

# Mitochondria-Targeting PCR and CO1 Barcode Sequence Analyses as Alternatives to Isoenzymology

Greg Sykes, M.S.

*Lead Biologist, Laboratory Testing Services, ATCC*

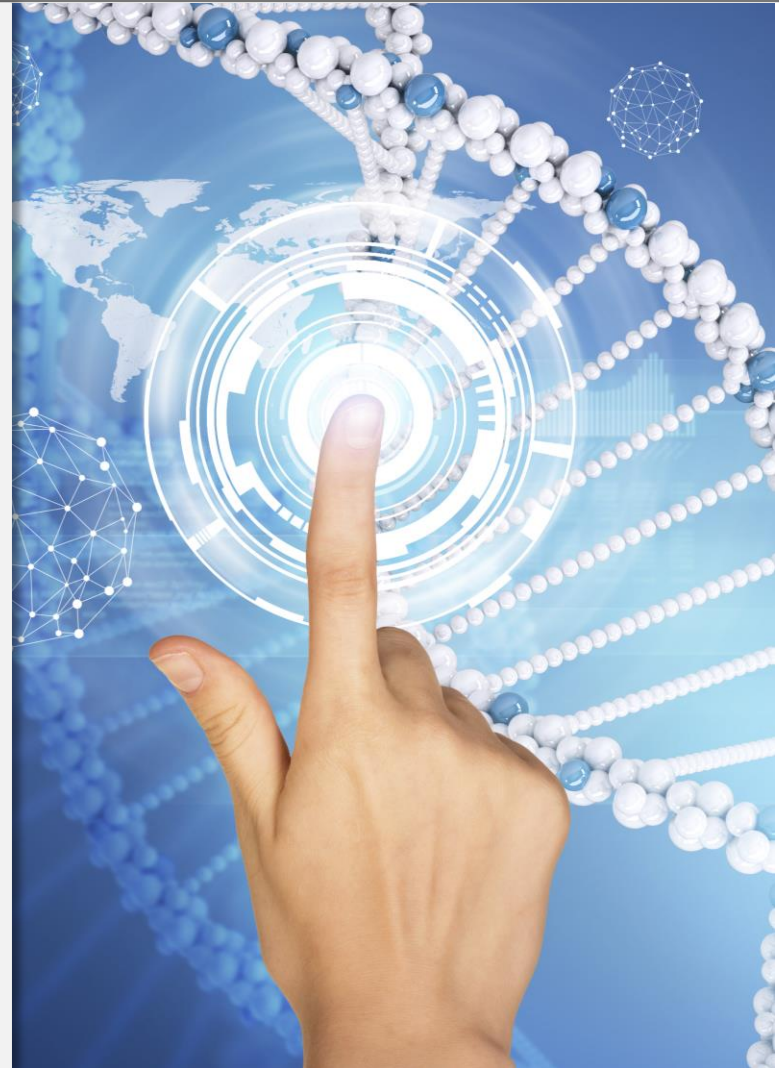
December 13, 2016



# Overview

## This presentation will cover:

- American Type Culture Collection (ATCC) overview
- Need for species identification
- Current DNA-based species ID at ATCC:
  - Multiplex PCR-Based Analysis
  - CO1 Barcode Sequencing
- Authentication

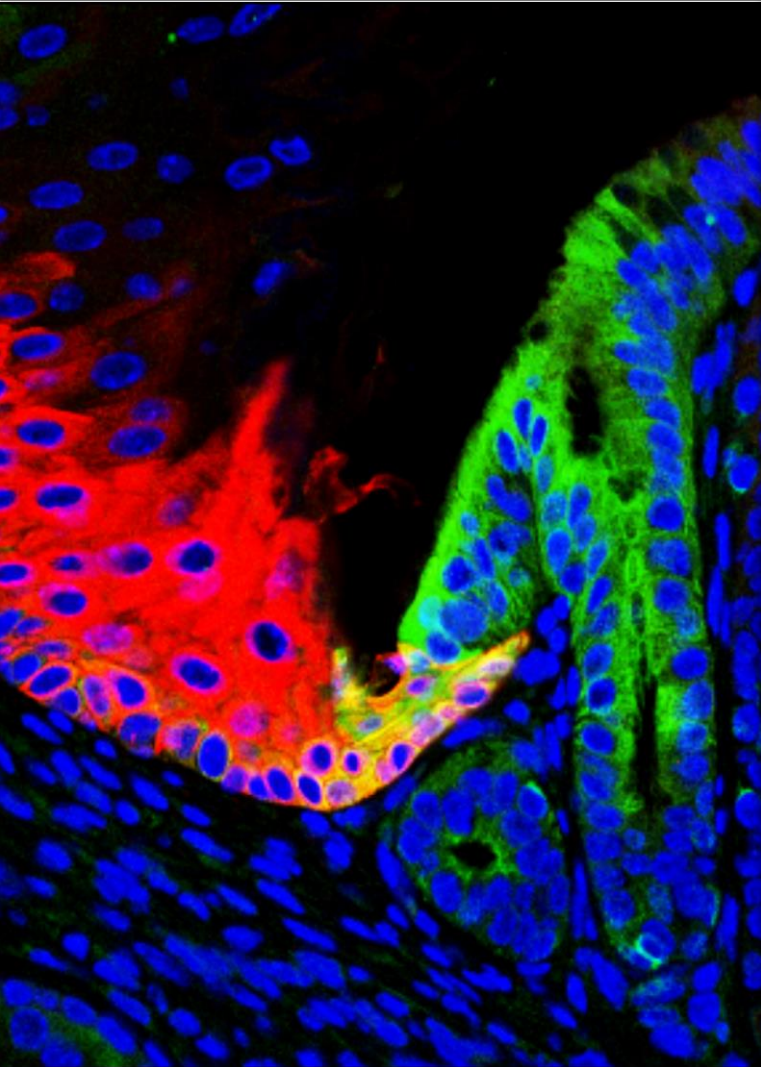


# About ATCC

- Founded in 1925, ATCC is a non-profit organization with headquarters in Manassas, VA
- World's premiere biological materials resource and standards development organization
- ATCC collaborates with and supports the scientific community with industry-standard biological products and innovative solutions
- Strong team of 400+ employees; over one-third with advanced degrees



# Scope of This Presentation



- Animal cell culture
- Interspecies cross-contamination and misidentification
- Other molecular-based detection techniques are not addressed here

## **Why perform species identification on cells?**

Because Bharati Hukku and colleagues (1984) found interspecies problems with 35% of cell lines examined!



# Historical Technique: Karyotyping

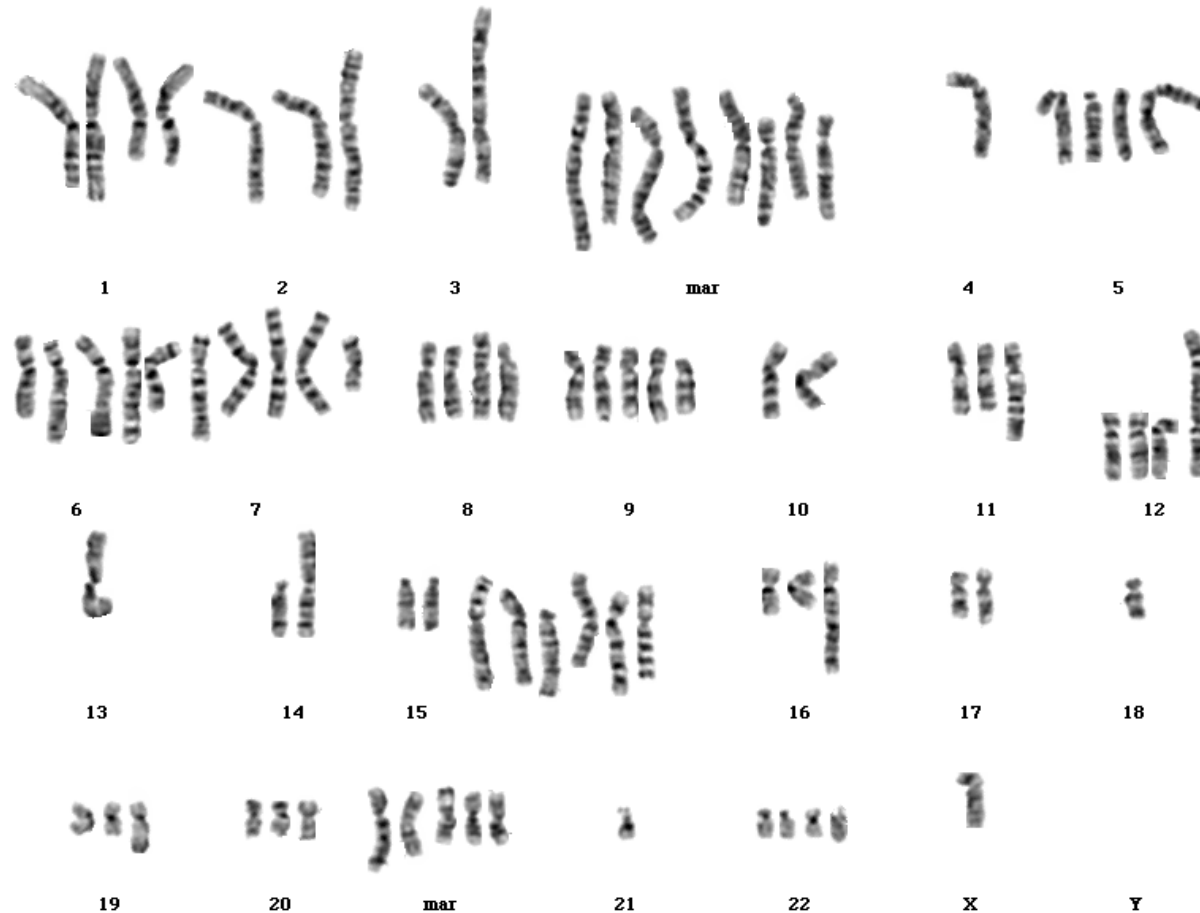


Figure 1. This complex karyogram of human cell line SJCRH30 (ATCC<sup>®</sup> CRL-2061<sup>™</sup>) is  $n=84$ , a significant departure from normal human  $n=46$ .

# Historical Technique: Isoenzyme Tests

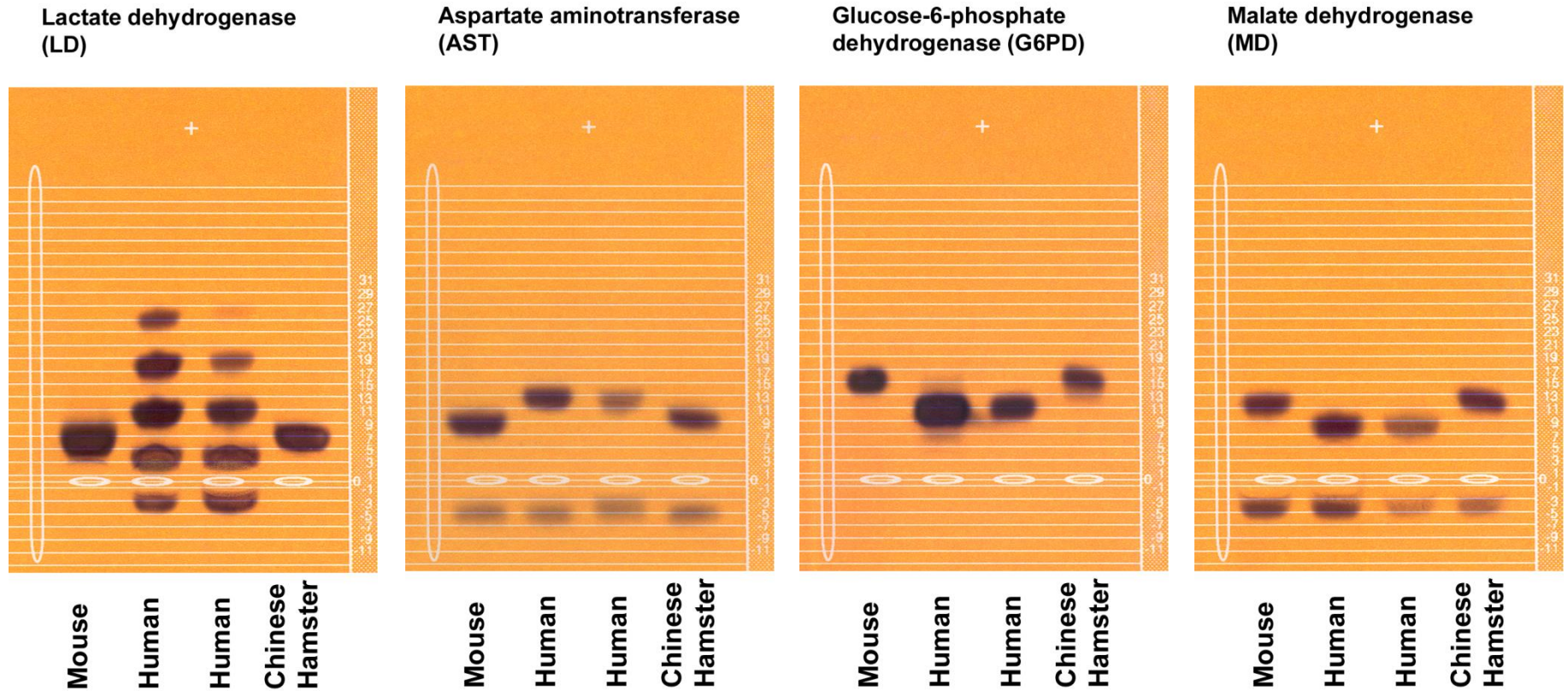


Figure 2. Four of the seven isoenzyme tests are seen here. Resolving related species may be challenging.

## The Big Question:

---

**I can't get a hold of  
isoenzyme reagents  
anymore!!**

***What do I do now?***

# Molecular-Based Species Identification

---



## **Designation: ASN-0004**

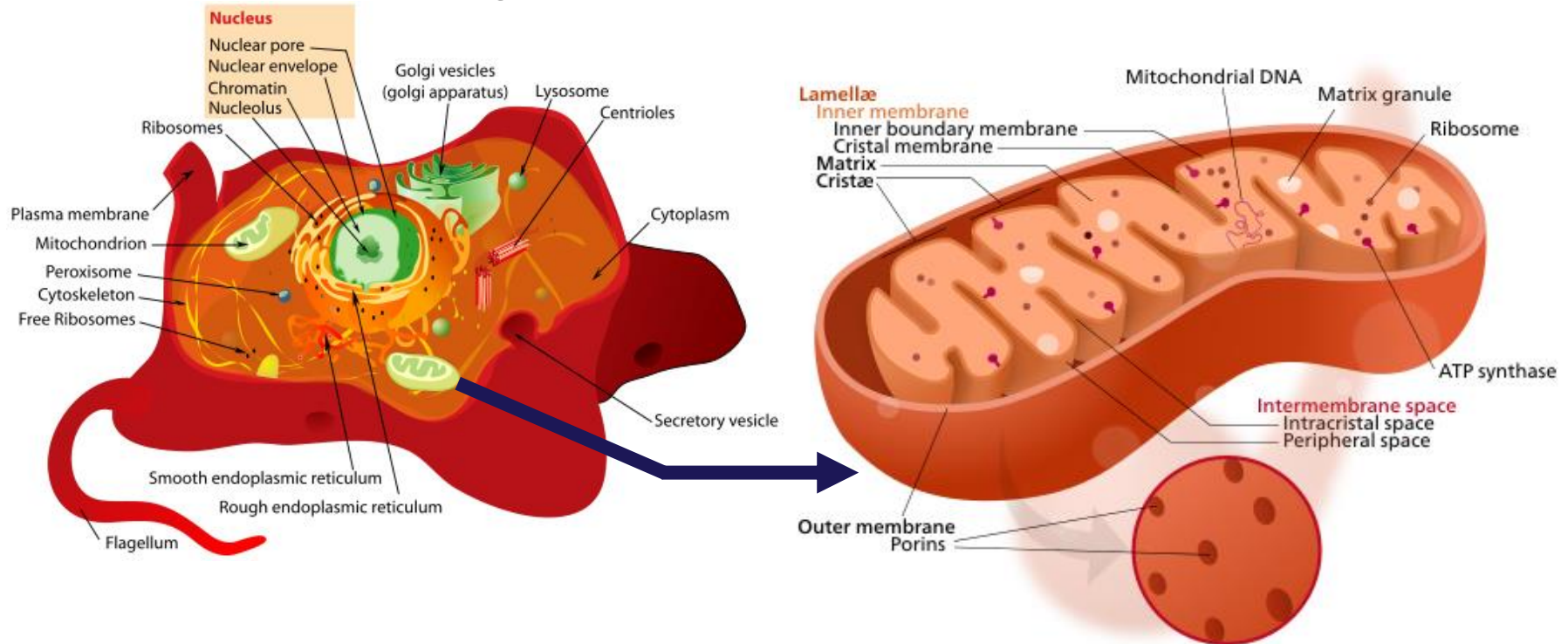
Species-Level Identification and Cross-Contamination Screening in Animal Cells by Multiplex PCR

*– in progress –*



# Molecular-Based Species Identification

Mitochondrial DNA regions are popular in identification



## Cytochrome c Oxidase I (CO1)

- Parodi et al. (2002): PCR fragment analysis
- Hebert et al. (2003): universal sequencing “barcode” region

# Fragment Analysis Assay



Target species and expected size fragments (tentative):

## Tier 1: Industry Focus Multiplex

Pig.....	480 bp
Human .....	393 bp
Chinese hamster.....	268 bp
African green monkey...	224 bp
Rat .....	206 bp
Dog.....	174 bp
Mouse.....	151 bp
Syrian hamster .....	124 bp
Bovine.....	103 bp

## Tier 2: Academic Focus Multiplex

Chicken.....	427 bp
Cat.....	378 bp
Guinea pig.....	335 bp
Rhesus monkey .....	289 bp
Horse.....	245 bp
Rabbit.....	138 bp

## Both Sets

Internal Control .....	70 bp
------------------------	-------

# Fragment Analysis Assay (early generation)

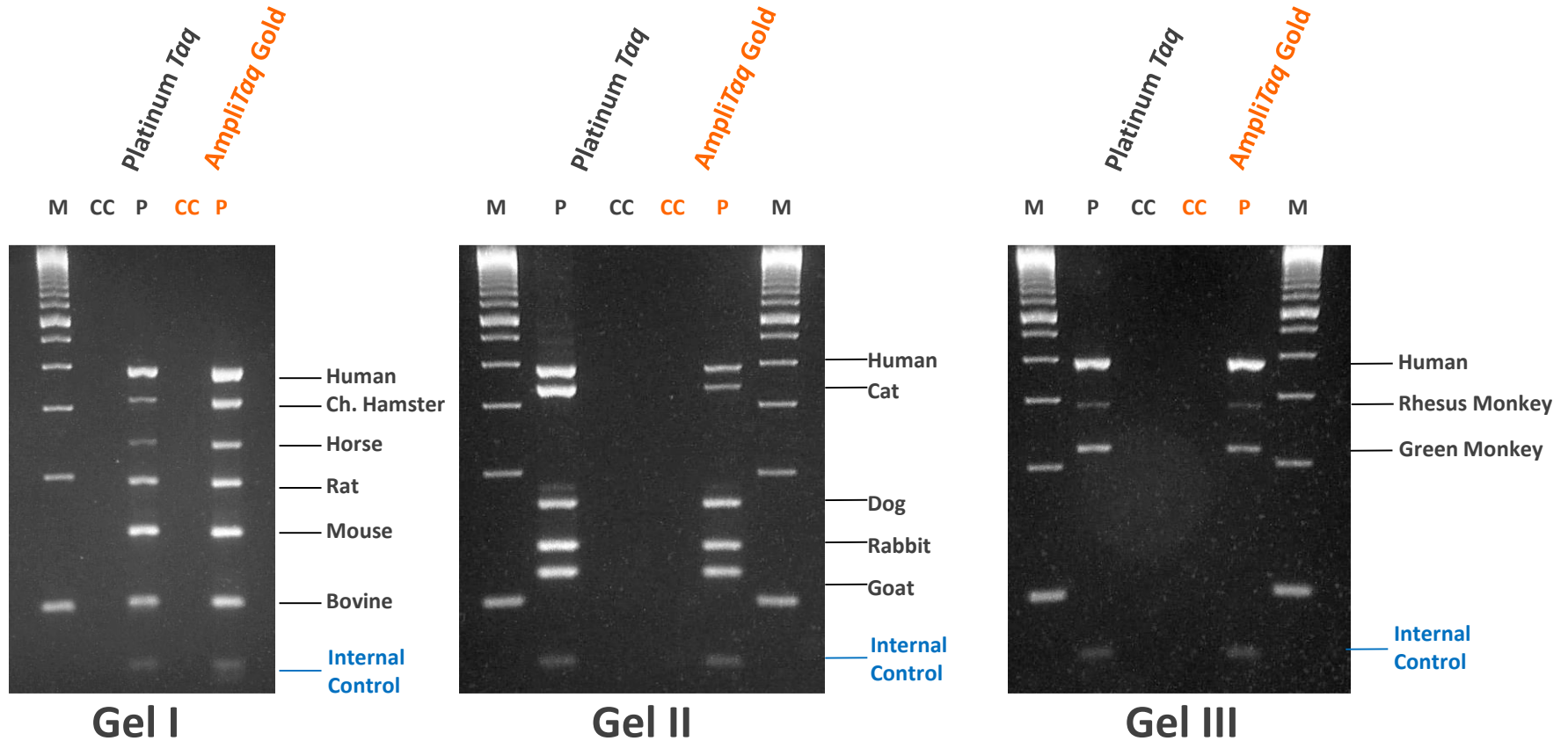


Figure 3. Three gels demonstrate how positive bands appear (P) along with the contamination check (CC). The marker (M) is a 100 bp ladder.

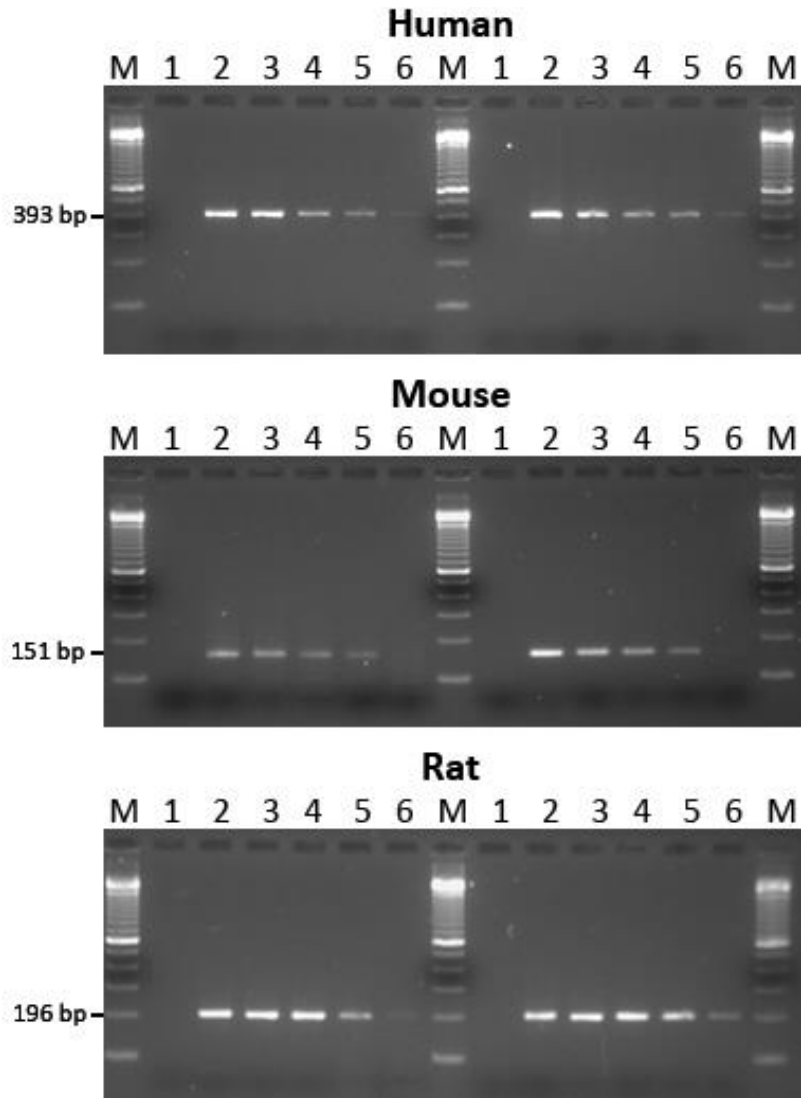
*Note: The Chinese hamster and rat bands here are from an earlier primer version; goat was later eliminated.*

# Fragment Analysis Assay Sensitivity (early generation)

Figure 4. Gels depicting CO1 Assay's sensitivity. It uses 5 ng of total genomic DNA per 25  $\mu$ L reaction. The rat band is from an earlier primer set.

## KEY

- 1)  $\emptyset$  control
- 2) 5.0 ng
- 3) 1.0 ng
- 4) 0.5 ng
- 5) 0.1 ng
- 6) 0.01 ng
- M) 100 bp



# Fragment Analysis Assay (early generation)

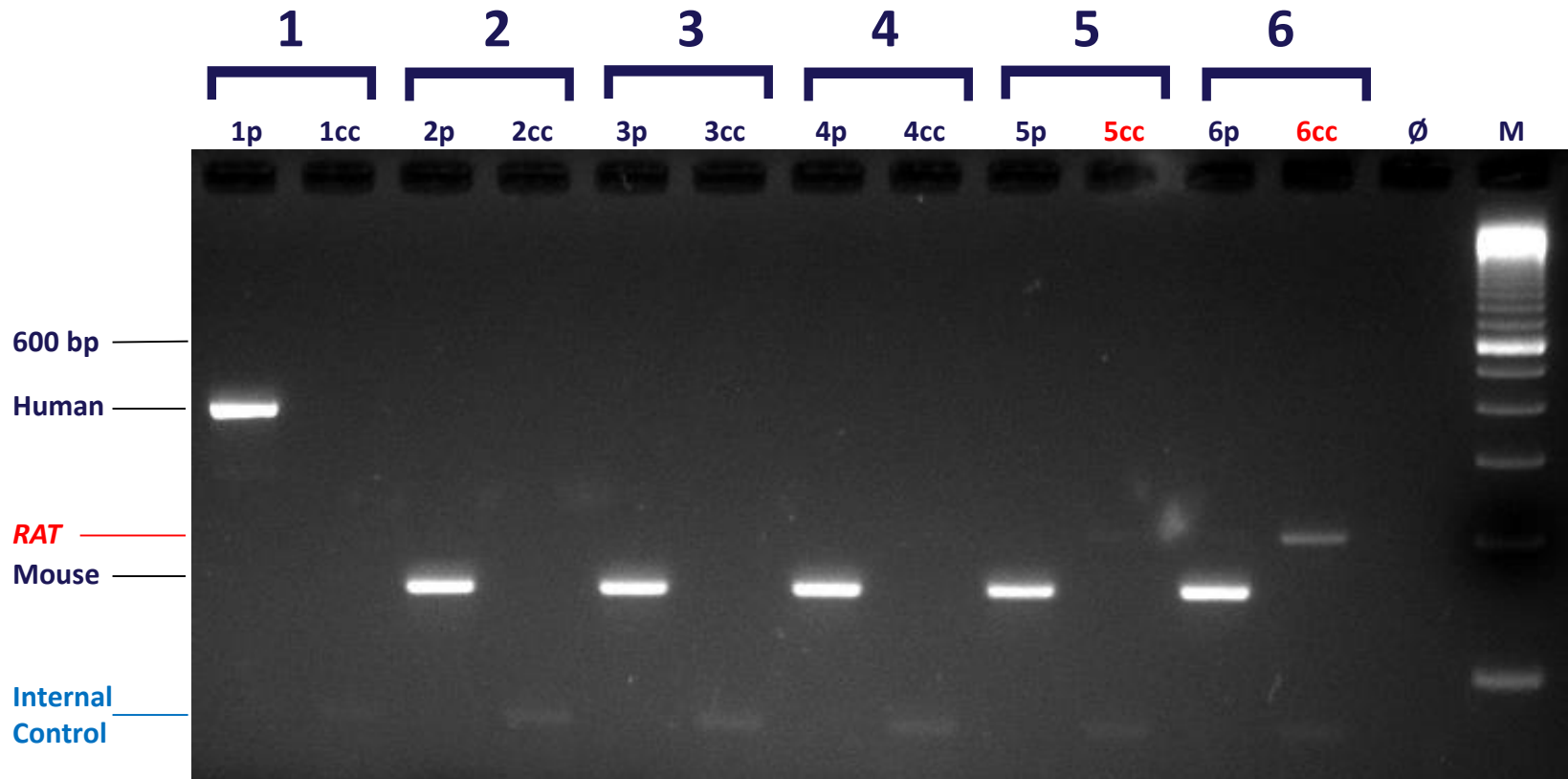


Figure 5. In this set, “p” PCRs contain the putative species’ primers and the “cc” contamination check has all primers except for the tested species. Set 1 tested for human; Sets 2-6 tested for mouse. Samples 5 and 6 are positive for mouse but also are contaminated with rat cells.



# Molecular-Based Species Identification



## **Designation: ASN-0003**

Species-Level Identification of  
Animal Cells through Mitochondrial  
Cytochrome *c* Oxidase Subunit 1  
(CO1) DNA Barcodes

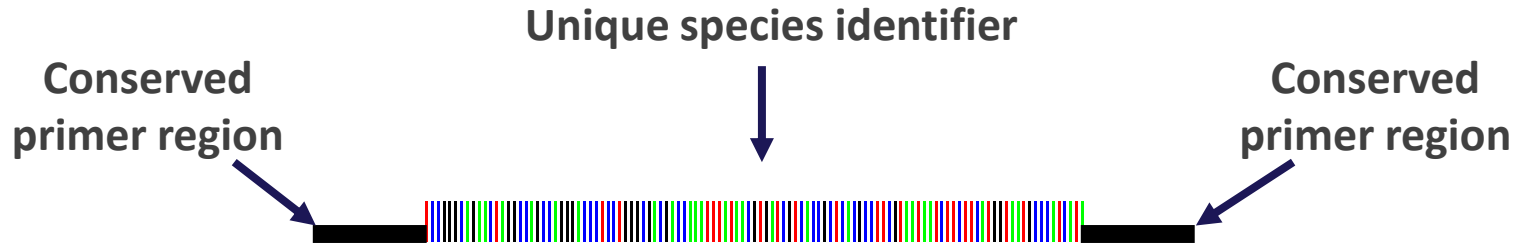
*– published –*

# The Genetic Barcoding Concept

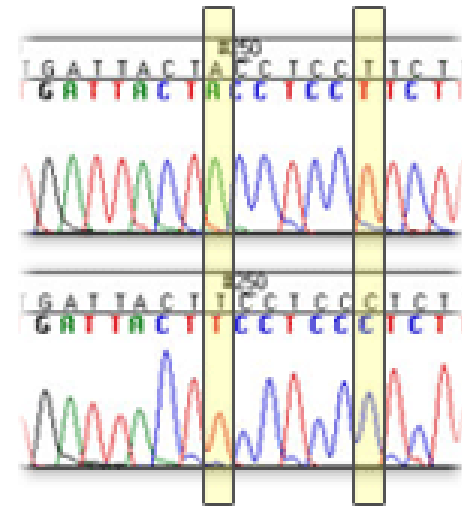
The Universal Product Code (UPC) system developed by the industrial sector employs 10 options at each of 12 positions to uniquely identify over 100 billion individual products.



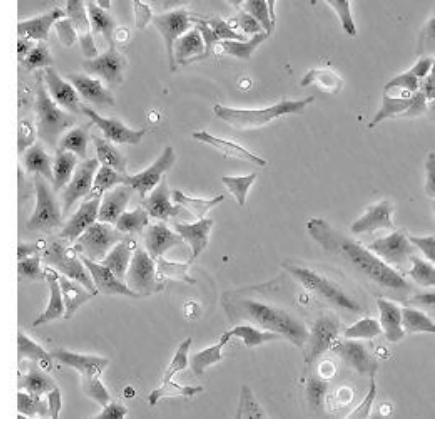
# The Genetic Barcoding Concept



Just like UPC barcodes, the DNA sequences within each species are unique.



# Applied Genetic Barcoding



## ATCC Collection

- Bacteria
- Fungi and Yeasts
- Protists
- *Animal Cells*

## Primary Molecular Target

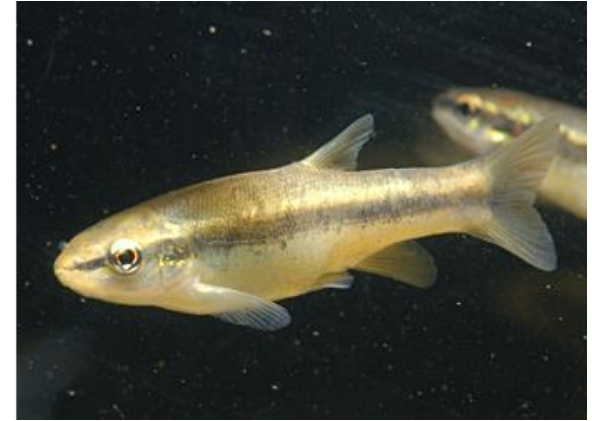
16S: 30S small ribosomal RNA subunit

ITS: Internal Transcribed Spacers;  
D1D2: large-subunit RNA gene

18S: small subunit nuclear ribosomal RNA

*CO1: cytochrome oxidase I*

# Consortium for the Barcode of Life (CBOL)



- The CBOL initiative
- Uses CO1 (648 bp)
- Major database efforts: insects, birds, and fish
- Websites:
  - Background: <http://www.barcodeoflife.org/>
  - Database Queries: <http://www.boldsystems.org/views/login.php>



# CBOL Search Query

IgH-2 (ATCC® CCL-108™)  
Organism: [iguana](#) /

GENERAL INFORMATION CHARACTERISTICS CULTURE METHOD HISTORY DOCUMENTATION

Permits and Restrictions [View Permits](#)

Organism	iguana
Product Format	frozen
Morphology	epithelial
Culture Properties	adherent
Biosafety Level	1
Age	immature
Gender	male
Storage Conditions	liquid nitrogen vapor phase

PCR and  
Sequencing

## CO1 Barcode for CCL-108

```
>CCL-108_sample
CACCCCTATACTTAGTCTTCGGTGCCTGAGCCGGCAGTTCGGAACTGCC
TCAGCCTGCTAATTCGAGCAGAACTCAGCCAGCCAGGGGCCCTTCTTGGT
GACGACCAAATTTACAACGTCATTGTAACCGCCCATGCCTTTGTTATAAT
TTTCTTCATAGTAATGCCCGTGATAATCGGAGGATTTGGAACTGATAG
TTCCCCATAAATCGGGCGCACCAGACATAGCCTTCCCCCGAATAAACAAC
ATAAGCTTCTGACTCCTACCCCATCCTTTCTACTCCTTTTAGCCTCCTC
TGGCATTGAAGCCGGAGCCGGCACAGGCTGAACTGTCTACCCCCACTAG
CGGGCAACCTAGCACACGCAGGCGCTTCAGTAGACCTTACAATTTTCTCC
CTCCACCTAGCCGGAATTCATCCATCCTAGGAGCAATCAACTTTATCAC
AACATCTATCAACATAAAGCCCCCTACAATAACCCAATATCAAACATCCT
TGTTTGTCTGATCCGTACTAATTACAGCCGTACTACTTCTGTTATCCCTA
CCCGTCTAGCAGCAGGTATTACAATACTACTCACCAGCCGCAACTTAAA
TACTTCATTCTTTGACCCGCGAGGAGGGGAGACCAATCCTTTACCAAC
ACTTATTT
```

# CBOL Search Query

www.boldsystems.org/index.php/IDS\_OpenIdEngine

**BOLD SYSTEMS** Databases | Taxonomy | Identification | Workbench | Resources Log In

## Identification Request Print

**Animal Identification [COI]** | **Fungal Identification [ITS]** | **Plant Identification [rbcL & matK]**

The BOLD Identification System (IDS) for COI accepts sequences from the 5' region of the mitochondrial Cytochrome c oxidase subunit I gene and returns a species-level identification when one is possible. Further validation with independent genetic markers will be desirable in some forensic applications.

**Historical Databases:** [Jul-2015](#) [Jul-2014](#) [Jul-2013](#) [Jul-2012](#) [Jul-2011](#) [Jul-2010](#) [Jul-2009](#)

Search Databases:

- All Barcode Records on BOLD (4,737,393 Sequences)**  
Every COI barcode record on BOLD with a minimum sequence length of 500bp (warning: unvalidated library and includes records without species level identification). This includes many species represented by only one or two specimens as well as all species with interim taxonomy. This search only returns a list of the nearest matches and does not provide a probability of placement to a taxon.
- Species Level Barcode Records (2,669,681 Sequences/174,315 Species/64,199 Interim Species)**  
Every COI barcode record with a species level identification and a minimum sequence length of 500bp. This includes many species represented by only one or two specimens as well as all species with interim taxonomy.
- Public Record Barcode Database (997,762 Sequences/83,882 Species/18,032 Interim Species)**  
All published COI records from BOLD and GenBank with a minimum sequence length of 500bp. This library is a collection of records from the published projects section of BOLD.
- Full Length Record Barcode Database (1,676,755 Sequences/157,283 Species/55,678 Interim Species)**  
Subset of the Species library with a minimum sequence length of 640bp and containing both public and private records. This library is intended for short sequence identification as it provides maximum overlap with short reads from the barcode region of COI.

Enter sequences in fasta format:

```
>CCL-108_sample
CACCCCTACTTAGTCCTCGGTGCCTGAGCCGGCATAGTCGGAACTGCC
TCAGCCTGCTAATTGGAGCAGAAGCTCAGCCAGCCAGGGGCCCTTCTGGI
GACGACCAAATTTACAACGCTATTGTAACCCGCCATGCCCTTTGTATAAT
TTTCTTCATAGTAATGCCCGTGAATAATCGGAGGATTTGGAACTGATTAG
TTCCCTAATAATCGGGCCACAGACATAGCCCTCCCGGAAATAACAAC
ATAAGCTTCTGACTCTACCCCATCCCTTCTACTCCTTTAGCCCTCCTC
TGGCATTGAAGCCGGAGCCGACAGGCTGAAGTGTCTACCCGCCACTAG
CGGGCAACCTAGCACAGCGAGGGCTTCAGTAGACCTTACAATTTCTCC
CTCCACCTAGCCGGAATCTCATCCATCTAGGAGCAATCAACTTTATCAC
AACATCTATCAACATAAAGCCCTCAGCAATAACCCAATATCAAGATCCT
TGTITGCTGATCCGTACTAATTACAGCCGTAAGTCTTCTGTTATCCCTA
CCCGTCTAGCAGCAGGTATTACAATCTACTCACCAGCCGCAACTTAA
TACTTCTATTCTTTGACCCCGCAGGAGGGGGAGACCAATCCTTTACCAAC
ACTTATT
```

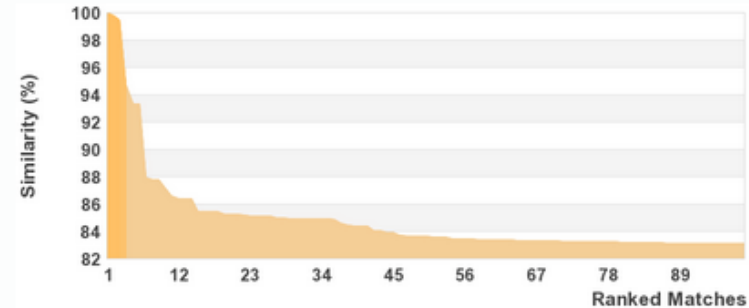
Submit

# CBOL Search Results

## Identification Summary:

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Chordata	100
Class	Reptilia	100
Order	Squamata	100
Family	Iguanidae	100
Genus	Iguana	100
Species	Iguana iguana	100

## Similarity Scores of Top 99 Matches:



## TOP 20 Matches :

Display option:

Phylum	Class	Order	Family	Genus	Species	Similarity (%)	Status
Chordata	Reptilia	Squamata	Iguanidae	Iguana	<i>iguana</i>	100	Private
Chordata	Reptilia	Squamata	Iguanidae	Iguana	<i>iguana</i>	99.82	Private
Chordata	Reptilia	Squamata	Iguanidae	Iguana	<i>iguana</i>	99.44	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Iguana	<i>iguana</i>	94.75	Private
Chordata	Reptilia	Squamata	Iguanidae	Iguana	<i>iguana</i>	93.36	Early-Release
Chordata	Reptilia	Squamata	Iguanidae	Iguana	<i>iguana</i>	93.33	Private
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>ater</i>	87.96	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>ater</i>	87.81	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>ater</i>	87.81	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>varius</i>	87.19	Early-Release
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>ater</i>	86.57	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>ater</i>	86.42	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	<i>ater</i>	86.42	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Amblyrhynchus	<i>cristatus</i>	86.42	Private
Chordata	Reptilia	Squamata	Iguanidae	Amblyrhynchus	<i>cristatus</i>	85.45	Published <a href="#">↗</a>
Chordata	Reptilia	Squamata	Iguanidae	Amblyrhynchus	<i>cristatus</i>	85.45	Published <a href="#">↗</a>

# Genetic Barcoding Catches a Misidentification

The ATCC website lists horse...

Horse (ATCC® CRL-6583™)

Organism: *Equus caballus, horse* / Tissue: **unknown** /

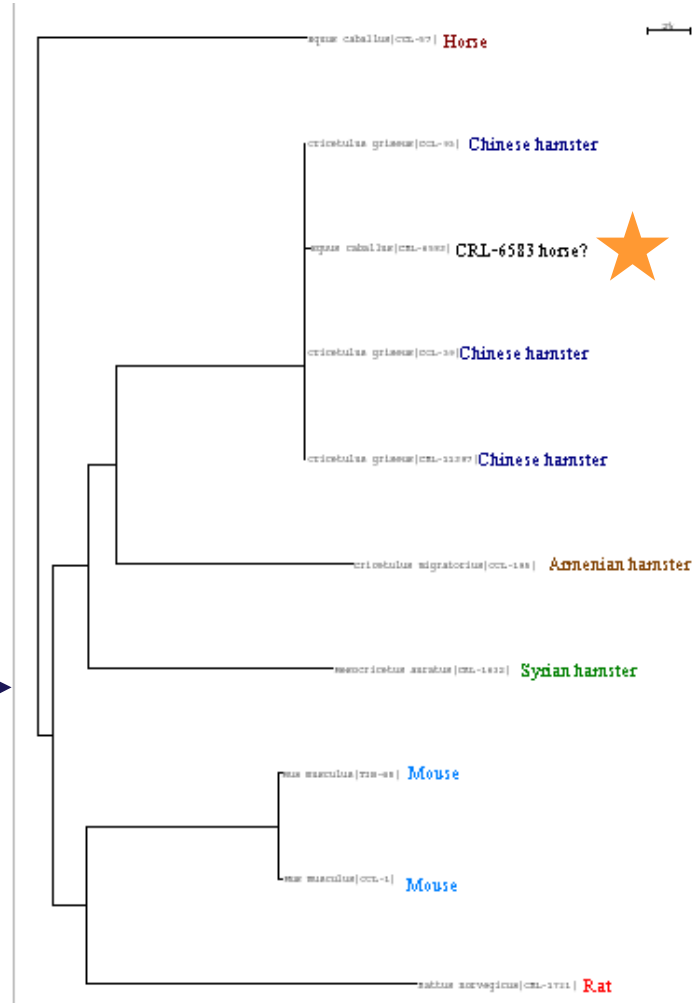
GENERAL INFORMATION CHARACTERISTICS CULTURE METHOD HISTORY DOCUMENTATION

Permits and Restrictions [View Permits](#)

Organism	<i>Equus caballus, horse</i>
Tissue	unknown
Product Format	flask
Biosafety Level	1

...but DNA Barcode tests show that ATCC® CRL-6583™ is actually Chinese hamster. →

**NOTE: CRL-6583 is part of the NBL collection, neither produced nor authenticated by ATCC.**



# Genetic Barcoding





# Genetic Barcoding Cautions

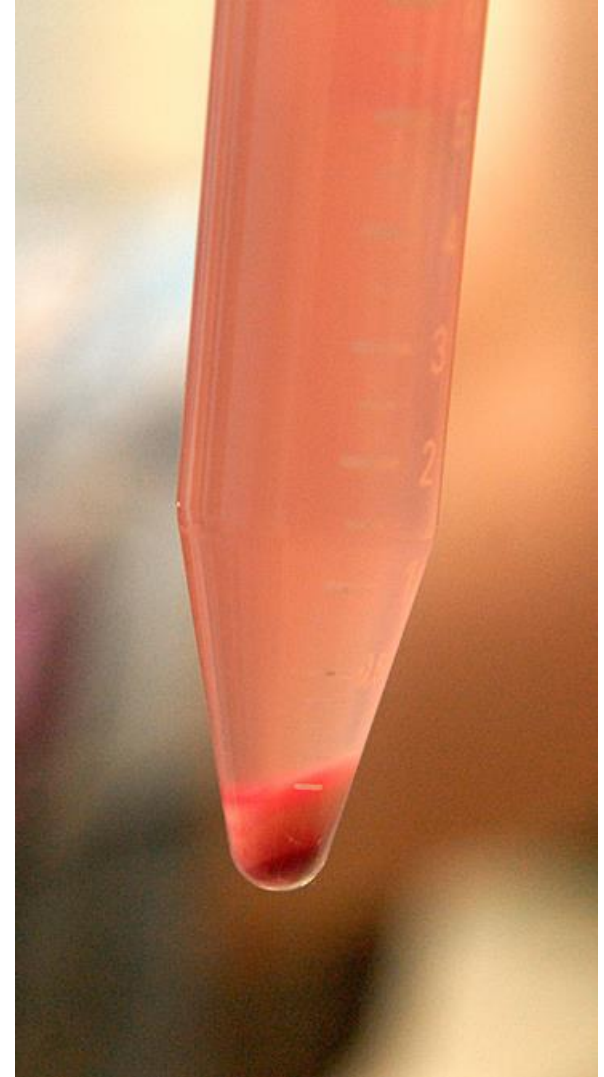
## Cell Culture Mixes and Low Level Contamination



# *EXTREMELY IMPORTANT: AUTHENTICATION*

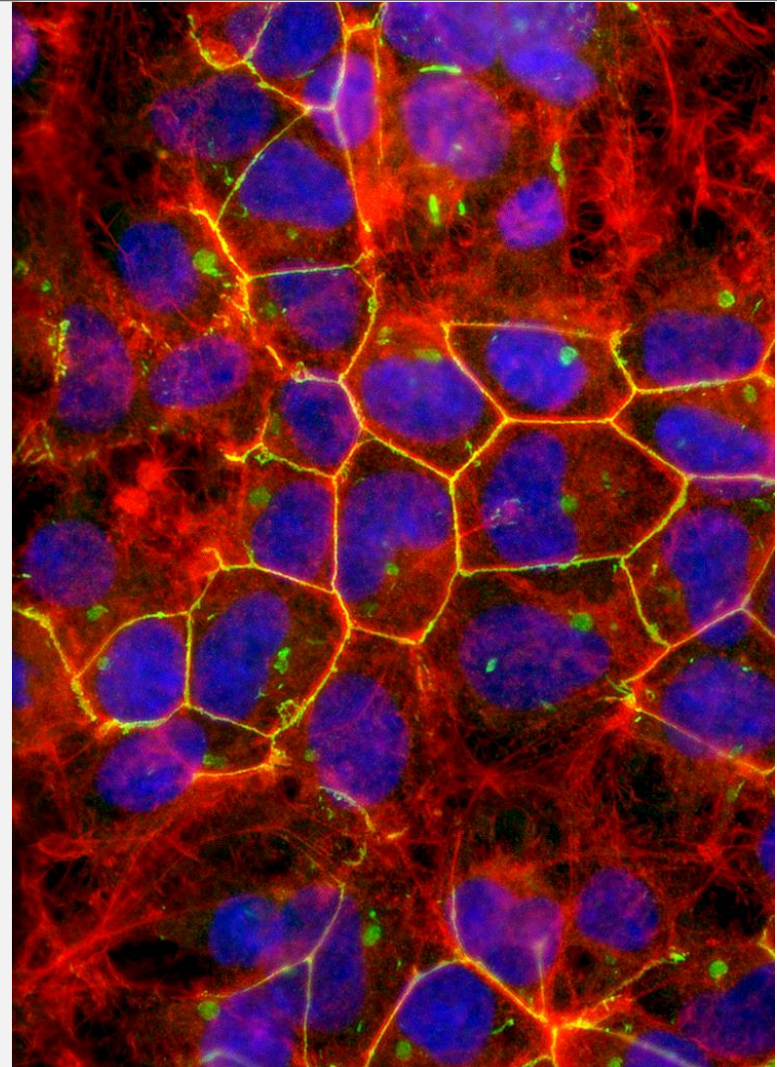
**AUTHENTICATE  
NOW!**

Use only authenticated cell lines  
in all of your research.



# Summary

- ATCC is a biorepository performing cell line authentication
- Genetic methods are preferable species determination approaches
- PCR-based CO1 Assay + DNA Barcoding = complimentary approaches to identify nearly every animal species
- **Use authenticated cell lines**
- Further reading:  
Cooper J, *et al.* 2007. Species identification in cell culture: a two-pronged molecular approach. *In Vitro Cell Dev Biol – Animal* 43(10):344-351.







# Acknowledgements

Jason Cooper

Pranvera Ikonomi

Maryellen de Mars

Yvonne Reid

Karin Kindig

Trudy Correia

Steve King

Balsam Shawky

# *Thank You*