Standard Operating Procedure (SOP) for Confirming Specimen Identity Using The Identifier Sequence-Specific Tandem Repeat (SSTR) Multiplex

I. SCOPE AND PURPOSE

The AmpF*l*STR® Identifiler® PCR Amplification Kit is a short sequence-specific tandem repeat (SSTR) multiplex assay that co-amplifies 15 SSTRs and the Amelogenin marker in a single PCR amplification reaction. The Amelogenin locus is used for gender identification as PCR amplification products of different lengths are generated from the X and Y chromosomes. The multiplex produces quality results necessary to uniquely identify a sample. The average match probability of identity profiles for 2 randomly selected unrelated Caucasian individuals is 5.01×10^{18}. The widely accepted tetranucleotide SSTRs co-amplified in the Identifilier Kit include the thirteen core SSTRs as required for sample entry into the Combined DNA Index System (CODIS) (Budowle et al., 1998). The data generated from these loci also satisfy the recommendations for several worldwide human identification databases including the European Network of Forensic Science Institutes (ENFSI) and Interpol organizations.

This procedure is used as a back-up method to SOP M010, "Tissue Matching by SNP Analysis" to confirm that normal and tumor samples are from the same patient as a quality control metric. It may also be used for cases in which a sample swap is suspected or to confirm whether DNA from two subjects are co-mingled in one sample.

II. PROCEDURE

A. Safety Procedures

1. Use Standard Precautions when handling all body fluids, tissues and cell cultures.
2. Wear Personal Protective Equipment (PPE), including a lab coat and nitrile gloves.

B. Reagent Preparation

1. AmpF*l*STR PCR reaction mix, Identifiler® Primer Set, and Identifiler® Allelic Ladder are received frozen. They are stored in a -20°C and refrozen after each use.
2. AmpliTaq Gold® DNA Polymerase is stored in a -20°C and refrozen after each use.
3. AmpF*l*STR Control DNA 9947A is stored in a -20°C and refrozen after each use.
4. IMPORTANT: The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpF*l*STR Identifiler Primer Set, Allelic Ladder, GeneScanTM-500 LIZTM Size Standard and amplified, fluorescently labeled PCR products from light.

C. Quality Control

1. Any deviations from the protocol as written should be documented at the sample level in LabVantage. Protocol deviations that have the potential to compromise sample quality or results should also be documented with an incident report.
2. Patient cases for which the genetic profile of the tumor tissue does not correlate with that of the normal control will be considered genotyped mismatches.
3. All new lots of reagents are testing in parallel with the one in current use before being put into use. All kit components must be quality control tested and used together thereafter. All reagents supplied in a kit must be used only with other reagents in the same kit lot number; reagents with identical lot numbers cannot be used

interchangeable between kit lot numbers. All QC results are recorded in the Quality Control notebook.

4. Separate designated areas will be used for pre- and post-PCR to avoid amplicon contamination of new reactions. The thermal cycler and ABI 3730 are located in the post-PCR area. Filtered pipet tips and pre-PCR-only designated pipettes will be used to set up all PCRs.
5. Aerosol-resistant tips are used for reaction assembly and sample analysis to prevent possible contamination.
6. Controls:
   a. Negative controls (e.g., a sample containing nuclease-free water with all reaction components except DNA) are run with each assay to detect potential contamination
   b. A single master mix is made containing all reagents necessary for the run except the template to control for individual well contamination.
   c. The cell line DNA, TCGA-AV-A03D-20A-01D is used as a positive control.
   d. The accuracy and batch-to-batch reproducibility of the assay is assessed by confirmation of the positive control genotype calls.

D. Required Equipment, Supplies, and Reagents

1. Equipment

a. 96-well formatted centrifuge
b. Vortex
c. ABI 3730 Genetic Analyzer
d. Genotyper® software
e. 96-well formatted Thermal cycler
f. Pipettors (2 µL, 20 µL, 200 µL, 1,000 µL)
g. Cap-It-All
h. Heat Block (95°C)

2. Supplies

a. ABI 3730 48 capillary array, 36 cm (Applied Biosystems, catalog# 4331247)
b. Filtered pipet tips
c. Labeling tape
d. Personal Protective Equipment (PPE)
e. Microseal F film (Biorad, catalog# MSF-1001)
f. Microseal B film (Biorad, catalog# MSB-1001)
g. Microcentrifuge tubes, 1.5 mL
h. Microcentrifuge tubes, 2.0 mL
i. MicroAmp optical 96 well reaction plate (Applied Biosystems, catalog# N801)
j. Plate Septa-96 Well (Applied Biosystems, Inc., Catalog# 4315933)

3. Reagents

a. Water, Molecular Biology Reagent (Sigma #W4502)
b. Deionized, Distilled Water – Type I (AquaSolutions)

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c. 1X or 0.1X Tris-EDTA (TE), pH 8, diluted from 100X TE (Sigma, catalog# T9285)
d. 10X Genetic Analyzer Buffer (Applied Biosystems, catalog# 402824)
e. POP7 Polymer (Applied Biosystems, catalog# 4335615)
f. Hi-Di ™ formamide (Applied Biosystems, catalog# 4311320)
g. AmpF*l*STR® Identifiler® kit (Applied Biosystems, catalog# 4322288)
h. GeneScan 500 LIZ internal lane standard

E. Procedure

1. Tumor, normal, and control DNA are diluted to a final concentration of 10 ng/µL. following the instructions below.
    a. Remove the Matrix rack containing the stock samples from the liquid nitrogen, -80°C freezer, or the refrigerator.
    b. When the samples are completely thawed, centrifuge the stock samples briefly to collect all the liquid at the bottom of the tube.
    c. Label a 96-well unskirted PCR plate with the plate ID, 10 ng/µl, and the date.
    d. Calculate the amount of water and DNA to add to create a 10 ng/µl dilution. This would be 14 µl of nuclease-free water and 1 µl of DNA if the stock DNA was at 150 ng/µl.
    e. Add the nuclease-free water to each well.
    f. Use the Cap-It All to remove the caps of the tubes in the Matrix rack containing the stock samples. Samples will be kept uncapped only long enough to remove the sample.
    g. Transfer the stock samples immediately to the appropriate wells of the labeled 96-well unskirted PCR plate using a multi-channel pipet.
    h. Use the Cap-It All to recap the stock sample Matrix rack.
    i. Seal the dilution plate with a sheet of adhesive foil.
    j. Vortex the plate for 5 seconds, while holding firmly at the edges.
    k. Briefly centrifuge the plate in a bench top centrifuge at 2200 rpm for 30 seconds.
2. Prepare the master mix, taking care to avoid exposing fluorescent dyes to light.
    a. Determine the total number of samples, including controls (one positive and one negative) and 10% extra to account for pipetting error.
    b. Vortex the following reagents for 5 seconds to mix and briefly centrifuge: AmpF*l*STR PCR Reaction Mix, and AmpF*l*STR Identifiler Primer Set.
        i. NOTE: Do not vortex AmpliTaq Gold DNA Polymerase enzyme.
    c. Calculate the required amount of components as follows:
        i. Number of samples x 5.25 µL of AmpF*l*STR PCR Reaction Mix
        ii. Number of samples x 0.25 µL of AmpliTaq Gold DNA Polymerase
        iii. Number of samples x 2.75 µL of AmpF*l*STR Identifiler Primer Set
        iv. Number of samples x 4 µL of Nuclease-free Water
    d. Vortex the master mix at medium speed for 5 seconds.
    e. Dispense 12.5 µL of master mix per PCR tube.

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3. Add 1 µL of sample, previously diluted to the appropriate PCR wells.
4. Add 1 µL of control DNA to the appropriate well.
5. Add 1 µL of Nuclease Free water to the negative control well.
6. Cover the plate with an adhesive clear plastic cover, vortex slightly and spin down.

F. PCR Amplification

1. Select program "SSTR" on the thermal cycler:

Initial Incubation	Denature	Anneal	Extend	Final Extension	Final Step
HOLD	CYCLE (28 cycles)			HOLD	HOLD
9°C 11 minutes	94°C 1 minute	59°C 1 minute	72°C 1 minute	60°C 60 minutes	4°C (forever)

2. Place the tray in the thermal cycler and close the heated cover.
3. Start the thermal cycler.
4. Remove the tubes from the instrument block after the PCR is complete.
5. Store the amplified DNA in a -20°C freezer, or proceed directly to the ABI for genotyping.
   IMPORTANT: Protect the amplified products from light.

G. ABI 3730 Set-up

1. Dilute PCR products 1:10 with Nuclease-free Water.
2. Combine 0.5 µL of diluted PCR product with 9 µL Hi-Di™ Formamide and 0.5 µL GS500 LIZ (internal lane standard). Cover with plate septa and spin down briefly in a plate centrifuge.
3. Heat denature for 3 minutes at 95°C, and immediately chill on ice for 2 minutes.
4. Assemble plate, tray cover, and plate base. Place on autosampler.
5. Follow the AB 3730 Genetics Analyzer procedure (EQP-35) for instrument preparation and use.
6. Complete plate record and link to plate assignment.
7. Press the green arrow.
8. Review extracted sample files in Genotyper software.
9. Print results from each case. Normal DNA should be on one page and tumor DNA on another for comparison. Each color is represented on its own line, and peaks should be labeled with base pair size. The base pair size peaks for each marker should match between the normal and the tumor. If a sample is mixed (more than two alleles for each marker), or if the tumor does not match the normal, then the case will fail for identity.

H. Amplified Loci

Locus Designation	Chromosome Location	Dye Label
D8S1179	8	6-FAM

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D21S11	21q11.2 – q21	
D7S820	7q11.21 – 22	
CSF1PO	5q33.3 – 34	
D3S1358	3p	VIC
TH01	11p15.5	
D13S317	13q22 – 31	
D16S539	16q24 – qter	
D2S1338	2q35 – 37.1	
D19S433	19q12 – 13.1	NED
vWA	12p12 – pter	
TPOX	2p23 – 2per	
D18S51	18q21.3	
Amelogenin	X: p22.1 – 22.3 Y: p11.2	PET
D5S818	5q21 – 31	
FGA	4q28	

III. REFERENCES

A. AmpF*l*STR® Identifiler PCR Amplification kit instructions. August 2012.
B. Application Note for AmpF*l*STR® Identifiler PCR Amplification Kit
C. Budowle, B. et al. 1998a. CODIS and PCR-Based Short Tandem Repeat Loci: Law Enforcement Tools. Second European Symposium on Human Identification. 73-88.

IV. COMPREHENSIVE REVISION HISTORY

A. Version 2, Effective Date 4/25/2016
1. Made title lower case
2. Added wording to clarify how profiles are compered ("average probability of identity profiles").
3. Reagents are stored in a "-20°C freezer"
4. Added wording for QC and use of reagents that come in a kit.
5. Added heat block as required equipment
6. Added steps for creating dilution plate.
7. Added precautionary statement: do not vortex enzyme.
8. Added step to spin down plate after adding sample and reagents.

B. Version 1, Effective Date **08/26/2014** - New

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Signatures

Approved By: **Signature on file** **Date:** **Date on file**

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