Appendix D: Example DNA Extraction Protocol

	Division of Tuberculosis Elimination Applied Research Laboratory		
Mycobacterium tuberculosis **complex (MTBC) DNA Isolation Using the ZR Fungal/Bacterial DNA MicroPrep Kit**			
Doc. No. ART.DR.C.0005	**Rev. No.** 02	**Effective Date:** 10/14/2014	**Page 1 of 12**

1.0 **Purpose/Principle**

The ZR Fungal/Bacterial DNA MicroPrep Kit is used for the simple, rapid isolation of DNA from M. *tuberculosis.* The purified DNA can be used for downstream molecular-based applications.

2.0 **Scope**

This procedure is used by the ART personnel for making MTBC DNA preparations by this method.

3.0 **Related Documents**

Title	Document Control Number
DNA Preparation Worksheet using the ZR Fungal/Bacterial DNA MicroPrep Kit	ART.DR.C.0005.F01
Guide for DNA Isolation Using the ZR Fungal/Bacterial DNA MicroPrep Kit	ART.DR.C.0005.J01
Training Checklist for the ZR Fungal/Bacterial DNA MicroPrep Kit	ART.HR.F.0007
ISAW Camera Operation	MLB.EQ.C.0002

4.0 **Responsibility**

Position	Responsibility
ART Staff	Only staff trained and cleared to work in the BSL-3 area and trained to perform this procedure may do so.
ART Team Lead or Designee	Oversees all ART activities.

5.0 **Definitions**

Term	Definition
BSL	Biological Safety Level - MTBC isolate manipulations must be done in the BSL-3 laboratory using good laboratory practices and wearing appropriate PPE for the job.
DSR	Division of Scientific Resources
FSE	Four Seasons Environmental Services is responsible for maintaining, monitoring and repairing both the building systems and laboratory (freezers and refrigerators) equipment.
MTBC	*Mycobacterium tuberculosis* complex includes *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii*, *M. mungi*, *M. orygis* and *M. canetti*.
PAPR	A powered air purifying respirator consists of a facepiece, breathing tube, battery-operated blower, and particulate filters. The PAPR uses a blower to pass contaminated air through a HEPA filter which removes the contaminant and supplies purified air to a facepiece.

6.0 Equipment

Item	Any required maintenance - See ART General Equipment Calibration and Maintenance (ART.EQ.C.0002) for additional information.
Aerosolve Canisters or Equivalent	Used for centrifuging viable biological agents in the Allegra centrifuge. A base that holds a tube rack with a cap and O-ring seal.
Allegra 6R Bench Top Centrifuge with Rotor GH-3.8 Horiz with 4 Arm Bkt or Equivalent	See separate procedure for maintenance requirements**.**
Autoclave	FSE is responsible for autoclave maintenance. Any run that has a process failure must be re-autoclaved after the problem is resolved, unless failure occurred while opening the door on a process complete run. A biological indicator is tested monthly as part of the monthly autoclave monitoring program (LB and DSR staff). The BSL-3/3+ laboratory area has a pass-through autoclave.
Biological Safety Cabinet (BSC)	Magnehelic gauge reading and direction of tape on glass shield is recorded before using BSC. Decontaminate the BSC work surfaces before and after each use. BSCs are re-certified each year by a qualified technician. Motor or filter problems must be corrected and re-certified before the BSC can be used. Contact ESHCO for BSC issues.
Camera (ISAW)	See the *ISAW Camera Operation* (MLB.EQ.C.0002*)* for additional information.
Eppendorf 5418 Microfuge 120V or Equivalent	See separate procedure for maintenance requirements**.** In *BSL-3+ laboratory, the microfuge is equipped with an aerosol rotor.*
MPBio Fast Prep 24	FastPrep 24 should be checked for damage before using.
Freezer	Set to -19 to -25°C. Freezer should be defrosted if frost/ice buildup interferes with storage useable space. Four Seasons Engineering services (FSE) is responsible for changing filters on each unit. The freezer temperature is monitored by FSE.
Freezer (Ultralow)	Freezer set at -65^{o} to -75 ^{o}C. Freezer should be defrosted if frost/ice buildup interferes with storage useable space. The unit must be wiped down with a tuberculocidal disinfectant whenever contaminated, defrosted (at least annually), moved, or if repairs are needed. Freezer temperatures are monitored by FSE.
Incubator	Incubator set at 35 to 38^{o}C. Incubator should be wiped down with a tuberculocidal disinfectant annually or whenever contaminated, moved, or if repairs are needed. The incubator temperatures are monitored by FSE.
Micropipettes	Calibration and checks for any needed repairs are done

	annually.
Refrigerator	Set to 2° to 8°C. Refrigerator should be wiped down with a tuberculocidal disinfectant whenever contaminated, moved, or if repairs are needed. Refrigerator temperatures are monitored by FSE.
Spectrophotometer (Nanodrop 2000 or Qubit 2.0)	
Vortex mixer	Clean if spill occurs.

7.0 Reagents and Media

Item	Storage requirements
Middlebrook 7H9 Broth (6 mL)	Store at 2 to 8°C. May be used until expiration date. Either made by DSR or by ART staff.
Nuclease free water	Store at RT.
Tuberculocidal Disinfectant (Vesphene or Lysol III)	Store at RT. May be used until expiration date.
ZRFungal/Bacterial DNA MicroPrep Kit	Store at RT. Integrity of kit components is guaranteed for up to one year from date of purchase.

ZR Fungal/Bacterial DNA MicroPrep Kit Components (50 preps.)	Quantity
Collection Tubes	150
DNA Elution Buffer	10 ml
DNA Pre-Wash Buffer*	15 ml
Fungal/Bacterial DNA Binding Buffer**	100 ml
Fungal/Bacterial DNA Wash Buffer	50 ml
Lysis Solution	40 ml
ZR BashingBead Lysis Tubes	50
Zymo-Spin IC Columns	50
Zymo-Spin IV Spin Filters (orange tops)	50

* A precipitate may have formed in the DNA Pre-Wash Buffer during shipping. To completely re-suspend the buffer, incubate the bottle 30 – 37 ˚C for 30 minutes and mix by inversion. DO NOT MICROWAVE.
**For optimal performance, add beta-mercaptoethanol to 0.5% (v/v) i.e., 500 µL per 100 mL

8.0 Supplies, Other Materials

Item
1.5 mL microtube
10 mL serological pipette
15 mL polypropylene centrifuge tubes
Appropriate PPE for the BSL-3+ and BSL-2 laboratories
Biological safety container or carrier (to move preparations from BSL-3 to BSL-2)

Electronic pipetting device
Screw cap glass test tube
Sterile filtered pipette tips
Test tube rack

9.0 **Safety Precautions**

9.1. *BSL-3+ Laboratories*:

9.1.1. Viable *Mycobacterium tuberculosis* is an infectious agent and must be handled with care by trained personnel working under appropriate conditions.

9.1.2. All procedures involving manipulation of live organisms (i.e., inoculum preparation, making dilutions, and inoculations of media) must be performed in a BSC (or equipment with aerosol containing devices) in a BSL-3+ laboratory using BSL-3 practices. Use appropriate PPE (scrubs, gown, PAPR, gloves, dedicated shoes, shoe covers and hair cap).

9.1.3. All containers and equipment must be sprayed with tuberculocidal disinfectant (Lysol III) before removing from the BSC.

9.1.4. Tubes should be contained within aerosolve containers or similar biosafety devices for all centrifugation steps. **Following centrifugation, these containers should be transferred into the BSC (microfuge is used in the BSC) before they are opened.**

9.1.5. *Recommended set up for BSC*: Items are arranged in the BSC to avoid contaminated items being passed over clean items. Items or equipment in direct contact with the etiologic agent remain in the BSC until surface decontaminated.

9.1.6. All biological waste is placed in a discard pan lined with a biohazard bag for autoclaving.

9.2. *BSL-2 Laboratories*:

9.2.1. A lab coat and gloves is worn when working in a BSL-2 laboratory with DNA preparations.

9.3. See the LB Site Specific BioSafety Document for PPE exceptions, biological spills, waste disposal and other biosafety information.

9.4. *PAPR requirements*

9.4.1. Inspect the breathing tube, body of the HEPA filter, hood for damage before using.

9.4.2. Use a battery that has sufficient charge for the activities to be performed.

9.4.3. Check for adequate airflow prior to use. Attach the breathing tube to the PAPR outlet then turn on the power. Insert the tapered end of the airflow meter.

9.4.4. The ball in the airflow meter should reach the level for the type of PAPR used (4 cfm for facepiece and 6 cfm for hood).

9.5. Verification testing has been done to show that MTBC organisms, at the volumes used in this procedure, are inactivated or depleted at the completion of the steps in this method. **If greater volumes or modifications are made to this method, verification testing must be performed. See the *Verification of An Inactivation or Depletion Method* procedure (ART.DR.C.0009) used by ART for MTBC Organisms.**

10.0 **Sample Information / Processing**
Frozen aliquot of MTBC organism of interest.

11.0 **Quality Control** – Not Applicable

12.0 **"Two Key" Approach to Critical Control Point**

12.1. The critical control point (step) for inactivation of the sample is the Fast Prep step.

12.2. To document this critical step, the ART staff member performing the procedure activates the camera to video Fast Prep steps. See the *ISAW Camera Operation* (MLB.EQ.C.0002) procedure for camera operation.

12.3. At the completion of the critical step, turn the camera off. Press the silver button on the top of the black box to transfer video.

12.4. Once BSL-3 work is complete, DNA samples are stored in the refrigerator in room 4079CC until the video is reviewed and the *DNA Preparation Worksheet using the ZR Fungal/Bacterial DNA MicroPrep Kit* (ART.DR.C.0005.F01) is signed off by the team lead or designee.

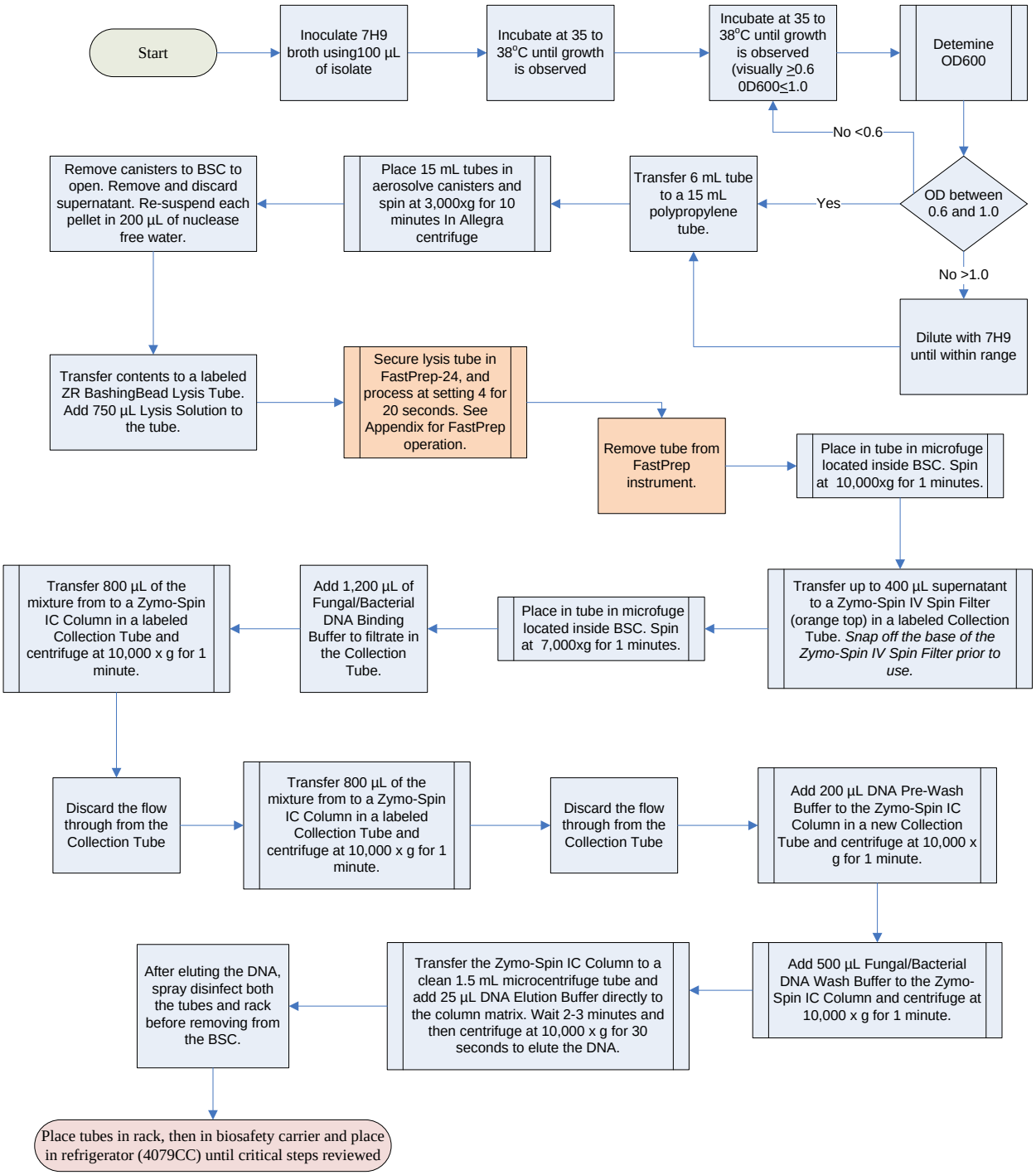
12.5. *Documentation of the critical step*:

12.5.1. The staff member attests that the materials were prepared (in the BSL-3+) according to the stated protocol on the worksheet.

12.5.2. The worksheet is scanned into the computer in room 4085 and emailed to the ART team lead or designee.

12.5.3. The ART team lead or designee documents on a copy of the scanned worksheet that they have reviewed the video and the samples can be moved to BSL-2.

13.0 Workflow Chart



Critical Steps in orange must be videoed.

14.0 Procedure

Step	Action
The following Steps are Performed in a BSL-3 Lab Wearing Appropriate BSL-3 PPE	
1	Working in a BSC, inoculate 100 µL of a frozen stock (thawed) to a labeled 7H9 broth (6 mL). *Return the aliquot stock to the freezer when BSL-3+ work is complete.*
2	Spray the tubes and the rack with tuberculocidal disinfectant before removing from the BSC. Place the rack inside the plastic container on the orbital shaker inside the 35 to 38˚C incubator. Proceed to step 3 when slightly turbid growth is observed.
3	When it appears to have achieved OD600 remove broth tube from incubator and proceed to step 4.
4	Determine OD600 using spectrophotometer using the 7H9 broth tube.
4.1	If OD600 is 0.6 to 1.0, proceed with purification (step 5).
4.2	If OD600 is <0.6, return broth tube to incubator until sufficient OD is achieved.
4.3	If OD600 is >1.0, dilute with 7H9 broth until OD is between 0.6 to 1.0. Proceed with purification (step 5).
4	Transfer 6 mL of culture to a labeled 15 mL polypropylene centrifuge tube. Spray disinfect the centrifuge tubes before removing from the BSC.
5	Place the centrifuge tubes in aerosolve canisters in the centrifuge making sure to balance the tubes. Replace the aerosolve caps. Ensure that swinging buckets are properly seated and spin at approximately 3,000 x g for 10 minutes to pellet the growth.
6	After centrifugation, the aerosolve canisters must be opened in the BSC. Remove the supernatant with a 10 mL pipette and discard into biohazard discard pan.
7	Re-suspend each pellet in 200 µL of nuclease free water and transfer contents to a labeled ZR BashingBead Lysis Tube. Add 750 µL Lysis Solution to the tube. Spray disinfect the lysis tube before removing from the BSC.
Start of '2 key' Critical Step	
8	Turn on the camera to record the critical step. (See appendix 23.2 for video recording steps.) See the *ISAW Camera Operation* (MLB.EQ.C.0002) procedure for detailed instructions on the camera/router/laptop operations.
9	Secure lysis tube in FastPrep-24. Distribute evenly amongst the available holes allowing at least one empty hole between tubes. Process at setting 4 for 20 seconds. See Appendix 23.1 for FastPrep operation.
10	Remove tube from FastPrep.

11	Turn off the camera. Press the silver button on the top of the black box to transfer video. **Seven minutes after the silver button is hit, the camera can start a new video if necessary.**
End of '2 key' Critical Step	
12	Place in microfuge located in BSC and centrifuge at 10,000 x g for 1 minute.
13	Transfer up to 400 µL supernatant to a Zymo-Spin IV Spin Filter (orange top) in a labeled Collection Tube. *Snap off the base of the Zymo-Spin IV Spin Filter prior to use.*
14	Centrifuge at 7,000 rpm for 1 minute.
15	Add 1,200 µL of Fungal/Bacterial DNA Binding Buffer to filtrate in the Collection Tube from Step 13.
16	Transfer 800 µL of the mixture from Step 15 to a Zymo-Spin IC Column in a labeled Collection Tube and centrifuge at 10,000 x g for 1 minute.
17	Discard the flow through from the Collection Tube and repeat Step 16.
18	Add 200 µL DNA Pre-Wash Buffer to the Zymo-Spin IC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.
19	Add 500 µL Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin IC Column and centrifuge at 10,000 x g for 1 minute.
20	Transfer the Zymo-Spin IC Column to a clean 1.5 mL microcentrifuge tube and add 25 µL DNA Elution Buffer directly to the column matrix. Wait 2-3 minutes and then centrifuge at 10,000 x g for 30 seconds to elute the DNA.
21	After eluting the DNA, spray disinfect both the tubes and rack before removing from the BSC.
22	When ready to leave the BSL-3+ lab, change outer gloves and place rack in biological safety carrier (on cart outside BSL-3+ lab). *At this point, the worksheet is signed for the BSL-3+ steps and scanned into the computer in room 4085.*
23	Email the ART team lead or designee requesting review of the video.
24	Place biological safety carrier in the refrigerator located in room 4079CC on the shelf labeled "ART DNA or Whole Cell Lysate Sample Storage - Pending Review of Critical Steps." **The video must be reviewed and the critical steps confirmed before the samples can be removed from the BSL-3 area.**
Documenting Review of the Critical Steps	
25	The team lead or his designee reviews the critical steps on the video.
25.1	*If the critical steps were performed correctly*, the team lead or designee signs and dates the emailed worksheet.
25.2	*If the critical steps were not performed correctly,* the samples must be

	discarded as biohazardous waste. Follow up actions are documented on the worksheet. **A review of the procedure and/or additional training is done before the individual can perform this procedure unsupervised.**
26	The file name of the video is changed to indicate the assay performed/tech.
27	The reviewed worksheet is kept in the office of the team lead for a minimum of 2 years.
If Critical Steps were Performed Correctly	
28	The team lead or designee notifies the person performing the assay that they can remove the samples to the BSL-2.
29	Place tube in either a 2 to 8^{o}C refrigerator (short term storage) or a $\leq$-20 ^{o}C freezer until ready to use.
30	Return the biological safety container to the BSL-3+ area.

15.0 **Method Performance Specifications -** Not Applicable

16.0 **Calculations** - Not Applicable

17.0 **Reference Values, Alert Values -** Not Applicable

18.0 **Interpretation of Results -** Not Applicable

19.0 **Results Review and Approval -** Not Applicable

20.0 **Reporting Results; Guidelines for Notification -** Not Applicable

21.0 **Sample Storage**
21.1. Short term storage is at 2 to 8°C
21.2. Long term storage is at $\leq$ -19°C

22.0 **References**
22.1. MPBio website Fast Prep-24 Instrument Product Information section. 2011.
22.2. MPBio Fast Prep Users Guide **(www.MPBio.com/product_info)**
22.3. ZR Fungal/Bacterial DNA MicroPrep Kit Instruction Manual. Ver. 1.0.4.

23.0 **Appendices**
23.1. **Fast Prep Operation (MPBio Fast Prep Users Guide)**
23.1.1. *Fast Prep Information*:
23.1.1.1. The FastPrep®-24 Instrument is a high-speed bench top homogenizer for the lysis of biological samples. The FastPrep®-24 Instrument uses a unique, optimized motion to

disrupt cells through the multidirectional, simultaneous beating of specialized Lysing Matrix beads on the sample material.

23.1.1.2. Specifications Controls: Microcontroller control panel, with LCD screen and membrane-printed keyboard

23.1.1.3. Microcontroller clock 16 MHz Time: 1- 60 seconds, programmable in 1 sec increments Speed: 4 - 6.5 m/s, programmable in 0.5 m/s increments

23.1.1.4. Acceleration: <2 seconds to maximum speed

23.1.1.5. Deceleration: <2 seconds to stop

23.1.1.6. Duty cycle: 6.5 m/s for 60 seconds with 60 seconds rest period between runs

23.1.1.7. Noise level: <70dB

23.1.1.8. Standard environmental temperature operating range: 4 – 40 °C

23.1.1.9. Safety features: automatic stop on lid opening; emergency stop button; sealing of the sample handling compartment

23.1.2. *Controls*

Set Key	Press this key to select: Speed - 4.0 m/n to 6.2 m/s (Default 4.0 m/s) Time – 1 sec to 60 sec (Default 20 sec)
Run Key	To start or stop the instrument.
▲ Key	To increase the selected value of speed, tube holder & time.
▼ Key	To decrease the selected value of speed, tube holder & time.

23.1.3. *Loading and securing the samples*

23.1.3.1. Remove the securing knob that holds down the sample holder by rotating counter-clockwise.

23.1.3.2. Remove the sample holder from the instrument.

23.1.3.3. Slightly lift the spoke plate (top portion of the sample holder) and rotate it clockwise until the retention spokes move away from the hole openings.

23.1.3.4. Load the sample tubes into the sample holder wells, so that they fit snugly. Make sure that the tubes are balanced.

23.1.3.5. Replace the sample holder in the instrument making sure to align the aluminum locking pin, on the aluminum assembly below the tube holder, to the hole in the tube holder.

23.1.3.6. Rotate the spoke plate counter-clockwise until the retention spokes are above each tube.

23.1.3.7. Replace the securing knob and tighten by rotating until secure.

23.1.3.8. Close the dome and secure the dome clip.

23.2. **Video Recording**

See (*MLB.EQ.C.0002) ISAW Camera Operation* for Detailed Setup Instructions for the Camera/Router/Laptop	
1	Check that the router is plugged in and turned on. Router lights should be lit if on.

2	Check the camera status and turn on if asleep. • *Awake* – the display on the back of the camera will show a picture. • *Asleep* – press the top button on camera briefly and firmly to change camera to awake status.
3	View the picture display to confirm that the camera location will capture the activity(s) that need to be recorded. If not, adjust the camera angle until correct. *Make sure that the location will not interfere with the activities to be performed.*
4	When ready to record make sure camera is awake. (See (MLB.EQ.C.0002) ISAW Camera Operation step 4 if no picture is displayed on back of camera).
5	When the camera is awake, press the top button on the camera and recording will start. The camera is recording when the timer begins recording and a red dot is seen in the lower right area of the display. **Do not bump or disturb the camera while recording.** *If a timer, thermometer or another item needs to be recorded, position the item until it is in view. If a critical step to be recorded involves a reagent, the reagent container should have the name of the reagent displayed.*
6	When finished recording, hold down the top button briefly (2 -3 seconds) to turn the camera off.
7	Press the silver button on the top of the black box. The video will transfer to the laptop to the predetermined directory for viewing. One hour of video transfers in approximately 1.5 minutes, 2 hours in 2.5 minutes, etc. ***Seven minutes after the silver button is hit, the camera can start a new video.***

24.0 Revision History

Rev #	DCR #	Changes Made to Document	Author	Date
01	2014-06	New document	Paige Chopra	7/29/2014
02	2014-31	Modified workflow charts and procedural steps to document storage of DNA samples in BSL-3 until after review of the critical step video. Added procedural steps of what occurs if critical steps not performed correctly. Added worksheet storage requirements. Added camera operation to appendices.	Lois Diem (Revised)	10/10/2014

25.0 Approval Signatures

Approved By: _____ Date: _____
Author or Revised By

_____
Print Name

Approved By: _____ Date: _____
Team Lead or Designee

_____
Print Name

Approved By: _____ Date: _____
QMS Officer

_____
Print Name