Syracuse University
College of Arts & Sciences
Forensic & National Security Sciences Institute

Sex-based targeted recovery of cells in a heterogeneous mixture: separating male and female like-cells

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Overview

- The Problem
- Technology
- Methods
- Labeling efficiency and cell targeting and recovery
- Mixtures
- Non-pristine samples
- How can this help you?

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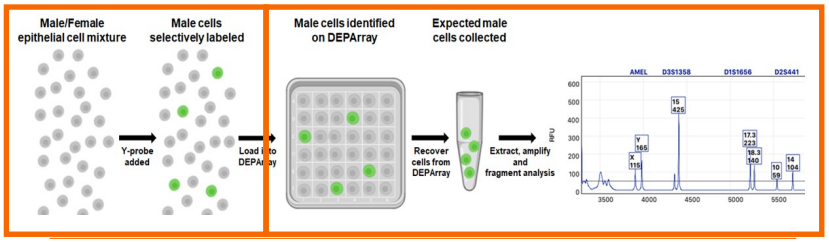
The Problem: Same Cell Mixtures

- Can we separate male and female cells **in *same* cell mixtures**?
  - –Targeting
  - –Recovery



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Targeted Cell Labeling and Recovery

Examples…

- Micromanipulation and microfluidic devices
  - Laser Capture Microdissection
  - Optical tweezers
  - (Huffman et.al. 2021, Farash et.al. 2018, Vandewoestyne et al. 2009, Anslinger et.al. 2006)
- FACS
  - (Verdon et.al. 2015, Stokes et.al. 2018)
- DEPArray™
  - (Williamson et.al. 2018, Watkins et.al. 2021, Fontana et.al. 2017, Anslinger et.al. 2019)

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Questions

1. Can a commercially available method for Y-chromosome labeling (**Abbott Molecular Vysis CEP Y DYZ1 probe**) be modified to successfully label male cells in **suspension**?
2. Can the method be used to successfully **detect/recover** the male cells using the DEPArray™ NxT or DEPArray™ PLUS?
   a. *Sensitivity* – What is the **labeling efficiency** (true positive rate) of probe binding and detection?
   b. *Specificity* – What is the **false positive and true negative** rate?
   c. *Mixture Study* – 1:1, 1:10 and 1:100
3. Is the method effective when targeting and recovering male cells in **non-pristine samples** (samples that are 10+ years old)?

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Methods

Samples

Cultured cells	• **RWPE prostate cells** • **HeLa cells (female)**	Initial "on slide" validation
Fresh epithelial cells	• **Male buccal swabs** • **Female buccal swabs**	Transition to suspension, DEPArray™ recovery & mixtures
20yr old epithelial cells	• **Male buccal swabs** • **Female buccal swabs**	Transition to casework-like samples

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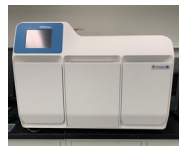
Methods

- Selective Labeling
- DEPArray™ NxT/PLUS
- Extraction - Menarini LysePrep Kit
- PowerPlex® Fusion 6C System
- Genetic Analyzer 3500xL



https://www.promega.com

https://assets.thermofisher.com/TFS-Assets/LSG/brochures/cms_078314.pdf 8

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Labeling

- Abbott CEP Y Spectrum Green and Orange probes
  - Yq12 satellite III region of the Human Y chromosome
  - Manufacturer's recommended protocol was modified and optimized
- Labeling efficiency
  1. Cultured cells on a slide
  2. Transition to in solution – "fresh" male and female epithelial cells
  3. Mixtures and aged samples

Fixation	Permeabilization	Denaturing	Hybridization	Counterstain
• 2% PFA • RT, 10 min	• 0.1% Triton x-100 (10% w/v)	• 73°C, 5 min • Probe & hyb buffer	• RT, overnight • Lab rotator	• DAPI • RT, 40 min

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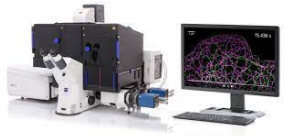
Y-Chromosome Labeling Procedure and Efficiency

- **Visualization**
  - Zeiss LSM 980 Airyscan Confocal 2 microscope (63x)
  - Olympus IX50 fluorescent microscope (400x)
  - Zeiss Axioscope 5 fluorescent microscope (400x)



- **DEPArray™ NxT and PLUS**
  - Identify and recover Y-probe labeled cells and DAPI (nucleus) stained cells following manufacturer recommended protocols.
  - DEPArray™ PLUS vs. NxT → increased sensitivity & lower signal to noise.
  - Cells were routed and recovered in two primary groups (1) single cells – male or female and (2) groups of cells – all Y-probe positive cells.

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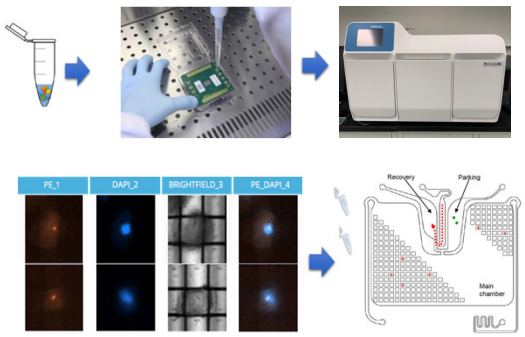
DEPArray

- Sample Prep – 20min
- Sample Scan – 40min
- Cell Selection – 30min
- Route/Recover – 2hr



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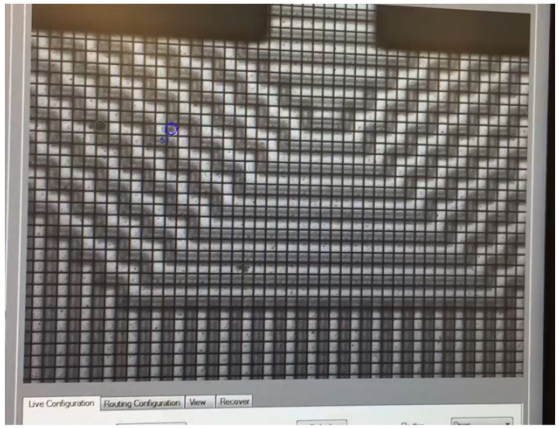

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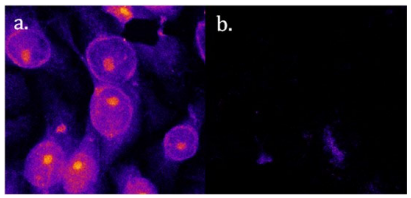
Labeling Efficiency - RWPE and HeLa Cells (on slide)

Sample	True Positives	False Negatives	% Efficiency*
0.2x RWPE	214	18	92.24
0.2x Hela	242	0	0.00

*5 trials

Male - RWPE | Female - HeLa



A single field of view on the LSM 980 Confocal microscope

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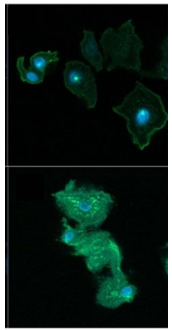
Transition to In-Solution – Fresh Male & Female Epithelial Cells

- Optimized:
  - –Probe concentration
  - –Fixative
  - –Hybridization buffer
  - –Hybridization time
  - –Hybridization temp
  - –Wash time/speed/#

Sample	DAPI +	Probe +	Probe binding efficiency (%)
3-hr 1% PFA	62	29	46.7%
3-hr 2% PFA	134	51	38.1%
overnight 1% PFA	161	133	82.6%
overnight 2% PFA	112	96	85.7%

Male / Female



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Results: Selective labeling – Y-probe Binding Optimization

- Cultured cells used to demonstrate the effectiveness of the labeling method (on a slide)
- Fresh male and female buccal epithelial cells labeled (separately) in suspension

Probe Color	Preparation	Cell Type	+ Count	Total Count	Efficiency (%)
Green	**Slide**	RWPE	214	232	92.24 ± 8.93
		HeLa	0	242	0.00 ± 0.00
Green	**Suspension**	Fresh Male	295	429	**68.76 ± 28.66**
		Fresh Female	1	207	0.48 ± 0.28
Orange	**Suspension**	Fresh Male	215	289	**74.39 ± 13.88**
		Fresh Female	0	224	0.00 ± 0.00

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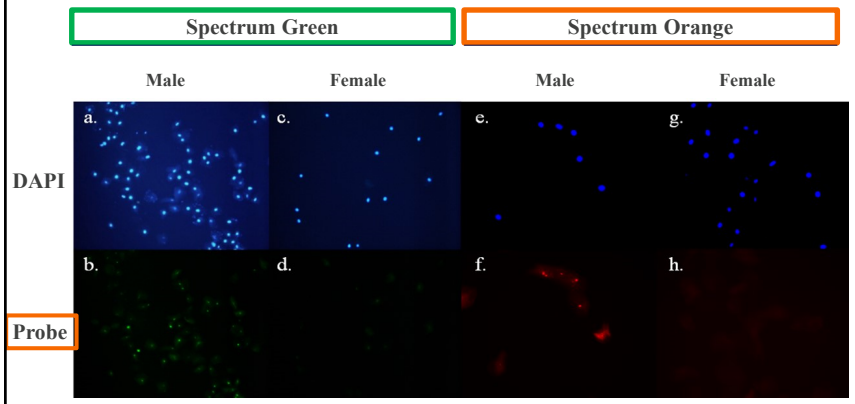

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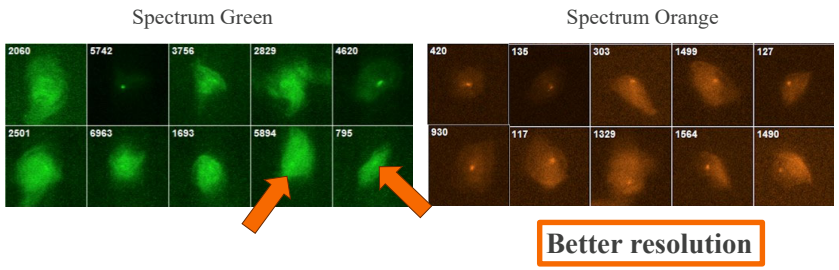
DEPArray™ and Probe Fluorophore

- Fresh male buccal cells



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Labeling Efficiency – Male : Female Epithelial Cell Mixtures

- Labeling efficiency in mixtures 1:1, 1:10 and 1:100
  - Expect→ 50%, 10% and 1% of male cells to be labeled, respectively
  - Example of 1:100 results

Ratio M:F	Trial	# Positively labeled cells	Total cell count	Expected labeling efficiency (%)	Observed labeling efficiency (%)
1:100	1	2	158	1	**0.86 ± 2.33**
	2	3	123	1	**4.00 ± 11.21**
	3	1	37	1	**3.33 ± 12.91**

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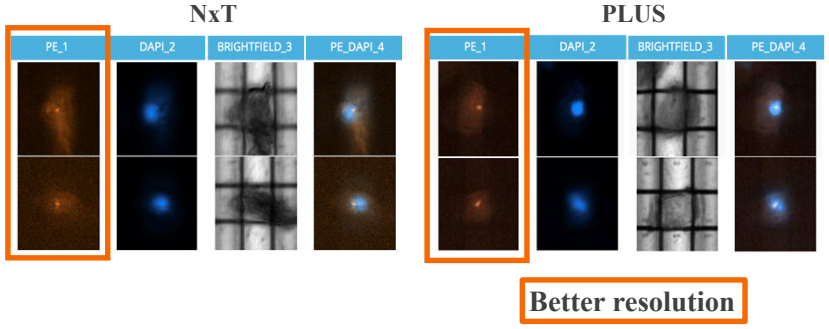
Transition to DEPArray™ - NxT vs PLUS



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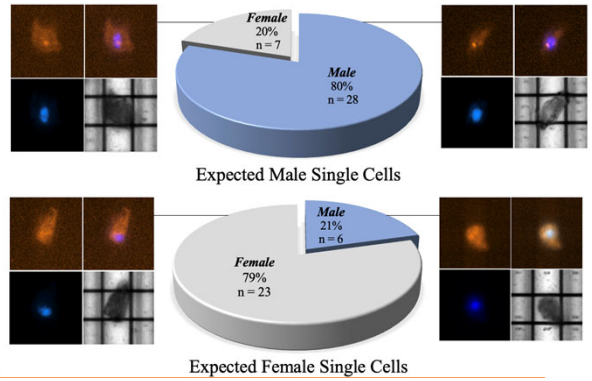
Cell Identification, Recovery and Profiling

- Evaluate success in routing expected cells
  - Single cells
  - 1:1 mixture
  - Verified by profiling



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1:1 Male to Female Mixtures

Mixture (male : female)	Sample type	n (cells)	Routable cells	Male		Female		Donor or ratio of M:F
				Proportion alleles present	Mean peak height	Proportion alleles present	Mean peak height	
1:1 - 1	Expected male	5	739	41/46	146 ± 75	0/43	0 ± 0	Single source - male
1:1 - 2	Expected male	2	258	24/46	55 ± 35	0/43	0 ± 0	Single source - male

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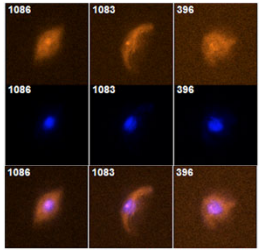
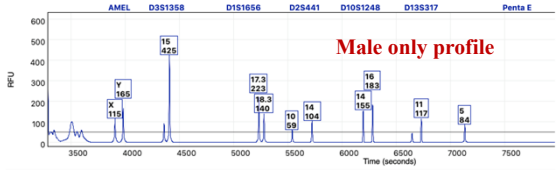
1:1 Male to Female Mixture

Target – Male cells



5 cells (only 3 shown)



Locus	Male Reference	Female Reference
AMEL	X,Y	X,X
D3S1358	15,15	16,17
D1S1656	17.3,18.3	17.3,17.3
D2S441	10,14	10,11
D10S1248	14,16	15,17
D13S317	9,11	11,11
Penta E	5,12	5,12

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1:10 Male to Female Mixtures

Mixture (male : female)	Sample type	n (cells)	Routable cells	Male		Female		Donor or ratio of M:F
				Proportion alleles present	Mean peak height	Proportion alleles present	Mean peak height	
1:10 - 1	Expected male	3	507	35/46	108 ± 48	25/43	98 ± 36	1.1
1:10 - 2	Expected male	4	1273	46/46	555 ± 287	0/43	0 ± 0	Single source - male
1:10 - 3	Expected male	2	258	28/46	128 ± 68	0/43	0 ± 0	Single source - male
1:10 - 4	Expected male	6	450	27/44	82 ± 49	31/42	97 ± 51	0.85
1:10 - 5	Expected male	7	1165	44/44	220 ± 169	0/42	0 ± 0	Single source - male

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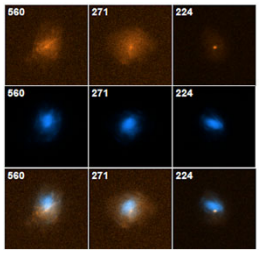
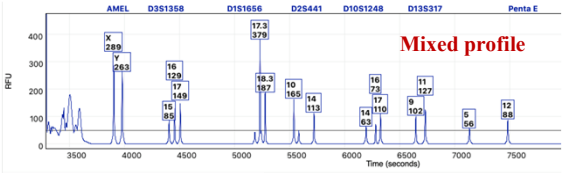
1:10 Sample 1 – Male cells

Target – Male cells



3 cells



Locus	Male Ref	Female Ref
AMEL	X,Y	X,X
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D1S1656	17.3,18.3	17.3,17.3
D2S441	10,14	10,11
D10S1248	14,16	15,17
D13S317	9,11	11,11
Penta E	5,12	5,12

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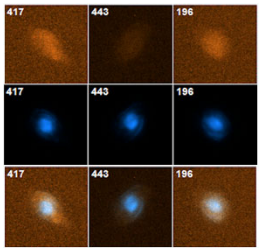
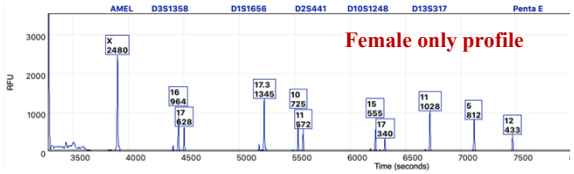
1:10 Sample 1 – Female cells

Target – Female Cells





Locus	Male Ref	Female Ref
AMEL	X,Y	X,X
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D1S1656	17.3,18.3	17.3,17.3
D2S441	10,14	10,11
D10S1248	14,16	15,17
D13S317	9,11	11,11
Penta E	5,12	5,12

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1:100 -1 Male to Female Mixture

Mixture (male : female)	Sample type	n (cells)	Routable cells	Male		Female		Donor or ratio of M:F
				Proportion alleles present	Mean peak height	Proportion alleles present	Mean peak height	
1:100 - 1	Expected male	1	648	0/44	0 ± 0	36/42	229 ± 131	Single source - female
1:100 - 2	Expected male	4	960	0/44	0 ± 0	42/42	223 ± 97	Single source - female
1:100- 3	Expected male	2	694	0/44	0 ± 0	37/42	109 ± 62	Single source - female
1:100 - 4	Expected male	7	1511	0/44	0 ± 0	33/42	123 ± 61	Single source - female

https://onesouthrealty.com/voices/houston-we-have-a-problem/

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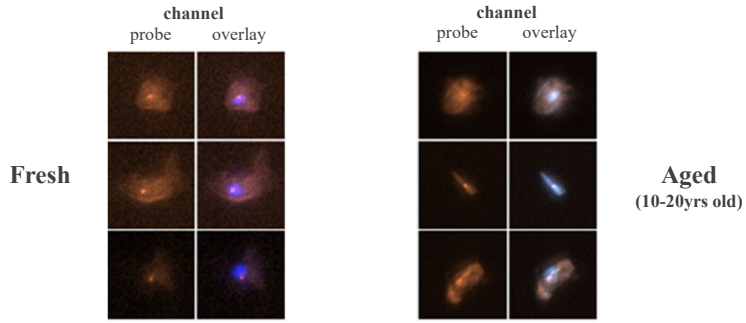
Non-pristine Sample Evaluation

How does the labeling compare to freshly collected?



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Non-pristine Sample Labeling Efficiency

Buccal swabs 10-20 years old, stored at room temp (unknown time) and then frozen

Sample	# Positively labeled cells	Total cell count	Expected labeling efficiency (%)	Observed labeling efficiency (%)
1:10 - 1	4	55	~10	13.00
1:10 - 2	0	26	~10	0.00
1:10 - 3	2	31	~10	12.00
1:10 - 4	0	45	~10	0.00

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How does this method help you?

- Improved ability to detect male target(s)
  - ✓ Male only profile - single cell or multiple cells
- Reducing female to male ratio
  - ✓ Potential to take a high female to male ratio and enrich for male → easier interpretation of male component
  - ✓ Example: 100:1→10:1
- Potential for CODIS eligible profiles when previously may not be
- Recommended as a last effort

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Conclusions

- **Method successfully transitioned to in suspension labeling**
  - ~ 75% labeling efficiency → fresh buccal cells
  - Expected level of staining in 1:1, 1:10 and 1:100 ratios of M to F → fresh and non-pristine
  - Spectrum Orange preferred
- **Recovery**
  - DEPArray™ PLUS preferred, but other means of recovery may be superior
  - Able to recover male cells at 1:1 and 1:10 ratios…and obtain male profiles…1:100, not so good
- **There are issues with false positives**
  - Visual identification, confirmation bias?
  - Additional optimization – hybridization temp/probe type/washes
  - BUT… Goal is to ID the male, with a high female to male ratio… selecting male cells at a rate of 80% will still greatly improve the ability to interpret the male component(s).
- **You don't need to select single cells… you can select groups of cells**
  - Reducing the ratio of female to male is a benefit

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References

- Kaitlin Huffman, Erin Hanson, Jack Ballantyne, Recovery of single source DNA profiles from mixtures by direct single cell subsampling and simplified micromanipulation, Science & Justice, Volume 61, Issue 1, 2021, Pages 13-25, ISSN 1355-0306, https://doi.org/10.1016/j.scijus.2020.10.005.
- V. R. Williamson, T. M. Laris, R. Romano, and M. A. Marciano, "Enhanced DNA mixture deconvolution of sexual offense samples using the DEPArrayTM system," Forensic Sci. Int. Genet., vol. 34, pp. 265–276, May 2018, doi: 10.1016/j.fsigen.2018.03.001.
- M. A. Marciano and J. D. Adelman, "PACE: Probabilistic Assessment for Contributor Estimation— A machine learning-based assessment of the number of contributors in DNA mixtures," Forensic Sci. Int. Genet., vol. 27, pp. 82–91, Mar. 2017, doi: 10.1016/J.FSIGEN.2016.11.006.
- Morrison et al., "Abstract 1717: Orthogonal identification of circulating tumor cells (CTCs) using single cell low pass whole-genome sequencing (WGS) and copy-number alteration (CNA) analysis," Cancer Res., vol. 77, no. 13_Supplement, pp. 1717–1717, Jul. 2017, doi: 10.1158/1538-7445.AM2017-1717.
- D. R. L. Watkins, D. Myers, H. E. Xavier, and M. A. Marciano, "Revisiting single cell analysis in forensic science," Sci. Reports |, vol. 11, p. 7054, 123AD, doi: 10.1038/s41598-021-86271-6.
- K. Anslinger, B. Bayer, B. Mack, and W. Eisenmenger, "Sex-specific fluorescent labelling of cells for laser microdissection and DNA profiling," Int. J. Legal Med., vol. 121, no. 1, pp. 54–56, 2007, doi: 10.1007/s00414-005-0065-7.
- F. Fontana et al., "Isolation and genetic analysis of pure cells from forensic biological mixtures: The precision of a digital approach," Forensic Sci. Int. Genet., vol. 29, pp. 225–241, Jul. 2017, doi: 10.1016/J.FSIGEN.2017.04.023.
- "Vysis-CEP-X-SpectrumOrangeY-SpectrumGreen-Direct-Labeled-Fluorescent-DNA-Probe-Kit @ www.molecularcatalog.abbott." [Online]. Available: https://www.molecularcatalog.abbott/us/en/Vysis-CEP-X-SpectrumOrangeY-SpectrumGreen-Direct-Labeled-Fluorescent-DNA-Probe-Kit.

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Thank You.

Questions?

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Syracuse Forensics program

Marciano Lab website

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