Y-Chromosome and Mitochondrial DNA Analysis

mitochondrial DNA

Goals and Objectives

- Overview and theory behind mtDNA analysis
- The science behind mtDNA sequencing.
- Forensic casework applications of mtDNA.
- Tools for mtDNA screening – Linear Arrays.
- Emerging mtDNA technologies – mtDNA genome sequencing for increased discrimination, mtDNA micro-chip technology.
- Summary and Questions

June 26, 2000

“A day for the ages”

Associated Press
You Say Tomato…

- Cowdry (1918) review of what microscopists called “mitochondria”

<table>
<thead>
<tr>
<th>Term</th>
<th>Synonym</th>
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<tbody>
<tr>
<td>Blepharoplasts</td>
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<td>Chondriokonts</td>
<td>Mitogel</td>
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<td>Chondriomites</td>
<td>Parabasal bodies</td>
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<td>Chondrioplasts</td>
<td>Plasmabioblasts</td>
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<td>Chondriosomes</td>
<td>Plastochondria</td>
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<td>Vermicules</td>
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<td>Fuchsinophilic</td>
<td>Sarcosomes</td>
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<td>Granules</td>
<td>Interstitial bodies</td>
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<td>Komera</td>
<td>Bioblasts</td>
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Mitochondrial Morphology

- Cytoplasmic organelle
- Double membrane
- Outer membrane – porin proteins for the transportation of materials.
- Inner membrane – highly folded (increased surface area) and highly impermeable.
- Inner Matrix – several copies of mtDNA

Mitochondrial Functions

- Cellular Respiration – ATP production via oxidative-phosphorylation (OX-PHOS).
- Apoptosis – programmed cell death
- Steroid synthesis
- Elongation of fatty acids
- Oxidation of epinephrine (adrenaline)
- Degradation of tryptophan
- Heme synthesis
- Heat production
Mitochondrial Evolution

• Endosymbiotic Theory – Ivan Wallin (1920s) and Lynn Margulis (1981).

• Proto-Eukaryotic cell incorporated a prokaryotic cell and formed a symbiotic relationship.
Support for the Endosymbiotic Theory

- Mitochondria have double membranes – and the inner membrane is rich in cardiolipin.
- Mitochondria have their own genome, which is circular like bacteria (no histones), and use a genetic code for amino acids different that the nuclear DNA.
- New mitochondria are formed by a process similar to binary fission.
- Mitochondrial ribosomes are very similar to bacterial ribosomes (affected by antibiotics such as linezolid).

Lucky Guess or Clairvoyant?

- 1890 – R. Altman writes that “bioplasts” (mitochondria) are, “autonomous, elemental living units, forming bacteria-like colonies in the cytoplasm of the host cell.”

Immo Scheffler, Mitochondria (1999)

Mitochondrial Evolution

<table>
<thead>
<tr>
<th>Complex</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
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<tr>
<td></td>
<td>NADH-CuQ</td>
<td>Succinate-CuQ</td>
<td>FAD-Cytochrome C</td>
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<td>ATP Synthase</td>
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<td>Inhibitor</td>
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<td>Amipril</td>
<td>Antimycin A</td>
<td>Carbon Monoxide</td>
<td>Azide</td>
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<td>Nuclear DNA Subunits</td>
<td>~42</td>
<td>4</td>
<td>10</td>
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<td>~16</td>
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<tr>
<td>mtDNA Subunits</td>
<td>ND6, ND4L</td>
<td>COX 1, 2, 3</td>
<td>COX 1, 2, 3</td>
<td>ATPase 6, 8</td>
<td>81</td>
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</table>

~81 subunits encoded by the nuclear genome
**mtDNA Genome**

- Coding Region
  - 13 polypeptides
  - 2 rRNAs
  - 13 tRNAs
  - All necessary for OXPHOS
  - Highly compact (few intergenic spaces)

**Different Inheritance Patterns**

- **Autosomal** (passed on in part, from all ancestors)
- **Y-Chromosome** (passed on complete, but only by sons)
- **Mitochondrial** (passed on complete, but only by daughters)

**Location and Copy Number of mtDNA**

- Found within the mitochondria in the cellular cytoplasm.
- On average 4-5 copies of mtDNA molecules per mitochondria (range of 1-15 mtDNA copies).
- Number of mitochondria vary by cell type (e.g., muscles have more...).
- Generally, hundreds of mitochondria per cell.

http://www.cstl.nist.gov/biotech/strbase/training.htm
mtDNA Is Not Always 16,569 bp …

- Dinucleotide repeat at positions 514-524 (near end of control region)
  - Usually ACACACACAC or (AC)₅ in most individuals
  - Can vary from (AC)₃ to (AC)⁷

- Other insertions and deletions may occur
  - 9 bp deletion (positions 8277 to 8285) in some individuals from Asia and Pacific Islands (haplogroup B) and Africans (haplogroup L).

Control Region (16024-576)

<table>
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<th>1,122 nucleotide positions</th>
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<tr>
<td>16024</td>
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<tr>
<td>HV1</td>
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</table>

Forensic Focus

Typically only 610 bases examined
- (HV1: 16024-16365; HVII: 73-340)

http://www.cstl.nist.gov/biotech/strbase/training.htm
“Heavy” vs. “Light” Strand

- The two strands (“inner” and “outer” loops) of mtDNA can be separated with an alkaline CsCl gradient.
- Heavy or H-strand contains a greater number of guanine nucleotides (largest molecular weight of the four nucleotides) – purine rich.
- Light or L-strand contains more C and T nucleotides and is thus physically lighter (pyrimidine rich).
- H-strand codes for 28 gene products while the L-strand is used to transcribe 8 tRNAs and the ND6 protein product.

Original Reference Sequence

- Human mtDNA was first sequenced in 1981 in Frederick Sanger’s lab located in Cambridge, England.
- Authors for this paper (Nature 1981, 290:457-465) were listed in alphabetical order so Stan Anderson was the first author.
- This sequence has come to be referred to as the “Anderson” sequence (GenBank accession: M63933).
- This first sequence is sometimes called the Cambridge Reference Sequence (CRS).

Re-Sequencing of CRS

- The 1981 sequence was derived primarily from a placenta of an individual with European ancestry; however, some HeLa and bovine sequence was used to fill in gaps due to early sequencing procedures performed.
- Re-analysis of original placental material by Andrews et al. (1999) found 11 nucleotides that differed from Anderson et al. (1981) sequence.
- This revised Cambridge Reference Sequence (rCRS) is now the accepted standard for comparison.
Evaluation of Sequence Differences
Between CRS (Anderson et al. 1981) and rCRS (Andrews et al. 1999)

<table>
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<th>Nucleotide Position</th>
<th>Region of mtDNA</th>
<th>Original CRS</th>
<th>Revised CRS</th>
<th>Remarks</th>
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<td>C</td>
<td>T</td>
<td>TATCTAC</td>
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</table>


Further Comparison of CRS and rCRS

- No differences seen between CRS and rCRS within the mtDNA control region.
- The original CRS contained a “CC” at positions 3106-3107 but rCRS was found to possess only a single “C”

```
TATCTACCTT Original CRS
TATCTAC - TT Revised CRS
```

- Thus, rCRS is only 16,568 bp!

Maternal Inheritance of mtDNA

- Fertilizing sperm contributes only nuclear DNA.
- Cellular components including the mitochondria in the cytoplasm come from the mother’s ovum.
- Any sperm mitochondria that may enter a fertilized egg are selectively destroyed due to a ubiquitin tag added during spermatogenesis.
- Barring mutation, a mother passes her mtDNA type on to her children.

http://www.cstl.nist.gov/biotech/strbase/training.htm
Maternal Inheritance of mtDNA

Note that mtDNA is not unique to an individual

Summary – mtDNA Characteristics

• High copy number of mtDNA.
• Maternal inheritance of mtDNA.
• Lack of recombination.
• High mutation rate compared to single copy nucDNA.

Methods for Measuring mtDNA Variation

• Low-resolution RFLP (1980s)
• High-resolution RFLP (1990s)
• Sequence analysis of HV1 and HV2 within control region (1991-present)
• Sequence analysis of complete mtDNA genome (2000-present)

http://www.cstl.nist.gov/biotech/strbase/training.htm
Mitochondrial DNA Sequencing in Forensic Casework

Issues and Examples

Role of mtDNA Compared to Autosomal STRs

- **Autosomal STRs provide a higher power of discrimination and are the preferred method whenever possible**

- **Due to high copy number**, mitochondrial DNA (mtDNA) may be the only source of surviving DNA in highly degraded specimens or low quantity samples such as hair shafts

- A mtDNA result is better than no result at all...

Comparison of Human nucDNA and mtDNA

<table>
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<th>Characteristics</th>
<th>Nuclear DNA (nucDNA)</th>
<th>Mitochondrial DNA (mtDNA)</th>
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<td>Size of genome</td>
<td>&gt;3 billion bp</td>
<td>~16,569 bp</td>
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<td>Cross-talk</td>
<td>1 hit allele per each</td>
<td>~1 hit allele per locus</td>
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<td>Presence of total DNA content per cell</td>
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<td>Structure</td>
<td>Linear, packaged in chromosomes</td>
<td>Circular, circular</td>
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<tr>
<td>Mismatch repair</td>
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<td>No</td>
</tr>
<tr>
<td>Unique</td>
<td>Unique to individual</td>
<td>Unique to individual (except identical family)</td>
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<tr>
<td>Mitochondrial genome size</td>
<td>All 5,000 base pairs</td>
<td>16,569 base pairs</td>
</tr>
<tr>
<td>Reference sequence</td>
<td>Derived from HUMAN mitochondrial DNA</td>
<td>Derived from HUMAN mitochondrial DNA</td>
</tr>
</tbody>
</table>


http://www.cstl.nist.gov/biotech/strbase/training.htm
Candidates for mtDNA Testing

- Shed hairs lacking root bulb or attached tissue
- Fragments of hair shafts.
- Aged bones or teeth that have been subjected to long periods of exposure.
- Crime scene stains or swabs that were unsuccessful for nuclear DNA testing.
- Tissues (muscle, organ, skin) that were unsuccessful for nuclear DNA testing.

mtDNA Testing on Hairs

- Human hair shafts contain very little DNA but because mtDNA is in higher copy number it can often be recovered and successfully analyzed
- Melanin found in hair is a PCR inhibitor

Important Publications:
  - Tissue grinding method described by FBI Lab
  - Obtained a full or partial mtDNA profile for >92% of hairs tested

The Mitotyping Experience

Terry Melton, Ph.D.; Gilmore, Daniel, M.S.; Ronnie Higgins, M.S.; Lynn Lindstrom, B.S.; and Kimberley Nelsen, Ph.D.

Forensic Mitochondrial DNA Analysis of 691 Casework Hairs

Mitochondrial DNA as a Means of Identification

When do you need it and why?

Why go to mtDNA?

- Disadvantages
  - mtDNA is not a positive form of identification (You have many maternal relatives!!)
  - Easily contaminated with modern DNA
Contamination

- Modern DNA can easily be introduced and overwhelm target DNA from the sample.
  - Due to the sensitivity of the reaction
    - Increased cycle number
    - Increased Taq
- Appropriate controls must be implemented to assure that the mtDNA sequence being reported is authentic.
- Laboratories need to be designed to lessen the chances of contamination.

Why go to mtDNA?

- Disadvantages
  - mtDNA is not a positive form of identification (You have many maternal relatives!!)
  - Easily contaminated with modern DNA
  - Time-consuming and costly

Nuclear DNA Analysis

- Sample Collection
- Laboratory
- 24-36 hours
- ~$100 per sample
- Use commercially available kits for processing
- Profile generation
MtDNA Analysis

Sample Collection → Laboratory

- 1-6 weeks post-submission to the laboratory
- ~$1,000 per sample
- Custom designed primers

Why go to mtDNA?

- Advantages
  - Maternally inherited
    - The pool of potential references is greatly increased.

Limited references available for nDNA
Maternal inheritance

Why go to mtDNA?

- Advantages
  - Maternally inherited
    - The pool of potential references is greatly increased.
  - Numerous copies of the mitochondrial DNA genome in each cell.
  - Small genome size and multiple copies increase chances of recovering DNA from degraded samples.
Laboratory Design

Organizing Your Space, People, and Samples

• All laboratories should be designed to be separated by use.
• At AFDIL, pre-PCR labs are physically separated from post-PCR by magnetically sealed doors and airlocks

Pre- and post-PCR Separation

• Separation of pre- and post-PCR areas prevents contamination.
  – Amplified product needs to be kept away from low quantity DNA areas.
  – Personnel flow from pre- and post-PCR areas needs to be controlled.
Pre- and post-PCR Separation

Pre-PCR
- Extractions & Amplification set-ups
- Amplification Reactions
- Amplified Product

Post-PCR
- Thermalcyclers & Sequencing

Pre-PCR Organization
- To further minimize contamination:
  - Pre-PCR lab space can be dedicated to teams of individuals.

http://www.cstl.nist.gov/biotech/strbase/training.htm
Staff Organization

• Now that the spaces are separated, what do you do with the people?
  – Staff organization can not only reduce contamination but increase efficiency.

Division of Labor

Evaluation of the Number of Sequencing Reactions Per Month in a Six Month Time Period
Sample and Contamination Tracking

- With this type of sample volume comes the additional issues of tracking your samples and contamination.
  - Even if the lab is fairly small, chain of custody issues and overall processing need to be tracked efficiently.
  - Contamination needs to be tracked, found and eradicated before it becomes an issue.

LIMS System

- An automated computer system is the most efficient method for accomplishing these goals.
- Many laboratory information management systems are available commercially.
- The name of our system is Laboratory Information Systems Application or LISA.

Case Accessioning

- Names and identifies each piece of evidence that is received.
- Assigns a sequential case number.
- Controls who has access to which samples based on set of ‘privileges’.
- Tracks Chain of Custody.
- Every step requires a password even once you are in the system.
Sample Storage

- Samples need to be stored at the appropriate temperatures.
  - Heat or large temperature fluctuations can cause further degradation of the DNA.
  - Bone material can be stored at ≤-20°C
  - Blood should be dried and stored at -20°C

Lab Processing

- Requires passwords throughout.
- Links all the forms and protocols used at AFDIL together.
- Procedures predicated on the completion of a step are not allowed until that step is finished.
- Designed to be compatible with sequencing equipment.

Contamination Tracking

- Contamination is a huge challenge in 'ancient' DNA laboratories.
- Must be able to guarantee that the sequence being generated is authentic and not modern.

How to control for contamination?
- Laboratory design
- Staff databases
- Contamination tracking via LISA
- Amplification controls
Control Databases

- All members of the staff, laboratory and administrative, at AFDIL have been profiled for both mitochondrial and nuclear DNA.
- The case management module of LISA has a separate database specifically for the sequences generated for contaminants.

Contamination Tracking

- Contamination can be tracked through the processing steps.
- LISA has a separate database specifically for the sequences generated for contaminants.
- Reports can be generated in LISA per primer pair and scientist to pinpoint a specific issue.

Degraded Skeletal Remains

What to choose and how to generate a full mtDNA profile.
Degraded Skeletal Remains

- Sample Selection
- Extraction Methods
- Amplification Strategies
- Sequencing Strategies

Degraded Specimens

- In general terms all skeletal remains are degraded.
- Some are more degraded than others due to environmental stressors.
- Prudent sample selection will increase the rate of success.

Environment

- Recovery sites vary
  - Extreme conditions
    - Salt-water marshes
    - Glaciers
  - High/Low temperatures
  - Repeated freezing and thawing
  - High/Low pH
  - High water levels
  - Salt or brackish water

http://www.cstl.nist.gov/biotech/strbase/training.htm
Environment

- Remains may be
  - On the surface
  - Buried in soil or other substrates
  - Highly fragmented
  - Subjected to burning or high heat
  - Exposed to fuel or other chemicals
  - Disturbed or moved by humans or animals
  - Animal destruction (feeding)

Storage Effects

- Handling of Remains
- Temperature
- Humidity
- Storage Container

Sample Selection

- Unknown skeletal remains
  - Remains are examined and samples selected by anthropologists or medical examiners
Selecting samples for analysis

- What are the best skeletal elements to use for analysis?

Bones Submitted for Analysis

Skeletal Success Rate

http://www.cstl.nist.gov/biotech/strbase/training.htm
Bone Structure

- Bones with dense cortical structure tend to have a greater success rate.
  - Compact bone may inherently afford greater protection for its deeper layers.
  - Trabecular bone and elements composed of thin cortical bone have a greater surface area.
- Cranial fragments vary in success
  - Formed of a layer of trabecular bone sandwiched between two layers of cortical bone.
  - Temporal and occipital tend to have denser cortical bone.

Cranial Success

- Overall success rates:
  - 90 – 100%
  - 80 – 89.9%
  - 70 – 79.9%
  - 60 – 69.9%
  - 50 – 59.9%
  - 40 – 49.9%
  - 30 – 39.9%
  - 0 – 20%

Dentition

- Dental remains provide a particular challenge
  - The enamel gives a greater protection to the dentin from which the DNA is extracted.
  - Anecdotally shown to provide copious quantities of DNA from even medieval era remains.
  - Require a lot of handling.
Extraction Methods

- Cleaning the samples – how much is too much?
- What protocols give the greatest yield of DNA?
- What method is right for you?
- Trouble-shooting the extraction.

Cleaning the Sample

- The exterior of the bone fragment needs to be cleaned of any possible contaminants:
  - Dirt
  - Plant material
  - Extraneous DNA
  - Dried Tissue

Cleaning

- An easy way to clean the surface is using a sanding bit in a Dremel tool.
How far to clean?

- Everything on the surface needs to come off, along with the spongy bone.
- But, you’ll hit a point where there is no solid bone left.

Other Cleaning Methods

- Bleaching
  - Bones can be subjected to a bleach sonication to remove external contaminants.
  - A fresh water sonication should follow to get rid of the bleach or DNA can be lost.
- “DNA Off” or other DNA removal products

Too much?

- Aggressive cleaning can remove or otherwise damage available DNA.
Extraction Methods

- Numerous extraction methods available.
- Involve different methods of –
  - pulverizing the samples
  - removing the DNA from the samples
- Different starting quantities of bone can also be used.

Pulverization Methods

- Freezer Mill
  - Uses liquid nitrogen and a magnet to pulverize the bone into a very fine powder.
  - Disadvantage:
    - Requires storage and handling of liquid nitrogen.
    - Grinders and sample vials are reused – potential contamination.

Pulverization Method

- Waring Blender Cup
  - Also grinds bone to a relatively fine powder
  - Disadvantage: Cups are reused, so there is a possibility of contamination.
"Freeing" the DNA

- Samples may be subjected to a decalcification step.
  - Demineralizes the bone matrix.
- Other chemical/physical treatments are commercially available to more easily acquire the DNA.
  - Silica gel
  - Charge Switch™
  - DNA IQ™

Extraction of Skeletal Remains

- The powdered bone is extracted with
  - 20mg/ml Proteinase K and extraction buffer
  - Overnight at 56°C
- DNA is removed from the extraction buffer with
  - a series of washes with Phenol/Chloroform/Isoamyl alcohol
  - Purification of product with filters.

There’s DNA, now what?

- Quantification –
  - At AFDIL, we do not quantify prior to amplification.
  - Can quantify using a 1% Agarose gel and ethidium bromide.
  - CalDOJ has a quantitation method for both nuclear and mtDNA using qPCR.
  
Amplification

- A standard program for amplification is used for the 9700's.
- The basic program is modified based on the primer pair used (Gabriel, et al. 2001)
  - 10-minute soak at 96.0°C
  - Followed by 38 cycles of
    - 20s at 94.0°C
    - 20s at 56.0°C
    - 30s at 72.0°C
  - Final hold at 4°C

PCR Amplification of mtDNA

- Usually performed with 34-38 cycles
- Some protocols may go to 42 cycles for highly degraded specimens

Primer Design

- Origin
- Primer locations:
  - HV1:
    - F15831/R158410
  - HV2:
    - F1584359

http://www.cstl.nist.gov/biotech/strbase/training.htm
Primer Design

miniPrimer Sets for HV1/HV2

Matthew N. Gabriel, M.S.; Edwin F. Huffman, M.S.; John H. Roux, Ph.D.; Mitchell M. Holland, Ph.D.; and Thomas J. Parsons, Ph.D.

Improved MitDNA Sequence Analysis of Forensic Remains Using a "Mini-Primer Set" Amplification Strategy

JFS (2001)
miniPrimer Sets for HV1/HV2

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<th>Sample</th>
<th>Primer Set</th>
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<th>Mini-Primer Set</th>
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<td>Mini-Primer Set</td>
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<tr>
<td></td>
<td>Sample</td>
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<td></td>
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Process for Evaluation of mtDNA Samples

1. Shaft (Telogen)
2. DNA Extraction
3. PCR Amplification
4. PCR Clean-up
5. DNA Sequencing
   - EXO/SAP
   - Centricon

Science of DNA Sequencing

- Fred Sanger – developed the dideoxy method of sequencing in the 1970s… still used today.
Sanger Sequencing

DNA template  
5' 3' -TAAATGATCC-5'

Primer anneals

Extension produces a series of ddNTP terminated products each one base different in length.

Each ddNTP is labeled with a different color fluorescent dye.

Sequence is read by noting peak color in electropherogram (possessing single base resolution).


Primers Used for Control Region Amplification and Sequencing

13 primers – 11 for routine sequencing

Why the Redundancy?

- Homopolymeric stretches of Cytosines (C-stretches).

16189T

HV1 AAAACCCCCCTCCCCCATG
5 Cs 4 Cs

16189C

AAAACCCCCCCCCCATG
10 Cs

Strand slippage can create 11+ tandem Cs

http://www.cstl.nist.gov/biotech/strbase/training.htm
Challenges with Sequencing Beyond the Polymeric C-Stretches in HV1 and HV2

Primer strategies typically used with C-stretch containing samples

Use of internal primers

Double reactions from the same strand


HV2 C-Stretch

*The rCRS has 5 Cs in this region

http://www.cstl.nist.gov/biotech/strbase/training.htm
A word about SRMs...

- NIST mtDNA SRM 2392 (1999) – contains 2 apparently normal cell lines (CHR and GM09947a) and a cloned DNA from CHR for HV1.

\[ 16189 \text{T-C} + 16193 \text{.1 C} \]

HV1 C-stretch mixture of templates having 11 and 12 C's

The cloned DNA has only 11 C's – so sequencing can continue without falling apart!

NIST SRM 2392-I


- SRM 2392-I complements SRM 2392 and was based on a suggestion from the FBI that this DNA would be particularly useful to the forensic community.

Process for Evaluation of mtDNA Samples

- Shaft (Telogen) DNA Extraction
- PCR Amplification
- PCR Clean-up
- DNA Sequencing
- Interpreting and Reporting of Results

http://www.cstl.nist.gov/biotech/strbase/training.htm 36