*draft report to the micronutrients initiative

field-appropriate technologies for micronutrient deficiency control programmes

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PATH CANADA
170 LAURIER AVENUE WEST, SUITE 902
OTTAWA, ONTARIO

(*) This report is satisfactory, and can serve as the Final Technical Report.
11/11/95 J. Chinsles

draft report:

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3.3 DIAGNOSTIC TESTS FOR ASSESSMENT OF MICRONUTRIENT DEFICIENCIES

3.3.1 introduction

A preliminary technical study was undertaken to determine the feasibility for development of rapid, simple, inexpensive diagnostic tests for use in the field assessment of micronutrient deficiencies including iodine, vitamin A, and iron. Such simple, rapid tests would be developed as self-contained kits designed for use by field evaluation teams and personnel working at or near the periphery of health care systems in developing countries. Availability of such tests would produce essential data more rapidly, reduce the reliance on centralized laboratory facilities, provide more effective tools for field recognition of micronutrient deficiencies in targeted populations and for monitoring interventions, and provide additional data for planning health interventions.

Over the last few years PATH/International's diagnostics group has developed a series of simple, rapid, potentially inexpensive immunodiagnostic technologies based on a dipstick-type format for detection of infectious diseases such as HIV, hepatitis B, and tuberculosis. Initial funding for research and development of the HIV dipstick test (see attachment 1) was provided in part by IDRC. The dipstick core technology has proven to be effective and a viable format upon which to develop other diagnostic tests. The dipstick technology is appropriately simple and has already been transferred for manufacturing in several developing countries. In addition, PATH is currently developing and/or optimizing other simple diagnostic technologies such as a second-generation dipstick based on a cassette format which is more suited for use as an individual test, and an immunochromatographic (IC) strip format similar to those which have been developed for home pregnancy tests.

Immunological assays in an ELISA format have been developed for assessment of iodine deficiency disease (IDD), including assays for thyroid stimulating hormone (TSH), and serum thyroglobulin (Tg); vitamin A deficiency, (retinol binding protein (RBP)); and iron deficiency, (serum ferritin (SF) and serum transferrin receptor (TR)). Test kits for many of these are now-- or will shortly be-- available commercially, while others are unavailable commercially and found only in specialized clinical reference laboratories.

While most ELISAs are reproducible and sensitive, there are many intrinsic disadvantages of ELISA tests, including the length of time needed to perform the assay, a precise dilution of components, need for refrigeration of reagents, the necessity to run the assay in centralized facilities, the cost/maintenance of relatively complex equipment, and a need for technically-skilled personnel to perform the assays. Traditional ELISA methods using microtiter plates are not adaptable for use in the field. To our knowledge there has been no commercial or academic effort to convert ELISA-based assays into simpler, more rapid test formats.

3.3.2 DEVELOPMENT OF PERFORMANCE SPECIFICATIONS

The first step in planning the feasibility study was to determine realistic performance specifications for the assays which are appropriate for their intended use, and then to design or adapt the candidate core technology best fitting the requirements specified. These test parameters include:

- a simple test, easily performed and interpreted by a peripheral health care worker after minimal instruction;
- specimen collection by minimally invasive means such as fingerstick blood using a simple device such as a lancet;
- minimal specimen preparation or purification;
- serum, plasma, or whole blood used as a specimen (other fluids which can be collected noninvasively are optional but not mandatory);
- visual, semi-quantitative reading and endpoint determination;
- appropriate nanogram or milligram sensitivity for an analyte using serum and/or reference standards;
- results available in 15 minutes or less;
- less than 10 percent error for the rapid test when compared with reference ELISA test methods and standards;
- positive control markers and/or reference calibrators included as an integral part of the assay system;
- low cost (preferably US$0.25-0.30 per test);
- stability of 18-24 months at ambient temperature (25-30 deg C);
- results can be stored and are permanent;
- simple to use and can be batched easily; and
- tests for different (micronutrient deficiency) markers can be run in parallel with no more than a 5 minute difference in reading the results of both tests.

Considering the product specifications listed above and the assay formats in development

and on hand, the immunochromatographic (IC) strip assay, an advanced diagnostic format, was selected for assay development over the HIV dipstick. The dipstick is relatively rapid, sensitive, and easy to use for serological testing (detection of specific antibodies); however, markers for micronutrient deficiencies are antigens. The disadvantages of the dipstick as an antigen capture test format include longer serum incubation times required for optimum sensitivity (compared with serological reactions), and difficulty in integrating a positive control reaction for each dipstick test as there is limited reaction area. For example, compared with HIV (a 20 minute test), the hepatitis B surface antigen dipstick test needs 60 to 90 minutes to complete. On the other hand, the IC strip method is more appropriate for antigen capture; test reactions are typically complete in 10 to 15 minutes and the strips will accommodate additional positive controls or calibrators. A key component shared by both the strip and dipstick tests is the use of colloidal gold conjugate as signal reagent. Since we have optimized larger scale production protocols for colloidal gold-protein A for use with the dipstick test, we were able to easily adapt these protocols to produce antibody conjugates for use in strip tests. The IC strip assay format is described in box 1.

It is estimated that strip test manufacturing costs will initially be somewhat higher compared to the dipstick, but not significantly more expensive per test if the IC strips are manufactured in large quantities. Both strips and dipsticks share simplicity for routine use, capability for batching, and permanent results can be obtained.

The IC strip pregnancy test is its most popular application and has been marketed and used extensively in the developing world. As an example, annex 1 shows the package insert from a HCG pregnancy test for home use which employs the IC strip method as produced for sale in Vietnam.

Other advantages of the IC strip over the dipstick format are:

- both systems use colloidal gold; however, the disptick employs a liquid form which is relatively labile, therefore the test needs refrigeration to retain shelf life; that in the IC strip is dried and incorporated into the pad. If kept in a foil package with a dessicant, it can be stored at ambient temperatures for a year or more;

- while the dipstick format has been accurately and reliably used in peripheral health centres, the IC strip test requires no laboratory backup or equipment.

- flexibility; depending on packaging, IC strip tests may be used individually.

3.3.3 RESULTS OF FEASIBILITY TESTING

Because key components and reagents are commercially-available, two systems were chosen to demonstrate initial feasibility: thyroglobulin (Tg) as a marker for IDD, and retinol binding protein (RBP) as a marker for vitamin A deficiency.

A. Optimization of the IC Strip Test Format

The IC strip test is a rapid, simple, one-step diagnostic technology which has been used for development of tests to detect ovulation and pregnancy (LH and HCG), infectious diseases such as chlamydia and hepatitis B, and drugs of abuse. The main advantage of the test is that all assay reagents are contained on the strip; to obtain a result only the specimen needs to be applied. It is more readily adapted to detection of antigens rather than serological detection of antibodies because the practical amount of signal reagent which can be used for each test is limited. When too much serum is applied, or if the specimen is high in immunoglobulin (Ig) levels there is competition for signal reagent which nonspecifically binds to the Ig, and a false negative test may result.

To perform the test, the specimen containing analyte is placed on the first pad and "wicked" by capillary action through the pad where it mixes with colloidal gold-signal antibody reagent. Specimen then proceeds through the nitrocellulose membrane where any specific complexes of analyte and colloidal gold-signal antibody combine with capture antibody which has been immobilized on the membrane. A positive reaction thus produces a red line or dot on the membrane, whereas a negative reaction remains colorless. Positive control reactions and/or other calibrators can also be included on the membrane. Typically, the reaction is complete after only 10 to 15 minutes, although sensitivity may increase (often, however, with reduced specificity) with further incubation time.

The basic technology for assembly of IC strip tests has been obtained by PATH for further optimization and adaptation for this and other diagnostics projects of interest.

B. Immunoassay for Thyroglobulin (Tg)

background

Experts in the field have called for a test to supplement the measurement of goiter size and urinary iodine. Recent field trials which examined epidemiological indicators in school-aged children indicated that serum Tg levels closely reflect thyroid volume, are a sensitive indicator, and should be practical in surveys where blood samples are available (Benmiloud et. al 1994). The same trials concluded serum TSH and T_4 were not useful indicators in this age group.

Urinary iodine levels reflect recent iodine ingestion and can change with variations in intakes, while iodine availability affects thyroid volume and serum thyroglobulin more chronically over months or years. Thus urinary iodine would be the most useful marker for assessing current iodine status, while serum Tg would be the best surrogate marker for assessing chronic IDD in children and adults (Benmiloud et. al 1994). Blood spots from fingerstick samples can be used to measure Tg.

Enhanced turn-over of thyroid cells from iodine deficiency-induced hyperplasia and hypertrophy leads to the release of Tg into the serum. Serum Tg changes inversely with iodine intakes in all age groups (WHO/UNICEF/ICCIDD 1993).ELISA kits have been developed for detection of Tg.

feasibility testing

Reference human Tg antigen and affinity-purified polyclonal rabbit anti-Tg antibody were obtained through commercial sources. Colloidal gold signal reagent was prepared in 20 nM and 30 nM particle sizes using purified anti-Tg by standardized procedures. Nitrocellulose membranes of 10 mM porosity were used in the assembly of IC strips used for the test. A 1:20 dilution of affinity-purified goat anti-rabbit IgG was used for the positive control indicator. To save on assay development and optimization time the colloidal gold-signal antibody solution was not sprayed and dried onto separate pads, but was mixed with specimen in a microtest plate immediately before running the strip test; we anticipate there to be little difference in the sensitivity limits using this preincubation method. Results were recorded 15 minutes after initiating the assay.

Initial feasibility experiments consisted of multiple dilutions of antiserum and reference antigen to determine relative sensitivity of the immunological system. For these initial feasibility studies, antibody was spotted on to the nitrocellulose membranes rather than producing lines with a fluid monitoring device. A small group of presumably normal sera of local origin were also tested.

The sensitivity limit of the assay using the present reagents was $\leq$2.8 ng/ml Tg using 1.5 microliters of a 1:20 dilution of antiserum as capture and $\leq$13 ng/ml using 1.5 microliters of a 1:40 dilution. At these capture antibody concentrations, the (+/-) reaction at the end point produced a very faint reaction, whereas at higher Tg concentrations, there was a stronger, easily interpretable reaction in the 1+ to 3+ range. Initial sensitivity was equivalent using either the 20 nM or 30 nM preparations of signal reagent. It was not difficult to semi-quantitate the intensity of the spots as from a +/- to 3+ reaction by visual comparison.

The consensus Tg values found in reference publications indicated that normal levels are 10-20 ng/ml, with approximately 20 ng/ml at the upper range of normal. The absolute assay sensitivity as developed was therefore within the physiologically normal range. This sensitivity level was also indicated with weak (+/-) but clearly visible reactions in six of seven "normal" sera obtained locally, with the seventh serum showing a slightly more intense 1+ reaction. This reaction disappeared when the serum was diluted. As expected, the higher dilutions of capture antibody were less sensitive for detection of Tg. For example, the endpoint for an antibody dilution of 1:200 was approximately 60-70 ng/ml.

field assay concept
For development of a field assay, two dilutions of capture antibody could be used:

- a relatively high concentration of specific antibody calibrated to produce a weak but clearly visible reaction within the normal limits of serum Tg found in well-nourished individuals, and

- a lower concentration of the same antibody preparation calibrated and titrated to produce a negative reaction at normal Tg levels, but be clearly visible in individuals with chronic, untreated IDD with elevated levels of Tg in the 50 to >200 ng/ml range.

A prototype IC strip test could contain three capture antibody lines (rather than only two as shown on the diagram in Box 1) including the control and two indicators. Results would be read according to the following:

- normal (<20 ng/ml)- Control line (or spot) clearly positive (1+ or more) with the first indicator line/spot weakly positive (+/-) to negative, and the second indicator line/spot negative;

- moderate IDD (20-50 ng/ml) - Control line/spot and the first indicator line/spot are clearly positive, while the second indicator line/spot is only weakly positive to negative;

- severe IDD (>50 ng/ml) - Strong positive; control line/spot positive, first and second indicator lines/spots both positive, with the first line producing a much stronger reaction than the second;

- technical error- no reactions visible, including the positive control. Repeat testing indicated.

Since the lower limit of the immunological detection system of the IC strip test proposed above falls within physiologically normal Tg limits, the development of a rapid test should be possible. This test would take advantage of higher Tg levels in iodine-deficient individuals producing a stronger reaction. By using two indicators, sera from both borderline and severe cases of IDD could be distinguished from sera from normal individuals.

C. IMMUNOASSAY FOR RETINOL BINDING PROTEIN (RBP)

background
Retinol (vitamin A) is transported in plasma bound to RBP. In vitamin A deficiency (VAD), the secretion of RBP is blocked, leading to lowered serum/plasma levels.

Measurement of serum retinol provides a useful indicator of vitamin A deficiency in populations, but not individuals. Retinol concentrations are affected by protein-energy malnutrition and infections, and caution is indicated when comparing serum levels in different populations. In contrast, changes in serum retinol over time within a population can be interpreted with greater confidence. Determination of retinol levels (high pressure liquid chromatography, fluorescence, and spectrophotometry) however, requires very considerable laboratory expertise, resources, and quality control (WHO/UNICEF 1994).

Levels of RBP in serum may be a suitable alternative to serum retinol, although more work is required to confirm its reliability in populations with varying degrees of infections and protein-energy malnutrition.

RBP can be measured by ELISA methods, but reliable ELISA techniques are not yet commercially available, and other immunoassay procedures are expensive.

feasibility testing

Rabbit and goat antisera to RBP and reference human RBP antigen (8.1 mg/ml) were obtained from commercial sources. Colloidal gold signal reagent was prepared using purified rabbit anti-RBP in 20 nM and 30 nM particle sizes by standardized procedures. Initial feasibility experiments also consisted of multiple dilutions of antiserum and reference antigen to determine relative sensitivity of the immunological system. Strip materials used, their assembly, and the assay conditions for RBP were identical to those indicated above for the Tg system. A small group of normal sera of local origin were also tested.

The sensitivity limit of the assay using the available reagents was found to be $\leq$9 ng/ml RPB using 1.5 microliters of a 1:20 dilution of sheep or rabbit antibody as capture and $\leq$45 ng/ml using 1.5 microliters of antibody at a 1:50 dilution. These values are approximately 1000-fold lower than levels of RBP found in sera. Mixing sheep and rabbit antibody together enhanced the intensity of the reaction appreciably at lower concentrations of RBP antigen, but did not contribute to increasing the level of sensitivity. Initial sensitivity was equivalent using either the 20 nM or 30 nM preparations of signal reagent. At these capture antibody concentrations, the (+/-) reaction at the end point produces a very faint reaction, whereas at higher concentrations, there is a stronger, easily interpretable reaction in the 1+ to 3+ range. Again, it was not difficult to visually semi-quantitate the intensity of the spots as from +/- to 3+ reactions.

A recent reference publication from the U.S. Centers for Disease Control and Prevention (Mueller et. al. 1992) indicates that RBP levels are approximately 50 microgm/ml in men and 25 microgm/ml in women. The absolute assay sensitivity as was developed fell well within and below the physiologically normal range. This sensitivity level was also indicated with clearly visible reactions in all eight "normal"

sera obtained locally. As was expected, the higher dilutions of capture antibody were less sensitive for detection of RBP.

field assay concept

For development of a useful field assay, the present assay must first be desensitized to detect normal levels of RBP. This may be easily accomplished either by diluting the capture antibody, using a larger porosity nitrocellulose membrane, using less signal antibody to sensitize colloidal gold conjugates, and/or employing a smaller volume of reactants. Capture antibody can be diluted to produce at least a 2+ test reaction (line or spot) to indicate normal levels of serum RBP. In addition, a positive control spot or line can be used in a dilution which would also produce a 2+ reaction for comparison of reaction intensities. Thus, at an RBP concentration of 25-50 microgm/ml, the intensity of the control and test reactions would be at least equal. However, if levels of RBP are lower, the intensity of the test reaction would be less intense compared with the control, indicating a deficient state. A prototype IC strip test would therefore contain two capture antibody lines (or spots), with results read according to the following interpretations:

- normal ($\geq$25 microgm/ml)- Control line (or spot) clearly positive (2+) with the test line/spot equal to or greater in intensity compared with the control;

- moderate VAD (<25 ng/ml) - Control line/spot is positive (2+), while the test line/spot is less reactive (clearly less than 2+) compared with the control;

- severe VAD (<<25 ng/ml) - Control line/spot clearly positive (2+), test line/spot only weakly positive (1+ to +/-) to negative;

- technical error - no reaction visible, including the positive control. Repeat testing indicated.

In summary, the lower limit of immunological detection of RBP in the IC strip test system was found to be well below physiologically normal limits. Thus, the development of a rapid test for RBP will be possible by first desensitizing the reaction and then calibrating the positive control to give a dependable, constant 2+ reaction. Levels of RBP would thus be semi-quantitated by comparing the control vs. test reactions. If the test reaction was seen as equal to or greater than the control reaction the patient's sera has normal levels of RBP. On the other hand, for sera mildly or severely deficient in RBP, the test reaction would appear to be slightly to significantly less when compared with the control.

C. IMMUNOASSAYS FOR IRON DEFICIENCY

background

Serum ferritin (SF) has been found to be a useful method for discrimination of iron-deficiency anemia from other anemias such as those produced by chronic disease or malaria. Low SF reflects depleted iron stores and is the most specific finding for iron deficiency. However, SF levels can also be elevated in response to infection or inflammation. In individuals, if normal SF levels are detected they do not always reflect adequate nutrition in individuals without taking these additional factors into account. On a population basis, however, there is good correlation of depleted iron stores or low serum ferritin with the severity of iron deficiency (WHO, 1994). WHO has proposed a classification of the severity of iron depletion using serum ferritin as an indicator.

More recently, serum transferrin receptor (TR) has been developed as an additional indicator of iron deficiency which directly measures the capacity of tissue to accept iron. Its advantages as an indicator include a rapid change in levels with change in iron status, little significant change with accompanying infections or inflammatory conditions, and the ability to distinguish iron deficiency anemia from other types. TR is a relatively late responder during the development of iron deficiency. However, TR levels may also be elevated in hemolytic anemia (WHO 1994).

Monoclonal antibody-based SF and TR ELISA assays have been used in tandem to some advantage. Since both tests have been refined and calibrated, the ratios determined from quantitative SF/TR ELISA assays can provide an accurate indication of iron status.

field assay concept

Assays for iron deficiency were not investigated in the current study, although these studies will continue using the IC strip format as research materials including monoclonal antibodies for SF and TR are received from academic collaborators and commercial sources. Based on the data generated above and the potential sensitivity demonstrated for specific antigen-antibody reactions using the IC strip format, it should be possible to devise semi-quantitative assays to detect relative levels of SF and TR as serum analytes.

For example, for development of a field assay to determine SF levels, capture antibody dilutions can be calibrated to produce a 2+ test reaction (line or spot) for "normal" SF levels of 20-30 ng/ml. The positive control spot or line would also be diluted to produce an equal 2+ reaction for comparison of reaction intensities. However, at SF levels of less than 20 ng/ml which is indicative of iron deficiency anemia, the test line would produce a visibly weaker reaction when compared with the control.

Similarly, since TR is elevated in iron deficiency anemia, a field assay could be developed which is similar to the Tg test model described above. For this assay two dilutions of capture antibody would be used:

- a relatively high concentration of specific antibody calibrated to produce a weak but clearly visible reaction within the normal limits of serum TR ($\leq$8 microgm/ml)[1] found in well-nourished individuals, and

- a lower concentration of the same antibody preparation calibrated and titrated to produce a negative reaction at normal TR levels, but clearly visible in individuals with elevated levels. Both tests for SF and TR could be formatted in tandem for use with the same specimen as a double indicator for determination of iron deficiency anemia.

3.3.4 CONCLUSIONS AND DISCUSSION

Laboratory feasibility has been established using the IC strip format for possible additional development and optimization of rapid, immunologically based assays for Tg and RBP which can serve as surrogate markers for iodine and vitamin A deficiency. In both assay systems investigated, titration endpoints were determined at or below physiologically normal ranges of serum analyte concentrations. With use of calibrator or reference markers integrated into the assay, either higher or lower concentrations of analytes can be determined which correspond to a micronutrient deficient status. In addition, similar assays could be developed for SF and TR since physiologically normal serum concentrations of these analytes are in the same range as those found for Tg and RBP.

The accuracy for rapid field assays will probably be adequate, although limited in being semi-quantitative and not able to produce values as precise as those from ELISA assays.

The small number of sera tested were obtained locally (Seattle) from a presumably normal donor group. Assay performance must be determined on sera from persons with micronutrient deficiencies and compared with reference ELISA methods to confirm the preliminary findings using the rapid IC strip test methods.

In addition, the use of whole blood specimens needs to be studied since validation of its use would provide a significant advantage over plasma or serum which requires an additional preparation step. This may be accomplished by including a red cell lysing agent and anticoagulant impregnated in the first sample pad. The resulting red cell stroma would be filtered by the pad and the hemoglobin would migrate through the nitrocellulose membrane with the plasma. Samples of peripheral (finger stick) blood could therefore be obtained with lancets rather than needles, syringes or vacutainers, thereby reducing costs and increasing

[1]WHO (1994) suggests a uniform cut-off value of 8.5 mg/l to define elevated TR

test acceptability. Alternatively, if there are interfering reactions from the red cell components, plasma from finger stick blood can be separated on a simple, inexpensive separator card for use in the assay.

PATH is unaware of a strip test formatted as a semi-quantitative (as opposed to a qualitative) indicator.

Although the feasibility of rapid test development has been demonstrated here, it is important to emphasize that this represents only a small fraction of the total effort required to complete initial research, assay optimization, product development, field evaluation, and product introduction. Even tests employing simple assay methods and technologies may take several years and several hundred thousand dollars in funding for full development and validation as a product.

On the other hand, in PATH's experience, formatting several related products on the same core technology will likely reduce the research and development time and costs following the initial effort. Once marketed as a product, the test must then be repeatedly evaluated for its value and cost-effectiveness in the role for which it was intended, expert troubleshooting must be performed if the test is reported to perform inadequately in the field, and additional improvements to the test or production methodology must be constantly considered. All these post-introduction activities require additional funding.

Beyond initial product development, the relative costs of production should be of concern. For example, HCG strip tests manufactured in quantities in excess of a million tests per year can be produced for US$0.25-0.30 each, or less. Cost savings are possible since production can be semi-automated, critical materials and components can be purchased in bulk, and production overheads are low per unit volume. However, a relatively low need/demand for a given test, say only 100,000-200,000 tests/year, provides correspondingly lower efficiencies of production and little economy of scale. In lower volumes, the assembly of devices is largely manual as the costs of capital equipment for automation cannot be justified. The per test cost will then likely be doubled or tripled, making the test relatively expensive for its intended use. This economic reality is not only for the IC strip format, but even for other simple tests such as the dipstick with seemingly low materials costs.

The IC strip format is considered adaptable to many different indicators or diagnostic systems, including hormones, drugs of abuse, small molecules, and infectious diseases. As more and different tests are formatted, production can be combined, lowering overhead and manufacturing costs.

While there are clear arguments in favor of the development of rapid test devices for field determination of micronutrient status, further refinement of the ELISA microplate methods, cumbersome and equipment-dependent as they may be, are also important. ELISA tests are essential as laboratory reference "gold standards" and for quality assurance to ensure that peripheral laboratories are performing to set standards. In addition, there should be further

development and validation of practical specimen field collection methods for blood, such as use of filter paper strips as is now done for HIV epidemiological surveillance studies. When completely dry, blood spots are stable for weeks or months at ambient temperatures and can be shipped to central laboratories for reference ELISA testing. While such procedures do not have a rapid turn-around times, they may be useful in project activities where time may not be a factor, such as monitoring or establishing baseline values for targeted populations, and could complement rapid field methods.

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FIELD TEST KITS FOR IODATE, IODIDE, IRON AND VITAMIN A

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ANNEX 1. DETAILS OF TECHNIQUES FOR FIELD TEST ANALYSIS

1. DETAILS OF TECHNIQUES FOR ANALYSIS OF IODIDE

Leuco crystal violet method

Procedure: STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER 17th edition, 1989

Measure 50 mL iodide solution into a 100 mL volumetric flask. Add 1.0 mL citric buffer and 0.5 mL of potassium peroxymonosulphate solution. Swirl to mix and let stand approximately 1 min. Add 1.0 mL leuco crystal violet indicator, mix, and dilute to 100 mL. For best results, read the absorbance (or compare the colour with standards) within 5 minutes after adding leuco crystal violet indicator solution.

CATALYTIC REDUCTION METHOD

Procedure: STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER 17th edition, 1989

Prepare 10 mL iodide solution in a 25 mL test tube, keeping the iodide content of the diluted sample in the range 2 to 6 μg/mL. Add reagents to the sample in the following order: 1 mL NaCl solution, 0.50 mL arsenous acid solution, and 0.5 mL concentrated sulfuric acid. Place the reaction mixture and the ceric ammonium sulphate solution into a water bath at 30 °C, and allow to come to temperature equilibrium. Add 1 mL ceric ammonium sulphate solution, mix the contents of the test tube by inversion and start the stopwatch to time the reaction. After 15 $\pm$ 0.1 min remove the sample from the water bath and add immediately 1.0 mL ferrous ammonium sulphate reagent with mixing, whereupon the yellow ceric ion colour should disappear. Then add, with mixing, 1.0 mL potassium thiocyanate solution. Replace sample in the water bath. Within 1 hr after the thiocyanate addition, read the red colour as percent transmittance in a photometric instrument.

IODIDE OXIDATION WITH BROMINE WATER

Procedure: OFFICIAL METHODS OF ANALYSIS of the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS 14th Edition Edited by Sidney Williams; Published by the AOAC, Inc. 1984;

Dissolve 50 gram sample in water and dilute to 250 mL in a volumetric flask. Take 25 mL aliquot for analysis. Place sample aliquot in 600 mL beaker and dilute to ca 300 mL. Neutralize to methyl orange with 85% phosphoric acid and add 1 mL excess. Add excess bromine water and boil solution gently until colourless, and than 5 min longer. Add few crystals salicylic acid and cool solution to ~ 20°C. Add 1.0 mL 85% phosphoric acid and ~ 0.5 gram potassium iodide, and titrate iodine with 0.005 N sodium thiosulphate solution, adding starch indicator solution when liberated iodine colour is nearly gone.
1 mL 0.005 N $Na_2S_2O_3$ = 0.1058 mg iodine.

IODIDE DECOMPOSITION WITH IODATE AND HYDROCHLORIC ACID

Procedure: STANDARD METHODS OF CHEMICAL ANALYSIS N.Y. D.VAN NOSTRAND Comp. Inc. SCOTT, Wilfred, W.

A known amount of 0.1 N potassium iodate is added to the iodide solution, in sufficient amount to liberate all of the iodine combined as iodide, with several mL in excess. Hydrochloric acid and a piece of calcite are added. The mixture is boiled until all of the liberated iodine has been expelled. To the cooled solution 2 or 3 grams of potassium iodide are added and the liberated iodine, corresponding to the excess of iodate in the solution, is titrated with standard thiosulphate. One mL of 0.1 N potassium iodate = 0.01058 gram iodine as potassium iodide.

IODIDE DECOMPOSITION BY FERRIC (Fe^{+++}) SALTS

Procedure STANDARD METHODS OF CHEMICAL ANALYSIS N.Y. D.VAN NOSTRAND Comp.

Inc. SCOTT, Wilfred, W. (SMCA)

An excess of ferric ammonium sulphate is added to the sample in a distillation flask. The solution is acidified with sulfuric acid, then heated to boiling, and the iodine is distilled into a solution of potassium iodide. The free iodine in the distillate is titrated with standard thiosulphate, or by arsenous acid in presence of an excess of sodium bicarbonate.

The reagent is added from a burette until the titrated solution becomes a pale yellow colour. About 5 mL of starch solution are then added and the titration continued until the blue colour of the starch fades and the solution becomes colourless.

2. DETAILS OF PROCEDURES FOR THE MEASUREMENT OF IODATE

REDUCTION OF IODATE TO IODINE BY POTASSIUM IODIDE
Procedure:

For soluble samples STANDARD METHODS OF CHEMICAL ANALYSIS N.Y. D.VAN NOSTRAND Comp. Inc. SCOTT, Wilfred, W.
The solution containing the iodate is allowed to run into an excess of potassium iodide solution containing hydrochloric acid. The liberated iodine is titrated with sodium thiosulphate as usual. One mL 0.1 N sodium thiosulphate = 3.567 mg potassium iodate.

Qualitative test for flour OFFICIAL METHODS OF ANALYSIS of the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS 14th Edition; S. Williams Ed.; AOAC, Inc. 1984
Reagent: 1 volume 1 % KI solution + 1 volume HCl (5%) solution.
Sift flour over surface of dry pan and spray mixed reagent onto flour from glass atomizer until particles are wetted. Black specks or purple spots indicate presence of iodate.

IODATE REDUCTION TO IODINE BY KSCN
Procedure: OFFICIAL METHODS OF ANALYSIS of the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS 14th Edition; Edited by Sidney Williams; Published by the AOAC, Inc. 1984

Qualitative test for flour
Reagent: 1 volume 1% KSCN + 4 volumes HCl (1%)
Distribute flour evenly over bottom of metal pan and cover with freshly prepared reagent solution. Break up any lumps with stirring rod and observe on white surface. Black specks or purple spots indicate presence of iodate.

3. DETAILS OF METHODOLOGIES FOR MEASURING IRON

PHENANTHROLINE METHOD FOR FERROUS IRON
Procedure: STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER - 17th edition 1989

To determine ferrous iron, acidify aqueous sample with 2 mL concentrated hydrochloric acid to prevent oxidation. Withdraw a 50 mL portion of the acidified sample into a 100 mL volumetric flask and add 20 mL phenanthroline solution and 10 mL ammonium acetate solution (buffer) with vigorous stirring. Dilute to volume and measure the colour intensity within 5 min.

PHENANTHROLINE METHOD FOR TOTAL IRON
Procedure: STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER - 17th

edition 1989

Measure 50 mL of the aqueous sample containing ferrous and ferric ion into a 125 mL Erlenmeyer flask. Add 2 mL concentrated hydrochloric acid and 1 mL hydroxylamine solution. Add a few glass beads and heat to boiling. To insure dissolution of all the iron, continue boiling until the volume is reduced to 15 to 20 mL. Cool to room temperature and transfer to a 100 mL volumetric flask or Nessler tube. Add 10 mL ammonium acetate buffer solution and 2 mL phenanthroline solution and dilute to the mark with distilled water. Mix thoroughly and allow at least 10 min. for maximum colour development

FERROZINE METHOD FOR TOTAL IRON

Procedure for aqueous samples: HACH WATER ANALYSIS HANDBOOK 1992 Hach Co. Loveland, Colorado U.S.A.

The procedure is based on a commercial portable kit including a spectrophotometer: Collect samples in acid washed glass or plastic bottles. To preserve samples, adjust the sample pH to 2.0 or less with nitric acid (about 2 mL per liter). Before testing digest samples in nitric/hydrochloric acid, then adjust the sample pH to 3 to 5 with ammonium hydroxide, ACS. Adjust wavelength of spectrophotometer to 562 nm. Fill a sample cell to the 25 mL mark with sample. Add the contents of one Ferrozine iron reagent pillow to the cell (the prepared sample). Swirl to mix. Let stand for five minutes. Read the display in mg/L Fe ferrozine.

THIOCYANATE METHOD FOR TOTAL IRON

Procedure: E.B. Sandell. - COLOURIMETRIC DETERMINATION OF TRACES OF METALS. Interscience Publishers, Inc., N.Y. 1959

To a 30 mL of sample add 20 mL of 0.4 N hydrochloric acid and heat in water bath just below the boiling point for 20 minutes. Cool to room temperature and add a drop of 0.5 per cent potassium permanganate or enough to maintain a colour in the solution for one minute. Add 5.0 mL of potassium thiocyanate solution (30 gram in 100 mL) and compare the colour at once with a suitable series of standards. If less than 0.4 ppm of iron is present use 50 mL Nessler tubes for the comparison.

TPTZ METHOD FOR TOTAL IRON

Procedure: HACH WATER ANALYSIS HANDBOOK, Hach Co. Loveland, Colorado U.S.A. 1992

The procedure is based on a commercial portable kit including a spectrophotometer:
Collect samples in acid washed glass or plastic bottles. To preserve samples, adjust the sample pH to 2.0 or less with nitric acid (about 2 mL per liter). Adjust the sample pH to 2 or less with nitric acid (about 2 mL/L). Before testing, adjust the pH of the stored sample to between 3 to 4 with 5.0 N sodium hydroxide solution. Do not exceed pH 5 as iron may precipitate. Rotate the wavelength dial to 590 nm. Fill a sample cell to the 25 mL mark with sample. Add the contents of one TPTZ iron reagent powder pillow (which also contains the reducing agent) to the cell (the prepared sample). Cap and shake for 30 seconds. Compare reading with blank Read the display in mg/L total iron.

4. REVIEW OF THE OFFICIAL METHODS FOR VITAMIN A

Vitamin A assay for tablets, capsules, oils and other pharmaceutical products (spectrophotometric method)
THE UNITED STATES PHARMACOPOEIA Nineteenth Revision 1975 (USP)

Principle: After sample preparation consisting of refluxing with water, saponification, extraction with ether, sample concentration and redissolution in isopropyl alcohol the vitamin A is determined by spectrophotometric measurement at 325 nm using background correction.

Procedure: An accurately weighed sample (containing approximately 0.2 mg retinol) is refluxed in 10 mL water on a water bath for ten minutes, then refluxed again with potassium hydroxide in ethanol for saponification for 30 minutes. The sample solution is cooled and the vitamin A is extracted with 3 portions of ethyl-ether. The ether is evaporated by a stream of inert gas (helium or nitrogen) or vacuum. The residue is dissolved in 5 mL of isopropyl alcohol, and the absorbance is measured at 325, 310 and 334 nm. A calculation using a correcting formula gives the vitamin A content of the sample.

VITAMIN A IN MARGARINE (SPECTROPHOTOMETRIC METHOD)
OFFICIAL METHODS OF ANALYSIS OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS
Fourteenth Edition 1984; Edited by Sidney Williams; Published by AOAC, Inc.

Principle: After sample preparation consisting, saponification, extraction with ether, sample concentration and redissolution in petroleum ether, the sample is fractionated by column chromatography using an alumina column followed by a magnesium column. Vitamin A is determined in the appropriate fractions by spectrophotometric measurement at 325 nm using background correction.

Procedure: The unsaponifiable portion of margarine is chromatographed on adsorption column consisting of 2 segments of activated alumina separated by a middle segment of alkaline alumina. If an eluate fraction containing vitamin A is coloured, it is further chromatographed on a column of magnesium oxide. The column chromatography technique achieves separation of vitamin A from impurities that cause erroneously high values. Adequacy of separation is determined from the ratio of absorbances of the vitamin A containing fraction at 310, 334 and 325 nm. Absorbance of sample solution at 325 nm multiplied by 18.3 gives the concentration of vitamin A in IU / mL.

VITAMIN A IN MIXED FEEDS, PREMIXES, AND FOODS (COLOURIMETRIC METHOD)
OFFICIAL METHODS OF ANALYSIS OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS
Fourteenth Edition 1984; Edited by Sidney Williams; Published by AOAC, Inc.

Principle: After sample preparation consisting of saponification, dilution with aqueous alcohol, extraction with hexane, the sample is centrifuged, and fractionated by column chromatography. Carotene is determined spectrophotometrically, as above, while the vitamin A is determined by the Carr-Price colourimetric method. Total vitamin A activity is determined by calculation.

Procedure: The sample is weighed into a 125 mL boiling flask. For low fat products, add 1 g fresh cottonseed oil to boiling flask before alkaline digestion. Add 5 mL concentrated potassium hydroxide solution and 30 mL ethanol. Reflux for 30 minutes at ca 2 drops/sec. Cool rapidly to room temperature. Add alcohol-water (3+1) solution to calibration mark. Let suspended matter settle. Transfer 10 mL supernatant to a 50 mL centrifuge tube. Add 2 mL water and 20 mL hexane. Extract vitamin A by shaking vigorously for more than one minute. et phases separate and centrifuge briefly. If yellow pigments are present, chromatograph sample on activated magnesia-diatomaceous earth mixture (1+1) using acetone-hexane (1+9) solvent, collect the entire eluate separately and determine carotene content photometrically. For vitamin A: evaporate eluate under vacuum in a water bath at 60-65°C. Dissolve residue in 1 mL chloroform. Adjust colourimeter to 620 nm using colour reagent (Carr-Price reagent:20 % antimony trichloride in chloroform) as blank. The method includes the quantitation of carotene, and the vitamin A activity calculated and reported as Retinol Equivalents.

Questionnaire:
performance of the rapid test kit for iodate in salt

We would be very grateful for your providing feedback on your experience with the rapid test kit for measuring levels of iodate in salt. The information you provide will be of great value to our efforts to identify improvements to the test method and kit, and may provide practical ideas for the design of new test kits.

PATH Canada is asking you to participate in this review because of the current use of the rapid test kit for measurement of iodate in salt in the national IDD programme with which you are involved. Many thanks in advance for your assistance.

1. How do you describe yourself ? (you may check more than one)

- ☐ a user of the test kit
- ☐ a national programme planner or manager
- ☐ regional or district-level health worker
- ☐ salt producer
- ☐ wholesale/retail trader
- ☐ other (please specify)_____

2. Please describe ways in which the rapid iodate test is <u>now</u> being used in the salt iodization programme in your country (please mark all answers that apply):

- ☐ for quality control at the production level
- ☐ for quality control at the distributor level
- ☐ in schools or classes for educational purposes
- ☐ for routine monitoring of iodate levels as part of an IDD control programme
- ☐ for spot-checking at community level
- ☐ other (please explain)

_____
_____
_____

3. What other ways could this test be used in the IDD control programme in your country (please mark all answers that apply):

- ☐ for quality control at the production level
- ☐ for quality control at the distributor level
- ☐ in schools or classes for educational purposes
- ☐ for routine monitoring of iodate levels as part of the IDD control programme
- ☐ for spot-checking at community level
- ☐ other (please explain)

_____
_____
_____
_____

4. What do you consider the biggest advantage of the rapid test kit ?

_____
_____
_____
_____

5. Please identify the most common problems with the use of the rapid iodate test kit. Please try to be specific. Please mark all answers that apply.

- ☐ the availability of the test kit (procurement and re-supply)
- ☐ the adequacy of the written instructions for use
- ☐ the ease of handling and use
- ☐ the visibility of the change in color
- ☐ the cost of the test kit
- ☐ the reproducibility of the test results
- ☐ other (please explain)_____

_____
_____

Please provide details regarding the above problems:

_____
_____
_____
_____

6. Please describe how the test results are used in your IDD control programme:

_____
_____
_____
_____

7. The rapid test gives information on the presence or absence of iodate in salt. For your activities, would information on the actual level of iodate be more useful ?

☐ yes
☐ no

8. Would you like to make specific suggestions for ways in which the rapid test kit could be modified in order to improve its performance ?

_____
_____
_____
_____
_____
_____

9. Any other comments.

completed by_____

micronutrient technologies

LIST OF PERSONS CONTACTED FOR EVALUATION OF THE PERFORMANCE OF THE RAPID TEST FOR IODATE IN SALT

name	title	address	number
Dr. Benny Kodyat	Dept of Community Nutrition, MoH	c/o Roger Shrimpton UNICEF Jakarta	6221 571 1326
Dr. Sangsom Sinawat	Dept of Health	c/o UNICEF THAILAND	662 281 6032
M. Baquer	Nutrition officer	UNICEF Dhaka	880 286 3678
Dr. Jane Seward	UNICEF consultant	UNICEF Beijing	861 532 3107
Dr. Judith Mutamba	Ministry of Health	Zimbabwe	263 4 793 634
Dr. Festo Kavishe	Regional nutrition advisor	UNICEF Nairobi	254 221 5584
Ernest Schoffelen	IDD Project officer	UNICEF La Paz	591 278 6701
Nimel Hettarachy	Programme officer	UNICEF Lagos	23 41 269 0726
Supachai Sathirasilapin		Public Welfare Dept. Division of Hill Tribes	662 281-5021
Dr Romsai Suwanik	retired	Mahidol U.	662 412 7165
Dr Umesh Kapil	AIIMS	c/o UNICEF New Delhi	91 11 462 7521
Gerardo Bayugo	Nutrition Service Department of Health	Manila	63 2 711 6235
Birthe Petersen		UNICEF Islamabad	92 51 219 565
Jonathan Gorstein		1130 Hill St Ann Arbor, Mich	mail
Per Engebak		UNICEF Guatemala	5022 336 317
Vulimir Srdanovic		UNICEF Copenhagen	4531 269 421

name	title	address	number
Mr. Charun Samibat	Nutritionist, Regional Office of Health Promotion Centre	Thailand UNICEF BAO/Bangkok	66-2-2816032
M. Asif (Abbottaford)		UNICEF Islamabad	92-51-219-565
Dr. M.H.K. Ammacha		UNICEF Islamabad	92-51-219-565
Wajid Alikhan		UNICEF Islamabad	92-51-219-565
Quazi Salamatullah	Professor, Inst. of Nutrition & Food Science, Dhaka Univer.	UNICEF Dhaka	880-286-3678
Md. Yeakule Patwary		UNICEF Dhaka	880-286-3678
Iris Rowena A. Bernardo	Nutritionist - Dietician II Nutrition Service - DOH	Philippines	mail
Florence Addo		Ghana	mail
Tatiana Ciomartan, MD		Institute for Mother & Child Care Bd Lacal Tei no 120, Sector 2 Bucuresti, Romania	mail
S. Sundaresan Dr. S. Mukerjee		Salt Commissioner's Organization 2A Jhalena Doongri, Lavan Jaipur, India	mail
Anno Brouowlie		Poland	mail
	Nutrition Service Department of Health	Manila	63 2 711 6235
Wang Jie		China	mail

name	title	address	number
Vincent Assey		Tanzania	mail
Ashraf rel Alam		UNICEF Dar-es-Salaam P.O. Box 4076 Dar-es-Salaam, Tanzania	mail
Anocha Wipulakorn	Nutritionist	Bangladesh Regional Office of Health Promotion Centre	mail

may 1994

NOTE ON FIELD TEST KITS FOR IODIZED SALT

The kits are manufactured by MBI Chemicals, Madras, India and are available through UNICEF Copenhagen.

The following information relates to the application of the kit (which I have put together based on discussions with MBI)::

1. The kits currently available work only on salt fortified with potassium iodate. I have provided MBI Chemicals with information to work on the development of a kit for salt fortified with potassium iodide. They expect have a kit ready by June '93.

2. The kits are designed to check the presence of iodine in salt(fortified with potassium iodate) in the range of 0-50 ppm Iodine and consist of two ampules of the test solution of 10 ml. each packed in a cloth pouch along with a stainless steel spoon and plate and with a colour chart and instruction manual.

3. Ten refill ampules are available in cardboard boxes along with a colour chart and instruction manual.

4. For countries that have set iodine dosage in salt at 100 ppm. MBI offer an alternate test solution for such users. These test solutions show colour contrast in the iodine content range of 20 to 100 ppm.

5. The solution has a shelf life of more than 18 months if unopened and 6 months after opening the ampoule.

6. Some salts contain alkalinity in the form of carbonates. The alkalinity could also be caused by the presence of free flow agents in the salt. In such circumstances the test solution may not release the blue colour to indicate the presence of iodine in the salt. To solve this problem a recheck solution has been developed. In cases where there is suspicion of a possible alkalinity or where the normal test solution does not indicate a change of colour, a drop of the recheck solution may be used and the normal test solution may be dropped over this spot to indicate the presence of iodine. However, if the recheck solution is used in a salt sample without alkalinity followed by the normal solution, it will still indicate the correct iodine level. The recheck solution is provided in the test kit if the buyer indicates the need. Two recheck solution ampules can also be provided in the refill ampule carton if requested. The recheck solution ampules can be recognised by the red cap and the label on the ampules.

List of Test Kits manufactured by MBI KITS

	USD
1. Test kit to check iodated salt with 2 Ampoules of test solution and 1 Ampoule of recheck solution, all packed in a plastic container with colour chart and instruction manual. Range 0-50 PPM	*0.40 per kit*
2. Test kit to check iodated salt with 2 Ampoules of test solution and 1 Ampoule of recheck solution , all packed in a plastic container with colour chart and instruction manual. Range 0-100 PPM	*0.40 per kit*
3. Test kit to check iodated salt packed in a cloth pouch containing 2 Ampoules of test solution, 1 instruction manual, 1 colour chart, 1 stainless steel plate and 1 stainless steel spoon	*1.80 per kit*
4. Test kit to check salt iodised with Potassium Iodide packed in a plastic container containing 3 Ampoules of test solution	*0.60 per kit*
5. Test kit to check salt iodised with Potassium Iodide packed in a cloth pouch containing 2 Ampoules of test solution 1 stainless steel plate, 1 stainless steel spoon, 1 instruction manual and 1 colour chart	*2.00 per kit*
6. Test kit containing only 3 Ampoules of recheck solution packed in a plastic container	*0.36 per kit*

Note: Item 1,2,3 pertain to salt iodised with KIO_3.

Item 4 and 5 pertain to salt iodised with KI.

7. **PROCUREMENT:** Usually a box of ten ampules (each ampoule has 10 ml. of test solution) is sufficient for one user for a whole year during which he/she can conduct around 400 tests. Hence a box of ampules may be planned for each user. I have requested MBI to offer the test solution in bulk packing of one litre (sufficient to fill 10 boxes of 10 ampules each). For every ten users one litre of test solution may be ordered as reserve. This may be used to refill the ampules of the ten users. care will need to be taken during the refilling to clean the ampules with boiled double distilled water so that the solution is not contaminated with the earlier stock. The bulk packing may be reordered as and when the stock depletes.

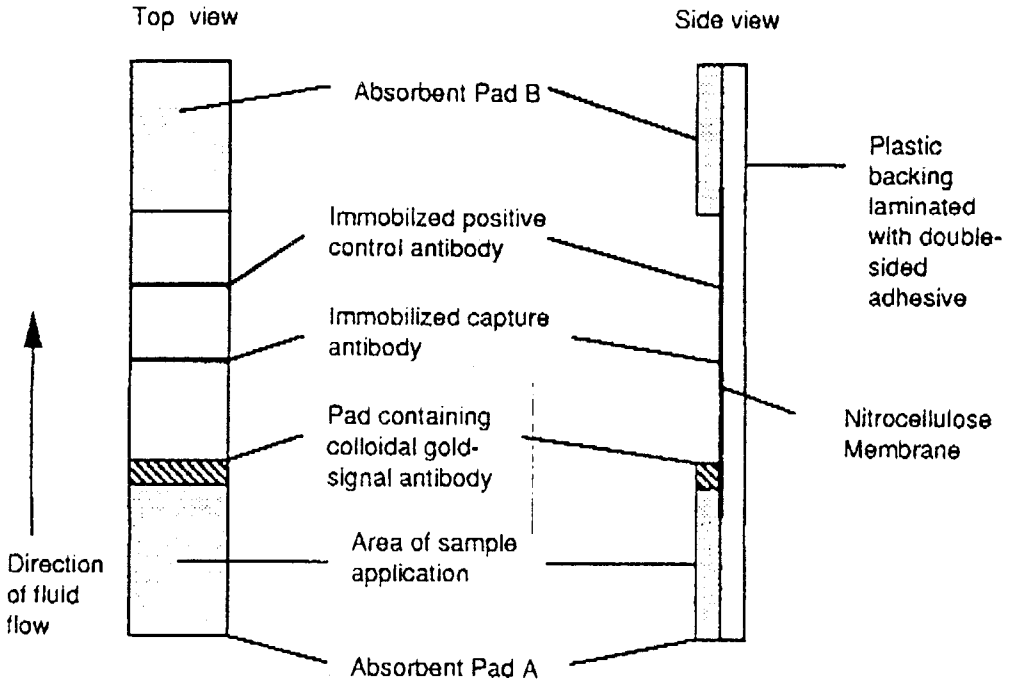
8. **PRICE:** MBI has provided the following prices for the kits (February 1993):

Pouch containing two ampoules, plate & spoon	$ 1.80
Cardboard box containing 10 ampoules, 1 colour chart & 1 instruction manual	$ 1.30
Plastic box containing 2 ampoules of test solution and 1 ampoule of recheck solution	$ 0.40

9. **DELIVERY:** MBI advises that a delivery lead time of 4 weeks may be assumed at the time of placement of the order.

10 May 1993

M.G.Venkatesh Mannar
IDD Consultant
Nutrition Cluster
UNICEF New York



Immunochromatographic Strip Assay Format

We are currently using the one-step format as depicted directly above in the schematic diagram. Nitrocellulose membranes (5 to 15 microns porosity) are first adhered to the plastic backing by double-sided adhesive tape. Colloidal gold signal reagent which has been impregnated into pads, dried, and cut to size, are attached onto the strip, and the two absorbent pads on each end are then added. Monoclonal or polyclonal antibodies used as capture and the anti-immunoglobulin used for the positive control are applied as lines using a fluid metering device, or for prototype development can be spotted using a volumetric pipetter. The individual strip tests are first formatted and assembled on a larger sheet and are then cut to size after final assembly of all components.