

# Standard Operating Procedure

**Children's Oncology Group  
Cell Culture and Xenograft Core  
Department of Cell Biology and Biochemistry  
Texas Tech University Health Sciences Center, Lubbock, Texas**

**Procedure Title: Procedure for Establishing Unique Genetic Identity of Cell Lines or Xenografts**

**Procedure #: COG-01**

## Standard Operating Procedure Staff Review Page:

I have read and understand the procedure listed above.

Employee name	Date SOP review complete

**Procedure Title: Procedure for Establishing Unique Genetic Identity of Cell Lines or Xenografts**

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**Effective Date: December 15, 2008**

## **Standard Operating Procedure Approval Page:**

**Prepared by** Charlie Linch **Dec 15, 2008**  
**Name** **Date**

**Name** **Date**

**Validated by** Charlie Linch **Dec 10, 2008**  
**Name** **Date**

**Laboratory Director:** C Patrick Reynolds, MD PhD **July 10, 2006**  
**Name** **Date**

<b>ANNUAL REVIEW REVISION DATES</b>	
<b>Initials</b>	
<b>Date</b>	

# Standard Operating Procedure

**Children's Oncology Group  
Cell Culture and Xenograft Core  
Department of Cell Biology and Biochemistry  
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**Laboratory Location: Room(s) 4C 134  
Lab Phone: 806-743-2707**

**Laboratory Director: C Patrick Reynolds, MD PhD**

**Purpose of Laboratory.** This laboratory establishes cell lines under TTUHSC-approved protocols and serves as a reference lab for the Children's Oncology Group (COG). Cells and tissues obtained from patients are placed into cell culture and/or xenografted into mice to enable their propagation and study in the laboratory.<sup>1,2</sup> Continuous cultures (cell lines) and xenografts are identified with a code that does not allow direct identification of the patient. The latter anonymized cell line can then be used for research by investigators obtaining the lines under approved COG procedures.

**Purpose of Protocol.** Growth of cells in culture or xenografts provides opportunities for mislabeling or cross-contamination (the latter referring to mistaken exchange of cells from different origins in a line or xenograft resulting in either a mixture, or outgrowth and replacement by more the aggressive, dominant line). Such "mistaken identities" in cell culture are all too common and can occur early in the process of establishing a cell line.<sup>3-7</sup> While the Children's Oncology Group lab has implemented a number of procedures to avoid such cross-contamination and mislabeling of cell lines and xenografts, the surest approach is to have a robust procedure by which the genetic identity of cells and tissues can be verified.

After study of various alternatives, we have chosen to implement the Applied Biosystems AmpF/STR® Identifier™ system<sup>8-12</sup> for genetic identification of the cell lines and xenografts. This PCR-based system uses 15 different short tandem repeat (STR) polymorphic loci, which are, after PCR, identified by fluorescently labeled dyes attached to the primers during capillary electrophoresis (CE). A 16<sup>th</sup> locus, intron 1 of the amelogenin gene, which is located on the X and Y chromosomes, provides gender identification. A 6 base pair deletion on the X chromosome allele of amelogenin allows electrophoretic distinction between DNA from X or Y chromosomes. Since females are X,X, only a single peak is observed when testing female DNA whereas males, which possess both X and Y chromosomes, exhibit two peaks with a standard amelogenin test. Then, proprietary software is used to establish numeric identity profiles and sex typing for a given sample. The probability of this particular assay identifying tissues from two different individuals as identical averages from 1 in  $7.64 \times 10^{17}$  to 1 in  $2.76 \times 10^{16}$ , depending on race.

## Required Materials

Qiagen® DNeasy® Blood and Tissue Kit, item # 69506

Tissue (~25 mg) or 1 to 5 million cells

Thermo Scientific NanoDrop™ 1000 Spectrophotometer

Applied Biosystems AmpFISTR® Identifiler™ Kit, item # 4322288

Applied Biosystems GeneAmp® PCR System 9700

Applied Biosystems 3130xl Genetic Analyzer

Applied Biosystems GeneMapper® ID Ver. 3.2.1 software

## Method

1. Begin with a cell pellet of 1-5 million cells, or ~25mg of minced tissue.
2. Extract DNA using the Qiagen® DNeasy® kit (see Appendix I). The kit manual is available for specific instructions regarding different tissue types. Final product will be about 200µl of eluted DNA, in solution.
3. Quantify DNA with the NanoDrop™ 1000 Spectrophotometer.
4. Dilute the DNA with TE buffer (pH 8.0) or sterile water to obtain a concentration 0.05-0.125 ng DNA/µl. A 1:100 dilution works well for pellets of 1-3 million cells. A 1:200 dilution is optimal for pellets between 3-5 million cells. If DNA is too concentrated, this may overload later PCR reactions and analysis.
5. Amplify DNA using Applied Biosystems AmpF/STR® Identifiler™ Kit, item # 4322288. PCR is performed using the GeneAmp® PCR System 9700 instrument. See kit for specific instructions and details, which are summarized in Appendix II.
6. Separate PCR fragment products by capillary electrophoresis using the ABI 3130xl Genetic Analyzer with Data Collection Software v3.0 (see Appendix III). Analyze results using GeneMapper® ID Ver. 3.2.1 software (See Appendix IV). Instruction manual provided. First check the raw data from each sample, and determine the proper range needed for analysis. The Analysis Method is HID\_Classic which differs from the factory default in that the partial range needs to be determined for each analysis, and the peak-amplitude thresholds are 100 for all dye colors. The Panel used is Identifiler\_v1. The Size Standard used is GS600Liz. Check internal standard for consistent results among samples from the same run. If no results appear, check for DNA overload. Try a more dilute sample. If unusual or off ladder peaks appear and

DNA is not too concentrated, check for dye pull-up (usually a product of overloading), or electronic spikes. Further descriptions of these and other troubleshooting issues are provided in the GeneMapper® user guide.

**Note:** Before performing genotyping, it is necessary to be familiar with the instructions for the Qiagen® DNeasy® kit and Identifiler® kits. A thorough reading of the ABI 3130xl Genetic Analyzer manual and the GeneMapper® manual is also necessary. Instructions for proper use of the NanoDrop™ Spectrophotometer can be obtained from laboratory staff. It is recommended to consult relevant chapters in Forensic DNA Typing by Dr. John M. Butler. Consult the appendices below for a comprehensive review of the genotyping process.

### 16 Loci Amplified by Identifiler™

#Loci	Loci type	Dye color
D8S1179	STR	Blue
D21S11	STR	Blue
D7S820	STR	Blue
CSF1PO	STR (intron)	Blue
D3S1358	STR	Green
TH01	STR (intron)	Green
D13S317	STR	Green
D16S539	STR	Green
D2S1338	STR	Green
D19S433	STR	Yellow
vWA	STR (intron)	Yellow
TPOX	STR (intron)	Yellow
D18S51	STR	Yellow
Amelogenin	Intron	Red
D5S818	STR	Red
FGA	STR (intron)	Red

CSF1PO	5q33.1; human c-fms proto-oncogene for CSF-1 receptor, 6 <sup>th</sup> intron.
TH01	11p15.5; human tyrosine hydroxylase, 1 <sup>st</sup> intron.
vWA	12p13.31; von Willebrand Factor, 40 <sup>th</sup> intron.
TPOX	2p25.3; human thyroid peroxidase, 10 <sup>th</sup> intron.
Amelogenin	Xp22.1-Xp22.3; Yp11.2; 1 <sup>st</sup> intron; X has 6 bp deletion. Protein found in developing tooth enamel.
FGA	4q28; human alpha fibrinogen gene, 3 <sup>rd</sup> intron.

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## **Appendix I: Cell pellet DNA extraction using the Qiagen® DNeasy® Blood and Tissue kit**

1. When opening a new kit, add the amount of 100% ethanol indicated on the bottles for the AW1 and AW2 buffers.
2. Set water bath or heat block to 56°C. Centrifuge pellet for 5 min at 300x g (2,200 rpm) if necessary to remove supernatant. Resuspend cell pellet in 200 µl sterile PBS.
3. Add 20 µl Proteinase K, 200 µl AL (lysis) buffer, vortex and incubate for 10 minutes at 56°C.
4. Add 200 µl 100% ethanol, vortex. Assemble a kit supplied filtration column into a kit supplied 2 ml collection tube for each sample. Label the filtration columns.
5. Pipette to filtration column (620 µl) and centrifuge at 8,000 rpm for 1 minute. Discard the 2 ml tube with spin product.
6. Place filtration column on another kit supplied 2 ml tube, add 500 µl of AW1 (wash) solution. Centrifuge for 1 minute at 8,000 rpm. Discard the 2 ml tube with spin product.
7. Place filtration column on another kit supplied 2 ml tube, add 500 µl of AW2 (wash) solution. Centrifuge for 3 minutes at 14,000 rpm. Discard the 2 ml tube with spin product.
8. Obtain and label a sterile lab 1.5 or 2 ml tube for each sample. Add 200 µl AE (elution) solution. Incubate at room temperature for 1 minute. Centrifuge 1 minute at 8,000 rpm. This is your final DNA product. Repeat step 8 for maximum DNA yield. A 2 ml tube must be used if the elution is performed twice.

For long-term DNA storage, store at -20°C. 4°C is sufficient for short-term storage. For quantification, TE buffer or sterile water can be used to blank spectrometers.

For other tissue types, refer to the Qiagen® DNeasy® kit instruction manual.

## **Appendix II: Amplification of DNA by Polymerase Chain Reaction using AmpFISTR® Identifiler™ PCR Amplification kit**

Prepare TE buffer by mixing 10 ml of 1 M Tris-HCl (pH 8.0) with .2 mL of .5 M EDTA and 990 mL glass-distilled or deionized water (EDTA can irritate skin, eyes, respiratory pathways, wear protective eye-wear and clothing, and gloves). Autoclave. Store at room temperature.

1. Quantitate DNA with the NanoDrop™ 1000 Spectrophotometer
2. Dilute extracted DNA with TE buffer or sterile water to a concentration of 0.05-.125 ng/μl. Keep chilled on ice.
3. Prepare the master mix. First centrifuge the tubes together in microcentrifuge tube to remove liquid from caps. Keep the primer set, master mix, and final PCR products away from light (fluorescent dyes attached to primers are light sensitive). Combine the three components of the master mix in the following proportions in a single 1.5 ml tube:

# samples x 10.5 μl AmpFISTR® PCR Reaction Mix

# samples x .5 μl AmpliTaq Gold® DNA Polymerase

# samples x 5.5 μl AmpFISTR® Identifiler™ Primer Set

The number of samples = each unknown sample + the positive and negative controls. Vortex the mix for 5 seconds. Return components of master mix to respective storage conditions (Reaction mix and primer set 2-8°C; AmpliTaqGold® polymerase -20°C).

4. Record a reference number for each sample and label PCR reaction tubes with reference numbers. PCR reaction tubes should be placed in a tube holder that has been chilled in the freezer. Dispense 15 μl of master mix to each reaction tube (add master mix first to avoid changing tips).
5. Add 10 μl of DNA from each sample to their respective reaction tubes according to previously recorded reference numbers, making sure to change pipette tips. Positive control DNA is included in the kit. Vortex the sample briefly and then centrifuge briefly to remove liquid from the cap. Negative control is whatever was used for the DNA dilutions.

*Program the GeneAmp® PCR System 9700 to the following conditions:*

<b>Initial Incubation Step</b>	<b>Denature</b>	<b>Anneal</b>	<b>Extend</b>	<b>Final Extension</b>	<b>Final Step</b>
HOLD	CYCLE (28 cycles)			HOLD	HOLD
95°C 11mins	94°C 1 min	59°C 1 min	72°C 1 min	60°C 60 mins	4-25°C (indefinite)

Close the heated cover and run PCR according to these specifications.

It takes about 3 ½ hours to complete the PCR.

When PCR is completed, store at 2-6°C for short term, or –20°C for long-term storage.

## Appendix III: Capillary Electrophoresis and Data Collection with ABI 3130xl Genetic Analyzer and Data Collection Software v3.0

Refer to the detailed instructions contained in the Applied Biosystems manuals for the 3130/3130xl Genetic Analyzers.

A. Getting Started Guide

B. User Bulletin

Turn on the computer first, then the 3130xl Genetic Analyzer (GA). Wait for the ready light on the GA to stabilize to green. Open the 3130xl Data Collection software and wait for all four red buttons in the Service Console to change to green. The Foundation Data Collection screen opens.

### 1. Plate Record creation-

GA Instruments ► ga3130xl ► Plate Manager → New → Complete dialog box as follows: Give the plate a Name. Application: Genemapper-lubcbb-5FH3BD1. Plate Type: 96-well. Owner → input initials. OK.

GeneMapper Plate Editor opens and is used for listing samples in their well positions. Complete table with Sample Type: Sample, Negative Control, Positive Control or Allelic Ladder. Panel: Identifiler v1. Analysis Method: HID Classic. Results Group1: select from drop down. Instrument Protocol1: Fragment G5 36 POP-7.

### 2. Preparing Samples-

The PCR products must be denatured prior to electrophoresis in the genetic analyzer 96-well plate. The ABI 3130xl is a 16-capillary (36 cm) machine and each capillary must be in use during a run. If you only have 7 samples, the other 9 wells in the plate must have 10 µl of Hi-Di™ Formamide in them. For 16 or less samples make up the size standard and formamide mix as follows in a 1.5 ml tube:

4 µl GS600 Liz size standard plus 174 µl Hi-Di™ Formamide, vortex, make sure the sample is at the bottom of the tube.

Each well gets 9 µl of the above mix and 1 µl of amplified DNA product. At least one well in each group of 16 must contain the DNA ladder that consists of known alleles that were amplified at the manufacturer. Like with the DNA sample wells, the ladder well gets 1 µl of ladder and 9 µl of formamide:size standard mix.

The sample wells are covered and heated in the thermal cycler at 95°C for 3 minutes after ensuring that the contents of each well are mixed and collected at the bottom.

The 96-well sample plate is then placed on ice for 3 minutes just prior to placement in the instrument. Carefully assemble the plate components and properly place in the

Genetic Analyzer.

3. Run Scheduler-

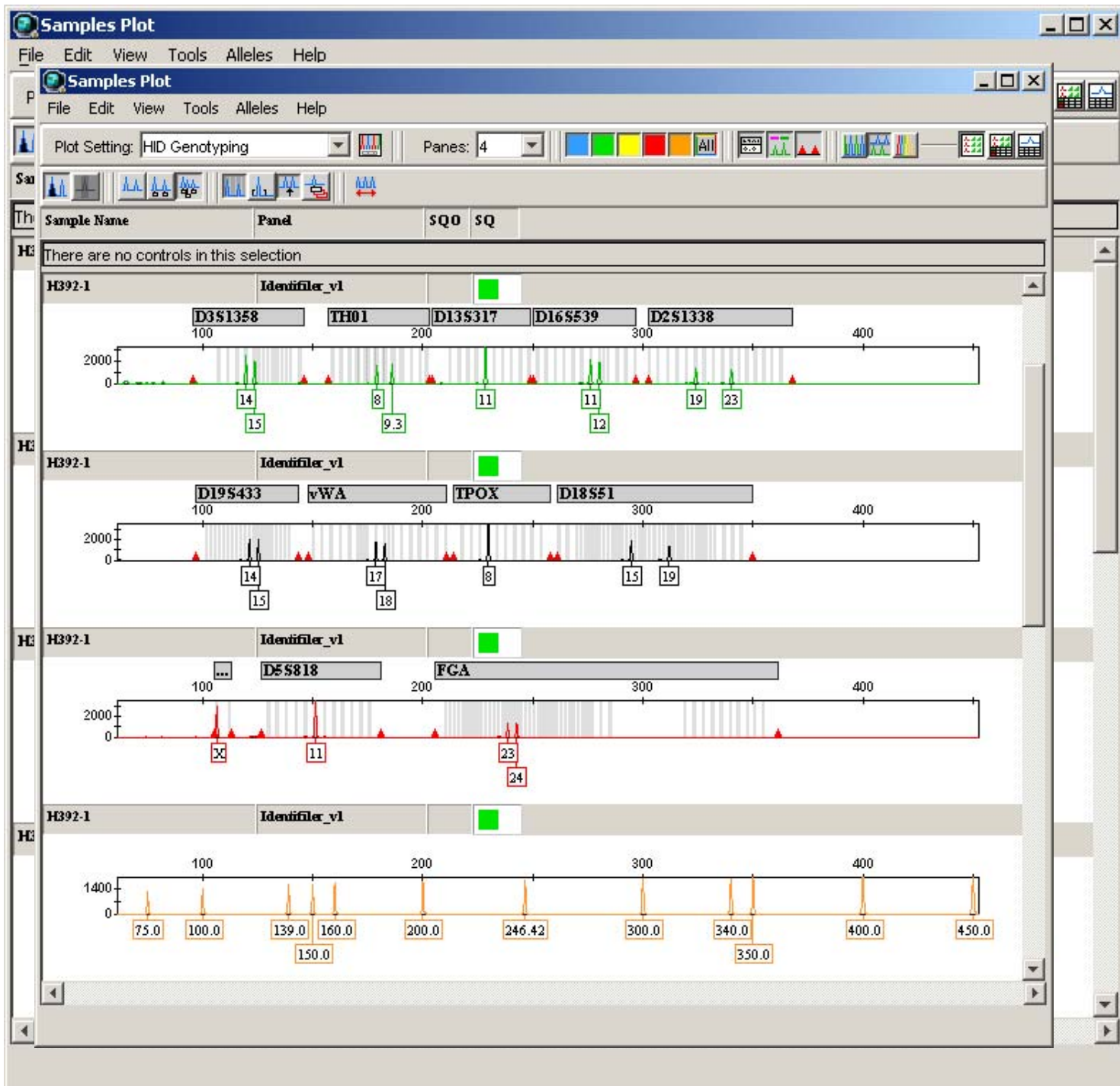
Click on "Seahorse" in the left panel. Click on Run Scheduler. Link plate. OK. To Run plate, click on green arrow. The 3130xl begins to process the plate(s). The run takes about 45 minutes with the 36 cm capillary.

4. Routine Maintenance-

Replace the sterile water and 1x running buffer (5 ml 10x ABI buffer and 45 ml of sterile water) in the reservoirs on the instrument before each run or at least weekly. Check the level of polymer (POP-7™) on the instrument before each run and change to fresh polymer at least every three weeks. Run the Replenish Polymer Wizard every 2 weeks and run the Water Wash Wizard and defragment the computer each month. Refer to the Applied Biosystems 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide for complete maintenance information.

#### **Appendix IV: Data Analysis with GeneMapper®**

1. Open GeneMapper. Select “File”, then select, “Add Samples to Project...” Select the folder that contains exclusively the files of the samples you wish to analyze. Once the folder has been highlighted, click on “Add to List.” This will copy the folder into the right-hand column. Now click “Add.”
2. The first step of analysis is to check the raw data from the Genetic Analyzer data collection software. First check the negative control. It should show no DNA in the allele range, only the orange peaks of the internal standard. Next, select the positive control. It should resemble the raw data readout of the samples, showing a large cluster of “junk” peaks to the left and smaller, neatly spaced peaks to the right (desired alleles). Thirdly, check the allelic ladder. This readout should show minimal “junk” DNA and densely packed short peaks spanning the allelic range. The allelic ladder contains all common alleles present for each locus examined leading to the large amount of DNA in the allele range. Lastly, view each sample to verify that they indeed contain analyzable DNA.
3. Return to GeneMapper and click on the “Analyze” button (green arrow ►). The program should immediately find and begin analysis on the allelic ladder. If it does not, make sure the sample type for the ladder is set at “Allelic Ladder”. Once analysis is completed, the final electropherogram for each sample can be viewed by highlighting the sample in the right column and then clicking on the “Display Plots” button. The red arrows on the bottom of each row serve to bracket each locus. The names of the loci are above. The peaks are labeled with the number of STRs at each locus. If the analysis failed, go to Step 4. Recheck electropherograms and genotyping results for each sample.
4. Record and catalogue the profiles (genotypes) of each sample. Enter genotypes into the Microsoft Office Access STR DataBase. Search the database for any matching samples as needed.



Electropherogram for Positive Control DNA