AGE-RELATED EYE DISEASE STUDY 2

MANUAL OF PROCEDURES

Age-Related Eye Disease Study 2 (AREDS2): A Multi-center, Randomized Trial of Lutein, Zeaxanthin, and Omega-3 Long-Chain Polyunsaturated Fatty Acids (Docosahexaenoic Acid [DHA] and Eicosapentaenoic Acid [EPA]) in Age-Related Macular Degeneration

IND # 74,781

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This study is being conducted in compliance with the protocol, FDA regulations (21 CFR Parts 50, 54, and 56, 312), Good Clinical Practices, applicable local regulations, and the Declaration of Helsinki.

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1. STUDY OVERVIEW

Eye disorders presenting in late adulthood contribute a substantial burden to society as the primary cause of blinding conditions and low vision. The Eye Disease Prevalence Research Group estimates that there are approximately 937,000 blind people residing in the United States; of these, approximately 841,000 (90 percent) are aged 60 years or older. Age-related eye disease will continue to be an issue of public health significance, since demographic shifts are projected to result in a 50 percent increase in the number of U.S. residents aged 65 years and older by the year 2020. The Age-Related Eye Disease Study 2 (AREDS2) is a study of nutrient-based factors that may influence the development and progression of the two most prevalent age-related eye diseases: age-related macular degeneration (AMD) and cataract.

Human and animal studies provide a reasonable basis for speculating that certain nutrients accreted to and concentrated in the eye have the capacity to modulate factors and processes implicated in the pathogenesis of AMD and cataract. Results from the Age-Related Eye Disease Study (AREDS) on the relationship of lutein/zeaxanthin and omega-3 long-chain polyunsaturated fatty acid (LCPUFA) intake with advanced AMD are informative, yet the non-experimental sampling design limits our strength of inference.

AREDS2 is a multi-center randomized study designed to assess the effects of oral supplementation of high doses of macular xanthophylls (i.e., lutein and zeaxanthin) and omega-3 LCPUFAs [i.e., docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)] for the treatment of AMD and cataract. All participants will be offered additional treatment with the AREDS formulation, which is now considered the standard of care. For those who will be taking these additional supplements, further randomization will be offered to evaluate the possibility of deleting beta-carotene and decreasing the original levels of zinc in the AREDS formulation for the treatment of AMD. The development of advanced AMD will be documented by fundus photography, and the lens opacity outcome will be documented by red reflex photographs (cortical and PSC opacities) and history of cataract surgery. Before randomization, participants will have a run-in period of at least one month and no more than three months, during which they will receive placebo tablets/capsules (Trial Supplement) and AREDS supplements for daily intake. Current smokers or former smokers who have quit during the past year will not receive the AREDS supplements because of the effect of beta carotene on lung cancer.

2. STUDY ORGANIZATION

2.1. Introduction

AREDS2 Principal Investigators (PIs) and centers collaborate through an organization designed to maintain a continuity of operations and to facilitate effective communication and cooperation among the units. The central resource units of AREDS2 include the Coordinating Center, located at The EMMES Corporation, the Fundus Photograph Reading Center, located at the University of Wisconsin, a nutritional biochemistry laboratory located at the Centers for Disease Control and Prevention (CDC), a drug distribution center located at the United States Public Health Service Supply Service Center, and the Nutrition Coordinating Center located at the University of Minnesota. The Study Chair and Project Office are located at the National Eye Institute (NEI), which is the funding agency for AREDS2. Committees that have been created to support AREDS2 include a Data and Safety Monitoring Committee (DSMC), an Executive Committee, an Operations Committee, an Advisory Group, and a Mortality and Morbidity Committee. The Operations Committee is comprised of the Study Chairperson, and representatives from the Coordinating Center, Reading Center, and the NEI. The Operations Committee is responsible for day-to-day operations. The AREDS2 Executive Committee consists of the members of the Operations Committee, and four to eight Clinic PI's (who will rotate on and off this committee for one-year terms). The Data and Safety Monitoring Committee and the NEI Project Team review major decisions. An Organizational Chart is available in Figure 2.1.

The success of a multi-center endeavor depends on the cooperation of the staff in all centers to perform their tasks and responsibilities in an efficient, effective, and timely manner. The participating clinical centers are shown in Figure 2.2.

2.2. NEI Study Chair's Office

The AREDS2 Study Chair, Dr. Emily Chew, is responsible for overseeing the scientific direction of the study and maintaining an effective working group of investigators.

In collaboration with the AREDS2 team, specific responsibilities include:

- Developing the study protocol
- Selecting clinical centers
- Developing study-related procedures, including examination and treatment procedures
- Developing project policies as they relate to clinical center participation and publication issues
- Communicating with FDA and industry
- Interpreting study data and writing manuscripts
- Creating standardized slides for presentations at Investigators' and scientific meetings
- Appointing study personnel and non-personnel to appropriate positions and committees

- Serving as Chairperson of the AREDS2 Executive Committee and Operations Committee and as a nonvoting member of the Data and Safety Monitoring Committee.

The Study Chairperson is appointed for the duration of the study. If the Study Chairperson is unable to serve because of resignation, death, or serious illness, the NEI Director will appoint a new chairperson. If the Study Chairperson is ill or unable to fulfill her obligation for a limited period (up to 6 months), she in conjunction with the NEI Director may appoint an Acting Chairperson for that period.

2.3. Clinical Centers

Clinical Centers are responsible for recruiting, enrolling, and examining AREDS2 study participants according to the AREDS2 Protocol and procedures. Each Clinical Center is supported by a separate Agreement with the Coordinating Center. Key center staff include the Clinic Principal Investigator (PI) and the Clinic Coordinator.

A PI, who will represent the center at Technical Group meetings, will head each Clinical Center. The PI will designate one person (the Clinic Coordinator) with adequate time commitment to AREDS2 to be responsible for supervising the day-to-day study operations. The PI retains the ultimate responsibility for the overall conduct of the study at his/her AREDS2 Clinical Center. Some large centers may require more than one Clinic Coordinator to fulfill the responsibilities of the position (this is dependent upon the number of enrolled study participants and the set-up of the center). Adequate provision must be made for backup during absences and replacement, if necessary.

The PI is responsible for recruiting eligible participants, conducting annual examinations, and answering study questions from participants. The Clinic Coordinator also attends the Technical Group meetings and is responsible for critical matters such as:

- Scheduling appointments for participants
- Obtaining informed consent if they have been designated to do so by their PI and this has been approved by their IRB
- Conducting study visits and telephone contacts with participants
- Maintaining rapport with participants and ensuring adherence to the Protocol and Manual of Procedures
- Ensuring the accuracy, completeness, and consistency of data in compliance with rules and regulations outlined under federal regulations
- Electronic key entry of data
- Maintaining an adequate supply of study supplements to distribute to participants
- Submitting fundus photographs to the Reading Center
- Collecting and submitting blood specimens (if applicable) to the Nutritional Biochemistry Laboratory (CDC)
- Resolving data queries, as appropriate, with the Coordinating Center and Reading Center

- Collecting hospitalization discharge summaries and death certificates
- Providing requested supporting information on reported serious adverse events
- Participating in regularly scheduled, structured telephone calls with the Protocol Monitor from the Coordinating Center and with the Coordinators' Group.
- Maintaining the AREDS2 Regulatory Binder

The responsibilities of the Clinic PI and Clinic Coordinator are further defined in Chapter 10.

2.4. Coordinating Center

The Coordinating Center has the responsibility for the overall coordination of study-related activities. Coordinating Center staff includes professionals in biostatistics, epidemiology, data processing and monitoring, administration, contracts, and communication coordination. Specific responsibilities include:

- Executing annual service agreements with Clinical Centers
- Coordinating the development of the study protocol
- Collaborating with study investigators in developing study procedures, case report forms, and the Manual of Procedures
- Developing and maintaining an Internet-based data capture system for the collection of study-related data
- Developing and maintaining a Web site for facilitating communication among the resource centers and the Clinical Centers
- Coordinating certification of Clinical Center staff
- Providing training to Clinical Center staff in the study-related procedures
- Reviewing electronically-submitted data for completeness, accuracy, and consistency and notifying Clinical Centers about sources of errors
- Maintaining a current computer master file of study data and conducting database assessments targeted at maintaining the integrity of the database
- Preparing periodic reports on the performance of the Clinical Centers including participant accrual and eligibility rates
- Preparing an analysis plan in conjunction with the Data and Safety Monitoring Committee and analyzing the frequency of specified events including adverse events, visual function parameters, and other outcomes covering both safety and efficacy and reporting to the Data and Safety Monitoring Committee
- Preparing recruitment, technical, and statistical reports for study committees and meetings
- Analyzing and interpreting data for Investigator meetings and presentations
- In collaboration with study leadership, preparing scientific reports for publication
- Tracking IRB approvals and expirations and providing other regulatory support, as necessary
- Arranging conference calls and study meetings
- Drafting and distributing summaries of conference calls and meetings
- Developing and maintaining roster of study personnel

- Coordinating site visits and creating site visit summaries

For meeting support, the Coordinating Center collaborates with AREDS2 leadership to:

- Determine optimal meeting dates
- Select meeting sites based on cost and convenience
- Reimburse expenses and provide honoraria when applicable
- Communicate information about meetings to participants
- Prepare and distribute meeting materials
- Provide logistical support on site
- Prepare and distribute minutes
- Follow-up on action items
- Coordinate conference calls

The Coordinating Center supports the preparation, duplication, and dissemination of administrative and technical reports and manuscripts. These documents include:

- AREDS2 Protocol
- Manual of Procedures (MOP)
- Data Collection User's Guide
- Participant recruitment materials
- Meeting minutes
- Participant and Staff Newsletters
- Statistical reports
- Bibliographies
- Abstracts
- Manuscripts for publication
- Roster of AREDS2 personnel

Additional Coordinating Center procedures are included in the Coordinating Center Standard Operating Procedures Manuals, which are maintained at The EMMES Corporation.

2.5. Fundus Photograph Reading Center

The AREDS2 Fundus Photograph Reading Center is responsible for the following:

- Participating in development of study design, protocol and Manual of Procedures
- Developing standardized procedures for fundus photography and other imaging techniques
- Developing and implementing training and certification procedures for the photographers and for the technicians who will perform the fundus photographs and other imaging techniques for AREDS2
- Developing and implementing quality control procedures for assessing fundus photographs and other imaging techniques

- Evaluating study photographs, fluorescein angiograms and optical coherence tomograms of study participants and providing data to the Coordinating Center for analysis
- Implement a grading system for red reflex photos for opacities and the fundus photographs for macular abnormalities
- Establishing secure methods for submission, authentication, and storage of image and non-image data, and establishing secure methods for transmission of image data between the Reading Center and Clinical Centers
- Participating in the analysis of study data and in the writing of manuscripts

The Reading Center is responsible for storing photographs or digital images obtained during the study. The procedures to be used are described in the AREDS2 Reading Center Procedures Manual.

2.6. National Eye Institute (NEI)

The National Eye Institute (NEI), the funding agency for AREDS2, is directly accountable to higher levels of the Executive Branch, the Congress, and the public for the use of Institute funds and programs. The NEI staff, therefore, will be involved in all major decisions affecting the course of the study. The NEI-assigned Project Officer serves on the AREDS2 Executive Committee and as a non-voting member of the Data and Safety Monitoring Committee. The NEI's Director makes final decisions on recommended protocol changes and on other issues of importance to the overall conduct of AREDS2.

2.7. National Institutes of Health Research Contracts

The responsibility of negotiating contracts with the AREDS2 Coordinating and Reading Centers, the CDC, and the Drug Distribution Center lies with the Office of the Director, Office of Administration, Office of Contracts Management, Division of Research Contracts, Contracting Officer.

2.8. Pharmaceutical Company

AREDS2 is partnering with various pharmaceutical companies that will provide either the raw material for making the study supplements or the actual study tablets and capsules. The Coordinating Center works with the pharmaceutical(s) to compile and submit necessary paperwork for filing IND applications to the FDA.

2.9. Drug Distribution Center

The United States Public Health Service (USPH) Supply Service Center located in Perry Point, Maryland receives study supplements and placebo from pharmaceutical companies and is responsible for packaging and distributing the supplements to the Clinical Centers. The distribution center is also responsible for building an electronic web-based system for Clinical Centers to use when ordering additional product.

2.10. Central Laboratory (Interagency Agreement)

A Central Laboratory is responsible for (1) training participating Clinical Center personnel in procedures for blood sample collection, processing, and shipping and (2) the receipt and analysis of blood samples. The results of the analyses will be forwarded to the Coordinating Center for collation with other data.

2.11. Nutrition Coordinating Center (Subcontract with Coordinating Center)

A Nutrition Coordinating Center (NCC) will produce nutrient values corresponding to the responses on the Harvard Dietary Assessment Questionnaire completed by AREDS2 participants.

2.12. NEI Project Team

The NEI Project Team consists of NEI staff from the Division of Epidemiology and Clinical Research (DECR). The Project Team monitors the study and advises the NEI Director on decisions relating to the conduct of the study.

The AREDS2 Project Officer has the overall responsibility for representing the NEI and is an ex-officio member of the Executive Committee; an ex-officio, nonvoting member of the Data and Safety Monitoring Committee; and a voting member of the Operations Committee.

The NEI Office of Health Education and Communication handles all publicity for AREDS2. Press inquiries about the study are referred to this office.

2.13. Data and Safety Monitoring Committee

The primary role of the AREDS2 Data and Safety Monitoring Committee (DSMC) is to monitor participant safety and study progress. The AREDS2 DSMC is also responsible for approving the AREDS2 Protocol prior to initiation of participant recruitment. Subsequent protocol changes that are substantive must be approved by the DSMC prior to implementation. The DSMC is expected to meet at least twice annually (at least one meeting will be in person) and will review all accumulating study data. The AREDS2 Coordinating Center will report to the DSMC within 15 days of notification any adverse experience that is associated with the use of the study supplements and that is both *serious and unexpected*. If the reported adverse event is an unexpected fatal or life-threatening experience associated with the use of the study supplements, the DSMC must be notified as soon as possible but no later than seven calendar days after the sponsor's initial receipt of the information. The DSMC may recommend to the NEI to suspend enrollment. Further details of the role of the DSMC and procedures for monitoring efficacy and safety in AREDS2 are included in the DSMC Charter.

2.14. Operations Committee

The AREDS2 Operations Committee assists the Study Chairperson in the scientific administration and the general operation of the study. The committee:

1. Reviews chapters of the Manual of Procedures, the Protocol, study forms, minutes, newsletters, and other materials.
2. Recommends changes in the specifications of treatment techniques as may be necessary or desirable.
3. Assists the participating units in the performance of their duties.
4. Monitors the performance of these units in accordance with the AREDS2 protocol.
5. Evaluates the efficiency and ability of the units to meet the needs of the study as defined by the protocol, the Study Chairperson, and the NEI.
6. Reviews and determines approval for ancillary studies.

The members of the committee are:

1. The Study Chairperson
2. AREDS2 Project Officer
3. Director, NEI Clinical Center
4. Co-directors, Coordinating Center and other Coordinating Center personnel
5. Director, Reading Center
6. Other NEI personnel
7. Coordinator Chairperson

The Operations Committee reviews the activities of all study units either by direct contact or from reports of groups responsible for monitoring specific aspects of study activities. Members of the Operations Committee frequently meet or confer by phone, and usually at least once a month.

If the performance of a study unit is unsatisfactory, the Operations Committee acts to remedy the situation. As needed, the Operations Committee will schedule problem-solving visits to selected Clinical Centers or other units. Any Clinical Center that is behind schedule in meeting its recruitment goals or that fails to otherwise adhere to the protocol will be reviewed by the Operations Committee as to whether that center should continue to participate in the study.

2.15. Executive Committee

The AREDS2 Executive Committee advises the study leadership in the scientific review and operational recommendations for the study. This committee provides broad representation in viewpoints, contributing to the perspective of those on the central standing committees. Teleconferences or in-person meetings (at the time of the annual Technical Group meetings) occur on a regular basis, at least as often as every six

months, with additional meetings proposed on an "as-needed basis." The committee reviews:

1. The ongoing progress of the study: recruitment, retention, retrospective eligibility
2. Reviews all ancillary study proposals and provides comments to the Operations Committee
3. Provides assistance in resolving operational issues brought to the Executive Committee by the Operations committee, Reading Center, Coordinating Center, or Clinical sites
4. Reviews changes to the Manual of Procedures or Protocol
5. Reviews and provides comments to the Operations Committee on all proposed scientific abstracts and publications

Four to ten clinical site PIs form this committee, and members serve a one-year term. Members of this committee are selected from a pool of volunteers. A list of alternate candidates is maintained. Any member who does not fulfill his/her committee responsibilities will be replaced by an alternate member at the direction of the Study Chair. Chairpersons of the Coordinators' Group serve on this committee as nonvoting members. Members of this committee receive an annual stipend of $250.

Appointment of New Members

Replacement of Executive Committee members occurs on an annual basis, determined by expiring terms for current members. The Coordinating Center tracks the Executive Committee membership and notifies the Operations Committee of expiring terms at least two months prior to expiration. The Coordinating Center requests volunteers from the AREDS2 roster. New members are appointed by the Operations Committee for a one-year term.

2.16. Technical Group

The Technical Group is composed of all study personnel, and its meetings are a forum for disseminating information and discussing study progress. Face-to-face discussion of study activities and the opportunity to interact personally contribute to the development of rapport among team members and encourage smooth operation in such a large multi-center clinical study. The units of the Technical Group are the Clinic PIs, the Coordinators' Group, and the technical committees.

The Technical Group meets at least annually. The Clinic PI (or a professional representative) and the Coordinator from each center attend all meetings. Each center must be represented. Technical Group meetings are open meetings and all members of the study are invited; however, Clinical Center funding is provided for only one Clinic PI and one Coordinator from each center. Exceptions may be made for special training sessions and are at the discretion of the Coordinating Center and the NEI. The Technical Group reviews all decisions of the Executive Committee concerning clinic procedures. Policy dictated by NEI requirements or data monitoring results does not

require approval by the Technical Group. Representatives from each center and the NEI Project Office can cast two votes at each meeting.

Technical Group responsibilities include the review of all AREDS2 manuscripts for publication and presentation. Periodically, the Operations Committee may form ad hoc subcommittees from the Technical Group to evaluate ancillary studies, focus on writing topics, or assist with study planning or other matters.

2.17. Coordinators' Group

The voting members of this group are one lead Coordinator from each Clinical Center; the nonvoting members are the Coordinator from the Reading Center and the Data Manager(s) and Protocol Monitor(s) from the Coordinating Center, as well as other study coordinators that may attend the meeting. The group, which meets at least annually in conjunction with meetings of the Technical Group, is responsible for providing information to the Study Chairperson about the logistical aspects of the study protocol and procedures as they relate to each Clinical Center. The Chairpersons of the Coordinators' Group, four Clinic Coordinators elected by the Clinic Coordinators, are responsible for preparing the agenda for the annual meeting, based on comments and suggestions solicited from the group. The Operations Committee reviews and comments on the draft agendas and on any recommendations from the Coordinators. The Chairpersons also are responsible for preparing the agenda and minutes and leading the bi-monthly conference calls of the Coordinators' Group. Each coordinator is paid a stipend of $10 for his/her participation on the bi-monthly coordinators' conference call.

Elections for Chairpersons are conducted annually during the Coordinators' Group meeting, with each Clinical Center having one vote. Each year four coordinators (2 from each of the two Clinical Center funding mechanisms) serve as co-Chairpersons. Nominations are solicited by the Coordinating Center a few months prior to each meeting. The elected persons serve a one-year term as Chairpersons beginning at the conclusion of the Technical Group meeting. Each Lead Coordinator receives a quarterly stipend of $125.

2.18. Training and Certification Committee

A Training and Certification Committee, appointed by the Study Chairperson, develops a training program and establishes certification criteria. Specifically, training and certification programs are developed for the following areas:

- Visual acuity (ETDRS and E-ETDRS) and Refraction
- Photography
- Blood drawing
- AdvantageEDC^{SM}
- Cognitive Function Telephone Battery

Training is provided for the following area:

- Forms administration and completion

2.19. Analysis Planning Committee

The Study Chairperson appoints members to the Analysis Planning Committee, which is a subcommittee of the Operations Committee. The functions of the Analysis Planning Committee include the following:

1. Make specific proposals or review submitted proposals for analysis of AREDS2 data
2. Set priorities for scheduling work on these analyses
3. Appoint writing teams to prepare manuscripts describing the results of these analyses
4. Designate AREDS2 reports as either Primary or Secondary Reports.

The Analysis Planning Committee reviews proposals for the analysis of data and preparation of manuscripts. Proposals must include a background and brief review of the literature, a clear statement of the research hypothesis, the specific data items to be used in the analyses, and a description of the proposed data analyses. The literature review should summarize relevant material but not be as detailed as the review that will appear in the manuscript. The major research questions to be answered should be identified clearly and succinctly. Table shells and suggested specific analyses also should be included.

Proposals are reviewed by mail by at least two members of the Analysis Planning Committee, who assign tentative priority scores. Subsequently, the proposals are reviewed by the Analysis Planning Committee at a meeting, during which the committee members assign a priority ranking to each proposal. Analyses are performed using available personnel and funds in the rank order assigned by the Analysis Planning Committee.

2.20. Other Committees

Other study committees may be formed as needed, with committee chairpersons appointed by the Study Chairperson.

FIGURE 2.1 AREDS2 ORGANIZATIONALCHART

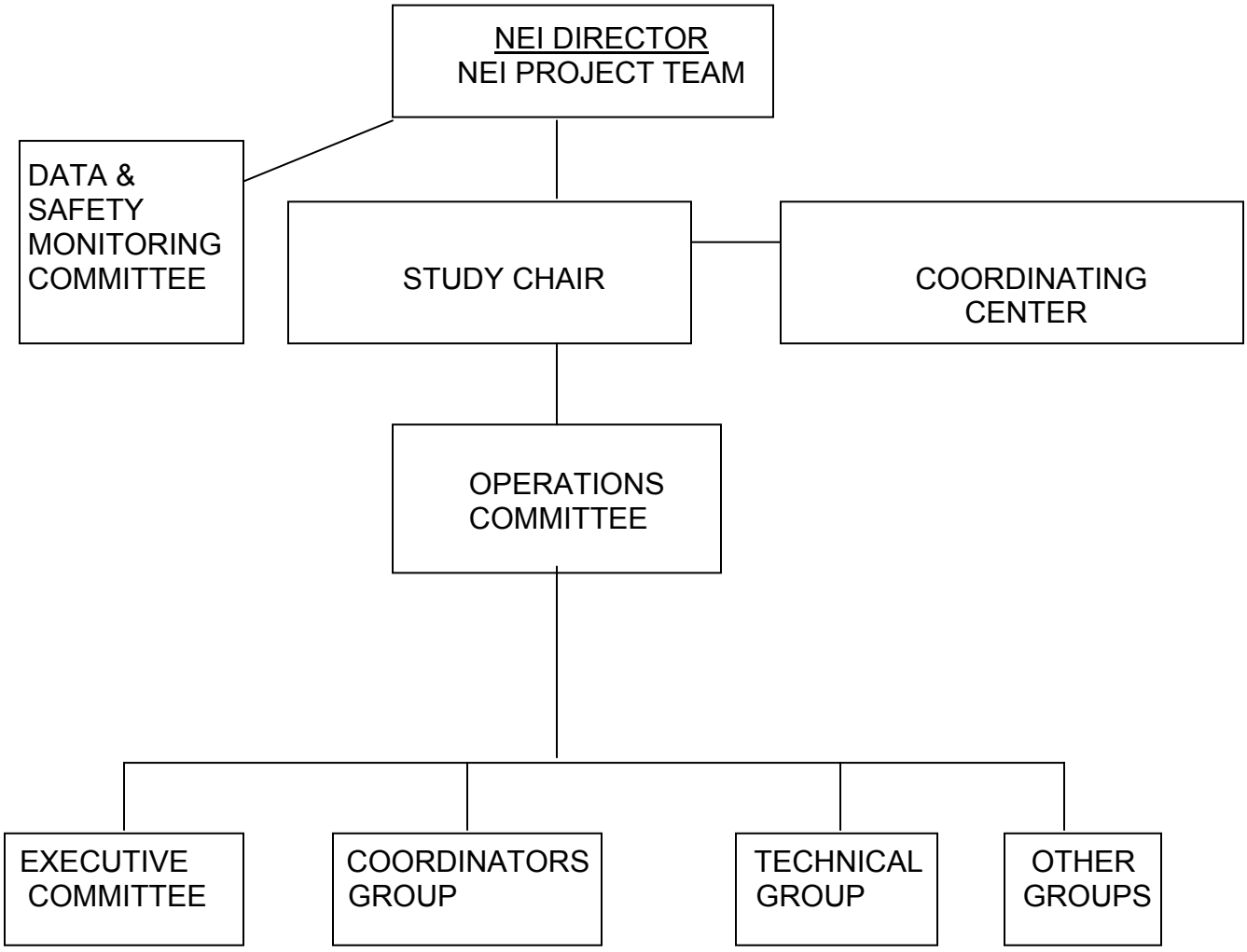


FIGURE 2.2 AREDS2 PARTICIPATING UNITS

Austin Retina Associates	Jose Martinez, M.D.
Barnes Retina Institute	Rajendra Apte, M.D., Ph.D.
Bascom Palmer Eye Institute	Philip J. Rosenfeld, M.D., Ph.D.
Baylor College of Medicine	Richard Alan Lewis, M.D., M.S.
Carolina Retina Center	Barron Fishburne, M.D.
Case/University Hospitals of Cleveland	Suber Huang, M.D.
Center for Retina and Macular Disease	Michael Tolentino, M.D.
Centura Health Research Center	Brian Joondeph, M.D.
Charlotte Eye Ear Nose and Throat Associates, PA	Andrew Antoszyk, M.D.
Dean McGee Eye Institute	Ronald Kingsley, M.D.
Delaware Valley Retina Associates	Darma Ie, M.D.
Devers Eye Institute	Michael Klein, M.D.
Doheny Eye Institute USC Keck School of Medicine	Jennifer Lim, M.D.
Duke University	Cynthia Toth, M.D.
Eldorado Retina Associates, P.C.	Mary Lansing, M.D.
Elman Retina Group, P.A.	Michael J. Elman, M.D.
Emory University Eye Center	Daniel Martin, M.D.
Eye Foundation of Kansas City at Truman Medical Center	Nelson Sabates, M.D.
Fletcher Allen Health Care – Univ. of Vermont	Robert Millay, M.D.
Georgia Retina, P.C.	Jay Stallman, M.D.
Henry Ford Health System - Eye Care Services	Michael Ober, M.D.
Ingalls Hospital	David Orth, M.D.
John Moran Eye Center, University of Utah	Paul Bernstein, M.D., Ph.D.
Jones Eye Institute, University of Arkansas for Medical Sciences	Ammar Safar, M.D.
Jules Stein Eye Institute, University of California at Los Angeles	Steven Schwartz, M.D.
Kresge Eye Institute	Robert Frank, M.D.
Loma Linda University	Michael Rauser, M.D.
Manhattan Eye Ear and Throat Hospital	Michael Cooney, M.D.
Massachusetts Eye and Ear Infirmary	Ivana Kim, MD
Mayo Clinic, Ophthalmology Department	Colin A. McCannel, M.D.
Medical College of Wisconsin	Judy Kim, M.D.
Mid-America Retina Consultants, P.A.	William Rosenthal, M.D.
National Eye Institute Clinical Center	Wai Wong, MD
New York Eye and Ear Infirmary	Richard Rosen, M.D.
Northwestern University, Ophthalmology	Alice Lyon, M.D.
Ohio State University/OSU Eye Physicians and Surgeons, LLC	Robert Chambers, M.D.
Ophthalmic Consultants of Boston	Jeffrey Heier, M.D.
Ophthalmic Consultants of Long Island	Glenn Stoller, M.D.
Pacific Eye Associates	Anne Fung, M.D.
Paducah Retinal Center	Carl Baker, M.D.
Palmetto Retina Center	John Wells III, M.D.
Pennsylvania Retina Specialists, P.C.	Michael Banach, M.D.
Penn State MS Hershey Medical Center	Ingrid Scott, M.D., M.P.H.
Retina Associates, PA	David Dyer, M.D.

Retina Associates of Cleveland	Michael Novak, M.D.
Retina Associates of Kentucky	Rick Isernhagen, M.D.
Retina Center Northwest	Todd Schneiderman, M.D.
Retina Consultants	Paul Beer, M.D.
Retina Diagnostic and Treatment Associates, LLC	Joseph Maguire, M.D.
Retina Group of Florida	Lawrence Halperin, M.D.
Retina Northwest, P.C.	Michael Lee, M.D.
Retina-Vitreous Associates Medical Group	David Boyer, M.D.
Retina Vitreous Consultants	Pamela Rath, M.D.
Sarasota Retina Institute	Keye Wong, M.D.
Scheie Eye Institute	Alexander J. Brucker, M.D.
Scott and White Memorial Hospital	Robert Rosa, M.D.
Southeastern Retina Associates, P.C.	John Hoskins, M.D.
Southern California Desert Retina Consultants, MC	Clement Chan, M.D.
Texas Retina Associates	Gary Fish, M.D.
The Institute of Ophthalmology and Visual Science (UMDNJ)	Neelakshi Bhagat, M.D.
The Research Foundation of SUNY/SB	Fadi El Baba, M.D.
The Retina Group of Washington	Richard Garfinkel, M.D.
The University of Chicago	William Mieler, M.D.
University Health Care- Mason Eye Institute	Dean Hainsworth, M.D.
University of Alabama at Birmingham	Cynthia Owsley, Ph.D., M.S.P.H.
University of California, Davis	Lawrence Morse, M.D.
University of California, San Diego	Barbara Brody, M.P.H.
University of Florida College of Medicine	Kakarla Chalam, M.D.
University of Illinois, Chicago	Lawrence Ulanski II, M.D.
University of Iowa/Dept. of Ophthalmology	James Folk, M.D.
University of North Carolina Department of Ophthalmology	Travis Meredith, M.D.
University of Tennessee Health Science Center	Alessandro Iannaccone, M.D., M.S.
University of Texas Southwestern Med. Center at Dallas	Yu-Guang He, M.D.
University of Wisconsin	Suresh Chandra, M.D.
University of Pittsburgh Medical Center Eye Center	Thomas Friberg, M.D.
VA Northern California Health Care System	Linda Margulies, M.D.
Vanderbilt Eye Institute	Anita Agarwal, M.D.
Vision Research Foundation	Alan J. Ruby, M.D.
Vitreoretinal Consultants	David M. Brown, M.D.
Wake Forest University Eye Center	Craig Greven, M.D.
West Coast Retina, Inc	J. Michael Jumper, M.D.
Western Carolina Retinal Associates	W. Copley McLean, Jr., M.D.
Wilmer Eye Institute, Johns Hopkins University	Susan Bressler, M.D.
Yale University Eye Center	Ron Adelman, M.D., M.P.H.

3. STUDY POLICIES

3.1. Introduction

The entire AREDS2 Research Group participates in the development, review, and acceptance of this Manual of Procedures. The manual is formally approved by the AREDS2 Operations Committee and the Data and Safety Monitoring Committee (DSMC). It is essential to the success of the study that all AREDS2 investigators adhere to the procedures outlined herein. If any AREDS2 investigator finds that, for whatever reason, adherence to these procedures is difficult or not possible, he or she should discuss the problem with the Study Chairperson or the Protocol Monitor.

3.2. Informed Consent

AREDS2 is implementing a dual informed consent process (if approved by the Clinical Center's IRB). Informed consent shall be obtained from each AREDS2 Study Participant prior to the Qualification phase. Additional informed consent shall be obtained prior to enrollment into the study. The Clinical Center staff must ensure that participants are adequately oriented with the objectives and procedures of AREDS2. The Principal Investigator and Clinic Coordinator communicate with the participant to ensure that he/she is aware that side effects may occur and that he/she will be monitored closely throughout participation in AREDS2. If the Principal Investigator is satisfied that the participant is aware of the risks and benefits of participating in AREDS2, written informed consent is obtained before the Qualification phase and again before the participant is randomized into the study if confirmed eligible.

Informed consent is also needed for research procedures that may be part of an ancillary study and may expose the participant to risk or discomfort.

The signed informed consent forms are placed in the participant's file at the Clinical Center. The sample informed consent forms are provided as appendices in the AREDS2 protocol.

3.3. Protection of Human Subjects

Prior to enrolling participants in the study each Clinical Center must submit to the Coordinating Center the initial local or central IRB approval letter and copies of the IRB approved informed consent statements. In addition, annual IRB approval letters must be submitted to the Coordinating Center. Every effort will be made to maintain the confidentiality of AREDS2 study participant records. On occasion, representatives from the AREDS2 Operations Committee and/or the Coordinating Center may wish to review the records.

3.4. Publicity

The AREDS2 Operations Committee should be informed of local publicity efforts to enroll study participants. Personnel at each Clinical Center should refer requests from news media for information about AREDS2 to the Clinical Center's PI.

Information given by Clinical Center PIs should emphasize the following:

- AREDS2 is a collaborative, multi-center study.
- The local center is only one of many.
- The study is funded by the National Eye Institute, National Institutes of Health, Department of Health and Human Services.
- Results of the clinical trial will not become available until the end of the trial or meaningful findings emerge, as determined by the Data and Safety Monitoring Committee.
- Inquiries for additional information not already in the public domain should be referred to the Study Chairperson.

3.5. Disclosure of Study Results

Because knowledge of interim results of the clinical trial could compromise the efforts by Clinical Centers to enroll and maintain follow-up of study participants, reports of such results are submitted by the Coordinating Center only to the Data and Safety Monitoring Committee (DSMC), which is responsible for monitoring the results for safety and efficacy.

The results of the trial will be made available to participating investigators at a time specified by the DSMC and as soon as beneficial or harmful effects clearly are established or the trial has concluded. Investigators should refrain from determining the overall results of the study from their own Clinical Center experience.

Disclosure of AREDS2 results, at appropriate times, to investigators, participants, the scientific community, and the public will be coordinated closely by the NEI and the AREDS2 Coordinating Center.

3.6. Scientific Publications and Presentations

3.6.1. Generation of Publications and Presentations

The Analysis Planning Committee will develop procedures for generating scientific publications and presentations emanating from the design and data collection of AREDS2. These procedures will be reviewed, amended, and approved by the Operations Committee. The Analysis Planning Committee will

also invite suggestions for additional papers from AREDS2 investigators. The Operations Committee appoints writing teams for developing AREDS2 reports and designates AREDS2 reports as either Primary or Secondary AREDS2 reports.

Primary AREDS2 Reports pertain to the primary AREDS2 objective; Secondary AREDS2 Reports deal with secondary AREDS2 objectives or ancillary studies. Before publication, copies of AREDS2 Reports are sent to all members of the Technical Group for information and approval. Reprints of published reports are mailed to each center for distribution to staff and outside consultants. Reprints of each report are sent to the Coordinating Center for the AREDS2 records.

3.6.2. Editorial Review

Abstracts of papers to be presented at scientific meetings and manuscripts to be submitted for publication that pertain to the design of AREDS2 or are based on AREDS2 data, whether they derive from a single AREDS2 center, several AREDS2 centers, or all AREDS2 centers, must be approved by the Operations Committee before presentation or publication. Reports on ancillary studies must be similarly approved. The only exception is oral presentations to local groups on the design of AREDS2, which do not need to be approved by the Operations Committee.

Chairpersons of writing teams, in submitting an AREDS2 report for publication, should include a copy of the approval letter from the Operations Committee.

3.6.3. Authorship

Primary AREDS2 Reports will be numbered serially and authored by the "Age-Related Eye Disease Study 2 (AREDS2) Research Group." For example:

Title: Progression to Advanced AMD.
AREDS 2 Report No. 5.

Author: Age-Related Eye Disease Study 2 (AREDS2) Research Group

Secondary AREDS2 Reports will also be numbered, but the members of the writing team and the AREDS2 Study Research Group will author them. For example:

Title: Changes in Visual Acuity in Persons Who Progress to Advanced AMD.
AREDS 2 Report No. 8.

Authors: Jefferson KL, Madison NO, Polk QR and Age-Related Eye Disease Study 2 (AREDS2) Research Group

3.6.4. Acknowledgements

Primary AREDS2 Reports will acknowledge the participation of the AREDS2 investigators, listed by center, who participated in AREDS2 for two or more years. Membership of major committees will also be acknowledged. Such acknowledgments are not required in Secondary AREDS2 Reports.

Primary and Secondary AREDS2 Reports will acknowledge support of the study by contracts from the National Eye Institute, National Institutes of Health.

3.7. Ancillary and Parallel Studies

Ancillary and parallel studies are investigations that are conducted concurrently with AREDS2 and involve AREDS2 participants. The AREDS2 Operations Committee (with assistance from the Executive Committee), the Data and Safety Monitoring Committee, and the National Eye Institute must approve these studies. Ancillary studies are AREDS2 studies: they involve participation by the AREDS2 Operations Committee and the Coordinating Center or Reading Center. Parallel studies are not AREDS2 studies: they do not involve participation by the AREDS2 Operations Committee, the Coordinating Center, or the Reading Center. Ancillary study protocols and summaries are kept in a separate volume available at the Coordinating Center.

3.7.1. Definitions

3.7.1.1. Ancillary studies

An ancillary study meets the following criteria:

(1) The research is conducted by AREDS2 investigators on AREDS2 participants.

(2) The goals of the study are consistent with AREDS2 objectives.

(3) The research requires supplementary clinical observations or procedures on AREDS2 participants.

(4) The AREDS2 Operations Committee, with NEI approval, has designated the study as an AREDS2 ancillary study, thus endorsing participation by (1) the AREDS2 Operations Committee and (2) the Coordinating Center or Reading Center in study development, conduct, data processing, and data analysis.

Ancillary studies by individual AREDS2 investigators or groups of AREDS2 investigators are encouraged because they can enhance the value of AREDS2 and increase the motivation and interest of investigators in AREDS2. However, to protect the integrity of AREDS2 and to prevent a drain on AREDS2 resources, all proposals for ancillary studies, whether or

not they involve the need for supplementary funds, must be submitted for approval to the Operations Committee and the DSMC.

3.7.1.2. Parallel studies

A parallel study meets the following criteria:

(1) The research is conducted on AREDS2 participants but does not need to be carried out by AREDS2 investigators or involve AMD or cataract. The research may have started before or after the inception of AREDS2.

(2) The AREDS2 Operations Committee, with NEI approval, has designated the study as a parallel study, thus precluding the participation in the study by the AREDS2 Operations Committee and by the Coordinating Center or the Reading Center.

3.7.2. Approval of Ancillary and Parallel Studies

3.7.2.1. Studies conducted by AREDS2 investigators.

The AREDS2 Operations Committee, with assistance from the Executive Committee, and the DSMC must approve an ancillary or parallel study conducted by AREDS2 investigators. Approval by the Operations Committee and the DSMC is contingent on local IRB approval. A copy of the local IRB approval must be sent to the Coordinating Center as well as subsequent approvals of modifications. Approval of ancillary and parallel studies by the AREDS2 Operations Committee and the DSMC is needed to assure that the studies will not:

- Adversely affect participant enrollment or cooperation
- Jeopardize the public reputation of AREDS2
- Result in premature release of AREDS2 outcome data
- Complicate the interpretation of AREDS2 results
- Substantially divert study resources at one or more Clinical Centers, the Reading Center, or the Coordinating Center.

Additionally, approval by the Operations Committee and DSMC of ancillary studies, but not of parallel studies, is needed to assure:

- The study's scientific merit
- The risks to participants do not outweigh potential benefits
- The participant's rights are not violated.

Such assurance is not needed for parallel studies because they are not AREDS2 studies: they do not involve participation by AREDS2 resource centers. The "approval" given to parallel studies by AREDS2 is limited to

indicating that the studies are not expected to jeopardize the scientific merit of AREDS2. Because AREDS2 plays no investigative role in parallel studies, AREDS2 has no responsibility for their scientific merit or ethical conduct.

AREDS2 investigators who wish to conduct an ancillary or parallel study should submit a proposal through the local Clinical Center PI to the Director of the Coordinating Center, who will distribute it to the Operations Committee. After review of the proposal for completeness and clarity by the Operations Committee, the Director of the Coordinating Center will summarize the committee's comments and forward the proposal and comments for review to the Executive Committee, with a copy of the comments to the proponent, and, if approved by the Executive committee, for review to the DSMC. If appropriate, the Director of the Coordinating Center, before forwarding the materials to the Executive Committee and DSMC, will give the applicant an opportunity to amplify, clarify, or withdraw the proposal. The Operations Committee and the DSMC will review amended proposals again. If additional funding is being sought through a peer review mechanism, a final review by the Operations Committee, Executive Committee and DSMC will be requested following notification of a funding award.

Proposals submitted for approval should:

- Specify whether the study is intended as an ancillary or parallel study (if the study is approved, the Operations Committee will determine the study's designation)
- Briefly describe the objectives, methods, and significance of the study and provide full details on procedures (e.g., examinations, tests, photographs, drawing of blood, additional questionnaires) to be carried out on participants. If intended as an ancillary study, the detail should be sufficient to evaluate the study's scientific merit, as in a National Institutes of Health grant application.
- State the numbers of AREDS2 and non-AREDS2 participants to be enrolled.
- State the amount of time by which AREDS2 clinic visits will be prolonged.
- State the number of non-AREDS2 clinic visits to be required for AREDS2 participants and their length of time.
- State whether participants will sign an informed consent and, if so, include a copy of the form.

3.7.3. Funding and Publication of Ancillary and Parallel Studies

3.7.3.1. Funding

NEI AREDS2 contract funds may not be used to support the conduct of ancillary or parallel studies. For additional funds, the investigator may wish

to submit a National Institutes of Health R01 grant application or to apply to another funding agency. If no additional funds are required, the investigator may proceed with the study as soon as the Operations Committee and DSMC approve it.

3.7.3.2. Publication

Abstracts and manuscripts authored by AREDS2 investigators. Abstracts and manuscripts of ancillary or parallel studies that are authored by AREDS2 investigators and are intended to be presented at scientific meetings or submitted for scientific publication must be reviewed by the AREDS2 Technical Group (Section 2.16). The AREDS2 Operations Committee with assistance from the Executive Committee will review these abstracts and manuscripts to assure that they do not compromise the conduct of AREDS2 or the interpretation of AREDS2 results.

For parallel studies, the review will also assure that the documents avoid mentioning AREDS2. The NEI may grant an exemption from full review.

Abstracts and manuscripts of parallel studies not authored by AREDS2 investigators. Because these abstracts and manuscripts are beyond the direct control of AREDS2, there is no official review role for AREDS2. Nevertheless, AREDS2 investigators should request their colleagues who are conducting these studies to avoid referring to AREDS2 in their publications and presentations.

3.8. Adverse Experience Reporting

Because this study is being conducted under an IND, any adverse experiences associated with the AREDS2 primary randomization supplements must be reported to the FDA. A description of Clinical Centers' requirements for reporting adverse experiences is provided in the AREDS2 Protocol.

3.8.1. Definition of the Adverse Experience

An adverse event by definition is “any unfavorable and unintended sign, symptom or disease temporally associated with the use of a medicinal product, whether or not considered related to the product”. Non-serious adverse events that are expected according to previous experience with the study supplement (as described in the protocol, consent materials, or any approved product labeling) can be collected in a routine manner using case report forms. If an expected event is observed to occur during the study at a greater frequency or severity than previously described, it becomes an unexpected event that requires further investigation, and it must be reported as an unexpected adverse event. Adverse events are defined in the AREDS2 Protocol Section 5.2.3. Events that require expedited reporting are all unexpected, serious, and related events. This

permits immediate investigation by the Coordinating Center and sponsor to determine the reporting requirements for regulatory authorities.

Progression or worsening of the medical condition under study, by itself, does not necessarily constitute an adverse event unless the change can be reasonably attributed to an action of the study supplements and not only to lack of efficacy. A hospitalization for an elective or cosmetic procedure unrelated to the medical condition under study, or a hospitalization for a procedure scheduled under this protocol, is excluded from this definition of an adverse event.

3.8.2. Adverse Experience Reporting and Management

FDA regulations describe conditions, responsibilities, and requirements related to the sponsorship of clinical trials for investigational drugs. These regulations mandate such responsibilities as monitoring for adherence with good clinical practice and record keeping; and documenting of supplement accountability and reporting of adverse experiences.

In AREDS2, the identification and assessment of an adverse experience related to a study intervention presents special problems for the investigator and company (IND sponsor) because many participants are likely to develop symptoms unrelated to the study supplements. Often this assessment cannot be made at the time of the adverse experience and requires a retrospective review of study data. The centralization of information on adverse experience makes possible a more accurate determination of the degree to which an adverse event was, in fact, study supplements-related.

The AREDS2 Adverse Experience Reporting (AER) system has been developed to ensure timely and accurate reporting of adverse experiences to comply with FDA regulations, to assess study risks, and, if necessary, disseminate information to the investigators and/or modify the study protocol. The AREDS2 AER system meets regulatory requirements and provides for evaluation of adverse experiences in the context of a multicenter clinical trial allowing for comparison of adverse experiences by intervention type. The adverse experience data will be used to prepare routine regulatory reports, study analyses, and other special reports.

4. EXAMINATION SCHEDULE

4.1. Overview of Schedule and Description of Participant Visits

This chapter presents the examination schedule for AREDS2 participants, highlighting the important features of each examination. The AREDS2 case report forms are available for printing from the Internet Data Entry System. Exhibit 4-1 provides an overview of visit requirements.

Candidates for AREDS2 are examined for eligibility during the Qualification Visit, at which time a participant ID is assigned and photographs of the retina are taken and forwarded to the Reading Center. Before randomization, participants take part in a run-in period for at least one month and not more than three months. During the run-in period, a participant receives a one-month supply of placebo lutein/zeaxanthin tablets and placebo DHA/EPA capsules and the actual AREDS-Type Supplement (ATS). Participants must sign an informed consent and authorization statement that describes their participation during the run-in period and authorizes the release of personal health information. If determined eligible for the study, participants are asked to return to the Clinical Center within three months for the Randomization Visit. Prior to randomization, a participant must sign a second informed consent statement (if approved by the IRB) that describes randomization and the responsibilities for continued participation in AREDS2.

Participants are randomized and assigned bottle codes when their eligibility information is entered into the AdvantageEDC^{SM} (Electronic Data Capture) System (described in detail in the AREDS2 Data Entry Guidelines). In-clinic follow-up visits occur annually post-randomization. Telephone contacts occur three and six months post-randomization and annually thereafter starting at 18 months post-randomization.

4.2. Qualification

The determination for eligibility during the Qualification Visit includes a comparison to the Inclusion and Exclusion criteria, a fundus examination and photographs, and optional refraction and visual acuity assessments. Participants who appear to be eligible during this visit will be assigned a participant ID.

4.2.1. Qualification Visit

Exhibit 4-1 summarizes the sequence of events during the Qualification Visit. A potential participant may be mailed the Participant Information Booklet and consent form(s) in advance of the visit. The Qualification Visit consists of the following examinations and procedures:

- Explanation of AREDS2 and a copy of the Participant Information Booklet (if not mailed ahead of the visit)
- Signing of the First Informed Consent and Authorization that describes participant rights during the qualification period and authorizes the release of personal health information to the study

- Complete medical and ocular history
- Manifest refraction – optional at Qualification
- Visual acuity examination via the Electronic Visual Acuity Tester (EVA) using the Electronic ETDRS (E-ETDRS) Visual Acuity Testing Protocol – optional at Qualification
- Dilated fundus examination
- Fundus photographs (3-standard field stereoscopic and red reflex).

Participants who still appear eligible will be given:

- Three bottles of run-in supplements (i.e., two bottles of placebo Study Supplements and also one bottle of AREDS type supplements if not a current smoker and if the participant has not smoked in the past year) and instructions for taking the supplements.
- A postcard to notify the clinic when the run-in supplements are stopped.
- A reminder to bring the run-in supplement bottles to the Randomization Visit.

If the Reading Center determines that the submitted photographs are not of suitable quality, retakes may be requested. Satisfactory photographs of the fundus are necessary prior to the Randomization Visit.

The assessments and procedures during the Qualification Visit are described below:

1. First Informed Consent: An informed consent statement that describes the participant's responsibilities during the run-in period must be obtained before the Qualification Visit examination begins. This consent process also includes a HIPAA authorization that is valid for the duration of the study.

2. Medical History: The interview addresses aspects of medical and ocular history that may indicate ineligibility. Medically related questions are found on the Qualification and portions of the Annual (In-clinic) Visit forms.

3. Visual Acuity and Refraction (optional at Qualification): A participant's current visual acuity and refraction are assessed using the electronic ETDRS Refraction and Visual Acuity Testing protocol. The AREDS2 Refraction and Visual Acuity Protocols are located on the study Web site at *http://www.areds2.org*.

4. Dilated Fundus Examination: A participant's ocular status (evaluated by ocular examination) is evaluated for conditions that may make the participant ineligible for the study. During this Qualification phase of AREDS2, clinical fellows may perform examinations to assess a potential participant's eligibility. Once the study visits begin, it becomes more critical that the AREDS2 ophthalmologist sees the participants. If the study

ophthalmologist is personally motivated to see the participant, talk to the participant, and answer any questions concerning the study, the participant is more likely to understand that he or she is a partner in the long-term study. Although AREDS2 ophthalmologists may change during the course of the study, it is certain that the fellows will only be in the clinic for a relatively short period of time during the follow-up period.

5. Fundus Photography: Good-quality color stereo photographs of the fundus of each eye are required for all participants before randomization. Imaging procedures involve only stereo color fundus photographs, taken at the 30°, 35° or 40° degree field-of-view, using color film or with a color digital capture system. Lens assessments are made using the fundus reflex photos taken as part of the standard set of images. Please refer to the *University of Wisconsin-Fundus Photograph Reading Center Imaging Procedures* for details on acceptable forms of photography.

6. Run-in Study Supplements: Participants are given:

   - AREDS2 Run-in Supplementation return postcard
   - Reminder for Next Visit card
   - Two bottles labeled RUN-IN SUPPLEMENT containing a one-month supply of 30 placebo lutein/zeaxanthin tablets and 60 placebo EPA/DHA capsules for daily intake
   - One bottle of Run-In Supplements containing 60 AREDS type supplement (ATS) capsules (Current smokers or former smokers who have quit during the past year will not receive the AREDS supplements.)
   - Instructions for taking the Run-in Supplements.

   Participants are instructed to take one tablet of lutein/zeaxanthin, two EPA/DHA capsules and, if a non-smoker, one ATS capsule each morning with food and one ATS capsule in the evening with food. Participants will be asked to refrain from taking multivitamins that contain the nutrients under study. Participants are requested to mail the AREDS2 Run-in Supplementation postcard to the Clinical Center when the Run-in Supplement is stopped and to bring all bottles when they return for the Randomization Visit. Adherence to the primary study Run-In Supplements will be assessed by estimated tablet/capsule counts if tablets/capsules remain, or date of completion of Run-In Supplement if the bottles are empty.

7. Qualification Visit Forms: Inclusion and exclusion criteria are captured on the Qualification form. Each of these criteria must be met in order to confirm eligibility. The interview and examination results are recorded on the Annual (In-clinic) Visit form.

4.2.2. Requalification Visit

Participants must be requalified if randomization does not occur within 3 months of the Qualification Visit. Requalification requires that the responses to each of the eligibility questions be verified and fundus photographs re-performed. The participant may be asked to return for the Randomization Visit as soon as the Reading Center's assessment of the photographs has been made.

4.3. Randomization Visit

Qualified participants are asked to return to the clinic within one to three months after the Qualification Visit. Randomization occurs only after the participant is confirmed to be eligible. Participants are considered eligible if they return to the clinic within three months following the Qualification Visit, have at least one study eye assessed by the Reading Center to be of adequate quality, consume at least 75 percent of the Run-In Supplements, and sign the randomization consent form.

The Electronic Data Capture system (AdvantageEDC^{SM}) assigns bottle numbers. The master randomization list for each center is maintained at the Coordinating Center. In the unlikely event of computer failure, randomization may be performed by calling the Coordinating Center Monday through Friday from 9:00 a.m. to 5:00 p.m. Eastern Time.

Study participants are assigned randomly to take one of the following Study Supplements on a daily basis: 1) Placebo, 2) Lutein/zeaxanthin, 3) DHA/EPA, or 4) Lutein/zeaxanthin and DHA/EPA. Participants are also offered an AREDS type supplement (ATS). Those who agree to take the AREDS formulation on a daily basis and consent to a second randomization are randomized to receive one of four alternative AREDS formulations in addition to the study supplements described above: 1) ATS, 2) ATS with no beta-carotene, 3) ATS with no beta-carotene and low zinc, or 4) ATS with low zinc. If the participant is a current smoker or a former smoker who has quit within the last year, he or she is randomized to one of the two arms without beta-carotene (Formulations 2 or 3). If a participant does not consent to randomization but wants to take the AREDS formulation, he or she is provided the supplements if he or she is not a current smoker or a former smoker who has quit within the last year.

All participants taking a daily multivitamin and/or multimineral supplement are asked to replace it with Centrum Silver®. This product is provided free-of-charge.

A brief description of the Randomization Visit is as follows:

1. Second Informed Consent: An informed consent statement that describes randomization and responsibilities for continued participation in AREDS2 must be obtained before the Randomization Visit begins. Consent is also obtained for the Nutritional Biochemistry study (at applicable clinics) and to receive a phone call about the Cognitive Function Study.

2. Determination of Adherence: Participants are asked to return the bottles of run-in supplements dispensed during the Qualification Visit. Adherence is assessed by estimating the number of tablets/capsules remaining of those dispensed. Participants who appear to have been unable or unwilling to take at least 75 percent of the run-in supplements are ineligible. The AdvantageEDC^{SM} System calculates adherence based on the number of days between the date the participant began the tablets/capsules and the day the run-in tablets/capsules were stopped and the estimated number of tablets/capsules remaining of those dispensed. Adjustments for days stopped due to illness or bottle misplacement are made. Tablets/capsules from partially full bottles must be disposed of in accordance with the medical waste procedures locally required.

3. Dilated Fundus Examination: A participant's ocular status (evaluated by ocular examination) is evaluated for conditions that may make the participant ineligible for the study.

4. Manifest Refraction and Visual Acuity: A participant's current visual acuity and refraction are assessed using the electronic ETDRS Refraction and Visual Acuity Testing protocol. The AREDS2 Refraction and Visual Acuity Protocols are located on the study Web site at www.areds2.org.

5. Blood Sample (selected clinics in the Nutritional Biochemistry Study): Blood samples for laboratory tests to be performed at the Central Laboratory are required for participating centers. See Chapter 13 for study procedures.

6. Food Frequency Questionnaire: A self-administered Harvard Dietary Assessment form may be mailed to the participant prior to the Randomization Visit. If not mailed, it will be completed by the participant during the Randomization Visit. At the end of each month, the Clinic Coordinator batches together all of the administered questionnaires and ships them to the Nutrition Coordinating Center.

7. Assignment of Bottle Number: A unique two-digit number sequence combined with a letter code identifying the formulation (e.g., LUT/ZEAyy where yy is a two-character value from 21 to 30) is used to minimize error. These codes are assigned by AdvantageEDC after the Randomization form is successfully key entered.

8. Distribution of Study Supplements and Centrum Silver®: Participants receive an adequate supply of AREDS2 supplements at the Randomization Visit to last until the next clinic visit and an AREDS2 bag to use for transporting the supplements. The bottles are identified with the assigned bottle number and are labeled "STUDY SUPPLEMENT." Participants currently supplementing with a multivitamin and/or multimineral formulation who wish to continue this practice are also provided Centrum Silver® and are reminded not to take other “non-study” multivitamins or extra supplements containing the nutrients used in AREDS2. Participants may continue to take nutritional supplements that are not

part of the randomized trial (e.g., calcium, herbal supplements), provided that these supplements are taken one to two hours before or after the study supplements. Participants are given (1) Instructions for taking the study supplements and (2) Reminder for Follow-up Visit. Every time study supplements are dispensed, the Coordinator must complete a Supplement Accountability Log for the Regulatory Binder.

Upon initial dispensation of the study supplements, participants should be educated as to the labeled storage instructions for each supplement. In general, supplements should be stored at room temperature in a dry place. An ideal place would be a kitchen pantry or a closet or cabinet that is not part of a bathroom. If the participant believes that the temperature or humidity of the storage environment may become compromised, even temporarily, the participant should consider storing the supplements elsewhere until the conditions of the primary storage area are corrected.

Additionally, some participants may have difficulty opening the child-resistant cap. A supply of non child-resistant caps will be provided upon request to the participant. Please note that the participant must first sign a waiver to receive the non child-resistant caps.

9. Family History Questionnaire: Participants are asked about family history of macular degeneration and cataract in order to understand more about the possible genetic factors that may be linked with these diseases.

4.4. Follow-Up Visits

The follow-up period begins on the day of randomization, and the timing of follow-up visits is based on that date. A telephone contact occurs three and six months post-randomization and annually thereafter starting at 18 months post-randomization. This telephone contact allows the participant to be asked about adverse events and compliance to study supplements. Participants return for an in-clinic follow-up examination at one year after randomization and annually thereafter.

After randomization, Clinic Coordinators may use the Protocol Calendar utility in the AdvantageEDC^{SM} system to view a listing of target dates and visit windows as well as procedures and/or forms to be completed at each participant visit. Every effort should be made to conduct each study visit as close to the target date as possible and before the expiration of the visit window.

If a visit is not conducted within the allowable time window, which is 1) two weeks on either side of the target date for the three and six months post randomization phone calls, and 2) three months on either side of the target date for all other visits and contacts, it will be designated as a **missed visit**. If any part of a visit is conducted within the allowable time window but the entire visit is not completed, it will be designated as an **incomplete visit**. AREDS2 source case report forms may be

completed manually or electronically. Data should be immediately keyed into the AdvantageEDC^{SM} following every visit.

4.4.1. Telephone Contact

Information to be collected by telephone includes adverse experiences, visual symptoms, hospitalization reports, protocol anomalies, and assessment of adherence.

4.4.2. In Clinic/Annual Visits

Information about important events occurring between study visits (e.g., ocular trauma, ocular surgery, or the development of major systemic diseases) is collected as part of the Annual (In-Clinic) Visit form.

A complete manifest refraction and visual acuity measurement using the electronic ETDRS Refraction and Visual Acuity Testing protocol are required at Annual Visits, as well as a dilated fundus examination, and fundus and red reflex photographs. If retakes are requested, Clinical Center staff should use their discretion as to whether a request to the participant for repeat photographs will jeopardize that participant's cooperation with the study. Other reports required at Annual Visits when applicable include hospitalization, adverse experience, study status, death, and protocol anomaly. Supplement bottles dispensed at the last study visit and that still contain study pills are collected and a new supply issued (empty bottles are not returned for collection); an adherence assessment is made by estimated pill count, and a Supplementation Record and Adherence Worksheet is completed. Returned bottles that have not been opened may be redispensed to the same participant, provided that they will not expire during the next visit window. Tablets/capsules from partially full bottles must be disposed of in accordance with the medical waste procedures locally required. Additional participant blood samples are required from centers participating in the Nutritional Biochemistry study. These samples are used to assess potential toxicity and adherence.

4.5. Missed Visits and Inactive Participants

If a participant misses a scheduled examination, Clinical Center staff contact the participant to reschedule the examination prior to the end of the visit window in which the examination may be completed. If the participant is unable to complete a scheduled Annual (In-clinic) follow-up examination within the acceptable visit window, a Missed Visit form for the visit is keyed to indicate that the information from the scheduled examination is unavailable. Missed telephone contacts are documented on the Telephone Contact form.

If an Annual Visit is missed, the clinic may attempt to reschedule the participant within the next scheduled visit as part of usual care. If the participant is contacted by telephone, any adverse experiences, hospitalizations, or other information available are recorded on the appropriate study forms.

For participants who have died, a Death Report form is completed and transmitted to the Coordinating Center along with a death certificate, if obtained.

For participants who are otherwise unable to visit their Clinical Center, see Section 5.4 regarding examination at other clinics or at home.

4.6. Hospitalization Discharge Summaries/Death Reports

Upon being notified of a hospitalization, key-enter the Hospitalization form within one week of notification. If the primary reason is cardiovascular in nature (see the Hospitalization Case Report Form for more details, obtain the hospital discharge summary and other supporting documents (if applicable) and mail all documents, along with a copy of this form, to the AREDS2 Coordinating Center. Before mailing, obscure the participant's name from all documents and add his or her Participant Study Number. The Coordinating Center collects the information and sends it to the Adjudication Committee on a monthly basis. The Adjudication Committee is comprised of two physicians who will review the information and determine if the hospitalization meets the definition of a cardiovascular outcome. The review and outcome are completed on the Adjudication form.

Upon being notified of a death, key-enter the Death Report within 24 hours of notification. Obtain the death certificate and mail a copy to the AREDS2 Coordinating Center. Before mailing, obscure the participant's name and add his or her Participant Study Number.

Exhibit 4-1. Scheduled Study Evaluation Flow sheet

	Qualification	Randomization	Telephone	Annual
Read and Sign Informed Consent	X	X		
General Assessment				
Inclusion/Exclusion Criteria	X			
Demographics	X			
Run-in Supplement Dispensing	X			
Study Supplement Dispensing/Accountability		X		X
Adverse Event Reports			X	X
Ophthalmic Assessment				
Best Corrected Visual Acuity using E-ETDRS Protocol	X-optional	X		X
Dilated Fundus Examination	X	X		X
Fundus Photographs	X			X
Other Assessments				
Family History Questionnaire		X		
Nutritional Biochemistry		X[1]		X[1]
Food Frequency Questionnaire		X		
Cognitive Function Telephone Battery		X[2]	X[2]	

[1] In selected clinics
[2] Administered via telephone within three months after randomization and every two years thereafter.

5. EXAMINATION PROCEDURES

The procedures for carrying out the examinations required in AREDS2 are described in this chapter. Required ocular examinations include dilated fundus exam, refraction and visual acuity measurements, and fundus photography. General characteristic assessment includes determination of past medical history. Risk factor assessments require the administration of a food frequency questionnaire, as well as collection of blood specimens from a sample of participants. Procedures for participant identification, masking, distribution and management of the study supplements, adherence assessment, and home visit examination are also described. Procedures for taking photographs are described in detail in the University of Wisconsin-Fundus Photograph Reading Center Imaging Procedures document. The schedule and description of participant visits in Chapter 4 outline the examinations required during each visit.

5.1. Dilated Fundus Examination

Ophthalmologists are required to perform dilated fundus exams during the Qualification Visit, Randomization Visit, and Annual Visits. This requires assessing a participant's drusen status, checking for pigmentary abnormalities, inquiring about surgical/non-systemic treatment for AMD and cataract surgery, and asking about enrollment in any studies that involve AMD or vision. Results are recorded in the appropriate fields on the Annual (In-Clinic) Visit form.

5.2. Refraction and Visual Acuity Measurements

Manifest refraction and visual acuity measurements must be performed during the Randomization Visit and Annual Visits. Participants' pupils should not be dilated at the time of visual acuity testing at any study visit. Pinhole acuity will not be tested as part of AREDS2.

Visual acuity testing is being performed with the Electronic Visual Acuity Tester (EVA) using a protocol called the Electronic ETDRS (E-ETDRS) Visual Acuity Testing Protocol. Documents describing the EVA system, the visual acuity and refraction protocols and certification procedures are available on the AREDS2 Web site at www.areds2.org. If the EVA is malfunctioning, manual ETDRS charts may be substituted for the assessment.

Record refraction and visual acuity data in the appropriate fields on the Annual (In-clinic) Visit form.

5.3. Fundus Photography

Good-quality color stereo photographs of the fundus of each eye are required for all participants at the Qualification Visit and at Annual Visits. Imaging procedures involve only stereo color fundus photographs, taken at the 30°, 35° or 40° degree field-of-view, using color film or with a color digital capture system. Lens assessments are made using the fundus reflex photos taken as part of the standard set of images. A Reading

Center Procedures manual detailing fundus photography procedures and the certification process required of AREDS2 photographers is available on the Reading Center's Web site.

5.4. Adverse Event Reporting Procedures

Each clinical site is responsible for reporting all adverse events that occur to AREDS2 participants, regardless of whether or not the adverse event is related to the study supplements or procedures. Reporting requirements and definitions are detailed in section 5.2 of the study protocol.

5.5. Procedures for Non-AREDS2 Participant Visits

During the course of AREDS2 some participants may relocate, become incapacitated, or experience other events that may make it difficult or impossible for them to visit the Clinical Center where they were enrolled for scheduled study visits. At a minimum, participants should be encouraged to be seen in the Clinical Center for the scheduled annual visits.

5.5.1. Participants Relocating Away from the Clinical Center

Whenever possible, clinic staff should attempt to have the participant return to the Clinical Center for the scheduled annual visits. Participants willing to fly to the clinic at their own expense should be informed that the airlines will not necessarily discount fares for persons traveling for medically-related reasons; however, some airlines will waive some, or all, restrictions. These might include, but are not limited to not requiring a Saturday night stay to be entitled to a less expensive fare, and allowing tickets to be purchased at any time prior to the flight at a fare equivalent to that of advance-purchase fares.

Some of the major airlines have representatives available to assist with special arrangements for persons traveling on medically related business. Clinic staff should contact the carriers serving their area for more information.

Participants that relocate may be reluctant or unable to travel long distances to the clinic that originally enrolled/randomized them; however, they may be willing to be seen at another AREDS2 Clinical Center if that clinic is closer or easier for them to reach. The participant should then be transferred from the original AREDS2 clinic to the Clinical Center where they will be followed.

When it is not possible for a participant to be followed at an AREDS2 Clinical Center, clinic staff should attempt to make arrangements for that participant to be seen by an area eye care specialist. The AREDS2 clinic staff should supply the physician with a copy of the AREDS2 Annual Follow-up forms prior to the date of the visit, with the appropriate sections highlighted for completion, and a self-addressed, stamped, return envelope. The eye care specialist should attempt to gather the following information:

- Visual acuity
- Clinical assessment of the retina
- Fundus photographs
- Red-reflex photographs

5.5.2. Home Visits

In the event that a participant is unable to be followed at an AREDS2 Clinical Center or other eye care specialist, he/she may give permission to be visited at home by an AREDS2 ophthalmologist and/or coordinator from the clinic. At the least, a visual acuity and refraction might be obtained utilizing either the ETDRS Charts R, 1, and 2, and a trial lens set, or some other, more portable, device for measuring visual acuity. In the event that an ophthalmologist participates in the home visit, it is expected that some assessment of retinal status might be obtained utilizing an ophthalmoscope. Other procedures might be undertaken at such a visit; however this would be dependent upon staff and instrumentation availability, in addition to the ability of the participant to tolerate the procedure(s).

5.6. End-of-Trial Clinic/Home Visit Guidelines

Home visits are a means for obtaining study data before the end of the clinical trial from participants who do not now come to the clinic, but who still live in the clinic area and with whom the study coordinator maintains some contact. The primary goal is for participants to come to the clinic for examination, and contacts by the coordinator including a visit to the home should have that as first priority. The coordinator knows her/his participants and should exercise judgment regarding participant cooperation and rapport. The home visit should be done by a team of two persons for security and to facilitate visual acuity examination in the home environment.

5.6.1. Clinic Visits

If in the coordinator's judgment it is reasonably likely that a participant will cooperate with at least one of the listed examination procedures once in the clinic, then the coordinator may provide for the necessary transport and other assistance. The clinic procedures for end-of-trial visit in priority order are:

- Visual acuity (with refraction if possible)
- Retinal photography

If participant refuses further follow-up:

- Collect study supplements
- Obtain address to which final trial information should be sent

Photography may not be done if dilation is refused or contraindicated. If photography cannot be done, clinical evaluation of the retina and lens should be

attempted, without dilation if necessary. Collection of other data should be done with discretion to maintain cooperation. Clinics are authorized to provide assistance to participants going to the clinic.

5.6.2. Home Visits

Whether or not the home visit leads to scheduling a clinic visit, visual acuity procedures should be done in the home. ETDRS Visual Acuity Charts 1 and 2 should be used, in a bright light setting if possible. Avoid direct light reflection off the surface of the chart toward the participant's eyes. The test should be done using as correction the refraction of record, updated at the visit if possible. Update refraction by using a trial frame and lenses for the refraction of record, and a limited set (at least the previous refraction and 1D increments of plus and minus sphere and cylinder) of trial lenses. Use Chart R. If a four-meter lane cannot be used, the test may be done at 3.2, 2.5, or 2 meters and the distance noted on the data form.

If the home visit team includes an ophthalmologist, clinical evaluation of the retina may be done through undilated or preferably dilated pupils of willing participants, by direct or indirect ophthalmoscopy, or hand-held slit illuminator. Collection of other data, including by interview (i.e., follow-up interview, annual visit form information), should be done with discretion to maintain cooperation.

Materials to bring for a home visit:

- Protocol anomaly and other appropriate study forms
- ETDRS charts 1, 2, and R; tape measure
- Equipment for ophthalmic exam (including dilating drops), if applicable
- Supplements and supplement accountability log, if applicable
- Trial frame and appropriate lenses for refraction.

6. CERTIFICATION PROCEDURES

6.1. Introduction

This chapter describes the AREDS2 activities that require certification and the procedures for obtaining certification. The AREDS2 Training and Certification Committee is responsible for approving all certification processes. Members of this committee include representatives from the NEI, the Coordinating Center, the Reading Center and the CDC.

6.1.1. Activities Requiring Certification

Certification is required for the following AREDS2 activities:

- Visual acuity (ETDRS and E-ETDRS) and Refraction
- Photography
- Blood drawing
- AdvantageEDC^{SM}
- Cognitive Function Telephone Battery (Coordinating Center staff activity)

One level of certification is required for each activity. Training manuals or tutorials are available on the AREDS2 Web site for each of the listed activities, and training is also available for administration and completion of case report forms.

A certification number is issued by the Coordinating Center when certification has been achieved in an activity. This number must be recorded on AREDS2 worksheets and case report forms and is used to document that certified study personnel performed a procedure. The number identifies the user so that the AdvantageEDCSM System may be accessed. A summary of a person's certification status in each activity (Exhibit 6-1) is provided to the individual upon request.

6.1.2. Obtaining and Maintaining Certification

The Clinic Coordinator should notify the Coordinating Center when a staff member wishes to begin the certification process. For electronic security and tracking purposes, the Clinic Coordinator must also notify the Coordinating Center of certified staff who are no longer with the study.

Certification is automatically renewed every year on January 1st, based on evidence that the staff member has performed the certified activity at least six times during the past calendar year on any NEI-sponsored trial. Around October 1st of each year, the Protocol Monitor notifies persons for whom the Coordinating Center lacks evidence that the minimum frequency requirement has been satisfied for the upcoming certification expiration.

To satisfy the maintenance requirement for AREDS2 examinations, the examinations need not be performed on AREDS2 participants. Upon notification,

Coordinating Center staff verify with other NEI-sponsored trials the conduct of certified activities needing renewal for identified study personnel.

To maintain certification, Clinical Center personnel must comply with the foregoing requirements and with the requirements that are specified for each activity in Sections 6.2 through 6.6. Individuals who allow their certification to lapse and wish to regain full certification will need to re-complete the certification curriculum for the activity(s) in question.

6.1.3. Decertification

Decertification may be necessary for a certified individual who performs in a substandard manner. A substandard performance may be determined on the basis of (a) review of forms submitted, (b) observation of performance during site visits, or (c) recommendation of the PI at the certified individual's Clinical Center. If there is concern about a person's performance on a certifiable activity, he/she will receive retraining. The issue will be reevaluated after three months, and if problems persist, the circumstances will be reported to the Operations Committee, and a final decision regarding certification status will be made by the study leadership.

6.2. Certification for Refraction and Visual Acuity

Certification is awarded separately for refraction and visual acuity and for the electronic and manual methods of collecting these data. The certification process and lane requirements are detailed in the AREDS2 Refraction and Visual Acuity Training Protocols provided on the study Web site at http://www.areds2.org/. The forms necessary to complete certification are also provided on the Web site.

6.3. Certification for Photography

The AREDS2 Reading Center (UW-FPRC) conducts a program to certify photographers and their digital camera system (in cases where sites elect to perform digital color imaging), and monitors the quality of their work. Certification status is established separately for photographers and digital systems used to perform color imaging. Sites are encouraged to perform color imaging using a suitable digital camera system, however 35mm color slides may be taken when sites do not have a suitable digital camera system.

Photographers taking images for AREDS2 must be certified for the Modified 3-Standard Field color imaging procedure, before submitting actual participant images. Only Reading Center certified photographers are allowed to take baseline (Qualification Visit) images, unless an exception to this rule is granted (on a case-by-case basis) by the study sponsor. A Clinical Center shall not be activated to begin participant enrollment if the site does not have a certified photographer available to take the baseline images. Only under extraordinary circumstances may follow-up visit images be taken by an uncertified photographer.

Photographers using digital color camera systems - Photographers already certified by the Reading Center to take digital color images for another study (provided their images are of good quality) using the identical Modified 3-Standard Field or Modified 7-Standard Field imaging procedures, are eligible for "automatic certification" and should submit to the Reading Center, a completed photographer certification request form along with a completed digital system certification request form found on the UW-FPRC Web site: *http://eyephoto.ophth.wisc.edu*. Photographers who are certified by the Reading Center but having difficulty maintaining good digital image quality may be asked to submit an abbreviated set of images before certification is granted.

Photographers joining the study who have not been previously certified by the Reading Center should submit digital color images of four eyes (two right eyes and two left eyes) taken using the Modified 3-Field imaging procedure. The color images may be taken of participants in whom imaging is being carried out for clinical purposes or in normal volunteers. These photographers should submit to the Reading Center a completed photographer certification request form along with a completed digital system certification request form. Photographers are certified if all of these are of good quality and were taken using a certified digital system.

Certification of digital color camera systems - The Reading Center accepts digital color images, provided they are of good color quality and taken with a 3-mega-pixel or higher resolution camera. Images should be uncompressed (preferred) though lossless compression is acceptable. Each digital system used for the study must be certified by the Reading Center before beginning study participant imaging. Certification begins with submission of a digital system certification request form. Each system requires a separate form and certification. If the digital system is new to the Reading Center, or if the system is certified with the Reading Center but hardware or software changes have been made since the system was last certified, the system must be evaluated. System specific procedures for certification and recertification are available on-line at http://eyephoto.ophth.wisc.edu.

Photographers using 35mm color slide film - Photographers electing to perform color imaging using 35mm film and who were previously certified in AREDS are eligible for "automatic certification". They should submit to the Reading Center a completed photographer certification request form. Photographers currently certified by the Reading Center and actively taking images for another study (provided their images are of good quality) using the identical Modified 3-Standard Field or Modified 7-Standard Field imaging procedures are also eligible for "automatic certification" and should submit to the Reading Center a completed photographer certification request form. Photographers who are currently certified by the Reading Center but having difficulty maintaining good image quality may be asked to submit an abbreviated set of images before certification is granted. Reading Center certification staff make this determination during the certification process following review of previous image quality.

Photographers should carefully monitor the quality of their images throughout the study. Their work is also monitored in detail at the Reading Center. Photographers whose

images are consistently of good quality (overall grade of Fair or better for at least 75 percent of a series of 20 or more eyes) maintain certification. If image quality for a certified photographer falls below this criterion, certification reverts to a "provisional" status. Such photographers are monitored closely and appropriate suggestions are made for improvement. If a photographer has problems that cannot be resolved in a reasonable period of time, a special site visit by a consulting AREDS2 photographer may be made to determine the nature of the problems and to identify possible solutions.

Event Fluorescein Angiogram and OCTs_ - In instances where a participant progresses to an advanced AMD event, the Reading Center should review any fluorescein angiograms or OCT scans that might be taken at the sites as part of the routine standard of care. The Reading Center has recommended imaging procedures for fluorescein angiography and OCT available on their Web site at http://eyephoto.ophth.wisc.edu (follow the AREDS2 link).

Imaging by Non-Certified Personnel - On rare occasions during **follow-up** visits, when a certified photographer is not available to take the images, an uncertified photographer familiar with the procedures may take the images. The uncertified photographer should review the imaging procedures before performing imaging to be certain he or she understands and follows the procedures. The name of the uncertified photographer should be entered on the image page labels or on the CD label when images are "burned" to CD. The Coordinating Center closely monitors the use of uncertified personnel to assure that it occurs only rarely.

6.4. Certification for Nutritional Biochemistry Study

Centers participating in the Nutritional Biochemistry Study must have personnel certified in blood drawing procedures. Staff are thoroughly trained in each aspect of the specimen collection, processing, and shipping procedures. Training takes place at an initial training session held at the Technical Group meeting and annually thereafter. Clinical Centers participating in the Nutritional Biochemistry Study are encouraged to have at least two AREDS2 staff members trained and certified in these procedures. It is expected that this helps to minimize any inconvenience to participants should a certified staff member be absent from the clinic. Training of personnel not attending this training session takes place at the Clinical Center by a staff member trained at the Central Laboratory training session and certified in conjunction with staff from the Central Laboratory. Members of the *Training and Certification Committee* who attended at least one of the annual Central Laboratory training sessions may assist in the training and certification process.

Following completion of training, clinic personnel applying for certification must take blood samples from two persons who are not AREDS2 participants. The samples should then be prepared according to the procedures outlined in Chapter 13 and shipped to the Central Laboratory. When the shipment is received at the Central Laboratory, the Logistics Supervisor reviews the documentation and evaluates the specimens. If the samples and documentation meet the protocol requirements, the

Protocol Monitor at the Coordinating Center is notified. The Protocol Monitor notifies the candidate that certification has been obtained.

6.5. Certification for the AdvantageEDC^{SM} Production System

Only personnel authorized by the Coordinating Center may use the AdvantageEDC^{SM} Production System. The Coordinating Center assigns a user name and temporary password that allows access to the AdvantageEDC^{SM} Production System. All users must establish a personalized password upon logging into the AdvantageEDC^{SM} Production System for the first time.

6.5.1. Users Training Practicum

All AREDS2 users are certified following successful completion of the training practicum. The training practicum consists of a series of case report forms completed with sample data. The candidate must be able to successfully enroll a study participant and utilize the assigned participant study number to enter subsequent case report forms in the AdvantageEDC^{SM} Training System. The training practicum for the CDC and the Reading Center personnel consists of case report forms to be used by these particular entities (e.g., enrollment is not required).

The Data Manager reviews the data for 100 percent accuracy, and the candidate is notified if any further demonstrations are required or if AdvantageEDC^{SM} certification is granted.

6.6. Certification for Cognitive Function Telephone Battery

Interviewers employed by the Coordinating Center are trained and certified to administer and score the Cognitive Function Telephone Battery. Interviewers are trained to follow a standardized script and on the administration techniques of each instrument. Two audiotaped mock interviews with non-AREDS2 participants are submitted to the Coordinating Center for review. Feedback is provided, and if necessary, additional mock interviews are requested. Certification is granted when errors are minimal.

6.7. Examination by Non-Certified Personnel

When an AREDS2 participant is scheduled for a study visit but no certified personnel are available to perform a task, then personnel familiar with the proper performance of the activity, but not certified, are permitted to perform the task. Tasks that may be performed by uncertified personnel in an emergency are fundus photography, refraction, visual acuity, and forms administration. Under no circumstances should uncertified personnel perform procedures relating to blood drawing/processing for the Central Laboratory, or enter data into the AdvantageEDC^{SM} system.

If an uncertified person performs a task, a certification number consisting of the clinic 3-digit number (e.g., 001, 002, etc.) followed by the digits "99" is recorded on all relevant forms. The Coordinating Center closely monitors the use of uncertified personnel to assure that it occurs only rarely.

6.8. Food Frequency Questionnaire Techniques

Certification is not applicable for the AREDS2 Food Frequency Questionnaire - the Harvard Dietary Assessment Questionnaire. The Harvard Dietary Assessment Questionnaire is a simple, self-administered questionnaire useful for service and research purposes that has the potential to provide important nutritional information for program planning, research, education, and intervention purposes.
These are recommended techniques to accurately complete the questionnaire.

The participant's ID number and the current date are recorded (top of page 1) before the questionnaire is given to the participant.

Distribution

Make sure the appropriate ID number and date have been filled in (top of page 1). Hand the questionnaire to the participant along with a number 2 pencil and state the following:

"Please complete this questionnaire. It takes most people between 20 and 30 minutes to complete. If you need help completing it please let me know. One of our staff can help. When you have finished the questionnaire please return it to me."

Responses to Questions Participants May Ask

Q: Can I use my own pen or pencil?
A: No. Please use the No. 2 pencil provided.

Q: Can I put a check mark or an "x" for my answer instead of darkening the circles?
A: No. Please darken the circles.

Q: I made a mistake and I want to change my answer. What do I do? Should I cross out the incorrect answer?
A: Please erase the incorrect answer.

Q: I'm not real sure how often I eat [ice cream, cream cheese, etc.]. What should I do?
A: Answer each question as best you can. Estimate if you are not sure. A guess is better than leaving a blank.

Q: I can't remember the [brand name of the multi-vitamin I use, brands of margarine I use, etc.]. What should I do?
A: Leave that answer blank.

Collection

Review the questionnaire page by page to identify pages left completely blank. Ask participants to complete any blank pages found.

Note: Some questions may be left blank due to the skip pattern in the questionnaire or the design of the questionnaire. Thus, it will not be unusual to see some responses left blank.

If you notice that the participant has completed the entire questionnaire using a pen instead of a pencil or using check marks or 'x' marks instead of darkened circles, leave as is. When the University of Minnesota Nutrition Coordinating Center (NCC) cleans and codes the questionnaires these types of errors will be corrected.

If you have any information you would like to relay to NCC, please do not write directly on the questionnaire. Stray marks on the questionnaire cause optical scanning problems. Instead, paperclip a note to the questionnaire (no staples please).

At the end of the month, all questionnaires received should be batched and logged within the Harvard Dietary Assessment tracking log located on the Main Menu page of the Advantage EDC system. Once logged, questionnaires should be mailed to the NCC at the following address:

Attn: Mary Stevens
Nutrition Coordinating Center
University of Minnesota
1300 South Second Street, Suite 300
Minneapolis, MN 55454

Exhibit 6-1. AREDS2 CERTIFICATION SUMMARY

PERSON CERTIFIED_____

AREDS2 CLINIC NAME AND NUMBER_____

CERTIFICATION TYPE	DATE CERTIFIED
REFRACTION	
VISUAL ACUITY MEASUREMENT	
BLOOD SPECIMENS	
AdvantageEDC^{SM} DATA ENTRY	
PHOTOGRAPHY	

_____ _____
Coordinating Center Protocol Monitor Date

7. DESCRIPTION OF INTERVENTION

7.1. Introduction

At the Qualification Visit, eligible participants receive a one-month's supply (i.e., two bottles) of placebo tablets/capsules. The bottle holding the placebo DHA/EPA capsules is 150 cc's and contains 60 capsules. The bottle of placebo lutein/zeaxanthin tablets is 100 cc's and contains 30 tablets. A third bottle, one that is 150 cc's and contains 60 capsules of AREDS formulation, is provided to all participants who are not smokers or former smokers who have quit for at least one year.

The design of AREDS2 includes a primary study of 4,000 participants with age-related macular changes who are randomized with 25 percent probability to each of four treatments: placebo, lutein/zeaxanthin (10mg/2mg), DHA/EPA (1gm), and lutein/zeaxanthin plus DHA/EPA. Participants are offered the AREDS formulation. Those who agree to take the AREDS formulation and consent to a second randomization are randomized to receive one of four alternative AREDS formulations in addition to the study supplements described above:

Formulations	Vitamin C	Vitamin E	Beta Carotene	Zinc Oxide	Cupric Oxide
1	500 mg	400 IU	15 mg	80 mg	2 mg
2	500 mg	400 IU	0 mg	80 mg	2 mg
3	500 mg	400 IU	0	25 mg	2 mg
4	500 mg	400 IU	15 mg	25 mg	2 mg

The bottles are labeled as illustrated in Exhibit 7-1. Each bottle has an expiration date. The AREDS2 Coordinating Center randomly assigns codes to each bottle to correspond to the type of supplement. A description of the design of the clinical trial is provided in the AREDS2 Protocol. A full description of the intervention and expected toxicities are provided below.

7.2. Description of Study Supplements

AREDS2 investigators are studying the effects of oral supplementation of high doses of macular xanthophylls, lutein and zeaxanthin and omega-3 long chain polyunsaturated fatty acids (LCPUFAs), specifically DHA and EPA, for the treatment of AMD and cataract. The study supplements are Kosher.

The ingredient list for the DSM lutein/zeaxanthin tablets includes:

- Lutein --211.5 mg
- Zeaxanthin --43.1 mg
- Microcrystalline cellulose --232.9 mg
- Magnesium Stearate --2.5 mg
- Croscarmellose Sodium --10.0 mg

The coating ingredients for the lutein/zeaxanthin pills include:

- Titanium dioxide --0.44 mg
- FD & C Red #40 Lake --3.11 mg
- FD & C Yellow #6 Lake --0.22 mg
- Methocel E5--6.67 mg
- Carbo Wax --0.67 mg
- Triacetin --0.02 mg
- Carnuba Wax --0.03 mg
- Talc --20.3 mg

The ingredient list for the Fish Oil /Placebo Oil Softgel Capsules includes:

- ROPUFA '75' N3 EE --853 mg contains 614 mg Omega-3 that provides 500mg EPA+DHA per capsule
- Gelatin—252.2 mg
- Corn Oil—147.0 mg
- Glycerin—140.1 mg
- Carob Liquid—19.6 mg
- Titanium Dioxide—2.10 mg
- Purified Water—46.0 mg

The placebo tablets and soft-gel capsules supplied for the run-in period are labeled RUN-IN SUPPLEMENTS. The primary study supplement tablets and capsules supplied for the clinical trial (placebo, lutein/zeaxanthin, DHA/EPA, and the combination of lutein/zeaxanthin and DHA/EPA) are labeled STUDY SUPPLEMENTS. The study supplements are identical in appearance, size, smell, and taste to their placebo counterparts. The formulation of the tablets and capsules is provided below in terms of the recommended dietary allowance (RDA).

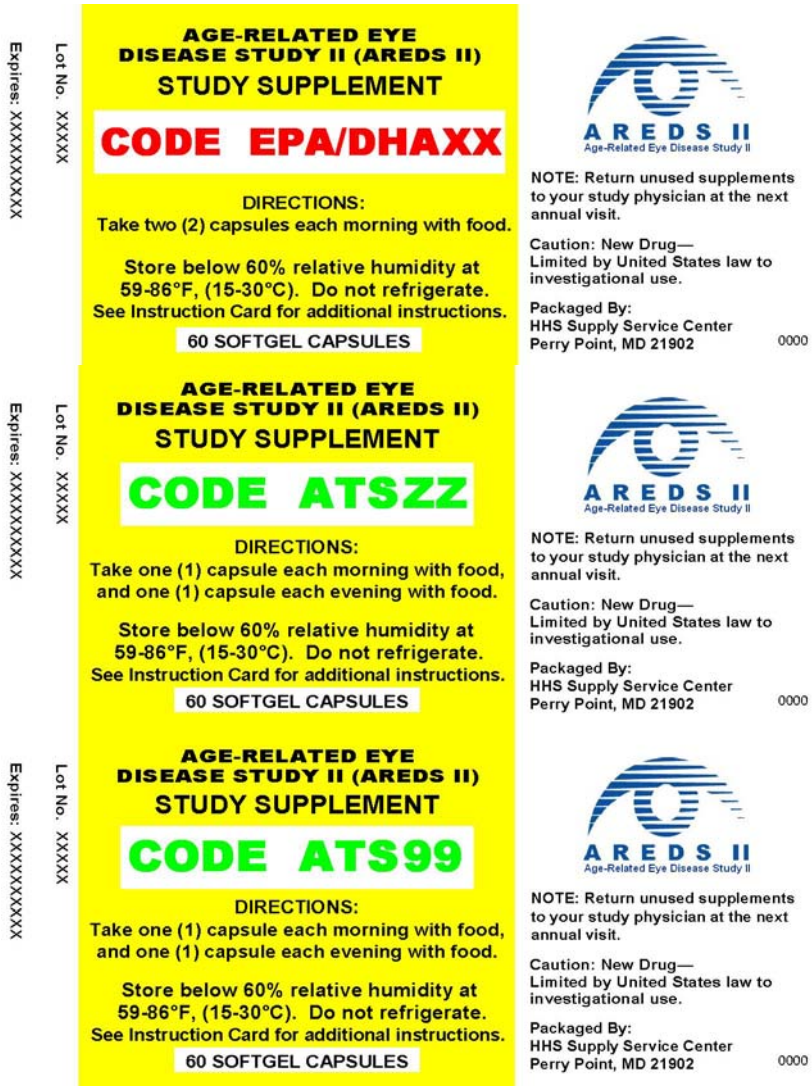
Placebo (Run-in Supplements and Study Supplements): Contains no vitamins or minerals and no active substances. The DHA/EPA placebo contains Fish Oil Essence with 0.0027mg of DHA/EPA per 3mg softgel and the source of the fish oil essence is salmon. The source of the lemon flavoring is 2.5 mg of Lemon Essence.

In addition, participants who wish to take a multivitamin and mineral supplement are provided Centrum Silver® free of charge.

Exhibit 7-1 AREDS2 Bottle Labels

RUN-IN Supplements

AREDS2 Randomization Supplements



7.3. Expected Toxicities

The primary study supplements do not have a RDA, but the ATS have much higher doses of vitamins and minerals than the RDA. The vitamin and mineral components used in the AREDS2 tablets/capsules are available in various concentrations in other marketed, over-the-counter, multivitamin and mineral products. Although these doses are believed to be safe, even in addition to a multivitamin and mineral tablet, it is possible that some participants may experience adverse effects. The pills do not contain lactose. A summary of possible side effects associated with each of the supplements is provided below:

7.3.1. Placebo

No adverse experiences are expected with either the placebo supplied for the run-in period (Run-in Supplements) or the placebo supplied for the clinical trial

(Study Supplements). The placebo has no nutrients or any other active substances.

7.3.2. Lutein/zeaxanthin

There is no known toxicity of lutein. Little is known about the toxicity of zeaxanthin.

7.3.3. DHA/EPA

Supplements containing DHA and EPA may be associated with side effects such as loose stool, abdominal discomfort, and unpleasant belching. In addition, they may slightly prolong bleeding time.

7.3.4. Antioxidants

Vitamin C. No side effects are expected at the dose that will be given. Persons who have had oxalate kidney stones or hemochromatosis (an iron disorder) are not eligible to participate in AREDS2 and should not take this large a dose.

Vitamin E. A meta analysis of 19 clinical trials that tested vitamin E found that high-dosage (≥ 400 International Unit) supplementation with vitamin E may increase all-cause mortality.[1] Of the 19 studies, AREDS and two other trials evaluated dosages of about 400 IU/d of vitamin E. Restricting data to these three studies, the group taking vitamin E was slightly more likely to be living after five years (801 deaths of 7564 persons in vitamin E group and 806 deaths of 7598 persons in the placebo group). A review of the mortality experience in AREDS showed that those taking the AREDS formulation (combination of antioxidants and zinc) had a 14% reduction in mortality risk after an average of 6.5 years of supplementation compared to placebo. No other adverse events are expected.

In other clinical studies of vitamin E, participants supplementing with E were observed to have greater risk of hemorrhagic strokes that those not taking vitamin E. They fewer other kinds of strokes and overall, there was no increase in strokes. In people who do not have enough vitamin K, high doses of vitamin E may slow the time it takes for blood to clot. Persons taking prescription blood-thinning medications should check with their medical doctor before enrolling in AREDS2.

Beta-carotene. In doses much higher than the study dosage some people find that their skin turns yellowish. The skin returns to a normal color when the dose is lowered. No problems are expected at the dosage to be used in this study. The effects on lung cancer of beta-carotene at a somewhat higher dose than used in AREDS2 was also tested in the Finnish study of 29,000 male cigarette smokers.[2] The study found no benefits and an increase in lung cancer for people taking beta-carotene. The number of new lung cancer cases per 1,000 smokers per year was about 6 for those taking beta-carotene and about 5 for those not

taking beta-carotene. There was also a somewhat smaller increase in the risk of heart disease and of total mortality in those taking beta-carotene.

The results of the Beta-Carotene and Retinol Efficiency Trial (CARET)[3], which followed over 14,000 current and former heavy smokers, also found an increased risk of lung cancer and mortality for participants taking beta-carotene at a higher dose than used in AREDS2. After four years of follow-up in CARET, participants taking beta-carotene (along with vitamin A) had 24 percent more lung cancers and 17 percent more deaths than the control group.

In contrast, the Physician's Health Study[4], which followed over 22,000 male physicians (11 percent of whom were smokers) for 12 years, found no increased risk of cancer, cardiovascular disease, or mortality for participants taking beta-carotene at a much higher dose than used in the Finnish Study or CARET.

In the Age-Related Eye Disease Study (AREDS) participants assigned to beta-carotene reported yellowing of the skin, but, overall, participants reported few side effects.

7.3.5. Zinc and Copper

Copper deficiency leading to anemia has been reported when high levels of zinc are given. For this reason, some copper is added to the zinc plus antioxidants and zinc tablets. It is expected that this addition will prevent anemia. A decrease in high-density lipoproteins (HDL), the beneficial or "good" part of the cholesterol measurement, in the blood has been observed in some persons taking about twice the dose of zinc being used in AREDS2. Some zinc compounds can cause stomach upset, but the form chosen for AREDS2 is less likely to do this. In AREDS there was a slight (2.5 percent) increase in the rate of urinary tract problems in participants who took the zinc formulation.

7.4. References

1. Miller ER, Pastor-Barriuso R, Darshan D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med*. 2005;143:37-46.

2. The Alpha-Tocopherol, Beta-Carotene Prevention Study Group: The effect of vitamin E and beta-carotene on the incidence of lung cancer and other cancers in male smokers. N Engl J Med 330:1029-35, 1994.

3. Omenn GS, Goodman GE, Thornquist MD, et al.: Effects of a combination of beta-carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 334:1150-5, 1996.

4. Hennekens CH, Buring JE, Manson JE, et al.: Lack of effect of long-term supplementation with beta-carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 334:1145-9, 1996.

8. DATA ANALYSIS AND REPORTING

The AREDS2 analysis plan is designed to carefully monitor participant accrual, data quality and timeliness, participant eligibility rates, adverse reactions, visual function parameters, and other outcomes. While detailed analyses of individual studies will be performed periodically, the overall progress of the study will be monitored continuously. Technical and administrative reporting requirements for AREDS2 consist of both interim and final reports on the scientific efforts. A complete discussion of the outcome variables and the sample size and statistical considerations is provided in the AREDS2 Protocol.

8.1. Analysis Plan

Data analysis in AREDS2 is designed to meet the study's scientific and regulatory objectives, which in general terms are to assess the efficacy and safety of lutein/zeaxanthin and/or omega-3 LCPUFA intake on the development and progression of age-related macular degeneration. The analysis plan for AREDS2 will be developed by the statisticians at the Coordinating Center, in collaboration with the Data and Safety Monitoring Committee (DSMC) and the Analysis Planning Committee. An initial plan will be designed during the first year of the study and subsequent modifications will be made as the study matures. Key aspects of the plan will include monitoring of data quality, study progress, safety, and efficacy. Analyses will be scheduled to coincide with the annual planned meetings of the DSMC and the planned meetings of the Technical Group. Database assessments will be performed monthly by the Coordinating Center to evaluate the quality of the database. In addition to these planned analyses, the Coordinating Center will conduct various unplanned analyses precipitated by evolving project needs. Requests for such analyses will likely come from the DSMC and the Operations Committee, and may be suggested by the statisticians at the Coordinating Center.

8.2. Specification of Analysis Database

Prior to performing a scheduled analysis, the master file will be copied into an analysis file. This analysis file is date-stamped with a closure date to indicate the last day for which data were included. The master file will continue to incorporate new data from the Clinical Centers, while the analysis file is frozen. The closure date provides a reference with regard to the currency of the data on which the analyses are based. Typically, the choice of a date to close the file for analysis depends on the type and quantity of the analyses to be performed. Files will likely be closed two to three months prior to a scheduled meeting.

8.3. Reports for Publication

The Coordinating Center will work with the Analysis Planning Committee to prepare a proposed schedule of analyses for disseminating information resulting from AREDS2 to the scientific community. This schedule will be based on the maturity of the data and the study. Presentations on study methods and baseline data will be scheduled during and after the conclusion of the recruitment phase. The timing of the release of reports on outcome data will be based on the recommendation of the DSMC.

8.4. Data Integrity

The Principal Investigator and Clinical Coordinator of each Clinical Center are responsible for the integrity of the information entered in the AdvantageEDC^{SM} System and recorded on hardcopy AREDS2 case report forms, if applicable. Data quality is maintained through a variety of analyses that target anomalies, delinquent data, and key-entry errors. A part of this process is to analyze the frequency of errors according to type to determine if certain types of errors are recurrent. Modifications to the data forms are made if the same types of errors occur frequently among Clinical Centers. If errors are localized within a Clinical Center, steps are taken to resolve the problems by providing additional training for Clinical Center staff and/or modifying the case report forms. In addition, random audits of the data collected on the forms may be performed by checking for accuracy and completeness.

Any Clinical Center staff member who is concerned about potential data anomalies at the Clinical Center that may jeopardize the integrity of the AREDS2 database must immediately bring these concerns to the attention of a member of the AREDS2 Operations Committee.

8.5. Reporting

A variety of scientific and administrative reports will be prepared for AREDS2, such as:

- Adverse Experience Reports (AER) to the Food and Drug Administration, sponsor, and DSMC
- Monthly reports for the Operations Committee, summarizing Clinical Center adherence to the study protocol
- Periodic reports on protocol adherence for Executive Committee and Technical Group meetings
- Semiannual reports on protocol adherence, data quality, and outcome results for the DSMC
- Protocol violation reports for the AREDS2 Operations Committee
- Reports for scientific publication to be reviewed by the Editorial Committee.

8.5.1. Adverse Experience Reports Procedure

Each clinical site is responsible for reporting all adverse events that occur to AREDS2 participants enrolled at their site, regardless of relatedness to study

supplementation. Reporting of all adverse events is expected upon recognition through the AdvantageEDC^{SM} System. Details of adverse event reporting are provided in the AREDS2 Protocol.

8.5.2. Reports to Operations Committee

The Coordinating Center will submit to the Operations Committee reports summarizing Clinical Center adherence to the study protocol and recruitment activities. These reports will include results of Protocol Review Telephone Calls, Protocol monitoring Visits, Clinical Center database quality and timeliness, and protocol violations. In addition, reports for the Clinical Centers will be prepared to provide them with similar data. A newsletter will serve to update the clinics on the study enrollment, protocol modifications, all personnel changes, and will provide a forum for circulating answers to protocol questions from the clinics. The newsletter will also serve to promote interest in the study.

8.5.3. Reports to Executive Committee and Technical Group

Protocol adherence reports will be prepared for periodic Executive Committee and Technical Group meetings. Copies will be forwarded to the AREDS2 Project Officer. These reports provide operational data to the Clinical Centers and serve to evaluate study progress. Outcome information will not be provided.

8.5.4. Reports to the Data and Safety Monitoring Committee

A comprehensive report will be prepared and submitted semiannually to the DSMC. Specific requirements for these reports are defined by the DSMC. The reports are in the form of tables and graphic displays summarizing administrative, adverse experience, and other outcome data.

8.5.5. Protocol Violation Reports

Protocol violations will be monitored continuously. Such violations can occur for a variety of reasons, and many of these violations can be avoided, such as carelessness of a clinic to thoroughly screen a participant prior to entry, administrative errors in allocating dietary supplements, and missed visits due to inadequate participant/clinic communication. Timely identification of such problems can prevent future violations. All violations, regardless of cause, will be reported to the AREDS2 Project Officer, Study Chairperson, and Operations Committee.

8.5.6. Scientific Reports

After approval of a scientific report by the Analysis Planning Committee, the Coordinating Center's statisticians will assist AREDS2 investigators in preparing scientific publications. In collaborating with clinicians on publications, the statisticians can provide tabular and graphic presentations of data, as well as a description of the study methods and results.

9. QUALITY ENHANCEMENT

9.1. Introduction

The goal of the AREDS2 quality enhancement program is to maintain the integrity of the study data. The principles of multi-center clinical trials that govern the AREDS2 quality enhancement program are:

- Providing uniform definitions
- Providing uniform criteria
- Maintaining uniform procedures
- Maintaining complete follow-up of all, or nearly all, participants
- Recruitment of adequate numbers of participants

9.2. Recruiting Adequate Numbers of Participants

A critical task for all clinical trials is the enrollment of adequate numbers of participants, a task that often proves to be more difficult than anticipated. A recruitment goal has been established for each AREDS2 Clinical Center. Recruitment strategies and successes will be a focus during initial meetings of the Technical Group and during Coordinator conference calls during the early years of the study.

9.3. Preventing Dropouts and Missed Visits

The primary objective of AREDS2 is to evaluate the effect of lutein/zeaxanthin and DHA/EPA on progression to advanced AMD. To achieve this objective, it is essential that each participant be examined regularly at follow-up visits until the study is terminated or until the participant dies. Missing information can bias the results of the study. Although occasional missed visits (for example due to illness) cannot be prevented, the data could be made invalid if there are many missed visits (for example, if numerous participants drop out). When data are incomplete, it is difficult to predict the direction of any bias resulting from the incompleteness. The only correct way to deal with missing information is not to have any.

Preventing dropouts and missed visits is a responsibility shared by the entire clinic staff, and this topic should be discussed frequently at staff meetings. When participants move to a location near another AREDS2 Clinical Center, efforts should be made to transfer them to that center. Clinic personnel can help prevent dropouts by doing the following:

- Explaining to each participant the procedures he or she will undergo.
- Minimizing waiting time and attending to each participant's comfort during waiting periods.
- Promptly following-up on all missed appointments. In some cases, a phone call from the Clinic Coordinator stressing the need for a follow-up examination may be enough. (Saying that "the protocol requires it" should never be given as a reason because it suggests that the participant's convenience is secondary to adhering to an unnecessarily rigid protocol.) Some participants will respond more favorably if

an ophthalmologist at the clinic calls them. Help in arranging transportation should be offered, and this help should include the payment of travel costs, if necessary and available. Participants who are unable to visit the clinic during regular hours may be willing to visit during evenings or weekends, or may be better able to attend another AREDS2 Clinical Center. Willingness to see a participant at his or her convenience, even at the inconvenience of clinic staff, will demonstrate the importance placed on follow-up and may improve a participant's cooperation.

In any long-term trial (particularly with an older study population such as in AREDS2), it is expected that continued follow-up would become more difficult as the study progresses. Infirmity, illness, institutionalization, and uncooperative families are some of the reasons given for the reluctance or inability of study participants to continue to visit the Clinical Center. However, it may be possible for clinic staff to gather data on the participant's status by employing one or more of the following strategies:

1. Interview a dependable family member.
2. Conduct a home- or health-care facility visit, if possible.

9.3.1. Use of Outside Services for Locating Participants Lost to Follow-up

Internet Services. The last known address and telephone number of many persons can be obtained through locator services at www.whitepages.com or www.switchboard.com. Staff can update information on participant contacts using these same services. Determining if a participant has died can often be accomplished by accessing the Social Security Death Index at URL www.ancestry.com or www.ssdi.rootsweb.com.

9.4. Study Monitoring

Study monitoring is performed by the AREDS2 Protocol Monitors and other Coordinating Center or NEI staff, as appropriate. The Protocol Monitor collaborates with the Data Manager in the editing of data received by the Coordinating Center, places regularly scheduled telephone calls (Protocol Review Calls) to each Clinical Center, participates in periodic Protocol Review Visits at each Clinical Center, monitors the certification status of each Clinical Center, and reports findings periodically to the Operations Committee. Each of these functions is described more specifically below.

9.4.1. Data Auditing

Data auditing at the Coordinating Center involves checking the data keyed by the Clinical Center for completeness, adherence to the Manual of Procedures, and internal consistency. This is performed via the AdvantageEDC^{SM} System. The Integrity data evaluation system checks for incomplete, questionable, or inconsistent data. If any of these are identified, they will be summarized in a report and will require corrections or clarification.

Part of the auditing process is to analyze the frequency of errors according to their type to determine if certain types of errors keep recurring. If they do, this

information is communicated to Clinical Centers. Also, the Coordinating Center will monitor for timeliness of data submissions. It is expected that the clinical sites will submit data as it is collected during all visits. Data may be entered directly into the computer using a wireless tablet or a personal computer (PC). Data entered directly without recording information on paper are considered 'source.' No paper records are expected at the site when the electronic entry is the source. For data that are first recorded on paper and then entered into the data entry system, the paper record is considered 'source.'

9.4.2. Protocol Review Visits

Another aspect of quality control is auditing a sample of data during Protocol Review Visits. The purpose of these visits is to exchange information, review the Clinical Center's operations, conduct a data audit, and discuss and resolve any problems encountered. Each site will be audited by a Protocol Review Visit team during the initial two years of the study and every two to three years thereafter. For cause audits may occur at any time. Typically, the Protocol Monitors conduct the monitoring visits; however, other Coordinating Center or NEI staff may choose to attend. The purpose of the monitoring visit may include a review of the Regulatory Binder, inventory of the study supplements and supplies, review the clinical center's operations, assist in the training and certification of staff, conduct a data audit of the source documents vis-à-vis the database and discuss problems encountered, with a view toward addressing issues of mutual concern. The agenda is sent in advance to all personnel involved in the site visit. Likewise, a summary report containing action items discussed during the visit is distributed within one month after the visit.

For a selected group of participants, the auditor confirms the presence of signed informed consent forms and compares information in a clinic's participant charts with information keyed into AdvantageEDC^{SM}. Any discrepancies are resolved by discussion with the clinic coordinator. The AREDS2 criteria for an acceptable data entry error rate is 0 – 1.0 errors per 1,000 keyed fields. Clinics with error rates above this range may be reaudited prior to their next expected audit. Other quality control aids, such as double key entry, will be implemented for sites that continue to exceed the acceptable data entry error rate.

9.4.3. Protocol Review Calls

The Protocol Monitors make regularly scheduled Protocol Review Calls to each Clinic Coordinator as a means of positive communication. Initially, these telephone calls are made monthly, and they gradually become less frequent. The calls follow a structured agenda that is sent in advance to the Study Coordinator. Rather than emphasize errors made by the clinical center, these calls provide the opportunity to report on the ways in which the clinic is functioning properly and successfully. The agenda includes the following:

- Staff changes and current or impending needs for training or certification
- Changes or impending changes in clinic facilities

- Functioning and calibration of equipment
- Participant enrollment
- Satisfaction of participants with their visits to the clinic
- Satisfaction of the staff with working conditions
- Problems with meeting the requirements of the study
- Problems with completing data forms
- Problems with the AdvantageEDC^{SM} System.

The Protocol Monitor prepares for all Protocol Review Calls by reviewing the data received from a Clinical Center, information about any errors made by the center, the certification status of new staff members, notes from previous calls, and recent correspondence from the Clinical Center.

The Protocol Monitor and Data Manager keep a log of telephone calls, correspondence, and site visits for each Clinical Center. The Protocol Review Calls are not a substitute for other telephone calls that may be needed to resolve problems as they occur. Such calls should be made as often as needed.

9.5. Quality Assessment of Pharmaceutical Manufacturing

The quality of pharmaceutical manufacturing and labeling will be monitored by randomly sampling a specific number of bottles containing the placebo or study supplements for the Central Laboratory. The Central Laboratory will perform on a limited basis, by techniques available in the Central Laboratory, qualitative and semi-quantitative tests of the samples of placebo and active study supplements to confirm the content and consistency of study supplements. This will serve as a secondary check of the quality of the study intervention. This independent analysis will verify whether or not the assigned intervention has been matched properly to the bottle numbers.

9.6. Participant Adherence Enhancement

Good participant adherence with the study protocol is essential for the success of the study. The best way of improving participant adherence is for the clinic staff to develop a good relationship with the participant, to show interest in the participant, and to emphasize repeatedly the importance of taking all prescribed medications and following directions. The benefits of participating in the study will be explained to each participant prior to enrollment and periodically during follow-up. Tools for adherence to the study protocol will be developed by each Clinical Center. These tools should convey appreciation for the individual's participation in AREDS2 and may include birthday or other greeting cards, calendars indicating clinic appointments, and clinic informational newsletters.

10. CLINICAL CENTER PROCEDURES

10.1. Staffing and Organization

Each AREDS2 Clinical Center is staffed, at a minimum, by

- Clinic PI (an ophthalmologist)
- Clinic Coordinator
- Ophthalmic technician
- Clinic Monitor
- Photographer.

One person may serve in multiple roles. There may be additional ophthalmologists, designated as co-investigators, additional technicians, and assistant Clinic Coordinators who simultaneously function as ophthalmic technicians. The Clinic PI designates one staff member to serve as Clinic Monitor.

10.2. Functions of the Clinic PI

The responsibilities of the Clinic PI, who is an ophthalmologist and the Principal Investigator named on the Clinical Center Letter of Agreement with The EMMES Corporation, are to do the following:

a. Direct the activities of AREDS2 personnel in the clinic;

b. Ensure adherence by clinic personnel to the procedures described in and required by the AREDS2 Manual of Procedures;

c. Spend adequate time in the clinic to observe study procedures and to hold regular discussions with staff to review all aspects of the study and to resolve any problems that may arise;

d. Represent the clinic at meetings of the Executive Committee and Technical Group; and

e. Perform ophthalmologic exams, introduce the study to potential participants, and ensure that the recruitment and retention goals for the clinic are obtained.

10.3. Functions of the Clinic Coordinator

The Clinic Coordinator is responsible for supervising day-to-day operations in the clinic and serves as the primary contact person for the participants in the study and for the Coordinating Center. The duties of the Clinic Coordinator are to do the following:

a. Ensure that potential AREDS2 participants receive appropriate written information about the study (e.g., Participant Information Booklet, Participant newsletters), including the Informed Consent statements;

b. Ensure that potential AREDS2 participants have the opportunity to ask questions about the AREDS I and 2 Studies;

c. Register participants in AREDS2;

d. Schedule participant appointments;

e. Prepare for participant visits, ensuring that the equipment needed for examinations is in place and functioning, and that clinic personnel are prepared to meet their responsibilities when a participant appears for an appointment;

f. Attend to a participant's comfort at the clinic and minimize a participant's waiting time;

g. Maintain good rapport with each participant;

h. Notify the Coordinating Center of changes or impending changes in the clinic personnel, address, or telephone number(s) of the clinic;

i. Maintain a calendar of participant visits, meetings, and scheduled telephone calls or visits from the Protocol Monitor, etc.;

j. Maintain a file of correspondence with Coordinating Center;

k. Obtain necessary information about deceased participants (e.g., death certificates) and hospitalizations (e.g., discharge summaries, if appropriate);

l. Maintain an inventory of study supplements and supplies and restock as needed;

m. Check completed data forms for accuracy and completeness and key data;

n. Prepare required photographs for submission to the Reading Center;

o. Respond to data queries from the Coordinating Center;

p. Oversee the collection and delivery of blood specimens to the CDC (if participating in the Nutritional Biochemistry Study).

Each Clinic Coordinator will be provided access to the AREDS2 Clinical Center Data Management Handbook for using the AdvantageEDC^{SM} System.

10.4. Functions of the Ophthalmic Technician

The ophthalmic technician performs visual acuity and refraction examinations on AREDS2 participants. The Clinical Director must notify the Coordinating Center before a new or

replacement technician is added to the study so that he/she can be certified for AREDS2 procedures.

10.5. Functions of the Clinic Monitor

The Clinic Monitor may be the PI, a co-Investigator, the Clinic Coordinator, or another staff member who is thoroughly familiar with the activities and equipment of the clinic and with the AREDS2 Protocol and Manual of Procedures.

The Clinic Monitor has the responsibility to monitor clinic activities for conformance to the requirements of the AREDS2 Manual of Procedures. The activities to be monitored include:

a. Ensure local/central IRB approval is obtained and a copy of the approval letter and IRB-approved informed consent statements are sent to the Coordinating Center;

b. Ensure that personnel performing AREDS2 procedures are properly trained and certified;

c. Ensure that equipment used to perform AREDS2 procedures is calibrated properly (e.g., illumination for visual acuity test);

d. Maintain awareness of regular data queries identified by the Coordinating Center;

e. Inform the local/central IRB of important study or protocol changes;

f. Participate in regularly scheduled telephone calls (Protocol Review Calls) with the Protocol Monitor;

g. Meet with the Protocol Monitor during Protocol Review Visits; and

h. Report irregularities that can affect the quality of the data to the Clinic PI and the Protocol Monitor

10.6. Functions of the Photographer

The photographer is responsible for taking quality fundus photographs on all AREDS2 participants at their Clinical Site. Roles of a photographer include:

- Provide quality color fundus and lens images
- Submit photographs in a timely manner according to the FPRC guidelines
- Know the photography visit schedule and naming conventions for the study
- Complete an online transmittal log to accompany a set of images

10.7. Recruitment

Each Clinical Center will develop a plan for meeting the recruitment goals and requirements of AREDS2. Specifically, each high-yield Clinical Center is expected to enroll approximately 150 participants and each fixed-fee Clinical Center is expected to enroll approximately 45 participants. Recruitment techniques may include:

- Chart reviews
- Listing of clinic patients with ICD codes for AMD
- Advertisements in community publications or presentations at community senior centers
- Brochure to referring/local ophthalmologists
- Presentations at local ophthalmologic society meetings

10.8. Eligibility Screening

If a participant appears to be eligible, the following steps are taken:

a. The plan of the study, as outlined in the Participant Information Booklet, is reviewed with the participant, and any questions by the participant are answered.

b. The participant is asked to read and sign the First Informed Consent form for the run-in period.

c. If the participant signs the consent form, the Qualification form is keyed into the AdvantageEDC^{SM} System, and a participant ID is assigned by the computer.

d. All Qualification Visit procedures as defined in Section 4.2.1 are performed.

e. Based on the participant schedule that is available in the AdvantageEDC^{SM} System, the participant is asked to return to the clinic within three months for final eligibility evaluation. The participant is provided a one-month supply of the Run-in Supplements and instructions for using the Run-in Supplements.

f. Participants who return to the clinic for the Randomization Visit and who satisfy the eligibility criteria and agree to participate are asked to read and sign the Second Informed Consent form. If the participant signs the consent form, he or she is randomized by keying the Randomization form into the AdvantageEDC^{SM} System. Bottle numbers are assigned by the computer. Initially, the participant is given an adequate supply of the AREDS2 Study Supplements until the next in clinic visit. The supplements are identified with the assigned bottle numbers. A follow-up appointment to return to the clinic in one year is scheduled using the Appointment schedule utility provided in the AdvantageEDC^{SM} System.

Once a participant has been assigned a participant ID, the number remains associated with the participant throughout the study and will not be reassigned.

10.9. Scheduling Participant Appointments

After a participant has been randomized, Clinic Coordinators may use the Protocol Calendar utility in the AdvantageEDC^{SM} system to view a listing of target dates and visit windows as well as procedures and/or forms to be completed at each participant visit. Every effort should be made to conduct each study visit as close to the target date as possible and before the expiration of the visit window. When scheduling appointments, the various time windows must be kept in mind. Efforts should be made to avoid missed visits and to keep scheduled visits as close to the target date as possible. This is the responsibility of the Clinic Coordinator.

10.10. Checking Completed Forms

Before and during the key entry of data into the AdvantageEDC^{SM} System, the Clinic Coordinator should carefully check all data for completeness and consistency. Some checking will be made automatically as data are entered. When necessary, anomaly reports are generated by the Coordinating Center and posted on the AdvantageEDC^{SM} for resolution.

10.11. Transferring Participants

When an AREDS2 participant relocates from one AREDS2 clinic to another clinic, the Clinic Coordinator from the sending site should contact the Clinic Coordinator at the receiving site to arrange the transfer. When the transfer has been arranged, the sending site should notify the Coordinating Center via telephone or email and the Participant Transfer form should be entered into the AdvantageEDC^{SM} System. The Clinic Coordinator at the sending site will be responsible for making copies of the participant's study records and sending the originals to the receiving site. If there are no pending issues at the sending site, the computer records will be transferred to the receiving site and the participant study number will be changed accordingly. After the computer records have been transferred, the new clinic will update the Participant Transfer form to confirm the transfer took place and be responsible for continued follow-up.

10.12. Preparing for the Participant Visit

Before a participant appears for a visit, the Clinic Coordinator should:

- Send a reminder card and call the participant to remind him or her of the appointment and to bring back all unused bottles of study supplements.
- Retrieve the participant’s file.
- Record the participant’s initials and registration number on all forms and worksheets to be completed during the visit.
- Be prepared to welcome the participant and inform him or her of what is going to happen.

- Ensure that all equipment is in place and functioning.
- Check that clinic personnel are prepared to complete their responsibilities.

11. INTERVENTION MANUFACTURERS' PROCEDURES

AREDS2 is supported in part by Alcon Laboratories, DSM, Tishcon Corporation, Wyeth Consumer Healthcare, and Bausch & Lomb. Alcon Laboratories is supplying the AREDS-Type Supplement (ATS) formulations for the second-tier randomization. DSM and Tishcon Corporation are supplying the main study treatments. Bausch & Lomb is providing the AREDS formulation for the run-in period and for participants (i.e., nonsmokers) who wish to take it during the trial and who do not want to be randomized to the various ATS formulations. Wyeth Consumer Healthcare is providing the Centrum Silver®.

Placebo study tablets, capsules and the actual AREDS formulation are provided during the run-in period. Study supplements and placebo, as well as Centrum Silver, all in the form of tablets and capsules, are provided during the clinical trial. Lutein/zeaxanthin, its placebo, and Centrum Silver are provided in tablet form. DHA/EPA as well as its placebo, and the ATS formulations are provided in capsule form. The placebo tablets/capsules supplied for the run-in period are labeled RUN-IN SUPPLEMENTS. The study supplement tablets/capsules supplied for the clinical trial are labeled STUDY SUPPLEMENTS. The study supplement tablets/capsules are identical in appearance, size, smell, and taste to the corresponding placebo tablets/capsules.

11.1. Manufacturing Procedures

Specific procedures for the manufacturing of the AREDS2 study tablets and capsules including quality control measures and stability testing were submitted to the FDA. This information is confidential.

11.2. Shipments to the Drug Distribution Center

Sponsors ship bulk containers of study supplements, 60-tablet bottles of Centrum Silver®, and bottles of the ATS formulation to the AREDS2 Drug Distribution Center located in Perry Point, Maryland. Shipments contain one lot number for each type of tablet or capsule, and bulk containers are marked with the name and strength of the agent, lot number, expiration date (if applicable), quantity, and manufacturer's name and address. Documentation summarizing the total amount of each supplement in the shipment accompanies the shipment.

12. COGNITIVE FUNCTION

The Cognitive Function Study measures the potential beneficial or deleterious effects of the AREDS2 study medications on cognitive function and investigates potential associations between cognitive function and the development or progression of AMD. A variety of instruments are used to measure the domains of cognitive function. The AREDS2 Cognitive Function Battery includes six validated and commonly used cognitive tests with eight components. A Telephone Battery was validated in AREDS and is used in AREDS2 because of the age of the study population. As the participants age, their mobility may be diminished, thus limiting or even preventing their ability to return for follow-up clinic visits. The Cognitive Function Telephone Battery is administered within three months after randomization and every two years thereafter by staff employed by the Coordinating Center.

The participant decides at Randomization whether to receive a phone call explaining the Cognitive Function Study. The Clinic Coordinator keys the participant's decision into AdvantageEDC^{SM}. If the participant consents, an administrator calls to explain the study and obtains a verbal informed consent or refusal and administers the Cognitive battery to each consenting AREDS2 participant over the telephone.

The ordering of the telephone battery is as follows (approximately 30 minutes to administer):

1. Hearing Handicap Inventory
2. Center for Epidemiologic Studies –Depression (interview-administered)
3. Initial reading of Wechsler Memory Scale (WMS) -R paragraph and immediate participant recall assessment
4. Telephone Interview Cognitive Status (TICS)
5. Letter Fluency
6. Category Fluency
7. Alternating Fluency
8. Backward Counting Task
9. Delayed recall of the WMS-R paragraph
10. TICS Recall

An abbreviated version of the Cognitive battery is also available (approximately 10-15 minutes to administer) and will be used with participants who have time constraints or other concerns about the full-length battery. The abbreviated battery consists of the following instruments:

1. Hearing Handicap Inventory
2. CES-D (interview-administered)
3. TICS

Additional information on the Cognitive Function Study can be found in the ARED2 Cognitive Function Study Protocol.

13. CARDIOVASCULAR OUTCOME STUDY

13.1. Introduction

After randomization, an AREDS2 participant may be hospitalized, experience a new or recurrent myocardial infarction or stroke, develop congestive heart failure (with subsequent hospitalization) or unstable angina, or undergo a cardiovascular procedure. These events will be part of the cardiovascular outcome study, a substudy of AREDS2, and will require physician adjudication, which is explained in this chapter of the MOP.

Since adjudication requires obtaining information from medical records, each AREDS2 Clinical Site should have the participant sign a "Release of Medical Records". A template form is provided in Appendix E of the AREDS2 Protocol.

13.2. Cardiovascular Outcomes of Interest

The primary endpoint for the AREDS2 cardiovascular sub-study is the composite outcome of CVD mortality (sudden death, death due to MI, heart failure, or stroke) and nonfatal CVD (MI, stroke, unstable angina, coronary revascularization, resuscitated cardiac arrest, cardiovascular procedure). The determination of these endpoints will be made by the AREDS2 cardiovascular outcome study adjudicators based on medical records documentation demonstrating that an event has occurred.

Adjudication of an endpoint will be triggered by the Clinical Site's completion of any of the following Case Report Forms:

1. Hospitalization
    a. Q4. Primary reason for hospitalization:
        i. 1- Cardiovascular Disease (includes myocardial infarction, angina, coronary artery disease, heart surgery, congestive heart failure, unexpected (sudden) death)
        ii. 2- Cerebrovascular disease (includes TIA, ischemic stroke, hemorrhagic stroke)

    b. Q5. Did the participant have any of the following events or procedures during hospitalization: (2-Yes to any of the following)
        i. Heart attack or myocardial infarction
        ii. Coronary bypass or heart surgery
        iii. Cardiac catheterization
        iv. Percutaneous transluminal coronary intervention (PTCI)
        v. Implantable cardioverter defibrillator (ICD)
        vi. Chest pain
        vii. Stroke
        viii. Carotid artery angioplasty
        ix. Peripheral artery angioplasty
        x. Congestive heart failure

2. Death Report
    a. Q4. Primary cause of death:
        i. 1- Cardiovascular Disease (includes myocardial infarction, angina, coronary artery disease, heart surgery, congestive heart failure, unexpected (sudden) death)
        ii. 2- Cerebrovascular disease (includes TIA, ischemic stroke, hemorrhagic stroke)

3. Outpatient Procedure (OPP)
    a. Q3. Outpatient procedure: (2-Yes to any of the following)
        i. Cardiac catheterization
        ii. Percutaneous transluminal coronary intervention (PTCI)
        iii. Implantable cardioverter defibrillator (ICD)
        iv. Carotid artery angioplasty
        v. Peripheral artery angioplasty

Supporting documents requested for each event (please see the specific Case Report Form) should be mailed to the Coordinating Center within 6 weeks of the date that the clinical site discovered the event. The clinical site should photocopy the material and store the originals in the participant's study chart at the clinical site and send the photocopied materials to the Coordinating Center. It is extremely important that Clinical Site personnel be diligent in identifying when one of these events has occurred and in collecting all relevant and requested data. Note that you will be asking the participant about hospitalizations and outpatient procedures every 6 months (In-clinic visit and 6-month phone call).

Using data and materials submitted by the AREDS2 Clinical Sites and applying the endpoint definitions defined later in this chapter, the Cardiovascular Outcome Study Adjudicators will review and classify each potential endpoint.

13.3. Preliminary Endpoint Notification

It is extremely important that the Coordinating Center be notified quickly that an AREDS2 participant has died or has had a cardiovascular/cerebrovascular event potentially meeting the endpoint definition. This is accomplished by completing one of the three forms noted in section 13.2.

Enter the appropriate form into the AREDS2 AdvantageEDC system within one week of notification that the event has occurred. Collect any necessary supporting documentation for the event(s) and mail these materials to the Coordinating Center within 6 week of notification of the event.

Guidelines for completing all AREDS2 forms can be found on the main menu of the AREDS2 AdvantageEDC under Study Documents.

13.4. Adjudication of Events

The following describes the procedures that will be followed in AREDS2 to classify the events that constitute the primary outcome for the AREDS2 Cardiovascular Outcome Study.

At the Clinical Site:

1. A coordinator at the clinical site who follows the participant will complete the appropriate CRF(s) (Hospitalization, Outpatient Procedure and/or Death) and Case Packet Checklist by entering the information in the AREDS AdvantageEDC system, and assemble the supporting documentation listed on the Case Packet Checklist.

2. Participant personal identifiers will be removed from supporting documents by a heavy black magic marker. Date of hospitalization, procedures, and lab reports should not be obscured.

3. A copy of the CRF(s), Case Packet Checklist and all supporting documents for each event will be sent to the Coordinating Center in a separate case packet. The site should ensure that all documents for each case packet are complete and clipped together.

4. The participant's six-digit identification number, the event description and the date of the event notification should be entered on the checklist.

At the Coordinating Center:

1. The form(s) and supporting documents are received by the Coordinating Center and reviewed for completeness and to ensure that all participant identifiers are removed from the documents.

2. Contents of the case packet will be entered into the AREDS2 AdvantageEDC by the data manager.

3. Missing information will be queried by the Coordinating Center.

4. Once the Coordinating Center is satisfied that all available information for each potential event has been obtained, the Adjudication Outcome form and the Participant Endpoint Status Report, if applicable, will be added to the Case Packet.

5. Three copies of the case packet will be made. Two copies of the case packet will be sent to two members of the Cardiovascular Outcome Study Adjudication Committee along with the Adjudication Outcome form and one copy will be kept at the Coordinating Center.

6. The Coordinating Center will select the pairs of reviewers with the guiding principle that the work will be evenly spread among members of the committee. If a participant has multiple events then these events will be sent to the same adjudicators.

7. Case packets will be sent on a quarterly basis to the adjudicators.

Review of an Event by the Adjudication Committee:

1. Using the definitions discussed later in this chapter the adjudicator will classify the endpoint event and submit the Adjudication Outcome form to the Coordinating Center via the AREDS2 AdvantageEDC.

2. If the two reviewers agree on the classification of the event, the classification will be considered final and will not be scheduled for review at a meeting or conference call of the entire committee.

3. If the two reviewers do not agree, the event will be scheduled by the Coordinating Center for an annual review of events by the full adjudication committee.

4. For those events that need to be re-reviewed by the full committee, the events will be classified by consensus or vote.

13.5. Event Definitions

The following definitions will be used by the Cardiovascular Outcome Study Adjudication Committee to determine whether a potential outcome meets the requirements of endpoint for the study.

13.5.1. Sudden death

Death of cardiac origin that occurred unexpectedly within 1 hour of the onset of new symptoms or a death that was unwitnessed and unexpected, unless a specific non-cardiac cause of death was confirmed.

13.5.2. Fatal myocardial infarction (MI)

Death within 7 days of the onset of documented MI (see 13.5.5)

13.5.3. Congestive Heart Failure (CHF)

Death due to clinical, radiological or postmortem evidence of CHF without clinical or postmortem evidence of an acute ischemic event.

13.5.4. Fatal Stroke

Death due to stroke occurring within 7 days of the signs and symptoms of a stroke (see 13.5.6)

13.5.5. Myocardial Infarction

(13.5.5.1) Q-wave MI: Diagnosis based on the occurrence of a compatible clinical syndrome with prolonged ischemic symptoms, associated with the development of new significant Q waves. Diagnostic elevation of cardiac enzymes will include: increase in CK-MB mass to a level > twice the upper limit of normal, and/or an increase in Troponin T or I to a level that indicates myonecrosis. Only the case that both Troponin and CK-MB mass measurements are not available, would the elevation of total CK to ≥ twice the upper limit of normal qualify for diagnosis

(13.5.5.2) Non Q-wave MI: Diagnosis based on the occurrence of a compatible clinical syndrome with prolonged ischemic symptoms, associated with elevation of serum enzymes, as for Q-wave MI.

(13.5.5.3) Probable non Q-wave MI: Diagnosis based on the occurrence of a compatible clinical syndrome with prolonged ischemic symptoms, without documentation of cardiac enzyme elevation, but associated with the development of new and persistent significant ST-T changes.

(13.5.5.4) MI after cardiovascular invasive interventions: Diagnosis based upon the occurrence of CK-MB or Troponin elevations to a level ≥ 3 times normal, occurring within 7 days of cardiac catheterization, arrhythmia ablation, angioplasty, atherectomy, stent deployment or other invasive coronary, carotid or peripheral vascular intervention.

(13.5.5.5) MI after coronary bypass graft surgery: Diagnosis based upon the occurrence of CK-MB or Troponin elevations to a level increased ≥ 5 times normal, occurring within 30 days of cardiac surgery.

(13.5.5.6) MI after non-cardiovascular surgery: MI (as defined above) occurring within 30 days of non-cardiovascular surgery.

13.5.6. Stroke

(13.5.6.1) Definite ischemic stroke: CT or MRI scan within 14 days of onset of a focal neurological deficit lasting more than 24 hours with evidence of brain infarction (mottled cerebral pattern or decreased density in a compatible location), no intraparenchymal hemorrhage by CT/MRI, no significant blood in the subarachnoid space by CT/MRI or by lumbar puncture. A nonvascular etiology must be absent.

(13.5.6.2) Definite primary intracebral hemorrhage: Focal neurological deficit lasting more than 24 hours. Confirmation of intraparenchymal hemorrhage in a compatible location with CT/MRI scan within 14 days of the deficit onset or by lumbar puncture.

(13.5.6.3) Subarachnoid hemorrhage: Sudden onset of headache, neck stiffness, loss of consciousness. There may be a focal neurological deficit, but neck stiffness is more prominent. Blood in the subarachnoid space by CT/MRI or lumbar puncture or intraventricular by CT/MRI.

(13.5.6.4) Stroke of unknown type etiology: Focal neurological deficit lasting more than 24 hour compatible with a stroke when CT or MRI are not done. Information is inadequate to diagnose ischemic (infarction), intracerebral hemorrhage, or subarachnoid hemorrhage. A nonvascular etiology must be absent.

(13.5.6.5) Non-fatal stroke after cardiovascular invasive interventions: Stroke (as defined in 13.5.6.1 – 13.5.6.4) associated with the intervention within 30 days of cardiovascular surgery, or within 7 days of cardiac catheterization, arrhythmia ablation, angioplasty, atherectomy, stent deployment or other invasive coronary or peripheral vascular interventions.

(13.5.6.6) Non-fatal stroke post non-cardiovascular surgery. Stroke (as defined in 13.5.6.1 – 13.5.6.4) occurring within 30 days of non-cardiovascular surgery.

13.5.7. Coronary revascularization

Includes:

a. Coronary artery bypass surgery
b. Percutaneous transluminal coronary intervention (PTCI) including angioplasty, placement of cardiac stents and atherectomy
c. Carotid artery angioplasty, stent placement, or endarterectomy
d. Peripheral artery angioplasty, bypass surgery or endarterectomy

13.5.8. Resuscitated cardiac arrest

(13.5.8.1) Definite resuscitated cardiac arrest. Discharged alive from hospital after witnessed cardiac arrest. Cardiac arrest is defined as no pulse or respiration and unresponsive lasting at least 30 seconds with documentation of any of the following cardiac rhythms at the time of unresponsiveness: ventricular fibrillation, ventricular tachycardia, asystole (absence of any QRS complex for 6 seconds), severe bradycardia (11-50 beats per minute), pulseless electrical activity. Cardiac arrest events occurring within one week of a documented MI will be excluded.

(13.5.8.2) Probable resuscitated cardiac arrest. Discharged alive from hospital after witnessed probable cardiac arrest. Cardiac arrest is defined as no pulse or respiration and unresponsive lasting at least 30 seconds. Documentation of cardiac rhythm at time of arrest is unavailable.

13.5.9. Placement of implantable cardioverter defibrillator (ICD)

Includes placement of any type of implantable cardiac defibrillator as either inpatient or outpatient.

13.6. Adjudication Procedures

13.6.1. Review of the Adjudication Case Packet

When reviewing the adjudication case packet the adjudicator will determine for each outcome whether the packet contains the information checked on the Case Packet Checklist. If the listed documentation is present the adjudicator may proceed in assigning an endpoint by completing the Adjudication Outcome form via the AREDS2 AdvantageEDC.

(13.6.1.1) Contents of Case Packet. The case packet consists of the following documents:

- Participant Endpoint Status Report. This report provides individual participate information about confirmed endpoints, outstanding endpoints, and pending adjudications. Enrollment status is also provided.*
- Case Packet Checklist. Lists specific information/materials provided within the case packet.
- Medical records documentation.
- Adjudication Outcome form*
- Relevant CRFs (hospitalization, death, OPP form)

* The Coordinating Center will provide in the case packet before sending to the adjudicators.

(13.6.1.2) Incomplete Adjudication Case Packets. If the adjudicator judges that the case packet is incomplete or needs additional information, s/he should indicate on the Adjudication Outcome form to request the missing information and/or documentation.

13.6.2. Completing the Adjudication Outcome Form

The adjudicator should read through the entire case packet carefully. Complete the Adjudication Outcome form. The form must include sufficient information (e.g. 6-digit participant ID, event description, and event date) to ensure they do not get mixed up with other cases.

All cardiovascular outcomes should be completed within 4 weeks of receipt by the adjudicator. Adjudicators may also request full committee review for any case by checking the box on the adjudication form.

14. PROCEDURES FOR CENTRAL LABORATORY SPECIMEN COLLECTING, SHIPPING AND PROCESSING

14.1. Introduction

Selected AREDS2 Clinical Centers are participating in the study of serum levels of vitamins A and E, carotenoids, selected fatty acids (see Exhibit 14-1), lipids, zinc and copper. The Centers include:

- Vision Research Foundation: Royal Oak, MI; Williamsburg, MI; Grand Rapids, MI
- The Retina Division at Wilmer Eye Institute, The Johns Hopkins University: Baltimore, MD
- The National Eye Institute: Bethesda, MD
- Texas Retina Associates: Dallas, TX
- Retina Vitreous Associates Medical Group: Beverly Hills, CA
- Bascom Palmer Eye Institute: Miami, FL

The procedures outlined below apply to these centers. For a long-term study such as AREDS2, it is very important to assure that continuity of all techniques is maintained throughout the study duration to minimize variability by clinic or by technician. This chapter is designed to provide all necessary information for correct collection, processing, and shipment of the AREDS2 blood specimens for analysis, as well as a summary of procedures used by the CDC Central Laboratory for processing the specimens. An overview of the collection, processing and shipment procedures is shown in Exhibit 14-2.

14.2. Centers For Disease Control (CDC)

The Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, serves as the AREDS2 Central Laboratory for the Nutritional Biochemistry Substudy. Serum and plasma samples for all AREDS2 participants at participating Clinical Centers are sent to the Central Laboratory for measurement of concentrations of vitamins A and E (alpha- & gamma-tocopherol), lutein, zeaxanthin, α- and β-carotene, β-cryptoxanthin, lycopene (trans- and total), selected fatty acids (including DHA and EPA), lipids (including cholesterol and triglycerides), zinc and copper.

14.3. Training and Certification of Clinical Center Staff

All Clinical Center staff participating in Central Laboratory studies are thoroughly trained in each aspect of the specimen collection, processing, and shipping procedures. Training takes place at an initial training session in Washington, DC on 21 April 2006 and annually or bi-annually thereafter. Clinical Centers participating in the Central Laboratory blood studies are encouraged to have at least two AREDS2 staff members trained in these procedures. This helps to minimize any inconvenience to participants should a staff member who has been trained in the Central Laboratory procedures be absent from the clinic. Training of any personnel not attending this training session will take place at the Clinical Center by a staff member trained at the Central Laboratory training session. This Chapter provides the guidance document for the training.

Following completion of training, each trained clinical staff member must take blood samples from two AREDS2 participants, prepare serum and plasma aliquots and ship the specimens to the Central Laboratory according to the procedures outlined in this chapter. The staff member should refrain from taking additional blood samples until receiving an official notice from the Coordinating Center that certification has been granted.

When the shipment is received at the Central Laboratory, the Logistics Supervisor reviews the documentation and evaluates the shipment with regard to the following parameters: appropriate number of vials, optimal specimen quality (non-hemolyzed; non-lipemic), correct specimen volume, correctly applied labels, correct packing of specimens, appropriate timing of shipment, verification of shipping manifest and package contents, and appropriate communication with the Central Lab about the shipment. If the samples and documentation meet the protocol requirements, the Protocol Monitor at the Coordinating Center is notified by the Central Lab Logistics Supervisor. The Protocol Monitor notifies the candidate that certification has been obtained. At any time, the clinical staff may call the Central Laboratory with questions about laboratory procedures for this study. Contact Charles Dodson, MT(ASCP), Logistics Supervisor at 770-488-4305.

14.4. Specimen Collecting and Processing

In selected Clinical Centers, blood specimens are obtained at the Randomization, 1-year, 3-year, and 5-year visits from fasting participants. If the Annual Visit is missed, the sample may be obtained the next time the participant is seen at the clinic for any reason.

It is recommended as good laboratory practice that all blood specimens, used needles and other items associated with the specimen collection should be treated as though they were infectious for HIV and hepatitis B virus. All used needles must be placed in puncture-resistant containers; then along with used gauze, Vacutainer tubes, pipettes, vials, etc, they must be autoclaved prior to disposal. Use of disposable gloves when collecting and processing blood is required. (See CDC recommendations for prevention of HIV transmission in health-care settings MMWR 1987;26 (suppl 2S) and CDC Updated US Public Health Service Guidelines for the Management of Occupational Exposures to HBV, HCV, and HIV and Recommendations for Post-exposure Prophylaxis MMWR 2001:50 (RR11):1-42.

Before the blood specimens are collected, have the participant rest in a seated position for at least 5 minutes and remain in this position during the venipuncture. Clothing should not restrict the arm. The participant should have been fasting for at least 8 hours and should not have smoked, been exposed to cold or exerted himself or herself for at least half an hour prior to the specimen collection.

Specimens are prepared for biochemical testing to be performed by the Central Laboratory. Test results are important to assure participant compliance with the study regimen and that the effective concentrations that were achieved with the various AREDS2 interventions. Data will also be used to examine the effect of supplementing with nutrients that may compete for uptake and to validate the FFQ.

The following equipment should be available at a participating center and assembled prior to sample collection:

1. blood-collection chair
2. centrifuge capable of 1,500 X G
3. freezer (not frost-free, at least -20 °C, with a high-temperature alarm)
4. powder-free disposable gloves (nitrile is always preferable to latex because staff or participants may be allergic to latex)
5. Vacutainer needle holder
6. gauze, alcohol swabs, bandages, and tourniquets
7. vortex mixer
8. test tube racks
9. permanent markers
10. disposable paper towels
11. biohazard disposal containers

In addition to the equipment listed above the following materials supplied by the Central Laboratory should be assembled:

1. 7-mL royal blue-top trace element Vacutainers (per participant: 2 for collection and for clean-up, if needed)
2. 4-mL EDTA purple-top Vacutainer (1 per participant)
3. 21g or 23g BD Butterfly Vacutainer needle
4. plastic rack for holding Vacutainers upright
5. prescreened, individually wrapped transfer pipettes
6. 2-mL high-density polypropylene Nalgene cryovials (one set will be sterile with caps attached for storing serum for Zinc/Copper analysis and one set will be bulk packed with caps detached for the other aliquots)
7. Cryovial rack for holding and storing cryovials
8. Cardboard boxes with 10 x 10 grid for storing and shipping cryovials

Do not use the Central Laboratory-supplied materials for collecting or processing blood for any other study tests; they have been prescreened for background trace element contamination and it is imperative to keep them as clean as possible.

Other miscellaneous phlebotomy supplies should be purchased and maintained by participating centers.

14.5. Preparation and Blood Collection

14.5.1. Summary

Collect and completely fill two **7-mL Royal Blue-top Vacutainers** and one **4-mL Purple-top Vacutainer** from each participant. See OSHA Regulations (Standards - 29 CFR) Bloodborne pathogens - 1910.1030 for information governing the use of

personal protective equipment when there is potential for occupational exposure to blood or other potentially infectious materials.

14.5.2. Preparation

Assemble blood collection materials such as needles, gauze pads, alcohol swabs, bandages, Vacutainers and holders, tourniquets, labels, vials, and needle disposal containers in advance. Wear disposable nitrile or latex gloves.

14.5.3. Blood Collection

1. Tie the tourniquet onto the upper arm, approximately 3 inches above the intended venipuncture site so that it can be quickly released with one hand.
2. Swab the venipuncture area with alcohol swabs using concentric circular motions.
3. Air dry excess alcohol.
4. Puncture the vein.
5. Push the first royal blue top tube into the needle holder.
6. After blood flow is established, loosen the tourniquet. Prolonged application of the tourniquet should be avoided to minimize hemoconcentration.
7. Allow the Vacutainer tubes to fill, following the instructions supplied with the tubes. (Training video tapes are available from the manufacturer, Becton-Dickinson, Rutherford, NJ.) Collect two 7-mL Royal blue top tubes followed by one 4-mL purple-top tube.
8. After the last tube has filled, remove it from the needle holder.
9. Withdraw the needle and dispose of in the sharps disposal container.
10. Apply pressure on the venipuncture site. Re-examine the puncture site to verify that any residual bleeding has ceased after five minutes, then apply a bandage.
11. Affix the bar-coded labels to the sides of the Vacutainer tubes.
12. Centrifuge the purple-top tube immediately.
13. Place the two royal blue top tubes upright in a tube rack and allow them to clot upright at room temperature for no longer than one hour. **Keep the tubes away from sunlight or any other strong light source, which may degrade the vitamin A and carotenoid content**. If necessary, the blood specimens may be allowed to clot for 30 minutes and then refrigerated for up to 2 hours to allow more time between the collection and processing phases. This is not recommended as a part of routine practice but for use in special circumstances.

14.5.4. Safety Note

Dispose of all needles and contaminated wastes properly in a puncture-resistant biosafety container. Containers should be autoclaved prior to disposal.

14.5.5. Errors

The clinic will report in writing any errors in preparation and/or collecting blood from participants. Communications about any such errors should be attached to the shipping manifest containing the specimen(s) in question.

14.6. Preparing Plasma and Serum

14.6.1. Summary

Centrifuge the purple-top tubes for 15 minutes at 1,500 x g (usually about 2,400-3,000 rpm; check a nomogram for appropriate speed for your centrifuge). Do not remove the tops of the tubes before centrifugation. Do not "rim" the tubes prior to centrifugation; this may introduce contamination.

After the blood has been allowed to clot at room temperature for a minimum of 30 minutes and up to 1 hour (2 hours if refrigerated), centrifuge the royal blue top tubes for 15 minutes at 1,500 x g (usually about 2,400-3,000 rpm; check a nomogram for appropriate speed for your centrifuge). Do not remove the tops of the tubes before centrifugation. Do not "rim" the tubes prior to centrifugation; this may introduce contamination.

Handle one participant's set of Vacutainers at a time. Line-up 7 cryovials in a rack for each participant. One vial (ZNCU) is a special type pre-screened for trace metals; it is pre-capped and empty. Six additional caps are needed.

The vials should always be lined up in order (according to the table below) to minimize chance for incorrect labeling.

The following is a table of cryovial aliquots that is expected to be created for each participant. The list is ranked by priority. Priority refers to the allocation of the sample to the tubes. Tubes 1 and 2 are considered the most important for the purposes of the study. The Zinc/Copper vial should always be filled first to avoid cross-contamination of the serum in this vial. If there is only sufficient sample to fill two tubes, then tubes 1 and 2 should be filled.

Plasma and Serum Vial Table

Priority	Analyte	Matrix	Minimum Volume	Desired Volume	Storage Container
1	Fatty Acids (FATA)	Plasma	0.5 mL	Up to 1.8 mL	2-mL cryovial
2	Fat-soluble vitamins (FATV)	Serum	0.5 mL	Up to 1.8 mL	2-mL cryovial
3	Zinc/Copper (ZNCU)	Serum	0.5 mL	Up to 1.8 mL	2-mL cryovial
4	Lipids (LIPD)	Serum	0.5 mL	Up to 1.8 mL	2-mL cryovial
5-7	Reserve vials (RESV)	Serum	0.5 mL	Up to 1.8 mL ea	2-mL cryovial

14.6.2. Errors

The clinic will report in writing any errors in preparing serum. Communications about any such errors should be attached to the shipping manifest containing the specimen(s) in question.

14.7. Preparing and Storing Specimen Vials

14.7.1. Fatty Acid Vial - plasma

Using a disposable pipette, transfer 1.8 mL clear plasma, free of red cells, to the FATA vial. Cap the cryovial and tighten to prevent leakage. Label as directed below (14.7.5). **Immediately freeze** cryovial between -20˚ to -80˚C. Discard the pipette and the Vacutainer.

14.7.2. Zinc/Copper Vial - serum

Analysis of serum specimens for zinc/copper concentrations requires special specimen preparation techniques. The Central Laboratory supplies pre-screened materials for this study. Do not use these materials for collecting or processing blood for any other study tests; they have been pre-screened for background zinc/copper contamination and it is imperative to keep them as clean as possible.

Remove 1 mL of serum from one of the royal blue top tubes and transfer it to a sterile, pre-capped 2-mL cyrovial specific for Zinc/Copper (ZNCU). Cap the vial immediately. If it appears that there will not be enough serum, the *Serum Vial Table* above lists priorities and minimum volumes required for testing. ZNCU should always be aliquoted before any other serum aliquot to prevent contamination.

14.7.3. Fat-soluble Vitamins, Fatty Acid and Reserve Vials - serum

After preparing the Zinc/Copper aliquot, use the same disposable pipette and transfer 1 mL clear serum, free of red cells, to the FATV, FATA and RESV vials. See the table for minimum amounts of serum for these vials. Any additional serum beyond the capacity of vial 7 may be equally divided among the vials up to the 1.8-mL fill mark. Cap all 2-mL cryovials and tighten to prevent leakage.

14.7.4. Lipids Vial - serum

After preparing the fat-soluble vitamins vial, re-use the disposable pipette and transfer 1 mL clear serum, free of red cells to the LIPD cryovial. See the table for minimum amounts of serum for lipid tests.

14.7.5. Plasma or Serum Clean-up

If any of the serum or plasma becomes mixed with red cells during the collection, transfer the bloody serum or plasma into a new royal blue top tube (not purple-top even for plasma) and re-centrifuge. Extra tubes and labels are provided for this purpose. Transfer the clear serum or plasma using a clean pipette.

14.7.6. Labeling

Working with Vacutainers and cryovials from one participant at a time, label the vials and the participant's visit record with bar-coded labels for those specimens that were actually collected, and place the vials in two 10 x 10 grid storage boxes. The bar-coded labels should be placed on the vials so that the barcode lies flat along the length of the vial. The left end of the label (as it is read) should be at the top of the vial and the right end towards the bottom. Oriented along this flat surface, the barcode can be properly scanned using a barcode reader. Consistent left-to-right alignment helps the analyst check the vial number against the run sheet during the assay. When the bar code is incorrectly wrapped around the tube, the scanner cannot see the complete SampleID. For every vial, a matching bar-coded label should be applied to the participant visit sample collection log (Exhibit 14-5) in order to document the collection of specimens. The labels on the paperwork are scanned to create a shipping manifest when the vials are shipped to the Central Laboratory. Extra labels must be discarded or saved elsewhere so that there is no chance for label mix-up the next time the participant visits the clinic.

Store vials in 10 x 10 grid boxes in at least a -20˚C freezer until ready to ship to the CDC. Place all of the aliquots for testing (4 vials) from one participant in one row in box 1. Prepare a second box with all of the aliquots for reserve (3 vials) and keep each participant's set in a separate row in box 2. Each column in box 1 should contain samples for a particular analysis (column 1: fat-soluble vitamins; column 2: fatty acids; column 3: zinc/copper; column 4: lipids). A similar arrangement should be used for box 2. This standardization will help the person receiving the specimens to quickly distribute the vials to the different analysts at the Central Lab.

14.7.7. Specimen Freezing and Vacutainer Disposal

Prior to shipment, secure the boxes in zip-loc bags. Freeze the specimens at least -20˚ C in an upright position so that serum does not collect in the vial cap. Maintain a freezer temperature log (Exhibit 14-6).

Discard Vacutainers and transfer pipettes into an autoclave bag and autoclave prior to disposal.

14.7.8. Errors

The clinic will report in writing any errors in preparing or storing specimen vials. Communications about any such errors should be attached to the shipping manifest containing the specimen(s) in question.

14.8. Shipping Specimens

Specimen shippers are supplied by the Central Laboratory; Federal Express labels will be supplied by each Clinical Center using a central account established by the Coordinating Center for the sole purpose of shipping items to and from the CDC. Use either cubed or pelletized dry ice (about 12 lbs per shipper). A completed shipping manifest should be

included with each shipment. Please also include a copy of the temperature logs for all months prior to the shipment or for all months between shipments

Add any comments regarding the collection, storage and shipment that might impact the analytical testing of the samples or account for missing sample. Issues such as patient refused, unable to find vein, inadequate blood draw, hemolysis, lipemia, unavoidable specimen freeze/thaw, Vacutainer spill, vial spill, etc should be entered into the tracking system. Corrective actions should also be indicated such as will reschedule patient ASAP or patient refuses to come back until next regularly scheduled visit.

A shipment schedule will be created by the Central Laboratory. It is currently expected that each Clinic will ship specimens approximately every two months.

1. Materials and Equipment Needed:

    - Saf-T-Pak shipping bags: internal plastic bag with biohazard symbol and outer Tyvek bag or two large zip bags
    - Absorbent gel strips or other absorbent material
    - Filled specimen storage boxes containing serum vials
    - Styrofoam shipping containers with outer cardboard liner
    - Dry ice
    - **Dry Ice** label
    - **Diagnostic Specimen** label
    - Completed FedEx airbill
    - Packing material (bubble wrap or newspaper)
    - Packing tape

2. Packing Instructions:

    - All specimens should be placed in an appropriate sized gridded storage box.
    - Place the specimen storage boxes inside one of the Saf-T-Pak plastic bags with the biohazard symbol along with an absorbent gel sheet and seal the bag.
    - Place the plastic bag with the biohazard symbol inside one of the Tyvek bags.
    - **OR** place each box inside a large zip bag with some absorbent material & seal bag.
    - Place the Tyvek bags or zip bags inside the Styrofoam shipping container with the outer cardboard liner. Insure the boxes are in an upright position.
    - Add 5-15 lbs of dry ice to the shipper containing the serum specimens and place extra packing material around the specimens (newspaper, paper towels, bubble wrap, etc.).
    - Prepare a Federal Express airbill for shipping and mark the appropriate boxes including the one for dry ice for the Serum shipment and overnight delivery.
    - Place a Dry Ice Label and a Diagnostic Specimen Label on the outside of the shipping box containing the Serum Specimens and write in the weight of the dry ice.

3. Shipping address:

Charles Dodson/Sherri Rule

Centers for Disease Control and Prevention
Building 110 Loading Dock
4770 Buford Highway NE
Atlanta, GA 30341
TEL: (770) 488-4305
TEL: (770) 488-0320
FAX: (770) 488-4301

In the event that the tracking system is non-functional, please phone, fax or email the information about the shipment to the above numbers or email to (wcd1@cdc.gov) or (dgq7@cdc.gov) the day that the package is shipped. Shipments should be made on Monday through Wednesday to insure that the shipment will arrive during a regular work day. Clinic staff should call or correspond with the CDC via e-mail prior to all shipments to determine whether the CDC staff will be available to receive the samples.

Any problems with the shipment that are discovered upon receipt are noted and reported to each clinic via the tracking system. Shipments are verified at the Central Laboratory against shipping lists.

14.9. Central Laboratory Procedures

14.9.1. Shipper Receipt at the Central Laboratory

Once a shipper arrives at the Central Laboratory, it is logged in by the Sample Logistics receiving personnel. Specimens are removed and placed at -20˚C for short-term storage (several days at most). Dry ice is removed from the shipper. Excess labels are removed; the shipper is resealed, and returned by surface mail to the originating clinic for reuse. Periodically, new outer cartons are provided for the Styrofoam inner shells. Because all shipments do not arrive at the Central Laboratory the same week of the month, the clerk holds all specimens for racking at one time each month.

Specimens are racked in sets of 20 for each analysis, with each set of 20 given a designated run name for data management purposes. The usual practice at the Central Laboratory is to designate the runs sequentially, according to a letter code indicating the month in which they are received. For example, specimens received in the first rack of 20 in January (A) 2007 would be identified as A07A01; those in the fourth rack of 20 in June (F) 2007 would be in rack A07F04, with the initial "A" indicating "AREDS2." If analysis will not be performed within a short period of time, the specimens are placed in -70˚C storage for maximum stability.

One in 20 specimens that are sent to the analysts is a blind QC specimen. Several years of blind QC are prepared at the beginning of the study and held at -70˚C in a Logistics Support freezer. Only the logistics personnel and supervisors are told which specimens are blind QC.

14.9.2. Assay Methods

Fat-soluble micronutrient concentrations are determined using a gradient high performance liquid chromatography (HPLC) method with UV/visible spectroscopy using Waters Alliance 2695 and Waters photodiode array 996 (1). Fatty acids are measured via gas chromatography with mass spectrometric detection using a modification of the method of Langerstadt et al. on a Thermo Electron TRACE DSQ GC/MS (2). Zinc and copper are measured using a Sciex 6100 ELAN Inductively Coupled Plasma Mass Spectrometry (3). Total cholesterol (4), HDL-cholesterol (5), total triglyceride (6) assays will be performed on a Roche Hitachi 912 Analyzer. After all analyses are completed, the reserve serum and plasma aliquots will be held at -70˚C at the Central Laboratory as an archival source, in case there are any questions about an analysis in the future, or if new technology emerges which may make additional analyses possible on these specimens to enhance the study database. Plasma and serum residues from vials 1-4 will generally be discarded once the data have been reported and sufficient time has elapsed for any questions about the data to be resolved.

14.9.3. Data Transfer

The Central Laboratory maintains a database of all blood level measurements obtained for each AREDS2 participant; the data are indexed by SampleID. The SampleID is barcoded on the vials (and records) as in the following example:

SampleID: 06-30-1234567-00-S5
06-30 is part of the CDC Lab study number in an abbreviated format (CDC Study Tracker 2006-0030)
1234567 is the 7 digit **Participant ID**
00 represents the visit number for the participant
S represents S serum
5 is vial 5 for a reserve vial (see Serum Vial Table in Section 14.6.1)

Data to be sent to the Coordinating Center are stored in a Microsoft Access database. The results are sent via the tracking system at regular, quarterly intervals in a comma-delimited text file (*.csv) attachment.

14.9.4. Reference Ranges for Serum Measurements

The reference ranges for serum measurements conducted by the Central Laboratory are shown in Exhibit 14-3.

14.9.5. Notification of Participants for Extreme Micronutrient Values

No participant or clinic will be informed of individual serum levels of micronutrients, except those participants found to have abnormal serum Vitamin A, as outlined in Section 14.10.

14.10. Abnormal Serum Vitamin A Notification Policy

In the event of the observation of serum vitamin A concentration ≥120 µg/dL and ≤ 150 µg/dL by the Central Laboratory, the Central Laboratory will analyze the specimen to determine the proportion of retinyl esters present. If less than 40 percent of the measured vitamin A content is present as retinyl esters, then no further follow-up is necessary, as such elevations have not, as yet, been associated with known pathological conditions.

The Central Laboratory will notify the appropriate Clinic PI of any of the findings listed below, with a suggestion for a confirmatory serum assay at the participant's next scheduled visit to the AREDS2 Clinical Center.

- Serum vitamin A concentration ≥ 120 µg/dL and the proportion of serum vitamin A present as retinyl esters is at least 40 percent

- Serum vitamin A concentration > 150 µg/dL regardless of proportion of retinyl esters present

- Serum vitamin A concentration < 10 µg/dL, regardless of proportion of retinyl esters present.

The Central Laboratory will notify the Clinic PI of the results of the analysis of the confirmatory specimen. If the result yields a serum vitamin A concentration of > 150 µg/dL or < 10 µg/dL, or a serum level of > 120 µg/dL with the proportion of retinyl esters present of at least 40 percent, then the Clinic PI will inform the participant. For high levels, the Clinic PI will inform the participant that the participant should discontinue taking CentrumSilver®. For low levels, the Clinic PI will advise participants taking CentrumSilver® to continue to do so. The Clinic PI will also inform the participant's personal physician of the results of the assays. The Central Laboratory will serve as a resource for the Clinic PI in determining the specific information to be communicated to the participant and the participant's personal physician.

14.11. Laboratory Methods References

1. Fat-soluble micronutrient assay CLIA method. Exhibit 14-4.

2. Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP. Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. Mol Genet Metab. 2001 May;73(1):38-45.

3. Taylor, H. E. *Inductively Coupled Plasma Mass-Spectrometry, Practices and Techniques*; Academic Press: New York, 2001

4. Cholesterol/HP, Roche Diagnostics Corporation, Indianapolis, IN 46256, Application Sheet: 03859304006 2005-01

5. HDL-C plus 2nd generation, Roche Diagnostics Corporation, Indianapolis, IN 46256, Application Sheet: 03860418006 - 2005-06

6. Triglycerides/GPO, Roche Diagnostics Corporation, Indianapolis, IN 46256, Application Sheet: 03856917006, 2005-10

EXHIBIT 14-1. SELECTED FATTY ACIDS TO BE EVALUATED

Analyte Code	Analyte Name
ALN	alpha-Linolenic acid (C18:3n3)
ARA	Arachidonic acid (C20:4n6)
DHA	Docosahexaenoic acid (C22:6n3)
DPA3	Docosapentaenoic acid (C22:5n3)
DPA6	Docosapentaenoic acid (C22:5n6)
DTA	Docosatetraenoic acid (C22:4n6)
EPA	Eicosapentaenoic acid (C20:5n3)
GLA	gamma-linolenic acid (C18:3n6)
HGLA	Homo-gamma-linolenic acid (C20:3n6)
LNA	Linoleic acid (C18:2n6)

Exhibit 14-2. PROCEDURES FOR CENTRAL LABORATORY SPECIMEN COLLECTING, SHIPPING AND PROCESSING: OVERVIEW

1. **MATERIALS PREPARATION** (Section 14.4)

Supplied by Clinical Center

blood collection chair
centrifuge (1500 x G)
-20˚C freezer with temperature alarm
powder-free nitrile or latex gloves
Vacutainer needle holders
gauze
alcohol swabs
Ziploc™ bags
bandages
tourniquets
vortex mixer
test tube racks
permanent markers
disposable paper towels
biohazard disposal containers

Supplied by Central Laboratory

7-mL royal blue-top trace metal Vacutainer
4-mL purple-top EDTA Vacutainer
21- or 23-g butterfly needles, pre-screened for trace metals
Transfer pipettes, pre-screened for trace metals
2-mL high-density polypropylene Nalgene storage cryovials, bulk packed
5-mL high-density polypropylene Nalgene storage cryovials, sterile, pre-capped & pre-screened for trace metals
10 x 10 grid cryovial storage boxes
Cryovial racks
Absorbant pads
Specimen shippers
Bar coded labels

2. **PARTICIPANT PREPARATION** (Section 14.5)

Fasting for at least 8 hours
Resting in seated position for at least 5 minutes
No smoking, exposure to cold, or exertion for at least 30 minutes
Clothing should not restrict arm

3. **BLOOD COLLECTION** (Section 14.5)

Collect and completely fill
- 2 7-mL royal blue-top Vacutainer
- 1 4-mL purple-top Vacutainer
- Spare 7-mL royal blue top Vacutainer for plasma or serum clean-up

4. **PREPARING PLASMA AND SERUM** (Section 14.6)

Centrifuge for 15 minutes at 1,500 x G. Do not “rim” the tubes.

5. **SPECIMEN PROCESSING AND STORAGE** (Section 14.7)

VIAL 1	(**"FATA"**)	plasma for selected fatty acids
VIAL 2	(**"FATV"**)	serum for fat soluble micronutrients
VIAL 3	(**"ZNCU"**)	serum for zinc/copper (special pre-screened materials for this vial)
VIAL 4	(**"LIPD"**)	serum for total cholesterol, HDL-cholesterol and total triglycerides
VIAL 5-7	(**"RESV"**)	excess serum in reserved or archived vials

6. **SHIPPING** (Section 14.8)

Use participant visit sample collection log to accumulate bar-coded labels of specimens that were actually prepared

Scan labels to create shipping manifest in the tracking system

Use specimen shippers to send to CDC Central Laboratory via Federal Express

Receive empty shippers from CDC within 5 days of receipt of specimens at Central Laboratory

Exhibit 14-3. CENTRAL LABORATORY REFERENCE RANGES FOR SUBJECTS AGES 61 - 86 YEARS FROM AREDS PLACEBO PARTICIPANTS (N=261)

Analyte/ Subjects (n)	Mean (SD)	5th Percentile	50th Percentile	95th Percentile
Retinol, µg/dL				
Men (105)	66 (15)	41	64	90
Women (156)	68 (16)	46	67	97
Vitamin E, µg/dL				
Men (105)	1500 (569)	873	1411	2358
Women (156)	1837 (674)	1041	1722	3493
Vitamin C, mg/dL				
Men (105)	0.77 (0.30)	0.36	0.74	1.27
Women (155)	1.01 (0.41)	0.35	1.04	1.60
Beta-carotene, µg/dL				
Men (105)	24.5 (14.9)	5	23.0	53.0
Women (156)	32.2 (25.8)	6	26.5	85.0
Lutein/zeaxanthin, µg/dL				
Men (105)	17 (9)	7	16	34
Women (156)	19 (9)	8	17	38
Beta-cryptoxanthin, µg/dL				
Men (105)	9 (6)	2	9	21
Women (156)	11 (7)	3	10	23
Lycopene, µg/dL				
Men (105)	19 (12)	4	17	41
Women (156)	16 (8)	6	15	31
Alpha-carotene, µg/dL				
Men (105)	5 (4)	1	4	14
Women (156)	6 (6)	1	5	17
Serum zinc, µg/dL				
Men (103)	84.0 (13.0)	63.0	83.0	109.0
Women (153)	79.6 (12.3)	64.0	78.0	101.0
Serum copper, µg/dL				
Men (103)	100.7 (20.4)	79.0	97.0	140.0
Women (153)	134.5 (31.6)	93.0	130.0	197.0

Exhibit 14-4. CENTRAL LABORATORY CLIA METHOD

Laboratory Account Number: 11D0668290
HCFA Analyte Code CLIA Number: 310 Routine Chemistry

ANALYTE: VITAMIN A/E/CAROTENOID PROFILE

MATRIX: SERUM

AS PERFORMED BY:

Inorganic Toxicology and Nutrition Branch
Division of Environmental Health Laboratory Sciences

CONTACT:

Dr. Eric J. Sampson, Director
Division of Laboratory Sciences
(770) 488-7950

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Serum concentrations of vitamins A (retinol) and E (α- and γ-tocopherol), two retinyl esters, and seven carotenoids are measured using high performance liquid chromatography with photodiode array detection. A small volume (100 μL) of serum is mixed with an ethanol solution containing two internal standards- retinyl butyrate and apo-8'-carotenal. The micronutrients are extracted from the aqueous phase into hexane and dried under vacuum. The extract is redissolved in ethanol and acetonitrile and is filtered to remove any insoluble material. An aliquot of the filtrate is injected onto a C18 reversed phase column and eluted with a gradient consisting of ethanol and acetonitrile. Absorbance of these substances in solution is linearly proportional to concentration (within limits), thus spectrophotometric methods are used for quantitative analysis. Three wavelengths, approximately corresponding to absorption maxima, namely, 300, 325, and 450 nm, are simultaneously monitored and chromatograms are recorded. Quantitation is accomplished by comparing the peak height or peak area of the analyte in the unknown with the peak height or peak area of a known amount of the same analyte in a calibrator solution. Calculations are corrected based on the peak height or peak area of the internal standard in the unknown compared with the peak height or peak area of the internal standard in the calibrator solution. Retinol and the retinyl esters are compared with retinyl butyrate at 325 nm, α- and γ-tocopherol are compared with retinyl butyrate at 300 nm, and the carotenoids are compared with apo-8'-carotenal at 450 nm.

Worldwide, vitamin A deficiency is the leading cause of preventable blindness. Although vitamin A deficiency is uncommon in the US, it is associated with excess morbidity and mortality from infectious disease in developing countries. Toxicity related to excess consumption of vitamin A can lead to permanent liver damage and death. Serum retinyl esters are of interest generally only in fasting specimens and are used to indicate potential hepatotoxicity in subjects with elevated serum retinol concentrations.

Vitamin E has low potential for toxicity. Elevated serum vitamin E concentrations are only of concern in people receiving anticoagulant therapy. Low serum concentrations are rarely observed, except in those with malabsorption syndromes.

A physiological need for the carotenoids, except as vitamin A precursors, has not been established. Excess consumption of carotenoids may cause red or orange discoloration of the skin as a result of carotenoid deposits in subcutaneous fat. Several xanthophylls are found in the macular pigment in the eye where they may protect against macular degeneration.

2. SAFETY PRECAUTIONS

All serum samples received for analysis must be considered potentially positive for infectious agents including HIV and hepatitis B viruses. **Universal Precautions** must be observed. Wear gloves, lab coat, and protective eyewear while handling all human blood products. [The hepatitis B vaccine series is recommended for all analysts working with blood, serum, or plasma.] Sample handling should be performed in a biological safety

cabinet, if available. Disposable plastic, glass, latex and paper items (pipette tips, autosampler inserts, gloves, etc.) that contact serum are to be placed in biohazard autoclave bags. These bags should be kept in appropriate containers until sealed and autoclaved. Periodically, wipe down all work surfaces with 10% bleach solution or an appropriate disinfectant solution such as ALL SAFE! (Momar, Inc., Atlanta, GA) when work is finished. Discard absorbent material used to cover work surfaces as often as needed. Organic solvents must be handled in a well-ventilated area, preferably in a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at either <<http://www.ilpi.com/msds/index.html>> or <<http://intranet.cdc.gov/ohs>>.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Management and calculation of the chromatography data are accomplished through Waters Millennium 32 software or Empower software installed on the personal computer connected via IEEE cable to the Alliance system. After a sample set ('run') is complete, the calculated amount data are exported to a floppy disk and to the network (I:\Instruments\Alliance) using a Millennium export method. The format is a single *.txt file. The *.txt is imported and transferred into a Microsoft Access database (I:\NHANESMain\). The bench QC data are reviewed by the analyst, along with other features associated with assay calibration and extraction recovery. Values that are outside of Critical Limits are scheduled for repeat analysis (with or without dilution). If the QC pools are within 2SD of the characterization mean, the data are ready to be submitted to the team leader. The team leader reviews the data in the Access Database on the NCEH/DLS Local Area Network (LAN) and prepares SAS data reports for each study or study segment. Either the supervisor, team leader, or analyst reviews replicate data and selects the appropriate final results. After the results are checked and integration or other corrections are made by the analyst, the supervisor approves the final values for release pending review and approval of the division statistician, Branch Chief and Division Director. The data are copied to a *.txt or *.xls file for release to the investigator. For long term studies, data are transmitted electronically during the course of the study. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file.

b. Files stored on the network are automatically backed up nightly by DLS LAN support staff. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a month onto a floppy disk or a CD-ROM using a CD writer.

c. Documentation for data system maintenance is contained in logs kept within the laboratory.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. For best results, a fasting sample should be obtained and care should be taken to avoid exposure of the serum to sunlight or other sources of full spectrum radiation.

b. Specimens for fat soluble vitamin analysis may be fresh or frozen. Serum should be harvested from blood collected in redtop or royal blue top Vacutainer brand tubes by standard venipuncture procedures.

c. A 500 μL sample of serum is preferable to allow sufficient material to conduct initial analysis and repeats, but a sample volume of as little as 100 μL may be analyzed.

d. The appropriate amount of serum is dispensed into a Nalgene cryovial or other plastic screw-capped vial labeled with the study and participant's ID.

e. Specimens collected in the field are frozen, and then shipped on dry ice by overnight mail. Frozen serum is stored at -70°C. Retinol and α-tocopherol are stable for at least 5 years at -70°C. The carotenoids are stable for 2 years at -70°C. The stability of the retinyl esters has not been determined. Sample quality may degrade with successive freeze-thaw cycles.

f. Specimens generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

g. Specimens which have been through more than five freeze-thaw cycles, which have been refrigerated for more than 24 hours, or which have undergone hemolysis may give inaccurate results for one or more of the primary analytes (i.e., retinol, α-tocopherol, or β-carotene). The retinyl ester concentration of non-fasting serum is generally non-informative.

h. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:\ITN\Nutrition Lab\CLIA). The document outlines the protocol for sample collection, transport of specimens, specimen storage, and the additional equipment required. In general, serum should be transported and stored at no more than 20°C. Generally, samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of serum should be transferred into a sterile Nalgene cryovial labeled with the study number, Astro number and SampleID.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. PREPARATION OF REAGENTS, CALIBRATORS, CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a. Reagent Preparation

(1) Mobile Phase. Ethanol (200 proof) and acetonitrile (HPLC grade) are filtered separately using 0.45 um pore size membranes (Cat. no. HVHP047; Millipore, Medford, MA). Triethylamine (TEA, 'Baker' grade) is added to each prior to filtering at 32 drops per liter using a glass Pasteur pipette to make 0.1% TEA solutions. The components are degassed and automated solvent blending takes place in the Alliance 2695 HPLC system.

(2) 10% Ascorbic Acid and 20% NaCl. 0.5 g L-Ascorbic Acid (ACS Certified) and 1.0 g NaCl (Analytical Grade) are dissolved in 5.000 mL deionized water in a 12 x 75 mm test tube. The ascorbic acid is relatively labile but normally can be used for 2 weeks or until there is a noticeable yellow color to the solution. The solution is capped with a disposable plastic cap, and stored at room temperature in yellow light. If there is significant headspace, flush with nitrogen or argon.

b. Standards Preparation

(1) Purified Stock Solutions. Stock solutions of all standards except retinyl butyrate, lutein, zeaxanthin, and apo-8'-carotenal are prepared using the following procedure:

A small amount of a standard is dissolved in chloroform. Using an autoinjector, 2-5 μL of the chloroform solution is injected on to a Burdick and Jackson OD5 octadecylsilane 15 cm x 4.6 mm, 5 μm particle size column and eluted with a 50:50 ethanol:acetonitrile mobile phase containing 0.1% TEA. The central third ("middle cut") of the peak is collected. Pooled middle cuts from several injections are diluted with mobile phase while maintaining minimal headspace. The absorbance of the solution is measured using a UV visible spectrophotometer. The concentrations are calculated based on the NHANES lab extinction coefficients (EC) listed in Table 1 (for SRM and NIST Round Robin exercises, NIST EC are used for retinol and retinyl palmitate). The resulting pooled solutions are diluted with mobile phase (50:50 ethanol:acetonitrile with 0.1% TEA) to approximately the concentrations shown in Table 1. Final working solution is aliqoted into prelabeled amber vials (National Scientific Co. part# C4012-2 12x32 mm). Lycopene is aliquotted into 500 μL vials (Sarated part# 72.730.009 cap# 65.716).

A small amount of retinyl butyrate is dissolved in ethanol. Using an autoinjector, 15-20 μL of the ethanol solution is injected on to a Luna C18 25 cm x 10.00 mm, 5μm particle size column and eluted with ethanol containing 0.1% TEA. The top three quarter of the peaks are collected. The absorbance of the pooled solution is measured with the spectrophotometer. The solution is diluted with 100% ethanol containing 0.1% TEA to obtain a solution with an absorbance of approximately 0.250 AU for retinyl butyrate at 325 nm.

Apo-8'-carotenal is purchased from Fluka (Cat.no.10810) at >98% purity. A small amount of standard apo-8'-carotenal powder is dissolved in 100% ethanol. Using an autoinjector, 30-40 μL of apo-8'-carotenal solution is injected on to a Luna C18 25 cm x 10.00 mm, 5μm particle size column and eluted with 100% ethanol containing 0.1% TEA. The top half of apo-8'-carotenal peak is collected. The absorbance of the pooled solution is measured with the spectrophotometer, and the solution diluted with 100% ethanol containing 0.1% TEA to obtain a solution with an absorbance of approximately 2.03 AU for apo-8'-carotenal at 450 nm. Approximately 17.85 mL of apo-8'-carotenal with AU = 2.03 is mixed with 500 mL of retinyl butyrate with AU = 0.25 to prepare an internal standard. The internal standard solution is stable for at least 24 weeks when blanketed with nitrogen or argon. The internal standard mixture is aliquoted into 20 mL glass vials with teflon lined caps (KIMBLE Glass Inc., Vineland, NJ. part # 74500-20) with minimal headspace. They are stored at -70°C freezer.

Lutein (Kemin Industries Reagent Grade) and Zeaxanthin (Extrasynthese) are separately diluted at 1 mg in 1.0 mL ethanol. Using a autoinjector, 25 μL of the ethanol solutions are injected on to a YMC C30 Carotenoid 15 cm x 4.6 mm column (cat# CT99S051546WT) and eluted with ethanol:acetonitrile (50:50) containing 0.1% TEA. The top third of the zeaxanthin and lutein peak is collected separately. The absorbance of the pooled middle cuts from several injections is measured with the spectrophotometer separately. The lutein solution is diluted with mobile phase to obtain a solution with an absorbance of approximately 0.0371 AU at 445 nm. Zeaxanthin solution is diluted with mobile phase to obtain a solution with an absorbance of approximately 0.160 AU at 454 nm.

TABLE 1. Extinction Coefficients Used to Calculate Concentrations of Standard Stock Solutions (NIST in ethanol unless otherwise indicated; NHANES in 50%:50% ethanol:acetonitrile or 100% ethanol)

Analyte	Extinction Coefficient* (dL/g·cm)	CDC Wavelength nm (NIST nm)	Target Concentration of Stock Solutions
Retinol	1850 CDC (1843 NIST)	325 (325)	50 μg/dL
Retinyl Palmitate	1850 CDC (975 NIST)	325 (325)	20 μg/dL**
Retinyl Stearate	1850 CDC	325	20 μg/dL**
γ-Tocopherol	91.4 CDC (91.4 NIST)	299 (298)	900 μg/dL
α-Tocopherol	75.8 CDC (75.8 NIST)	293 (292)	2110 μg/dL
Lutein	2550 CDC (2550 NIST)	445 (445)	17 μg/dL
Zeaxanthin	2540 CDC (2540 NIST)	454 (450)	63 μg/dL
β-Cryptoxanthin	2370 CDC (2356 NIST)	454 (452)	57 μg/dL
Lycopene (NIST in hexane)	3450 CDC (3450 NIST)	474 (472)	24 μg/dL
α-Carotene (NIST in hexane)	2725 CDC (2800 NIST)	447 (444)	36 μg/dL
β-Carotene	2560 CDC (2560 NIST)	454 (450)	23 μg/dL

*$A^{1\%}_{1cm}$ is defined as the theoretical absorbance of a 1 % solution (1g/100 mL) in a cell of 1 cm pathlength.
** Concentrations of retinyl esters are reported as retinol equivalents by the NHANES lab.

(2) Mixed Standards (Working Solutions)

A retinyl ester standard is prepared by mixing equal volumes of retinyl palmitate and retinyl stearate stock solutions using one volumetric pipette and rinsing the pipette with 50:50 ethanol:acetonitrile between each standard.

The vitamin E standard is prepared by mixing equal parts of α-tocopherol and γ-tocopherol using one volumetric pipette and rinsing the pipette with 50:50 ethanol:acetonitrile between each standard.

The carotenoid mixed standard is prepared by mixing equal volumes of α-carotene, zeaxanthin and β-cryptoxanthin using one volumetric pipette and rinsing the pipette with 50:50 ethanol:acetonitrile between each standard.

All working (mixed) standard solutions are stored in 1.8 mL μL aliquots in glass vials at -70°C and are stable for at least 24 weeks.

Retinyl stearate is synthesized using the following procedure:

To 1.0 g of retinol, add 10 mL of pyridine and 2.85 mL of anhydride in a round bottom flask. The reaction mixture is stirred overnight at room temperature (25 °C). When the reaction is complete, neutralize the retinyl ester and extract it from the pyridine solution by adding 100 mL of methylene chloride and 15 mL of dilute (5M) hydrochloric acid. Add 50 mL of 1M sodium hydroxide to neutralize any acid. Solvent layer is evaporated using Rotovap at 45 °C. The residue is chromatographed on a column of alumina grade III (or silica woelm 32-63) and the ester is isolated as a colorless material with greenish-yellow fluorescence.

Retinyl butyrate is synthesized using the following procedure:

To 1.0 g of retinol, add 4 mL of triethylamine (TEA), 40 mL of hexane, and 0.6 mL of butyric anhydride in a round bottom flask. The reaction mixture is stirred for 3.5 hours at 60°C. Hexane and TEA are evaporated using Rotovap at 45°C. Retinyl butyrate is isolated on the same column used to isolate retinyl stearate.

c. Preparation of Bench Quality Control (QC) Material

All serum pools are filtered through sterile gauze. One half gram of L-ascorbic acid per liter of serum is added and mixed overnight in a -4°C walk-in refrigerator. The serum is filtered through sterile gauze again prior to aliquotting into sterile 5-mL vials. Vials are blanketed with nitrogen or argon, and sealed. The QC pools are stored at -70°C for up to six years.

The low QC pool is prepared by selecting and blending sera that contain low levels of all analytes.

The medium QC pool is prepared by pooling sera that contain most of the analytes at levels close to the mean levels observed in normal subjects (NHANES III data).

The high QC pool is prepared by pooling sera that contain higher than normal levels of most analytes. Spiking is generally successful only for retinol. People who eat very large quantities of fresh fruits and vegetables and have high serum lipid concentrations are most likely to have high concentrations of fat-soluble micronutrients. In some instances dog serum, which typically has a high retinyl ester concentration, is added to the high pool. Other types of subjects useful for blending into the high pool are Type 2 diabetics who, in the absence of good glycemic control, may have high concentrations of lutein/zeaxanthin,

β-cryptoxanthin, and γ-tocopherol. Sera from individuals taking vitamin supplements are also used.

When preparing pools, filters that are hydrophilic should be used to minimize loss of the carotenoids and vitamin E during filtration. If the pool levels are unacceptably low, and spiking needs to be done, it is essential that the spiked, pooled sera be mixed overnight before the final filtration step.

d. Instrumentation

(1) Waters HPLC system (Waters Chromatography Division, Milford, MA)
  (a) Model 2695 Alliance system
  (b) Model 2996 or 996 photodiode array detector
  (c) Computer with the following specifications: Microsoft Windows 2000 Professional operating system, 1000 mHz, 260 MB RAM, 18 GB hard drive

(2) Cera column cooler 250 (Cera, Inc. Baldwin Park, CA) or equivalent

(3) Rack-type vortex mixer (American Scientific Products, McGaw Park, IL) or equivalent

(4) Cary 3E spectrophotometer (Varian Instruments, Palo Alto, CA) or equivalent

(5) Speedvac SC200 and SC210A Systems (Savant Instrument Co., Farmingdale, NY) or equivalent

(6) Precision Model VP-190 Direct Drive Vacuum Pump (Precision Scientific Inc., Chicago, IL) or equivalent

(7) Refrigerated vapor trap, model RVT-4104 (Savant Instrument Co., Farmingdale, NY) or equivalent

(8) Magnetic stirrer (American Scientific Products) or equivalent

(9) Digiflex Automatic Diluter/Dispenser, with 200-μL sampling and 2.0-mL dispensing syringes (Titertek Instruments, Inc., Huntsville, AL) or equivalent

(10) Gilson Microman positive displacement Pipettes (Gilson, Villiers-le, France) or equivalent

(11) Ranin Pipetman pipette (Ranin, Woburn, MA) or equivalent

e. Materials

(1) 15 cm x 4.6 mm Phenomenex Ultracarb 3 octadecylsilane, 3 μm particle size column (Phenomenex, Torrance, CA)

(2) 15 cm x 4.6 mm YMC C30 carotenoid column (YMC, Wilmington, NC)

(3) 15 cm x 4.6 mm Burdick and Jackson OD5 octadecylsilane 5μm particle size column (Burdick and Jackson Laboratories, Muskegan, MI)

(4) 25 cm x 10 mm Phenomenex Ultracarb 3μ m C18 column (Phenomenex, Torrance, CA)

(5) Hexane UV (Burdick and Jackson Laboratories, Muskegan, MI) or equivalent

(6) Acetonitrile HPLC grade (Burdick and Jackson Laboratories, Muskegan, MI) or equivalent

(7) Ethanol, absolute (U.S.P.), stored in glass, (Pharmco Products, Brookfield, CT) or equivalent

(8) Chloroform, spectrophotometric grade (Mallinckrodt, St. Louis, MO) or equivalent

(9) Triethylamine, 'Baker' grade (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent

(10) Retinol (Sigma Chemical Co., St. Louis, MO) or equivalent

(11) Retinyl Palmitate (Sigma Chemical Co., St. Louis, MO) or equivalent

(12) α-Tocopherol (Eastman Chemical Co, Kingsport, TN) or equivalent

(13) γ-Tocopherol (Sigma Chemical Co., St. Louis, MO) or equivalent

(14) Zeaxanthin (Extrasynthese S.A., Impasse Jacquard, B.P.62, 69730 Genay, France, Phone (33)78982034, FAX (33)7898l946, Telex 306231F) or equivalent

(15) Lutein (Kemin Industries, Des Moines, IA) or equivalent

(16) β-Cryptoxanthin (Extrasynthese) or equivalent

(17) Lycopene (Sigma Chemical Co., St. Louis, MO) or equivalent

(18) α-Carotene (Sigma Chemical Co., St. Louis, MO) or equivalent

(19) β-Carotene (Sigma Chemical Co., St. Louis, MO) or equivalent

(20) Trans-β-apo-8'-carotenal(Fluka Chemie AG Industriestrasse 25, CH-9471 Buchs, Switzerland) or equivalent

(21) L-Ascorbic acid, ACS grade (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent

(22) Stearic anhydride (Sigma Chemical Co., St. Louis, MO) or equivalent

(23) Butyric anhydride (Sigma Chemical Co., St. Louis, MO) or equivalent

(24) Triethylamine (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent

(25) Alumina, Grade III (obtained from various sources) or equivalent

(26) Methanol, HPLC grade, (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent

(27) Argon, Ultrapure (Air Products, Inc., Chamblee, GA) or equivalent

(28) Nitrogen (Air Products, Inc., Chamblee, GA) or equivalent

(29) 13x100mm disposable glass culture tubes (Corning Glassworks, Corning, NY) or equivalent

(30) Pipette tips for SMI Digitron pipette (American SMI, Emeryville, CA) or equivalent

(31) Plastic tuberculin syringes (obtained from various sources) or equivalent

(32) Serum extract filters, 0.45 μm pore size (cat. no. SJHV004NS, Millipore Corp., Bedford, MA) or equivalent

(33) Solvent filters, 0.45 um pore size (cat. no. HVHP047, Millipore Corp., Medford, MA) or equivalent

(34) 12x75 mm disposable glass culture tubes (Corning Glassworks, Corning, NY) or equivalent

(35) Autosampler vials and inserts (Wheaton Science Products, 12x31 mm widemouth vials (cat. no. TCW224626), 250 μL polypropylene inserts (cat. no. TCW225259), and screw caps with teflon/silicone septa (cat. no. TCW242762, Millville, NJ) or equivalent

(36) Actinic glassware (obtained from various sources) or equivalent

(37) Nunc vials (obtained from various sources) or equivalent

(38) Aluminum Foil (obtained from various sources) or equivalent

(39) Waters Guard-Pak Module (cat. no. WAT 88141) with Guard Pak filters (cat. no. WAT032472) or equivalent

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Before each run, duplicate sets of calibrators are prepared by combining 200 μL of the internal standard and 200 μL of working standard solutions using a positive displacement pipette. Thus, each calibrator contains half as much of each component as the working solutions (Table 2).

The standards are read as calibrators at the beginning of each run. These values are used to generate a one point, single concentration, linear, forced through zero, standard curve for each analyte. The calibrators are injected again at the end of the run and treated as unknowns by the processing software. As unknowns, their values must agree within 20 % of the calibrator values.

The Millennium 32 or Empower software performs all calculations. Calibration curves are linear, forced through zero, and based on single injection analysis of a single standard concentration. For each analyte not present in a standard solution, linkage to an appropriate standard is made and used to calculate concentrations.

The CDC laboratory participates in a proficiency testing program for retinol, retinyl palmitate, α- and γ-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, and α- and β-carotene sponsored by the National Institute of Standards and Technology (NIST, Gaithersburg, MD). Twice a year, Round Robin materials are sent by NIST to assess laboratory performance. At the same time, certified reference materials (SRM) for retinol, α-tocopherol and β-carotene, currently NIST SRM 968c (certificate of values kept by analyst), are analyzed to determine the agreement between results obtained with the CDC laboratory method and the certified values.

Table 2. Concentrations of assay standards

Analyte	**Stock Concentration** (purified unmixed)	**Working Concentration** (aliquoted frozen)	**Final Concentration** (plus internal standard)
Retinol	50 μg/dL ± 10%	50 μg/dL ± 10%	25μg/dL ± 10%
Retinyl Palmitate	20 μg/dL ± 10%	10 μg/dL ± 10%	5 μg/dL ± 10%
Retinyl Stearate	20 μg/dL ± 10%	10 μg/dL ± 10%	5 μg/dL ± 10%
α-Tocopherol	2110 μg/dL ± 10%	1055 μg/dL ± 10%	527.5 μg/dL ± 10%
γ-Tocopherol	900 μg/dL ± 10%	450 μg/dL ± 10%	225 μg/dL ± 10%
Lutein	17 μg/dL ± 10%	17 μg/dL ± 10%	8.5 μg/dL ± 10%
Zeaxanthin	63 μg/dL ± 10%	21 μg/dL ± 10%	10.5 μg/dL ± 10%
β-Cryptoxanthin	57 μg/dL ± 10%	19 μg/dL ± 10%	9.5 μg/dL ± 10%
α-Carotene	36 μg/dL ± 10%	12 μg/dL ± 10%	6 μg/dL ± 10%
Lycopene	24 μg/dL ± 10%	24 μg/dL ± 10%	12μg/dL ± 10%
β-Carotene	23 μg/dL ± 10%	23 μg/dL ± 10%	11.5 μg/dL ± 10%

Transfer the calibrators to autosampler vials and place them in the Alliance (20°C) as in Table 3.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

(1) Build Sample Sets using Millennium 32 or Empower Run Samples as described in section 8, part c. Participant ID numbers may be scanned into the computer if they are bar-coded.

(2) Allow frozen serum, quality control serum, working standards and the internal standard to reach ambient temperature but not to exceed 27°C.

(3) Prepare the mobile phase by adding 32 drops of TEA per liter of each solution, and by individually filtering the two components (acetonitrile and ethanol). Always prepare fresh mobile phase. Retention times shift when mobile phase picks up water from the room air.

(4) Turn on instrument as described in the instrument set- up section (section 8 c) of this document.

b. Sample Preparation

(1) Prepare one set of labeled 12 x 75 mm glass tubes and one set of labeled 13 x 100 mm glass tubes for the calibrators, pools and unknowns.

(2) Prepare a shallow (2-3'' deep) ethanol and dry ice bath for freezing samples.

(3) With an Eppendorf repeating pipette, or equivalent, dispense 10 μL of 10 % L-Ascorbic Acid/20 % NaCl solution into the bottom of each 13 x 100 mm glass tube.

(4) Using a micropipette, or equivalent, add 100 μL of serum to each tube containing the 10 % ascorbic acid/20 % NaCl solution. Cover the tubes with heavy duty aluminum foil and vortex for 60 sec.

(5) With the Digiflex Dilutor add 100 μL of the internal standard solution to each tube.

(6) Cover the tubes with foil and vortex the mixture for 60 seconds, being careful not to allow the liquids to touch the foil during mixing.

(7) With the Digiflex Dilutor, add 1.000 mL of hexane. Cover the tubes with foil and vortex for 60 seconds, being careful not to allow the liquids to touch the foil during mixing. Repeat this mixing cycle (60 sec) six times for a total of 6 min of extraction with hexane. For good recovery, it is important to mix as vigorously as possible without touching the foil (liquid should reach up to two-thirds of the tube while shaking).

(8) Allow the samples to rest for 10 min in order for aqueous and organic phases to separate.

(9) Carefully transfer the rack of tubes to the dry ice/ethanol bath, which is at least -70°C. Allow the aqueous phase to freeze (≥ 25 min). Pour the hexane (upper) layers into the second set of labeled 12x75 mm tubes and evaporate the hexane in the Speedvac (without heat) according to the number of tubes (9 minutes for 40 - 60 tubes, 10 minutes for 60 - 100 tubes, and 12 minutes for >100 tubes). Each Speedvac has slight differences in dry times, which each analyst has documented. Make sure not to over-dry the extracts.

(10) Add 50 μL of ethanol to the tubes containing the dried extracts.

(11) Vortex the tubes for 2 cycles of 60 seconds each.

(12) Add 50 μL of acetonitrile to each dissolved extract and vortex the tubes for 2 cycles of 60 seconds each.

(13) Draw each extract into a 1.0 mL tuberculin syringe taking care to leave an air space between the plunger tip and the solution, place a 0.45 μ pore-size syringe filter on the loaded syringe, and filter the extract into an autosampler vial.

(14) Cap the vials, tap the vial to remove any bubbles, and place them in the Alliance carousels at 20°C.

Table 3. Calibrators used in vitamin A/E/carotenoid assay contain the multiple components

Calibrators	Vial Position/Name
α-Carotene Zeaxanthin β-Cryptoxanthin Retinyl Butyrate Apo-8'-carotenal	97 / Carotenoids/Xanthophylls mixed
β-Carotene Retinyl Butyrate Apo-8'-carotenal	98 / β-Carotene
Lycopene Retinyl Butyrate Apo-8'-carotenal	99 / Lycopene
Retinol Retinyl Butyrate Apo-8'-carotenal	100 / Vitamin A
γ-Tocopherol α-Tocopherol Retinyl Butyrate Apo-8'-carotenal	101 / Vitamin E
Retinyl Palmitate Retinyl Stearate Retinyl Butyrate Apo-8'-carotenal	102 / Retinyl Esters
Lutein Retinyl Butyrate Apo-8'-carotenal	103 / Lutein

c. Instrument Preparation

(1) Load fresh mobile phase into reservoirs. Top off methanol in needle and seal wash reservoir.

(2) Turn on the Waters Alliance 2695. Set the refrigeration unit to maintain a temperature of 20°C in the autosampler compartment. Perform a wet prime for 5 mins at flow rate of 5 ml/min and a purge cycle at sample loop volume 6.0 mL.

(3) Turn on the Waters 2996 or 996 detector.

(4) Turn on the Cera column cooler 250 set at 25°C.

(5) Turn on the computer. Log onto Millennium 32 or Empower. Open the Project folder. Open Run Samples. Load the Instrument Method. Collect baseline absorbance data at normal operating flow (1.0 mL/min) and equilibrate with mobile phase (15% ethanol:85 % acetonitrile) for at least 1 hour.

(6) Load a method that contains the following parameters:

Acquisition Parameters for 2695 and 2996:

Run time: 30.0 min (usually)
Pump pressure limits: 20-3000 psi
Pump flow ramp: 3 min
Degas mode: continuous
PDA sampling frequency: 1.0 points per sec
PDA resolution bandwidth: 4.8 nm
PDA wavelengths: 270-480 nm
Data rate: 10.56 KB/min

Table 4. Waters 2695 gradient conditions

Time	Flow	% ethanol	% acetonitrile	Curve
	0.9	15	85	
8.0	0.9	50	50	9
8.5	1.0	50	50	6
18.9	1.0	50	50	6
19.0	1.5	15	85	6
29.0	1.5	15	85	2
31.0	0.0	15	85	6

Integration Parameters

The integration parameters will vary with lamp age, column characteristics and age, and other factors. In the component table (Table 5), all components are calibrated on the basis of height or area using a linear curve forced through the origin. The parameters in Table 5 are acceptable for use as a starting point from which to optimize the conditions. The retention times will vary with age of the column, from column to column, and from

instrument to instrument so those in the table should be taken only as a guide. Actual retention times for a given column/instrument combination should be determined individually, monitored on a regular basis, and the component table updated when necessary. Enter a manual response factor for *cis*–β-carotene that is equal to the calculated response factor for β-carotene under the same chromatographic conditions. Similarly, enter a response factor for α-cryptoxanthin that is equal to the calculated response factor for β-cryptoxanthin. NB: α-cryptoxanthin is tentatively identified as such and will not be reported until a positive identification is made.

(7) Edit the Sample Queue so that all of the samples are correctly identified and the appropriate vial numbers entered. Edit the calibrator concentrations in the Amount Table. The concentrations for the internal standards in all calibrator vials are entered as 1.00.

(8) Save the Sample Queue and the Sample Set Method.

(9) Start the run.

Table 5. Component Table Information. Retinyl butyrate is read as an internal standard at 300 nm (RB300) and 325 nm (RB325). Apo-8'-carotenal (Apo-8) is read at 450 nm.

Component Name	Retention time	Window	Channel	Quantitate	Internal Standard
Retinol	3.65	0.15	325	Yes	RB325
RB300	4.70	0.25	300	Int	
RB325	4.70	0.20	325	Int	
Lutein	5.19	0.29	450	Yes	Apo-8
Zeaxanthin	5.45	0.10	450	Yes	Apo-8
Apo-8'-carotenal	6.05	0.35	450	Int	
γ-Tocopherol	8.15	0.40	300	Yes	RB300
α-Cryptoxanthin	8.31	0.51	450	No	Apo-8
β-Cryptoxanthin	8.66	0.54	450	Yes	Apo-8
α-Tocopherol	8.66	0.40	300	Yes	RB300
trans-Lycopene	9.74	0.59	450	Yes	Apo-8
α-Carotene	12.41	0.30	450	Yes	Apo-8
β-Carotene	12.92	0.30	450	Yes	Apo-8
Retinyl palmitate	13.57	0.50	325	Yes	RB325
Cis-β-Carotene	13.52	0.25	450	Yes	Apo-8
Retinyl stearate	16.39	0.50	325	Yes	RB325

d. Maintenance

(1) Speedvac. The trap temperature is maintained between -110 °C to -97 °C and is checked daily before turning on the vacuum pump. The vapor trap of the Speedvac is emptied at least weekly when operating at full capacity. The vacuum pump oil is changed at least annually or when the quality of the vacuum deteriorates or the oil becomes turbid.

(2) HPLC System. Pre-column Guard-Pak inserts are changed monthly. Waters-specified preventive maintenance on the HPLC system is performed annually.

e. Calculations

(1) I.The Millennium 32 software and the Empower software performs all calculation. Calibration curves are linear, forced through zero, and based on single injection analysis of a single standard concentration. For each analyte not present in a standard solution, linkage to an appropriate standard is made and used to calculate concentrations.

(2) II.The concentration of the components of the mixed standards is equal to the concentration of the purified stock divided by the number of components of the solution, excluding the internal standards.

Example:

In a mixed standard containing zeaxanthin, β-cryptoxanthin, and α-carotene (3 components), if the concentration of the zeaxanthin in the purified stock solution is 64.0 μg/dL, then the concentration of zeaxanthin in the mixed standard (working concentration) is 64.0 μg/dL divided by 3, or 21.3 μg/dL. This concentration is entered in the amount table for zeaxanthin in RunSamples even though the actual concentration of zeaxanthin in the mixed standard is half of this after addition of the internal standards.

The following codes and integration parameters (called 'Metric' in the Access database; refers to the manner in which the baseline is drawn) are used for fat-soluble micronutrients in the Access database:

Final Quantitation Parameters:

Analyte	**Code**	**Metric**
α-carotene	ALC	Area
β-carotene	BEC	Area
cis-β-carotene	CBC	Area
β-cryptoxanthin	CRY	Height
γ-tocopherol	GTC	Height
lutein	LUT	Area
total lycopene	TLY	Area
retinyl palmitate	RPL	Height

retinyl stearate	RST	Height
trans-lycopene	LYC	Height
retinol	VIA	Area
α-tocopherol	VIE	Area
zeaxanthin	ZEA	Area

f. CDC Modifications

This method is a modification of a method described by Sowell et al. (1).

9. REPORTABLE RANGE OF RESULTS

This method is linear for the carotenoids in the range 1-150 μg/dL, for retinol and the retinyl esters in the range 1-150 μg/dL, and for α-tocopherol in the range 100-6000 μg/dL. The CVs for vitamins A and E and beta-carotene are generally less than 5 %. The CVs for the minor carotenoids are generally less than 20 %. Analysis is repeated on any sample for which either vitamin A, vitamin E, or β-carotene are outside of the normal range (Table 6).

10. QUALITY CONTROL (QC) PROCEDURES

a. Blind controls

For most studies blind controls are inserted prior to the arrival of the samples in Inorganic Toxicology and Nutrition Branch. Blind controls are prepared at two levels to emulate patient samples, including the use of labels identical to those used in the study.

Table 6. Updated Ranges for 98-99% of US population (NHANES III), μg/dL

VITAMIN A (4-9 yr)	19-63
VITAMIN A (10-19 yr)	24-84
VITAMIN A (20-49 yr)	25-103
VITAMIN A (50+ yr)	24-128
VITAMIN E (4-9 yr)	504-1479
VITAMIN E (10+ yr)	512-2875
β-CAROTENE	2-90
LUTEIN/ZEAXANTHIN	6-59
β-CRYPTOXANTHIN	1-33
LYCOPENE	3-56
α-CAROTENE	1-21
RETINYL ESTERS	1-18

b. Bench controls

Three serum pools are normally used as bench controls. These controls represent high, medium, and low levels of the analytes in serum. Duplicates of these pools are extracted exactly like patient samples and analyzed as part of each run. The run is considered 'out of control' based on the quality control scheme detailed below. The initial limits are established from the results of analyzing pool material in twenty consecutive runs and then are updated as needed.

Quality Control Scheme for HPLC Fat-Soluble Vitamin Analysis

The HPLC fat-soluble vitamins assay measures vitamins A and E and carotenoids. Each of these classes of nutrients is measured at a different wavelength. While there are some common characteristics, each class of analytes has distinct chemical properties and physiological functions. The following five analytes: retinol, α-tocopherol, lutein/zeaxanthin, lycopene, and β-carotene are generally present in significant amounts in most sera. These analytes are either required nutrients or have been associated with health effects in epidemiological studies. Much less is known about health effects associated with the other analytes. Most methodological problems with an analysis affect all analytes in a class in a similar manner, though not necessarily all classes of analytes.

Standard Shewhart QC charts are maintained for this internal QC specimen. A separate QC chart is to be maintained for each QC material used for bench QC specimen. Standard criteria for analyte rejection based on statistical probabilities are used to declare an analyte either in-control or out-of-control. These rules are:

Analytical run with 3 QC results:

1) If all 3 QC run means are within 2 S_m limits and individual results are within 2 S_i limits, then accept the run.
2) If 1 of the 3 QC run means is outside a 2 S_m limit - reject run if:
   a) Extreme Outlier - Run result is beyond the characterization mean ± 4 S_i **or**
   b)1 3S Rule - Run mean is outside a 3Sm limit **or**
   c)2 2S Rule - 2 or more of the 3 run means are outside the same 2 S_m limit **or**
   d)10 X_{bar} Rule - Current and previous 9 run means are on same side of the characterization mean
3) If one of the 6 QC individual results is outside a 2 S_i limit - reject run if:
   a) Extreme Outlier - One individual result is beyond the characterization mean ± 4 S_i **or**
   b) R 4S Rule - 2 or more of the within-run ranges in the same run exceed 4 S_w (i.e., 95% range limit) (Since runs have multiple results per pool for 3 pools, the R 4S rule is applied within runs only).

S_i = Standard deviation associated with an individual QC result; S_m = Standard deviation associated with a run mean; and S_w = Within-run standard deviation. These standard deviations as well as the QC pool mean are calculated during a characterization period after the measurement system has been optimized. Because some QC rules involve the use of multiple pools with different concentration and measurement error levels, individual QC results and run means are also transformed into standardized units by subtracting the characterization mean from the QC result or run mean and then dividing the resulting subtractand by the characterization S_i or S_m. When multiple QC pools are used these standardized results for all pools are displayed on a single standardized deviation chart along with the separate un-standardized QC charts for each pool.

A QC program written in SAS is available from the DLS Quality Assurance Officer. The program applies these rules to QC data and generates Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared out-of-control for that analyte as assessed by internal ("bench") QC.

If the blind QC is out of control, all or part of the run is declared out of control.

If the recovery of the internal standards from any sample is above or below three standard deviation from the mean recovery for the run, the sample is reanalyzed.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. **Chromatography** - Check the chromatography for faulty integration. Correct the integration if indicated. This is an uncommon event for controls, unless the column is new and the retention times/windows are not yet stable.

b. **Comment Codes -** Check sample preparation notes for comment codes indicating a spill or some other lab error involving the QC samples

c. **Pipetting** - If analytes are uniformly high or low in the QC, check fluid dispensing devices (Digiflex, pipettes) for accuracy. If not accurate, arrange for recalibration.

d. **Extraction** - Look for extraction problems by assessing the recovery of the internal standards in the calibrators, QC samples and unknowns. Isolated poor recovery suggests a sample spill. Poor recovery for the entire run suggests analyte degradation. Carotenoids are sensitive to air, light and heat. Was the room too warm? Were the samples at room temperature for longer than necessary? Were the samples dried in SpeedVac too long? Were the samples exposed to full-spectrum light for longer than necessary? Was the ascorbic acid antioxidant prepared fresh and added to the serum samples according to directions? Check the temperature log for the room and the current temperature. If the room was too warm when the samples were processed, and is still warm, call the building manager.

e. **Calibration** - Check the lot of calibrator used to quantitate the analyte that failed QC. Is it more than 6 months old? Check the chart showing peak height for that calibrator on the current column. Is there a trend showing decreasing peak height? You might need to schedule calibrator purification. Alternatively, the detector lamp energy may be inadequate. Run lamp diagnostics. Replace lamp about every 6 - 12 months. Check the column history for the number of injections run on that column. Is it above average? Are the smaller peaks no longer recognized? If so, you probably need a new column.

f. **Hardware** - Check to make sure that the HPLC system hardware is functioning properly. Make sure the pump is operating at the appropriate pressure with steady delivery. Check the autosampler and the run sheet to make sure the injections are being made as programmed. Look at vial volumes and puncture marks. Make sure that the run sheet reflects the actual vial sequence by cross-checking sample numbers. Check the temperature of the autosampler. If you suspect that the injection volumes are too variable (check the internal standard peak height in the calibrators), you should run a test of the reproducibility of the injection volume using a standard. Check the Instrument Malfunction Log for past hardware/software problems. Check the Delta Pressure Log. When the ripple pressure (delta) exceeds 2.5% of the baseline system pressure, the check valves in the pump probably need to be replaced.

g. **Freezer Log** - Check the freezer logs for stable temperatures for assay materials requiring -70°C storage. Obtain NIST SRM material if you suspect that your QC pools or calibrators may have degraded (or after a significant change in the system).

h. **Miscellaneous** - Many obscure problems can be corrected by re-booting the HPLC/detector system. Every time the system is re-booted, diagnostic checks are automatically made. The system should be re-booted at least once a week.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Phytofluene, a UV absorbing carotenoid (~maximum at 300nm) found in tomatoes, which has been found in some sera, co-elutes with retinyl palmitate at (~maximum at 325nm) and spills over from 300nm to 325nm. In a situation of elevated retinyl palmitate without elevation of other retinyl esters, the extent of interference can be subjectively determined by viewing the 300 nm chromatogram of the sera. The chromatography should be examined to determine if the peak maximum is higher at 300nm than at 325nm and at the same retention time. The peak maxima will typically be higher at 300nm than at 325nm if phytofluene is present. The palmitate value is unreliable in these samples.

b. Under conditions where the retention times for β-carotene and retinyl palmitate are similar, and β-carotene is elevated, the carotenoid absorption at 325 nm may cause overestimation of retinyl palmitate. The palmitate value is unreliable in these samples.

c. Ideally the column cooler should be at 25°C for 24 hours to allow the column to stabilize. The autosampler refrigeration unit needs approximately 45 minutes to stabilize. The lamp should have 1 hour to stabilize. The column should be under flow for at least 60 minutes before the first injection is made. In actual practice the system is only turned completely off if it will be idle for more than three days, except for the lamp, which is turned off when not in use.

d. The following substitutions may be made for the specified instrumentation:

(1) Instead of drying the hexane extracts with a Speedvac system, the samples may be dried under a stream of nitrogen without heating.

(2) Instead of the Waters 2695, two Waters 730 data modules may be used by setting the detector to 0.01 AUFS at 450 nm, 0.05 AUFS at 325 nm, and 0.02 AUFS at 300 nm and connecting channel 1 to pen 1 and channel 2 to pen 2 of one data module and channel 3 to pen 1 of the second data module. When this is done it is necessary to manually measure the peak heights or peak areas and calculate the concentrations and it is not possible to measure normal levels of retinyl esters.

e. All of the HPLC equipment is attached to line conditioners to minimize the effects of fluctuations of electrical current.

13. REFERENCE RANGES (NORMAL VALUES)

Reference ranges have not been established for retinyl esters in serum. We are using the values in Table 6 for the reference ranges for retinol, α-tocopherol, and the carotenoids. These values are approximately based on the 1-99 percentile ranges for 8,284 specimens analyzed for NHANES III.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Any sample with a vitamin A result that is <10 μg/dL or greater than the 99.5% age-specific limit (from NHANES III), or any vitamin A/retinyl ester profile that suggests hypervitaminosis A with hepatotoxicity, i.e. fasting serum with retinol elevated for age/sex and total retinyl esters > 40% of serum retinoids, is repeated and reported to the responsible party for the study (Project Officer) as soon as possible.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, the prepared samples are placed in the Alliance at 20°C. The unused portion of the patient specimen is returned to the -70°C freezer ASAP.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Since the analysis of serum for fat-soluble vitamins is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, then storage at $\leq$ -20°C of the extracted specimens is recommended until the analytical system is restored to functionality.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

For studies other than NHANES, where the survey physician is notified by Westat, the collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX or e-mail of any Vitamin A result that is < 10 μg/dL, or of any Vitamin A/Retinyl Ester profile that suggests hypervitaminosis A as determined by the supervisor. Test results that are not abnormal are reported to the collaborating agency at intervals and by a method determined by the study coordinator.

Data are transmitted via the Director of the Division of Laboratory Sciences, NCEH, CDC after review by the Section Supervisor, Branch Chief, and a CDC statistician.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If serum vitamin A analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab\AECarDocuments on the DLS LAN. We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

19. REFERENCES

1. Sowell AL, Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, β-carotene, trans-β-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. Clin Chem. 1994; 40(3): 411

Exhibit 14-5 Participant visit sample collection log

AREDS2 **NUTRITIONAL BIOCHEMISTRY STUDY LOG**

PARTICIPANT STUDY # | INITIALS | VISIT #

This log is completed each time a participant has blood drawn for the Nutritional Biochemistry Study.

This form is not key-entered into AdvantageEDC.

1. Date of specimen draw: _____/_____/_____
   mm dd yyyy

Place duplicate labels for all stored vials in the spaces below

2. Certification number of Phlebotomist:

3. Name of Phlebotomist: _____

AREDS2 Nutritional Biochemistry Study
Monthly Temperature Log

Investigator Name: _____ Site ID #: _____

Study Name/Type: A Multi-center, Randomized Trial of Lutein, Zeaxanthin, and Omega-3 Long-Chain Polyunsaturated Fatty Acids (Docosahexaenoic Acid [DHA] and Eicosapentaenoic Acid [EPA]) in Age-Related Macular Degeneration

IND #: 74,781

Month/Year →		**Material Stored →**	❑ **Plasma** ❑ **Serum**
Location →		**Temperature range →**	

Date	Day of Week	Temperature Reading	Recorder	Comment
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
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26				
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30				
31				

Instructions: Verify temperature in area indicated at least weekly. Send a copy of the log to the CDC with each shipment.