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

Title Slide

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

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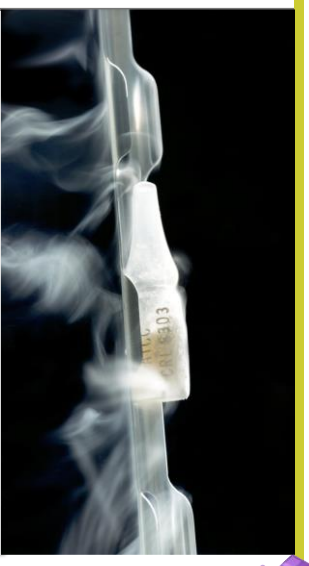
This presentation will have a brief introduction to the ATCC, discuss the need for species identification in cell culture, cover some of the historical approaches used in species determination, detail current DNA-based species identification employed at ATCC, and drive home the need to authentic all materials studied.

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



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Additional comments:

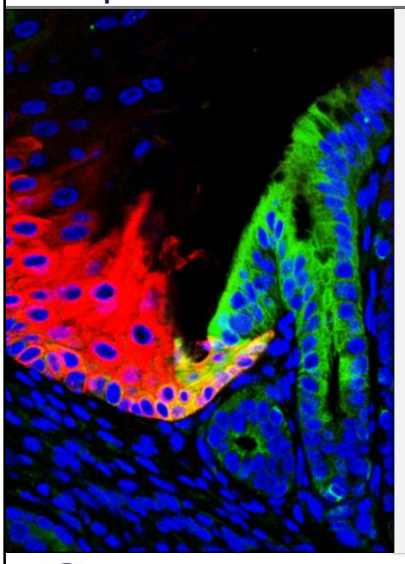
•Provides the essentials of life science research to the global scientific community – 145 countries in 2010

•Development, assembly, and global delivery of critical reagents

•ATCC scientists conduct diverse research that allows us to respond to public health issues (e.g., swine flu diagnostic kits during the outbreak)

•ISO 9001certified and ISO Guide 34 accredited; ANSI accredited SDO

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!

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Image Source: courtesy of Xia Wang, www.cellimagelibrary.org/images/36293

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Other molecular-based detection techniques of *Mycoplasma*, viruses, and species outside of the Animal Kingdom are not addressed in this talk

- One slide will place animal DNA barcoding into context of barcoding other major taxonomical groups

Reasons for species identification:

- Part of cell line authentication
- Verify the putative species without having the whole animal available for morphological confirmation
- Check for cross-contamination and misidentification
- Example: Virus Host—Confirm that only the proper viral host cell is present

Complete reference:

Hukku, Bharati, David M. Halton, Michael Mally, and, Ward D. Peterson Jr. 1984. Cell characterization by use of multiple genetic markers. *Advances in Experimental Medicine and Biology* 174:13-31.

Image (http://www.cellimagelibrary.org/images/36293) is of mouse Barret's esophagus cells near ES junction by Wang et al. (June 2011) Residual embryonic cells as precursors of a Barrett's-like metaplasia. Cell *24;145(7):1023-35.*

Historical Technique: Karyotyping

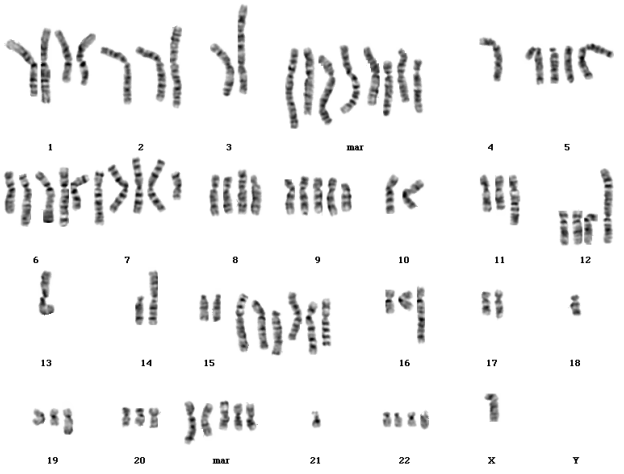

Figure 1. This complex karyogram of human cell line SJCRH30 (ATCC® CRL-2061™) is n=84, a significant departure from normal human n=46.

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Karyotyping Pros and Cons:

Pros

•Able to distinguish certain cultured species and even individual cell cultures within a species

Cons

•Growing the cells and preparing the slides takes a long time

•Data analysis requires a large amount of expertise

•Resolving closely related species may be difficult, especially when chromosome abnormalities are present

Historical Technique: Isoenzyme Tests

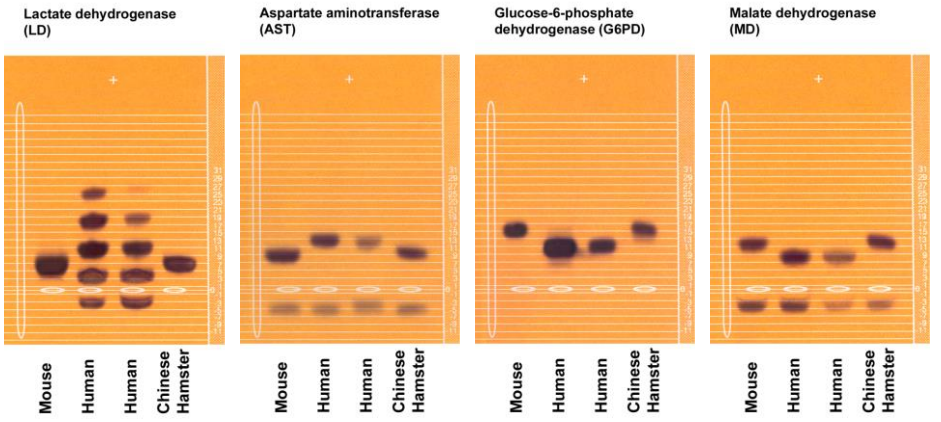

Figure 2. Four of the seven isoenzyme tests are seen here. Resolving related species may be challenging.

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Isoenzyme (aka "Isozyme") Pros and Cons:

Pros

- Able to distinguish certain cultured species
- Requires basic laboratory equipment
- Enduring technique used for over 30 years

Cons

- Low sensitivity, requiring large cell counts (~5.0×10^6 cells)
- Cell preparation can only be used for isoenzyme analysis
- Reliant upon protein expression
- Requires a fair amount of expertise
- Gels may be difficult to interpret or resolve
- Resolving closely related species may be difficult if not impossible
- Turnaround time: 3 days + required time for cell growth
- Adding to the time is the frequent need to repeat the assay for a given protein test(s)—makes for an extremely long assay and unpopular with many laboratory technicians!
- <u>No longer commercially available</u>

The Big Question:

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

What do I do now?

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Molecular-Based Species Identification

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Designation: ASN-0004

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

– in progress –

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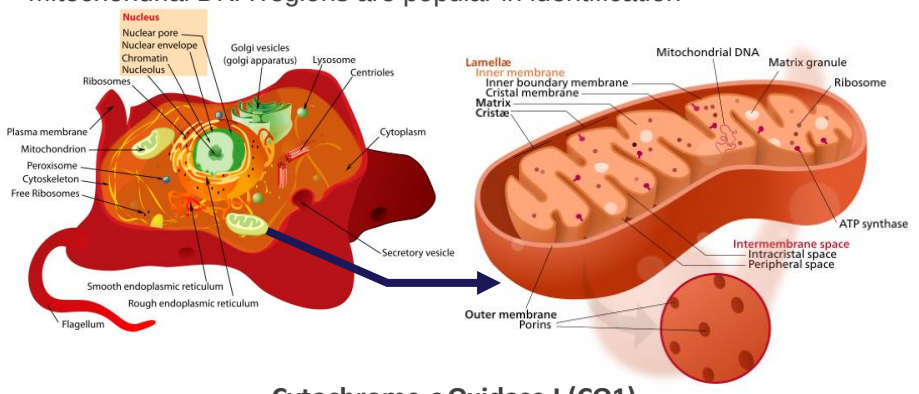
Molecular-Based Species Identification

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

Image Sources: https://en.wikipedia.org/wiki/Cell_(biology); https://en.wikipedia.org/wiki/Mitochondrion

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Most research targets conservative mitochondrial DNA regions

- All animal cells have mitochondria
- Animal cells usually have hundreds or thousands of mitochondria in each cell, depending on species and cell type
  - Each animal cell mitochondrion has usually 5-10 copies of the mitochondrial genome
  - Many mitochondria and multiple copies of the genome result in many more gene copies available for targeted detection compared to a normal diploid nuclear genomic DNA target
- Small (~16.5 kb), well-described genome
- Mitochondrial genes lack introns, have limited exposure to recombination and have a haploid mode of inheritance
- Coding genes have few insertions or deletions (indels) that complicate sequence alignments

Parodi et al. (2002) published nine *fragment* primer sets targeting cytochrome c oxidase subunit I (CO1) and cytochrome b genes

- Good for a few known species

Hebert et al. (2003) published universal *sequencing* primers targeting CO1 to genetically "barcode" the Animal Kingdom

- Broad species identification platform using 648 bp sequence

Pros

- Requires small number of cells (direct testing from the ampoule, cell pellet, or other original source)
- DNA preparations may be used for other genetic procedures and stored indefinitely
- Turnaround time: ~2 days
- Low cost

Cons

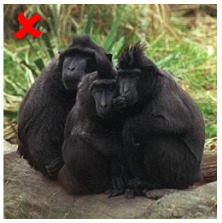
- Hybrid cell lines may detect only one species if nuclear transfer was involved
- Fused cells (e.g., mouse-human) may appear contaminated

Complete references:

Hebert Paul D.N., Alina Cywinska, Shelley L. Ball, and Jeremy R. deWaard. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Science* 270(1512):313-321.

Parodi B., O. Aresu, D. Bini, R. Lorenzini, F. Schena, P. Visconti, M. Cesaro, D. Ferrera, V. Andreotti, and T. Ruzzon. 2002. Species identification and confirmation of human and animal cell lines: a PCR-based method. *Biotechniques* 32(2):432–440.

Fragment Analysis Assay

Target species and expected size fragments (tentative):

Tier 1: Industry Focus Multiplex		Tier 2: Academic Focus Multiplex	
Pig	480 bp	Chicken	427 bp
Human	393 bp	Cat	378 bp
Chinese hamster	268 bp	Guinea pig	335 bp
African green monkey	224 bp	Rhesus monkey	289 bp
Rat	206 bp	Horse	245 bp
Dog	174 bp	Rabbit	138 bp
Mouse	151 bp		
Syrian hamster	124 bp	**Both Sets**	
Bovine	103 bp	Internal Control	70 bp

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PCR components:

- Template
- PCR buffers and *Taq* polymerase
- Primer mixes and internal control
  - Confirmation set contains primers targeting the putative species
  - Contamination check has all primers except for the putative species
- IC (targets the 18S gene)
- Appropriate controls

Fragment Analysis Assay (early generation)

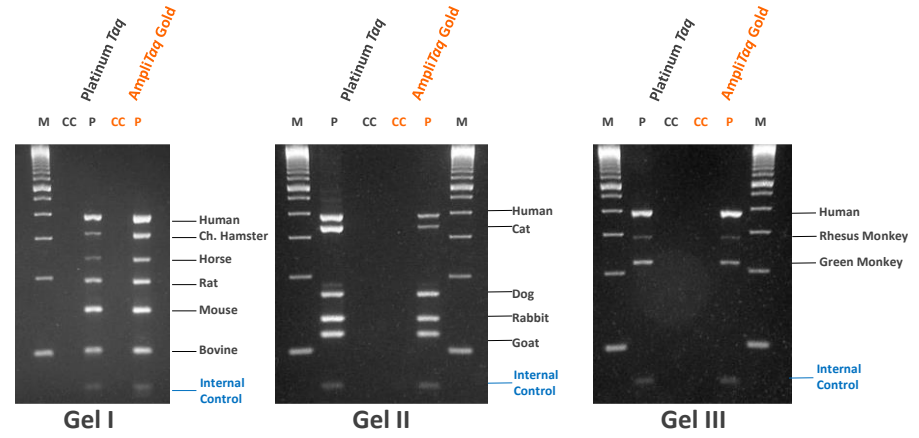

Figure 3. Three gels demonstrate how positive bands appear (P) along with the contamination check (CC). The marker (M) is a 100 bp ladder.

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

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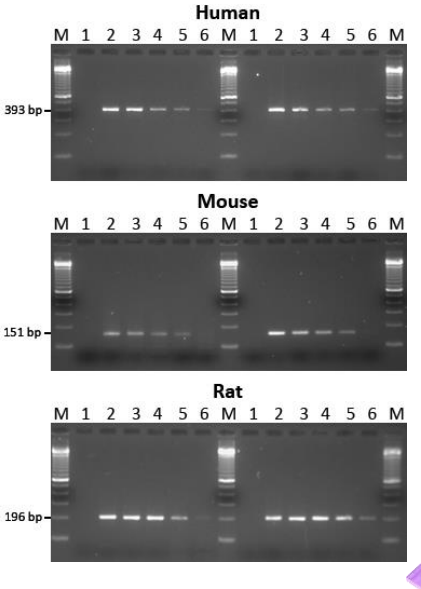
Two different *Taq* polymerases were tested.

Fragment Analysis Assay Sensitivity (early generation)

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

KEY
1) Ø control
2) 5.0 ng
3) 1.0 ng
4) 0.5 ng
5) 0.1 ng
6) 0.01 ng
M) 100 bp



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Sensitivity amongst the primers is sufficiently similar; we must consider that unlike normal nuclear DNA, mitochondria numbers vary based on cell type.

Fragment Analysis Assay (early generation)

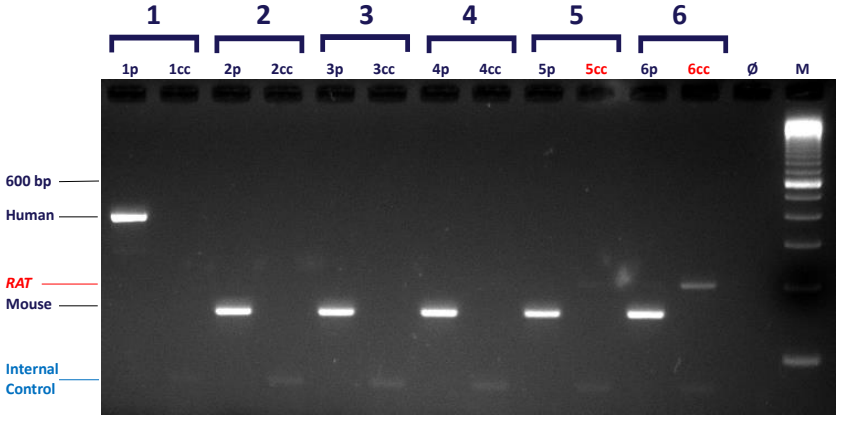

Figure 5. In this set, "p" PCRs contain the putative species' primers and the "cc" contamination check has all primers except for the tested species. Set 1 tested for human; Sets 2-6 tested for mouse. Samples 5 and 6 are positive for mouse but also are contaminated with rat cells.

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Pros

- Multiplex PCR identifies animal species common in cell research
- High sensitivity
- Able to detect low levels or percentages of a contaminating species
- Can view data on agarose gels or genetic analyzers
- Affordable, using common molecular equipment and supplies

Cons

- Limited to detecting 15 species
- A contaminating cell line from a species outside of these 15 may go undetected
- Multiple master mixes needed for contamination check

Molecular-Based Species Identification

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Designation: ASN-0003

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

– published –

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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.

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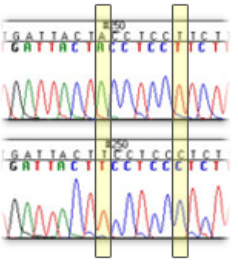
The Genetic Barcoding Concept

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



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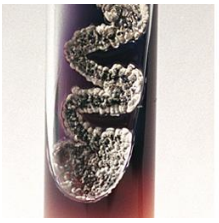
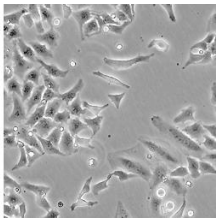
In theory, the sequence analysis of a gene segment of the genome will permit the reliable diagnosis of most species.

Applied Genetic Barcoding



ATCC Collection	**Primary Molecular Target**
▪ Bacteria	16S: 30S small ribosomal RNA subunit
▪ Fungi and Yeasts	ITS: Internal Transcribed Spacer; D1D2: large-subunit RNA gene
▪ Protists	18S: small subunit nuclear ribosomal RNA
▪ ***Animal Cells***	***CO1: cytochrome oxidase I***

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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

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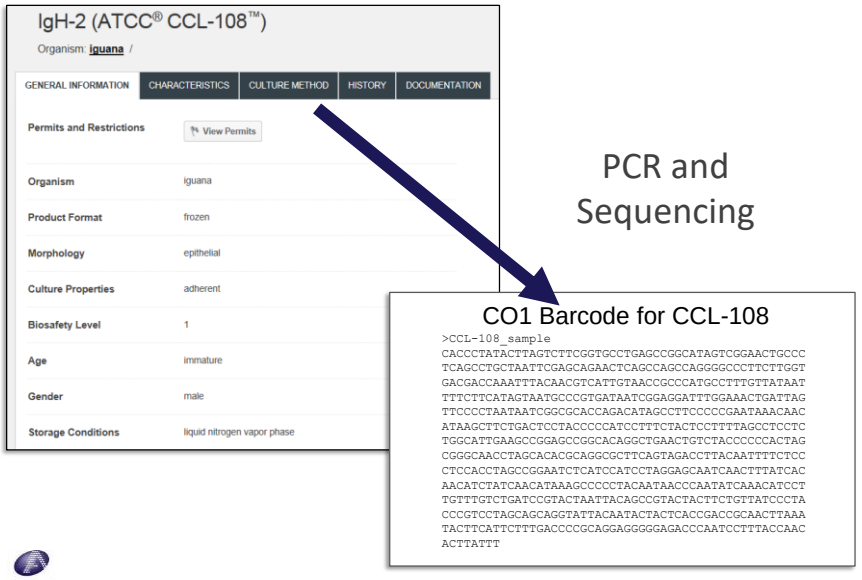
The **CBOL** is an international initiative devoted to developing DNA barcoding using CO1 (**648 bp**)

CBOL includes natural history museums, zoos, research organizations, governmental and intergovernmental agencies, private companies, and other organizations involved in taxonomic research and biodiversity issues

Database reference data are from voucher specimens

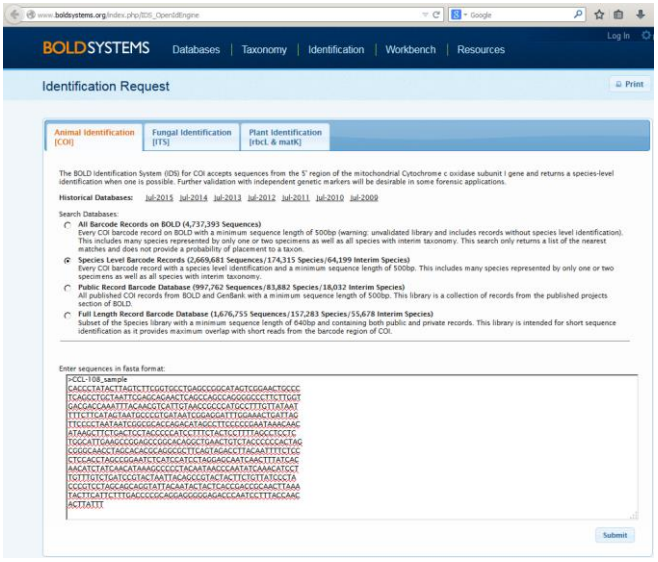
- The CBOL database uses quality-score sequence data in its references and can include voucher-derived specimens.

CBOL Search Query



In this example, CCL-108 is amplified and sequenced with barcode primers.

CBOL Search Query



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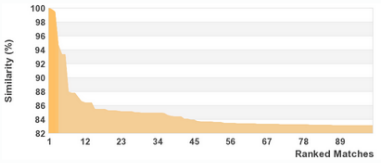
The sequence data are uploaded into the CBOL query field.

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: Top 20

Phylum	Class	Order	Family	Genus	Species	Similarity (%)	Status
Chordata	Reptilia	Squamata	Iguanidae	Iguana	*iguana*	100	Private
Chordata	Reptilia	Squamata	Iguanidae	Iguana	*iguana*	99.82	Private
Chordata	Reptilia	Squamata	Iguanidae	Iguana	*iguana*	99.44	Published
Chordata	Reptilia	Squamata	Iguanidae	Iguana	*iguana*	94.75	Private
Chordata	Reptilia	Squamata	Iguanidae	Iguana	*iguana*	93.36	Early-Release
Chordata	Reptilia	Squamata	Iguanidae	Iguana	*iguana*	93.33	Private
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*ater*	87.96	Published
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*ater*	87.81	Published
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*ater*	87.81	Published
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*varius*	87.19	Early-Release
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*ater*	86.57	Published
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*ater*	86.42	Published
Chordata	Reptilia	Squamata	Iguanidae	Sauromalus	*ater*	86.42	Published
Chordata	Reptilia	Squamata	Iguanidae	Amblyrhynchus	*cristatus*	86.42	Private
Chordata	Reptilia	Squamata	Iguanidae	Amblyrhynchus	*cristatus*	85.45	Published

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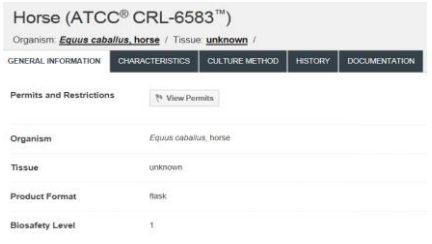
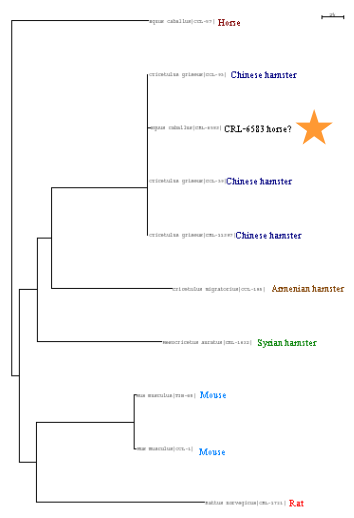
CBOL report confirms that CCL-108 is *Iguana iguana* (green iguana).

Genetic Barcoding Catches a Misidentification

The ATCC website lists horse…



…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.

https://www.atcc.org/Support/Technical_Support/About_NBL_Collection.aspx

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CRL-6583 is listed as horse but the neighbor joining tree analysis shows it to be Chinese hamster.

Special note on ATCC's NBL Collection (from https://www.atcc.org/Support/Technical_Support/About_NBL_Collection.aspx):

> ***Because these cell lines are neither fully characterized nor accessioned by ATCC, we do not warrant them to the same extent that we warrant our other cell lines. ATCC does not guarantee they will maintain a specific morphology, purity, or any other property upon passage. The information provided on these cell lines was not generated nor warranted by ATCC. As noted in the Material Transfer Agreement, it is the purchaser's responsibility to assess and interpret this information in consideration of the use, selection, application, or suitability of these cell lines.***

Accurately identifies animal species using a standard locus (CO1)
DNA Barcoding may be applied to any animal tissue

Genetic Barcoding Cautions

Cell Culture Mixes

and

Low Level Contamination

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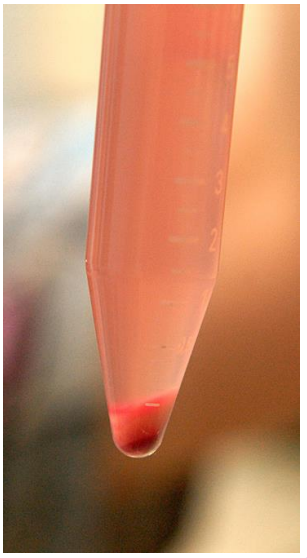
Originally designed for whole animals, low level cell contaminations may go unnoticed
Lower animals (jellyfish, coral, etc.) are difficult to resolve

EXTREMELY IMPORTANT: **AUTHENTICATION**

AUTHENTICATE NOW!



Use only authenticated cell lines in all of your research.

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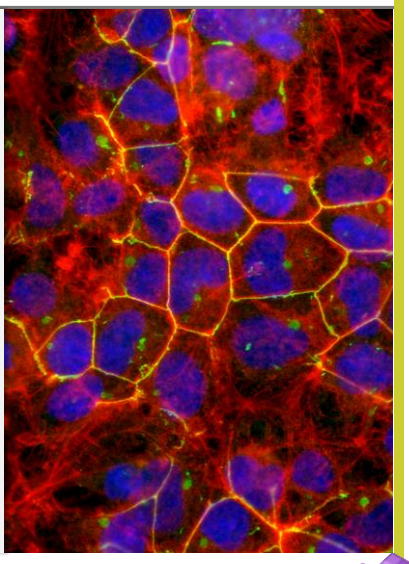
Use only authenticated cell lines in all of your research

- Obtain tested and verified material from a reputable source
- Check continuously passaged cell lines routinely
- When creating a new cell line, immediately establish its baseline profile upon which all future confirmations will be compared
    - If possible, obtain material other than the studied biopsy from the donor (e.g., blood, buccal cells) as a secondary reference

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.



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Several species determination methods exist but genetic approaches are relatively quick, affordable, and extremely accurate
CO1 Assay enables cross-contamination check from common animal origins

Image is of HEK-293 (ATCC® CRL-1573™) cells stained for ZOI and phalloidin

Jason Cooper…….. Barcode R&D, CO1 tech transfer, barcoding demo slides contributor
Pranvera Ikonomi… supervised Barcode and CO1 development
Maryellen de Mars……Senior Director, Standards Resource Center
Yvonne Reid……initiated the CO1 pilot project
Karin Kindig……. CO1 service development
Trudy Correia……Barcode and CO1 technician
Steve King…….. Barcode and CO1 technician
Balsam Shawky…… CO1 standard technician