# Authenticating human cell lines using CLA IdentiFiler and CLA GlobalFiler kits on capillary electrophoresis platforms

## In this application note, we show:

- The identity of cell lines grown *in vitro* can be verified using the Applied Biosystems™ Cell Line Authentication (CLA) IdentiFiler™ Plus and Direct kits, and CLA GlobalFiler™ PCR Amplification Kit

- Cell lines can be authenticated using as little as 100 pg of purified genomic DNA (gDNA)

- Cell lines can be authenticated directly from punches of cells spotted onto NUCLEIC-CARD™ sample collection devices

- The Applied Biosystems™ SeqStudio™ Genetic Analyzer gives high performance with low concentrations of gDNA and cells

## Introduction

The study of human diseases relies heavily on the analysis of dissociated human cell lines grown in culture. However, an increasingly acknowledged problem is that cells grown *in vitro* can be misidentified or become contaminated with other, unrelated cell lines [1]. Misidentification of cell lines produces misleading results, confusion, and added costs to research [2-4]. Journals and funding agencies now require researchers to ascertain that the cell lines they use are authentic, and to identify strategies for ensuring they remain so over the course of a study (for examples, see Yu et al., 2015 [5] and Neimark, 2015 [6]).
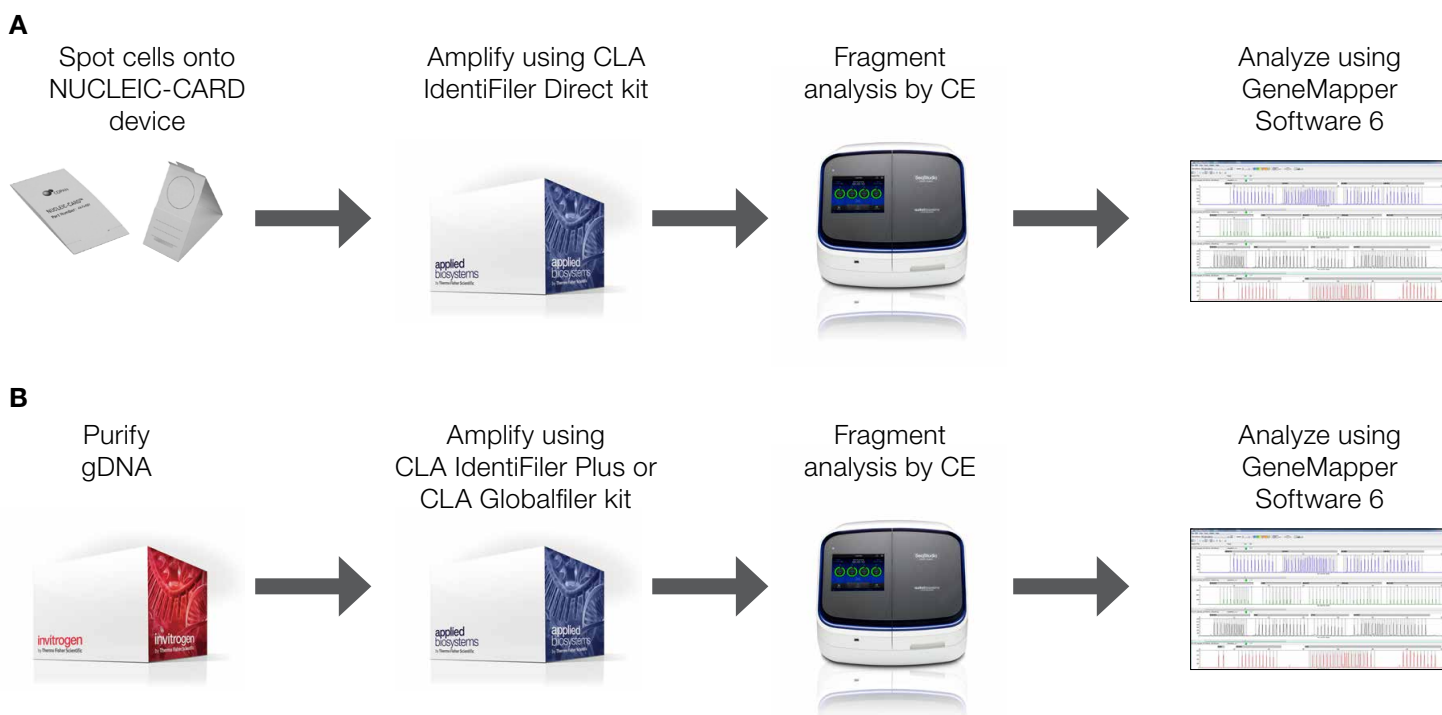
Establishing the authenticity or provenance of a cell line often involves analysis of variants or alleles of several loci and making sure these variants match expected alleles. There are several methods for analyzing variant loci, ranging from electrophoretic analysis of isozyme variants through analysis of restriction fragment length polymorphisms (RFLPs) and amplified fragment length polymorphisms (AFLPs), to next-generation sequencing (NGS) and analysis with MALDI-TOF mass spectrometry. However, many of these methods suffer from at least one drawback, ranging from insufficient complexity to high costs. Nevertheless, analysis of highly variable short tandem repeat (STR) markers, a well-established technique commonly used in DNA forensic analysis, can provide a simple, inexpensive, and highly specific genetic "fingerprint" of a cell line. Comparing a profile of alleles present at these highly variant loci to known, standardized samples of a cell line provides confidence that the cell line is authentic. Organizations such as ATCC and Leibniz-Institute DSMZ—German Collection of Microorganisms and Cell Cultures provide online access to searchable databases that allow investigators to query known cell types. Alternatively, researchers can establish an allelic profile of cell lines unique to their lab, and over time compare the allelic profiles of the cells to their own internal standards to ensure that the cell identities are true.

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S C I E N T I F I C

Typically, analysis of STRs is performed by capillary electrophoresis (CE) of fragments amplified from microsatellite loci with varying number of repeats. We offer instruments that are optimized for researchers' needs in sensitivity and throughput. Furthermore, the Applied Biosystems™ product portfolio has several different kits for PCR-based STR fingerprinting for use on CE instruments. The CLA IdentiFiler Plus PCR Amplification Kit has been optimized to analyze 16 highly variant human STRs over a wide range of purified gDNA preparations. The CLA IdentiFiler Direct PCR Amplification Kit was first developed to analyze the same 16 STR loci, starting from dried blood or buccal spots (for example, on NUCLEIC-CARD devices) or buccal swabs. For the NUCLEIC-CARD device, a 1.2 mm punch from the card is placed directly into a PCR tube or well, and amplified without any further purification. When extra levels of discrimination are needed, the CLA GlobalFiler PCR Amplification Kit allows 6-dye analysis of 24 loci, 16 of which are included in the IdentiFiler kits.

Finally, Applied Biosystems™ GeneMapper™ Software 6 and the cloud-based microsatellite analysis (MSA)

software solutions facilitate analysis of STRs by making use of pre-established allelic ladders and sizing bin sets for the various STR alleles covered by the IdentiFiler kits. An illustration of the complete workflows for cell line authentication is shown in Figure 1.

The latest member of our CE instrument family—the SeqStudio Genetic Analyzer—has several new features aimed at making CE analysis easier. The SeqStudio Genetic Analyzer has a completely redesigned user interface driven by an on-instrument touchscreen and integrated computer, facilitating setup and runs. Embedded run modules and a removable slide-in cartridge allow the SeqStudio Genetic Analyzer to be used for either Sanger sequencing or fragment analysis without any reconfiguration. This genetic analyzer provides maximum flexibility and ease of use, making it an ideal platform for many applications, such as cell line authentication when coupled with the CLA IdentiFiler and GlobalFiler kits.



**Figure 1. Workflows for cell line authentication.** Two methods are available for cell line authentication. **(A)** Cells can be spotted onto NUCLEIC-CARD devices, punches of the cards amplified directly using the CLA IdentiFiler Direct kit, and fragments analyzed on Applied Biosystems™ CE instruments using GeneMapper Software 6 or the MSA cloud application. **(B)** Alternatively, gDNA can be purified from cell lines, amplified using the CLA IdentiFiler Plus or CLA GlobalFiler kit, and fragments analyzed by capillary electrophoresis and GeneMapper Software 6 or the MSA cloud application.

## Results

### Use of CLA IdentiFiler Plus or GlobalFiler kit, and gDNA

GeneMapper Software 6 simplifies the calling of STR alleles by aligning unknown fragments with a ladder of STR fragments of known allele sizes (Figure 2, top). For example, the sample containing gDNA from M4A4GFP cells has two peaks from the sample at the D7S820 locus. GeneMapper Software 6 aligns these two peaks to alleles 8 and 10 in the allelic ladder, with sizes of approximately 263 and 271 bp, respectively, without any other significant peak being present (red circle, Figure 2, bottom). Thus, at this locus, the cells are heterozygous for these two alleles. At the D19S433 locus, a single peak is present at allele 14, indicating that these cells are homozygous for this allele.

The combination of all the alleles gives a unique fingerprint to this cell culture, and can be compared to other cultures or known samples to establish authenticity.
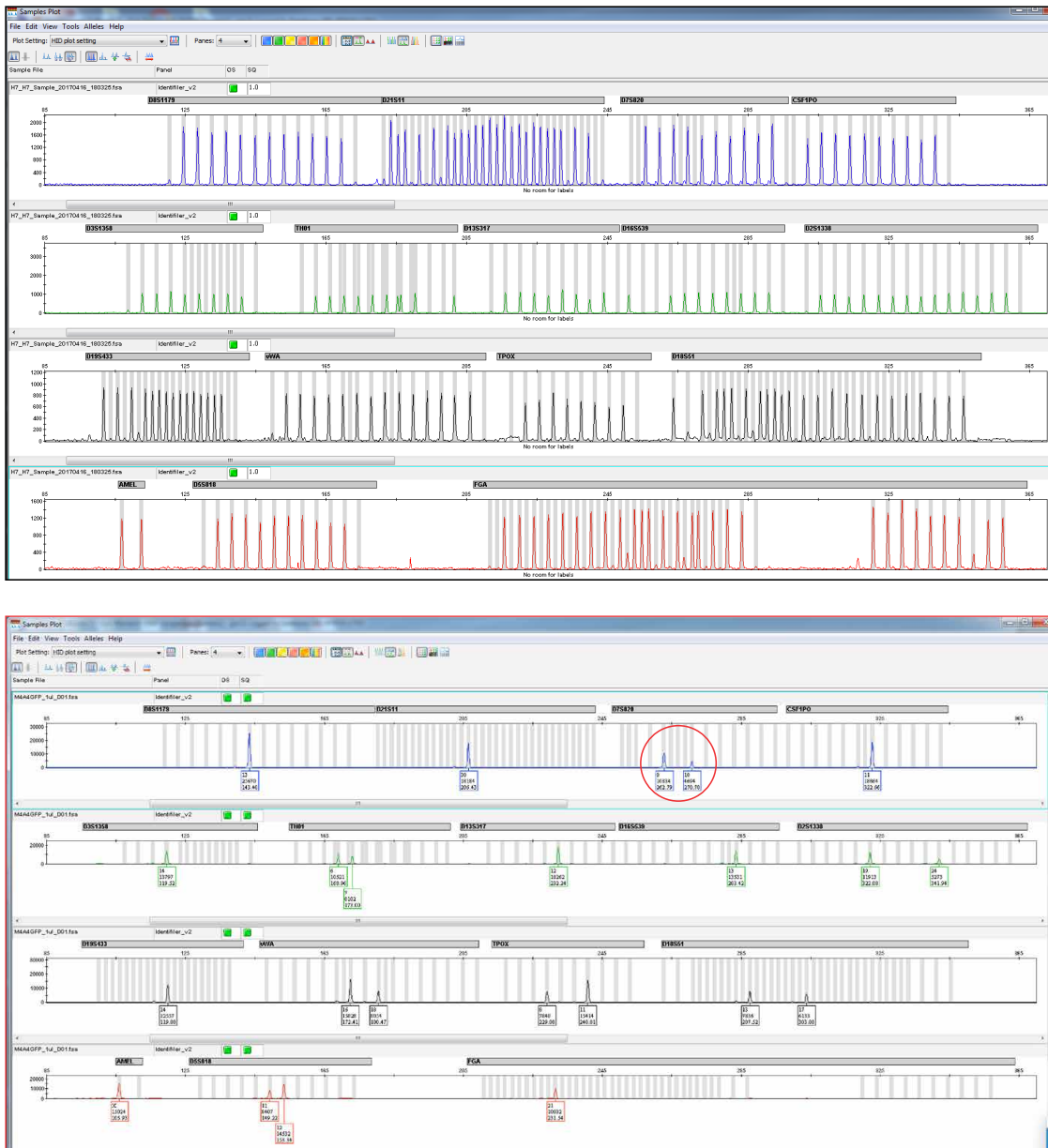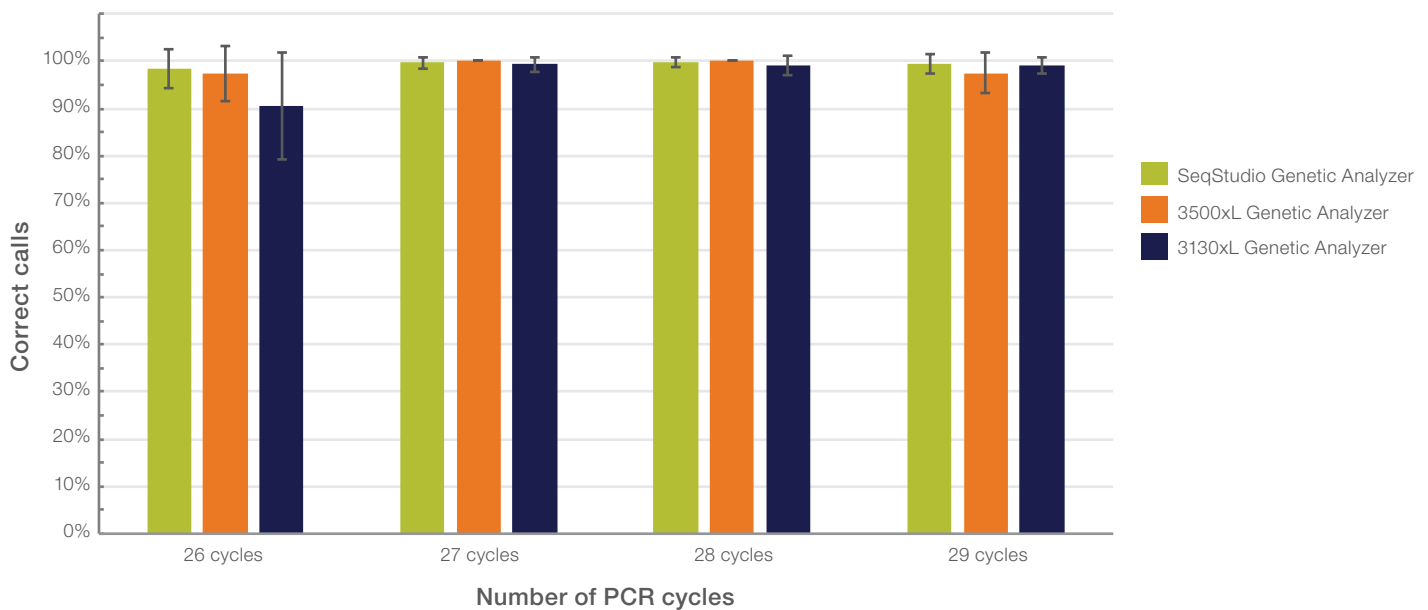


**Figure 2. GeneMapper Software 6 analysis that compares the Applied Biosystems™ IdentiFiler™ Plus Allelic Ladder (top) and purified gDNA from M4A4GFP cells grown in culture (bottom).** GeneMapper Software 6 uses the allelic ladder provided in the IdentiFiler kits to assign the alleles present in an unknown sample to known STR alleles. The boxes below the peaks show the allele number, the height of the peak, and the size of the fragment in base pairs.

To show the utility of the CLA IdentiFiler Plus kit and SeqStudio instrument for cell line authentication, we analyzed DNA from cultures of five different commonly used cell lines. Briefly, gDNA was purified from cell pellets using the Invitrogen™ RecoverAll™ Total Nucleic Acid Isolation Kit for formalin-fixed, paraffin-embedded (FFPE) samples. However, since deparaffinization was not necessary for this sample type, we skipped those steps in the protocol. The DNA amount was determined using the Applied Biosystems™ Quantifiler™ Human DNA Quantification Kit. One nanogram of purified DNA in 10 µL water, or 3-fold serial dilutions of gDNA starting at 3 ng/µL in water, were prepared and analyzed with the CLA IdentiFiler Plus kit according to the protocol found in the User Guide (Pub. No. 4440211, Revision F). Following PCR, 1 µL of the reaction was denatured in Applied Biosystems™ Hi-Di™ Formamide, and loaded onto the instrument for capillary electrophoresis. Fragment peaks were analyzed in GeneMapper Software 6 using an imported human identification (HID) analysis method (see Appendix).

Initial experiments were performed to determine the number of PCR cycles needed for optimal allele detection on the different instrument platforms (Figure 3). Accuracy was determined by comparing against the alleles present in the ATCC database (see later section, Verifying cell authenticity against known standards) for these five cell lines. On the Applied Biosystems 3130xl instrument, 27–29 cycles generated the highest percentage of accurate calls, whereas on the Applied Biosystems™ 3500xL and SeqStudio instruments, 27–28 cycles produced highest-confidence data. Using these results as a guide, we recommend determining the optimal number of cycles for the CE system that will be used in your lab.

To determine the minimal amount of DNA that can be used with the CLA IdentiFiler Plus kit, we also performed serial dilutions of purified M4A4GFP gDNA (data not shown). The most accurate results were obtained when 0.3–3 ng of gDNA was used in the amplification reaction. By using the higher number of PCR cycles, we were able to accurately profile small amounts of DNA; however, at higher DNA concentrations, more PCR cycles also resulted in higher numbers of spurious peaks and reduced accuracy. Therefore, for highest confidence in the allelic calls, we recommend using 1 ng of purified gDNA in a cell line authenticity analysis.
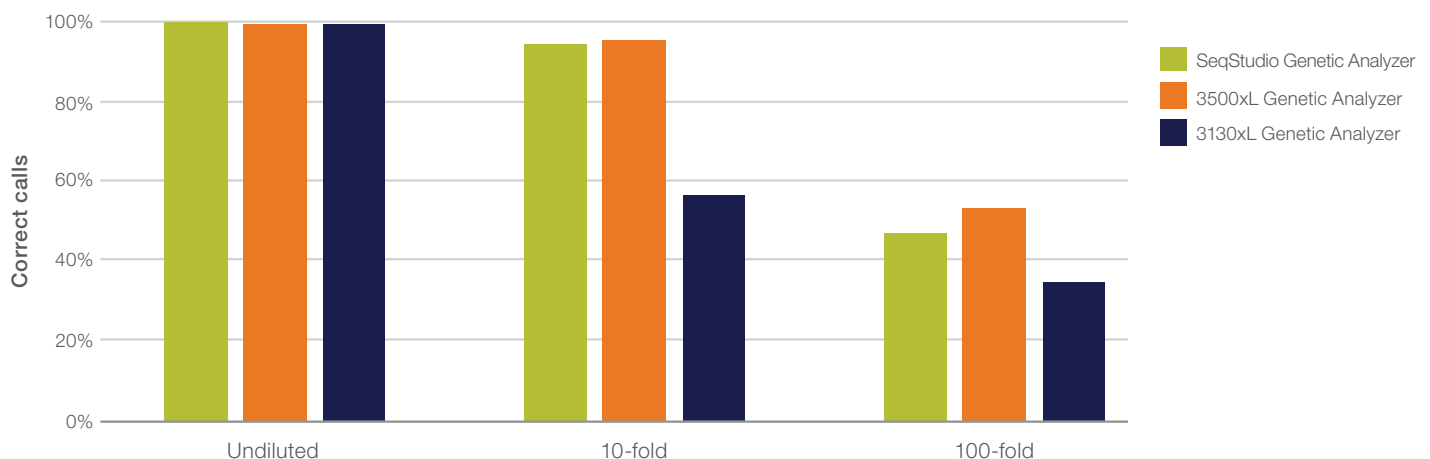


Figure 3. Analysis of gDNA using the CLA IdentiFiler Plus kit. One nanogram of DNA from five different cell lines was analyzed using varying numbers of PCR cycles. Overall, the most accurate results were obtained when PCR was performed for 27–29 cycles.

## Use of CLA IdentiFiler Direct kit and NUCLEIC-CARD device

An alternative method for authenticating cell cultures makes use of the NUCLEIC-CARD device. The matrix in these cards is chemically treated to enable cell lysis and protein denaturation so that the DNA on the card is immobilized and preserved for long-term storage at room temperature. To demonstrate the performance of these cards, we prepared suspensions of several different human cell lines in PBS (approximately $5 \times 10^5$ cells/mL; see Figure 4 for exact concentrations). One hundred microliters of suspension were spotted directly onto the NUCLEIC-CARD device and dried overnight. Single 1.2 mm punches were taken from the area with the dried suspension and placed into a well of a 96-well PCR plate. Controls and reagents from the CLA IdentiFiler Direct kit were added to the plate according to the protocol supplied in the user guide (Pub. No. 4415125, Revision J), and amplified for 29 cycles. As described above, following PCR amplification, 1 µL of product was denatured in Hi-Di Formamide and analyzed on a SeqStudio, 3500xL, or 3130xl Genetic Analyzer using GeneMapper Software 6.

The minimum amount of cell suspension that can be analyzed was determined by performing serial dilutions of the starting cell suspension into PBS (Figure 4). Complete and accurate profiles were obtained from the undiluted samples across all cell lines. Diluting the suspension 10-fold before spotting onto the NUCLEIC-CARD device resulted in dropout of some alleles. The number of allele dropouts inversely correlated with the concentration of the suspensions—those with slightly higher concentration had fewer dropouts, while lower concentrations had more dropouts. At a 100-fold dilution, an average of about 50% of the alleles were detectable. For highest confidence in allele calls, we therefore recommend spotting a suspension of around $5 \times 10^5$ cells/mL onto the NUCLEIC-CARD device.

| Cell line | Starting concentration (cells/mL) |
|-----------|-----------------------------------|
| A549 | $2.6 \times 10^5$ |
| M4A4GFP | $4.7 \times 10^5$ |
| U2OS | $7.2 \times 10^5$ |
| HeLa | $2.1 \times 10^5$ |
| HEK293 | $5.0 \times 10^5$ |



Figure 4. Titration of cell suspensions dried onto NUCLEIC-CARD device. Cell suspensions were prepared at the concentrations shown in the table, then serial dilutions of those suspensions were analyzed using NUCLEIC-CARD devices and the CLA IdentiFiler Direct kit. The average percentages of correct allele calls across all cell lines at the indicated dilutions are shown.

## Verifying cell authenticity against known standards

Several research organizations have recognized the need for matching unknown samples against known cell lines to establish authenticity. For example, ATCC has set up a web-based query against their database of cell line STRs (Figure 5, **atcc.org/STR_Database.aspx**). To use it, simply enter the alleles from the sample and choose the stringency of the query. A list of cell lines in the ATCC database that match the alleles present in the sample will be returned. Similarly, the Leibniz-Institute DMSZ has a web-based STR query system in place (**dsmz.de/fp/cgi-bin/str.html**). Note that both of these pages query only 9 loci (8 autosomal and 1 sex-linked), and therefore not all of the loci in the CLA IdentiFiler or CLA GlobalFiler kits are used for this external database comparison. However, the additional loci are enormously useful for comparisons against locally generated controls or when establishing the provenance of a new cell line. The identity of each of the cell lines described in this application note was verified using both databases. These organizations have simplified the cell line authentication process by making it possible to compare alleles present in an unknown sample to those of known, commonly used cell lines. The International Cell Line Authentication Committee has set guidelines for interpretation: cells with 80–100% allelic matching come from the same donor, and a <50% match generally means that cells come from different donors or have different origins [7].

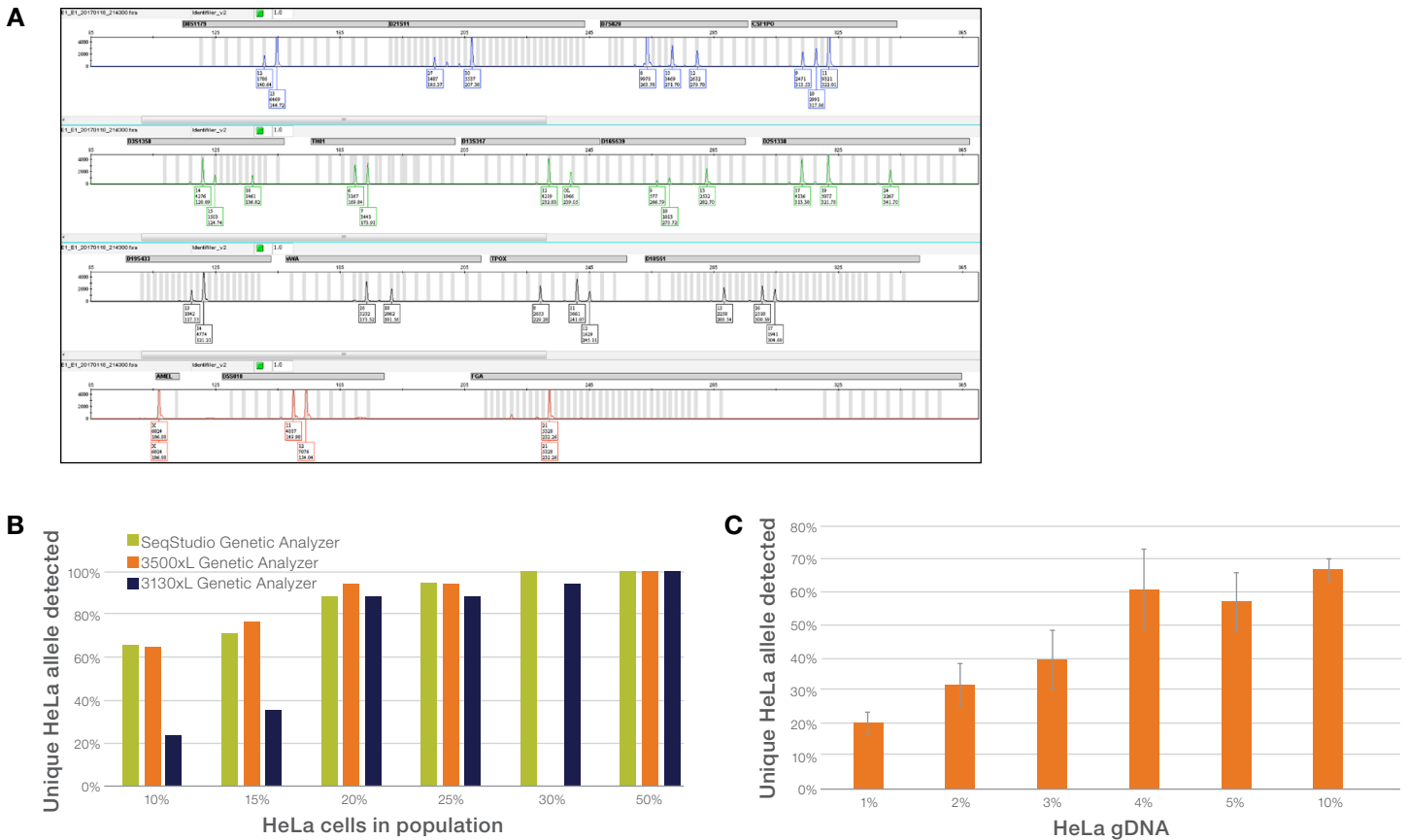## Identification of contaminating cells in a culture

One of the objectives of a cell line authentication solution is to determine whether a cell line is contaminated with unrelated cells. This is easily achievable, since the contaminating cells are likely to have a different STR profile than the parent line. In a mixture of cell lines, the final profile will reflect the combination of all cells present. For example, a single peak at a locus could represent both cell types being homozygous for the same allele, or one cell type being homozygous for the allele and the other homozygous for a deletion that removes the locus. Two peaks could mean both cell types are homozygous for different loci, or heterozygous for the same loci, etc. Although the interpretation of aberrant peaks at a single locus could be challenging and ambiguous, analysis of 16 different loci will likely identify distinct peaks that clearly point to the presence of a contaminating cell line, even if its genomic makeup might not be fully discernible.

To test the limit of detection of contaminating cells in a culture, we prepared cell suspensions of $5 \times 10^5$ cells from M4A4GFP cells and HeLa cell cultures, then mixed the suspensions such that they contained 10%, 15%, 20%, 25%, 30%, and 50% HeLa cells; 100 µL of each mixed cell suspension was spotted onto the NUCLEIC-CARD device. Card punches (1.2 mm) were obtained once the suspensions were dried, amplified with CLA IdentiFiler Direct reagents for 29 PCR cycles, and analyzed on the three different instrument platforms. We also mixed HeLa gDNA with M4A4GFP gDNA at 1%, 2%, 3%, 4%, 5%, and 10% proportions. One nanogram of each mixture was amplified using the CLA IdentiFiler Plus kit and 29 PCR cycles, and analyzed on the SeqStudio Genetic Analyzer.



**Figure 5. Cell line identities can be verified by uploading allele calls to the ATCC cell line STR database.** Once the alleles for each locus are entered, cell lines that match the STR profile are shown. Note that the ATCC database requires queries for 8 loci, all of which are covered in the IdentiFiler kits. The database can be accessed at **atcc.org/STR_Database.aspx**. For details, see the tutorial on the ATCC STR database page.

An equal mixture of M4A4GFP and HeLa cells produces a profile in which extra peaks are clearly present (Figure 6A). For example, at the locus D7S820, three alleles were positively located, whereas two alleles, at most, would be expected in a homogeneous diploid cell population. Several other loci also have three alleles present. Although conclusions can't be drawn solely from the one- and two-peak loci, the presence of three alleles at multiple loci definitely points to a heterogeneous cell population or a DNA mixture. On all platforms, we could clearly detect heterogeneity in the mixture containing 20% HeLa cells (Figure 6B). As the percentage of HeLa cells dropped in the mixture, the number of HeLa-specific alleles detected also decreased. However, the decrease was less marked on the SeqStudio and 3500xL platforms—even at 10% of the cell mixture, about 65% of the HeLa-specific alleles were still detectable. A similar analysis was performed using lower percentages of gDNA from HeLa cells mixed with M4A4GFP DNA (Figure 6C). Over half of the HeLa-specific alleles could be detected in mixtures containing as little as 4% HeLa cells, demonstrating the analytical sensitivity of the CLA IdentiFiler Plus kit.

Contaminating cells can be detected with the highest confidence if they represent >20% of the total population. However, indicator alleles may be detectable if the population contains as little as 4% of a contaminating cell line.
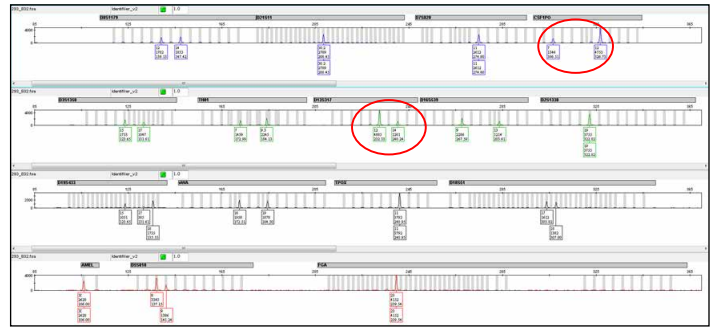


Figure 6. Detection of contaminating cells in a culture. (A) Extraneous peaks in a culture may be an indication of genomic heterogeneity, or the presence of contaminating cells. In this case, a 1:1 mixed suspension of M4A4GFP and HeLa cells was analyzed using the NUCLEIC-CARD device and CLA IdentiFiler Direct kit. The profile of a contaminated culture can vary, depending on the allelic makeup of the host and contaminating cells. (B) Analysis of mixed samples on the NUCLEIC-CARD device. HeLa cells and M4A4GFP cell suspensions were diluted to $5 \times 10^5$ cells/mL each, mixed in the indicated proportions, and spotted onto the NUCLEIC-CARD device. Identical aliquots were analyzed on the indicated instrument types. Note that the 30% population was not analyzed on the 3500xL platform. (C) Analysis of gDNA from mixed samples. gDNA from HeLa cells was mixed with gDNA from M4A4GFP cells at the indicated proportions. More than half of the alleles unique to HeLa cells can be detected in a mixture containing at least 4% HeLa gDNA using the 3500xL instrument.

## Allelic imbalance in cell lines

In STR analyses, normal genotypes present themselves as either a single peak (homozygous) or two balanced peaks with equal height (heterozygous). However, cell lines passaged *in vitro* demonstrate genomic instability, and the peak heights of sister alleles can differ due to duplication or deletion of loci, portions of chromosomes, or entire chromosomes. For example, the HEK293 cells used in this study are balanced at the D8S1179 locus, but show significantly different peak heights at the CSF1P0 and D13S317 loci (Figure 7). Moreover, when the DNA quantity added to the amplification reaction is 100 pg or less, stochastic effects may introduce imbalances in peak height.

To demonstrate that the CLA IdentiFiler kits are detecting true allelic imbalances, we analyzed 8 different standardized gDNA preparations (1 ng) from normal human donors. We determined the allelic balance by calculating the ratio of peak heights of alleles detected at each locus. For heterozygous loci, this ratio is 1.0; for homozygous loci we defined the ratio as 1.0 for this study. We simultaneously analyzed 1 ng of gDNA from the cell lines described above.

Most of the loci from the standardized human gDNA showed allelic balance, with ratios very close to 1.0 when 1 ng of DNA was used (Figure 8). There were a few exceptions—two individual donors had a locus with a ratio of 1.5, suggesting they might contain a duplication of an allele (3:2), and one individual had a manifold duplication of the TH01 allele locus. However, the cell lines showed a range of allele ratios: 0.2 to 4.5. Since the kit was able to accurately measure normal allele ratios, this potentially reflects genomic instability of the cell lines through passaging over the years. Conversely, cells that have not been passaged over long periods, such as primary cells or uninduced stem cells, are not likely to demonstrate the same allelic imbalance.
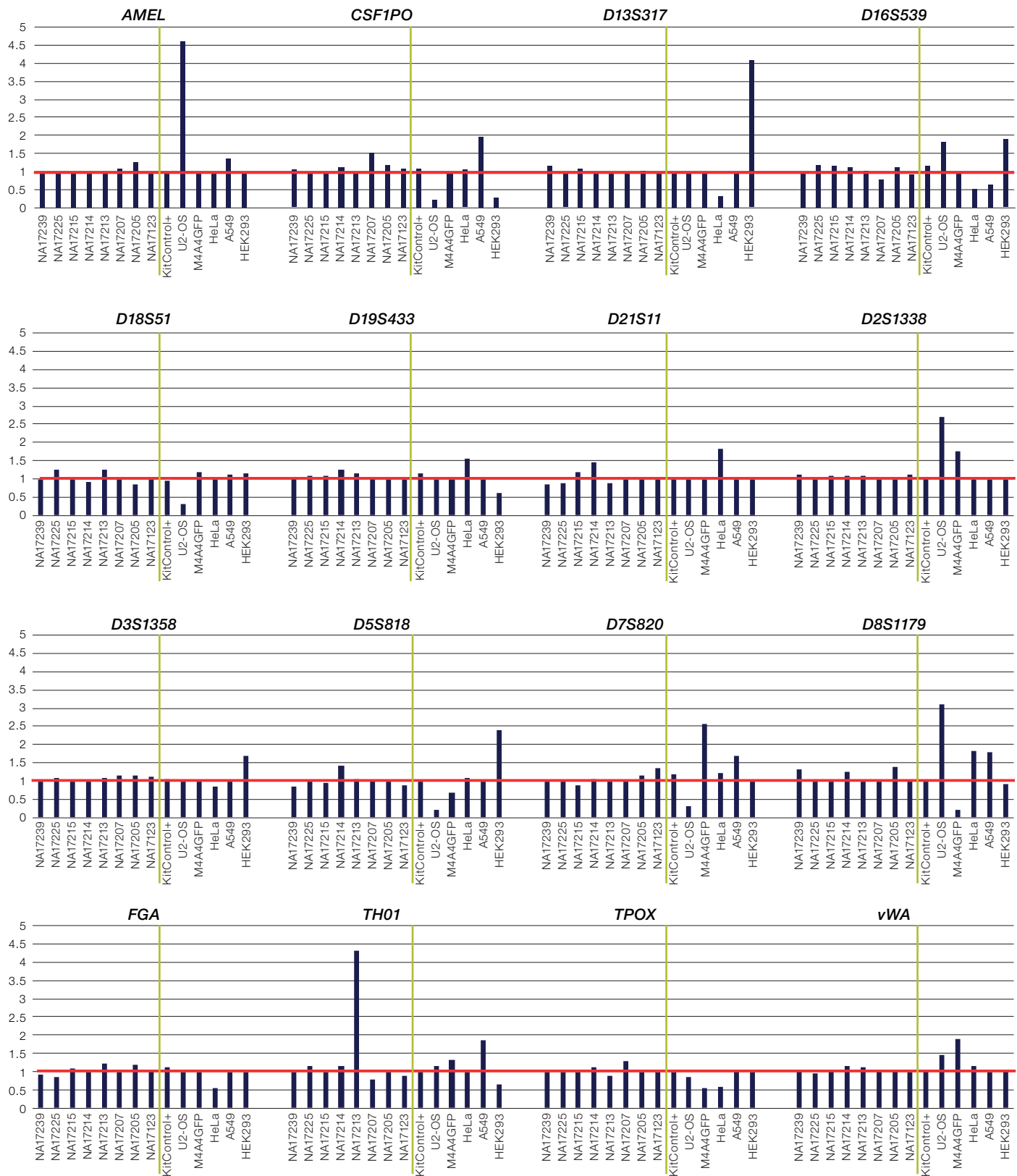


**Figure 7. Allelic imbalance in cell lines.** In normal cells, the peak height of heterozygotes is very similar; in cell lines, peak heights can often differ at a locus (red circles). This is possibly due to genomic instability of the cell line, leading to duplication and deletions of loci and therefore different abundances of alleles.

## Conclusions

In this application note, we have described simple and inexpensive workflows for cell line authentication. We demonstrated that the CLA IdentiFiler Plus and Direct kits can be used to distinguish STR allelic "fingerprints" in *in vitro*–cultured cells. In fact, because the CLA IdentiFiler kits query 16 STR loci on 15 different chromosomes, they provide exceptional flexibility for detecting changes in ploidy in cell lines, when querying DNA from degraded samples, and in future analyses as cell line derivatives and databases become more numerous and complex. We showed that results can be obtained from as little as 0.1 ng of gDNA. However, to help ensure optimal results with highest confidence, we recommend 1 ng of gDNA. We also showed that authentication results could be obtained from 100 µL of a suspension of 5 x 10⁵ cells/mL immobilized on a NUCLEIC-CARD device. We demonstrated that our CE instruments perform equivalently when gDNA or cell concentrations are relatively high. Finally, we showed that the SeqStudio Genetic Analyzer gives higher performance with lower concentrations of gDNA and cells compared to the 3130xl Genetic Analyzer. Notably, the initial sample preparation, PCR amplification, preparation for CE, and downstream analysis methods are nearly identical across the entire CE instrument line. This provides an opportunity for researchers to choose the platform most appropriate for their laboratory's throughput and budget.

**Figure 8. Cell lines demonstrate measurable allelic imbalance.** Eight standardized gDNA samples from human donors (left of the green line) and five cell lines (right of the green line) were analyzed with the CLA IdentiFiler Plus kit. Allele ratios were calculated using the heights of peaks in heterozygotes, or defined as 1.0 in homozygotes (red line). Note that most of the samples from normal human donors have allele ratios close to 1.0, whereas the cell line samples often have deviations from unity.

# Appendix

## GeneMapper modules and settings needed for analysis

Instructions below are given for importing into GeneMapper Software 6. For use with the cloud-based MSA software, please contact your local field applications scientist.

Before analyzing the FSA files for a cell line authentication project, the appropriate BIN files must be imported into GeneMapper Software 6. To import the files needed, follow the instructions below.

• AmpFℓSTR_Bins_v2.txt and AmpFℓSTR_Panels_v2.txt can be downloaded here: **thermofisher.com/us/en/ home/technical-resources/software-downloads/ genemapper-id-software.html**

• Launch GeneMapper Software 6.

• Select "Tools" from the menu bar.

• Select "Panel Manager".

**To install the "Panel File":**

– Single-click on "Panel Manager" in the left-hand window pane.

– Select "File" and "Import Panels" from the menu bar.

– A dialog box will appear. Navigate to the location of the "Panel" file on your computer. If you placed it in the default "Panels" folder, the dialog box should open to the correct folder.

– Select the file titled AmpFℓSTR_Panels_v2.txt
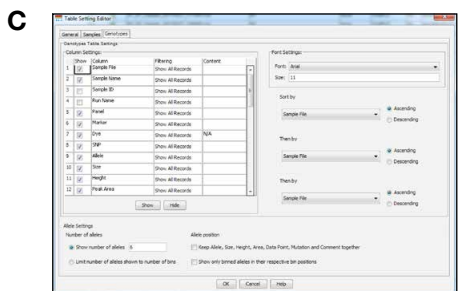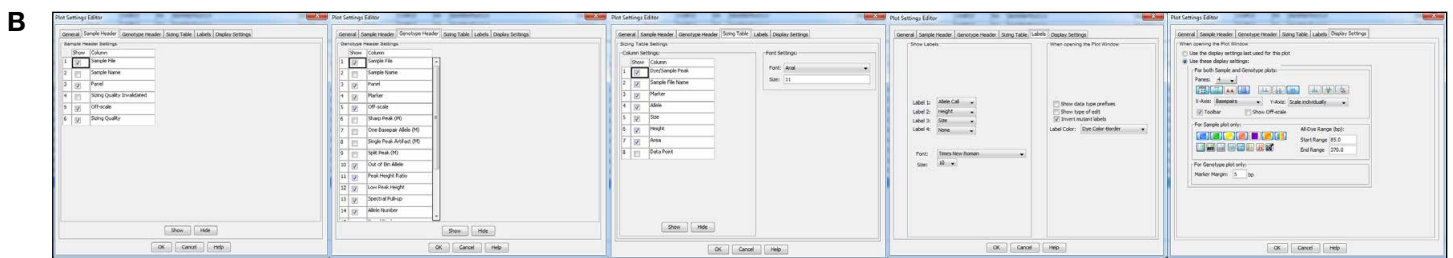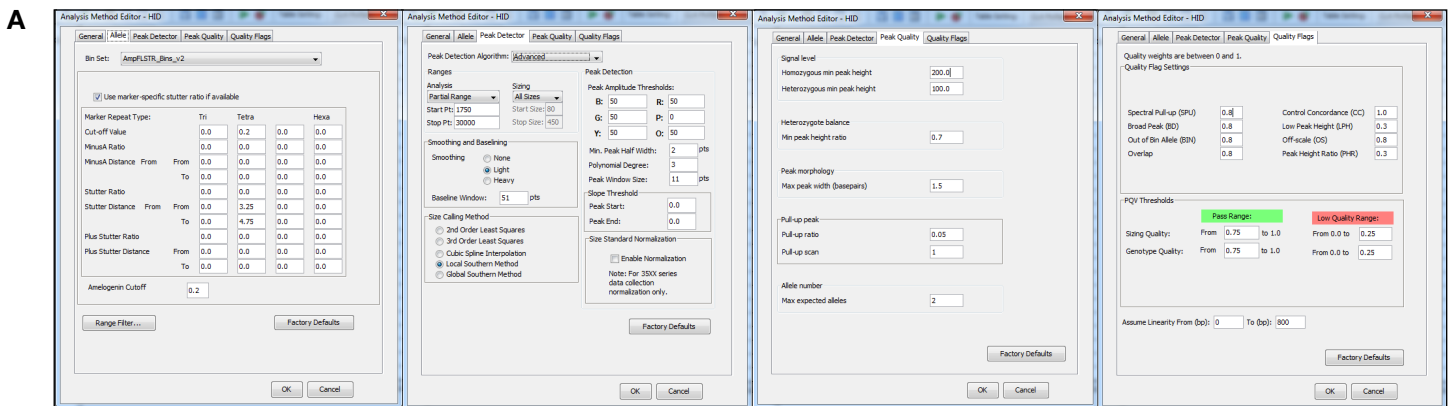
– This will install the "Panel" in the left window pane.

**Run modules for each instrument used in this study.**

| Instrument | Polymer used | Injection voltage (kV) | Injection time (sec) | Run voltage (kV) | Run time (sec) | Capillary length (cm) | Run module |
|---|---|---|---|---|---|---|---|
| 3130xl | POP-7™ | 1.2 | 23 | 15.0 | 1,200 | 36 cm | FragmentAnalysis36_POP7 |
| 3500xL | POP-7™ | 1.6 | 15 | 19.5 | 1,330 | 50 cm | FragmentAnalysis50_POP7xl |
| SeqStudio | POP-1™ | 2.0 | 10 | 9.0 | 1,440 | 28 cm | FragAnalysis |

**To install the "Bin File":**

– Single-click on "AmpFℓSTR_Panels_v2" in the left-hand window pane.

– Select "File" and "Import Bin Set" from the menu bar.

– A dialog box will appear. Navigate to the location of the "Bin" file on your computer. If you placed it in the default "Panels" folder, the dialog box should open to the correct folder.

– Select the file titled AmpFℓSTR_Bins_v2.txt

– This will install the "Bins" in the pull-down menu at the top titled "Bin Set".

- Click on "Apply", followed by "OK" to return to GeneMapper Software 6. The IdentiFiler_v2 panel and associated "Bin Set" can be chosen under "Panel" when performing analysis.

- To set up a cell line authentication analysis method, either modify an existing method or create a new one using the values in Figure 9A as a guide.

- To set up a plot setting to visualize the alleles detected, create a new setting and enter the parameters shown in Figure 9B.

- To view more than two alleles in the genotyping tables, change the "Allele Settings" in the "Genotypes" tab of the "Table Setting Editor" (Figure 9C).



**Figure 9. GeneMapper Software 6 settings for (A) cell line authentication analysis method, (B) cell line authentication plot settings, and (C) viewing more than two alleles in the genotyping tables.** These screens can be accessed by opening the "Manager Tool" and choosing the appropriate tab.

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## Ordering information

| Product | Quantity | Cat. No. |
|---|---|---|
| SeqStudio Genetic Analyzer System with SmartStart orientation (Includes SeqStudio Genetic Analyzer, SeqStudio Genetic Analysis Software, SmartStart 1-day training, 1-year warranty) | | A35644 |
| SeqStudio Genetic Analyzer System with SmartStart orientation plus 1-year extended warranty (Includes all items from A35644 plus additional 1-year warranty) | | A35645 |
| SeqStudio Genetic Analyzer System with SmartStart orientation plus 3-year extended warranty (Includes all items from A35644 plus additional 3-year warranty) | | A35646 |
| SeqStudio Cartridge v2 | 1,000 reactions | A41331 |
| 3500 Genetic Analyzer | 1 system | 4440466 |
| 3500xL Genetic Analyzer | 1 system | 4440467 |
| CLA IdentiFiler Direct PCR Amplification Kit | 200 reactions | A44661 |
| NUCLEIC-CARD COLOR matrix, 4 spots | 50 cards | 4473978 |
| NUCLEIC-CARD matrix, 1 spot | 100 cards | 4473973 |
| Prep-n-Go Buffer (for use with buccal swab substrate) | 200 reactions | 4471406 |
| Prep-n-Go Buffer (for use with untreated paper substrates) | 1,000 reactions | 4467079 |
| CLA IdentiFiler Plus PCR Amplification Kit | 50 reactions | A47624 |
| CLA IdentiFiler Plus PCR Amplification Kit | 200 reactions | A44660 |
| CLA GlobalFiler PCR Amplification Kit | 200 reactions | A44662 |
| RecoverAll Total Nucleic Acid Isolation Kit for FFPE | 40 reactions | AM1975 |
| GeneMapper Software 6 | 1 license | A38892 |
| GeneScan 600 LIZ v2.0 | 800 reactions | 4408399 |

## References

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## Find out more about the SeqStudio Genetic Analyzer at
**thermofisher.com/seqstudio**

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