

# Appropriate uses of Vitamin A Tracer (Stable Isotope) Methodology

## Vitamin A Tracer Task Force\*

### Vitamin A deficiency contributes to illness, blindness, and death

Vitamin A deficiency (VAD) is second to iron deficiency anemia as being the nutritional health problem of highest public health significance in developing countries. Globally, more than 200 million children are vitamin A deficient, and VAD is still the leading cause of blindness in children. Women in developing countries are also at risk of VAD, especially during pregnancy and lactation. Vitamin A is essential for growth, reproduction, immunity, and vision. VAD is associated with a higher risk of death in preschool-age children, presumably because of vitamin A's role in immune function and in maintaining the integrity of epithelial tissue. Supplementation with vitamin A reduces the risk of child and may reduce maternal mortality. It also reduces the risk of severe diarrhea and measles, both of which are important and sometimes serious illnesses in developing countries. Because the vitamin A content of breast milk is often low in vitamin A-depleted women, infants of these women are at greater risk of becoming VAD early in life. If left untreated, this can result in a vicious cycle of deficiency that is not resolved. Correcting VAD in populations at risk of deficiency is an investment in improving human development.

Public health programs to control VAD include supplementation with therapeutic doses of the vitamin, food fortification, and dietary diversification. More recently, biofortification to enhance provitamin A in staple crops has been recognized as a viable and feasible option, and research is under way to develop this approach. Supplementation with fortified food or pharmaceutical doses of vitamin A have resulted in a significant improvement in reducing overt clinical deficiency; however, the effect of supplementation on improving marginal vitamin A status, which is more prevalent, is difficult to assess. This is important because populations with marginal vitamin A status are more susceptible to infection, and a single bout of infection can rapidly deplete vitamin A stores in the body.

## Lack of ocular signs does not mean the problem is solved

Traditionally, clinical eye signs and symptoms of xerophthalmia are used to determine whether a population group is vitamin A deficient. However, because clinical signs are now rare as a result of intervention programs and economic development, thousands of children would need to be examined to determine the population prevalence of xerophthalmia. Night blindness, a clinical symptom of deficiency, can be a useful assessment tool in populations in which night blindness is a recognized condition. Severe forms of xerophthalmia also do not necessarily respond quickly to treatment with vitamin A, especially when other micronutrient deficiencies co-exist. Biochemical or marginal deficiency, in which clinical eye signs and symptoms are absent, is more prevalent and also has negative consequences on human health.

VAD without clinical manifestations can be assessed using biochemical assessment techniques. The serum vitamin A (retinol) concentration is the most commonly used biochemical technique, but is only useful at the population level. Moreover, because serum retinol concentration is homeostatically controlled and negatively affected by subclinical infection, it is not an optimal indicator for assessing the change in vitamin A status in response to an intervention. Serum retinol concentration would only be expected to increase in response to an intervention when initial values are low and subclinical infection is not present. Because of the detrimental effects that VAD has on human health, assessing VAD at both the individual and population level is important. Accurate assessment of VAD is necessary for evaluating vitamin A interventions, to provide insight into the effect of VAD on human health, and to justify allocation of government funds for intervention programs.

## Vitamin A program evaluation

Few vitamin A programs have been appropriately evaluated, often because of resource constraints. Most of the evaluations that have been done have relied on serum retinol concentrations to assess

change in vitamin A status in response to an intervention. Because serum retinol concentration is not an optimal indicator for assessing change in status, as described above, the results of these evaluations are difficult to interpret. Measuring the true change in vitamin A status in response to an intervention is important so that program managers and policy makers can avoid drawing incorrect conclusions about the efficacy or effectiveness of interventions.

## No change in serum retinol concentration in response to an intervention does not always indicate that the program was ineffective

Because vitamin A is stored in the liver, direct measurement of the liver vitamin A concentration is considered to be the best indicator of vitamin A status. For obvious reasons, this is not a feasible method for routinely assessing status. Several indirect assessment techniques exist to determine the vitamin A status of populations, as shown on the left in Figure 1. Night blindness during pregnancy and dark adaptometry testing are methods for assessing clinical symptoms and an adverse functional effect of VAD, respectively. However, it is not certain whether these tests are useful in populations in which marginal VAD is prevalent without clinical symptoms of night blindness. Serum retinol concentrations have been used extensively to identify populations at risk of VAD. However, serum retinol concentrations are homeostatically controlled and do not reflect liver vitamin A stores until liver reserves are dangerously low. Serum retinol is also affected by recent dietary vitamin A and provitamins A intake, and may change due to seasonal variation in fruit and vegetable intake even though a true change in liver stores of the vitamin does not occur. In addition, the protein that carries retinol in plasma, retinol-binding protein (RBP), is a negative-acute-phase protein; thus, serum retinol and RBP concentrations decline during subclinical infection. A deficiency of other nutrients, particularly iron, may also negatively affect serum retinol concentrations.

Vitamin A STATUS	Deficient	Marginal	Adequate	Sub-toxic	Toxic
LIVER Vitamin A ( $\mu\text{mol/g}$ )	< 0.07	0.07 — 0.1	0.1 — 1.0	> 1.0	?
<b>INDICATOR</b>					
Clinical signs and tests					
Serum retinol					
Breast milk retinol					
Dose response tests					
Isotope dilution					
Liver sample					

**Figure 1: Vitamin A Status Continuum**

The association between vitamin A status, liver reserves, and the indicators currently used. Clinical signs, symptoms and functional tests include xerophthalmia, night blindness, and dark adaptometry. Breast milk retinol is specific to lactating women, but extrapolation to infants is possible. "Dose response tests" include both the RDR and the MRDR. The tracer dilution technique is the only method that provides a quantitative estimate of total body vitamin A pool size.

During the past two decades, other methods to determine vitamin A status have been developed to better reflect liver reserves of vitamin A, the best indicator of VAD. The relative dose response (RDR) and modified relative dose response (MRDR) tests, which consist of giving a small oral dose of retinyl or 3,4-didehydroretinyl ester, respectively, assess the adequacy of liver vitamin A reserves. The dose response tests are based on the principle that during vitamin A depletion the apo-RBP not bound to retinol accumulates in the liver. By giving a challenge dose, the retinol or dehydroretinol will bind to the excess RBP and be secreted into the blood as the holo-RBP-retinol complex. Because circulating concentrations of dehydroretinol are very low in human plasma, a single blood sample for the MRDR, as opposed to two in the RDR test, is all that is required 4 to 7 hours after dosing, after which the ratio of dehydroretinol to retinol is calculated. An elevated ratio of dehydroretinol to retinol is indicative of inadequate liver vitamin A reserves. The MRDR test has been used extensively throughout the world to assess marginal vitamin A status in populations and to determine the efficacy of interventions.

## Tracer methodology using dilution of stable isotopes

Although very useful, the relative dose response tests do not provide a quantitative estimate of the total amount of vitamin A in the body. A tracer dilution technique has emerged as a select method for estimating total body vitamin A pool size, and for answering specific biological questions related to vitamin A metabolism. The tracer dilution technique consists of: 1) administering an oral dose of an appropriate tracer to subjects, 2) collecting a blood sample after the tracer has mixed with endogenous vitamin A (Figure 2), 3) measuring the plasma isotopic ratio of tracer to tracee (unlabelled vitamin A), and 4) estimating the total amount of vitamin A in the body using a prediction equation. The plasma isotopic ratio of tracer to tracee can be measured using gas chromatography-mass spectrometric methods.

The tracer dilution technique is the only indirect assessment technique that provides a quantitative estimate of total body vitamin A pool size. Because the technique is responsive to food or pharmaceutical supplementation with vitamin A, it can be used to evaluate the efficacy or effectiveness of intervention programs by assessing quantitatively the change in total body vitamin A stores in response to an intervention. An added advantage is that the technique can estimate total body vitamin A from the range of deficient to sub-toxic levels (Figure 1). Additionally, it is not necessary to select subjects with deficient or marginally depleted initial status to detect a change in vitamin A status in response to an intervention. Thus, the tracer dilution technique can be useful for assessing change in vitamin A status in populations with low but adequate initial status, whereas the other indirect assessment techniques are only useful for detecting a change in status when initial status is deficient or marginally depleted.

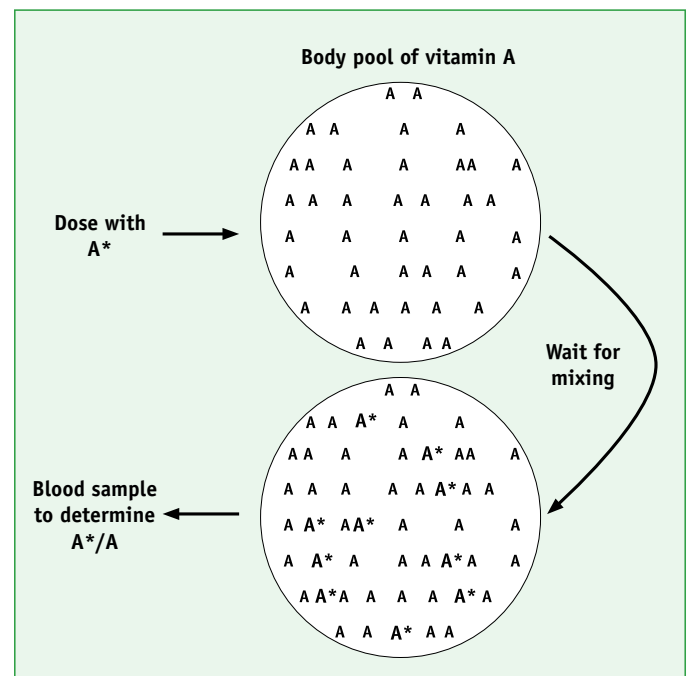
Vitamin A tracer studies have been successfully applied for assessing vitamin A status of populations, and for assessing efficacy and effectiveness of interventions in population groups at risk of deficiency in several different countries. With improvements in the sensitivity of mass spectrometers, the tracer dilution technique has gained momentum and is now available to program managers who wish to

use it to evaluate interventions. The degree of sophistication of the laboratory and resources available will dictate which method is chosen for population assessment and intervention evaluation.

## Implications for monitoring and evaluation

When looking at the continuum of vitamin A assessment methods (Figure 1), the more sensitive the indicator is in reflecting total body reserves of vitamin A, the smaller the sample size required. Moreover, the more sensitive the method is to changes in status, the shorter the study duration; however, this is somewhat dictated by the baseline vitamin A status of the community to be measured.

Although tracer methodology is a powerful tool for assessing vitamin A status and the efficacy and effectiveness of interventions, the costs are not trivial. Therefore, studies must be carefully designed and implemented to obtain meaningful results. A gram of commercially available, stable isotope-labeled vitamin A costs about \$6500, and the cost of analyzing the plasma isotopic ratio of tracer to tracee is approximately \$150 per sample. While these costs may seem high, the sample size required to evaluate interventions using the tracer dilution technique are usually much lower than the samples sizes required for other indirect assessment techniques. Because fewer subjects are needed and because the technique provides an accurate, quantitative estimate



**Figure 2: The principles of tracer methodology**

To estimate how much vitamin A (A) is in the entire body (the body pool), a dose of labeled vitamin A is orally administered, A\*. After a sufficient amount of time is allowed for A\* to mix with the body pool of A, a blood sample is drawn to determine the plasma ratio of A\* to A (A\*/A). From A\*/A, the total body pool of vitamin A can be calculated and liver reserves can be estimated. Depending on the tracer used or the design of the intervention, baseline blood samples may also be necessary.

of change in vitamin A status in response to an intervention, the time required for field operations (which incur a significant cost in serum retinol surveys) is much reduced, making this approach both attractive and feasible (Table 1).

Because the costs are not trivial, evaluators should only embark on a tracer study once all of the plans have been made to ensure that the doses are properly prepared and that the analysis can be done in a timely manner after the samples have been collected.

The method chosen to determine the vitamin A status of a population will also depend upon its initial vitamin A status. For example, an evaluation of the vitamin A-sugar fortification program in Nicaraguan children<sup>1</sup> showed using tracer methodology that the baseline mean liver retinol reserve was 0.57 µmol/g liver, well above deficient (0.07 µmol/g liver) (Figure 1). All of the children had serum retinol concentrations between 0.74 and 1.31 µmol/L, considered a marginal to adequate level. One year after sugar fortification was implemented, liver reserves increased to an average value of 1.2 µmol/g liver (median 0.78 µmol/g), and 9 of the 21 children had liver concentrations ≥1.05 µmol/g liver. The upper safe limit of liver vitamin A stores is unknown; thus this suggests that the program needs to carefully monitor the concentration of retinol in both the fortified sugar and in young children to ensure

that liver retinol concentrations remain at levels that ensure no risk of adverse health effects. It is noteworthy that the MRDR test would not have been a good choice for evaluating this program because the children enrolled in the study were not marginally depleted. Serum retinol concentrations increased but did not reflect any potential sub-toxicity that tracer methods can detect.

## Benefits and limitations of tracer methods versus other methods

The continuum of vitamin A status has five sub-categories: 1) clinical deficiency, 2) marginal deficiency, 3) normal, 4) sub-toxic, and 5) toxic. Figure 1 shows the estimated range of vitamin A status that each indicator is useful in determining. Not all indicators have been compared with direct measurement of liver reserves of vitamin A. Indeed, only vitamin A tracer studies and the relative dose response tests have been validated against liver reserves in either animals or humans. However, if individuals have clinical eye signs and abnormal functional tests, they are already vitamin A deficient. Thus, if a population group has a high incidence of night blindness or impaired dark adaptation, liver stores of vitamin A are already seriously depleted to the

	Method		
	Serum Retinol	MRDR	Isotope Dilution
<b>Dose cost</b>	Not applicable	\$4,000/g DR <sup>†</sup> equivalents	\$6,500/g d4 <sup>†</sup> -retinyl acetate \$10,800/g d8 <sup>†</sup> -retinyl acetate <sup>13</sup> C not yet commercially available
<b>Number of individuals per gram</b>	Not applicable	~600 children (~350 women)	~200 children (100–200 adults)
<b>Projected n/group</b> - Food-based - Supplementation	Depends on status, often thousands	80-90 20-50	15-30 15-30
<b>Average cost per sample analysis</b>	\$25	\$25	~\$150
<b>Cost for acute-phase protein analysis</b>	Highly recommended <sup>‡</sup> ~\$15	Not applicable	Recommended <sup>‡</sup> (but association unknown) ~\$15
<b>Measures status</b>	No <sup>§</sup>	Yes	Yes
<b>Minimum no. of blood samples for an intervention evaluation</b>	2	2	Two if different isotopes; three if same isotope is used
<b>Cost per child</b>	\$80	\$64	\$417 for two isotopes \$560 for three isotopes
<b>Cost per intervention (for pre-and post-intervention assessments)</b>	n = 1000 \$80,000	n = 90 \$5,760	n = 30 \$12,510 for two isotopes \$16,800 for three isotopes
<b>Time to collect blood samples each time</b>	>4 weeks	1 week	<1 week
<b>Measures total body reserves</b>	No	No	Yes
<b>Determines vit. A bioequivalents</b>	No	No	Yes

**Table 1: Attributes and Comparative Costs of Different Methods for Assessing Vitamin A Status.**

<sup>†</sup> DR=dehydroretinol; d-4=tetra-deuterated; d-8=octa-deuterated

<sup>‡</sup> Cost to measure both alpha-1-acid glycoprotein and C-reactive protein

<sup>§</sup> Indicative of deficiency only when <0.35 µmol/L and C-reactive protein is low

point that normal function is not maintained, although overt clinical signs are absent. If serum retinol concentrations are low in the absence of infection, liver stores are depleted.

Breast milk retinol concentrations are useful in determining the vitamin A status of groups of lactating women and appear to be responsive to vitamin A supplementation. The dose response tests, RDR and MRDR, are more sensitive than the above-mentioned indicators of vitamin A status. Therefore, if the only outcome measure is vitamin A status of a population, then the MRDR test on a subsample of the population would be more appropriate and much less expensive than tracer methodology.

The relative dose response tests are not, however, useful in determining total body reserves and do not differentiate between different degrees of adequate and toxic levels of vitamin A. As stated previously, tracer methods provide a quantitative estimate of total vitamin A pool size. Direct measurement of liver reserves of vitamin A status through biopsy is rarely an option and therefore has no utility globally.

## Stable isotope safety for human studies

The use of stable isotopes in research is safe for human subjects and acceptable to institutional review boards, making this technique realistic for program evaluation and monitoring. However, institutional review boards that are not familiar with stable isotopes may often need further documentation and explanation that the tracers used are not radioactive and are safe to be used even in children and women of childbearing age. Because many international research studies have now been published with institutional review board approval, this task is not as onerous as it was a decade ago.

## When tracer methodology is the indicator of choice

The tracer dilution technique is the method of choice when a quantitative estimate of vitamin A pool size is important and when critical decision-making is needed. For example, the tracer dilution technique can be used to estimate quantitatively the amount of vita-

min A retained in the body in response to supplementation with different dietary sources of vitamin A (e.g., animal source versus plant source foods) and to estimate the relative impact of consumption of these foods on total body vitamin A stores. The MRDR test would not be as useful in this situation because it can only determine if vitamin A status has improved in response to a food-based intervention; it cannot estimate and compare how much retinol was derived from the various vitamin A-containing foods. Determination of the amount of vitamin A retained in the body in response to an intervention is required for this type of study and tracer methodology is the only way to obtain this information.

## Requirements for successful use of tracer methodology

Any group interested in using tracer methodology to evaluate interventions must first have a good understanding of vitamin A chemistry and analysis of retinol in serum. In terms of equipment, the use of gas chromatography-mass spectrometry is rapidly increasing and will soon be as standard as high-pressure liquid chromatography (HPLC). However, the use of such instrumentation is much more technically challenging than that for HPLC, and therefore setting up appropriate collaborations with expert consultants or contract laboratories is important at the outset to ensure the integrity of the data.

Technical training and some expertise in mathematical modeling are also necessary to further the understanding of tracer methodology, so it will be important for each country to choose technically skilled individuals and seek resources to build their capacity to use and understand both vitamin A metabolism and tracer methodology.

## Regional resource base—centers of excellence

As tracer studies gain in popularity and become more widely used, it may be more cost-effective in both the long- and short-term to set up regional centers of excellence in several continents. Regional centers would allow for more quality assurance, less downtime on the instrument, and more intensive training for the operators than would be available if each country had their own instrumentation.

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