

# Amplification of Nuclear DNA

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# Amplification of Nuclear DNA

## 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 the PowerPlex® Y23 System for amplification.

## 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 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
  - 007, quantified and diluted as necessary, Applied Biosystems
- PowerPlex® Y23 System kit(s), Promega
  - 2800M, quantified and diluted as necessary, Promega
- 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™ kit is 007. The positive control for the Y23 kit is 2800M. 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 additional 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.	<p>Ensure the Tecan is prepared to run:</p> <ul style="list-style-type: none"><li>• Prior to daily use:<ul style="list-style-type: none"><li>• Make ~100 mL of 3% bleach and replace bleach in front trough</li><li>• Clean the outside of the Tecan tips with 70% isopropyl alcohol</li><li>• Decontaminate the Tecan work deck with 10% bleach</li><li>• Run the appropriate daily start up script<ul style="list-style-type: none"><li>◦ 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.</li></ul></li></ul></li><li>• Prior to each run:<ul style="list-style-type: none"><li>• 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.</li><li>• Check volume of waste container and empty if needed</li></ul></li></ul>
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	<ul style="list-style-type: none"> <li>• Ensure the tubing is properly submerged in the intake water and not coiled or kinked.</li> <li>• As needed: <ul style="list-style-type: none"> <li>• Clean barcode scanners with a lint-free cloth.</li> </ul> </li> </ul>
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## 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.	<p>Place tubes into Tecan sample rack(s):</p> <ul style="list-style-type: none"> <li>• A ladder place holder tube (i.e., uncapped empty tube with a unique “BL” barcode) is in position 1 of sample rack 1.</li> <li>• Sample tubes start in position 2 of sample rack 1 and continue on to additional racks as needed. <ul style="list-style-type: none"> <li>• 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.</li> </ul> </li> <li>• A tube of positive control DNA with an appropriate barcode (e.g., MC for Globalfiler) is in the sample position immediately after the last sample tube. <i>Note: For Y23, the appropriate barcode is dependent upon the target concentration selected during the QC.</i></li> <li>• A new barcoded amplification blank (“AMPBLANK”) tube is in the sample position immediately after the positive control DNA.</li> <li>• A ladder place holder tube is immediately after the amplification blank tube.</li> <li>• Any rack position(s) unfilled by a tube as described above must contain empty tubes with unique “BL” barcodes.</li> </ul>
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“BL” barcode tubes may be reused; however, each “BL” barcode on the Tecan must be unique.

2.	<ul style="list-style-type: none"> <li>• Load the sample racks onto the Tecan.</li> <li>• Use the appropriate script to scan the sample racks and generate a .csv scan file.</li> <li>• Import the scan file into STACS.</li> </ul>
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## 5.3 Master Mix Preparation

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

1.	<ul style="list-style-type: none"> <li>• Create master mix based on the following volumes.</li> <li>• Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.</li> <li>• Generate the Amp File in STACS.</li> </ul>
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### Amplification Master Mix Components

GlobalFiler™ (GF)		Y23 (Y)	
	µL per well*		µL per well*
GF Master Mix	7.5	Y23 Master Mix	5.0
GF Primer Set	2.5	Y23 Primer Pair Mix	2.5
Low TE Buffer	5.0	Water	7.5

\*Number of wells = number of samples + extra 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.	<b>Water Rack:</b> <ul style="list-style-type: none"> <li>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).</li> </ul>
2.	<b>Plate Rack:</b> <ul style="list-style-type: none"> <li>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</li> <li>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.</li> </ul>
3.	<b>Master Mix Rack:</b> <ul style="list-style-type: none"> <li>Place the two tubes (with “C1” barcodes) containing equal volumes of master mix in positions 3 and 4.</li> <li>Place empty tubes (with unique “BL” barcodes) in positions 1 and 2, and 5 through 16.</li> </ul>

### 5.5 Tecan Amplification Plate Set-up

1.	In the current version of FBI AMPSTR select the applicable process for Globalfiler or Y23. Then run the appropriate Amp Script for Globalfiler or Y23 in EvoWare. <ul style="list-style-type: none"> <li>Follow the prompts to ensure the racks are on the deck as required</li> </ul>
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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.
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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.
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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.	<ul style="list-style-type: none"> <li>Remove the dilution plate from the Te-shake and invert several additional times, attempting to shake the liquid off the bottom of the plate.</li> <li>Centrifuge the dilution plate for ~1.5 minutes at ~2000 rpm.</li> <li>Remove the dilution plate from the centrifuge and carefully remove the foil seal.</li> <li>Return the dilution plate to its original deck position <b>in the correct orientation</b>.</li> <li>Ensure that the dilution plate is properly positioned <b>BEFORE</b> selecting “OK” to continue with the script.</li> </ul>
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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, or diluted sample) or 10 µL of the appropriate control. (See the Calculations and Limitations section for more information.)

3.	<ul style="list-style-type: none"> <li>Upon completion of the Tecan run, seal the amplification plate with a clear seal.</li> <li>Ensure that the edges of each well are well-sealed.</li> <li>Quick spin (generally ~2,000 rpm for 5 seconds).</li> </ul>
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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.
2.	Select the applicable amplification method.

Each thermal cycler is programmed with at least 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 ∞

### Y23™

HOLD 96°C 2 minutes  
 CYCLE 94°C 10 seconds  
           61°C 1 minute  
           72°C 30 seconds  
 Repeat for 30 total cycles  
 HOLD 60°C 20 minutes  
 HOLD 4°C ∞

3.	<p>Follow the prompts to start the method.</p> <ul style="list-style-type: none"> <li>For GlobalFiler:           <ul style="list-style-type: none"> <li>Ensure the reaction volume is 25 µL.</li> <li>Ensure the ramp speed is <b>9700 Simulation</b> mode.</li> </ul> </li> <li>For Y23:           <ul style="list-style-type: none"> <li>Ensure the reaction volume is 25 µL.</li> <li>Ensure the ramp speed is in <b>No Simulation</b> mode.</li> </ul> </li> </ul>
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Upon completion of the PCR amplification, the amp plate should be stored until needed for capillary electrophoresis (CE). Globalfiler amp plates should be stored refrigerated. Y23 amp plates should be stored frozen for overnight or longer periods of time.

## 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 and/or reagent blank amplified, as needed, provided the reagent blank is not diluted more than the associated sample. 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 for GlobalFiler:

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 STACS. STACS uses the quantitation results and the target DNA amount to determine the volume of input of DNA or the default dilution calculation; however, the input volumes may be adjusted by an examiner such that more or less DNA sample is used.
  - Generally, 1 ng of DNA is targeted for GlobalFiler. For Globalfiler, samples that quant at less than 0.1 ng/μL, the default is the maximum input of 10 μL.
  - The target for Y23 is between 0.2 ng and 0.5 ng as determined during the QC of each kit lot. For Y23, samples that quant at less than the target concentration (i.e., 0.02, 0.03, 0.04, or 0.05 ng/μL), the default is the maximum input of 10 μL.
- The Tecan is not maintained to pipette less than 2 μL. As a result, with a target of 1 ng per 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., diluent plus sample) and the accepted maximum volume of diluent is 1200 μL.
  - The Tecan has a maximum dilution that can be made. Generally, samples with quant values that would require exceeding the maximum dilution need to be manually diluted and requanted prior to amplification.
    - For Globalfiler with a 1 ng input target, this value is >300 ng/μL.
    - For Y23, the maximum quant value will depend on the target concentration for the kit lot. As an example, with a 0.2 ng input target, this value >60 ng/μL.
- Replicate amplifications of Globalfiler 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 the sensitivity conditions as required by the 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

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## 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.
01	09/04/2024	Revised all Y-STR procedures to change to Y23 kit. Removed all references to 9700.

## 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 ProFlex™ Thermal Cycler - Cleaning and Performance Verification

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

- A. Cleaning – Refer to the chapter for maintaining the instrument for instructions on how to clean the sample wells and heated cover.
- B. Self Verification Test – This test checks the instrument hardware and should be performed periodically or whenever there is an intermittent instrument error. The test is found in the Self Verification Test screen.
- C. Performance Verification is performed by an external vendor.

### 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™ 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. Multiple positive control tubes from the same amplification kit lot may be combined prior to quantitation, if necessary.
  - 2. If necessary (i.e., when the average concentration is greater than the target of ~0.10 ng/μL for GlobalFiler), a portion of the positive control DNA will be adjusted with an appropriate diluent (e.g., TE-4 or molecular grade water) to the target concentration. 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/μL 007 from a GFE amplification kit) may be used and diluted to ~0.10 ng/μL.

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).
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 (i.e., average peak heights between 1900-6800 RFU). 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.

**11.2.2 PowerPlex® Y23 System**

**A. Normalization of Positive Control DNA**

1. The positive control DNA (i.e., 2800M) from each new lot of an amplification kit will be quantitated, generally, in triplicate, and the quant values averaged. Multiple positive control tubes from the same amplification kit lot may be combined prior to quantitation, if necessary.
2. Using the quant value of the stock, the 2800M will be diluted with TE-4 to three consecutive concentrations in the validated range (i.e., 0.02, 0.03, 0.04, or 0.05 ng/μL). These dilutions will be requanted to check the concentrations and will be adjusted, if necessary.

3. The positive control DNA, at the above concentrations, 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 seven replicates of the three concentrations of the positive control, and a negative amplification/TE control. This procedure will be done robotically.
2. Run the amplified control samples on each in use capillary electrophoresis instrument(s) at current conditions using the new lot of allelic ladder and WEN size standard. Refer to the procedure for capillary electrophoresis of nuclear DNA (i.e., BIO-560).
3. Evaluate the CE data.
  - i. Calculate the average peak height across all loci. Generally, the input amount that generates a profile with an average peak height closest to ~9000 rfu's (as determined during the Y23 validation) will be chosen as the target input amount for the amplification kit lot.
  - ii. The amplification kit 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)
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 dilution of the positive control DNA will be stored in 1.5 mL screw cap tubes affixed with appropriate positive control barcodes for casework usage.
6. The approved target concentration will be updated in STACS prior to use of the new amplification kit lot.