Direct Amplification of DNA from Storage Card Punches and Swabs Using the VersaPlex™ 27PY System

Promega Corporation

Direct Amplification from Storage Card Punches
Materials to Be Supplied by the User:

- ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- PunchSolution™ Kit for non-lytic card punches (Cat.# DC9271)
- 5X AmpSolution™ Reagent for lytic card punches (Cat.# DM1231; also supplied with the PunchSolution™ Kit)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

Direct Amplification from Swabs
Materials to Be Supplied by the User:

- ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

Introduction

The VersaPlex™ 27PY System (Cat.# DC7020) is a 27-locus multiplex system for human identification applications including forensic analysis, relationship testing and research use. This six-color system allows co-amplification and fluorescent detection of the 20 autosomal loci in the expanded CODIS core loci (CSF1PO, FGA, TH01, TPOX, vWA, D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11 and D22S1045) as well as Amelogenin and DYS391 for gender determination. The Penta D, Penta E and D6S1043 loci are also included to increase discrimination and allow searching of databases that include profiles with these loci. Finally, two rapidly mutating Y-STR loci, DYS570 and DYS576, are included in the multiplex panel. This extended panel of STR markers is intended to satisfy both CODIS and ESS recommendations (1).

Direct amplification of known standard samples has become the preferred analysis method in laboratories. The ability of a laboratory to conduct direct amplification using the same kit utilized in casework can significantly streamline their workflow while stretching their budget. For this reason, the direct amplification application was examined for use with the VersaPlex™ 27PY System. Testing shows the VersaPlex™ 27PY System with the ProFlex® PCR System enables direct amplification with 12.5µl reactions from numerous sample types, including Whatman FTA® card punches, S&S 903 Paper, Bode Buccal DNA Collector™ devices, OmniSwabs and cotton swabs. This Application Note provides example protocols for direct amplification of DNA from storage card punches and swabs.

Direct Amplification of DNA from Storage Card Punches in a 12.5µl Reaction Volume

Materials to Be Supplied by the User:

- ProFlex® PCR System (Applied Biosystems)
- Centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- Aerosol-resistant pipette tips
- PunchSolution™ Kit (Cat.# DC9271) for nonlytic card punches
- 5X AmpSolution™ Reagent for lytic card punches (Cat.# DM1231, also supplied with the PunchSolution™ Kit)
- 1.2mm Harris Micro-Punch or equivalent manual punch and cutting mat or automated punch system

Lytic Card Sample Types Include:

- Buccal cells collected on FTA® cards with Whatman EasiCollect™ or Fitzco Sampact™ devices
- Buccal cells collected with swabs transferred to FTA® or Indicating FTA® cards
- Liquid blood (from collection or storage Vacutainer® tubes or finger sticks) spotted onto FTA® cards

Nonlytic Card Sample Types Include:

- Buccal samples and Bode Buccal DNA Collector™ Devices
- Blood and buccal samples on nonlytic cards (e.g., S&S 903)

Pretreat nonlytic sample types with the PunchSolution™ Kit (Cat.# DC9271) to lyse samples before adding the PCR amplification mix. For more information, see the *PunchSolution™ Kit Technical Manual* #TMD038.

! Failure to pretreat these samples may result in incomplete STR profiles.

Use a manual punch tool with a 1.2mm tip to manually create sample disks from a storage card. Select an appropriate area of the stain, and with a twisting or pressing action, cut a 1.2mm sample disk. Use the plunger to eject the disk into the appropriate well of a reaction plate.

Automated punchers can also be used to create sample disks. Refer to the user's guide for your instrument for assistance with generating 1.2mm disks, technical advice and troubleshooting information.

Note: Static electricity may be a problem when adding a punch to a well. For lytic card punches, adding PCR amplification mix to the well before adding the punch may help alleviate this problem. For nonlytic card punches, adding PunchSolution™ Reagent to the well before adding the punch during pretreatment may help alleviate this problem.

Amplification Setup

1. At the first use, thaw the VersaPlex™ 27PY 5X Master Mix, VersaPlex™ 27PY 5X Primer Pair Mix and Amplification-Grade Water completely. After the first use, store the reagents at 2–10°C.

   Note: Centrifuge the tubes briefly to bring the contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add one or two reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
3. Use a clean reaction plate for assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 1 to a clean tube.

Table 1. PCR Amplification Mix for Direct Amplification of DNA from Storage Card Punches Using a 12.5µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	5.0µl	×		=	
VersaPlex™ 27PY 5X Master Mix	2.5µl	×		=	
VersaPlex™ 27PY 5X Primer Pair Mix	2.5µl	×		=	
5X AmpSolution™ Reagent	2.5µl	×		=	
Total reaction volume	12.5µl				

[1]Add Amplification-Grade Water to the tube first, then add VersaPlex™ 27PY 5X Master Mix, VersaPlex™ 27PY 5X Primer Pair Mix and 5X AmpSolution™ Reagent. For lytic card punches, the template DNA will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, and then pipet 12.5µl of PCR amplification mix into each reaction well.

! Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the punches as soon as possible to each well and follow immediately by thermal cycling.

Amplification Setup (continued)

6. For lytic cards, add one 1.2mm punch from a card containing buccal cells or whole blood to the appropriate wells of the reaction plate. For nonlytic card punches, add the PCR amplification mix to the PunchSolution™ Reagent-treated punch.
   Note: It also is acceptable to add the lytic card punch to the plate first, and then add the PCR amplification mix.
7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then add 1µl (10ng) to a reaction well containing 12.5µl of PCR amplification mix.
   Notes:
   1. You may need to optimize the amount of control DNA, depending on cycling conditions and laboratory preferences.
   2. When performing more than 25 cycles of PCR with 12.5µl volume reactions, you may need to dilute the 2800M Control DNA to 5ng/µl prior to adding 1µl (5ng) to positive control reactions.
   3. Do not include blank storage card punches in the positive control reactions.
8. Reserve a well containing PCR amplification mix as a negative amplification control.
   Note: An additional negative control with a blank punch may be performed to detect contamination from the storage card or punch device.
9. Seal or cap the plate, or close the tubes. Briefly centrifuge reactions to bring storage card punches to the bottom of the wells and remove air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the number of storage card punches, cycle number (24–26 cycles), injection time and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for a variety of sample types. Buccal samples may require more amplification cycles than blood samples. Nonlytic card punches may require fewer amplification cycles than lytic punches. Cycle number should be optimized in each laboratory for each sample type.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 1. The total cycling time is approximately 1 hour.
   Note: When using the ProFlex® PCR System, use the default ramping mode (no emulation).

Thermal Cycling Protocol

96°C for 5 minutes, then:
96°C for 5 seconds 60°C for 1 minute for 25 cycles, then:
60°C for 10 minutes
4°C soak

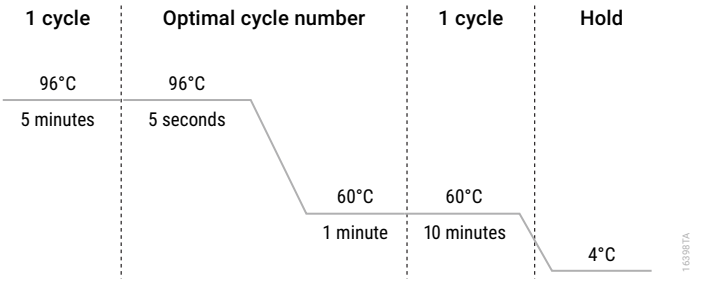

Figure 1. Thermal cycling protocol for the ProFlex® PCR System.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C protected from light.
   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Place one 1.2mm storage card punch containing buccal cells or whole blood in each well of a reaction plate. Be sure to pretreat nonlytic samples with the PunchSolution™ Kit (Cat.# DC9271).
3. Prepare three identical reaction plates with punches from the same samples.
4. Amplify samples using the thermal cycling protocol provided above but subject each plate to a different cycle number (24–26 cycles).
5. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

Direct Amplification of DNA from Swabs in a 12.5µl Reaction Volume

Materials to Be Supplied by the User:

- ProFlex® PCR System (Applied Biosystems)
- Centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- Aerosol-resistant pipette tips
- SwabSolution™ Kit (Cat.# DC8271)

Pretreat OmniSwabs or cotton swabs with the SwabSolution™ Kit (Cat.# DC8271) as described in the *SwabSolution™ Kit Technical Manual* #TMD037 to generate a swab extract.

Amplification Setup

1. At the first use, thaw the VersaPlex™ 27PY 5X Master Mix, VersaPlex™ 27PY 5X Primer Pair Mix and Amplification-Grade Water completely. After the first use, store the reagents at 2–10°C.
   Note: Centrifuge the tubes briefly to bring the contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.
2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add one or two reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.
3. Use a clean reaction plate for assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.
4. Add the final volume of each reagent listed in Table 2 to a clean tube.

Table 2. PCR Amplification Mix for Direct Amplification of DNA from Swabs Using a 12.5µl Reaction Volume.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	5.5µl	×		=	
VersaPlex™ 27PY 5X Master Mix	2.5µl	×		=	
VersaPlex™ 27PY 5X Primer Pair Mix	2.5µl	×		=	
swab extract	2.0µl				
Total reaction volume	12.5µl				

[1]Add Amplification-Grade Water to the tube first, then add VersaPlex™ 27PY 5X Master Mix and VersaPlex™ 27PY 5X Primer Pair Mix. The swab extract will be added at Step 6.

5. Vortex the PCR amplification mix for 5–10 seconds, and then pipet 10.5µl of PCR amplification mix into each reaction well.
   Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.
   Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add the swab extract as soon as possible to each well and follow immediately by thermal cycling.
6. Pipet 2.0µl of swab extract for each sample into the appropriate well of the reaction plate or 0.2ml tube.
7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 5.0ng/µl. Add 2µl (10ng) to a reaction well containing 10.5µl of PCR amplification mix.
   Note: You may need to optimize the amount of control DNA, depending on thermal cycling conditions and laboratory preferences.
8. For the negative amplification control, pipet 2.0µl of Amplification-Grade Water or TE^{-4} buffer instead of swab extract into a reaction well containing PCR amplification mix.
   Note: Additional negative controls can be included. Assemble a reaction containing the swab extract prepared from a blank swab, or assemble a reaction where the SwabSolution™ Reagent is processed as a blank without a swab.
9. Seal or cap the plate, or close the tubes.
   Optional: Briefly centrifuge reactions to bring contents to the bottom of the wells and remove air bubbles.

Thermal Cycling

Amplification and detection instrumentation may vary. You will need to optimize protocols including the amount of template DNA, cycle number (24–26 cycles), injection time and loading volume for your laboratory instrumentation. Testing at Promega shows that 25 cycles works well for buccal swabs. Cycle number will need to be optimized in each laboratory.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided below and in Figure 2. The total cycling time is approximately 1 hour.
   Note: When using the ProFlex® PCR System, use the default ramping mode (no emulation).

Thermal Cycling Protocol

96°C for 5 minutes, then:
96°C for 5 seconds 60°C for 1 minute for 25 cycles, then:
60°C for 10 minutes
4°C soak

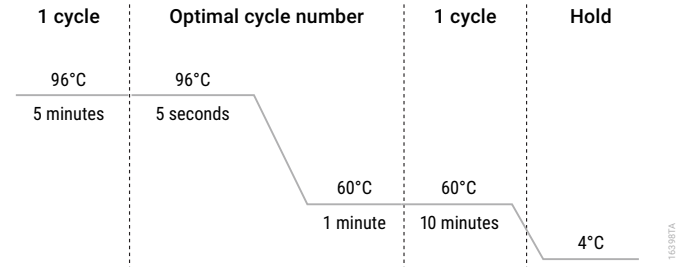

Figure 2. Thermal cycling protocol for the ProFlex® PCR System.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at –20°C protected from light.
   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.

PCR Optimization

Cycle number should be optimized based on the results of an initial experiment to determine the sensitivity with your collection method, sample types and instrumentation.

1. Choose several samples that represent typical sample types you encounter in the laboratory. Prepare them as you would using your normal workflow.
2. Prepare three identical reaction plates with aliquots of the same swab extracts.
3. Amplify samples using the thermal cycling protocol provided above, but subject each plate to a different cycle number (24–26 cycles).
4. Following amplification, use your laboratory's validated separation and detection protocols to determine the optimal cycle number for the sample type.

Results

Various direct amplification substrates (blood on FTA® cards, buccal samples on Indicating FTA® cards, Bode Buccal DNA Collectors™, blood on S&S 903 paper, cotton swabs and OmniSwabs) were tested using three cycle numbers: 24 cycles; 25 cycles; and 26 cycles.

Three donors in replicates of four were tested from each sample type, except for the buccal samples on Indicating FTA® cards which had seven donors in replicates of four. Aside from the cycle number, amplification was performed using the cycling parameters using a 12.5µl reaction volume recommended in the *VersaPlex™ 27PY System for Use on the Applied Biosystems® Genetic Analyzers Technical Manual* #TMD055.

The results of direct amplification (Figures 3 and 4) show some expected variability, given the potential for variation in donor cell count, collection technique and differences in cell density from punch to punch. This occurred more frequently with buccal samples on FTA® cards and on Bode Buccal DNA Collectors™.

Blood on FTA® cards yielded 99% (556/564), 99% (560/564) and 100% (564/564) of expected alleles at 24, 25 and 26 cycles, respectively. Buccal on Indicating FTA® cards yielded 89% (1190/1331), 96% (1374/1425) and 95% (1271/1332) of expected alleles at 24, 25 and 26 cycles, respectively. Bode Buccal DNA Collectors™ yielded 90% (504/560), 100% (560/560) and 98% (502/513) of expected alleles at 24, 25 and 26 cycles, respectively.

Blood on S&S 903 paper yielded 100% (518/518), 98% (554/564) and 100% (564/564) of expected alleles at 24, 25 and 26 cycles, respectively. Both cotton swab extracts and OmniSwab extracts yielded 100% of expected alleles at all cycle numbers tested.

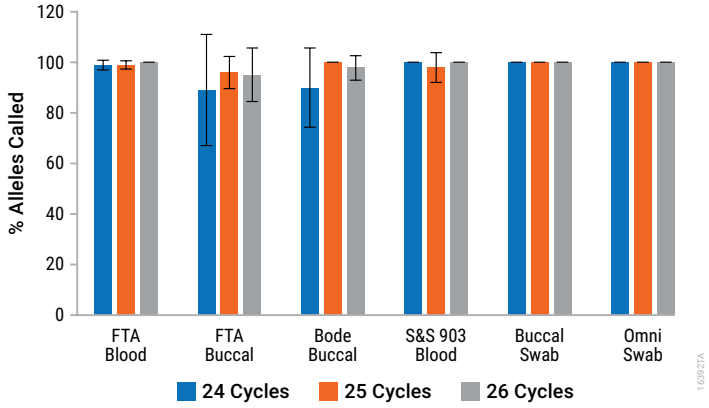

Figure 3. Percent profiles observed. Amplification of various substrates were tested using differing cycle numbers. Amplification reactions (12.5µl) was performed using the Applied Biosystems® ProFlex® PCR System for 24, 25 and 26 cycles in replicates of four. After amplification, 1µl of each reaction was analyzed on an Applied Biosystems® 3500xL Genetic Analyzer using the recommended protocol in the *VersaPlex™ 27PY System for Use on the Applied Biosystems® Genetic Analyzers Technical Manual* #TMD055. Results were analyzed using GeneMapper® ID-X software, version 1.5 and VersaPlex™ 27PY panels and bins text files, at a 175RFU threshold and a 20% global stutter filter. The average percentage of the expected alleles detected at a given substrate and cycle number is shown. Error bars represent ±1SD of replicate results.

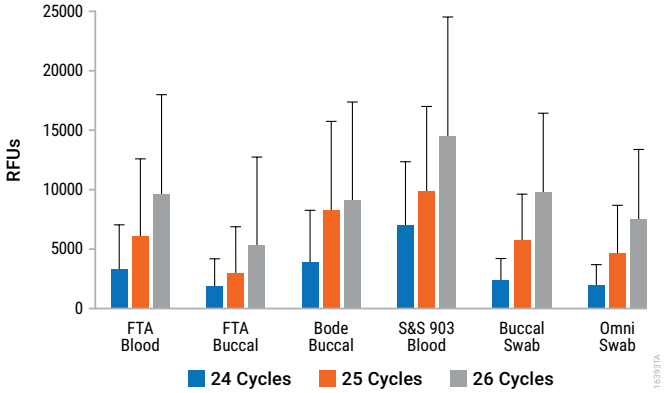

Figure 4. Average peak heights. Amplification of various substrates was tested using differing cycle numbers. Amplification reactions (12.5µl) were performed using the Applied Biosystems® ProFlex® PCR System for 24, 25 and 26 cycles in replicates of four. After amplification, 1µl of each reaction was analyzed on an Applied Biosystems® 3500xL Genetic Analyzer using the recommended protocol at a 175RFU threshold and a 20% global stutter filter. The average peak heights are shown. Error bars represent +1SD of replicate results.

Average peak heights were also determined in relative fluorescence units (RFU) at each cycle number for each substrate. Blood on FTA® cards averaged 3,363, 6,156 and 9,636RFU at 24, 25 and 26 cycles, respectively. Buccal samples on Indicating FTA® cards averaged 1,938, 2,993 and 5,320RFU at 24, 25 and 26 cycles, respectively. Bode Buccal DNA Collectors™ averaged 3,905, 8,318 and 9,182RFU at 24, 25 and 26 cycles, respectively. Blood on S&S 903 paper averaged 7,031, 9,936 and 14,508RFU at 24, 25 and 26 cycles, respectively. Cotton swabs averaged 2,396, 5,777 and 9,765RFU at 24, 25 and 26 cycles, respectively. OmniSwab averaged 2,050, 4,620 and 7,510RFU at 24, 25 and 26 cycles, respectively.

Sample-to-sample and punch-to-punch variability were the main sources of variability seen in this experiment. Samples amplified at 26 cycles often exhibited much more pull-up and saturation compared to the 24- and 25-cycle data. Overall, 25-cycle amplifications worked well across all sample types.

Additional testing was conducted to assess whether or not pre-incubating punches from buccal samples on Indicating FTA® cards with the PunchSolution™ Kit (Cat.# DC9271) helped to improve results from difficult samples. The seven donor samples from above were incubated in PunchSolution™ Reagent following the *PunchSolution™ Kit Technical Manual* #TMD038.

The results showed increased percent profiles obtained in every sample tested that did not yield 100% profiles (Figure 5). No incubation with PunchSolution™ Reagent yielded 89% (1190/1331), 96% (1374/1425) and 95% (1271/1332) of expected alleles at 24, 25 and 26 cycles, respectively. Pre-incubation with PunchSolution™ Reagent yielded 96% (1417/1472), 100% (1472/1472) and 100% (1472/1472) of expected alleles at 24, 25 and 26 cycles, respectively.

One donor had 3 replicates at 24 cycles, 1 replicate at 25 cycles, and 2 replicates at 26 cycles that did not yield any alleles above calling threshold. The samples from the same donor, when pre-incubated with PunchSolution™ Reagent, yielded 100% of expected alleles being called in 8 of 9 replicates. One replicate yielded 87% (41/47) of expected alleles being called.

Average peak heights were also determined in relative RFU at each cycle number for each condition tested. No incubation with PunchSolution™ Reagent averaged 1,938, 2,993 and 5,320RFU at 24, 25 and 26 cycles, respectively. Pre-incubation with PunchSolution™ Reagent averaged 3,299, 5,083 and 9,392RFU at 24, 25 and 26 cycles, respectively. It appears that pre-incubation with PunchSolution™ Reagent may help in the recovery of buccal samples on Indicating FTA® cards. It is important to note that sample-to-sample and punch-to-punch variability could also be a source of variability in results.

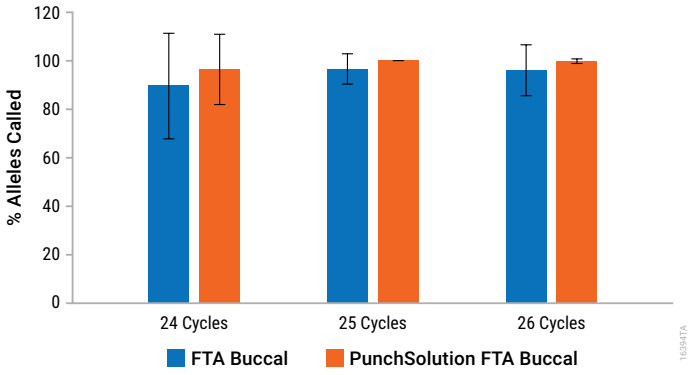

Figure 5. Percent profiles observed. Amplification of buccal on Indicating FTA® card samples with no pre-incubation vs. being pre-incubated with PunchSolution™ Reagent using differing cycle numbers. Amplification reactions (12.5µl) were performed using the Applied Biosystems® ProFlex® PCR System for 24, 25 and 26 cycles in replicates of four. After amplification, 1µl of each reaction was analyzed on an Applied Biosystems® 3500xL Genetic Analyzer using the recommended protocol at a 175RFU threshold and a 20% global stutter filter. The percentage of the alleles detected at a given substrate and cycle number is shown. Error bars represent ±1SD of replicate results.

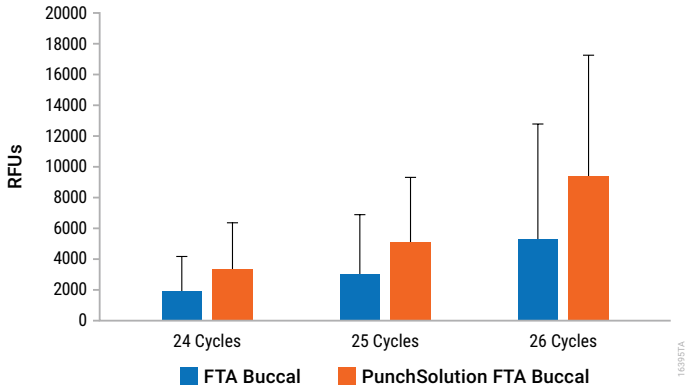

Figure 6. Average peak heights. Amplification of buccal samples on Indicating FTA® cards with no pre-incubation vs. being pre-incubated with PunchSolution™ Reagent using differing cycle numbers. Amplification reactions (12.5µl) were performed using the Applied Biosystems® ProFlex® PCR System for 24, 25 and 26 cycles in replicates of four. After amplification, 1µl of each reaction was analyzed on an Applied Biosystems® 3500xL Genetic Analyzer using the recommended protocol at a 175RFU threshold and a 20% global stutter filter.The average peak heights are shown. Error bars represent +1SD of replicate results.

Conclusion

Testing shows the VersaPlex™ 27PY System with the ProFlex® PCR System enables direct amplification with 12.5µl reactions from numerous sample types, including Whatman FTA® card punches, S&S 903 Paper, Bode Buccal DNA Collector™ devices, OmniSwabs and cotton swabs. Further testing shows that results can be improved for some buccal samples on Indicating FTA® cards by implementing a pre-incubation with PunchSolution™ step. Optimization of cycle number is still recommended for best results. Utilization of these direct amplification protocols can allow laboratories to streamline their workflow while stretching their budget.

Reference

1. *VersaPlex™ 27PY System for Use on the Applied Biosystems® Genetic Analyzers Technical Manual* #TMD055, Promega Corporation.

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