Chapter 12

DNA Profiling by Multiplex PCR Amplification and Genotype Determination by Reverse Dot-Blot Hybridization to Sequence-Specific Oligonucleotide Probes

Amplitype® PM & DQA1 Amplification and Analysis

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Introduction

The use of the polymerase chain reaction (PCR; Saiki *et al.* 1988) for human DNA profiling is one of the most significant developments in forensics (von Beroldingen *et al.* 1989; Reynolds *et al.* 1991). This exercise provides students the opportunity to gain first-hand experience with procedures that are currently used to extract DNA from their own cells, quantify the DNA in the extract, perform a multiplex PCR amplification of several loci used in forensic analysis (Budowle *et al.* 1995), and determine their own genotype at those loci. In addition, methods for analyzing results relative to existing population databases are discussed (Perkin Elmer, AmpliType® User Guide, Version 2, 1990). The exercise is normally presented in the context of a laboratory course in Forensic DNA Analysis that presents students with a variety of techniques that have been and/or continue to be employed in forensic laboratories. It is divided into several independent but related activities. The initial activities of DNA extraction and quantification can be used in a variety of settings with a wide range of organisms as the source of the DNA. While presented in the context of a forensic exercise using the AmpliType® Pm & DQA1 system, the DNA, once extracted, can be used for a broad range of activities. Some alternatives are discussed in the Notes for the Instructor section.

This exercise works best in laboratory situations where the number of students is limited to 16 or less. To control costs, the students usually work in pairs, and certain aspects of the exercise (*e.g.*, the quantification of the extracted DNA by a slot-blot hybridization procedure) are performed by the

class as a whole. The exercise, as presented here, takes approximately 5 weeks to complete, including time for initial discussion and for analysis of the results. Collection of buccal cells and isolation of the DNA can be done in one 3-hour lab period. Quantification of the DNA using the QuantiBlot® system requires at least a complete 3-hour lab period (maybe a little more). The PCR amplification reaction is set up during the third lab period but does not require more than about an hour. The rest of the time can be used for discussion, or for another, overlapping exercise. Hybridization and colorimetric detection occurs in the fourth lab period and generally requires up to 4 hours to complete. Students then complete the worksheet at home and this is discussed in the fifth and final lab period devoted to this exercise. In order to accommodate the extra time needed for some phases of the exercise, we usually schedule the lecture (75 minutes, twice a week) immediately before the lab (3 hours, once a week) and use the entire period (4 hours 15 minutes) for lab activities when extra time is needed. Alternatively, this semester we have scheduled the entire class (lecture and laboratory) on one day (Friday) from 8:00 am until 2:45 pm, and integrate lecture and lab activities as necessary. We have found both of these scheduling alternatives to be useful in courses where the lab exercises do not fit into neat, definable time blocks, or for exercises where there is a lot of long incubation/wait times. These times can now be used for class discussion.

The human genome contains a variety of length-specific and sequence-specific polymorphic loci that can be used to distinguish individuals (Weir, ed. 1995). Allelic frequencies for many of these polymorphic loci have been determined and can be used to calculate the genotypic frequency (a.k.a. random match probability) for any individual whose profile has been determined. For extensive review of issues associated with the use of polymorphic loci in forensic DNA testing see National Research Council Reports I (1992) and II (1996), and *An Introduction to Forensic DNA Analysis* by Rudin and Inman (2002).

Materials

The materials needed for this exercise and their suppliers are listed below. All buffers and reagents are supplied with the various kits or are specified within the product literature for the kits, and are prepared according to the specifications within that product literature. The list is categorized by procedure.

Collection of Buccal Cells:

- FTA Classic Cards
- Foam Applicators
  - Whatman Bioscience® (can be ordered from Fisher Scientific)

Extraction of DNA

- 2 mm Harris Micro Punch with Pad
  - Whatman Bioscience® (can be ordered from Fisher Scientific)
- Buccal Amp™ Quick Extract DNA Extraction Kit (includes nylon brushes)
  - Epicentre Technologies
- DNA IQ™ System for DNA Extraction
- IQ™ Spin Baskets
- Magnetic Separation Stands (Promega™)

Quantification of DNA:

- QuantiBlot™ Human DNA Quantitation System
- Chromagen:TMB
- Hybridization Trays
- Hybridzation Tray Retainer (Applied Biosystems, Inc.)
- Slot Blot Unit
- Zeta Probe Membranes (BioRad; other suppliers have similar items)

PCR Amplification of the AmpliType Polymarker loci and the DQA1 locus:

- AmpliType® PM & DQA1 System
- AmpliType Typing Trays (Applied Biosystems, Inc.)

Other Specific Materials Needed:

- Bellco Shaking Water Bath (or equivalent)
- Polaroid Photo Documentation System (or equivalent)
- Thermal Cycler (for PCR amplification)
- Dry Block Incubators
- Microcentrifuge
- Micropipettors, Pipet Tips, Microcentrifuge Tubes, PCR Reaction Tubes, and various chemicals (Fisher Scientific or other general suppliers)

Notes for the Instructor

1. All solutions should be prepared according to the suppliers' product literature.

2. The Buccal Amp™ procedure (Epicentre Technologies) is included for reference. However, it frequently results in DNA preparations that do not adequately amplify in the subsequent PCR reactions. Therefore, it is seldom used. Instead, DNA extracted by the DNA IQ™ system from Promega™ is normally used for all subsequent procedures.

3. The hybridization tray retainer from Applied Biosystems, Inc. is designed to be used with the Bellco Shaking Water Bath. However, any shaking incubator that can be set for 50^{o}C can be used. Depending on the particular shaker system used, the retainer may not be necessary.

4. We have made a retainer out of Plexiglas that is designed to hold two of the AmpliType® Typing Trays in the Bellco Shaking Water Bath. However, depending on the particular shaker system used, such a retainer may not be necessary.

5. DNA extracted by the Promega™ DNA IQ™ system is suitable for any downstream PCR amplification process that uses single-stranded DNA as its starting material. Therefore, we have used DNA extracted this way for the following PCR-based analyses:
    1. AmpliType® PM and DQA1 Typing (this exercise)
    2. Short Tandem Repeat (STR) Typing using the Green I, Profiler Plus, and COfiler kits from Applied Biosystems, Inc.
    3. PCR Amplification of the *Alu* insert in the tissue plasminogen activator (TPA) gene on chromosome 8 (kit available from BioRad) – this is particularly useful for looking at allele distribution as a means of determining if a particular population is in Hardy-Weinberg Equilibrium

6. The quantification of DNA extracted by the Promega™ DNA IQ™ system prior to PCR amplification is not absolutely necessary. Because the resin is saturable, the use of 8-12 x 2mm diameter punches should yield approximately 100 ng DNA. You can use this as an estimate and will generally get useable results.

Student Outline

Overview of the Exercise

The AmpliType® PM & DQA1 system from Applied Biosystems, Inc. (ABI) is a modification of one of the first systems to become available for forensic analysis of PCR-amplified DNA (Budowle *et al.* 1995). The loci involved in this system are all sequence polymorphisms that are detected/delineated by hybridization to immobilized allele-specific (*i.e.,* sequence-specific) oligonucleotide (ASO) probes (Saiki *et al.* 1989). The discriminatory power of this system is more limited (approximately 1:2000) than that obtained with either RFLP or STR analysis because, for each of the six loci, only a limited number of alleles (and therefore, a limited number of genotypes) exist in the population. Nevertheless, the system is useful when evidence samples yield limited amounts of DNA and/or DNA that is too degraded for RFLP analysis, and other factors interfere with STR analysis. The DNA to be analyzed is isolated from buccal cells that are obtained by swabbing the inside of the cheeks and stored on Whatman® FTA paper. Two methods of extraction are presented. The chelex-based method (Epicentre Technologies Buccal Amp™ system) is quick. However, DNA extracted by this method sometimes fails to amplify in subsequent PCR reactions. A relatively new procedure available from Promega (DNA IQ™ System) involves the use of a magnetic resin for separation and purification of DNA. It is a little more time-consuming, but yields are more predictable and consistent, and the samples can be used for subsequent PCR amplification without quantification, if necessary.

The AmpliType® PM & DQA1 system tests for polymorphisms at 6 loci:

- Low Density Lipoprotein Receptor (LDLR)
- Glycophorin A (GLYPA)
- Hemoglobin G Gamma Globulin (HBGG)
- D7S8
- Group-Specific Component (GC)
- HLA-DQA1

The basic steps in the procedure are:

1. Extraction of DNA from buccal (cheek) cells
2. Quantification of extracted DNA (Walsh *et al.* 1992)
3. Simultaneous PCR amplification of the six loci using primers that have been conjugated to biotin
4. Hybridization (allele-specific) of the PCR products using a reverse dot-blot method in which the ASO probes have been impregnated onto membrane filter strips in specific locations. Two strips are used: one for the DQA1 locus and one for the remaining 5 loci of the Polymarker (PM).

5. Binding of a horseradish peroxidase/streptavidin conjugate to the hybridized biotinylated PCR product.
6. Colorimetric detection of the hybridized PCR product-biotin/HRP-streptavidin complex using a chromagenic substrate for the HRP.
7. Interpretation of results
8. Completion of a worksheet

Procedures

I. Collection of Cheek Cells – Whatman® BioScience FTA Cards

1. Thoroughly rinse out your mouth twice with water.
2. Moisten the inside of your cheek on the left side of your mouth with saliva.
3. Take the Foam Applicator and rub one side of it on the inside of your left cheek. Use about ten circular strokes (soak up as much saliva as possible).
4. Press the applicator firmly onto the upper left circle of the FTA card. Rock back and forth to transfer the material from the applicator to the card.
5. Using the other side of the foam applicator repeat the process for the right cheek and press the applicator onto the upper right circle of the FTA card.
6. Repeat steps 1-5 using the lower circles of the FTA card.
7. Air-dry in the open position for at least 30 minutes at room temperature.
8. Fold the card closed and store at room temperature, protected from light, until needed.

II. Extraction of DNA – Epicentre Technologies Buccal Amp™ System

1. Punch 8 x 2 mm diameter samples from the FTA card using the Harris Micro Punch.
2. Place the punch samples into the tube containing the QuickExtract DNA Extraction Solution.
3. Screw the cap on the tube tightly and vortex mix for 10 seconds.
4. Incubate the tube at 65ºC for 30 minutes.
5. Vortex mix for 15 seconds.
6. Incubate at 98^{o}C for 8 minutes.
7. Vortex mix for 15 seconds.
8. Incubate for an additional 8 minutes at 98^{o}C.
9. Vortex mix for 15 seconds.
10. Transfer as much of the supernatant as possible to a clean microcentrifuge tube.
11. Centrifuge @ 14,000 rpm for 2 minutes to pellet any residual chelex resin and any of the filter punches that may have been transferred.
12. Transfer the supernatant (contains the DNA) to a clean microcentrifuge tube.
13. Store the DNA at –20^{o}C until needed.

III. Extraction of DNA – Promega™ DNA IQ™ System

1. Punch 8 x 2 mm diameter samples from the FTA card using the Harris Micro Punch.
2. Place the punch samples into a 1.5-ml microcentrifuge tube with 150 µl of Lysis Buffer.
3. Close the lid and incubate @ 95^{o}C for 30 min in a dry-block incubator.

4. Transfer the Lysis Buffer and punch samples to a Spin Basket.
5. Centrifuge at room temp for 2 min @ 14,000 rpm.
6. Remove the Spin Basket.
7. Vortex the stock Resin bottle to resuspend the Resin.
8. Add 7 µl of Resin to the DNA solution.
9. Briefly vortex.
10. Incubate at room temp for 5 min.
11. Place the tube in the magnetic stand; separation will occur immediately.
12. Carefully remove all of the solution without disturbing the Resin on the side of the tube.
13. Remove the tube from the stand; add 100 µl of Lysis Buffer; vortex briefly.
14. Return the tube to the magnetic stand and discard the Lysis Buffer after separation occurs.
15. Remove the tube from the stand; add 100 µl of Wash Buffer; vortex briefly.
16. Return the tube to the magnetic stand and discard the Wash Buffer after separation occurs.
17. Repeat steps 15 & 16 twice more for a total of three washes; make sure that all of the Wash Buffer has been removed after the third wash.
18. With the lid open, air-dry the Resin in the magnetic stand for 5 min.
19. Remove the tube from the stand; add 50 µl of Elution Buffer; vortex briefly.
20. Close the lid and incubate @ 65^{o}C for 5 min in a dry-block incubator.
21. Remove the tube from the dry-block incubator; vortex briefly; immediately place on the magnetic stand.
22. After separation, transfer the DNA solution to a clean microcentrifuge tube and store until needed for DNA quantification and/or AmpliType PM & DQA1 PCR amplification.

IV. Quantification of Extracted DNA – Applied Biosystems, Inc® QuantiBlot™ System

A. Preparation of Samples and Membranes

1. Each membrane can easily accommodate a set of standards, a set of three samples of known concentration (calibrators), and a set of dilutions from 8 students as indicated in the table below (actually more can be accommodated if all slots are used but the pictured arrangement results in each set of student samples being clearly delineated from one another). Therefore, you should prepare as many membranes as needed to accommodate all of the students in the class.
2. Prepare (as indicated below) a set of 2-fold serial dilutions of the Human DNA Standard (2 ng/µl) provided with the QuantiBlot™ Kit
    - Set up 7 microcentrifuge tubes labeled A through G
    - Put 60 µl TE Buffer in tubes B through G
    - Put 120 ml DNA Standard in Tube A
    - Remove 60 µl from Tube A and put into Tube B; Mix
    - Remove 60 µl from Tube B and put into Tube C; Mix
    - Remove 60 µl from Tube C and put into Tube D; Mix
    - Remove 60 µl from Tube D and put into Tube E; Mix
    - Remove 60 µl from Tube E and put into Tube F; Mix
    - Remove 60 µl from Tube F and put into Tube G; Mix

3. There is now a series of standards: A = 2 ng/µl; B = 1 ng/µl; C = 0.5 ng/µl; D = 0.25ng/µl; E = 0.125 ng/µl; F = 0.0625 ng/µl; G = 0.03125 ng/µl
4. Prepare (as indicated below) two serial 5-fold dilutions of your extracted DNA
    - Set up 3 microcentrifuge tubes labeled *Undil*; 5X; and 25X
    - Put 20 µl TE Buffer into the tubes labeled 5Xand 25X
    - Put 10 µl of your extracted DNA into the tube labeled Undil
    - Remove 5 µl from that tube and add it to the 5X tube; Mix
    - Remove 5 µl from the 5X tube and add it to the 25X tube; Mix
    - Pipet 5 µl of the 3 samples into 3 clean microcentrifuge tubes (one sample per tube)
    - To each of the 3 tubes add 150 µl Spotting Solution (SS)
    - **NOTE:** The instructor will do (or assign to specific students to do) the standards, two calibration samples, and a water sample (negative control) for each of the membranes (5 µl of each standard/sample + 150 µl Spotting Solution)
12. Set up a Hybridization Tray with 50 ml Pre-Wetting Solution (PWS).
13. Obtain as many Bio-Rad Zeta Probe Membranes as needed.
14. Cut a small diagonal notch in the lower left corner of membrane #1.
15. If a second membrane is needed, cut a small square notch in the lower right corner of it.
16. Repeat for as many membranes as necessary using a different notch for each membrane so that each can be uniquely identified.
17. Incubate at room temp. in PWS for 5 minutes.
18. For each membrane, set up the Slot Blot unit as follows (if only one unit is available then membranes will have to be processed sequentially):
    - Wet 3 sheets of blotting paper in PWS
    - Place sheets on bottom unit
    - Put pre-wet membrane on top of blotting paper
    - Place top unit over membrane and tighten using a diagonal fastener pattern
12. Attach the unit to a vacuum pump but do not pull vacuum through the membrane yet.
13. Pipet each sample (155 µl) into the appropriate slot as indicated in Table 1 below:

Table 1: Placement of Samples in a Slot Blot Apparatus

	1	2	3	4	5	6
A	Dil A 10 ng	DNA Cal 1 3.5 ng	Stud #1 Undil	Stud #3 Undil	Stud #5 Undil	Stud #7 Undil
B	Dil B 5 ng	-----	Stud #1 5X	Stud #3 5X	Stud #5 5X	Stud #7 5X
C	Dil C 2.5 ng	DNA Cal 2 0.5 ng	Stud #1 25X	Stud #3 25X	Stud #5 25X	Stud #7 25X
D	Dil D 1.25 ng	-----	----	----	----	----
E	Dil E 0.625 ng	Neg Control	Stud #2 Undil	Stud #4 Undil	Stud #6 Undil	Stud #8 Undil
F	Dil F 0.3125 ng	-----	Stud #2 5X	Stud #4 5X	Stud #6 5X	Stud #8 5X
G	Dil G 0.15625 ng	-----	Stud #2 25X	Stud #4 25X	Stud #6 25X	Stud #8 25X
H	-----	-----	----	----	----	----

14. Slowly turn on the sample vacuum and leave it on until all samples have been drawn through the membrane.
15. Remove from vacuum; disassemble; remove membrane.

B. DNA Hybridization (All steps are to be done for each membrane)

1. Place the membrane into a Hybridization Tray with 100 ml pre-warmed Hybridization Solution (Hyb Soln).
2. Add 5 ml 30% Hydrogen Peroxide (this step bleaches the dye that is on the membrane from the Spotting Solution so that it does not interfere with post-hybridization color development).
3. Place the lid on the Hybridization Tray.
4. Incubate with shaking (50-60 rpm) @ 50^{o}C for 15 min.
5. Discard the solution.
6. Add 30 ml pre-warmed Hyb Soln.
7. Tilt the tray to one side and add 20 µl of QuantiBlot D17Z1 Probe.
8. Place the lid on the tray.
9. Incubate with shaking (50-60 rpm) @ 50^{o}C for 20 min.
10. Discard the solution.
11. Rinse with pre-warmed Hyb Soln for 1 min.
12. Discard the solution.
13. Add 30 ml pre-warmed Wash Solution (WS) to the tray.
14. Tilt the tray to one side and add 180 µl Enzyme-Conjugate-HRP-SA.
15. Place the lid on the tray.
16. Incubate with shaking (50-60 rpm) @ 50^{o}C for 10 min.

17. Discard the solution.
18. Rinse 2 x 1 min (100 ml WS) with shaking (100-125 rpm) @ room temp.
19. Rinse 1 x 15 min (100 ml WS) with shaking (100-125 rpm) @ room temp.
20. Discard the solution.
21. Rinse briefly in 100 ml Citrate Buffer (CB).

C. Detection

1. Prepare the Color Development Solution (CDS) by adding the following, in order, to a glass flask **(NOTE:** Mix but do not Vortex).
   - 30 ml Citrate Buffer
   - 1.5 ml Chromagen:TMB
   - 30 µl 3% Hydrogen Peroxide
2. Discard the CB from the Hybridization Tray.
3. Add the CDS to the Hybridization Tray.
4. Incubate with shaking (50-60 rpm) @ room temp for 30 min.
5. Discard the solution.
6. Wash 3 x 5 min (100 ml dH_2O) with shaking (50-60 rpm) @ room temp.
7. Wrap the membrane in Saran Wrap.
8. Place on white background and photograph with white light epi-illumination.
9. Look at the results: Do the relative intensities of the DNA standards & DNA calibrators make sense?
10. If they do then make a table in your notebook as indicated below.
11. Fill in the table by estimating the amount of DNA in each dilution that is within the range established by the standards.
12. Then calculate the concentration (ng/µl) and fill in the appropriate boxes in the table.
13. You will need these data in subsequent parts of this exercise.

Table 2: Calculation of DNA Concentration in Student Samples

Sample	Volume Tested	Amount of DNA in Diluted Sample (estimate)	Concentration in Undiluted Sample (ng/µl)
DNA (undil)	5 µl		
DNA (5X dilution)	5 µl		
DNA (25X dilution)	5 µl		

V. Adjustment of DNA Concentration

1. For best results the extracted cheek cell genomic DNA should be at a concentration of 0.1-0.5 ng/µl so that a 20 µl aliquot added into the PCR reaction tube will contain 2-10 ng of DNA.

2. Dilute your extracted cheek cell genomic DNA with dH_2O to an approximate concentration of 0.5 ng/µl (use the following as an example):
    - Suppose you obtained the following results from the QuantiBlot™ determination
        - Dilution that was within range of standards ➔ 5X
        - Volume tested = 5 µl
        - Approximate amount of DNA = 4 ng
        - Conc (ng/ul) = (amount of DNA in sample/volume tested) X dilution factor ➔ (4 ng/5 µl) X 5 = 4.0 ng/µl
        - 2.5 µl = 10 ng
    - Mix 2.5 µl IQ™-extracted cheek cell DNA with 17.5 µl dH_2O = 10 ng/20 µl = 0.5 ng/µl

NOTES:
1. If the concentration of your undiluted DNA is less than (or equal to) 0.5 ng/µl, then do not dilute it. Instead, use 20 µl of the undiluted DNA in the PCR reaction tube in order to deliver as much DNA as possible (up to 10 ng) to the amplification reaction; record the amount of DNA that is in the 20 µl volume.
2. Examples
    - Conc = 0.4 ng/µl; 20 µl = 8 ng
    - Conc = 0.1 ng/µl; 20 µl = 2 ng
    - Conc = 0.02 ng/µl; 20 µl = 0.4 ng

VI. PCR Amplification of the AmpliType® PM & DQA1 Loci

A. Each student will set up one reaction in a PCR reaction tube to amplify the six loci of the AmpliType® PM & DQA1 system from the extracted cheek cell DNA that you have prepared. In addition, the Instructor will set up a positive control and a negative control as follows:
- Positive Control (K562 DNA): 20 µl (ng) of the K562 control DNA that is supplied with the AmpliType® PM & DQA1 kit
- Negative Control: 20 µl dH_2O instead of a DNA sample

B. Reaction Set-Up
1. In a 0.2 ml (or 0.5 ml) PCR reaction tube, add the following:
    - 40 µl AmpliType® PM & DQA1 PCR Reaction Mix (AmpliType® Taq DNA Polymerase; $MgCl_2$; dNTPs)
    - 40 µl AmpliType® PM & DQA1 biotinylated Primer Set (12 primers; 2 for each of the 6 loci)
    - 20 µl Extracted cheek cell DNA (as prepared in Step IV)
2. Mix briefly and set in the Thermal Cycler
3. Use the following program for PCR amplification:
    - Pre-Heat Block to 95^oC
    - Initial Denaturation - 95^oC for 4 minutes
    - Cycles X 32

- Denaturation - 95°C for 60 sec
- Annealing - 63°C for 30 sec
- Extension - 72°C for 30 sec

- Run-Off Completion Hold - 72°C for 10 min
- Final Hold - 4°C indefinitely

4. Store @ 2-8°C until ready to proceed.

VII. Hybridization of PCR Amplification Products to AmpliType® PM & DQA1 Probe Strips

- The AmpliType® hybridization trays hold eight strips each. Therefore, one tray will be needed for the PM (PolyMarker) hybridization for every eight students
- Strips for the DQA1 typing must be in separate wells from those used for the PM (PolyMarker) loci (it is best to use separate trays). Therefore, another tray (or set of trays) will be needed for the DQA1 hybridization.
- The shaking water bath can only hold two AmpliType® trays at a time. Therefore, the hybridization protocol will have to be repeated for every two trays to be processed.
- The procedure is outlined below.

Procedure for PM Typing

1. Retrieve the PCR reaction tube containing your AmpliType® PM + DQA1 reaction, and transfer the reaction mix to a clean 1.5-ml microcentrifuge tube.
2. Add 5 µl of 0.2 M EDTA, pH 8.0
3. Mix and place the tube in a dry-bath incubator @ 95°C (**NOTE:** Keep the tubes in the dry-block incubator except when pipetting out a sample).
4. Obtain a PM Probe Strip and place it in one well of an AmpliType® hybridization tray; Record which well contains your strip.
5. Tilt the tray toward the left end of the strip.
6. Add 3.0 ml of pre-warmed Hyb Soln to the lower end of each well; *DO NOT WET THE REMAINDER OF THE STRIP.*
7. *For each student, one at a time* remove the tube containing your PCR reaction from the dry-block incubator; open it; pipet 20 µl into the Hyb Soln in the tilted tray.
8. Cap the tube and return it to the dry-block incubator @ 95°C.
9. When all of the samples have been loaded into a tray put the lid on the tray and rock it to mix the samples.
10. Place the tray into the 55°C rotating water bath and incubate with shaking (50-60 rpm) for 15 minutes (± 2 minutes).

11. After 10 minutes, prepare the Enzyme Conjugate as follows:
    - To a 50-ml conical centrifuge tube, add 49.5 ml pre-warmed Hyb Soln
    - Add 405 µl Enzyme Conjugate HRP-SA
    - Mix and let stand at room temp.
12. After hybridization remove the trays from the shaking bath and remove the lids.
13. Pour off the Hyb Soln from the trays and wipe the condensation off the lids with a Kim-Wipe; DO NOT USE PAPER TOWELS.
14. Add 5.0 ml pre-warmed AmpliType Wash Solution (AWS; 2.5 X SSPE, 0.1% SDS) to each well of the tray.
15. Rock gently for 15 seconds and pour the solution off.
16. Add 3.0 ml of the Enzyme Conjugate prepared in step 12 to each well of the tray.
17. Incubate with shaking (50-60 rpm) @ 55^{o}C for 5 minutes ($\pm$ 1 minute).
18. Pour off the Enzyme Conjugate solution from the trays and wipe the condensation off the lids with a Kim-Wipe; DO NOT USE PAPER TOWELS.
19. Pipet 5.0 ml pre-warmed AWS into each well of the tray.
20. Rock gently for 15 seconds and pour the solution off.
21. Again pipet 5.0 ml pre-warmed AWS into each well of the tray.
22. Incubate with shaking (50-60 rpm) @ 55^{o}C for 12 minutes ($\pm$ 1 minute); *THE TEMPERATURE & TIMING OF THIS STEP ARE CRITICAL*.
23. The remaining steps are performed at room temperature; therefore after this step (#23) you may begin the DQA1 hybridization while completing the Color Development for the PM strips.
24. Pour off the AWS.
25. Add 5.0 ml Citrate Buffer (CB) to each well of the tray.
26. Incubate with shaking (50-60 rpm) at room temp for 5 minutes.
27. During this incubation prepare the Color Development Solution (CDS) as follows
    - To a 125 ml Ehrlenmeyer Fask add 75.0 ml CB
    - Add 75 µl 3% Hydrogen Peroxide
    - Add 3.75 ml Chromagen:TMB solution
    - Mix well; DO NOT VORTEX
28. Pour off the CB.
29. Add 5.0 ml CDS to each well of the tray.
30. Place the lid on the tray; cover with aluminum foil to protect from strong light.
31. Incubate with shaking (50-60 rpm) at room temp for 20-30 minutes (or until the "S" spot is visible).
32. Pour off the CDS.
33. Wash 3 x 5 min with dH_2O with shaking (50-60 rpm) at room temp.
34. Record your results.

Procedure for DQA1 Typing

1. If multiple trays are needed for each probe (PM & DQA1), then after Step #23 in the PM Hybridization procedure, you may begin the DQA1 hybridization procedure.
2. The procedure is the same (Steps 4-34) except:
    - At Step #4, obtain a DQA1 Typing strip
    - At Step 31, the control spot is labeled "C" instead of "S"

VIII. Interpretation of Results

A. Basis of the AmpliType PM® & DQA1 Typing System

- Six (6) loci were amplified simultaneously using 12 primers (6 pairs of primers) with one pair specific for each of the 6 loci.
- At each locus, two or more alleles are possible in the population.
- Any individual can be either homozygous for any one of the possible alleles or heterozygous for any two of the possible alleles .
    - Low Density Lipoprotein Receptor (**LDLR**) - **Alleles = A or B**
    - Glycophorin A (**GLYPA**) - **Alleles = A or B**
    - Hemoglobin G Gamma Globulin (**HBGG**) - **Alleles = A, B or C**
    - **D7S8** - **Alleles = A or B**
    - Group-Specific Component (**GC**) - **Alleles = A, B, or C**
    - **HLA-DQA1** - **Alleles = 1.1, 1.2, 1.3, 2, 3, 4.1, 4.2, or 4.3** (**NOTE: 4.2 and 4.3 cannot be distinguished in this system**)

- One primer in each pair is modified by having the molecule **"biotin"** covalently attached.
- The detection is by means of a **reverse dot blot hybridization** in which the biotinylated amplification products are hybridized to **allele-specific oligonucleotide probes (ASO probes)** that have been attached to a membrane probe strip.
- Hybridized amplimers are reacted with the enzyme **"horseradish peroxidase" (HRP)** which has been covalently coupled to **streptavidin (SA).**
- The **HRP-SA conjugate** can only **bind** to hybridized amplimers through a **streptavidin-biotin interaction.**
- Therefore, if hybridization does occur between the PCR amplimers and one of the ASO probes, then the HRP-SA will bind to that location and a blue color will develop when the substrate (**3,3',5,5' - tetramethylbenzidine; TMB**) for the HRP is reacted to form an insoluble *blue product that precipitates onto the test strip in the location where it was formed.*
- Alternatively, **if no hybridization takes place for a particular ASO probe** then the **HRP-SA** cannot bind in that specific location of the test strip and no color will develop there.
- A **control spot** (**S on the PM strip; C on the DQA1 strip**) is included on each strip; it is used to determine the least amount of color development needed for a positive reaction

- Any spot lighter than the control spot is considered negative
- Any spot darker than the control spot is considered positive

- Please remember that loci chosen for forensic analysis are **not chosen for any functional significance**.
- In fact, with a few exceptions loci are chosen primarily because they are non-functional.
  - Non-functional loci are free to diverge evolutionarily with no selective pressure for (or against) particular allelic variants.
  - Therefore, non-functional loci are more likely to be polymorphic because of the lack of selective pressure to maintain a particular allelic form and thereby maintain a specific phenotype.
- Even the functional loci such as five of the six loci used in the AmpliType® PM & DQA1 system are chosen because the alleles found in the population have no known selective advantage or disadvantage.

B. Interpretation of Results:

The results from a positive control reaction using DNA supplied with the AmpliTyp®e PM & DQA1 kit are shown below. They illustrate the way in which the results are interpreted for the five polymarker (PM) loci (Figure 1) and the DQA1 locus (Figure 2).

Figure 1. Polymarker (PM) Results

- LDLR ==> B,B homozygous
- GYPA ==> A,B heterozygous
- HBGG ==> A,A homozygous
- D7S8 ==>A,B heterozygous
- GC ==> B,B homozygous

Figure 2. DQA1 Results (interpreted from left to right)

- Spots "1" and "4" show that the individual is heterozygous for one of the #1 alleles (1.1, 1.2 or 1.3) and one of the 4 alleles (4.1, 4.2 or 4.3)
- Spot "1.1" shows that it is specifically the 1.1 allele
- Therefore, Spot "1.2, 1.3, 4" must be positive because of the 4 allele
- Spot "All but 1.3" is uninformative in this case
- Spot "4.1" indicates that it is the 4.1 allele

Therefore the individual is a 1.1, 4.1 heterozygous individual

VIII. Complete the following worksheet:

Forensic DNA Analysis
AmpliType® PM & DQA1 Worksheet

Name: _____ Score: _____

1. Attach a photograph of your AmpliType® PM & DQA1 results. If a photograph is not available then draw a representation of the results.

Summarize the **results** of this exercise by answering the following questions:

2. Indicate your genotype for each locus tested and indicate if you are homozygous or heterozygous.
   - LDLR
   - GYPA
   - HBGG
   - D7S8
   - GC
   - DQA1 (Explain your reasoning)

3. Calculate the "**Random Match Probability**" (i.e. **Genotype Frequency**) for your genotype profile using the appropriate allele frequencies from the table on the next page. (**Show All Work**)

   For each locus, the genotype frequencies are calculated according to the following formulas:

   If Homozygous ➔ genotype freq = $p^2 + (p)(1-p)(\theta)$; $\theta = 0.01$
   (adjusts for deviation from Hardy-Weinberg Equilibrium)

   If Heterozygous ➔ genotype freq = 2pq

4. If, in analyzing evidence from a crime scene, using the AmpliType® PM & DQA1 system that we used, two or more suspects showed the same allelic pattern (*i.e.* genotype profile) as the perpetrator, what could you do to resolve the question?

5. List two reasons why **STR** analysis has largely replaced the **AmpliType® PM & DQA1** analysis in forensic work.

Allele Frequency Table* for the PM & DQA1 Loci

Genetic Marker	Allele	Frequency (U.S. Caucasians)	Frequency (African Americans)	Frequency (U.S. Hispanic)	Frequency (Japanese)
DQA1	1.1	0.158	0.125	0.105	0.084
	1.2	0.190	0.329	0.130	0.118
	1.3	0.073	0.058	0.053	0.236
	2	0.145	0.130	0.115	0.006
	3	0.192	0.090	0.218	0.444
	4.1	0.214	0.185	0.269	0.073
	4.2/4.3	0.028	0.083	0.110	0.039
LDLR	A	0.448	0.235	0.485	0.202
	B	0.552	0.765	0.515	0.798
GYPA	A	0.530	0.527	0.615	0.517
	B	0.470	0.473	0.385	0.483
HBGG	A	0.537	0.439	0.375	0.331
	B	0.450	0.228	0.580	0.669
	C	0.013	0.333	0.045	0.000
D7S8	A	0.610	0.655	0.622	0.612
	B	0.390	0.345	0.378	.0388
GC	A	0.275	0.090	0.203	0.287
	B	0.178	0.720	0.335	0.471
	C	0.547	0.190	0.462	0.242

* Reprinted from the product literature supplied by Applied Biosystems, Inc.® with the AmpliType® PM & DQA1 kit.

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