PROTOCOLS FOR FORENSIC PCR ANALYSIS
HLA DQα

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General PCR Guidelines

1. The general laboratory policy is to identify physiological fluids (see the Forensic Biochemistry and Hematology Laboratory Manual) before individualization is attempted. However, circumstances will exist when this is not be possible.

2. Since PCR consumes only a small amount of most samples, repeat analyses should be performed whenever possible. The first analysis can be used as a guide for conditions for the second analysis. At least one of the analyses should have an extraction reagent control with no visible dots. If neither analysis produces typeable strips, additional analyses should be performed until there is either two sets of typeable strips, it is not possible to get another set of typeable strips, or 75% of the original sample is consumed.

3. To minimize the potential for carry-over contamination, the laboratory is organized so that the areas for handling amplified DNA, for DNA extraction and for PCR set-up are physically isolated from each other. Each of the three areas is in a separate room. Dedicated equipment such as pipettors should not leave their designated areas. Only the samples should move between areas.

4. Samples that have not yet been amplified should never come in contact with equipment in the amplified DNA work area. Samples that have been amplified should never come in contact with equipment in the unamplified work area.

5. Handle all samples aseptically to prevent contamination by extraneous DNA.

6. The DNA extraction and PCR setup of evidence samples should be performed at a separate time from the DNA extraction and PCR setup of reference samples. This precaution will help to prevent potential cross-contamination between evidence samples and reference samples.

7. Change gloves frequently to avoid sample-to-sample contamination. Change them whenever they might have been contaminated with DNA and whenever exiting a work area.

8. Always change pipette tips between handling each sample.

9. Never "blow out" the last bit of sample from a pipettor. Blowing out increases the potential for aerosols, which may contaminate a sample with DNA from other samples. The accuracy of liquid volume delivered is not critical enough to justify blowing out.

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10. Use filter pipet tips for pipeting all DNA and use whenever possible for other reagents. Use the appropriate filter tips for the different sized pipetmen. The tip of the pipetman should never touch the filter.

11. Avoid splashes. Centrifuge all liquid to the bottom of the closed tube before opening it.

12. Avoid touching the inside surface of the tube caps.

13. Use disposable bench paper to prevent the accumulation of human DNA on permanent work surfaces. Bleach should be used periodically to decontaminate exposed work surfaces.

14. Limit the quantity of samples handled in a single run to a manageable number (approximately 16). This precaution will reduce the risk of sample mix-up and the potential for sample-to sample contamination.

15. Store the DNA Amplification Reagents together in the box provided which will serve as a barrier to possible contamination by exogenous DNA. The box should be stored in PCR set-up refrigerator.

16. Store evidence and unamplified DNA in a separate refrigerator or freezer from the amplified DNA. Do not replace tubes of amplified DNA in the amplification reagents box.

17. Each sample handling area should have its own microfuge racks. The racks should only leave their designated area to transport samples to the next area. Immediately after transporting samples, the racks should be returned to their designated area.

18. Avoid exposing mineral oil to UV light. Exposure to UV light causes the mineral oil to inhibit PCR.

19. Use the Thermal Cycler only for amplification and denaturation of amplified DNA for typing. Never use the Thermal Cycler for incubation of tubes containing unamplified DNA.

20. Keep bleach away from the color development area. Small quantities of bleach can inhibit dot color development.

21. Make sure lab coat sleeves do not touch the caps of open tubes.

22. Discard pipette tips if they accidentally touch the bench paper or any other surface.

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23. Wipe the outside of the pipetman with 10% bleach solution if the barrel goes inside a tube.

24. All evidence, unamplified DNA, and amplified DNA should be stored refrigerated or frozen. Freezing is generally better for long term storage.

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DNA Extraction

Slightly different extraction procedures may be required for each type of specimen. Due to the varied nature of evidence samples, the user may need to modify procedures. See the trouble shooting guide (page 42) for suggestions on how to modify procedures.

1. Perform DNA extraction from samples containing high levels of DNA (for example, whole blood) separately from samples containing a low level of DNA (single hairs, small bloodstains, etc.) to minimize the potential for sample-to sample contamination.

2. Prepare evidence samples at a separate time from reference samples.

3. Use a clean cutting surface for each sample such as a Kimwipe.

4. Clean scissors thoroughly with ethanol and water or use fresh razor blades after cutting each evidence sample.

5. Swabs should be dissected into two or three pieces of equal size with a fresh disposable razor blade. Sections which are not to be analyzed immediately should be stored frozen.

6. Only one tube should be uncapped at a time. When a sample is added to a tube, the tube should be re-capped and the scissors and work area cleaned before the next tube is uncapped.

7. Never consume more than 75% of the sample if at all possible.

8. When pipetting Chelex solutions, the resin beads must be distributed evenly in solution. This can be achieved by one of the following methods: (1) Pour approximately 15 mL of Chelex stock solution into a 50 mL beaker containing a stir bar. Pipette the volume needed for each sample directly from the beaker while the stir bar is mixing. (2) Shake or vortex the tubes containing the Chelex stock solution before aliquoting. (3) Pipet the Chelex stock solution up and down several times before aliquoting.

9. For pipeting Chelex, the pipette tip used must have a relatively large bore--1 mL pipetman tips are adequate.

10. Keep the chelex extraction reagents and equipment separated from the rest of the laboratory equipment.

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11. Use Kimwipes to open sample tubes and blood tubes.

12. After closing each blood tube, wipe around the rim with a Kimwipe to remove residual blood.

13. Be aware of small particles of fabric which may cling to the outsides of tubes.

14. Include substrate controls (if applicable) and an extraction reagent control with each batch of extractions to demonstrate extraction integrity (see Table I for the proper extraction reagent control or substrate control). Substrate controls are obtained from unstained fabric or substrate as close to each stain as possible or from an unused swab. The extraction reagent control contains water in place of biological fluids or stains. Substrate and extraction reagent controls are treated as normal samples and extracted, amplified and typed along with the test samples. If a substrate control is not available and an extraction reagent control is used, a second extraction reagent control is not necessary.

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Table I- Guide to Appropriate Substrate and Extraction Reagent Controls

Sample	Control
Liquid or Frozen Blood, Semen, or Saliva Fresh, Dried, or Frozen Tissue	One extraction reagent control per batch of extractions
Exemplar Stain, i.e. Post-Mortem Stain	One extraction reagent control per batch of extractions
Scraped Blood or Biological Fluid	If possible, use a scraping of an unstained portion of the substrate. Scrapings submitted by outside agencies may not have an appropriate substrate control included. An extraction reagent control is then appropriate.
Swab of Dried Blood, Saliva or Semen	Swab an unstained portion of the substrate. If this is not possible, include an unused swab or unstained piece of filter paper. Swabs or filter paper submitted by outside agencies may not have an appropriate substrate control included. An extraction reagent control is then appropriate.
Bloodstain on fabric or soft surface	Substrate Control for each stain. On old cases without an appropriate control, retrieve the item from the Evidence Unit or Police Department and re-sample.
Bloodstain with no unstained piece of substrate available	One extraction reagent control per batch of extractions

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Chelex DNA Extraction From Whole Blood, Bloodstains, or Blood Scraped or Swabbed Off a Surface

1. Fill out the extraction worksheet.

2. Pipette 1 mL of sterile deionized water into a 1.5 mL microcentrifuge tube for each sample. Cap all of the tubes.

3. A. Open one tube at a time with a Kimwipe and add one of the following:
   a) 3 μL whole blood
   b) portion of bloodstain or swab about 3 mm square
   c) enough scrapings to give a light straw colored extract

   B. Cap the tube before adding sample to the next tube.

4. Mix the tubes by inversion or vortexing.

5. Incubate at room temperature for 15 to 30 minutes. Mix occasionally by inversion or vortexing.

6. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.

7. Carefully remove supernatant (all but 20 to 30 μL). If the sample is a bloodstain or swab, leave the substrate in the tube with pellet. (The supernatant can be frozen and retained for analysis according to the Forensic Biochemistry and Hematology Manual or it can be discarded if it is not needed).

8. Add 175 μL of 5% Chelex.

9. Incubate at 56°C for 15 to 30 minutes.

10. Vortex at high speed for 5 to 10 seconds.

11. Incubate in a boiling water bath for 8 minutes using a screw down rack.

12. Vortex at high speed for 5 to 10 seconds.

13. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.

14. It is recommended that 20 μL of the supernatant be added to the PCR Mix.

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15. Store the remainder of the supernatant at 2 to 8°C or frozen. To re-use, repeat steps 11 through 13.

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Chelex DNA Extraction From Liquid Semen

1. Fill out the extraction worksheet.

2. Add 200 μL of 5% Chelex into a 1.5 mL microcentrifuge tube for each sample.

3. Add 2 μL of 10 mg/mL Proteinase K and 7 μL of 1 M DTT to each of the tubes. Cap all of the tubes and mix gently.

4. Open one tube at a time with a Kimwipe and add 3 μL of whole semen. Cap the tube before adding sample to the next tube.

5. Mix gently.

6. Incubate at 56°C for 30 to 60 minutes. Vortex at high speed 5 to 10 seconds.

7. Spin in a microcentrifuge for 10 to 20 seconds at 10,000 - 15,000 x g.

8. Follow the protocol for Whole Blood/Blood Stains (page 9) beginning with Step 10. Use 20 μL of the supernatant for PCR amplification.

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Chelex DNA Extraction from Semen Stains or Swabs

1. Fill out the extraction worksheet.

2. Pipette 1 mL of PBS into a 1.5 mL microcentrifuge tube for each sample. Cap all of the tubes.

3. A. Open one tube at a time with a Kimwipe and add one of the following:
   a) portion of semen stain about 3 mm square
   b) one third of a swab
   c) scrapings of a stain

   B. Cap the tube before adding sample to the next tube.

4. Mix by inversion or vortexing

5. Incubate at room temperature for 30 minutes.

6. Vortex or sonicate the substrate or swab for at least 2 minutes to agitate the cells off of the substrate or swab.

7. Remove the swab or other substrate from the sample tube, one tube at a time, using sterile tweezers and close tubes. Sterilize tweezers with ethanol before the removal of each sample.

   It is advisable not to discard the substrate until microscopic analysis (Step 11) shows that the sample contains sperm. Store swab or substrate in a sterile tube.

8. Spin in a microcentrifuge for 5 minutes at 10,000 to 15,000 x g.

9. Without disturbing the pellet, remove all but 50 μL of the supernatant. (The supernatant may be frozen or lyophilized and retained for analysis according to the Biochemistry and Hematology Laboratory Manual or discarded if not needed).

10. Resuspend the pellet in the remaining 50 μL by stirring with a sterile pipette tip.

11. Remove about 3 μL of the resuspended sample for a Christmas Tree Stain (see Biochemistry and Hematology Laboratory Manual). After staining, the slide should be labeled and saved as evidence. If sperm are not visible microscopically, the substrate may be put back into the PBS and vortexed more vigorously (step 6) to try to dislodge additional sperm.

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Note: If epithelial cells are detected, proceed with the differential extraction procedure beginning with step 12. If no epithelial cells are observed, the differential extraction procedure may be omitted and the sample may be processed beginning with step 21.

12. To the approximately 50 μL of resuspended cell debris pellet, add 150 μL sterile deionized water (final volume of 200 μL).
13. Add 2 μL of 10 mg/mL Proteinase K. Vortex briefly to resuspend the pellet
14. Incubate at 56°C for about 60 minutes to lyse epithelial cells, but for no more than 75 minutes, to minimize sperm lysis.
15. During the incubation step do the following:
    a. Label a new tube for each sample. Mark each tube as an epithelial cell fraction
    b. Add 50 μL of 20% Chelex to each epithelial cell fraction tube
    c. Close tubes
16. Spin the extract in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
17. Add 150 μL of the supernatant from each sample to its respective epithelial cell fraction sample tube. Store at 4°C or on ice until step 22.
18. Wash the sperm pellet with Digest Buffer as follows:
    a. Resuspend the pellet in 0.5 mL Digest Buffer.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 μL of the supernatant and discard the supernatant.
    e. Repeat steps a-d for a total of 5 times.
19. Wash the sperm pellet once with sterile dH_2O as follows:
    a. Resuspend the pellet in 1 mL sterile dH_2O.
    b. Vortex briefly to resuspend pellet.
    c. Spin in a microcentrifuge at 10,000 to 15,000 x g for 5 minutes.
    d. Remove all but 50 μL of the supernatant and discard the supernatant.
20. Resuspend the pellet by stirring with a sterile pipette tip. Remove about 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas Tree stain (see Biochemistry and Hematology Laboratory Protocol). After staining the slide should be labeled and saved as evidence.

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21. To the approximately 50 μL resuspended sperm fraction, add 150 μL of 5% Chelex, 2 μL of 10 mg/mL Proteinase K, and 7 μL of 1M DTT. Mix gently.

22. Vortex both the epithelial cell and sperm fractions. The following steps apply to both fractions

23. Incubate at 56°C for approximately 60 minutes.

24. Vortex at high speed for 5 to 10 seconds.

25. Incubate in a boiling water bath for 8 minutes using a screw down rack.

26. Vortex at high speed for 5 to 10 seconds.

27. Spin in a microcentrifuge for 2 to 3 minutes at 10,000 to 15,000 x g.

28. It is recommended that 20 μL of the supernatant be added to the PCR Mix.

29. Store the remainder of the supernatant at 2 to 8°C or frozen. To re-use, repeat steps 26 through 28.

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Chelex DNA Extraction from Semen Stains or Dried Semen Inside a Condom- No Differential Lysis

1. Fill out the extraction worksheet.

2. Pipette 1 mL of sterile deionized water into a 1.5 mL microcentrifuge tube for each sample. Cap all of the tubes.

3. Dissect a substrate into thirds using a new razor or scalpel blade or scrape or swab dried semen from a surface such as a condom and use one third. Use a clean cutting surface for each different sample.

4. Open one tube at a time with a Kimwipe and add the substrate. Cap the tube before adding sample to the next tube.

5. Mix by inversion or vortexing

6. Incubate at room temperature for 30 minutes.

7. If there is a substrate or swab, vortex or sonicate the substrate or swab for at least 2 minutes to agitate the cells off of the substrate or swab.

8. Spin in a microcentrifuge for 2 minutes at 10,000 to 15,000 x g.

9. Without disturbing the pellet, remove all but 50 μL of the supernatant. (The supernatant may be frozen and retained for analysis according to the Biochemistry and Hematology Laboratory Manual or discarded if not needed). Resuspend the pellet in the remaining 50 μL by stirring with a sterile pipette tip.

10. Remove about 3 μL of the resuspended sample and spot on a glass microscope slide for examination. Perform Christmas tree stain (see Biochemistry and Hematology Laboratory Manual). After staining the slide should be labeled and saved as evidence.

11. To the approximately 50 μL resuspended cell pellet, add 150 μL of 5% Chelex, 2 μL of 10 mg/mL Proteinase K, and 7 μL of 1M DTT. Mix gently.

12. Incubate at 56°C for 30 to 60 minutes.

13. Follow the protocol for Whole Blood/blood Stains (page 9) beginning with Step 10. Use 20 μL of the supernatant for PCR amplification.

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Non-Organic Extraction of DNA From Blood

1. Process a Cell Pellet Control and an Extraction Reagent Control (0.5 mL) with every batch of extractions.

2. Add 0.5 mL or of well mixed blood to a microcentrifuge tube.

3. Add 1.0 mL ice cold Cell Lysis Buffer (CLB). Vortex at high speed for one minute.

4. Centrifuge samples 5 minutes, 2700 x G at 4°C.

5. Decant supernatant. (With a quick motion, hold tubes upside down to decant the supernatant.) Blot each tube on absorbent tissue. Store tubes on ice.

6. Repeat steps 3-5 two (2) more times with CLB.

7. Repeat steps 3-5 one more time substituting ice cold Protein Lysis Buffer (PLB) for the CLB. Keep tubes on ice.

8. Thaw a tube of Proteinase K and mix thoroughly by gentle inversion of the tube.

9. Prepare a master mix of PLB and Proteinase K for N+2 samples.

   Master mix for 1 sample.
   225 μL PLB
   25 μL Proteinase K (10 mg/mL)

10. Process one sample at a time:
    Add 250 μL of master mix. Pipet up and down to resuspend pellet. Mix well. Place tube in 65°C heat block.

11. Incubate each tube for 2-2.5 hour. Vortex at high speed for $\approx$ 30 seconds every 15-20 minutes to insure nuclear pellet is resuspended.

12. Vortex at high speed for $\approx$ 30 seconds following complete incubation.

13. Centrifuge samples 5 minutes in a microcentrifuge at room temperature to remove particles. Transfer supernatant to a new tube.

14. Run a yield gel (see next page) to determine the DNA concentration of each sample.

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14 Store DNA at 4°C. It is stable for several months.

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Estimation of DNA Quantity and Quality From a Yield Gel

1. Preparation of 20 x 25 cm yield gel (BRL Apparatus).

   BRL gels are poured with a 30 lane, 2 mm comb. The gel may have 2 or more origins if many samples are analyzed. The BRL gels are cast in the electrophoresis tank using the combs and dams in the tank.

   a. For each gel, add 20 mL 10X TAE, 180 mL dH_2O and 1.6 g agarose to a flask of at least twice the liquid volume.
   b. Bring to a boil to dissolve agarose.
   c. Add 20 μL of ethidium bromide (10 mg/mL)
   d. Equilibrate at 56°C.
   e. Pour agarose into gel form (be sure comb is in place).
   f. Let stand 30-90 minutes to gel.

2. Pour 1x TAE into electrophoresis tank. Enough buffer should be present to cover the gel. Remove comb.

3. Vortex samples including cell pellet and Extraction Reagent controls, Yield Calibrators, Calibration Control and Lambda Marker tubes for 15 seconds. Microcentrifuge briefly to bring contents to the bottom of the tube.

4. Incubate 5 minutes at 65°C.

5. Microcentrifuge briefly to bring contents to the bottom of the tube.

6. Add 10 μL of DNA sample and 2 μL of Yield Gel Loading Buffer to a well of a microtiter plate or an eppendorf tube. Store unused sample at 4°C.

7. If using an eppendorf tube, microfuge briefly .

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8. Load each row of each gel as follows

Lane	Volume	Material	Description
2	10 μL	Lambda Marker	Hind III digested λ DNA
3	10 μL	Yield Calibrator A	30 ng/μL λ DNA (300 ng total)
4	10 μL	Yield Calibrator B	20 ng/μL λ DNA (200 ng total)
5	10 μL	Yield Calibrator C	10 ng/μL λ DNA (100 ng total)
6	10 μL	Yield Calibrator D	5 ng/μL λ DNA (50 ng total)
7	10 μL	Yield Calibrator E	2.5 ng/μL λ DNA (25 ng total)
8	10 μL	Yield Calibrator F	1 ng/μL λ DNA (10 ng total)
9	10 μL	Cal. Control	50 ng high M.W. human DNA
10-29	12 μL	Samples	Unknown samples

Remember to include the cell pellet and extraction reagent controls as samples.

9. Set the voltage at 100 volts on the dial. When the bromophenol blue tracking dye has moved 1-2 cm from the origin, the run can be stopped.

10. Switch off the power supply. Remove the gel from the tank. Examine the gel on the ultraviolet light transilluminator. Take a photograph of the gel using Polaroid 667 film. DO NOT EXPOSE YOURSELF TO THE UV LIGHT FOR AN EXCESSIVE AMOUNT OF TIME. ALWAYS WEAR U.V. GOGGLES WHEN WORKING WITH THE TRANSILLUMINATOR.

11. Interpretation:

A. Quality- Intact DNA will move as a band not far from the origin. A smear from the origin to, or past the dye front indicates that the DNA has been fragmented.

B. Compare the intensity of the calibration control (lane 9) to that of the yield calibrators. If the intensity between Yield Calibrator's D and E (lanes 6-7), the result is valid. Otherwise the gel must be repeated.

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C. Estimate the quantity (ng) of high molecular weight DNA loaded for each sample by comparing the band size of the high molecular weight band of the unknowns with those of the Yield Calibrators. High molecular weight DNA should appear as a tight band slightly above the uppermost band of the Lambda Marker. If the sample shows "trailing", use only the region above the upper band of the Lambda Marker for quantifying high molecular weight DNA. If the band size is between two yield calibrators match the sample to the yield calibrator with the lowest amount of DNA. See Table II for interpretation.

12. Save 25 μL of each sample for PCR analysis.

TABLE II- INTERPRETATION OF YIELD GEL

	DNA CONCENTRATION[b]	
Sample Intensity[a]	2 μL Sample Loaded On Yield Gel	10 μL Sample Loaded On Yield Gel
$\geq$ A	see note c below	Repeat yield gel loading 2 μL of sample
B	100 ng/μL	20 ng/μL
C	50 ng/μL	10 ng/μL
D	25 ng/μL	5 ng/μL
E	12.5 ng/μL	2.5 ng/μL
$\leq$ F	Repeat yield gel loading 10 μL of sample	$\leq$ 1 ng/μL

a. Yield Calibrator of closest intensity

b. For diluted samples, multiply DNA concentration by 10.

c. Dilute 5 μL of sample with 45 μL of TE and mix. Run 2 μL on a new yield gel. Reserve 25 μL of the dilution for PCR analysis and save the rest of the dilution at 4°C.

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Chelex Extraction From Non-Organically Extracted Samples

The amount of DNA in the non-organic samples is quantitated by yield gel (see above). The target amount of DNA to add to each amplification tube is 5 ng in 20 μL. The amount of DNA is normalized by adding different volumes of non-organic extract to each Chelex tube, depending upon the yield of the sample. Table III lists the volumes of non-organic extract to add to the Chelex tube for the various yield gel concentrations.

Table III: Non-Organic Extract for Amplification

yield gel conc (ng/μl)	volume dH_2O (μL)	volume non-organic extract (μL)	volume Chelex (μL)
100	49.5	0.5	150
50	49	1.0	150
25	48	2.0	150
20	47.5	2.5	150
12.5	46	4.0	150
10	45	5.0	150
5.0	40	10	150
2.5	30	20	150
$\leq$ 1.0	25	25	150

1. Fill out the extraction worksheet.

2. Vortex and briefly microfuge non-organic extract tubes.

3. Add in order, the appropriate amount of dH_2O, non-organic extract, and 5% Chelex solution to each sample tube (Table III) for a final volume of 200 μL.

4. Incubate at 56°C for 15-30 minutes.

5. Vortex.

6. Incubate in a boiling water bath for 8 minutes.

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7. Vortex.

8. Microfuge for 2-3 minutes.

9. Add 20 μL of the supernatant to the PCR reaction tube.

10. Store the remaining sample at 2-8°C or frozen. To re-use, repeat steps 3-8.

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PCR DQα Test

Amplification

A positive control, an extraction reagent control and substrate controls, if applicable, should be included with each batch of samples being amplified to demonstrate procedural integrity.

1. Turn on the Perkin Elmer Thermal Cycler. (See manufacturer's instructions). File #14 on both machines should include the following:

   Denature at 94°C for 1 minute
   Anneal at 60°C for 30 seconds
   Extend at 72°C for 30 seconds

   Repeat the above for 32 cycles

   Link to Time Delay File #13 on both machines for an additional 7 minutes incubation at 72°C.

   Link to Soak File #12 on both machines for a 4°C soak.

   If Files #12, 13, or 14 are not correct, bring this to the attention of the QC/QA coordinator and a supervisor. Re-program the incorrect file(s) and note that the file(s) was changed in the log book for that run.

2. Determine the number of samples to be amplified, including controls.

3. Fill out the amplification worksheet and record the appropriate lot numbers.

4. Ensure that the solution is at the bottom of each PCR reaction mix tube by tapping the tube down onto a clean work surface or by centrifuging briefly. Label the caps of the PCR Reaction Mix tubes. Open caps using the microcentrifuge tube de-capping tool or a new Kimwipe. **Avoid touching the inside surface of the tube caps.**

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5. **Note: It is important to begin the cycling processing within 30 minutes after addition of the $MgC1_2$ Solution to the PCR Reaction Mix.**

   Pipet 50 μL of the $MgC1_2$ Solution provided into each tube including controls, with a sterile pipet tip. Pipet carefully at a slight angle to minimize mixing and to avoid splashing of solution .Use either a filter tip or a combi tip on the pipettor.

6. Carefully add 2 drops of the Mineral Oil from the dropper bottle provided in the kit to all tubes including the controls. **Do not actually touch tube.**

7. **Note: Use a new sterile filter pipet tip for each sample addition.**

   Test Sample Tubes:
   Add 2-40 μL* of sample DNA to each labeled tube by inserting the pipet tip through the mineral oil layer. After the addition of the DNA, cap each sample before proceeding to the next tube. **Do not vortex or mix.**

   *See the appropriate section of the DNA extraction section for the recommended amount of sample DNA to add to each tube.

   Positive Control Tubes:
   Add 20 μL of the 100 ng/mL Genomic Control DNA to the designated PCR Reaction Mix tube.

   The final aqueous volume in the PCR reaction Mix tubes will vary between 100 μL and 140 μL, depending on the volume of DNA added.

8. **Note: PCR Reaction Mixes should be amplified in rows A-D in the Thermal Cycler**

   For each well of the Thermal Cycler heat block which will be used to amplify samples, add one drop of mineral oil to the well. Place the PCR Reaction Mix tubes into the Thermal Cycler. Push the tubes down completely into the heat block. Record the heat block position of each tube.

9. Start the Thermal Cycler amplification program. Verify the cycling parameters by monitoring the first cycle. The tubes should be checked after the first cycle and pressed further into the heat block so that they fit tightly.

10. Return the microtube rack used to set-up the samples for PCR to the PCR Set-Up Area.

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11. Wash the microtube rack in which the DNA extractions were performed with 10% bleach and return the rack to the Extraction Work Area.

12. After the amplification process, the samples are ready for DNA Hybridization and Color Development or they may be stored at 2-8°C for at least seven days, or at -20° for extended periods. **Do not store amplified DNA samples in the same box with unused DNA Amplification Reagents or unamplified DNA.**

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Optional Step to Verify Amplification

The presence of amplified DQα sequences can be verified by gel electrophoresis. The Amplitype™ HLA DQα gene amplification product will be either 239 or 242 base pairs long, depending on the particular allele.

Agarose Gel Preparation

1. Weigh out agarose for a 3% Nusieve™ plus 1% Seakem™ GTG gel
2. Add agarose to the appropriate amount of 1X TAE in a flask. Volume required will vary depending on size of the minigel apparatus. Prepare enough agarose to pour a 3 mm gel.
3. To dissolve agarose, heat in a microwave oven for 1 to 2 minutes. Swirl flask to aid in dissolving agarose.
4. Cool the solution to 55°C in a water bath.
5. Add a volume of 10 mg/ML Ethidium Bromide to the molten agarose to achieve a final concentration of 1 μg/mL Ethidium Bromide.
6. To form the gel, pour liquified agarose/ethidium bromide solution in minigel apparatus for a 3 mm thick gel. Immediately insert slot forms and adjust to assure that the apparatus is level.
7. Allow the gel to set for 20 minutes at room temperature, or until completely solidified.
8. When gel is ready, add a sufficient volume of 1X TAE to fill the buffer tanks and cover the gel to a depth of about 1 mm.
9. Carefully remove slot forms. Avoid touching wells.

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Gel Loading and Electrophoresis

1. Add 5 μL DNA sample to a 0.5 mL microcentrifuge tube. Add 1 μL Analytical Gel Loading Buffer and mix by tapping gently.

2. Do not use the outside lanes of the gel. In the first and last useable lanes of the gel, pipet 6 μL of the Phi X Marker.

3. Carefully pipette samples into the remaining wells.

4. Connect leads so that the DNA migrates toward the positive (+) electrode. Run the minigel at 50 to 100 volts, (or 5 to 15 v/cm) at room temperature for about one to two hours or until the bromophenol blue (faster-migrating) dye is one to two cm from the end of the gel.

5. Check for the extent of DNA migration by examining visually on a 300 nm UV transilluminator. **WEAR PROTECTIVE EYEWEAR AND HANDLE THE GEL WITH GLOVES.**

   To photograph gel, place on a UV transilluminator box under a stationary camera with a Kodak 23A Wratten orange filter. Photograph in the dark under UV illumination.

   Note: Sample evaluation gels and PCR-product gels are both run in the DNA Amplification Work Area. However, the pre-amplification samples must never be brought into that area. Only the aliquot of sample prepared for gel loading should be removed from the DNA Extraction Work Area.

Interpretation of Gel Electrophoresis Patterns

The HLA DQα amplification products will appear between the 281 bp/271 bp doublet (sixth band from the origin) and the 234 bp fragment (seventh band from the origin) of the Phi X Marker. A "primer dimer" band will be frequently observed at slightly lower molecular weight than the 72 bp marker fragment. Unincorporated primers will appear as a broad band at still lower molecular weight.

Occasionally the HLA DQα amplification product will appear as a doublet band.

Initials: RCS Date: 3/5/93

DNA Hybridization

1. Heat a shaking water bath to 55°C. The water level should be 1/4 - 1/2 inch above the shaking platform. The temperature should not go below 54°C or above 56°C. It is essential to check the temperature with a calibrated thermistor probe before the hybridization is performed. The water level should not be higher than 1/2 inch, as higher levels may result in water splashing into wells.

2. Heat a stationary water bath to 37-65°C. Warm the DQα Hybridization Solution and the DQα Wash Solution in the water bath. All solids must be in solution before use.

   Do not proceed to step 3 until the water baths in steps 1 and 2 are at the proper temperature.

3. Fill out the hybridization worksheet.

4. Using filter forceps, remove the required number of DNA Probe Strips from the glass tube. Place one Probe Strip in each clean well of the Amplitype DNA Typing Tray. With a waterproof marking pen, label each strip in the space provided.

5. Prepare the Perkin Elmer Cetus DNA Thermal Cycler to denature the amplified DNA. Start the program (File # 15 on both machines) to hold the Thermal Cycler Temperature at 95°C.

6. Place the tubes in the Thermal Cycler. Press the tubes down tightly in the heat block. Denature the amplified DNA by incubation at 95°C for 3-10 minutes. Keep each tube at 95°C until use.

Initials: RC Date: 4/13/92

DNA Hybridization

1. Heat a shaking water bath to 55°C. The water level should be 1/4 - 1/2 inch above the shaking platform. The temperature should not go below 54°C or above 56°C. It is essential to check the temperature with a calibrated thermistor probe before the hybridization is performed. The water level should not be higher than 1/2 inch, as higher levels may result in water splashing into wells.

2. Heat a stationary water bath to 65 ± 5°C. Warm the DQα Hybridization Solution and the DQα Wash Solution in the water bath. All solids must be in solution before use.

   Do not proceed to step 3 until the water baths in steps 1 and 2 are at the proper temperature.

3. Fill out the hybridization worksheet.

4. Using filter forceps, remove the required number of DNA Probe Strips from the glass tube. Place one Probe Strip in each clean well of the Amplitype DNA Typing Tray. With a waterproof marking pen, label each strip in the space provided.

5. Prepare the Perkin Elmer Cetus DNA Thermal Cycler to denature the amplified DNA. Start the program (File # 15 on both machines) to hold the Thermal Cycler Temperature at 95°C.

6. Place the tubes in the Thermal Cycler. Press the tubes down tightly in the heat block. Denature the amplified DNA by incubation at 95°C for 3-10 minutes. Keep each tube at 95°C until use.

Initials: RCJ Date: 4/13/92

7. **Note: Do not prepare the DQα Hybridization/Enzyme conjugate Solution more than 15 minutes before use.**

Prepare the hybridization solution in a glass flask either according to Table IV or the following formula:

Strips x 3.3mL pre-warmed DQα Hybridization Solution
strips x 27 μL Enzyme Conjugate

Mix by swirling.

Table IV- DQα Hybridization Solution

number of strips	hyb solution	enzyme conjugate
4	13.2 ml	108 μl
6	19.8 ml	162 μl
8	26.4 ml	216 μl
10	33.0 ml	270 μl
12	39.6 ml	324 μl
14	46.2 ml	378 μl
16	52.8 ml	432 μl

8. Tilt the Typing Tray towards the labelled end of the strips. Add 3 mL of the freshly prepared DQα Hybridization/Enzyme Conjugate Solution at the labelled end of each strip.

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9. **Note: Perform the following steps for each tube of amplified DNA. When pipeting amplified extract, wait for the bead of mineral oil to expel from the tip before drawing up. For each tube, perform steps a-c within 30 seconds.**

   a. Remove the tube from the 95°C heat block.
   b. Carefully open the tube. (Use the microcentrifuge tube decapping device or a new Kimwipe).
   c. Withdraw 35 μL amplified DNA from the aqueous (bottom) layer and immediately add to the contents of the well (pipet below the surface of the hybridization solution) at the labeled end of the corresponding Probe Strip.
   d. Cap the tube and set aside.
   e. Repeat until each amplified DNA sample has been added to the corresponding well. Use a new pipet tip for each addition.

   The remaining amplified DNA samples can be stored at -20°C.

10. Mix the tray by carefully rocking and place the clear plastic lid on the tray. Put the tray into the 55°C shaking water bath. Place a weight (e.g., lead ring) on the covered tray to prevent the tray from sliding or floating.

    Note: Do not shake the tray after the samples have been added to the strips. It is important that the strips are completely submerged in the hybridization solution before placing the tray in the water bath, but cross contamination can occur at this step if the solution is shaken out of the wells. Gentle rocking of the tray is sufficient to moisten the strips.

11. Hybridize the amplified DNA samples to the Probe Strips by incubating at 55°C for 20 minutes at 50-90 rpm. Adjust the water level and check the tray position so that water does not splash into the wells of the tray.

12. After hybridization, remove the tray from the water bath and pour out the contents of each well. Wipe the tray lid with a Kimwipe or paper towel.

13. **Note: DQα Wash Solution solids must be in solution before use.**

    Dispense 10 mL of DQα Wash Solution into each well. Rinse by rocking for several seconds, then pour the solution from each well.

Initials: LCS Date: 4/13/92

14. **Note: The temperature and timing of the Stringent Wash are CRITICAL.**

    Stringent Wash:
    Dispense 10 mL of DQα Wash Solution into each well. Cover tray with lid and weight. Place into the 55°C shaking water bath for 12 minutes, ±2 minutes, at about 50 rpm.

15. Remove the tray from the water bath, take off the lid and pour the solution from each well.

16. Dispense 10 mL of DQα Wash Solution into each well. Cover and place on an orbital shaker at room temperature for 5 minutes at about 50 rpm.

17. Remove the tray from the orbital shaker, take off the lid and pour the solution from each well.

Initials: RC Date: 4/13/92

Color Development

1. Dispense 10 ml of citrate buffer into each well. Wipe the tray lid with a Kimwipe or paper towel. Cover tray and place on an orbital shaker for 5 minutes at about 50 rpm.

2. **Note: Do not prepare the Color Development Solution more than 10 minutes before use. Use a new tube of hydrogen peroxide for each batch of Color Development Solution. Discard the remaining hydrogen peroxide after use. Parafilm the chromogen bottle after each use to prevent oxidation.**

   During this wash step, prepare the Color Development Solution in an Erlenmeyer flask either according to Table V or the following formula. Add the reagents in order:

   # strips x 10 mL DQα Citrate Buffer
   # strips x 10 µL 3% Hydrogen Peroxide
   # strips x 0.5 mL Chromogen Solution

Mix by swirling 2-3 times. Do Not Vortex

Table V- Development Solution

number of strips	citrate buffer	hydrogen peroxide	chromogen
4	40 ml	40 µl	2 ml
6	60 ml	60 µl	3 ml
8	80 ml	80 µl	4 ml
10	100 ml	100 µl	5 ml
12	120 ml	120 µl	6 ml
14	140 ml	140 µl	7 ml
16	160 ml	160 µl	8 ml

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3. **Note:** **Place lid on tray and cover lid with aluminum foil during steps, 3,5 and 6 to protect from strong light.**

   Remove the tray from the orbital shaker, remove the cover and slowly pour off the DQα Citrate Buffer. Add 10 mL of the newly prepared Color Development Solution to each well. Develop the strips at room temperature by shaking on the orbital shaker at about 50 rpm for 20-30 minutes.

4. Remove tray from shaker and slowly pour off the contents from each well.

5. Stop the color development by washing the strips in deionized water. Dispense approximately 10 mL of water into each well. Place tray on orbital shaker at about 50 rpm for 5-10 minutes. Slowly pour off the contents of each well.

6. Repeat Step 4 and Step 5 twice for a minimum of three water washes. Additional 5-10 minutes washes will reduce the potential for developing background color.

   As long as the strips are protected from light, the dot intensity does not fade when they are washed in distilled water, even for extended periods. Insufficient washing results in a blue background when the strips are dried.

7. Photographs must be taken for a permanent record (see the next section).

8. Determine and record the HLA DQα type for each sample from the photograph. (See Results Interpretation section.)

9. Any discrepancies between the strips and photographs should be documented on the hybridization worksheet.

Initials: RCJ Date: 3/5/93

Photography And Storage

1. Place wet strips on a flat surface as close together as possible. Keep strips wet throughout the photographic procedure. Minimize exposure to strong light.

   For casework photograph up to 8 strips at a time. For QC and validation photograph up to 16 strips at a time.

2. Use a Polaroid MP4 camera system with type 667 or 665 film and a Wratten 23A orange filter. Type 667 is the preferred film.

3. Turn on the flood lights. Adjust the height of the camera and focus so that the strips fill the **entire** viewing frame.

4. Photograph at 1/125 second and f22 for type 667 film. Photograph at 1/2 second and f16 for type 665 film.

5. Develop at room temperature for ≈ 30-60 seconds.

6. Label the photograph using a permanent marking pen with the date, analyst's initials, and case number.

7. If the photograph is not exposed properly or does not accurately record the dots on the strips, vary the exposure conditions and re-photograph.

   Make sure there is at least one good photograph of each set of strips.

8. Attach the photographs to the hybridization worksheets and file in the appropriate binder or folder. For cases the worksheets are retained in the case file.

9. For cases also photograph on color 135 slide film following the exposure directions posted. The slides are retained in the case file.

10. Following photography, store the strips in a plastic folder in the appropriate binder or folder. Protect from light and oxidizing agents. Color may fade somewhat on drying.

Initials: JCJ Date: 4/13/92

Photography And Storage

1. Place wet strips on a flat surface as close together as possible. Keep strips wet throughout the photographic procedure. Minimize exposure to strong light.

   For casework photograph up to 8 strips at a time. For QC and validation photograph up to 16 strips at a time.

2. Use a Polaroid MP4 camera system with type 667 film and a Wratten 23A orange filter.

3. Turn on the flood lights. Adjust the height of the camera and focus so that the strips fill the **entire** viewing frame.

4. Photograph at 1/125 second and f22.

5. Develop at room temperature for ≈ 30-60 seconds.

6. Label the photograph using a permanent marking pen with the date, analyst's initials, and case number.

7. If the photograph is not exposed properly or does not accurately record the dots on the strips, vary the exposure conditions and re-photograph.

   Make sure there is at least one good photograph of each set of strips.

8. Attach the photographs to the hybridization worksheets and file in the appropriate binder or folder. For cases the worksheets are retained in the case file.

9. For cases also photograph on color 135 slide film following the exposure directions posted. The slides are retained in the case file.

10. Following photography, store the strips in a plastic folder in the appropriate binder or folder. Protect from light and oxidizing agents. Color may fade somewhat on drying.

Initials: RU Date: 4/13/92

Results Interpretation

Results are interpreted by reading the pattern of blue dots on the Polaroid photograph of the DNA Probe Strips to determine which DQα alleles are present in the DNA sample. Examples of 21 HLA DQα genotypes are shown on the Abbreviated Protocol Card provided in the Amplitype™ Kit.

1. The "C" Dot

The strip is read by first examining the "C" dot. **DNA Probe Strips with no visible "C" dot should not be HLA DQα typed.** Those dots equivalent to or stronger than the "C" dot are considered positive. Dots with signal less than the "C" dot should be interpreted carefully.

The "C" probe serves two functions.

A. To indicate adequate amplification and typing of the DQα alleles in the sample.

The "C" dot is usually the weakest on the strip. If the "C" dot is absent, an accurate determination of the type cannot be made since there is a possibility that other probe signals are also below the threshold of detection. The presence of a "C" dot provides assurance that the appropriate typing and sub-typing dots should be clearly visible.

B. To indicate a possible procedural error, mixed sample, or DNA contamination.

The presence of visible dots with a signal intensity less than the "C" dot may indicate any of the above or amplification of DXα, DQα type 1.3,4, or sub-types of the DQα 4 allele (See page 38).

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2. Typing and Sub-Typing Probes

The typing and sub-typing probes are used to determine the DQα genotype of the DNA sample analyzed. Each positive dot indicates the presence of the corresponding DQα allele (see Table VI).

The "1" dot is positive in the presence of the DQα 1.1, 1.2, and 1.3 alleles.
The "2" dot is positive only in the presence of the DQα 2 allele.
The "3" dot is positive only in the presence of the DQα 3 allele.
The "4" dot is positive only in the presence of the DQα 4 allele (4.1, 4.2, and 4.3 alleles).

The four DQα sub-typing probes differentiate DQα 1.1, 1.2, and 1.3.
The "1.1" dot is positive only in the presence of the DQα 1.1 allele. (see discussion of DXα, page 40)
The "1.3" dot is positive only in the presence of the DQα 1.3 allele.
There is no probe unique to DQα 1.2. The "1.2, 1.3, 4" dot is positive in the presence of DQα 1.2, 1.3, and 4 alleles.
The remaining sub-typing probe, "All but 1.3", is necessary to differentiate the 1.2, 1.3 genotype from the 1.3, 1.3 genotype. This dot is positive in the presence of all DQα alleles EXCEPT 1.3.

NOTE: **The "All but 1.3" dot can be weaker than the "C" dot with the 1.3, 4 genotype. The "All but 1.3" probe has a base pair mismatch with both the DQα 1.3 sequence and the DQα 4 sequence. The mismatch with the DQα 4 sequence pairs a G with a T. This mismatch is only partially destabilizing, and allows the 4 sequence to give signal at this dot, albeit a weak signal. The "All but 1.3" dot is used only to distinguish the 1.2, 1.3 from the 1.3, 1.3 genotype. The "All but 1.3" may also sometimes be weaker than the "C" dot for the 4,4 genotype.**

There is also variation among the typing dots on a single probe strip. For instance, for the genotype 1.1, 3 the "1" dot is often darker than the "3" dot for high, medium and low signal levels. The difference is most pronounced at the high level. These differences in intensity are quite consistent from typing to typing.

Amplified samples which have been stored for even a short time often show low intensity dots which were not visible during the first hybridization. These dots are less intense than the "C" dot and do not affect the typing results.

Initials: RCJ Date: 4/10/92

TABLE VI

PROBE	ALLELES DETECTED
1	DQα 1.1, 1.2, 1.3
2	DQα 2
3	DQα 3
4	DQα 4 (4.1, 4.2, 4.3)
C	DQα 1.1, 1.2, 1.3, 2, 3, 4 (All DQα Alleles)
1.1	DQα 1.1
1.2, 1.3, 4	DQα 1.2, 1.3, 4
1.3	DQα 1.3
All but 1.3	DQα 1.1, 1.2, 2, 3, 4

3. Extraction Reagent and Substrate Controls

The extraction reagent control is a check for the possible contamination of the reagents in the DQα test by other human DNA or by amplified DQα DNA. The extraction reagent control is performed by carrying out the extraction in a tube containing no sample.

The substrate control is a check for the possible contamination of substrate by an undetected stain containing biological material (e.g. human DNA). This contamination could be pre-existing, it could be deposited on the substrate during the commission of the crime, or it could be deposited during the handling and processing of the evidence. The substrate control is also a check for the contamination of the reagents in the DQα test by other human DNA or by amplified DQα DNA. In addition, the substrate control extract can be used to verify that the substrate contains an extractable PCR inhibitor. The substrate control is performed by carrying out the DNA extraction on unstained substrates (e.g. piece of fabric) located as close to each stain as possible.

Initials: fcj Date: 4/13/92

The extraction reagent and substrate controls are amplified and typed along with the test samples. The appearance of signals in the typing of these controls indicates any or all of the following:

a. The sample preparation reagents may have been contaminated.

b. Cross-contamination between samples may be occurring during preparation.

c. The substrate might have an undetected stain containing biological material.

d. Human DNA or amplified HLA DQα DNA may be getting into the samples from some other source.

Clearly, if the test sample does not show any probe signal in common with the controls, the test sample is not affected by the same source of contamination.

If the signals on the typing strips for the extraction reagent or substrate controls are very faint with the "C" dots not visible, and the test samples are easily typeable with clearly visible "C" dots, the contamination problem is not serious. If the "C" dot is visible on the typing strip of the extraction reagent or substrate controls, the contamination problem is more serious. **See Table VII for interpretation guidelines.**

The appearance of signals in extraction reagent or substrate controls does not necessarily mean that the types obtained for the test samples are incorrect because of the following:

a. The contamination might be due to a single event limited to the control.

b. The level of contamination might be inconsequential compared to the amount of DNA being amplified and typed in the test samples.

c. The contamination might be easily distinguished from the test samples because the contamination and test samples do not have any alleles in common.

However, further testing may be necessary to support these possibilities.

See Table VII for interpretation guidelines.

Initials: RCJ Date: 9/03/92

Table VII- Guideline to the Interpretation of Visible Dots in the Extraction Reagent and Substrate Controls

Control	"C" dot visible?	All test sample alleles found in the control?*	Interpretation of Test Sample
Reagent Extraction	yes	yes/no	All test samples inconclusive
	no	yes	Test samples with alleles found in the reagent extraction control are inconclusive. Re-test those samples using a new reagent extraction control.
	no	no	Interpret with caution
Substrate	yes	yes/no	The corresponding stain is inconclusive. Re-test the same stain with a new substrate control
	no	yes	Interpret with caution. Probably the result of substrate contamination with stain. Re-test the same stain with a new substrate control.
	no	no	Interpret with caution. Re-test the same stain with a new substrate control.

*Alleles refer only to the primary alleles dots "1", "2", "3", "4", "1.1", or "1.3"

Initials: KC Date: 4/13/92

4. Amplification Positive Control

The DQα 1.1, 4 control DNA provided in the Amplitype™ HLA DQ kit is a positive control which is used with each batch of samples typed to demonstrate that the kit is performing properly.

If the positive control does not produce a correct and readable type, the amplification must be repeated and the test samples are considered inconclusive.

5. Species Specificity

A positive and interpretable DQα result can be considered primate positive. Identification of the specific physiological fluid may be accomplished using the procedures described in the Forensic Biochemistry and Hematology Laboratory Manual.

6. Population Frequencies

To interpret the significance of a match between genetically typed samples, it is necessary to know the population distribution of alleles at the locus in question. For example, if the DQα genotype of the relevant evidence sample is different from the type of the suspect's reference sample, then the suspect is "excluded", and cannot be the donor of the biological evidence being tested. An exclusion is independent of the frequency of the two genotypes in the population.

If the suspect and evidence samples have the same genotype, then the suspect is "included", and could be the source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is equal to the frequency of that genotype in the relevant population.

Initials: JC) Date: 4/10/92

Interpretation of Complex Results

Occasionally typing results may appear markedly different from the standard 21 patterns. Such results could be due to a procedural error, mixtures of DNAs (multiple contributors to the sample) the presence of the uncommon subtypes 4.2 or 4.3, or the presence of an allele of the related pseudogene, DXα.

1. Mixtures of DNA: more than one genotype present in the DNA sample.

   A. General Mixtures

   Evidence samples may contain DNA from more than one individual either because of the nature of the sample or from contamination. The possibility of multiple contributors should be considered when interpreting the HLA DQα typing results. For any typing system in which heterozygous genotypes are analyzed, the detection of more than two alleles indicates a mixed sample. Furthermore, there is a possibility that a phenotype read for example as DQα 3,4 is a mixture of approximately equal contributions from a homozygous 3,3 individual and a homozygous 4,4 individual or from {3,3 + 3,4} or {4,4 + 3,4} mixtures. Such mixtures would not be detected from typing results alone, as they would reveal only two alleles.

   There are 210 possible mixtures of two different DQα genotypes. Of these, 166 are mixtures that would contain three or more alleles and 44 are mixtures of the type that may not be easily determined to be mixtures as demonstrated by the example above.

   B. Mixtures containing the 1.2 allele.

   Since there is no probe uniquely specific for the 1.2 allele, it could be overlooked in some three-allele mixtures. There are five hybridization patterns corresponding to normal DQα genotypes in which a three allele mixture could contain an undetected 1.2 allele. These five patterns are listed in Table VIII. These five patterns correspond to 25 of the 166 possible mixtures of two genotypes containing three alleles in total.

Initials: RCJ Date: 4/13/92

Table VIII: DQα phenotypes that can contain hidden 1.2 alleles

three allele combinations in which 1.2 can be present but not specifically detected	apparent heterozygous type
1.1, 1.2, 4	1.1, 4
1.1, 1.2, 1.3	1.1, 1.3
1.2, 1.3, 2	1.3, 2
1.2, 1.3, 3	1.3, 3
1.2, 1.3, 4	1.3, 4

C. Mixtures with different level of starting DNA

Mixtures may be present in unequal amounts. The typing result of a mixture of ten parts type DQα 3,3 and one part of type 4,4 is usually recognizable as being different than a true heterozygous type 3,4. When the dot intensities from the mixture are compared to the dot intensities in a heterozygous individual, the dot or dots corresponding to 3 are darker than they should be relative to the other dots, and the dots corresponding to 4 may be fainter than the "C" dot. This result is a flag for the presence of a mixture or contamination.

The best possible controls for such interpretation are reference sample DNAs of the same apparent type and about the same "C" dot intensity assayed at the same time. Because this type is probably not known before the samples are analyzed, a second typing using "leftover" amplified DNA from the potentially mixed sample in parallel with the reference sample can be performed if a mixture is suspected.

D. Apparent mixtures containing a weak "1.1" allele

The presence of a weak "1.1" allele may not be the result of a mixture or contamination but instead could be due to the amplification of the related DXα pseudogene (see page 40 for a discussion of this topic).

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2. Subtypes of the DQα 4 allele

Occasionally, the signal intensity of the "1.2, 1.3, 4" dot will be much weaker than the "C" dot, even though a strong "4" dot is present. This phenomena is due to the relatively rare subtypes of the DQα 4 allele which are not discriminated by this kit. The 4.2 and 4.3 allele sequences contain a single mismatch to the "1.2, 1.3, 4" probe. This mismatch, close to one end of the probe sequence, is not completely destabilizing so that a reduced signal is obtained with these alleles.

3. Weak Amplification of the Related DXα allele

Occasionally a very weak signal, much weaker than the "C" dot, is seen at the "1.1" dot of a probe strip on which other signals are strong, even when there is no dot present for the "1" probe. This is due to the relatively low-level amplification of a related gene, DXα. **DXα can not be used for comparison of samples.**

DXα is a non-expressed pseudogene that has some sequence similarity to DQα. Sequence information shows that one DQα primer has two base-pair mismatches with the second exon of DXα and the other DQα primer has a one base-pair mismatch. Under the primer annealing conditions of this kit, the efficiency of DXα amplification is always very low compare to DQα; however signals at the "1.1" dot which can be attributed to weak DXα amplification are observed sporadically. The amplified segment of DXα will hybridized to the DQα "1.1" dot but not to the "1" dot. This hybridization pattern - "1" dot negative, "1.1" dot weak - is an indication of DXα amplification. This signal should not confuse the typing result since 1.1 signal due solely to DXα will always be much weaker than the "C" probe and as such should be noted but not considered as part of the genotype.

Another possible indication of DXα amplification is the presence of a weak "1.1" and strong hybridization to the other dots in types {1.2, 1.3}, {1.2, 1.2}, {1.3, 1.3}, {1.2, 3}, {1.2, 4}, {1.3, 3}, or {1.3, 4}. The "1" dot is positive (darker than the "C" dot) in all of these types because they contain the 1 allele. However the "1.1" dot is weaker than the "C" dot. Therefore the presence of a weak "1.1" may indicate amplification of the DXα pseudogene and does <u>not</u> necessarily suggest that a sample is composed of a mixture or is contaminated.

Initials: RO Date: 4/10/92

4. Amplified Samples Which Have Been Stored in the Refrigerator Prior to Hybridization

Amplified samples which have been stored for even a short time at 4°C often show low intensity dots which were not visible if the samples had been hybridized immediately after amplification. These dots are less intense than the "C" dot and do not affect the typing results.

Initials: RCJ Date: 4/13/92

Troubleshooting

If the Chelex extracted DNA samples fail to amplify try one or several of the following: The choice of which procedure to use is up to the discretion of the Analyst in consultation with a Scientist or Assistant Laboratory Director.

(1) Amplify using an additional 10 units of Taq polymerase.

(2) Amplify a smaller aliquot (2-3 μL) of the DNA extract to dilute potential Taq polymerase inhibitors.

(3) Amplify up to 40 μL of the DNA extract to ensure sufficient high molecular weight DNA is present.

(3) Re-extract the sample using a smaller area of the stain or less biological fluid to prevent saturation of the Chelex and to dilute potential Taq polymerase inhibitors.

(4) Re-extract the sample using a larger area of the stain or more biological fluid to ensure sufficient high molecular DNA is present.

(5) Re-extract the samples following the appropriate Organic DNA Extraction Procedure from the RFLP Protocol. Amplify 10 ng of material as calculated by the yield gel.

(6) Add BSA to a final concentration of 160 μg/mL in the amplification reaction.

(7) Add Ficoll to a final concentration of 0.5%, in the amplification reaction.

(8) Prior to amplification, heat treat the extracted DNA at 94°C for 30 minutes.

Initials: EC Date: 4/13/92

(9) Purify the extracted DNA on a Centricon-100 ultrafiltration device as follows:

a) Assemble the Centricon-100 unit according to the manufacturer's directions and label each unit.

b) Add 1.5 mL TE Buffer to the upper Centricon-100 reservoir.

c) Add the entire extracted DNA sample to the top of the TE Buffer. Cover the tube with Parafilm™. Use a sterile needle to punch a pinhole in the Parafilm being careful not to touch the solution with the needle. Centrifuge in a fixed-angle rotor (see Centricon instructions) at 1000 x g at room temperature for 20 minutes. The DNA sample will remain concentrated in about 20 to 40 μL of TE Buffer in the bottom of the upper Centricon reservoir and molecules with molecular weights of less than about 100,00 daltons will pass through the filter. Discard the effluent in the lower reservoir.

Note: The Centricon units are sensitive to rotor force. Do not centrifuge above 1,000 x g. Centrifugation time can be increased if the volume does not reduce to 40 uL in the specified 20 minutes.

d) Add 2 mL of TE Buffer to the concentrated DNA solution in the upper Centricon reservoir.

e) Repeat the centrifugation and wash steps in c through d twice for a total of 3 washes. After the last wash cycle collect the approximately 40 μL concentrated DNA sample (as per Centricon instructions) by inverting the upper reservoir into the provided retentate cup, and centrifuging at 500 x g for 2 minutes to transfer concentrate into cup. Label retentate cup. The sample is now ready for the PCR amplification process. Store the sample at 2 to 8°C or freeze at -20°C until ready to perform PCR.

Initials: RCl Date: 4/13/92

Problem	Possible Cause	Remedy
1. No or faint signal from both the Genomic Control DNA and DNA test samples	No or insufficient amplification	Run Amplified DNA on agarose gel (see page 23)
		If no product on gel see below
No product on gel: Repeat test from Amplification Step	No or insufficient DNA added to PCR Reaction Mix	Add >2 ng DNA
	$MgCl_2$ not added to PCR Reaction Mix	Add $MgCl_2$ Solution
	Thermal Cycler failure or wrong program	See Thermal Cycler Manual
	Tubes popped up from Thermal Cycler heating block during amplification	Push tubes firmly into contact with Thermal Cycler heat block after first cycle.
		If 239-242 bp product visible on gel see other possible causes
Product visible on gel: Repeat test from Hybridization Step using new Probe Strips	Hybridization or Stringent Wash temperature too high	Check water bath temperature, should be 55°C
	DQα Hybridization or DQα Wash Solution salt concentration too low	Check DQα Hybridization and DQα Wash Solutions; prepare new solutions
	Stringent Wash time too long	Wash for correct time
	Inadequate agitation of the DNA Probe Strips during hybridization	Check rotation speed of shaking water bath
	Amplified DNA was not added to Probe Strips	Add Amplified DNA
	Amplified DNA was not denatured	Check heat block temperature (95°C) and complete DNA addition within 30 seconds

Initials: RCJ Date: 4/13/92

Problem	Possible Cause	Remedy
	Enzyme Conjugate was not added to the DQα Hybridization/ Enzyme Conjugate Solution	Make new DQα Hybridization/ Enzyme Conjugate Solution. Add Enzyme Conjugate
Product visible on gel: Repeat test from Color Development Steps using previously developed strips	Hydrogen peroxide was not added or too much was added to Color Development Solution	Use 3% hydrogen peroxide. Make new Color Development Solution
	Hydrogen peroxide inactive	Use new bottle of hydrogen peroxide. Make new Color Development Solution
	Chromogen was not added to the Color Development Solution	Make new Color Development Solution. Add Chromogen
2. Positive signal from Control DNA, no signal from DNA test sample	The quantity of DNA test sample is below the assay sensitivity	Repeat amplification increasing DNA volume, if possible
	The test sample contains PCR inhibitors e.g. heme compounds	Try adding additional Taq Polymerase or further purification procedures
3. High Probe Strip background color	Low or lack of SDS in DQα Hybridization and/or DQα Wash Solutions	Prepare new DQα Hybridization and DQα Wash Solutions with correct amount of SDS
	Inadequate agitation of the DNA Probe Strips during hybridization and washing steps	Check rotation speed of shaking water bath
	Excess amounts of Enzyme Conjugate added to DQα Hybridization Solution	Make new DQα Hybridization/ Enzyme Conjugate Solution with correct amount of Enzyme Conjugate
	Exposure to light during color development	Cover tray lid with foil

Initials: RCS Date: 4/13/92

Problem	Possible Cause	Remedy
4. High Probe Strip background color upon storage	Exposure to strong light and oxidizing agents	Store strips in the dark away from oxidizing agents
	Insufficient water washes after Color Development	Increase number of water washes
5. Presence of wrong alleles in Control DNA	Hybridization or Stringent Wash temperature too low	Check shaking water bath temperature, should be 55°C
	DQα Hybridization or DQα Wash Solution salt concentration too high	Check DQα Hybridization and DQα Wash Solutions; prepare new solutions
	Stringent Wash time too short	Wash for correct time
6. Signal weaker than "C"	Hybridization or Stringent Wash temperature too low or high	Check shaking water bath temperature, should be 55°C
	DQα Hybridization or DQα Wash Solution salt concentration too low or high	Check DQα Hybridization and DQα Wash Solutions; prepare new solutions
	Stringent Wash time too short or long	Wash for correct time
	Mixed sample or contamination	see page 38
	DXα	see page 38
	Subtypes of the DQα4 allele	see page 38
"All but 1.3" signal weaker than "C" for a 1.3,4 or 4,4 genotype	Inherent property of type	see page 33
"1,2, 1.3, 4" signal weaker than "C" and "4" signal stronger than "C"	Subtype of the DQα4 allele (Inherent property of subtype)	see page 38

Initials: RCJ Date: 4/13/92

Problem	Possible Cause	Remedy
Weak "1.1" signal with a strong signal for 1-2 additional alleles	May be DXα	see page 38
"1" dot darker than "3" dot	Inherent property of type	see page 33
More than two alleles present	Hybridization or Stringent Wash temperature too low	Check shaking water bath temperature, should be 55°C
	DQα Hybridization or DQα Wash Solution salt concentration too high	Check DQα Hybridization and DQα Wash Solutions; prepare new solutions
	Stringent Wash time too short	Wash for correct time
	Mixed sample or contamination	see page 38

Initials: RC Date: 4/13/92

References

1. Higuchi, R., von Beroldingen, C.H., Sensabaugh, G.F., and Erlich, H.A. "DNA typing from single hair". Nature 332: 543-546, 1988.

2. Gyllensten, U.B., Erlich, H.A. "Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA DQα locus". Proc. Natl. Acad. Sci USA 85: 7652-7656 1988.

3. Saiki, R.K., Walsh, P.S., Levenson, C.H., and Erlich, H.A. "Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes". Pro. Natl. Acad. Sci USA 86: 6230-6234 1989.

4. Helmuth, R, et.al. "HLA DQα allele and genotype frequencies in various human population determined by using enzymatic amplification and oligonucleotide probes." Am. J. Hum. Genet. 47: 515-523.

5. von Beroldingen, C., Blake, E.T., Higuchi, R., Sensabaugh, G.F., Erlich, H. "Applications of PCR to the analysis of biological evidence" (Chapter 17, p 209), in PCR Technology: Principles and Applications for DNA Amplification, ed. Erlich, H., 1989.

6. Huguchi, R., Kwok, S. "Avoiding False Positive with PCR". Nature 339: 237-238, 1989.

7. Amplitype™ Users Guide, Version 2, Cetus Corporation. 1990.

8. HLA DQα Forensic DNA Amplification & Typing Kit, 50 Test Kit, Package Insert. Catalog No. N808-0056. 1991

9. Walsh, P.S., Metzger, D.A., and Higuchi, R. "Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10: 506-513. 1991.

Initials: RCJ Date: 4/13/92

APPENDIX A-Preparation and Storage of Reagents Supplied With the HLA DQα Kit

Chromogen Solution

When reconstituting chromogen, rinse the graduated cylinder several times with 100% ethanol to remove traces of water. Bring the bottle of AmpliType™ Chromogen to room temperature. Before opening, tap bottle on the lab bench to shake to Chromogen (TMB) to the bottom of the bottle. Remove the stopper carefully to prevent loss of the powder. Reconstitute by slowly adding 30 mL of room temperature reagent grade 100% ethanol to the bottle. **Note: Do not use ethanol that has been stored in a metal container. Do not use 95% ethanol.** Recap the bottle. Seal the stopper with parafilm. Shake the bottle to remove any remaining Chromogen from cap. Shake on orbital shaker for 30 minutes to solubilize. Record the reconstitution date on the bottle. Protect from rust. Store at 2-8°C. Stable for four months after reconstitution.

PCR Reaction Mix

Upon receipt of the Kit, remove the PCR Mix bottle. Carefully aliquot 50 μL PCR Mix into each of the reaction tubes provided using a repeat pipettor or a P-200. **Caution: This should be done in the PCR set-up room which is free from amplified DNA.**

Place PCR Reaction Mix tubes in a rack not used for DNA preparation or amplified DNA handling.
Store tubes separated from any source of DNA at 2 to 8°C.

Initials: RC Date: 4/13/92

Storage and Stability of the Amplitype HLA DQα Kit

The AmpliType™ HLA DQα Forensic DNA Amplification and Typing Kit should be stored at 2-8°C.

The Kit should be isolated from any sources of contaminating DNA, especially amplified PCR product.

The DNA Probe Strips should be stored with the desiccant in the glass tube with the screw-cap securely tightened, protected from light, at 2-30°C.

Under these conditions, components of the kit are stable until the printed expiration date.

Initials: RG Date: 4/17/92

Washing and Re-Use of the DNA Typing Trays

1. To each well of the DNA typing tray, add approximately 10 mL of 95-100% ethanol.

2. Cover the DNA typing tray with the lid and carefully agitate for 15 to 30 seconds to dissolve any residual Chromogen.

3. Remove the lid and pour off the ethanol from each well. Visually inspect each well for the presence of Chromogen (faint blue color). If necessary repeat steps a and b to remove any residual Chromogen.

4. Rinse each tray well and the tray lid with dH_2O.

5. Allow trays and lids to air dry. The trays are now ready for re-use.

Initials: RCJ Date: 9/10/92

Appendix B- Equipment and Supplies for PCR Typing

General Equipment and Supplies

Autoclave
Balance
Deionizer column for water
Calibrated Thermocouple
Labware, glassware including beakers, graduated cylinders, Erlenmeyer flasks
Freezer, -80°C and -20°C
Refrigerator
Lab Coat
Microwave oven
pH meter with calomel (Tris) electrode
Reference buffers
Polaroid Camera with type 667 film
Stir plate, Stir bar
Thermometer (N.B.S or equivalent)
Timer, 60 minute (±1 minute)
Parafilm
U.V. Transilluminator
Absorbent bench paper
Absorbent tissues (e.g., Kimwipes)
Evidence bags or envelops (for storage)
Filter paper, Whatman 3M, Whatman #1
Lab marker, waterproof ink
Microscope slides and cover slips
Paper, white
Paper towels
Parafilm
Plastic disposable pasteur pipettes
Pipettes, plastic, sterile disposable to deliver 1 to 10 mL
Pipette bulbs or electric pipettors
Wash bottles

Initials: LCJ Date: 4/13/92

DNA Extraction Work Area Dedicated Equipment and Supplies

This work area should be used only for evidence handling and for extraction and isolation of DNA. Microscopy, photography, and any other activities that involve the handling of evidence samples before amplification should also be performed in this work area. These items should never leave the DNA extraction work area.

1. Pipettors: Adjustable 2 to 20 μL, 20 to 200 μL and 200 to 100 μL. If possible, reserve one set of pipettor for reagent use only, and another set for the handling of samples containing DNA.
2. Microcentrifuge tube racks
3. Microcentrifuge tubes (1.5 mL)
4. Microcentrifuge
5. Refrigerated Centrifuge and Rotors
6. Scissors
7. Disposable razor blades
8. Pipette tips and Filter pipette tips
9. Pipette bulb
10. Refrigerator
11. Hot/Stir plate
12. Laboratory glassware
13. Disposable gloves
14. Forceps
15. Vortex mixer
16. Freezer to -20°C
17. Heat blocks and Heater
18. Laminar flow/biological/biosafety cabinet with U.V. source
19. Centricon-100 microconcentrators
20. Toothpicks
21. Boiling Water bath racks
22. 1 L Pyrex glass beakers
23. Ice
24. Ice bucket

Initials: EC Date: 4/13/92

PCR Set Up Work Area Dedicated Equipment and Supplies

This work area is used only for adding $MgC1_2$ Solution, mineral oil, and sample DNA to the PCR Reaction Mix. These items should never leave the PCR Setup Work Area

1. Combitip™ repeat pipettor with combitips
2. Dedicated pipettor (adjustable 2 to 20 μL and 20 to 1000 μL for adding DNA samples to the PCR Reaction Mix.
3. Sterile filter pipette tips
4. Microtube de-capping devices, autoclavable
5. Microtube rack
6. Disposable gloves
7. Microcentrifuge
8. Heatblock and heater
9. Refrigerator
10. Vortex Mixer
11. Kimwipes

Initials: RCJ Date: 3/5/93

Amplified DNA Work Area Dedicated Equipment and Supplies

This work area is used only for those activities that involve the handling of amplified DNA. This includes DNA typing (Hybridization and Color Development), gel electrophoresis of amplified DNA, waste disposal of amplified DNA solutions, and storage of amplified DNA. Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of Amplified DNA Work Area.

Samples that have not yet been amplified should never come in contact with this equipment.

1. PE Cetus DNA Thermal Cycler
2. 1 to 20 μL and 20 to 200 μL adjustable pipettors
3. Disposable gloves
4. Microtube de-capping device
5. Disposable serological pipettes
6. Pipettor for serological pipettes
7. Towel wipes
8. Kimwipes
9. Microtube racks
10. Filter Pipette tips
11. Laboratory glassware
12. Gel electrophoresis apparatus
13. Power supply for electrophoresis
14. Disposable gloves
15. Sink
16. Refrigerator
17. Aluminum foil
18. Seal-a-meal bags
19. Waterproof marking pen
20. Water bath, stationary
21. Weight, (approx. 1kg)
22. Shaker, variable speed, orbital platform
23. Shaker, water bath
24. Filter forceps
25. Microcentrifuge
26. Heat block and heater
27. Vortex Mixer
28. Microcentrifuge tubes, 0.5 mL & 1.5 mL
29. 2 to 10 mL multi-dispensers

Initials: CCJ Date: 4/13/92

Amplified DNA Work Area Dedicated Equipment and Supplies

This work area is used only for those activities that involve the handling of amplified DNA. This includes DNA typing (Hybridization and Color Development), gel electrophoresis of amplified DNA, waste disposal of amplified DNA solutions, and storage of amplified DNA. Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of Amplified DNA Work Area.

Samples that have not yet been amplified should never come in contact with this equipment.

1. PE Cetus DNA Thermal Cycler
2. 1 to 20 μL and 20 to 200 μL adjustable pipettors
3. Disposable gloves
4. Microtube de-capping device
5. Disposable serological pipettes
6. Pipettor for serological pipettes
7. Towel wipes
8. Kimwipes
9. Microtube racks
10. Filter Pipette tips
11. Laboratory glassware
12. Gel electrophoresis apparatus
13. Power supply for electrophoresis
14. Disposable gloves
15. Sink
16. Refrigerator
17. Aluminum foil
18. Seal-a-meal bags
19. Waterproof marking pen
20. Water bath, stationary
21. Weight, (approx. 1kg)
22. Shaker, variable speed, orbital platform
23. Shaker, water bath
24. Filter forceps
25. Microcentrifuge
26. Heat block and heater
27. Vortex Mixer
28. Microcentrifuge tubes, 0.5 mL & 1.5 mL