FIRST EUROPEAN-AMERICAN INTENSIVE COURSE IN PCR BASED CLINICAL AND FORENSIC TESTING

September 23-October 3

1997

Laboratory for Clinical and Forensic Genetics
Clinical Hospital, Split
Spinciceva 1, Split 21 000
Croatia
Tel/FAX 385 21 365 057

We would to thank the following sponsors for their kind support for this workshop. This list is not complete and we hope all will be acknowledged at a later date.

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Note from the editor:

I wish to thank all of the people who contributed to this lab manual. I apologize for the misspeklling of people's name, but the e-mail converter had a grand time. I also made the assumption that all manuscripts were spell checked before submission.

This lab manual is a working manual; the spirit of the conference is to ask questions, receive answers and record them to take home to your labs. Please feel free to write on the backs of the pages in the workshops and lectures. The manual was designed so that the blank page opposite of the procedure being studied can be used as writing space. Record your observations, tricks of the trade, and contact numbers. Enjoy the workshops.

I wish to also thank Dr. Moses Schanfield and Dr. Dragan Primorac for allowing me the opportunity to work on this manual.

Dr. Dean S. Burgi

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8:00 a.m. Welcome VD Drs. Shchanfield, Andelinovic and Primorac
Hon. I.Skaric Mayor of Split
Hon. B. Luksic Prefect, County of Split and Dalmatia
Dr. M. Biocic, Director of CHS

Dean of Medical School

8:25 a.m. Searching for missing and imprisoned persons: Organization of the identification in the Republic of Croatia. Dr.I. Kostovic

8:45 a.m. DNA analysis in criminal investigation Dr.H. Lee

9:30 a.m. High profile cases Dr.M.Baden

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11:25 a.m. PE and its role in the biological revolution Dr.K-H.Franzen

11:55 a.m. Fluorescent detection methods Dr.D. Burgi

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12:35 a.m. Morning section adjourns

2:05 p.m. Implication for gene therapy of dominantly inherited Dr.D.Rowe diseases of connective tissues

2:50 p.m. Dendritic cell education for T-cell mediated imunotherapy Dr.S.Vuk-Pavlovic

3:35 p.m. Molecular basis of genetic predisposition to genetic diseases Dr.M.Radman

4:10 p.m. Cystic fibrosis and mutation analysis Dr.P F Pignatti

4:55 p.m. The use of antisense hammerhead ribozymes to selectively Dr.P.Tsipouras target single nucleotide mutations

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CLINICAL SECTION

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8:20 a.m. PCR analysis of gene expression in rare cell populations Lec Dr.A.Dietz

9:05 a.m. PCR analysis of gene expression in rare cell populations Lab L 1&2 NMS Dr.A.Dietz

10:45 a.m. Coffee break

11:00 a.m. Developments in automated human genetic analysis Lec MR NMS Dr.D.Schuster

11:45 a.m. Morphogenic proteins: From basic discovery to clinical r vehicles for somatic gene Therapy of bone Lec MR NMS Dr. D.Rowe

12:30 p.m. Morning section adjourns

2:00 p.m. Interon gamma deficiency and susceptibility to mycovacterial diseases: a possibility for prenatal diagnostic Lec MR NMS Dr. Z. Dembic

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3:45 p.m. Detection and scanning of mutations and polymorphism using immobilized mismatch binding protiens Lec MR NMS Dr. M. Radman

4:30 p.m. Coffee break

4:45 p.m. Th eIGF pathway: A therapeutic target for rhabdamyosarcoma Lec MR NMS Dr. T. Kalebic

5:15 p.m. Demonstration of the Guest Elchrom Scientific Program Lab Dr. B. Kozulic

7:00 p.m. Adjourns

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8:00 a.m. Molecular diagnosis and monitoring of leukemia Lec MR NMS Dr. A. Biondi

8:45 a.m. Glycosphingolipid phenotype of TNF receptor I knock out mice Lec Drs. A. Marusic, J. Muething
9:30 a.m. Diagnosis of germ cell tumors Lec MR MNS Dr. I. Damjanov
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4:30 p.m. Premature STOP coden and its influence to mRNA transport Lec MR NMS
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Dr. D. Ramljak
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2:45 p.m. PCR genotyping of CYP2D6 gene Lab L 3 NMS I.Zuntar,M.Stefanovic
3:30 p.m. RFLP and SSCP of the HGF gene (IV exon PCR product) Lab L 3 NMS
I.Zuntar,M.Stefanovic
4:30 p.m. Detection of minimal residual disease in CML by PCR Lab L 3 NMS Dr. B.Grahovac
5:30 p.m. STR analysis to look for Aneuplody Lec MR NMS Dr. M.Schanfield
6:15 p.m. Adjourns

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8:00 a.m. Single strand confirmation polymorphism analysis
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8:45 a.m. PCR-SSCP method for the detection of point mutation in
the LDLR gene Lab MR NMS R. Zrinski, MS
9:30 a.m. Detection of BCR-ABL transcript by RT-PCR transcript Lab L 3 NMS Dr.R. Zadro
10:15 a.m. Coffee break
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11:45 a.m. Genetic research and clinical testing and quality
assurance and informed consent: International
Science and human rights perspectives. Lec MR NMS Dr. Gale Gardiner
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1:30 p.m. History of human genetics in Croatia Lec MR NMS Dr. Lj.Zergolern-Cupak
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deficiency alleles Pi Z and Pi S Lab L 3 NMS I.Zuntar, M.Stefanovic
3:30 p.m. Adjurn
6:00 p.m. Farewell Party

Key to activities and locations:
Lab: Laboratory exercises and/or demonstrations
Lec: Lecture
CHS: Clinical Hospital Split, Spinciceva 1
MR NMS: Main Room at New Medical School, Soltanska 28, 1th floor

MA NMS: Main Auditorium at New Medical School, Soltanska 28, 1th floor
L 1 NMS: Laboratory #1 at New Medical School, Soltanska 28, 1th floor
AR CHS: Autopsy Room at Department of Pathology at Clinical Hospital Split, Spinciceva 1, 1th floor
MR CHS: Main Room at Department of Pathology, Clinical Hospital Split, Spinciceva 1, 1th floor
VD: Villa Dalmatia, Marjan, Split

Restaurant with fast food and drinks will be avilable at the NMS during the whole Intensive Course

Exchange office will be open at the Hotel Split and at the NMS during the whole Intensive Course
RATE: 1 USA $ = 6.29 kn, 1 DEM = 3.48 kn, 1 FRF 1.01 kn, 100 ITL= 0.35 kn, 1 GBP= 10.01 kn

Scientific and Organizing Commitiee (available 24 hours-during the Intensive Course):
Dr. M. Schanfield : 098 264 064 (if you call from Croatia)
Dr. D. Primorac : 098 264 844
Dr. S. An l elinovic : 099 478 635
Social program:

Wednesday, September 24
Sightseeing of Split and visit to Exhibition of Postmodern painting
Cocktail Party (8:00 p.m. Diocletian Palace)
Friday, September 26
Welcome/Farewell Party (8:00 p.m. Villa Dalmatia)
Saturday, September 27
Cruise along the Adriatic Coast
Sunday, September 28
Sightseseeing of Split or Trogir
or
Visit to Me l ugorje
Wednesday, October 1
Farewell Party (8:00 p.m. at Hotel Split)

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4:30 p.m. DNA quantification Lab L 1 NMS Dr. M. Schanfield
5:00 p.m. PM and DQA1 testing Lab L 1 & 2 NMS Dr. D. Primorac, I. Drmic
6:30 p.m. Adjourn

Collection and Analysis of Evidence in Sexual Assault Cases

Manfred Hochmeister, MD
Forensic DNA Identification Laboratory
Insitute of Legal Medicine, Bern, Switzerland

Procedures to be taught in the course:

1. The new sexual assault evidence collection kit **RAPE CARE®**
   A complete kit for medical / forensic examination and evidence colletion for the female and male Patient
2. **RAPE CARE®** one hour teaching video demonstrating an actual examination and evidence collection of a female and male patient
3. The **"SWAB BOX"**, a new device for collection and storage of evidence at a crime scene, in sexual assault investigations and for paternity testing and DNA-databasing
4. The acid phosphatase (**ACP**) presumptive test Phosphatesmo KM
5. The prostate specific antigen (**PSA**) confirmatory test PSA-check-1
6. The seminal vesicle specific (**MHS-5**) confirmatory test SEMA™
7. The human **hemoglobin** specific blood confirmatory test HEXAGON-OBTI
8. The **amylase** presumptive test GRANUTEST 3 MERCK
9. The Oppitz stain (**Christmas-Tree-Stain**) for microscopy
10. The recovery of sperm cell DNA and vaginal cell DNA from mixed stains **(differential lysis)**
11. **The Slot-Blot quantification** QuantiBlot™ Human DNA Quantitation Kit
12. **RFLP and PCR systems** currently in use for DNA-databasing

1. The new sexual assault evidence collection kit RAPE CARE®
(A complete kit for medical / forensic examination and evidence colletion for the female and male Patient)
This new kit focuses on the latest developments in DNA techology. In a simple eight step checklist the medical / forensic examination of the patient and the forensic evidence collection are carried out. The contents of the kit are:
"General Information and Assault History" checklist
1. Collection of oral swabs and oral smear
2. Collection of clothing
3. Collection of evidence on patient's body such as matted hairs, head hair combing, saliva and semen on skin, bite mark evidence, fingernails and foreign debris
4. Physical head to toe examination
5. Anal examination and collection of anal swabs and anal smear
6. Genital examination including inspection, collection of matted pubic hairs, pubic hair combing, external vaginal swab, speculum examination, collection of vaginal swabs, colletion of cervical swabs, vaginal smear, diagnostic procedures, hymen examination.
7. Collection of blood and urine
8. Checklist for diagnosis, treatment, follow up

The evidence collected on cotton swabs is packed in so called "SWAB BOXES". The kit and clothing bags will be stored at room temperature (saving the necessity for freezer space). Blood and urine are frozen.

The **RAPE CARE® KIT** is available in January 1998 and can be ordered from the Institute of Legal Medicine, University of Bern, Switzerland, Bühlstrasse 20, CH-3012 (Fax: ++41 31 631 38 33).

2. RAPE CARE® ONE-HOUR TEACHING VIDEO

In order to teach providers who have never cared for a sexually assaulted patient and to improve the technique of experienced providers a one hour teaching video presents in detail the medical / forensic examination of the male and femlae patient, including a view of an actual genital examination of the female and male patient. It will also demonstrate the complete evidence collection process, examples of physical findings on the body, and the subsequent analysis of evidence in the forensic laboratory. The video is available in PAL and NTSC System in January 1998 and can be ordered from the Institute of Legal Medicine, University of Bern, Switzerland, Bühlstrasse 20, CH-3012 (Fax: ++41 31 631 38 33). It will only be sold to registered health care providers (Universities, hospitals, physicians, forensic nurses).

3. The "SWAB BOX", a new device for collection and storage of evidence at a crime scene, in sexual assault investigations and for paternity testing and DNA-databasing

The proper collection and preservation of biological evidence is a critical point for a successful DNA analysis. The method of collection depends largely on the type of substrate and the state and condition of the biological evidence. In general, liquid body fluids can be recovered using dry cotton swabs. Dried stains of these body fluids can be eluted onto sterile cotton swabs moistened with 0.9 % saline by rubbing the swabs on the stained area.

In order to facilitate the proper collection and preservation of biological evidence recovered on cotton swabs, a simple foldable "SWAB BOX" made of cardboard for drying and long - time storage of swabs should be used. This box can act as a substitute for a swab dryer or freezer, if such equipment is not available, and it can replace expensive shipment, courier service, or cooling devices.

The box is made of 1 millimeter corrugated cardboard and can be sealed on both ends with a special non-removable security seal. Immediately after collection swabs are placed into the drying racks within the cardboard box, which is subsequently folded, labeled, sealed and initialed. Depending on temperature and relative humidity swabs completely air dried within the sealed box in 4 - 9 hours. Swabs air dried in this box can be shipped to the forensic laboratory and stored at room temperature.

In the "SWAB BOX" the evidence can be immediately packed, labeled and sealed after collection, thus preventing sample switch, cross contamination, and degradation. The simple "SWAB BOX" has now been extensively tested by crime scene investigators in Switzerland. It is a valuable device for the collection of biological evidence at a crime scene, during sexual assault examinations, and for PCR-based data basing and paternity testing.

4. The acid phosphatase (ACP) presumptive test Phosphatesmo KM

Phosphatesmo KM test paper indicates the presence of acid phosphatase by developing a violet spot against a white background. The carrier is normally subjected to a macroscopic examination for suspicious spots. Textiles generally assume boardlike consistency and yield a bluish, white or yellow fluorescene under UV-light. Phosphatesmo KM offers the possibility to determine, on the spot, the presence of ACP.

A test paper is lightly moistened with sterile water to serve as a neagtiv control. A second test paper is lightly moistened with sterile water and either one fiber of the stain in question (or one fibre from a cotton swab) is placed onto the paper or the paper is directly pressed onto an edge of the stain in question or onto the cotton swab. It has been demonstrated that the contact of the reagents present on the test paper does not interfere with subsequent DNA testing. A clear violet coloration indicates the presence of ACP.

In current forensic practice, the test for prostatic acid phosphatase (ACP) is considered a presumptive test. ACP activity from endogenous vaginal sources and plant materials which contain the enzyme may give false positive results. False negative results may be obtained if degradation of the enzyme occured. Initially high postcoital ACP levels in vaginal fluid soon decline into the endogenous range. Acid phosphatase levels from postcoital swabs showed a mean decay time of 8 - 14 hours.
The sensitivity of the test is in the range of a 1:320 to 1:1000 fold dilution. Basically the ACP method is very sensitiv, but in the Phosphatesmo KM strips this sensitivity is attentionally not used to avoid unspecific reactions from low concentrations of ACP in vaginal fluid.

5. The prostate specific antigen (PSA) confirmatory test PSA-check-1

Prostate specific antigen (PSA, also known as p30), a glycoprotein produced by the prostatic gland and secreted into seminal plasma, is now accepted as a marker for detecting semen in criminal cases involving vasectomized or azoospermic males. It is accepted as a confirmatory test for the presence of seminal fluid. The reported frequency of azoospermia of 1-9% in seminal stains or swabs examined in sexual assault cases can be expected to rise, since the frequency of contraceptive vasectomy has been estimated to be 750 000 to 1,000 000 per year in the United States.
Successful isolation and purification of PSA from human semen has made it possible to develop immunological methods for its detection. Methods for the detection of PSA include Ouchterlony double diffusion, crossover electrophoresis, rocket immunoelectrophoresis, radial immunodiffusion, and ELISA. The extremely sensitive ELISA technique can detect PSA in body fluids at concentrations as low as Å 4 ng per milliliter. A disadvantage of all techniques is that they are either not sensitive enough or cumbersome and time consuming to perform in forensic laboratories dealing only with a few cases per week.
PSA specific membrane tests offer a reliable approach for the forensic casework laboratory to test for the presence of PSA within 10 minutes. The sensitivity of the test is in the range of a 1: 100 000 - 1 million fold dilution. All tests use monoclonal antibodies directed against constant epitopes in free and complexed PSA as well as all its isoforms.
Here the PSA-check-1test (VED-LAB France) is described, but all tests offer the same sensitivity. The test is carried out as described by the manufacturer.
Extraction of specimens is performed in 550 µl of HEPES buffered saline for 1 h at 4°C (distilled water or other buffers suitable for further DNA extraction may be used as well). This procedure recovers approximately 99 % of the extractable PSA on the swab. After a 3 min. centrifugation step 200 µl of the supernatant are removed for the PSA test. A positive test result (> 4 ng PSA / ml) is indicated by the formation of a red line in the test and control region of the membrane and the result is read after 10 minutes.
The membrane tests does not detect PSA in any samples from women. Besides semen from both normal and vasectomized men, positive results are only obtained from post-ejaculate urine and male urine from adult men, when the urine samples are directly added to the membrane tests. However, it is well established that PSA does occur in these fluids. The sensitivity and detection limits of the rapid PSA specific membrane tests are equal to an enzyme-linked immunoabsorbent assay.
Compared to time consuming ELISA-based measurements of PSA, rapid membrane tests offer the same sensitivity (4 ng PSA / ml) within 10 minutes using 200 µl of supernatant from the DNA extraction procedure. They are a reliable and extremely sensitive tool for the identification of seminal fluid from vasectomized individuals. If the presence of male urine is in question, additional testing using the seminal vesicle specific antigen MHS-5 (SEMA test kit) can be useful. The mean vaginal decay time of PSA is 27 hours (13-47 hours). After 48 hours p30 concentrations drop to undetectable leves.

6. The seminal vesicle specific (MHS-5) confirmatory test SEMA™

The MHS-5 antigen is detected using the qualitative SEMA kit (Humagen Inc., Charlottesville) as described by the manufacturer. The sensitivity of the test is in the range of a 1:75 000 - 160 000 fold dilution. The antigen has been detected in no human biological fluid other than semen and is not present in male urine. SEMA is an enzyme-linked immunosorbant assay, utilizing a soluble complex (biotinylated anti-MHS-5 and streptavidin-horseradish peroxidase) to detect traces of seminal fluid. Proteins in the ejaculate bind nonspecifically to the test immunoplate plastic wall. If seminal plasma antigens are present, the soluble complex will bind to them and remain bound following removal of the unbound complex by several washings. Bound complexes are detected by the addition of H_2O_2 and ABTS (2.2'-azino-di[3-ethylbenzthiazoline sulfonic acid-6], the chromogenic substrate for the enzyme peroxidase. The absorbance of the blue-green color is measured at 405 nm by an ELISA reader.
The mean vaginal decay time of MHS-5 has not been determined.

7. The human hemoglobin specific blood confirmatory test HEXAGON-OBTI
This immunochromatographic 1-step occult blood test (Hexagon Obti Test) offers an extremely high sensitivity and specificity for the forensic identification of human blood. The Hexagon Obti Test (Human Gesellschaft für Biochemica und Diagnostica mbH, Taunusstein, Germany, Fax: ++49 61 288 87 51 00) is a immunochromatographic 1-step test for the detection of fecal occult blood. As used in a clinical setting, stool is transferred into a 1 ml sample tube with transport medium (Tris buffer, pH 7.5) and extracted. One drop of this mixture is added to the test device. Human hemoglobin (hHb) in the sample reacts with a conjugate consisting of blue colored particles coated with anti-human Hb antibodies. The immune complex thus formed migrates to the test zone where it is captured by a second antibody directed against hHb forming a blue test line to indicate a positive result. Blue particles that did not react migrate further and are bound to a second line by immobilized anti-conjugate antibodies. This control line indicates proper function and correct handling of the test.
A blood stained fiber from clothing, a small blood particle scrapped off a stain, a fiber from a moistened cotton swab used to collect a stain, and liquid blood may be used. Extraction of specimens is performed either on a slide or in a 1.5 ml tube by adding 1-2 drops of the provided Tris-buffer. Fresh blood stained material can be used immediately for the test, while old bloodstains (stored up to 5 years at room temperature were tested) should be soaked in the buffer for approx. 15-30 minutes in order to extract the hemoglobin. The extract is then added to the Hexagon Obti test. A positive test result is indicated by the formation of a blue line in the test and control region of the membrane and the result is read after 10 minutes. Very small blood stains on clothing ($< 1 \times 1$ mm) can be cut, soaked in the buffer (the length of the soak depending on the age of the stain), subjected to a 3 min. centrifugation step and 100 µl of the supernatant is used for the confirmatory test. The samples is then subjected to DNA extraction.
Using the test, > 0.1 µg Hb / ml buffer, corresponding to a blood dilution of 1: 1 000 000 or 1 µl in 1 liter can be detected. The test was found to be specific for human hemoglobin subtypes HbAo, HbA2, HbF, and HbS. No cross reactivity to hemoglobin from different species, including sheep, turkey, pork, beef, goat, horse, rabbit has been observed up to 1000 µg Hb / ml each. No cross reactivity to horse radish peroxidase has been observed up to 1000 µg / ml. Also no prozone effect has been detected up to a hemoglobin concentration of 2000 µg / ml in the buffer. However, care should be exercised when obtaining a negative test result from an old and encrusted blood stain, because it is necessary to soak the stain properly in order for the test to perform correctly.
It should be remembered, that because of the extreme sensitivity of the test trace levels of hemoglobin might be occasionally detected in body fluid samples other than blood (e.g. urine, semen, stool). However, knowing this fact, this limitation has no practical impact in the vast majority of cases. Compared to another fecal blood test reported for forensic use in 1994

(HemeSelect™ test, pos. results up to 1: 100 000 dilution) this immunochromatographic test offers now an increase in sensitivity of more than ten orders of magnitude.

8. The a-amylase presumptive test GRANUTEST 3 MERCK

The a-Amylase Granutest® 3 Merck (E.Merck, Darmstadt, Germany) is used to confirm presumptively the presence of saliva by the employment of a rapid and sensitive a-amylase assay, which neither consumes parts of the sample nor adversely affects the yield or quality of DNA. Here a combined a-amylase - DNA extraction protocol is described.

With the introduction of a new substrate (2-Chloro-4-nitrophenyl-b-D-maltoheptaoside) in 1984 [22], a sensitive colorimetric test kit is available (a-Amylase Granutest® 3 Merck). The principle of the test is the production of dyed low molecular weight sugars (Cl-PNP-G_2, Cl-PNP-G_3, Cl-PNP-G_4 [2-Chloro-4-nitrophenyl-b-D-maltoside, 2-Chloro-4-nitrophenyl-b-D-maltotrioside, 2-Chloro-4-nitrophenyl-b-D-maltotetraoside]) as a result of amylase action on Cl-PNP-G_7 (2-Chloro-4-nitrophenyl-b-D-maltoheptaoside). Cl-PNP-G_2 + Cl-PNP-G_3 are transformed by a-glucosidase to Cl-PNP-G_1 (2-Chloro-4-nitrophenyl-b-D-glucoside), which is converted by b-glucosidase to 2-chloro-4-nitrophenol + G1. The rate of formation of 2-chloro-4-nitrophenol is directly proportional to the a-amylase activity in the sample and is determined by measuring the increase in absorbance at 405 nm. Reference values provided in the kit are up to 200 U/l for serum / plasma and up to 1150 U/l for urine samples (reference values for saliva are not provided).

Determination of a-Amylase Activity: The tip of one cotton swab obtained from a bite mark or from the skin after oro-genital sexual acts is cut into small pieces and placed in a sterile Spin-EASE™ extraction tube (Gibco BRL). After addition of 400 µL buffer provided in the kit, the tube is vigorously shaken at 4°C for at least 2 hours to overnight and then subjected to centrifugation at 10 000 g for 5 min. The a-Amylase Granutest® 3 Merck is used for the determination of the enzymatic activity of a-amylase.The test kit contains vials with reagents (2-Chloro-4-nitrophenyl-b-D-maltoheptaoside, a-glucosidase, and b-glucosidase) and a solvent (50 mM KCl, 50 mM phosphate buffer, pH 6.8). The contents of one vial were dissolved using 3 mL of the solvent. To 1 mL of prewarmed (37°C) a-Amylase reagent 10 µL of the supernatant from the sample were added in a 1 cm light path, thermostated cuvette. The contents were mixed and after 5 min. the absorbance was measured against the solvent and again read after 1, 2, and 3 minutes in a filter photometer (Hg 405 nm) or spectrophotometer (405 nm). The increase in absorbance, DA / min, was calculated. According to the manufacterers protocol, when DA / min exceeded 0.16, the sample was diluted and the assay repeated. To determine the enzyme activity (U/l) in the sample, DA / min was multipled by a factor provided in the kits (9099). A reaction is considered positive, when DA / min in the sample exceeded three times the DA / min of the blank reagent control. Negative control samples (reagents) and positive control samples are processed in the same manner. For calibration, human control sera with an a-amylase activity of 208 U/l and 438 U/l at 37°C (Qualitrol® Human Serum Normal and Qualitrol® Human Serum Pathological; Merck) are used.

DNA Extraction after determination of a-amylase activity: After an amylase assay had been carried out, 200 µL of prewarmed 3 x stain extraction buffer (30 mM Tris, 30 mM EDTA, 300 mM NaCl, 6 % SDS, pH 10.2) and 15 µL Proteinase K (20 mg / mL) are added to the 390 µL amylase buffer (total volume 605 µL, pH Å 8.0), and the tube is incubated overnight at 56°C. On the following day an additional 15 µL Proteinase K (20 mg/mL) is added, and the sample is incubated for 2 more hours at 56°C. The cuttings are then removed with sterile forceps and placed into the spin insert. The spin insert is placed into the tube from which the material came and subjected to centrifugation at 10 000 g for 5 min. Then the spin insert with the material is removed and discarded. After addition of 700 µL phenol / chloroform / isoamylalcohol (25:24:1), the tube is vigorously shaken by hand for 2 min. to achieve a milky emulsion and subsequently subjected to centrifugation at 10 000 g for 3 min. The aqueous phase (top layer) is transferred to a new 2.0 mL Saarstedt tube with a screw cap, taking care not to transfer the interphase. To the aqueous phase 700 µL of water-saturated n-butanol are added in order to remove traces of phenol. The tube is vigorously shaken by hand for 2 min. and then subjected to centrifugation at

10 000 g for 3 min. The aqueous phase (bottom layer) is transferred to a Centricon™ 100 microconcentrator tube. After the transfer, the volume is brought up with sterile water to 2.0 mL. The sample reservoir is sealed with parafilm, and after punching a hole in the parafilm (using a sterile needle), the tube is subjected to centrifugation at 1000 g for 30 min. Then, 2 mL of sterile water are added to the sample reservoir, and the reservoir is sealed with new parafilm. Again the tube is centrifuged at 1000 g for 30 min. The DNA is recovered by back centrifugation at 1000 g for 5 min. The final sample volume is approximately 25 - 40 µl. Ten percent of the retentate is used to determine the quantity of human DNA by slot blot analysis.

9. The Oppitz stain (Christmas-Tree-Stain) for microscopic determination of the presence of spermatozoa

During the DNA extraction (see next paragraph) 1 µl of the pellet is spotted on a slide and briefly air dried. The staining procedure described by Oppitz uses Nuclear Fast Red (calcium red), or "Kernechtrot", (stain for 15 minutes) with indigo carmine in saturated picric acid as counterstain (stain for 15 seconds). Sperm cell heads are stained red, midpieces pink to green, and tails green with this procedure. Vaginal epithelial cells and their nuclei stain green.

10. Recovery of Sperm Cell DNA and Vaginal Cell DNA from Mixed Stains (Differential Lysis)

This method, reported by Gill et al in 1985 is one of the key methods in forensic applications. Stains or swabs containing sperm undergo differential lysis, i.e. the vaginal epithelium cells are lysed in a first step and the DNA which they release is removed. Then DNA is released from the spermatozoal heads using dithiothreitol (DDT) in a second step. In this way the (female) epithelial cell fraction can be separated from the (male) spermatozoal fraction. The following protocol has proved itself in practice for RFLP and PCR-based typing. It is a standard protocol for isolation of DNA in sexual assault cases dealing with vaginal swabs or stains. In order to perform a PSA confirmatory test, the following modifications have been made:
The swab or stain is placed into a sterile Spin-EASE™ extraction tube (Gibco BRL) and 550 µl of HEPES buffered saline, pH 7.5 is added. Shake for 1 h at 4°C (distilled water or other buffers suitable for further DNA extraction may be used as well). After a 3 min. centrifugation step 200 µl of the supernatant are removed for the PSA test. The PSA test isonly performed after a Christmas-Tree stain confirmed the lack of spermatozoas.
Then 50 µl 20% sarcosyl are added and the tube is shaken vigorously over night at 4°C. The swab is placed into the spin basket and the tube centrifuged for 3 minutes. The supernatant fluid in the tube is discarded. 1 µl of the pellet is spotted on a microscopic slide and stained using the Christmas Tree Stain. Then the swab is placed back into the tube and 200 µl Tris-EDTA-NaCl, 50 µl 10% SDS, 245 µl sterile water, 5 µl Proteinase K (20 mg/ml) are added. The tube is incubated at 37°C for 2 hours to isolate the DNA from the epithelial cells. Again, the swab is placed into the spin basket and the tube is cenrifuged for 5 minutes. The swab is then discarded and the supernatant fluid saved as "epithelial cell fraction". The sperm cell pellet is washed 3 x with 1 ml of HEPES buffered saline in order to remove female DNA. To the sperm cell pellet 200 µl TNE, 125 µl 10 % sarcosyl, 50 µl 0.39 M dithiothreitol (DTT), 115 µl sterile water, 10 µl Proteinase K (20 mg/ml) are added and the tube is incubated at 37°C for two hours to isolate the DNA from the sperm cells. This fluid is called "sperm cell fraction". Both fractions are extracted with an equal volume of phenol / chloroform / isoamylalcohol and purified using a Centricon-100 tube.
A carry-over of male DNA to the epithelial cell fraction may occur from white blood cells present in semen.

11. The Slot-Blot quantification

The quantitation of DNA is highly desirable for enhancing the quality of results when performing DNA typing, particularly for the PCR. Since only 1-5 ng of DNA template are required for PCR, determination of initial template DNA quantities can reduce undue consumption of biological evidence. Agarose gel electrophoresis / ethidium bromide staining fails to distinguish between

human and non human genomic DNA and generally cannot detect less than 5 ng of DNA. If a small aliquot of a sample contains a quantity of DNA just below the treshold of ethidium bromide staining, no assay result can be obtained. Yet the sample may contain sufficient human genomic DNA for several assays by PCR.

The QuantiBlot™ Human DNA Quantitation Kit provides reagents and protocols necessary for quantitation of human DNA. The procedure is based on the hybridization of a biotinylated probe to DNA samples immobilized on a nylon membrane (Walsh et al., 1992). The DNA probe is complementary to a primate-specific alpha satellite DNA sequence at the locus D17Z1. Subsequent binding of enzyme conjugate:HRP-SA (horseradish peroxidase-streptavidin) to the biotin moiety of the probe allows for either colorimetric or chemiluminescent detection. In the case of colorimetric detection, the oxidation of tetramethylbenzidine catalyzed by the horseradish peroxidase results in the formation of a blue-colored precipitate directly on the membrane. For this detection method a darkroom equipment is not necessary. Alternatively, for chemiluminescent detection the oxidation of a luminol based reagent catalyzed by the horseradish peroxidase results in the emission of photons that are detected on standard autoradiograph film (Whitehead et al., 1983) which can act as a permanent record. This process is called enhanced chemiluminescence (ECL™). In both cases the quantity of sample DNA is determined by comparsion of the signal intensity to human DNA standards. Using the protocol supplied in the kit 0.15-10.0 ng of human DNA can be quantitated in less than 2 hours. With both methods the results are interpreted by comparing the signal intensity of the DNA test sample to the signal intensity obtained for the DNA standards. The concentration of the DNA test sample in ng / µl is derived by dividing the observed quantity by the volume of DNA test sample.

Based on experience in casework, the colorimetric detection method is not the first choice for stains on substrates with blue, green or black dyes (e.g blue denim), since those colors may interfere with the blue colored results on the membrane. For those items the chemiluminescent detection method is preferable. The method is exactly performed as described in the manufacturer's manual.

12. RFLP and PCR systems currently in use

Currently, the following DNA polymorphism are widely used in forensic laboratories:

RFLP-Typing Hae III: D2S44 (yNH24), D10S28 (TBQ7), D17S26 (EFD52), D4S139 (pH30), D1S7 (MS1), D5S110 (LH1), D17S79 (V1), D14S13 (CMM101). Hinf I: D1S7 (MS1), D7S21 (MS31), D12S11 (MS43), D7S22 (g3), D5S43 (MS8), D11S97 (MS51)

PCR-based systems used or evaluated for nationalwide forensic DNA-databases:

British Home Office: **Quadruplex:** VWA, TH01, F13A, FESFPS; **SGM:** D21S11, D8S1179, D18S51, FGA, TH01, VWA, Amelogenin; **TGM:** D3S1358, D10S516, TH01, D1S518, D14S306, GGAA3A09, GATA4F03

Promega: **Powerplex:** CSF1PO, TPOX, TH01, VWA (CTTV) + D16S539, D7S820, D13S317, D5S818 (GammaSTR); **FFFL:** F13A, FESFPS, F13B, LPL.

Perkin Elmer: **Blue Kit:** FGA, VWA, D3S1358; **Green I:** TH01, TPOX, CSF1PO; **Yellow:** D7S820, D13S317, D5S818; **Profiler:** FGA, VWA, D3S1358, TH01, TPOX, CSF1PO, D7S820, D13S317, D5S818.

Due to fluorescent labeling of primers, ome of these systems can only be used with a 373, 377 Genescanner, Capillaryelektrophoresis or Hitachi machine.

DNA EXTRACTION PROCEDURES

WHOLE BLOOD

Supplies and Reagents

15 ml conical polypropylene centrifuge tubes
Red Cell Lysis buffer
Nuclei Lysis buffer
10% SDS
Proteinase K
Proteinase K Buffer
Saturated NH4 Acetate
Absolute Ethanol
TE Buffer
1.5 ml Eppendorf Tube
1 ul innoculation loop

1. Spin tube with anticoagulated blood, 20 minutes at 2000 RPM. Transfer buffy coat and approximately 1 ml of underlying red cells to labeled 15 ml conical polypropylene centrifuge tube.

2. Add red cell lysis buffer to 9 ml mark on tube. Mix well by inverting tube and leave at room temperature for 15 minutes.

3. Spin tube for 20 minutes at 2000 rpm and discard supernatant. Add 3 ml of nuclei lysis buffer to white cell button and mix well to resuspend cells. Agitate or use plastic disposable pipette to breakup button.

4. Add 200 ul of 10% SDS and 600 ul of protease K solution (use fresh mixture of 2 mg protease K/1 ml protease K buffer). Mix by inverting tube and incubate overnight at 37 C or 2 hours at 50-55 C.

5. Add 1 ml saturated NH4 Ac to tube and shake vigorously for 15 seconds. Incubate at room temperature for 15 minutes, then centrifuge for 15 minutes at 2500 RPM.

6. Pipette supernatant containing DNA into another 15 ml polypropylene tube and add two volumes (approximately 8 ml) of room temperature absolute ethanol. Mix gently by inverting tube until DNA precipitates and floats to top of tube.

7. Gently spool precipitated DNA from tube with 1 ul innoculation loop and transfer to 1.5 ml eppendorf tube containing 200-300 ul of TE buffer.

8. Vortex 8-10 seconds and incubate at 50 C for a minimum of two hours. Check to make sure DNA is dissolved before quantitating. If not dissolved add another 100 ul of TE buffer and reincubate.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells. Nucleic Acids Research Vol. 16, No. 3, (1988). U.S. Patent Application Number 07/154024.

Sambrook, J, Fritsch, E F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd ed. Vol. 2. Cold Spring Harbor Laboratory Press, New York. (1989) E10-E14.

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BLOODSTAINS

Reagents and Supplies

1.5 ml Eppendorf tube
1.0 M DTT
Digest Buffer
Proteinase K solution (2mg/ml)
Saturated Ammonium Acetate
TE Buffer
Mini-gel
1% Ethidium Bromide
Centricon 100 Microconcentrator
UV Transilluminator

1. Place 1.0 x 1.0 cm blood stained cloth into a 1.5 ml Eppendorf tube. Add 300 ul of Digest Buffer, 100 ul of Proteinase K solution (2 mg/ml concentration), and 20 ul of 1.0 M DTT. Vortex briefly and incubate at 37 C overnight. NOTE: Be sure that the cloth material is completely submerged in solution.

2. Punch 3 holes in cap of the tube, place cloth in cap and centrifuge at 10,000 RPM for 10 minutes. NOTE: If cap is not deep enough, punch hole in bottom of second eppendorf tube, transfer cloth and piggyback spin remaining extract into a second tube. Add 100 ul of saturated ammonium acetate. Vortex vigorously for 30 seconds and allow sample to sit for 10-15 minutes. Centrifuge for 10 minutes at 10,000 RPM.

3. Transfer supernatant to a Centricon 100 microconcentrator and add approximately 1.5-2.0 ml of TE buffer to the top reservoir. Parafilm the top of the reservoir, punch several holes in the parafilm, and centrifuge at 3500 RPM in a fixed angle rotor for 20 minutes. Repeat three more times adding 1.5-2.0 ml of TE buffer at each wash. The final volume of sample should be somewhere between 20 to 50 ul. Cap the top of the reservoir, invert, and centrifuge the reservoir at 3500 RPM for 5 minutes to recover retentate. NOTE: Do not exceed 3500 RPM when centrifuging. The Centricon membrane may collapse at higher speeds).

4. Run a yield gel on 1 ul of the sample, stain with 1% ethidium bromide, and observe gel for the presence of high molecular weight DNA under UV light. Transilluminator should emit at 356 nm wavelength for best results.

NOTE: After supernatant has been removed at the end of step #2, the extraction procedure can be discontinued temporarily at this point.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells. Nucleic Acids Research Vol. 16, No. 3, (1988). U.S. Patent Application Number 07/154024.

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HAIR

NOTE: These extraction procedures may also be performed on tissue and bone sample.

Reagents and Supplies

1.5 ml Eppendorf Tube
1.0 M DTT
Digest Buffer
Proteinase K solution (2 mg/ml)
Saturated Ammonium Acetate
TE Buffer
Mini-Gel
1% Ethidium Bromide
Centricon 100 Microconcentrator
UV Transilluminator

1. Wash hair to remove surface dirt and contaminants as follows: For loose, found hairs - fill a 10 x 75 mm test tube with sterile, deionized water. Wash each hair to be analyzed in a separate test tube containing fresh water. Lift hair from evidence container with a clean forceps (ie. flame sterilized or alcohol storage). Wash hair by immersing in water.

For mounted hairs - freeze the slide in a -20 C freezer for 20 minutes. Remove coverslip by prying it off using a scalpel. Alternatively, the coverslip may be removed by soaking the slide in xylene for several hours after cracking the coverslip with a diamond scribe. Using a Pasteur pipet, wash away the residual mounting media by squirting with xylene. Pick up hair with clean forceps and wash in a microfuge tube containing 100% ethanol. Then wash in microfuge tube containing sterile deionized water.

2. Cut off about five to ten mm of the proximal (root) end for digestion. It the root is not visibly apparent with the naked eye, the end of each hair can be positioned together under a coverslip on a microscope slide and examined microscopically for the presence of a root. Because hair may contain cellular material on the surface that may or may not originate from the hair donor, it is advisable to cut off about five to ten mm of the shaft adjacent to the root for separate analysis as a control. The shaft may be retained for remounting.

3. Add the hair sample to the microcentrifuge tube. Add 300 ul of digest buffer to the tube. Add 100 ul of Proteinase K (2 mg/ml) solution. Add 20 ul of 1.0 M DTT. Vortex briefly to mix. Be sure hair sample is completely suspended in the solution. Incubate overnight at 37 C. Hair will usually soften but not dissolve after this initial incubation.

4. The next day, add to the sample an additional 20 ul of 1.0 M DTT and 15 ul of Proteinase K (10 mg/ml) solution. Vortex briefly to mix. Be sure hair is completely suspended in the solution. Incubate overnight at 37 C. Hair should be completely dissolved following this incubation period.

5. Add 100 ul of saturated ammonium acetate to tube and vortex vigorously for 30 seconds and allow sample to sit for 10 to 15 minutes. Centrifuge for 10 minutes at 10,000 RPM.

6. Transfer supernatant to a Centricon 100 microconcentrator and add 1.5-2.0 ml TE buffer to the top reservoir. Parafilm the top of the reservoir, punch several holes in the parafilm, and centrifuge at 3500 RPM in a fixed angle rotor for 20 minutes. Repeat three more times adding 1.5-2.0 ml of TE buffer at each wash. The final volume of sample should be somewhere between 25 to 50 ul. Cap the top reservoir, invert, and centrifuge the reservoir at 3500 RPM for five minutes to recover retentate. NOTE: Do Not exceed 3500 RPM when centrifuging. The Centricon membrane may collapse at higher speeds).

7. Run a yield gel on 1 ul of the sample, stain with 1.0% ethidium bromide, and observe gel for the presence of high molecular weight DNA under UV light. Transillumintor should emit at 356 nm wavelength for best results.

NOTE: After supernatant has been removed at the end of step #5, the extraction procedure can be discontinued temporarily at this point.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells. Nucleic Acids Research Vol. 16, No. 3, (1988). U.S. Patent Application Number 07/154024.

Sambrook, J, Fritsch, E F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd ed. Vol. 2. Cold Spring Harbor Laboratory Press, New York. (1989) E10-E14.

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TISSUE

Reagents and Supplies

15 ml Polypropylene tube
10% SDS
1.0 M DTT
Nuclei Lysis Buffer
Proteinase K solution (2 mg/ml)
Saturated Ammonium Acetate
TE Buffer
Mini-gel
1% Ethidium Bromide
Centricon 100 Microconcentrator
UV Transilluminator
PBS (0.01 M pH 7.3)

1. Remove approximately 100.0 mg of tissue material and place in small 10 x 75 mm test tube. Wash sample in PBS. Remove PBS. Place washed tissue sample in a petri dish and mince the sample as fine as possible using a sterile scalpel. Place minced sample in a 15 ml polypropylene tube. NOTE: If 100.0 mg of tissue sample is not available, refer to the DNA extraction of hair procedure and follow that protocol.

2. Add 3.0 ml of Nuclei Lysis buffer to the sample and vortex vigorously to homogenize sample further. Add 600.0 ul of Proteinase K (2.0 mg/ml), 200.0 ul of 10% SDS, and 20 ul of 1.0 M DTT. Vortex briefly and incubate at 37 C overnight.

3. Check the sample the next day. If there appears to be undigested tissue remaining, add 15.0 ul of Proteinase K (10.0 mg/ml) and 20.0 ul of 1.0 M DTT, vortex briefly and reincubate overnight at 37 C. If tissue sample appears to be adequately digested, proceed to step #4.

4. Add 1.0 ml of saturated ammonium acetate to the sample and vortex vigorously for 30 seconds and allow sample to sit for 10-15 minutes. Centrifuge for 20 minutes at 2500 RPM. Remove supernatant and place in another polypropylene tube. Add two fold volume of absolute ethanol to the tube and invert gently. Spool precipitated DNA from the tube with a 1.0 ul inoculation loop and transfer to an Eppendorf tube containing TE buffer. The amount of TE buffer will be dependant upon the amount of the precipitated DNA removed. If no DNA precipitates, the total volume of sample in the ethanol precipitation tube can be run through a Centricon 100 microconcentrator (see step #6 in DNA extraction of hair).

5. Vortex the precipitated DNA in the TE buffer for ten seconds, then incubate at 50 C for a minimum of two hours to dissolve DNA.

6. Run a yield gel on 1 ul of the sample, stain with 1.0% ethidium bromide, and observe gel for the presence of high molecular weight DNA under UV light. The Transilluminator should emit at 356 nm wavelength for best results.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells. Nucleic Acids Research Vol. 16, No. 3, (1988). U.S. Patent Application Number 07/154024.

Sambrook, J, Fritsch, E F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd ed. Vol. 2. Cold Spring Harbor Laboratory Press, New York. (1989) E10-E14.

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SALIVA IDENTIFICATION

AMYLASE DIFFUSION

Purpose

The enzyme amylase is found in many body fluids including saliva, urine, feces, perspiration, semen, and vaginal secretion. The highest concentration of amylase is found in saliva followed by feces, milk, perspiration, blood serum, semen, and vaginal secretion.

Amylase diffusion is a test to indicate whether there is an elevated amylase level in vaginal swabs, semen stains, or other body fluid stains that could indicate the presence of saliva.

Buffer

0.1 M Phosphate Buffer pH 6.9

2.7 gms NaH_2PO_4 anhydrous (0.045 M)

3.9 gm Na_2HPO_4 anhydrous (0.055M)

0.2 gm NaCl (7 mM)

500.0 ml deionized H_2O

Type/Strength/Volume of Gel

Prepare a 1% agarose 0.1% soluble starch gel by boiling gel solution and pouring into a plastic Petri dish (20.0 ml for a 14.0 cm diameter round Petri dish). Allow to cool and solidify. Note: In order to protect from amylase contamination by aerosol spray, avoid talking, coughing, or sneezing over the gel. Do not touch gel.

0.2 gm Agarose (Sigma Type I)

0.04 gm Soluble Starch

20.0 ml Buffer pH 6.9

Application

Punch approximately 1.5 mm to 2.0 mm holes in gel with a gel punch connected to an aspirator. Leave at least 1.5 cm between holes. Use the template (for the 14.0 cm diameter circular Petri dish diffusion plate) to evenly space sample wells. This template provides for 16 sample wells. Caution: It is advisable to wear a mask that covers the nose and mouth while loading samples into the wells. This can prevent amylase contamination of the gel by aerosol spray. Using a 0.5 ul to 10.0 ul pipettor, place 2.0 ul of sample into the well (controls and unknowns). Record protocol and place an orientation mark on the plate.

Controls

1:100 saliva dilution

1:500 saliva dilution

neat plasma or serum

neat semen

neat urine

vaginal secretion (swab extract)

Temperature and Conditions

Cover Petri dish and place in a 37 degree C incubator overnight.

Staining

Pour a 1:100 dilution (0.5 ml + 49.5 ml DI H_2O) of a saturated iodine solution (use GLO1 iodine solution). Clear circles around holes indicate amylase. Measure diameter of clear circles (in mm) and record.

Reaction

amylase

Starch ----> Dextrins ----> Maltose ----> < 4 glucose units

(8-12 glucose units) red   (4-8 glucose units) yellow   (colorless)

Interpretation Cautions

It has been reported that Thiocyanate ions sometime occurring at higher levels in saliva from smokers increases decoloration of the starch-iodine complex which may lead to overestimations of amylase activity. Remember that this is a preliminary test for amylase ativity. It does not confirm the presence of saliva without additional test procedures.

Reducing agents also reduce iodine therefore extracts used for amylase tests should be prepared in D/I H_2O or physiological saline solutions without reducing agents such as dithiothreitol.

Mapping

Panty crotches, whole garments, bed sheets, etc. may be tested for the presence of amylase using "plates" of amylase diffusion gels as follows:

1. Prepare a 50 ml 1% Agarose 0.1% Soluble Starch gel (0.5 gms agarose 0.05 gms starch) on a 20 x 20 cm framed glass plate. Allow to solidify.

2. For small areas or garments spread the material over the gel, weigh lightly, leave in contact with the gel for 10-15 minutes.

Large areas may be surveyed by placing multiple gel plates in logical sequence across the area of interest. Be sure to use some system of orientation or marking to be able to relate positive amylase patterns on the gel plates back to their stain position on the garment, etc.

3. Place the exposed gel in an appropriate size tray and stain with enough 1:100 Iodine Solution to cover the plate. Positive amylase patterns will show as clear areas on the dark blue gel.

Control Ranges

Sample	Diameter of Ring
1:100 Saliva dilution	
1:500 Saliva dilution	
Neat urine	
Neat serum	
Neat semen	
Vaginal secretion	

References

1. Nelson & Kirk, Identification of Saliva Stains by Amylase Activity, J. of Fores. Med., 10, 1, 14-20, (1963)

2. Schill & Schumacher, Radial Diffusion in Gel for Micro Determination of Enzymes..., Anal. Biochem., 46, 502-533, (1972).

3. Stiefel & Keller, Preparation and Some Properties of Human Pancreatic Amylase Including a Comparison With Human Parotid Amylase, Biochem, Biophys Acta (1973).

4. Metropolitan Police Forensic Science Laboratory, Biology Methods Manual, 3-9 to 3-10, London, England (1978).

5. Lathia & Brendeback, Influence of Thiocyanate Ions on Starch-Iodine Reaction Used for the Estimation of a-Amylase Activity, Clinica Chimica Acta, 82, 209-214, (1978).

	Lab Director	Supervisor
Reviewed by:	____________	____________
Date:	____________	____________

AMY 1 & AMY 2 AGAROSE ISOELECTRIC FOCUSING (0.5 mM)

Reagents & Supplies

AGAROSE IEF (PHARAMACIA)	.225 gms
SUCROSE	2.7 gms
DI H2O	20 mls
AMPHOLINE (LKB)	
pH 6 - 8	1.0 ml
pH 7 - 9	0.4 ml
ELECTROLYTE STRIP SOLUTIONS	
ANODAL STRIP (mixture)	0.025 M Aspartic Acid 0.025 M Glutamic Acid
CATHODAL STRIP	0.2 N NaOH
GEL CASTING PLATE	
GELBOND	
AMYLASE CONTROLS:	
AMY1 SALIVA POOLED 1:500 DILUTION	
AMY1 SALIVA POOLED 1:100 DILUTION	
AMY2 URINE UNDILUTED	
HB CONTROL FOR ELECTROPHORESIS	
AMYLASE DIFFUSION BUFFER:	
NaH2PO4 (anhydrous) (0.045 M)	2.7 gms
Na2HPO4 (anhydrous) (0.055 M)	3.9 gms
NaCl (7 mM)	0.2 gms
DI H2O	500 mls
SUBSTRATE SOLUTION:	
1 gm soluble starch in 100 mls of AMYLASE DIFFUSION BUFFER.	
NOTE: MUST BRING TO BOIL TO DISSOLVE STARCH THEN COOL TO ROOM TEMPERATURE.	
IODINE SOLUTION:	
Potassium Iodide	2 gms
Iodine	1 gm
DI H2O	200 mls
STAIN SOLUTION:	
1 ml of IODINE SOLUTION in 100 mls DI H2O.	
POLAROID DS34 CAMERA	
POLAROID TYPE 667 FILM	
WHATMAN #1 FILTER PAPER	

continued .... page 1 of 4.

GEL ELECTROPHORESIS

1. Gel is prepared by mixing 20.0 ml deionized water, 2.7 gm Sucrose and 0.225 gm Agarose IEF and heating until dissolved. Reduce temperature to 75°_C as described by manufacturer and add the appropriate ampholyte.

2. Pour molten solution (70-75°_C) onto preheated (60-65°_C) gel casting plate using the flap technique (see gel casting procedures). Cover with a glass plate upon which a piece of Gel Bond Film has been rolled. Let gel set at room temperature for 15 minutes, then cool at least 1 hour at 4°_C.

3. Using an I.C.E. EC 1001, place the gel on the cooling plate which is maintained at 5°_C. Blot gel briefly for using Whatman #1 filter paper 5 x 10 inches for 3-5 seconds. Saturate electrode strips with appropriate electrolyte solutions and place on gel surface at correct electrode contact sites.

4. Saturate sample wicks, Whatman #1 filter paper 3 X 4 mm, with the appropriate specimens and place the sample wick 1.5 cm from the anodal elec trolyte strip. Leave a 3 mm space between each sample wick. **Use the controls listed above in the procedure.**

5. Set voltage at 2000 volts, 20 ma, rampling only the watts as follows:

T0	5 watts	
T15	7 watts	
T30	10 watts	remove sample wicks
T75	off	

DEVELOPMENT:

1. Remove gel sandwich from electrophoresis tank and carefully separate the glass plates. Wearing gloves, place the gel in a clean tray. **Do not touch the gel with bare hands.**

2. Add substrate solution to the gel tray and incubate at room temperature with gentle agitation for 30 minutes.

continued ....

3. Rinse gel with DI H2O three times.

4. Bathe gel in STAIN SOLUTION until gel turns dark blue.

5. Place gel on glass plate, cover and incubate at 37°_C for 1 hour check gel every 15 minutes. Amylase activity is detected by clear (unstained) areas. The length of time of the second incubation depends on sample activity. Low activity samples may be incubated as long as overnight in humid atmosphere. Sample activity diffuses over time making the bands larger.

INTERPRETATION:

1. Amylase activity is detected by clear bands in a blue background.

2. Control samples of pooled saliva and urine demonstrate the location of the AMY1 and AMY2 bands. Reactions in these locations are indicative of the presence of either AMY1 or AMY2. Quantitative saliva standards provide some information on the quantity of saliva, which should have been previously determined.

   Note: Due to polymorphism at both AMY1 and AMY2 it is possible that additional bands of activity may be detected in some cases.

3. HB control verifies separation took place according to IEF.

continued..........

References:

Blake ET (1977) *Genetic Markers in Human Semen*, University of California, Berkeley, Dissertation Abstracts International. Vol XXXVIII, #2, 1977. Order #77-15, 567, 277 pages. Order from University Microfilms International, Dissertation Copies, P.O. Box 1764, Ann Arbor, MI 48106.

IEF Procedure based on AGTC HB procedure, unpublished.

Lab Director Supervisor

Reviewed by:____________________ ____________________

Date:____/____/____ ____/____/____

written 06/11/97
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AMYLASE VERTICAL GEL ELECTROPHORESIS

Purpose

Separation of the Amy_1 and Amy2 isozymes, and for Amy2 phenotype determination.

Method

*Prepare a 6.5% acrylamide gel in a vertical gel "sandwich" plate as follows:

1. Mix: 6.5 mls 30% Stock Acrylamide

8.5 mls H_2O

15.0 mls Gel Buffer pH 8.8

2. Add 20.0 mgs Ammonium Persulfate and degas the solution using a vacuum pump or a good aspirator.

3. Add 20 ul TEMED, swirl gently to mix and pour between "sandwich" plates immediately.

4. Insert comb to form sample wells and top off acrylamide solution carefully with DIH_2O.

5. Allow to polymerize completely (approx. 1/2 hour). Store in refrigerator until ready to use; will keep for 48 hours if protected from dehydration.

* Or use Premade 6.5% Acrylamide minigel.

Sample Preparation

1. Determine the volume of sample to be used based on Amylase Diffusion test results; 2-5 uls for strong samples, 5-10 uls for moderate samples, 15-20 uls for weak amylase activity samples. Place each in separate sample tube.

Controls	Volume

Saliva 1:500 2-5 uls

Amy 2A Standard Neat 15-10 uls

Amy 2B Standard Neat 15-20 uls

2. Add 10 ul of 10% sucrose to each sample. Also add 5 ul Bromophenol Blue, saturated solution, to one of the controls (i.e. 1/500 saliva control) as a position marker.

Gel Loading and Electrophoresis

1. Remove the sample comb and secure the sandwich gel in the vertical gel tank. Fill the anode and cathode chambers with Tank Buffer.

2. Using double drawn pipettes, fill each sample well with predetermined sample volume. Be careful to insert pipette tip and expel sample near bottom of well. The sucrose solution will weight the sample so that it sinks to the bottom. Do not blow air bubbles into the wells.

3. Connect power supply and allow to run until the Bromophenol Blue marker runs off the bottom of the plate.

Run Conditions

30 mA constant currect at 5-8 degree C.

(approximately 100V start to 270V finish).

(3-4 hours)

Gel Development

1. Remove gel sandwich from electrophoresis tank and carefully separate the glass plate from the acrylamide gel. Wearing gloves, place the gel in a clean tray. ***Do not touch the gel with bare hands!***

2. Add Substrate Solution to the gel tray and incubate at room temperature with gentle agitation for one (1) hour.

3. Rinse gel with DIH_2O approximately three times.

4. Place gel on glass plate, cover and incubate at 37 degree C for one (1) hour. The length of time of the second incubation depends on sample activity. Low activity samples may be incubated as long as overnight in humid atmosphere.

5. After second incubation, bathe gel in stain solution until gel turns dark blue with clear (unstained) bands of amylase activity.

Reagents

Electrophoresis

Stock Acrylamide

Acrylamide	30.00 g
N,N' -methylenebisacrylamide	0.82 g
DI H_2O	100.00 mls

Gel Buffer (pH 8.8)

Tris (0.75 M)	90.83 g
DI H_2O	1.0 liter
Titrate to pH 8.8 with 1:1 HCl	

6.5% Acrylamide Gel

Stock Acrylamide	6.5 mls
Gel Buffer	15.0 mls
DI H_2O	8.5 mls
Ammonium persulfate (APS)	20.0 mgs (de gas)
TEMED (initiates polymerization)	20.0 ul

Tank Buffer pH 8.4

Glycine (0.192 M)	57.60 g
Tris (0.025 M)	12.10 g
DI H_2O	4.0 liters

Development

Amylase Diffusion Buffer pH 6.9

NaH_2PO_4, anhydrous (0.045 M)	2.7 gms
Na_2HPO_4, anhydrous (0.055 M)	3.9 gms
NaCl (7mM)	0.2 gms
DI H_2O	500.0 mls

Substrate Solution

1 g soluble starch in 100 mls of Amylase Diffusion buffer.

Must bring substrate to boil to dissolve starch then cool to room temperature.

Iodine Solution

Potassium Iodide	2 g
Iodine	1 g
DI H_2O	200 mls

Stain Solution

1 ml Iodine Solution in 100 mls DI H_2O

Materials List

Vertical Electrophoresis Tank Set-up

Sandwich Gel Pouring Apparatus (or Premade 6.5% Acrylamide Gels)

Double Drawn Glass Pipettes

Disposable Gloves

Development Trays

Notes

1. The 6.5% acrylamide gels are also available as premade minigels. They fit standard Pharmacia or Isolab Vertical gel apparatus.

2. Take care not to blow bubbles into wells or agitate the gel which may contaminate samples in adjacent wells. Good pipetting techniques are in order here.

3. After electrophoresis is run, carefully separate glass plates from gel. <u>USE GLOVES</u> when handling gel in all development steps to avoid perspiration contamination.

Interpretation Cautions

1. Overloaded samples may need to be repeated at a lower concentration or less sample volume to see a clearly defined band pattern.

2. Weak samples can be concentrated or repeated with more sample volume. Weak samples may also be enhanced by increased 37 degree C incubation time.

3. Degrading samples, especially older urines, may show intense anodic band patterns. Be careful not to interpret these as unusual variant.

References

1. Blake, E.T. Genetic Markers in Human Semen, University of California, Berkeley (1976) Dissertation Abstracts International, Vol. XXXVIII, Number 2, 1977, Order No. 77-15, 567, 277 pages. Order from University Microfilms International, Dissertation Copies, P.O. Box 1764, Ann Arbor, MI 48106.

	Lab Director	Supervisor
Reviewed by:	______________	______________
Date:	______________	______________

SEMEN METHODS

SCREENING TESTS

BRENTAMINE REACTION

Purpose

Spot test for the enzyme Acid Phosphatase. Used to indicate a semen stain, but not to identify semen. Make fresh each time used.

Spot Test

Moisten a piece of filter paper; press on suspected semen stain (or swab). Remove and add a small drop of Brentamine test reagent to the filter paper. Color change to purple within a few minutes is a positive reaction. Also, a small cutting of stain may be tested directly.

Mapping

Whole garments, bedsheets, panty crotch panels, etc. may be assayed for the possible presence of seminal stains by "mapping" the item as follows:

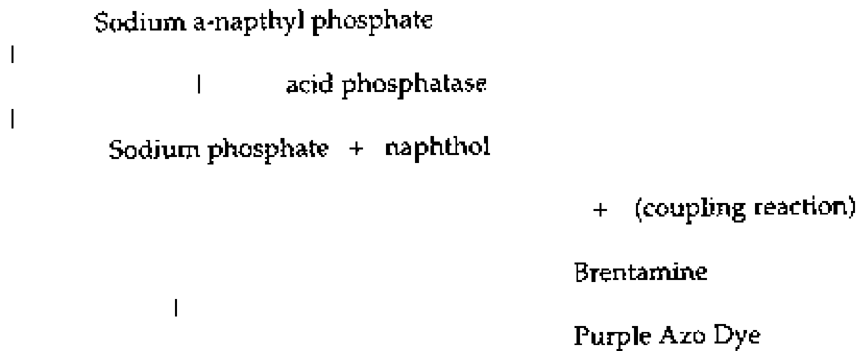
1. Cut Whatman 1 filter paper to appropriate lengths. Saturate the filter paper with deionized water using a spray bottle.

2. Lay the wet filter paper over the area of interest and press onto the item for 30-60 seconds. A glass or plastic plate and weight (i.e. books or bricks) on the paper may be useful at this step. Large areas may require two or more filter paper overlays laid side by side to map efficiently. If multiple sheets are used they should be marked in a manner to facilitate their orientation to one another and to relate positive test reactions back to specific areas of the evidence item.

3. After pressing, hang the filter paper in a fume or safety hood and spray with Brentamine test reagent.

Reaction

Liberation of napthol from sodium a-naphthyl phosphate by the enzyme and the formation of a purple azo dye by the coupling of napthol with buffered fast blue B.



Semen stains should give a fast deep purple color reaction.

"False" Positives

The enzyme acid phosphatase occurs in other body fluids as well as bacteria, fungi, and many plants. Common false positive reactions may be seen in:

1. Vaginal secretions; especially during pregnancy or with bacterial infection. The reaction is slow and faint.

2. Fecal stains - slow, faint pink reaction.

3. Certain plants and fungi.

Intertretation Cautions

Remember that this is a presumptive test only. Positive reactions should be confirmed by P30 Crossover or Rocket immunoelectrophoresis. Slow or weak reactions and different color reactions may not be seminal fluid.

References

1. Metropolitan Police Forensic Science Laboratory Biology Methods Manual, 3-17 to 3-19, London, England (1978)

ACID PHOSPHATASE REAGENT

Sodium Citrate (dihydrate)	17.65 gms
Citric Acid (free anhydrous)	4.2 gms
alpha-naphthyl phosphate (disodium salt)	1.0 gms
o-dianisidine (tetrazotized)	4.0 gms

Mix all reagents thoroughly. Store frozen.

The above amounts will make 1.0 liter of aqueous reagent.

The amount can be scaled up or down as needed.

Recommended working solution for AP quantitative assay or qualitative assay (spot test) is 13.0 mg/1.0 ml of D/I H20.

The above reagents can be ordered through the following suppliers if so desired:

Sodium Citrate (dihydrate)	J.T. Baker Cat.# 3646-01
Citric Acid (free anhydrous)	Sigma Cat. # C-0759
alpha-naphthyl phosphate (disodium salt)	Sigma Cat. # N-7255
o-dianisidine (tetrazotized)	Sigma Cat. # D-3502

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Reviewed by:	______________	______________
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CONFIRMTORY TESTS

NUCLEAR FAST RED PICROINDIGOCARMINE STAIN FOR SPERMATOZOA

This stain was developed for spermatozoa and for seminal vaginal mixtures. It consists of a Nuclear Fast Red (CI 60760) stain followed by an indigocarmine (CI 73015) in picric acid counterstain. Nuclear Fast Red is also called Calcium Red, and its German name is Kernechtrot. This stain is sometimes called "Christmas Tree" because the spermatozoa are stained different colors in different parts. Heads are reddish, midpieces blue and tails a yellowish green. These colors also contrast well with the green to blue-green shades of the epithelial cells.

Preparation of Stain

1. Nuclear Fast Red (May be called "Kernechtrot Solution" or "KS"): 5 g. $Al_2(SO4)3$ in 100 ml hot distilled water, and add 0.1 g Nuclear Fast Red. Stir, allow to cool, and filter.

2. Picroindigocarmine (PICS): 4 g picric acid in 300 ml water is covered and allowed to stand overnight, giving a saturated solution. 1 g indigocarmine is dissolved in this solution and the resulting solution filtered. A few picric acid crystals may remain.

Staining Technique

1. Pipet the cellular debris from the body fluid extract onto a glass slide; fix by heating at 60 degrees C for at least 30 minutes.

2. Cover sample area with KS solution and place in moisture chamber for 15 min. (may be placed under inverted Petri dish).

3. Wash with water by gentle flooding.

4. Cover sample area with PICS solution for 15 seconds or less.

5. Wash and fix by gently flooding the slide with absolute ethanol.

6. Allow to dry.

Appearance of Stained Preparations

Sperm:	anterior head	blue green
	posterior head	dark red
	midpiece	blue
	tail	yellowish green
Epithelial Cells:		
	nucleus	green-blue green
	cytoplasm	light green-blue green

References

1. Lee, Gaensslen, Mertens, Stolorow, Analysis of Sexual Assault Evidence, Sampling and Staining Techniques from Oppitz, E. 1969, Arch Kriminol. 144: 145-148

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CHRISTMAS TREE STAIN

Reagents and Supplies

NUCLEAR FAST RED (NFR) STAIN (AGTC) SPRAY FIXATIVE (OPTIONAL)
PICROINDIGO CARMINE (PIC) STAIN (AGTC) MOISTURE CHAMBER
ABSOLUTE ETHANOL OR METHANOL MICROSCOPE SLIDES
PIPET MICROSCOPE (20-100X)

This stain was originally developed to detect spermatozoa in semen and semen vaginal cell mixtures. The procedure consists of staining with Nuclear Fast Red (NFR) followed by counter staining with Picroindigo Carmine (PIC). The results of this procedure are sometimes referred to as "Christmas Tree", because different parts of the spermatozoa are stained different colors. The heads are reddish, the midpiece and the tails green. These colors contrast well with the pink and blue-green colors of the epithelial cells.

Procedure

1. Pipet the cellular debris from the body fluid extract onto a glass microscope slide; fix by heating at 60°C for at least 30 minutes.

   (optional) Smear may be covered with SPRAY FIXATIVE following heat fixation.

2. Cover area of smear with NFR solution and place in moisture chamber for a minimum of 15 minutes. (An inverted petri dish may be used.)

3. Wash with water by gentle flooding.

4. Cover smear area with PIC solution for 5- 10 seconds, but no longer.

5. Wash and fix by gently flooding the slide with ABSOLUTE ETHANOL or METHANOL.

   Note: Avoid over washing, in that it may cause destaining of the smear.

6. Allow to air dry.

Appearance of stained preparation:

Sperm: Anterior Head - faint pint to translucent
Posterior Head - dark red
Midpiece - green
Tail - green

Epithelial Cells: Nucleus - pink
Cytoplasm - light green-blue to green

References

Adapted by Lee, H, Gaensslen, RE, Mertens, and Stolorow, M from Oppitz E. (1969) Analysis of sexual assault e, sampling and staining techniques. *Arch. Kriminol.* *144*: 145-148.

	Laboratory Director	Supervisor
Reviewed by:	______________	______________
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A GRAM MODIFIED CHRISTMAS TREE STAIN

Purpose

To elucidate cell material and components for microscopic examination and determination.

Method

1. Fix cells to a microscope slide in a circulating 55 degree Coven for 30 minutes. Use Cytoprep. (Instructions on can)

2. Stain with Aqueous Crystal Violet for 1 minute, then rinse with water.

3. Stain with Gram's Iodine for 1 minute, rinse with acetone, then dry.

4. Stain with Nuclear Fast Red for at least 5-10 minutes, then wash with water.

5. Stain with Picroindigocarmine for 5-10 <u>seconds</u>, then rinse with EtOH, dry and mount with Permount and coverslip.

6. Ovserve at 400X; use oil immersion if necessary.

Reagents

0.5% Crystal (Methyl) Violet in water

<u>Gram's Iodine</u>: 1.0 g Iodine, 2.0 Potassium Iodide, 300 ml water

<u>Nuclear Fast Red Stain</u>: Dissolve 5.0 g Aluminum Sulfate in 100 ml of hot distilled water and add 50 mg of Nuclear Fast Red. Stir and allow to cool, then filter and store at 8 degree C.

<u>Picroindigocarmine Stain</u>: Dissolve 1.3 g of Picric Acid in 100 ml warm distilled water. Add 0.33 g Indigo Carmine and stir over-night. Filter and store at 8 degrees C.

Supplies

3"x 1" x 1mm Microscope Slides, precleaned (Fisher 12-544-1)

Cytoprep

Permount

22 x 22mm glass cover slips

Acetone

Ethanol

Distilled/Deionized Water

Notes

Nuclear material is stained red by the Nuclear Fast Red dye. Sperm heads are usually well differentiated with the acrosome staining significantly less densely than the distal region of the head. Epithelial membranes are stained green by the picroindigocarmine. Nuclei inside epithelial cells appear purple. Yeast cells also stain red, however, the stain is uniform throughout the cells and extends into polyp-like structures which are occasionally observed with yeast cells.

Gram positive bacteria stain deep violet; Gram negative bacteria are unstained.

References

1. Edward T. Blake, Charles E. Cook, Jr., Forensic Science Associates and Jan Bashinski, Oakland Police Department, presented at the 67th Semi-annual seminar, California Association of Criminalists, May 15-17, 1986.

2. Oppitz, E., Arkiv Fur Krimin., 144, 1969, 145.

3. Luna, L.G., Ed., Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, McGraw-Hill Book Co., New York, 1968.

	Lab Director	Supervisor
Reviewed by:	____________	____________
Date:	____________	____________

IDENTIFICATION OF SEMEN USING ANTI-P30

Purpose

P30 is a seminal plasma protein with a molecular weight of approximately 30,000 daltons.[1] This protein is not present in other body fluids or secretions. The level of P30 in normal semen (as assayed by a quantitative immunoradial diffusion technique) is in a range of 0.145 to 5.9 mg/ml with a mean of 0.800 mg/ml.[4] The prostate gland is the likely tissue of origin. Antisera against P30 is made in rabbits and used in an immunologic technique to detect the antigen. AGTC uses either a Laurel rocket or a crossover electrophoresis technique for the detection of this protein. It is used to confirm and to estimate the amount of semen present in sexual assault evidence.[2]

P30 Rocket Electrophoresis

The technique of Rawlinson and Wraxall is used.[3] For a small rocket plate (7.5 cm x 5.0 cm), 80 uls of AGTC anti-P30 is added to 8.0 mls of gel buffer containing 1% Agarose (Sigma Type I) at 56 - 59 degrees C just prior to pouring on to a clear polyester film support.

pre-cut Gel Bond 7.5 x 5.0 cm (3" x 2")

0.08 gms Agarose (Sigma Type I)

8.0 mls of gel buffer

80 ul of AGTC anti-P30

8 samples can be run on the gel

For a larger rocket plate (10.0 cm x 7.5 cm); 160 uls of AGTC anti-P30 is added to 16.0 mls of 1% Agarose in gel buffer at 56-59 degrees C just prior to pouring. 12 to 16 samples can be run.

Note: Higher, but weaker, peaks will be obtained using smaller amount of anti-P30.

Tank and Gel Buffer

25.2 gms Trizma base

2.5 gms EDTA free acid (or 2.8 gms of disodium salt)

1.9 gms Boric Acid

Q.S. to 1 liter with deionized water

Adjust pH to 9.1 with 10% or 20% w/v NaOH

Store at 4 degrees C

Sample Application

Sample wells are punched approximately 1.5 cm from the cathodic edge of the plate. The wells should be 2 mm in diameter and 0.5 cm apart. 2 uls of sample extract and 2 uls of control/calibration standard are pipetted into the appropriate sample well.

Control/Calibration Standards

Calibration standards are prepared from the AGTC Semen Standard by making the appropriate dilution (see reagent insert) to yield an approximate P30 concentration of 50 ug/ml. Doubling dilutions can be prepared to obtain the 25 ug/ml, 12.5 ug/ml, and 6.25 ug/ml standards.

P30 rocket height to antigen concentration is a log-normal function. Measurements of the calibration standards are used to plot a calibration line (antigen concentration in ug/ml vs. peak height in mm). The peak height of an unknown sample extract is found on the calibration line and the corresponding P30 concentration is determined from the graph.

Peak height is measured from the center of the sample well to the tip of the rocket peak.

Note: The precision of your standard line can be checked quickly by utilizing the Linear Regression Program found in many calculators.

Unknown Samples

Extracts of stains should be run neat, and at times at a 1:5 dilution. Liquid semen samples should be run at 1:50, 1:100, or 1:200 dilutions.

Electrophoresis Running Conditions

40 Volts (at power supply) for 16 hours.
No cooling necessary.

Staining

Rocket immunoelectrophoresis gels can be stained without washing because of low protein content. Gels can be pressed, dried, and stained immediately after electrophoresis using Coomassie Blue staining or Coomassie Blue followed by silver staining[5] and blue toning.[6] There is little or no background after destaining.

P30 Concentration

The resultant P30 concentration can be used to estimate a range of semen concentrations present in the sample. For example, by using both the high and low ends of the range of P30 concentrations in the male population, a feel is obtained for the range of semen concentrations that might be represented in one sample. By using the mean value, insight is gained as to the semen dilution assuming an average male semen donor. Having all three calculations enables the analyst to evaluate the semen evidence using best case, worst case, and average case assumptions. This allows the analyst to make decisions about the advisability of obtaining a reference semen sample from any possible semen donor(s) for further evaluation of inclusion or exclusion.

References

1. Sensabaugh, Isolation and Characterization of a Semen Specific Protein From Human Seminal Plasma: A Potential New Marker For Semen Identification, Journal of Forensic Science, 23, No. 1, 1978

2. Blake, Sensabaugh, Bashinski, A Systematic Approach To The Analysis of Semen Evidence, CAC Meeting, 6 November 1980

3. Rawlinson, Wraxall, Semen Quantification Utilizing P30 Antigen, Interamerican Congress of Forensic Sciences, 2 November 1982

4. Blake, Gibbons, Sensabaugh, Bashinski, Population Survey And Stability Studies Of P30 In Semen, CAC Meeting, November 1981

5. White, J.M., Silver Staining Of Agarose Gels, CAC meeting, May 1985 Modification of the Method of Willoughby and Lambert (Anal. Biochem. 130, 353 (1983). After Dykes (Electrophoresis, 6, 90 (1985) and Budowle (Electrophoresis, 5, 174 (1984)

6. Berson (Anal. Biochem. 134, 230 (1983)

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P-30 SEMEN STANDARD

Reagent

AGTC semen standard is a pool of human seminal plasma in isotonic saline (0.9% NaCl).

Storage

AGTC recommends aliquoting the semen standard immediately upon receipt and freezing. If the product becomes turbid or smells, the product should not be used.

FOR FORENSIC USE ONLY NOT FOR CLINICAL USE

Use

ACTC Semen standard has been quantitated at 100 ug/ml. Doubling dilutions of the 100 ug/ml semen standard can be prepared to obtain calibration standards for P-30 rocket electrophoresis.

ACTC recommends the semen standard be diluted with isotonic saline (0.9% NaCl). If silver staining methods are to be employed with rocket P-30 gels, any phosphate present in diluents will precipitate out the silver.

AGTC recommends a 1.0% anti-P30 concentration in the rocket gel. For example, a small (7.5 cm. x 5.0 cm.) gel would contain 80 ul of AGTC anti-P30 in 8.0 mls of 1.0% agarose. A 50.0 ug/ml AGTC semen standard will produce peaks 34.0-42.0 mm in height. Higher peaks will be obtained if smaller amounts of anti-P30 are used in the gel.

The AGTC semen standard may also be used as a positive control in P-30 counter immunoelectrophoresis (crossover) and in radial immunodiffusion methods.

DETECTION OF P30 BY CROSS-OVER ELECTROPHORESIS

Tank Buffer

25.2 gm Trizma base

2.5 gm EDTA free acid

1.9 gm Boric Acid

1.0 liter of D/I water pH to 9.1 with 20% w/v NaOH

Gel Buffer

Same as tank buffer

Gel Preparation

Prepare a 1.0% agarose gel as follows:

0.08 gm of agarose (EEO of 0.25)

8.0 ml of gel buffer

Melt agarose and pour on to a pre-cut piece of Gel Bond (FMC) 3" x 2" size. Note: Be sure to pour on to the hydrophilic side.

Sample Application

Sample wells are prepared by punching a pair of holes at right angles to the direction of the current. Remove agar from the holes by aspiration.

Anodic well: Fill well with antiserum

Cathodic well: Fill well with sample extract (antigen)

Controls: Positive: semen stain extract

Negative: vaginal swab extract (semen-free)

Electrophoresis

Place gel into chamber upside down on to the wicks. Apply 120 Volts to gel and run for 30 minutes at room temperature; no cooling of gel is recquired during the run.

Detection

Upon completion of run, remove gel from chamber and wash in 1.0 Molar saline overnight with gentle rotation /rocking. Cover gel with moistened piece of Whatman #1 filter paper and several pieces of dry Whatman #3. Add weight and press for 5-10 minutes. Remove filter paper and dry in 56 C oven.

Staining

0.1% Coomassie Blue or Serva Blue stain may be used. Stain gel for 15 minutes. If using Serva Blue, destaining can be performed in D/I water. If Coomassie Blue stain is used, destaining must be accomplished using the following destain solution:

50 volumes of Methanol
50 volumes of D/I water
10 volumes of glacial acetic acid

Destain until background is clear.

Interpretation

Positive: Precipitin line between antigen and antibody well.

Negative: No precipitin line between antigen and antibody well.

References

1. Saferstein, Richard, Ed., Forensic Science Handbook, Prentice-Hall, Inc. Englewood Cliffs, New Jersey, pp. 283-97

2. Culliford, B.J., "Examination and Typing of Bloodstains in the Crime Laboratory," LEAA 1977 pp. 62-66

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Reviewed by:	__________	__________
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P-30 DETECTION BY IMMUNODIFFUSION (OUCHTERLONY)

Reagent Preparation:

2.55 gm of Type IV Agar

300 ml physiologic saline

40 mg thiomersal (preservative)

1. Mix above in a 1000 ml flask
2. Heat on a hotplate until melted (continue swirling solution)
3. Place disposable Petri dishes (35 mm x 10 mm) on a level table with lids off.
4. Pipette 5.0 mls of melted agar into each dish.
5. Swirl plates to evenly distribute agar.
6. Let stand for 1 hour to completely solidify.
7. Punch holes (sample wells) in the agar. The holes should be approximately 0.5 cm in diameter and 2.0 cm apart. The template format most utilized is one in which six sample wells surround a central well with all sample wells being equidistant from the center well. This minimizes the quantity of antisera used.
8. Vacuum agar from holes by aspiration and replace lids.
9. Store in refrigerator upside down in a moisture box.

Procedure

1. Extract stain/swab in PBS (phosphate buffered saline).
2. Centrifuge extract.
3. Fill center well with anti-p30.
4. Pipette small amount of sample extract to fill sample well. One well should contain known human semen; the positive control. The negative control should be known semen-free vaginal secretion. Number the wells for sample reference.
5. Let stand for 30 minutes; then invert and place in 37 degree C oven overnight.

Interpretation

The appearance of a white precipitin line is indicative of a positive result. Reading of the dish may be faclitated by observation against a black background with oblique backlighting.

References

1. Forensic Science Handbook, Richard Saferstein, Ed., Prentice Hall, Inc., Englewood Cliffs, New Jersey, 1982.

2. O. Ouchterlony, "Antigen-antibody Reactions in Gels," Acta Pathol. Microbiol. Scand., 26 (1949), 507.

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SPERM DIGESTION (DIFFERENTIAL EXTRACTION)

Reagents and Supplies

Soft-Top microfuge tubes
TES (Tris EDTA Saline)
Digest Buffer
Proteinase K solution (2 mg/ml)
1.0 M DTT
DI H2O
Saturated Ammonium Acetate
TE Buffer
Mini-gel
1% Ethidium Bromide
Centricon 100 Microconcentrator
UV Transilluminator
PBS (0.01 M pH 7.3)

1. Extract stain/swab in TES solution for 1-2 hours at room temperature or overnight at 4 C. Use polypropylene microfuge tubes with soft polyethylene tops. Vortex samples periodically to enhance extraction process.

2. Punch 3 holes in cap of tube; remove cloth/swab material and place in cap. NOTE: If cap is not deep enough, punch a hole in the bottom of a second microfuge tube, transfer cloth/swab to this tube and piggyback spin). Centrifuge at 10,000 RPM for 5-10 minutes. Remove cloth/swab and save in freezer if extraction to be performed at a future time.

3. Remove all but approximately 30 ul of supernatant and place in another labeled microfuge for conventional genetic marker testing. Resuspend pellet and remove 1.0 ul for preparation of a smear to confirm the presence of spermatozoa microscopically.

4. If spermatozoa are identified in the smear, vortex briefly to resuspend and replace the microfuge cap (a cap without holes punched in it). Add 300 ul of Digest Buffer to the pellet. Add 100 ul of Proteinase K solution. Add the cloth/swab material (removed previously) to the solution and vortex briefly. Make sure material is completely submerged in solution and incubate for 1-2 hours at 50 C (but no longer than 2 hours to minimize loss of sperm).

5. Punch 3 holes in cap of tube and repeat step #2. Remove supernatant for epithelial cell DNA analysis or discard. Remove substrate material and store frozen at -20°C.

6. Wash pellet 2 times with Digest Buffer and discard supernatant. Wash once with deionized water and discard all but approximately 30 ul of supernatant. NOTE: Perform this wash quickly to prevent possible sperm lysis. The wash steps are critical, particularly when the ratio of sperm cells to epithelial cells is small.

7. Resuspend pellet in residual supernatant and remove 1.0 ul for preparation of a smear for microscopic exam to verify digestion of epithelial cells and recovery of sperm. If performed successfully, you should observe sperm and no epithelial cells (or "ghost" epithelial cells). If epithelial cells persist repeat steps 4 through 7 but incubate for 15-30 minutes only at step #4.

8. Add 300 ul of Digest Buffer to the sample and vortex briefly to resuspend pellet. Add 100 ul of Proteinase K solution and 20 ul of 1.0 M DTT. Vortex briefly. Incubate for a minimum of 6 hours at 50 C (but no longer than 16 hours).

Optional: A direct digest can be performed on the substrate material (cloth, swab, etc.) Often times the quantity of DNA recovered is higher than that obtained with the differential digest. Be aware that the DNA recovered may contain both sperm and epithelial cell DNA.

9. Add 100 ul of saturated NH4 acetate to sample and vortex <u>vigorously</u> for 15 seconds. Allow to stand for 15 minutes at room temperature. Centrifuge sample at 10,000 rpm for 10 minutes.

10. Label a Centricon 100 microconcentrator and add 1.5 ml of TE buffer to the top reservoir. Remove sample supernatant from step #9 and place in the top reservoir of the Centricon 100. Save the protein pellet, since it may contain the sperm if the digest was not done correctly. Parafilm the top of the reservoir, punch several holes in the parafilm, and centrifuge at 3500 rpm in a fixed angle rotor for 20 minutes. Repeat 3 more times adding 2.0 ml of TE buffer at each wash. The final volume of sample should be somewhere between 25 and 50 ul. Cap the top of the reservoir, invert and centrifuge the reservoir at 3500 rpm for 5 minutes to recover retentate. NOTE: Do not exeed 3500 rpm when centrifuging. The centricon membrane may collapse at higher speeds.

11. Run a yield gel on 1 ul of the sample, stain with 1% ethidium bromide, and observe gel with UV light. Estimate quantity and quality of the DNA in the sample.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells. Nucleic Acids Research Vol. 16, No. 3, (1988). U.S. Patent Application Number 07/154024.

Sambrook, J, Fritsch, E F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd ed. Vol. 2. Cold Spring Harbor Laboratory Press, New York. (1989) E10-E14.

	Laboratory Director	Supervisor
Reviewed by:	______________	______________
Date:	______________	______________

Solutions For Sperm Digest Extraction

DIGEST BUFFER Store at R.T.

100.0 ml	Nuclei Lysis Buffer
25.0 ml	10% SDS

PROTEINASE K SOLUTION (2 mg/ml) Store frozen in 800 ul aliquots

2.0 mg	Proteinase K
1.0 ml	Proteinase K Stock Buffer

PROTEINASE K SOLUTION (10 mg/ml) Store frozen in 100 ul aliquots

10.0 mg	Proteinase K
1.0 ml	Proteinase K Stock Buffer

1 M DTT (DITHIOTHREITOL)

Dissolve 1.542 gms DTT in 10.0 ml D/I H_2O
Add 100 ul of 1M Sodium Acetate. Store frozen in 250 ul aliquots - Use fresh.

SATURATED NH_4 ACETATE (19.2 M) Store at R.T.

148.0 gm NH_4 Acetate

Q.S. to 100.0 ml with deionized water

NUCLEI LYSIS BUFFER Store at 4°C

10.0 mM	Tris	1.21 gm
400.0 mM	NaCl	23.40 gm
2.0 mM	EDTA·Na_2	0.74 gm

Q.S. to 1 liter with distilled water

1 M SODIUM ACETATE, pH 5.2 (100 ml) Store at R.T.

13.6 gms Sodium Acetate in 80.0 ml D/I H_2O
Adjust pH to 5.2 with glacial acetic acid
Q.S. to 100.0 ml

Solutions For Sperm Digest Extraction (cont'd)

PROTEINASE K STOCK BUFFER Store at 4°C

2.0 mM EDTA-Na_2 (0.5 M Na2 EDTA Stock) 2.0 ml
1.0% SDS (10% SDS Stock) 50.0 ml
Deionized Water 448.0 ml

TRIS BUFFER (For dialyzing & concentration of forensic DNA extracts - PCR)

10.0 mM Tris 1.21 gm

pH to 7.5 with HCl
Q.S. to 1 liter with deionized water

CENTRICON-100 MICRONCENTRATOR (For small volume concentration)

Amicon Division of W. R. Grace & Company - Conn.
Danvers, MA 01923 USA

0.01 M PHOSPHATE BUFFERED SALINE (pH 7.30)

Solution Volume: 1.0 liter

#1 Na2HPO4 1.11 gm
#2 KH2PO4 0.3 gm
#3 NaCl 8.5 gm
#4 D/I H_{20} 1.0 liter
#5 NaOH 1.0 N used to adjust pH

Dissolve #1, #2, and #3 in 1.0 liter of D/I H_2O
Adjust pH to 7.3 with #5
Store at R.T.

TE (TRIS-EDTA) BUFFER

10.0 mM Tris 1.21 gm
0.2 mM EDTA-Na_2 0.074 gm

pH to 7.5 with HCl
Q.S. to 1 liter with deionized water

DNA QUANTITATION

SPECTROPHOTOMETER

Needed UNDIGESTED MALE GENOMIC DNA (AGTC)
- TE BUFFER
- UV CUVETTES
- ADJUSTABLE PIPETTORS
- DISPOSABLE TIPS
- 10 X 75 OR 12 X 75 DISPOSABLE TUBES
- MILTON ROY UV SPECTROPHOTOMETER

1. Plug the instrument into a grounded outlet.

2. Turn the spectrophotometer on, using the POWER switch on the right side of the lower control panel. Although the instru ment can be used almost as soon as it is turned on, best performance is achieved following a warmup period of 15 to 30 minutes.

3. Select the desired wavelength with the wavelength selector. Wavelength is indicated on the dial located to the left of the wavelength selector (260 or 280 nm).

4. Adjust the mirror position for the lamp required by pulling the mirror lever out when using the deuterium lamp (UV) and pushing the lever in when using the tungsten lamp (VIS).

5. Choose the correct lamp for the selected wavelength by flip ping the lamp power switch to TUNGSTEN-VIS or DEUTERIUM-UV.

6. For UV wavelength analysis, ignite the deuterium lamp by pushing the starter button for approximately two (2) seconds and releasing. The deuterium lamp requires a warmup of at least ten(10) minutes before readings are taken. Maximum lamp stability takes longer. Do this after the 30 minute warmup period in step #2.

7. Select the operating mode - Transmittance, Absorbance, or Concentration - using the mode selector.

8. Set the sensitivity switch located in the center of the lower control panel to LO.

9. Add TE BUFFER to microcuvette and set 100% T, 000 A, or 000 C for the blank using the 100% T/000 A control located on the far left side of the lower control panel (do this at 260 nm).

10. Prepare standards as follows:

Standard	TE(ul)	Human DNA (ul)	Spec Reading at 260 nm	DNA (ug)
1	983	17 (0.731ug/ul)	0.250	1.25
2	500	500 (STD 1)	0.125	0.625
3	500	500 (STD 2)	0.062	0.313

11. Vortex and briefly spin DNA samples. Transfer 5 ul sample to 495 of TE BUFFER and briefly vortex.

12. Read and record sample absorbance at 260 nm

13. To obtain concentration of sample, multiply A260 by five(5). This value is obtained as follows:

$$\text{Dilution factor} = \frac{495 \text{ (ul H20)}}{5 \text{ (ul DNA)}} = 100$$

DNA Constant= 0.05 (If A260=1 then [DNA]=50ug/ml=0.05ug/ul)

A260 (sample) X dilution factor X DNA Constant = ug/ul

A260 (sample) X 100 X 0.05 = ug/ul

A260 (sample) X 5 = ug/ul

14. To obtain total amount (ug) of sample multiply ug/ul by total volume of sample in TE buffer.

References

Milton Roy SPECTRONIC 21 Spectrophotometers. Operator's Manual. Copyright 1988 Milton Roy Company.

	Lab Director	Supervisor
Reviewed by:	____________	____________
Date:	___/___/___	___/___/___

DNA QUANTITATION USING YIELD GEL KIT

Reagents & Supplies

- YIELD GEL KIT:
  - DNA STANDARDS
  - 5X LOADING BUFFER
  - KNOWN CONTROL SAMPLE
  - LAMBDA HIND III VISIBLE LADDER
- EPPENDORF TUBES
- PIPETTOR (1-100 ul)
- TE BUFFER
- HEAT BLOCK (65°c)
- ETHIDIUM BROMIDE (10 mg/ml)
- POLAROID DS34
- POLAROID TYPE 667 FILM

1. Make 0.6% gel according to mini-gel electrophoresis procedure.

2. Use neat forensic samples from blood or semen stains. Do not dilute.

3. Dilute genomic DNA samples from whole blood extractions 1:40 (4ul : 156ul) with TE buffer.

4. Fill out "mini-gel recording form" for yield gel using lanes 12-28 for the unknown samples.

5. Melt 6 ul of Lambda Hind III at 65°C for ten (10) minutes and then load it into lane #1.

6. Load 6 ul of each genomic DNA standard as well as the known control, being sure to vortex and pulse spin each standard before loading, into lanes 2-11 as per yield gel recording form.

7. In an Eppendorf tube, add 2 ul of 5X loading buffer to 4 ul of each un known DNA sample to be tested and load the 6 ul into lanes 12-28.

8. Electrophorese at 50 volts until bromophenol blue marker has migrated 1.5 cm from the origin.

9. At the end of the electrophoresis, stain the gel with ethidium bromide (using ethidium bromide at a final concentration of 0.5 µg/ml) for 20-30 minutes. **Wearing gloves,** transport the gel in its casting tray to the transillluminator.

   Warning: Ethidium Bromide is a known carcinogen, treat with cau tion. Always wear gloves, wash if it is spilled.

10. Carefully slide the gel off the casting tray. **Using UV eye protection,** turn on the transilluminator to visualize the DNA.

11. Photograph using DS34 camera set at F 5.6, for one (1) second using an orange Tiffen 15 filter. Attach photograph to worksheet for interpreta tion.

Irena Drmi , Boja Re?i , Davorka Sutlovi and Dragan Primorac

PCR genotyping of the AmpliType Polymarker

Introduction

One of the useful PCR amplification and typing systems in forensic medicine is Polymarker (PM), which contains specific regions of the six genetic loci: HLADQA1 (Human Leukocyte Antigen), LDLR (Low Density Lipoprotein Receptor), GYPA (Glycophorin A), HBGG (Hemoglobin G Gammaglobin), D7S8, and GC (Group Specific Component).
AmpliType PM System, the Perkin-Elmer Kit, developed and manufactured by Roche Molecular Systems uses the amplification and reverse dot blot typing system.

PROCEDURE

1.Sample preparation

Prepare genomic DNA from 10 ml of whole blood in EDTA obtained from family members, using any of the standard methods.
Determine the amount and purity of DNA spectrophotometrically (260 nm, 260/280 nm) as well as quality of DNA by electrophoresis on 1.0% agarose with ethidium bromide and photograph by a Fotodyne camera using a Polaroid 667 film.

2.DNA Amplification (PCR)

Reagents	Volume
1. AmpliType PM PCR Reaction Mix	20 ml
2. AmpliType PM Primer Set	20 ml
3. DNA sample, diluted 1:10 (5-50 ng)	2-10 ml
4. Sterile H2O	Ad 50ml

For amplification of AmpliType PM perform the following program on the GeneAmp PCR System 9600 (Perkin Elmer):

Preheat block 95°C, 5 min (using by "Pause During Run" step of the program)

Denaturation	95°C, 30 s	
Annealing	63°C,30 s	No. of cycles 32
Extension	72°C,30 s	

Final Extension 72°C, 10 min

Check the PCR products (138-239/242 bp) by electrophoresis on 1.0% agarose with ethidium bromide and photograph by a Fotodyne camera using a Polaroid 667 film.

3.DNA Hybridization

Before starting DNA hybridization and color development procedures, assemble the required reagents and equipment as follows:

Enzyme conjugate: HRP-SA
Chromogen : TMB solution (Add 30 mL of 100% ethanol to the bottle)
Hybridization solution (5XSSPE and 0.5% w/v SDS)
Wash solution: 2.5XSSPE, 0.1% w/v SDS)
Citrate buffer (0.1 M sodium citrate, pH 5.0)
3% Hydrogen peroxide
AmpliType PM DNA Probe strips
AmpliType DNA Typing trays

Gloves should be worn throughout the whole procedure!

1. Heat the rotating water bath to 55 °C (+/_ 1°C). The water level should be between 0.6 and 1.2 cm above the shaker platform.

2. Pre-warm the Hybridization solution and Wash solution to 37°-55°C. Both solutions should be completely dissolved and mixed well.

3. Place required number of labeled DNA probe strips (at right edge), and one strip for positive and one for negative control.

4. Prepare the Gene Amp PCR System 9600 and place the tubes to denature the amplified DNA (95°C, 5 minutes). Maintain each tube at 95 °C until use.

5. Add 3 mL of pre-warmed hybridization solution to each well at the labeled end of each strip. DNA Typing trays should be tilted towards the labeled end of the strips.

6. Add 20 mL of amplified DNA, below the surface of the hybridization solution in the well.

7. Put the plastic lid on the tray and mix by carefully rocking the tray. Transfer the tray to the 55°C water bath, and hybridize the samples at 50-90 rpm, 15+/_2 minutes.

8. Few minutes before the end of previous step, prepare the enzyme conjugate solution (3.3 mL of hybridization solution and 27 mL of enzyme conjugate, per each strip).

9. Stop the rotation (but maintain the temperature) and remove the tray. Aspirate the contents of each well from the labeled end while tilting the tray slightly.

10. Dispense 5 mL of pre-warmed wash solution into each well. Rinse by rocking the tray for several seconds, then aspirate the solution from the each well.

11. Dispense 3 mL of the enzyme conjugate solution into each well and transfer the tray to the 55°C water bath. Rotate the tray at 50-70 rpm for 5 minutes.

12. Remove the tray and aspirate the contents of each well from the labeled end of the strips.

13. Dispense 5 mL of pre-warmed wash solution into each well. Rinse by rocking the tray for several seconds, then aspirate the solution from the each well.

14. Dispense 5 mL of pre-warmed wash solution into each well and transfer the tray to the 55°C water bath. Rotate the tray at 50-70 rpm for 12+/_1 minutes. This is a critical step!

15. Remove the tray and aspirate the contents of each well from the labeled end of the strips.

16. Dispense 5 mL of pre-warmed wash solution into each well. Rinse by rocking the tray for several seconds, then aspirate the solution from each well. Remove any solution from the tray and from the tray lid.

4.Color Development

1. Dispense 5 mL of citrate buffer into each well and transfer the tray on an orbital shaker set. Rotate at room temperature (15°-30°C), at 50 rpm for 5 minutes.

2. During the previous step prepare the color development solution (5 mL citrate buffer; 5 mL 3% hydrogen peroxide; 0.25 mL chromogen TMB solution per strip). Do not prepare the color development solution more than 10 minutes before use!

3. Remove the tray from orbital shaker and aspirate the buffer from each well. Add 5 mL of the freshly prepared color development solution.

4. Develop the strips at room temperature by rotating on an orbital shaker set at 50 rpm, until the adequate color intensity of "S" dot (control probe).

5. Remove the tray from orbital shaker and aspirate the contents from each well.

6. Stop the color development: dispense approximately 5 mL of deionized water into the well. Place the tray on an orbital shaker, at 50 rpm for 10 minutes.

7. Remove the tray from orbital shaker and aspirate the contents from each well.

8. Repeat the steps 6 and 7 at least two times.

9. Record the pattern of blue dots from each wet strip before the photography and interpretation of results. Photographs must be taken while DNA probe strips are still wet.

Literature:

1. Perkin Elmer: AmpliType User Guide, 2.0, 1993.
2. Perkin Elmer. Good Laboratory Practices for Gene Amp PCR Instruments. Forensic Forum. Issue 4 (1993):1-2.
3. Keys KM, Budowle B, Andjelinovic S, Definis Gojanovic M, Drmic I, Marcikic M, Primorac D. Northern and southern Croatian population data on seven PCR based loci. For Sci Int 81(1996):191-199.

Irena Drmi , Boja Re?i , Davorka Sutlovi and Dragan Primorac

PCR Genotyping of the AmpliType HLA DQA1

Introduction

AmpliType HLA DQA1Forensic Kit, amplifies a region of the of Human Leukocyte Antigen DQA1 gene (6. chromosome). This system uses the amplification and reverse dot blot typing system and distinguishes six alleles, which define twenty-one different genotype.

PROCEDURE

1.Sample preparation

Prepare genomic DNA from 10 ml of EDTA whole blood obtained from family members, using any of the standard methods.
Determine the amount and purity of DNA spectrophotometrically (260 nm, 260/280 nm) as well as quality of DNA by electrophoresis on 1.0% agarose with ethidium bromide and photograph by a Fotodyne camera using a Polaroid 667 film.

2.DNA Amplification (PCR)

Reagents	Volume
1. AmpliType HLADQA1 PCR Reaction Mix	20 ml
2. DNA sample, diluted 1:10 (2-50 ng)	2-10 ml
3. Sterile H2O	Ad 50ml
4. 6 mM MgCl2	20 ml

For amplification of AmpliType HLADQA1 perform the following program on the GeneAmp PCR System 9600 (Perkin Elmer):

Preheat block	95 °C, 3 min	
Denaturation	94 °C,1 min	
Annealing	60 °C,30 s	No. of cycles 32
Extension	72 °C,30 s	
Final Extension	72 °C, 10 min	

Check the PCR products (239/242 bp) by electrophoresis on 1.0% agarose, stain with ethidium bromide and photograph by a Fotodyne camera using a Polaroid 667 film.

3.DNA Hybridization

Before starting DNA hybridization and color development procedures, assemble the required reagents and equipment as follows:

Enzyme conjugate: HRP-SA
Chromogen : TMB solution (Add 30 mL of 100% ethanol to the bottle)
Hybridization solution (5XSSPE: 3.6 M NaCl, 200 mM NaH2PO4xH2O, 20mM EDTA, pH 7.4; and 0.5% w/v SDS)
Wash solution: 2.5XSSPE, 0.1% w/v SDS)
Citrate buffer (0.1 M sodium citrate, pH 5.0)
3% Hydrogen peroxide
AmpliType HLADQA1 DNA Probe strips
AmpliType DNA Typing trays

Gloves should be worn throughout the whole procedure!

1. Heat the rotating water bath to 55 oC (+/_ 1oC). The water level should be between 0.6 and 1.2 cm above the shaker platform.

2. Prewarm the Hybridization solution and Wash solution to 37o-55oC. Both solutions should be completely dissolved.

3. Place required number of labeled DNA probe strips (at the right edge), and one strip for positive and one for negative control.

4. Prepare the Gene Amp PCR System 9600 and place the tubes to denature the amplified DNA (95oC, 10 minutes). Maintain each tube at 95 oC until use.

5. Few minutes before the end of previous step, prepare enzyme conjugate solution (3.3 mL of hybridization solution and 27 mL of enzyme conjugate, per each strip). Do not prepare this solution more than 15 minutes before use!

6. Add 3 mL of freshly prepared Hybridization/enzyme conjugate solution to each well at the labeled end of each strip. DNA Typing trays should be tilted towards the labeled end of the strips.

7. Add 35 mL of amplified DNA, below the surface of the hybridization solution in the well.

8. Put the plastic lid on the tray and mix by carefully rocking the tray. Transfer the tray to the 55°C water bath, and hybridize the samples at 50-90 rpm, 20 minutes.

9. Stop the rotation (but maintain the temperature) and remove the tray. Aspirate the contents of each well from the labeled end while tilting the tray slightly.

10. Dispense 10 mL of pre-warmed wash solution into each well. Rinse by rocking the tray for several seconds, then aspirate the solution from the each well.

11. Dispense 10 mL of pre-warmed wash solution into each well and transfer the tray to the 55 °C water bath. Rotate the tray at 50 rpm for 12+/_1 minutes. This is a critical step!

12. Remove the tray and aspirate the contents of each well from the labeled end of the strips.

13. Dispense 10 mL of pre-warmed wash solution into each well. Rotate the tray at 50 rpm for 5 minutes, then aspirate the solution from each well. Remove any solution from the tray and from the tray lid.

4.Color Development

1. Dispense 10 mL of citrate buffer into each well and transfer the tray on an orbital shaker set. Rotate at room temperature (15 °-30 °C), at 50 rpm for 5 minutes.

2. During the previous step prepare the color development solution (10 mL citrate buffer; 10 mL 3% hydrogen peroxide; 0.5 mL chromogen TMB solution per strip). Do not prepare the color development solution more than 10 minutes before use!

3. Remove the tray from orbital shaker and aspirate the buffer from each well. Add 10 mL of the freshly prepared color development solution.

4. Develop the strips at room temperature by rotating on an orbital shaker set at 50 rpm, until the adequate color intensity of "C" dot (control probe).

5. Remove the tray from orbital shaker and aspirate the contents from each well.

6. Stop the color development: dispense approximately 10 mL of deionized water into each well. Place the tray on an orbital shaker, at 50 rpm for 10 minutes.

7. Remove the tray from orbital shaker and aspirate the contents from each well.

8. Repeat the steps 6 and 7 at least two times.

9. Record the pattern of blue dots from each wet strip before the photography and interpretation of results. Photographs must be taken while DNA probe strips are still wet.

Literature:

1. Perkin Elmer. AmpliType User Guide, 2.0, 1993.

2. Perkin Elmer. Good Laboratory Practices for Gene Amp PCR Instruments. Forensic Forum. Issue 4 (1993):1-2.

3. Keys KM, Budowle B, Andjelinovic S, Definis Gojanovic M, Drmic I, Marcikic M, Primorac D. Northern and southern Croatian population data on seven PCR based loci. For Sci Int 81(1996):191-199.

Wednesday, September 24 (Day 2)

7:30 a.m. Registration MA NMS
8:00 a.m. Development and validation of the AMPFL STR
human identification system Lec MR NMS Dr.N. Oldroyd
8:40 a.m. Application of mtDNA to forensic case work Lec MR NMS Dr. M.Holland
9:30 a.m. Mt DNA extraction and quantification Lab L 1 NMS Dr. Ed Huffine
10:30 a.m. Coffee break
12:30 a.m. Morning section adjourns
2:00 p.m. Mt DNA amplification Lab L 3 NMS Dr. Ed Huffine
4:15 p.m. Sequencing of Mt DNA L 2 NMS Dr. Ed Huffine
5:30 p.m. Identification of war victims by DNA Lec MR NMS Dr. M.Kubat
6:00 p.m. Identification of war victims Lec MR NMS Dr. D. Zecevic
7:30 p.m. Adjourn

DoD DNA Registry
Office of the Armed Forces Medical Examiner
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Adopted By ______________________ Date __________
Reviewed By ______________________ Date __________
Version 2.0 Replaces Version 1.0

Organic Extraction of DNA from Hair

PRINCIPLE

To provide instructions for isolating DNA from hair. The basic procedure consists of breaking disulfide bonds with dithiothreotol (DTT) and lysing nucleated cells with sodium dodecyl sulfate (SDS) coupled with protein digestion with proteinase K. After digestion is complete, a series of phenol/chloroform/isoamyl alcohol extractions is performed. The DNA is recovered by precipitation with cold ethanol or Centricon® purification and brought to the desired volume with Tris-EDTA (TE) buffer. Precipitation with ethanol is suitable for specimens suspected of containing high levels of DNA (e.g., anagen hairs). Centricon® purification is recommended for those specimens suspected of containing low levels of DNA (e.g., catagen and telogen hairs).

SPECIMEN

Hair specimen(s)

REAGENTS, SUPPLIES, and EQUIPMENT

Reagents
Bleach, 10% commercial (7 mM sodium hypochlorite solution)
1-Butanol
Dithiothreotol (DTT), 1 M
Ethanol, absolute
Ethanol (70% v/v)
Extraction buffer (10mM Tris, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS)
Mounting media solvent (e.g., Toluene, 100% Xylenes)
Phenol/chloroform/isoamyl alcohol (25:24:1)
Proteinase K (20 mg/ml)
TE buffer (10 mM Tris; 1 mM EDTA, pH 7.5)
Water, sterile, distilled

Supplies

Centricon-100® concentrators
Coverslips, microscope slide
Forceps
Kim-wipes
Microcentrifuge tubes (1.5-2.0 ml)
Pipette tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettors)
Racks, tube
Slides, microscope, glass
Scissors
Tube, conical (15-50 ml)
Waste containers (general, biohazard, organic, Sharps™)

Personal Protective Equipment

Gloves (Viton™ or 6 mil or greater nitrile required for certain steps)
Laboratory coat
Mask, surgical
Safety glasses
Sleeves, disposable

Equipment

Centrifuge (e.g., IEC)
Evaporator/concentrator
Freezer, -20°C
Incubator, 56°C (or 56°C heat block)
Laboratory hoods (laminar flow, chemical fume)
Microcentrifuge (e.g., Sorvall MC 12V, Eppendorf Centrifuge 5402)
Microscope
Pipettors (e.g., P-10, P-100, P-1000)
Refrigerator, 4°C

QUALITY ASSURANCE

1. Extraction of specimens is to be performed in the following designated laboratories:

blood references and whole blood specimens	Lab 3
specimens suspected of containing high levels of DNA	
-non-organic extraction	Lab 4
-organic extraction	Lab 5
specimens suspected of containing low levels of mitochondrial DNA	Labs 9 and 10

2. Ensure that the following worksheet is completed for this procedure and placed in the appropriate case folder:

"Organic Extraction of DNA from Hair"/DNA Form 213

3. Ensure that all reagents satisfy the minimum standards for quality control, where appropriate.

4. Ensure that all instruments and equipment satisfy the minimum standards for quality control, where appropriate.

5. In order to prevent contamination, all steps in this procedure should be performed, where appropriate, in the proper laboratory hoods.

6. Any laboratory workspace and all pipettors and racks to be used in this procedure must be cleaned with 10% commercial bleach and thoroughly dried before beginning. When using a laminar flow hood, expose to ultraviolet light for a minimum of 10 minutes after cleaning.

7. Before use, the forceps and scissors must be cleaned with 10% commercial bleach. Dry thoroughly. In addition, they should be cleaned in the same manner following the processing of each individual specimen (if used).

8. To prevent contamination, microcentrifuge tubes (closed) should be irradiated in an ultraviolet crosslinker with 2 J/cm^2. The extraction buffer and TE buffer (in 50 ml conical tubes) must be irradiated with 6 J/cm^2. Follow "Irradiation of Reagents and Supplies in the Ultraviolet Crosslinker" SOP for appropriate method of irradiation.

   Note: Do not include the proteinase K or DTT in the extraction buffer when irradiating.

9. To prevent contamination, use Centricon-100® concentrators that have been pre-assembled in a laminar flow hood.

10. No reagents, supplies, or equipment used for the extraction of DNA from specimens will be allowed in a post-amplification room.

11. Any supplies or equipment taken from a post-amplification room to a pre-amplification room must be sterilized with 10% commercial bleach before removal from the post-amplification room and again in the pre-amplification room before use.

12. When extracting specimens suspected of containing low levels of DNA:

    A surgical face mask and disposable sleeves will be worn at all times to prevent contamination.

    Gloves and disposable sleeves will be changed between extraction set-up of each specimen.

13. Only one evidence specimen will be open at any one time. Only evidence specimens from one case will be extracted together.

14. A maximum of four hair specimens are to be processed at any one time.

15. If only a portion of a single hair is to be extracted, sectioning should be performed outside of a hood to prevent loss of any part of the specimen due to air flow in the hood.

16. A minimum of one reagent blank and one substrate control (if available) must be carried throughout the extraction procedure and assayed in parallel with the evidentiary samples.

    NOTE: If it is suspected that one or more of the specimens may require additional incubations with proteinase K and DTT, it is recommended that these samples be extracted at a different time.

17. Change pipet tips between each transfer or addition of sample or reagent, unless otherwise noted.

18. No aliquot of any processed sample may be returned to its storage container or to the original evidence container.

19. No aliquot of any reagent may be returned to the original stock container.

20. For mitochondrial cases only: When a sufficient amount of sample is remaining, a second extraction, performed at a different time (e.g., the day following completion of the first extraction), will be conducted on each evidentiary specimen.

SAFETY

1. All appropriate MSDS sheets must be read prior to performing this procedure.

2. Treat all biological specimens as potentially infectious. Gloves, safety glasses, and a laboratory coat must be worn at all times.

3. Follow the "Exposure Control Plan for Occupational Exposure to Bloodborne Pathogens" (DoD Forensic Advisory Committee).

4. When using mounting media solvents, 6 mil or greater nitrile gloves must be worn and each hand must be double-gloved. The outer glove on each hand must be removed immediately if an organic solvent is observed on the glove. If a significant amount of solvent is observed, both gloves on that hand must be replaced. Inspect gloves approximately every five minutes for wetness or spots from solvents.

5. When performing the organic extraction steps of this procedure, either Viton™ or 6 mil or greater nitrile gloves must be worn. If using nitrile gloves, each hand must be double-gloved. The outer glove on each hand must be removed immediately if an organic solvent is observed on the glove. If a significant amount of solvent is observed, both gloves on that hand must be replaced. Inspect gloves approximately every five minutes for wetness or spots from solvents.

6. All organic extraction procedures are to be performed in a chemical fume hood.

7. Avoid direct exposure to ultraviolet light when using the germicidal lamp in the laminar flow hood.

8. Distinguish all waste as general, biohazard, organic, or Sharps™ and discard appropriately. Since Xylenes waste and toluene waste are recycled, each must be disposed of in a separate waste container from other liquid organic waste.

PROCEDURE

Begin at Step 1 if hairs are mounted. If hairs are loose, begin at Step at 11.

Note: A "specimen" refers to a single hair, root end, or pool of approximately 15 hairs.

At the examiner's discretion, it is allowable to extract only a portion of a single hair specimen.

If necessary, a "bulk extraction" (approximately 0.1 g of hair) may be performed. Adjust extraction volumes and sample containers accordingly.

If microscopic analysis is necessary, it must be performed by a qualified hair examiner.

Mounted Hair(s)

1. If necessary, a qualified hair examiner will observe hair(s) microscopically.

Perform in a Laminar Flow Hood

2. Place several drops of the appropriate mounting media solvent around the perimeter of the coverslip.

   Note: 100% Xylenes dissolves Permount; toluene dissolves Cytoseal.

   Note: It may be necessary for sample to incubate at room temperature for several minutes to allow mounting media to dissolve.

CAUTION: Perform Steps 3 and 4 near the center of the hood to avoid losing sample in the down draft of laminar flow at the front and back.

3. Carefully remove coverslip.

4. Using forceps, carefully remove hair(s) and place in a sterile, labelled microcentrifuge tube containing 1.0 ml mounting media solvent. Recap tube.

   Note: Initiate reagent blank(s) at this step. Set up the evidentiary specimen(s) and then the reagent blank(s).

5. Incubate at room temperature for approximately five (5) minutes. Gently agitate several times during incubation.

6. Carefully remove the mounting media solvent.

7. Add 1.0 ml sterile, distilled water. Gently agitate hair(s) to rinse off the mounting media solvent.

8. Carefully remove water.

9. Repeat wash step one time and remove water.

10. Proceed to Step 16.

Loose Hair(s)

<u>Perform in the General Laboratory</u>

11. If necessary, a qualified hair examiner will observe hair(s) microscopically.

<u>Perform in a Laminar Flow Hood</u>

12. Carefully remove hair(s) and place in a sterile, labelled microcentrifuge tube.

13. Add 1.0 ml sterile, distilled water.

    Note: Initiate reagent blank(s) at this step. Set up the evidentiary specimen(s) and then the reagent blank(s).

14. Incubate at room temperature for approximately five (5) minutes. Gently agitate several times during incubation.

15. Carefully remove water. Repeat Steps 13-15 one time.

All samples

16. Add 1.0 ml absolute ethanol. Recap tube.

17. Incubate at room temperature for approximately five (5) minutes. Gently agitate several times during incubation.

18. Carefully remove absolute ethanol.

19. Add 1.0 ml 70% ethanol. Gently agitate.

20. Carefully remove 70% ethanol.

21. Preparation of hair lysis buffer (for 10 samples):

Volume	Component
1.87 ml	extraction buffer
80 _l	1 M DTT
50 _l	20 mg/ml proteinase K

Note: Hair lysis buffer must be prepared fresh daily.

22. Add 200 &l hair lysis buffer. Mix thoroughly.

23. Incubate at 56°C for 2-3 hours.

24. Add 5 &l 20 mg/ml proteinase K and 8 &l 1 M DTT.

25. Incubate at 56°C overnight.

26. Observe each sample for complete extraction. Proceed to Step 28 if all sample extracts appear homogeneous.

27. If a sample extract does not appear homogeneous, add 5 _l 20 mg/ml proteinase K and 8 _l 1 M DTT and incubate at 56°C for 2-3 hours. Repeat as necessary (maximum of 5 times).

    Note: Additional incubations with proteinase K and DTT must be performed with each sample, including the reagent blank(s) and substrate control(s).

Perform in a Chemical Fume Hood

28. Add 200 &l phenol/chloroform/isoamyl alcohol (25:24:1). Mix thoroughly. Spin for 2 minutes at 10,000-15,000 rpm in a microcentrifuge. Transfer upper aqueous layer to a sterile, labelled microcentrifuge tube. Repeat extraction with phenol/chloroform/isoamyl alcohol until the interface is clean. Dispose of phenol waste in the appropriate waste container.

Option: To recover DNA by ethanol precipitation, follow Steps 29 through 38.
To recover DNA by Centricon® purification, begin at Step 39.

Ethanol Precipitation

Perform in a Laminar Flow Hood

29. Add 1.0 ml cold absolute ethanol to the tube containing the aqueous layer. Mix gently.

30. Incubate at -20°C for a minimum of 30 minutes.

31. Spin for 15 minutes at 10,000-15,000 rpm in a microcentrifuge.

    Note: If a refrigerated microcentrifuge is available, perform spin at -20°C.

32. Remove alcohol by decanting or carefully pipetting.

33. Add 1.0 ml room temperature 70% ethanol.

34. Spin for 5 minutes at 10,000-15,000 rpm in a microcentrifuge.

35. Remove alcohol by decanting or carefully pipetting. If there is any residual alcohol after decanting, remove the remainder by careful pipetting.

36. Spin samples in the evaporator/concentrator approximately 10 minutes or until dry. Be careful not to overdry.

37. Add approximately 50 &l TE Buffer to resolubilize DNA. Record total volume. Incubate at 56°C for a minimum of 2 hours.

    Note: Final volume of extract depends upon the level of DNA presumed to be present based on visual observation of the evidence and/or specific case history.

38. Proceed to Step 45.

Centricon® Purification

<u>Perform in a Chemical Fume Hood</u>

39. Add 200 &l 1-butanol. Mix thoroughly. Centrifuge 2 minutes at 10,000-15,000 rpm in a microcentrifuge. Remove and discard most of the 1-butanol upper layer into the appropriate waste container.

<u>Perform in a Laminar Flow Hood</u>

40. Label a sufficient number of pre-assembled Centricon-100® concentrators.

41. Add 1.5 ml TE buffer to the sample reservoir of the Centricon-100®. Recap before transfer to the chemical fume hood.

Perform in a Chemical Fume Hood

42. Transfer the lower aqueous layer to the sample reservoir of the Centricon-100®. Avoid pipetting any residual 1-butanol. Spin column at 1000 x g (IEC 2600 rpm) for 15-30 minutes or until sample has spun through. Discard filtrate.

    Note: Wipe pipet tip with a kim-wipe to remove any carry-over 1-butanol before dispensing aqueous layer.

Perform in a Laminar Flow Hood

43. Add 2 ml TE buffer and spin 15-30 minutes at 1000 x g or until the buffer has spun through.

44. Pipette the 40-50 &l of retentate up and down 8-10 times. Transfer retentate to a sterile. labelled microcentrifuge tube. Adjust final volume to approximately 50 &l. Record total volume.

    Note: Final volume of extract depends upon the level of DNA presumed to be present based on visual observation of the evidence and/or specific case history.

45. Store samples at 4%C if to be amplified within 3 weeks or used routinely. Store at -20%C for long-term storage, but limit the number of freeze/thaw cycles.

INTERPRETATION/ACCEPTABLE VALUES/TROUBLESHOOTING

Not applicable. Samples are ready for evaluation by either extraction yield gel or slot blot quantitation, if desired.

REFERENCES

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FBI Laboratory, "Isolation of DNA from Liquid Blood Samples," in Procedures for the Detection of Restriction Fragment Length Polymorphisms in Human DNA, December 7, 1990.

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ANNUAL REVIEW

Reviewed By ______________________ Date __________

Reviewed By ______________________ Date __________

Reviewed By ______________________ Date __________

Reviewed By ______________________ Date __________

Armed Forces DNA Identification Laboratory
DoD DNA Registry
Office of the Armed Forces Medical Examiner
Washington, D.C.

Adopted ________ By ______________
Reviewed ________ By ______________
Version: 3.0 Replaces Version 2.1

Extraction of DNA from Dried Skeletal Remains

PRINCIPLE:

This procedure outlines the steps for the extraction of DNA from skeletal remains. This procedure is to be used with those remains which are dry, i.e. no fresh tissue adhering to the specimen and no marrow inside the bone.

SPECIMEN:

Bone specimen (usually 0.5 g to 2.5 g) - specimen extraction to be repeated when possible

REAGENTS AND SPECIAL SUPPLIES:

Agarose (DNA Grade)
Agarose Gel Loading Buffer, 6X (50% glycerol, 1.5 mM bromophenol blue, 100 mM EDTA)
Aluminum Oxide Grinding Stones
Beckman GPR Centrifuge
N-Butanol
Centricon-100 Concentrator
10% Commercial Bleach (7 mM Sodium Hypochlorite Solution)
Dremel Tool
95% Ethanol
Ethidium Bromide (5mg/ml)
Emery Wheel (0.25" thick)
Extraction Buffer (10mM Tris, pH 8.0, 100 mM NaCl, 50 mM EDTA, pH 8.0, 0.5% SDS)
IEC Centra MP4 Centrifuge
Micro-Mill Grinder or Waring Blender
Nutator
Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
Proteinase K (20 mg/ml)
Robbins Scientific Hybridization Incubator
1X TBE (89 mM Tris HCl, pH 8.3, 89 mM Boric Acid, 2 mM EDTA)
TE Buffer (10 mM Tris, 1 mM EDTA, pH 7.5)
Sizing Ladder (123 base pair ladder)
Sterile Deionized Water

QUALITY ASSURANCE:

1. Ensure that all reagents/buffers satisfy the minimum standards for quality control, see SOP "Quality Control Procedure for DNA Extraction Reagents and Buffers."

2. Ensure that all instruments and equipment comply with the minimum standards for quality control, where appropriate.

3. All aluminum oxide sanding bits will be scrubbed with 10% commercial bleach and a brush, soaked in bleach for approximately 10 minutes, rinsed with distilled water, dried at room temperature and autoclaved.

4. One reagent blank must be carried throughout the extraction procedure and assayed in parallel with the test samples.

5. In order to prevent contamination, all steps in this procedure should be performed, where appropriate, in the proper hoods.

6. No specimens or materials used for the extraction of DNA from skeletal remains will be allowed in a post-amplification PCR product room.

7. Only one case will be opened at any one time per analyst and a maximum of four skeletal specimens are to be processed at any one time.

8. Any specimens suspected of containing large quantities of DNA, e.g. fresh bone and whole blood, will not be allowed in the bone extraction area. Additionally, dry bone or old specimens that are suspected of having large quantities of DNA will not be allowed in the bone extraction area. Exception: the sanding of old bone can be performed in the chemical fume hood in Lab 10.

9. Gloves and disposable sleeves will be changed after working with each bone specimen.

10. A surgical face mask will be worn at all times to prevent contamination.

11. A second extraction, independently performed at a different time (e.g. the day following the completion of the first extraction), will be conducted on each bone specimen when at least 0.5 g of bone is remaining.

SAFETY:

1. Gloves, safety glasses, and laboratory coats must be worn at all times.

2. Surgical face masks will be worn while sanding and grinding bone specimens.

3. Follow the Exposure Control Plan for Occupational Exposure to Bloodborne Pathogens (DoD Forensic Advisory Committee).

4. All organic extraction procedures are to be performed in a chemical fume hood.
5. Discard organic waste in a hazardous waste container.

6. All appropriate MSDS sheets must be read prior to using this protocol.

PROCEDURE:

1. Follow the SOP for "Chain of Custody" and the SOP for "Photodocumentation" before processing any evidentiary material.

2. The hoods and equipment must be cleaned appropriately prior to use for the extraction of each specimen. The minimum standards of cleaning are described and are to be followed when

"clean . . . appropriately" is cited in the protocol. The grinders/blenders must be cleaned with 10% commercial bleach, sterile distilled water, and 95% ethanol in the laminar flow hood. The hoods and all other equipment must be cleaned with 10% commercial bleach. The cleaned equipment should be thoroughly dried before use.

3. While following this procedure, complete the "Preparation of Dried Skeletal Remains for Extraction, " DNA Form 2 and the "Extraction of DNA from Skeletal Remains," DNA Form 3 (see Pages 8 and 9).

4. Obtain bone specimen. Record gross weight of bone specimen received. If bone specimen is approximately 8 grams or less or if the analyst chooses, proceed to Step 5. Clean mortar and chisel appropriately. Allow the chisel and mortar to dry. With a chisel and hammer, break bone specimen in a mortar to obtain a fragment of approximately 4.0 grams.

Performed in Bone Sanding Hood

5. Clean bone extraction hood appropriately. Sand all exposed surfaces (outer and inner layers) with a cleaned and autoclaved aluminum oxide sanding bit fitted to a rotary tool. Use an Emery Wheel to cut the bone, if necessary. Clean hood appropriately and change Dremel bit, gloves, and disposable sleeves between each specimen.

6. Clean mortar and chisel as in Step 2.

7. Obtain approximately 2.0 g of bone specimen. If necessary, break the specimen into 2 or 3 fragments with a clean chisel and hammer in a mortar. Weigh the bone specimen. Repackage bone specimens not to be used. Place bone fragment in weigh boat and carry to laminar flow hood.

Performed in Laminar Flow Hood

8. Clean specimen of powdered debris by placing the sanded skeletal fragments into a 50 ml conical tube containing approximately 25 ml of bottled sterile deionized water. Shake the tube approximately 10 times. Decant into a waste container. Repeat twice.

9. Cover the specimen in the conical tube with 95% ethanol. Shake the tube at least 10 times. Decant into waste container. Repeat twice. Label and clean a weigh boat with 10% commercial bleach and ethanol. Pour fragments into the cleaned, labelled weigh boat and allow to air dry in the laminar flow hood.

10. Clean the Micro-Mill Grinder (Step12a) or the Waring blender (Steps 12d) appropriately.

Performed in the Bone Sanding Hood

11. Pulverize fragments using either the Micro-Mill Grinder (Steps 12b and 12c) or the Waring Blender (Steps 12f and 12g).

Performed in the Laminar Flow Hood

Micro-Mill Grinder

12a. Clean the entire grinding chamber appropriately. Do not allow any fluid to leak near or on the contact points. Allow the interior surfaces to dry thoroughly. Place specimen fragments into the lower portion of the grinding chamber.

Note: The specimen must be dry prior to being milled.

Place the upper portion of the grinding chamber onto the lower portion by inserting the pins through the holes in the back plate on the lower portion of the grinding chamber. Fasten the left latch and then the right latch.

Performed in Bone Sanding Hood

12b. Set the grinding chamber onto the base of the grinder. Set timer to one minute, then run for another minute. Tap the upper chamber gently to remove pulverized bone from the cover.

12c. Remove the grinding chamber from the base. Tilt container at approximately a 45 degree angle and tap gently on bench top to accumulate the specimen to one side. Wait approximately 30 seconds for bone powder to settle then gently remove lid. Pour powder into a clean weigh boat or funnel for transfer to a labelled sterile, preweighed or tared 15 ml polypropylene tube.

OR

Performed in the Laminar Flow Hood

_____ Waring Blender

12d. Clean grinding chamber and lid: fill pulverizer ~1/3 full with 10% bleach, attach lid and run unit approximately 10 seconds; remove lid without touching inside of lip, rinse lid and cup with sterile distilled water, then 95% ethanol. Drain off the excess ethanol. Allow the interior surfaces to dry thoroughly.

12e. Place specimen fragments into the lower portion of the grinding chamber. Place lid on blender without touching inside lip of lid or cup.

Performed in the Bone Sanding Hood

12f. Take sealed blender cup to base unit. Run blender for up to one minute, monitoring extent of pulverization through translucent lid. If sample is not completely ground after one minute, shut off blender, tap the container, and wait approximately 30 seconds. Repeat if necessary. When starting the unit, the bone may become wedged between a blade and the cup wall; if so, shut off power, remove cup and dislodge bone by tapping or rotating the blade spindle from below.

12g. Tilt grinding chamber at approximately a 45 degree angle and tap gently on bench top to accumulate the specimen to one side. Wait approximately 30 seconds for bone powder to settle then gently remove lid. Pour powder into a clean weigh boat or funnel for transfer to a labelled sterile, preweighed or tared 15 ml polypropylene tube.

Performed in the General Laboratory

13. Determine the weight of the pulverized bone specimen in the conical tube.

14. Clean remaining bone dust from the grinding chamber at the sink. Additionally, for the Waring blender, wash grinding chamber and lid with running water and wiping, then fill 2/3 full with water and a squirt of Liquinox soap, replace lid, run approximately 30 seconds on power base and repeat with water, then 10% commercial bleach. For both units perform a final rinse

with 10% commercial bleach, distilled water, then 95% ethanol. Proceed to Step 4 if processing additional specimens.

Note: You may stop at this point for the day. Store pulverized bone sample in a cool dry dark environment (for example, your personally assigned drawer in Laboratory 9) for a maximum of 5 days. For longer storage, place pulverized bone sample in -20%C freezer.

15. Initiate a tube labelled "RB#" for the reagent blank sample which must be carried through the extraction procedure and assayed in parallel with the test samples. The reagent blank should be the last specimen processed for the remaining steps.

Performed in the Laminar Flow Hood

16. Add 3 ml Extraction Buffer (assure that the Extraction Buffer is homogenous) and 100 &l 20 mg/ml Proteinase K to the specimens and the reagent blank. Suspend the bone dust thoroughly in the reagents. Incubate overnight at 56%C on a tilted Nutator ensuring that the reagents do not reach the cap of the tube. After one hour of incubation, mix thoroughly and re-secure the caps of the tubes.

Performed in Chemical Fume Hood

17. Extract with 3 ml Phenol/Chloroform/Isoamyl Alcohol (25:24:1). Mix thoroughly. Centrifuge 2 minutes at 4950 x g using the Beckman GPR Centrifuge (6400 RPM) or the IEC Centra MP4 (5000 RPM). Transfer upper aqueous layer to a clean labelled tube. Repeat extraction with Phenol/Chloroform/Isoamyl Alcohol until the interface is clean, or a minimum of two times. Dispose of phenol waste in the appropriate waste container.

18. Extract with 3 ml n-Butanol. Mix thoroughly. Centrifuge 2 minutes at 4950 x g using the Beckman GPR Centrifuge (6400 RPM) or the IEC Centra MP4 (5000 RPM). Remove and discard most of the n-Butanol upper layer into the appropriate waste container.

Performed in Laminar Flow Hood

19. Assemble a sufficient number of Centricon-100 concentrators and label appropriately.

Performed in Chemical Fume Hood

20. Transfer the lower aqueous layer to the corresponding Centricon-100 concentrators.

Note: Avoid pipetting any residual n-Butanol, and wipe pipet tip with a Kim-Wipe to remove any carry-over n-Butanol.

Centrifuge approximately 30 minutes, or longer if necessary, at 1000 x g (2600 RPM) using the IEC Centra MP4. Discard filtrate.

Performed in Laminar Flow Hood

21. Add 2 ml of sterile TE Buffer to sample reservoir. Centrifuge approximately 30 minutes, or longer if necessary, at 1000 x g (2600 RPM) using the IEC Centra MP4. Discard filtrate. Repeat.

22. Pipette extract directly from the sample reservoir and transfer to a sterile labelled microcentrifuge tube. Rinse the Centricon filter by adding an aliquot of TE Buffer to sample reservoir, pipette up and down several times. Add this liquid to the labelled microcentrifuge tube and resuspend extract to a final volume of approximately 100 &l.

Note: Take extreme care in not allowing the pipettor to touch the sides of the sample reservoir.

23. Evaluation of Extracted DNA

a. Prepare 1% agarose according to the size of the gel tray being used.

*Small tray - 0.3g agarose with 30ml 1X TBE; 3&l EtBr (5mg/ml)

*Large tray - 0.8g agarose with 80ml 1X TBE; 8&l EtBr (5mg/ml)

b. Add 5&l of the extract to 1&l of 6X agarose gel loading buffer. Load the samples and electrophorese at a constant voltage (100 Volts) for approximately 30 minutes. Be sure to flank samples with a sizing ladder (1&l of 123 base pair ladder, 4&l dH_2O and 1&l of 6X loading buffer).

c. Visualize gel on a trans-illuminator. Photograph the gel using a Polaroid camera (suggested settings - shutter speed 1 second and f-stop at 8-11) and type 667 film. Attach photo to "Extraction Yield Gel" Worksheet (Page 10).

Note: Optimal amount of template DNA for amplification = 200 pg total DNA.

24. Store at 4%C if amplified within 3 weeks or at -20%C for long-term storage.

25. Repeat the extraction procedure if at least 0.5 g bone specimen is remaining.

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Preparation of Dried Skeletal Remains for Extraction

Case Number:______________________ Date/Time:________________ Scientist:____________________

Specimen	Gross Weight (g)	Sanded Weight (g)	Weight of Fragment to be Pulverized (g)	Weight of Powder to be Extracted (g)	Hood #	Grinder #	Comments
Specimen #:							
Specimen #:							
Specimen #:							
Specimen #:							
Reagent Blank #:							

Item	Source	Lot #
Distilled Water		
Ethanol		

1. Remove outer and inner layer of bone with Dremel tool.
2. Obtain a fragment of bone approximately 2.0 grams.
3. (PERFORM IN A BIOHAZARD HOOD): Place bone specimen in a 50 mL conical tube and fill with water, allowing space at the top for movement. Place cap on tube and gently agitate the bone in the water. Discard water and repeat wash with ethanol. Allow bone to dry at room temperature in the biohazard hood.
4. Pulverize to a powder using a Micro-Mill Grinder or Waring Blender.
5. Transfer bone powder to a labelled 15 mL conical tube.

Comments: ____

____

____

____

Location of Re-packaged Bones ____________________

DNA Form 2 Revised 09/26/95

Extraction of DNA from Dried Skeletal Remains

Case Number:____________________Date:__________________Scientist:__________________

Extraction

Item	Source	Lot #
QC Extraction Lot No.	AFDIL	
Centricon-100	Amicon	

Centricon Purification

#	Specimen	Approximate Volume Recovered	Approximate Final Volume	Comments
	Specimen #:			
	Specimen #:			
	Specimen #:			
	Specimen #:			
	Reagent Blank #:			

1. Add 3 mL Extraction Buffer to pulverized specimen in 15 mL conical tube.
2. Add 100 uL of 20 mg/mL Proteinase K.
3. Incubate at 56°C overnight.
4. Add 100 uL of 20 mg/mL Proteinase K.
5. Incubate at 56°C for 3 hours.
6. Add 3 mL phenol:chloroform:isoamyl alcohol.
7. Centrifuge 2 minutes at 4950 x g (Beckman 6400 rpm; IEC 5000rpm).
8. Transfer upper aqueous layer to a clean 15 mL conical tube.
9. Repeat steps 7 and 8 until interface is clean.
10. Add 3 mL butanol.
11. Centrifuge 2 minutes at 4590 x g.
12. Add lower aqueous layer from butanol extract to sample reservoir of Centricon-100.
13. Spin column at 1000 x g for 30-60 minutes or until sample has spun through. Add 2 mL TE Buffer and spin 30-60 minutes or until the buffer has spun through. Discard filtrate and repeat 2 mL TE Buffer wash.
14. Pipette the 40-50 uL of retentate up and down 8-10 times. Transfer retentate to a sterile, labelled tube. Rinse membrane with a volume of TE Buffer necessary to adjust final volume to approximately 100 uL. Transfer rinse volume to the sterile, labelled tube.

Comments: ____

Location of DNA Extracts ____________

DNA Form 3 Revised 07/14/95

Extraction Yield Gel

Case Number/Scientist: ____________ Date:

Gel Number: ____________ Gel Type: 1% Agarose

Lane	Specimen	Case	uL	Comment	Lane	Specimen	Case	uL	Comm
1					16				
2					17				
3					18				
4					19				
5					20				
6					21				
7					22				
8					23				
9					24				
10					25				
11					26				
12					27				
13					28				
14					29				
15					30				

Voltage: ____________ Time On: ____________

PHOTO:

DNA Form 6 Revised 03/24/95

Armed Forces DNA Identification Laboratory
DoD DNA Registry
Office of the Armed Forces Medical Examiner
Washington, D.C.

Adopted ________ By ____________________

Reviewed ________ By ____________________
Version: 2.0 Replaces "Extraction of Mitochondrial DNA from Teeth", Version 1.0

Extraction of DNA from Teeth

PRINCIPLE:

This procedure outlines the steps for the extraction of mitochondrial DNA from tooth specimens.

SPECIMEN:

Human Tooth

SPECIAL SUPPLIES AND REAGENTS:

Agarose (DNA Grade)
Agarose Gel Loading Buffer, 6X (50% glycerol, 1.5 mM bromophenol blue, 100 mM EDTA)
Beckman GPR Centrifuge
N-Butanol
Centricon-100 Concentrator
10% Commercial Bleach (7 mM Sodium Hypochlorite Solution)
Dental Drill
Drill Bits
95% Ethanol
Ethidium Bromide (5mg/ml)
Extraction Buffer (10mM Tris, pH 8.0, 100 mM NaCl, 50 mM EDTA, pH 8.0, 0.5% SDS)
IEC Centra MP4 Centrifuge
Metal File
Micro-Mill Grinder or Waring Blender
Nutator
Osteotome (surgical chisel)
Parafilm
Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
Proteinase K (20 mg/ml)
Robbins Scientific Hybridization Incubator
Sizing Ladder (123 base pair ladder)
Spoon Excavator
Sterile Deionized Water

SPECIAL SUPPLIES AND REAGENTS continued:

1X TBE (89 mM Tris HCl, pH 8.3, 89 mM Boric Acid, 2 mM EDTA)
TE Buffer (10 mM Tris, 1 mM EDTA, pH 7.5)

QUALITY ASSURANCE:

1. Ensure that all reagents/buffers satisfy the minimum standards for quality control, see SOP "Quality Control Procedure for DNA Extraction Reagents and Buffers."

2. Ensure that all instruments and equipment comply with the minimum standards for quality control, where appropriate.

3. One reagent blank must be carried throughout the extraction procedure and assayed in parallel with the test samples.

4. In order to prevent contamination, all steps in this procedure should be performed, where appropriate, in the proper hoods.

5. No specimens or materials used for the extraction of DNA from skeletal remains will be allowed in a post-amplification PCR product room.

6. Only one case will be opened at any one time per analyst and a maximum of four skeletal specimens are to be processed at any one time.

7. Any specimens suspected of containing large quantities of DNA, e.g. fresh bone or teeth and whole blood, will not be allowed in the bone\tooth extraction area. Additionally, dry bone or old specimens that are suspected of having large quantities of DNA will not be allowed in the bone extraction area. Exception: the sanding of old bone can be performed in the chemical fume hood in Lab 10.

8. Gloves and disposable sleeves will be changed after working with each specimen.

9. A surgical face mask will be worn at all times to prevent contamination.

SAFETY:

1. Gloves, safety glasses, and laboratory coats must be worn at all times.

2. Surgical face masks will be worn while cleaning and grinding tooth specimens.

3. Follow the Exposure Control Plan for Occupational Exposure to Bloodborne Pathogens (DoD Forensic Advisory Committee).

4. All organic extraction procedures are to be performed in a chemical fume hood.

5. Discard organic waste in a hazardous waste container.

6. All appropriate MSDS sheets must be read prior to using this protocol.

PROCEDURE:

1. Follow the SOP for "Chain of Custody" and the SOP for "Photodocumentation" before processing any evidentiary material.

2. The hoods and equipment must be cleaned appropriately prior to use for the extraction of each specimen. The minimum standards of cleaning are described and are to be followed when "clean . . . appropriately" is cited in the protocol. The grinders/blenders must be cleaned with 10% commercial bleach, sterile distilled water, and 95% ethanol in the laminar flow hood. The hoods and all other equipment must be cleaned with 10% commercial bleach. The cleaned equipment should be thoroughly dried before use.

3. While following this procedure, complete the "Preparation of Dried Skeletal Remains for Extraction, " DNA Form 2 and the "Extraction of DNA from Skeletal Remains," DNA Form 3 (see Pages 8 and 9).

4. Obtain tooth specimen. Record the gross weight of the tooth on the worksheet.

Performed in Laminar Flow Hood

5. Clean specimen by placing the tooth into a 50 ml conical tube containing approximately 25 ml of bottled sterile deionized water. (Be sure to set aside any dried tissue or loose bone adhering to the tooth, as this material contains a relatively large amount of DNA. It can be extracted separately, or combined with the pulp and dentin and extracted as a single specimen.) Shake the tube approximately 10 times. Decant into a waste container. Repeat twice.

6. Cover the specimen in the conical tube with 95% ethanol. Shake the tube at least 10 times. Decant into waste container. Repeat twice. Label and clean a weigh boat with 10% commercial bleach and ethanol. Pour specimen into the cleaned, labelled weigh boat and allow to air dry.

Performed in Bone Sanding Hood

7. Clean bone extraction hood appropriately. Using a metal file, carve a groove at the midline of the tooth where the crown meets the roots. This groove provides a place to rest the osteotome so it will not slide off that surface causing damage to the roots or the crown.

8. Place the tooth into a clean mortar. Cover with parafilm. Place the osteotome in the groove made with the metal file. Strike the osteotome with a hammer to split the tooth horizontally, being careful not to damage the crown as this may still be of importance to the case. Once the section has been made, set aside the roots for weighing and subsequent pulverization.

9. Remove any visible pulp from either half of the tooth with a pair of tweezers or a spoon excavator. Large pieces of pulp can either be extracted separately or with all other material. Place into a weigh boat and set aside.

10. Using the dental drill, drill out all dentin from within the crown, using caution so as not to disrupt any morphological features of the crown. Be sure to hold the specimen over a large weigh boat so that no sample is lost. (A clamp may be used at this time to hold the specimen.) Drill until all dentin and pulp are absent (approximately 1-2 millimeters), leaving only the enamel.

11. Clean the Micro-Mill Grinder (Step 13a) or the Warring blender (Steps 13d) appropriately.

Performed in the Bone Sanding Hood

12. Pulverize root fragments using either the Micro-Mill Grinder (Steps 13b and 13c) or the Warring Blender (Steps 13f and 13g).

Performed in the Laminar Flow Hood

Micro-Mill Grinder

13a. Clean the entire grinding chamber appropriately. Do not allow any fluid to leak near or on the contact points. Allow the interior surfaces to dry thoroughly. Place root fragments into the lower portion of the grinding chamber.

Note: The specimen must be dry prior to being milled.

Place the upper portion of the grinding chamber onto the lower portion by inserting the pins through the holes in the back plate on the lower portion of the grinding chamber. Fasten the left latch and then the right latch.

Performed in Bone Sanding Hood

13b. Set the grinding chamber onto the base of the grinder. Set timer to one minute, then run for another minute. Tap the upper chamber gently to remove pulverized tooth from the cover.

13c. Remove the grinding chamber from the base. Tilt container at approximately a 45 degree angle and tap gently on bench top to accumulate the specimen to one side. Wait approximately 30 seconds for the powder to settle then gently remove lid. Pour powder into a clean weigh boat or funnel for transfer to a labelled sterile, preweighed or tared 15 ml polypropylene tube.

OR

Performed in the Laminar Flow Hood

Warring Blender

13d. Clean grinding chamber and lid: fill pulverizer ~1/3 full with 10% bleach, attach lid and run unit approximately 10 seconds; remove lid without touching inside of lip, rinse lid and cup with sterile distilled water, then 95% ethanol. Drain off the excess ethanol. Allow the interior surfaces to dry thoroughly.

13e. Place specimen fragments into the lower portion of the grinding chamber. Place lid on blender without touching inside lip of lid or cup.

Performed in the Bone Sanding Hood

13f. Take sealed blender cup to base unit. Run blender for up to one minute, monitoring extent of pulverization through translucent lid. If sample is not completely ground after one minute, shut off blender, tap the container, and wait approximately 30 seconds. Repeat if necessary. When starting the unit, the bone may become wedged between a blade and the cup wall; if so, shut off power, remove cup and dislodge large fragments by tapping or rotating the blade spindle from below.

13g. Tilt grinding chamber at approximately a 45 degree angle and tap gently on bench top to accumulate the specimen to one side. Wait approximately 30 seconds for the powder to settle then gently remove lid. Pour powder into a clean weigh boat or funnel for transfer to a labelled sterile, preweighed or tared 15 ml polypropylene tube.

Performed in the General Laboratory

14. Determine the weight of the pulverized tooth specimen in the conical tube.

15. Clean remaining dust from the grinding chamber at the sink. Additionally, for the Warring blender, wash grinding chamber and lid with running water and wiping, then fill 2/3 full with water and a squirt of Liquinox soap, replace lid, run approximately 30 seconds on power base and repeat with water, then 10% commercial bleach. For both units perform a final rinse with 10% commercial bleach, distilled water, then 95% ethanol. Proceed to Step 4 if processing additional specimens.

Note: You may stop at this point for the day. Store pulverized sample in a cool dry dark environment (for example, your personally assigned drawer in Laboratory 9) for a maximum of 5 days. For longer storage, place pulverized sample in -20%C freezer.

16. Initiate a tube labelled "RB#" for the reagent blank sample which must be carried through the extraction procedure and assayed in parallel with the test samples. The reagent blank should be the last specimen processed for the remaining steps.

Performed in the Laminar Flow Hood

17. Add 3 ml Extraction Buffer (assure that the Extraction Buffer is homogenous) and 100 &l 20 mg/ml Proteinase K to the specimens and the reagent blank. Suspend the tooth dust thoroughly in the reagents. Incubate overnight at 56%C on a tilted Nutator ensuring that the reagents do not reach the cap of the tube. After one hour of incubation, mix thoroughly and re-secure the caps of the tubes.

Performed in Chemical Fume Hood

18. Extract with 3 ml Phenol/Chloroform/Isoamyl Alcohol (25:24:1). Mix thoroughly. Centrifuge 2 minutes at 4950 x g using the Beckman GPR Centrifuge (6400 RPM) or the IEC Centra MP4 (5000 RPM). Transfer upper aqueous layer to a clean labelled tube. Repeat extraction with Phenol/Chloroform/Isoamyl Alcohol until the interface is clean, or a minimum of two times. Dispose of phenol waste in the appropriate waste container.

19. Extract with 3 ml n-Butanol. Mix thoroughly. Centrifuge 2 minutes at 4950 x g using the Beckman GPR Centrifuge (6400 RPM) or the IEC Centra MP4 (5000 RPM). Remove and discard most of the n-Butanol upper layer into the appropriate waste container.

Performed in Laminar Flow Hood

20. Assemble a sufficient number of Centricon-100 concentrators and label appropriately.

Performed in Chemical Fume Hood

21. Transfer the lower aqueous layer to the corresponding Centricon-100 concentrators.

Note: Avoid pipetting any residual n-Butanol, and wipe pipet tip with a Kim-Wipe to remove any carry-over n-Butanol.

Centrifuge approximately 30 minutes, or longer if necessary, at 1000 x g (2600 RPM) using the IEC Centra MP4. Discard filtrate.

Performed in Laminar Flow Hood

22. Add 2 ml of sterile TE Buffer to sample reservoir. Centrifuge approximately 30 minutes, or longer if necessary, at 1000 x g (2600 RPM) using the IEC Centra MP4. Discard filtrate. Repeat.

23. Pipette extract directly from the sample reservoir and transfer to a sterile labelled microcentrifuge tube. Rinse the Centricon filter by adding an aliquot of TE Buffer to sample reservoir, pipette up and down several times. Add this liquid to the labelled microcentrifuge tube and resuspend extract to a final volume of approximately 100 &l.

Note: Take extreme care in not allowing the pipettor to touch the sides of the sample reservoir.

24. Evaluation of Extracted DNA

a. Prepare 1% agarose according to the size of the gel tray being used.

*Small tray - 0.3g agarose with 30ml 1X TBE; 3&l EtBr (5mg/ml)
*Large tray - 0.8g agarose with 80ml 1X TBE; 8&l EtBr (5mg/ml)

b. Add 5&l of the extract to 1&l of 6X agarose gel loading buffer. Load the samples and electrophorese at a constant voltage (100 Volts) for approximately 30 minutes. Be sure to flank samples with a sizing ladder (1&l of 123 base pair ladder, 4&l dH_2O and 1&l of 6X loading buffer).

c. Visualize gel on a trans-illuminator. Photograph the gel using a Polaroid camera (suggested settings - shutter speed 1 second and f-stop at 8-11) and type 667 film. Attach photo to "Extraction Yield Gel" Worksheet (Page 10).

Note: Optimal amount of template DNA for amplification = 200 pg total DNA.

25. Store at 4%C if amplified within 3 weeks or at -20%C for long-term storage.

26. Return remaining crown to its proper packaging for storage.

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I. Deoxyribonucleic Acid (DNA) Analysis," *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 2, March 1991, pp. 320-330.

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Preparation of Dried Skeletal Remains for Extraction

Case Number:____________________ Date/Time:________________ Scientist:____________________

Specimen	Gross Weight (g)	Weight of Fragment to be Pulverized (g)	Weight of Powder to be Extracted (g)	Hood #	Grinder #	Comments
Specimen #:						
Specimen #:						
Specimen #:						
Specimen #:						
Reagent Blank #:						

Item	Source	Lot #
Distilled Water		
Ethanol		

1. (PERFORM IN A BIOHAZARD HOOD): Place specimen in a 50 mL conical tube and fill with water, allowing space at the top for movement. Place cap on tube and gently agitate the tooth in the water. Discard water and repeat wash with ethanol. Allow tooth to dry at room temperature in the biohazard hood.
2. (PERFORM IN CHEMICAL FUME HOOD): Section tooth. Set roots aside.
3. Remove pulp and dentin from crown with a Dental Drill.
4. Pulverize the roots to a powder using a Micro-Mill Grinder or Waring Blender.
5. Transfer all powder to a labelled 15 mL conical tube.

Comments:

____

____

Location of Re-packaged Crown ____________

DNA Form 21 Revised 11/2/95

Extraction of DNA from Dried Skeletal Remains

Case Number:____________ Date:____________ Scientist:____________

Extraction

Item	Source	Lot #
QC Extraction Lot No.	AFDIL	
Centricon-100	Amicon	

Centricon Purification

#	Specimen	Approximate Volume Recovered	Approximate Final Volume	Comments
	Specimen #:			
	Specimen #:			
	Specimen #:			
	Specimen #:			
	Reagent Blank #:			

1. Add 3 mL Extraction Buffer to pulverized specimen in 15 mL conical tube.
2. Add 100 uL of 20 mg/mL Proteinase K.
3. Incubate at 56°C overnight.
4. Add 100 uL of 20 mg/mL Proteinase K.
5. Incubate at 56°C for 3 hours.
6. Add 3 mL phenol:chloroform:isoamyl alcohol.
7. Centrifuge 2 minutes at 4950 x g (Beckman 6400 rpm; IEC 5000rpm).
8. Transfer upper aqueous layer to a clean 15 mL conical tube.
9. Repeat steps 7 and 8 until interface is clean.
10. Add 3 mL butanol.
11. Centrifuge 2 minutes at 4590 x g.
12. Add lower aqueous layer from butanol extract to sample reservoir of Centricon-100.

13. Spin column at 1000 x g for 30-60 minutes or until sample has spun through. Add 2 mL TE Buffer and spin 30-60 minutes or until the buffer has spun through. Discard filtrate and repeat 2 mL TE Buffer wash.

14. Pipette the 40-50 uL of retentate up and down 8-10 times. Transfer retentate to a sterile, labelled tube. Rinse membrane with a volume of TE Buffer necessary to adjust final volume to approximately 100 uL. Transfer rinse volume to the sterile, labelled tube.

Comments: ____________________

____

____

____

____

Location of DNA Extracts ____________________

DNA Form 3 Revised 07/14/95

Extraction Yield Gel

Case Number/Scientist: ____________________ Date:

Gel Number: ____________________ Gel Type: 1% Agarose

Lane	Specimen	Case	uL	Comment	Lane	Specimen	Case	uL	Comm
1					16				
2					17				
3					18				
4					19				
5					20				
6					21				
7					22				
8					23				
9					24				
10					25				
11					26				
12					27				
13					28				
14					29				
15					30				

Voltage:______________ Time On:______________

PHOTO:

Service and Genetic Systems Branch
DoD DNA Registry
Office of the Armed Forces Medical Examiner
Armed Forces Institute of Pathology

Adopted By ______________________ Date __________
Reviewed By ______________________ Date __________
Version 3.0 Replaces Version 2.0

Amplification of Mitochondrial DNA

PRINCIPLE

To provide instructions for the amplification of the mitochondrial DNA (mtDNA) control region using the polymerase chain reaction (PCR). The control region of mtDNA contains two hypervariable segments which comprise the majority of the polymorphic information found in mtDNA. The hypervariable regions are amplified in two or four pieces. For poor quality DNA, two overlapping segments of each hypervariable region are amplified, for a total of four amplification reactions. For good quality DNA, the two hypervariable regions are amplified completely in two separate reactions. Following amplification, the product is purified prior to automated sequence analysis.

SPECIMEN

The DNA extract of any appropriate biological specimen and the accompanying reagent and substrate controls as detailed in the DNA extraction protocols located in this manual. The optimum starting template is 10-1000 pg, however amplification can be performed on less than 10 pg.

REAGENTS, SUPPLIES, and EQUIPMENT

Reagents
Agarose, DNA grade
Agarose gel loading buffer, 6X (50% glycerol, 1.5 mM bromophenol blue, 100 mM EDTA)
Bleach, 10% commercial (7 mM sodium hypochlorite solution)
Bovine Serum Albumin (4 &g/&l), DNA grade
DNA, positive control
dNTP mix, 2.5 mM
Ethanol, absolute (optional)
Ethidium bromide (5 mg/ml)
PCR buffer, 10X (1X is 10mM Tris-HCl,pH 8.3; 50 ml KCl; 1.5 mM $MgCl_2$)
Primers, 10 _M
Size ladder (123 base pair)
Reagents (continued)
Taq DNA Polymerase
1X TBE (89 mM Tris HCl, pH 8.3; 89 mM boric acid; 2 mM EDTA)

Water, sterile, distilled

Supplies
Bottle, Pyrex® (250 ml)
Centricon-100® concentrators
Comb, well-casting
Film, Polaroid type 667
Graduated cylinders (100, 1000 ml)
Kim-wipes
Microtitre plate
Racks, tube
Tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettors)
Tubes, thin-walled PCR (0.2 ml)
Waste containers (general, biohazard, Sharps™)

Personal Protective Equipment
Gloves
Laboratory coat
Mask (surgical or dust)/optional
Safety glasses
Sleeves, disposable/optional

Equipment
Camera, Polaroid
Centrifuge (e.g., IEC, Dynac)
Freezer, -20°C
Gel electrophoresis apparatus
Laminar flow hood (or dead space hood)
Microwave
Pipettors (e.g., P-10, P-100, P-1000)
Refrigerator, 4°C
Thermal cycler (e.g., Perkin-Elmer 9600)
UV transilluminator

QUALITY ASSURANCE

1. Amplification set-up of samples must be performed in a pre-amplification laboratory as follows:

specimens suspected of containing high levels of DNA	Lab 5
specimens suspected of containing low levels of DNA	Lab 9

2. Ensure that the following worksheets are completed for this procedure and placed in the appropriate case folder(s) and/or notebook(s):

"Amplification"/DNA Form 7 (or "Amplification Using Master Mix"/DNA Form 15)

3. Ensure that all reagents satisfy the minimum standards for quality control, where appropriate.

4. Ensure that all equipment satisfies the minimum standards for quality control, where appropriate.

5. In order to prevent contamination, all steps in this procedure should be performed, where appropriate, in the proper laboratory hoods.

6. Any laboratory workspace and all pipettors and racks to be used in this procedure should be cleaned with 10% commercial bleach and thoroughly dried before beginning.

7. To prevent contamination, thin-walled PCR tubes (closed) should be irradiated in an ultraviolet crosslinker with 2 j/cm^2. Master mix should be irradiated with 6 j/cm^2.

   Note: Include only those primers that have been validated for irradiation in the master mix (see Appendix A).
   Do NOT include Taq polymerase in the master mix.

   Follow "Irradiation of Reagents and Supplies in the Ultraviolet Crosslinker" SOP for the appropriate method of irradiation.

8. The lamp intensity of the ultraviolet crosslinker should be calibrated once every two weeks. Verify that the posted value is current before irradiation of reagents and supplies.

9. Any supplies or equipment taken from a post-amplification room to a pre-amplification room must be cleaned with 10% commercial bleach before removal from the post-amplification room and again in the pre-amplification room before use. Reagents should never be transferred from a post-amplification room to a pre-amplication room.

10. Change pipet tips between each transfer or addition of sample or reagent, unless otherwise noted.

11. No aliquot of any processed sample may be returned to its storage container or to the original evidence container.

12. No aliquot of any reagent may be returned to the original stock container.

SAFETY

1. All appropriate MSDS sheets must be read prior to performing this procedure.

2. Treat all biological specimens as potentially infectious. Gloves, safety glasses, and a laboratory coat must be worn at all times.

3. Follow the "Exposure Control Plan for Occupational Exposure to Bloodborne Pathogens" (DoD Forensic Advisory Committee).

4. Avoid direct exposure to ultraviolet light when using the germicidal lamp in the biological hood or the transilluminator.

5. The heat block in the thermal cycler can become very hot. Be careful not to touch the heating surfaces while in operation.

6. Distinguish all waste as general, biohazard, organic, or Sharps™ and discard appropriately.

PROCEDURE

1. Program the thermal cycler to a 96° C preheat cycle before setting up amplification reactions.

2. Prepare master mix for each hypervariable region and/or primer set being amplified as follows:

VOLUME	VOLUME PER RXN.	COMPONENT
(n+1)5 ul	5 ul	10X PCR Buffer
(n+1)4 ul	4 ul	2.5mM dNTPs
(n+1)2 ul	2 ul	4 ug/ul BSA
(n+1)2 ul	2 ul	Forward Primer(10 uM)
(n+1)2 ul	2 ul	Reverse Primer(10 uM)
(n+1)__ul	0.5-2.5 ul	Taq polymerase(5 units/ul)
qs to (n+1)40 ul	qs to 40 ul	sterile, distilled water
(n+1)40 ul	40 ul	total volume

Note: n = number of samples being amplified per region or set

2. (continued)

Note: Use BSA for amplification of extracts of degraded DNA (bone, tissue, etc.). BSA is not required for amplification of extracts from whole blood or bloodstains.

Note: When the amount of template to be amplified exceeds 10 ul/reaction, adjust master mix total volume accordingly.

Note: Appendix B contains the sequences of primers validated for mitochondrial DNA amplification and the hypervariable regions and/or primer sets for which they are specific. Alternate primers may be used following proper validation and quality control.

3. Prepare each amplification reaction in the order shown and combine with 40 ul of master mix in a 0.2 ml thin-walled PCR tube:

Volume	Component
10 ul sterile, distilled water	Negative 1
1-10.0 ul Reagent Blank q.s. to 10 ul with sterile, distilled water	Reagent blank(s): volume added should be equal to the maximum sample volume added
1-10.0 ul sample DNA (10-100 ng) q.s. to 10 ul with sterile, distilled water	sample DNA extract(s)
10 ul Positive Control DNA	Positive: 200 pg/10 ul for low quantity samples; 1 ng/10 ul for high quantity samples
10 ul sterile, distilled water	Negative 2

Note: Total reaction volume should be 50 ul.

4. Place tubes in the thermal cycler and perform the PCR according to the appropriate thermal cycling parameters for the primers being employed (see Appendix C).

INTERPRETATION, ACCEPTABLE VALUES, and TROUBLESHOOTING

Not applicable. Samples are ready for evaluation by amplification product yield gel (see "Amplification Product Yield Gel" SOP).

REFERENCES

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APPENDIX A

PCR master mix (without Taq polymerase) containing designated primer pairs is validated for irradiation in the ultraviolet crosslinker as a means to decrease or eliminate contaminating DNA.

Primer pairs that show no decrease in sensitivity upon irradiation:

F16144/R16410
F15/R285

Primer pair that shows detectable, but very slight decrease in sensitivity upon irradiation:

F155/R484

Primer pairs that show significant decrease in sensitivity upon irradiation:

F15971/R16258
F15989/R16251
F15/R274
F155/R389

NOTE: Since amplification sensitivity may be affected, the addition of primers to the master mix for irradiation is up to the discretion of the individual scientist based on the specific circumstances of the sample(s) to be amplified.

APPENDIX B

Hypervariable Regions and Primer Sets:

HV1	F15971, F15989, F16190 / R16410
HV2	F15 / R484
PSI	F15971, F15989, F16190 / R16175, R16251, R16255, R16258
PSII	F16144, F16190 / R16410
PSIII	F15, F29 / R270, R274, R285
PSIV	F155 / R381, R389, R484

Primer Sequences:

F15971	5'	TTA	ACT	CCA	CCA	TTA	GCA	CC	3'
F15989	5	CCC	AAA	GCT	AAG	ATT	CTA	AT	3'
F16144	5	TGA	CCA	CCT	GTA	GTA	CAT	AA	3'
F16190	5'	CCC	CAT	GCT	TAC	AAG	CAA	GT	3'
R16175	5'	TGG	ATT	GGG	TTT	TTA	TGT	A	3'
R16251	5'	GGA	GTT	GCA	GTT	GAT	GT		3'
R16255	5'	CTT	TGG	AGT	TGC	AGT	TGA	TG	3'
R16258	5	TGG	CTT	TGG	AGT	TGC	AGT	TG	3'
R16410	5'	GAG	GAT	GGT	GGT	CAA	GGG	AC	3'
F15	5'	CAC	CCT	ATT	AAC	CAC	TCA	CG	3'
F29	5'	CTC	ACG	GGA	GCT	CTC	CAT	GC	3'
F155	5'	TAT	TTA	TCG	CAC	CTA	CGT	TC	3'
R270	5'	TGG	AAA	GTG	GCT	GTG	CAG	AC	3'
R274	5'	TGT	GTG	GAA	AGT	GGC	TGT	GC	3'
R285	5'	GTT	ATG	ATG	TCT	GTG	TGG	AA	3'
R381	5'	GCT	GGT	GTT	AGG	GTT	CTT	TG	3'
R389	5'	CTG	GTT	AGG	CTG	GTG	TTA	GG	3'
R484	5'	TGA	GAT	TAG	TAG	TAT	GGG	AG	3'

Primer sequences are based on the numbering system of Anderson et al. Numbering begins at the 5' end of each primer.

HV1 = Hypervariable Region 1, HV2 = Hypervariable Region 2.

APPENDIX C Thermal Cycler Programs and Conditions

PROGRAM #20 96°C SOAK

Program	Conditions	Use / Cycles
PROGRAM #111	94°C X 30 SEC	HV1, HV2 32 CYCLES
	94°C X 20 SEC 56°C X 10 SEC 72°C X 30 SEC 5°C SOAK	
PROGRAM #113	94°C X 30 SEC	Primer Sets I, II, III, IV 38 CYCLES
	94°C X 20 SEC 56°C X 10 SEC 72°C X 30 SEC 5°C SOAK	
PROGRAM #117	94°C X 30 SEC	Primer Set I* 38 CYCLES
	94°C X 20 SEC 62°C X 10 SEC 72°C X 30 SEC 5°C SOAK	

*use only when F15971 is paired with R16258 or R16255

DoD DNA Registry
Office of the Armed Forces Medical Examiner
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Adopted By ______________________ Date __________
Reviewed By ______________________ Date __________
Version 4.0 Replaces Version 3.0
seqmtdna.040

Sequencing of Mitochondrial DNA

I. PRINCIPLE

To provide instructions for the sequencing of PCR product amplified from the mitochondrial DNA (mtDNA) control region. Cycle sequencing is performed using either the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS or the Applied Biosystems (AB, a Division of Perkin-Elmer) PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit. The resulting product is loaded on an AB 373 DNA Sequencer.

II. SPECIMEN

The PCR product from any appropriate biological specimen as detailed in the "Amplification of Mitochondrial DNA" procedure. The optimum starting template is 20-200 ng if using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit or 1-20 ng if using the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS. Include all appropriate amplification controls.

III. REAGENTS, SUPPLIES, and EQUIPMENT

Reagents
Acrylamide/Bis Premix 19:1, 40% solution, electrophoresis purity grade
Ammonium persulfate , 10% (make fresh weekly)
Bleach, 10% commercial (7 mM sodium hypochlorite solution)
Detergent, liquid, 1% (e.g., Liquinox, Alconox)
DNA sequencing kit (choose one):
  ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS
  PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit
EDTA, 50 mM (either with or without 3 mg/ml Dextran Blue)
Ethanol, absolute (optional)
Formamide, deionized
Reagents (continued)
Ice
Primers, 10 uM

TBE, 10X (89 mM Tris-HCl, pH 8.3; 89 mM Boric Acid; 2 mM Na_2EDTA)
TBE, 1X (prepare a 1:10 dilution of 10X TBE)
TEMED
Urea, ultrapure, DNA grade
Water, sterile, distilled

Supplies
Clamps, butterfly (optional)
Comb, shark's tooth
Comb, well-casting
Dialysis columns (i.e., Quick Spin™ G-50 or AGTC Centriflex™ Gel Filtration Cartridge)
Filter units, 0.2 micron, disposable
Ice bucket
Kim-wipes (no-lint type recommended for certain steps)
Pipets, disposable, serological (5, 10, 25 ml)
Pipets, disposable, transfer
Plate set, DNA sequencing (for the appropriate sequencer)
Racks, tube
Spacers, 1 mm
Syringe, plastic
Tape, 2-inch wide (optional/e.g., Permacel)
Tips, aerosol-resistant (e.g., for P-10, P-100, P-1000 pipettors)
Tubes, thin-walled PCR (0.2 ml)
Waste containers (general, biohazard, Sharps™)

Personal Protective Equipment
Gloves
Laboratory coat
Safety glasses

Equipment
Aspirator, water-driven (or electric vacuum pump)
Evaporator/concentrator
Freezer, -20°C
Heat block, 95°C
Heat plate
Microcentrifuge (e.g., Eppendorf)
Pipettors (e.g., P-10, P-100, P-1000, PipetAid)
Refrigerator, 4°C
Sequencer, DNA (e.g., AB 373A or 373 Stretch) and accessories
Thermal cycler (e.g., Perkin-Elmer 9600)

IV. QUALITY ASSURANCE

1. Sequencing of samples must be performed in either post-amplification laboratory 8 or 12.

2. Ensure that the following worksheets are completed for this procedure and placed in the appropriate case folder(s) and/or notebook(s):

   "Centricon Product Purification"/DNA Form 10
   "Dye Deoxy Terminator Sequencing Reaction"/DNA Form 11
   "Gel Pouring"/DNA Form 12
   "Sequence Gel Load Sheet"/DNA Form 13

3. Ensure that all reagents satisfy the minimum standards for quality control, where appropriate.

4. Ensure that all equipment satisfies the minimum standards for quality control, where appropriate.

5. Any laboratory workspace and all pipettors and racks to be used in this procedure should be cleaned with 10% commercial bleach and thoroughly dried before beginning.

6. Any supplies or equipment taken from a post-amplification room to a pre-amplification room must be cleaned with 10% commercial bleach before removal from the post-amplification room and again in the pre-amplification room before use. Reagents should never be transferred from a post-amplification room to a pre-amplication room.

7. Change pipet tips between each transfer or addition of sample or reagent, unless otherwise noted.

8. No aliquot of any processed sample may be returned to its storage container or to the original evidence container.

9. No aliquot of any reagent may be returned to the original stock container.

10. Set up at least one (1) pGEM®-3Zf(+) DNA (pGEM) sequencing control per sequencing gel.

11. Two forward (or two reverse) reactions of the same hypervariable region or primer set should not be loaded in adjacent lanes on the sequencing gel.

12. Positive Amplification and Sequencing Controls:

The positive amplification control for each hypervariable region or primer set must be sequenced each time the unknown samples are sequenced UNTIL the positive control sequence is determined to be consistent with the known sequence for a specific amplification set-up (e.g., .1, .2...). If hypervariable regions or primer sets are to be run without the corresponding positive controls, all sequencing primer lot numbers must remain the same as those used when the positive control was confirmed; all other reagents should remain the same when possible.

Note: See the "Specimen Numbering" protocol for explanation of amplification set-up designations and the "Analysis of Mitochondrial DNA Sequencing Data" protocol for sample confirmation criteria.

V. SAFETY

1. All appropriate MSDS sheets must be read prior to performing this procedure.

2. Treat all biological specimens as potentially infectious. Gloves, safety glasses, and a laboratory coat must be worn at all times.

3. Follow the "Exposure Control Plan for Occupational Exposure to Bloodborne Pathogens" (DoD Forensic Advisory Committee).

4. Acrylamide is a neurotoxin and can be absorbed through the skin and mucous membranes. Always use proper protective equipment when handling acrylamide. All liquid acrylamide must be polymerized before disposal.

5. The heat block in the thermal cycler can become very hot. Be careful not to touch the heating surfaces while in operation.

6. The heat plate can become very hot. Be careful not to touch the heating surfaces while in operation.

7. Use proper protective equipment to prevent burns when handling hot solutions.

8. Gel electrophoresis is run at very high voltages (e.g., 980-1600 volts). Do not open gel apparatus or touch gel buffer during electrophoresis.

9. The laser is hazardous to the eyes and skin. Do not lift the lid on the DNA sequencer while instrument is in operation.

10. Distinguish all waste as general, biohazard, organic, or Sharps™ and discard appropriately.

VI. PROCEDURE

Purification of Amplification Products to be Sequenced

Note: Begin at Step 6 if samples are purified already.

1. Add PCR product to 2 ml of sterile, distilled water in a Centricon-100 spin dialysis column.

2. Spin column at 1000 x g (IEC 2600 RPM) for 15-30 minutes or until sample has spun through.

3. Add 2 ml sterile, distilled water and spin column at 1000 x g (IEC 2600 RPM) 15-30 minutes or until water has spun through.

4. Flip column and spin column at 1000 x g (IEC 2600 RPM) 2 minutes to recover product.

5. Adjust final volume to approximately 40-50 ul for low quantities of DNA or greater volumes for high quantities of DNA.

   Note: Refer to amplification product yield gel results to estimate quantity of sample product DNA and determine final volume.

   Note: Store purified PCR product samples at 4° C.

Cycle Sequencing of Samples

6. Program the thermal cycler to a 96° C preheat cycle before setting up sequencing reactions.

7. Prepare each terminator cycle sequencing reaction as follows in a 0.2 ml thin-walled PCR tube:

ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS

Volume	Component
1 ul	sequencing primer (10 uM)
1-11.0 ul (1-20 ng)	purified PCR product
8.0 ul	ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS
q.s. to 20 ul	sterile, distilled water

OR (continued on next page)

8. (continued)

PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit

Volume	Component
1 ul	sequencing primer (10 uM)
1-9.5 ul (20-200 ng)	purified PCR product
9.5 ul	PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit
q.s. to 20 ul	sterile, distilled water

Option: Sample tubes may be kept on ice when setting up sequencing reactions.

Note: Appendix A contains the sequences of primers validated for mitochondrial sequencing and the hypervariable regions and/or primer sets for which they are specific. Alternate primers may be used following proper validation and quality control.

9. Prepare the pGEM control sequencing reaction as follows:

Add 8.0 ul of reagent from the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (or 9.5 ul of reagent from the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit) and complete as listed below.

Volume	Component
1 ul	-21 M13 primer
2 ul or 4 ul	pGEM®-3Zf(+) DNA
q.s. to 20 ul	sterile, distilled water

Note: Use 2 ul with ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS. Use 4 ul with PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit.

10. Place tubes in the thermal cycler and cycle under the following conditions:

25 cycles at: 96° C for 15 seconds
50° C for 5 seconds
60° C for 2 minutes

4° C hold

Purification of Samples

Option: To purify samples using Quick Spin™ G-50 columns, follow Steps 11 through 18.
To purify samples using AGTC Centriflex™ Gel Filtration cartridges, begin at Step 19.

Purification with Quick Spin™ G-50 Columns

11. Allow Quick Spin™ dialysis columns to reach room temperature (one for each sequencing reaction performed).

12. Dislodge air bubbles from dialysis columns.

13. Remove the cap carefully from each column using caution not to draw any air bubbles into the bottom of the column. Remove the stopper located on the tip and allow column to drain completely.

14. Fill each column with sterile, distilled water and allow to drain.

    Note: For best results, do not repeat this step.

15. Place each column into a collection tube and spin at 3100 rpm for two minutes in a microcentrifuge.

    Note: Do not spin at a higher rpm or for longer than two minutes as over-drying of the sephadex beads in the gel matrix of the column may occur.

    Note: If preparing columns before cycle sequencing is complete, do not allow them to sit for a long period of time before proceeding to Step 6 or over-drying may occur.

16. Transfer each column into a fresh, labelled collection tube. Pipet the entire sequencing reaction product onto the gel matrix of the corresponding column. Spin at 3100 rpm for two minutes in a microcentrifuge.

    Note: Do not allow the pipet tip to touch the gel matrix when pipetting the sequencing reaction product.

    Note: With a kim-wipe (preferably no-lint), attempt to remove any sequencing reaction product that may be pipetted onto the wall of the column. If not successfully filtered through the gel matrix, the resulting sample may contain high background in the sequence data.

17. Remove and discard each column from the sample collection tube.

18. Proceed to Step 23.

Purification with AGTC Centriflex™ Gel Filtration cartridges

19. Spin unit (cartridge and microtube) at 750 x g (Eppendorf 3000 rpm) for 1 minute.

20. Transfer cartridge to a fresh, labelled microtube. Pipet the entire sequencing reaction product onto the packed column.

21. Close cap. Spin at 750 x g (Eppendorf 3000 rpm) for 2 minutes.

22. Remove and discard the cartridge from each microtube.

23. Spin samples in the evaporator/concentrator for 30-45 minutes or until dry.

    Note: Severe over-drying of the samples may present difficulties when resuspending in loading buffer.

    Note: Dried samples may be stored for one month at -20° C before being run on a sequencer.

Automated DNA Sequencing

24. Clean a set of DNA sequencing plates with liquid detergent. Rinse thoroughly with distilled water (dH_2O). Dry completely using kimwipes (preferably no-lint). Do not allow to air dry.

    Option: Rinse with 100% ethanol after the distilled water rinse, followed by a distilled water rinse.

    Note: If ethanol is not completely removed, a blue fluorescence may result when plates are scanned.

25. With plates in a horizontal position, sandwich 1 mm spacers between the two plates and tape the sides and bottom to stop the gel mix from leaking when poured.

    Option: Instead of tape, butterfly clamps can be placed on the side edges to hold plates together (no clamps on the bottom).

    Note: Ensure that the same side of each plate always faces outward based on the etched lines that can be found on the outside bottom edges (also found on the outside top edge on unnotched plates).

26. Prepare polyacylamide gel as follows:

Reagent	24 cm well-to-read gel (Sequencers A and B)	34 cm well-to-read gel (Sequencers C and D)
DNA grade, ultrapure urea	30 g	30 g
19:1 Acrylamide/Bis solution, 40%	9 ml	7.2 ml
10X TBE	6 ml	6 ml
sterile, distilled water	22 ml	25 ml

Note: Insure that the 19:1 Acrylamide/Bis is completely in solution before adding to the gel mixture. If not, the gel percentage may be too low and result in band broadening during sequencing.

27. Gently stir on a heat plate until the urea begins to go into solution (e.g., Corning/set no higher than 3). Remove from heat.

Note: Do not heat solution above room temperature.

28. Stir until urea is completely in solution. Filter the solution through a 0.2 micron filter unit.

29. Degas the gel solution for 3 minutes using a bench-top water-driven aspirator (or electric vacuum pump).

30. Add 300 ul 10% ammonium persulfate and 34 ul TEMED to the gel mix and gently swirl into solution to avoid introducing air bubbles.

Note: Both reagents should be at room temperature before adding to the gel solution.

31. Pour the gel immediately. Dislodge any bubbles, then insert the well-casting comb at the top. Clamp the comb three times on the glass plates along the white edge of the comb that is inserted into the plates. Allow the acrylamide to polymerize for at least 1 hour at room temperature.

Note: Ensure that plates are on a level surface during polymerization.

32. Once the gel is polymerized, remove the tape from the edges (if appropriate) and the well-casting comb. Clean the well with distilled water and ensure that it is free of small gel pieces. Clean the outside of the gel plates with distilled water.

Option: If residue appears to be present on the exterior of the plates after cleaning with distilled water an ethanol wash may be performed, followed by a distilled water rinse.

Note: Be sure to remove the ethanol completely or a blue fluorescence may result when plates are scanned.

33. Turn off file sharing, then restart the computer to clear the RAM. In addition, verify that at least 25 mb of hard disk space is available for the collected data.

34. Perform plate check as follows: Place the lower buffer chamber in the sequencer. Put the gel plates into the sequencer and secure in place with the beam-stop bar. Close the lid. Scan the plates for dust or dirt by pressing *Pre-Run* on the instrument keypad, followed by *Plate Check*, then Full Scan. Open the Collection software. Click the *Scan* and the Map icons on the Collection window to observe plate check. Clean plates will produce a flat scan. Press Main Menu, then Abort Run to stop scanning.

Note: If the scan is not flat, remove the plates and clean again. Repeat this process until a clean scan is observed. A gel with a small number of peaks in the scan may still be used if the lanes corresponding to the location of the peaks are not loaded. See Appendix B for "Channel Lane Assignments" table.

Note: If the gel is not acceptable due to excessive air bubbles or lint deposits, then a new gel should be poured.

35. Put the upper gel buffer reservoir in place and lock the gel plates into the instrument.

36. Fill both buffer reservoirs with 1X TBE.

Note: Check to make sure that the upper reservoir gasket is not leaking.

Note: Buffer should cover electrodes in the lower reservoir and the top of the gel in the upper reservoir.

37. Flush the sample wells with buffer from the upper reservoir using a transfer pipet or plastic syringe.

38. Insert the sharks-tooth well-forming comb into the top of the gel. Ensure that the teeth of the comb do not penetrate more than 1-2 mm into the gel.

Option: The well-forming comb may be inserted before filling buffer reservoirs.

39. Press Start Pre-run, then Pre-Run Gel and allow the gel to run for at least 10 minutes at a constant power of 30 watts for the 373A model or 28 watts for the 373 Stretch model.

Note: Readings of 18-21 milliamps, 980-1600 volts, and a temperature approaching 40° C are indications that electrophoresis is running properly.

40. While the gel is pre-running (or during the 10 minutes between loading lanes), set the run parameters for the Collection software and complete a sample sheet as follows:

Select *Edit*, then *Settings*. Change the settings to reflect specific information about the gel (e.g., run length, 24- or 36-well format, terminator or primer chemistry) and the information necessary to identify the gel file (i.e., MnDyYr-A/PM). If desired, set the Analysis software to analyze the raw data by selecting *Analyze All Samples* in the *Settings* file.

Note: The gel run length should be at least 8 hours for primer sets and 12 hours for full hypervariable regions.

Select *File*, then *New Sample Sheet*. Insert the appropriate sample name, including primer designation, next to the corresponding lane number in the *Sample Name* column. Also, insert the appropriate case number and scientist's initials in the Comments column. Save the sample sheet under the gel file name plus the suffix SS (i.e., MnDyYr-A/PM SS).

Note: Do not load two forward (or two reverse) reactions of the same hypervariable region or primer set in adjacent lanes.

Note: To ensure better tracking of the gel, it is recommended that Lane Contains Sample is de-selected for all empty lanes in the Auto Settings column.

Note: Verify that the information selected in the *Settings* file is reflected on the sample sheet before saving.

41. Press Main Menu, then Abort Run to stop the pre-run. Verify that the Collection software window is open.

42. Preparation of DNA Sequencing Loading Buffer (for 60 samples):

Volume	Component
40 ul	50 mM EDTA (either with or without 30 mg/ml Dextran Blue)
200 ul	deionized formamide

Note: Loading buffer must be prepared fresh daily.

43. Resuspend each sample in 4 ul loading buffer.

44. Denature samples at 96° C in a heat block for 2 minutes, then place on ice until loaded.

Note: Samples should be loaded within two hours. If samples are not loaded within two hours, repeat this step.

45. Flush the sample wells with buffer from the upper reservoir using a transfer pipet or plastic syringe.

46. Load alternate numbered samples, preferably avoiding the first and last lanes of the gel, as well as any lane(s) that did not scan clean. This will aid in proper tracking of the lanes by the computer software.

47. Press Main Menu, followed by Choose Run, Sequence Run, then Full Scan to begin sequencing run. Run gel for at least 10 minutes. Press Interrupt Run to stop the run.

Note: On Sequencer B, press Main Menu, followed by Start Run to begin.

Note: Ensure that the sequencer is set to the same length run as the computer.

48. Load the remaining samples.

Option: Flush the wells again prior to loading the second set of samples.

49. Press Resume Run to continue the sequencing run.

Note: The run parameters should be the same as listed for the pre-run. The PMT value should be between 600 and 800 depending on the instrument's calibration (see assigned value written on the side of each instrument) and the blue base line value between 500 and 1000.

50. Click on the *Collect* icon in the Collection software window. The raw sequencing data automatically will be collected and the data stored in the designated gel file.

VII. INTERPRETATION

See the "Analysis of Mitochondrial DNA Sequencing Data" protocol for detailed instructions on saving and analyzing gel files and individual sample sequence information. Sample confirmation criteria is included in that protocol.

VIII. ACCEPTABLE VALUES

At least one of the following criteria must be met for the data from a sequencing gel to be acceptable:

- pGEM control sequence is determined to be consistent with the known pGEM sequence
- a positive control sequence is determined to be consistent with the known positive control sequence

If neither criteria is met, no data for any sample run on that sequencing gel may be included in the sample analysis layout (as detailed in the "Analysis of Mitochondrial DNA Sequencing Data" protocol).

IX. TROUBLESHOOTING

Adjust run parameters if not in the acceptable range through selections under the Main Menu on the sequencer. See the appropriate sequencer manual for further instructions.

If the sequencer instrument screen reads "Electrophoresis Power Off" and is accompanied by a series of 3 beeps, check the level of buffer in each of the reservoirs to verify that the electrodes and/or the bottom of the gel are sufficiently covered.

If a power outage occurs during a sequencing run, the instrument may automatically restart. If it does not, manually restart it if possible.

X. REFERENCES

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XI. ANNUAL REVIEW

Year 1 Reviewed By ______________________________ Date ____________

Year 2 Reviewed By ______________________________ Date ____________

Year 3 Reviewed By ______________________________ Date ____________

Year 4 Reviewed By ______________________________ Date ____________

APPENDIX A

Hypervariable Regions and Primer Sets:

HV1	F15971, F15989, F16190 / R16410
HV2	F15 / R484
PSI	F15971, F15989, F16190 / R16175, R16251, R16255, R16258
PSII	F16144, F16190 / R16410
PSIII	F15, F29 / R270, R274, R285
PSIV	F155 / R381, R389, R484

Primer Sequences:

F15971	5'	TTA	ACT	CCA	CCA	TTA	GCA	CC	3'
F15989	5	CCC	AAA	GCT	AAG	ATT	CTA	AT	3'
F16144	5	TGA	CCA	CCT	GTA	GTA	CAT	AA	3'
F16190	5'	CCC	CAT	GCT	TAC	AAG	CAA	GT	3'
R16175	5'	TGG	ATT	GGG	TTT	TTA	TGT	A	3'
R16251	5'	GGA	GTT	GCA	GTT	GAT	GT		3'
R16255	5'	CTT	TGG	AGT	TGC	AGT	TGA	TG	3'
R16258	5	TGG	CTT	TGG	AGT	TGC	AGT	TG	3'
R16410	5'	GAG	GAT	GGT	GGT	CAA	GGG	AC	3'
F15	5'	CAC	CCT	ATT	AAC	CAC	TCA	CG	3'
F29	5'	CTC	ACG	GGA	GCT	CTC	CAT	GC	3'
F155	5'	TAT	TTA	TCG	CAC	CTA	CGT	TC	3'
R270	5'	TGG	AAA	GTG	GCT	GTG	CAG	AC	3'
R274	5'	TGT	GTG	GAA	AGT	GGC	TGT	GC	3'
R285	5'	GTT	ATG	ATG	TCT	GTG	TGG	AA	3'
R381	5'	GCT	GGT	GTT	AGG	GTT	CTT	TG	3'
R389	5'	CTG	GTT	AGG	CTG	GTG	TTA	GG	3'
R484	5'	TGA	GAT	TAG	TAG	TAT	GGG	AG	3'

Primer sequences are based on the numbering system of Anderson et al. Numbering begins at the 5' end of each primer.

HV1 = Hypervariable Region 1, HV2 = Hypervariable Region 2.

APPENDIX B

Channel Lane Assignments

24-Well Comb		32-Well Comb		36-Well Comb	
Lane	Channel	Lane	Channel	Lane	Channel
1	7	1	5	1	4
2	15	2	11	2	9
3	23	3	17	3	14
4	30	4	23	4	20
5	38	5	29	5	25
6	46	6	35	6	30
7	54	7	41	7	36
8	62	8	47	8	41
9	69	9	53	9	46
10	77	10	58	10	52
11	85	11	64	11	57
12	93	12	70	12	62
13	101	13	76	13	68
14	107	14	82	14	73
15	116	15	88	15	78
16	124	16	94	16	84
17	132	17	100	17	89
18	140	18	106	18	94
19	148	19	112	19	100
20	155	20	117	20	105
21	163	21	123	21	110
22	171	22	129	22	116
23	179	23	135	23	121
24	187	24	141	24	126
		25	147	25	132
		26	153	26	137
		27	159	27	142
		28	165	28	148
		29	171	29	153
		30	177	30	158
		31	183	31	164
		32	189	32	169
				33	174
				34	180
				35	185
				36	190

Thursday, September 25 (Day 3)

7:30 a.m. Analysis of Mt DNA Lab L 4 NMS Dr. M.Holland
8:45 a.m. Sampling collections Lab AR CHS Dr. H.Lee
11:00 a.m. STR analysis by means of direct blotting electrophoresis Lec MR NMS Dr. H. Jurgen
11:45 a.m Demonstration of Direct Blotting Electrophoresis System Lab L 4 NMS Dr. G. Bothe
12:55 p.m. Morning section adjourns
1:30 p.m. STR analysis-Forensic Sciences Services Lec MR NMS Dr. R. Sparkes
2:20 p.m. STR analysis with the 310 Genetic Analyzer Lab L 2 NMS Dr. N. Oldroyd
4:20 p.m. STR-s analysis (AGTC) Lab L 2 NMS Dr. M. Schanfield
5:30 p.m. Identification of human remains from mass graves Lec MR NMS Dr. S.Andelinovic
6:00 p.m. Adjourn

DoD DNA Registry
Office of the Armed Forces Medical Examiner
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Adopted By ______________________ Date __________
Reviewed By ______________________ Date __________
Version 2.0 Replaces "Analysis of Mitochondrial DNA Sequencing Data," Version 1.0

Analysis of Mitochondrial DNA Sequencing Data

PRINCIPLE

To provide instructions for the analysis of deoxyribonucleic acid (DNA) sequencing data generated from the polymerase chain reaction (PCR) products of mitochondrial DNA (mtDNA). Sequence information is reported with respect to a consensus sequence termed Anderson (Anderson, et al.) and is documented on a sequence analysis layout created with the Sequence Navigator (SeqNav) software program. References to specific mtDNA positions are based on the Anderson sequence. The data analysis procedure, criteria for acceptance of data, and troubleshooting guidelines are provided.

SPECIMEN

Not applicable. This procedure requires mtDNA sequencing data generated on an Applied Biosystems (ABI) 373 DNA Sequencer.

INSTRUMENTS/SPECIAL SUPPLIES

Macintosh computer (e.g., Quadra 650, Centris 650)
"Sequence Navigator DNA and Protein Sequence Comparison" software (Version 1.0.1b15 or more current)
Black-and-white printer (e.g., Hewlett Packard LaserJet 4)
Color printer (e.g., Canon Color Pass)

QUALITY ASSURANCE

1. Before reporting sequence information from evidentiary samples, the authenticity of the positive amplification control(s) and the pGEM sequencing control(s) should be confirmed. Control samples should consist of a sufficient number of bases to confirm authenticity relative to the known sequence.

2. Sequence information at each base position should be confirmed by data from both DNA strands when possible. A specific exception is when a poly-cytosine stretch prohibits confirmation in both directions. In these instances, confirming sequence information from a single DNA strand will be acceptable. In general, data should be obtained from at least two independent amplifications when sequence information from only one strand is being reported.

3. A second scientist must independently analyze all data before sequence information is reported.

4. Base positions that are not agreed upon by the two scientists will be called "N" and the basis of the disagreement will be documented.

5. Sequence analysis layouts will be saved to the appropriate case folder and to a disk (floppy or optical) on a daily basis. When complete, the full case folder will be saved to the "Completed Cases" folder on the file server and to the scientist's personal optical disk.

SAFETY

1. When analyzing or editing sequence data on a computer, the font size should be no smaller than 15. Look away from the monitor every 15 minutes and focus on an object at least 10 feet away for 10-15 seconds.

2. A break of at least one hour must be taken for each uninterrupted two hours of computer analysis. A maximum of six hours per day and no more than three consecutive six-hour days is permitted.

3. All sequence analysis layouts must be printed in font size 15 or higher.

4. When analyzing sequence data on a printed electropherogram, look away every 15 minutes and focus on an object at least 10 feet away for 10-15 seconds.

PROCEDURE

After the sequencing gel has finished running, there are several options for data transfer and performing gel analysis. These are listed below (detailed instructions follow):

At the file server: Download the gel folder directly from the sequencer hard drive via Ethertalk to the appropriate monthly folder on the file server. Complete gel and gel image analysis at the file server, then transfer lane data to the case folder.

OR

At a workstation on the Ethertalk network: Connect to the appropriate sequencer hard drive via Ethertalk and open the gel image file. Complete gel and gel image analysis (do not download gel folder to the workstation.). Transfer lane data to the case folder. Gel folder must still be downloaded from the sequencer hard drive to the appropriate monthly folder on the file server (this must be performed at the file server).

OR (IF the Ethertalk network is not functioning)

At a sequencer workstation in Lab 8: Download the gel folder from the sequencer hard drive to an optical disk. Date and initial the "Sequence Gel Load Sheet" (DNA Form 13).

At the file server: Download the gel folder from the optical disk to the appropriate monthly folder on the file server. Complete gel and gel image analysis at the file server, then transfer lane data to the case folder. Date and initial the"Sequence Gel Load Sheet" (DNA Form 13).

1. **Communication on the Ethertalk Network between the File Server/Workstations and the Sequencers**

   NOTE: When switching between Localtalk and Ethertalk (or vice-versa), verify that no one is using the established network before proceeding. In addition, DO NOT attempt to communicate via Ethertalk with a sequencer if it is in use.

   The file server and designated workstations are on the Ethertalk Network. Perform the following at one of these locations to establish communication:

   a. To connect to Ethertalk, choose "Network" from the Apple Menu (upper left corner) OR choose "Control Panel," then "Network." Double-click on "Ethertalk Slot 1." Double-click "OK" to confirm switch to Ethertalk.

   b. To connect to the appropriate sequencer hard drive, choose "Appleshare Sequencers" from the Apple Menu, then "Sequencer (A, B, C, or D) HD Alias" OR choose "Chooser" from the Apple Menu, then "Appleshare," then the appropriate sequencer. Either method will open the directory window for the sequencer hard drive chosen.

   c. Perform gel folder consolidation, gel and gel image analysis, and data transfer as necessary via Ethertalk to the sequencer hard drive.

   d. Reconnect to Localtalk by following instructions given in Step 1a. Choose "Localtalk Built In" in the Network window.

   NOTE: Always allow at least one minute when switching between Localtalk and Ethertalk (or vice-versa) before attempting to switch back.

2. **Gel Folder Management and Data Transfer**

   a. Consolidate sequence data into the gel folder (e.g., 051795-B/PM; this folder is created by the collection software and contains all the lane data). Double-click on the hard disk icon to open the directory window. Drag the gel image from the 373 Software folder to the appropriate gel folder. Drag the sample sheet for that gel from the sample sheets folder to the gel folder.

   b. To copy data, drag the gel folder from the sequencer hard drive to the appropriate monthly folder on the file server.

   c. Verify that all information was copied successfully to the appropriate monthly folder on the file server. Date and initial the gel loading "Sequence Gel Load Sheet" (DNA Form 13)..

3. **Gel and Gel Image Analysis**

   a. Double-click on the gel image file in the gel folder. This will open the 373A Analysis program and the gel image. If gel was analyzed at the time of sequencing, proceed to Step 3c.

   b. Choose "Gel," then "Track and Extract Gel" (this operation will take 10-20 minutes).

   c. Check the <u>full length</u> of the tracking line for each lane on the enlarged gel image. Sometimes the lane tracking is not consisent with the lane position and must be modified (e.g., often tracks incorrectly if all lanes are not loaded). Failure to retrack in such instances may result in data which is not valid. If lane tracking is acceptable, proceed to Step 3e.

d. To retrack a lane on the gel image, click on the triangle below the lane number for the lane to be modified. The following options are available:

Modify lane numbering: To renumber lanes usually requires moving a lane or lanes from the extreme left to the extreme right or vice-versa. Place cursor on the triangle below the lane number to be moved and drag the tracking line to the desired position.

Shift entire track left or right: Place cursor on the triangle for the tracking line to be modified and move it in the desired direction.

Align tracking line at a node (place where tracking changes in vertical alignment): Click on the portion of the tracking line to be modified and move it to the desired location.

Remove node: Choose "Edit," then "Clear." Click on the node to be removed.

Create a node to move part of the tracking line left or right: Click on the Scissors icon (OR Choose "Edit," then "Cut"), then click on the tracking line at the spot where a node is wanted. Click on the portion of the tracking line to be modified and move it to the desired location.

e. Date and initial the "Sequence Gel Load Sheet" (DNA Form 13) on the "Lane Tracking" line. Have a second scientist verify the tracking and date and initial on the "Tracking Verification" line. Make enough copies for each scientist. Put the original in the appropriate sequencer notebook in the data analysis room.

f. Choose "Gel," then "Generate New Sample Files." Select either "All Lanes" or "Modified Lanes Only" as appropriate with the following settings:

For Extracted Lanes: Analyze Samples
Save Gel File Before Extraction

Analysis operation will take 10-20 minutes.

g. When analysis is complete, verify that lane data is present in the gel folder. If present, it will be necessary to get rid of older (unmodified) lane data files with the same label as modified lane data files. Highlight these lanes in the directory and drag to the "Trash."

h. Copy gel folder to a "Gel Folder" optical disk for permanent storage.

4. **Case Folder Set-Up and Transfer of Lane Data**

a. Create a case folder on a workstation other than the file server. Label it with the case number (e.g., 94T-009). All lane data and sequence analysis layouts for the case should be saved to this folder.

b. Create a lane data folder in the case folder. Label it with the gel name (e.g., 051795-B/PM).

c. Copy **ONLY** the appropriate lane data and the gel sample sheet from the gel folder on the file server to the lane data folder in the case folder just created. Do not copy the gel image to the case folder.

d. For all subsequent gels run in the same case, create lane data folders in a similar manner.

e. Create an additional folder within the case folder into which all sequence analysis layouts can be saved (e.g., call it "SeqNav").

5. **Printing Electropherograms**

Double-click on the "Sequence Analysis" icon to start the program. Electropherograms can be printed as a batch file or individually (base calls appear in corresponding colors if printed individually or in black-on-white if printed as a batch file).

a. Electropherograms should be printed on a color printer. Choose "File," then "Page Setup" to designate printer settings. Use settings as instructed from the appropriate printer manual.

b. Choose "Edit," then "Preferences." Recommended settings are as follows:

Basecaller: ABI50
Panels per Page: 4 (Primer Sets) or 5 (Hypervariable (HV) Regions)
Points per Panel: 1000 (Primer Sets) or 1100 (HV Regions)
Print First Page Only
Electropherogram
Sequence File Format: ABI

c. To print as a batch file, choose "Window," then "Open Sample File Queue." Click on "Add Lanes to be Printed" and select lanes from the directory box that appears. Click on "Done," then "Start."

d. To print individually, double-click on the lane to be printed to open electropherogram file. Printer settings and preferences must be designated each time an electropherogram is printed (see Steps 4a and 4b). Choose "File," then "Print." Close electropherogram window when printing is finished. Choose "Do Not Save" when prompted at this point. Saving causes the sample name on the sample sheet to be replaced with the gel name/lane number designation. Repeat for each lane to be printed.

NOTE: Whenever possible, electropherograms should be printed from lane data in case folders on individual workstations to avoid tying up the file server.

6. **Criteria for Acceptance of Data (Part I)**

Lane data should be discarded under the following conditions:

a. Insufficient data or no data: Analysis software does not create a lane file with a ".seq" extension.

b. Visual inspection of electropherogram: Majority of sequence information is off scale, at baseline, or contains excessive background noise.

e.g., off scale

e.g., at baseline

e.g., excessive background noise

7. **Create a Sequence Analysis Layout**

For each primer set, create a separate layout for each evidentiary sample, the positive amplification control, and any reagent blanks which amplified. In addition, create one layout for all pGEM sequencing controls run in lieu of a positive amplification control.

a. Double-click on the Sequence Navigator icon to start the program. A blank layout should appear into which sequences can be imported. If not, choose "File" from the "Main Menu," then "New Layout."

b. Choose "View," then "Preferences (ID) Panel." Select only the following:

ID Number
Reverse Complement Indication
Filename and Nickname (set "length" to 31)

Importing sequences:

c. Choose "Sequence," then "Import Sequence."

d. Choose the appropriate Anderson sequence from the mtDNA Standards folder on the desktop. Double-click on the sequence to import it.

e. Repeat for each additional sequence, clicking through the message box until reaching the "Lane Data Folder" containing the sequence desired.

OR

Open the appropriate lane data folder/case folder and highlight the lanes to be put in the new layout. Drag the highlighted lanes to the Sequence Navigator icon. A new layout will be created.
It is not possible to import the Anderson sequences from the mtDNA Standards folder in this manner.

Importing lanes into an existing sequence analysis layout:

f. Double-click on the file name of the layout to open, OR start Sequence Navigator and choose "File," then "Open." Select layout to be opened from the directory box that appears.

g. Import lanes as given in Step 7c-7e.

8. **Initial Set-up of the Sequence Analysis Layout**

   a. Place the Anderson sequence at the top, followed by each of the sample sequences in chronological order, placing the forward sequence before the reverse for each gel.

   b. Save the sequence analysis layout to the SeqNav folder in the appropriate case folder as follows: case number/primer set/sample/initials (e.g., 94T-009/PSIII/01A1/PGF).

   Manipulation of sequence information usually requires that the sequence be highlighted. To highlight, click on each desired sequence while holding the "Shift" key.

   c. Highlight all sequences. Choose "Sequence," then "Offset Sequence." Offset "to the right" as follows:

      HV1/PSI 16023
      PSII 16164*
      HV2/PSIII 72
      PSIV 174*

      * or one (1) base downstream of the 3' end of the sequencing primer

   d. Highlight all reverse sequences. Choose "Sequence," then "Reverse Complement."

   e. Align each sequence with Anderson using one of the following methods (note that most require a certain amount of trial and error):

      Place cursor at beginning of the sequence to be lined up. Choose "Find," then "Find." Type in a short base combination of sequence known in the Anderson sequence. If present in the sample sequence, it will be highlighted.

      Use "Find" to locate a base combination of sequence in Anderson known to be in the sample sequence.
      Visually inspect the sample sequence for a base combination known to be in the Anderson sequence.

      Once a base combination is located, use it to help in lining up the two sequences.

   f. Delete portions of sequence data that are outside the desired HV Region or Primer Set.

9. **Criteria for Acceptance of Data (Part II)**

   Lane data for a sample should be discarded under the following conditions:

   a. Sequence information appears poor and cannot be lined up with Anderson.

   b. Sequence information appears good, but cannot be lined up with Anderson.

      There are several reasons why this may occur, including incorrect tracking of the gel image, competition from a nonspecific amplification product, and primer switch. Try to identify the cause by reviewing the sequencing gel image, reviewing the product gel, or reverse complement the sequence for that sample, respectively. When the problem is

identified, take the appropriate corrective action and continue data analysis if possible. Where necessary, document the problem, cause, and corrective action taken.

10. **Analysis of Sequence Information**

Each sequence must be independently analyzed.

a. Highlight the sequence to be analyzed.

b. To compare electropherogram data, highlight at least 10 bases of sequence on the sample. Choose "Sequence," then "Display Electropherogram" (OR, press the "Apple" and "D" keys simultaneously). The electropherogram for the highlighted sequence will appear.

c. Visually analyze the electropherogram data, reviewing <u>all</u> positions within the HV Region or Primer Set.

d. Edit sequences as necessary (See Step 11, "Criteria for Accepting or Editing a Base Call"). **All edited bases should appear in the lower case.**

It is sometimes helpful to create a shadow between two of the sequences for the sample being analyzed.

e. Highlight the two sequences to be compared.
f. Choose "Sequence," then "Create Shadow." Click "OK" to affirm the sequences to be compared. An asterisk will appear at any position where the two sequences differ.

11. **Criteria for Accepting or Editing a Base Call**

It is necessary either to accept or edit the base call at each position made by the "Analysis" software program. The term "authentic" refers to a base that, in the educated opinion of the scientist, is the base present in the sample sequence.

General Rules:

a. All sequence acceptance or editing must be performed without comparing individual lanes of sequence data. However, general knowledge and understanding of problematic positions can be used as a basis for editing base positions.

b. A base call must not be edited without proper justification.

c. Two or more peaks of approximately equal intensity and spacing at a single base position (assessed as not the result of a mixture) must be called a "N/n."

d. Significant differences in relative peak intensity may be used as a criterion for editing a base call. However, substantial weight should be given to the base call made by the analysis software.

e. Significant differences in sequence spacing may be used as a criterion for editing a base call. However, spacing should be considered a weak justification for editing a base call and cannot be used as the sole criterion.

f. Discrete peaks are not necessary to justify keeping sequence data. However, data should be excluded where peaks become continuous (i.e., no definition between shoulders).

g. The base calls on forward strands agree with each other, but are clearly different from the base calls on reverse strands, which also agree with each other. Leave bases as called, but highlight the position on the sequence analysis layout and include as "N" (unconfirmed) in the summary.

h. There are certain patterns that can be expected when employing dye-terminator chemistry to generate DNA sequence information that may justify editing a base call. However, this should not be the sole criterion for base editing.

Note: The patterns that may be observed with dye-terminator chemistry are dependent on which type of Taq polymerase is used (standard AmpliTaq® v. AmpliTaq® FS).

Specific justifications that support both base acceptance and base editing are given below. Examples are included with each.

i. The authentic base is <u>clearly</u> apparent and is called by the analysis program.

j. The authentic base is <u>clearly</u> apparent, but is obscured or has a lower signal than (an)other base(s), which is/are <u>clearly</u> background. Change call to the authentic base.

A base of good intensity and spacing is visible where there is a consistent pattern of background in a particular area of sequence.

Clear mitochondrial sequence is visible below an artifactual signal due to excess fluorescence of a single base over a range of bases.

k. The authentic base is <u>clearly</u> apparent, however, it is off-scale and an underlying base is recognized by the computer.

l. An additional base is present in the authentic sequence (often due to peak broadening near the ends of sequence data). Delete the additional base.

m. The authentic base is omitted from the sequence, however, there is a <u>discrete</u> peak in the electropherogram to which it can be assigned. Insert the authentic base.

n. Polycytosine stretches are often difficult to interpret. A possible cause may be the presence of a mixture of length variants in the mtDNA of an individual. A predominant length species is often apparent; however, the frequency of a particular length species cannot be determined accurately and may vary between maternal relatives. The sequence reported for Hypervariable Region 1 represents the first 10 cytosines observed, beginning at position 16184. The sequence reported for Hypervariable Region 2 represents the number of cytosines present in the predominant base sequence. When no predominant base sequence is observed, the insertions that could not be confirmed are designated by a "N."

The patterns described in "o" through "v" may be observed when employing dye-terminator chemistry with standard AmpliTaq® polymerase.

o. C's following G's are weak. This effect may be even more pronounced when the C is the first in a string of C's following a G.

May be seen at the following positions: 80, 91, 186, 264, 317, 324, 330

p. In a string of four or more G's, the third G may show a reduced signal.

May be seen at the following positions: 16035, 16049

q. The first A in a string of A's will be strong and subsequent A's in the string will be weak.

May be seen at the following positions: 285, 16162

r. A C following a string of two or more T's can display an enhanced signal.

May be seen at the following positions: 174, 16357

s. T's following G's often display a weak signal.

May be seen at the following positions: 131, 172, 226, 16097, 16126

t. A G following a string of T's may display an enhanced signal and an underlying peak. This G signal can sometimes be so strong as to obscure signal from adjacent peaks.

May be seen at the following positions: 136, 225, 16047

u. The first T in a string of T's will be strong and subsequent T's in the string will be weak.

May be seen at the following positions: 133, 292, 16027, 16044, 16092

v. A's following T's can show reduced signal.

May be seen at the following positions: 234, 237, 240, 16137, 16162

The patterns described in "w" through "z" may be observed when employing dye-terminator chemistry with standard AmpliTaq® polymerase.

w. G's following C's are small.

x. G's following A's are small.

y. G's, A's, and T's following G's are large.

z. C's following T's are large.

12. **Criteria for Acceptance of Data (Part III)**

Portions of sequence information for a sample should be discarded under the following conditions:

a. Sequence information is off-scale, at baseline, or contains excessive background (see Step 6b).

b. Poor spacing of peaks (too close or too broad). Fully discrete peaks are not necessary to justify keeping sequence data. However, data should be excluded where peaks become continuous (e.g., no definition between shoulders).

13. **Comparison of the Sample Sequence to the Anderson Sequence**

a. Choose "View," then "Preferences (Main Panel)." Change "Insertion" designation to a "*" and "Deletion" designation to a "blank."

b. Choose "Sequences," then "Designate Reference Sequence." Type in lane number for the Anderson sequence (e.g., usually 1). The lane number will now be in bold.

c. Highlight all sample sequences. Choose "Sequences," then "Create Shadow...to Reference Sequence."

d. Review each position where an asterisk appears in the shadow comparison to Anderson on the sample electropherograms. Visually confirm the validity of all polymorphisms.

e. At positions where an insertion (or possible insertion) occurs, type an asterisk (*) into the Anderson sequence. Similarly, type a hyphen into the sample sequences at positions where a deletion occurs (note: position numbers will have to be corrected to reflect this insertion on the sequence analysis layout).

14. **Creation of a Sample Consensus Sequence** (OPTIONAL)

a. Highlight all sample sequences. Choose "Sequences," then "Create Shadow...Compute Consensus Sequence." The consensus sequence will appear as the last lane.

b. Create a shadow between the consensus sequence and the Anderson sequence (see Step 13c).

15. **Final Sequence Analysis Layout Organization**

a. If sequences have been rearranged for ease of review, place back in order (see Step 8a). The consensus sequence and its shadow, if created, should be placed before all sample sequences.

b. Choose "View," then "Home."

c. Choose "View," then "Renumber Sequences."

Print the final sequence analysis layout on a black-and-white printer

d. Choose "File," then "Page Setup." Select landscape orientation.

e. Choose "File," then "Print Layout." Click on "Print."

16. **Summary of the Sequence Analysis Layout**

a. Highlight <u>all</u> polymorphisms relative to the Anderson sequence. Highlight that same position in Anderson.

b. Highlight any positions that are "N/n," IUB coded, or are discrepant (e.g., base calls on forward strands disagree with base calls on reverse strands).

c. For each sample, including the positive amplification control, document the range of sequence confirmed (e.g., 73-340), all polymorphisms (e.g., 263 A-G), and all positions that are "N/n," IUB coded, or are discrepant.

d. Date and initial each page of the sequence analysis layout.

17. **Documentation of Electropherograms**

a. All electropherograms for evidentiary samples must be labelled with the following information:

- beginning and end of confirmed sequence used
- polymorphisms present in all bones and reagent blanks extracted together
- unconfirmed, IUB coded, and discrepant positions for that sample
- mark one position in the middle if only the ends are designated

b. All electropherograms for reagent blanks must be labelled with the following information:

- beginning and end of confirmed sequence used
- polymorphisms present in all bones and reagent blanks extracted together
- mark one position in the middle if only the ends are designated

c. Positive amplification control electropherograms do not have to be labelled.

d. Staple the associated electropherograms in the order in which they appear behind the final sequence analysis layout for each sample. Staple electropherograms from any unused sequence data at the very back. Document on the electropherogram that it was not included. If necessary, document why data was not included. Never discard any unused sequence electropherograms.

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FORENSIC APPLICATIONS OF DNA TYPING

Collection and Preservation of DNA Evidence

Henry C. Lee, Ph.D., Carll Ladd, Ph.D.,
Carol A. Scherczinger, Ph.D., and Michael T. Bourke, Ph.D

I. Introduction

In the last ten years, a number of DNA typing methods have been introduced into forensic science with considerable success and yet considerable controversy (1,2,3). DNA draws importance from its tremendous discriminating power and its stability. The ability to differentiate between individuals using genetic markers can be pivotal to the successful investigation of many crimes. This very strength is, though, also a prime reason that DNA has been challenged so vigorously in court. While most courts accept the basic methodology behind DNA analysis, some Defense objections regarding DNA evidence continue to be effective. Indeed, some of the few remaining challenges to the admissibility of DNA testing in court actually predate the methodology. They involve the initial collection, preservation, and subsequent handling of the biological evidence--issues that appear prosaic, but clearly are not.

Successful criminal investigation requires that proper procedures be employed for the collection and preservation of biological evidence. Evidence integrity begins with the first investigator at the crime scene. The issue of sample contamination, both at the scene and in the laboratory, is revisited regularly. Recent court cases such as the O.J. Simpson trial underscore this fact, but reveal nothing new. Nonetheless, even greater scrutiny of evidence collection and preservation methods is clearly forthcoming. If the evidence is mishandled during the initial stages of the investigation, salvaging the case can be problematic. This paper addresses forensic applications of DNA typing. It will discuss steps which help ensure that the application of DNA typing in criminal investigations progresses as smoothly as possible.

A. Universal Precautions

When collecting any type of biological material, the universal precautions for blood and body fluids should be followed. Blood-borne pathogens are prevalent today. Hence, it is prudent to assume that all biological samples are infectious regardless of the source. There are many publications which discuss chemical and biological safety issues in great detail (4,5,6,7,8) In general, follow departmental safety guidelines, including avoiding direct contact with biological evidence and refraining from eating and drinking. Wear gloves and any additional protective equipment as appropriate when handling biological specimens.

B. Sources of DNA

Many different types of physical evidence are commonly submitted to forensic science laboratories for subsequent examination. Evidence that could be subjected to DNA analysis (with the exception of mitochondrial DNA analysis) is limited to biological substances that contain nucleated cells. The following is a list of biological materials from which DNA has been successfully isolated and typed:

1) Blood and bloodstains
2) Semen and seminal stains
3) Tissues and organs

4) Bones and teeth
5) Hairs and nails
6) Saliva, urine and other biological fluids

Other types of biological evidence, such as tears, perspiration, serum, and other body fluids without nucleated cells are not amenable to standard DNA analysis. DNA has been isolated from materials such as gastric fluids and fecal stains. However, it is difficult to obtain sufficient DNA from these sources in case samples. It should also be noted that while DNA can often be typed from the specimens mentioned above (1-6), in many cases the quality and/or quantity of the sample proves inadequate for DNA analysis.

Several factors affect the ability to obtain a DNA profile. The first issue is sample quantity. DNA tying methods (especially PCR-based tests) are very sensitive, but not infinitely so. The second factor is sample degradation. For example, prolonged exposure even of a large blood stain to the environment or to bacterial contamination can degrade the DNA and render it unsuitable for further analysis. The third consideration is sample purity. While most DNA typing methods are robust, dirt, grease, some dyes in fabrics, etc., can seriously compromise the DNA typing process (9,10,11,12,13,14).

Collection and preservation procedures can seriously impact upon these three issues. In general, collect as large a quantity of material as possible to ensure recovery of sufficient DNA for testing purposes. It is essential to avoid collecting additional dirt, grease, etc., from the surrounding area since these impurities are known to inhibit the DNA typing process (especially PCR). Once the samples have been collected, they should be promptly delivered to the forensic laboratory. Store the items in a *cool, dry* environment until they are submitted.

II. Modes of Evidence Transfer

The six different types of biological evidence listed above can be used to associate an individual to (or dissociate an individual from) a crime. DNA can link one individual to another, to an object, or to a location. DNA evidence may also help to establish if the crime scene is a primary or secondary scene. Generally, biological evidence can be transferred by either of two means: direct deposit or secondary transfer (sometimes referred to as indirect).

A. Direct Deposit

Blood, semen, body tissue, bone, hair, urine and saliva can be transferred to an individual's body or clothing, to an object, or to a crime scene by direct deposit. Once liquid biological materials are deposited, they adhere to the surface or the substratum and become stains. Non-fluid biological evidence, such as tissue, bone, or hair can also be transferred by direct contact. Direct transfer could result from any of the following situations:

1) Suspect's DNA deposited on a person's body or clothing
2) Suspect's DNA deposited on an object or location
3) Victim's DNA deposited on a person's body or clothing
4) Victim's DNA deposited on an object or location
5) Witness' DNA deposited on victim or suspect
6) Witness' DNA deposited on an object or location

B. Secondary Transfer

Blood, semen, body tissue, hair, saliva or urine could be transferred to a victim, suspect, witness, object or location through an intermediary. With the secondary transfer mode, there is no direct contact between the original source (donor of the DNA evidence) and the target surface. For example, a rape victim's clothing bearing seminal fluid may rub against a car seat and subsequently be transferred to a second individual who sat in the same vehicle. Another example of secondary transfer is a person who picks up a victim's hair from the suspect's vehicle and then

deposits it in another car. The transfer intermediary can be a person or an object. The secondary transfer of physical evidence may, but does not *necessarily* establish a direct link between an individual and a specific crime.

III. Collection and Preservation of Biological Evidence

The ability to successfully perform DNA analysis on biological evidence recovered from a crime scene depends greatly on the types of specimens collected and how they are preserved. Thus, the technique used to collect and document such evidence, the quantity and type of evidence that should be collected, the way the evidence should be handled and packaged, and how the evidence should be preserved, are some of the critical points for a forensic DNA testing program. Unless the evidence is properly documented, collected, packaged, and preserved, it will not meet the legal and scientific requirements for admissibility into a court of law. If the DNA evidence is not properly documented prior to collection, its origin can be questioned. If it is improperly collected or packaged, cross contamination may occur. Finally, if the DNA evidence is not properly preserved, sample degradation may result. Although these scenarios will not change DNA profile 'A" into profile 'B', they can adversely affect the outcome of DNA typing or its admissibility. Given the sensitivity of DNA analysis methods, it is essential that all implements used for collection be absolutely clean. The following are general recommendations for the documentation, collection, packaging, and preservation of DNA evidence.

A. Documentation of DNA Evidence

The initial stages of physical evidence examination, including evidence documentation, may occur at the crime scene, the autopsy room, or the forensic laboratory. In any criminal or civil investigation, documentation is important from both the legal and the scientific perspectives. Each item seized should be thoroughly documented. Nothing should ever be processed until its original condition and other relevant information have been recorded. Several different means of documentation are available. Generally, the use of more than one method is recommended.

1. Evidence at a Crime Scene

1) Photograph the evidence before it is touched, moved, or collected.
2) Videotape the evidence and its relative position at the crime scene.
3) Document the location and condition of the evidence.
4) Note and sketch the spatial relationship of the evidence relative to other objects at the scene.
5) Label, initial, and seal the evidence package.

2. Evidence at the Forensic Laboratory

1) Note the package, label, and seal condition of the item.
2) Label the package with initials, laboratory case number, and date.
3) Check the item number and compare it to the submission form to ensure that the correct item has been received. Also verify that the description of the item is accurate.
4) Note, sketch, and/or photograph the contents of the package.
5) Document the location and condition of biological evidence on the item prior to any sampling. Note when secondary cuttings of the evidence are taken; include the area where the cutting was collected.

6) If any preliminary testing is done, record the nature of the test and the results obtained. When handling the evidence, always wear clean, disposable gloves to avoid possible contamination.

3. Evidence at the Autopsy Room

1) Photograph the body and any additional evidence before cleaning the body.
2) Note and sketch the evidence.
3) Systematically collect each piece of evidence with clean tools.
4) Separately package each item in a proper container.
5) Label the container and note the quantity of sample collected. Do not add preservatives such as formaldehyde to the specimen.
6) Store the item appropriately.
7) Carefully collect the clothing to avoid losing trace evidence and to avoid contamination with other biological samples.
8) Release the evidence according to proper procedures.

B. Collection, Packaging and Preservation of Evidence

Once the biological evidence is transferred through direct deposit or secondary transfer, it can remain on the target surface either by absorption or by adherence. In general, liquid biological evidence will be absorbed , while other evidence (non-liquid) will adhere. The method of collection depends largely on the state and condition of the biological evidence. Listed below are general recommendations for the collection of biological evidence for DNA analysis.

1. Blood and Bloodstains

A) Liquid Blood Samples

(1) Blood from a Person

a) Blood from a person should be collected by qualified medical personnel.

b) At least one vial of blood, (5 ml) should be collected in a vacuum collection tube with ethylenediaminetetraacetic acid (EDTA) as anticoagulant(purple-capped); EDTA tubes are best for the preservation of DNA. If conventional serological analysis and/or drug or alcohol analyses are to be conducted, additional tubes of blood should be collected. Generally, blood samples for conventional serological analyses should not contain a preservative (red-capped), and the tube for drug/alcohol testing should contain NaF (sodium fluoride).
c) Each tube should be labeled with the date, time, subject's name, location, collector's name, quantity collected, case number, and item number.
d) Blood samples should be refrigerated and submitted to the laboratory as soon as possible. Note that the blood should not be frozen if serological tests are desired. If the blood tubes are mailed, they should be sent by registered mail or overnight delivery service. Dry ice should never be used to cool the blood vials.

2. Liquid Blood Specimens at Crime Scenes

a) Liquid blood should be collected with a clean (preferably sterile) syringe or disposable pipette and transferred to a clean (preferably sterile) test tube. Do not mouth pipette any body fluid regardless of the source.

b) A blood clot can be transferred to a clean test tube with a clean spatula. Between samples, thoroughly clean the instrument with 10% bleach and alcohol. Note that the instrument must be free of bleach prior to use, as bleach destroys DNA.

c) A clean cotton cloth can be used to soak up liquid blood or a blood clot. Avoid areas that contain only serum. The blood should be air-dried (not in direct sunlight) prior to its packaging and submission to the laboratory.

d) Label the specimens with case number, item number, date, time, location, and evidence collector's initials.

e) Any liquid blood samples should be preserved in a suitable anticoagulant (EDTA for DNA), refrigerated, and submitted to the laboratory as soon as possible.

3. Liquid Blood Specimens in Snow/Ice or Water

a) Blood samples found on snow or in water should be collected immediately to avoid further dilution.

b) The most concentrated samples and the largest possible quantity of these samples should be collected in a clean, suitable container (e.g., specimen cups), avoiding impurities as much as possible.

c) Label the items as previously indicated.

d) Freeze the samples if possible.

e) Submit the items to the laboratory as soon as possible.

B. Wet Bloodstains

1. Garments with Wet Bloodstains

a) Clothing with wet bloodstains should be placed on a clean surface and allowed to air dry (not in direct sunlight). Do not use a heat source, such as a hair dryer, to speed the process.

b) Never collect a wet garment or garment with a wet bloodstain in a sealed, air tight container or plastic bag. This practice causes the samples to retain moisture, thus promoting bacterial growth and sample degradation.

c) Once the garments and stains are dry, they should be packaged using clean paper (or in a paper container) and properly labeled. Store the items in a dry environment (cool when possible) prior to their submission to the laboratory.

d) Avoid excessive handling of the garments, as such action may cause the transfer of blood crusts.

2. Objects with Wet Bloodstains

a) Small objects bearing wet bloodstains should be allowed to air dry, and then collected "as is".

b) An effort should be made to preserve the integrity of any bloodstain patterns during packaging and transportation.

c) Large objects that cannot be removed from a crime scene may have wet bloodstains on them. The wet blood should be transferred onto clean cotton cloth.

d) Bloodstained cotton cloth must be allowed to air dry before packaging in a paper container.

e) Each object must be packaged separately and its container properly labeled.

C. Dried Bloodstains

1. Dried Bloodstains on Removable Items:

a) Dried bloodstains on weapons, garments and other movable objects should be collected separately. Retain the entire item.
b) Each item should be placed in its own container, then sealed and properly labeled.

2. Dried Bloodstains on Large or Immovable Objects, Walls, Concrete, etc.

a) Document, photograph, and sketch the bloodstain pattern.
b) The bloodstain may be scraped off the object directly onto a clean piece of paper. Alternatively, the stain can be lifted from the surface using high quality tape. If tape is not available, the sample can be eluted by rubbing the stained area with clean, cotton swatches/sterile swabs moistened with distilled water. Lastly, the stains can be eluted from the object by pipetting a small volume of distilled water onto the stain several times and transferring the sample to a clean test tube.
c) The cotton swatch/swab is then allowed to dry and placed in a paper "druggist fold".
d) The packet is then placed in an envelope, sealed, and properly labeled.
e) An "unstained" portion of any item may be collected and submitted. However, since a small amount of DNA may actually be present, the "substrate control" is just another piece of evidentiary material, and not a *true* control for DNA analysis.

3. Dried Bloodstains on Objects that Can be Cut, e.g., Carpet, Upholstery

a) The stained area should be documented as previously described.
b) A portion of the item containing the bloodstain can be removed by cutting with a clean, sharp instrument.
c) Each cutting should be packaged separately and labeled accordingly.

4. Dried Small Blood Spatters

a) Small blood spatters are often difficult to remove from their surfaces. After proper documentation, it may be possible to collect them using high quality tape or a small, moistened sterile cotton thread. Try to avoid collecting dirt, etc., from the surrounding area.
b) With finely textured surfaces, it is often useful to collect the samples by pipetting a small quantity of distilled water up and down several times and transferring the sample to a clean test tube.
c) Label the items appropriately.

5. Bloodstains on Vehicles Involved in Hit and Run Cases

a) The exterior surface of the vehicle should be thoroughly examined for the presence of fabric impressions, imprint evidence, paint transfer, hairs, tissue, fibers, blood, and other trace evidence. All findings should be documented properly.
b) The undercarriage of the vehicle should also be examined for the presence of tissue, hair, blood, fibers, and other evidentiary material.
c) Photograph all imprint and impression evidence.
d) Remove and properly package all trace evidence.
e) Bloodstains are removed from painted surfaces preferably by chipping them with a sharp object such as a scalpel, chisel, or screw driver. The retained paint sample can be analyzed and compared to any paint samples found on the victim. If no paint sample is required, the stain can be scraped into a clean paper fold using a sterile scalpel.

f) As discussed above, if the scraping method is unsatisfactory, the blood stain can be removed using a moistened cotton swatch. Air dry the swatch before packaging.
g) Label and seal the items accordingly.
h) This type of evidence should be collected prior to using any method of latent fingerprint detection, with the exception of lasers, alternate light sources, or other non-destructive methods.

6. Bloodstains on the Body

a) Bloodstains on a body should be thoroughly documented before sampling.
b) Note the location, size, quantity, shape, and pattern of the stain.
c) When feasible, collect the bloodstain prior to removing the body from the scene.
d) Gently remove the stain to minimize collecting skin cells from the body. This can be accomplished by tape lifting or lightly swabbing the stained area.
e) Bloodstains under fingernails should be scraped with a clean tooth pick. Collect the nails with a clean nail clipper. Avoid using excessive force and clip each nail carefully to minimize getting the decedents blood/tissue on the stain. Package each nail and tooth pick separately.

2. Semen and Seminal Stains

A. Liquid Semen Evidence Found at the Scene

1) Document the semen evidence by notes, photography, videotape and/or sketches.
2) Use a clean syringe or disposable pipette to transfer liquid semen to a clean, sterile, test tube. Again, if non-disposable implements are used for collection, thoroughly clean them between items with 10% bleach and alcohol.
3) Label the tube as previously described.
4) Keep the specimen refrigerated and submit it to the laboratory as soon as possible.
5) Alternatively, liquid semen can be transferred onto a clean cotton cloth/swab by absorption. The item is then air dried, packaged, sealed, and labeled properly. Refrigerate the sample if possible.

B. Seminal Stains on Movable Objects

1) Seminal stains on panties, clothing, bedsheets, pillows and other movable objects should be collected "as is."
2) If an article has a wet stain on it, the stain must be allowed to air dry thoroughly prior to its collection.
3) Each item must be packaged **separately** in a clean paper container.
4) The packaging material must be properly sealed and labeled.
5) Packaged items should be refrigerated if possible, and submitted to the laboratory as soon as possible.

C. Seminal Stains on Large Objects That Can Be Cut

1) Examples of large objects that can be cut are carpets, bedding, and upholstery.
2) Document the evidence as previously described.
3) Use a clean scalpel to cut the semen stain onto clean paper, and fold the paper into a druggist fold.
4) Clean the scalpel thoroughly with bleach and alcohol between uses to avoid any possibility of cross contamination.
5) Each druggist fold should be placed into its own container, e.g., coin envelop.

6) Properly seal and label each container. Refrigerate the sample.

D. Seminal Stains on Immovable, Nonabsorbent Surfaces

1) Examples of these surfaces are floors, counters and metal surfaces.
2) Document the seminal stain evidence as previously described.
3) Use a clean scalpel to scrape the semen stain onto clean paper, and fold the paper into a druggist fold. Alternatively, collect the sample by tape lifting or swabbing the stained area.
4) Clean the scalpel between each use.
5) Each druggist fold should be placed into its own container.
6) Seal and label each container accordingly. Store the item in the refrigerator.

E. Seminal Evidence from Sexual Assault Victims

1) Sexual assault victims are examined in a hospital emergency room, rape crisis center, or physician's office.
2) Physical evidence, e.g., clothing, should be collected using established protocols as previously described.
3) A standard sexual assault kit should be used to collect vaginal, oral and anal evidence as indicated by the assault. Follow the collection steps sequentially as described in the Sexual Assault Kit.
4) Each item of evidence should be packaged, sealed, and labeled properly. Refrigerate the kit and all other items.
5) Deliver the physical evidence to the laboratory as soon as possible.

3. Tissue, Organ and Bone

A. Fresh Tissue, Organ or Bone

1) Each item of evidence should be described by notes and documented by photography, sketches, or videotape.
2) Collect the item with a clean pair of forceps. Clean the forceps between samples as described above.
3) Each item should be placed in a clean container without fixatives.
4) Each container should be properly sealed, labeled, and stored in the cold. [Note that for DNA storage, the best type of freezer is one that does not have an automatic defrost cycle].
5) Evidence should be submitted to the laboratory as soon as possible.

B. Old Tissue, Organ or Bone

1) Each item of evidence should be photographed and sketched before collection. The size, shape, pattern, and spatial relationships to the rest of the scene should be properly documented.
2) Each item can be picked up with a clean pair of forceps. Evidence still physically joined should be collected together.
3) Be careful not to contaminate any item with material from another item. Clean the forceps between samples as discussed above.
4) Each item should be placed in a separate clean container, the container then sealed and properly labeled as previously described.
5) Evidence should be refrigerated if possible, and promptly submitted to the

laboratory

4. Urine, Saliva and Other Body Fluids

A. Liquid Samples

1) Liquid urine or saliva should be transferred to a clean, sterilized container (plastic or glass specimen cup) as soon as possible.
2) Each container should be sealed and properly labeled.
3) Evidence should be stored in a refrigerator, and submitted to the laboratory as soon as possible.

B. Stains

1) Urine stains, saliva stains, and other body fluid stains can be collected "as is", or removed from their substrata by scraping or cutting.
2) Make sure the evidence is thoroughly dry and place it in a clean, paper container. Scrapings or cuttings should be collected in a druggist fold made from clean paper. The druggist fold is then placed into a secondary paper container.
3) Containers should be sealed and properly labeled.
4) Refrigerate the items when possible. Promptly transport the evidence to the laboratory.

5. Hair Evidence

1) Hair evidence can be picked up using a clean pair of forceps
2) Each item or group of hairs should be packaged separately, and the package sealed and properly labeled.
3) Exercise care in collection so as not to damage any hair root tissue present.
4) Hairs mixed with blood, tissue, or other body fluids should be treated with care. Each item should be placed in a clean container that is then sealed and labeled properly.
5) Evidence should be stored in a refrigerator and submitted to the laboratory as soon as possible.
6) If hairs are mixed with wet body fluids, they should be air dried thoroughly prior to their submission.

IV. Laboratory Processing of DNA Evidence

A. Laboratory Receipt of Evidence

Once items have been collected and transported to the forensic science laboratory, the following guidelines are recommended for processing evidence submitted for DNA analysis.

1) Physical evidence should be submitted to the laboratory with a transmittal letter, inventory sheet, and notation of the type of examination requested for each item.
2) All evidence should be received according to standard laboratory procedure, and a unique case identifier should be assigned.
3) All identifying information on the physical evidence should be checked against the submission forms. Any discrepancies should be noted and corrected.
4) Each package should be properly packaged, sealed, and labeled. Any sign of improper packaging, sealing, or labeling should be noted.
5) Any sign of sample leakage or contamination should also be noted.
6) Any special requests/instructions regarding the DNA testing should be recorded on the submission form.

7) A receipt for evidence showing the date, time, submitting agency, submitter's name, case number, item numbers, and the receiver's name should be issued.
8) Physical evidence submitted for DNA analysis should be transmitted as soon as possible to the DNA unit and stored as previously described.

B. Laboratory Initial Processing Procedures

Once the DNA unit has received the case and it has been assigned to an examiner, the following guidelines are recommended for the initial processing of evidence prior to DNA analysis.

1) An evidence examination form should be used to record the preliminary processing of each item. Relevant information includes:
   a) Package description and actual contents
   b) Label information
   c) Description/condition of evidence
   d) Local case number
   e) Laboratory case and item number
   f) Date and initials of examiner
2) Document the size, location, pattern, and condition of the stained area.
3) Weigh biological evidence such as bone, teeth, nail, and tissue as necessary. Note the quantity used for DNA analysis.
4) Record any preliminary test results and any trace evidence recovered.
5) A worksheet should be used to record information about each sample subjected to DNA analysis. Information that should be documented upon DNA extraction includes:
   a) Case number
   b) Item description and number
   c) Examiners' initials
   d) Reagent lot numbers
   e) Protocol followed
   f) Quantity of sample consumed
6) Testing results on each item should be entered on the appropriate worksheets.
7) Each sample should be handled carefully to avoid mislabeling or cross contamination.
8) Whenever feasible, a portion of the sample should be preserved for possible future analysis. These specimens should be stored in a freezer. However, in many instances the item cannot be divided due to insufficient quantity. In this event, the sample should be processed according to standard forensic laboratory guidelines.
9) Any secondary cutting for DNA analysis should be placed in a separate container, package, or tube (labeled accordingly).
11) A positive control sample should be subjected to the same protocols/reagents as the question samples beginning with DNA extraction. This applies to both RFLP and PCR methods. There are additional positive and negative controls unique to PCR.
12) Unused DNA should be properly labeled and stored in a freezer.

V. Conclusion

Physical evidence has become increasingly important to criminal investigation. Eye witness accounts are often seen as unreliable or biased. Physical evidence such as DNA may **independently** and **objectively** link a suspect/victim to a scene or to each other. The converse is equally true. However, physical evidence that is not properly recognized, documented, collected, and preserved may ultimately be of no value to a criminal investigation. DNA analysis of

biological material is an extremely powerful tool for identification purposes. Nonetheless, if the above parameters are not followed, the forensic scientist may not be able to use this tool in the interest of justice.

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STR analysis by means of direct blotting electrophoresis

L. Henke (1), J. Henke (2)

(1) Institut for Blutgruppenforschung, Abtlg. Forensische Blutgruppenkunde und Molekulargenetik, Otto-Hahn-Str. 39, P.O.Box 130468, D-40554 Dosseldorf, Germany
(2) Institut for Blutgruppenforschung, Hohenzollernring 57, P.O.Box 190420, D-50501 Koln, Germany

STR polymorphisms are powerful tools in forensic casework and parentage testing. Poor separation of alleles and interalleles however renders it difficult to apply highly polymorphic systems to practical work. Up to now, these problems could be solved by the use of sequencing gels. Many laboratories however are reluctant to work with radioactivity or to by an "automatic sequencer". These problems can be overcome by means of direct blotting electrophoresis(DBE) in combination with highly sensitive, non radioactive detection protocols. DBE easily allows to resolve DNA molecules up to 800 bp in 1 basepair steps. Instead of a computer-generated result, a hardcopy is produced.

Demonstration of the "Direct Blotting Electrophoresis System"
G. Bothe, GATC GmbH, Fritz-Arnold-Str. 23, D-78467 Konstanz, Germany

The Direct Blotting Electrophoresis (DBE) method combines electrophoresis and blotting. DNA samples (single- or double-stranded) are simultaneously separated through conventional gels (polyacrylamide or agarose) and are blotted as they leave the gel onto a nylon membrane passing underneath the gel. Since the elution time of the individual bands is proportional to their molecular weight, the result is a linear band pattern. This patented method takes full advantage of non-radioactive labeling without sophisticated technology. The DBE system is optimized for DNA sequencing, PCR analysis, STR analysis, fingerprinting, differential display and Southern blotting.

GATC GmbH
Fritz-Arnold-Str. 23
D-78467 Konstanz

Tel.: 07531-81 60 0
Fax : 07531-81 60 81
email service@gatc.de

MULTIPLEX DNA TEST FOR CYSTIC FIBROSISDNA TEST FOR CYSTIC FIBROSIS

Dr. Edwin Roovers. Edwin Roovers
Perkin-Elmer Netherlands
P.O. Box 305
2910 AH Nieuwerkerk aan den Ijssel
Netherlands
Phone: 31 180331400
Fax: 31 180331409

Cystic Fibrosis Assay Reference Guide

This new assay technology employs a rapid, single-tube, PCR/OLA multiplex, followed by 4-color electrophoresis, to identify normal and mutant Cystic Fibrosis Transmembrane Conductance Regulator gene alleles. The one-day assay uses a single reaction tube and a single lane of an electrophoresis run or a single capillary injection. The test panel screens for 31 possible CF mutations, including the 24 most common CF mutations, world-wide, as identified by the CF Consortium. These 31 mutations are: F508, G542X, G551D, W1282X, 3905 ins T, N1303K, 3849+10kb C to T, R553X, 621+1 G to T, 1717-1 G to A, 1078 del T, 2789+5 G to A, 3849+4 A to G, 711+1 G to T, R1162X, 1898+1 G to A, R117H, 3659 del C, G85E, 2183 AA to G, I507, R347P, R560T, A455E, R334W, Y122X, S549R, Q493X, V520F, S549N, and R347H. ABI software, GeneScan and Genotyper, analyze the electrophoresis data and identify a CF genotype.

Description of Cystic Fibrosis (CF) Starter Kit (P/N K0013)

- Cystic Fibrosis Assay (P/N K0002) Sufficient material for 24 tests.
  CF Sample Preparation Module - M0015, contains two buffers for processing purified DNA, fresh EDTA blood, and a positive control sample. Required storage at 2 to 8 C
  CF PCR/OLA Module - M0009, contains reagents for PCR amplification (including primers and AmpliTaq Gold), oligonucleotide probes and r*Tth* ligase for Oligonucleotide Ligation Assay.
  Required storage at -15 to -25 C
  MicroAmp Tubes with Caps - M0035
  Parts M0015, M0009, and M0035 Not Sold Separately
- OLA Gel loading Module (P/N M0013), contains formamide, loading buffer, and OLA-Tamra size standards.
  Required storage at 2 to 8 C
- Cystic Fibrosis Assay Protocol (P/N L0002)
- CF Genotyper Template (P/N L0088)

Materials Required but Not Provided

PE Applied Biosystems GeneAmp ® PCR System 2400 or 9600
ABI PRISM 377, 373 DNA Sequencer or ABI PRISM 310 Genetic Analyzer
Software: ABI PRISM GeneScan Analysis (version 2.1) or 672 GeneScan (version 1.1) and Genotyper (versions 1.1, 1.1.1, or 2.0)

CF Assay Protocol

User Notice:

A more detailed description of the Cystic Fibrosis Assay is found in the Cystic Fibrosis Assay Protocol, P/N L0002. Refer to the Cystic Fibrosis Assay Protocol pages cited within each step for more information.

Step 1: Prepare a set of MicroAmp tubes (P/N M0035) by placing a label on a tube for each sample to be analyzed, plus a positive and negative control. Place the tubes into the MicroAmp tray. Cystic Fibrosis Assay Protocol (P/N L0002) - p. 23

Step 2: Prepare one negative control by adding 15 µL of deionized water to one tube containing 60 µL of appropriate buffer, either Buffer for EDTA Blood (P/NT0020) or Buffer for Purified DNA (P/N T0022). Use the buffer corresponding to buffer used for samples. Prepare one positive control by adding 15 µL of Human DNA, male, to one tube containing 60 µL of Buffer for Purified DNA (P/N T0022). Cystic Fibrosis Assay Protocol (P/N L0002) - p. 23 for blood or p. 25 for purified DNA.

Step 3: Add 60 µL of the appropriate buffer, either Buffer for EDTA Blood (P/NT0020) or Buffer for Purified DNA (P/N T0022) into each tube. Close the cap for each tube. Cystic Fibrosis Assay Protocol - p.23

Step 4: Add 15 µL of a test sample (a test sample is either 15 µL of EDTA whole blood, or 500 ng of purified DNA in 15 µL volume) to the MicroAmp tube containing the appropriate buffer, resealing the cap after each operation. Cystic Fibrosis Assay Protocol - p. 23 for blood or p. 25 for purified DNA

Step 5: Place the tubes into the thermocycler and heat to 97 C for 40 minutes. Cool the solution to 4 C before proceeding to the next step. Cystic Fibrosis Assay Protocol - p. 17

Step 6: Centrifuge the sample preparation tubes for 10 minutes. While the sample is centrifuging, thaw and gently mix the CF PCR Reagent (P/N T0030). Quick spin the CF PCR Reagent tube before opening.

Step 7: Set up a MicroAmp tray with a fresh set of labeled tubes.

Step 8: Place 5 µL of the CF PCR Reagent into each tube, closing the cap after each addition. Cystic Fibrosis Assay Protocol - p. 28

Step 9: Add 5 µL of each test sample preparation solution (from Step 6) to the corresponding labeled tube, closing the cap after each addition. Cystic Fibrosis Assay Protocol - p. 28

Step 10: Place the tray with the MicroAmp tubes into the thermocycler and heat under the following conditions:

94 C for 12 mins
5 cycles: 98 C for 15 sec
58 C for 30 sec
72 C for 1.5 min
22 cycles: 94 C for 15 sec
58 C for 2 min
72 C for 5 min
99.9 C for 30 min

Cool the tubes to 4 C before proceeding to the next step. Cystic Fibrosis Assay Protocol - p. 17

Step 11: Thaw and gently mix the CF OLA reagent (P/N T0028). Combine the entire contents of the CF OLA reagent with the tube containing *rTth* ligase (P/N T0029). Gently mix contents, then quick spin before opening the tube.

Step 12: Add 10 µL of the OLA/ligase mix from Step 11, to each PCR test sample, capping the tube after each addition. Place the tubes into the thermocycler and heat under the following conditions:

32 cycles: 90 C for 5 sec
46.5 C for 45 sec
99 C for 10 min

Cool solution to 4 C before proceeding to next step. Cystic Fibrosis Assay Protocol - p. 17

Step 13: Combine the formamide (P/N T0023), OLA-TAMRA Size Standard (P/N T0033), loading buffer, and the sample according to one of the instrument-specific recipes below:

373 DNA Sequencer - loading volume is 5 µL
- 0.5 µL OLA-TAMRA Size Std
- 0.5 µL loading buffer
- 2.5 µL formamide
- 2.0 µL sample

377 DNA Sequencer - loading volume is 2 µL
- 0.5 µL OLA-TAMRA Size Std
- 0.5 µL loading buffer
- 3.5 µL formamide
- 2.0 µL sample

310 Genetic Analyzer - loading time is10 seconds
- 0.5 µL OLA-TAMRA Size Std
- 7.5 µL formamide
- 2.0 µL sample

Step 14: Load all solutions onto the appropriate DNA Sequencer and run the instrument as described on pages 38 (for 373), 43 (for 377) or 31 (for 310).

Step 15: Process the data using both GeneScan Analysis and CF Genotyper Template software (P/N L0088). Cystic Fibrosis Assay Protocol pp. 49, 52, 65, and 85.

Step 16: Review the data from CF Genotyper Template and validate the results according to the procedures specified in the Cystic Fibrosis Assay Protocol, p. 101, for each sample beginning with the negative and positive controls.

CTTV and FFFL

Reagents Needed:

GenePrint™ Fluorescent STR Multiplex F13A01-FESFPS-F13B-LPL, Catalog # DC6310 (Promega Corp., Madison, WI)
GenePrint™ Fluorescent STR Multiplex CSF1PO, TPOX, THO1, vWA, Catalog # DC6300 (Promega Corp., Madison, WI)
AmpliTaq DNA Polymerase™ (Perkin Elmer, Norwalk, CT)
Bovine Serum Albumin (BSA, 4 mg/ml, Sigma,)
White, light mineral oil, molecular biology grade (Sigma,)

Storage Conditions: Store all kits at -20C.

Materials needed for amplification: Dedicated pipettors (0.5ul - 1000ul), sterile pipette tips, sterile PCR tubes, PCR tube openers, sterile 0.5 ml tubes, latex gloves, thermal cycler.

Materials needed for electrophoresis: Gel electrophoresis chamber, power pack capable of 3000 volts, HR 1000 6% (Genomyx, Foster City, CA), TEMED (Amresco, Solon, OH) 10% Ammonium Persulfate (Amresco, Solon, OH), 0.5-10ul pipettor.

Equipment needed for detection: Genomyx Scanner (Genomyx, Foster City, CA).

Directions for use:

PCR amplification

Important: Use physical methods and careful technique to avoid PCR crossover contamination.

Note: For forensic samples, quantification of human DNA should be performed using hybridization with human (primate) specific oligo probes on a slot blot device.

1. The PCR reactions are set up for CTTv and /or FFFL multiplex amplification according to the manufacturer's recommendation as outlined in the Technical Manual: Geneprint™ Fluorescent STR Systems (Part # TMD006, revised 2/96, (Promega Corp., Madison, WI)

2. Use a fresh pair of gloves. In a separate area prepare a master mix tube with a volume appropriate to amplify the number of samples in the run, plus a negative control and one extra samples worth. For example, to amplify ten samples, two positive controls and two negative controls prepare a 15x master mix.

3. Prepare a master mix following the table below (aanexample based on 15 reactions is included).

Component	Final Conc.	1x Vol	15x vol
deionized water	to a final volume of 25ul/reaction		
STR 10x buffer	1x	2.5ul	37.5ul
Multiplex 10x primer pair mix	1x	2.5ul	37.5ul
AmpliTaq DNA Polymerase	1 unit	0.2ul	3.0ul
BSA	0.16 mg/ml	1.0ul	15.0ul

DNA Template	1-25 ng	*	*

Calculate volumes before adding any reagents. Add master mix components in the order they are listed.

* Amplification can occur with more or less DNA, however 5-25 ng is a good target. Adjust the amount of DNA template and deionized water added to the reactions so that the final volume of the reaction is 25ul.

4. Ensure that the reagents are well mixed by repeated pipetting, or by quickly vortexing the tube. Pulse spin the master mix.

5. Label the required number of tubes.

6. Add the required volume of master mix to each 0.5 ml PCR tube. Close each tube.

7. Using a fresh sterile pipette tip each time, open a single DNA template tube. Add the required volume of DNA to each PCR tube. Add one drop of white, light mineral oil. Close the PCR tube. Vortex to mix and pulse spin each tube.

8. Amplifications are performed in a Bioscycler Oven (Bios,) using the following cycling parameters:

94C for 2 min

10 cycles:
94C for 1 min, 60C for 1 min, 70C for 1.5 min

20 cycles:
90C for 1 min, 60C for 1 min, 70C for 1.5 min

1 cycle:
60C for 30 min.

9. Following the completion of thermocycling remove the tubes and pulse spin.

Electrophoresis:

1. Clean two low flourescent glass plates (one regular, one dog eared) with deionized water. Dry well using ethanol and Kim Wipes.

2. Lay the regular glass plate on the gel-pouring apparatus, wet two clear spacers with water and place them on the long edges of the glass plate.

3. Add 30ul of TEMED and 30ul of 10% Ammonium Persulfate to 30 ml of HR 1000 6% (Genomyx, Foster City, CA). Mix gently and pour across the regular plate approximately 1/3 of the way from the end of the plate.

4. Use the dog eared plate to spread the gel evenly on the plate and carefully line up the two plates. Clamp the long sides of the plates with binder clamps. Insert the flat edge of the sharkstooth comb and clampp the top of the gel. Allow the gel to polymerize for 1 hour prior to running.

5. Prepare the samples to be loaded on the gel. In a clean 0.5 ml tube add 2.0ul of 2x STR loading buffer to 2.0ul of PCR product. Add 2.0ul of 2x STR loading buffer to 2.0ul of the appropriate flourescently labeled allele ladder. Heat each tube in a 95C heating block for at least two minutes prior to loading.

6. Place the glass plates in the electrophoretic chamber. Add 1x TBE to the upper and lower chambers. Remove the sharkstooth comb, clean the well, and replace the comb with the teeth touching the gel.

7. Load 4ul of each sample or ladder into the appropriate lanes.

8. Electrophorese the gel at 100 watts and 50C constant temperature for 1 hour.

Flourescent Detection

1. Remove the glass plates from the electrophoretic apparatus. Clean the glass plates as above.

2. Scan the gel on the Genomyx SC Scanner according to the manufaturer's instructions.

Reviewed by:____________________ ____________________
Laboratory Director Supervisor

Date:____-____-____

Amelogenin

Reagents Needed:

Male control template 1ng/ul
Female control template 1ng/ul
10X Reaction Mix 100 mM TrisHCl Ph 8.3
500 mM KCl
15 mM MgCl2
Amelogenin Primers 5 uM
dNTPs 5 mM
AmpliTaq Polymerase 5 U/ul
BSA 4 mg/ml
Sterile Deionized Water

Storage Conditions: Store all reagents at 4°_C, except AmpliTaq is stored at -20°_C.

Materials Needed For Amplification: Dedicated pipettors (0.5 ul - 1000ul), sterile pipette tips, sterile PCR tubes, PCR tube openers, sterile 1.5 ml tubes, latex gloves, thermal cycler.

Materials needed for electrophoresis: Gel Electrophoresis chamber, power pack capable of 0 - 250 volts, agarose (Metaphor or NuSieve), pipettors, 10 X Loading Buffer, Ethidium Bromide, transilluminator, Polaroid camera and film.

Directions for use:

Important: Use physical methods and careful technique to avoid PCR cross- and carryover contamination.

1. Assess DNA concentration by spectrophotometric, or yield gel or Quanti-Blot methods, adjust DNA concentration to 1ng/ul. Note: For forensic samples, human quantification should be performed using hybridization with human (primate) specific oligo probes on a slot blot device.

2. Use a fresh pair of gloves. In a separate area, prepare master mix tube with a volume appropriate to amplify the number of samples in the run, plus a negative control and one extra samples worth. For example, to amplify 10 (ten) samples, two positive controls, and two negative con trols, prepare 15X master mix.

3. Prepare master mix following the table below (an example based on 15 reactions is included).

Component	Stock	Final 1X	1X Vol.	15X Vol.
Deionized Water		up to 100 ul	72.8 ul	1092
Amelogenin Primers	5.0 uM	0.2 uM	4.0 ul	60 ul
dNTPs	5.0 mM	0.2 mM	4.0 ul	60 ul
BSA	4 mg/ml	16 ug	4.0 ul	60 ul
PCR Rxn buffer 10X		1.5 mM	10 ul	150 ul
Amplitaq	5 U/ml	1.0 U	0.2 ul	3.0 ul
DNA template	1ng/ul	5 ng	5.0 ul	----

For a total volume of 100 ul add 95 ul of master mix per reaction and 5 ul of template DNA into a PCR reaction tube.

Calculate volumes before adding any reagents. Add master mix components in the order they are listed.

Assume 5 ul of template DNA (1 ng/ul) is used. Amplification can occur with more or less DNA, however 5 ng is a good target. More then 5 ul of DNA may be added by adjusting the amount of Deionized Water to get a final volume of 100 ul.

Use AmpliTaq DNA Polymerase (distributed by Perkin-Elmer). AmpliTaq is a registered trade mark of Hoffmann-La Roche, Inc.

4. Following the addition of master mix reagents, mix by repeated pipetting times) using sterile tips, or by quickly vortexing the tube, to make sure the enzyme is in homogeneous solution. Pulse spin the master mix.

5. Using a fresh sterile pipette tip each time, add the required volume of master mix to each PCR tube. Close each PCR tube.

6. Opening only a single DNA tube at a time, using a fresh sterile pipette tip each time, add the required volume of DNA to each PCR tube. Close PCR tube. Vortex to mix and pulse spin each tube.

7. Check the thermocycling oven parameters. The following thermal profile is recommended for Amelogenin amplification.

   Initial soak at 94°_C for 2 min
   3 cycles of 94°_C 1 min, 55°_C 15 sec, 72°_C 15 sec
   27 cycles of 94°_C 30 sec, 55°_C 15 sec, 72°_C 15 sec

   Note: These parameters are based on a thermal cycler controlled by a probe monitoring reaction temperature. Thermal cyclers based on block temperature should adjust parameters accordingly (i.e. lengthen time at sample temperature, usually 1 min is plenty).

8. Following completion of thermocycling, remove tubes and pulse spin to collect 100 ul reactions in the bottom of the tube. Either remove a 20 ul ul aliquot and add 2.5 ul of 10X loading buffer or add 12 ul of 10X Loading Buffer to the 100 ul samples.

Detection of Amelogenin

1. Using 1X TBE, pour either a 3.5% Metaphor gel or 4% (1:3) NuSieve gel.

   Use 1X TBE as the running buffer.

2. Load 10 - 15 ul of amplified sample. It may also be helpful to load a molecular weight ladder along with your samples. Electrophorese the samples at approximately 7 volts/cm (based on inter-electrode distance).

3. Monitor separation by using ethidium bromide and a transilluminator.

4. Electrophoresis may be stopped when interpretation can be easily per formed. It will take approximately 2 to 4 hours to complete electropho resis.

5. Take a Polaroid picture of the gel to show results.

Interpretation:

Females will show one band at 106 bp

Males will show two bands, one at 106 bp and the second at 112 bp.

References:

Sullivan KM, Mannucci A, Kimpton CP, Gill P (1993) A rapid and quantitative DNA set test: Flouresence-based PCR analysis of X-Y homologous gene Amelogenin. *Biotechniques 15: 636-641.*

Reviewed by:_________________________ _________________________

Laboratory Director Supervisor

Date: ____/____/____ ____/____/____

WS\SOP.MAN\AMELXY.WS6

Friday, September 26 (Day 4)

8:00 a.m. Welcome VD Drs. Shchanfield, Andelinovic and Primorac
Hon. I.Skaric Mayor of Split
Hon. B. Luksic Prefect, County of Split and Dalmatia
Dr. M. Biocic, Director of CHS
Dean of Medical School
8:25 a.m. Searching for missing and imprisoned persons: Organization of the identification in the Republic of Croatia. Dr.I. Kostovic
8:45 a.m. DNA analysis in criminal investigation Dr.H. Lee
9:30 a.m. High profile cases Dr.M.Baden
10:15 a.m. Coffee break
10:40 a.m. Mt DNA sequence analysis in USA Military Dr.M.Holland
11:25 a.m. PE and its role in the biological revolution Dr.K-H.Franzen
11:55 a.m. Fluorescent detection methods Dr.D. Burgi
12:25 a.m. Discussion
12:35 a.m. Morning section adjourns
2:05 p.m. Implication for gene therapy of dominantly inherited Dr.D.Rowe
diseases of connective tissues
2:50 p.m. Dendritic cell education for T-cell mediated imunotherapy Dr.S.Vuk-Pavlovic
3:35 p.m. Molecular basis of genetic predisposition to genetic diseases Dr.M.Radman
4:10 p.m. Cystic fibrosis and mutation analysis Dr.P F Pignatti
4:55 p.m. The use of antisense hammerhead ribozymes to selectively Dr.P.Tsipouras
target single nucleotide mutations
5:35 p.m. Discussion
5:45 p.m. Adjourn
5:45 p.m. Croatian Society for Human Genetics Annual Meeting
8:00 p.m. Welcome/Farewell Party VD

CLINICAL SECTION

Monday, September 29 (Day 1)

7:00 a.m. Registration MA NMS
8:00 a.m. Opening Remarks MR NMS Dr. M. Schanfield
8:20 a.m. PCR analysis of gene expression in rare cell populations Lec Dr.A.Dietz
9:05 a.m. PCR analysis of gene expression in rare cell populations Lab L 1&2 NMS Dr.A.Dietz
10:45 a.m. Coffee break
11:00 a.m. Developments in automated human genetic analysis Lec MR NMS Dr.D.Schuster
11:45 a.m. Morphogenic proteins: From basic discovery to clinical r vehicles for somatic gene Therapy of bone Lec MR NMS Dr. D.Rowe
12:30 p.m. Morning section adjourns
2:00 p.m. Interon gamma deficiency and susceptibility to mycovacterial diseases: a possibility for prenatal diagnostic Lec MR NMS Dr. Z. Dembic
2:45 p.m. PCR analysis of gene expression in rare cell populations Lab L 1&2 NMS Dr.A.Dietz
3:45 p.m. Detection and scanning of mutations and polymorphism using immobilized mismatch binding protiens Lec MR NMS Dr. M. Radman
4:30 p.m. Coffee break
4:45 p.m. Th eIGF pathway: A therapeutic target for rhabdamyosarcoma Lec MR NMS Dr. T. Kalebic
5:15 p.m. Demonstration of the Guest Elchrom Scientific Program Lab Dr. B. Kozulic
7:00 p.m. Adjourns

Analysis of Gene Expression in Rare Cell Types

Allan B. Dietz, Ph. D.
Stem Cell Laboratory, Mayo Cancer Center, Mayo Clinic and Mayo Foundation, Rochester, MN, 55905, USA

Introduction

The technique for analysis of temporal and spatial patterns of gene expression have vastly increased our understanding of biological processes. Recent improvements in these techniquies have allowed us to differentiate ever more closely related cells. Although one can now identify transcription on the course of interactions among cells and interactions of cellls with their environment. Because cellular interactions are often intimately related to and dependent on patterns of gene ecpresion, further refinement of techniques for analysis of gene expression remains a quest of the molecular biologist.

An ideal, highly sensitive molecular tool for analysis of gene expression in rare cell types (i.e., when only few cells are available and/or when phenotypic differences among cells are rather small) is RT-PCR, a combination of reverse transcription (RT) and polymerase chain reaction (PCR). In this method, RT converts transcripts into cDNA, while PCR amplifies a small amount of template cDNA into quantities sufficient for analysis by standard analytical methods. RT-PCR has been instrumental in our efforts to understand the dynamics of gene expression and its relationship to gene function. It can be used for amplification of all transcripts expressed in the cell with the aim of developing cDNA libraries for gene cloning and subsequent analysis. The following protocol is a n example of quanlitative RT-PRC analysis of a gene expressed in most tissues. The protocol is quite basic, but can adapted to fit the needs of most transcript analysis.

Overview of the Protocol

RT-PRC consists of three distinct steps, each important to the success of the method. *Isolation of RNA* from cultured cells or tissue is the first step. Success of RT- PCR depends on teh amount, purity and integrity of isolated RNA. For most applications, isolation of total RNA suffices and isolation of mRNA us not required; this is quite advantageous, since isolation of total RNA is simpler, takes fewer steps and generally results in more usable target molecules. Generally the amounts of RNA isolated from rare cells are not sufficient for photometric and/or electrphoretic characterization of quantity and quality; consequently, RNA extracttion and all other steps should be optimized using RNA from a known source. Thus, RT-PCR analysis of transcripts from rare cells is often based on the assumption that the amounts of RNA in analzed and known (control) cells, together with the efficiency of reverse transcription and PCR, are commensurate.

The second step in the protocol is *conversion of target RNA to cDNA.* Entire mRNA can be converted to cDNA by the use of poly-T primer (to anneal to the poly-A end of the transcript). Alternatively, a primer or primers for specific transcription of one or more RNA species can be used to prime reverse transcriptase. Use of primers specific for analyzed genes results in higher yields of transcrip-specific cDNA and, consequently, greater sensitivity of RT-PCR. Therefore, when feasible, the use of gene-specific primers is highly recommended. When both RT and PCR are performed in the same single test tube, one should use gene-specific primers only.

The third step in RT-PCR amplifies cDNA; the cDNA species to be amplified is selected by the use of specific primers. Primers are designed to amplify the target cDNA with annealing temperatures as similar to each other as possible for all primers in the reaction. To prevent undetected amplification of residual genomic DNA, primers are selected one from each side of an intron in the genomic sequence; thus, the larger size of amplified genomic DNA (because it

contains the intron) will be clearly distinguished from the smaller amplification product of the intronless cDNA.

Experiment

The following experiment is an example of RT-PCR. Its purpose is to provide some experience in RT-PCR with a core protocol that can be used as the essence of future protocols for qualitative and quantitative analysis of RNA. Total human thymus RNA is provided as the starting material. We will dilute this material into progressively smaller quantities of RNA to evaluate the minimum level of total RNA needed to detect the transcript glyceraldehyde 3-phosphate dehydrogenase (G3PDH). G3PDH is expressed in most tissues at levels easily detectable by RT-PCR. The presence of expressed G3PDH in most tissues will allow scientists to use this protocol as a reference method for other RT-PCR protocols.

General considerations

When dealing with RNA, care should be taken to prevent contamination with RNases which swiftly destroy RNA. RNases are present on human skin, so always wear gloves. Treat all glassware and plastic to eliminate RNase. Stored DNA keeps better than RNA, so convert RNA to cDNA immediately after RNA extraction. Keep all solutions and mixtures on ice until needed. This protocol also uses PCR to amplify specific DNA fragments. Routine use of PCR to analyze the same fragment requires measures to control contamination. Separate areas for reaction setup and electrophoresis, aerosol control tips, and appropriate reaction controls are useful to prevent contamination problems.

Protocol

Elimination of RNases

Treat each tube to be used in the protocol to eliminate RNases. Apply one to two drops RNASEZAP (Ambion, Austin, Texas; catalog# 9780), to the tubes, cap and vortex.

Remove solution and rinse three times with distilled water.

Dry briefly and use immediately.

RNA isolation

One of the best methods for rapid and efficient isolation of RNA is the method of Chomczynski & Sacchi. Also, kits for isolating total RNA are commercially available. These products are reliable and produce very clean RNA. One of the products used routinely in our lab, Rneasy, is available through Qiagen (Chatsworth, California). This experiment will use previously prepared total RNA from human thymus. RNA can be obtained commercially (Clontech, Palo Alto, California; catalog #64028-1). For this experiment we will use 1 mg, 50 ng, and 1 ng of total RNA to determine the sensitivity of the protocol for one-tube RT-PCR.

RT-PCR

Thaw all materials on ice.

Label two nuclease-free 1.5 ml tubes "Mix 1" and "Mix 2" respectively, and place them on ice.

To the tube labeled "Mix 1" add:

	Volume per reaction (Stock concentration)	Volume for 5.5 reactions*	Final concentration
Sterile H2O	14.2 ml	78.1 ml	
dNTPs	0.8 ml (Mix of 25 mM each)	4.4 ml	0.4 mM each
downstream primer	1.0 ml (20 mM)	5.5 ml	0.4 mM
upstream primer**	1.0 ml (20 mM)	5.5 ml	0.4 mM
DTT	2.5 ml (100 mM)	13.8 ml	5.0 mM
MgCl2	3.0 ml (25 mM)	16.5 ml	1.5 mM
Rnase Inhibitor	0.5 ml	2.8 ml	5-10 Units
Total	23.0 ml		

To the tube labeled "Mix 2" add:

	Volume per reaction	Volume for 5.5 reactions*
Sterile H2O	14.0 ml	77.0 ml
5X buffer***	10.0 ml	55.0 ml
Enzyme mix****	1.0 ml	5.5 ml
Total	25.0 ml	

*Although 5 reactions will be run for this experiment, the solution is made up contaning an additional volume to insure enough reaction components

**Primers for amplification of G3PDH are: Upstream 5'TGAAGGTCGGAGTCAACGGATTTGGT and Downstream 5'CATGTGGGCCATGAGGTCCACCAC. These primers are located at bp 71 and bp 1053 of the cDNA, respectively. The primers are available comercially as a set (Clontech, Palo Alto, California; catalog # 5406-3).

***5X buffer is 20 mM Tris-HCL, 100 mM Kcl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Tween 20 (v/v), 0.5% Nonidet P40 (v/v), pH 7.0.

****The enzyme mix used for this experiment is a mixture of AMV reverse transcriptase and the Expand High Fidelity enzyme mix. This enzyme mix as well as the 5X buffer are available from Boehringer Mannheim as the Titan One Tube RT-PCR System.

Vortex the tubes (Mix 1 and Mix 2) and briefly centrifuge.

Preheat the thermocycler to 50 oC.

Aliquot 23 ml of Mix 1 into each of the five PCR tubes.

Pipet 2 ml of total RNA into three PCR tubes. (The three RNA reactions will include two dilutions of the original total RNA as well as a sample of original RNA. One of the remaining reactions will be a negative control and one will be a genomic DNA control. The negative control will substitute water for the volume of RNA. The genomic control will use 2 ml of genomic DNA (100 ng) in place of total RNA.)

Add 25 ml of Mix 2 to each PCR tube.

Cap the PCR tubes and immediatly transfer to the thermocycler.

Proceed with the following RT-PCR thermal cycle pattern:

Number of Cyles	Temperature (°C)	Time (minutes:seconds)
1	50	30:00
1	94	2:00
	94	0:30
20	60*	0:30
	68	1:30**
	94	0:30
15	60*	0:30
	68	1:00**
1	68	7:00

*Annealing temperature must be determined for each primer pair. Alterations in the MgCl2 concentration will affect annealing temperature.
**Initially, extension is longer to insure complete extension of target DNA in the critical first cycles. Later, excess extension time is not necessary because enough template DNA is available. Also, extension time must be long enough to ensure complete extension. A good estimate is one minute per kilobase of target DNA.
This amplification profile is for a Perkin Elmer 9600 thermocycler. Adjustments in incubation times may be needed depending on the efficiency of thermocycling for other machine types.

After amplification, add 5 ml loading dye and load the sample on a 1.5% agarose gel made with TAE, load an appropriate DNA size marker, and electrophoresis.

Expected Results

Electrophoresis products of G3PDH cDNA should be seen in amplified dilutions of the thymus RNA to levels of less than 50 ng total RNA per RT-PCR reaction. The amplified G3PDH cDNA fragment should be 983 bp. Little amplified product should be seen in the genomic DNA control using the amplification parameters listed. The primers span seven introns adding 2,331 bp to the size of the cDNA making a 3,314 bp genomic DNA sequence. The extension time parameter listed above is not long enough to allow efficient DNA amplification of the genomic sequence. No amplified product should be seen in the negative control reaction.

Potential Pitfalls

Problem: No amplification products

Solution: Confirm the PCR conditions. Use the primers with genomic DNA and increase extension times (3 minutes and 45 seconds) to confirm the fidelity of the amplification reaction. Variations in thermocyclers may require optimization of annealing temperatures. Lower the annealing temperature by three degree increments and repeat amplification. Alternatively, increase the magnesium chloride concentration in increments of 2 mM.

Solution: Confirm RNA quality. If amplification of genomic DNA is possible but RT-PCR is still not successful, check the quality and quantity of RNA. This may not be possible with rare RNAs. Therefore, use RNA purified from lymphocytes with the above reaction. G3PDH is easily detectable in RNA from lymphocytes and the method of RNA isolation can be tested for quality and quantity. If quantity is acceptable (as measured by absorbance) but quality is not (as determined by gel electrophoresis), then additional measures to eliminate RNases are required. Keep in mind that material carried over from the purification of RNA (such as phenol, ethanol, and EDTA) can inhibit RT-PCR.

Solution: Increase sensitivity of detection. G3PDH expression is detectable with agarose gel electrophoresis and ethidium bromide staining when starting with as little as 50 ng total RNA. Other transcripts in rare cell types may require increased sensitivity to detect RT-PCR products. Transcript detection sensitivity can be improved by incorporation of a radionucleotide (followed by polyacrylamide electrophoreses and autoradiograph detection) or by the reamplification of the PCR product.

Problem: Amplification of genomic DNA

Solution: Design primers to amplify across introns. This will not be a problem with the above protocol. The size of genomic DNA amplification product is substantially larger than amplified cDNA, easily distinguishing it from the target amplification. However, if starting a system de novo, many frustrating problems can be eliminated if primer sequences are designed to flank introns.

Solution: Use DNase to eliminate DNA. When primers cannot be designed to differentiate genomic DNA from cDNA, use RNase free DNase to eliminate contaminating DNA. When analyzing rare RNAs, this step should be avoided when possible. It requires additional manipulation of isolated RNA and reduces the specificity of RT-PCR.

Problem: Amplification in the negative control

Solution: Prevent product carryover. Use aerosol tips, separate preparation and electrophoresis areas, and use dedicated pipettes to prepare the reactions.

Modifications to this protocol

The protocol above is an example of basic RT-PCR. It was developed with two purposes in mind. First, it can be used as a control reaction to confirm RT-PCR in most tissues. Second, it provides researchers with a template to develop their own customized RT-PCR. The following are comments regarding customization of the protocol for qualitative and quantitative RT-PCR.

To customize this protocol, first design and optimize amplification of the desired transcript. Design the primers to flank an intron if possible. Most software for primer design will provide good starting estimates of annealing temperatures which can be further optimized empirically. Other optimization is possible by altering Mg^{++} concentration. The present protocol is suitable for amplification products up to one kilobase. By modifying extension time, amplification of cDNAs up to 5 kilobases in length is possible. These changes are all that will be necessary for modifications if qualitative RT-PCR is required.

Quantitative analysis of transcripts is challenging. It requires internal controls for both the reverse transcriptase and PCR portions of the experiment. One of the most widely used techniques for quantification is competitive RT-PCR. Competitive RT-PCR uses a competitor template with the same primer binding sites as the template to be quantitated. Therefore, to quantitate an unknown template, a competitor of known concentration containing the same primer binding sites is added to the RT-PCR. The best template to use for quantification of RNA is RNA. When competitor RNA is used, it is added directly into RT-PCR cocktail. Adding competitor RNA into the RT-PCR reaction with target RNA provides a control for fidelity of reverse transcription and allows one-tube quantitative RT-PCR. Competitor RNA is obtained by cloning the gene of interest, making a small deletion, and transcribing the gene in vitro. In vitro transcribed RNA is quantitated, diluted, and spiked into the reaction. The competitor template is modified by size to distinguish it from the target. Analysis after amplification of the ratio of target to competitor will allow determination of the concentration of target molecules. The most difficult step for reproducible results with this procedure is measuring the RNA. It is very difficult to measure small amounts of RNA reliably without destroying the sample making measurement of RNA in rare cells nearly impossible. One approach to quantitate transcript expression in rare cells is to measure the input RNA by counting the number of input cells. This can only be used after validation of the RNA isolation technique. Likewise, the competitor RNA must be accurately measured before it can be spiked into the reaction. Accurate routine measurements of target and competitor RNA are difficult. It is needed only if absolute quantitation (number of molecules) is desired. Measurements can be relative (setting the highest expression seen to 100%) if the experiment can be controlled to measure changes in the same cells in a defined setting. For example, experiments measuring changes in gene expression over time or after perturbation of a biological system are best (and most easily) measured relative to an arbitrary starting point. Therefore, although this

technique can be used for absolute quantitation, it is best suited for relative quantitation of target transcripts.

Classic References

RNA isolation ADDIN ENRef (Chomczynski and Sacchi 1987)_ ADDIN ENRef (Chirgwin, Przbyla et al. 1979; Maniatis, Fritsch et al. 1982)_

RT-PCR ADDIN ENRef (Mocharla, Mocharla et al. 1990)_ ADDIN ENRef (Brenner, Tam et al. 1989; Montarras, Pinset et al. 1994)_

Competitive RT-PCR ADDIN ENRef (Becker-Andre and Hahlbrock 1989; Wang, Doyle et al. 1989)_

Recent References for RT-PCR

Competitive RT-PCR ADDIN ENRef (Tarnuzzer, Macauley et al. 1996; Saric and Shain 1997)_

Removal of contaminating DNA in RT-PCR ADDIN ENRef (Huang, Fasco et al. 1996)_

Making competitors for quantitative RT-PCR ADDIN ENRef (Schneeberger and Zeillinger 1996; Ali, Sarto et al. 1997)_

Isolation of RNA ADDIN ENRef (Reno, Marchuk et al. 1997; Su, Vivier et al. 1997)_

Commercial Addresses

Ambion	2130 Woodward St., Austin, TX, 78744 USA	800-888-8804
Clontech	1020 E. Meadow Circle, Palo Alto, CA, 94303 USA	800-424-8222
Boehringer Mannheim	9115 Hague Rd. PO Box 50414, Indianapolis IN,46250	800-262-1640
Qiagen	9600 De Soto Ave., Chatsworth, CA, 91311	800-426-8157

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Reno, C., L. Marchuk, et al. (1997). "Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues." BioTechniques 22: 1082-1086.

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Schneeberger, C. and R. Zeillinger (1996). "PCR-mediated synthesis of exogenous competitors for quantitative RT-PCR." BioTechniques 20: 360-362.

Su, S., R. G. Vivie BioTechniques 22: 1107-1113.

Tarnuzzer, R. W., S. P. Macauley, et al. (1996). "Competitive RNA templates for detection and quantitation of growth factors, cytokines, extracellular matrix components and matrix metalloproteinases by RT-PCR." BioTechniques 20: 670-674.

Wang, A. M., M. V. Doyle, et al. (1989). "Quantitation of mRNA by the polymerase chain reaction." Proceedings of the National Academy of Science, USA.

Figure captions

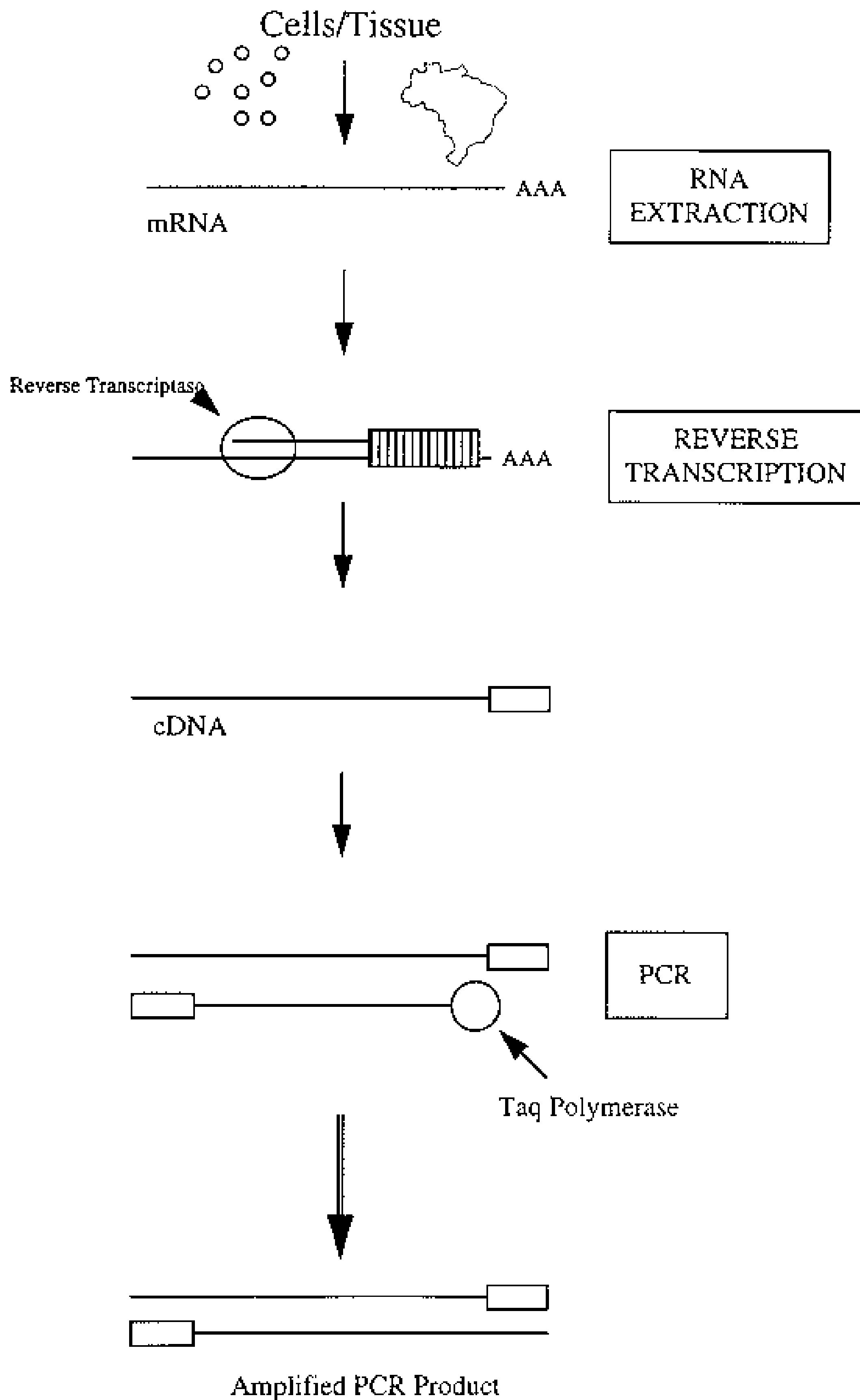
RT-PCR

RT-PCR consists of isolation of RNA from cells or tissues, converting the RNA to cDNA using reverse transcriptase, amplifying the target cDNA, and analyzing the amplified product.

Quantitative RT-PCR

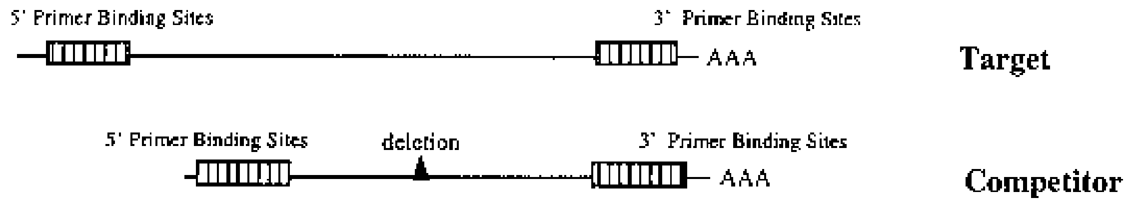
This schematic represents an example of quantitative RT-PCR. A competitor molecule is engineered to contain the exact primer binding sites of the target cDNA as well as a small deletion to distinguish the amplified competitor from amplified target DNA. This molecule undergoes in vitro transcription to obtain RNA. In vitro transcribed RNA, quantitated, and added in known increasing amounts to RT-PCR reactions containing equal amounts of RNA to be analyzed. After RT-PCR, the reactions are electrophoresed and the ratio of amplified target to competitor is determined. The target molecule is quantitated by determining the amount of added competitor that results in equivalent amplification products.

RT-PCR

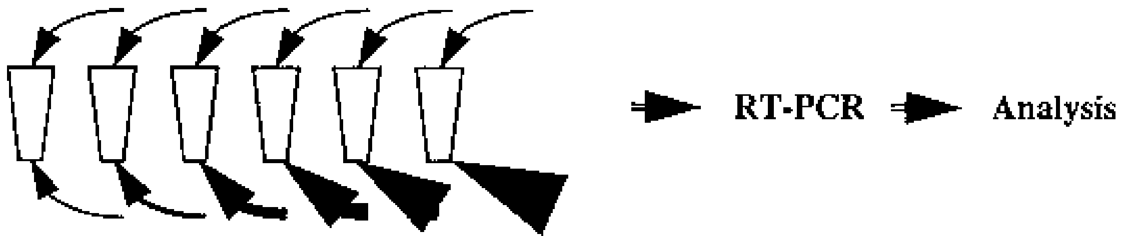


Quantitative RT-PCR

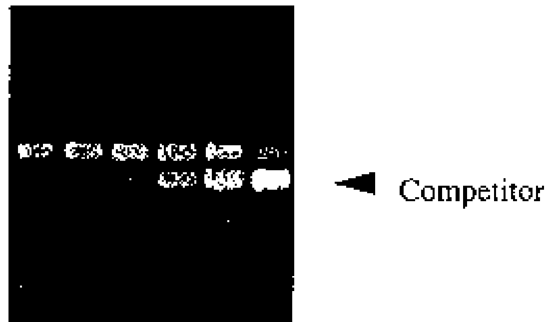
Competitor Design



Equal amounts of Target RNA



Increasing amounts of Competitor



Analysis of Competitive RT-PCR

LABORATORY PROCEDURES USING ELCHROM SUBMARINE ELECTROPHORESIS PRODUCTS

1. Checking the Success of PCR with Reusable CLEAROSE BG - Ethidium Bromide Gels

Clearose BG - EtBr gels are intended for fast analysis of dsDNA fragments in the size range from 50 to 4,000 bp. The gels are pre-equilibrated with ethidium bromide, and are run in 30 mM TAE buffer containing 0.5 micrograms/ml of EtBr. The electrophoresis time is 10 min for checking PCRs in which a single amplification product is expected. After electrophoresis, without destaining, the gel is viewed and/or photographed. Precast Clearose BG gels are available with 8, 26, 50, 52 and 100 sample wells.

Sequential loading of PCR samples containing a single amplification product increases the throughput. When several bands are present in the samples, a gel can be re-used after driving the fragments out of the gel by reverse electrophoresis.

A companion product is Bind-ET, an ethidium bromide removal system. The electrophoresis buffer containing EtBr will be passed before disposal over a cartridge able to adsorb at least 1 g of ethidium, equivalent of 2,000 l of the used running buffer.

2. Analysis of STRs with Precast Spreadex Gels

Tetranucleotide repeats in the 100-300 bp range are separated on 4 cm long Spreadex Mini gels. Electrophoresis will be carried out at 55oC using Elchrom's SEA 2000 submerged gel electrophoresis apparatus equipped with an external temperature probe immersed in the running buffer. The gels will be stained with SYBR Green I and photographed using a Polaroid direct screen camera. Allele scoring will be done by comparison with allelic ladders. The analysis of suitable STR samples provided by participants is solicited.

3. Recovery of Individual STR Alleles

For construction of an allelic ladder, and also for DNA sequencing, individual STR alleles can be recovered from Spreadex gels by a simple procedure. Using a gel puncher, a small gel piece is excised from the band of interest and placed in a thin-wall tube. Following the addition of PCR reagents and 15 cycles, the recovered single allele is ready for further work. This procedure is successful because the gel piece does not melt during thermal cycling, and because it does not inhibit the PCR.

4. SSCP Analysis (Optional)

The SEA 2000 submarine gel electrophoresis apparatus is designed to maintain the temperature of running buffer constant (within 0.5oC). Precise temperature control is essential for the success of SSCP. Experimental precast SSCP gels will be available, as well as SYBR Green II, for testing a limited number of samples from participants.

Tuesday, Septmeber 30 (Day2)

7:00 a.m. Registration MA NMS
8:00 a.m. Molecular diagnosis and monitoring of leukemia Lec MR NMS Dr. A. Biondi
8:45 a.m. Glycosphingolipid phenotype of TNF receptor I knock out mice Lec Drs. A. Marusic, J. Muething
9:30 a.m. Diagnosis of germ cell tumors Lec MR MNS Dr. I. Damjanov
10:45 a.m. Coffee break
11:00 a.m. Multiplex DNA test for cystic fibrosis Lab L2 NMS Dr. E. Roovers
1:00 p.m. Morning section adjourns
2:00 p.m. The use of neutrophil alkaline phosphatase (NAP) as a marker for prenatal screening of Down syndrome Lec MR NMS Dr. P. Tsipouras
2:45 p.m. The use of neutrophil alkaline phosphatase (NAP) as a marker for prenatal screening of Down syndrome Lab L3 NMS Dr. T. Triantafyllos
4:30 p.m. Premature STOP coden and its influence to mRNA transport Lec MR NMS Dr. D. Pirmorac
5:15 p.m. The cell cycle and retinoblastoma pathway in cancer cells Lec MR NMS Dr. D. Ramljak
6:05 p.m. Adjourns

MULTIPLEX DNA TEST FOR CYSTIC FIBROSISDNA TEST FOR CYSTIC FIBROSIS

Dr. Edwin Roovers. Edwin Roovers
Perkin-Elmer Netherlands
P.O. Box 305
2910 AH Nieuwerkerk aan den Ijssel
Netherlands
Phone: 31 180331400
Fax: 31 180331409

Cystic Fibrosis Assay Reference Guide

This new assay technology employs a rapid, single-tube, PCR/OLA multiplex, followed by 4-color electrophoresis, to identify normal and mutant Cystic Fibrosis Transmembrane Conductance Regulator gene alleles. The one-day assay uses a single reaction tube and a single lane of an electrophoresis run or a single capillary injection. The test panel screens for 31 possible CF mutations, including the 24 most common CF mutations, world-wide, as identified by the CF Consortium. These 31 mutations are: F508, G542X, G551D, W1282X, 3905 ins T, N1303K, 3849+10kb C to T, R553X, 621+1 G to T, 1717-1 G to A, 1078 del T, 2789+5 G to A, 3849+4 A to G, 711+1 G to T, R1162X, 1898+1 G to A, R117H, 3659 del C, G85E, 2183 AA to G, I507, R347P, R560T, A455E, R334W, Y122X, S549R, Q493X, V520F, S549N, and R347H. ABI software, GeneScan and Genotyper, analyze the electrophoresis data and identify a CF genotype.

Description of Cystic Fibrosis (CF) Starter Kit (P/N K0013)

- Cystic Fibrosis Assay (P/N K0002) Sufficient material for 24 tests.
  - CF Sample Preparation Module - M0015, contains two buffers for processing purified DNA, fresh EDTA blood, and a positive control sample. Required storage at 2 to 8 C
  - CF PCR/OLA Module - M0009, contains reagents for PCR amplification (including primers and AmpliTaq Gold), oligonucleotide probes and r*Tth* ligase for Oligonucleotide Ligation Assay. Required storage at -15 to -25 C
  - MicroAmp Tubes with Caps - M0035
  - *Parts M0015, M0009, and M0035 Not Sold Separately*
- OLA Gel loading Module (P/N M0013), contains formamide, loading buffer, and OLA-Tamra size standards. Required storage at 2 to 8 C
- Cystic Fibrosis Assay Protocol (P/N L0002)
- CF Genotyper Template (P/N L0088)

Materials Required but Not Provided

PE Applied Biosystems GeneAmp ® PCR System 2400 or 9600
ABI PRISM 377, 373 DNA Sequencer or ABI PRISM 310 Genetic Analyzer
Software: ABI PRISM GeneScan Analysis (version 2.1) or 672 GeneScan (version 1.1) and Genotyper (versions 1.1, 1.1.1, or 2.0)

CF Assay Protocol

User Notice:

A more detailed description of the Cystic Fibrosis Assay is found in the Cystic Fibrosis Assay Protocol, P/N L0002. Refer to the Cystic Fibrosis Assay Protocol pages cited within each step for more information.

Step 1: Prepare a set of MicroAmp tubes (P/N M0005) by placing a label on a tube for each sample to be analyzed, plus a positive and negative control. Place the tubes into the MicroAmp tray. Cystic Fibrosis Assay Protocol (P/N L0002) - p. 23

Step 2: Prepare one negative control by adding 15 µL of deionized water to one tube containing 60 µL of appropriate buffer, either Buffer for EDTA Blood (P/NT0020) or Buffer for Purified DNA (P/N T0022). Use the buffer corresponding to buffer used for samples. Prepare one positive control by adding 15 µL of Human DNA, male, to one tube containing 60 µL of Buffer for Purified DNA (P/N T0022). Cystic Fibrosis Assay Protocol (P/N L0002) - p. 23 for blood or p. 25 for purified DNA.

Step 3: Add 60 µL of the appropriate buffer, either Buffer for EDTA Blood (P/NT0020) or Buffer for Purified DNA (P/N T0022) into each tube. Close the cap for each tube. Cystic Fibrosis Assay Protocol - p.23

Step 4: Add 15 µL of a test sample (a test sample is either 15 µL of EDTA whole blood, or 500 ng of purified DNA in 15 µL volume) to the MicroAmp tube containing the appropriate buffer, resealing the cap after each operation. Cystic Fibrosis Assay Protocol - p. 23 for blood or p. 25 for purified DNA

Step 5: Place the tubes into the thermocycler and heat to 97 C for 40 minutes. Cool the solution to 4 C before proceeding to the next step. Cystic Fibrosis Assay Protocol - p. 17

Step 6: Centrifuge the sample preparation tubes for 10 minutes. While the sample is centrifuging, thaw and gently mix the CF PCR Reagent (P/N T0030). Quick spin the CF PCR Reagent tube before opening.

Step 7: Set up a MicroAmp tray with a fresh set of labeled tubes.

Step 8: Place 5 µL of the CF PCR Reagent into each tube, closing the cap after each addition. Cystic Fibrosis Assay Protocol - p. 28

Step 9: Add 5 µL of each test sample preparation solution (from Step 6) to the corresponding labeled tube, closing the cap after each addition. Cystic Fibrosis Assay Protocol - p. 28

Step 10: Place the tray with the MicroAmp tubes into the thermocycler and heat under the following conditions:

94 C for 12 mins
5 cycles: 98 C for 15 sec
58 C for 30 sec
72 C for 1.5 min
22 cycles: 94 C for 15 sec
58 C for 2 min
72 C for 5 min
99.9 C for 30 min

Cool the tubes to 4 C before proceeding to the next step. Cystic Fibrosis Assay Protocol - p. 17

Step 11: Thaw and gently mix the CF OLA reagent (P/N T0028). Combine the entire contents of the CF OLA reagent with the tube containing r*Tth* ligase (P/N T0029). Gently mix contents, then quick spin before opening the tube.

Step 12: Add 10 µL of the OLA/ligase mix from Step 11, to each PCR test sample, capping the tube after each addition. Place the tubes into the thermocycler and heat under the following conditions:

32 cycles: 90 C for 5 sec
46.5 C for 45 sec
99 C for 10 min

Cool solution to 4 C before proceeding to next step. Cystic Fibrosis Assay Protocol - p. 17

Step 13: Combine the formamide (P/N T0023), OLA-TAMRA Size Standard (P/N T0033), loading buffer, and the sample according to one of the instrument-specific recipes below:

373 DNA Sequencer - loading volume is 5 µL
- 0.5 µL OLA-TAMRA Size Std
- 0.5 µL loading buffer
- 2.5 µL formamide
- 2.0 µL sample

377 DNA Sequencer - loading volume is 2 µL
- 0.5 µL OLA-TAMRA Size Std
- 0.5 µL loading buffer
- 3.5 µL formamide
- 2.0 µL sample

310 Genetic Analyzer - loading time is10 seconds
- 0.5 µL OLA-TAMRA Size Std
- 7.5 µL formamide
- 2.0 µL sample

Step 14: Load all solutions onto the appropriate DNA Sequencer and run the instrument as described on pages 38 (for 373), 43 (for 377) or 31 (for 310).

Step 15: Process the data using both GeneScan Analysis and CF Genotyper Template software (P/N L0088). Cystic Fibrosis Assay Protocol pp. 49, 52, 65, and 85.

Step 16: Review the data from CF Genotyper Template and validate the results according to the procedures specified in the Cystic Fibrosis Assay Protocol, p. 101, for each sample beginning with the negative and positive controls.

Wednesday, October 1 (Day 3)

9:45 a.m. Detection of Hepatitis C virus-RNA in serum Lec MR NMS Dr. B.Grahovac
10:00 a.m. Coffee break
10:15 a.m. Detection of Hepatitis C virus-RNA in serum Lab L 3 NMS Dr. B.Grahovac
11:00 a.m. RT in situ PCR with direct incorporation of digoxigenin
-11-dUTP using specific AML1-ETD fusion transcript Lab L 3 NMS Dr. M.Sucic
12:30 p.m. Morning section adjourns
2:00 p.m. Pharmacogenetics: Clinical laboratory linkage between
genetics and therapeutic management Lec Dr. E.Topic
2:45 p.m. PCR genotyping of CYP2D6 gene Lab L 3 NMS I.Zuntar,M.Stefanovic
3:30 p.m. RFLP and SSCP of the HGF gene (IV exon PCR product) Lab L 3 NMS
I.Zuntar,M.Stefanovic
4:30 p.m. Detection of minimal residual disease in CML by PCR Lab L 3 NMS Dr. B.Grahovac
5:30 p.m. STR analysis to look for Aneuplody Lec MR NMS Dr. M.Schanfield
6:15 p.m. Adjourns

Detection of Hepatitis C virus - RNA in serum
(" in house" RT- PCR method)

Bla?enka Grahovac, Jasna Bingulac-Popoviæ
Croatian Institut of Transfusion Medicine
10000 Zagreb, Petrova 3

INTRODUCTION

Hepatitis C virus (HCV) is considered as the major etiological agent of 90-95% of post-transfusion non-A non-B hepatitis cases. A very high number of HCV-infected patients develop chronic hepatitis, which often results in liver chirosis and occasionally progresses to hepatocellular carcinoma(1). The HCV genome is linear positive single stranded RNA molecule comprising about 10000 nucleotides and show an organization comparable to those of the genome of pestiviruses and flaviviruses . HCV encodes a polyprotein precursor of ~ 3000 amino acids, which is cleaved by host or viral proteases into three structural and six non-structural proteins(2). Since its discovery in 1989, at least 28 HCV genotypes have been reported, which differ by >20% in the nucleotide sequence of the entire genome.(3) Different HCV genotypes have distinct geographical distributions, and may be associated with variations in viral replications and disease-inducing activity, as well as poor response to interferons in patients with chronic hepatitis C(4).

Several serological assays based on the nucleotide sequence of the HCV genome have been developed(5). One of the most important findings of this first HCV serology was that a high proportion of anti-HCV positive cases was related to parenteral transmission of the virus. Nevertheless screening of blood donors showed the existence of HCV asymptomatic carriers without a history of transfusion or intravenous drug use.Furthermore anti-HCV antibodies were also observed in sporadic hepatitis independently of any transfusion history .This observation suggested that the HCV could be transmitted by another route than the parenteral way.

Serological immunoassays have markedly diminished the incidence of post-transfusional hepatitis but, because of the long delay required for seroconversion, these tests are not sufficient to remove completely the risk of this disease(6). The most sensitive diagnosis test of HCV infection can be obtained by the polymerase chain reaction (PCR), which allows the detections of viral nucleic acid in liver and serum at very low virus load(7). The specificity of HCV RNA amplified by PCR is generally verified by oligonucleotide probe hybridization(8).

We describe here a RT-PCR method for HCV-RNA detection, developed in our laboratory, which allows identification of HCV in serum and plasma.

RT-PCR METHOD

The detection of HCV- RNA in serum consists of :

a) isolation of viral HCV-RNA
b) reverse transcription of RNA®cDNA
c) "nested" PCR
d) evaluation of results by agarose gel-electrophoresis of PCR products
e) DNA-enzym immunoassay (hybridization with biotinylated oligonucleotide probe)

a) Isolation of viral RNA
(addapted after Chomczynski and Sacchi method (1))

1. In an Eppendorf tube (1.5 ml) add 500 ml chilled D+ solution (mixture of 2 ml 4M guanidinyum thyocianat and 14,4 ml b-mercaptoethanol) and 200 ml serum. Mix well.
2. Add 50 ml 2M sodium acetat (pH 4,0) and mix by inverting the tube.
3. Add 500ml of phenol and mix well.
4. Add 200ml of solution chlorophorm / isoamyl alcohol and mix by inverting the tube.
5. Vortex and incubate for 15 minutes on ice.
6. Centrifuge for 15 minutes on 12 000 rpm, at 4°C.
7. Remove supernatant , add the same volume of isopropanol and mix well.
8. Precipitate for 1 hour at - 20°C.
9. Centrifuge for 15 minutes on 12 000 rpm, at 4°C.
10. Remove carefully the supernatant by pipeting and add 300ml 75% ethanol.
11. Centrifuge for 15 minutes on 12000 rpm ,at 4°C.
12. Remove carefully the supernatant by pipet, dry in vacuum centrifuge for 5 minutes.
13. Add 20ml of redestilled water treated with DEPC.
14. Dissolve the pelet for 10 minutes at 55-60 °C in waterbath.
15.

b) Reverse Transcription (RT-PCR kit " Perkin- Elmer ")

This enzyme reaction perform transcription of RNA to cDNA using Murine Leukemia Virus (MuLV) Reverse Transcriptase. A recombinant RNA-se Inhibitor is included for inhibition of certain mammalian RNAses.

1. Add :

Random Hexamer, 50mM	1ml
RNA	9 ml

Incubate for 10 minutes at 65 °C.

2. Put on ice for 2 minutes and for 5 minutes at room temperature.
3. Prepare a Master Mix by adding the following reagents in proportions per one RT-PCR reaction ("Perkin Elmer") :

10x PCR Buffer II	2 ml
25 mM MgCl2	4 ml
dATP	1 ml
dCTP ,10 mM	1 ml
dGTP	1 ml
dTTP	1 ml
RNA se Inhibitor, 20 U / ml	1 ml
MuLV Reverse Transcriptase, (50 U/ml)	1 ml

4. Add 10 ml of Master Mix to all tubes and let cDNA to syntetize for 45 minutes at 42°C in thermal cycler. Inactivate the enzyme(RT) for 5 minutes at 95 °C.

c) PCR amplification (" in house" protocol)

Viral RNA in serum is amplifyed with primers located in core region of HCV. The sensitivity of reaction is enhanced by using " nested " PCR.

The primer sequences used :

CORE REGION (5)

23on=5'-TAG ATT GGG TGT GCG CGC GA-3' (position 467)
186= 5'-ATG TAC CCC ATG AGG TCG GC-3' (position 751)
185= 5'-GCA (CT)GT (AG)AG GGT ATC GAT GAC (CT)T-3' (position 725)
104= 5'-AGG AAG ACT TCC GAA CGG TC -3' (position 477)

PCR single reaction mix

10xPCR Buffer	5ml
2.5mM dNTP mix	4ml
primer 5'	1ml
primer 3'	1ml
TAQ Polimerase (5U /L)	1ml
redestilled water	28,8ml
	40 ml mix + 10ml viral cDNA

For reamplification (second step of "nested " PCR) use the same volume of reagents , except 5ml product of first PCR and 5ml of redestiled water.

SORIN-BIOMEDICA DIAGNOSTICS - PROTOCOL
(Saluggia - ITALY)
BIOMEDICA GmbH Graz, Austria

5' UNTRANSLATED REGION (5'UTR) - SORIN-BIOMEDICA

1CH 5'- GGTGCACGGTCTACGAGACCTC-3'
2CH 5'- AACTACTGTCTTCACGCAGAA -3'
1TS 5'- GCCACCCAACACTACTCGGCT-3'
4CH 5'- ATGGCGTTAGTATGAGTG -3'

Probe: 5' biotinylated oligonucleotide (SORIN-BIOMEDICA)

5'- CGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCT-3'
(position nt-185/nt-143)

HYBRIDIZATION FORMAT

GEN-ETI-K DEIA (DNA Enzyme Immunoassay) is a non-isotopic methodology to detect double-stranded DNA formed by the hybridization of target DNA with specific probe, done on microtitre plate(9).

Assay procedure:

Hybridization: An oligonucleotide probe coated on the wells of microtitre plate by a streptavidin-biotin bridge is hybridized to a denatured DNA sample.

Immune reaction: The anti-double-stranded monoclonal antibody is added and the complex is recognized by the enzyme-conjugated antibody (mouse anti-IgG antibody conjugated to peroxidase).

Colorimetric reaction: The chromogen/substrate solution is read with a spectrophotometer at 450/630 nm.

PCR Program (for both amplification- In house method and SORIN-BIOMEDICA)

94°C 3 minutes Hold

94°C 30 seconds
55°C * 30 seconds n 30 cycles * for 5'UTR annealing temp. is 52°C
72°C 45 seconds

72°C 7 minutes Hold
4°C Y Hold

PCR amplification is performed by thermal cycler "Perkin Elmer" Gene Amp PCR System typ 9600 or 2400,
After amplification, the PCR products are subjected to electrophoresis (1.5% agarose / with ethidium bromide).
The final PCR product of CORE region is 236 bp and of 5'UTR region is 187 bp.

References:

1. Choo QL, Kuo G, Weiner A , et al. Isolation of a cDNA clone derived from a blood-borne non-A,non-B, viral hepatitis genome. Science 1989,244:359-362.
2. Choo QL, et al. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci 1991; 88:2451-2455.
3. Bukh J,Purcell RH, Miller RH. Sequence analysis of the core gene of 14 hepatitis C virus genotypea. Proc Natl Acad Sci 1994; 91:8239-8243
4. McOmish F, Yap PL, Dow BC, et al. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J Clin Microbiol 1994;32:884-892.
5. Kuo G, Choo QL, Alter HJ, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis.Science1989;244:362-364.
6. Zaaijer HL, Vrielink H, van Exel-Oehlers PJ, et al. Confirmation of hepatitis C infection:a comparison of five immunoblot assays. Transfusion 1994;34:603-607.
7. Yoshioka K, et al. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C

virus. Hepatology 1992;16:293-299.
8. Imberti L, Cariani E, Bettinardi A, Zonaro A, Albertini A, Primi D. An immunoassay for specific amplified HCV sequences. J Virol Method 1991; 34:233-243.
9. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.

Mirna Suèiæ,MD,PhD
Koraljka Gjadrov-Kuve?diæ,MD
Clinical Hospital Centre Zagreb
Clinical Institute for Laboratory Diagnostics
Department of Cytology
Zagreb University School of Medicine
Ki?patiæeva 12, 10000 Zagreb, Croatia
tel&fax : +385-1-212-079
Head: prof.dr. Ana Stavljeniæ-Rukavina, PhD

Protocol of RT in situ PCR with direct incorporation of digoxigenin-11-dUTP using specific AML1/ETO fusion transcript

The (8;21) translocation is frequent karyotypic abnormality detected in acute myeloid leukemia, consists of a reciprocal translocation with breakpoints at band q22 on chromosome 8, involving ETO gene, and q22 on chromosome 21 involving AML1 gene, producing AML1/ETO chimeric gene (1). Transcript is most common in AML M2 (upon FAB system), and is presented in 5-10% of adult AML M2 cases (2). Using RT in situ PCR method, which combines ISH as a method that allows localisation of nucleic acid sequence in an individual cell, and PCR which permits amplification of single copy gene, it is posible to detect sequence in intact cell, with high sensitivity. I will describe RT in situ PCR protocol with direct incorporation of digoxigenin-11-dUTP, to allow detection of PCR amplified RNA, via cDNA(3), that is currently is beeing used in our laboratory.

Procedure:

Sample preparation:
1. Obtain approximatly 1 ml of bone marrow in EDTA(K3) coated vial.

Slide preparation:
1. Using sterile stick drop three drops on specific places on one glass slide (Perkin Elmer in situ PCR glass slide, coated with aminoalkylsilane). Air dry. Store 24 hours at +4° C.
2. Fix slides in 10% buffered formalin 4 hours. Air dry.
3. You can store fixed slides at +4°C several weeks.

RT preparation:
1. Digest samples with pepsin (Sigma) 2 mg/ml at room temperature 15 minutes.
2. Inactivate pepsin by washing for 1 min in diethylpyrocarbonate treated water and for 1 min in 100% ethanol. Air dry.
3. Digest samples with Dnase, Rnase free (Boehringer Mannheim; this eliminates the nonspecific DNA amplification) on two of three samples:

10xconc PCR buffer		4uL (PerkinElmer)
Dnase,Rnase free		4uL
DEPC'd water		32uL
total	40uL	

4. Place slides in assembly tool, and cover them with amplicover discs and clips (Perkin Elmer). Incubate over night at 37° C.Remove discs and clips.
5.Wash for 1 min in DEPC'd water and 1 min in 100% ethanol.Air dry.

Reverse transcription:

1. To one of two samples treated with DNase add:

MgCl2 (stock sol. 25uM)	8uL (PerkinElmer)
10xconc PCR buffer	4uL
dNTPs (stock sol. 10mM)	4uL each
DEPC´d water	6uL
3´ primer	2uL
Rnase inhibitor	2uL
Reverse transcriptase	2uL
total	40uL

2. Place slide on assembly tool, cover them with amplicover discs and clips.
3. Place slides in sample block in GeneAmp in situ PCR system 1000 (120V, Perkin Elmer) and incubate at 42°C for 30 min.
4. Remove discs and clips, wash in 100% ethanol for 5 minutes.

PCR:
1. Prepare master mix with PCR DIG Probe synthesis kit by Boehringer Mannheim:

Sterile H2O	34,25uL
PCR buffer with MgCl2	5uL
PCR dig mix,10xconc	2,5uL
dNTP´s (stock sol,10xconc)	2,5uL
enzyme mix expand	0,75uL
total	50uL

2. Repeat the procedure with assembly tool
3. Place slides in sample block, then perform the amplification with step program consisting:

	94°C	55°C	72°C
1 cycle	2 min	1 min	1 min
14 cycles	1	1	1
14 cycles	1	1	2
1 cycle	1	1	7 min

4. Repeat the procedure using set of nested primers again upon same protocol (all primers sintesised by MWG BIOTECH)

Detection of results:
1. Detect incorporated digoxigenin with alkaline phosphatase conugated antidigoxigenin diluted 1:50 in TBS buffer (4 mg NaCl, 0.30 TRIS, 2.2 HCL in 500 ml dest. H2O). Incubate for 45 minutes. Wash in TBS.
2. Make the reaction visible by using Fast red. Incubate for 15 minutes. Wash in dest. H2O.
3. Stain with Hematoxylin Mayer 5 minutes. Air dry.

It is neceseary to use positive and negative control on the same slide to eliminate nonspecific DNA amplification. In addition to qualitative controls such as samples that lack nucleic acid sequence of interest, additional controls specific to in situ PCR should be added to each experiment, including omission of DNA polymerase from reaction mixture to detect

nonspecific sticking of detection probes, omission of primers to detect artifacts related to "DNA repair", and "endogenous priming" in direct PCR experiments,
and RNase pretreatment of samples and the omission of the RT step in experiments designed to detect (m)RNA sequence(4).

References:

1. J.R.Downing et al.: An AML1/ETO fusion transcript is constantly detected by RNA-based polymerase chain reaction in acute myelogenous leukemia containing the (8;21)(q22;q22) translocation. Blood,81:2860,1993.
2. D. Campana, C. Pui: Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance. Blood,85:1426,1995.
3. Gerard J. Nuovo: RT in situ PCR with direct incorporation of digoxigenin-11- dUTP,protocols and applications. Biochemica,11:4,1994.
4. PCR application manual. Boehringer Mannheim,1995.

PCR genotyping of CYP2D6 gene

The Cytochrome P 450 (CYP) family is the most important oxidative enzyme system involved in the metabolism of numerous widely used drugs including antiarrhythmics, antidepressants, beta-blockers, and neuroleptics. More than thirty CYP isoenzymes have been characterized in humans,each with distinct catalytic specificity and unique regulation. The genetic polymorphism has been linked to three classes of phenotypes relating to drug metabolism; extensive (EM),poor(PM) and ultraextensive (UEM) metabolizers resulting in normal,high and low blood levels of therapeutics.

The PCR method is a screening method for genetic mutations associated with altered metabolism of drugs by amplification of a specific region of the gene of interest, followed by digestion of the amplified DNA product with restriction endonucleases and comparison of the size of the digestion products generated from a DNA substrate amplified from control subject DNA vs. study subject DNAs.

Differences in the size of DNA fragments generated as a result of endonuclease digstion, commonly referred to a restriction fragment length polymorphism (RFLP), can be easily evaluated by agarose gel electrophoresis with ethidium bromide staining and UV transillumination. The protocol for the two mutations of the most common allele (CYP2D6A-2,7% and CYP2D6B - 28,6%) of CYP2D6 gene is described.

PROCEDURE

1.Sample preparation

Prepare genomic DNA from 10 ml of EDTA whole blood obtained from patients and healthy volunteers, using any of the standard methods.
Determine the amount and purity of DNA spectrophotometrically (260 nm, 260/280 nm) as well as quality of DNA by electrophoresis on 0.3% MP-agarose with ethidium bromide and photograph by a Polaroid DS-34 camera using a Polaroid 667 film.

Prepare the 0.3% MP-agarose solution, 0.4 cm thick:
dissolve 0.1 g MP-agarose in 32.3 ml H2O by heating in a boiling-water bath
Cool the agarose solution to about 50oC and than add 700ml TAE 50X and 1.6 ml ethidium bromide (final concentration of 0.5 mg/ml)
poor the agarose into the gel tray (76x100 mm) and place the gel comb
After the gel is completely set, remove the gel comb and load samples with loading buffer (bromphenol blue and glycerol 1+1)
run "submarine" gel electrophoresis (Pharmacia apparatus GNA-100) in 1x TAE buffer at 100 mA/60 V/ 1 hour
After electrophoresis, detect bands under UV light and take the photo of the gel.

2.DNA Amplification (PCR)

The specific CYP2D6 gene fragments (A and B) are amplified with the folowing primes:

CYP2D6A O 5' GATGAGCTGCTAACTAGCCC 3'
5' CCGAGAGCATACTCGGGAC 3'

CYP2D6B O 5' GCCTTCGCCAACCACTCCG 3'
5'AAATCCTGCTCTTCCGAGGC 3'
Continue with the amplification of DNA in microtube folowing the next procedure:

Reagents	Final Concentration
1. Sterile H_2O	Ad 50ml
2. $MgCl_2$	7 mM
3. KCl	50 mM
4. Tris HCl pH 9	50 mM
5. dNTP mix (10 mM of each dNTP)	200 mM
6. primers	0.2 mM
7. template DNA	0.1 mg
7. Taq DNA polymerase	2 U

At the end add two drops of mineral oil.

For amplification of CYP2D6A perform the following program:

	No. of cycles		
	1X	40X	1X
Denaturation	94°C 5 min	94°C 1 min	
Annealing		61°C 1 min	
Extension		72°C 30s	72°C 10 min

For amplification of CYP2D6B perform the following program:

	No. of cycles		
	1X	30X	1X
Denaturation	94°C 5 min	94°C 1 min	
Annealing		60°C 1min	
Extension		72°C 30s	72°C 10 min

Store the amplicon on +4°C.

Check the amplicon electrophoretically on 1.5% MP-agarose with ethidium bromide and phptograph by Polaroid DS-34 camera using a Polaroid 667 film.

-Follow previously described example for the gel preparation and gel electrophoresis.

3.RFLP

Cleave each 10ml of the amplicon with 5 U RE Mspl (Boehringer Mannheim, c cgg) and 5 U RE Mval (Boehringer Mannheim, cc (a,t)gg) at 37°C/3 h. Than do the separation of the restriction fragments electrophoretically on 2 % MP Agarose (Boehringer Mannheim) and detect the

References

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2. Heim M, Meyer UA,. Genotyping of poor metabolizers of debrisoquin by allele - specific PCR amplification. Lancet :336;1990.529-532
3. Douglas AM, Atchison BA,Somogy AA, Drummer OH. Interpretation of a simple PCR analysis of the CYP2D6A and CYP2D6B null alleles associated with the debrisoquine/sparteine genetic polymorphism. Pharmacogenetics: 4;1994.154-8.

RFLP and SSCP of the HGF
(IV exon PCR product)

Hepatocyte growth factor (HGF) is a multifunctional cytokine and a major factor of liver regeneration, regulating the cell growth, development, motility and morphogenesis. Active HGF consists of a (64kDa) and b (34 kDa) chains linked by disulfide bond. The gene is located on chromosome 7, locus q 11.1-21, and consists of 18 exons and 17 introns of approximately 70 kb. The structure of HGF contains four kringle domains, each of them encoded by two exons. The first and second kringle domain, and hairpin loop are essential for HGF recognition by c-met/HGF receptors, as well as for HGF structural domain. The forth and fifth exons ancode for the first structural domain. As mutations in functionally and biologically relevant domains lead to a pathologic process, poymorphism studies in healthy subjects and patients with liver diseases, and determination of their association with the occurrence of disease are of utmost importance. The polymorphism of DNA fragment obtained by polymerase chain reaction (PCR) can be determined by two established methods, i.e. restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP). Here we described the PCR-SSCP protocol for the polymorphism analysis of exon 4th of the HGF gen.

PROCEDURE:

1. Sample preparation

Prepare genomic DNA from 10 ml of EDTA whole blood obtained from patients and healthy volunteers, using any of the standard methods.
Determine the amount and purity of DNA spectrophotometrically (260 nm, 260/280 nm) as well as quality of DNA by electrophoresis on 0.3% MP-agarose with ethidium bromide and photograph by a Polaroid DS-34 camera using a Polaroid 667 film.

Prepare the 0.3% MP-agarose solution, 0.4 cm thick:
Dissolve 0.1 g MP-agarose in 32.3 ml H_2O by heating in a boiling-water bath
Cool the agarose solution to about 50oC and than add 700ml TAE 50X and 1.6 ml ethidium bromide (final concentration of 0.5 mg/ml)
Pour the agarose into the gel tray (76x100 mm) and place the gel comb
after the gel is completely set, remove the gel comb and load samples with loading buffer (bromphenol blue and glycerol 1+1)
Run "submarine" gel electrophoresis (Pharmacia apparatus GNA-100) in 1x TAE buffer at 100 mA/60 V/ 1 hour
after electrophoresis, detect bands under UV light and take the photo of the gel.

2. DNA Amplification (PCR)

Prepare the amplification of DNA (214bp) in microtube following the next procedure:

Reagents	Volume	Final Concentration
1. Sterile H_2O	Ad 100 ml	
2. PCR buffer, 10xconc.	10 ml	1 X
3. dNTP mix (10 mM of each dNTP)	2 ml	200 mM
4. primer 1*	1ml	0.1 mM

5. primer 2*	1ml	0.1 mM
6. template DNA	variable	0.3 mg
7. Taq DNA polymerase	2 ml	2 U/100 ml

P1: 5' - ctgcatatgttttgcatagttgc - 3

P2: 5' - catgtaaaaaagacagaggcttc - 3'

At the end add two drops of mineral oil.

Perform amplification according to the program:

	No. of cycles		
	1X	35X	1X
Denaturation	95oC 2 min	95oC 20s	95oC 20s
Annealing	53oC 2o s	53oC 20s	53oC 20s
Extension	72oC 30 s	72oC 30s	72oC 5 min

Store the amplicon on +4oC.

Check the amplicon electrophoretically on 1.5% MP-agarose with ethidium bromide and phptograph by Polaroid DS-34 camera using a Polaroid 667 film.
Follow previously described example for the gel preparation and gel electrophoresis.

3. RFLP

Cleave 10 ml of the amplicon with 10 U RE MvaI (Boehringer Mannheim, cc (a,t) gg) and 15 U RE AluI (Boehringer Mannheim, ag ct) and DdeI (Boehringer Mannheim, c nag) at 37oC/3h. Than do the separation of the restriction fragments electrophoretically on 15% PAGE and detect the fragments by the method of silver staining.

4.SSCP

Denature 8 ml of the amplicon with 8 ml 95% formamide loading buffer at 95oC/5 min. Quickly chill on ice where the samples should sty until loading on the gel. Than, separate ssDNA fragments (obtained by Denaturation) electrophoretically on 10% PAGE (pattern depends on electrophoresis temperature) and perform the detection by the silver staining method.

4. PAGE

To detect the DNA fragments (either RFLP or SSCP) the poliacrilamide gel has to be prepared. Glass mould is prepared of two glass plates well washed and dried. N one plate attach a plastic Gel bond film using a thin layer of water for the adhesion. Than put the two glass plates (one of them with the Gel bond film) together with a half milimeter U - shaped spacer between them and clamp them with a clamps. In thus obtained mould, pour a previously prepared gel and live on a cool flat surface allowing gel to polymerize. Be sure that no air bubbles are left in the gel.
The 50 ml of the 15% PAG is preparing by the following protocol:

Mix:

12.5 ml 30% acrilamid/bis-acrilamid

1.3 ml 20X TBE
10.3 ml H2O
Just before pouring the gel into the mould, add:
200ml ammonium - persulphate (10%) and 10ml TEMED.

After about an hour when the gel is polymerized, put it out of the mould and set it on the horizontal electrophoresis system (1X TBE electrophoresis buffer) with the wells turned to the cathode side.
After 10 minutes of prerun (500V/300mA) load the gel with your samples (about 15ml of RFLP or SSCP) - and set the elektrophoresis until the bromphenol-blue reaches the end of the gel (400V/150mA - 15oC).

References:
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Detection of Minimal Residual Disease in Chronic Myeloid Leukemia Using the Polymerase Chain Reaction

Bla?enka Grahovac
Croatian Institut of Transfusion Medicine, 10000 Zagreb, Petrova 3

INTRODUCTION

The first tumor-specific chromosomal aberation, the shortened chromosom 22 (22q-) or Philadelphia (Ph) chromosome was observed in chronic myeloid leukemia (CML) (1).The Ph chromosome, the result of a translocation between the abl proto-oncogene on chromosome 9 and the bcr gene on chromosome 22, is found in more than 95% of CML, and also between 10% and 25% of acute lymphoblastic leukemias(ALL(2). These leukemic cells express BCR/ABL transcript in which either exon2 (bcr2/abl2 junction) or exon 3 (bcr3/abl 2 junction) from the major breakpoint cluster region of the BCR gene is spliced to exon 2 of ABL gene(3,). The bcr3/abl2 and bcr2/abl2 fusion mRNAs both encode 210-Kd chimeric BCR/ABL proteins with oncogenic activity(4). In the remaining Ph-positive ALLs, the first exon of the BCR gene is spliced to exon 2 of ABL, leading to the production of a smaller 185-Kd fusion protein. Since the BCR-ABL gene is specific to the leukemic cells, and its acquisition is probably the initiating event in CML, it is an excellent marker to identify minimal residual disease (MRD) after therapy(5). Despite impressive advances in leukemia treatment, disease recurrence following a successful remission induction,represents a major problem. Since more relapses orginate from neoplastic cells escaping therapeutic intervention, the development of adequately sensitive technique to detect MRD is an important task. Therefore, a number of different techniques including cytogenetics, Southern blotting and polymerase chain reaction(PCR) analysis have been applied to detect MRD in order to define patients who are at high risk of relapse(6,7). Cytogenetic and Southern blotting techniques may not detect leukemic cells comprising less than 1% of the total cell population and therefore, lack the sensitivity necessary to study MRD. In contrast, because of its enormous sensitivity, PCR method, by allowing the identification of as few as one leukemic cell in 105 to 106 normal cells, has been used for diagnostics, for monitoring individual responses to therapeutic efforts, and to predict impeding relapse prior to clinical manifestation of the disease(8).

The PCR has become a standard method for highly sensitive detection of BCR/ABL transcript in patients with CML, after chemotherapy, immunotherapy and bone marrow transplantation(BMT)(9).

Material and Methods

We have developed the reverse transcription(RT)-PCR method by using GENEAMP RNA PCR kit (Perkin-Elmer). Total RNA was isolated from 0,5-1,0 ml of bone marrow and peripheral blood , by the acid guanidium- thiocyanate/phenol/chloroform method (9).

Reverse Transcription (GENEAMP RNA -PCR kit " Perkin- Elmer ")

Reverse transcription of 500 ng - 1 mg of total RNA to cDNA was performed in total volume of 20 ml.

1. **Add :**

Random hexamer, 50mM	1ml
RNA (0,5-1,0 mg)	9 ml

Incubate for 10 minutes at 65 °C.

2. Put on ice for 2 minutes and for 5 minutes at room temperature.
3. Prepare a Master Mix by adding the following reagents in proportion per one RT-PCR reaction (Perkin- Elmer) :

10x PCR Buffer II	2 ml
25 mM MgCl2	4 ml
dATP 10 mM	1 ml
dCTP "	1 ml
dGTP "	1 ml
dTTP "	1 ml
RNA se Inhibitor, 20 U / ml	1 ml
MuLV Reverse Transcriptase, (50 U/ml)	1 ml

4. Add 10 ml of Master Mix to all tubes and let stay for 45 minutes at 42°C in thermal cycler. Inactivate the enzyme(RT) for 5 minutes at 95 °C.

PCR amplification (" in house" protocol)

Oligonucleotide primers:

outer primers

bcr1	5'- GCTGCTGCTTATGTCTCCCAG-3'	3068Æ3088
abl1	5'- GGCGTGATGTAGTTGCTTGGG-3'	717Æ697

nested primer

bcr2	5'-GATCTCCTCTGACTATGAGCGTG-3'	3137Æ3159
abl2	5'-CCATTTTTGGTTTGGGCTTCACACCATTCC-3'	684Æ658

PCR single reaction mix

10xPCR Buffer	5ml
2.5mM dNTP mix	4ml
primer 5' (20mM)	1ml
primer 3' (20mM)	1ml
TAQ Polimerase (5U /L) Perkin-Elmer	1ml
redestilled water	28,8ml
	40 ml mix + 10ml cDNA

For reamplification (second step - nested PCR) use the same volume of reagents , but 5ml product of first PCR and 5ml of redestiled water.

PCR Program (for both amplifications)

94°C 3 minutes Hold

94°C 30 seconds
55°C 30 seconds n 30 cycles
72°C 45 seconds

72°C 7 minutes Hold
4°C Y Hold

PCR amplification is performed by thermal cycler "Perkin Elmer" Gene Amp PCR System typ 9600 or 2400,
After amplification, the final PCR products are subjected to electrophoresis (1.5% agarose / with ethidium bromide). The size of PCR product is 481 bp in case of bcr3/abl2 transcript and 406 bp in case that bcr2/abl2 transcript is amplified.

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7. Negrin RS, Blume KG. The use of the polymerase chain reaction foe the detection of minimal residual malignant disease. Blood 1991;78:255
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Buccal Swab Extraction

Reagents and Supplies

Soft-top microfuge tubes (1.5 ml) - 2 per sample
2 ml polypropylene centrifuge tube
Digest Buffer, prewarmed
Proteinase K solution (10 mg/ml), freshly thawed
1.0 M DTT, freshly thawed
Protein Precipitating Reagent (SQG Kit)
Absolute ethanol
Tris Buffer

Note: 2 swabs per person are processed and pooled in step 9.

1. Take 2 buccal swabs, slice along side and tip and remove outer "shell". Place each "shell" in a separate labeled 1.5 ml soft-top microfuge tube.

2. Warm Digest Buffer in 55°C. water bath to dissolve SDS. Place swab material in 300 µl Digest Buffer. Vortex and make sure swab is covered with liquid.

3. Add 20 µl Proteinase K solution (10 mg/ml) and 20 µl 1.0 M DTT. Using swab stick swirl the substrate material and mixture thoroughly. Make sure substrate material is completely submerged. Incubate both tubes at 37°C overnight.

4. Using forceps cleaned in HCL/methanol punch 3 holes in cap of tube; remove swab material; wring out on inside of tube and place in cap. Centrifuge at 7,500 RPM for 5 minutes.

5. Add 100 µl of Protein Precipitating Reagent to the tube and vortex _vigorously_ for 20 seconds. Allow to stand for 15 minutes at room temperature.

6. Centrifuge sample for 10 minutes at 7500 - 10,000 rpm.

7. Transfer the **_supernatant_** from each tube to 2 clean 2 ml tubes, add two (2) volumes of absolut ethanol(approximately 1 ml to each tube) and gently rock to mix.

8. Place at -20°C 30 minutes to overnight.

9. Centrifuge at 10,000 RPM for 10 minutes. Decant ethanol and air dry. Add 50 µl of Tris buffer or DI H2O to dried pellet in the first tube, mixing to dissolve, transfer to second tube and repeat.

continued......... page 1 of 2

Buccal Swab Extraction (continued) page 2 of 2.

Note: Postive and negative controls for extraction must be run. Check sample swab is the positive control. Clean swab is the negative control

References

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Sambrook, J, Fritsch, E F. and Maniatis, T. *Molecular Cloning: A_Laboratory Manual 2nd. ed. Vol. 2.* Cold Spring Harbor Laboratory Press, New York. (1989) E10-E14.

	Lab Director	Supervisor
Reviewed by:	____________	____________
Date:	____________	____________

1% FICIN SOLUTION

Reagents and Supplies

CRUDE FICIN LATEX (SIGMA F-8629)
PHOSPHATE BUFFERED SALINE (0.01m PBS, ph 7.4)
COFFEE FILTER OR EQUIVALENT
ERLENMEYER FLASK
MAGNETIC STIRRER
CENTRIFUGE TUBES (DISPOSABLE)
CENTRIFUGE
POWDER FUNNEL

This recipe is for 100 mls of 1% Ficin. The recipe can be scaled up or down as needed

1. To an Erlenmayer flask on a magnetic stirrer containing 100 mls of pH 7.4 PBS, slowly add 1 gram ficin latex.

   Note: Ficin latex does not go into solution well. Make sure all of the ficin wets.

2. Mix on low speed for 1 hour.
3. Incubate overnight in the refrigerator.
4. Transfer ficin/PBS to centrifuge tubes and centrifuge at 1-2000 RCF for 15 minutes.
5. Pour the supernatant through a coffee filter, or similar filter mounted in a powder funnel.
6. Quality control the 1% ficin solution before aliquoting and freezing.

   Quality Control:

   A. Dilute 1% ficin 1/10 in PBS.

   B. Treat 2% Group O RH+ RBC as in RBC/LEWIS protocol. Wash 3 (three) times in PBS.

   C. Add 50 uls of non-agglutinating anti-D to 50 uls of enzyme treated O RH+ RBC to a 10 x 75 or 12 x 75 centrifuge tube and mix well. Use untreated O RH+ RBC as a negative control.

   Note: GM/KM coating antigen works well for this, commercial RH D typing reagent cannot be used.

   D. Centrifuge immediately in a clinical centrifuge on high speed for 20 seconds, record results.

   The enzyme treated RBC should be 4+, negative control should be -.

1% FICIN SOLUTION (continued)

7. Aliquot the 1% ficin solution in convenient volumes, in appropriately labeled plastic tubes with stoppers.

8. Store frozen at -10°C or colder.

9. This product has a storage life of approximately 1 year. A lot number should be assigned and the outdate noted on the storage container.

10. For use, the tubes should be thawed and mixed before diluting 1/10 in PBS.

11. Discard unused material at the end of the day.

Fill out appropriate solution manufacture worksheet and file in solution manufacturing file.

References

Issitt, P, and Issitt, C H (1975) *Applied Blood Group Serology* 2nd ed. Spectra Biologicals, Oxnard. pp 25-26.

	Laboratory Director	Supervisor
Reviewed by:	______________	______________
Date:	______________	______________

DNA PROCEDURES for AMNIOTIC CELLS

Required Specimen: 4 T-25 flasks grown to confluency.

Reagents & Supplies

TRIS EDTA SALINE
(warmed to 37°c)
FICIN SOLUTION
- **1.0 ml freshly thawed 1% Ficin solution**
- **9ml TrisEDTA solution**

DTT
- **20 ul of 1M DTT**

SUPER QUIK GENE EXTRACTION KIT
- **1,500 ul SQG White Cell Lysis Buffer**
- **200 ul SDS**
- **500 ul PPR**

ABSOLUTE ETHANOL
TE BUFFER
50 ml CONICAL POLYPROPYLENE TUBES LABELED WITH SAMPLE NUMBER
15 ml CONICAL POLYPROPYLENE TUBES LABELED WITH SAMPLE NUMBER
BULB PIPETTE (POLYPROPYLENE, TIP BENT)

Note: Cells are maintained in a 37°c oven until growth is confluent. Flasks should never be refrigerated.

1. Pour the media from the flasks into labeled 50 ml polypropylene tubes equally as to balance for centrifugation.

2. Using a serological pipette add 10.0 ml, pre-warmed to 37°c, TrisED TA saline into each flask and set-aside until step #6.

3. Centrifuge the balanced 50 ml tubes at 2000 RPM for 20 minutes.

4. Suction off the supernatant leaving approximately 1.0 ml in each tube.

5. Resuspend buttons and pool into two(2) of the 50 ml tubes. Rinse with TrisEDTA saline to be certain all cells are transfered into the pooling tubes.

6. Transfer TrisEDTA saline from flasks in step #2 to pooling tubes.

7. Add one bulb pipette full of pre-warmed ficin solution to each flask. Gently rotate solution about flask. Check for cell detach ment from flask using the scope. A curved pipette may be useful in flushing cells from flask surface.

8. When all cells are detached, transfer all cells and solution to the pooling flasks using a curved pipette. Rinse flasks with 2-3 ml TrisEDTA and add this to pooling tubes. Under scope, check to be certain all cells have been transfered.

9. Centrifuge balanced pooling tubes 20 minutes at 2000 RPM.DNA PROCEDURES for AMNIOTIC CELLS (continued)

10. Suction balanced pooling tubes down to approximately 1.0 ml. Resus pend and transfer to a pre-labeled 15 ml conical polypropyline tube.
Rinse pooling tubes with 1-2 ml TrisEDTA and add to centrifuge tubes.

11. Centrifuge 15 ml conical tube 20 minutes at 2000 RPM.

12. Rinse once with TrisEDTA to remove any residual fetal calf serum in original media.

13. Centrifuge 15 ml conical tube 20 minutes at 2000 RPM.

14. Remove as much supernatant as possible without endangering cell pellet and procede immediately with extraction.

Extraction

1. Add to the cell pellet 20 ul 1M DTT and 1,500 ul SQG White Cell Lysis Buffer. Mix the solution to resuspend all cells. Incubate in a 55^oC water bath for 60 min.

2. Add 200 ul 10% SDS and 500 ul PPR. Shake vigorously for 30 seconds. Incubate in a 55^oC water bath for 15 minutes.

3. Centrifuge @ 3000 rpm for 20 min. Transfer the supernatant to a new polyprolene tube, 15 ml. Add 2 volumes of absolute ethanol. Mix by gentle inversion 5 minutes at room temperature.

4. Centrifuge @ 3000 rpm for 15 min. Decant ethanol, invert tube and air dry 15 minutes at 42^oC. Add 25 ul increments of TE buffer to dissolve the DNA pellet.

If NO VISIBLE DNA IS NOTED IN STEP 4 ABOVE:

1. Using two(2) Amicon Centricon 100's, place 1.0 ml of TE buffer in the tube then add 1.0 ml of sample directly from the 15 ml conical tube being careful not to suck any of the salt pellet from the bottom.

2. Spin for 20 minutes at 3500 RPM (savant).

3. Repeat steps 3 and 4 using a 1:1 ratio until the sample has all been centriconed.

4. Put 1.0 ml of TE buffer into the centricon as a wash, centricon 20 minutes at 3500 RPM. Repeat this two(2) more times until a total of three(3) washes have been accomplished.

5. Flip over the filter, throwing away the "catch" tube and centricon 20 minutes at 3500 RPM into the sample tube.

6. Pool the samples, making note of total volume, into a 1.5 ml Eppen dorf tube.

Note: Do Not heat the sample in a heat block, this is unnecessary and the sample could evaporate. Run sample on a yield gel (see Yield Gel procedures).

References

Cytogenetics Technical Manual, Association of Cytogenetic Techonologist, 1982 Preprint. Section Two 2.91.5 - Enzymatic Dispersal.

SUPER QUIK-GENE, DNA Isolation Kit, Product Information, Analytical Genetic Testing Center, Denver.

Lab Director Supervisor

Reviewed by:________________________ ________________________

Date:____/____/____ ____/____/____

Thursday, October 2 (Day 4)

8:00 a.m. Single strand confirmation polymorphism analysis
and its use in mutation detection in FH Lec MR NMS Dr.A. Stavljenic-Rukavina,
8:45 a.m. PCR-SSCP method for the detection of point mutation in
the LDLR gene Lab MR NMS R. Zrinski, MS
9:30 a.m. Detection of BCR-ABL transcript by RT-PCR transcript Lab L 3 NMS Dr.R. Zadro
10:15 a.m. Coffee break
10:30 p.m. Genomyx LR DNA Sequencing System Lab L 2 NMS Dr. D.Burgi
11:45 a.m. Genetic research and clinical testing and quality
assurance and informed consent: International
Science and human rights perspectives. Lec MR NMS Dr. Gale Gardiner
12:30 p.m. Morning section adjourns
1:30 p.m. History of human genetics in Croatia Lec MR NMS Dr. Lj.Zergolern-Cupak
2:15 p.m. PCR genotyping of the two common a1-antitrypsin
deficiency alleles Pi Z and Pi S Lab L 3 NMS I.Zuntar, M.Stefanovic
3:30 p.m. Adjurn
6:00 p.m. Farewell Party

PROTOCOL OF PCR-SSCP METHOD FOR THE DETECTION OF POINT MUTATIONS IN THE LDLR GENE.

[Dragan Primorac]

Mutations in the low-density lipoprotein receptor (LDLR) gene cause the autosomal dominant inherited disorder familial hypercholesterolemia. More than 200 mutations in the LDLR gene have been characterized at the molecular level. The majority of mutations are point mutations and minor deletions and insertions. PCR-SSCP is one of the several methods that can be used to screen DNA fragments for small sequence changes. This technique involves denaturing double-stranded DNA into single stranded DNA, followed by electrophoresis on non-denaturing polyacrylamide gel. Sequence differences may alter the secondary structure, resulting in detectable mobility shift during gel electrophoresis. Here we described the PCR-SSCP protocol for the analysis of exon 4b of the LDLR gene.

Procedure:

Sample preparation:
Prepare genomic DNA from peripheral blood leukocytes using any of the standard methods.

PCR: 1. Prepare master mix using Gene Amp PCR Reagent Kit (Perkin Elmer):

Sterile H2O	72,75 ml
PCR buffer	10ml
DNTP mix(1,25mM of each dNTP)	10ml
Primer 1	2,5ml
Primer 2	2,5ml
AmplyTaq polymerase	0,25ml

2. Mix 98 ml master mix with 2 ml DNA, add 2 drops of mineral oil.

3. Perform amplification according to the program:

	94oC	57oC	72oC	30oC
1 cycle	2 min	1 min	1 min	-
30 cycles	1 min	1 min	1 min	-
1 cycle	-	-	1 min	45 s

4. Store PCR products at +4oC.

Gel preparation: 1. Treat the glass plate with bind-silane and treat the thermostatic plate with gel slick.

2. Prepare gel solution:

MDE gel	12,5 ml
Dest. H2O to	35 ml
10XTBE buffer	3 ml
dest . H2O	50 ml
TEMED	20ml
10% ammonium persulphate	200ml

3.Pour the gel solution and polymerize for 1,5-2 hours.

SSCP elektrophoresis:

1. Fix the gel in the Macrophor system (Pharmacia, Biotech), rinse the sample wells with buffer and prerun electrophoresis according to the following instructions.

3 W 7oC
700 V
5 mA
1 hour

2. During this time prepare the sample: 2ml stop mix solution 6ml PCR product and denature double-stranded DNA at 94oC for 6 minutes.

3.Load the samples and run the electrophoresis for 17 hours according to the previously mentioned instructions.

Silver staining: 1. Place the glass plate with the gel still attached in an exposure box on the shaker.

2. Change solutions according to the following protocol:

40% methanol	30 minutes
10% ethanol	15 minutes
10% ethanol	15 minutes
oxidizer	3 minutes
deionized H2O	10 minutes
deionized H2O	10 minutes
deionized H2O	10 minutes
10% silver reagent	15 minutes
deionized H2O	1 minute
developer	7-8 minutes
5%acetic acid	1 minute
deionized H2O	1 minute

3. Air dry the gel

References:

1. H.K. Jensen et al.: High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. Clin Chem 1996; 42:1140-6.
2. J. Geisel et al.: Screening for mutations in exon 4 of the LDLR receptor gene in German population with severe hypercholesterolemia.Hum.Genet 1995;96:301-4.
3. Macrophor system manual. Pharmacia Biotech 1994.
4. Silver stain manual. Bio-Rad.

DETECTION OF BCR-ABL TRANSCRIPT BY RT-PCR

CLINICAL HOSPITAL CENTRE ZAGREB
CLINICAL INSTITUTE OF LABORATORY DIAGNOSIS
ZAGREB UNIVERSITY SCHOOL OF MEDICINE
Ki?patiÊeva 12, 10000 Zagreb, Croatia
tel & fax +385-1-212-079
Head: Professor Ana StavljeniÊ-Rukavina, PhD
Dr Renata Zadro
Division of Hematology and Coagulation
Isolation of mRNA using QuickPrep Micro mRNA Purification Kit (1-3)

Preparation of reagents

Approximately 20-30 minutes before the sample will be ready for extraction, remove the kit from storage at +4 °C and place it at room temperature. Remove the Extraction Buffer and place it at 37 °C until all crystalline material is dissolved. Cool to room temperature.
Place 400 mL of Elution Buffer (per purification) at 65 °C until needed.

Extraction

Place an aliquot of suspension containing between one and 1x107 cells (25-50 mL aspirated bone marrow) in a microcentrifuge tube and add 400 mL of Extraction Buffer. Vortex until a homogenous suspension is achieved.
Dilute the sample by adding 800 mL of Elution Buffer and mix using a vortex mixer.

Preparation of Oligo(dT)-Cellulose

Gently swirl the Oligo(dT)-Cellulose slurry to obtain a uniform suspension.
Immediately pipette 1 mL of suspension in clean microcentrifuge tube.

Isolation of mRNA

Centrifuge both the sample and the tube containing the Oligo(dT)-Cellulose for 1 min at top speed (between 5000xg and 16000xg).
Remove the buffer from the Oligo(dT)-Cellulose pellet by aspiration or by using a 1 mL pipetting device.
Place 1 mL of the cleared cellular homogenate on top of the pellet of Oligo(dT)-Cellulose.
Close the tube and invert to resuspend the Oligo(dT)-Cellulose.
Gently mix for 3 minutes by inverting the tube manually.
Centrifuge at a maximum of 16000xg for 10 seconds.
Remove the supernatant by aspiration or pipetting.

Washing steps

Add 1 mL of High-Salt Buffer. Close the tube and resuspend the Oligo(dT)-Cellulose. Spin for 10 seconds. Remove the supernatant by aspiration or pipetting.
Repeat the wash using High-Salt Buffer four more times, exactly as described in the step above.
Add 1 mL of Low-Salt Buffer to the Oligo(dT)-Cellulose pellet, close the lid and resuspend the resin by inversion (tap the bottom of the tube, if necessary). Spin for 10 seconds and remove the supernatant.
Repeat the wash using Low-Salt Buffer one more time.

Resuspend the resin in 300 mL of Low-Salt Buffer and transfer the slurry to MicroSpin Column, placed in microcentrifuge tube.
Spin at full speed for 5 seconds.
Discard the effluent from the collection tube, replace the column in the tube and add 500 mL of Low-Salt Buffer. Avoid disturbing cellulose bed. Centrifuge for 5 seconds at full speed.
Repeat the step above additional two times, emptying the collection tube each step, if necessary.

Elution Step

Remove the column and place it in the sterile microcentrifuge tube.
Place this tube in the microcentrifuge and add 200 mL of prewarmed Elution Buffer to the top of the resin bed.
Centrifuge at top speed for 5 seconds. The eluat contains mRNA. Add a second 200 mL aliquot of warm Elution Buffer to the top of the resin bed and centrifuge again.
Remove the column and place the tube containing the eluted mRNA on ice. Add 10 mL of Glycogen Solution and 40 mL Potassium Acetate Solution to the 400 mL of sample. Add 1 mL of 95% ethanol (chilled to -20 °C) and place the sample at -20 °C for a minimum of 30 minutes.
Centrifuge the precipitated mRNA at +4 °C for 5 minutes. Store at -80°C under ethanol if not to be used immediately.
Decant the supernatant, invert the tube over the clean paper towel and redissolve the precipitated RNA in 20 mL of Elution Buffer.

Transcription of RNA to cDNA (Perkin Elmer)

master mix I
10 x PCR buffer II 2 mL
25 mM MgCl2 4 mL
dNTPs 8 mL
Program:

42 °C 15 min.
99 °C 5 min.
5 °C 5 min.
RNA-se inhibitor 1 mL
hexamers 1 mL
transkriptase 1 mL
Total 17 mL
+ 3 mL RNA

1. amplification

master mix II
10 X PCR buffer II 8 mL
Second-strand synthesis: Amplification:

94 °C 10 min 94 °C 45 s
55 °C 5 min 55 °C 45 s
72 °C 2 min 72 °C 1 min

Total of 1 cycle Total of 34 cycles
25 mM MgCl2 4 mL
primer BCR-A 1 mL
primer BCR-B 1 mL

Taq DNA polymerase	0.5mL
dd H2O	65.5 mL
Total	80 mL

Add under oil to the transcription reaction

2. amplification

master mix III (per sample)
Program:

94 °C 45 sek
55 °C 45 sek
72 °C 1min

Total of 34 cycles

10 X PCR buffer	10 mL
dNTPs	8 mL
Taq DNA polymerase	0.5mL
primer BCR-D	1 mL
primer BCR-E	1 mL
dd H2O	80 mL
Total	100 mL

2.5 mL 1.PCR product + 95 mL mm III

Amplified products are visualised after electrophoresis on 2-3% agarose gel with ethidium bromide.

Primers (4):

BCR-A: 5'- TGT GAT TAT AGC CTA AGA CCC GGA GCT TTT -3'
BCR-B: 5'- GAG CGT GCA GAG TGG AGG GAG AAC ATC CGG -3'
BCR-C: 5'- TTC AGC GGC CAG TAG CAT CTG ACT T -3'
BCR-D: 5'- GAC CCG GAG CTT TTC ACC TTT AGT T -3'
BCR-E: 5'- GAA GAA GTG TTT CAG AAG CTT CTC C - 3'

Literature:

1. Chirgwin, JM et al, Biochemistry 1979; 18:5294.
2. Pharmacia P-L Biochemicals, Analects 1989; 17.1
3. Pharmacia P-L Biochemicals, Analects 1988; 16.1
4. Hughes, TP et al, Blood 1991; 77:874.

PCR genotyping of the two common a1-antitrypsin deficiency allels PiZ and PiS

a1 - antitripsin is important protease inhibitor. The gene is located on chromosome 14. Some deficiency alleles are associated with low levels of a1 - antitripsin in serum and can either be linked to liver disease and/or to early onset emphysema. The most common deficiency allele are PiZ (single base substitution in exon 5) and PiS (single base substitution in exon 3). Here we described the PCR-RFLP protocol for the detection of Z and S mutation in a1 - antitripsin gene using mutated primers.

PROCEDURE:

1.Sample preparation
Prepare genomic DNA from peripheral blood leukocytes using any of the standard methods.

2. DNA amplification (PCR)
Prepare the amplicon in microtube following the next procedure:

Reagents	Volume	Final Concentration
1. Sterile H2O	Ad 100 ml	
2. PCR buffer, 10xconc.	10 ml	1 X
3. dNTP mix (10 mM of each dNTP)	2 ml	200 mM
4. primer 1* (P1)	0.3ml	0.3 mM
5. primer 2* (P2)	0.3ml	0.3 mM
6. template DNA	variable	0.3 mg
7. Taq DNA polymeraze	1.3 ml	1.3 U/100 ml

At the end add two drops of mineral oil.

For PiZ: P1 5' - ggctgtgctgaccatcgtc-3' and
P2 5' - aactcttctttaatgtcatcgagg - 3'

For PiS: P1 5' - aggggaaactacagcacctcg - 3' and
P2 5' - tgggtactgttctcctcatcgagcatg-3'

Perform amplification according to the program:

	No. of cycles		
	1X	38X	1X
Denaturation	95°C 5 min	95°C 45s	
Annealing		53°C 45s	
Extension		72°C 30s	72°C 5 min

Store the amplicon on +4°C.

Check the amplicon electrophoretically on 1.5% MP-agarose with ethidium bromide and phptograph by Polaroid DS-34 camera using a Polaroid 667 film.

3.RFLP

Cleave 15 mlof the amplicon with 10 U RE Taq I (Boehringer Mannheim;tc ga) at 65°C/1 hour (mineral oil has to be added).
Separate the restriction fragments electrophoretically on 4% Meta Phor agarose (BioProducts) and documented by taking Polaroid 667 photograph.

References:

1. Crystal RG. Alfa-1 - antitrypsin deficiency, emphysemia, and lever disease. Genetic bases and strategies of therapy. J Clin Invest 1990:85;1343-52.
2. Kurachi K, Chandra T, Degen SJF, White TT, Marchioro TL, Woo SLC, Davie EW. Cloning and sequence of cDNA coding for alfa - 1 - antitrypsin. Proc Nat Acad Sci USA 1981; 78:6826-30.
3. Braun A, Meyer P, Cleve H, Roscher AA. Rapid and simple diagnosis of the two common a1- proteinase inhibitor deficiency alleles PiZ and PiS by DNA analysis. Eur J Clin Chem Clin Biochem 1996;34:761-4.

TISSUE DNA EXTRACTION

SUPER QUIK GENE PROCEDURE

Reagents and Supplies

Petri dish
Scalpel
Polypropylene conical tubes (at least 3.5 ml size)
WBC Lysis buffer
10% SDS
Protein Precipitating Reagent
1.0 M DTT
1.5 ml microfuge tubes
Absolute ethanol
Tris buffer (or Tris-EDTA buffer)
Plastic inoculation loops
Mini-gel apparatus and power supply
Ethidium Bromide (10 mg/ml conc.)
Centricon 100 microconcentrator
UV Transilluminator
Centrifuges - microfuge and fixed angle rotor
PBS (0.01 M pH 7.3)

1. Remove approximately 100.0 mg of tissue material and place in a 1.5 ml microfuge tube. Wash sample in PBS. Remove PBS. Place washed tissue sample in a Petri dish and mince the sample as fine as possible using a sterile scalpel. Place minced sample in a polypropylene conical tube (at least 3.5 ml in size).

2. Add 1.5 ml of WBC lysis buffer to the cell pellet. Vortex vigorously for 30 seconds to enhance homongenization of tissue material. Add 20.0 ul of 1.0 M DTT to the sample and vortex briefly. Incubate for 1 hour in a 55°C water bath.

3. At the end of the incubation, add 0.2 ml 10% SDS and 0.5 ml Protein Precipitating Reagent. Vortex vigorously for 30 seconds. Incubate for 15 minutes in a 55°C water bath.

4. Centrifuge at 2500 rpm (or greater) for 20 minutes. Precipitated protein will appear as a crystalline pellet in the bottom of the tube.

5. Transfer the clear aqueous supernatant, containing the DNA, to a polypropylene conical tube (at least 3.5 ml in size). Add 2 volumes of room temperature absolute ethanol. Mix by gentle inversion until the DNA precipitates as white stringy fibers. It may be helpful to let the sample stand at room temperature for about 5 minutes to allow the DNA to fully precipitate.

NOTE: If no DNA precipitates, transfer the supernatant to a Centricon 100 microconcentrator and proceed as in step #3 of ProK/SDS digestion procedure for bloodstains.

6. Spool out precipitated DNA with a plastic inoculation loop and transfer to a 1.5 ml microfuge tube containing TE buffer (Tris-EDTA). Dissolving of the DNA may be facilitated by modest vortexing and should go back into solution quickly. The sample can be incubated at 37°C for 1 hour. If the DNA is still not fully dissolved, add additional TE buffer in increments of about 50 ul, gently vortex, and briefly incubate at 37°C.

WARNING: Avoid dissolving DNA for extended periods (> 2 hours) at temperatures exceeding 37°C, as these conditions may prove deleterious to high molecular weight DNA.

7. Run a yield gel on 1 ul of sample as per step #6 of ProK/SDS digest procedure.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells.
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CONFIDENTIAL DEVELOPMENTAL PRECEDURE

BUCCAL SWAB
SUPER QUICK GENE PROCEDURE

Kit Contents

160 ml 10X RBC Lysis Buffer
150 ml WBC Lysis Buffer
20 ml 10% SDS
50 ml Protein Precipitating Reagent

Storage Requirements

Reagents should be stored at room temperature. Note: For best results, diluted RBC Lysis buffer should be stored and used at 4°C.

Equipment and Supplies

1 M 2-Mercaptoethanol
1.5 ml microfuge tubes "Soft-Top"
Tris buffer (no EDTA added)
Absolute ethanol
Microcentrifuge (capable of 5000 - 10,000 rpm)

Procedure

1. Slice swab with sterile clean scalpel, and peel off outer layer of swab.

2. Put swab "Soft-Top" microfuge tube and add 400 µl of WBC lysis buffer and 40 ul of 1 M 2-mercaptoethanol, vortex vigorously, and incubate in a water bath at 55°C for 30 minutes.

2. Punch 3 holes in cap of tube; remove swab material and place in cap. Centrifuge at 10,000 RPM for 10 minutes.

3. Add 40 µl of 10% SDS, and 100 µl of Protein Precipitating Reagent, replace with a new cap, vortex vigorously for 30 seconds. Incubate at 55°C for 15 minutes.

4. Centrifuge sample for 5 minutes at 7500 - 10,000 rpm.

NOTE: After centrifugation, there should be a white pellet in the tube; if not vortex and centrifuge again.

5. Transfer the **supernatant** to a clean 1.5 ml tube, add two (2) volumes of absolute ethanol. Centrifuge at 10,000 RPM for 10 minutes.

6. Decant ethanol and air dry.

7. If there is a visible pellet add 100 µl of Tris buffer to dried pellet. If no pellet is visible, add 25 µl of Tris.

8. Quantitate using PE Quantiblot

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells.
Nucleic Acids Research Vol. 16, No. 3, (1988). U.S. Patent Application Number 07/154024.

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Reviewed by:	____________	____________
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MODIFIED DIFFERENTIAL EXTRACTION METHOD FOR SPERM DIGESTION USING SUPER QUIK GENE

1. Conduct steps 1 through 5 in ProK/SDS differential extraction procedure.

2. Wash pellet 3X with sterile PBS to exchange Protein Precipitating Reagent and detergents.

3. Remove 1 ul of pellet and prepare a smear. Perform Christmas Tree stain on smear and conduct microscopic exam to verify the presence of sperm and digestion of epithelial cells.

4. Add 20 ul of neat 2-mercaptoethanol, vortex gently and incubate at room temperature for 2 minutes.

5. Add 750 ul of WBC lysis buffer, vortex gently, and incubate in a water bath at 55°C for 30 minutes. NOTE: For direct digest of substrate material (i.e., E3 fraction) add 750 ul of WBC lysis buffer and 20 ul of 1.0 M DTT and incubate in a water bath at 55°C for 30 minutes.

6. Add 100 ul of 10% SDS, and 250 ul of Protein Precipitating Reagent, vortex vigorously for 30 seconds. Incubate at 55°C for 15 minutes.

NOTE: For direct digest samples, prior to addition of reagents in step #6, punch 3 holes in cap, remove substrate material, place in cap and centrifuge for 5 to 10 minutes as 7500 to 10,000 rpm. Then add reagents in step #6, place new cap on tube and proceed.

7. Centrifuge sample for 10 minutes at 7500 - 10,000 rpm.

NOTE: After centrifugation, there should be a white pellet in the tube; if not vortex and centrifuge again.

8. Transfer the supernatant to a Centricon 100 microconcentrator. Add Tris buffer (no EDTA) to reservoir. Conduct microconcentration of sample as per ProK/SDS procedure (see differential extraction for sperm digestion).

Optional: The pellet can be examined microscopically to verify that all cellular components have been digested. If sperm are still present, the procedure may be repeated.

BLOODSTAINS

SUPER QUICK GENE PROCEDURE

Kit Contents

160 ml 10X RBC Lysis Buffer
150 ml WBC Lysis Buffer
20 ml 10% SDS
50 ml Protein Precipitating Reagent

Storage Requirements

Reagents should be stored at room temperature. Note: For best results, diluted RBC Lysis buffer should be stored and used at 4°C.

Equipment and Supplies

3.5 ml polypropylene conical tubes
1.5 ml microfuge tubes
TE buffer (Tris-EDTA buffer)
Tris buffer (no EDTA added)
Plastic inoculation loops
Absolute ethanol
Centricon 100 microconcentrators
Centrifuge (fixed angle rotor capable of 3500 rpm)
Parafilm
Microcentrifuge (capable of 5000 - 10,000 rpm)

Procedure

1. Cut a 1.0 cm^2 (or up to 2.0 cm^2) portion of a a bloodstain into quarters and place in a polypropylene conical tube.

2. Add sufficient 1X RBC lysis buffer to saturate stain material (approx. 2-4 ml).

3. Incubate overnight at room temperature with agitation.

4. After overnight incubation, centrifuge at 7500 rpm for 10 minutes. Leave stain substrate in tube during centrifugation.

5. Decant supernatant.

6. Add 750 ul of WBC lysis buffer to tube. Briefly vortex to resuspend pellet and stain substrate insuring that both are saturated with the buffer.

7. Incubate in a water bath at 55° for 30 minutes.

8. Punch 3 holes in cap and transfer stain substrate to cap. Parafilm top of cap and centrifuge at 7500 - 10,000 rpm for 5 to 10 minutes.

9. Add 100 ul of 10% SDS and 250 ul of Protein Precipitating Reagent. Place new cap on tube and vortex vigorously for 30 seconds.

10. Incubate in a water bath at 55°C for 30 minutes.

11. Centrifuge tube for 10 minutes at 7500 rpm.

12. Remove supernatant and place in a Centricon 100 microconcentrator. Add Tris buffer (no EDTA) to reservoir. Conduct microconcentration of sample as per ProK/SDS procedure (see bloodstain procedure).

13. Optional Method (no microconcentration)

a. Decant supernatant into a new 3.5 ml conical tube.

b. Add 2 volumes of ethanol to supernatant and rock gently for 5 minutes at room temperature.

c. Centrifuge sample at 10,000 rpm for 10 minutes.

d. Decant ethanol and air dry the tube inverted in a 42°C oven for 15 minutes.

e. Add 25 ul of Tris-EDTA (or Tris buffer), cap the tube (or parafilm) and dissolve at 37°C for 1 hour.

f. Briefly pulse spin tube, remove and quantitate volume, place in Click Seal tube.

References

Miller, S A, Dykes, D D, and Polesky, H F. A Simple Salting Out Procedure for Extracting DNA from Human Nucleated Cells.
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Lab Director Supervisor

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