complexity of the immune system, there are a number of ways to categorize its components for the purposes of simplifying reasoning and analysis. One common schema divide between innate and adaptive immunity (Blackwell et al., 2010; Garcia, Blackwell, et al., 2020; McDade, Georgiev, & Kuzawa, 2016). Innate immunity does not require experience with a pathogen, while adaptive immunity is acquired through exposure. Adaptive immunity primary involves T and B cells (as well as immunoglobulins), which undergo recombination of their receptors to generate novel variants capable of recognizing new pathogens. Some approaches further divide adaptive responses into cell-mediated and humoral responses, reflecting the differing functions and costs of leukocytes versus antibodies (McDade, 2003, 2005a). However, the term cellular immunity is also often used to encompass all cellular responses, both innate and adaptive, and the term humoral immunity sometimes refers to all factors in the serum, not just antibody (Ruoss et al., 2019).

Other approaches group aspects differently, for example a pattern of gene expression dubbed the conserved transcriptional response to adversity (CTRA; Cole, 2019) has components representing antibody response, type 1 interferon responses (viral responses), and general proinflammatory responses. Approaches also sometimes differentiate between constitutive defenses and induced defenses (Heinrich et al., 2017; Schmid-Hempel & Ebert, 2003). Still other approaches look at responses to specific classes of infection, for example, viral versus bacterial stimuli or indicators of responses specific to parasitic infections (Blackwell et al., 2010; Harrison et al., 2019; Trumble et al., 2016).

2.3 | Tradeoffs and defense portfolios

Classifying aspects of immunity is important from both a theoretical and a practical perspective. Many studies in ecoimmunology and evolutionary anthropology use life history theory as an organizing framework. Life history theory examines the trade-offs organisms make between competing demands on time, energy, and other resources. Most life history approaches decompose these demands into the categories of growth, reproduction, and maintenance, with the maintenance category encompassing tissue repair, immune response, and other factors.

Studies examining trade-offs between immune function and other life history demands are often most interesting in quantifying broad measures of immunity, for example, proxies for immunocompetence or measures

associated with the overall cost of immunity. However, most researchers recognize that immunocompetence is not unidimensional. Different individuals or populations may be more resistant to one kind of threat than to another. Thus, a more nuanced approaches examines trade-offs between competence or investment in different categories of immune response, or in specific immune responses to specific threats. Other approaches think of the suite of investments and individual has as a defense portfolio (Schmid-Hempel & Ebert, 2003), or as an immunophenotype,¹ which can be thought of as a snapshot of an individual's immune system allocations (McDade, Georgiev, & Kuzawa, 2016).

3 | THE RIGHT MEASURES FOR THE QUESTION

An important consideration in designing a study investigating immune function is to measure the right aspect or aspects of immune function for the question under investigation. A well-designed study cannot simply pick the most convenient measure and assume it represents "immune function," since there are many facets to the immune system which may not be correlated or for which correlations may depend on context. We therefore review some general areas of inquiry and the markers researchers have used in those areas, before turning to practical concerns and field friendly methods.

3.1 | Life history allocations, energetics, and individual variation

Central to the field of ecoimmunology is the study of how the energetic demands of immune defense and activation trade-off against other demands such as growth and reproduction. Thus, the measures that are most appropriate for these studies are measures that (1) vary between individuals or populations and (2) are indicative of the energetic demands of immunity. In some contexts, indicators of acute infection may be most relevant, for example, CRP, while in others indicators of chronic infection or immune activation might be better, for example, concentration of immunoglobulin E (Blackwell et al., 2010; McDade et al., 2008). As we might expect from the lability of these measures, these indicate investments on different timescales, and thus trade-offs on different timescales. Urlacher et al. (2018) found that CRP, which varies on short timescales, was associated with growth over a 1-week period, while IgG was associated with growth over a 3-month period, and IgE with growth over much longer periods.

Leukocyte counts are also likely to represent energy investments, since these cells are costly to manufacture and operate (Straub et al., 2010). However, some cell types may be costlier than others, particularly those involved in adaptive immunity, due to the need to maintain a wide repertoire of cells and antibodies with different receptors. For example, Garcia, Blackwell, et al. (2020) found that T cells and B cells were negatively associated with child height-for-age. Naïve subsets of adaptive cell types, or markers of thymic output like sjTREC may further be interpreted as investment into future immune defense, since these represent the production of new cell lines in preparation for new infections.

3.2 | Immunosenescence and immunocompetence

Measures related to immunosenescence may also be useful for comparing individuals and making life history inferences. Naïve cell counts and sjTREC may be useful in this regard, as well as counts of senescent T cells (Franceschi et al., 2000). Methylation assays have been used to develop a so-called “epigenetic clock” related to immune aging (Horvath et al., 2016).

Finally, many measures are intended to compare individuals in terms of immunocompetence—the hypothetical capacity of an individual to respond to a randomly encountered pathogen. The most direct assays of immunocompetence are functional assays such as antigen stimulation assays and bacterial killing assays. However, we caution that for the most part the results of these assays have not been directly validated against real-life morbidity and mortality from illness. It is also probably impossible to create a single, global measure of immunocompetence since individuals will differ in response to different pathogens (Schmid-Hempel & Ebert, 2003).

In animals, measures of immune response, such as the hemolysis-hemagglutination assay (HHA), have been used as measures of immunocompetence and related to sex, reproductive status, and other factors (Gilot-Fromont et al., 2012; Ruoss et al., 2019). These are usually used in concert with other measures such as cell counts and antibody levels in order to provide a multifaceted assessment.

3.3 | The role of inflammation in disease

In the biomedical literature measures of inflammation have gained prominence due to their associations with what tend to be considered chronic diseases of modernity such as obesity, heart disease, and type-2 diabetes (Gurven et al., 2009, 2016; Hersoug & Linneberg, 2007;

West-Eberhard, 2019). These are often straightforward to collect given their ease of measurement in DBSSs. Measures usually include CRP and inflammatory cytokines such as IL-6 and TNF- α . However, these measures are quite labile and care must be taken to distinguish between baseline levels and acute elevations due to infection. Inflammatory markers may need to be interpreted differently in high- and low-pathogen contexts: in low-pathogen contexts baseline elevations of these markers are indicative of nonspecific inflammation and a variety of chronic diseases, whereas in high-pathogen contexts these are more likely to indicate recent or ongoing acute infections (McDade et al., 2012).

3.4 | Old friends, hygiene, and mismatches with ancestral environments

Complementary to the study of diseases of modernity is the study of how exposure to parasites and pathogens may regulate immune responses and limit disease. These studies are informed by the old friends and hygiene (i.e., biodiversity) hypotheses and often focus on the mismatch between modern and ancestral environments (Blackwell, 2022). These studies often involve measures of the disease state in question, often linked to either excess inflammation or to misdirected immunity, for example, autoimmunity or allergy. Measures of inflammation have been noted above, and for information on measures of autoimmunity, see Cepon-Robins (2021). Additionally, measures related to the regulation of immunity are important for this area of study, including counts of regulatory T cells through flow cytometry and measurement of cytokines characteristic of Th1 or Th2 immune responses (e.g., IL-10, IL-4, IFN- γ). Parasite exposure can be indirectly inferred through immune responses to parasites, including IgE levels, eosinophil counts, and the expression of genes related to both (Table 2).

3.5 | Stress, social inequality, status

Studies examining the regulation of immunity in relation to social environment have used several different approaches. Studies of chronic stress or inequality often focus on inflammatory markers, as above, as these relate to chronic diseases which are often associated with economic and social inequality. A number of studies (e.g., Dieckmann et al., 2020; Levine et al., 2017; Powell et al., 2013) have examined patterns of RNA expression associated with the “conserved transcriptional response to adversity” (Cole, 2019), which is characterized by upregulation of pro-inflammatory responses and downregulation

TABLE 2 Selected genes whose expression may be useful as immunological biomarkers

Category	Gene name	Description	CTRA ^a
Cell type	CD3D	CD3 (T cell marker)	
	CD4	CD4 (helper T cell marker)	
	CD8A	CD8 (killer T cell marker)	
	FCGR3A	CD16 (neutrophil and natural killer cell marker)	
	CD14	CD14 (monocyte marker)	
	CD19	CD19 (B cell marker)	
	NCAM1	CD56 (natural killer cells)	
Humoral	IGHM	IgM heavy chain	
	IGHG1/2/3/4	IgG1 heavy chain 1/2/3/4	
	IGHE	IgE heavy chain	
	CRP	C-reactive protein	
	IGJ	Joining chain of Multimeric IgA and IgM	Antibody
	IGLL1/3	B cell development	Antibody
Cytokines	IFNG	IFN- γ	
	IFNB1	Interferon Beta 1	Type 1 IFN Antiviral
	TNF	TNF- α	Pro-inflammatory
	IL1A	Interleukin-1A	Pro-inflammatory
	IL1B	Interleukin-1B	Pro-inflammatory
	IL4	Interleukin-4	
	IL6	Interleukin-6	Pro-inflammatory
	CXCL8	Interleukin-8	Pro-inflammatory
	IL10	Interleukin-10	
Eosinophil	EPX	Eosinophil peroxidase	
	RNASE3	Eosinophil cationic protein	
	CCL11	Eotaxin (Eosinophil chemokine)	
Pro-inflammatory	PTGS1/PTGS2	Prostaglandin-endoperoxide synthase	Pro-inflammatory
	FOS/B, FOSL1/L2, JUN/B/D, NFKB1/2, REL/A/B	Transcription factors	Pro-inflammatory
		Transcription factors	Pro-inflammatory
		Transcription factors	Pro-inflammatory
Antiviral	GBP1, IFI16, IFI27, IFI27L1/2, IFI30, IFI35, IFI44, IFI44L, IFI6, IFIH1, IFIT1/2/3, IFIT5, IFIT1L, IFITM1-3, IFITM4P, IFITM5, MX1/2, OAS1/2/3, OASL	Interferon induced proteins	Type 1 IFN antiviral
	IRF2/7/8	Interferon regulatory factors	Type 1 IFN antiviral

^aGenes associated with one of the three conserved transcriptional response to adversity dimensions (Cole, 2019).

of type I interferon antiviral response genes and antibody synthesis genes (Table 1). Measures related to the reactivation of latent retroviruses such as Epstein–Barr virus and cytomegalovirus have also been used as proxy

measures for stress related immunosuppression (Glaser et al., 1991; McDade et al., 2000). However, anti-EBV antibody is an indirect measure and findings have been mixed (see below).

3.6 | Choosing the right marker

In choosing biomarkers, often researchers will face a trade-off between scientific and practical concerns. For example, the best measure is often not the most field friendly or cost-effective. Thus, choosing the right measure is a matter of picking something that is a close proxy for what you really want to investigate, but which fits within logistical and financial constraints. Table 1 gives a general overview of the aspects of immunity we discuss in this review, as well as some general ideas for the theoretical concepts these might proxy. In the following sections we discuss more of the practical details for each of these measures when used in the field.

4 | POINT OF CARE DEVICES

Point-of-care devices are typically small, portable devices that provide immediate results. When available, point-of-care devices have a number of advantages over other approaches since they eliminate the need for sample storage and transport and allow the researcher to provide immediate results to participants and local health professionals. To date, however, point-of-care devices are available for only a very narrow range of immune markers, and some of these devices have significant drawbacks.

For quantifying peripheral blood cells, there are two main point-of-care devices on the market that are compact enough to be convenient for field work. Quantitative buffy coat (QBC) dry hematology analyzers (Autoread Plus and STAR; Drucker Diagnostic, Inc) provide a 2-part differential with nine parameters: hematocrit, hemoglobin, MCHC, white blood cell count, granulocyte count, lymphocyte/monocyte count, and platelet count. The QBC analyzers require 70 or 111 μl of capillary or venous blood (tube type dependent). Once blood is collected (or pipetted) into a capillary tube, a float is inserted, and the tube is then centrifuged in a system-specific capillary centrifuge. The differing densities of blood components will cause them to separate into layers during centrifugation. This is optimized by the addition of the float—which physically expands the cell and platelet layers, and a metachromatic acridine orange stain that the capillary tubes are coated with which uniquely dyes the cell types. The resulting layers can then be read by the analyzer using a dual light source and optical detector (<https://druckerdiagnostics.com/qbc-autoread-plus-dry-hematology-system/>). The QBC is generally stable under hot and humid conditions and does not require refrigerated or liquid reagents. It does require AC electrical power but can be run off a battery with an AC adapter. The QBC provides high consistency across

repeat measures taken by different individuals (Laney et al., 2019). Currently the QBC system (analyzer and centrifuge) runs around \$10 000. Tests kits run about \$8 per sample.

The HemoCue WBC System (Hemocue AB) provides only total white blood cell (WBC) counts. It offers some advantages over the QBC as it is smaller in size, has battery-powered capacity, and requires less blood (10 μl vs. 70–111 μl). In controlled conditions the HemoCue WBC works well and provides high correlations with reference methods (Osei-Bimpong et al., 2009). However, in our experience with this system in the field we found that it often fails when temperature and humidity are high. We learned a few hacks that did not affect the read reliability—like keeping the HemoCue WBC in a cooler with ice packs between tests—however these are less than ideal and data loss can still occur with higher than acceptable frequency. The HemoCue WBC system costs around \$2000, while test cuvettes cost around \$3.25 per sample. The same company also offers the HemoCue WBC Diff, which provides a 5-part differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). It is not currently available in the United States, and we have not had an opportunity to test it. Some anthropologists have successfully used this device in the field (Page et al., 2016). However, other reports in the literature suggest it may be somewhat inaccurate with finger prick samples (Karawajczyk et al., 2017).

Apart from white blood cell differentials, the only analyte directly relevant to immune function that can now be measured by a point-of-care device is CRP, using the NycoCard Reader II (Abbott). However, it has a measuring range of only 8 to 200 mg/L, making it functional for detecting acute infections (typical CRP >10 mg/L) but ineffective for detecting lower-level variation in CRP, which is often of interest for assessing cardiometabolic risk, recent infection, or testing theories from eco-immunology and developmental origins of health and disease. It is also not currently available in the United States. A number of other point-of-care devices are available for clinical settings, some of which measure high sensitivity CRP (hs-CRP), but they are generally much larger. Given that CRP can be cheaply and easily measured in DBSs (see next section) we generally prefer this approach.

In addition to these general measures, there are a number of point-of-care options for diagnosing specific ailments. Most of you reading this (in 2022) are by now familiar with rapid tests for detecting SARS-CoV-2. Whether these tests will remain available or will be effective for future variants after the COVID-19 pandemic remains to be seen. Rapid tests are also available for influenza A & B, for example with the BD Veritor Plus

System (Becton, Dickinson and Company) which is around \$300, with tests running around \$12 each. Test kits are also available to use this system for respiratory syncytial virus (RSV), SARS-CoV-2, and Group A Streptococcus. The supplier Thermo Scientific makes rapid tests under the Xpect brand which can detect Flu A & B, RSV, *C. difficile*, rotavirus, giardia, cryptosporidium, and legionella, some of which work with nasal or throat swabs and other fecal samples. However, all are fairly expensive (around \$40 per test). These kind of point-of-care rapid tests are likely to always be less sensitive than PCR analysis. If immediate results are not necessary, studies have shown that nasal swabs are stable for at least 21 days at room temperature and probably longer if refrigerated, at least for SARS-CoV-2 (Skalina et al., 2022). These can therefore be stored for later PCR analysis.

5 | DRIED BLOOD SPOTS

DBSs are a favorite for minimally invasive measurements of immune function. Relative to other methods, they are easy to collect, easy to store, and easy to transport. The use of DBS has been reviewed extensively elsewhere (McDade et al., 2007) so we will highlight only a few key measures. DBS are best utilized for measuring the levels of stable and prevalent protein products, while more care must be used in measuring proteins that are less concentrated or less stable, for example many cytokines. Some examples of stable proteins for which enzyme-linked immunosorbent assays (ELISAs) of DBS are particularly useful include:

5.1 | Immunoglobulins

The major subclasses of immunoglobulins (IgG, IgA, IgE, and IgM) are robust against freeze–thaw cycles and are easily measured in DBSs with only slight modifications to commercially available kits (Tanner & McDade, 2007). In general, this means that DBS can be used to measure not only total immunoglobulin levels, but also specific antibodies against things such as Epstein–Barr virus capsid antigen (EBV), which is commonly measured as an index of stress-related reactivation of latent virus and possibly T-cell suppression (Glaser et al., 1991; McDade et al., 2000). However, anti-EBV antibody may be a more complicated measure than is typically appreciated (e.g., Cacioppo et al., 2002) and many studies, particularly those including other biomarkers, do not find associations between EBV and other stress-related measures (e.g., Panter-Brick et al., 2020; Rudzik et al., 2014) raising the possibility of publication bias for positive findings.

Specific antibodies against a number of pathogens have been measured in DBS, for example, *Toxoplasma gondii* (Lebech & Petersen, 1995), malaria (Corran et al., 2008), and measles (Colson et al., 2015). In principle antibodies should have similar properties in DBSs regardless of target, so adapting ELISAs devised for measuring immunoglobulins in serum for use with DBSs is often possible. DBSs can also be used to measure immunoglobulin subtypes, that is, sub-classes of IgG (Andersen et al., 2014).

5.2 | C-reactive protein

CRP is a favorite measure in human eco-immunology given its stability and the availability of established ELISA protocols (i.e., Brindle et al., 2010; McDade et al., 2004). However, these protocols were published a number of years ago, and some of the antibodies used by them are no longer available commercially. The capture antibody used by Brindle et al., 2010 is currently available from Meridian (clone C5 anti-CRP MAb, M86005M), however, the detection antibody is no longer listed. An alternate detection antibody (M01239M) is given as pairable with M86005M and will likely work similarly to the original antibody from the protocol. However, we would recommend validating this or any other replacement antibody pair before using them to analyze DBS since it is always possible for confirmational changes, elution buffer differences, or matrix effects to make a DBS assay behave differently from serum assays. DBS measures of CRP have been used extensively in a number of papers (e.g., Blackwell et al., 2010; Urlacher et al., 2018).

5.3 | Cytokines

Cytokines are small molecules used for signaling between cells of the immune system. Because each cytokine has its own molecular structure, cytokines differ significantly in their stability at room temperature, loss due to freeze–thaw cycles, and recovery from DBS. One study found that interleukin (IL)-6 and IL-10 are stable in serum through freeze–thaws, but levels of IL-4, IL-13, IL-15, IL-17, tumor necrosis factor (TNF) α , interferon (IFN) γ and CXCL8 all changed with freeze–thaw cycles (De Jager et al., 2009). Skogstrand et al. (2005) used a Luminex xMAP assay to measure 25 cytokines and other biomarkers in DBS. The results show that in principle many cytokines can be measured in DBS and that cytokines in DBS are stable when kept frozen at -80°C , but the study did not compare values in plasma to DBS or assess the effect of freeze–thaw cycles. A second study (Skogstrand et al., 2008) assessed stability at room temperature,

35 and 4°C and found that a few cytokines, such as IL-8, IL-10, and TNF- α appear to be relatively stable, even up to 30 days at room temperature, but the majority were not. Another concern is that the variability in cytokine levels due to differences in collection or storage is not consistently directional (i.e., values may go up or down). There may be a variety of reasons for this including differential degradation of cytokine molecules and release of cytokines from intracellular locations, binding molecules, or the DBS matrix. The study did find that one advantage of DBSs is that cytokine levels change rapidly in venous blood that is kept at room temperature or refrigerated, as the living cells in the blood become activated by the novel environment (Keustermans et al., 2013; Skogstrand et al., 2008). Since DBS are usually collected and dried immediately this is less of an issue.

More recently McDade et al. (2021) used a multiplex electrochemiluminescent immunoassay to simultaneously measure IL-6, IL-10, IL-8, and TNF- α in matched DBSs and plasma. They obtained good correspondence for IL-6, IL-10, and TNF- α , but poor correspondence for IL-8. They speculate this may be due to IL-8 stored in red blood cells which is released on lysing, and which would not be present in plasma. An ELISA protocol for just IL-6 has also been validated, with good correlation between DBS and serum (Miller & McDade, 2012).

In conclusion, DBSs seem to be reliable for the measurement of higher concentration, higher stability cytokines such as IL-6, IL-10, and TNF- α . However, caution may be needed when measuring other cytokines in DBS, particularly those with lower concentrations or shorter half-lives. An alternative may be to measure cytokine related gene (mRNA) expression (see below).

5.4 | CD4 lymphocytes

For many assays where serum equivalents are desired the inclusion of red and white blood cells may be a nuisance. However, the fact that DBS contain whole blood presents an opportunity as well. Mwaba et al. (2003) tested an ELISA kit for measured CD4 protein in DBS and showed a reasonable correspondence between estimated CD4 counts from this method, and counts obtained by flow cytometry. Likely estimation of other cell types using ELISAs and DBS is possible, though we are not aware of publications validating these methods.

5.5 | Single versus multiplex platforms

ELISAs can be used to measure many single analytes in DBS. ELISAs have the advantage of using relatively

common equipment (an ELISA plate reader, at the minimum) and having a lower cost than multiplex assays. DBS kits typically cost around \$300 to \$700 for a 96-well plate, good for running about 35 samples in duplicate, after accounting for standards and controls. Multiplex kits may be more than five times as expensive as ELISA kits. However, when measuring many analytes multiplex assays become more efficient, in terms of labor, sample used, and cost. However, multiplex assays also sometimes sacrifice sensitivity, particularly for lower concentration analytes, since they must compromise on assay dilutions that are effective for the many things being measured, which may differ in concentration by orders of magnitude. Common multiplex platforms include the MagPix xMAP instrument (Luminex Corp) which uses magnetic beads with bound antibodies, instruments from Meso Scale Diagnostics which use electrochemiluminescence, and flow cytometry-based bead assays such as the Luminex 200 System and BD Cytometric Bead Array kits (Becton, Dickinson and Company) which can be used with any number of flow cytometers. However, only a handful of multiplex assays have been validated with DBS (McDade et al., 2021; Skogstrand et al., 2008), so other assays will require protocol development for use.

5.6 | Complications for DBS samples

Although DBSs are convenient, recent studies have shown that there are a number of ways to introduce errors into blood spot analyses. In addition to the aforementioned effects of freeze-thaws and storage temperatures, humidity may also impact certain analytes measured in DBS. DBSs differ from serum measures due to the inclusion of white and red blood cells, which lyse, and the filter paper matrix itself which may create assay nonlinearities. At a minimum, the analysis of whole blood, rather than serum, will dilute concentrations by a factor of about 2 to 2.5, equivalent to the proportional volume of red blood cells in blood (hematocrit). Because of this and other factors, many protocols will include conversion equations for estimated serum equivalent values from DBS results. However, in the absence of data on serum equivalence it is problematic to compare DBS and serum values, so caution is needed. Most commercial kits are developed for analysis of serum and thus it is also inadvisable to perform DBS assays without validating the protocol against serum, given the issues noted above. Considerations and requirements for protocol validation has been described in a number of papers (e.g., McDade et al., 2007; Sultana et al., 2022). Additionally, the use of small blood spots, overlapping drops, smeared drops or the edges of blood spots can create issues, as these all

affect the concentration per unit of filter paper and may also affect matrix effects and elution (Crimmins et al., 2020). Finally, because most commercial assay kits are not designed for DBS, maintaining consistency in the kit manufacturer used across a study sample is necessary. This means that if a researcher shifts to a different antibody or assay manufacturer (e.g., if one brand goes off the market) the burden is on them to do a new validation. To ensure continuity and comparability of biomarker data across a study sample, researchers should purchase enough antibody or kits for their entire study, while also needing to be aware of expiration dates. Planning out the materials needed and timeline to conduct laboratory analyses are as important as planning the data collection itself.

6 | FLOW CYTOMETRY

Flow cytometry is used to analyze the relative proportions of different white blood cells by tagging cells with fluorescently marked antibodies for specific cell-surface (or sometimes interior) proteins. Cells are suspended in a salt-based buffer and single cells are then rapidly analyzed as they flow past a single (or set of) laser(s). Cells are analyzed based on the presence or absence of each tag, and the visible light scatter, which roughly corresponds to size and shape of the cell.

Blackwell et al. (2016) used flow cytometry to characterize leukocyte subtypes in participants as part of the Tsimane Health and Life history Project (THLHP). For that project, a small Accuri C6 (Becton, Dickinson and Company) flow cytometer was taken to Bolivia for use in the THLHP field clinic (Figure 1D). In this study, venous blood was collected from participants and analyzed fresh using flow cytometry. While analyzing fresh samples is ideal and it is certainly more convenient to avoid transporting samples, this setup presents a number of challenges. First, a flow cytometer must be available for transport, which precludes the use of core facilities. Second, reagents require refrigeration, and may be unavailable in country, and thus require temperature-controlled transport and storage. Third, if the instrument requires maintenance, it may be difficult to obtain service in country (team members took ours from Bolivia to Chile for service). Finally, a clean area with regular power is needed for analyses.

Given the difficulty of this procedure, we recently validated a field protocol for freezing finger prick blood samples in the field for later flow cytometry (Blackwell et al., 2021). The procedure utilizes a liquid nitrogen (LN₂) storage or shipping tank, sometimes called a dewar

(Figure 1C), which can keep samples frozen without electricity for a month or more. Such tanks can be bought from scientific suppliers like Thermo Scientific, but may also be sold in some countries for the purpose of livestock breeding (e.g., sementanks.com). The key is to freeze samples in the vapor phase, which freezes at a slower rate and causes less lysing than freezing in the liquid. In short, the steps are as follows:

1. Prepare aliquots of 20% DMSO/80% RPMI 1640 medium. These may be prepared early and stored frozen in the LN₂ tank.
2. Collect blood from a finger prick in in a heparin coated tube, such as the BD Microtainer Capillary Blood Collector (Becton, Dickinson and Company).
3. In a cryotube, mix 100 ul blood +100 ul RPMI/DMSO. Do not invert to keep blood from getting stuck in the cap. Instead, gently tap, vortex, or pipette to mix.
4. Do not allow to sit for more than 5 min before freezing
5. Freeze in the vapor phase of a LN₂ tank with ~1 inch LN₂ in the bottom, using only the top two cane positions in the tank, or in a LN₂ shipper with the liquid absorbed.
6. After ~6 to 24 h, move to liquid phase in second tank

Frozen blood can be transported in the liquid nitrogen shipping tank or on dry ice and then stored in a freezer at -80°C . When ready to analyze, cells are thawed in a water bath and quickly washed with fresh medium (full protocol details are available in Blackwell et al., 2021).

Overall, analyses with previously frozen cells produce equivalent results to those with fresh cells (Figure 2). However, there are some notable exceptions. First, freezing lyses granulocytes more than lymphocytes, and may alter the lymphocyte percentage in samples. Thus, measuring white blood cell differentials at the time of collection with a point of care device (see above) can be very useful if absolute differential counts are desired. Major lymphocyte subsets (i.e., CD4 T cells, CD8 T cells, natural killer cells, and B cells) can be measured accurately in frozen cells. However, some minor subsets, such as the percent naïve and senescent were altered with freezing. Additionally, some antibody-fluorochrome combinations performed poorly with previously frozen cells. We recommend testing these on frozen control samples before analyzing precious field samples.

Apart from the cost of the flow cytometer, expenses for flow cytometry can be minimized by using small samples and no more reagents than are necessary. Typical flow cytometry protocols call for 100 μl of blood and 5 μl

FIGURE 1 Methods for measuring immune function and collecting samples. (A) Dried blood spots covered by a net to prevent mosquitos from landing (photo: Lawrence Sugiyama). (B) Field antigen stimulation, in which a candle is lit and then sealed in a plastic container to deplete oxygen and increase carbon dioxide. (the jaguar skull looks cool but has not been shown to improve results). (C) a liquid nitrogen shipping tank for sample transport. (D) THLHP field setup for flow cytometry and antigen stimulation

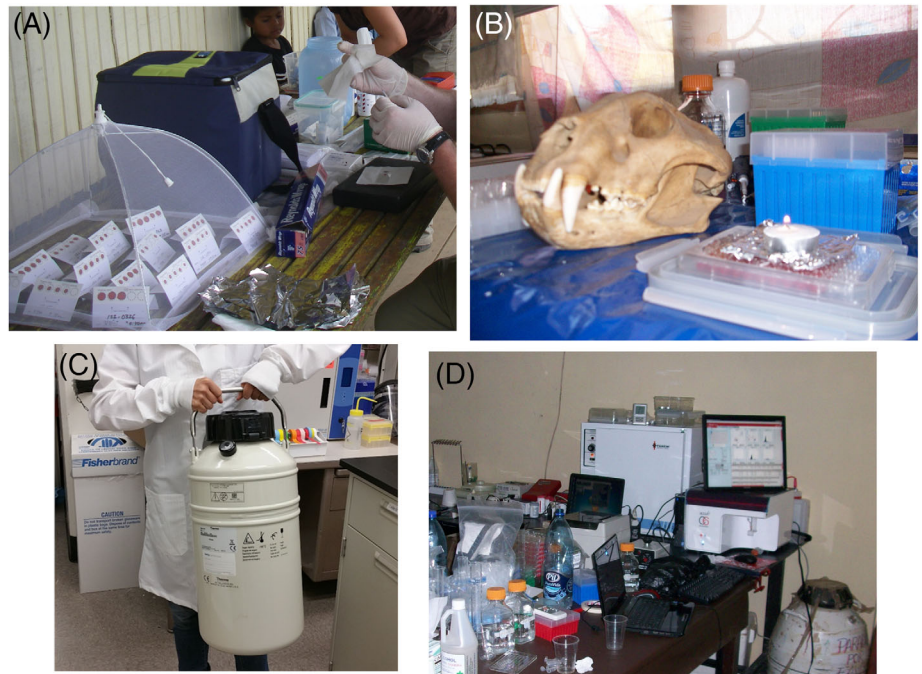
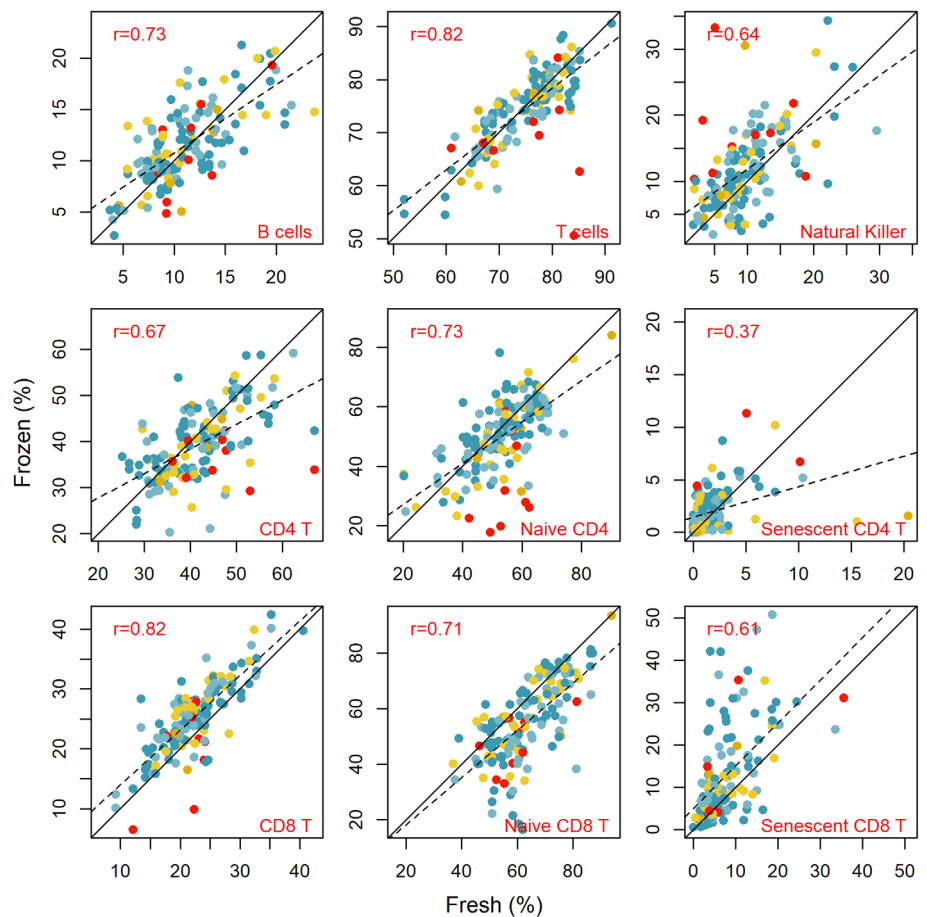


FIGURE 2 Percent of CD45+ cells or percent of respective T cell subset (CD4 or CD8 for naive and senescent cells) in matched fresh and frozen samples analyzed by flow cytometry. Black line is the 1:1 slope. Dashed line is the best linear fit line. Reprinted from (Blackwell et al., 2021). See original reference for further details



of each labeled antibody. However, we have had good results with as little as 10 μ l of blood and 0.5 μ l of each antibody per sample, cutting the sample required and expense by a factor of 10 (Blackwell et al., 2021).

7 | RNA SEQUENCING

RNA sequencing (RNA-seq) allows for the quantification of mRNA transcripts and is used to compare gene



expression between individuals or experimental conditions. RNA-seq relies on next generation sequencing (NGS), a technology that sequences multiple DNA fragments (reverse transcribed from mRNA) in parallel. Because most immunological processes of interest require mRNA transcription for protein synthesis, gene expression levels are likely to be correlated with many other biomarkers and are informative of a wide range of processes. As such, RNA sequencing may prove a more cost effective and informative method than direct measurement of isolated biomarkers.

RNA-seq data can be analyzed in several ways, for example, to infer broad patterns of immune investment, to infer the representation of different leukocyte proportions, or to infer cytokine or antibody production (Table 1). Cell proportions can be inferred through RNA transcripts for canonical cell type markers, for example, CD14 for monocytes, CD3D and CD3E for T-cells, CD4 for helper t-cells, CD8A for cytotoxic t-cells, CD19 for B cells, CD16 (FCGR3A) for neutrophils and monocytes, and CD56 (NCAM1) for natural killer cells. They can also be inferred through transcript origin analysis which uses full profiles of gene expression in multiple tissues (e.g., Cole et al., 2011; Palmer et al., 2006; Su et al., 2004).

Functional genomics also uses RNA-seq data to study gene and protein expression and function on a global (genome- or system-wide) scale. Research by Cole (2019), focusing on individuals exposed to chronic psychosocial threats has led to the identification of a pattern of gene expression that associates with such experiences. This pattern, referred to as the CTRA, involves upregulation of pro-inflammatory responses and downregulation of type I interferon antiviral response genes and antibody synthesis genes (Table 1). Differences in patterns of gene expression across these markers has been increasingly used to investigate the biological effects of psychosocial stress (Dieckmann et al., 2020; Levine et al., 2017; Powell et al., 2013).

Finally, with network-based approaches RNA-seq data can be used to assess the effects of different environmental exposures on the function of biological pathways. For example, PathOlogist, an analytical tool developed by Greenblum et al. (2011), utilizes pathway structure to integrate biological information, and assesses differences in activity and consistency states of networks of genes within pathways of known biological processes and functions. While such network-based genomic approaches tend to be used to assess differences by disease state (e.g., gene expression profiles in healthy versus tumorous tissue), applying them to questions relevant to eco-immunology opens opportunities for in-depth investigation of how the environment influences biologic pathways and processes related to life history processes (e.g., cellular energy metabolism or immune investment).

7.1 | Isolating RNA from finger-prick blood samples

Typical RNA-seq blood collection protocols, such as the PAXgene Blood RNA extraction system (Qiagen, Inc) call for the collection of 2.5 ml venous blood into PAXgene vacutainers. PAXgene documents report that 2.5 ml blood yields ≥ 3 μg RNA for $\geq 95\%$ of samples (mean ~ 8 μg) (PreAnalytiX., 2020). However, RNA sequencing requires only 50 to 100 ng RNA for evaluation of transcripts, and only 500 to 1000 ng for de novo transcriptome assembly, suggesting that 2.5 ml produces roughly $60\times$ the minimum RNA yield needed for 3' sequencing and that less blood could be collected.

Krawiec et al. (2009) developed a protocol for isolation of RNA from small samples of blood, to be used with mice who cannot spare 2.5 ml. Although not yet validated with human blood, for mice 50 μl of blood yielded 2300 ng of RNA, more than sufficient for sequencing. ADB also successfully collected and extracted RNA from great-tailed grackles, with yields from 50 μl blood averaging 3152 ng of RNA. While birds have nucleated red blood cells, mice and humans have roughly similar white blood cell counts, suggesting results for humans may be similar to mice. While reported RNA yields from PAXgene documents suggest possible yields as low as 60 ng from 50 μl blood, other references suggest typical RNA levels for humans are actually higher than this, with a mean of 15 $\mu\text{g}/\text{ml}$ (range 7–23 $\mu\text{g}/\text{ml}$) (Chomczynski et al., 2016), which would yield 750 ng from 50 μl . Lower yields in some PAXgene protocols may in fact be due to a ceiling effect from the RNA spin columns used, with higher recovery possible for lower volumes of blood (Krawiec et al., 2009).

Together, these results suggest that blood collected via finger-prick should produce usable yields for RNAseq in humans. We plan to validate this procedure in the near future, but prior to that we recommend verifying RNA yields before using this protocol. An example finger-prick collection protocol, modified from the protocols used for mice and birds, might read as follows: (1) Aliquot PAXgene reagent into smaller tubes at a ratio of 2.76 reagent/blood (e.g., 138 μl PAXgene/50 μl blood); (2) Pierce the fingertip with a sterile lancet and collect blood into a finger-prick blood collection tube; (3) pipette 50 μl of blood from the collection tube into the PAXgene aliquot; (4) store at room temperature for at least 2 h (no longer than 72 hrs), then freeze. PAXgene aliquots may be frozen at -20C , -80C , or placed in liquid nitrogen. PAXgene-preserved samples are also stable across at least three freeze-thaws, making them ideal for easy transport. Extraction and analysis should follow standard protocols.

7.2 | Isolated RNA from DBS

Multiple authors have successfully sequenced RNA from DBS (McDade, Ross, et al., 2016; Reust et al., 2018). In these studies, relative transcript reads derived from DBS were highly correlated with PAXgene derived values and yielded largely similar associations with predictors, though with some discrepancies. Most of these discrepancies may be due to the small amount of RNA recovered from DBS. For example, McDade, Ross, et al. (2016) did not quantify RNA yields since these were expected to be too low for accurate quantification. The authors note that due to the increased noise from a small RNA sample, a sample size $\sim 5\%$ greater than in PAXgene studies might be required to maintain statistical power. These studies require a large number of blood spots, in the range of two to four complete blood spots (McDade, Ross, et al., 2016) or $8 \times 3 \text{ mm}^2$ punches (Reust et al., 2018). Overall, DBS are likely to be simpler to store and transport, but small liquid aliquots with PAXgene are likely to yield better results.

7.3 | Sample preparation and sequencing for RNA-seq

Sequencing is typically done either by reading from the 3' end of each cDNA transcript, which only partially reads the transcript, or by fragmenting the mRNA into smaller pieces for whole transcript sequencing before reverse transcribing into cDNA. Each approach has its own advantages and disadvantages, which we will briefly detail in the next sections (for a more thorough comparison see Ma et al., 2019). For both methods, after collecting the sample, RNA is isolated and purified, usually with a kit such as the RNeasy kit (Qiagen).

Due to the high volume of RNA in red blood cells, globin-based reads can consequently comprise a large proportion ($>80\%$) of sequencing reads. Thus, when preparing whole blood for 3' RNA sequencing, it may be advisable to include a globin depletion step in the protocol. However, globin reads can also be removed from analyses after sequencing, and depletion may introduce biases to other reads (Harrington et al., 2020). Consequently, there are costs and benefits to globin depletion with the primary benefit being a reduction in sequencing depth and expense, and the cost being an increase in protocol complexity and read bias. Globin reads may also sometimes be of interest.

For whole transcript sequencing an enrichment step is commonly added after RNA isolation and purification to remove ribosomal RNA repeats which would otherwise swamp the sequencing and be uninformative for

many purposes. Some protocols also enrich for RNAs with poly-adenine tails, which helps to eliminate immature RNAs which may contain introns that have not yet been spliced out. These steps are not necessary for 3' sequencing, as 3' sequencing only targets the poly-adenine tails at the 3' ends of mature mRNAs. After enrichment the RNA is converted to complementary double-stranded DNA (cDNA) via a reverse transcriptase enzyme and prepared for sequencing.

Whole transcript sequencing generates more cDNA fragments for longer genes, and consequentially more reads for these genes, relative to shorter genes. In contrast 3' sequencing sequences from the 3' end of the mRNA. Since mRNA is not fragmented, gene length does not influence sequencing reads and the number of reads is proportional to the original mRNA transcripts. Ma et al. (2019) compared the two methods, and as expected found that 3' sequenced performed better for shorter transcripts (particularly $<1000 \text{ bp}$) because they receive equal reads. Many genes of interest for quantifying immune function are small genes, for example, cytokines (e.g., the mRNA for IL-10 is approximately 500 bases). 3' can also be substantially cheaper than whole transcript sequencing (Sholder et al., 2020). There are, however, advantages of whole transcript sequencing. First among these is that whole transcript sequencing allows for the discovery and counting of alternative splice variants and noncoding RNAs. Depending on the study aims this may or may not be relevant.

Another important consideration—which depends on the sequencing technique and the question—is sequencing depth. For RNA-seq, sequencing depth refers to the total number of reads obtained in a sequencing run. For example, if a gene of interest is lowly expressed, it may appear in only one out of a million transcripts. If a sequencing depth of 5 million is used, on average there will be 5 reads, but with a 95% confidence interval ranging from 1 to 10 reads, leading to an estimated relative expression of between 2 and 20 per 10^7 reads. Increasing sequencing depth to 20 million narrows this range to 6 to 14.5 per 10^7 reads. A main disadvantage of higher sequencing depth is increased cost.

The required read depth also depends on whether one is doing whole transcriptome sequencing or expression profiling sequencing (i.e., 3' sequencing). Because only a single fragment is generated for a transcript with 3' sequencing, this method attains a similar coverage as whole transcriptome sequencing at a fraction of the depth, and there are diminishing returns for increasing depth past ~ 10 million reads for 3' sequencing (Moll et al., 2014). Thus, depending on the study design and budget a researcher might have to choose between sequencing many samples at low depth or few at high



depth. One analysis of this question found that sequencing more replicates at lower depth provided more power than sequencing fewer replicates at higher depth (Liu et al., 2014). Thus, if the object of a study is to compare two groups of individuals, a lower sequencing depth and a greater sample might be preferred. If, however, it is essential to quantify the gene expression for a particular individual (rather than a group of individuals) a greater sequencing depth might be more important.

7.4 | Post-sequencing processing and analysis workflow

After samples are sequenced (i.e., “read”) raw read data go through multiple processing steps before they are converted to raw count data, and ready for downstream analysis. Post-sequencing processing steps include quality assessment, transcript assembly, alignment, analysis of raw counts, and annotation. For the trainee interested in integrating RNA-seq into their project, an important consideration is that each of these steps relies on bioinformatic tools and pipelines, which require a level of fluency and comfort with programming languages (e.g., R, Python) and statistical software and packages. As an example, here is an abbreviated description of an RNA-seq pipeline for samples sequenced on an Illumina sequencing platform (full pipeline available at <https://github.com/heinin>):

1. Raw RNA-seq data exist as FASTQ files, which contain the short sequence reads and quality scores. The quality of the FASTQ files is assessed with a tool called FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)
2. The raw sequence reads often contain adapter sequences that were used to create the Illumina sequencing libraries. These adapters and low-quality bases at the ends of each read are trimmed using Trimmomatic (<https://anaconda.org/bioconda/trimmomatic>). Trimming improves read mapping and affects all downstream analyses.
3. After trimming, the FASTQ quality is assessed again, until quality checks are passed, after which we continue with read mapping. The trimmed reads are mapped to the human reference genome using HISAT2 (<https://ccb.jhu.edu/software/hisat2/index.shtml>) (this is one of many tools for DNA and RNA-seq alignment). The result is one SAM file (Sequence Alignment Map) for each sample. The SAM file is converted to a binary version using SAMtools (<http://samtools.sourceforge.net/>; <https://samtools.github.io/hts-specs/SAMv1.pdf>), and these BAM files are sorted

based on genomic coordinates (also with SAMtools). Most tools that take BAMs as input files require them to be coordinate sorted.

4. Next, the expression levels of genes are obtained by quantifying the read counts mapping to each gene. One commonly used tool is called featureCounts (<http://bioinf.wehi.edu.au/featureCounts/>). For each sample, the output is a text file containing the read counts in each gene. These files can then be combined to create a count table with samples in columns and genes in rows, and that count table is used in downstream analyses (e.g., differential expression).

For researchers new to RNAseq, the learning curve for bioinformatic processing can be steep but may be worthwhile depending on your approach to a research question. There are a number of resources that describe workflows, pipelines, and workflow managers, including <https://bioinformatics.uconn.edu/resources-and-events/tutorials-2/rna-seq-tutorial-with-reference-genome/> and <https://rnaseq.uoregon.edu/>. The Biostar Handbook is also an invaluable guide to bioinformatics that is updated multiple times a year and has topic-specific (<https://www.biostarhandbook.com/>). The learning curve can also be buffered by teaming up with other researchers that are experts in the use of a particular method(s), as they can guide you through parts of the process and direct you to relevant resources. It is also possible to have a majority of these steps done “behind the scenes” (e.g., among the services offered by a Core Lab), though we suggest familiarizing yourself with the overall process in order to better understand the data you are working with, including its limitations.

7.5 | Statistical analysis

The statistical approach for downstream analysis of count data will depend on the specific goals and research question being asked and is thus outside the scope of this review. Here we briefly discuss the process of count normalization and its importance for the interpretation of RNA-seq data in field studies. In both whole transcript sequencing and 3' RNA-seq reads must be normalized for between sample variation in library size, that is, the total of all reads for a sample. For whole transcript sequencing counts are also normalized for gene length because gene length affects the number of cDNA fragments produced and thus the number of reads mapped to a particular gene; this is not usually necessary for 3' sequencing because only one fragment is generated per transcript (Moll et al., 2014). Because library size is more likely to reflect variation in sequencing depth due to

methodological stochasticity, rather than real-world variation in total mRNA expression, counts can be normalized in a number of ways which reflect different assumptions (Evans et al., 2018).

One approach is to normalize to total library size, which converts values into counts per million reads (CPM). When using whole blood rather than isolated PBMCs normalized values will reflect the total proportion of all transcripts in all blood cells including red blood cells which contain RNA from a large number of genes (Kabanova et al., 2009). If red blood cell globin transcripts are depleted (either before sequencing or by removing these reads after sequencing) the interpretation of CPMs will change to represent the proportion of nonglobin reads. However, in some circumstances, normalization by read count may lead to faulty conclusions if the total mRNA differs between samples for biological reasons; for example, if some prevalent genes are upregulated in one sample this could instead be interpreted as a downregulation of the remaining genes, since these transcript proportions would decrease. To avoid this issue, a number of other normalization procedures are possible, including normalizing or controlling for sample RNA concentration while also normalizing for library size. Other approaches include normalization for housekeeping genes (genes thought to be expressed at relatively constant levels across cells), normalization based on distribution (quantile normalization), normalization based on cell-type specific genes, and normalization based on cell counts measured independently in the sample (Dillies et al., 2013; Eisenberg & Levanon, 2013; Evans et al., 2018).

The choice of normalization procedure should depend on: (1) the initial sample (e.g., whole blood), (2) whether the RNA was enriched before sequencing, (3) whether the desired end product is mRNA/cell, mRNA/leukocyte, mRNA/total transcript, mRNA per volume of blood or some other value. These values will have different interpretations, for example, mRNA/leukocyte may be most appropriate for inferring leukocyte differentials, while mRNA/volume of blood may speak more to cell counts or systemic expression levels for the whole organism. Like most statistical analyses, there are numerous ways to approach normalization and we strongly advise investigating the advantages and constraints of each method before deciding which method is most appropriate for answering a specific question. There are a number of studies that describe the details of each method and provide useful insights on appropriate choice of RNA-seq normalization and analysis methods (Abrams et al., 2019; Evans et al., 2018; Zypych-Walczak et al., 2015).

In sum, the decision to incorporate RNA-seq into one's research should be driven by a triangulation between the question, the cost, and the learning curve.

All specific methods and analyses will follow from the question.

8 | DNA-BASED ASSAYS

8.1 | Thymic output

Like RNA, it is also possible to extract DNA from DBS for analysis. In addition to genotyping, there are several assays of interest when measuring immune function. When T cells mature, they undergo a process wherein the DNA coding for the T-cell receptor is randomly rearranged to create a unique receptor for each T cell lineage. Leftover DNA from this process is excised from the T cell receptor arrangement and circularized into DNA molecules called signal joint TCR excision circles (sjTRECs). New T cells leave the thymus with one or two sjTREC molecules, and as they mature and divide these become distributed among the daughter cells (Lorenzi et al., 2008). As such, sjTREC levels in the blood directly correspond to thymic output, that is, production of new naïve T cell lines by the thymus. sjTREC levels correlate with naïve cell counts measured in the blood, but imperfectly so, as naïve cells may also replicate in the periphery (though divergences are themselves informative) (den Braber et al., 2012). Thymic output declines with age in a consistent enough fashion that sjTREC has been used to estimate age from bloodstains (Ou et al., 2011; Ou et al., 2012). However, thymic output can also be temporarily upregulated during infections (Permar et al., 2003) and is influenced by various immunological (Cho et al., 2017) and physiological (Tarcic et al., 1998) conditions. These divergences from general age patterns are probably of the most interest in eco-immunological studies, as they are useful measures of variation in immune investment. However, this means that analyses using sjTREC need to consider how to parse out infection or exposure-related variation from effects due to initial thymic size and typical age-related decline.

Quantitative PCR (qPCR) is used to quantify sjTREC DNA, as well as levels of a housekeeping gene for normalization. To quantify sjTREC in DBS, DNA is first extracted with a kit such as the QIAamp 96 DNA Blood Kit or Genra Puregene Tissue Kit (Qiagen); Qiagen also provides an adapted protocol specifically for DBS. Extracted DNA samples are analyzed for quantity and quality with a spectrometer (e.g., NanoDrop, Thermo Scientific). qPCR is then performed on an instrument such as the Bio-Rad CFX96 (Bio-Rad Laboratories) using appropriate dyes and primers for sjTREC and any normalization genes (see Lang et al., 2011 for primer sequences).



Lang et al. (2011) used ALB2 for initial normalization, to obtain a ratio of sjTREC per total nucleated cells. They also quantified the unrearranged configuration of the VD junction region (VD-J) of the TCR- β , which is the version present in non-T cells. By subtracting the estimate of non-T cells from total cells they were able to obtain an estimate of T cells, and ultimately calculate an sjTREC/T cell ratio. As with the RNA-seq assays above, the choice of normalization alters interpretation. If peripheral cells replicate, for example in response to infection, then the sjTREC/T cell ratio will go down, even though thymic output might actually increase. Thus, some advocate for measuring sjTREC/ml (Lorenzi et al., 2008). An alternative approach when blood volume is not precise is to measure sjTREC/cell by PCR and use a point-of-care device to measure total leukocyte count. Multiplying sjTREC/cell times cell count will yield sjTREC/ml.

8.2 | Lymphocyte cell subsets

As should be apparent from the sjTREC protocol above, qPCR can also be used to estimate the proportion of non-T cells (and by extension T cells) in a sample. Though we know of no validations, a similar approach is likely possible for estimating B cells using primers for the non-rearranged heavy and light immunoglobulin chains. Assessment of rearrangements is currently described for monitoring of B cell disorders (Cho et al., 2017). Measurement of non-rearranged sequenced should be considerably simpler.

8.3 | Methylation

DNA methylation can be measured in minimally invasive blood samples, like DBS, but can also be measured in buccal swabs or saliva (Non & Thayer, 2015). However, because buccal swab and saliva samples contain epithelial cells as well as white blood cells, blood may be the best sample for measuring immune function directly. Methylation is stable in DBS for considerable lengths of time, even at room temperature (Sasaki et al., 2020), and protocols for measuring methylation in DBS are similar to those for fresh blood (Walker et al., 2019). DNA is first extracted with a DNA purification kit before analysis. Methylation analyses may look at global methylation, may compare groups or individuals for differentially methylated genes, or may look at methylation of specific genes. The choice of assay method will differ depending on which of these approaches a study takes; methylation assays may be done through sequencing, mass spectrometry, the reading of microarrays, or through qPCR (Bock

et al., 2016; Kurdyukov & Bullock, 2016; Non & Thayer, 2015). We refer the reader to the citations referenced here for more information.

For the purposes of eco-immunology, methylation may be useful for examining particular immune related genes, particularly with regard to chronic effects due to lifestyle (Lighthart et al., 2016; McDade et al., 2019; Nakajima et al., 2010). Methylation is part of the regulatory pathway for gene expression, and may be important, for example, in TH1/TH2 balance (Brand et al., 2012). Patterns of methylation have also been found to associate with levels of CRP (Sun et al., 2013). However, methylation is not the only factor in gene expression as transcription factors and other regulators make many short-term adjustments (Makar & Wilson, 2004). Thus, for measuring expression at a particular timepoint the measurement of RNA expression or protein product level is probably complementary. Similar to RNA-seq data, methylation results can also be used to infer underlying cell subset proportions and thus may be another useful alternative to flow cytometry (Titus et al., 2017).

9 | IMMUNE RESPONSE

The methods described above measure the state of the immune system at the time a blood sample is taken. However, interpretation of such measures is sometimes difficult. Does a high value indicate an immune system more prepared to face an infection? Or does it represent the consequence of an infection that evaded initial immune defenses and triggered a more widespread response? In some cases, the difference between baseline immune levels and activated responses can be identified, but in others they cannot. For this reason, it is sometimes informative to measure immune responses to experimental stimuli. In humans, in vivo tests are occasionally possible, usually in the case of vaccination campaigns in which response to a vaccine is measured. However, generally in vitro stimulations provide more flexibility and are more feasible.

9.1 | Antigen stimulation

Antigen stimulation assays involve mixing blood with an antigen or mitogen and incubating in medium, followed by measurement of the response to the treatment. Common antigens include lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria that initiates B-cell division and activation of antigen presenting cells, and phytohemagglutinin (PHA) a mitogen that activates T cells; other studies have also used antigens ranging

from fragments of helminths (van Riet et al., 2009) to H1N1 flu vaccine (Schneider-Crease et al., 2021). Typical protocols call for incubation in a CO₂ enriched incubator at 37°C. However, May, van Bodegom, et al. (2009) developed a field-friendly method for whole blood stimulation that does not require the CO₂ tank. They created a CO₂ enriched environment by sealing the samples in a plastic container containing water and a burning candle. The candle depletes the oxygen and enriches the CO₂ in the contained before going out (Figure 1B). This procedure has been successfully carried out for a number of studies (May, van Den Biggelaar, et al., 2009; Schneider-Crease et al., 2021; Trumble et al., 2016), however, because it still requires the ability to maintain samples at 37°C—usually in an incubator—it is not the most field-friendly protocol. The incubation time ranges from 24 to 72 h, depending on whether the assay is designed to capture initial, innate responses, or slower adaptive immune responses.

Antigen stimulations are usually done with 100 µl of whole blood per sample, so it is feasible to conduct them with finger-prick collections. However, the blood must be fresh (ideally stimulation is done within an hour of collection) and cannot be frozen. After stimulation, the supernatant is collected and frozen (which necessitates a freezer or liquid nitrogen tank). The supernatant is typically transported frozen and assayed for cytokines using ELISA or multiplex assays. Cytokine levels in stimulated supernatants are usually much higher than they are in circulating blood samples. In principle, therefore, assays could be developed which avoid freezing, for example, by spotting stimulated samples on filter paper, or by preserving stimulated cells with PAXgene reagent and performing RNA-seq instead of cytokine measurement (e.g., Meitern et al., 2014; Pulido-Salgado et al., 2018).

One important caveat about antigen stimulation assays is to remember that the assays measure the response to stimulation, but not how effective that response is in fighting the infection. Cytokines are signaling molecules and measuring them is equivalent to measuring how loud the immune system yells when it feels threatened. Indeed, in some cases strong inflammatory responses may be detrimental or represent a failure of specific responses, so care in interpretation is needed.

9.2 | Bacterial killing assays

Bacterial killing assays involve in vitro introduction of microbes to blood, plasma, or saliva in a dish or well. Microbes such as *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus* can be obtained as lyophilized pellets and reconstituted as needed. Liebl and Martin II (2009) report on a procedure designed for small birds that

uses only 1.5 µl of plasma or blood. The sample is diluted in medium and mixed with the microbe solution, then incubated for 30 min to 1 h at a temperature optimal for the particular microbe used. The microbes are then diluted in nutrient medium and incubated for an additional 12 h. The bacterial concentration is then measured with a small Nanodrop Spectrophotometer (Thermo Scientific). As an alternate measurement, bacteria can also be spread on plates and the colonies counted (Millet et al., 2007). Since bacterial killing largely depends on the action of whole cells which are likely to be lysed or stressed by freezing it is unlikely to produce useful results from frozen blood, though we are not aware of protocols that have directly evaluated this.

9.3 | Hemolysis-hemagglutination assay

The HHA is an assay utilized by field biologists to measure complement and natural antibody activity via the ability of plasma to lyse or agglutinate foreign red blood cells. Natural antibodies are antibodies produced without previous exposure to an antigen and which are genetically encoded rather than requiring gene rearrangement to produce, while the complement system consists of a number of small molecules that bind to invaders and trigger further responses to clear infections. The HHA assay is similar to the hemolytic complement assay (HCA) developed in the 1960s to measure complement activity in humans and aid in diagnosing complement related disorders (Kirschfink & Mollnes, 2003; Mayer, 1961; Morgan, 2000; Rapp & Borsos, 1970). However, it is simpler to perform in the field with small volumes and results that are read by eye or with a scanner or camera, rather than a spectrophotometer. The HCA assay uses antibody-sensitized sheep erythrocytes to evaluate the classical complement pathway and rabbit erythrocytes to assess the alternative complement pathway; the HHA does not distinguish lysis by pathway, however it does assess agglutination which requires natural antibodies.

The HHA was first developed in birds, in which bird plasma was treated with a sample of rabbit blood (Matson et al., 2005), but has since been used for mammals, with chicken blood replacing rabbit blood for the assay (Gilot-Fromont et al., 2012; Heinrich et al., 2017; Ruoss et al., 2019). To the best of our knowledge the HHA has not yet been used to assess human immune response, but its use in other mammals suggests it should produce results. As little as 10 µl of plasma is serially diluted in PBS into 11 wells in a 96-well plate. A 1% suspension of chicken red blood cells is added. Plates are incubated at 37°C for 110 min and then photographed or scanned to measure agglutination due to natural



antibodies and then again after 70 more minutes to measure lysis, which is caused by complement. The scans are scored manually based on appearance. As with the other response assays, this assay requires an incubator or other method of maintaining a controlled temperature. Most assays use commercially available red blood cells, which may be difficult to obtain and maintain in the field. However, it is likely possible to use fresh erythrocytes which have been washed and resuspended in an appropriate buffer. Given the relatively complexity of performing the assay in the field, it is possible to use frozen plasma for the assay. Most protocols caution against freeze–thaw cycles, however evaluations of freeze–thaw cycles on complement activity show that these have little effect (Hegemann et al., 2017; O’Shaughnessy et al., 2012; Yang et al., 2015).

10 | FURTHER CONSIDERATIONS

10.1 | Technical variation (batch effects)

For a number of assays, samples are analyzed in a series of “batches.” For example, ELISA and microarray assays are typically run on 96-well plates that hold anywhere between ~36 and 96 samples, depending on protocols; qPCR assays are run on a 384-well plate, and other genomic assays that use NGS technologies (e.g., RNA-seq) are run on flowcells that have between one and eight lanes, and can analyze up to 400 samples. For ELISAs, metrics like the coefficient of variation (CV) are typically used describe variation within and between plates. The CV is the ratio of the standard deviation to the mean (typically multiplied by 100 to report as a percentage). Within each plate the CV is assessed for each sample, run in duplicate, to identify idiosyncrasies, many of which are due to pipetting errors or samples that are on the high or low end of the assay range. Samples with high CVs (>10%) are usually run again, sometimes with a different dilution. “High” and “low” controls, which have a known quantity of the analyte being measured, are included in most assay kits and should be run on each plate. CVs for the control duplicates are used to report variation across plates, due to minor differences in handling and processing. This noise is known as “technical variation.” It can be minimized through careful laboratory technique, but some variation is usually unavoidable. In some cases, it may be useful to statistically control for batch in the data analysis step, however this is only feasible if batch is not correlated with another variable of interest. For example, if using two plates to compare samples from two villages, it would be better to run each plate with a mix of samples from both villages, rather than one plate for each village, in order to minimize confounding of village and batch.

Unfortunately, the practice of including something like a “control” in next-gen sequencing assays has yet to be adopted, and it remains unclear how best to accomplish this given the way NGS operates. There can be a significant amount of variation between batches due to technical factors in RNA-seq and other NGS data. There are some statistical packages (e.g., Combat-seq, variance-Partition [Hoffman & Schadt, 2016; Zhang et al., 2020]) that quantify sources of variation that may be used to adjust the substantive NGS data for better comparability. Still, it is important to try to sequence all samples for a specific project together to limit such batch effects.

10.2 | Age, sex, and reproductive status

Many immunological values vary with age, sex, and reproductive status. Age-related patterns are apparent in many measures due to development and late-life senescence (e.g., Blackwell et al., 2011, 2016; McDade, 2005b). Studies should evaluate whether it is appropriate to use age-standardized z-scores or to otherwise control for age. Note, however, that many age-related changes are not strictly linear, so linear terms in regression models may not be sufficient. Differences by sex and reproductive status (e.g., menstrual cycle, pregnancy) are also apparent (Aghaeepour et al., 2017; Hové et al., 2020; Klein & Flanagan, 2016; Natri et al., 2019; Wander et al., 2008) and so studies should take these into account as part of study design and analysis.

10.3 | Diurnal patterns

Many papers studying immune function do not report or control for time of blood collection. However, many components of the immune system vary diurnally, with many being lower in the morning and higher in the afternoon, opposite the diurnal rhythm of cortisol (Ackermann et al., 2012; Labrecque & Cermakian, 2015; Petrovsky & Harrison, 1998). Diurnal regulation of immunity may be relevant for many research questions (e.g., Garcia, Blackwell, et al., 2020), but even when it is not the focus, time of sample collection is an important variable that should be captured and accounted for in study designs.

10.4 | Chronic versus acute elevations

Some immunological measures are likely to be much more variable within an individual. McDade et al. (2012) analyzed repeat samples of CRP in an Amazonian population. Even though 35% had elevated CRP at one time-point, no participant had more than one elevated

measure, indicating that these were acute elevations rather than chronic elevations. Blackwell et al. (2016) calculated intra-class correlations for 19 immune measures based on repeat samples. Some measures, such as IgE concentration, CD4 counts, and CD8 counts were relatively stable across repeat measures, while others such as neutrophil count and natural killer cell count appeared more labile. The stability of a measure should also be considered in study design (repeat vs. single measures) and interpretation (see Table 1).

10.5 | Capillary versus venous blood

For the most part, measures from capillary and venous blood correlate strongly. However, some differences have been noted (Siteo et al., 2011; Srisala et al., 2019). Study designs should consider whether this is relevant to a particular study design and may wish to validate against serum measures.

10.6 | Measures in urine or feces

Blood is the optimal tissue in which to measure systemic immunity, since blood distributes white blood cells, cytokines, and other factors throughout the body. However, if blood is unavailable, what, if any, immunological measures are available? The short answer is: it is difficult to measure systemic immunity in other fluids. The long answer is that while many things can be measured, care must be taken in interpretation. This is because markers in urine or feces are much more indicative of localized bladder or renal (in the case of urine) or gut (in the case of feces) infections than systemic immune allocations. Higham et al. (2015) compared serum measures with several fecal and urinary measures. They found that while serum and urinary neopterin were correlated ($r = 0.664$), fecal concentrations were not correlated with serum. Serum CRP was not correlated with urinary or fecal CRP levels, but there was a short-term rise in fecal and urinary CRP following a surgery. Serum and urinary haptoglobin were also not correlated, and haptoglobin could not be measured in feces.

10.7 | Measures in saliva and breastmilk

Many studies have measured secretory IgA in saliva, breastmilk, and other secreted substances (e.g., Hodges-Simeon et al., 2017; Miller & McConnell, 2011; Van Anders, 2010). However, this is probably best interpreted as a marker of mucosal immunity, a complex system in

its own right. Caution should be used when interpreting whether secretory IgA is indicative of overall immune status since mucosal immunity is not necessarily correlated with systemic immunity, and can be indicative of better health or worse health depending on the circumstances (Drummond & Hewson-Bower, 1997; Gornowicz et al., 2014; Koss et al., 2016; Nakamura et al., 2006; Reid et al., 2001; Thaweboon et al., 2008). Cytokines can also be measured in saliva, but salivary cytokine levels are generally uncorrelated with serum levels (Riis et al., 2014) and may be indicative of periodontal disease (Riis et al., 2014). Saliva is also a source of white blood cells, which might be used for RNA-seq, methylation, or other assays. The concentration of leukocytes in saliva is $\sim 1.6 \times 10^5$ cells/ml (Vidović et al., 2012), meaning ~ 2 ml of saliva would be equivalent to 50 μ l blood. However, cells in saliva are only $\sim 60\%$ leukocytes with the remaining 40% being buccal cells (Theda et al., 2018), which may change the interpretation of these assays. Salivary leukocytes are also affected by gingivitis and other oral conditions, and in general proportions differ from blood, with monocytes overrepresented. Because of these differences, further validation is needed to understand how best to interpret measures in saliva, and how they correlate (or perhaps trade-off) with measures in blood.

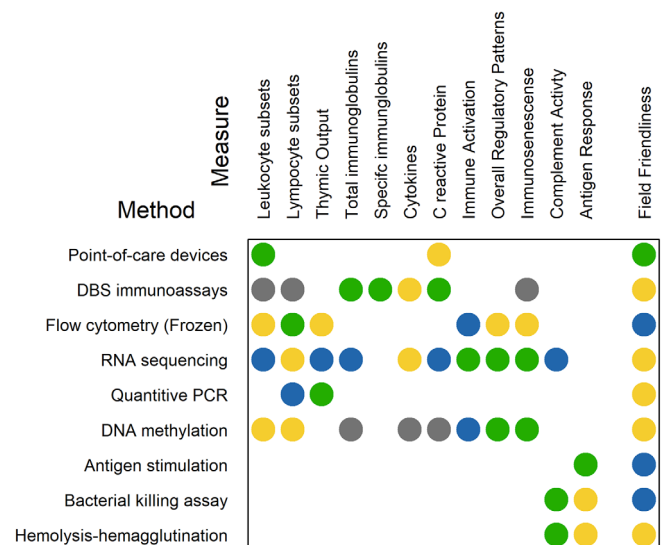


FIGURE 3 Summary of ecoimmunology methods. Colors indicate our subjective assessment of the suitability of a method for a particular measure. Green = optimal measure. Yellow = good measure but may be error prone, difficult, or indirect. Blue = indirect measure that can still be used to infer values. Gray = untested or unvalidated, but perhaps possible. For field friendliness: Green = immediate results, no sample transport; yellow = sample transport or equipment needed; blue = equipment needed and or difficult sample handling

TABLE 3 Key equipment, expenses, and references for each method

Assay type	Major equipment	Cost of equipment ^a	Cost per sample ^b	Expertise	References
Point of care					
White blood cell differential	QBC hematology analyzer; microcapillary tube centrifuge	\$9–\$14 k	\$6–\$8	Minimal	Laney et al., 2019
Influenza A & B	HemoCue WBC system	\$2.5 k	\$3–\$4	Minimal	Osei-Bimpong et al., 2009; Karawajczyk et al., 2017
Immunoassays	BD veritor system plus	\$300	\$20	Minimal	Hassan et al., 2014
Immunoglobulins	Microplate reader; software	\$6–\$56 k	\$14–\$18	Intermediate	Tanner & McDade, 2007; Andersen et al., 2014
C-reactive protein	Microplate reader	\$6–56 k	\$1–\$15	Intermediate	McDade et al., 2004; Brindle et al., 2010
Cytokines (single)	Microplate reader	\$6–56 k	\$9–\$15	Intermediate	McDade et al., 2007, 2021
Cytokines (multiplex)	Multiplex reader		\$40–\$80	Intermediate	Skogstrand et al., 2005; McDade et al., 2021
CD4 Lymphocytes	Microplate reader; software	\$6–56 k	\$12–20	Intermediate	Mwaba et al., 2003
Flow cytometry					
Sample collection	Liquid nitrogen tank/dry shipper	\$300–\$1200	\$2	Intermediate	
Analysis	Flow cytometer	\$40–\$60 k	\$3–\$10	Intermediate	Blackwell et al., 2021
Nucleic acid assays					
Collection, extraction	Qubit fluorometer	\$3200	\$20–40	Intermediate	Krawiec et al., 2009
3' QuantSeq	Next generation sequencer	\$100–\$500 k	\$40–\$100	Advanced	Liu et al., 2014; Moll et al., 2014; Zypych-Walczak et al., 2015; McDade, Ross, et al., 2016; Evans et al., 2018; Reust et al., 2018; Abrams et al., 2019; Cole, 2019; Ma et al., 2019; Harrington et al., 2020; Sholder et al., 2020; https://rnaseq.uoregon.edu/
Whole transcript RNA-Seq	Next generation sequencer	\$100–\$500 k	\$200–\$400	Advanced	
sjTREC, lymphocyte subsets	qPCR machine	\$15–90 k	\$2–\$10	Intermediate	Lorenzi et al., 2008; Lang et al., 2011; den Braber et al., 2012; Cho et al., 2017
DNA methylation	Pyrosequencer; qPCR; mass spectrometer; microarray scanner ^c	\$15–\$500 k	\$50–\$1000	Advanced	Non & Thayer, 2015; Bock et al., 2016; Kurdyukov & Bullock, 2016; Sasaki et al., 2020
Immune response					
Antigen stimulation	Incubator	\$500–\$2000	\$2–\$5 ^d	Intermediate	May, van Den Biggelaar, et al., 2009; van Riet et al., 2009; Trumble et al., 2016; Schneider-Crease et al., 2021
Bacterial killing	Nanodrop spectrophotometer; incubator	\$12–\$17 k	\$5–\$10	Intermediate	Liebl & Martin II, 2009

TABLE 3 (Continued)

Assay type	Major equipment	Cost of equipment ^a	Cost per sample ^b	Expertise	References
HHA	Flatbed scanner; incubator	\$500–2000	\$5–\$10	Intermediate	Matson et al., 2005; Gilot-Fromont et al., 2012; Heinrich et al., 2017; Ruoss et al., 2019

Note: Cost per sample is an estimate as of time of writing and excludes cost of major equipment. Expertise needed is coded as follows: minimal = able to be performed by a novice, with some practice on elements of protocol; intermediate = bench-based laboratory training; statistical analysis; advanced = some bench-based laboratory training, fluency in software and programming, statistical packages, computational analysis.

^aMore expensive equipment is often available at core facilities for a recharge fee, which is usually much more cost effective than purchasing for an individual lab.

^bExcludes the cost of equipment.

^cThere are several different methods, each using different equipment.

^dDoes not include the cost of immunoassays to measure cytokines.

11 | CONCLUSIONS

Many studies have relied on single measures of immunity to draw broad conclusions about the regulation of immune function and how immune function trades-off with other life history demands. However, immunity is multifaceted and single measures are unlikely to accurately capture the full range and plasticity of human immune function. Luckily, the repertoire of methods for the minimally invasive measurement of immune function is rapidly expanding. These measures differ in the aspects of immunity they measure, as well as in the inferences that can be made based on them (Figure 3). They also differ in cost and complexity, which are summarized in Table 3. Traditional measures, such as ELISAs for DBSs remain the simplest and cheapest to implement. These measures are accurate and easy to implement but are limited in scope since they typically measure only one analyte at a time. Multiplex immunoassays overcome this limitation, but usually with a cost to sensitivity, which may be particularly detrimental to measures of low-concentration analytes such as cytokines. Point-of-care devices are often the most convenient because they require no sample transport. However, sensitive point-of-care devices are only available for a few measures, though many more will likely become available in the relatively near future.

Apart from these practical concerns, we have given a general overview of the theoretical contexts in which different biomarkers may be useful. However, biomarkers are never just markers but are part of the biological system in which they are embedded. As such these should not be used as proxies without considering how their meaning might vary with context and what the implications of an interpretation are for the underlying biological process.

At present we are most excited by measures that allow for broad characterizations of immunity. RNA-seq and DNA methylation, in particular, can be used in an exploratory fashion, but also with targeted analyses that can be used to infer cell compositions, cytokine expression levels, and many other aspects of immunity. The cost of sequencing continues to decline precipitously, while sensitivity and analysis tools increase. That said, many analyses require further validation, for example, against gold standard methods such as flow cytometry for immunophenotyping of leukocytes. RNA-seq and methylation analysis are also limited in their ability to differentiate expression by cell type, which can be done with flow cytometry. Single-cell RNA-seq is becoming commonplace (Luecken & Theis, 2019), yet for field studies single cell analysis runs into the same limitation that flow cytometry has, namely the need to preserve and transport blood without cellular lysing.

The measures we catalog here are primarily intended to measure human immune function. However, these methods have much in common with those used in other species (e.g., Boughton et al., 2011; Prall & Muehlenbein, 2014). Methods designed for small animals like mice and birds are easily translatable to finger prick samples of capillary blood from humans. Point-of-care devices designed for medical professions are also likely to become increasingly useful in the future. The suite of tools available for eco-immunology in the field continues to rapidly expand meaning that future studies will increasingly be able to integrate multiple measures of immunity with less effort.

ACKNOWLEDGMENTS

Thanks to Josh Snodgrass and Geeta Eick for organizing this special issue and the AAPA session on which it is based. We also thank two anonymous reviewers for their helpful comments.

ENDNOTE


¹ To avoid confusion, we also note that the term immunophenotype also often refers to the phenotype of individual immune cells, as determined by flow cytometry or other methods.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Aaron D. Blackwell  <https://orcid.org/0000-0002-5871-9865>

Angela R. Garcia  <https://orcid.org/0000-0002-6685-5533>

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How to cite this article: Blackwell, A. D., & Garcia, A. R. (2022). Ecoimmunology in the field: Measuring multiple dimensions of immune function with minimally invasive, field-adapted techniques. *American Journal of Human Biology*, *34*(11), e23784. <https://doi.org/10.1002/ajhb.23784>