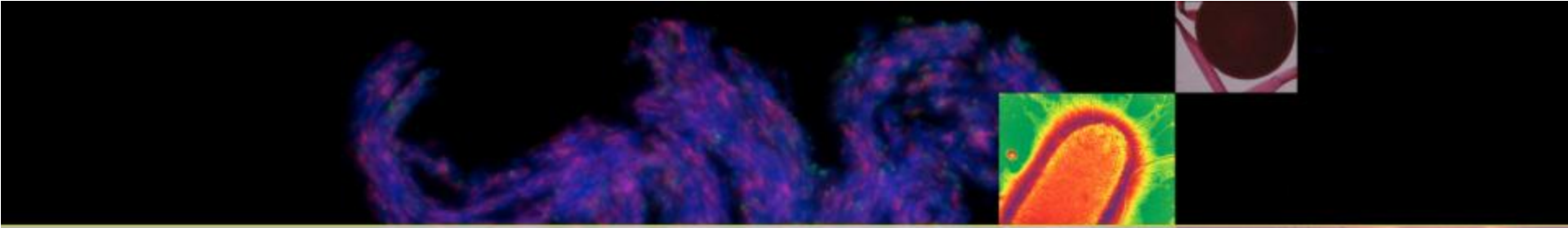

AUTHENTICATION AND CHARACTERIZATION OF ANIMAL CELL LINES: TOWARDS BEST PRACTICES IN CELL CULTURE

Yvonne A. Reid, Ph.D.

Manager/Scientist, Cell Biology Program, ATCC

February 27th 2014

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- Founded in 1925, ATCC is a non-profit organization with headquarters in Manassas, VA
- ATCC serves and supports the scientific community with industry-standard products and innovative solutions
- World's leading biological resource center and provider of biological standards
- Broad range of biological materials
  - Microorganisms
  - Cell lines
  - Derivatives
  - Bioproducts

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Outline

- Monitoring cell morphology
- Optimizing cell growth
- Applying the ‘Seed Stock’ concept
- Using cells at low passage or PDL
- Checking for microbial contamination
- Confirming species (misidentification or cross-contamination)
- Confirming characteristics/functionality
- History of misidentified cell lines – impact on research
- Reducing cellular and microbial contamination
- Receiving a cell line into the laboratory

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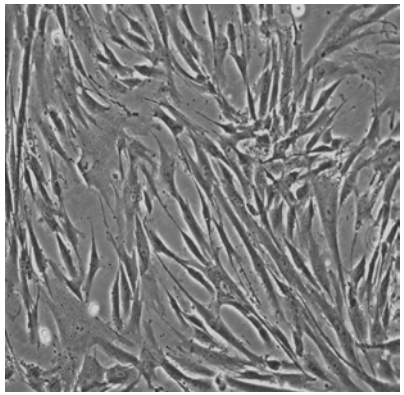
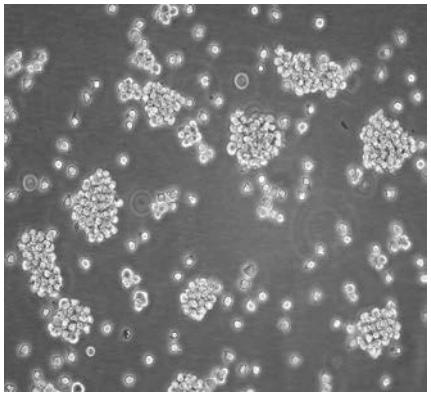
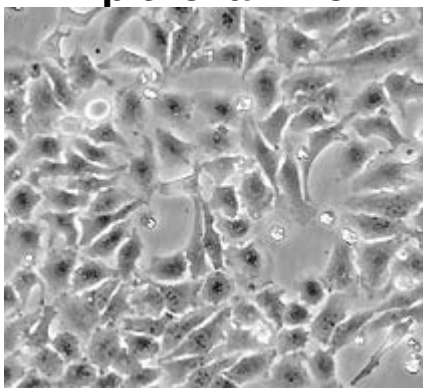
Monitoring cell morphology

Fibroblast-like	Lymphoblastoid-like	Epithelial-like	Senescing cells
			
Adherent	**Suspension**	**Adherent**	β- galactosidase

- **Monitor morphology:** shape, membrane structure, optical properties – at various stages of cell growth
  - **Detecting senescing cells:** larger, vacuolated, more heterogeneous
  - **Detecting overt microbial contamination**

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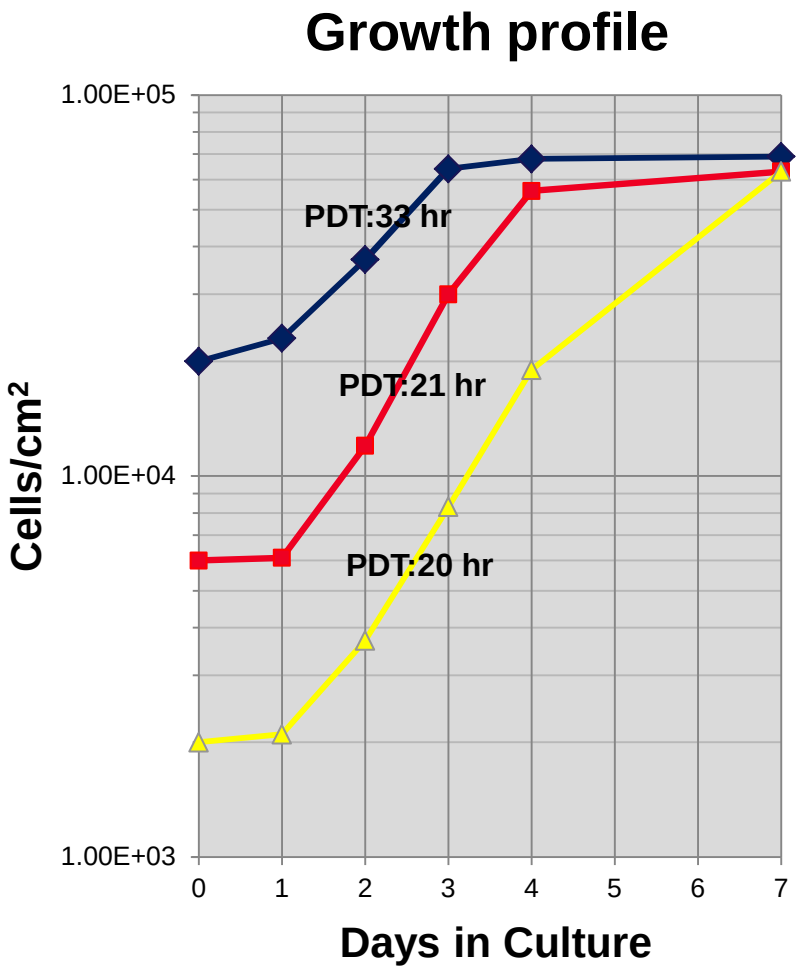
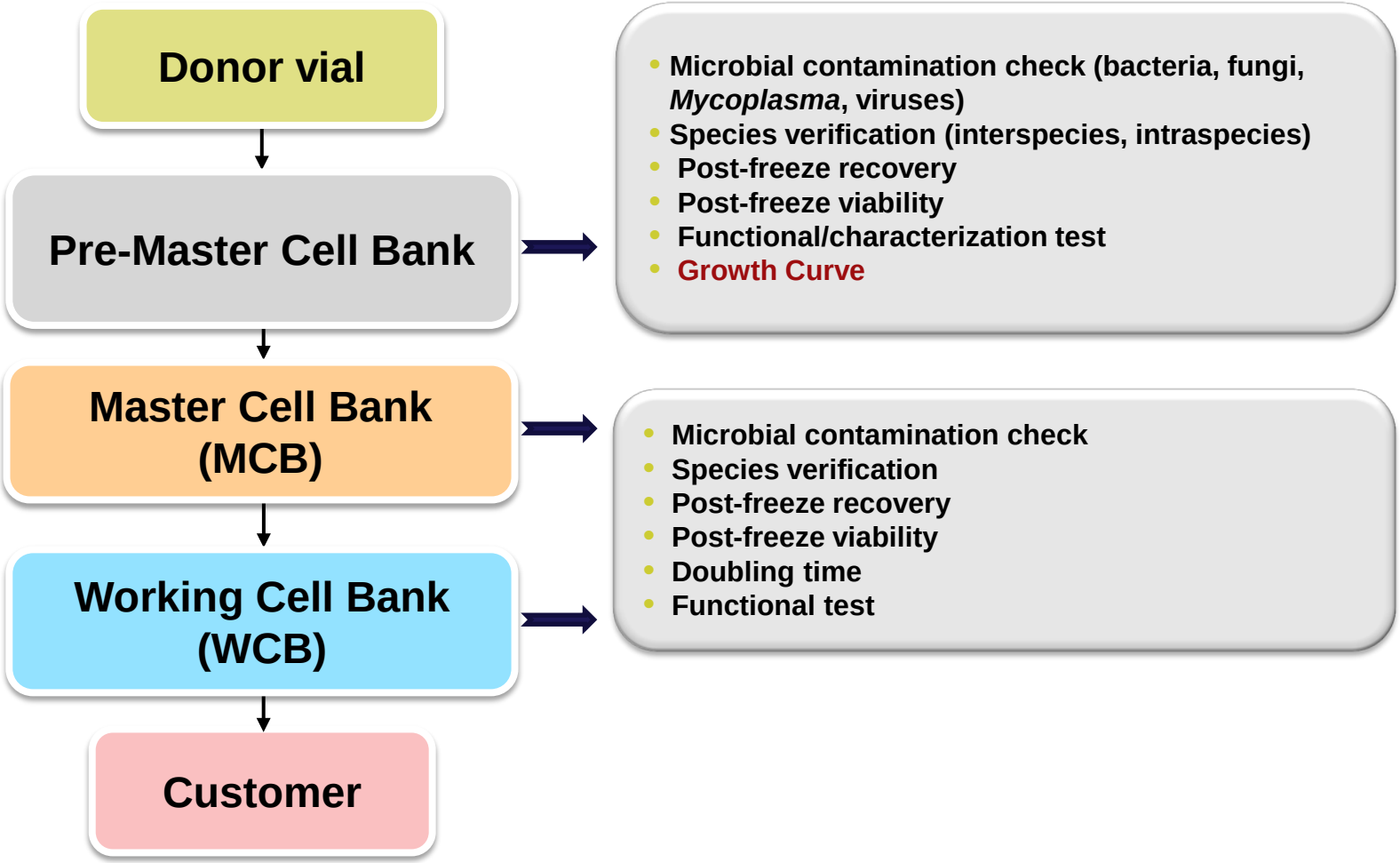
Optimizing cell growth

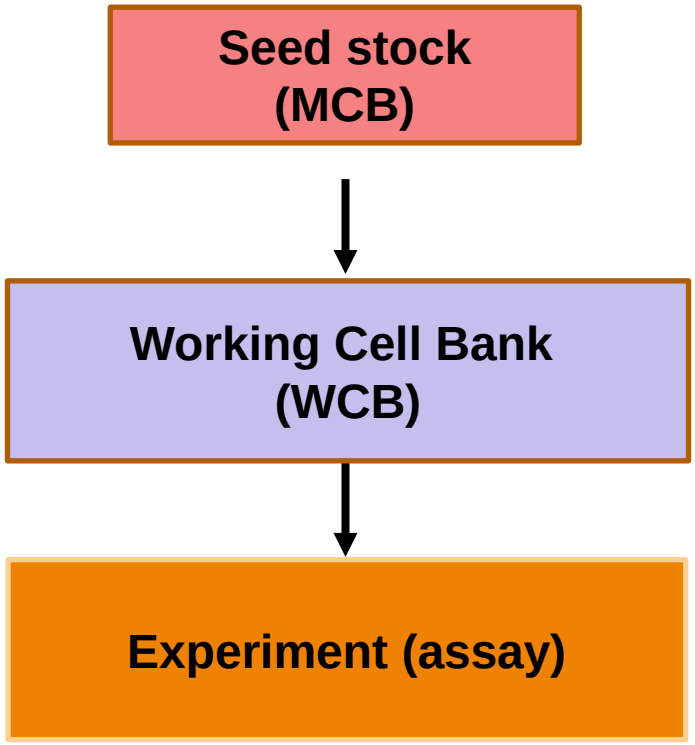


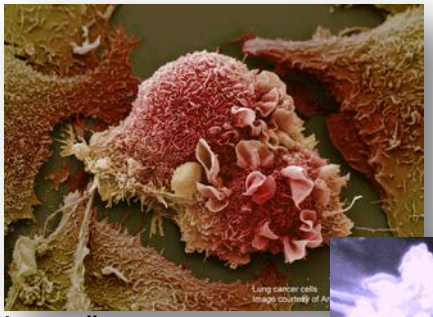
- Recommended inoculum
- Population Doubling Time (PDT)
- Optimum concentration range for maintenance and subculture
- Observe for changes in growth rate (crisis)

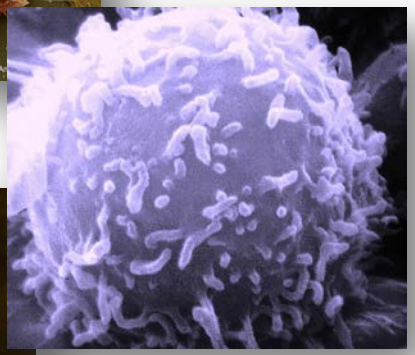
Applying the ‘Seed Stock’ concept to manage cell banks



Managing your cells – apply the ‘Seed Stock’ concept



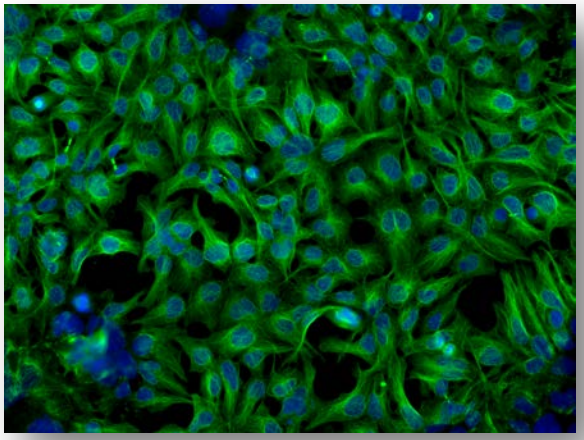

Lung cells


Lymphocyte cell


Pancreatic cells

Advantages of the ‘Seed Stock’ concept

- Ensures safe stocks of early material (Master Cell Bank)
- Reduces passage number (prevents genotypic and phenotypic drift)
- Prevents lot-to-lot variability
- Reduces the chance of cellular and microbial contamination
- Ensures reproducibility of experimental data



HEK-293 cells stained with beta tubulin

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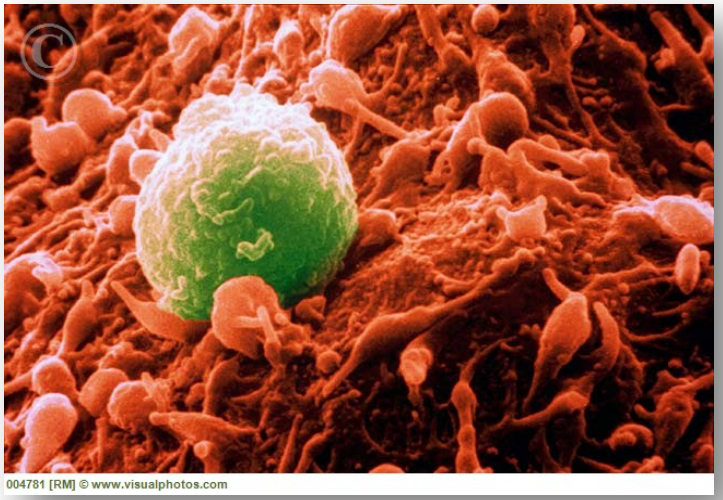
Use cell lines at low passage number

- Mimics tissue of origin
- Reduce phenotypic drift
- Reduce genotypic drift
- Reproducible results



Lymphocyte

Effects of high passage number

Several characteristics were tested to determine the effect of various passage numbers on Syrian hamster embryo (SHE) cells

Characteristic	Low passage no. (3)	Intermediate passage no. (43, 48)	High passage no. (64)
Plating efficiency			
Doubling time			
Cell growth			

Chang-Liu, CM et al. Cancer Letters. 26;113:77-86. (1997)

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Test for microbial contamination

Bacteria and Fungi

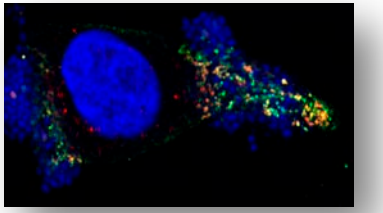
- Microbiological culture (Aerobic, Anaerobic)
- PCR

Mycoplasma

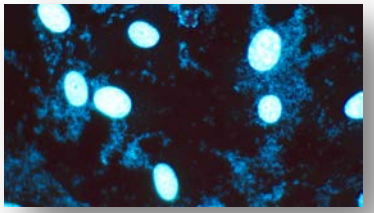
- Direct agar culture
- Indirect Hoechst stain
- PCR

Viruses

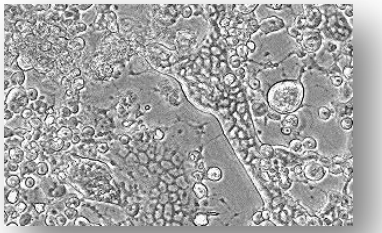
- Cytopathic effect (CPE)
- Indirect immunofluorescent antibody (IFA)
- Enzyme immunoassay (EIA)
- PCR



HeLa contaminated with *E. coli*



***M. hyorhinis* infection**



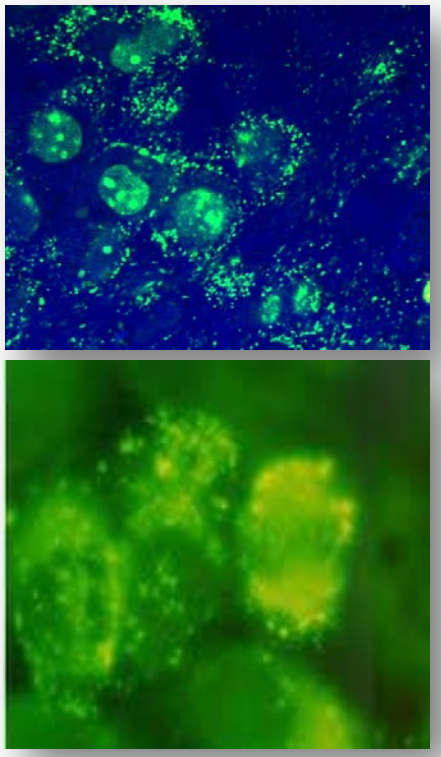
CPE of human herpes virus

Tests for bacterial/fungal contamination

Medium	Temperature	Atmosphere	Incubation time	Detection
Brain Heart Infusion (BHI)	26°C/37°C	Aerobic	14 days	Wide variety of fastidious bacterial
Thioglycollate broth	26°C/37°C	Aerobic	14 days	Obligate aerobes (O_2); Obligate anaerobes (no O_2 to grow) Facultative anaerobes (growth with or without O_2)
Trypticase Soy broth (TSB)	26°C/37°C	Aerobic	14 days	Wide variety of aerobic and facultative anaerobic microorganisms (S. aureaus, B subtilis, P. aeruginosa, C. albicans, A. niger)
Blood agar	37°C	Aerobic Anaerobic	14 days	Wide variety of fastidious microorganism (bacteria)
Sabouroud broth	26°C/37°C	Aerobic	14 days	Molds, yeasts, fungi (Aspergillus, Saccharomyces, Candida)
HTYE broth	26°C/37°C	Aerobic	14 days	Water-borne organisms

Consequences of bacterial and fungal infection

- Interferes with the growth rate of cells (by depleting medium of nutrients)
- Cell-cell interaction (interaction of NK cells and dendritic cells
- Interferes with invasion assay
- Interferes with protein synthesis, RNA synthesis and DNA synthesis

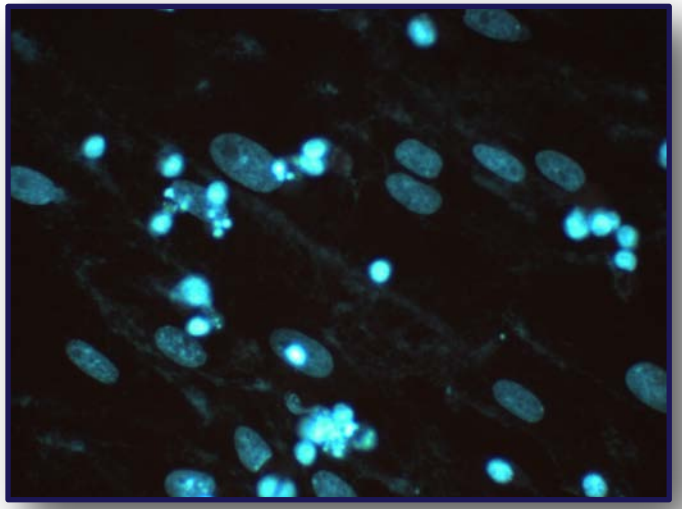


Cells infected with Mycoplasma

Tests for *Mycoplasma* contamination

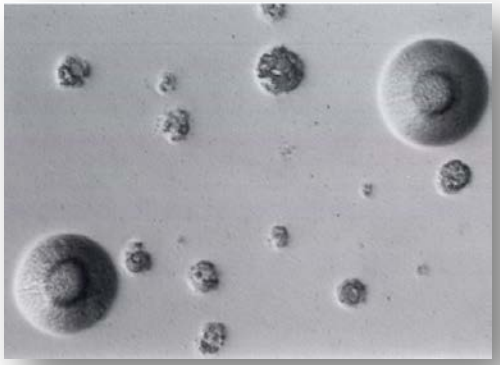
Mycoplasma

- Small bacteria without cell wall
- Growth to a high concentration without visible morphological changes
- Alter cell growth characteristics
- Easily detected
  - Indirect Hoechst stain method
  - Direct agar method
  - PCR

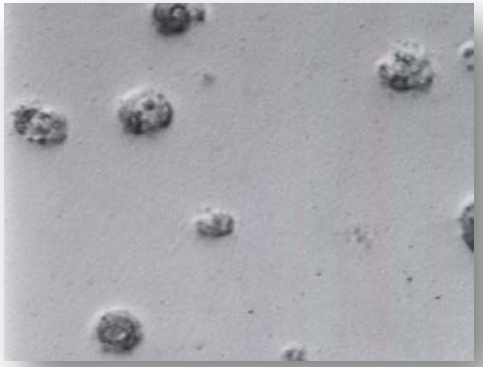


Tests for *Mycoplasma* contamination

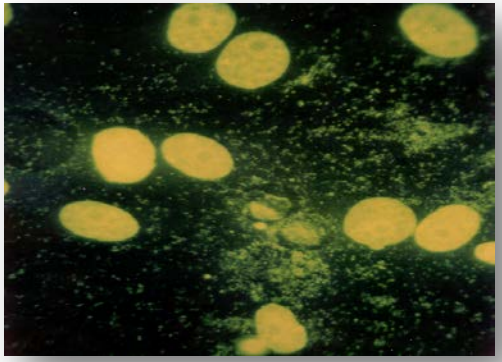
Direct agar (positive)



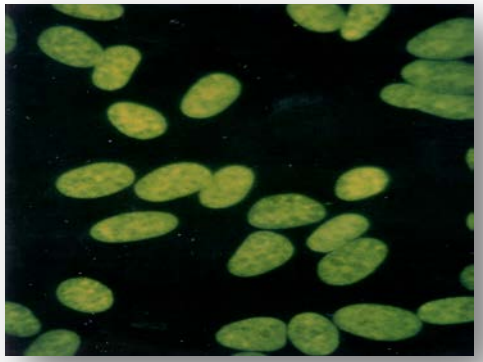
Direct agar (negative)



Indirect Hoechst stain* (positive)



Indirect Hoechst stain (negative)



* FDA Points to Consider (POC)

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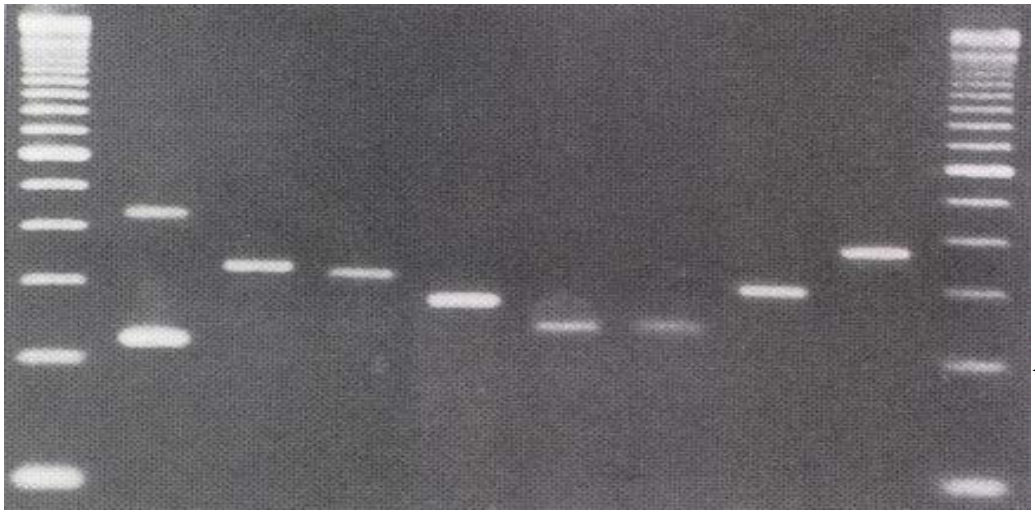
Tests for *Mycoplasma* contamination

PCR



1. 100 bp DNA ladder
2. *A. laidlawii*
3. *M. pirum*
4. *M. hyorhinis*
5. *M. salivarium*
6. *M. arginini*
7. *M. hominis*
8. *M. orale*
9. *M. fermentans*
10. 100 bp DNA ladder

Universal primers designed specific for the 16S rRNA coding region in the *Mycoplasma* genome will detect a wide variety of *Mycoplasma* species

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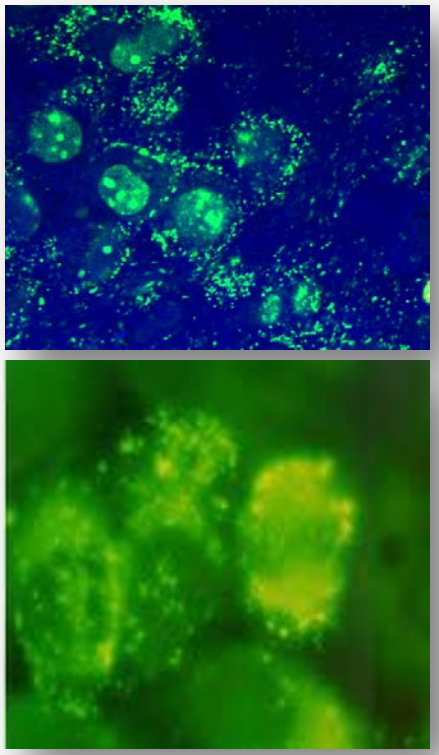
Consequences of *Mycoplasma* infection

- Interferes with the growth rate of cells
- Interferes with lymphocyte transformation
- Induction of morphological alteration (CPE)
- Alters DNA, RNA, and protein synthesis
- Interferes with the selection of mutant cells
- Induces chromosomal aberrations (e.g., *M. orale*, and *M. arginini*)
- Depletes essential amino acids (e.g., arginine from culture medium)
- Interferes (inhibit or enhance) with viral replication
- Alters host plasma membrane (interferes with receptor studies)



Cells infected with Mycoplasma

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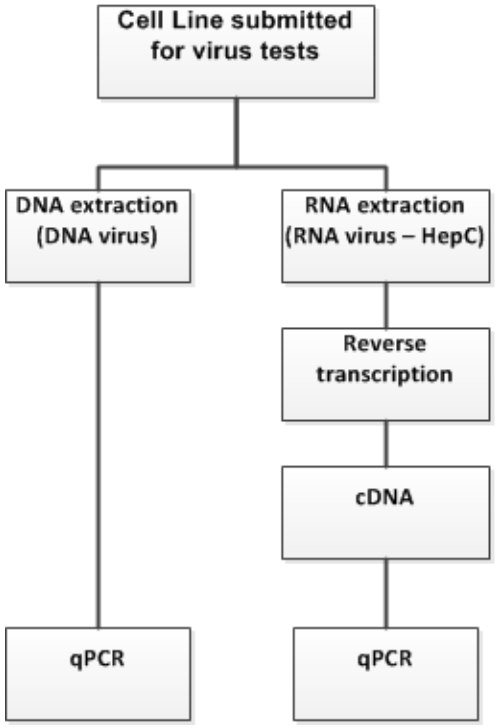
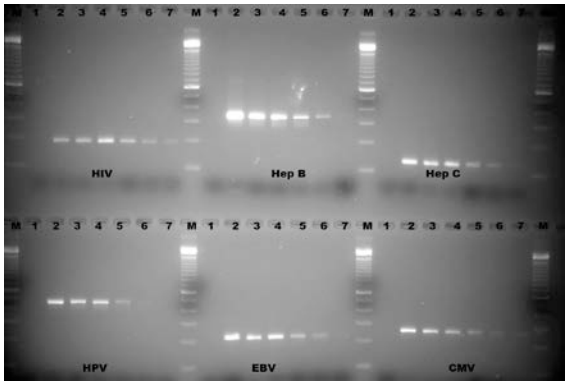
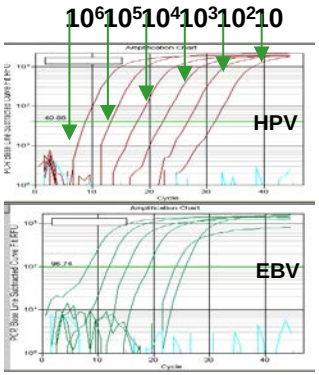
Test for viral contamination

Detection of human viruses by qPCR



DNA virus	RNA virus
Human Papilloma Virus **(HPV)**	Human Immunodeficiency Virus **(HIV)**
Epstein-Barr Virus **(EBV)**	Hepatitis C Virus **(HepC)**
Cytomegalovirus **(CMV)**	
Hepatitis B virus **(HBV)**)	





Sensitivity test: amplicons separated on agarose gel; qPCR

Consequences of viral infection

- Induces morphological changes (cytopathic effect – CPE)
- Changes metabolic activity of cells
- Interferes with receptor studies
- Health hazard

Note: *Special safety precautions should be employed when working with cells from humans to avoid possible transmission of viral infection from the cultures to the technician. Contact your safety office for additional information on appropriate practices for your institution.*

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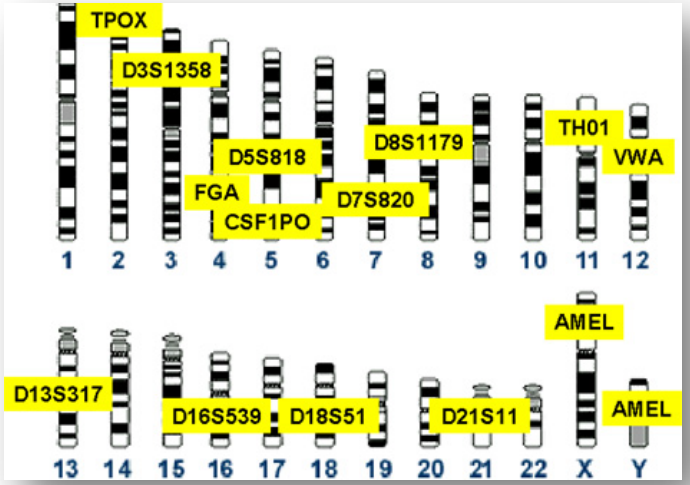
Confirm species and identity of cell line

- Cytochrome C Oxidase subunit 1 (COI) for interspecies identification
- STR analysis (DNA profiling) for intraspecies identification and authentication of human cell lines

Barcode of Life
Barcoding for species Identification



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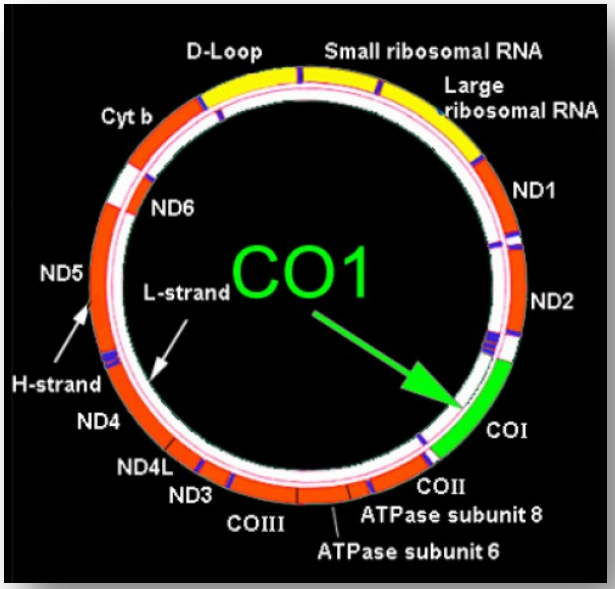
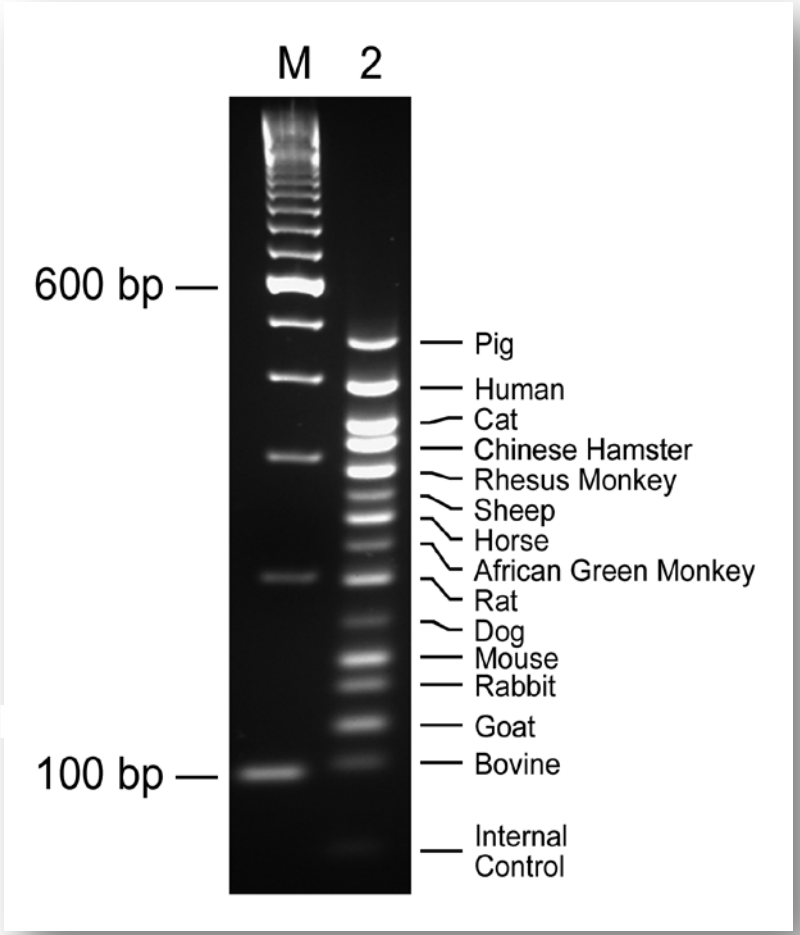
Confirm interspecies identity of cell line

Cytochrome Oxidase I (COI) Assay





Amplification of ~650 bp (base pairs) target sequence in the mitochondrial gene for cytochrome *c* oxidase subunit 1 (CO1)

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Short Tandem Repeat (STR) analysis for intraspecies identification of human cell line

DNA location	Degree of repetition	Number of loci	Repeat unit length
Satellite DNA (centromere)	10^3 to 10^7	1 to 2	2 to several thousand bp
Minisatellite DNA (telomere)	2 to several hundred	Many thousands	9 to 100 bp
Microsatellite DNA (STRs); randomly scattered	5 to about a hundred	10^4 to 10^5	1 to 6 bp

STR profiling a method for cell line authentication!

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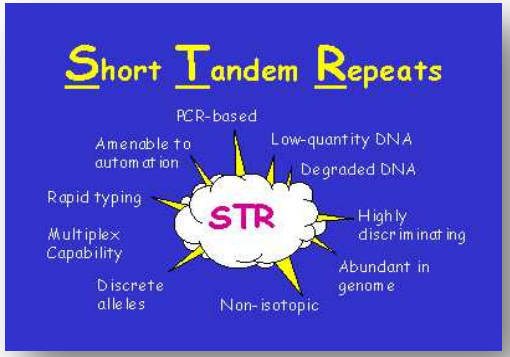
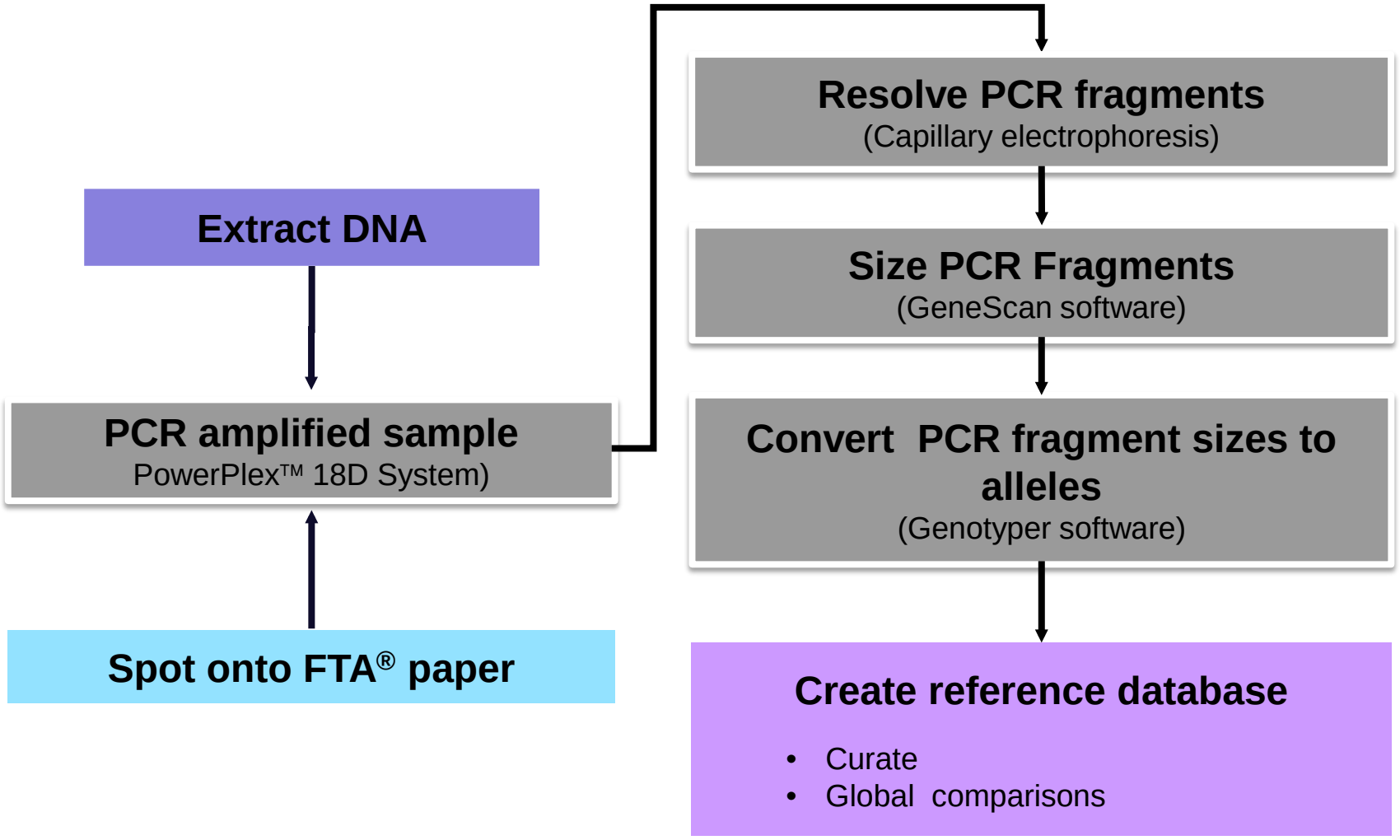
STR analysis for human cell line identity

- Target sequence consists of microsatellite DNA
- Typically use 1-2 ng DNA
- 1 to 2 fragments; discrete alleles allow digital record of data
- Markers distributed throughout the genome
- Highly variable within populations; highly informative



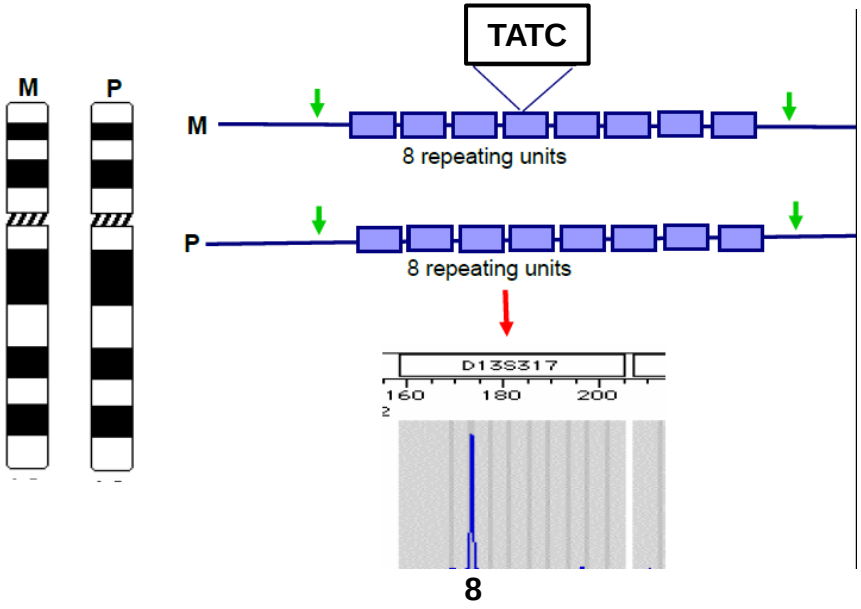
Probability of two cell lines having identical STR profile at 18 markers is 1×10^{-15}

Outline of STR profiling procedure



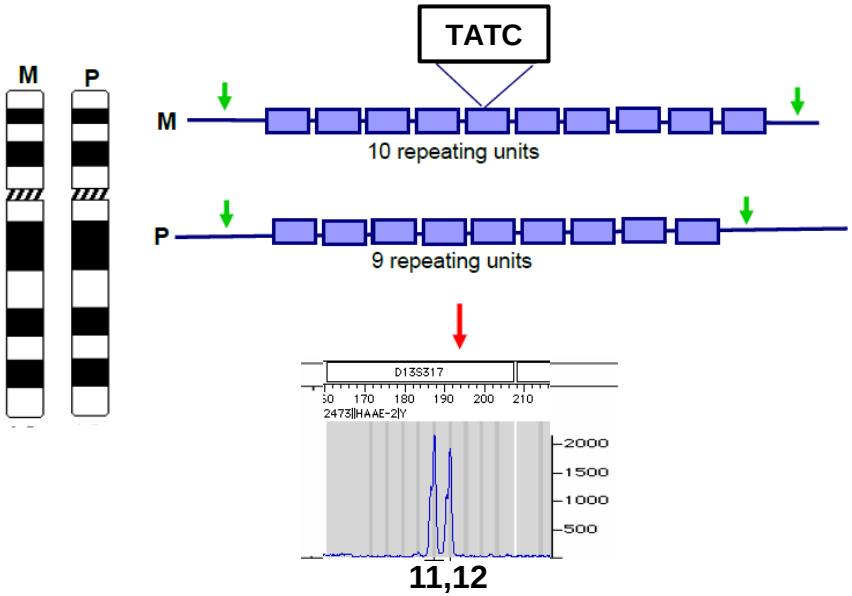
STR polymorphism

Homozygous



Cell line A

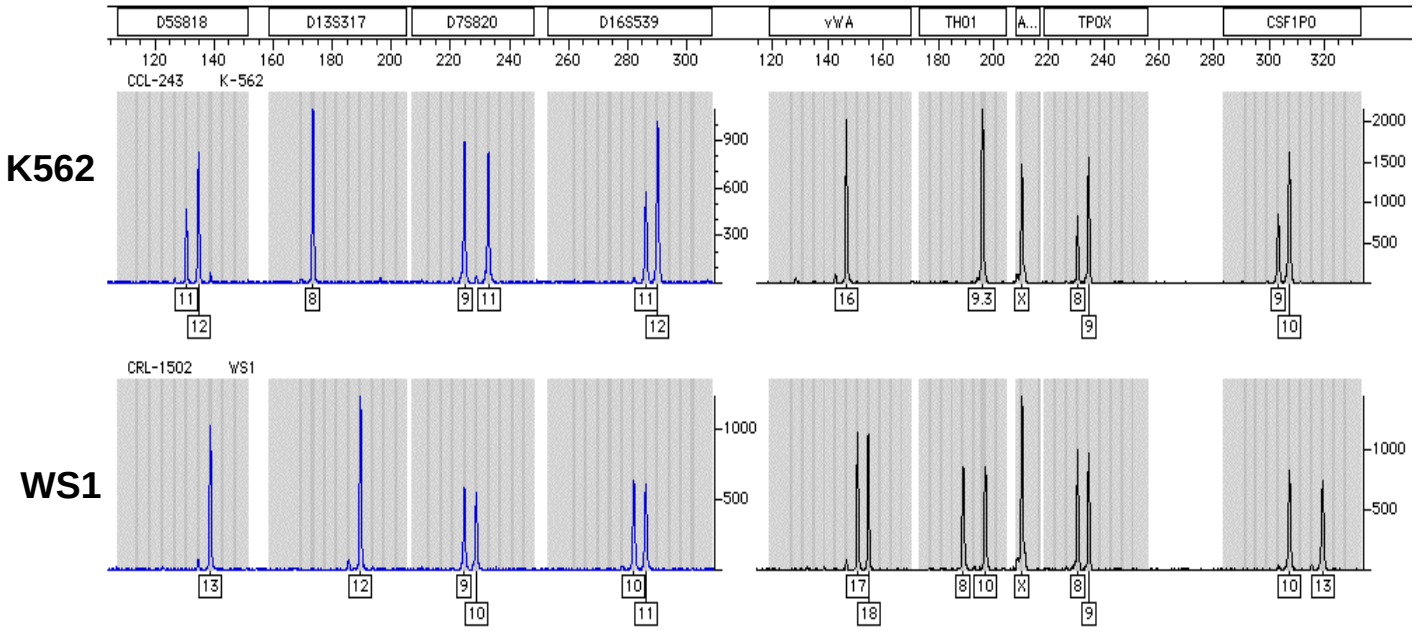
Heterozygous



Cell line B

Human cell line identification: STR analysis

2 unrelated cell lines (separate individuals, female in origin)



	D5S818	D13S317	D7S820	D16S539	vWA	THO1	Amel.	TPOX	CSF1PO
K562	11, 12	8	9, 11	11, 12	16	9.3	X	8, 9	9, 10
WS1	13	12	9, 10	10, 11	17, 18	8, 10	X	8, 9	10, 13

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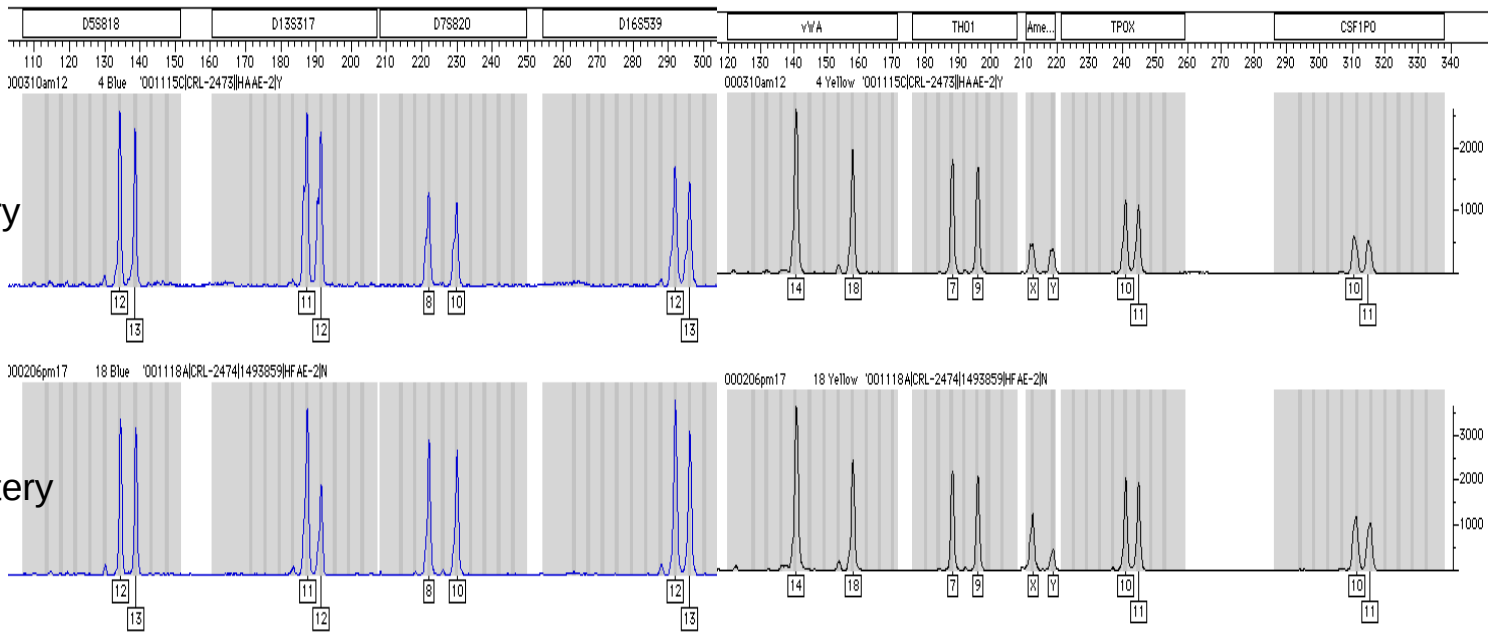
Human cell line identification: STR analysis

2 related cell lines (same individual; male in origin)

HAAE-2
aortic artery

HFAE-2
femoral artery



	D5S818	D13S317	D7S820	D16S539	vWA	THO1	Amel.	TPOX	CSF1PO
HAAE-2	12,13	11,12	8,10	12,13	14,18	7,9	X,Y	10,11	10,11
HFAE-2	12,13	11,12	8,10	12,13	14,18	7,9	X,Y	10,11	10,11

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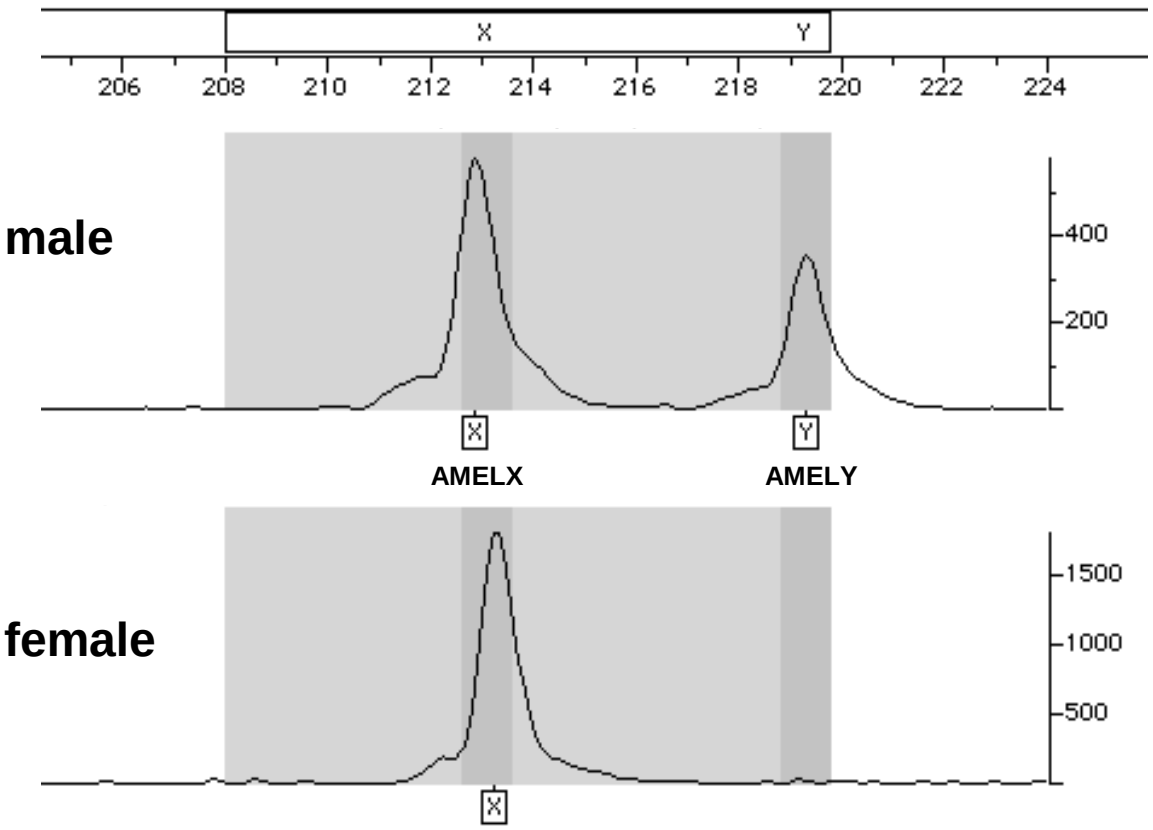
Gender is important for identification (amelogenin gene)



AMELX gene contains a 6 bp deletion in the intron 1

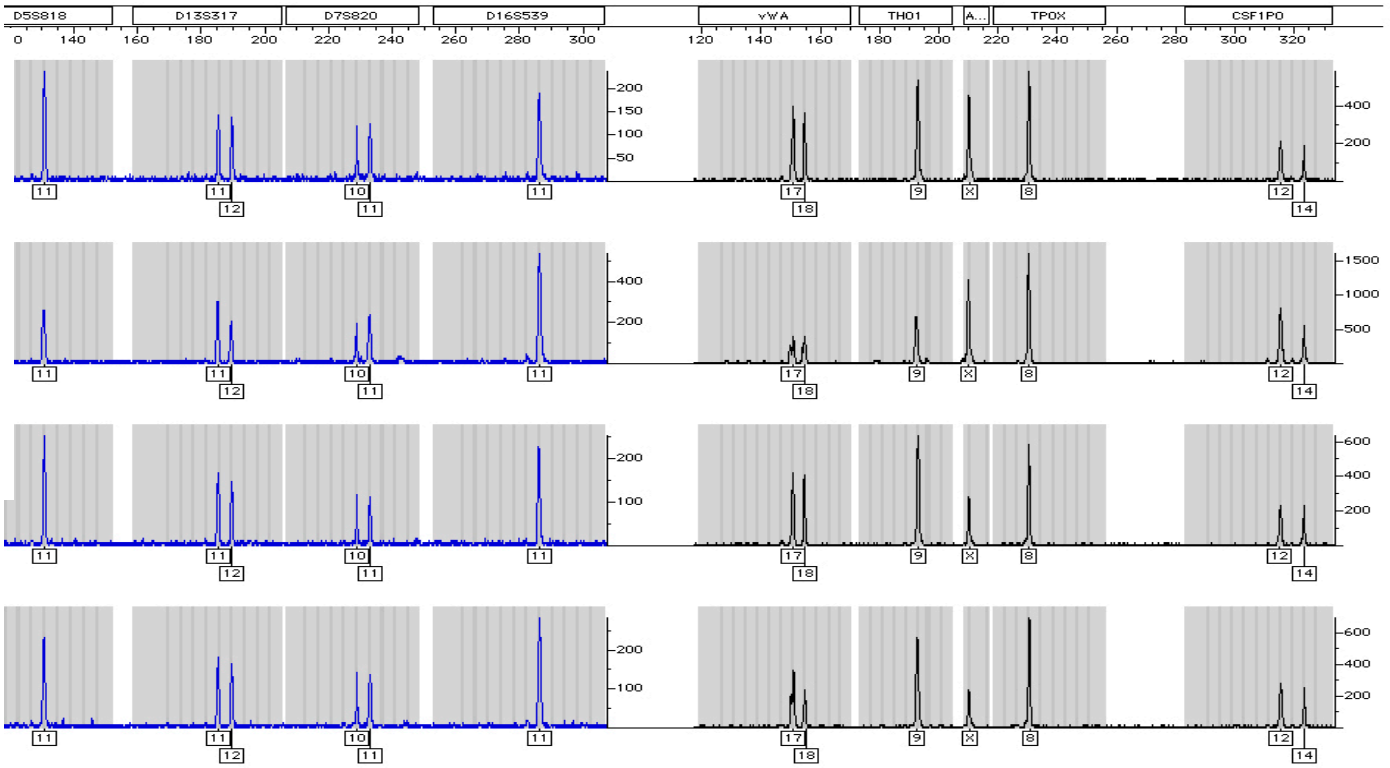
STR analysis to monitor genomic stability

Donor

Token (Pre-MCB)

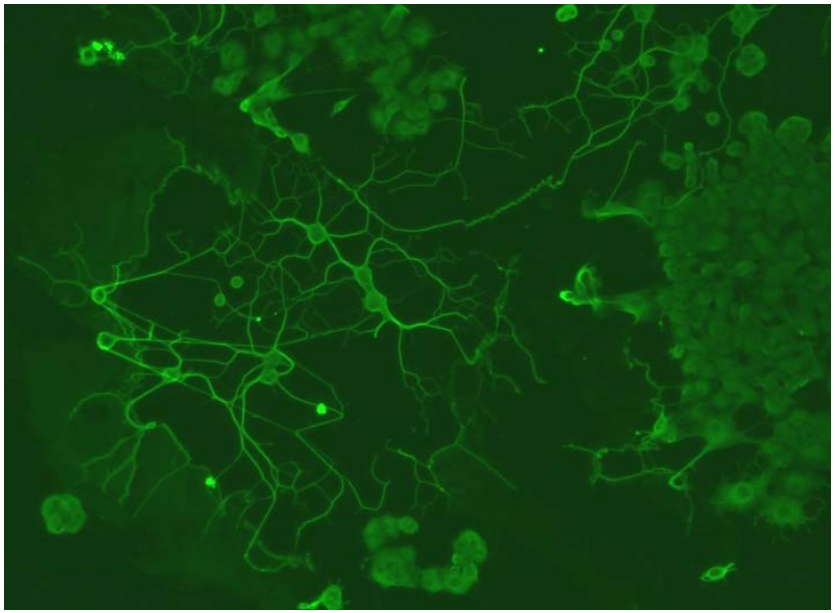
Seed (MCB)

Distribution (WCB)



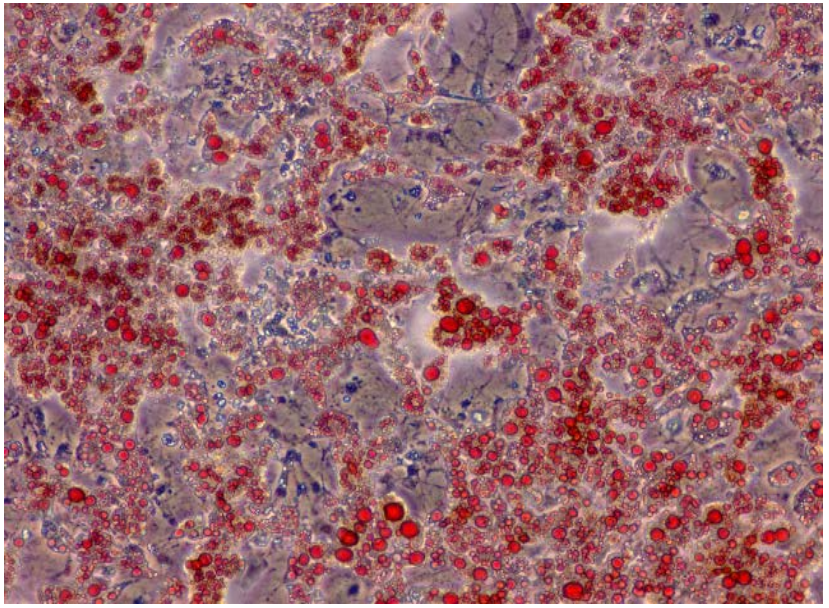
Various cell lines function confirmed

ATCC® CRL-1712.1™



ATCC® CRL-1712.1™ (PC-12 Adh) rat pheochromocytoma cells form beta-tubulin in the presence of NGF

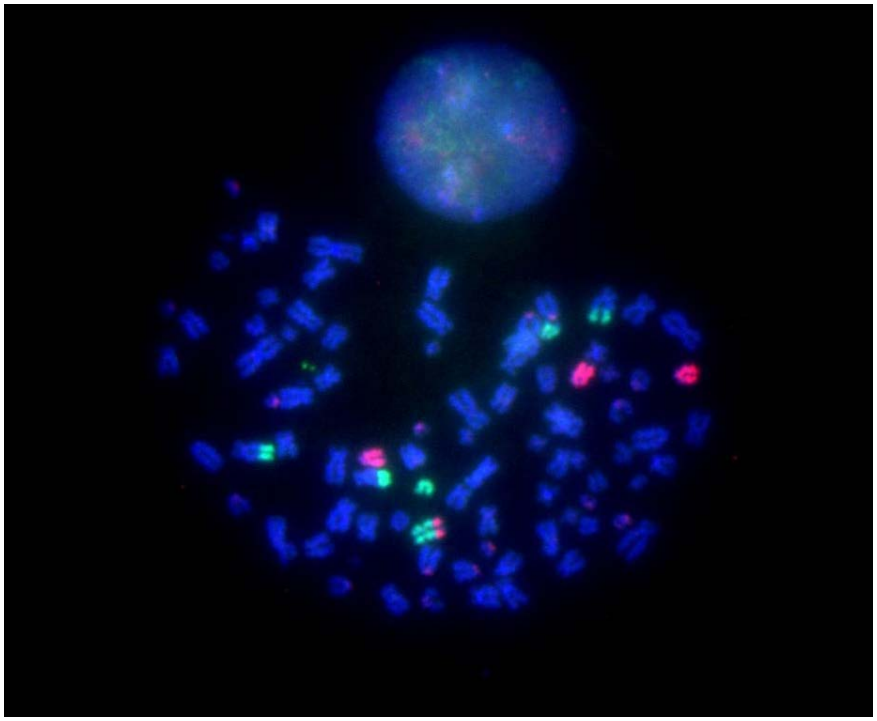
ATCC® CL-173™



ATCC® CL-173™, 3T3 L1, mouse preadipocyte, induced with dexamethasone and BMX to differentiate into adipocyte and produce fat droplet

Markers confirm tumor type

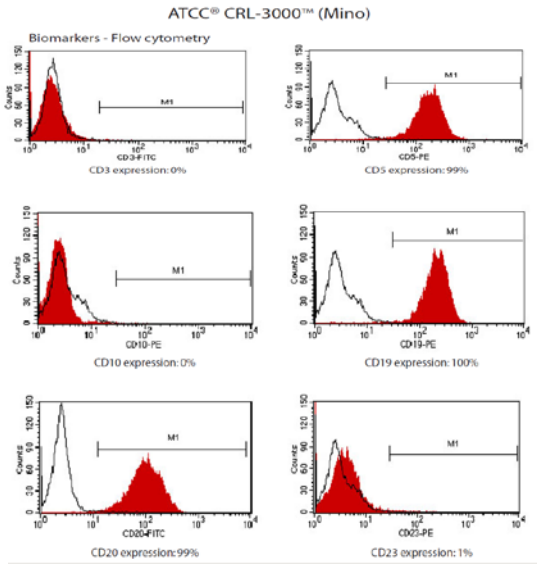
Mino cell line, ATCC® CRL-3000™ (Mantle Cell Lymphoma; Non-Hodgkin's lymphoma



Cytogenetic marker: t(11;14) (q13:q32)

Overexpression of Cyclin D1 protein - contributes to cell malignancy

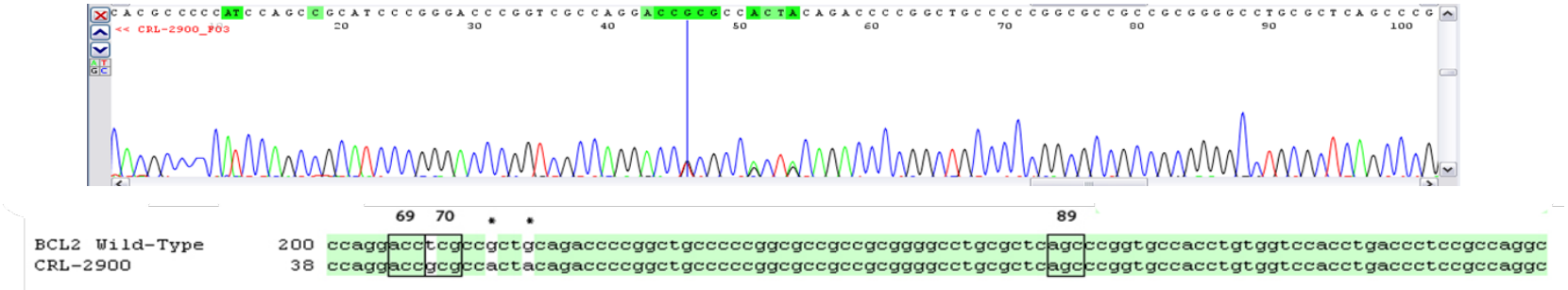
Immunophenotyping profile of Mino Cell Line	
CD3	Negative
CD5	Positive
CD10	Negative
CD19	Positive
CD20	Positive
CD23	Negative



Sequencing to confirm mutations

BCL-2 Family Cell Panel 1 (ATCC® TCP-2100™)

Item	Designation	Expected DNA/Base Change	Observation	Coverage Level
CRL-2898™	Neo Jurkat	None	Wild Type	8x
CRL-2899™	BCL2 Jurkat	BCL2 JurkatNone	Wild Type	8x
CRL-2900™	BCL2 (S70A) Jurkat	c.208 T>G (p.Ser70Ala)	Heterozygous	8x
CRL-2901™	BCL2 (S87A) Jurkat	c. 259 A>G, c. 260 G>C (p. Ser87Ala)	Heterozygous	8x
CRL-2902™	BCL2 (AAA) Jurkat	c. 205 A>G (p. Thr69Ala); c. 208 T>G (p. Ser70Ala); c. 259 A>C, c. 260 G>C (p. Ser87Ala)	Heterozygous	8x



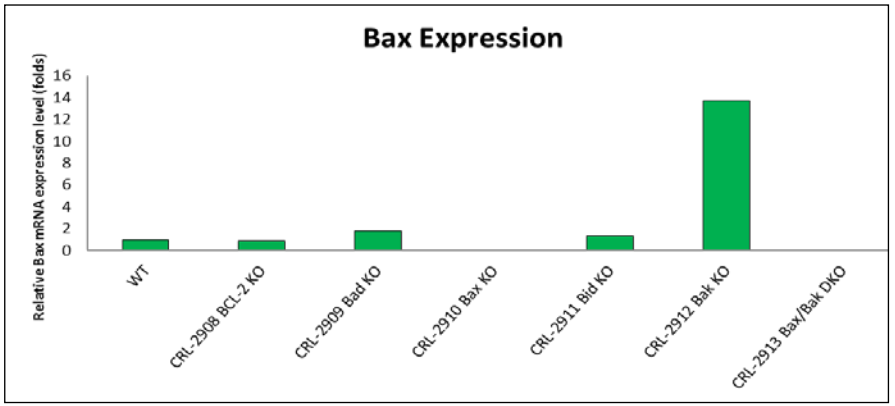
At position 46 the presence of both G and T (heterozygous alleles). This substitution results in an amino acid substitution of Serine to Alanine at codon 70.

qPCR to confirm gene knock-outs

BCL-2 Family Cell Panel 2 (ATCC® TCP-2110™)

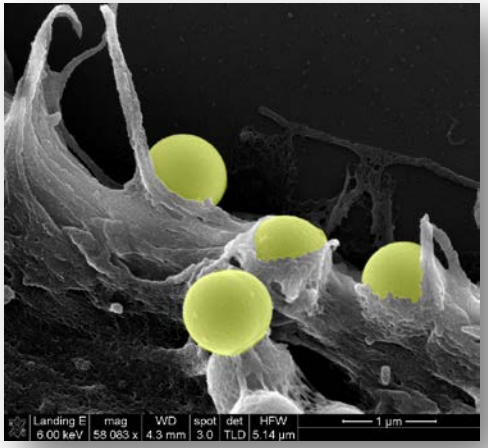
Item	Designation	Expected Gene Knock Out	Result
CRL-2907™	WT SV40 MEF	None	Wild Type
CRL-2908™	Bcl-2 KO SV40 MEF	BCL-2	No expression of BCL-2
CRL-2909™	Bad KO SV40 MEF	BAD	No expression of BAD
CRL-2910™	Bax KO SV40 MEF	BAX	No expression of BAX
CRL-2911™	Bid KO SV40 MEF	BID	No expression of BID
CRL-2912™	Bak KO SV40 MEF	BAK	No expression of BAK
CRL-2913™	Bax Bak DKO SV40 MEF	BAX & BAK	No expression of BAX or BAK

Real time qPCR analysis of mRNA levels for Bax: Relative Bax mRNA expression of the BCL-2 Family Cell Panel 2 (ATCC® TCP-2110™) cell lines after normalization to the housekeeping gene 36B4.



Minimum requirements for reproducible results

- Monitor morphology
- Optimize growth conditions
- Use cells at low passage or PDL
- Check for microbial contamination
- Confirm species (misidentification)
- Confirm functionality/characteristics



Silica beads on ATCC® SCRC-1041 ™ human foreskin fibroblast (HFF-1) cell membrane

The use of misidentification of cell lines is widespread!

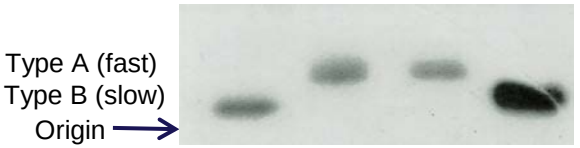
It is estimated that 1/3 of all cell lines used in the life sciences are misidentified

Everything was going along fine until they discovered their HeLa cell line expressed Y chromosome markers.

Courtesy of Promega.com

Stanley Gartler describes HeLa contamination of cell lines

2nd Bicennial Review Conference on cell tissue and organ culture, 1962, Bedford, PA



Isoenzyme Analysis: glucose-6-phosphate dehydrogenase (G6PD)

Name	Description	ATCC catalog no.	Origin	G6PD variant
HeLa	Cervical adenocarcinoma; human	ATCC® CCL-2™	African	Type A (fast)
KB	Oral epidermoid carcinoma, human	ATCC® CCL-17™	Caucasian	Type A (fast)
HEp-2	Larynx epidermoid carcinoma, human	ATCC® CCL-23™	Caucasian	Type A (fast)
Chang liver	Liver, human	ATCC® CCL-13™	Caucasian	Type A (fast)
Int-407	Embryonic intestine; human	ATCC® CCL-6™	Caucasian	Type A (fast)


Dr. Stanley Gartler

Conclusion: 90% (18/20) human cell lines are 'HeLa'

Gartler SM, NCI Monogr. 26:176, 1967; Gartler, SM, Nature 217:750, 1968

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1980-2003: interspecies and intraspecies cross-contamination

Year	No.	%	Type of contam.	Technology	Reference
1981	466	9.2%	Interspecies	Karyotyping	*Nelson Rees, WA, et al. Science 212, 446, 1981.*
1984	275	35%	Interspecies	Karyotyping	*Hukku, B. et al. Eukaryotic cell culture. Plenum Press, 1984*
1999	252	18%	Intraspecies	STR profiling	*Drexler, HG et al. Leukemia 13:1999.*
2003	550	15%	Intraspecies	STR profiling	*Drexler, HG et al. Leukemia 17:2003*

"Less than 50% of researchers regularly verify the identities of their cell lines using standard methods such as DNA fingerprinting by STR analysis"

Buehring, G.C., et al. (2004) In Vitro Cell Dev Biol 40:211

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2004-2010: Cellular cross-contamination persists…

Year	Title of article	Reference
2004	LCC15-MB cells are MDA-MB-435: a review of misidentified **breast and prostate cell lines**.	Clin Exp Metastasis. 21(6):535, 2004.
2007	MDA-MB-435: The Questionable Use of a Melanoma Cell Line as a Model for **Human Breast Cancer** is Ongoing	Cancer Biology & Therapy 6:9, 1355, 2007.
2008	Deoxyribonucleic Acid Profiling Analysis of 40 **Human Thyroid Cancer Cell Lines** Reveals Cross-Contamination Resulting in Cell Line Redundancy and Misidentification.	J Clin Endocrinol Metab. 93(11):4331, 2008.
2009	Genetic Profiling Reveals Cross-Contamination and Misidentification of 6 **Adenoid Cystic Carcinoma Cell Lines**: ACC2, ACC3, ACCM, ACCNS, ACCS and CAC2.	PLoS one. 4(6):e6040, 2009
2010	Verification and Unmasking of Widely Used **Human Esophageal Adenocarcinoma Cell Lines.**	JNCI. 102(4):271, 2010

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Impact of cellular contamination on research

Misidentification of frequently used esophageal adenocarcinoma cell lines (EAC)			
	Purported	STR confirmed (ATCC STRProfile database)	
SEG-1	Esophageal adenocarcinoma cell line	H460 (ATCC® HTB-177™)	Lung carcinoma (large cell lung cancer)
BIC-1	Esophageal adenocarcinoma cell line	SW620 (ATCC® CCL-227™)	Colorectal adenocarcinoma
SK-GT-5	Esophageal adenocarcinoma cell line	SK-GT-2	Gastric fundus carcinoma

Experimental results based on contaminated cell lines…

- Clinical trail recruiting EAC patients
- 100 scientific publications
- At least 3 NIH cancer research grants
- 11 US patents

Boonstra, J.J., et al. (2010) JNCI.102(4):271

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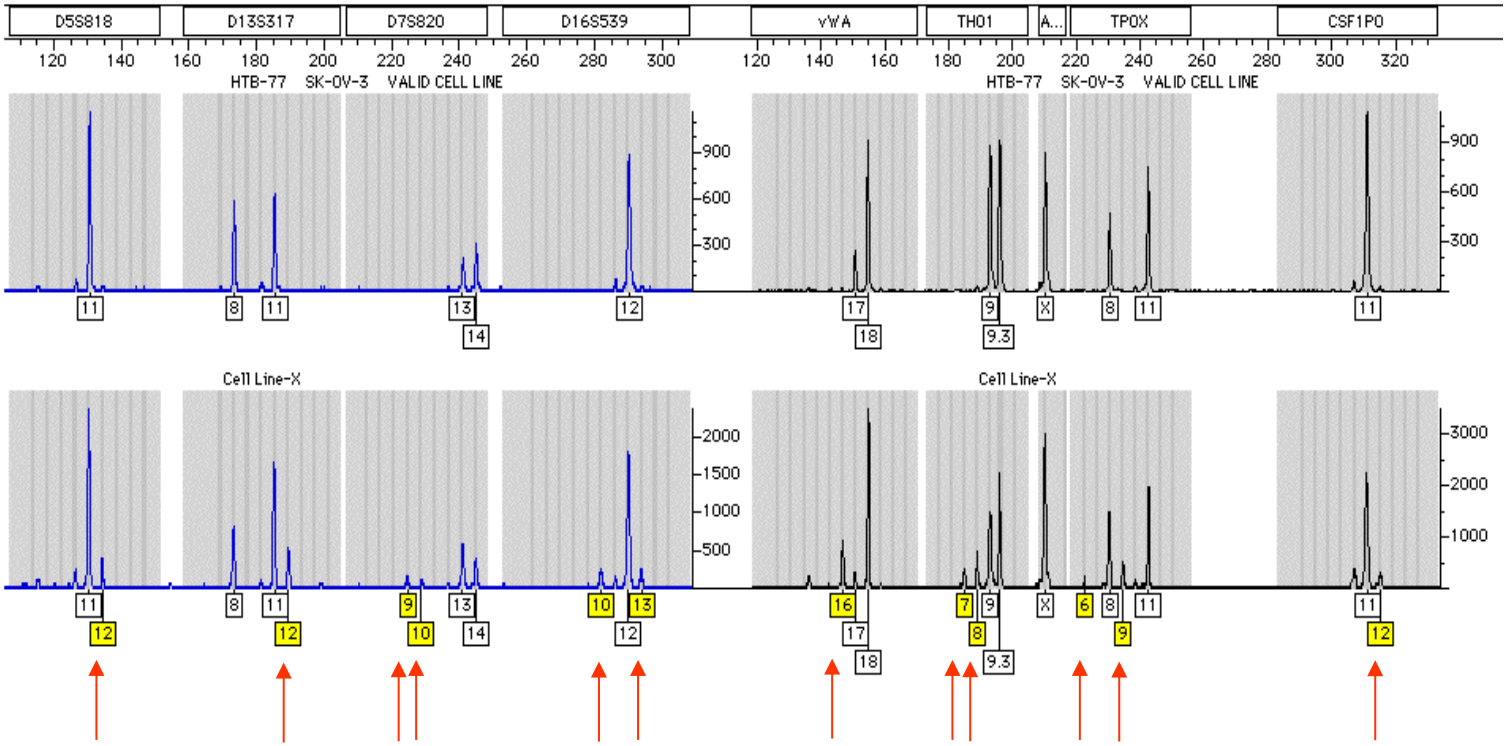
Case study 1: Cellular cross-contamination

SK-OV-3

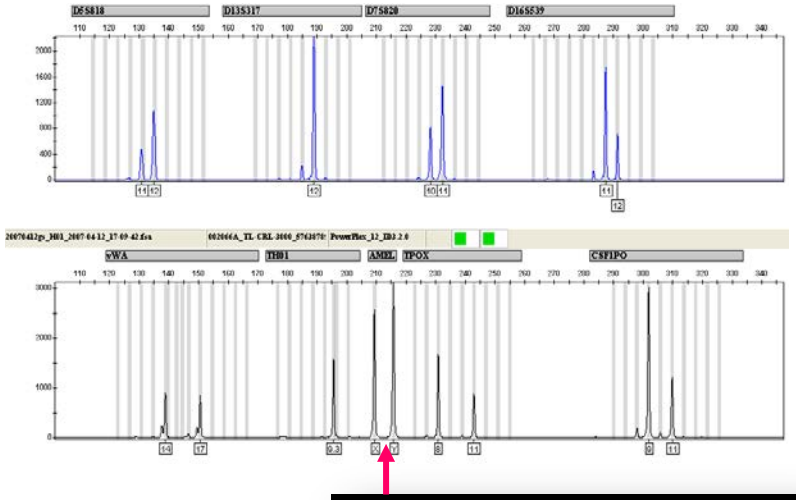
SK-OV-3 +
cell line X

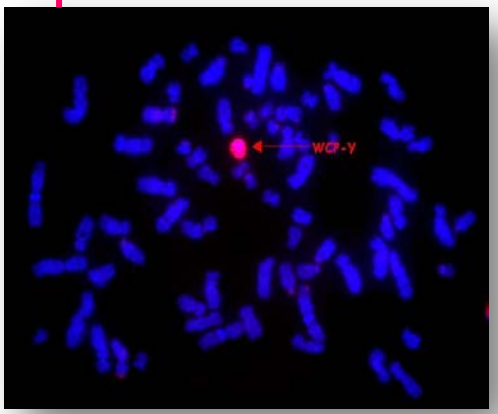


Case study 2: gender misidentification

Human cell line purported to be of female origin

STR analysis





Y paint

G-banding

Are you sure you are working with the correct cells?

Common sources of cellular contamination

- Getting cell lines from colleague down the hall
- Continuous culturing of working cell banks
- Use of feeder cells
- Mislabeling of culture flasks
- Working with multiple cell lines, concurrently

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Consequences of using misidentified cell lines

- Loss of cell line
- Loss of time and money
- Misinformation in the public domain
- Discordant or irreproducible results
- Private embarrassment /public humiliation

"For decades, biologists working with contaminated or misidentified cell lines have wasted time and money and produced spurious results; journals and funding agencies say it's not their job to solve this problem"

Rhitu Chatterjee. Cases of Mistaken Identity (2007) Science 15:928

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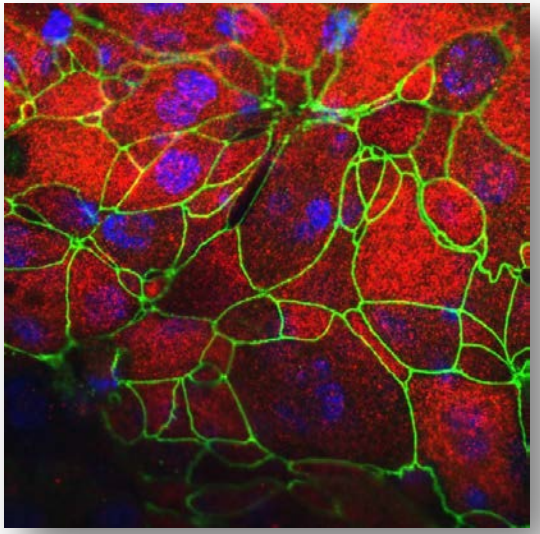
Steps to reduce cellular/microbial contamination

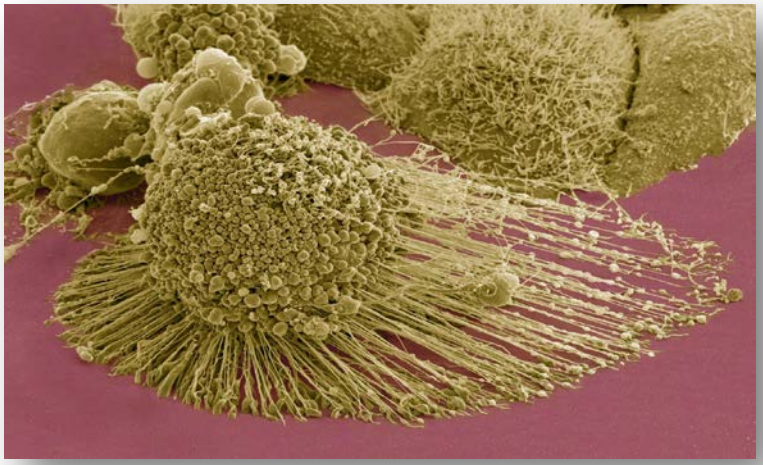
- Good documentation
- Highly trained technicians
- Good aseptic techniques
- Use one reservoir of medium per cell line
- Aliquot stock solutions/reagents



ATCC® HTB-174™, NCI-H441, human papillary adenocarcinoma differentiated under air-liquid interface conditions

Steps to reduce cellular/microbial contamination

- Label flasks (name of cell line, passage number, date of transfer (use barcoded flasks when available).
- Work with one cell line at a time in biological safety cabinet.
- Clean biological safety cabinet between each cell line
- Allow a minimum of 5 minutes between each cell line



ATCC® CCL-2™; Hela, cervical carcinoma. Scanning EM of cultured HeLa cell undergoing apoptosis.

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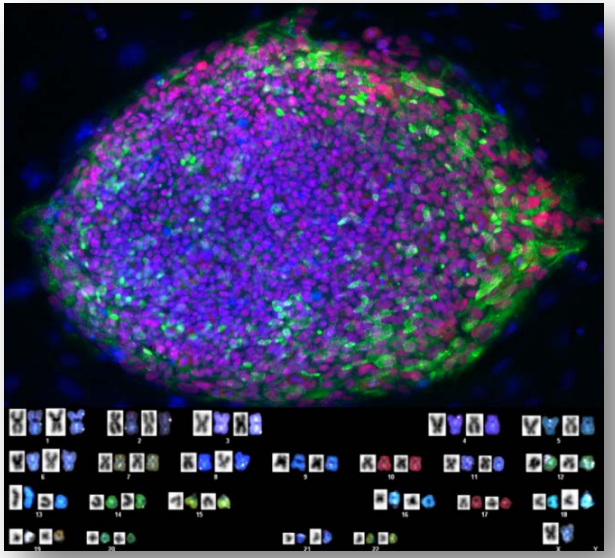
Steps to reduce cellular/microbial contamination

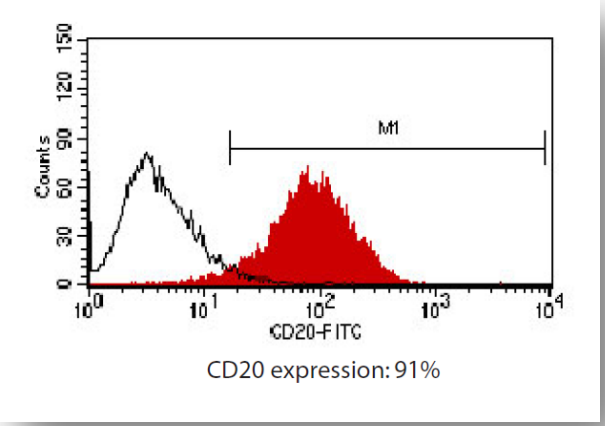
- Quarantine “dirty” cell line from “clean” cell line
- Manageable work load (reduce accidents)
- Clean laboratory (reduce bioburden)
- Legible handwriting (printed labels)



IPSC colony, on mouse feeder cells, derived from ATCC® CCL-65™, turner syndrome fibroblasts, expressing OCT4, SOX2, KLF4 and cMYC.

Steps to reduce cellular/microbial contamination

- Monitor for cell line identity and characteristics contamination, routinely
- Use seed stock (create master stocks)
- Create “good” working environment
- Review and approve laboratory notebook



CRL-3002™, JVM-2, mantle cell lymphoma cell line

Receiving a new cell line into the laboratory

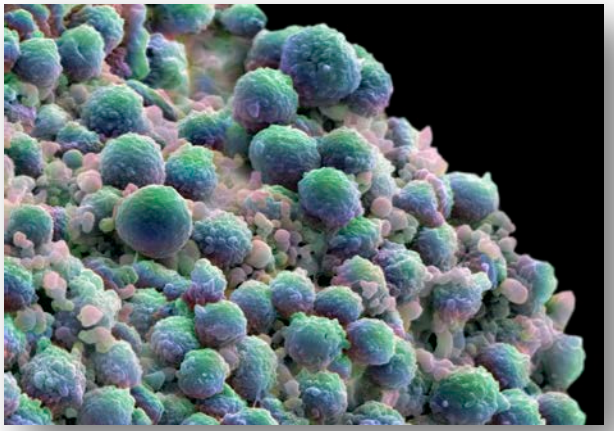
Record history of cell line

- Originator
- Institution/laboratory
- Date of origin
- Publication on deriving the cell line

Receiving a new cell line into the laboratory

Record all background information pertaining to cell line

- Cell line name
- Tissue of origin
- Species, strain
- Passage number
- Population doubling level
- Unique characteristics
- Unique function



Prostate cancer cells

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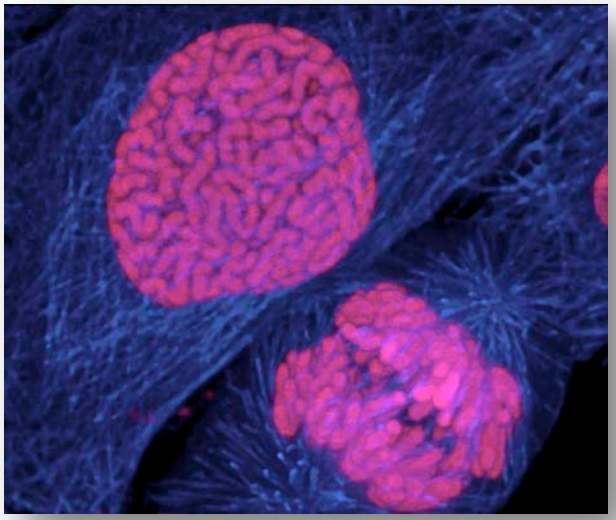
Receiving a new cell line into the laboratory

Record information on how to grow cell line

- Complete growth medium (include additives)
- Type of serum (to include source)
- Source of additives, concentration
- Procedure for thawing cells
- Procedure for subculturing
- Cryopreservation medium and procedure
- Doubling time
- Expected pre-freeze, post-freeze viability
- Storage temperature



Human breast cancer cells

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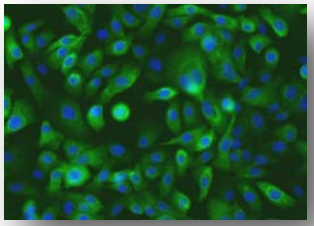
Thank you!

Register for more webinars in the ATCC "*Excellence in Research*" webinar series at www.atcc.org/webinars.

March 13, 2014
10:00 AM, 3:00 PM EST
Dr. Cara Wilder will provide an overview on drug-resistant *Acinetobacter baumannii*



March 27, 2014
10:00 AM, 3:00 PM EST
Dr. Chengkang Zhang will discuss hTERT immortalized cell lines and their use as physiologically relevant models for cancer research

Thank you for joining today!
Please send additional questions to tech@atcc.org

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