Procedures for Amplification of Nuclear DNA

1 Scope

These procedures apply to DNA personnel who prepare plates for the amplification of nuclear DNA (nDNA) from evidence and reference samples and DNA personnel that perform the associated quality control procedures. The DNA Casework Unit (DCU) and Biometrics Analysis Unit (BAU) use Sample Tracking and Control Software (STACS) and robotic workstations to automate the set-up of the amplification (aka amp) plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- Tecan robot model 'Freedom EVO'
  - Tecan EvoWare software, version 2.3 or higher
- STACS, version 3.2.920 or later
- Thermal Cycler, GeneAmp® PCR System 9700
- General laboratory supplies (e.g., pipettes, tubes)
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear and foil plate seals
- Thermal Microplate Sealer

Reagents

- AmpF*l*STR® PCR Amplification Kit(s) (Applied Biosystems)
  - Identifiler® Plus
  - Yfiler™
    - 9947A, 10 ng/µL
- GlobalFiler™ PCR Amplification Kit (Applied Biosystems)
- 007 or 9947A sample, quantified and diluted as necessary (Applied Biosystems)
- Buffer, Low TE (aka TEKnova DNA Suspension Buffer) (Fisher Scientific or equivalent)
- 3% bleach (household or equivalent)
- 10% bleach (Daigger or equivalent)
- Isopropyl alcohol, 70%
- Water (reagent grade or equivalent)
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)

3 Standards and Controls

A positive amplification control and negative amplification control (also referred to as the amplification blank) must be processed in parallel with each set or batch of evidentiary samples

subjected to polymerase chain reaction (PCR) amplification. 9947A DNA is the positive control for the Identifiler® Plus kit and 007 DNA is the positive control for the GlobalFiler™ and Yfiler™ kits. The maximum volume of reagent grade water that can be accommodated by the PCR volume (i.e., 10 µL) is the negative control. The positive and negative amplification controls must be amplified concurrently (i.e., in the same instrument and with the same primers) with the forensic samples to which they will be associated.

At least one reagent blank (RB) from an extraction batch must be amplified using the same primers, same instrument model, and same concentration conditions as required by the sample(s) in the extraction batch containing the least amount of DNA. For extraction batches with multiple RBs, at least the RB that demonstrates the greatest signal, if any, must be amplified. An amplification test kit may not be utilized if no RB(s) associated with the extraction batch or sample being amplified remains.

Refer to the appropriate interpretation procedure of the *DNA Procedures Manual* for interpretation guidelines of these control samples.

4 Procedures

Refer to the DNA Introduction Procedure (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water, available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

4.1 Preparation of the Tecan Robotic Workstation

If necessary, turn on the Tecan, which will undergo an initialization routine. Log on to the Tecan computer, launch and log on to the current Tecan software.

4.1.1	Ensure the Tecan is prepared to run: Prior to daily use: • Make ~100 mL of 3% bleach to replace bleach in front trough • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script Prior to each run: • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. *If replacing carboy, rerun daily start up script. When a carboy is refilled, it should be allowed to de-gas overnight*	

	before use. • Check volume of waste container and empty if needed As needed: • Clean barcode scanners with a lint-free cloth.	

The daily start up script prompt “Check syringes and tips,” refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

RoboScrub cleaning should be performed weekly, generally at the end of the workday:

- Make ~3.5 L of diluted Liquinox solution (see instructions on the label of the bottle for preparation)
- ~3.5 L purified water in a separate container is needed
- Run the RoboScrub Clean script, and follow the prompt

4.2 Preparing Sample Racks/Creating a Scan File Import

Ensure all DNA extracts and reagent blanks (aka DNA sample tubes) are in Tecan compatible tubes and appropriately barcoded.

4.2.1	Place tubes into Tecan samples rack(s) as described below and load the sample racks onto the Tecan. • A ladder place holder tube (i.e., uncapped empty tube with a unique “BL” barcode) is in position 1 of sample rack 1. • DNA sample tubes start in position 2 of sample rack 1 and continue on to additional racks as needed. o When more than 4 sample racks are used, a ladder place holder tube must be added between the first and last samples. For a full 96-well amplification plate, this is generally position 13 of rack 3. • A tube of positive control DNA (with an appropriate [i.e., FC, MC] barcode) is in the sample position immediately after the last DNA sample tube. o 007 ("MC...") is used for GlobalFiler™ and Yfiler™ o 9947A ("FC...") is used for Identifiler® Plus • A new barcoded amplification blank ("AMPBLANK") tube is in the sample position immediately after the positive control DNA. • A ladder place holder tube is immediately after the amplification blank tube. • Any rack position(s) unfilled by a tube as described above must contain empty tubes with unique “BL” barcodes.	

“BL” barcode tubes may be reused; however, each “BL” barcode on the Tecan must be unique.

4.2.2	Use the current appropriate script to scan the sample racks and generate a .csv scan file. Import the scan file into STACS.	

4.3 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan robot.

4.3.1	Create master mix based on the following volumes. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin. Ensure the appropriate barcodes are entered into STACS. Generate Amp File in STACS.	

Amplification Master Mix Components

GlobalFiler™ (GF)		Yfiler™ (Y)		Identifiler® Plus (ID)	
	µL per well*		µL per well*		µL per well*
GF Master Mix	7.5	Y Reaction Mix	9.2	ID Master Mix	10
GF Primer Set	2.5	Y Primer Set	5	ID Primer Set	5
Low TE Buffer	5	AmpliTaq Gold®	0.8		

*Number of wells = number of samples + ~11-12 (for controls, ladders, and overage)

4.4 Preparing the Tecan Deck

The below steps may be performed in any order prior to running the Tecan robot.

Positions of racks may vary between instruments. The robotic script will direct the placement.

4.4.1	Water Rack: • Prior to each use, replace the ~200 mL reagent grade water in the center trough.	

Maximum penetration depth for the robot tips is 0.75 inches from the bottom of the trough (threshold line is on the trough). Observe the water level during processing and replenish if necessary.

4.4.2	Bleach Rack: • Ensure the 3% bleach solution in the front trough was replaced prior to daily use.	

4.4.3	Plate Rack: • Place a 96-well plate into a base. Place into the back position of the plate rack. Ensure an amplification batch barcode label is on the right side of the base or the plate.	

	• Label the right side of a 96-well 2.0 mL deep well plate with a working plate barcode and place it into the center position of the plate rack.	

4.4.4	Master Mix Rack: • Place the two tubes (with "C1" barcodes) containing equal volumes of master mix in positions 3 and 4. Place empty tubes (with unique "BL" barcodes) in positions 1 and 2, and 5 through 16.	

4.5 Tecan Amplification Plate Set-up

4.5.1	Run the current version of FBI AMPSTR then run appropriate Amp Script. Follow the prompts to ensure the appropriate racks are on the deck as required.	

Based on each sample's quantification (quant) result and any adjustments made by an examiner, the Tecan will create the necessary dilutions using reagent grade water. The dilution plate can take more than 30 minutes to generate.

4.5.2	Follow the script prompts for the dilution plate. The script will include prompts to perform the following tasks. *If no dilutions were prepared, the dilution plate does not need to be manipulated while following the script prompts.*	

4.5.2.1	Seal the dilution plate with a foil cover.	

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are well-sealed.

4.5.2.2	Invert the dilution plate several times, attempting to shake the liquid off the bottom of the plate.	

4.5.2.3	Firmly seat the dilution plate on the te-shake and follow computer prompts to turn on the plate shaker.	

The plate will shake for ~1.5 minutes.

4.5.2.4	Remove the dilution plate from the te-shake and invert several additional times, attempting to shake the liquid off the bottom of the plate. Centrifuge the dilution plate for ~1.5 minutes at ~2000 rpm. Remove the dilution plate from the centrifuge and carefully remove the foil seal. Return the dilution plate to its original deck position **in the correct orientation**.	

Ensure that the plate is properly positioned **BEFORE** selecting "OK" to continue with the script.

The Tecan will prepare the amp plate so that each well contains 15 µL of master mix and 10 µL of DNA template (i.e., neat sample, sample plus water, diluted sample) or 10 µL of the appropriate control. See the calculations and limitations section for more information.

4.5.3	Upon completion of the Tecan run, seal the amplification plate with a clear seal.	

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are well-sealed.

4.5.4	Quick spin (generally ~2,000 rpm for 5 seconds).	

Sample tubes should be removed from the Tecan deck and capped prior to proceeding to the Amplification (Amp) room.

4.5.5	Ensure the amp batch barcode is on a side of the amplification plate and proceed to the Amp room.	

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

4.6 PCR Amplification

4.6.1	Place the amp plate in an appropriate thermal cycler. Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely.	

4.6.2	Select the programmed amplification method to run (e.g., "GlobalFiler").	

Each thermal cycler is programmed for one of the following methods.

GlobalFiler™

HOLD	95°C	1 minute
CYCLE	94°C	10 seconds
	59°C	90 seconds
Repeat for 28 total cycles		
HOLD	60°C	10 minutes
HOLD	4°C	∞

Identifiler® Plus

HOLD	95°C	11 minutes
CYCLE	94°C	20 seconds
	59°C	3 minutes
Repeat for 27 total cycles		
HOLD	60°C	10 minutes
HOLD	25°C	∞

Yfiler™

HOLD	95°C	11 minutes
CYCLE	94°C	1 minute
	61°C	1 minute
	72°C	1 minute
Repeat for 28 total cycles		
HOLD	60°C	80 minutes
HOLD	25°C	∞

4.6.3	Follow the prompts to start the method. • For GlobalFiler: Ensure the reaction volume is 25 µL and the ramp speed is MAX. • For Identifiler Plus and Yfiler: Ensure the reaction volume is 25 µL and the ramp speed is 9600.	

Upon completion of the PCR amplification, the plate should be stored refrigerated until needed for capillary electrophoresis (CE).

5 Calculations

The volume of DNA extract used in the amplification is calculated using the quant results of the sample, the maximum volume of sample allowed in the amplification reaction, and the target DNA amounts. An examiner may adjust the volume of sample amplified, as needed. An examiner can override or adjust the dilutions that the Tecan will make within STACS.

The following is the basis for determining the volume of sample to use for amplification:

$$C_1V_1 = C_2V_2 \rightarrow V_1 = (C_2V_2)/C_1$$

Where C_1 = Quant result (ng/µL)
V_1 = Volume of sample to add to amplification reaction (µL)
C_2 = Target Concentration (i.e., 0.1 ng/µl)
V_2 = Maximum input volume (i.e., 10 µL)

Example inputs:

DNA sample quant result (ng/µl)	Volumes to add to amplification for target concentration of 1 ng per 10 µL
<0.1	10 µl sample
0.1	10 µl sample
0.25	4 µl sample + 6 µl water
0.5	2 µl sample + 8 µl water

6 Sampling or Sample Selection

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 Successful amplification is dependent upon the quantity or quality of DNA in the sample.

8.2 Target DNA amounts are determined during validation and set in the software. The software uses the quantitation results and the target DNA amount to determine the volume of input of DNA or the default dilution calculation. Generally, 1 ng of DNA is targeted but the input volumes may be adjusted by an examiner such that more or less DNA sample is used. For samples that quant at less than 0.1 ng/µL, the default is the maximum input (i.e., 10 µL).

8.2.1 The Tecan is not maintained to pipette less than 2 µL. As a result, with a target of 1 ng/10µL, the default is to make a dilution for any sample with a quant value >0.5 ng/µL. An examiner should adjust the amplification setup sheet information, as appropriate, to prevent the Tecan from using more sample than necessary to make a dilution while ensuring an appropriate dilution volume is queued.

8.2.1.1 When adjusting the amplification setup information, generally the minimum volume accepted for a dilution is 40 µL total (i.e., dilution buffer plus sample) and the accepted maximum volume of dilution buffer is 1200 µL.

8.2.2 The Tecan has a maximum dilution that can be made. Generally, any sample with a quant value >300 ng/µL will need to be manually diluted and requanted prior to amplification.

8.3 Replicate amplifications may assist with statistical interpretation using STRMix. Replicate amplifications are not required to be at the same input concentration.

8.4 At least one reagent blank from an extraction batch must be amplified using the same primers (i.e., amp kit), same instrument model, and same concentration conditions as the sample(s) containing the least amount of DNA. An additional amplification kit may not be utilized if no reagent blank(s) associated with the extraction batch or sample being amplified remains.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the "Safe Work Practices Procedures," "Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)," "Personal Protective Equipment Policy," and "Chemical Hygiene Plan" sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the "Hazardous Waste Disposal" section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories.

10 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *GeneAmp® PCR System 9700 User's Manual Set.* 1997.

Applied Biosystems. *GlobalFiler™PCR Amplification Kit User's Guide*, P/N 4477604 Rev E, 2016.

Applied Biosystems. *AmpFlSTR® Identifiler® Plus PCR Amplification Kit User's Guide*, P/N 4402743, 2008.

Applied Biosystems. *AmpFlSTR® Yfiler™ PCR Amplification Kit User's Manual*, P/N 4358101 Rev. A 4305246, Foster City, CA.

ARTEL. *MVS Multichannel Verification System User Guide.* 2006

Budowle, B. STR allele concordance between different primer sets: a brief summary. *Profiles in DNA* (2000) 3: 10-11.

Budowle B, Defenbaugh DA, and Keys KM. Genetic variation at nine short tandem repeat loci in Chamorros and Filipinos from Guam, *Legal Medicine* (2000) 2: 26-30.

Budowle B, Masibay A, Anderson SA, Barna C, Biega L, Brenneke S, Brown BL, Cramer J, DeGroot GA, Douglas D, Duceman B, Eastman A, Giles R, Hamill J, Haase DJ, Janssen DW, Kupferschmid TD, Lawton T, Lemire C, Llewellyn B, Moretti T, Neves J, Palaski C, Schueler S, Sgueglia J, Sprecher C. Tomsey C, and Yet D. STR primer concordance study, *Forensic Science International* (2001) 124: 47-54.

Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Smerick JB, and Budowle B. Validation of short tandem repeat (STRs) for forensic usage: Performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples, *Journal of Forensic Sciences* (2001) 46: 647-660.

Moretti TR, Koons BW, and Budowle B. Enhancement of PCR amplification yield and specificity using AmpliTaq Gold™ DNA Polymerase, *BioTechniques* (1998) 25: 716-722.

Mulero JJ, Chang CW, Calandro LM, Green RL, Li Y, Johnson CL, and Hennessy LK.

Development and validation of the AmpFlSTR YFiler PCR amplification kit: a male specific, single amplification 17 Y-STR multiplex system, *J. Forensic Sci.* (2006) 51:64-75.

Wallin JM, Holt CL, Lazaruk KD, Nguyen TH, and Walsh PS. Constructing universal multiplex PCR systems for comparative genotyping, *J. Forensic Sci*. (2002) 47: 52-65.

Rev. #	Issue Date	History
11	05/25/16	Complete revision for simplification of procedure. Changed from nDNAU to DCU throughout. Changed from nDNAU LIMS to STACS thoughout and made necessary adjustments for STACS. Moved QC procedures to Appendix and simplified.
12	12/30/16	Updated to add GlobalFiler and remove MiniFiler. Incorporated BAU for when Huntsville lab is authorized to perform procedure on casework.
13	02/28/18	1 Adjusted scope. 4.4.3 Added allowance for different placement of the barcode based on the Tecan plate holder. Appendix A, 2 Critical Reagents, section C made triplicate for consistency.

Approval

Redacted - Signatures on File

DNA Technical Leader Date: 02/27/2018

DCU Chief Date: 02/27/2018

BAU Chief Date: 02/27/2018

DSU Chief Date: 02/27/2018

QA Approval

Quality Manager Date: 02/27/2018

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verifications and additional requirements.

A. Performance Verification (PV) of the Tecan Robotic Workstation

Refer to the nuclear DNA quantification procedure (i.e., nDNA 226) for instructions for the PV of the Tecan Robotic Workstation.

B. Performance Verification of the 9700 Thermal Cycler

1. Cleaning - Refer to the instructions for cleaning the sample wells and cleaning the sample block cover described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.

2. Temperature Verification Test - This procedure verifies that the thermal cycler remains within the temperature accuracy specification. Refer to the instructions for running the Temperature Verification Test described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.

3. Temperature Non-uniformity Test - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to the instructions for running the Temperature Non-uniformity Test described in the maintenance section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.

4. Rate Test and Cycle Test - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to the instructions for running system performance diagnostics described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

Qualification of GlobalFiler™, AmpF*l*STR® Identifiler® Plus, and AmpF*l*STR® Yfiler™ Amplification Kits

A. Normalization of Positive Control DNA

1. The positive control DNA (e.g., 007, 9947A) from each new lot of an amplification kit will be quantitated. Generally, in triplicate and the quant values averaged.

2. If necessary (i.e., when the average concentration is greater that ~0.10 ng/µL), a portion of the positive control DNA will be adjusted with an appropriate diluent (e.g., TE^{-4} or reagent grade water) to a concentration of ~0.10 ng/µL.

3. The positive control DNA, at the ~0.10 ng/µL concentration, will be used for the assessment of the sensitivity of detection and kit performance.

B. Sensitivity of Detection

1. Using an amplification kit from the new lot, amplify the normalized positive control DNA (generally in triplicate), a negative amplification control, and a diluent control (if applicable). Run the samples on the CE instrument at current conditions.

2. Evaluate the CE data and compare the data generated for the positive control to the expected sensitivity results. Sensitivity expectations for a given amplification kit are based on the evaluation of multiple lots of the kit and positive control during validation.

3. The sensitivity of the new lot will be accepted if the average allelic peak heights for all successful injections of the positive control DNA are generally consistent with sensitivity expectations for the given amplification kit.

4. If the amplification kit does not meet sensitivity expectations, the assessment will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

C. Positive Control and Kit Performance

Once a new lot of an amplification kit has demonstrated an acceptable sensitivity of detection, a batch of the normalized positive control DNA will be prepared for use in casework analysis.

1. Using an amplification kit from the new lot, amplify, generally in triplicate, a sample from this batch of normalized positive control DNA, a negative amplification control, and a diluent control (if applicable). For a new lot of Yfiler™ Amplification Kit, the 9947A control DNA (10 ng/µL) is run as an additional negative control sample. Run the samples on the capillary electrophoresis instrument at current conditions using the new lot of allelic ladder.

2. Evaluate the CE data. Only 1 replicate of each sample is needed for the evaluation.

3. The amplification kit lot and the positive control DNA preparation will be approved for casework analysis if:
   a. all allelic ladder peaks are present for at least one injection of the allelic ladder
   b. correct and interpretable typing results are obtained for the positive control DNA
   c. no allelic peaks, other than those attributable to the amplified positive control DNA, are detected
   d. no allelic peaks are detected in the negative control and diluent control (if applicable)
   e. the allelic peak heights of the positive control DNA are generally consistent with sensitivity expectations for the given amplification kit.

4. If the injection set does not meet the above listed criteria, the assessment will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

5. The approved neat or adjusted preparation of the positive control DNA will be stored in 1.5 mL screw cap tubes affixed with appropriate positive control barcodes for usage.