External Quality Assurance

Laboratory Handbook and SOPs

Laboratory Methods for Onchocerciasis Elimination

Version: May, 2023

Contributors

Thomas R. Unnasch

Hassan K. Hassan

Reviewed and edited

Vitaliano Cama

Financial Support

The Carter Center

The Mectizan Donation Program

NOTE: These protocols will be updated periodically. The most recent versions may be found at https://health.usf.edu/publichealth/onchocerciasis

Table of Contents

Acronyms

CDC	Centers for Disease Control and Prevention
DBS	Dry blood spots
ELISA	Enzyme-linked Immunosorbent Assay
EC	External Control
EQA	External Quality Assurance
EQAL	External Quality Assessment Laboratory
IC	Internal Control
IQC	Internal Quality Control
MDP	Mectizan Donation Program
MoH	Ministry of Health
NEC	Negative extraction control
NTC	Negative template control
PCR	Polymerase Chain Reaction
PEC	Positive extraction control
PPE	Personal protective equipment
PTC	Positive template control
QA	Quality Assurance
QC	Quality Control
QI	Quality improvement
RT-PCR	Real-Time Polymerase Chain Reaction
SME	Subject matter expert
SOP	Standard Operating Procedure
USF	University of South Florida
USFCC	USF WHO Collaborating Center for Onchocerciasis Diagnostics
WHO	World Health Organization

List of SOPS

1. USF-002 Ov16 ELISA Protocol (OEPA Version)– Detection of antibodies against *O. volvulus*
2. USF-003 DNA extraction from *Simulium* black flies to detect *Onchocerca volvulus*
3. USF-004 Endpoint O-150 PCR Amplification of *Onchocerca volvulus* DNA
4. USF-005 ELISA based detection of O-150 PCR products
5. USF-006 *E. coli* ELISA Protocol – Detection of Antibodies for Sample Acceptance

1. USF-002 Ov16 ELISA Protocol (OEPA Version)– Detection of antibodies against *O. volvulus*

Version May 2023

1. Purpose

This document describes the detection of antibodies against *Onchocerca volvulus* in dry blood spot samples collected from children under 15-years of age. The determination whether a sample is positive is based on ELISA testing.

2. Scope

This procedure applies to the laboratories that belong to the Onchocerciasis Laboratory Diagnostic Network managed by the USF Onchocerciasis Reference Laboratory.

The prevalence of antibodies to the Ov16 in children 5-10 years old is one of two metrics used recommended by WHO to determine if transmission has been suppressed and therefore treatments may be stopped. This is also a supplemental metric recommended by WHO for the verification of elimination if some evidence for ongoing transmission is detected in the post MDA entomological surveys. Ov16 is a 16 kDa antigen found in all lifecycle stages of *Onchocerca volvulus*. IgG4 class antibodies against this antigen have been to be a highly specific indicator of exposure to the parasite. This ELISA protocol is the one used successfully by the countries in Latin America which have verified elimination of onchocerciasis. It is also widely used in Africa.

If this protocol is to be run in a laboratory that is also running the O150 PCR ELISA to detect the presence of *O. volvulus* L3 in vectors, the Ov16 ELISA should be carried out in the post-PCR area where the ELISA plate reader is located. Post PCR pipettors and can be shared between the PCR ELISA and this ELISA protocols. However, it is important that you DO NOT work in the pre-PCR/PCR area after you have done an Ov16 ELISA, as you will be risking contamination by moving from the post PCR area to the pre-PCR/PCR area, as discussed in the O150 PCR ELISA protocol.

For the results of this assay to be valid, the dried blood spots (DBS) that have been collected must contain active antibodies. DBS are stable for relatively long periods (e.g. days) at room temperature, but are very susceptible to degradation if not kept completely dry. Thus prior to beginning the analysis of DBS for Ov16 antibodies, it is necessary to determine if the DBS still contain active antibodies. You do this by first testing a random selection of DBS for the presence of antibodies against *Escherichia coli.* Please refer to the accompanying protocol on how to do this.

3. Related Documents

Title	Document Control Number
External Quality Assurance Manual	USF-001
E. coli ELISA Protocol – Detection of Antibodies for Sample Acceptance	USF-006

4. Responsibility

Position	Duties
Laboratory testing personnel	• Complies with laboratory policies and procedures • Read and understand protocol • Perform testing according to protocol • Record results according to protocol
Supervisor	• Develop and implement policies, processes, and procedures to ensure all critical laboratory functions are carried out under controlled conditions • Ensure all testing personnel are trained and knowledgeable • Review and approve results

5. Definitions

ELISA: Enzyme linked immunosorbent assay
USF: University of South Florida
MDP: Mectizan Donation Program
USFCC: USF Onchocerciasis Reference Laboratory

6. Equipment/Materials (if applicable)

Item
-70^{o}C freezer
-20^{o}C freezer
4^{o}C refrigerator
ELISA plate reader capable of reading at 405 nm
Top loading balance accurate to 10mg
Analytical balance accurate to 0.1mg

pH meter
Vortex mixer
Magnetic Stir plate and stirrers
1-20 µL, 20-200 µL, 200-1000 µL adjustable micropipettes
20-200µL multichannel micropipette (either 8 or 12 channel)
Squirt bottles 500ml size
¼ inch whole punchers

7. Reagents and Supplies

Item	Recommended Supplier	Catalog Number
Latex or nitrile gloves	Any	
Aerosol barrier tips (20µL, 200µL, 1000µL)	Any	
Plate Sealers	Any or Fisher	3501
One liter Ziplock plastic bags	Any	
Polystyrene 96 well plates for DBS elution	Any or Fisher	3370
Immunolon 2HB flat bottomed plates	Fisher	3455
Paper Towels	Any (local)	
50ml Reagent Reservoirs	Any	
Coating Buffer	prepared in lab	
Ov16-GST Antigen	USFCC	
GST Antigen	USFCC	
Humanized Ov16 IgG4 monoclonal antibody (McAb)	BioRad	
anti-human IgG_4 antibody conjugated to biotin	Sigma Invitrogen	Sigma B3648 Invitrogen A10663
Phosphate Buffered Saline (PBS)	prepared in lab	
PBST	prepared in lab	
PBST-BSA	prepared in lab	
Strepavidin-AP conjugate	Fisher	434322
pNPP Substrate and buffer kit	Fisher	37620
Sodium Chloride (NaCl)	Any	
Potassium Chloride (KCl)	Any	
Monobasic potassium phosphate (KH_2PO_4)	Any	
Dibasic Sodium Phosphate (Na_2HPO_4)	Any	
Tween 20	Any	

Sodium Bicarbonate ($NaHCO_3$)		
Bovine Serum Albumin, Fraction V (BSA)	Any or Genesee	2Genessee 5-529
Sodium Hydroxide (NaOH)	Any (locally obtained)	
Stop Solution	prepared in lab	

8. Supplies, Other Materials

Item
Distilled water

9. Safety Precautions

9.1. Procedures should be performed in compliance with all institutional safety policies and procedures, in line with the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC).

9.2. **Personal Protective Equipment:** Eye protection, gloves and lab coat are to be worn at all times

9.3. **Containment Requirements:** Universal precautions apply

9.4. **Spill Response:** n/a

10. **Specimen Collection, Storage, Preservation and Transportation** Specimens will be dry blood spots, individually separated, stored in plastic bags with silica desiccant, and a humidity color indicator.

10.1. All batches of DBS to be tested must comply with acceptance test criteria:

10.2. Proper packaging

10.3. Properly identified with an accompanying spreadsheet to identify all samples

10.4. Pass the *E. coli* batch screening

11. **Specimen Accessioning** The testing laboratory enters the batch information into a master "Ov16 ELISA" Excel document. Each batch of samples will be assigned a unique identifier that allows sample and batch tracking.

12. Quality Control

Positive and negative controls as provided by the USFCC

13. Workflow or Process Table (optional)

13.1. Indicate the building and room numbers where the samples will be processed, and the ELISA will be read.

13.2. Indicate the building and room numbers where the freezers will be located.

14. Procedure

14.1.

Step	Action
1	Prepare a plate map using the following template:

STD 640	STD 640	Blank	Blank	S7	S7	S15	S15	S23	S23	S31	S31
STD 320	STD 320	Blank	Blank	S8	S8	S16	S16	S24	S24	S32	S32
STD 160	STD 160	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
STD 80	STD 80	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
STD 40	STD 40	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
STD 20	STD 20	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
STD 10	STD 10	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
STD 5	STD 5	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38

STD = Standards
Blank = wells with PBST 5% BSA added in place of serum
S1-S38= Eluted serum samples from the DBS (in duplicate)

14.2 Making and Eluting DBS punches

Step	Action
1	Punch out duplicate spots from the dried blood samples collected on the filter papers using a standard 6mm paper punch. Using your map, place the duplicate punches of the blood spots into the sample wells of a DBS elution plate. Add 200 µl of PBST-BSA to each sample. Push the punches to the bottom of the well and then mix 10 times by pipetting. Cover the plates with a plate sealer and incubate them at 4°C overnight.
2	Store the eluted serum samples short term (up to two weeks) at 4°C and long term at -20°C. Minimize the number of freeze thaw cycles.
3	Coating plate with antigen: Dilute Ov16-GST antigen to 2.0ug/ml in coating buffer. Add 100 µL to each well of an Immunolon 2HB plate. Place the plate in a ziplock bag and incubate it overnight at 4°C.
4	Wash 4 times with PBST, using a squirt bottle. Do not dry between washes. Blot dry the plate on a stack of paper towels after last wash.
5	Add 100 µl of PBST-BSA, place the plate in a ziplock bag and incubate it at 4°C for at least 1 hour.

6	During the incubation of step 4 prepare 250µL of a 250ng/ml of the McAb in PBST-5% BSA. This is equivalent to 640 units (per 50µL). Prepare a series of seven two-fold dilutions of the McAb. Start with 125µL of the 250 ng/ml stock and add it to 125µL of PBST-5% BSA. This will be a solution of 125 ng/ml or 320 units/50µL. Continue making the additional dilutions until you have a total of 8 dilutions in the series, which will range from 640 units/50µL to 5 units/50µL. Set aside 250µL of PBST-5% BSA to use as blanks.
7	After the incubation of step 4, empty the PBST-BSA in the sink and blot dry the plate on a stack of paper towels. Do not wash.
8	Using your map (described in 14.1), add 50µl of each serum sample (positive control dilution, blanks and eluted samples) to the corresponding wells on the plate map. Place the plate in a ziplock bag and incubate it at room temperature for 2 hours.
9	Wash 4 times with PBST, using a squirt bottle. Blot dry the plate after the first wash, then carry out the remaining three washes without drying the plate between the wash steps. Blot dry the plate after the 4^{th} wash.
10	10 minutes before the time is up for the last incubation, prepare the conjugate. Dilute the Invitrogen anti-human IgG_4 antibody conjugated to biotin 1:1000 in PBST. If using Sigma IgG4 dilute 1:10,000. Add 50µl of the diluted conjugate to all wells. For a full plate you will need 5.5ml of th working solution. Place the plate in a ziplock bag and incubate it at room temperature for 1 hour.
11	Wash 4 times with PBST, using a squirt bottle. Do not dry between washes. But dry the plate after last wash.
12	10 minutes before the time is up for the last incubation, prepare the streptavidin-AP. Dilute Streptavidin-AP 1:2000 in PBST. For one plate, add 2.75µL of the strepavidin-AP to 5.5ml PBST. Add 50µl to all wells cover the plate and incubate it at room temperature for 1 hour.

13	Wash 4 times with PBST, using a wash bottle. Do not dry between washes. Blot dry the plate after last wash.
14	5 min before the incubation period is over switch on the ELISA plate reader and set it to read at 405nm. Prepare pNPP solution with the substrate buffer by dissolving 1 tablet in 5ml of 1X substrate buffer. Add 50µl to each well.
15	If your reader reads the whole plate at once, then read the plate until the 640 standard is around OD 1.5.
16	If your reader reads well by well (BioTek), then read only the two wells of the 640 standard every minute, and shake the plate between readings. When they are around 0.9 OD, start reading more frequently. When you reach OD 1.1, stop the reaction by adding 25µl of 3M NaOH to each well. Shake the plates to stop the reaction. Incubate the plate for 5 minutes at room temperature and read it at 405nm.

15. Internal Quality Control

The following assumes that you have determined that the DBS have been stored properly and contain active antibodies. Unless this is done, the results of the Ov16 ELISA cannot be validated.

Analysis/Calculations

The Ov16 ELISAs should be analyzed using the following criteria; if the ELISA run does not meet these criteria it must be repeated.

The standard curve dilutions are assigned the following arbitrary units:

Dilution	Units
250ng/ml	640
125ng/ml	320
62.5ng/ml	160
31ng/ml	80
15.5ng/ml	40
8ng/ml	20
4ng/ml	10
2ng/ml	5

A plot of OD versus units should be linear in the range of 10-160 units, with an r^2 value for the line of >0.95. The dynamic range for the linear part of the curve should be at least 5 fold, i.e. the OD value for the 160 unit points should be at least four times greater than the 10 unit points. The 40 unit points should be at least twice the blank ODs. Duplicate wells in the standard curve should be within 10% of one another.

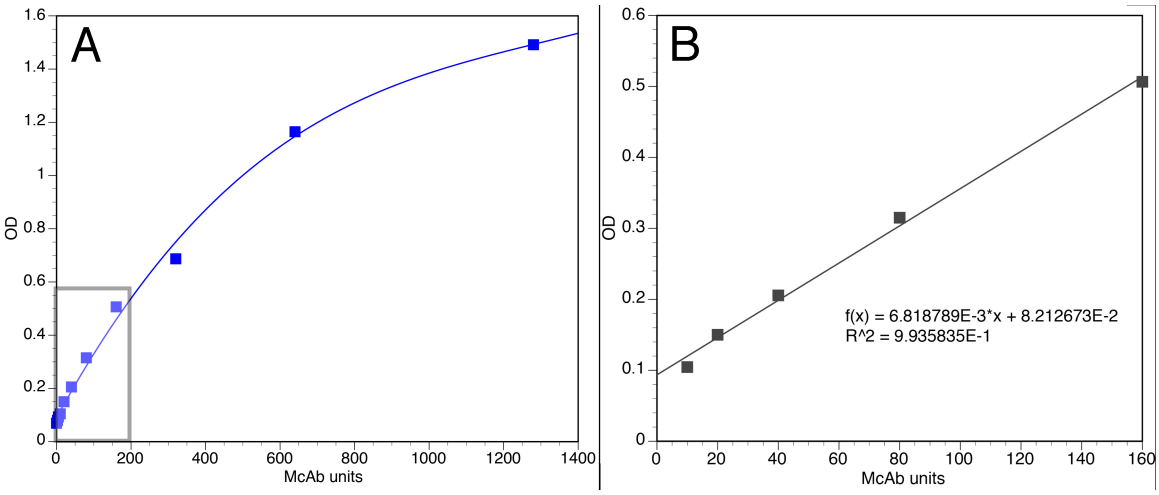

Figure 1: An example of a standard curve that has passed QC standards. Panel A: Plot of all OD values versus McAb units. Note the OD for the 640 unit point is approximately 1.1 indicating the assay was terminated at an appropriate time. The boxed area indicates the linear area of the curve. Panel B: A re-plot of the linear area of the curve. Note the r^2 value is 0.993, which is greater than 0.95. The OD value of the 160 unit point is 0.51, which is more than four times the value of the 10 unit point (0.10).

16. Reference Values/Alert Values

The cutoff is set at 40 arbitrary units.

17. Interpretation of Results

Samples with values equal or greater than 40 arbitrary units are considered positive. Samples with less than 40 arbitrary units are considered negative.

Any sample for which both duplicate samples give a reading at or above this point is considered putatively positive. If the sample is putatively positive, repeat the assay. Samples giving OD values in both assays that are at least 40 units should be then tested in the same way on wells coated with GST. In running the GST plate, the first column should be coated with Ov16 and the standard curve run as usual. Coat the remaining wells to be tested with the serum samples with GST alone. Samples giving an OD value greater than 40 units in both of the assays when tested against Ov16 and which give an OD corresponding to less than 20 units when tested against GST alone are confirmed positives.

18. Results Review and Approval

Laboratory data will be verified and signed off by the personnel who conducted the assay. The results will then be reviewed by the laboratory supervisor. The laboratory director will review and grant approval to the results.

19. Reporting Results; Guidelines for Notification

Enter the results in the laboratory designated file tracking system. Report the results as per laboratory reporting procedures to the submitter.

20. Sample Retention and Storage

Eluted DBS and leftover DBS will be properly labelled and stored in the designated freezer.

21. Records Management

Data sheets from all steps of the process shall be maintained in the laboratory as described in their Quality Management Systems. These records shall be readily available for audit or review at all times.

22. References

22.1. CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC). Accessed April 3, 2023

23. Attachments/Appendices

Appendix 1

Preparation of Coating Buffer (0.1 M $NaHCO_3$, pH 9.6):

1. Dissolve 8.4g of $NaHCO_3$ in 900ml of distilled or deionized water.
2. Check the pH of the solution with the pH meter. It should be pH 9.5-9.7.
3. Add water to reach a final volume of 1 liter.
4. Label including preparation and expiration date (7 days after preparation). Store at 4°C. Keep for no longer than a week.

10X PBS:

1. Weigh:
   - NaCl 80 g
   - KCl 2.0 g
   - KH_2PO_4 2.4g
   - Na_2HPO_4 14.4 g
2. Dissolve all ingredients in 800ml in distilled or deionized water , and adjust the final volume to 1l.
3. Check the pH with the pH meter. It should be pH 7.0-7.4.
4. Label with preparation and expiration date. Store at room temperature.

Appendix 2

Preparation of PBS Tween (PBST):

1. Measure and mix:

10X PBS	100ml
Tween 20	0.5ml
Distilled or deionized water	900ml

Note: Tween 20 is very viscous and may be hard to pipet accurately. To make this easier, thin it out by heating it for a short period in a microwave, the 56°C incubator or a water bath.

2. Label with preparation and expiration date. Keep in a squirt bottle at room temperature.

Appendix 3

Preparation of PBST-BSA

1. Dissolve 5g of BSA in 100ml PBST.
2. Label with preparation and expiration date.
3. Store at 4^{o}C

Stop Solution (3M NaOH)

1. Dissolve 12g of NaOH in 80ml of deionized or distilled water.
2. Adjust final volume to 100ml.
3. Label with preparation and expiration date.
4. Store at room temperature.

24. Revision History

Rev #	DCR #	Changes Made to Document	Date
New		New Document	01/

25. Approval Signatures

Approved By: _____

Author Date

_____

Print Name and Title

Approved By: _____

SME Reviewer Date

_____

Print Name and Title

Approved By: _____

USF Onchocerciasis Reference Laboratory Date

_____

Print Name and Title

2. USF-003 DNA extraction from *Simulium* black flies to detect *Onchocerca volvulus*

1. Purpose

This protocol describes the DNA extraction from *Simulium* black flies for in the detection of infective larval stages of *Onchocerca volvulus*, the causative agent of Onchocerciasis (river blindness). DNA extraction will be from individual pools of up to 100 wild caught blackflies. The prevalence of flies carrying infective larvae is a primary metric recommended by WHO for stop MDA surveys and is the sole metric used by WHO to verify transmission elimination.

2. Scope

This procedure applies to the laboratories that belong to the Onchocerciasis Laboratory Diagnostic Network managed by the USFCC that will test batches of flies for infections with *Onchocerca volvulus*.

Flies should be collected in the field according to standard collection methods and preserved in at least 70% ethanol or isopropanol. Sort the flies according to morphometric criteria when in the lab and only process those identified as *Simulium damnosum sensu lato*. Record the number of *S. damnosum s.l. i*n each pool in an Excel spreadsheet prior to beginning processing.

NOTE 1:

This protocol involves opening the PCR plates and analyzing the sample post PCR. This means you are working with post PCR amplicons, which are a major risk for back contamination, which creates technical issues that can take months to solve. **IT IS CRITICAL** that all steps prior to the PCR be strictly isolated from those carried out after the PCR. This means that **NOTHING** should move from the post-PCR/ELISA area to the pre-PCR/PCR area. To ensure this does not happen you need to physically separate the pre-PCR/PCR areas and the post PCR area, ideally in separate rooms. **DO NOT initiate this protocol if you have been working in the post PCR/ELISA area that day.** Wear gloves throughout the procedure.

NOTE 2:

The laboratory must have room designated for each activity, with dedicated a) personal protective equipment including lab coats, gloves and goggles, b) instruments, c) supplies and d) sets of pipettes for the DNA isolation.

Laboratories must have an unequivocally defined workflow, and personnel should not move from the post PCR area to the pre-PCR/PCR area during the day. If you are going

to do both a PCR and post PCR ELISA on the same day, set up the PCR first and THEN do the PCR ELISA.

All laboratory work must use aerosol barrier tips to prevent contamination.

Note 3:
It is also best to separate the DNA isolation and PCR steps by time. For example, prepare DNA from all the pools first, and once the DNA is prepared, then proceed to the PCR and post PCR analyses.
All reagents and buffers should be prepared with high quality distilled or deionized water.

3. Related Documents

Title	Document Control Number
External Quality Assurance Manual	USF-001

4. Responsibility

Position	Duties
Laboratory testing personnel	• Complies with laboratory policies and procedures • Read and understand protocol • Perform testing according to protocol • Record results according to protocol
Supervisor	• Develop and implement policies, processes, and procedures to ensure all critical laboratory functions are carried out under controlled conditions • Ensure all testing personnel are trained and knowledgeable • Review and approve results

5. Definitions

DNA: Deoxyribonucleic Acid
ELISA: Enzyme linked immunosorbent assay
PCR: Polymerase chain reaction
USF: University of South Florida

MDP: Mectizan Donation Program
USFCC: USF Onchocerciasis Reference Laboratory

6. Equipment/Materials for separation of heads and bodies of the flies

Item	Recommended Supplier	Catalog Number
96-Well Flipper tube Rack	Any	
Dry Block Heater	Any	
-70°C freezer	Any	
-20°C freezer	Any	
1-20µL, 20-200µL, 200-1000µL adjustable micropipettes	Any	
20-200µL multichannel micropipette	Any	
microcentrifuge (12 or 24 tube capacity) capable of 14,000xg	Any	
tube rocker	Any	
Vortex tube mixer	Any	
Thermocycler	Any	
96 well magnetic separator	Any or Fisher	12331D
pH meter	Any	
top loading balance accurate to 10mg	Any	
Analytical balance accurate to 0.1mg	Any	

7. Reagents and Supplies

Item	Recommended Supplier	Catalog Number
Latex or nitrile gloves	Any	
1.5-mL or 1.7ml microcentrifuge tubes	Any	
Disposable blue plastic homogenizers	Fisher	
Aerosol barrier tips (20µL, 200µL, 1000µL)	Any	
96-well PCR plate plates	Any	
PCR plate sealers	Any	
95% ethanol	Any	
TE buffer (10mM Tris-HCl, 1mM disodium EDTA, pH 8.0)	prepared in lab Or Fisher	BP2473
10mg/ml proteinase K	Fisher	BP1700-100
1M DTT	Fisher	BP172
1M Tris HCl (pH 7.5)	Lab prepared	

4M NaCl	Lab prepared	
0.5uM OVS2-biotin. (5´B-AATCTCAAAAAACGGGTACATA-3´, where B = biotin)	USFCC provided	
Dynabeads M-280 Streptavidin coated	Invitrogen	#112-05D
Bead binding buffer (100mM Tris-HCl (pH 7.5) 100mM NaCl).	Lab prepared	
PCR grade water	Any	
Tris Base	Any	
Disodium EDTA dihydrate	Any	
DTT	Fisher	BP172
NaCl	Any	

8. Supplies, Other Materials

Item	Recommended Supplier	Catalog Number
Distilled or deionized water. Reagent or molecular grade water is also acceptable.	Any	

9. Safety Precautions

9.1. Procedures should be performed in compliance with all institutional safety policies and procedures, in line with the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC).

9.2. **Personal Protective Equipment:** Eye protection, gloves and lab coat are to be worn at all times

9.3. **Containment Requirements:** Universal precautions apply

9.4. **Spill Response:** n/a

10. Specimen Collection, Storage, Preservation and Transportation

Flies should be collected in the field according to standard collection methods and preserved in 95% ethanol or 70% isopropanol whichever is locally available. Sort the flies according to morphometric criteria when in the lab and only process those identified as *Simulium damnosum sensu lato.*

11. Specimen Accessioning

Specimen Accessioning The testing laboratory enters the batch information into a master “O-150 PCR ELISA ” Excel document. Each batch of samples will be assigned a unique identifier that allows sample and batch tracking.

12. Quality Control

Positive and negative controls as provided by the USFCC.

13. Workflow or Process Table (optional)

13.1. Indicate the building and room numbers where the fly samples will be processed.

13.2. Indicate the building and room numbers where the freezers will be located.

14. Procedure for black fly processing, in black fly processing room.

14.1. Plan DNA extractions in batches of 12 pools at the time, considering that each batch will include two sham extractions. The number of pools can be adjusted based on **the capacity of your centrifuge**. The two-sham extractions are empty tubes that are carried through the process exactly as if they contained flies. **These are required to ensure that the DNA extraction process remains free of contamination.**

14.2. Rinse the flies in 95% ethanol. Pour the flies out into a petri dish. Allow the ethanol to evaporate until the flies appear to be dry. Do not allow the flies to desiccate completely, or the head purification will not work.

14.3. Place the flies in a clean dry 15 ml polypropylene conical centrifuge tube. Label the tube.

14.4. Place the tube at -70oC for at least 2 hours, or in the vapor phase of liquid nitrogen (if available) for at least 30 minutes.

14.5. Snap off the heads by pounding the tube vigorously on the foam mouse pad placed on the bench. This will separate the heads from the bodies.

14.6. Resuspend the separated heads and bodies in 95% ethanol and remove them from the tube with a wide plastic transfer pipet. The amount of ethanol is not important but try to get all of the fly material.

14.7. Pour the ethanol/fly mix through a 25-mesh sieve connected to a pan. The bodies will collect on the sieve, and the heads will pass through. Rinse the flies collected on the sieve to ensure that all the heads pass through.

14.8. Collect the heads from the pan and the bodies from the sieve using a transfer pipet with a wide opening and put them into separate 35mm petri dishes. Examine them with the microscope to confirm that the separation was successful.

14.9. Collect the separated heads from the petri dish using a wide-opening transfer pipette and transfer them to a 1.5ml snap-cap tubes, labeled as follows: HNNN, where H=Head, and NNN = is the pool sample number. Label another tube, B001 (B=Body, 001= same sample number).

14.10. After the heads are collected, proceed in a similar way to collect the separated bodies from the petri dish: use a wide-opening transfer pipette and transfer them to a 1.5ml snap-cap tubes, labeled BNNN, where B=Body, and NNN = is the corresponding to pool sample number. The pool sample number must be the same on the H and B tubes.

14.11. The samples can be kept at 4oC indefinitely or you can proceed directly to the DNA isolation section.

15. Procedure for DNA extraction from black flies

15.1. Plan DNA extractions in batches of 12 pools at the time, considering that each batch will include two sham extractions. The number of pools can be adjusted based on **the capacity of your centrifuge**. The two-sham extractions are empty tubes that are carried through the process exactly as if they contained flies. **These are required to ensure that the DNA extraction process remains free of contamination.**

15.2. Rinse each of the selected pool sample tubes three times with 95% ethanol. Remove as much of the ethanol as possible using a narrow tip pipet, or 200µL tip.

15.3. Allow the ethanol to evaporate for about 10 minutes at room temperature.

15.4. Add 200µL TE buffer to the heads or bodies, Homogenize the heads or bodies with a disposable blue plastic homogenizer. Continue until the flies are completely broken up.

15.5. Add 40µL 10mg/ml proteinase K.

15.6. Incubate 2 hours at 56°C in the dry block heater.

15.7. Add 20µL of 1M DTT and add 240µL TE to bring the total volume to approximately 500µL.

15.8. Pierce the top of the tube with a needle to provide a vent and incubate at 95°C for 30 minutes. You need to observe the tubes closely, as some might still open during the incubation. If they did, close them immediately to avoid cross contamination.

15.9. Freeze-thaw the extract two times.

15.10. Freeze tubes at -70oC for at least 15 min and thaw on the bench (for approximately another 15 minutes or until they thaw completely). DO NOT quick thaw by warming in your hand or in a water bath. Slow thawing is necessary to allow the ice crystals to lyse the cells and release the DNA.

15.11. Centrifuges the samples for 5 minutes at maximum speed (14,000 xg). Transfer the supernatant to a new 1.5ml tubes.

15.12. Add 50µL 1MTris HCl (pH 7.5) and 12.5µL 4NaCl to each sample.

15.13. Add 5µL 0f 0.5uM OVS2-biotin to each sample.

15.14. Incubate the tubes at 95°C for three minutes in dry block heater and allow them to cool slowly at room temperature on the bench top. This slow cooling allows the oligonucleotides to find and hybridize to the parasite DNA in the sample.

15.15. Preparation of the Dynabeads M-280 Streptavidin. While the samples are cooling down, prepare the beads as they are sent in a solution with preservative.

15.16. Calculate the number of beads required for the total number of samples that you are working with. You need 10µL per sample from the stock solution of 10mg/ml; for example, for 12 samples you will need 120µL of beads.

15.17. Resuspend the beads in its original vial by vortexing for >30 sec. or tilt and rotate the vial for 5 minutes.

15.18. Transfer the desired volume in batches of 50µL into the first wells in row A of microtiter plate (A1,A2,A3,A4,etc.). Place the plate on the magnet and allow the beads to collect for 2 minutes.

15.19. Wash the beads with 200µL binding buffer. Allow the beads to collect for 5 minutes on the magnet and carefully remove and discard the supernatant. Repeat washing four times for a total of five washes.

15.20. Remove the plate from the magnet and resuspend the washed beads in the same volume of binding buffer as the initial volume of beads taken from the vial.

15.21. Add 10µL of the washed bead solution to each sample. Incubate the samples on the tube rocker overnight at room temperature. (Note: procedure can be stopped here at the end of the first day).

15.22. Pipet 100µL of the samples into Rows B-H of the same flat bottom microtiter plate you used above to prepare the beads. Place the plate on the magnet. Allow the beads to collect in the magnetic field for five minutes.

15.23. Carefully remove the supernatant with a micropipette, changing tips between each sample. Sequentially pipet another 100µL of the sample into the well, allowing five minutes for the beads after each addition to collect on the side of the tube facing the magnet before removing the solution and adding the next aliquot. Be careful not to disturb the beads during this process.

15.24. Wash the beads 6 times with 150µL of binding buffer per wash, using a multi-channel pipet. To do so, remove the plate from the magnet, pipet the wash into each well with the multichannel pipet and pipet up and down to mix. Make sure to use aerosol barrier tips.

15.25. Once the beads are resuspended, place the plate back on the magnet and allow the beads to collect for 5 minutes as before. Carefully remove the wash as above. Repeat this process five times more for a total of 6 washes.

15.26. After the last wash, carefully remove as much solution from the beads collected on the side of the wells as possible. Resuspend the beads in 20µL of PCR water. Transfer the samples (solution and beads) to a PCR plate. Seal the plate with a plate sealer.

15.27. Place the plate in the thermocycler and run the following program:
- 80°C for 2 minutes
- 4oC hold.

This will release the captured DNA from the magnetic beads.

15.28. Place the plate on top of the magnet for 2 minutes. This step is to collect the magnetic beads.

15.29. Carefully remove the supernatant using a 200µL multichannel pipette and transfer the extracted DNA to a new plate. Place a seal on the plate. Label the plate including sample information, laboratory personnel initials and date. Reminder: this plate contains your purified DNA.

15.30. Store the plate at -20^{o}C until used for PCR.

16. Internal Quality Control

The two sham tubes will be tested by PCR.

17. Analysis/Calculations

Not applicable

18. Reference Values/Alert Values

Not applicable.

19. Interpretation of Results

If any of the sham tubes has positive PCR results, the PCR will be repeated only with the sham tubes. If the PCR results continue to be positive, this could invalidate the DNA extraction process due to potential contamination.

20. Results Review and Approval

This is the first step of a procedure and results will be reported after PCR amplification.

21. Reporting Results; Guidelines for Notification

Any findings from DNA extraction will be reported with results from PCR amplification.

22. Sample Retention and Storage

Extracted DNA and leftover DNA will be properly labelled and stored in the designated -70C freezer for a minimum of three years.

23. Records Management

Data sheets from all steps of the process shall be maintained in the laboratory as described in their Quality Management Systems. These records shall be readily available for audit or review at all times.

24. References

24.1. CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC). Accessed April 3, 2023

25. Attachments/Appendices

Appendix 1

Preparation of 1M Tris HCl, pH 7.5:

1. Dissolve 121.1g of Tris base in 800ml distilled or deionized water.
2. Adjust the pH to 7.5 using the pH meter and HCl.
3. Adjust the final volume to 1 liter with distilled or deionized water.
4. Label and store at room temperature.

Appendix 2

Preparation of 1M Tris HCl, pH 8.0:

1. Dissolve 121.1g of Tris base in 800ml distilled or deionized water.
2. Adjust the pH to 8.0 using the pH meter and HCl.
3. Adjust the final volume to 1 liter with distilled or deionized water.
4. Label and store at room temperature.

Appendix 3

Preparation of 500mM EDTA:

1. Dissolve 186.1g of disodium EDTA dihydrate in 350ml of distilled or deionized water.
2. Add HCl or NaON to adjust the final pH to 8.0, using the pH meter.
   2.1. Note the EDTA will take a long time to dissolve, and as it dissolves, the pH move away from 8.0.
3. Keep adding acid or NaOH until it fully dissolves and the pH is 8.0.
4. Adjust the final volume to 500ml with distilled or deionized water.
5. Label and store at room temperature.

Appendix 4

4M NaCl:

1. Dissolve 116.9g of NaCl in 300ml of distilled or deionized water.
2. Adjust the final volume to 500ml using distilled or deionized water.
3. Label and tore at room temperature.

Appendix 5

1M DTT:

1. Dissolve 154.3 mg of DTT in 900µL distilled or deionized water.
2. Adjust the final volume to 1ml with distilled or deionized water.
3. Label and store at -20^{o}C.

Appendix 6

TE buffer (10mM Tris-HCl, 1mM disodium EDTA, pH 8.0)

1. Measure and mix:

1M Tris-HCl, pH 8.0	5 ml
500mM EDTA	1 ml
distilled or deionized water	494 ml

2. Place a stir bar in the container and mix on a stir plate for at least one minute
3. Label and store at room temperature.

Appendix 7

Bead binding buffer (100mM Tris-HCl (pH 7.5) 100mM NaCl):

1. Measure and mix:

1M Tris-HCl, pH 7.5	50 ml
4M NaCl	12.5 ml
distilled or deionized water	437.5 ml

2. Place a stir bar in the container and mix on a stir plate for at least one minute
3. Label and store at room temperature.

Appendix 8

10mg/ml Proteinase K:

1. Measure and mix

proteinase K (weigh)	50mg
TE buffer	5ml

2. Place a stir bar in the container to dissolve and mix on a stir plate for at least three minutes
3. Label 10 1.5 microcentrifuge tubes for making aliquots
4. Using a 1000 µL pipette and tips, aliquot into 10 x 500µL aliquots.
5. Store at -70^{o}C.
6. **Special instructions**
   6.1. Aliquots must remain frozen until use. Once thaw, the aliquots cannot be frozen again.
   6.2. Each aliquot (in the 1.5 ml microcentrifuge tube) will be enough to process 12 samples.
   6.3. Once thawed, the letforvers of the aliquot must be discarded.
   6.4. Do not re-freeze at any time.

26. Revision History

Rev #	DCR #	Changes Made to Document	Date
New		New Document	

27. Approval Signatures

Approved By: _____

Author Date

_____

Print Name and Title

Approved By: _____

SME Reviewer Date

_____

Print Name and Title

Approved By: _____

USF Onchocerciasis Reference Laboratory Date

3. USF-004 Endpoint O-150 PCR Amplification of *Onchocerca volvulus* DNA

1. Purpose

This protocol describes in the detection of *Onchocerca volvulus* infective stage larvae, the causative agent of Onchocerciasis (river blindness), in DNA extracted from individual pools of up to 100 wild caught blackflies (SOP USF-003). The prevalence of flies carrying infective larvae is a primary metric recommended by WHO for stop MDA surveys and is the sole metric used by WHO to verify transmission elimination.

2. Scope

This procedure applies to the laboratories that belong to the Onchocerciasis Laboratory Diagnostic Network managed by the USF Onchocerciasis Reference Laboratory. These laboratories will perform PCR on DNA extracted from heads or bodies of *Simulium* flies as part of the program assessments towards the elimination of *Onchocerca volvulus*.

3. Related Documents

Title	Document Control Number
External Quality Assurance Manual	USF-001
DNA extraction from *Simulium* black flies to detect *Onchocerca volvulus*	USF-003
ELISA based detection of O-150 PCR Products	USF-005

4. Responsibility

Position	Duties
Laboratory testing personnel	• Complies with laboratory policies and procedures • Read and understand protocol • Perform testing according to protocol • Record results according to protocol
Supervisor	• Develop and implement policies, processes, and procedures to ensure all critical laboratory functions are carried out under controlled conditions • Ensure all testing personnel are trained and knowledgeable • Review and approve results

5. Definitions

DNA: Deoxyribonucleic Acid
ELISA: Enzyme linked immunosorbent assay
PCR: Polymerase chain reaction
USF: University of South Florida
MDP: Mectizan Donation Program
USFCC: USF Onchocerciasis Reference Laboratory

6. Equipment/Materials for separation of heads and bodies of the flies

Item	Recommended Supplier	Catalog Number
PCR clean hood	Any	
96-Well Flipper tube Rack	Any	
-70°C freezer	Any	
-20°C freezer	Any	
1-20µL, 20-200µL, 200-1000µL adjustable micropipettes dedicated to master mix preparation	Any	
1-20µL adjustable micropipette dedicated to positive control addition (post PCR area)	Any	
20-200µL multichannel micropipette	Any	
microcentrifuge (12 or 24 tube capacity) capable of 14,000xg	Any	
Vortex tube mixer	Any	
pH meter	Any	
top loading balance accurate to 10mg	Any	
Thermocycler, conventional	BioRad	

7. Reagents and Supplies

Item	Recommended Supplier	Catalog Number
Latex or nitrile gloves	Any	
1.5-mL or 1.7ml microcentrifuge tubes	Any	
Aerosol barrier tips (20µL, 200µL, 1000µL)	Any	
96-well PCR plate plates	Any	
PCR plate sealers	Any	
10X PCR buffer	lab prepared	
2mM dNTP mix	lab prepared (stocks from Invitrogen)	
dNTP stock solutions	Thermo Fisher	
20uM O-150 primers	Thermo Fisher	
Taq polymerase	Any	
O-150 positive control	USFCC	
Ammonium Sulfate $(NH_4)_2SO_4$	Any	
Tris Base	Any	
$MgCl_2$	Any	

8. Supplies, Other Materials

Item	Recommended Supplier	Catalog Number
PCR or molecular grade water.	Any	

9. Safety Precautions

9.1. Procedures should be performed in compliance with all institutional safety policies and procedures, in line with the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition.

9.2. **Personal Protective Equipment:** Eye protection, gloves and lab coat are to be worn at all times

9.3. **Containment Requirements:** Universal precautions apply

9.4. **Spill Response:** n/a

10. Specimen Collection, Storage, Preservation and Transportation

DNA extracted and stored as per SOP USF-003

11. Specimen Accessioning

Not applicable, however, the testing laboratory must have a master “O-150 PCR ELISA” Excel document that allows sample and batch tracking.

12. Quality Control

Positive controls as provided by the USFCC.

13. Workflow or Process Table (optional)

13.1. Indicate the building and room numbers where the master mix will be prepared, the PCR will be setup and run and where the ELISA will be read.

13.2. Indicate the building and room numbers where the freezers will be located.

14. Preparation of mastermix (pre-PCR/PCR area)

14.1. DO NOT initiate this protocol if you have been working in the post PCR/ELISA area that day.

14.2. Wear pre-PCR/PCR room designated lab coats, gloves, and goggles throughout the procedure.

14.3. The master mix should be prepared in the PCR clean hood using pipets and aerosol barrier tips solely dedicated to master mix production.

14.4. Bring all the PCR reagents (10X buffer, primers, dNTPs, Taq polymerase) on ice bucket from the freezer to the pre-PCR/PCR area and allow them to thaw. Keep the Taq polymerase on ice.

14.5. Centrifuge all reagents for 30 sec.

14.6. Prepare the amount of master mix according to the number of samples you will be testing. See table below for examples for 1 reaction and 100 reactions (one plate). Make about 10% extra to account for losses during pipetting.

14.7. Mix the contents of the master mix tube by gentle vortexing.

14.8. Remove the master mix from the PCR clean hood. Aliquot 45µL of the master mix into each well of a 96-well PCR plate.

14.9. Keep the plate on ice and return the reagents to the freezer.

Reagent	Volume needed for 1 reaction	Volume needed for 100 reactions
PCR water	32µL	3.2ml
10X buffer	5µL	500µL
2mM dNTPs	5µL	500µL
20uM 1632 primer	1.25µL	125µL
20uM 1633 biotin primer	1.25µL	125µL
Taq polymerase	0.5µL	50µL
Total volume	45µL	4.5ml

15. Procedure for adding extracted DNA for PCR amplification (pre-PCR/PCR area)

15.1. Negative controls must be added before any sample or DNA positive control. Each negative control reaction should contain 5 µL of PCR water in place of the DNA.

15.2. Add 5µL of the selected purified DNA samples using multichannel pipette. Use the following template:

Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	pos + DNA
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham
Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	Sampl	sha	sham

15.3. Each complete PCR plate will have up two positive control reactions (in wells A11 and A12) and 10 negative control reactions (in rows A1-A10). Overall, you should be able to do 84 PCRs on each ELISA plate. In total, this means a single plate will contain DNA from 70 head pools, 14 sham extractions, 10 negative controls and two positive controls.

16. Adding positive controls

16.1. Cover the plate and move the plate to the **post PCR area.**

16.2. In the post PCR area, dedicate a small area of the bench away from all other activities as the positive control addition area.

16.3. Place the plate in this area and add the positive control (see **Appendix 1** for preparation of positive control) using the pipet dedicated to this purpose.

16.4. In well A11, add 2.5 µL of the PCR positive control DNA plus 2.5 µL of DNA from a preparation from a pool that has previously tested negative. Alternatively, 2.5 µL of DNA that was prepared from a pool of 100 flies known to be uninfected (collected from a confirmed non endemic region). T

16.5. In well A12, add 2.5 µL of the positive control DNA (from 16.3 above).

16.6. Seal the with a plate sealer.

16.7. Change your gloves and **return to the pre-PCR area.**

16.8. Place the plate in the thermocycler and proceed to PCR amplification. The cycling parameters for the PCR amplifications are as follows:

Step 1: 94° C for 1 min
Step 2: 37° C for 2 min
Step 3: 72° C for 30 seconds
Step 4: Repeat steps 1–3 for a total of 5 cycles
Step 5: 94° C for 30 seconds
Step 6: 37° C for 30 seconds
Step 7: 72° C for 30 seconds
Step 8: Repeat steps 5–7 for a total of 35 cycles
Step 9: Hold at 4C

17. Analysis/Calculations

Not applicable

18. Reference Values/Alert Values

Not applicable.

19. Interpretation of Results

If any of the sham tubes has positive PCR results, the PCR will be repeated only with the sham tubes. If the PCR results continue to be positive, this could invalidate the DNA extraction process due to potential contamination.

The positive control in well A12 will tell whether the PCR reaction worked as efficiently as possible. The positive control in well A11 (well with 2.5 µL of DNA from known negative flies) will demonstrate that fly DNA preparations are no inhibiting the PCR.

20. Results Review and Approval

This is the first step of a procedure and results will be reported after PCR amplification.

21. Reporting Results; Guidelines for Notification

Any findings from DNA extraction will be reported with results from PCR amplification.

22. Sample Retention and Storage

Extracted DNA and leftover DNA will be properly labelled and stored in the designated -70C freezer for a minimum of three years.

23. Records Management

Data sheets from all steps of the process shall be maintained in the laboratory as described in their Quality Management Systems. These records shall be readily available for audit or review at all times.

24. References

24.1. CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC). Accessed April 3, 2023

25. Attachments/Appendices

Appendix 1

Preparation of O-150 positive control.
Upon receipt from USF, the O-150 positive controls need to be calibrated through PCR reactivity of serial dilutions.

1. Work and prepare the dilutions in the post PCR/ELISA area using the pipet dedicated to positive control addition.
2. Set up six 10-fold serial dilutions of the positive control stock to be used. To do this, begin diluting 2.5 µL of the undiluted positive control from USF in 22.5 µL of TE 8.0 buffer. This is your 1/10 dilution. Take 2.5 µL of the 1/10 dilution and add it to 22.5 µL of TE 8.0 buffer. This is your 1/100 dilution. Continue making dilutions in the same way until you have made a total of 6 10-fold dilutions. Dilutions could be prepared in PCR plates or PCR amplification tubes.
3. Prepare master mix for 10 reactions following the instructions from #14 above.
4. Dispense 47.5 µL of mastermix to nine wells (if running a plate) or PCR tubes.
5. Add 1.5 µL of PCR water to each well/tube
6. 1µL of each serial dilution of the positive control DNA. See proposed plate template below

1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				
1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	Neg	Neg				

7. Run PCR using the amplification conditions described in #15 above.
8. Identify the highest dilution that reliable gives a PCR positive result. Using the dilution that is 100-fold more concentrated than this dilution (e.g. if you detect 1/100,000 reliably, take 1/1000) and prepare 100 µL of this dilution by diluting 10 µL of the 100 fold dilution into 90 µL TE 8.0 buffer.
9. Use 2.5µL of this dilution as positive control in all subsequent experiments. Store all dilutions at -20^{o}C.

Appendix 2

Preparation of 1M Tris HCl, pH 9.0:

1. Dissolve 121.1g of Tris base in 800ml distilled or deionized water.
2. Adjust the pH to 9.0 using the pH meter and HCl.
3. Adjust the final volume to 1 liter with distilled or deionized water.
4. Label, autoclave, and store at room temperature.

Appendix 3

Preparation of 1M Ammonium Sulfate ($(NH_4)_2SO_{4)}$):

1. Dissolve 13.2g of Ammonium Sulfate in 80ml of distilled or deionized water.
2. Adjust the final volume to 100ml with distilled or deionized water.
3. Label, autoclave, and store at room temperature.

Appendix 4

Preparation of 1M Magnesium Chloride ($MgCl_2$):

1. Dissolve 9.5g of Magnesium Chloride in 80ml of distilled or deionized water.
2. Adjust the final volume to 100ml with distilled or deionized water.
3. Label, autoclave, and store at room temperature.

Appendix 5

Preparation of 10X PCR Buffer:

1. Prepare 50ml from autoclaved solutions by mixing:

Reagent/concentration	Final concentration	Volume to add
1M Tris-HCl (pH9.0)	600mM	30 ml
1M $(NH_4)_2SO_4$	150mM	7.5 ml
1M $MgCl_2$	20mM	1.0 ml
PCR water	---	11.5 ml

2. Aliquot in 1.5ml tubes.
3. Label and store at -20^{o}C.

Appendix 6

Preparation of dNTP, 2mM each

1. Prepare 5ml with the following reagents:

Reagent/concentration	Final concentration	Amount you add
100mM dATP	2mM	100µL
100mM dATP	2mM	100µL
100mM dATP	2mM	100µL
100mM dATP	2mM	100µL
PCR water	---	4.6 ml

2. Make 1ml aliquots in 1.5ml tubes.
3. Label and store at -20°C.

Appendix 7

Preparation of primers:

1. Primer sequences for this assay are:
   1632: 5' GATTYTTCCGRCGAANARCGC 3'
   1633 biotin: 5' biotin-GCNRTRTAAATNTGNAAATTC 3'
   Where, N = A, G, C OR T, Y = C OR T, R = A OR G
2. The primer spec sheet will indicate the total number of nmol that they gave you. Dissolve the primers in 10 times the number of µL of PCR water as there are nmol in the tube. For example, if they say there are 38 nmol in the tube on the spec sheet, dissolve the primer in 380µL of PCR water. This produces a 100uM stock solution.
3. Aliquot the stock solutions into 100µL aliquots in screw capped microfuge tubes and store at -80°C. Add 400µL of PCR water for a 20uM working solution.
4. Label and store the working solutions at -20°C.

Appendix 8

10mg/ml Proteinase K:

1. Measure and mix
   proteinase K (weigh) 50mg
   TE buffer 5ml
2. Place a stir bar in the container to dissolve and mix on a stir plate for at least three minutes
3. Label 10 1.5 microcentrifuge tubes for making aliquots

26. Revision History

Rev #	DCR #	Changes Made to Document	Date
New		New Document	01/

27. Approval Signatures

Approved By: _____

Author Date

_____

Print Name and Title

Approved By: _____

SME Reviewer Date

_____

Print Name and Title

Approved By: _____

USF Onchocerciasis Reference Laboratory Date

4. USF-005 ELISA based detection of O-150 PCR products

1. Purpose

This protocol describes the ELISA based detection of DNA from *Onchocerca volvulus* infective stage larvae, after O-150 PCR amplification from individual pools of up to 100 wild caught blackflies (SOP USF-004).

2. Scope

This procedure applies to the laboratories that belong to the Onchocerciasis Laboratory Diagnostic Network managed by the USF Onchocerciasis Reference Laboratory. These laboratories will perform PCR on DNA extracted from heads or bodies of *Simulium* flies as part of the program assessments towards the elimination of *Onchocerca volvulus*.

3. Related Documents

Title	Document Control Number
External Quality Assurance Manual	USF-001
DNA extraction from *Simulium* black flies to detect *Onchocerca volvulus*	USF-003
Endpoint O-150 PCR Amplification of *Onchocerca volvulus* DNA	USF-004

4. Responsibility

Position	Duties
Laboratory testing personnel	• Complies with laboratory policies and procedures • Read and understand protocol • Perform testing according to protocol • Record results according to protocol
Supervisor	• Develop and implement policies, processes, and procedures to ensure all critical laboratory functions are carried out under controlled conditions • Ensure all testing personnel are trained and knowledgeable • Review and approve results

5. Definitions

DNA: Deoxyribonucleic Acid
ELISA: Enzyme linked immunosorbent assay
PCR: Polymerase chain reaction
USF: University of South Florida
MDP: Mectizan Donation Program
USFCC: USF Onchocerciasis Reference Laboratory

6. Equipment/Materials for separation of heads and bodies of the flies

Item	Recommended Supplier	Catalog Number
Incubator set at 37°C	Any	
Incubator set at 42°C	Any	
Incubator set at 56°C	Any	
Refrigerator at 4°C	Any	
-20°C Freezer	Any	
Squirt bottles 500ml size	Any	
1-20µL, 20-200µL, 200-1000µL adjustable micropipettes	Any	
1-20µL adjustable micropipette dedicated to positive control addition (post PCR area)	Any	
20-200µL multichannel micropipette	Any	
ELISA plate reader capable of reading at 650 nm	Any	
Vortex tube mixer	Any	
pH meter	Any	
top loading balance accurate to 10mg	Any	

7. Reagents and Supplies

Item	Recommended Supplier	Catalog Number
Latex or nitrile gloves	Any	
Aerosol barrier tips (20µL, 200µL, 1000µL)	Any	
Plate Sealers		
Streptavidin 1mg/ml	Thermo Fisher	434301
Immunolon 2 HB round bottom plates	Fisher	
Paper Towels	Any	
50ml Reagent Reservoirs	Any	
Coating buffer	Prepared in lab	
TBST solution	Prepared in lab	
Hybridization buffer	Prepared in lab	
Sodium Hydroxide (NaOH)	Any (local)	
OVS2-Fl probe	USFCC	
Anti-fluorescein-AP conjugated Fab fragments	Thermo Fisher	11426338910
Blue Phos detection system	KPL	50-88-22
AP Stop solution	KPL	50-89-00
SSPE/SDS wash buffer	Prepared in lab	
Sodium bicarbonate ($NaHCO_3$)	Any	
Sodium Carbonate (Na_2CO_3)	Any	
Tris base	Any	
Tween 20	Any	
NaCl	Any	
Bovine Serum Albumin, Fraction V (BSA)	Any	
Sodium Phosphate dibasic (NaH_2PO_4)	Any	
Disodium EDTA dihydrate	Any	
polyvinylpyrrolidone	Any	
Ficoll 400	Any	
Sodium dodecyl sulfate (SDS)	Any	
N-lauryl sarcosine	Any	

8. Supplies, Other Materials

Item	Recommended Supplier	Catalog Number
Distilled or reagent grade water.	Any	

9. Safety Precautions

9.1. Procedures should be performed in compliance with all institutional safety policies and procedures, in line with the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition

9.2. **Personal Protective Equipment:** Eye protection, gloves and lab coat are to be worn at all times.

9.3. **Containment Requirements:** Universal precautions apply

9.4. **Spill Response:** n/a

10. Specimen Collection, Storage, Preservation and Transportation

This SOP is to detect DNA in plates that underwent PCR amplification as described in SOP USF-004. Plates must be read the same day or next day after PCR amplification. Plates must remain covered and stored at 4^{o}C until ELISA testing starts.

11. Specimen Accessioning

Not applicable, however, the testing laboratory must have a master "O-150 PCR ELISA" Excel document that allows sample and batch tracking.

12. Quality Control

Positive controls were included in the PCR amplification procedure (SOP USF-004) and previously provided by the USFCC.

13. Workflow or Process Table (optional)

13.1. Indicate the building and room numbers where the master mix will be prepared, the PCR will be setup and run and where the ELISA will be read.

13.2. Indicate the building and room numbers where the freezers will be located.

14. Preparation for ELISA (post-PCR area)

14.1. When the PCR is complete. remove the plate from the thermocycler and move to the post PCR area without opening the plate. DO NOT open the plate until in the post PCR area. Once you have opened the plate, DO NOT return to the pre-PCR/PCR area that day. If it is necessary to return to the pre-PCR/PCR area, go home first, change your clothing and take a shower before returning to the lab.

14.2. Wear gloves throughout the procedure. Remove gloves and discard them before leaving the post PCR/ELISA area.

14.3. Coat one plate (or the required number of wells) with streptavidin by adding 100ul of a working solution of 1ug/ml streptavidin in coating buffer to each well. Cover the plate with plate sealer.

14.4. Incubate the plate at 4°C overnight.

14.5. Empty the plate into the sink. Wash the well six times with TBST at room temperature. Invert the plate between washes on to a paper towel to remove the residual wash fluid.

14.6. Add 20ul of hybridization buffer to each coated well using multichannel pipette.

14.7. Add 5ul of each PCR product to the appropriate wells.

14.8. Incubate the plate at room temperature for 30 minutes.

14.9. During the incubation period, prepare the working solution of the OVS2-FL probe (100 ng/ml in hybridization buffer).

14.10. Empty the plate into the sink. Wash the well six times with TBST at room temperature.

14.11. Add 100ul IM NaOH to each well and incubate for one minute at room temperature.

14.12. Empty the plate into the sink. Wash the well six times with TBST at room temperature.

14.13. Add 50ul of the working solution of the OVS2-FL probe and incubate at 42°C for 15 minutes.

14.14. Empty the plate into the sink. Wash the well six times with TBST solution at room temperature.

14.15. Add 100ul of warmed (56°C) SSPE/SDS wash buffer to each well.

14.16. Incubate the plate at 56^{o}C for 10 minutes.

14.17. During the incubation period, prepare working solutions of the Anti-fluorescein-AP conjugated Fab fragments (1ul of the stock solution in 10ml antibody dilution buffer).

14.18. Empty the plate into the sink. Wash the well six times with TBST.

14.19. Add 50ul of the diluted Anti-fluorescein-AP conjugated Fab fragments to each well.

14.20. Incubate the plate at 37^{o}C for 15 minutes.

14.21. During the incubation period, prepare working solutions of the BluePhos detection system by mixing equal volumes of solution A and solution B in 15 ml tube. Use within 30 minutes of mixing. You need 10ml of the mixture for one full plate.

14.22. Empty the plate into the sink. Wash the well six times with TBST solution at room temperature.

14.23. Add 100ul of freshly mixed BluePhos solution to each well. Tap gently to Mix. Incubate at room temp for 20-30 min. Blue color will start to appear.

14.24. Dilute one part of AP Stop solution with 9 parts of high-quality water to make 1X stop solution at room temperature.

14.25. For one plate 1 ml AP stop + 9ml H_2O.

14.26. Add 100ul of AP stop solution to each well to stop color development at desired time.
14.27. Read plates at 650nm.

15. Analysis/Calculations

15.1. Take the mean of all ten negative control wells and determine the standard deviation of the negative controls. Add a value equal to three standard deviations to the mean of the negative control wells.

15.2. We use two different cutoffs for the assay depending upon what the mean plus 3 SD of the negatives are. If this value is above 0.1, use the mean plus 3 SD as the cutoff. If it is below 0.1, set the cutoff at 0.1. The reason for this is that as you do the assay, your consistency will improve, and the blank wells will begin to show very little variation. The major source of variation that you see when this happens arises from background contributed by the DNA samples themselves and not variation in the assay. If you get very tight low blanks, the cutoff will not be sufficient to account for this underlying source of variation, and you will score a number of wells as false positives. However, this underlying variation never exceeds 0.1, so 0.1 is the cutoff to use in this situation.

15.3. Any well with an OD value below the cutoff is scored as negative and anything with a value above the cutoff is scored as a putative positive. When a putative positive is detected, repeat the entire assay for the putative positive samples, beginning with a new PCR reaction. Any sample that scores above the cutoff in two independent PCRs is scored as a confirmed positive.

16. Reference Values/Alert Values

Not applicable.

17. Interpretation of Results

18. Data analysis of experimental plates:

Take the mean of all ten negative control wells and determine the standard deviation of the negative controls. Add a value equal to three standard deviations to the mean of the negative control wells.

We use two different cutoffs for the assay depending upon what the mean plus 3 SD of the negatives are. If this value is above 0.1, use the mean plus 3 SD as the cutoff. If it is below 0.1, set the cutoff at 0.1. The reason for this is that as you do the assay, your

consistency will improve and the blank wells will begin to show very little variation. The major source of variation that you see when this happens arises from background contributed by the DNA samples themselves and not variation in the assay. If you get very tight low blanks, the cutoff will not be sufficient to account for this underlying source of variation, and you will score a number of wells as false positives. However, this underlying variation never exceeds 0.1, so 0.1 is the cutoff to use in this situation.

Look at the OD values in the positive control wells (A11 and A12). Both should be above the positive cutoff as determined above. It is normal for the well containing the fly DNA to be somewhat less than the well without the fly DNA, but both must be above the cutoff for the result on the plate to be valid. Any well with an OD value below the cutoff is scored as negative and anything with a value above the cutoff is scored as a putative positive. When a putative positive is detected, repeat the entire assay, beginning with a new PCR reaction. Any sample that scores above the cutoff in two independent PCRs is scored as a confirmed O-150 positive. Confirmed positive DNA samples should be sent to the Unnasch laboratory for independent confirmation.

All the negative control wells and the sham DNA extractions should have OD values that are at background level (i.e. less than 0.1). If any of the negative control wells or sham extractions have positive OD values, it is an indication that you have contamination in your system somewhere. If the sham reactions re positive and the negative control reactions are all negative, this points to contamination in the DNA extraction process. If a negative control is positive, you likely have contamination in the PCR process. Contamination issues are very hard to diagnose and correct. Contact the reference lab for advice on how to identify the source of contamination and correct the problem.

19. Results Review and Approval

Laboratory data will be verified and signed off by the personnel who conducted the assay. The results will then be reviewed by the laboratory supervisor. The laboratory director will review and grant approval to the results.

20. Reporting Results; Guidelines for Notification

Enter the results in the laboratory designated file tracking system. Report the results as per laboratory reporting procedures to the submitter.

21. Sample Retention and Storage

Extracted DNA and leftover DNA will be properly labelled and stored in the designated -70C freezer for a minimum of three years.

22. Records Management

Data sheets from all steps of the process shall be maintained in the laboratory as described in their Quality Management Systems. These records shall be readily available for audit or review at all times.

23. References

23.1. CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC). Accessed April 3, 2023

24. Attachments/Appendices

Appendix 1

Preparation of 10x TBS:

1. Dissolve 30.2g Tris base and 43.8gNaCl in 350ml deionized or distilled water.
2. Adjust pH to 7.5 with HCl. Adjust volume to 500ml.
3. Label and store at room temperature.

Appendix 2

Preparation of 1 L of TBST:

1. Measure and mix
    a. 10x TBS 100ml
    b. Tween 20 0.5ml
    c. distilled or deionized water to 1 liter (ca. 900ml)

    Note: Tween 20 is very viscous and may be hard to pipet accurately. To thin it out heat it for a short period in a microwave or the 56°C incubator.
2. Keep in squirt bottles
3. Label and store at room temperature.

Appendix 3

Preparation of Coating Buffer:

1. Prepare 50mM $NaHCO_3$, 2mM $Na2CO_3$.
    a. 4.2g Sodium bicarbonate
    b. 0.211g Sodium carbonate
    c. Dissolve to make one liter
2. Make the coating buffer fresh weekly as the pH goes off during storage.
3. Label with an expiration date of 1-week, and store at 4°C

Appendix 4

Preparation of Hybridization buffer

1. Make 500ml stock solution using the following solutions:

Reagent	Amount to add
20X SSPE	120ml
10X Denhardt's	50ml
1% (w/v) N-lauryl sarcosine	50ml
10% SDS	1ml
distilled or deionized water	279ml

2. Label, autoclave, and store at room temperature.

Appendix 5

Preparation of OVS2-Fl probe:

5' AATCTCAAAAAACGGGTACATA-fl 3' where fl = fluorescein.

1. When you receive this, the spec sheet should indicate the exact amount they shipped to you in micrograms.
2. Dissolve the entire amount in enough hybridization buffer to give you a 100x stock solution of 10ug/ml. Aliquot the 100x stock solution into aliquots of 55ul each and store them at -70°C.
3. To prepare the working solution, remove an aliquot from the freezer, thaw and add 5.45ml of hybridization buffer. This will produce 5.5ml of working solution, enough to do one full plate.
4. Store any unused working solution at -20°C and use within a month.

Appendix 6

Preparation of 20X SSPE:

1. Dissolve
    a. 174 g NaCl,
    b. 27.6g NaH_2PO_4,
    c. 7.4g disodium EDTA
    d. in 700ml distilled or deionized water.
2. Adjust pH to 7.4 with NaOH.
3. Bring volume to 1 liter.
4. Store at room temperature.

Appendix 7

Preparation of 10X Denhardt's

1. Weigh out 0.2g of each of the three ingredients below and dissolve them in 100ml distilled or deionized water.
2. 0.2% (w/v) bovine serum albumin
3. 0.2% (w/v) polyvinylpyrrolidone
4. 0.2% (w/v) Ficoll 400
5. Label and store at -20°C

Appendix 8

Preparation of 1% N-lauryl sarcosine

1. Dissolve 1g N-lauryl in 80ml distilled or deionized water.
2. Adjust volume to 100ml
3. Label and store at room temperature.

Appendix 9

Preparation of 10% SDS:

1. SDS is very light and the crystals are very irritating if inhaled. Wear a dust mask when weighing it out.
2. Dissolve 100g of SDS in 800ml of distilled or deionized water.
3. Adjust the final volume to 1 liter.
4. Label and store at room temperature.

Appendix 10

Preparation of SSPE/SDS wash buffer:

1. Measure and mix:
    a. 20X SSPE 5ml
    b. 10% SDS 1ml
    c. Distilled or deionized water 94ml
2. Label and store at 56^{o}C.

Appendix 11

Preparation of Antibody dilution buffer

1. Measure and mix the following:

Reagent	Amount to add
4M NaCl	10ml
1M Tris HCl (pH 7.5)	10ml
BSA	0.5g
distilled or deionized water.	80ml

2. Label and store at -20^{o}C

25. Revision History

Rev #	DCR #	Changes Made to Document	Date
New		New Document	

26. Approval Signatures

Approved By: _____

Author Date

_____

Print Name and Title

Approved By: _____

SME Reviewer Date

_____

Print Name and Title

Approved By: _____

USF Onchocerciasis Reference Laboratory Date

5. USF-006 *E. coli* ELISA Protocol – Detection of Antibodies for Sample Acceptance

1. Purpose

This document describes the detection of antibodies against *Escherichia coli* in dry blood spot samples collected from children under 15-years of age. The determination whether a sample is positive is based on ELISA testing.

2. Scope

This procedure applies to the laboratories that belong to the Onchocerciasis Laboratory Diagnostic Network managed by the USFCC.

In order for the results of the Ov16 ELISA assay to be valid, the dried blood spots (DBS) that have been collected must contain active antibodies. DBS are stable for relatively long periods (e.g. days) at room temperature, but are very susceptible to degradation if not kept completely dry. Thus prior to beginning the analysis of DBS for Ov16 antibodies, it is necessary to determine if the DBS still contain active antibodies. You do this by first testing a random selection of DBS for the presence of antibodies against *Eschereria coli*. *E. coli* is a bacterium that is univerally found in our intestines and is a common source of infections (of cuts or the urinary tract), as feces contain large quantities of *E. coli*. Thus, antibodies to E. coli are almost universal. This protocol describes how to detect *E. coli* antibodies in the eluted blood spots.

If this protocol is to be run in a laboratory that is also running the O150 PCR ELISA to detect the presence of *O. volvulus* L3 in vectors, the Ov16 ELISA should be carried out in the post-PCR area where the ELISA plate reader is located. Post PCR pipettors and can be shared between the PCR ELISA and this ELISA protocols. However, it is important that you **DO NOT** work in the pre-PCR/PCR area after you have done an Ov16 ELISA, as you will be risking contamination by moving from the post PCR area to the pre PCR/PCR area, as discussed in the O150 PCR ELISA protocol.

3. Related Documents

Title	Document Control Number
External Quality Assurance Manual	USF-001
Ov16 ELISA Protocol (OEPA Version)– Detection of antibodies against *O. volvulus*	USF-002

4. Responsibility

Position	Duties
Laboratory testing personnel	• Complies with laboratory policies and procedures • Read and understand protocol • Perform testing according to protocol • Record results according to protocol
Supervisor	• Develop and implement policies, processes, and procedures to ensure all critical laboratory functions are carried out under controlled conditions • Ensure all testing personnel are trained and knowledgeable • Review and approve results

5. Definitions

ELISA: Enzyme linked immunosorbent assay
USF: University of South Florida
MDP: Mectizan Donation Program
USFCC: USF Onchocerciasis Reference Laboratory

6. Equipment/Materials (if applicable)

Item
-70^{o}C freezer
-20^{o}C freezer
4^{o}C refrigerator
ELISA plate reader capable of reading at 405 nm
Top loading balance accurate to 10mg
Analytical balance accurate to 0.1mg
pH meter

Vortex mixer
Magnetic Stir plate and stirrers
1-20 µL, 20-200 µL, 200-1000 µL adjustable micropipets
20-200ul multichannel micropipette (either 8 or 12 channel)
Squirt bottles 500ml size
¼" notebook hole punch

7. Reagents and Supplies

Item	Recommended Supplier	Catalog Number
Latex or nitrile gloves	Any	
Aerosol barrier tips (20ul, 200ul, 1000ul)	Any	
Plate Sealers	Any or Fisher	3501
One liter Ziplock plastic bags	Any	
Polystyrene 96 well plates for DBS elution	Any or Fisher	3370
Immunolon 2HB flat bottomed plates	Fisher	3455
Paper Towels	Any (local)	
50ml Reagent Reservoirs	Any	
Coating Buffer	prepared in lab	
E. coli Antigen	USFCC	
GST Antigen	USFCC	
Goat Anti-Human IgG AP conjugate	Jackson Immuno Research	109-055-003
Phosphate Buffered Saline (PBS)	prepared in lab	
PBST	prepared in lab	
PBST-BSA	prepared in lab	
pNPP Substrate and buffer kit	Fisher	37620
Sodium Chloride (NaCl)	Any	
Potassium Chloride (KCl)	Any	
Monobasic potassium phosphate (KH_2PO_4)	Any	
Dibasic Sodium Phosphate (Na_2HPO_4)	Any	
Tween 20	Any	
Sodium Bicarbonate ($NaHCO_3$)	Any	
Bovine Serum Albumin, Fraction V (BSA)	Sigma recommended	
Sodium Hydroxide (NaOH)	Any (locally obtained)	
Stop Solution	prepared in lab	

8. Supplies, Other Materials

Item
Not applicable

9. Safety Precautions

9.1. Procedures should be performed in compliance with all institutional safety policies and procedures, in line with the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC).

9.2. **Personal Protective Equipment:** Eye protection, gloves and lab coat are to be worn at all times

9.3. **Containment Requirements:** Universal precautions apply

9.4. **Spill Response:** n/a

10. Specimen Collection, Storage, Preservation and Transportation Specimens will be dry blood spots, individually separated, stored in plastic bags with silica desiccant, and a humidity color indicator.

10.1. All batches of DBS to be tested must comply with acceptance test criteria:

10.2. Proper bag packaging: intact bag, fully labelled, properly sealed, presence of desiccant with color indicator representing dry, card humidity indicator showing <20% humidity.

10.3. Each bag in the batch properly identified with an accompanying spreadsheet to identify all samples

10.4. If these requirements are not met, the bags could not be further processed for testing. The submitter will be notified and the bags returned to the submitter.

11. Specimen Accessioning The testing laboratory enters the batch information into a master “Ov16 ELISA” Excel document. Each batch of samples will be assigned a unique identifier that allows sample and batch tracking.

12. Quality Control

Positive and negative controls as provided by the USFCC.

13. Workflow or Process Table (optional)

13.1. Indicate the building and room numbers where the samples will be processed, and the ELISA will be read.

13.2. Indicate the building and room numbers where the freezers will be located.

14. Procedure

14.1. Randomly choose a selection of DBS to test for *E. coli* antibodies. You should choose 10% of the total number of samples from a given collection bag that was delivered to the lab, or at least 10 DBS if there are less than 100 DBS in a collection bag. Each ELISA plate will be able to analyze 40 samples in this protocol (see below)

14.2. Punch out a spot from the dried blood samples collected on the filter papers using a standard 1/4" paper punch. Using your map, place the duplicate punches of the blood spots into the wells of a 96 well DBS elution plate. Add 200 µL of PBST-BSA to each sample. Push the punches to the bottom of the well and then mix 10 times by pipetting. Cover the plates with a plate sealer and incubate them at 4°C overnight. Store the eluted serum samples short term (up to two weeks) at 4°C and long term at -20°C. Minimize the number of freeze thaw cycles.

14.3. Prepare two antigen solutions for coating the plate: One a solution of 2µg/ml *E. coli* antigen in coating buffer, and the second containing 2µg/ml BSA.

14.4. Following the map below, add 100 µL of each coating solution as indicated to the wells of an Immulon II HB plate, coating columns 1-6 with BSA and 7-12 with *E. coli* antigen. Seal the plate and incubate at 4°C overnight.

	BSA						***E.coli***					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	BLK	S1	S9	S17	S25	S33	BLK	S1	S9	S17	S25	S33
B	BLK	S2	S10	S18	S26	S34	BLK	S2	S10	S18	S26	S34
C	BLK	S3	S11	S19	S27	S35	BLK	S3	S11	S19	S27	S35
D	BLK	S4	S12	S20	S28	S36	BLK	S4	S12	S20	S28	S36
E	BLK	S5	S13	S21	S29	S37	BLK	S5	S13	S21	S29	S37
F	BLK	S6	S14	S22	S30	S38	BLK	S6	S14	S22	S30	S38
G	BLK	S7	S15	S23	S31	S39	BLK	S7	S15	S23	S31	S39
H	BLK	S8	S16	S24	S32	S40	BLK	S8	S16	S24	S32	S40

S= DBS (sample)

BLK= Blank (no serum will be added)

14.5. Wash the 4 times with PBST, using a squirt bottle. Do not dry between washes. Blot dry the plate on a stack of paper towels after last wash.

14.6. Add 100 µL of PBST-BSA to each well and incubate at 4°C for 1 hour.

14.7. After incubation, remove the plate from the refrigerator and discard the buffer. Don't wash the plate but tap on a stack of paper towels to dry.

14.8. Add 50 µL eluted DBS in duplicate to each well according to the map. For blanks add PBST-BSA.

14.9. Seal the plate and incubate at room temperature for 2 hours.

14.10. Wash the plates four times with PBST. Tap the plate after final wash on paper towels to dry.

14.11. Add 50 µL of AP conjugated goat anti-human IgG diluted 1:5000 in PBST to each well. Seal the plate and incubate the plate at room temperature for one hour.

14.12. Wash the plates four times with PBST. Tap the plate after final wash on paper towels to dry.

14.13. Prepare pNPP solution with the substrate buffer by dissolving 1 tablet in 5ml of 1X substrate buffer. Add 50µl to each well. Incubate at room temperature covered with foil or in a drawer for 20-30 minutes.

14.14. Add 25 µL of stop solution (3M NaOH) to each well and read the plate immediately at 405nm.

15. Quality Control

The blank wells and the BSA coated wells should all have OD values that are 0.1 or less.

16. Analysis/Calculations

A sample is scored as positive for *E. coli* antibodies if the OD in the *E. coli* coated well is 5-fold or more than the OD seen in the corresponding BSA coated well.

At least 70% of your samples from a collection bag should be positive for *E. coli* antibodies. A lower level of reactivity to *E. coli* suggests that the DBS were spoiled, and all samples in such bag should not be used in the Ov16 ELISA. The submitter will be notified and the bag with its contents will be returned to the submitter.

17. Reference Values/Alert Values

Not applicable.

18. Results Review and Approval

Laboratory results will be evaluated by the laboratory supervisor and the director.

19. Reporting Results; Guidelines for Notification

Enter the results in the laboratory designated file tracking system. Bags that pass the test will be allowed to be further tested. Results from failing bags will be processed as per laboratory reporting procedures to the submitter.

20. Sample Retention and Storage

Eluted DBS and leftover DBS will be properly labelled and stored in the designated freezer.

21. Records Management

Data sheets from all steps of the process shall be maintained in the laboratory as described in their Quality Management Systems. These records shall be readily available for audit or review at all times.

22. References

22.1. CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition | CDC Laboratory Portal | CDC). Accessed April 3, 2023

23. Attachments/Appendices

Appendix 1

Preparation of Coating Buffer (0.1 M $NaHCO_3$, pH 9.6):

1. Dissolve 8.4g of $NaHCO_3$ in 900ml of distilled or deionized water.
2. Check the pH of the solution with the pH meter. It should be pH 9.5-9.7.
3. Add water to reach a final volume of 1 liter.
4. Label including preparation and expiration date (7 days after preparation). Store at 4°C. Keep for no longer than a week.

10X PBS:

1. Weigh:
   - NaCl 80 g
   - KCl 2.0 g
   - KH_2PO_4 2.4g
   - Na_2HPO_4 14.4 g
2. Dissolve all ingredients in 800ml in distilled or deionized water , and adjust the final volume to 1l.
3. Check the pH with the pH meter. It should be pH 7.0-7.4.
4. Label with preparation and expiration date. Store at room temperature.

Appendix 2

Preparation of PBS Tween (PBST):

1. Measure and mix:

10X PBS	100ml
Tween 20	0.5ml
Distilled or deionized water	900ml

Note: Tween 20 is very viscous and may be hard to pipet accurately. To make this easier, thin it out by heating it for a short period in a microwave, the 56°C incubator or a water bath.

2. Label with preparation and expiration date. Keep in a squirt bottle at room temperature.

Appendix 3

Preparation of PBST-BSA

1. Dissolve 5g of BSA in 100ml PBST.
2. Label with preparation and expiration date.
3. Store at 4°C

Stop Solution (3M NaOH)

1. Dissolve 12g of NaOH in 80ml of deionized or distilled water.
2. Adjust final volume to 100ml.
3. Label with preparation and expiration date.
4. Store at room temperature.

24. Revision History

Rev #	DCR #	Changes Made to Document	Date
New		New Document	

25. Approval Signatures

Approved By: _____

Author Date

_____

Print Name and Title

Approved By: _____

SME Reviewer Date

_____

Print Name and Title

Approved By: _____

USF Onchocerciasis Reference Laboratory Date

_____

Print Name and Title