Amplification of Nuclear DNA

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1 INTRODUCTION

These procedures describe the process for the amplification of short tandem repeats (STR) in the nuclear DNA (nDNA) extracted from evidence and reference samples using polymerase chain reaction (PCR) and the GlobalFiler™ PCR Amplification Kit and/or AmpFLSTR™ Yfiler™ PCR Amplification Kit.

2 SCOPE

These procedures apply to DNA personnel who perform amplification of nuclear DNA, evaluate data, and/or perform the associated quality control (QC) procedures.

3 EQUIPMENT

3.1 Equipment/Materials

- Tecan robot model 'Freedom EVO'
  - Tecan EvoWare software, version 2.8 or higher
- STACS™ Casework Software (STACS), Sample Tracking and Control Solutions [STACS DNA Inc.] part of InVita Healthcare Technologies, version 5.0 or higher
- Thermal Cycler, Applied Biosystems GeneAmp® PCR System 9700 or ProFlex™ PCR System
- General laboratory supplies (e.g., pipettes, tubes)
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- 96-well Deep Well plates (2.0mL)
- Clear and foil plate seals
- Thermal Microplate Sealer

3.2 Reagents

- GlobalFiler™ PCR Amplification Kit, Applied Biosystems
- AmpF/STR® Yfiler™ PCR Amplification Kit(s), Applied Biosystems
- 007, quantified and diluted as necessary, Applied Biosystems
- 9947A, 10 ng/µL (For QC only)
- Buffer, Low TE (aka TEKnova DNA Suspension Buffer), Fisher Scientific or equivalent
- 3% bleach (reagent grade or equivalent)
- Water (molecular grade or equivalent)
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution, Liquinox™ or equivalent

4 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. The positive control for the GlobalFiler™ and Yfiler™ kits is 007. The negative control is the maximum volume of molecular grade water that can be accommodated by the PCR volume (i.e., 10 µL). 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. The RB is not required to be reamplified with samples in the associated extraction batch as long as an RB has been amplified using the same typing test kit, instrument model, and sensitivity conditions as the samples within the extraction batch. 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 applicable DNA interpretation procedure (i.e., BIO-570 or BIO-572) for interpretation guidelines of the control samples.

5 PROCEDURE

Refer to the DNA Introduction Procedure (i.e., BIO-100) and follow applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), molecular 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, as necessary.

5.1 Preparation of the Tecan Robotic Workstation

1.	Ensure the Tecan is prepared to run:
	• Prior to daily use:
	  • Make ~100 mL of 3% bleach and 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 appropriate daily start up script
	  ○ The 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.
	• Prior to each run:
	  • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. If replacing the carboy, rerun the 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.

5.2 Preparing Sample Racks/Creating a Scan File Import

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

1.	Place tubes into Tecan sample rack(s): • A ladder place holder tube (i.e., uncapped empty tube with a unique “BL” barcode) is in position 1 of sample rack 1. • Sample tubes start in position 2 of sample rack 1 and continue on to additional racks as needed. • 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., MC] barcode) is in the sample position immediately after the last sample tube. • 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.

2.	• Load the sample racks onto the Tecan. • Use the appropriate script to scan the sample racks and generate a .csv scan file. • Import the scan file into STACS.

5.3 Master Mix Preparation

Master Mix may be prepared at most convenient point prior to loading on the Tecan robot.

1.	• Create master mix based on the following volumes. • Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin. • Generate the Amp File in STACS.

Amplification Master Mix Components

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

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

5.4 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.

1.	Water Rack: • Prior to each use, replace the molecular grade water in the center trough. Ensure the water is filled above the line (i.e., contains at least ~200mL).
2.	Plate Rack: • Place a 96-well plate into a base, if applicable. Place into the back position of the plate rack so that well A1 is in the back-left. Ensure an amplification batch barcode label is on the right side of the base or the plate, as appropriate • 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 so that well A1 is in the back-left.
3.	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.

5.5 Tecan Amplification Plate Set-up

1.	Run the current version of FBI AMPSTR then run appropriate Amp Script. • Follow the prompts to ensure the 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 molecular grade water. The dilution plate can take more than 30 minutes to generate.

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 as the prompts are clicked through.

2A.	Seal the dilution plate with a foil cover.

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually.

2B.	Ensure that the edges of each well are well-sealed. Invert the dilution plate several times, attempting to shake the liquid off the bottom of the plate.
2C.	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. If necessary, the dilution plate may be manually vortexed with a corresponding batch comment in the notes.

2D.	• 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 dilution 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.)

3.	• Upon completion of the Tecan run, seal the amplification plate with a clear seal. • Ensure that the edges of each well are well-sealed. • Quick spin (generally ~2,000 rpm for 5 seconds).

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually.

Sample tubes should be removed from the Tecan deck and capped prior to proceeding to the Amplification (Amp) room. Ensure the amp batch barcode is on a side of the amplification plate and proceed to the Amp room.

5.6 PCR Amplification

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.
2.	Select the programmed amplification method to run.

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	∞

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	∞

3.	Follow the prompts to start the method.
	• For GlobalFiler: Ensure the reaction volume is 25 µL.
	  • On a 9700: Ensure the ramp speed is MAX.
	  • On a ProFlex: Ensure the ramp speed is 9700 simulation mode.
	• For Yfiler: Ensure the reaction volume is 25 µL.
	  • On a 9700: Ensure the ramp speed is 9600.
	  • On a ProFlex: Ensure the ramp speed is 9600 simulation mode.

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

6 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

7 LIMITATIONS

- Successful amplification is dependent upon the quantity or quality of DNA in the sample.
- 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).
    - 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.
        - 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.
    - 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.
- Replicate amplifications may assist with statistical interpretation using STRMix. Replicate amplifications are not required to be at the same input concentration.
- 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.

8 SAFETY

- 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.

- Refer to the FBI Laboratory Safety Manual for information on personal protection, the proper disposal of the chemicals used in these procedures, as well as the biohazardous wastes generated.
- Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories.

9 REFERENCES

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

Applied Biosystems. *Proflex™ PCR System User Guide*. 2016.

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

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.

10 REVISION HISTORY

Revision	Issued	Changes
00	09/01/2022	Reformatted DNA 213-13 into new template and assigned new Doc ID. Removed Identifiler Plus. Minor edits throughout.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., BIO-104) for minimum frequency of performance verifications and additional requirements.

11.1.1 Tecan Robotic Workstation

A. General Maintenance of the Tecan Robotic Workstation
   1. RoboScrub cleaning should be performed weekly, generally at the end of a workday:
      i. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
      ii. ~3.5 L purified water in a separate container is needed
      iii. Run the RoboScrub Clean script, and follow the prompts

B. Refer to the nuclear DNA quantification procedure (i.e., BIO-520) for instructions for the Performance Verification (PV) of the Tecan Robotic Workstation

11.1.2 Thermal Cycler - Cleaning and Performance Verification

A. 9700

Refer to the GeneAmp® PCR System 9700 User's Manual Set 96-Well Sample Block Module User's Manual for instructions on how to perform the following procedures.

1. Cleaning - Refer to the instructions for cleaning the sample wells and cleaning the sample block cover.
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. 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. 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.

B. ProFlex™

Refer to the ProFlex™ PCR System User Guide for instructions on how to perform the following procedures.

1. Cleaning – Refer to the chapter for maintaining the instrument for instructions on how to clean the sample wells and heated cover.

2. Verify Block Temperature –These tests are found in the Block Verification Test screen and requires the use of a Temperature Verification System for the following test types:
    i. Heated Cover Test - This test verifies the proper functioning of the heated cover.
    ii. Verification Test - This procedure verifies that the thermal cycler remains within the temperature accuracy specification.
    iii. Temperature Non-Uniformity Test - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler.

11.2 Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., BIO-103) for additional requirements.

11.2.1 GlobalFiler™ and AmpFlSTR® Yfiler™ PCR Amplification Kits

A. Normalization of Positive Control DNA
    1. The positive control DNA (i.e., 007) 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 molecular grade water) to a concentration of ~0.10 ng/µL. If the positive control from the new lot of amplification kit does not quantify at or above 0.10 ng/ul, a positive control with a higher concentration (e.g. 2 ng/ul 007 from GFE amplification kits) may be used and diluted to 0.10 ng/ul.
    3. The positive control DNA, at the ~0.10 ng/µL concentration, will be used for the assessment of the kit performance and sensitivity of detection.

B. Verification of the Positive Control DNA and Kit Performance and Sensitivity
    1. Using an amplification kit from the new lot, amplify a sample from the batch of normalized positive control DNA (generally in triplicate), a negative amplification control, and a diluent control (if applicable).
        i. For a new lot of Yfiler™ Amplification Kit, the 9947A control DNA (10 ng/µL) is run as an additional negative control sample.
    2. Run the amplified control samples on the capillary electrophoresis instrument at current conditions using the new lot of allelic ladder. Include additional wells of the positive control to fill the injection. Refer to the procedure for capillary electrophoresis of nuclear DNA (i.e., BIO-560).
    3. Evaluate the CE data. The new amplification kit lot and the positive control DNA preparation will be approved for casework analysis if:
        i. all allelic ladder peaks are present for at least one injection of the allelic ladder
        ii. correct and interpretable typing results are obtained for the positive control DNA
        iii. no allelic peaks, other than those attributable to the amplified positive control DNA, are detected

iv. no allelic peaks are detected in the negative control and diluent control (if applicable)
v. the allelic peak heights of the positive control DNA are generally consistent with sensitivity expectations for the given amplification kit. Sensitivity expectations for a given amplification kit are based on the evaluation of multiple lots of the kit and positive control during validation.

4. If the data 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 casework usage.