CALIBRATION AMONG BLOOD SAMPLES
(DRIED BLOOD SPOT - LIQUID SERUM)
FROM CAPILLARY AND VENOUS SITES:
EXTENSION TO POPULATIONS WITH CONTEMPORARY
RISK OF HYPOVITAMINOSIS A.

(Centre File: 5600-0007-19-300)

Final Narrative Report

Submitted to

The Micronutrient Initiative
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by

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SYNTHESIS

The specific subcontract to the International Nutrition Foundation, Inc for the Central American institutions (CeSSIAM, UNAN) related to the design, planning, staging and execution of sample collections and transport and the ancillary capacitation of individuals and institutions in relation to the technical objectives of the project: DRIED BLOOD SPOT - LIQUID SERUM FROM CAPILLARY AND VENOUS SITES: EXTENSION TO POPULATIONS WITH CONTEMPORARY RISK OF HYPOVITAMINOSIS A. The site of collection was changed from Chiapas, Mexico to the provinces of Granada and Esteli in the Central American republic of Nicaragua, based on reasons of convenience, coordination and institution building. This allowed for collection of specimens of liquid serum and dried blood spots for comparing across sites of extraction (capillary, venous) for assessing inter-site and inter-method correspondence in circulating retinol measurement and for determining the stability of stored samples of dried blood spots on filter paper for retinol measurement at different storage temperatures (-20, 4, 24, and 37°C) for up to 50 days of storage. The array of samples were collected in Nicaragua in Mar, 2001 and transported within 48 to North Carolina, USA for the chemical analysis phase. Several Nicaraguan professionals received capacitation through the design, planning and execution phases. Inspection of the preliminary biochemical data suggests: 1. high inter-site (capillary, venous) correspondence of retinol values and 2. "workable" stability (relatively minor deterioration) of retinol values on the stored filter paper samples for refrigeration and temperate ambient room temperatures. [Once the analyzed samples' values are tabulated and subjected to mathematical analyses in the collaborating center with the separate subcontract: Craft Technologies.] However, despite expectations to the contrary, the population chosen for study in Nicaragua's "CONTEMPORARY RISK OF HYPOVITAMINOSIS A" was low, and the yield of retinol values in the low range was short of the prevalence needed for a sensitivity and specificity analysis for the DBS methods. Major new insights were gained about the logistical and technical issues of DBS approaches to retinol-status surveys in remote populations.

Technical report is satisfactory as per contractual obligations under INF's responsibilities. The lack of serum retinol result is under Craft Tech's scope of work. Please make final payment to INF. Oct 16, 01
RESEARCH PROBLEM

Plasma or serum concentrations of retinol are the time-honed measurements for the assessment of the risk of hypovitaminosis A at the population level. The criteria to define diverse degrees of severity (mild, moderate, or severe) of vitamin A deficiency at the population level is that different proportions of the surveyed subjects (have plasma or serum retinol concentration of <20 µg/dL (<0.70 µmol/L) (WHO, 1996). Clearly, the method or methods that be used to survey populations must be accurate and, as a screening test, have a high sensitivity and specificity for classification of individuals in the population into the below- and above-cut-off-criterion classes.

Beyond accuracy and reliability of classification, certain number of additional advantages might be achieved with the use of alternative methods of blood/serum collection already available and proven in service to public health and epidemiology; the advantages include acceptability by the population, especially when dealing with children; for logistical convenience, handler safety, and overall cost among other considerations.

In order to collect blood to measure analytes in the circulation, certain degree of violation of the integrity of the skin is required. If anything other than a few 100 µL is to be collected for analysis then a venous sample is necessary, which requires the use of a hypodermic needle and syringe or vacutainer. In many cultures, drawing volumes of blood that require syringe often provokes strong adverse cultural reactions, with subsequent rejection of blood sampling. When liquid samples of blood are collected, centrifugation is required to separate serum or plasma and the storage conditions are constrained: generally, the material must be frozen, with temperatures from -20°C (household fridge freezer compartment) to -40°C (deep freeze) to -70°C (ultra-cold) depending upon the analyte of interest.

In contrast, for several analytes the use of dried blood spot techniques involve collecting capillary blood samples followed by drying at room temperature and often storage can be done in a refrigerator or simply at ambient temperature.

Since 1981, the AIDS epidemic has extended throughout the world. The human immunodeficiency virus (HIV) is transmitted in human blood exchanged in transfusions, in intense sexual contact, during childbirth, and, by inadvertent exposure, in analytical laboratories. The agents of chronic hepatitis (C, D) are also bloodborne and a hazard for the laboratory handlers. The absolute amounts of blood involved in capillary sampling are lower; hence only lower amounts of virus are potentially in play. Data from the CDC (Knudsen, 1993) suggest that the very process of drying on filter paper renders HIV non-inflective.

The cost of obtaining a final accurate and reliable value for the analyte of interest is the resultant of many factors. In the use of miniaturized processes, substantial investment in instrumentation and labor is required. The analytical costs for reporting a given value are relatively high. However, savings in the field at the site of collections can be somewhat compensatory as no outlays for a centrifuge, or for sterile hypodermic
needles plus syringes are needed. Only lancets and filter paper cards are needed to proceed, and often only postage stamps may be needed to get the samples to a reference analytical laboratory.

So, the use of the minuscule amounts (drops) of blood obtained from a finger-prick or heel-prick provides for acceptability, logistical convenience, handler safety, and possibly, comparable overall costs.

The field of miniaturization of analyses for retinol has accelerated on two fronts. One can use advances in protein analytical chemistry to detect and quantify retinol binding protein (RPB), as a proxy measure for retinol with which it circulates in a ~1:1 molar ratio in the bloodstream. Recently, improvements in detectors in high precision liquid chromatography (HPLC), however, allow for direct quantification by the absorbance, chromogenic or fluorescent properties of retinol in samples as low as 5µL.

We have demonstrated a strong correlation between serum retinol measured from venous blood (standard method) and retinol measured in a dry single drop of blood, spotted on filter paper (DBS), from normal individuals (Craft et al 1997, 1998, Bulux et al 1999, Craft 2000). A critical condition for this correlation seems to be the keeping of the cold-chain for transportation of samples from the collection site to the analytical laboratory. Thus, it is critical to establish whether this association stands in deficient to marginally retinol deficient subjects. In addition to fully take advantage of the convenience of dried samples, it is necessary to determine the minimum transportation and storage conditions required to preserve the analyte.
OBJECTIVES

**Overall aim:**

To determine the correlation and concordance coefficients in bivariate manner between venous serum retinol (standard) versus capillary DBS retinol (primary surrogate), both in the (expected) low range of retinol values.

To compare the means and distributions, as well as the to determine the concordance correlation coefficients of the DBS samples with a) no cold chain (ambient temperature); b) a "refrigeration" cold chain; or c) a "frozen" cold-chain.

**Specific objectives:**

To enroll 40 subjects from a geographical area with a high probability of endemic low retinol levels.

To collect blood samples from two sites (antecubital vein; volar finger surface) and prepare four types of samples: liquid venous serum; venous DBS; liquid capillary serum; capillary DBS.

To analyze the samples by miniaturized HPLC techniques.

To determine the correspondence of distributions and rank-orders among the four site-state combinations, as well as their concordance correlation coefficient.

To determine the correspondence of distributions and rank-orders among the three sets of transportation/storage conditions of the DBS samples.
Selection of geographical area

An original site proposed for this study was the south-eastern region of Mexico, specifically in the area of Chiapas. The probability of endemic low levels of circulating vitamin A in that population was assumed because of relative marginal health attention to this area, internal migration and displacement, and social and economical problems. There was no real (clinical or biochemical evidence for vitamin A deficiency) but academic authorities and colleagues from the region supported such assumptions. During the process of obtaining permission and planning the possibility of performing this research in the area we obtained information from an ongoing study in the Republic of Nicaragua, in which certain areas showed low values of circulating retinol among children followed in sentinel sites. The sentinel sites with the highest prevalences of low retinol were two hamlets (San Caralampio and Caña de Castilla) in the health district of Diriomo, in the province of Granada (with 51% of subjects with <20 μg/dL), and the health district of San Nicolas, in the province of Estelí (with 4.2% children <20 μg/dL and 49% <30 μg/dL).

Besides these latter data, an ongoing collaborative study in coordination with the Universidad Nacional Autónoma de Nicaragua, the opportunity to strengthen this collaboration and the opportunity to increase the training objectives lead the researchers team to obtain permission to shift the study site to these areas of Nicaragua.

All procedures of this study were approved by the CeSSIAM's Committee for Studies in Human Subjects and by the corresponding IRB of the Universidad Nacional Autónoma de Nicaragua.

Subjects

Since preschoolers were already under surveillance in this region; the collaborating field workers were instructed to recruit pregnant or lactating women, women of childbearing age and school-children (in that rank of priority), all living in the area and willing to participate in the study.

Procedures

Preparatory field visits were performed by our local collaborator (Yadira Medrano) to set the recruitment process, to give instructions to our field collaborators.
and set the places for procedures. Information was also delivered to the potential candidates to participate to explain the nature of the study and the procedures.

Subjects willing to participate were invited to arrive to the study site (a private house of one of the field collaborators in Diriomo, Granada; and the health center in San Nicolás, Esteli), they were not asked to come in fasting state due to distances the subjects had to walk to the study site. At arrival they were again informed about the study and procedures, and they were asked to sign/fingerprint an informed consent form.

Collection procedures were performed one site per day in two subsequent days. Twenty subjects were recruited per site (see annex No. 2 for description of subjects characteristics). All subjects were served a meal after their samples were collected.

Collection and preparation of venous samples.

Approximately eight mL of blood were drawn from an antecubital vein by using disposable sterile syringe and needles. Immediately after the blood was drawn, DBS were prepared on 17 previously labeled (subject id, date, and type of preparation) filter paper cards ((Schleicher & Schuell, Keene, NH, USA) per person. These DBS were prepared letting the drops to fill the pre-labeled circles on the filter paper (five circles per card), these samples were know as VDBS (venous DBS). Once prepared the DBS cards were stored in black boxes to avoid exposition to light and to let them dry. The remaining blood was then transferred to non-heparinized vacutainers, left to coagulation and subsequently centrifuged. Serum was then transferred to pre-labeled (id, date; and type of preparation) plastic screw-top cryotubes (Vanguard-Cryos, Sumitomo Bakelite Co, Japan), stored in coolers for transportation to the laboratory in Managua in the following five to seven hours. These samples, called VLS (venous liquid serum) were collected for determination of retinol concentration by standard HPLC methods.

Collection and preparation of capillary samples.

After collection of venous samples, a fingerprick was performed using a special lancet (Microtainer, Becton-Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) on the volar surface of the subject’s finger. A pre-labeled filter paper card was prepared with five DBS from blood flowing freely from the fingerprick. This card was known as CDBS (capillary dried blood spot). Additional blood was obtained from the fingerprick to fill one non-heparinized micro-collector tube (Microtainer, Becton-Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) with approximately one mL of whole blood. After coagulation, this tube was centrifuged and the serum was transferred to pre-labeled (id, date; and type of preparation) plastic screw-top cryotubes (Vanguard-Cryos, Sumitomo Bakelite Co, Japan), stored in coolers for transportation to the laboratory in Managua in the following five to seven hours. These samples, called CLS (capillary liquid serum) were collected for determination of retinol concentration by standard HPLC methods, as for the case of VLS samples.
Storage and transportation of samples.

Venous and capillary serum samples were transported to Managua within seven hours after collection in coolers, on ice and protected against light. They were stored at -20°C until transportation within 48 hours to North Carolina (Craft Technologies, Inc.). Transportation was done on dry ice. Once the samples arrived at North Carolina they were stored at -70°C until analysis.

Dried blood spots, were left to dry over a period of approximately 10 to 12 hours in the black boxes. When they arrived to the laboratory in Managua and after checking that they were dry, they were packed in self sealing plastic bags, one bag per subject, each card separated with wax paper to avoid inter-card contamination. A bag of desiccant was also added to each individual pack to avoid further humidity collection after full extraction of air by compression and complete sealing of the bag.

All packed dried blood spots were transported on dry ice to North Carolina (Craft Technologies, Inc.) within 48 hours after collection. Immediately after arrival to the laboratory in North Carolina the CDBS and one third of the VDBS were stored at -70°C, another portion of the VDBS were kept at refrigeration temperature, and others were kept at room temperature.

The samples kept at different storage conditions were analyzed on the following 15 days after collection according to a pre-established schedule; using the method developed at Craft Technologies, Inc.

Results:

A total of 40 subjects were recruited for this study. Half of the participants were from the hamlets of San Caralampio and Caña de Castilla in Diriomo, in the province of Granada. The other participants were from the health district of San Nicolás, in the province of Estelí. The ages of the participants ranged from 12 to 65 y with a median of 22.5 y. Among the participants there were 13 lactating and 3 pregnant women, Gender distribution was 29 female and 11 male.

To the date of this report we the results of the biochemical analyses are still pending fullest analysis. We did have the opportunity to discuss the findings in qualitative terms in face-to-face discussions with our collaborator, Dr. Neal Craft, at a recent encounter at the International Nutrition Congress in Vienna, Austria, however.

In qualitative terms, the values of retinol by both the DBS and liquid serum methods from the Nicaraguan sample were higher than anticipated, without values in the <30 µg/dL or <20 µg/dL ranges that would have permitted an analysis of sensitivity and specificity based on the categorical classifications of: abnormal/normal. As reported, the inter-collection-site correspondence remains as tight as was found in our Guatemala-based forerunner project published as Craft et al. 2000b.
Also, in qualitative terms, the findings for stability of DBS results at different temperatures revealed a graded and differential temperature effect. The Craft Tech laboratory went further than stipulated with their temperature-time matrix. It included four temperature conditions: -20°, 4°, 24°, and 37°. This latter condition was added to simulate tropical conditions by placing the filter paper under humid conditions in a humidor at 37°. Also, the serial measurements of DBS retinol were carried out to 50 days of storage, well beyond the 20 days of the original protocol's design. Visual (eyeballing) observations on the contours of the retinol conservation curves showed the expected stability over time of samples preserved frozen at -20°. With maintenance at 4° (refrigeration) and 24° (temperate climate's ambient temperature), there was an initial and early decline in DBS retinol values followed by a stable plateauing at a constant value. With the simulated tropical conditions, a progressive deterioration and erosion of retinol values proceeded through the 50 days of storage. Without detailed analysis at both a group-wise and an individual basis, the feasibility of using common correction-factors to extrapolate back to a true (valid) initial value (i.e. mathematical simulation of conventional frozen storage) cannot be concluded at this time.
PROJECT OUTPUTS AND DISSEMINATION

Training:

In terms of training, the Nicaraguan laboratory student, Ms Hernandez, participated during her time in Guatemala with the staging and logistics of the project. She had been involved in the "routine" collection of DBS as part of a distinct (Sentinel Study of the Impact of Sugar Fortification) and during her time in Guatemala, the discussions of this projected enhanced the theoretical bases of her understanding to complement an extensive experience base acquired in the field participation in "routine" DBS sampling. The participation of Yadira Medrano in the preparation steps, planning in coordination with the principal investigator and her involvement in the field collection procedures was also a great opportunity to enhance skills in epidemiological work in the area of nutrition.

Knowledge creation:

The findings of the study, in response to the objectives, are expected to create new "understanding" (rather than new knowledge) providing:

1. Understanding of the reliability and validity of the dried blood spot method in the lowest range of vitamin A concentrations, and its sensitivity and specificity for a categorical diagnosis of low-retinol and normal-retinol.  
   COMMENT: Because the range of retinol concentrations was higher than expected, this resulted only in another inter-method validation exercise

2. Understanding of the need to preserve (and not discard) the requirement for maintaining a (frozen) cold-chain for the preservation of DBS samples for retinol.
   COMMENT: This objective has been realized in all of its expected comprehensiveness.

Information sharing and dissemination:

To date, no abstracts or papers have been produced. Plans would be to present the work at scientific meetings both local (Guatemala, Nicaragua) and international. The most important vehicle, however, would be a publication -- most appropriately to an analytical chemistry or nutrition journal -- presenting the findings as a guide to procedures for successful ferritin surveying at the community and field level. In fact, the previous collaborative research on Guatemalan adults (Craft 2000b) will serve as a template for the form and message of the sequel paper, but dealing with the issue of temperature sensitivity of stored DBS samples.
CAPACITY BUILDING

We cannot speak for the Craft Technologies, Inc. (which has itself an opportunity to respond in the complementary report on the same project). A very important result for the CESSIAM was to build a strong bridge of collaboration with the Universidad Autónoma Nacional de Nicaragua (UNAN) which is a platform for ongoing relations, many funded by Micronutrient Initiative through IDRC. UNAN came out with the opportunity to observe the execution of a complex, field experiment.

Both the formal submission within the IDRC system of the grant proposal and the experience in preparing this detailed narrative report for IDRC were learning experiences in the management of internationally-funded projects. Although CESSIAM can only tangentially speak for our Nicaraguan counterparts, it is our assessment that the training received by Ms Hernandez in Guatemala and the activities performed by Yadira Medrano in Nicaragua in preparation for the retinol study has enriched the skill-base of the UNAN.

In terms of women, Ms Hernandez of Nicaragua (a biology student), Ms. Yadira Medrano (a faculty member of the UNAAN) and Licda Romero-Abal (a graduate professional) were benefitted by participating actively in the process of discovering that understanding. Honestly, marginalized social groups did not gather increased capacity as the population studies was more passively than actively involved.

In terms of lessons learned, we experienced what it means to work with a collaboration group (the University of Chiapas) with which we had no prior experience of direct working relations. We see in retrospect that interest in the collaboration was lukewarm at certain levels, and getting the institutional assurances across the border in Mexico was a frustrating attempt, at times. Another lesson learned, a hard lesson, is that our basing our projection of nutritional status in March 2001 on the values of retinol found in the same region in September 2001 so that the objective of examining a low ranges of retinol could be explored for the sensitivity and specificity analysis, was a failed notion. As such, the objective of assessing sensitivity and specificity prospectively was not achieved as planned.

PROJECT MANAGEMENT

From the administrative point of view, the decision taking and distribution of contracts took longer than was convenient for the parties in Central America as the final calendarization was less convenient here than the projected calendar at the moment of application.

In terms of the Project Officers from Ottawa, the project began under the charge of Dr. France Begin, and it was transferred to the responsibility of Dr. Erick Boy when the field site was changed to Nicaragua. Both professionals are well rooted in issues of

Venous vs. capillary dried blood spots (DBS) for retinol analysis - Calibration study
micronutrient diagnosis, and have worked and studied in Central America. MI showed flexibility in allowing a change of venue and in anticipating the consequences for the original time-line, initiatives of Dr. Begin. Dr. Boy was flexible in recognizing that the budget items for international travel and international shipping could be creatively condensed and combined to better achieve the goals of assured and rapid transport of the samples for field to lab for the temperature-dependence protocol. He was also especially helpful in clarifying points of inquiry and his suggestions and demystifying of the technical reporting process has improved our ability to write this narrative technical report.

IMPACT

Clearly, hypovitaminosis A iron deficiency and iron deficiency anemia are the most widespread of nutritional deficiencies, and among the most difficult to address with public health measures. It is the marginalized social groups who are differentially susceptible to iron deficiency, although it is found in all social strata, especially among fertile-age women. Knowing where to apply effort and assessing the return on one's public health investment -- and sometimes in which individuals among the group -- are critically important to health systems and funding agencies.

This model should encourage the public health community to seek ways to avoid the need for freezing and frozen transport and storage for biological materials now that the conditions of airline security have changed so drastically, no longer permitting easy shipping of specimens at frozen temperatures. Room-temperature shipping is becoming an imperative for the movement of samples from sites of collection to sites for sophisticated quantitative analysis. Successful ventures will transform the landscape for transport and collaboration along the North-South axis.

OVERALL ASSESSMENT

As stated, the objectives were concrete and practical. [The project contributed, through its new understanding of the universality of the capacity of the DBS to substitute for the more traditional (and invasive) venous-blood, liquid-sample assays for retinol.] To the extent that the text takes on widespread use (reach), the ripple effect of the investment will be ever more important. The duration of the project's execution rapid, inherent to the exigencies to move the samples from the field in Nicaragua to the laboratory in North Carolina. The investment in assured transport by hand-carrying site-to-site (rather than the more risk, insecure, and prolonged transport on dry-ice by a cargo firm) was worth the slightly higher cost. The funding was modest for the Central American components.

This research was performed in Central America, and will be known about here first. For instance, in Guatemala in 2003, the next periodic maternal and child micronutrient nutrition survey will be conducted, as announced by Dr. Francisco Chew
of the Ministry of Public Health. Well, if not to replace the traditional (venous sample) approach for retinol, it would be taking advantage of a real opportunity for a "demonstration" sub-sample. This might constitute a step toward building the experience and confidence in decision-makers to shift from the liquid serum to the DBS methods for retinol in survey and surveillance.

RECOMMENDATIONS

IDRC through MI should continue to be vigilant for opportunities to support innovative inquiries to make diagnostic tests more field friendly for both examiners and examined.

Specifically in the case of national surveys in developing countries, IDRC might make the effort to promote (and support monetarily) the pilot introduction of these techniques of capillary blood and dried blood/serum on filter paper.

Continued capacity building at the Universidad Autónoma Nacional de Nicaragua (UNAN) is recommended as it is an institution with the potential to foster development in Nicaragua and reach out to the public health needs of the marginalized of that society.

Place special effort in mathematical approaches to extrapolate the values from DBS stored at room-temperature back to original (frozen) values as the newer airline security precautions, introduced after September 11, 2001 may have forever altered any practicality for frozen cold-chains for biological samples collected in remote locations for analysis in industrialized countries such as the U.S. or Canada.
LITERATURE CITED:


Craft NE, Haitema T, Brindle LK, Yamini S, Humphrey JH, West KP, Jr. Retinol Analysis in Dried Blood Spots by HPLC. J Nutr 2000; 130:882-885


Annexes

Annex 1: Informed consent form
Annex 2: Data-sheet
Annex No. 1. Informed consent.

 Centro de Estudios en Sensoriaptas, Senscud e Impedimentos y Alteraciones Metabólicas  

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 Calibración entre muestra sanguínea venosa y capilar para la determinación de vitamina A

La deficiencia de vitamina A sigue siendo un problema que afecta a muchas personas en países en desarrollo, especialmente entre los grupos más desprotegidos socioeconómicamente. Una de las formas más utilizadas para saber el estado de vitamina A es mediante el análisis de sangre venosa, pero por varias razones, la obtención de sangre de la vena no es muy aceptada por la población. Se ha encontrado una forma más sencilla para analizar la vitamina A usando sangre obtenida del dedo y colocada sobre un papel especial. Entre las ventajas de este método están: la menor cantidad de sangre extraída, el manejo más fácil de la muestra, es menos impresionante y molesta menos que la punción de la vena.

Para comparar este método sencillo con la forma tradicional, pedimos la colaboración de personas ya sea mayores o niños, que no estén recibiendo tratamiento con vitaminas para que permitan la obtención de una muestra de sangre de la vena del brazo y una muestra de sangre del dedo. La muestra de vena se saca con jeringa y aguja estériles descartables y solamente se obtiene menos de 10 mililitros (menos de una cucharada). La muestra del dedo se hace por un pinchazo en el dedo y se recogen las gotas en unos tubos pequeños además se colocan unas gotas sobre el papel especial que luego se deja secar. Las muestras son luego preparadas para ser enviadas a un laboratorio especializado.

No existen peligros agregados con este tipo de pruebas. La molestia producida es mínima y parecida a cualquier otra prueba de laboratorio. Las personas que quieran participar lo harán voluntariamente y nadie debe sentirse obligado a participar. No se necesita estar en ayunas. Los niños deberán contar con la autorización de sus padres o encargados.

He sido informado de los objetivos y procedimientos de este estudio y voluntariamente decido participar en el mismo:

Nombre del(a) participante: _______________________________ Edad (años) ______

Firma del(a) participante si es mayor de edad; o del encargado, en caso de menores:

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Fecha: __________________

Solo en caso de menores: Nombre del firmante y su relación social con el menor:

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Clave (ID): CL-________
### Annex No. 2. Data sheet

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