CHAPTER 03 - FOODBORNE BIOLOGICAL HAZARDS

SUBJECT:	**IMPLEMENTATION DATE**
Domestic Food Safety (FY 07-08) This program has completed a Good Guidance Practices clearance by CFSAN's ORP and OC/DFP/CPB in November 2006.	TBD **COMPLETION DATE** 11/9/08

DATA REPORTING

PRODUCT CODES	PRODUCT/ASSIGNMENT CODES
ALL HUMAN FOOD CODES	INSPECTION 03803 Foodborne Biological Hazards 04803 Chemical Contamination 09803 Food and Color Additives
USE APPROPRIATE PRODUCT CODES	SAMPLE COLLECTION AND ANALYSIS 03803B Filth 03803C Decomposition 03803D Microbiological 04803 Chemical Contamination 09803E Color Additives 09803F Food Additives
	OTHER 21005 NLEA

NOTE: Material this is not releasable under the Freedom of Information Act (FIOA) has been redacted/deleted from this electronic version of the program. Deletions are marked as follows: (#) denotes one or more words were deleted; (&) denotes one or more paragraphs were deleted; and (%) denotes an entire attachment was deleted.

NOTE: The allergen guidance previously provided as Attachment F has been removed. At such time as new allergen program guidance is established, it will be in a separate program. All allergen work ("for cause" and other) should continue to be reported under PAC 03803E.

FIELD REPORTS TO HEADQUARTERS
SUBMIT COMPLETED REPORTS TO:

A. Food and/ or Color Manufacturers Inspections

- Send copies of the entire Establishment Inspection Report (EIR) for only those inspections conducted at food additive manufacturers [Program and Project Structure (PPS) 09] which are classified as 'Official Action Indicated (OAI)' to:

CFSAN/ Compliance Programs Branch, HFS–636
5100 Paint Branch Parkway
College Park, MD 20740-3835

EIR's for color manufacturers should be submitted to CFSAN (at the above address). Documents may also be submitted via common carrier such as FedEx to CFSAN/Office of Cosmetics and Colors (OCAC) at the address below (this is not a post office mailing address):

CFSAN/ Office of Cosmetics and Colors (OCAC), HFS – 100
4300 River Road
College Park, MD 20740-3835

PROGRAM ASSIGNMENT CODE REPORTING

- Do not report any coverage of domestic fish or fishery products under this Compliance Program. See Compliance Program 7303.842, "Domestic Fish and Fishery Products Inspection Program". Use the appropriate PACs identified.

- Do not report any coverage of domestic cheese products for filth, decomposition, microbiological, phosphatase analysis or good manufacturing practices (GMP) inspections under this program. See Compliance Program 7303.037, "Domestic and Imported Cheese and Cheese Products Program". Use the appropriate PACs identified.

  NOTE: If cheese manufacturers are covered for incorrect application and storage of industrial/household chemicals and misuse of food/color additives, see Attachment B and C of this program. Resources for these areas will still be reported under the appropriate PACs listed in this Domestic Food Safety Compliance Program 7303.803.

- Do not report any coverage of Juice HACCP under this Compliance Program. See Compliance Program 7303.847 "Juice HACCP Inspection Program" for coverage of Juice HACCP. Use the appropriate PACs identified. Juice beverages (less than 100% juice) are not subject to Juice HACCP regulations and therefore will continue to be inspected under this Domestic Food Safety compliance program (CP 7303.803). However, 100% juice, and juice beverages for which the juice portion is manufactured at the same location as the juice beverage are subject to Juice HACCP and will be covered entirely under the Juice HACCP compliance program (CP 7303.847). All firms are currently subject to Juice HACCP and will be covered entirely under the Juice HACCP Compliance Program (CP 7303.847) for both HACCP and GMP violations.

- Refer to Compliance Program 7304.004, "Pesticides and Industrial Chemicals in Domestic Foods" for coverage of pesticides incurred during farming/growing of raw and finished agricultural product. In-plant misuse of chemicals, such as the problem of incorrect application and storage of industrial and household chemicals in food processing plants, warehouses, and transport vehicles, and coverage of food/color additives by the firm for all products, other than seafood and juice HACCP, are covered under this Domestic Food Safety Compliance Program 7303.803.

- See the following attachments for FACTS reporting requirements for sample collection and analysis:

Attachment A – Project 03 – Foodborne Biological Hazards
Attachment B – Project 04 – Chemical Contaminants
Attachment C – Project 09 – Food and Color Additives

PART I - BACKGROUND

This compliance program provides inspectional and analytical coverage of food products, in domestic commerce, for foodborne biological hazards, including unsanitary conditions (e.g., filth, and microbiological contamination), and decomposition, under Program and Project Structure (PPS) 03, adulteration with pesticides and industrial chemicals under PPS 04, and illegal use of color or food additives under PPS 09. This program also provides coverage of selected color manufacturers. A significant portion of the program's resources is devoted to inspections of firms producing products, which if abused, could be health hazards.

Inspectional and analytical resources for juice, cheese and seafood products are covered in the Juice HACCP Inspection Program (7303.847), Domestic and Imported Cheese and Cheese Products Program (CP 7303.037) and Domestic Fish and Fishery Products Inspection Program (CP 7303.842) programs, respectively. Domestic coverage of pesticides in raw agricultural products incurred during farming/growing is provided in “Pesticides and Industrial Chemicals in Domestic Foods”, (CP 7304.004). This (Domestic Foods Safety) program covers the problem of incorrect application and storage of industrial and household chemicals in food processing plants, warehouses, and transport vehicles for all products except seafood and juice.

PART II – IMPLEMENTATION

OBJECTIVE

To conduct food safety inspections of domestic establishments involved in the production, storage, and distribution of food and to document any deficiencies that are observed.

PROGRAM MANAGEMENT INSTRUCTIONS

When appropriate:

- Have FDA laboratory personnel, such as microbiologists, chemists (chemical, food additive, etc. problems), and entomologists accompany investigators during complex inspections.

- Districts should coordinate inspectional operations with the appropriate Federal, State, and Local agencies.

A. Program Priorities and Resources Utilization

Resources shown in the ORA Workplan can be used to cover foodborne biological hazards and chemical contaminants (PPS 03, 04, and/ or 09) as described in the Attachments A, B, and C, of this program. When determining how this resource pool should be expended, use the priorities listed below. The core inspection is to be reported under PAC 03803, however, inspectional deficiencies observed or suspected, or necessary follow-up, in the areas of pesticide application or food and color additive usage should be reported under PAC 04803 or PAC 09803, respectively.

1) High-Risk Foods

For more information on identifiers of high risk foods refer to the high risk food instructions as updated at #

The term “High Risk Foods” is used to denote foods that may present hazards, which FDA believes, may present a high potential to cause harm from their consumption. The firms that produce high risk foods have priority for inspectional purposes. #

It is important to recognize that the number of establishments that FDA identifies associated with these foods will always be an estimate because: 1) food manufacturing business is subject to growth, mergers and/ or failure; 2) the Agency establishes new rules and further instructions to reduce foodborne hazards which may expand existing programs; and 3) as underlying scientific information improves to help target the source of the hazard, the number and types of firms applicable to the high risk definition will change.

A number of the “High Risk” firms and products, such as soft cheese, seafood, and juice are covered under other Compliance

Programs. These programs should be consulted prior to inspections.

NOTE: Dairy Plants on the Interstate Milk Shippers (IMS) list that meet the "High Risk Foods" definition will be inspected by a Regional Milk Specialist or identified for state contract inspections as directed under the Milk Safety Compliance Program (7318.003) #. For interstate milk producers listed in the Interstate Milk Shippers (IMS) list of Grade "A" milk and milk producers, refer to "Memorandum of Understanding with the National Conference of Interstate Milk Shippers" (formerly CPG 7158.01).

2) Firms classified "Official Action Indicated" (OAI), one or more times during the past three (3) years. Also consider coverage of firms with a "borderline" compliance profile over this time period, some of which may or may not have been classified as "OAI" during this period.

3) Nonfat Dry Milk Products

NOTE: Inspect *ALL* operating dried milk product manufacturers not inspected by USDA or not on the IMS List.

Refer to "Memorandum of Understanding between USDA/ AMS and FDA concerning *Salmonella* in Nonfat Dry Milk Products," (formerly CPG 7155a.07) when considering coverage of nonfat milk producers. For interstate milk producers listed in the Interstate Milk Shippers (IMS) list of Grade "A" milk and milk producers, refer to "Memorandum of Understanding with the National Conference of Interstate Milk Shippers" (formerly CPG 7158.01). These documents can be obtained through the Federal Cooperative Agreements Manual, August 1996, which can be obtained through the Office of Regulatory Affairs (ORA), Office of Enforcement (OE), Division of Compliance Policy (HFC-230) at 301-827-0420.

Inspect firms participating in the USDA *Salmonella* Surveillance Program or Resident Inspection and Grading Program for problems other than *Salmonella*. Base the extent and frequency of surveillance on the firm's past sanitation history and violative sample record (e.g., antibiotics, phosphatase, pesticides, etc.).

Inspections of plants processing non–grade "A" milk and milk products, specifically ice cream, frozen desserts, and dried milk should be emphasized (see IOM 538 – Other Government Inspection). Plants targeted for coverage under this special emphasis should include:

- Milk processing plants that are NOT listed in the IMS list that receive or sell milk or milk product(s) interstate;
- Non – USDA inspected portions of USDA inspected plants;

Special Note: Any plant covered under a current special emphasis FDA Compliance Program (e.g., Domestic and Imported Cheese and Cheese Products, the Milk Safety Program and Grade "A" IMS listed plants) should be specifically **EXCLUDED** from coverage under this special emphasis initiative.

4) Firms covered by this program, other than warehouses, that do not fall under priority 1, 2 or 3, should be considered under the following criteria for coverage:

- Firms never inspected by the FDA or never inspected under the Domestic Food Safety Program should be given precedence over firms previously inspected or inspected under this program and classified as "No Action Indicated" (NAI).

- Violative history of the overall industry.

NOTE: The violative history of the overall industry will be summarized and issued periodically to the field by the Center.

5) Do not **routinely** conduct **surveillance** inspections of firms that are designated as establishment type "**warehouse**" (W), **only**. The Field will conduct follow–up inspections of warehouses that were classified "OAI" during the last inspection, or where intelligence indicates significant foodborne hazards.

B. Food Defense Measures and Food Registration

A Food Defense component should be included in all inspections conducted under this program. Reconciliation exams should also be covered (Please see IOM section 5.4.1.4.2)

C. Interaction with Other Programs

FDA staff should refer to the on-line FDA Compliance Program Guidance Manual Master List for the latest issuance of all CFSAN programs at:#

1. Milk Safety Program, CP 7318.003

Consult with the Regional Milk Specialist to determine if a dairy plant is on the IMS list prior to identifying this plant for inspection. Only those areas of a milk processing plant that process Grade A listed products should be excluded from coverage under this special initiave. Under the IMS agreement, regulatory inspection and enforcement action are the responsibility of state agencies for Grade "A" products, which are IMS listed. Grade A products that are not covered by the IMS, or Non–Grade A products are appropriate for FDA coverage. See IOM section 5.4.9.3 "Grade A Dairy Plant Inspections" for additional information

The USDA has two voluntary inspection programs for dry milk plants; the Plant Inspection Program (PIP) and The Resident Inspection and Grading Program. If a firm is operating in compliance with either of these two programs they will appear on the USDA's List of Dairy Plants Surveyed and approved for USDA Grading Service. Under MOUs with USDA (CPG #'s 7155a.01 and 7155a.07), FDA agrees not to (routinely) inspect dry milk product plants that are covered by one of the USDA inspection programs, or routinely sample dry milk for salmonella testing if the plant is operating under one of these two programs. See IOM section 3.2.1.5 #8 for additional information.

2. Domestic and Imported Cheese and Cheese Products Program, CP 7303.037. CP 7303.037 directs sampling of domestic and imported cheese for filth, decomposition, microbiological contamination and phosphatase, and good manufacturing practice (GMP) inspections of domestic cheese establishments.

   NOTE: Coverage of domestic cheese establishments for incorrect application and storage of industrial/ household chemicals and misuse of food/ color additives will still be reported under the appropriate PACs listed in this Domestic Food Safety program.

3. Domestic and Import NLEA, Nutrient Sample Analysis and General Food Labeling Program, CP 7321.005

   Coverage of the NLEA and FALCPA is to be accomplished during ALL routine Domestic Food Safety inspections of firms that are manufacturing and/ or labeling or re-labeling food products at the site inspected.

4. Domestic Fish and Fishery Products Inspection Program, CP 7303.842

5. Pesticides and Industrial Chemicals in Domestic Foods, CP 7304.004

   CP 7304.004 directs coverage of pesticides in domestic raw agricultural products incurred during farming/growing. The problem of incorrect application and storage of industrial and household chemicals in food processing plants, warehouses, and transport vehicles for all products except seafood and juice is covered under the Domestic Food Safety Program.

PART III – INSPECTIONAL AND PART IV - ANALYTICAL

To emphasize coverage of all relevant Program and Project Structure (PPS) areas during a food safety inspection, this program includes a separate Attachment for each project and analytical procedure not covered elsewhere.

Attachment A - PPS 03 - Foodborne Biological Hazards

Attachment B - PPS 04 - Chemical Contaminants

Attachment C - PPS 09 – Food Additives and Color Additives

Attachment D - Polymerase Chain Reaction (PCR) for *Vibrio cholerae* Enterotoxigenic Strains

Attachment E - Detection of *Shigella* by the Polymerase Chain Reaction (PCR)

PART V – REGULATORY/ ADMINISTRATIVE STRATEGY

A. Regulatory/ Administrative Follow-up

This Compliance Program addresses various food adulterations under section 402 of the Act. Food misbranding is covered under the "Domestic and Import NLEA Nutrient Sample Analysis and General Food Labeling Program" (C.P. 7321.005). Refer to Part II, C. Interaction with Other Programs.

Foods contaminated with harmful microorganisms and adulterated under 402(a)(1) should be removed from commerce either through voluntary recalls by the responsible firm(s) or FDA civil legal actions, such as seizures or injunctions. Further instructions on recalls and model class I press releases for these situations can be accessed at http://www.fda.gov/opacom/7alerts.html and http://www.cfsan.fda.gov/~lrd/inspect.html. Foods adulterated under other sub-sections of 402 should be handled similarly; however, districts should determine appropriate regulatory strategy depending on the situation. Districts should also review the Foods, Color and Cosmetics Chapter 5 of the Compliance Policy Guides for FDA compliance policy of foods, http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/default.htm.

Food defense observations should not be listed on form FDA-483, Inspectional Observations, unless they constitute deviations from Current Good Manufacturing Practice.

Adverse findings from a reconciliation examination should be immediately relayed by telephone to FDA Emergency Operations (301-443-1240) and CFSAN Emergency Coordination and Response Staff (301-436-2700). The findings will be evaluated on a case-by-case basis.

Enforcement action recommendations should be submitted directly to Division of Enforcement via the "Compliance Management System" link located on Inside FDA's IT Applications Page under CFSAN Applications # prior to issuance of any Warning Letter. Contact one of the Regulatory Contacts from the Domestic Branch for early discussion concerning seizures, injunctions, or prosecutions under this program:

- Donald Greaves at (301) 436-2057 or Donald.Greaves@FDA.HHS.GOV
- Dwayne Johnson at (301)436-1782 or Dwayne.Johnson@FDA.HHS.GOV

Districts are also encouraged to provide early alerts on potential legal action recommendations to the Branch Chief, Domestic Branch (HFS-607), William Correll at (301)436-1611 or by e-mail at William.Correll@FDA.HHS.GOV.

If a transport vehicle is shown to be the source of contamination, a Warning Letter may be considered. If further action is necessary, contact CFSAN/ Division of Enforcement/ Domestic Branch regulatory contacts identified in Part VI of this Compliance Program.

In reference to a carrier company and adulterated food, refer to CPG

7117.08 Foods, Rail Car Sanitation – Adulteration, section 545.300 at http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/cpg545-300.html.

Refer, as necessary, samples showing non-certified colors or labeling violations to the home district of the color manufacturer.

When unsanitary conditions are encountered in a warehouse containing USDA-donated surplus foods, refer to FMD #126 (Sanitation Problems in Warehouses Storing USDA - Donated Surplus Foods) (http://www.fda.gov/ora/inspect_ref/fmd/fmd126.htm).

B. **Compliance Achievements**

Encourage responsible individuals to correct deficiencies during an inspection. Explain the significance of the observations. Verify corrections made during the inspection. Ask management to notify the district in writing of additional corrections they intend to take, including the time frame for completion of corrections. Report compliance achievements/ voluntary corrections into FACTS using the Compliance/Compliance Achievements screens after the corrections have been certified (i.e., by written format or physical observation of correction).

C. **Federal/ State Relations**

Contracts exist with many states to inspect food establishments. This program is incorporated by reference in these contracts as further instructions in conducting sanitation inspections. Some states may also inspect firms where there are greater risks as identified in this program. State inspection assignments should follow the priorities in this program and should be coordinated with the districts selection of firms for FDA inspections. The selection of Dairy Firms should be coordinated with the Regional Milk Specialist.

PART VI – ATTACHMENTS, REFERENCES AND PROGRAM CONTACTS

A. ATTACHMENTS

1. Attachment A – Project 03 – Foodborne Biological Hazards
2. Attachment B – Project 04 – Chemical Contaminants
3. Attachment C – Project 09 – Food Additives and Color Additives
4. Attachment D – Polymerase Chain Reaction (PCR) for *V. cholerae* Enterotoxigenic Strains
5. Attachment E – Detection of *Shigella* by PCR

B. REFERENCES

Refer to the on-line FDA Compliance Program Guidance Manual Master List for FDA Staff for the latest issuance of all CFSAN programs @ #

FDA Investigations Operations Manual, most current issue, http://www.fda.gov/ora/inspect_ref/IOM/default.htm

"FDA's Catalog of Information Materials for the Food and Cosmetics Industries" is available from CFSAN/ Office of Constituent Operations/ Industry Activities Staff, HFS–565, (301) 436–1727.

DFI Inspections Guides, available hard copy from DFI, 301-827-5639, or #

Compliance Policy Guides, http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/default.htm

C. PROGRAM CONTACTS

General:	Monali Yajnik, Office of Compliance, Division of Field Programs, Compliance Programs Branch, HFS–636, (301) 436–1616, Fax: (301) 436–2657, Monali.Yajnik@FDA.HHS.GOV
Center Filth Analysis:	Michael McLaughlin (Acting), Office of Plant and Dairy Foods, Division of Natural Products, Micro-analytical Branch, HFS–345, (301) 436–1958, Michael.Mclaughlin@FDA.HHS.GOV
Center Phosphatase Analysis:	George C. Ziobro, Office of Plant and Dairy Foods, Division of Natural Products, Micro-analytical Branch, HFS–315, (301) 436–1965, George.Ziobro@FDA.HHS.GOV
Center Microbiological Analysis:	Keith Lampel, Office of Plant and Dairy Foods, Division of Microbiological Studies, HFS–515, (301) 436–2007, Fax: (301) 436–2644, Keith.Lampel@FDA.HHS.GOV
CFSAN Methods Contact:	Peter Feng (*E. coli* O157:H7), Office of Plant and Dairy Foods, Microbiology Methods Research Branch, HFS-515, and (301) 436–1650, Peter.Feng@FDA.HHS.GOV

	Wallace Andrews (*Salmonella*), Office of Plant and Dairy Foods, Microbiology Methods Research Branch, HFS–515, (301) 436–2008, Wallace.Andrews@FDA.HHS.GOV
	Reginald Bennett, (*Staphylococcus)* Office of Plant and Dairy Foods, Division of Microbiological Studies, HFS–515, (301) 436–2009, Fax: (301) 436–2644, Keith.Lampel@FDA.HHS.GOV
	Keith Lampel (*Shigella*), Office of Plant and Dairy Foods, Division of Microbiological Studies, HFS–515, (301) 436–2007, Fax: (301) 436–2644, Keith.Lampel@FDA.HHS.GOV
	Mahendra Kothary (*V. cholerae*), Office of Science, HFS–237, (301) 827-8616, Mahendra.Kothary@FDA.HHS.GOV
	William L. Payne (ETEC and *V. Cholerae* PCR), Office of Science, HFS–237, (301) 827-8623, William.Payne@FDA.HHS.GOV
	Anthony Hitchins (*Listeria*), Office of Plant and Dairy Foods, Microbiology Methods Branch, HFS–516, (301) 436-1649, Anthony.Hitchins@FDA.HHS.GOV
Center Chemical Contaminants Analysis:	Steve Capar, Office of Plant and Dairy Foods and Beverages, Division of Pesticides and Industrial Chemicals, HFS–335, (301) 436–2003, Steve.Capar@FDA.HHS.GOV
Center Food Additives Analysis:	Gregory Diachenko, Office of Food Additive Safety, Division of Chemistry Research and Environmental Review, HFS–245, (301) 436–2387, Gregory.Diachenko@FDA.HHS.GOV
Center Color Additives Analysis:	Alan Scher, Office of Cosmetics and Colors, Division of Science and Applied Technology, Color Technology Branch, HFS–126, (301) 436-1139, Alan.Scher@FDA.HHS.GOV
Regulatory:	Donald Greaves, OC/ DB, HFS–607, (301) 436–2057, Donald.Greaves@FDA.HHS.GOV
	Dwayne Johnson, OC/ DB, HFS-607, (301) 436-1782, Dwayne.Johnson@FDA.HHS.GOV
Investigations:	Barbara Marcelletti, Division of Field Investigations, Investigations Branch, HFC–132, (301) 827–5635,

	Barbara.Marcelletti@ORA.FDA.GOV
ORA Analytical Contacts (Microbiology):	Marsha Hayden, Division of Field Science, HFC-141, (301) 827-1039, Marsha.Hayden@ora.fda.gov
	Lydia Rosas-Marty, Division of Field Science, HFC-141,(301) 827-6624, Lydia.Rosas-Marty@ora.fda.gov
	Carl Sciacchitano, Division of Field Science, HFC-141, (301) 827-1028, Carl.Sciacchitano@ora.fda.gov
	Atin Datta (PFGE) , Division of Field Science, HFC-141, (301) 827-1030, Atin.Datta@ora.fda.gov
ORA Analytical Contact (Filth):	Helen Jones, Division of Field Science, HFC-141, (301) 827-1233, Helen.Jones@ora.fda.gov

PART VII – CENTER RESPONSIBILITIES

PROGRAM EVALUATIONS

The Director, Office of Plant and Dairy Foods (OPDF) has the responsibility to prepare periodic formal evaluations of this compliance program. (%)

DOMESTIC FOOD SAFETY PROGRAM

PROJECT 03 – FOODBORNE BIOLOGICAL HAZARDS

OBJECTIVE

To emphasize coverage of all relevant Program and Project Structure (PPS) areas during a food safety inspection. This attachment covers filth, decomposition and microbiological contamination.

FACTS REPORTING REQUIREMENTS:

- Report resources utilized for filth, decomposition, microbiological contamination into the FACTS as appropriate using the following Program Assignment Codes (PAC) and Problem Area Flags (PAF). Use the appropriate secondary PAF when needed:

PAC	PAF	Problem Area	Sub-PAF	PAF Description
03803B (Filth)	FIL	ANI	--	Animal Filth
		ART	--	Arthropod Filth
		BIR	--	Bird Filth
		EXH	--	Filth Exhibit
		FOR	--	Foreign material
		OTH	--	Other, Not Listed, Identify in Remarks
03803C (Decomp)	FIL	DEC	--	Decomposition
		EXH	--	Filth Exhibit
		GEO	--	Geotrichum Mold
		HMC	--	Howard Mold Count
		MAC	--	Macroscopic Mold
		YEA	--	Yeast
		OTH	--	Other, Not Listed, Identify in Remarks
03803D (Micro)	MIC	MIC	--	Microbiological analysis (included rapid test kits for micro analysis)
		PHOS	--	Phosphatase analysis
		ANT	SAL	Salmonella serotyping
		ANT	ABR	Antibiotic resistance testing

- Refer to page 2, Program Assignment Code Reporting, of this Compliance Program for more information regarding reporting requirements.

B. Inspectional Instructions:

1. Inspectional

A top priority of the Agency is to decrease foodborne illness caused by microbial contamination. Use the following as directions for conducting inspections of firms manufacturing products susceptible to microbial contamination.

- **Care should be taken during the inspection to fully identify sources of and possible routes of contamination to the product.** For example, report the number of flies and times when they were on the product, open doors or damaged screens providing the flies a route to a toilet or to the outside, and the specific distances to animal feces, garbage or decaying animals. If a swab is taken of a floor drain, report if any flies that landed on that drain or splash from that drain go to the finished food or food contact surfaces. In addition, depending on the nature of the adulteration, in–line bacteriological sampling may be indicated, For further instructions on conducting inspections involving microbial contamination, refer to the current edition of the Investigations Operations Manual (IOM) (http://www.fda.gov/ora/inspect_ref/iom/), Sections 5.4.7.2, "Inspectional Guidance for Firms Producing Products Susceptible to Contamination with Non – Pathogenic or Pathogenic Microorganisms", and 4.3.7.7, "Products Susceptible to Contamination with Pathogenic Microorganisms" for sampling instructions.

- Product(s) covered should be selected based on health risk and seriousness of objectionable conditions observed. However, within reason, the investigator should select as many products as necessary to establish the firm's overall compliance picture.

- Cover railcars or trucks that are off–loading or being loaded to insure food products are shipped in compliance. When the condition of a transport vehicle may lead to food contamination, attempt to dissuade the shipper from using it. If unsuccessful notify the destination District so they may follow–up. See PART V Regulatory/ Administrative Strategy of the "Domestic Food Safety Compliance Program" for more information.

  For firms that receive, via tanker trucks, product components such as liquid egg-, dairy-, soy-, corn - or sugar- syrup based products (e.g., products that can support microbial growth), and are producing food products that are susceptible to microbial contamination that would not normally be cooked or prepared in a manner that would be lethal to harmful microorganisms [Category I and II foods of IOM "Sample Schedule Chart 1" {IOM Chapter 4}, check to see that controls are in effect to prevent contamination of the finished products. These controls would consist of microbial testing or heat-treating to affect a 5-log kill of the incoming product component(s) or of the finished product(s), use of dedicated tankers for incoming food components, etc. If such controls are not in place, collect samples of the incoming and/ or finished product for pathogens causing foodborne illness testing.

- Conduct follow–up inspections for all positive findings of

pathogenic microorganisms in a processed food product.

Before beginning a follow-up inspection, the District should discuss plans for the inspection with CFSAN/ Division of Enforcement to ensure that evidence/samples obtained will support further regulatory action. Depending on the nature of the adulteration, in–line bacteriological sampling may be indicated. In addition to analyzing in–line and finished product samples for the specific microorganisms whose association with the firm's product generated the inspection, the product should also be analyzed for microorganism such as *E. coli*, which could be an indicator of the plant's potentially unsanitary condition(s), and *Staphylococcus aureus*, which could be an indicator of possible temperature abuse of products made by hand.

- Follow-up activities associated with "import for export" articles are to be conducted during domestic inspections when the inspection is conducted at a manufacturer's facility where an imported article is being further processed or incorporated into a product intended for exportation. Refer to the Regulatory Procedures Manual, Chapter 9, Import for Export, Follow-up section. See IOM section 5.1.1.14.

2. Sample Collection

Detailed instructions for sample collection, including collection technique, aseptic sampling, field examination, selective sampling, in-line sampling and sampling of products susceptible to contamination with pathogenic organisms is contained in IOM Subchapter 4.3. See also IOM sample schedule chart 1 and applicable DFI Inspectional Guidance Documents, # for applicable sample size. If you are unsure of the proper sample size, call your servicing laboratory listed in the current ORA Field Workplan, Appendix III, "Servicing Laboratories".

A. Filth/ Unsanitary Conditions

- Collect raw materials, in-line samples, and finished product samples if contaminated raw materials were believed used.

- Do not sample finished products to support contamination in the finished product if inspectional evidence indicates little likelihood of detecting this contamination in the finished product. Instead, collect physical samples of contaminated raw material(s) and/or exhibits with documentation that the finished product moves interstate. Fully document the use of these contaminated raw materials in the manufacture of a specific lot or lots of finished products.

  The Center will consider cases based solely on detailed observations, photographs, etc., which would convince the ordinary consumer that a condition or practice is filthy). Obtain physical samples of raw materials, in-process sub-samples, and other items such as filth exhibits showing routes of contamination to clearly show that conditions of manufacture or storage are such that there is a reasonable possibility that contamination may have occurred.

  Obtain swab samples (i.e., environmental) of surfaces that are likely to come in contact with food if there is a suspicion that

they might contaminate the food with pathogenic organisms such as *Listeria monocytogenes*.

B. Microbiological

- Collect samples for microbiological analyses only if:

  1) The firm has a previous history of microbiological contamination (e.g., follow – up to illness or injury, complaint, recalled/ seized product, previous inspectional history, etc.)

  or

  2) Sampling is conducted "for cause" during an inspection (e.g., inspectional observations warrants collection for microbiological analyses).

- General Information

  Pay particular attention to the presence of potential vectors of microorganisms. Some of these vectors would be flies, roaches, and other filth insects. Also document the source of filth such as the presence of cats and dogs and their feces. Document unsanitary conditions outside of the plant.

- Nonpathogenic Microorganisms

  See the appropriate Sections of the IOM, Chapter 4 and # for sampling instructions. If a CPG does not exist and there is no reason to suspect contamination with pathogens, consider regular filth cases if conditions indicate abuse of susceptible products/raw materials. Include observation concerning organoleptically detectable filth or decomposition and time/ temperature parameters. Refer to IOM (Section 4.3.9) and (http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/default.html) for specific information regarding filth and specific commodities.

- Pathogenic Microorganisms

  See the current edition of the IOM, Chapter 4 for sampling instructions and # for sample size. For Salmonella, see IOM "Sample Schedule Chart 1" {IOM Chapter 4}. If unsure of sample size, call your servicing laboratory.

- Animal Drug Residue

  When inspecting firms that produce dry milk products (including dry whey, a by-product of cheese manufacturing), determine if the firm tests for animal drug residue. Review the firm's procedure to assure proper operation of analytical equipment, both mechanical and biological, used in the analysis. If the firm tests for animal drug residue, review the quality control records. (Antibiotic contamination may be seasonal because the heaviest usage in animals occurs in late winter and spring.) If animal drug residue is being found or testing is not performed on every lot, collect a sample.

Sample size: # retail units of at least 120 grams (4oz.) each if the sample is being analyzed for animal drug residue only. If the sample is also being analyzed for phosphatase, collect # retail units of at least 120 grams each in triplicate. If it is necessary to sample from bulk, use aseptic sampling techniques.

- Phosphatase

  Pasteurization of milk products destroys organisms that cause serious diseases (tuberculosis, Q fever, etc.) and inactivates the enzyme phosphatase. Measurement of phosphatase activity indicates that some or all of the milk used may not have been properly pasteurized. Phosphatase testing is to be performed only on fluid milk, dried milk products, ice cream and cheese – not on any cultured dairy products.

  Sample size: as directed under "Animal Drug Residue"

3. Sample Submission

   Refer to the current ORA Field Workplan, Appendix III, "Servicing Laboratories", for field servicing laboratories.

C. Analytical Instructions

Conduct and confirm analyses in field laboratories. Some confirmations or speciations must be performed at Headquarters. Use the methodology, special instruction or sample preparation cited below as necessary for each type of analysis.

1. Filth, Mold and Foreign Objects: Microscopic and Macroscopic

Methodology:	AOAC, 17th Ed. or most current Ed., Chapter 16 Extraneous Materials: Isolation JAOAC {Interim Official First Action Methods} Macroanalytical Procedures Manual (MPM), FDA Technical Bulletin, No. 5 Laboratory Information Bulletins (LIBs)
Comments:	For Identification (as needed) submit to CFSAN's Division of Natural Products, Microanalytical Branch, HFS–345, Douglas L. Park, (301) 436–2401

2. Decomposition

Methodology:	Organoleptic; AOAC, 17th Ed. or most current Ed., for chemical indices; mold counts, etc.
Field Laboratories:	Refer to the current ORA Field Workplan, Appendix III, "Servicing Laboratories".
Comments:	Consult ORO/DFS, HFS–140 for servicing laboratories, if necessary

3. Microbiology

Methodology:	Bacteriological Analytical Manual (BAM), 8th Ed., Revision A, 1998 or most current Ed. (http://www.cfsan.fda.gov/~ebam/bam-toc.html); AOAC, 17th Ed. or most current Ed., Chapter 17 "Microbiological Methods".
Comments:	Final identification (if needed) submit to CFSAN/ Office of Plant, Dairy Foods and Beverages/ Division of Microbiological Studies, HFS-515. Reginald Bennett at (301) 436 - 2009

- ***E. coli* and coliform detection** [See Ch. 4, eBAM Section I) (http://www.cfsan.fda.gov/~ebam/bam-toc.html)].

  Alternatively, LST MUG method (Ch. 4, eBAM Section II) may be used to examine for coliforms when both *E. coli* and coliform analysis are required in **chilled and frozen foods ONLY and exclusive of bivalve molluscan shellfish**. The presumptive test for coliforms can be performed in conjunction with the test for *E. coli* by preparing tubes of LST – MUG medium with gas tubes (i.e., using the LST MUG for both *E. coli* and coliform determination).

- **Enterotoxigenic *E. coli* (ETEC), Gene Probe**

  Do not perform ETEC analysis unless *E. coli* is greater than or equal to 10,000 / gram.

  Method: eBAM, Ch. 24, "Identification of Foodborne Bacterial Pathogens by Gene Probe", "Enterotoxigenic E. coli", (http://www.cfsan.fda.gov/~ebam/bam-toc.html)

  PRL-SW will provide radioactive probes for ETEC. The laboratory contact is Michael Kawalek at 949-608-3505.

- **Enterohemorrhagic *E coli* (*E coli* O157:H7, EHEC) – Individual Sub–samples.**

  Remove 25g from a sub–sample then add 225ml of EHEC Enrichment Broth (EEB) and place into a sterile blender. Blend (10,000 – 12,000 rpm for 60 seconds at a minimum). Include positive and negative controls. See eBAM, Ch. 4A Section M and N (http://www.cfsan.fda.gov/~ebam/bam-toc.html)

Incubate with vigorous shaking for 24h (i.e., 130–140rpm on an orbital shaker at 37°C. Proceed with the method as described in eBAM, Ch. 4A Section O http://www.cfsan.fda.gov/~ebam/bam-toc.html).

Positive *E. coli* O157:H7

Submit *E. coli* 0157:H7 isolates for Pulse Field Gel Electrophoresis (PFGE) assay to respective servicing laboratories.

- ***Shigella***

PCR primers and the positive control will be supplied to each District. Contact Keith Lampel, CFSAN's scientific contact for *Shigella* at (301) 827-8617 for primers and positive control.

Shigella will be done on a composite basis (i.e., 2 composites per sample). Each composite for _Shigella_ analysis will consist of 250mL.

Prepare each composite by removing 50mL from each of five (5) sub–sample rinses into a sterile beaker/flask and mix thoroughly.

Remove 100mL (of each composite) and place it into centrifuge tubes and spin at 2000rpm for 3 minutes to pellet plant material. Then follow methodology as outlined in Attachment E, "Detection of *Shigella* by Polymerase Chain Reaction".

This method is to be used as presumptive evidence and confirmation for the presence of *Shigella*. Retain the PCR products in the event that DNA sequencing is required. In this case, send the remaining PCR product to Keith Lampel, the CFSAN Scientific contact for *Shigella*.

- ***Listeria***

General Method

BAM 8th Ed., Revision A, or most current Ed., Ch. 10 "*Listeria monocytogenes*", pages 141 – 151 and Ch. 11, "Serodiagnosis of *Listeria monocytogenes*", pages 153 – 160 (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

SAFETY PRECAUTIONS: Media preparation for *L. monocytogenes* directs the use of cycloheximide, which is an extremely toxic chemical, and acriflavine, which is a powerful mutagen (use caution).

Since the *L. monocytogenes* method gives the option of using α-naphthol, do not use α-naphthylamine. All analysts should take extreme safety precautions when handling these chemicals (e.g., weigh in a containment hood free of drafts; wear gloves and face masks). Those

laboratories with pesticide capabilities should take additional precautions against possible contamination, as cycloheximide is a fungicide.

Compositing/ Sample Preparation Instructions

Listeria analysis will be done on ready–to–eat food products that require none or minimal processing (no adequate kill step by the preparer).

The analysis will be conducted on composite basis **ONLY** (e.g., analyze two (2) composites per sample).

This includes all follow–up samples collected based on an initial positive finding (if appropriate).

Use the following procedure for preparing each composite:

6-subs/ sample – Remove 83.3g from each of three (3) sub-samples. Each composite size is 250g.

10-subs/ sample – Remove 50g from each of five (5) sub-samples. Each composite size is 250g.

Once the two composites have been prepared, remove 25mL or g from each composite for analysis. Mix the 25mL or g with 225mL *Listeria* enrichment broth.

Incubate the enrichment broth (EB) mixture for a total of 48hrs at 30°C. Proceed with the method in the BAM 8th Ed., Revision A, or most current Ed. Ch. 10, page 10.04, section D "Isolation Procedure" (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

NOTE: IF THE SAMPLE IS TO BE ANALYZED FOR BOTH *LISTERIA* AND *SALMONELLA* THEN COMPOSITE SUB-SAMPLES FOR *SALMONELLA* AS OUTLINED IN BAM 8TH ED., REVISION A, OR MOST CURRRENT ED. CHAPTER 1, PAGE 3, THEN RANDOMLY SELECT TEN (10) SUB-SAMPLES FROM THE ORIGINAL SAMPLE TO PREPARE THE TWO COMPOSITES FOR *LISTERIA* ANALYSIS AS OUTLINED ABOVE.

Isolation and Identification: BAM 8th Ed., Revision A, or most current Ed. Ch. 10 and 11 (http://www.cfsan.fda.gov/~ebam/bam-toc.html). Additionally, Rapid Test Kits mentioned in the July 9, 1998 memo, "Guidance for the Use of *Listeria* Rapid Methods for Food Microbiology", may be used #. If the laboratory does not have a copy of the memo, they should request a copy from the Division of Field Science, HFC–140.

Enumeration

Perform using the cultural method.

Cultural Enumeration Method: This can be readily accomplished by the enumeration method in the current version of BAM 8th Ed., Revision A or most current Ed., Ch. 10, (http://www.cfsan.fda.gov/~ebam/bam-10.html). The Simultaneous Detection and Enumeration method is strongly recommended. Note:

Use of BCM or ALOA media is preferred to use of Oxford and other esculin media because the former are more specific for *L. monocytogenes*. [Contact: A.D. Hitchins, CFSAN at (301) 436-1649]

Positive *Listeria*

Submit *Listeria* isolates for Pulse Field Gel Electrophoresis (PFGE) assay to respective servicing laboratories.

Salmonella

Isolation and Identification

Refer to the BAM online, October 2001, Ch. 5, *Salmonella* (http://www.cfsan.fda.gov/~ebam/bam-5.html). Additionally, Rapid Test Kits mentioned in the April 24, 1998 memo, "Guidance for the Use of Rapid Methods for Food Microbiology", may be used. If the laboratory does not have a copy of this memo, it should be requested from the Division of Field Science, HFC–140.

Speciation

If the sample is positive for *Salmonella*, prepare slants and provide hard copy information requested under BAM online, Ch. 5, section E – 11, "Submission of cultures for serotyping" (http://www.cfsan.fda.gov/~ebam/bam-toc.html). Send to Denver Laboratory (DEN) or designated lab by Division of Field Science (DFS) for speciation. Laboratories should submit appropriate form when sending isolates for Salmonella speciation.

Salmonella Positive Isolates

ARL will submit one (1) positive *Salmonella* isolate of each somatic group recovered from each sub-sample to Denver District Laboratory's (DEN-LAB) microbiology laboratory for antibiotic sensitivity assay. Isolates, which were found to be multi-drug resistant, will be shipped to Regional ORA lab for PFGE analysis.

Prepare cultures for shipment according to requirements for shipment of etiological agents. Submit cultures on brain heart infusion (BHI) agar slants in screw – cap tubes (13mm x 100mm) with caps securely tightened. Label each tube with the sample and sub-sample numbers. Submit copies of the collection report and analytical worksheets. Place the cultures in a culture container with an official FDA seal. Place the accompanying record inside the shipping carton but not within the officially sealed culture container. Label the secondary shipping container according the service needed (see below). Send the container by the most rapid means available. Maintain duplicate cultures for all cases, which are under consideration for legal action.

Microbiology Field laboratories should follow the following instruction in sending Salmonella isolates for serotyping (for epidemiological purposes):

Isolates from NRL, WEAC, SRL and ARL will be serotyped in ARL:

Arkansas Regional Laboratory
3900 NCTR Road Building 26
Jefferson, AR 72029
Attention: Gwendolyn Anderson
Tel# 870-543-4621
Fax# 870-543-4041

Isolates from SAN, PRL-NW, PRL-SW and DEN will be serotyped in DEN:

Denver District Laboratory
6th Ave & Kipling St,DFC,
DFC Building 20
Denver, CO 80225-0087
Attention: Doris Farmer
Tel# 303-236-9604
Fax# 303-236-9675

ARL submit *Salmonella* isolates for antibiotic sensitivity assays to:

FDA/ ORA/ DEN
ATTN: Connie Kiessling
6th Ave & Kipling St,DFC,
Building 20, ENT-10
Denver, CO 80225-0087

Submit *Salmonella* isolates for Pulse Field Gel Electrophoresis (PFGE) assay to respective servicing laboratories.

- **Staphylococcal Enterotoxin**

General

Perform enterotoxin testing if:

1. Product abuse (e.g. temperature, outbreaks, etc.) is suspected

or

2. Instructed by Compliance Program or Field Assignment to analyze the sample for *Staphylococcus*.

If viable *Staphylococcus sp.* colonies are observed by:

- most probable number (MPN) when performed as directed per BAM Chapter 12
- (http://www.cfsan.fda.gov/~ebam/bam-12.html)where the results are >11,000

and

- direct plate counts when performed as directed per BAM chapter 2 (http://www.cfsan.fda.gov/~ebam/bam-2.html)indicates a level of 10,000/ gram

Enterotoxin Analysis:

Follow the methodology outlined in BAM 8th Ed., Revision A, 1998 or most current Ed., Ch. 13, "Staphylococcal Enterotoxins" (http://www.cfsan.fda.gov/~ebam/bam-toc.html), beginning on page 13.01.

The laboratory will individually test each sub-sample using the TECRA™ ELISA with proper procedures followed accordingly.

NOTE: Under **no** circumstances should positive TECRA™ ELISA results be conveyed to a regulated firm or consumer without confirmation. The TECRA™ ELISA is intended as a screening method only.

NOTE: The total contents of each subsample should be retained until the original analyses are completed to ensure that a sufficient amount of product is available for subsequent additional and confirmation tests, if necessary.

TECRA™ ELISA Test Results

1. Negative result - the laboratory need not conduct further analysis for enterotoxin. The sample is considered "negative" and no other regulatory or follow-up action is warranted.

2. Positive result – the laboratory should analyze the original sample using the VIDAS method for confirmation refer to BAM 8th Ed., Revision A, 1998 or most current Ed., Ch. 13A, "Staphylococcal Enterotoxins: Micro-slide Double Diffusion and ELISA Based Methods"(i.e., VIDAS)". See (http://www.cfsan.fda.gov/~ebam/bam-toc.html)

NOTE: If the District or Regional Laboratory cannot perform the VIDAS, contact Reginald Bennett, HFS–516 at (301) 436–2009 to arrange for shipment of portions of the actual sub-samples to CFSAN for confirmation.

TECRA™ ELISA **and** the VIDAS Tests Results

1. When the TECRA™ ELISA **and** the VIDAS Tests are **positive**, the following **should be sent to CFSAN for re-confirmation analysis**:

   (a) The remaining portion of the original TECRA™ ELISA positive tested extract(s)

   (b) The remaining portion of the positive tested extract(s) from the VIDAS System

   (c) "all" of the remaining reserves portion of the

positive subsample(s)

(d) a copy of the analytical worksheets

(e) a copy of the collection report

to

FDA/ CFSAN/ Microbiology Methods Research Branch
Attention: Reginald Bennett, HFS-516
5100 Paint Branch Parkway
College Park, MD 20740.

NOTE: Please notify the Division Director at 301.436.2007 that the sample is being sent to CFSAN for confirmation analyses.

If additional information concerning sample preparation, handling or shipping to CFSAN is needed contact Reginald Bennett at 301.436.2009.
The Microbiology Methods Research Branch will perform confirmation analyses on the extract and reserve subsample(s) as appropriate and provide sample(s) to Dr. A. Rasooly for SDS-PAGE immunoblot analyses as indicated.

Districts should wait for Center confirmation before recommending any regulatory action.

2. When the result of the TECRA™ ELISA is **positive** and the VIDAS is **negative** then:

Check for the presence of peroxidase.

NOTE: Some foods contain peroxidase which can cause a false-positive reaction with the TECRA™ ELISA; therefore if this scenario presents itself the analyst should check for the presence of peroxidase and if present, inactivate the peroxidase and retest.

To determine peroxidase presence, refer to the method outlined in BAM Ch. 13A, "Staphylococcal Enterotoxins: Micro-slide Double Diffusion and ELISA Based Methods, Section "Extraction of Enterotoxins from Foods for ELISA Assays", A. General Precautions. If peroxidase is present, inactivate the peroxidase using the methods outlined in the General Precautions section and retest.

NOTE: Under **no** circumstances should positive TECRA™ ELISA results be conveyed to a regulated firm or consumer without confirmation. The TECRA™ ELISA is intended as a screening method only.

If the TECRA™ retest remains positive, send the extract used for the TECRA™ ELISA and the reserve portion for all of the original sub-samples to:

FDA/ CFSAN/ Microbiology Methods Research Branch

Attention: Reginald Bennett, HFS-516
5100 Paint Branch Parkway
College Park, MD 20740.

The Microbiology Methods Research Branch will consolidate CFSAN's analyses results and provide the finding to CFSAN/ Office of Compliance/ Division of Enforcement. The Division of Enforcement will contact the District's Compliance Branch with the results for appropriate follow-up.

- ***V. cholerae***

General Instructions

Each sample will be examined on an individual sub-sample basis except for the analysis using the Polymerase Chain Reaction (PCR) method for *V. cholerae* Enterotoxigenic strains (see *V. cholerae* section below). When the PCR method is used, the sample will be analyzed on a composite basis (see below for instructions).

Methods

General Method:
BAM 8th Edition, Revision A or most current Edition, Ch. 9, "*Vibrio cholerae, V. parahaemolyticus, V. vulnificus,* and Other *Vibrio* spp" (http://www.cfsan.fda.gov/~ebam/bam-toc.html)

PCR Methods for *V. cholerae*:
Attachment D "Polymerase Chain Reaction (PCR) for *V. cholerae* Enterotoxigenic Strains" of this program. Alternatively, BAM 8th Edition, Revision A or most current Edition, Ch. 28 "Detection of Enterotoxigenic *Vibrio cholerae* in foods by the Polymerase Chain Reaction" may be used.

V. cholerae: "Isolation, identification, pathogenicity and PCR"

A. Each sample will be analyzed using the General Method and one of the PCR Methods for *V. cholerae* methods referenced above.

B. If the sample is found to be positive for *V. cholerae*, send one set of ALL isolates of *V. cholerae* 01 or non-01 for confirmation to:

FDA/ CFSAN/ Virulence Mechanisms Branch
ATTN: Mahendra Kothary (HFS – 025)
MOD 1
Laurel, MD 20708
(301) 827 - 8616

C. Alkaline Peptone Water (APW) Lysate Preparation for PCR analysis

NOTE: THE FOLLOWING INSTRUCTIONS ARE TO BE USED IN LIEU OF ATTACHMENT D (PAGE 3, STEP C).

1. Once the appropriate dilutions have been prepared for each

of the individual ten (10) sub-samples using the BAM method, the laboratory will **prepare two (2) APW Lysate composites from the original 1:10 APW dilutions** (e.g., the blended solution) **PRIOR** to incubation.

NOTE: APW Lysate composites will be prepared from the original 1:100 APW dilutions for products (e.g., products with possible high concentration of microflora) with a potential inhibitory effect to the PCR reaction.

2. One APW Lysate composite will be prepared by removing 1.0mL from each of the 1:10 or 1:100, as appropriate, for dilutions for sub-samples 1 thru 5 (e.g., composite #1A) and the second APW Lysate composite will reflect 1.0mL being removed from each of the 1:10 or 1:100, as appropriate, for dilutions for sub-samples 5 through 10 (e.g., composite #1B).

3. These APW Lysate composites will be **designated as zero (0) times lysates, (e.g., composites 1A and 1B).** Boil for 5 min, and then freeze.

   NOTE: THIS "0" TIME ALIQUOT WILL BE USED FOR PCR TESTING ONLY IF THE 6 – 8 HOUR OR 16 – 24 HOUR INCUBATED LYSATE SHOWS A POSITIVE REACTION ON THE PCR TEST.

4. A second set of APW Lysate composites will be prepared using step (2) above from the original 1:10 or 1:100 dilutions AFTER the 6 – 8 hour incubation period at 37°C.

   If the sample is a frozen food product, then the APW Lysate composites will be prepared using step (2) above from the original 1:10 or 1:100 dilution AFTER the 16 – 24 hour incubation.

   NOTE: THIS LYSATE WILL BE TESTED FIRST USING THE PCR TEST. IF THIS LYSATE CANNOT BE TESTED IMMEDIATELY, THEN FREEZE UNTIL THE PCR TEST CAN BE PERFORMED.

5. See the PCR Methods for *V. cholerae* referenced above for further instructions for PCR analysis.

- **Animal Drug Residue in Milk**

  Refer to the Analytical Section of the National Drug Residue Milk Compliance Program (CP 7303.039) for instructions.

- **Phosphatase**

  Methodology

  Dried Milk – AOAC, 17th Ed. or most current Ed., 979.13 (section 33.2.50), Phosphatase (Residual) in Milk, Method V

Fluid Milk and **Ice Cream Products** – AOAC 17th Ed. or most current Ed., 946.01, (section 33.2.47) Phosphatase (Residual) in Milk, Method II and 946.04 (section 33.8.12) Phosphatase (Residual) in Ice Cream and Frozen Desserts.

Analyzing Laboratories

Refer to Appendix III "Servicing Laboratories", of the current ORA Field Workplan for field servicing laboratories

Comments

Consult ORO/ DFS, HFC–141, for servicing laboratories as needed.

- **Analytical Reporting**

Report all analytical results (filth, decomposition, microbiological) into FACTS using the following Problem Area Flags (PAF) and PACs:

PAC	PAF	Problem Area	Sub-PAF	PAF Description
03803B (Filth)	FIL	ANI	--	Animal Filth
		ART	--	Arthropod Filth
		BIR	--	Bird Filth
		EXH	--	Filth Exhibit
		FOR	--	Foreign material
		OTH	--	Other, Not Listed, Identify in Remarks
03803C (Decomp)	FIL	DEC	--	Decomposition
		EXH	--	Filth Exhibit
		GEO	--	Geotrichum Mold
		HMC	--	Howard Mold Count
		MAC	--	Macroscopic Mold
		YEA	--	Yeast
		OTH	--	Other, Not Listed, Identify in Remarks
03803D (Micro)	MIC	MIC	--	Microbiological analysis (included rapid test kits for micro analysis)
		PHOS	--	Phosphatase analysis
		ANT	SAL	Salmonella serotyping
		ANT	ABR	Antibiotic resistance testing

DOMESTIC FOOD SAFETY PROGRAM
PROJECT 04 – CHEMICAL CONTAMINANTS

Domestic coverage of pesticides in raw agricultural products is provided in "Pesticides and Industrial Chemicals in Domestic Foods", CP 7304.004. This attachment covers the problem of incorrect application and storage of industrial and household chemicals in food processing plants, warehouses, and transport vehicles.

FACTS REPORTING REQUIREMENTS

- Report all analytical results (Chemical contaminants) into FACTS using the following Problem Area Flags (PAF) and Program Assignment Codes (PAC).

PAC	PAF	PAF Description
04803	PES	Pesticide analysis

- Report resources utilized for all operations conducted in conjunction with a Food Safety inspection (includes sample collection and analysis) under PAC 04803 – Chemical Contaminants.

- Refer to page 2, Program Assignment Code Reporting, of this Compliance Program for more information regarding reporting requirements.

INSPECTIONAL INSTRUCTIONS

1. Inspection: Refer to the current edition of the IOM, Section 5.4.7.1: "Routes of Contamination".

   Portions of the Memorandum of Understanding with USDA (FSIS and AMS) and EPA Regarding Regulatory Activities Concerning Residues of Drugs, Pesticides, and Environmental Contaminants in Foods pertain to this program. See Federal Cooperative Agreements Manual, August 1996.

   Also relevant to this program is ORA Field Management Directive No. 129, Interagency Pesticide Referrals Between EPA and FDA.

2. Sample Collection: Refer to the current edition of the IOM, Sample Schedule; Chart 3 "Pesticide Sampling Guidance". Check with the lab (refer to Appendix III, "Servicing Laboratories", of the current ORA Field Workplan) for field servicing laboratories to determine sample size and the proper type of collection container. This is especially important with volatile compounds such as chlorinated solvents (e.g., chloroform and trichlorethylene).

3. Sample Submission: Submit samples to the collecting District servicing lab (see Appendix III, "Servicing Laboratories", of the current ORA Field Workplan).

ANALYTICAL INSTRUCTIONS

Analysis and Reporting: See PART IV "Pesticides and Industrial Chemicals in Domestic Foods" CP, 7304.004.

DOMESTIC FOOD SAFETY PROGRAM

PROJECT 09 – FOOD AND COLOR ADDITIVES

The Center is prepared to move quickly against products containing banned, illegal, or improperly used food or color additives.

In addition to the general coverage given to food and color additives, give priority coverage to the additives highlighted in this attachment.

FACTS REPORTING REQUIREMENTS

- Report all analytical results (food and color additives) into FACTS using the following Problem Area Flags (PAF) and Program Assignment Codes (PACs):

PAC	PAF	PAF Description
09803E	COL	Color Additive analysis
09803F	FAD	Food Additive analysis

- Report resources utilized for operations other than 31 (Sample Collection) and 41 (Sample Analysis) under PAC 09803, "Food and Color Additives".

- Report resources expended for:
  1. 09803E - sample collection/ analysis for color additives
  2. 09803F – sample collection/ analysis for food additives

- Refer to page 2, Program Assignment Code Reporting, of this Compliance Program for more information regarding reporting requirements.

INSPECTIONAL INSTRUCTIONS

Inspection: Refer to the current edition of the IOM, Section 5.4.6.3, "Food Additives" through 5.4.6.4, "Color Additives". See the following website for a list of approved food and color additives at:
http://www.cfsan.fda.gov/~dms/opa-appa.html

1.

A. Food Additives

- The functions of common categories of food chemicals are given in 21 CFR 170.3(o). Substances specifically prohibited from use in human food are listed in 21 CFR 189.

  NOTE: The **saccharin warning label** requirement under section 403(o)(2) of the Federal Food, Drug and Cosmetic Act **was repealed** by Public Law 106-554, December 21, 2000. Food containing saccharin **no longer requires** the warning statement. The interim regulation requirements for saccharin under section 180.37 remain in effect.

  21 CFR 172.804(d)(2) requires that the label or labeling of any food containing aspartame to bear either on the principal display panel or the information panel the following statement: "PHENYLKETONURICS: CONTAINS PHENYLALANINE".

The regulation for aspartame further requires the statement to appear in the labeling prominently and conspicuously as compared to other words, statements, designs or devices and in bold type on clear contrasting background.

Foods containing aspartame but without the required label being prominent and conspicuous may be brought to the attention of management. For questions on the labeling requirements for aspartame-containing foods, contact Felicia Billingslea, CFSAN/ONPLDS at (301) 436-2371 or via email at: Felicia.Billingslea@fda.hhs.gov.

Sulfiting Agents

Refer to 21 CFR 182.3616, 182.3637, 182.3739, 182.3766, 182.3798, and 182.3862, for categories of foods that are excluded from using sulfiting agents as chemical preservatives.

For more detailed information on sulfiting agents see, http://www.cfsan.fda.gov/~dms/fdpreser.html.

The use of sulfiting agents as chemical preservatives in foods (other than the aforementioned categories) is Generally Recognized As Safe (GRAS) when used in accordance with Good Manufacturing Practices (GMPs). Refer to 21 CFR 101.100(a)(4) for additional requirements applicable to sulfite usage in foods.

Sulfur dioxide used as a fumigant for table grapes is officially defined as a pesticide and is required by the U.S. Environmental Protection Agency (EPA) to be at less than detectable levels (less than 10 ppm).

For additional details see “Sulfites: An Important Food Safety Issue” at http://www.cfsan.fda.gov/~dms/fssulfit.html.

B. Color Additives

- Firms may use unlisted or restricted color additives through ignorance of changes in regulations or by deliberately using up old stock to avoid financial losses. Firms may use technical grades of color additives rather than certified lots as required because they are cheaper. Check whether color additives are used in accordance with legal restrictions or tolerances.

  During inspection, check whether certified lots of certifiable colors are being used. This cannot be determined analytically.

  Certification lot numbers should be declared on the labeling of bulk color additives that have been certified by the FDA.

  Check the labeling of color additives and color additive mixtures against the requirements of 21 CFR 70.25 and 80.35(b).

  21 CFR 101.22(k) and CPG 7127.01, section 587.100 should be used as directions for the declaration of both certified colors and

colors exempt from certification in food.

The NLEA requires that all certified color additives used in food be declared by the name of the color additive listed in 21 CFR 74 or 82. Abbreviations for certified colors may be declared in accordance with 21 CFR 101.22(k)(1). Also use 21 CFR 74.705 for the declaration of FD&C Yellow No 5. Colors exempt from certification must be declared in accordance with 21 CFR 101.22(k)(2).

FD&C Red No 3 is currently permitted for use in foods only when used in the form of the straight dye. However, all “lakes” of the dye are not permitted in foods manufactured on/or after January 29, 1990. Due to analytical difficulties in distinguishing between the straight dye and its lakes when in a food matrix, the suspected use of FD&C Red No. 3 lake in a domestic food should be documented at the manufacturer through record collection and observations.

- **Joint Inspections (Field/ CFSAN personnel) of Color Manufacturers**

1. **Joint Field/ CFSAN inspections** will be conducted at selected color-manufacturing firms during the fiscal year. Color manufacturer inspections are being conducted in order to ensure compliance with all CFR requirements. These regulations include requirements for packaging, labeling, storage, documentation of GMP and record keeping for distribution of color additives.

2. CFSAN/ Office of Cosmetics and Colors/ Division of Cosmetics and Compliance, HFS–125, **will be contacting the District directly** to arrange for the inspection of selected firms. Approximately # firms will be inspected per year from a list that will be provided to the districts.

3. Staff members of the CFSAN/ OCAC will accompany the investigator assigned to perform the unannounced inspections of the identified manufacturers.

   The CFSAN/ OCAC personnel will be comparing requests for certification with those documents maintained at the plant. In addition to this review, the CFSAN personnel will also review the manufacturers color batch distribution records.

4. The FDA investigator will perform a GMP inspection of the facility.

5. The FDA investigator may be collecting official samples of certified colors at the direction of the CFSAN/ OCAC personnel.

   If it is determined that samples will be collected, each sample will consist of 100g (i.e., 4 ounces).

   The color sample must be collected in a plastic/ glass jar and the container must be securely sealed with tape around the lid to prevent moisture pickup and to prevent dye from escaping.

   The investigator will send the samples to CFSAN by Federal Express (Fed Ex).

C. Check products containing

- Aspartame/ Nutrasweet for the required statement (21 CFR 172.804(d)(2)
- Sulfites for the sulfite ingredient declaration
- Color additives for the ingredient declaration

Send copies of the entire EIR made for the food and color manufacturer inspections to CFSAN/Compliance Programs Branch, HFS-636 at the address on page 1 of this program. Also send copies of color manufacturer EIR's to OCAC at the address on page 2 of this program.

2. Sample Collection

A. Food Additives – Refer to the current edition of the IOM, section 5.4.6.3or applicable DFI Inspectional Guidance Documents. If a physical sample of the finished product containing the additive is collected, always collect a physical sample of the pure additive and its label.

In most cases, the size of a sample collected for filth analysis will be sufficient for the food additive analysis as well. However, it may be necessary to consult with the analyzing laboratory on the amount of sample required for analysis of specific food additives.

B. Color Additives – Refer to the current edition of the IOM, Section 5.4.6.4 or Sample Schedule Chart 9 for the color containing products. When sampling, collect 4oz of the liquid, paste or powdered product. Document inspectional observations of the use of non-permitted colors in foods.

NOTE: Do not collect samples of food product containing a suspected FD&C Red No. 3 lake for analysis. Due to analytical difficulties, the straight dye and its lakes, when in a food matrix, cannot be distinguished. See Attachment C, page 2, B "Color Additives", for obtaining record collection and inspectional observations of suspected use.

C. Sample Shipment – Refer to the current ORA Field Workplan, Appendix III, "Servicing Laboratories", for field servicing laboratories for Food and Color Additives samples.

ANALYTICAL INSTRUCTIONS

1. Analytical Methodology

Report all analytical results (food and color additives) into FACTS using the following Problem Area Flags (PAF) and Program Assignment Codes (PACs):

PAC	PAF	PAF Description
09803E	COL	Color Additive analysis
09803F	FAD	Food Additive analysis

A. Food Additives

- AOAC, Official Methods of Analysis, 17th Ed., or the most current Ed., Chapter 47 and 48.
- Food Additives Analytical Manual, Vol. I and II, 1983 and 1987.
- Food Chemicals Codex 4th Ed., or more recent.
- Antioxidants in Fats and Oils: AOAC, 17h Ed, or most current Ed., 02, "Antioxidants in Oils and Fats, Liquid Chromatographic Method.
- Sulfites: AOAC, 17h Ed. or most current Ed., 990.28 (section 47.3.43), "Sulfites in Foods, Optimized Monier – Williams Method", Final Action, 1994.

B. Color Additives

- AOAC, Official Methods of Analysis, 17th Ed. or the most recent Ed., Chapter 46
- Attempt to identify any non-permitted/ non-listed color (s) present. If non-permitted/ non-listed color(s) are found, check analysis must be performed. If one non-permitted/ non-listed color is confirmed, identification of the remaining color components in the product is not necessary. The field should also be aware of possible inappropriate use of certain color additives (e.g., the use of colors listed only for drug and cosmetic use of external drug and cosmetic use in food products).
- NLEA requires the declaration of all certified color additives by name (21 CFR 101.22(k)(1)). If undeclared certified colors are found in a sample, perform check analyses to confirm the presence of each undeclared certifiable color additive as appropriate depending on the investigational evidence developed. (See Inspectional Guidance section).
- When a certifiable color additive is not declared on the label, FDA may or may not have certified it for use in food products, without evidence that the color came from a batch that has been certified (e.g., FDA certification lot number, record review, investigational observations that a certified color additive was at the manufacturing site and/or used in the finished product, etc.).
- The original or check analyses for the identification of *non-permitted/ non-listed* or undeclared color additives should always include visible spectra of the isolated color additive, ideally under acidic, basic and neutral conditions. Standard reference spectra in the same solvent as those for the isolated color should be attached to the analytical worksheets. Confirmatory analyses should include different characterizing data (e.g., TLC Rf-value; HPLC retention times, etc.). TLC confirmation should include either tables of Rf-values, or high quality reproductions of the TLC plates with spots clearly circled and labeled. The color of the spots and streaks should also be reported, especially if black and white reproductions are submitted. In addition, spots should

be checked under UV light. The presence or absence of fluorescence as well as the visual color of the fluorescence should be reported to support the identity of fluorescent dyes.

- Do not routinely quantitate the colors for which no limits have been established.

- When samples are analyzed for color additives, it is imperative that the reports of analysis not convey erroneous information. Since no analytical method is capable of determining that a color additive in **a food matrix is from a certified lot**, the conclusion should not state, for example, "contains FD&C Yellow No 5". The following language is suggested instead to more accurately describe a color additive that has not been shown to come from certified color lots: Tartrazine (C.I. 17140, certifiable as FD&C Yellow No 5).

  1. Refer to the list of approved colors at: http://www.cfsan.fda.gov/~dms/opa-appa.html. Be aware that the following color additives are not on the list for use in food products in the United States.

     a. Amaranth (C.I. 16185, EEC No E123, formerly certifiable as FD&C Red No 2)

     b. Azorubine (C.I. 14720, EEC No E122, formerly certifiable as Ext D&C Red No 10); also called Azo Rubine and Carmoisine.

     c. Ponceau 4R (C.I. Acid Red No 18, C.I. 16255, EEC No E124, no certifiable equivalent); also called Cochineal Red A, Brilliant Scarlet 3R and Brilliant Scarlet 4R, but not Brilliant Scarlet which is a different color (C.I. 15585:1, formerly certifiable as D&C Red No 8).

     d. Quinoline Yellow (resembles C.I. 47005, EEC No E104, C.I. Acid Yellow No 3, C.I. Food Yellow No 13).

     e. D&C Yellow No 10: In the United States, primarily monosulfonated quinoline yellow is certifiable as D&C Yellow No 10 for use in drug and cosmetics, but is not permitted in foods at this time. In European and other countries, primarily disulfonated quinoline yellow may be used as a color additive in foods.

  2. FD&C Red No 3, Citrus Red 2, FD&C Red No 40, FD&C Blue No 1, FD&C Blue No 2, FD&C Green No 3, Orange B, FD&C Yellow No 5, and FD&C Yellow No 6 are color additives authorized by regulation for safe use in food products. The colors must be from batches that have been certified by the Food and Drug Administration. Analytically, it cannot be determined if a color additive subject to certification has in fact been certified. Analysts should report their findings of these colors as follows:

     a. Allura Red AC (C.I. 16035, EEC No E129, certifiable as FD&C Red No 40)

     b. Brilliant Blue FCF (C.I. 42090, EEC No E133, certifiable as

FD&C Blue No 1)

c. Citrus Red 2 (C.I. 12156, certifiable as Citrus Red 2, for use only for coloring the skins of oranges that are not intended or used for processing)

d. Erythrosine (C.I. 45430, EEC No E127, the disodium salt is certifiable as FD&C Red No 3)

e. Fast Green FCF (C.I. 42053, No EEC designation, certifiable as FD&C Green No 3)

f. Indigotine (C.I. 73015, EEC No E132, certifiable as FD&C Blue No 2)

g. Orange B (C.I. 19235, No EEC designation, for use only in coloring sausage casings)

h. Sunset Yellow FCF (C.I. 15985, EEC No E110, certifiable as FD&C Yellow No 6)

i. Tartrazine (C.I. 17140, EEC No E102, certifiable as FD&C Yellow No 5)

- Be aware of the presence and possible separation of subsidiary and isomeric dyes, which are permitted in many of the FD&C colors (see 21 CFR Part 74). Their presence may be more evident when high-resolution techniques are employed such as HPLC and HPTLC. Excessively high levels of subsidiaries in tartrazine, Sunset Yellow FCF and Allura Red AC may indicate the use of non-certified batches of these dyes and should be noted as a possibility on the analytical screen.

REPORTING

- Report all analytical results (food and color additives) into the FACTS as appropriate using the following PAFs and PACs:

PAC	PAF	PAF Description
09803E	COL	Color Additive analysis
09803F	FAD	Food Additive Analysis

POLYMERASE CHAIN REACTION (PCR) FOR *V. CHOLERAE* ENTEROTOXIGENIC STRAINS

Method Contact:
Mahendra Kothary
FDA/ CFSAN/ Office of Science
(301) 827 - 8616

General Information

Fruits and vegetables are the easiest foods to survey by this PCR method since only surface contamination is examined and the APW washes are virtually devoid of a food matrix. In contrast, seafood is far more challenging since the food matrix is blended at 1 – 10% weight per volume of APW. Using artificially seeded molluscan shellfish/ crustaceans, the crab blends of up to 10% are acceptable for amplification while oysters and shrimp can be assayed only at 1% (w/v) blends with this method. After 8hr enrichments, as few as 1 cell/10g can be detected from lettuce while approximately 10 cells/g of artificially contaminated oysters and shrimp are routinely detected.

PCR ANALYSIS FOR FOODS NOT PREVIOUSLY TESTED WITH THE *ctxAB* PRIMER SETS

Data obtained from the "*V. cholerae* in Domestic and Imported Foods Assignment" has shown that the PCR method was used for various foods which have not been previously tested for potential inhibitory effects on the PCR reaction (e.g., various fish and other seafood). Since APW food blends behave differently (e.g., a 10% blend of shrimp or oyster inhibits the PCR reaction while a 10%crab blend does not), it is imperative to run proper controls for possible inhibitory effects for every new food blend tested. Minimally this would entail spiking 1mL of a 1:10 and 1:100 APW food blend post-enrichment with approximately 5 x 10^6 organisms/mL (or an equivalent amount of positive control lysate). A direct comparison of these spiked samples with the APW (no food) Lysate containing identical numbers of *ctx*+ control cells allows one to determine if any inhibition occurs at either of the two food blend concentrations and prevents the occurrence of false negatives. These controls should be done for any new food blend for which PCR analyses are being attempted (e.g., any seafood other than shrimp, oyster, crab or other novel foods like coconut products). It is unlikely that food washes (e.g., fruits and vegetables) inhibits the PCR reaction unless the fruits are bruised and washed releasing excessive acidity to the APW wash.

METHODOLOGY

A. EQUIPMENT

1. Electrophoresis apparatus

2. Constant voltage power supply

3. Thermocycler: Perkin – Elmer Cetus

4. Comb: The choice of comb is left to the discretion of the analyst. The comb should hold at least 20μL.

B. REAGENTS

1. Two microcentrifuge tubes containing PCR primers for amplification of ***ctxAB*** sequences from Enterotoxigenic *V. cholerae*. The tubes contain:

   - 4000pmol of WK74 (5'-TGAAATAAAGCAGTCAGGTG-3')
   - sense strand, bp 611 – 630

   OR

   - WK75C (5'-GGTATTCTGCACACAAATCAG-3')
   - Antisense Strand, BP 1368 - 1388

   All oligonucleotides are numbered according to Mekalanos, et. al. (1983), however, **NOTE** that bp 1386 and 1387 are inverted in this reference. These PCR primers were sent to the appropriate analyzing laboratories from Dr. Walter H. Koch, FDA/ CFSA/ Division of Molecular Biological Research/ Molecular Biology Branch, HFS–237.

   Hydrate the primers with **400µL** sterile water of the highest purity available. Store at -20°C (final concentration 10pmol/µL). If 10pmol of each primer per 50µL PCR reaction is used, then it is sufficient to perform 400 reactions.

2. Electrophoresis Buffer

BUFFER	WORKING SOLUTION	CONCENTRATION STOCK SOLUTION (PER LITER)
Tris-borate (TBE)	1X: 90mM Tris-borate 2mM EDTA	5X: 54g Tris base 27.5g boric acid 20mL 0.5M EDTA (pH 8.0)

   NOTE: Some precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid, store the 5X solution in glass bottles at room temperature and discard any batches that develop a precipitate.

   Premixed TBE Concentrate or powered blends may also be purchased from several venders (e.g., Sigma 1-800-325-3010, Gibco BRL 1-800-828-6686, Digene 1-800-DIGENE-1, etc.).

3. Gel Loading Buffer

BUFFER TYPE	6X BUFFER	STORAGE TEMPERATURE
I	0.25% Bromophenol Blue 0.25% Xylene Cyanol FF 4.0% (w/v) Sucrose in Water	4°C
II	0.25% Bromophenol Blue 0.25% Xylene Cyanol FF 15.0% Ficoll (Type 400; Pharmacia) in water	Room Temp.
III	0.25% Bromophenol Blue 0.25% Xylene Cyanol FF 30.0% Glycerol in Water	4°C
IV	0.25% Bromophenol Blue 40.0% (w/v) Sucrose in Water	4°C

NOTE: These gel-loading buffers serve three purposes: (1) increase the density of the sample, ensuring that the DNA drops evenly into the well, (2) add color to the sample, thereby simplifying the loading process, (3) contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels approximately 2.2 folds faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5X TBE at approximately the same rate as linear double-stranded DNA 300pb in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5% to 1.4%. **The type of loading dye used is a matter of personal preference.**

4. Ethidium Bromide

   Stock solution: 10mg/mL in water, stored at room temperature in dark bottles or bottles in aluminum foil. This may be purchased as a 10mg/mL solution from #.

5. Agarose

   Any agarose for analytical nucleic acid electrophoresis is suitable when molecular weight standards are used (e.g.,#).

C. PREPARATION OF ALKALINE PEPTONE WATER (APW) LYSATES

1. Once the appropriate dilutions have been prepared for the sample using the BAM 8th Ed., Revision A or most current Ed., 1998, Ch. 9, "*Vibrio cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio spp*" (http://www.cfsan.fda.gov/~ebam/bam-9.html), **prepare two (2) APW lysates from the original 1:10 APW dilutions** (e.g., the blended solution).

   NOTE: For products with potential inhibitory effect of the PCR reaction (e.g., oyster, raw shrimp, or products with possible

high concentration of microflora) two (2) APW lysates will be prepared from the original 1:100 APW dilutions.

2. Remove 1.0mL from the original 1:10 dilution (e.g., the blended solution) or undiluted APW rinse, Prior to incubation. Boil the 1.0mL aliquot immediately for 5 min, and then freeze.

   NOTE: THIS "0" TIME ALIQUOT WILL BE USED FOR PCR TESTING ONLY IF THE 6 – 8HR OR 16 – 24HR INCUBATED LYSATE SHOWS A POSITIVE REACTION ON THE PCR TEST.

3. A second APW lysate will be prepared (using the instructions above) from the original 1:10 dilution (e.g., blended solution) **AFTER** the 6 – 8 hrs incubation period at 37°C.

   NOTE: THIS LYSATE WILL BE TESTED FIRST USING THE PCR TEST. IF THIS LYSATE CANNOT BE TESTED IMMEDIATELY, THEN FREZE UNTIL THE PCR TEST CAN BE PERFORMED.

   If the sample is a **frozen food product**, then the second APW lysate will be prepared and tested **after** the 16 – 24hr incubation period.

D. PREPARATION OF PCR REACTION MIXTURES (50µl)

NOTE: TAKE GREAT CARE TO AVOID CONTAMINATING REAGENTS; PREFERABLY MAKE MASTER MIXES AND ADD LYSATES LAST.

- PCR Buffer (1X)
  - 50mM KCL
  - 10mM Tris – HCL, pH 8.4
  - 1.5mM $MgCl_2$

  NOTE: 10X PCR buffers can be purchased from Perkin-Elmer, cat. no. N808-006.

- 200µM each dATP, dCTP, dGTP, dTTP (United States Biochemical)

- 2 – 5% v/v APW lysate

  NOTE: If lysate is frozen, thaw for approximately 10 minutes prior to adding to the PCR reaction mixture. When thawed, store on ice at room temperature.

- 1.25U Amplitaq (Perkin-Elmer Cetus)

  NOTE: 10X PCR buffers are supplied free with the purchase of Amplitaq.

- 0.2 – 0.5µM of each primer

E.PCR AMPLIFICATION OF CRUDE APW LYSATES

Take the PCR reaction mixture and perform twenty–five to thirty cycles of amplification using a programmable thermocycler (Perkin–Elmer Cetus)

NOTE: DO NOT ATTEMPT TO INCREASE THE SENSITIVITY OF THIS METHOD BY INCREASING PCR CYCLES NUMBERS BEYOND 30 – 35 CYCLES AS THIS OFTEN LEADS TO THE FORMATION OF NONSPECIFIC AMPLIFICATION PRODUCTS, INCLUDING PRIMER DIMERS.

One-minute denaturation, annealing and extension steps at 94°C, 55°C, and 72°C, respectively, are used.

PCR reaction products are resolved on a 1.8% agarose gel in 1X TBE (0.09M Tris-borate, 0.002mM EDTA, pH 8.3), and visualized by UV-induced fluorescence after staining with 1μg/mL ethidium bromide. The identity of the 999bp fragments by Southern Blot Hybridization with an internal *ctxA*-specific oligonucleotide probe has been confirmed.

F. PREPARATION OF AGAROSE GEL

1. Seal the edges of a clean, dry, glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with autoclave tape so as to form a mold. Set the mold on a horizontal section of the bench (check with a level).

2. Prepare sufficient electrophoresis buffer (1X TBE) to fill the electrophoresis tank and to prepare the gel. Add the correct amount of powered agarose (1.8g/100mL, e.g., 1.8%) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle with a loose-fitting cap. The buffer should not occupy more than 50% of the volume of the flask or bottle.

   NOTE: It is important to use the same concentration of electrophoresis buffers in both the electrophoresis tank and the gel. Small differences in ionic strength or pH create fronts in the gel that can greatly affect the mobility of DNA fragments.

3. Loosely plug the neck of the Erlenmeyer flask with kimwipes. When using a glass bottle, make sure the cap is loose. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves.

   NOTE: Heat the slurry for the minimum time required to allow tall of the grains of agarose to dissolve. Wearing an oven mitt, gingerly swirl the bottle or flask from time to time to make sure that any grains sticking to the walls enter the solution. *Take care – the agarose solution can become superheated and may boil violently if it has been heated for too long in the microwave oven*. Undissolved agarose appears as small “lenses” floating in the solution. Check that the volume of the solution has not been decreased by evaporation during boiling; replenish with water if necessary.

4. Cool the solution to 60°C, and add ethidium bromide (from a stock solution of 10mg/mL in water) to a final concentration of 0.5μg/mL and mix thoroughly.

   CAUTION: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be

decontaminated by one of the methods described on pages 6.16 – 6.17.

Stock solution of ethidium bromide should be stored in light - tight containers (e.g., in a bottle completely wrapped in aluminum foil) at room temperature.

5. Using a Pasteur pipette, seal the edges of the mild with a small quantity of the agarose solution. Allow the seal to set. Position the comb 0.5 – 1.0mm above the plate so that a complete well is formed when the agarose is added. If the comb is closer to the glass plate, there is a risk that the base of the well may tear when the comb is withdrawn, allowing the sample to leak between the gel and the glass plate.

   NOTE: Some combs are designed with two outer teeth slightly longer than the internal teeth. When the comb is positioned above the plate, it is supported on the outer teeth; the inner teeth are automatically lifted clear of the glass plate. The disadvantage of this design is that eh wells made by the outer teeth cannot be used during the subsequent electrophoresis.

6. Pour the agarose solution into the mold. The gel should be between 3mm and 5mm thick. Check to see that there are no air bubbles under or between the teeth of the comb. Make sure that both gels are made from the same batch of buffer and the ethidium bromide is added to both gels or to neither.

7. After the gel is completely set (30 – 45 minutes at room temperature), carefully remove the comb and autoclave tape and mount the gel in the electrophoresis tank.

8. Add just enough electrophoresis buffer to cover the gel to a depth of approximately 1mm. Add ethidium bromide to a final concentration of 0.5μg/mL and mix thoroughly.

9. Mix the 10 – 20μL of the PCR reaction mixture with the gel-loading buffer. Slowly load the mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or, a Pasteur pipette.

   NOTE: Gel loading buffers are usually made up as six-fold concentrated solutions.

   The dimensions of the slot determine the maximum volume of solution that can be loaded. [A typical slot (0.5cm x 0.5cm x 0.15cm) will hold amount 37.5μL].

   Because of the extreme sensitivity of PCR, it is required to use a fresh pipette tip for every sample to avoid causing appearance of false positives.

   1μg Marker DNA of known size (which can be purchased from commercial sources) should be loaded into slots on both the right and left sides of the gel. This makes it easier to determine the sizes of the gel. This makes it easier to determine the sizes of unknown DNA is any systematic distortion of the gel should occur during electrophoresis.

The recommended marker is the 123bp DNA ladder from BRL (Cat. No. 5613SB, 250μg/$126.00).

10. **ALWAYS INCLUDE POSITIVE AND NEGATIVE CONTROLS AND THE MOLECULAR WEIGHT SIZE MARKER (E.G., "MARKER LADDER") ON EACH AGAROSE GEL USED.**

11. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage of 5 – 10V/cm (measured as the distance between the electrodes). [**NOTE**: Lower voltage of 1 – 5V/cm may be used since it provides better resolution of amplicons.] If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and, within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance through the gel.

12. Turn off the electric current and remove the leads and lid from the gel tank since ethidium bromide was present in the gel and electrophoresis buffer. Since ethidium bromide was present in the gel and electrophoresis buffer, the gel may be examined directly by ultraviolet light, and photographic (Polaroid) records should be made. See method described under "**PHOTOGRAPHY OF GELS**".

    NOTE: Solutions containing ethidium bromide should be decontaminated by one of the methods described under "**DECONTAMINATION OF ETHIDIUM BROMIDE SOLUTIONS**".

G. PHOTOGRAPHY OF GELS

Photographs of gels may be made using transmitted or incident ultraviolet light. Most commercially available ultraviolet light sources emit ultraviolet light at 302nm. The fluorescent yield of ethidium bromide: DNA complexes are considerably greater at this wavelength than at 366nm and slightly less than at short-wavelength (254nm) light. However, the amount of nicking of the DNA is much less at 302nm than at 254nm.

The most sensitive film is Polaroid Type 57 or 667 (ASA 3000). With an efficient ultraviolet light source (> 2500μW/cm^2), a Wratten 22A filter, and a good lens (f = 135mm), an exposure of a few seconds is sufficient to obtain images of bands containing as little as 10ng of DNA. With a long exposure time and a strong ultraviolet light source, the fluorescence emitted by as little as 1ng of DNA can be recorded on film. For detection of extremely faint bands, a lens with a shorter focal length (f = 75mm) should be used in combination with conventional wet-process film (e.g., Eastman Kodak No. 4155). This allows the lens to be moved closer to the gel, concentrates the image on a smaller area of film, and allows for flexibility in developing and printing the image.

CAUTION: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

H. RESULTS/ REPORTING REQUIREMENTS

- If the PCR test shows a **positive** reaction:

  - **Immediately** call FDA/ CFSAN/ DOEP/ Case Processing Branch or FDA/ FSAN/ CFSAN/ DOEP/ Import Branch, as appropriate,
  - Perform the PCR reaction of the "0" time APW lysate(s) **AND**
  - **Continue with the BAM method for biochemical, serological and biotype analysis (http://www.cfsan.fda.gov/~ebam/bam-toc.html).**

- If the PCR test shows a **negative** reaction of the lysate that was incubates of 6 – 8hr or 16 – 24hr incubations (e.g., a frozen product):

  - The 0 – hr lysate does not have to be analyzed using the PCR method;
  - If the sample was collected in import status, the sample can be released;
  - The **laboratory can discontinue with the BAM method.**

- The analyzing laboratory should save Photograph(s) of all the PCR agar gel reactions so that the photographs can be used as laboratory reference material for future PCR analyses.

  NOTE: ENSURE THAT THE PHOTOGRAPHS ARE CLEARLY IDENTIFIED WITH APPROPRIATE SAMPLE NUMBER.

I. DECONTAMINATION OF CONCENTRATED SOLUTIONS OF ETHIDIUM BROMIDE (i.e., SOLUTIONS CONTAINING > 0.5mg/mL)

- METHOD 1

  This method (Lunn and Sansone, 1987, Analytical Biochemistry 162:453) reduces the mutagenic activity of ethidium bromide in the *Salmonella/* microsome assay by approximately 200 – fold.

  1. Add sufficient water to reduce the concentration of ethidium bromide to < 0.5mg/ mL.

  2. To the resulting solution, add 0.2 volumes of fresh 5% hypophosphorous acid and 0.12 volumes of fresh 0.5M sodium nitrite. Mix carefully.

     IMPORTANT: Check that the pH of the solution is < 0.3.

     Hypophosphorous acid is usually supplied as a 50% solution, which is corrosive and should be handled with care. Is should be freshly diluted immediately before use.

     Sodium nitrite solution (0.5M) should be freshly prepared by dissolving 34.5g of sodium nitrite in water to a final volume of 500mL.

  3. After incubation for 24 hours at room temperature, add a large excess of 1M sodium bicarbonate. The solution may now be discarded.

- METHOD 2

This method (Quillardet and Hofnung 1988, Trends in Genetics 4:89) reduces the mutagenic activity of ethidium bromide in the Salmonella/ microsome assay by approximately 3000 – fold. However, there are reports (Lunn and Sansone, 1987) of mutagenic activity in occasional batches of "blanks" treated with the decontaminating solutions.

1. Add sufficient water to reduce the concentration of ethidium bromide to < 0.5mg/ mL.

2. Add 1 volume of 0.5M $KMnO_4$. Mix carefully, and then add 1 volume of 2.5N HCL. Mix carefully, and allow the solution to stand at room temperature for several hours.

3. Add 1 volume of 2.5N NaOH. Mix carefully, and then discard the solution.

J. DECONTAMINATION OF DILUTED SOLUTIONS OF ETHIDIUM BROMIDE
(e.g., ELECTROPHORESIS BUFFER CONTAINING 0.5μg/mL ETHIDIUM BROMIDE)

- METHOD 1

The following method is from Lunn and Sansone, 1987.

1. Add 2.9g of Amberlite XAD-16 for each 100mL of solution. Amberlite XAD-16, a nonionic, polymeric absorbent, is available from Rohin and Haas.

2. Store the solution for 12 hours at room temperature, shaking it intermittently.

3. Filter the solution through a Whatman No. 1 filter, and discard the filtrate.

4. Seal the filter and Amberlite resin in a plastic bag, and dispose of the bag in hazardous waste.

- METHOD 2

The following method is from Bensaude (1988, Trends in Genetics 4:89).

1. Add 100mg of powdered activated charcoal for each 100mL of solution.

2. Store the solution for 1 hour at room temperature, shaking it intermittently.

3. Filter the solution through a Whatman No. 1 filter, and discard the filtrate.

4. Seat the filter and activated charcoal in a plastic bag and dispose of in hazardous waste.

NOTE:
i. Treatment of dilute solutions of ethidium bromide with hypochlorite (bleach) is not recommended as a method of

decontamination. Such treatment reduces the mutagenic activity of ethidium bromide in the Salmonella/ microsome assay by about 1000-fold, but it converts the dye into a compound that is mutagenic in the absence of microsomes (Quillardet and Hofnung, 19688)

ii. Ethidium bromide decomposes at 262°C and is unlikely to be hazardous after incineration under standard conditions.

iii. Slurries of Amberlite XAD-16 or activated charcoal can be used to decontaminate surfaces that become contaminated by ethidium bromide.

DETECTION OF *SHIGELLA* BY POLYMERASE CHAIN REACTION

NOTE: Previous steps are located in **Attachment A - Analytical Instructions** of this program. This method is to be used for detection only.

1. Decant supernatant into another set of centrifuge tubes and spin at 8000 rpm for 10 minutes to pellet bacterial cells. Remove as much of the supernatant as possible (to avoid leaving behind any inhibitory compounds; aspiration may be a better alternative than decanting the supernatant).

2. Add 50-100 µl of 1 X phosphate buffer solution (PBS) (this will depend upon the size of the pellet). If the pellet is hardly visible, then add the smallest amount of 1 X PBS. Also, the total amount of PBS added should be 50-100 µl; therefore if two tubes were used for centrifugation, one tube should be suspended in 1 X PBS and this suspension added to the other tube(s).

3. For PCR template preparation: boil the cell suspension in a water bath for 5 minutes, cool on ice and centrifuge at 8000 rpm for 5 minutes. Transferring the supernatant to another tube is not necessary; use the supernatant as template without disturbing the pellet.

4. **PCR setup**

   The following is a typical set up for PCR methodology.

dH2O	13 µl
Buffer	2.5 µl 10 X stock (*polymerase buffer)
dNTP	2.0 µl (1:10 dilution of 10 mM dNTP stock)
Primers	2.5 µl each primer (10 nmoles/ul stock) (will be provided)
Template	2.5µl (e.g., positive control or PCR template prep)

   (It is recommended to use the polymerase buffer that is supplied by the manufacturer of the Taq Polymerase; **NOTE**: not all buffers contain $MgCl_2$; if not provided, final concentration is 1.5 mM).

   Add 1-2 drops of mineral oil (if necessary) and place in thermocycler.

   Note: A strain of *S. flexneri* (2457M) is provided on a slant and should be streaked out onto nutrient agar plate (e.g., isolated colonies). This strain is a positive control, which can be used, with the PCR primers. However, this strain can be differentiated from any other *Shigella* isolates with another set of primers (607, 608) and its resistance to the antibiotic kanamycin [50 ug/ml]. PCR template is prepared by boiling a colony from an agar plate in 150 µl dH20 and using 1 µl in a PCR control tube. Another set of primers specific for this strain is available.

   It is recommended that the positive control be used to ensure that the reagents are working properly.

5. Hot start PCR

This step can be eliminated if using Taq polymersases that require heat activation.

Set one file as follows: 80°C for 10 minutes (this amount of time can be increased if warranted). After thermocycler reached 80°C, 0.3 µl of Taq Polymerase (#) can be added.

6. PCR Amplification cycles and steps

Each cycle consists of the following steps:

1. 94°C for 1 minute (denaturation)
2. 60°C for 1 minute (annealing)
3. 72°C for 1 minute (extension)

Total number of cycles is 30.

7. Agarose Gel Analysis of PCR Products

After amplification, transfer 10 µl of the PCR products to another microcentrifuge tube containing 2 µl of tracking dye and load on 1% agarose gel.

NOTE: Do not add dye directly to the PCR product.

A 100 base pair ladder is used as a molecular weight standard. A 620 base pair product is expected from the positive samples. When reactions are completed, keep PCR product at 4°C or stored at -20°C.

If a 620 bp amplicon is seen on the agarose gel, proceed to the nested PCR step below.

Note: PCR primers

PCR primers (ipaH-F and ipaH-R) are targeted to the ipaH genes; there are multiple copies of this gene residing in the chromosome and one copy in the virulence plasmid of *Shigella*.

Nested PCR protocol

Primers ipaH3 and ipaH4 are directed to internal sequences within the 620 bp amplicon generated from PCRs using primers ipaHF and ipaHR. Using primers ipaH3 and ipaH4, a 290 bp product should be amplified if the 620 bp fragment was generated from *Shigella* DNA. The objective of using the nested PCR assay is to confirm that the 620 bp fragment was amplified from *Shigella*.

The PCR assay is as follows:

1. Distilled water	16 µl	
2. 10 X buffer	2.5 µl	(see note I below)
3. dNTP	0.3 µl	(stock is 10 mM dNTP)
4. Primers		
	2.5 µl ipaH3	(stock is 10 pmol/µl)
	2.5 µl ipaH4	(stock is 10 pmol/µl)
5. Template	1.0 µl	(see explanation II below)

6. Mineral oil	2 drops	
7. Taq polymerase	0.25 µl	(see explanation III below)

PCR conditions (cycles and temperatures) are identical to amplification using PCR primers ipaHF and ipaHR.

After reactions are complete, run 8-10 µl through a 1% agarose gel in 0.5X Tris-acetate EDTA buffer, pH 8.3. Each gel should have a 100 bp ladder as molecular weight marker. A positive reaction generates a 290 bp fragment.

Notes:

I. Certain buffers, # , contain 1.5 mM $MgCl_2$; other buffers may not; therefore, add $MgCl_2$ to a final concentration in the reaction of 1.5 mM.

II. Usually if 1 µl of the PCR product is used directly from the reaction that amplified the 620 bp fragments, then 3 bands may be seen on the gel; the correct band at 290 bp and 2 other approximately 400 and 500 bp. The larger 2 bands are due to primers ipaHF and ipaHR being carried over from the first reaction. The larger amplified bands were generated from the combinations of ipaHF-ipaH4 and ipaHR-ipaHF. To avoid this, dilute the reactions that yielded a presumptive positive product 1:10 and 1:100 in separate tubes with dH_2O. Use 1 µl of the diluted products as template. In some cases, faint bands around 400 and 500 bp may be seen on agarose gels using the 1:10 diluted product as template; this is explained above. A band at 290 bp is confirmation for the presence of *Shigella*.

III. The DNA polymerase used to develop this assay was from Qiagen. Others are probably suitable.

Primer sequence

ipaH3: 5'-CCA CTG AGA GCT GTG AGG
ipaH4: 5'-TGT CAC TCC CGA CAC GCC