



University of  
**Strathclyde**

# **A Single Step Multiplex PCR to Identify Mammalian Species in the United Kingdom**

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# Need for Species Identification

- Non-human biological evidence encountered:
  - Abuse (both against people and animals)
  - Poaching
  - Components in food
  - Linking suspects to crimes
  - As components in TCM
  - Other applications

# Current Methods

- Gross
  - Microscopy
  - Osteology
  
- Trace
  - Antigen-antibody
  - DNA analysis
    - Sequencing

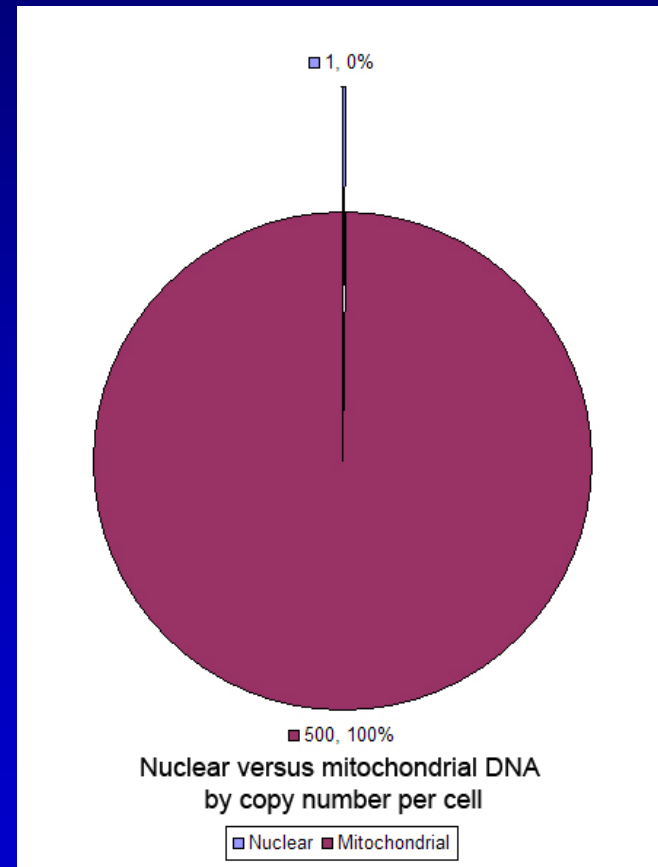
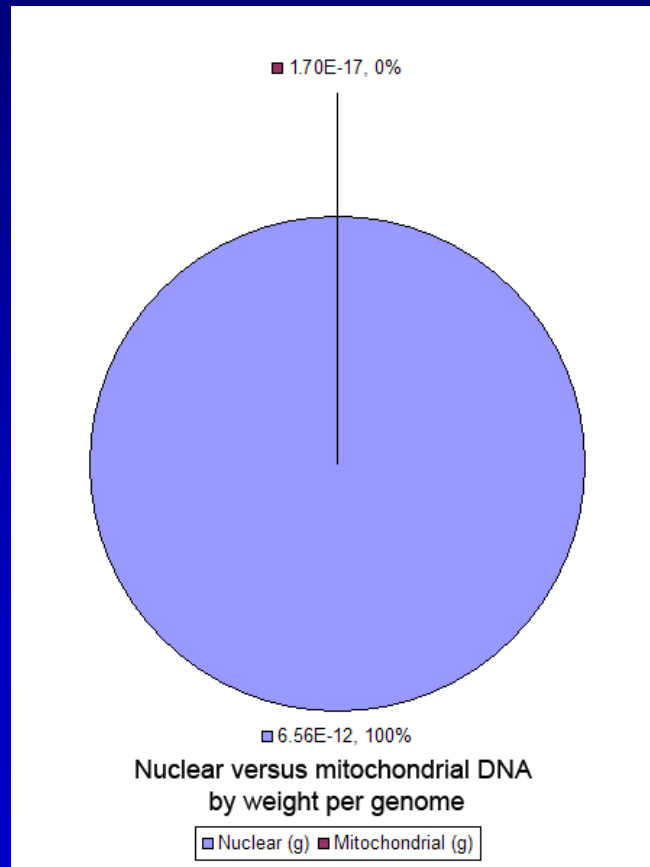
# Problems With Sequencing

- Costly and labour intensive
- Mixtures cannot be separated reliably
- Degraded samples may not yield enough sequence data for identification

# Requirements For Identification

- Any test must work
  - on poor or powdered remains
  - on samples that have been subjected to environmental insult
  - on mixtures
  
- The test must also be cost and labour effective

# Nuclear vs. Mitochondrial DNA



# Other Advantages of mtDNA

- Most of the mtDNA is encoding
  - 13 genes for proteins
  - 24 RNA molecules encoded
- The order of genes on the mitochondrial DNA is very similar for most species
- Order and structure of the vertebrate mtDNA used in taxonomic studies

# Locus Qualities Required For Species Identification

- Locus must show inter species variation
- Locus must also show little intra species variation



# Choosing a Locus

- Several genes show inter species variation but little intra species variation
- Cytochrome b is one such gene
  - commonly used for taxonomy
- It is situated near to the D-loop and encodes a 380 amino acid protein and is ~1,140 bp in size

# Cytochrome *b*

- The DNA sequence for many animal and plant species is known for the cytochrome b gene
- DNA Databases exist
  - EMBL DNA Database ([www.ebi.ac.uk](http://www.ebi.ac.uk))
  - GenBank® ([www.ncbi.nih.gov](http://www.ncbi.nih.gov))
  - Currently there are over 32 million sequence records on these databases

# Taxonomy of Mammals

	Wolf	Dog	Fox	Cat	Human
Phylum	Chordata				
Class	Mammalia				
Order	Carnivora				Primate
Family	Canidae			Felidae	Hominid
Genus	<i>Canis</i>		<i>Vulpes</i>	<i>Felis</i>	<i>Homo</i>
Species	<i>lupus</i>	<i>familiaris</i>	<i>vulpes</i>	<i>catus</i>	<i>sapiens</i>

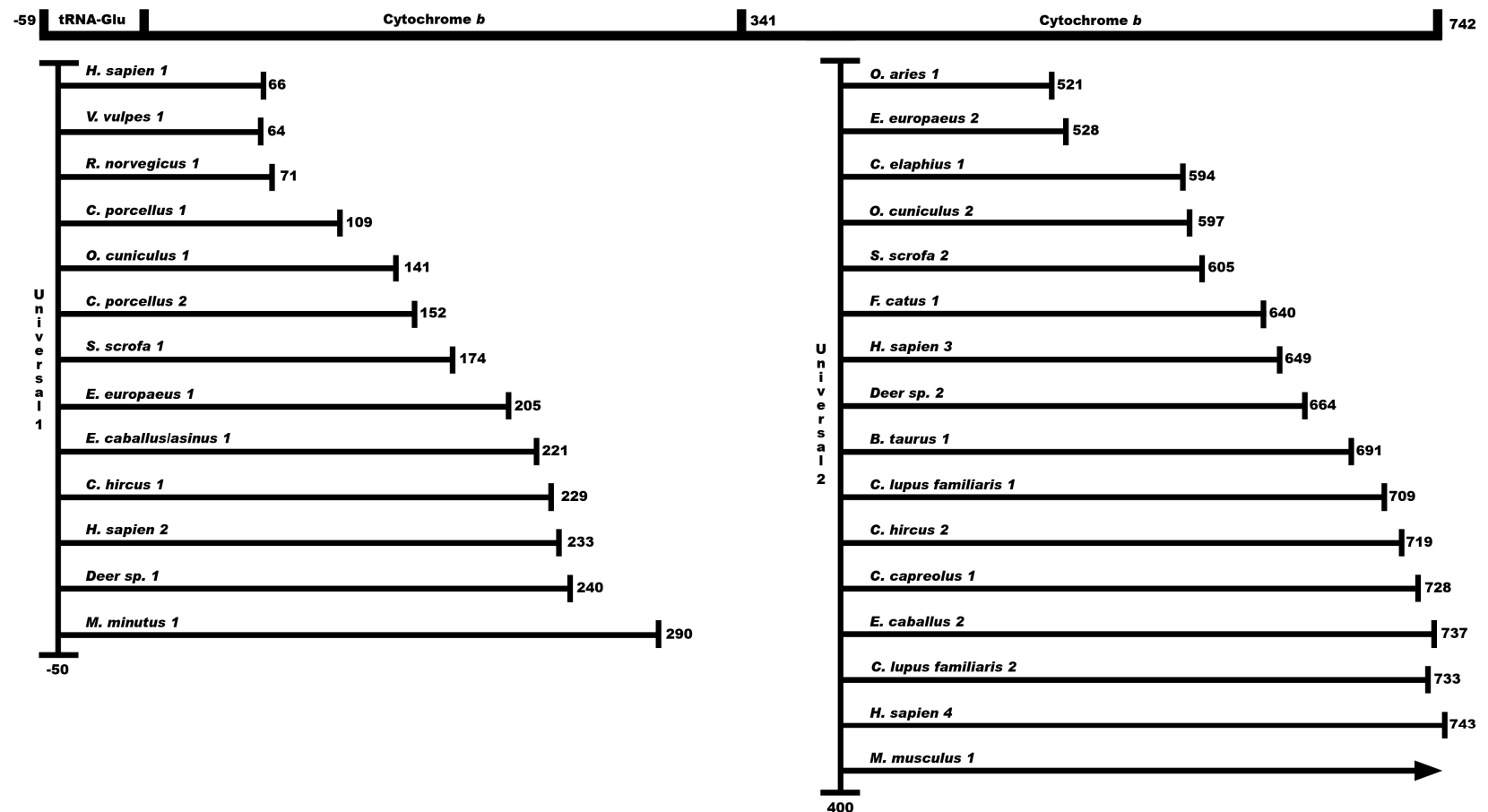
# Base Pair Differences At The Cytochrome *b* Gene

	Wolf	Dog	Fox	Cat	Human
Wolf		99.6%	84.0%	79.0%	74.5%
Dog	4		83.7%	78.9%	74.3%
Fox	182	186		78.6%	73.8%
Cat	239	240	244		76.6%
Human	291	293	299	267	

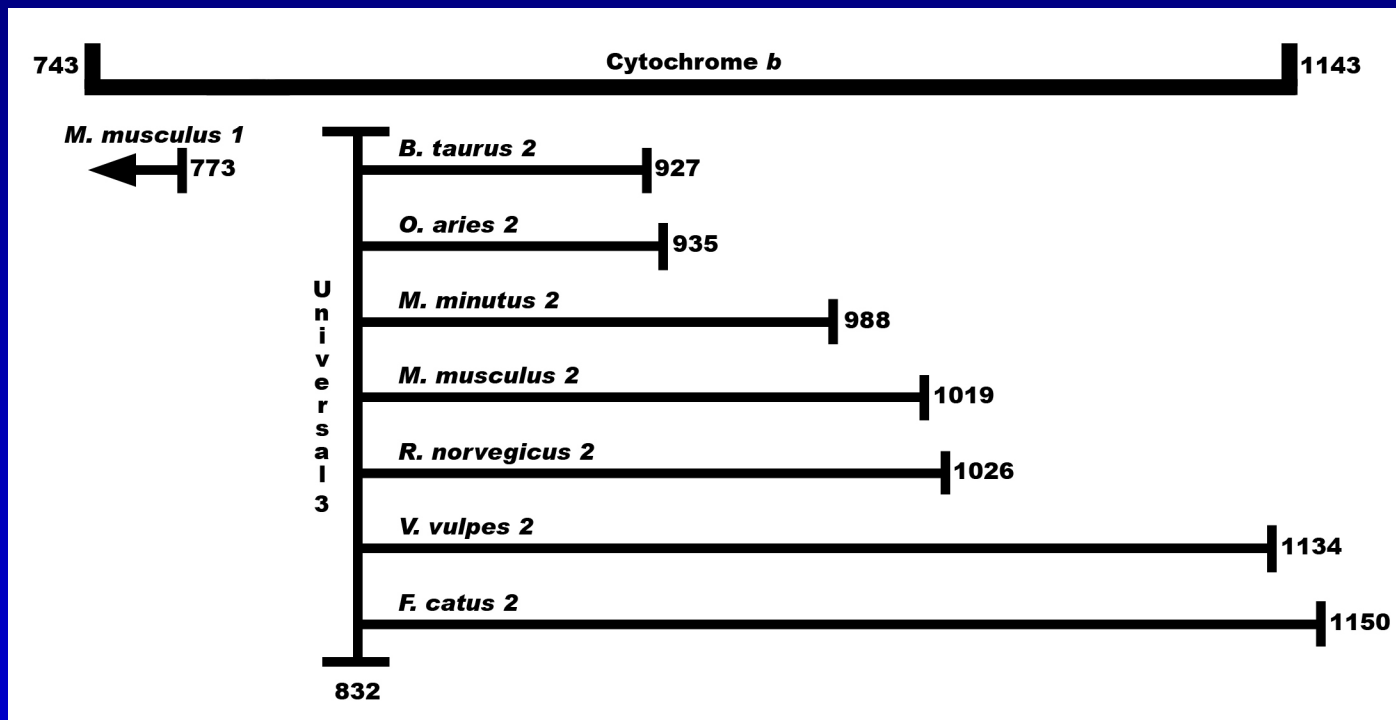
# Primer Design

<i>B. taurus</i>	TCACAGTAATAGCCACAGCATTTATAGGATACGTCTACCATGAGGACAAATATCATTCT	479
<i>H. grypus</i>	TCACCATCATAGCCACGGCATTTCATGGGCTACGTCTACCATGAGGACAAATATCATTCT	479
<i>S. scrofa</i>	TTACCGTTATAGCAACAGCCTTCATAGGCTACGTCTGCCCCTGAGGACAAATATCATTCT	479
<i>F. catus</i>	TTACAGTCATAGCCACAGCTTTTATGGGATACGTCTACCATGAGGCCAAATGTCCTTCT	478
<i>H. sapien</i>	TTGCAACTATAGCAACAGCCTTCATAGGCTATGTCCTCCCGTCAGGCCAAATATCATTCT	479
<b>Universal 2</b>		
<i>B. taurus</i>	GAGGAGCAACAGTCATCACCAACCTCTTATCAGCAATCCCATACATCGGCACAAATTTAG	539
<i>H. grypus</i>	GAGGGGCAACAGTCATTACCAATCTACTATCAGCAATCCCTATATCGGAACCGACCTTG	539
<i>S. scrofa</i>	GAGGAGCTACGGTCATCACAAATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCG	539
<i>F. catus</i>	GAGGAGCAACCGTAATCACTAACCTCCTGTGACCAATCCATACATCGGGACTGAACCTAG	538
<i>H. sapien</i>	GAGGGGCACAGTAATTACAAACTTACTATCCGCCATCCCATACATTGGGACAGACCTAG	539
<i>B. taurus</i>	TCCAATGAATCTGAGGCGGATTCTCAGTAGACAAAGCAACCCCTTACCCGATTCTTCGCTT	599
<i>H. grypus</i>	TACAATGAATCTGAGGAGGATTTTCAGTAGACAAAGCAACCCCTAACAGGATTCTTCGCCT	599
<i>S. scrofa</i>	TAGAATGAATCTGAGGGGGCTTTTCCGTGACAAAGCAACCCCTCACACGATTCTTCGCCT	599
<i>F. catus</i>	TAGAATGAATCTGAGGGGGCTTCTCAGTAGACAAAGCCACCCCTAACACGATTCTTTGGCT	598
<i>H. sapien</i>	TTCAATGAATCTGAGGAGGCTACTCAGTAGACAGTCCCACCCCTCACACGATTCTTTACCT	599
<b>H. grypus 1 - 187bp</b>		
<i>B. taurus</i>	TCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCC	659
<i>H. grypus</i>	TCCACTTCATCCTACCATTCCTAGTATTAGCACTAGCAGCAGTCCACCTACTATTCCTAC	659
<i>S. scrofa</i>	TCCACTTTATCCTGCCATTTCATCATTACCGCCCTCGCAGCCGTACATCTCCTATTCTCTG	659
<i>F. catus</i>	TCCACTTCATTCTTCCATTTCATTATCTCAGCCTTAGCAGGAGTACACCTCTTATTCCTTC	658
<i>H. sapien</i>	TTCACTTCATCTTACCCTTCATTATTGACGCCCTAGCAGCACTCCACCTCCTATTCTTGC	659
<b>H. sapien 3 - 246bp</b>		
<i>B. taurus</i>	ACGAAACAGGCTCCAAACAACCCCAACAGGAATTTCTCAGACGTAGACAAAATCCCATTCC	719
<i>H. grypus</i>	ACGAAACAGGATCAAAACAACCCCTCCGGAATCATACCCGACTCAGACAAAATCCCATTCC	719
<i>S. scrofa</i>	ACGAAACCGGATCCAAACAACCCCTACCGGAATCTCATCAGACATAGACAAAATTCATTTC	719
<i>F. catus</i>	ATGAAACAGGATCTAAACAACCCCTCAGGAATTACATCCGATTTCAGACAAAATCCCATTCC	718
<i>H. sapien</i>	ACGAAACCGGATCAAAACAACCCCTAGGAATCACCTCCCATTCCGATAAAATCACCTTCC	719
<i>B. taurus</i>	ACCCCTACTATACCATTAAGGACATCTTAAGGGGCCCTCTTACTAATTCTAGCTCTAATAC	779
<i>H. grypus</i>	ACCCGTACTATACAATTAAGGACATCCTAGGAGCCCTGCTTCTCATTCTAGTCCTGACAC	779
<i>S. scrofa</i>	ACCCATACCTACTATTAAAGACATTCCTAGGAGCCCTATTTTATAATACTAACTACTAA	779
<i>F. catus</i>	ACCCATACCTATACAATCAAGACATCCTAGGTCCTTCTAGTACTAGTTTAAACCTCATAC	778
<i>H. sapien</i>	ACCCTTACTACACAATCAAGACGCCCTCGGCTTACTTCTCTTCTTCTCTCTTAATGA	779

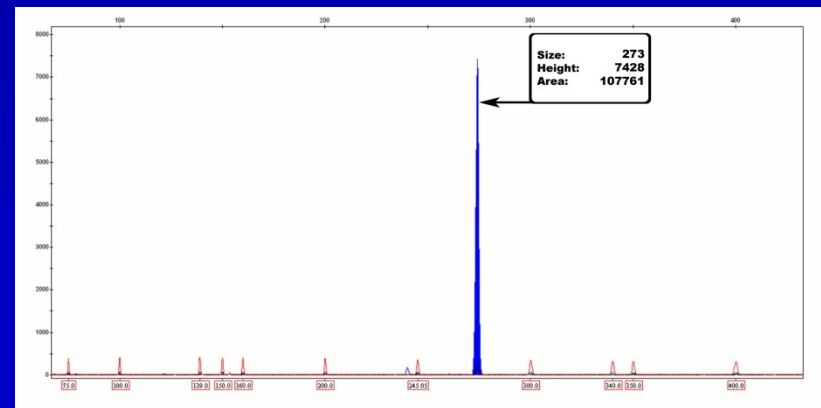
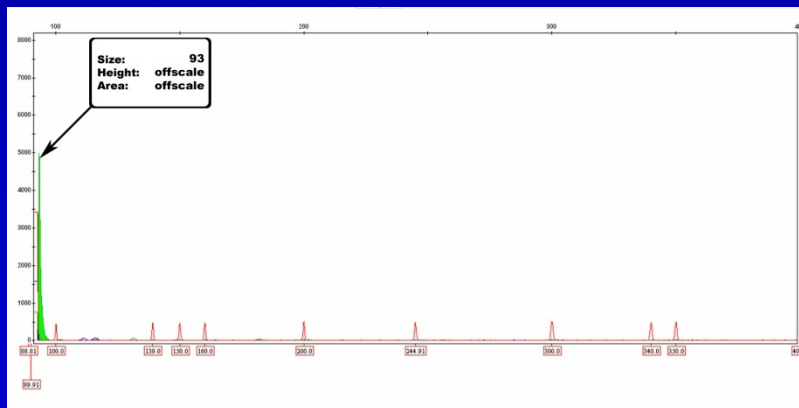
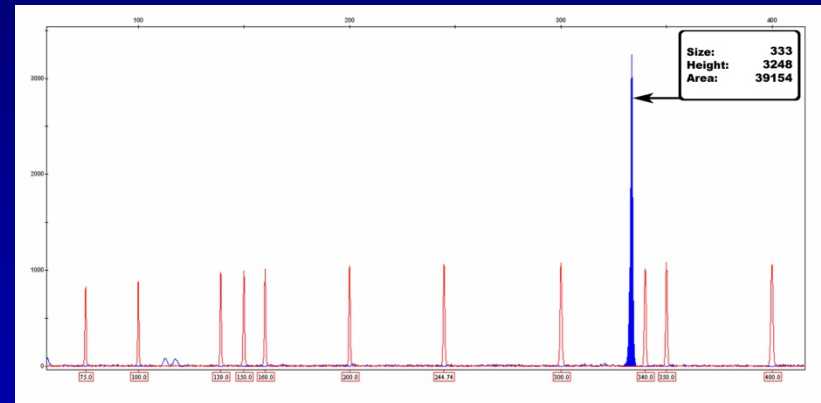
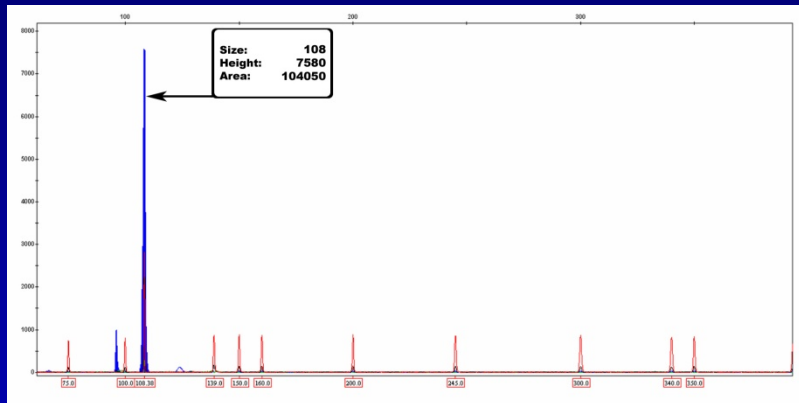
# Species-Specific and Universal Primers



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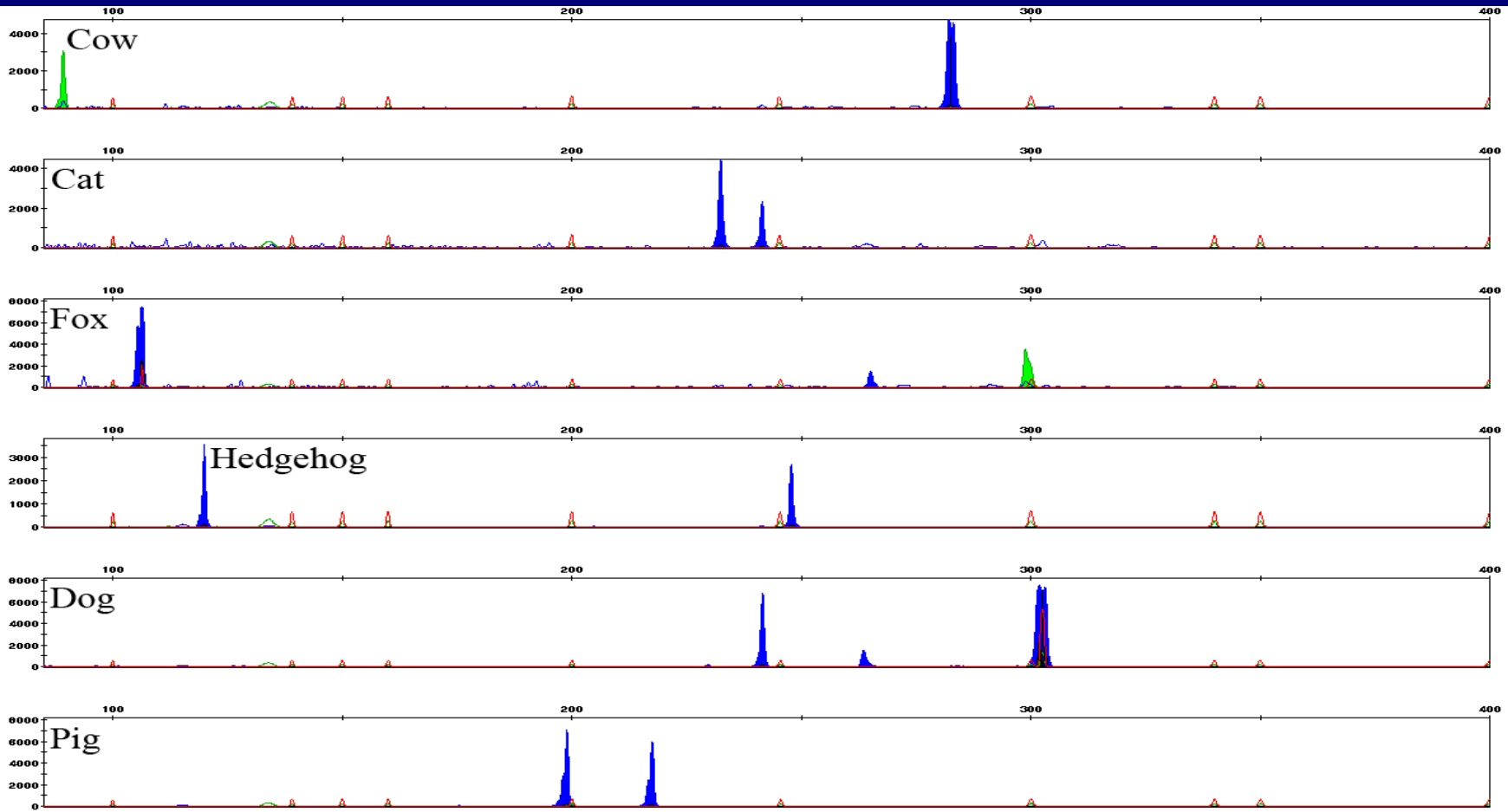
# Single Animal Reactions



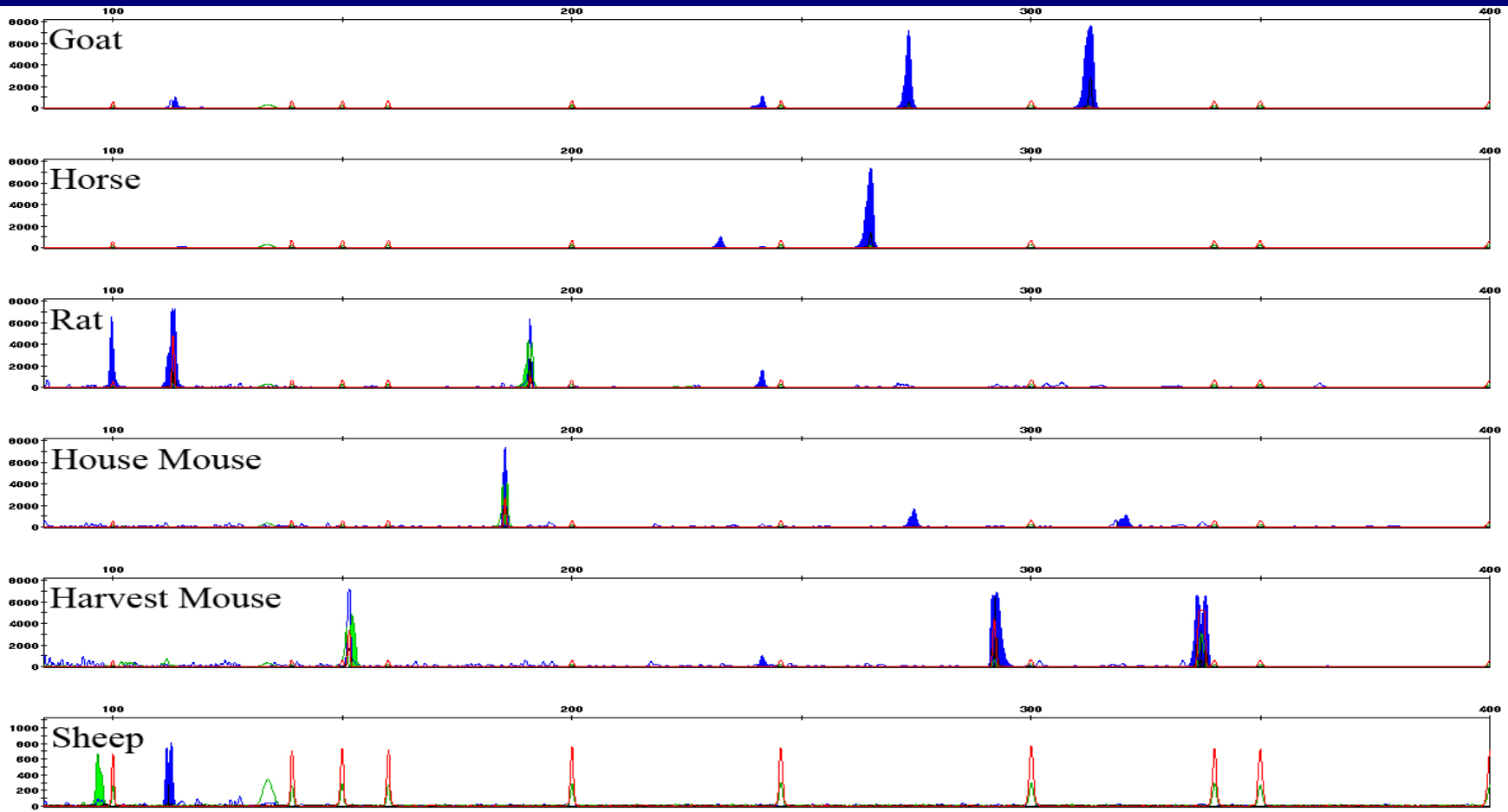
From top left: Human, Horse, Cow and Goat



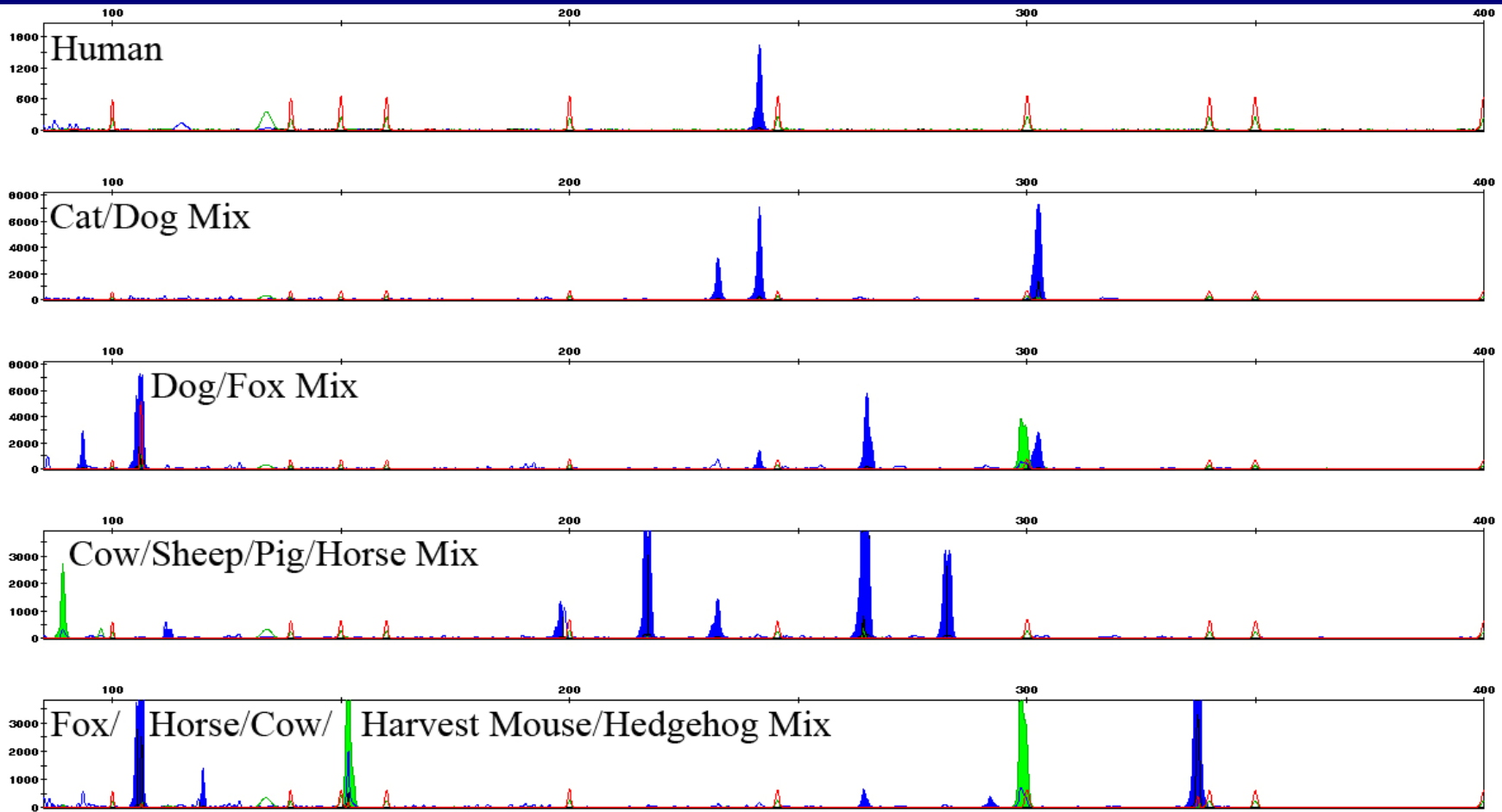
# Multiplex Species Test



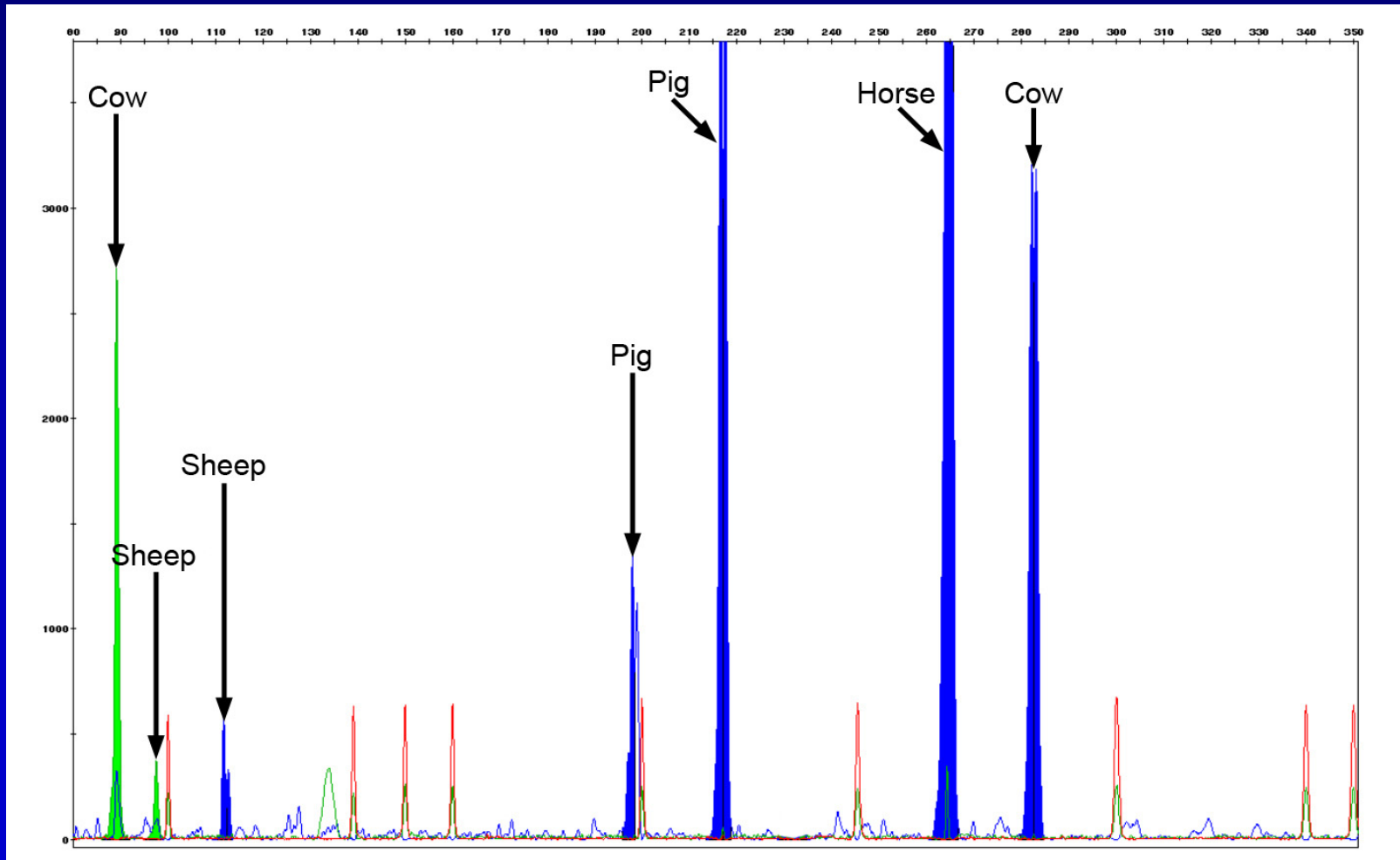
# Multiplex Species Test



# Multiplex Species Test



# Cow/Sheep/Pig/Horse Mixture

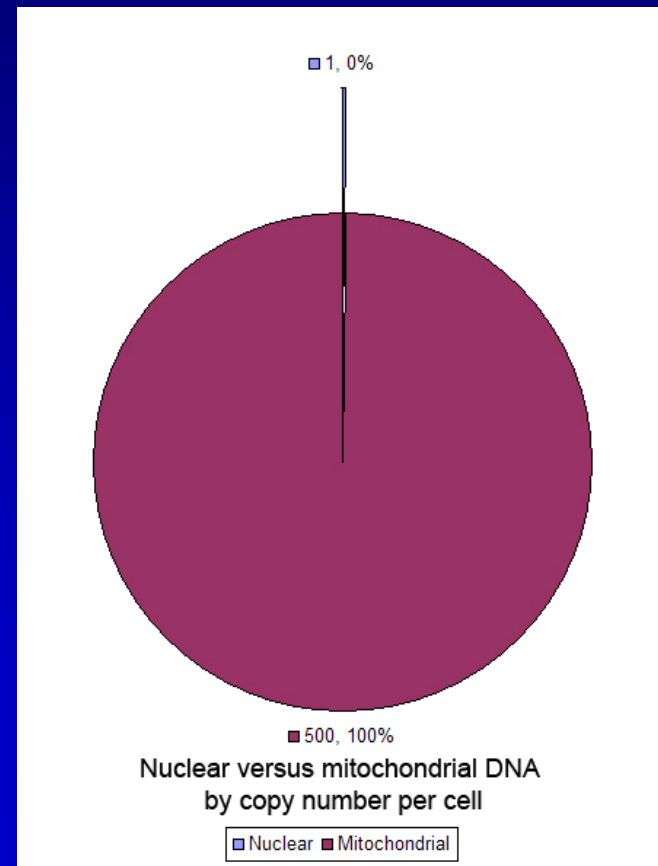
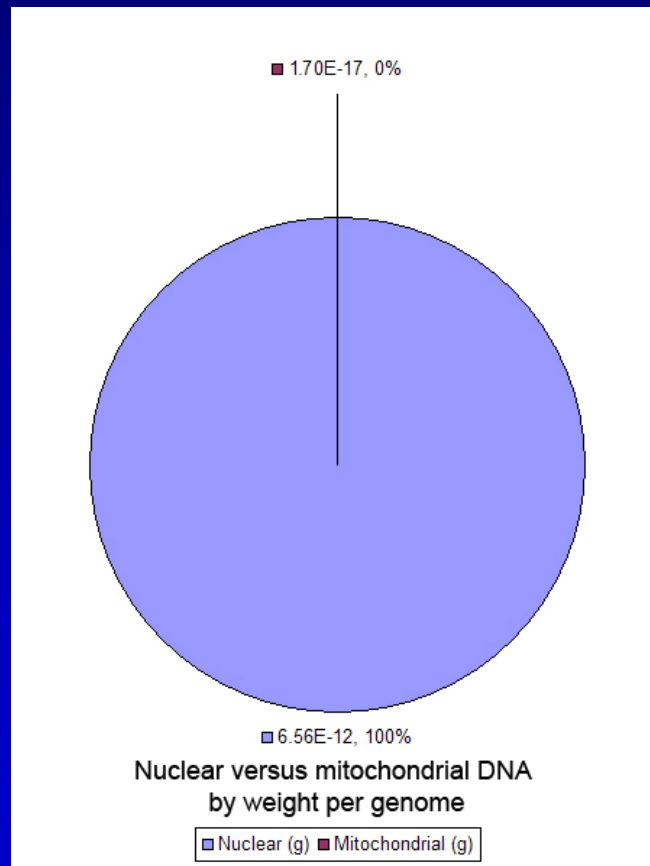


# Quantification

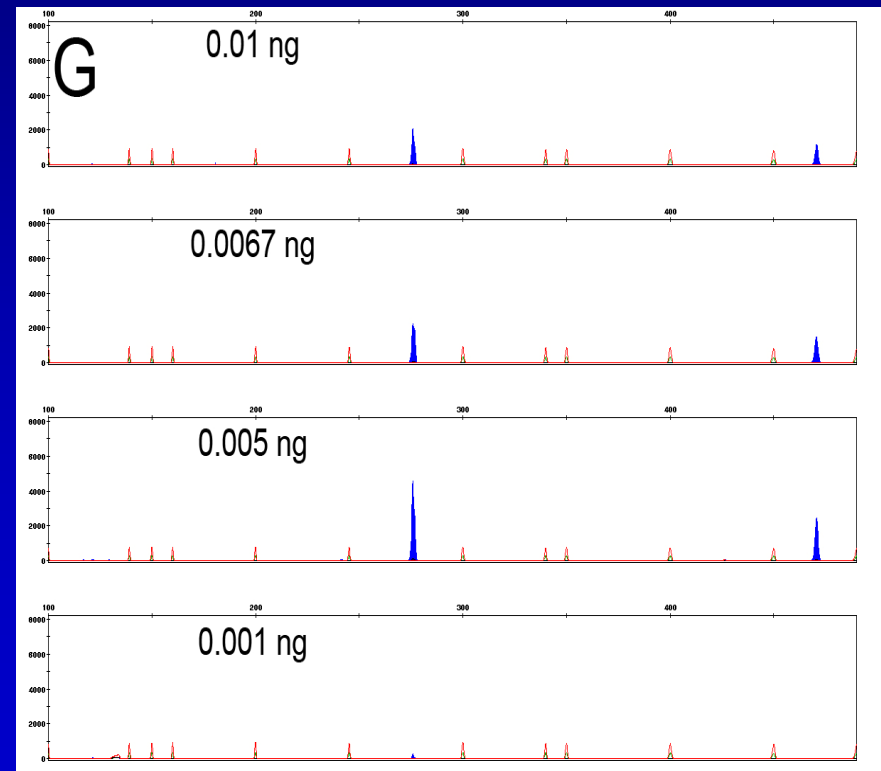
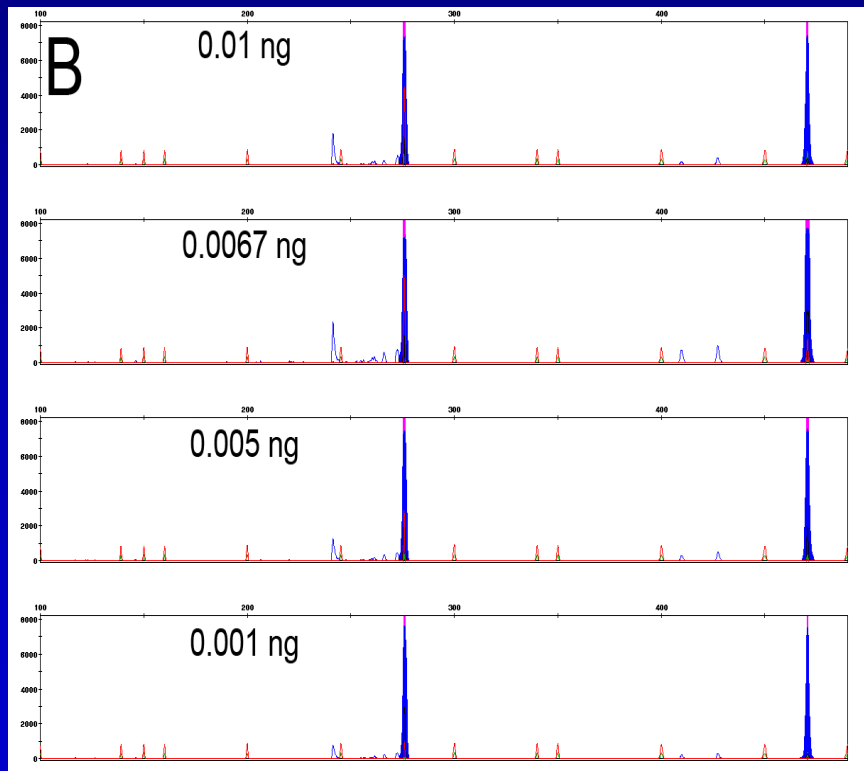
Two methods of quantification were attempted:

- Based on peak height ratio
- Based on real time PCR

# Nuclear vs. Mitochondrial DNA



# Quantification by Peak Height Ratio



# Quantification by Peak Height Ratio

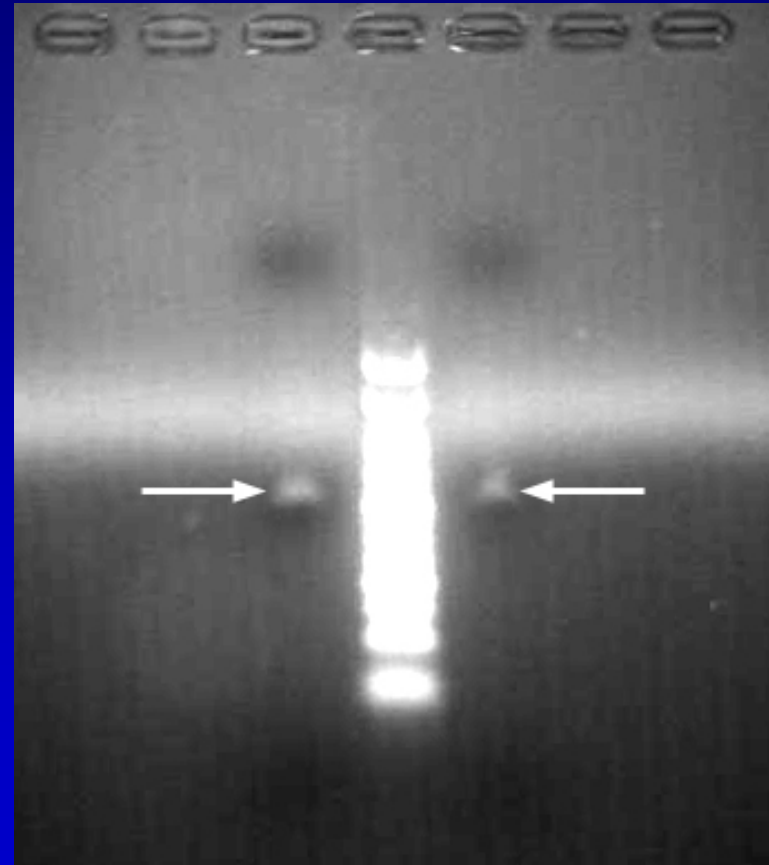
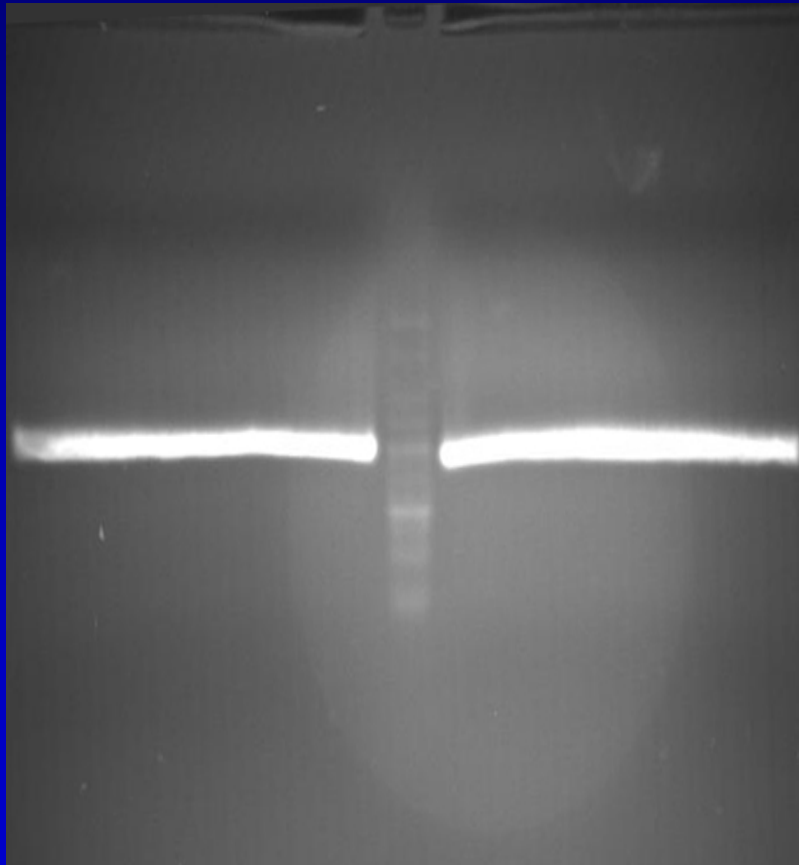
- 10 human samples analysed
  - Human/Universal ratio calculated
    - Peak Height Human/Universal
      - Average  $2.044 \pm 1.13$
    - Peak Area Human/Universal
      - Average  $1.9 \pm 0.56$



# RT-PCR Quantification of mtDNA

- Two methods investigated: SYBR Green and TaqMan
- SYBR Green Cheaper
  - Not as specific
    - This can be addressed by having highly efficient primer sets

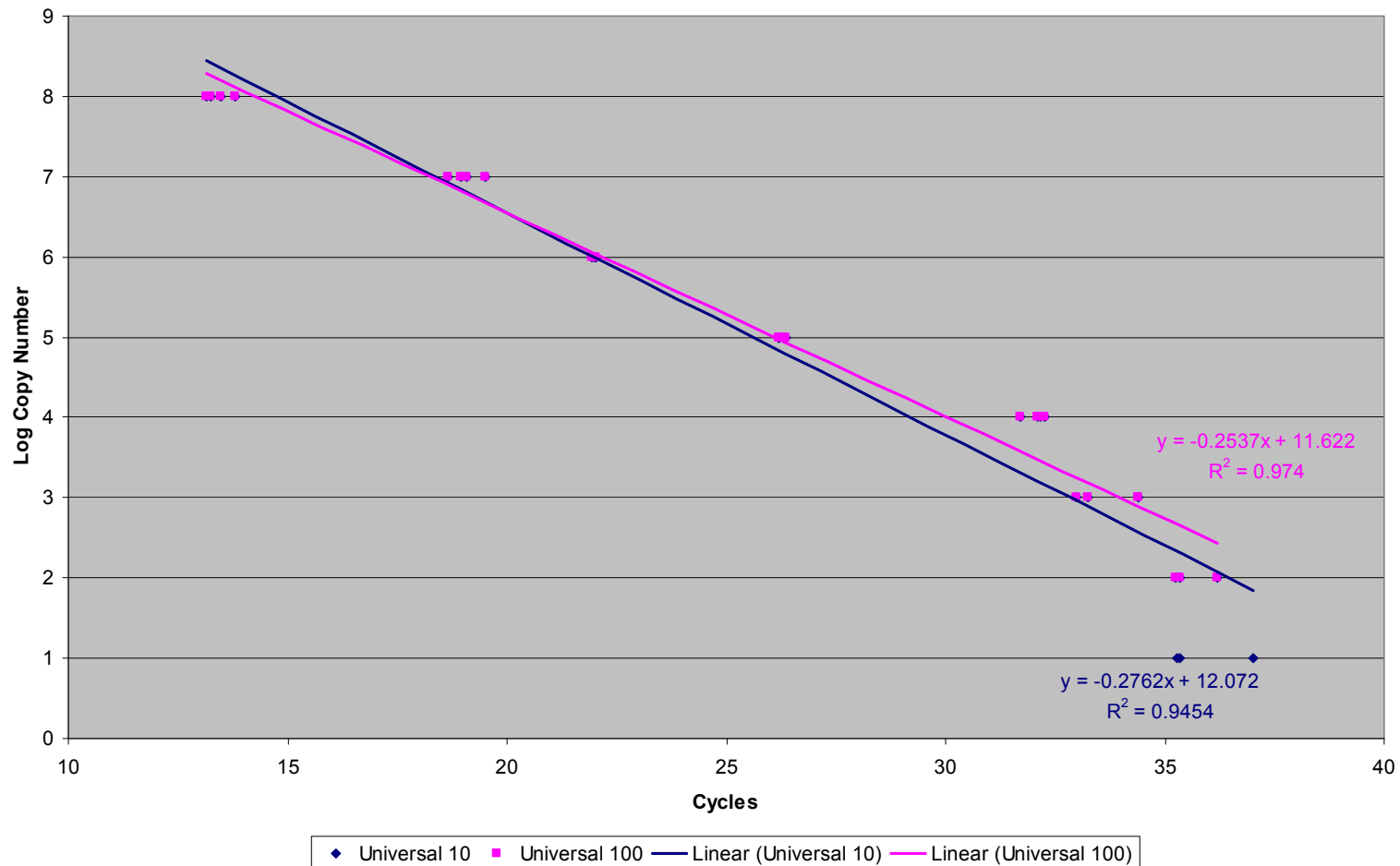
# Specificity of Primers



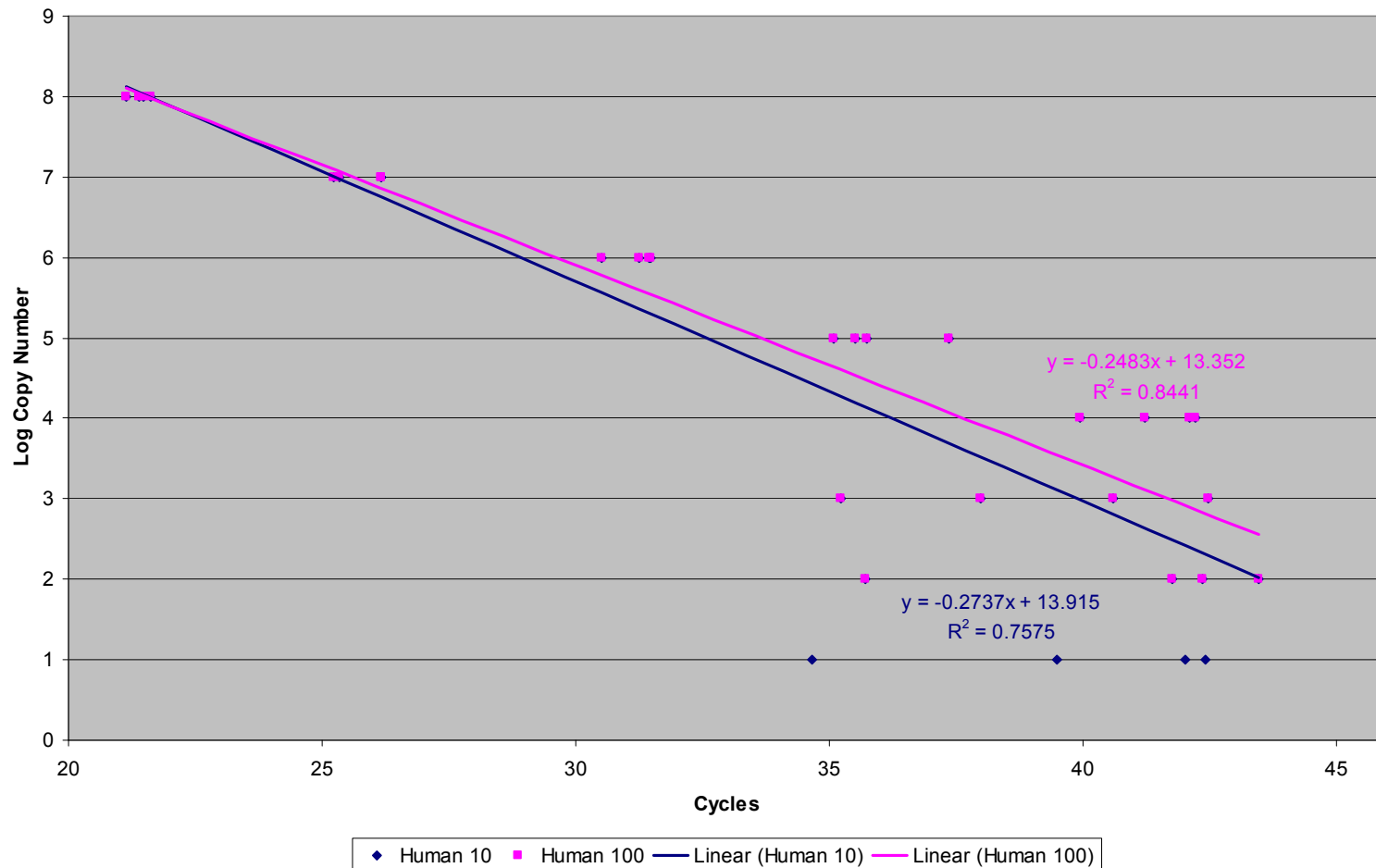
# SYBR Green

- Human and Universal primer sets developed and tested
- Fragments extracted and quantified using UV Vis
- Dilution series created and amplified with SYBR Green

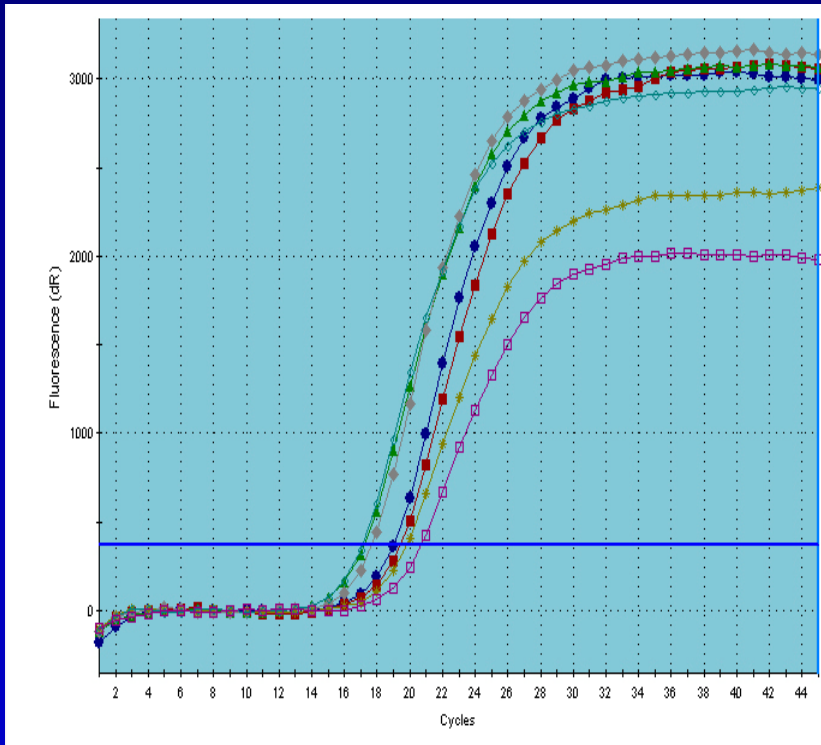
# Standard Curve for Universal Fragment



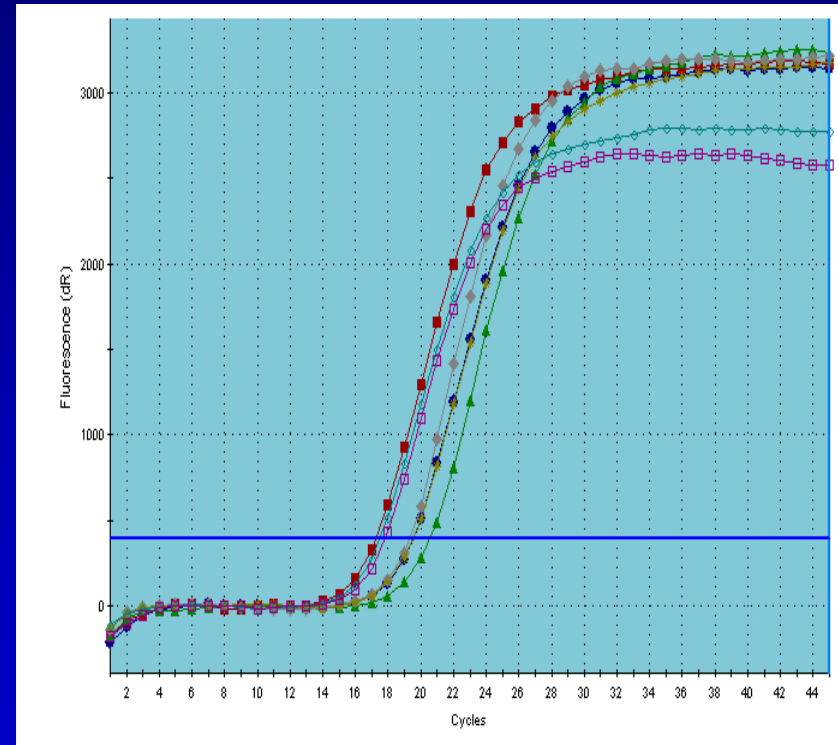
# Standard Curve for Human Fragment



# Human DNA Test



Human DNA with Human  
Primers



Human DNA with Universal  
Primers

# Quantification by RT-PCR

- 7 Human samples tested with human specific and universal primers
- Human/Universal Ratio
  - Average  $0.787 \pm 0.016$

# Additional Work

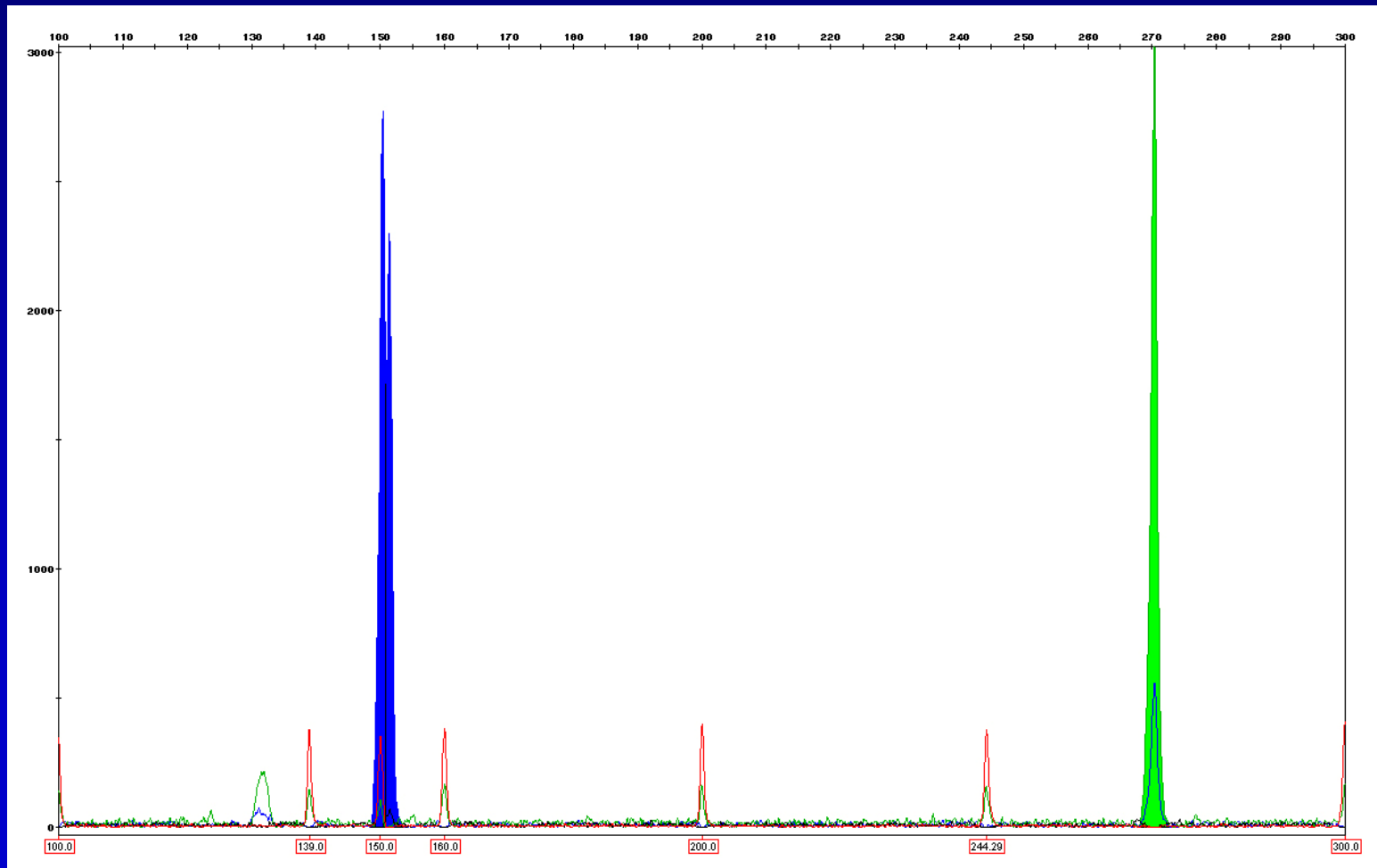
- Traditional Chinese Medicine (TCM)
  - Bears
  - Tigers
  - Leopards
  - Musk Deer
  
- Casework



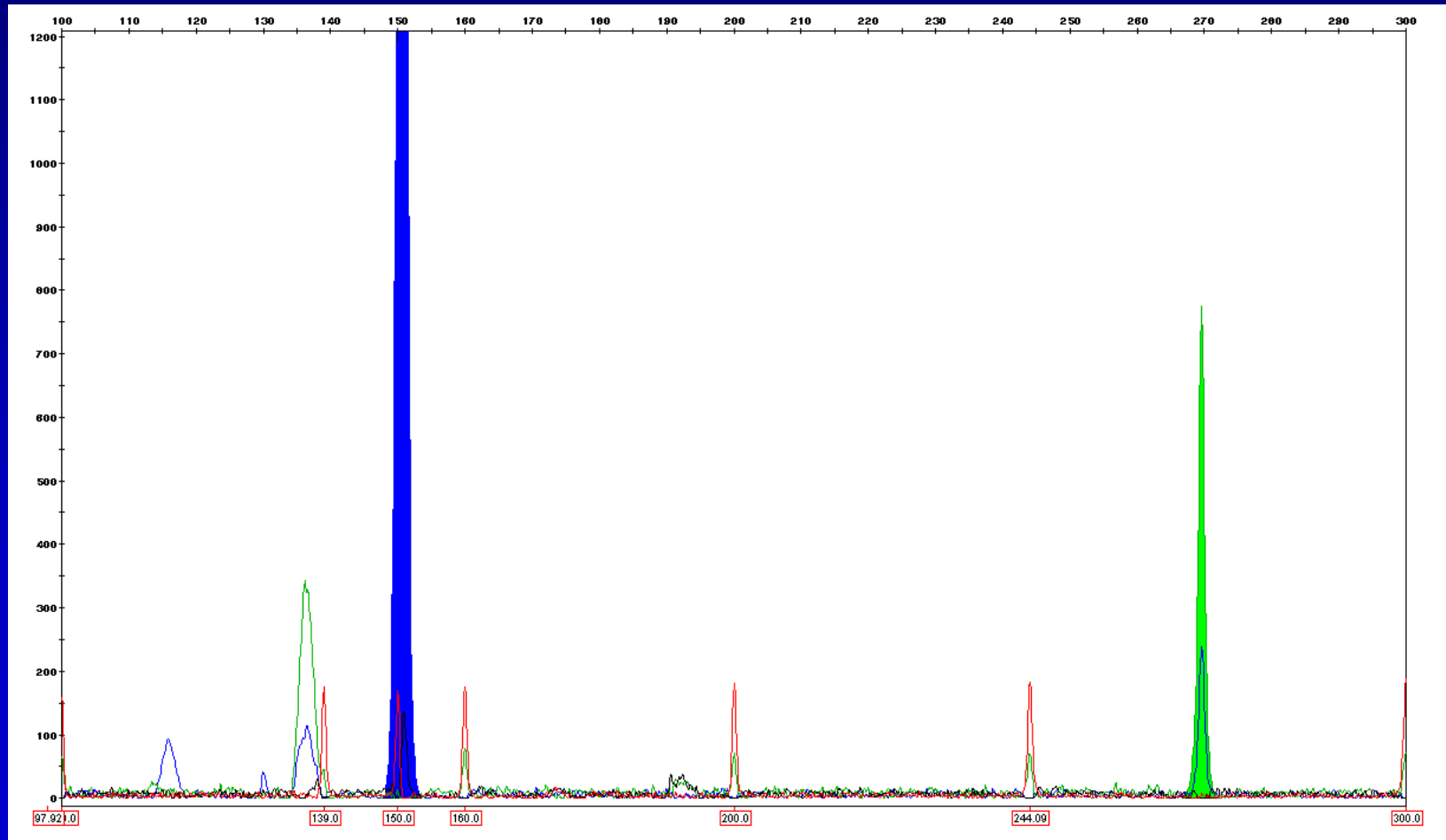
# Additional Work: TCM

- Bear, tiger, leopard and musk deer parts used in many products and TCM
- Bears farmed for their bile and other parts
- Inhumane treatment
- CITES listed species

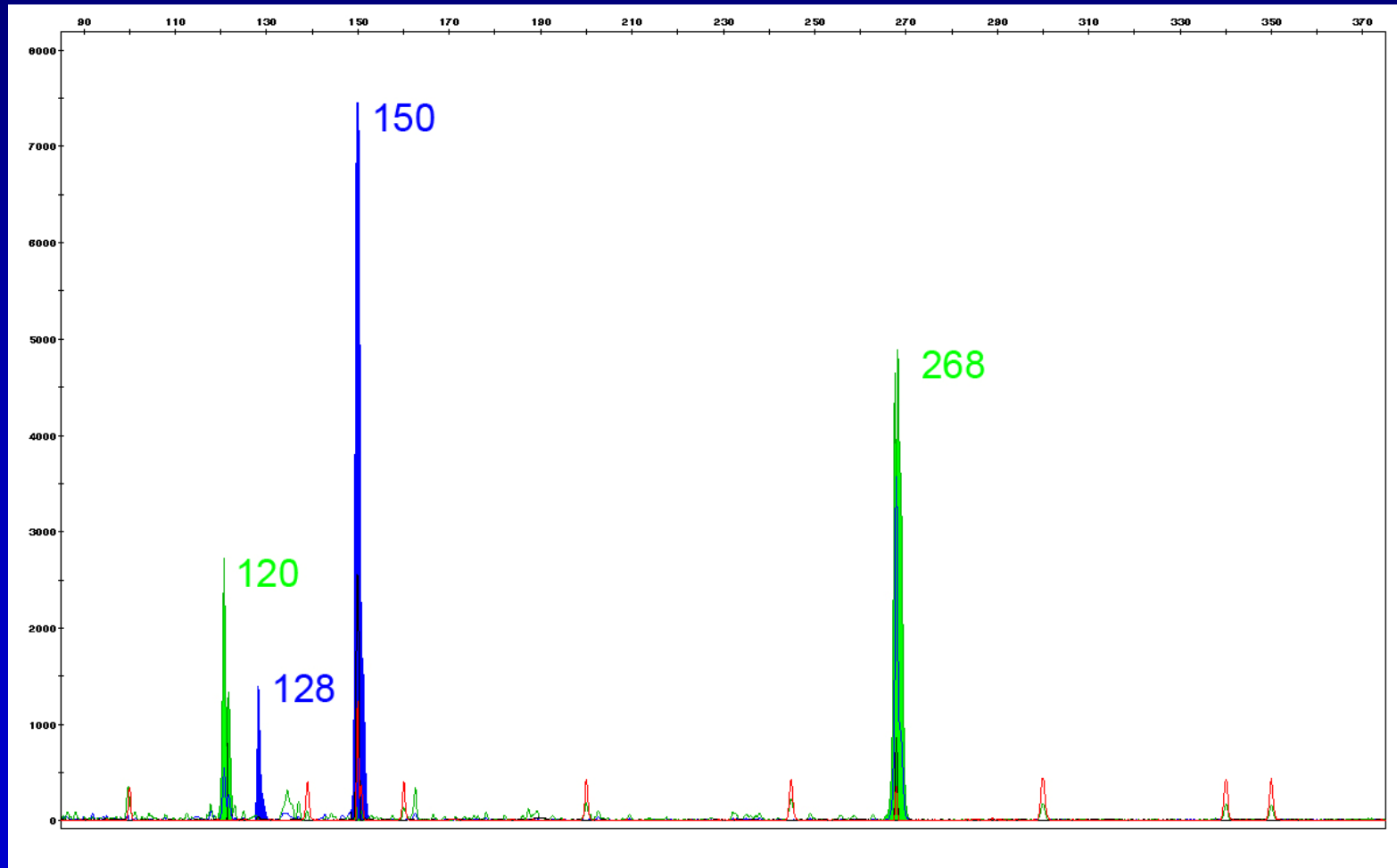
# Canadian Black Bear



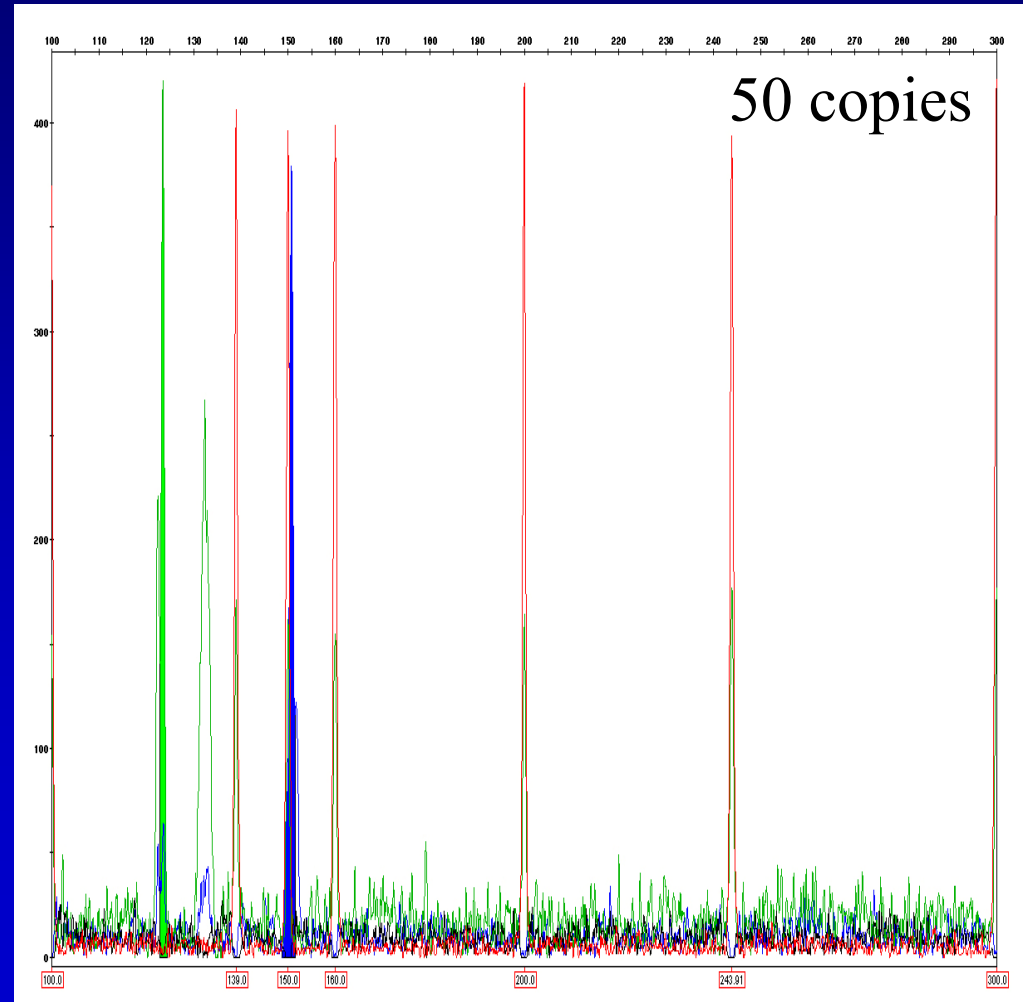
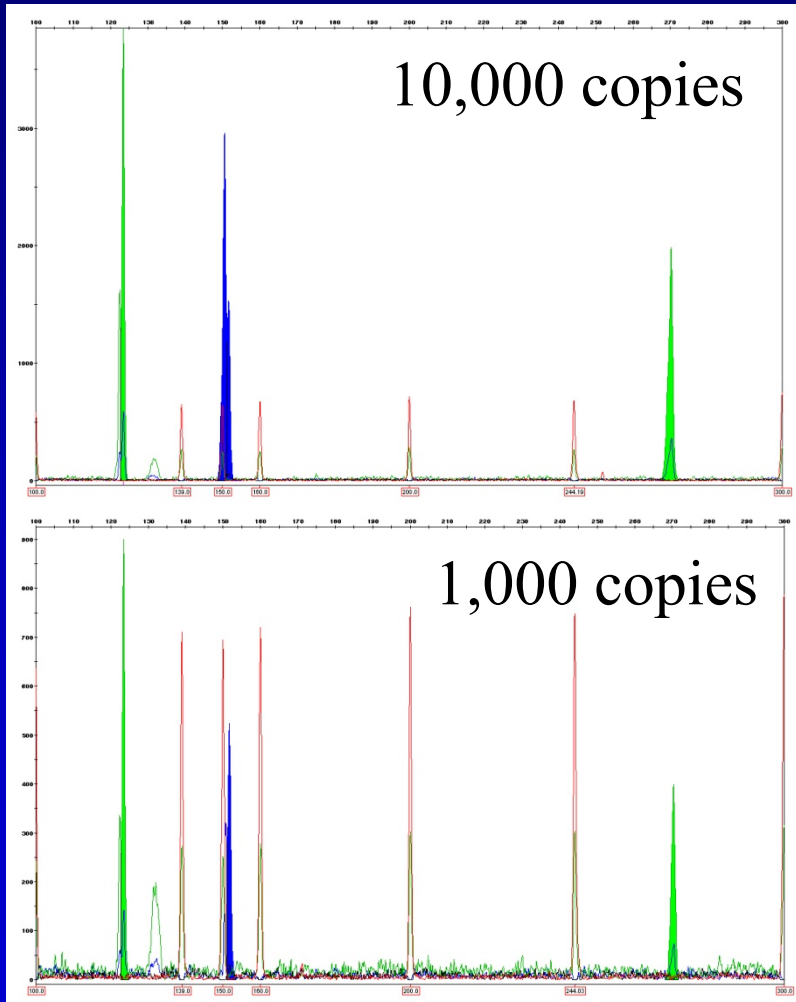
# Polar Bear



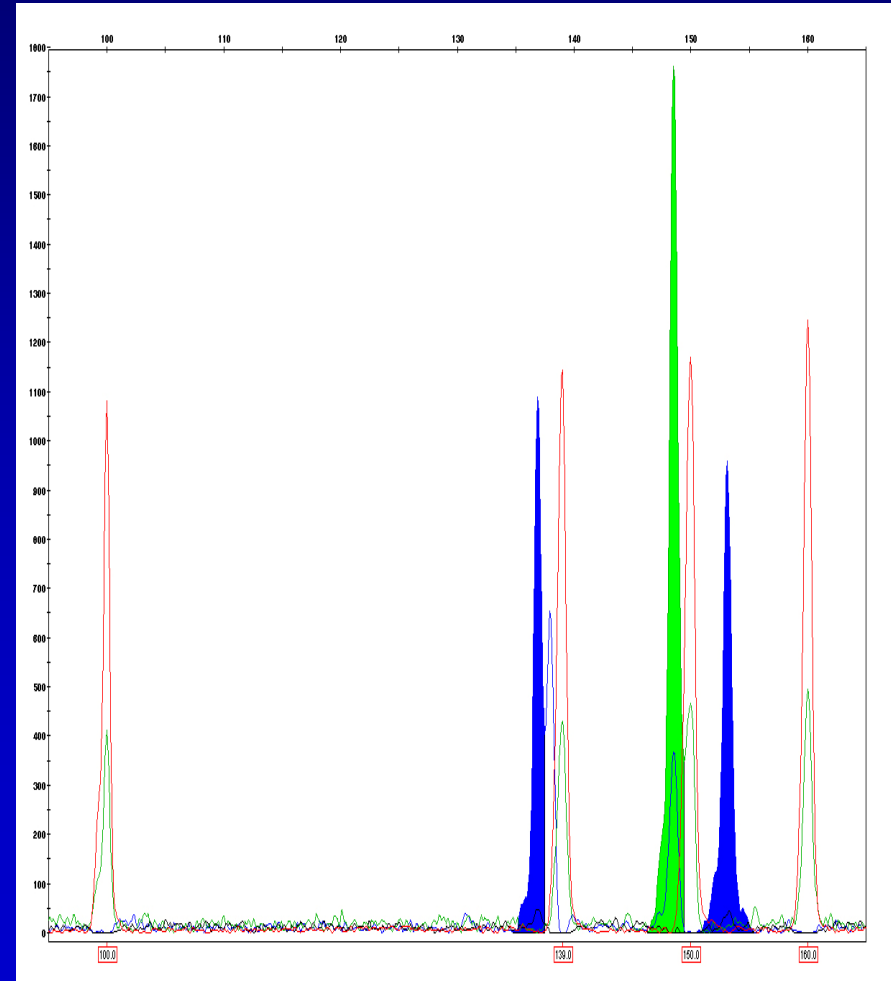
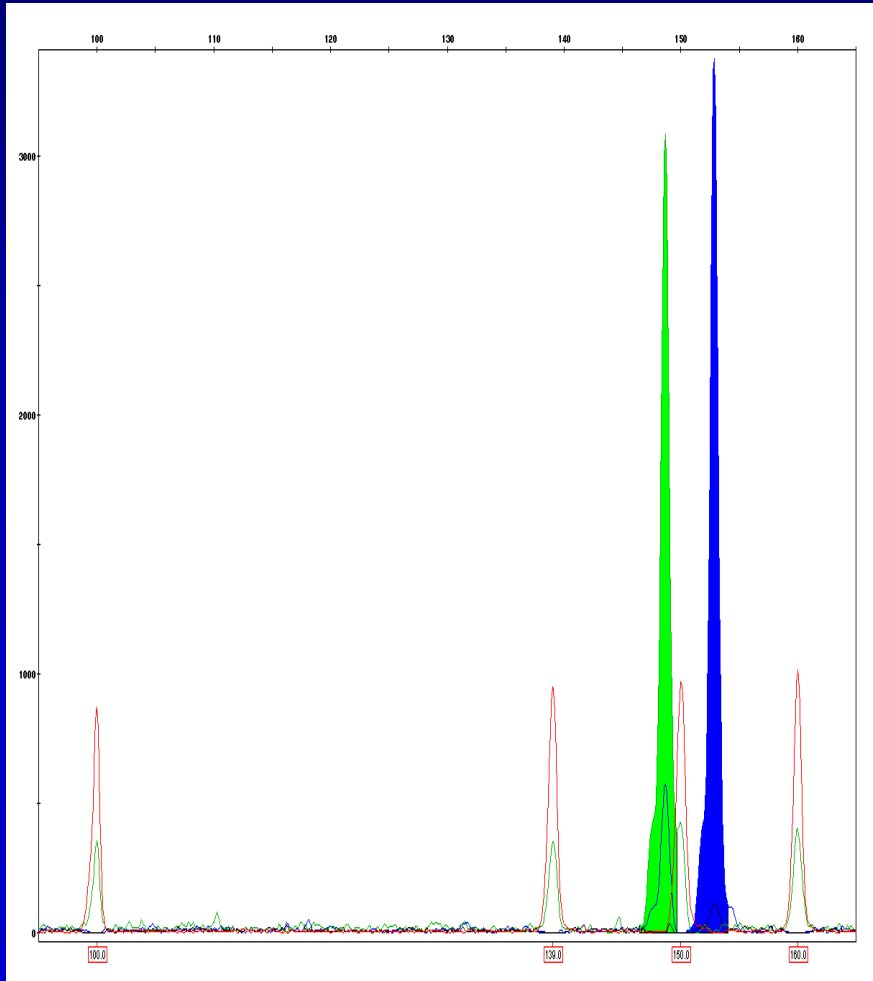
# Asiatic Black Bear



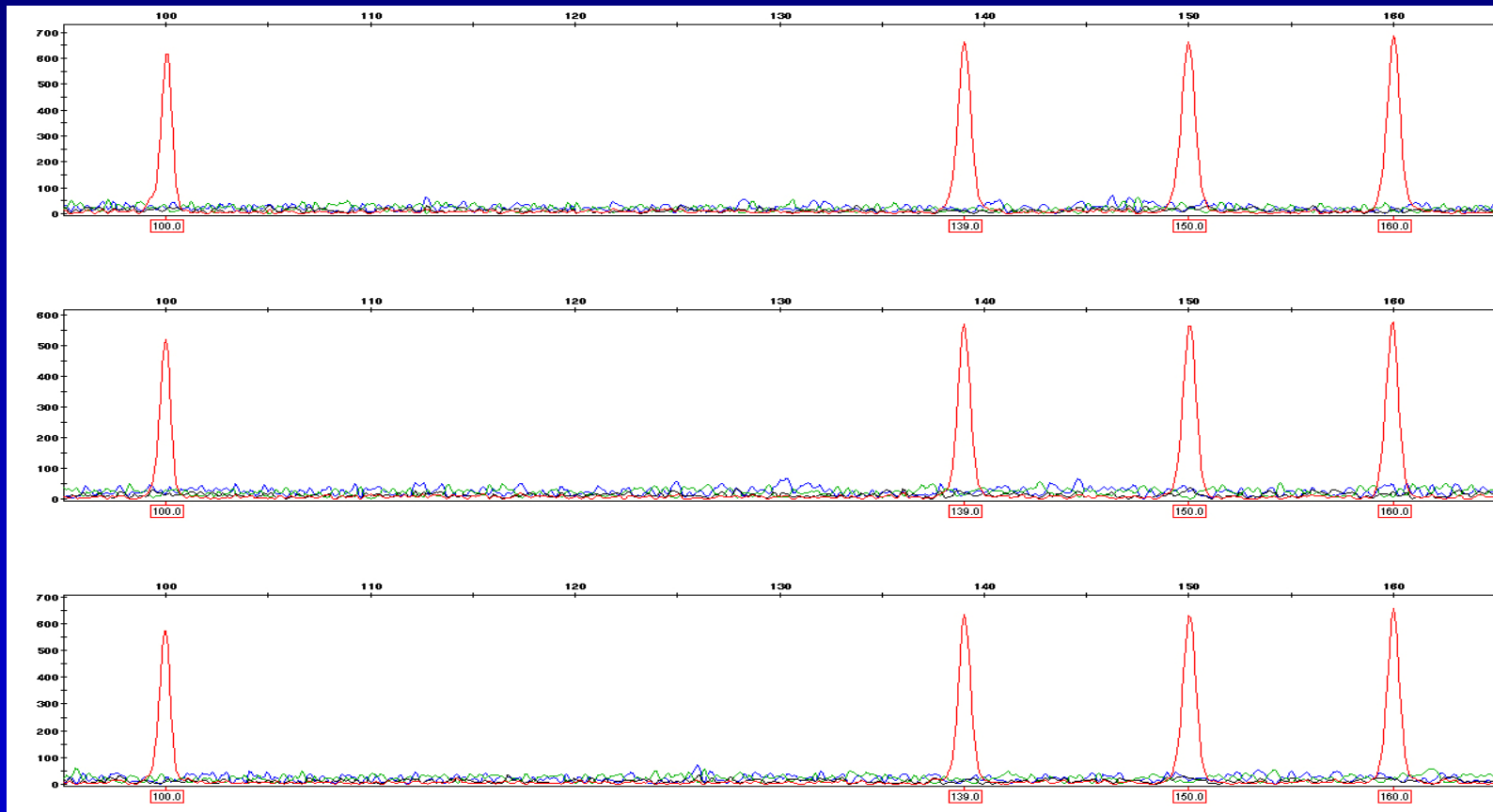
# Asiatic Black Bear



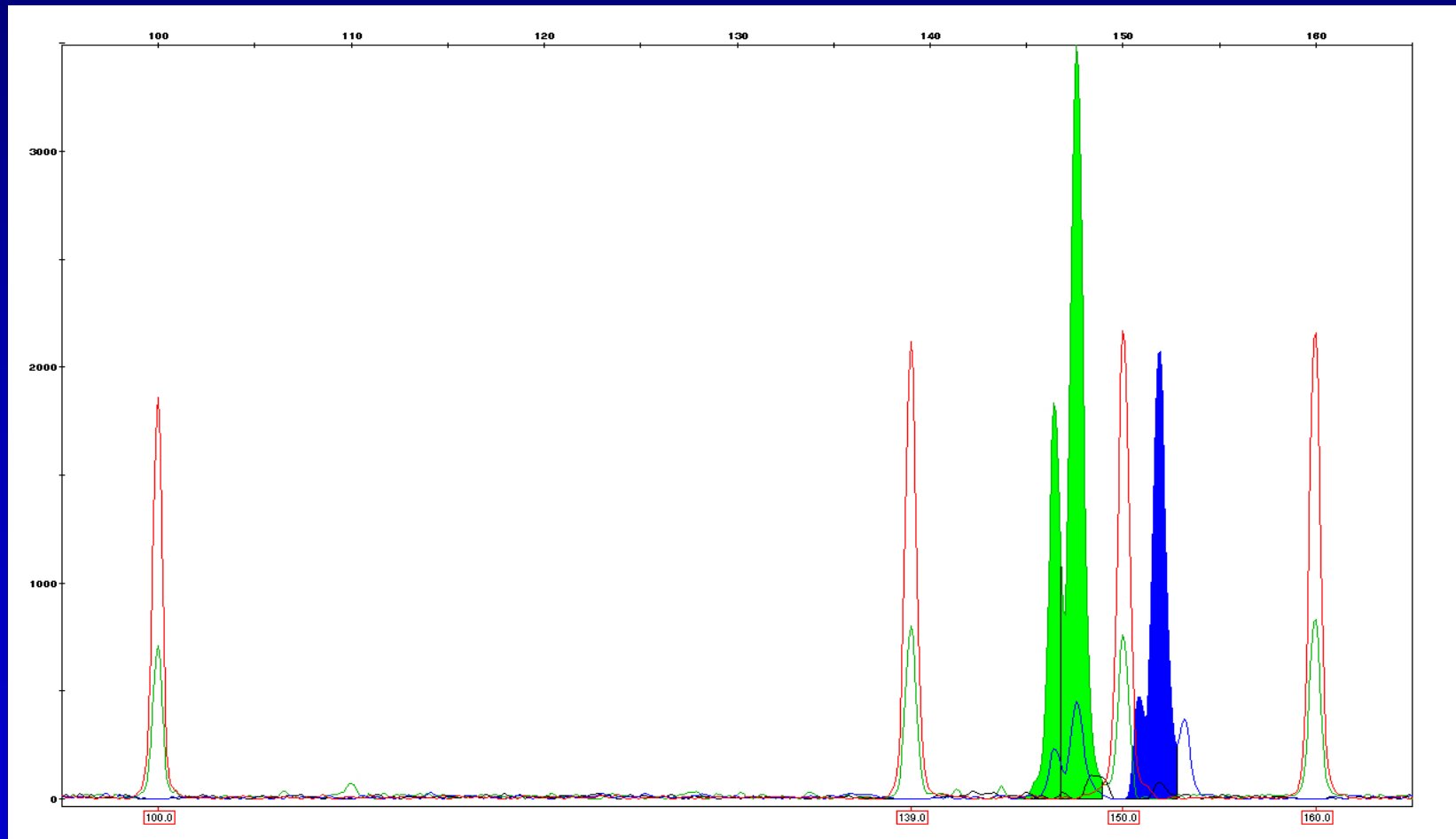
# Big Cats



# TCM Samples

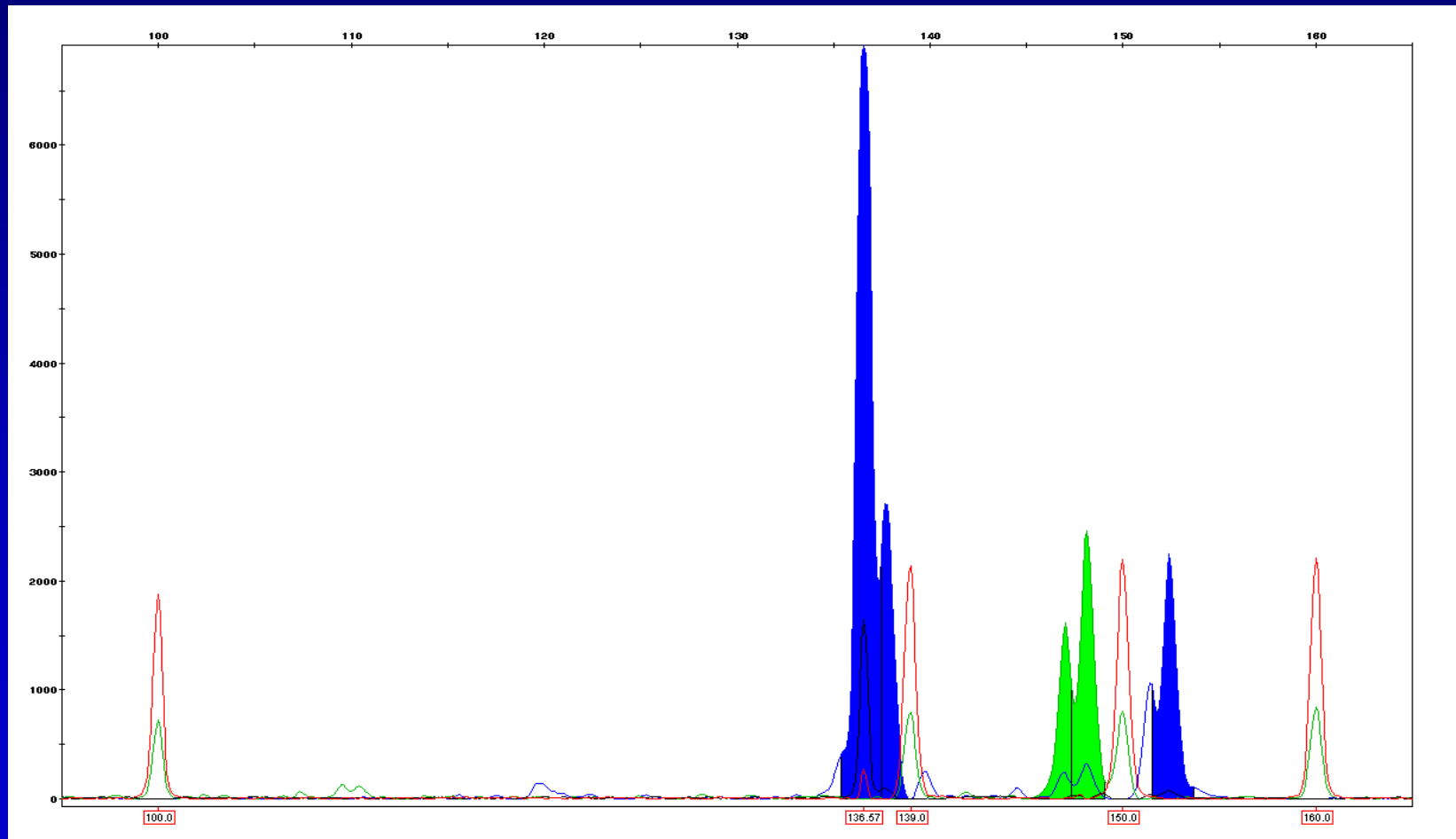


# TCM: Sample

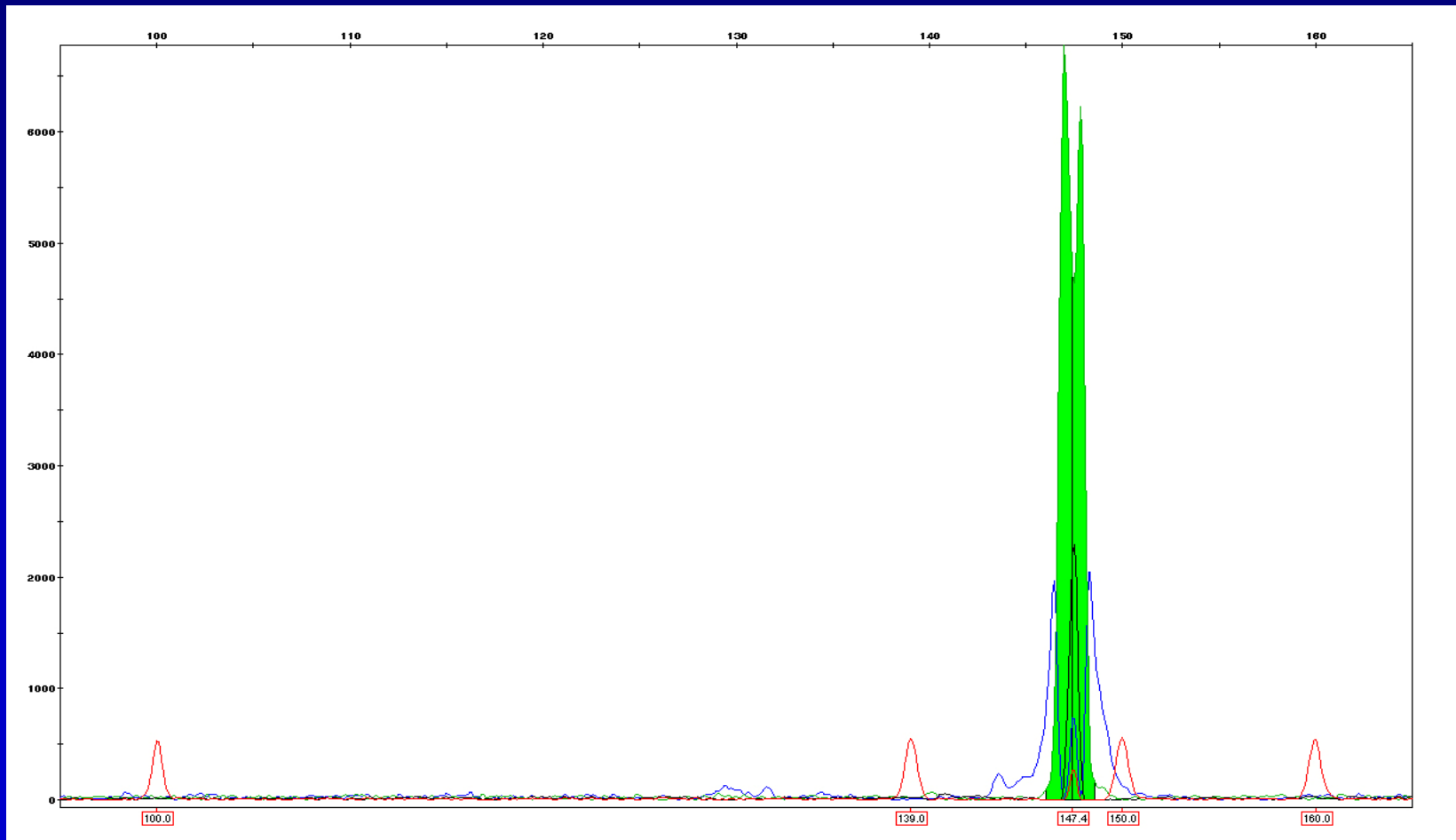




# TCM: Sample



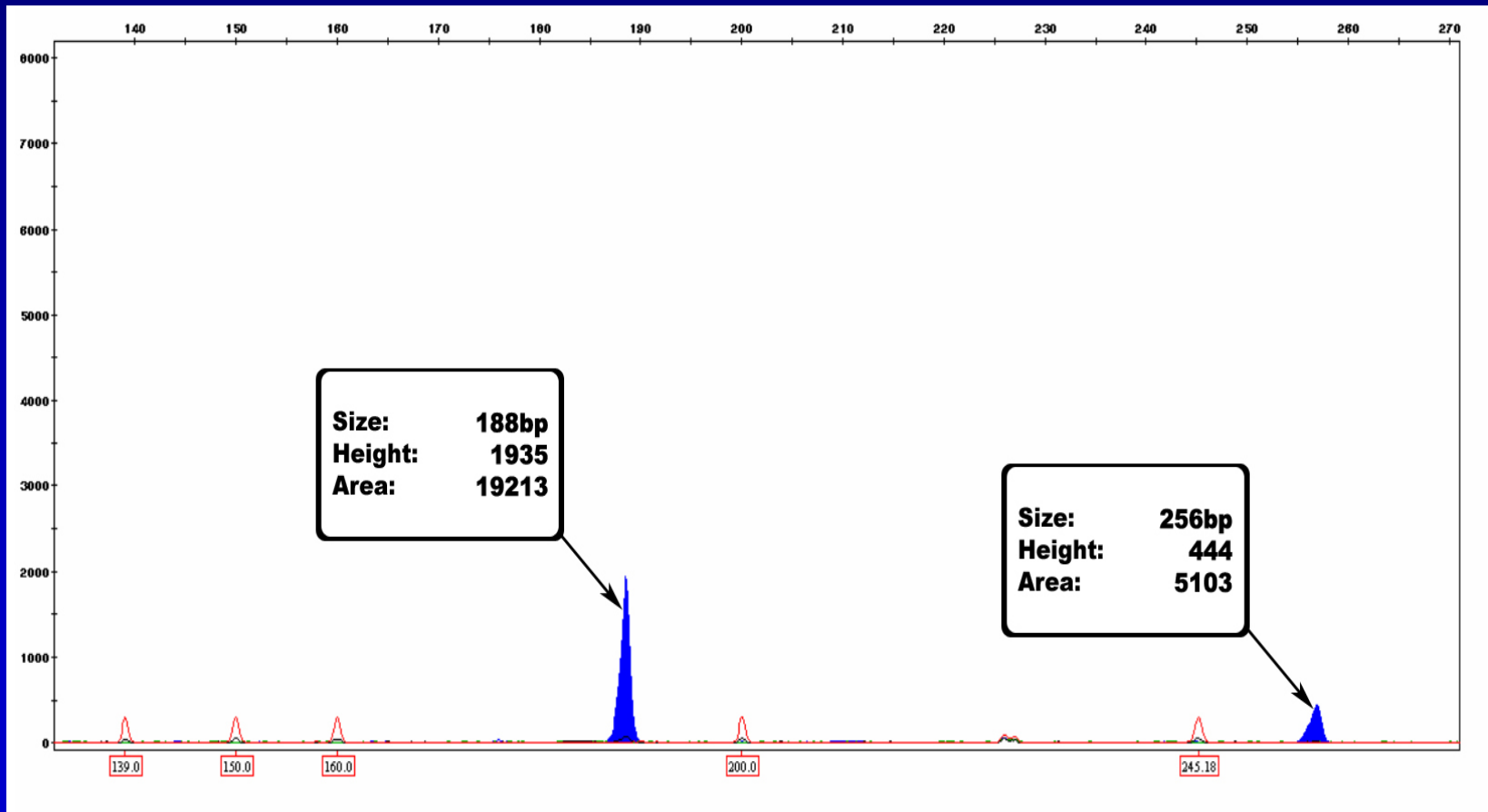
# Human with Big Cat



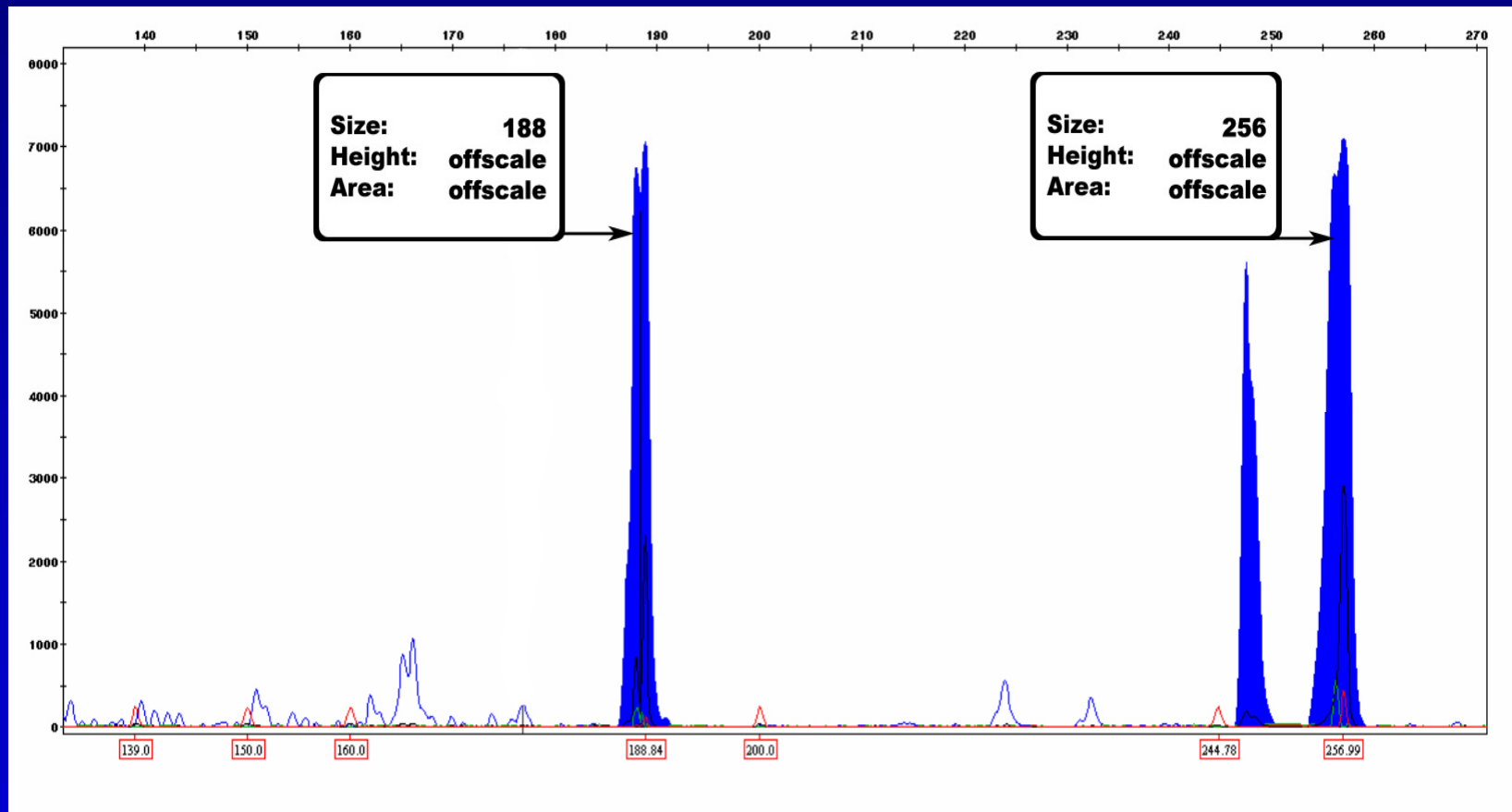
# Case Example

- Man found in army fatigues with blood spatter.
- Suspected of poaching red deer.
- Police were interested if the blood was that of red deer.

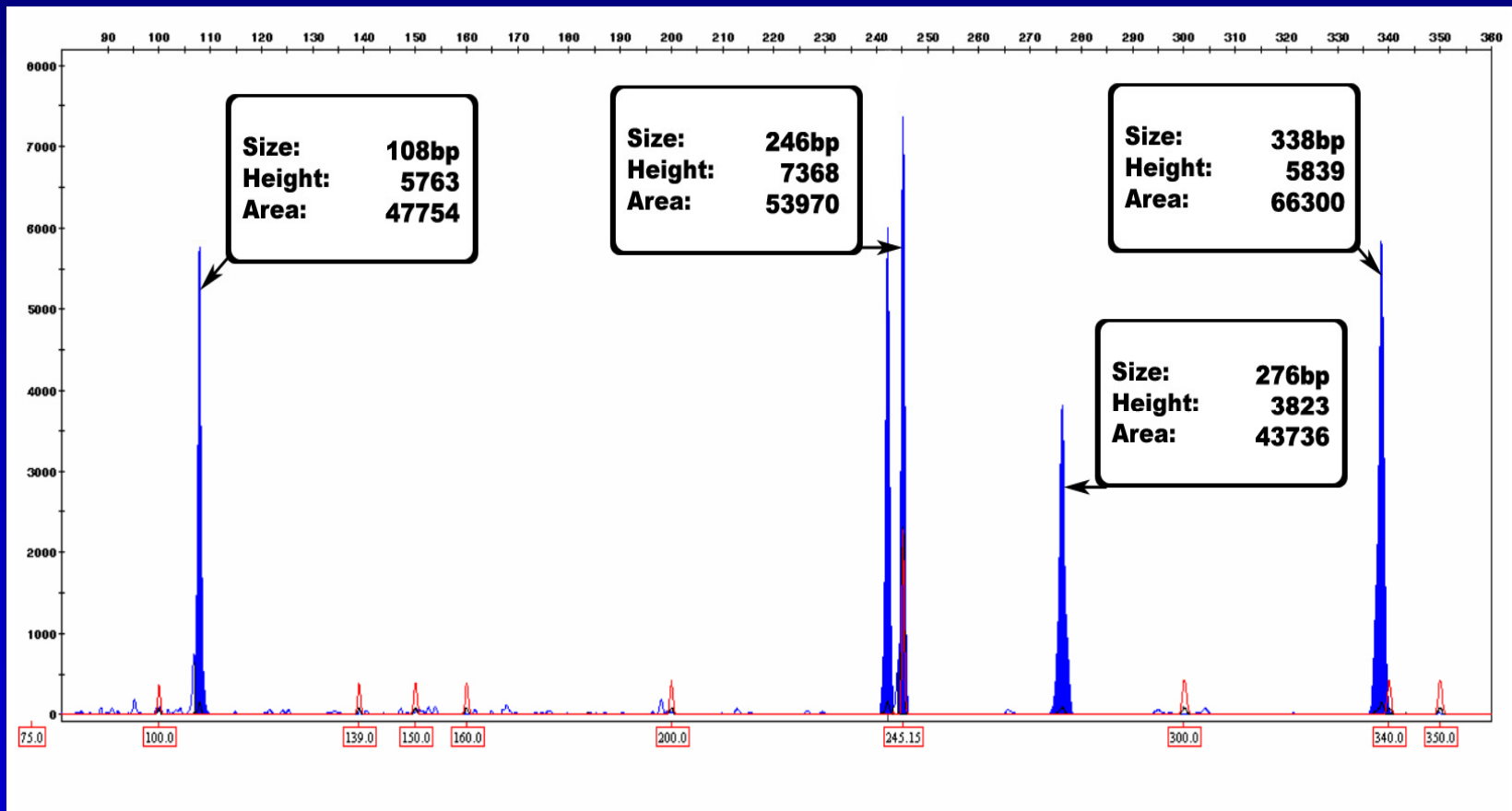
# Red Deer Control



# Unknown Sample With Red Deer Primers



# Unknown Sample With Human Primers



# Case Conclusions

- Expect a human result due to epithelial cells on trousers.
- Red deer result only obtained if blood was that of deer.
- Obtained a mixture of human and red deer DNA.
- Blood on the trousers was from a red deer.

# THANK YOU