Making the best use of all your curves:

SRM 2372a Human DNA Quantitation Standard

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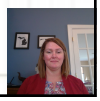
Forensics@NIST 2020



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Outline

- Why qPCR?
- Standards: here to help!
- Remediation of some sources of bias
- FAQ!

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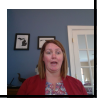
Why Quantitative PCR?

- Goal: quantify the amount of DNA recovered from extraction
- Why: the next step (PCR amplification) requires a specific range of input amounts – if not met, interpretation may be complex
  - To satisfy the FBI QAS requirement
- Quantitation methods can also inform your workflow
  - Is there enough for one test or many?
  - Low amounts of DNA that can be further concentrated
  - Extent of DNA degradation
  - The ratio of total DNA to male DNA (Y chromosome) *Y-Screening*
  - Degree of inhibition (agents in the sample that reduce PCR amplification efficiency)
  - "Stop at Quant"
  - Go back and re-sample and/or re-extract



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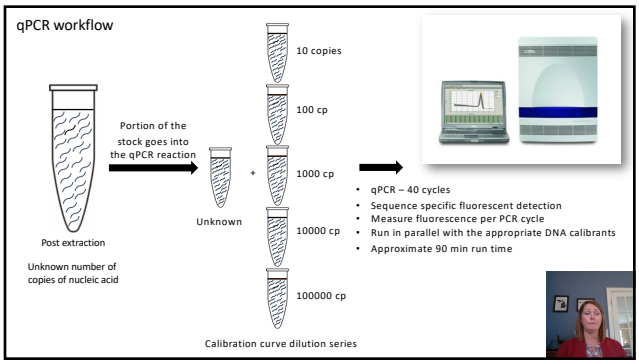
qPCR workflow
Post extraction
Unknown number of copies of nucleic acid
Portion of the stock goes into the qPCR reaction
Unknown
10 copies
100 cp
1000 cp
10000 cp
100000 cp
Calibration curve dilution series
qPCR – 40 cycles
Sequence specific fluorescent detection
Measure fluorescence per PCR cycle
Run in parallel with the appropriate DNA calibrants
Approximate 90 min run time

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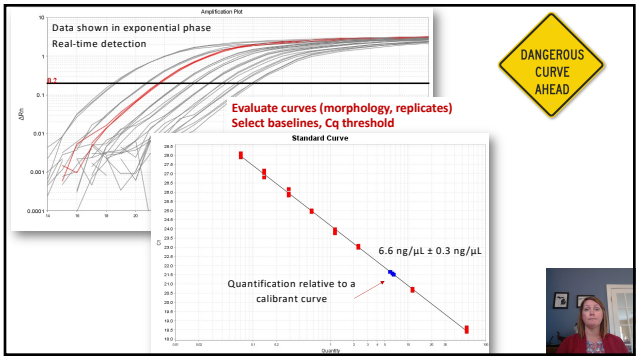
Data shown in exponential phase
Real-time detection
Evaluate curves (morphology, replicates)
Select baselines, Cq threshold
Standard Curve
6.6 ng/µL ± 0.3 ng/µL
Quantification relative to a calibrant curve
DANGEROUS CURVE AHEAD

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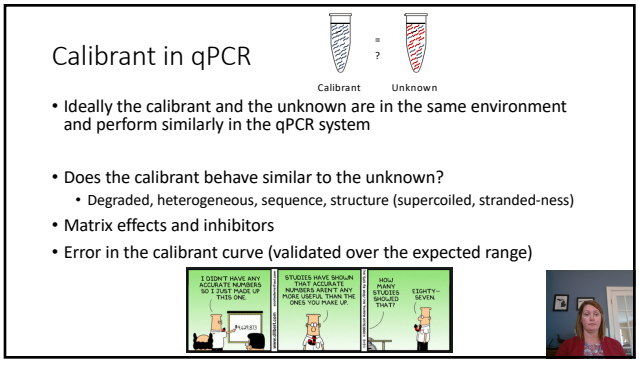
Calibrant in qPCR
Calibrant
Unknown
Ideally the calibrant and the unknown are in the same environment and perform similarly in the qPCR system
Does the calibrant behave similar to the unknown?
Degraded, heterogeneous, sequence, structure (supercoiled, stranded-ness)
Matrix effects and inhibitors
Error in the calibrant curve (validated over the expected range)

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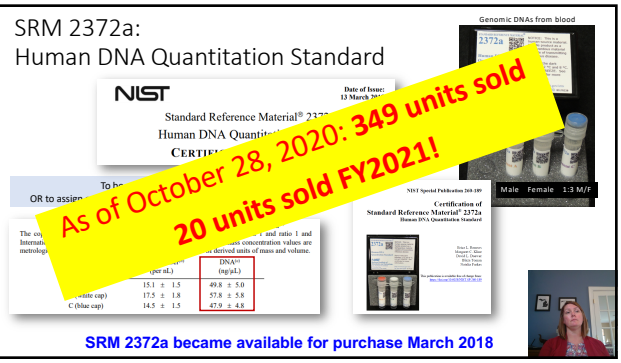
SRM 2372a:
Human DNA Quantitation Standard



As of October 28, 2020: 349 units sold

20 units sold FY2021!

SRM 2372a became available for purchase March 2018

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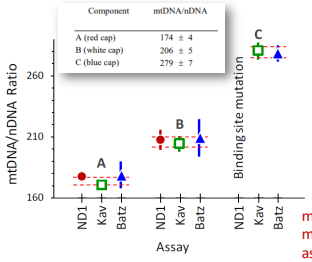
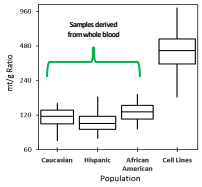
SRM 2372a Includes Mitochondrial Value

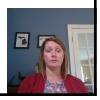
Component	mtDNA/nDNA
A (red cap)	174 ± 4
B (white cap)	206 ± 5
C (blue cap)	279 ± 7



Originating source of standards matter



mtDNA/nDNA ratio for three mitochondrial quantification assays optimized for dPCR.



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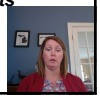
Digital PCR at NIST

- Digital PCR has become our 'go to' method for the quantification of nucleic acid-based materials
- Replacing UV spectroscopy (indirect method)
- The typical downstream application of our ***reference materials*** is PCR or sequencing-based

  We care about ***intact (and accessible) genomic targets***

This assures our standard is more commutable with qPCR



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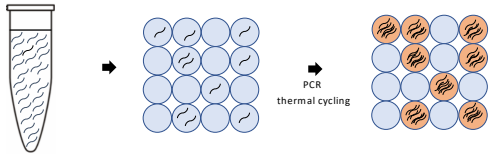
What is Digital PCR?



Post extraction

Unknown number of copies of nucleic acid

Portion of the stock goes into a dPCR mastermix (similar to a qPCR mastermix)

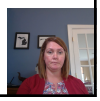
The sample is partitioned (e.g. droplets)

1000s of droplet are generated (micro reactors)

Post PCR read out of droplets

Endpoint detection of fluorescence

Counting positive/negative droplets



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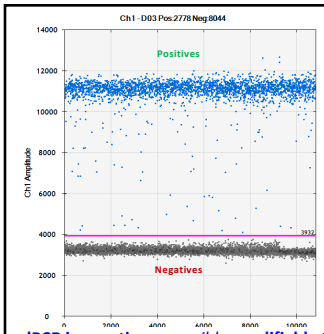


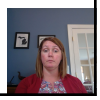
$\lambda = -\ln(N_{neg}/N_{tot})$

λ = number of targets per partition

From here, concentration can be calculated.

- Select a threshold to separate positive and negative partitions
- Positives and negative events are counted
- 2778 positive, 8044 negative, (10822 total)

dPCR is counting *accessible* amplifiable targets



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$\lambda = -\ln(8044/10822)$

$C = 0.2967/(0.85e^{-9}L * 100e^{-6}L)$

$= 3.46e^{6}$ copies/µL

λ = number of targets per partition

V = mean partition volume

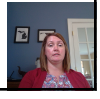
F = volume fraction of sample in the reaction mixture

C = target concentration in a sample

2778 positive

8044 negative

10822 total



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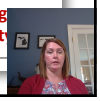
Converting copies per nanoliter to nanograms nuclear DNA per microliter

$$[\mathrm{nDNA}]\frac{\mathrm{ng}}{\mu\mathrm{L}} = \left(\frac{\lambda \text{ copies of target}}{\text{droplet}}\right)\left(\frac{\mu\mathrm{L\ mixture}}{F\ \mu\mathrm{L\ sample}}\right)\left(\frac{\text{droplet}}{V\ \text{mixture}}\right)\left(\frac{\mathrm{HHGE}}{r\ \text{target}}\right)\left(\frac{n\ \text{base pairs}}{\mathrm{HHGE}}\right)\left(\frac{\overline{m}\ \mathrm{g}}{\text{mol base pairs}}\right)\left(\frac{\text{mol base pairs}}{6.022\ 10^{23}\ \text{base pairs}}\right)\left(\frac{10^3\ \mathrm{nL}}{\mu\mathrm{L}}\right)\left(\frac{10^9\ \mathrm{ng}}{\mathrm{g}}\right) \quad (1)$$

DNA in ng/µL = 3.301 x (λ/(Dilution * Droplet Vol)

$$\frac{u([\mathrm{nDNA}])}{[\mathrm{nDNA}]} = \sqrt{\left(\frac{u(\lambda)}{\lambda}\right)^2 + \left(\frac{u(F)}{F}\right)^2 + \left(\frac{u(V)}{V}\right)^2 + \left(\frac{u(r)}{r}\right)^2 + \left(\frac{u(n)}{n}\right)^2 + \left(\frac{u(\overline{m})}{\overline{m}}\right)^2}$$

Calculating Uncertainty



Duewer DL, Kline MC, Romsos EL, Toman B. Anal Bioanal Chem. 2018 May;410(12):2879-2887

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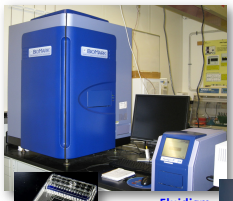
dPCR platforms at NIST

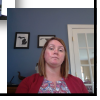
Droplet Digital - ddPCR

BIO-RAD QX200

Chamber Digital - cdPCR



Fluidigm BioMark



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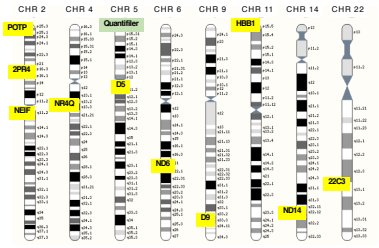
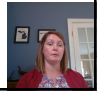
Optimized Assays for SRM 2372a

10 assays across 8 different chromosomes

All assays are single copy, and Human, or Primate specific





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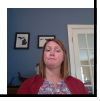
Why use dPCR for certification?

- No need for an external calibrant
- Multiple dPCR assays can be used for characterization
  - Establish reasonable estimates of uncertainty
- More accurate form of concentration measurement for end user



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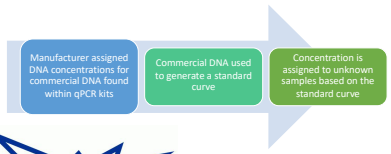
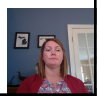
Forensic Need for SRM 2372a



Is the manufacturer assigned concentration accurate?



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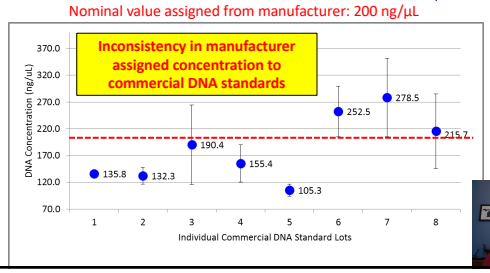
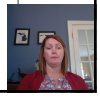
8 Commercial DNA Standards

DNA Standard derived from a human cell line found in commercial qPCR kits
Nominal value assigned from manufacturer: 200 ng/µL





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That might have you saying…

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Example of DNA Standard Bias

- Use of cell lines for production of commercial DNA standards—deviation from wild type DNA due to characteristics of cell lines
- Example: Raji cell line used for a commercial DNA standard
  - More copies in ~85% of all tested immortalized cell lines


Advances in Brief

Frequent Amplification of the *Telomerase Reverse Transcriptase* Gene in Human Tumors[1]

ScienceDirect

Comparison of five DNA quantific

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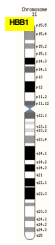
qPCR vs. Digital PCR

- Quantification of the same 8 DNA standards
- No calibration curve – absolute quantification
- Alternate target from hTERT
  - HBB1 – housekeep gene on chromosome 11



Assay Target	Chromosome, Band Accession #	Primers and Probe [a]	Amplicon Length, bp
HBB1 Gene HBB	Chr 11, p15.5 NC_000011.10	F gctgagggtttgaagtccaactc R ggtctaagtgatgacagccgtacct P[†] agccagtgccagaagagccaagga	76

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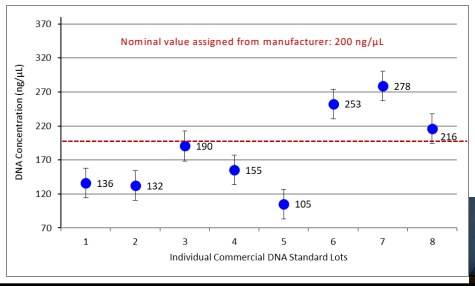
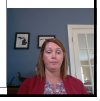
qPCR measurements from eight independent lots of a commercial DNA calibrant





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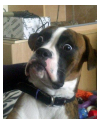
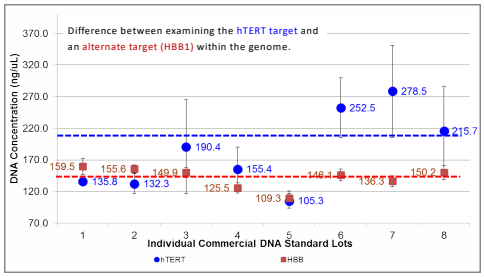
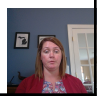
hTERT (qPCR) vs. HBB1 (ddPCR)







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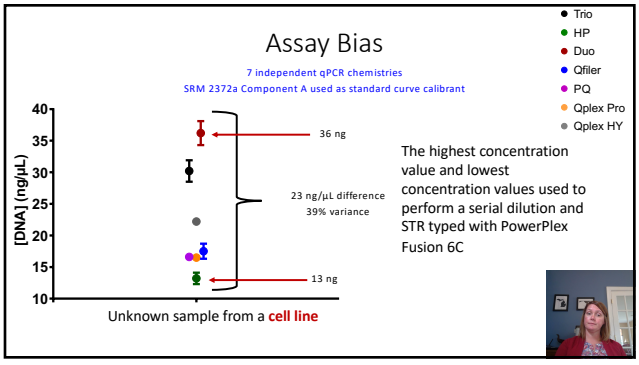
Assay Bias



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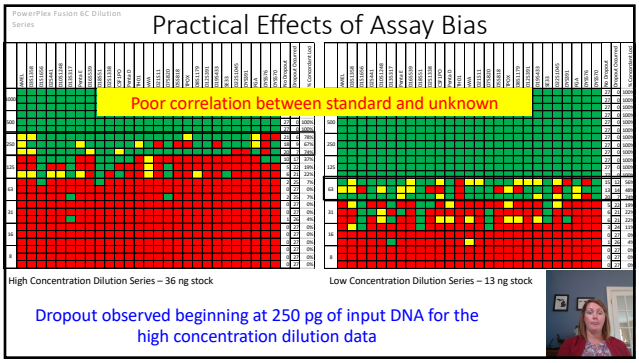
Practical Effects of Assay Bias



Dropout observed beginning at 250 pg of input DNA for the high concentration dilution data

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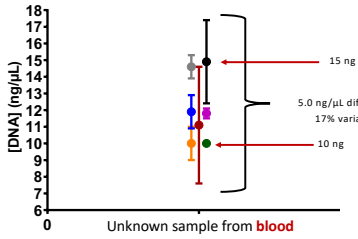
Assay Bias

7 independent qPCR chemistries
SRM 2372a Component A used as standard curve calibrant



- Trio
- HP
- Duo
- Qfiler
- PQ
- Qplex Pro
- Qplex HY

The highest concentration value and lowest concentration values used to perform a serial dilution and STR typed with PowerPlex Fusion 6C

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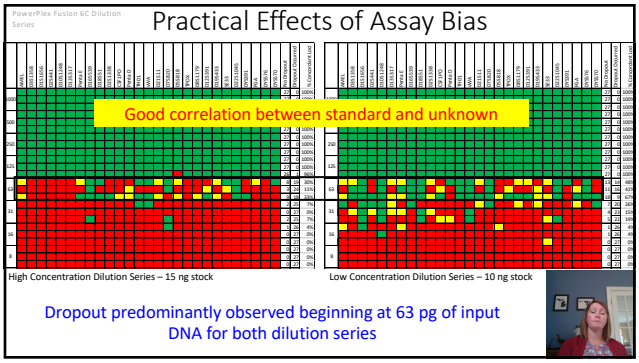
Practical Effects of Assay Bias



Dropout predominantly observed beginning at 63 pg of input DNA for both dilution series

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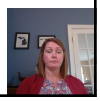
Potential Remediation of Bias

Does it really matter?



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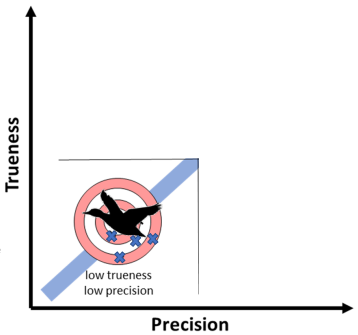
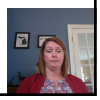
Hitting where you aim

Aim is "off" each time

Incapable of doing the same thing twice





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Hitting where you aim

Aim is "off" each time

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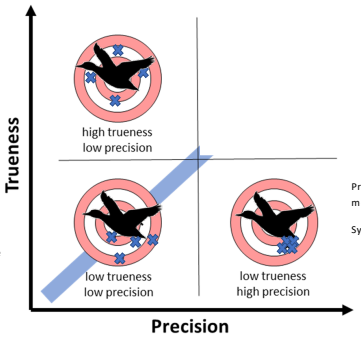
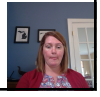


Precise but not true measurements

Systematic bias present



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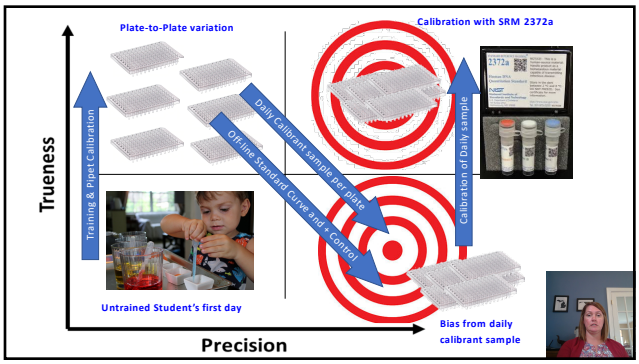
Plate-to-Plate variation
Calibration with SRM 2372a
Trueness
Training & Pipet Calibration
Daily Calibrant Sample Per Plate
Offline Standard Curve and + Control
Calibration of Daily Sample
Untrained Student's first day
Bias from daily calibrant sample
Precision

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where do you want to go?
it's easier to get where you want to go when you know where you want to go.

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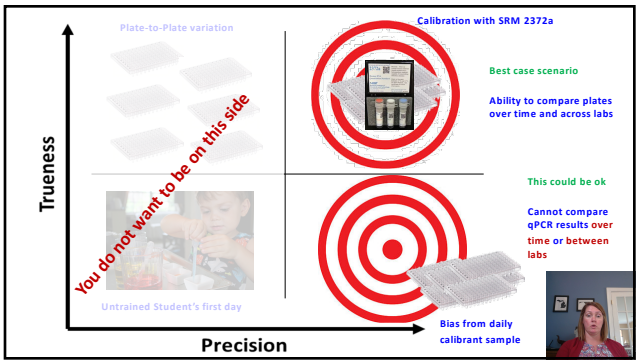
Plate-to-Plate variation
Calibration with SRM 2372a
Best case scenario
Ability to compare plates over time and across labs
Trueness
You do not want to be on this side
This could be ok
Cannot compare qPCR results over time or between labs
Untrained Student's first day
Bias from daily calibrant sample
Precision

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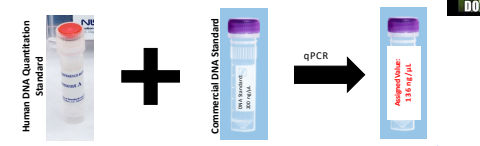
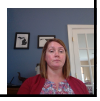
Most Frequently Asked Question:

- The certified value is wrong, what do I do?



Standard Curve **"Unknown"** Reassign Commercial DNA Standard



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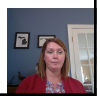
Second Most Frequently Asked Question:

- Can you please provide me with the genotypes of the samples? I am using them in my validation.

Unfortunately, **no** we cannot provide (nor do we have) the genotypes for these samples.

You can however, purchase SRM 2391d with its highly characterized genotypes.



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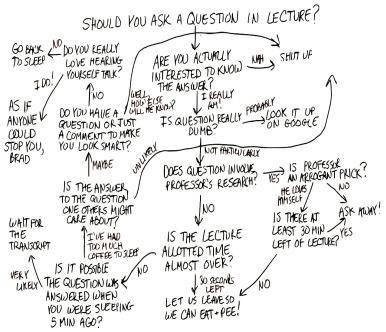

THANK YOU FOR YOUR ATTENTION

Questions?
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1-301-975-5107

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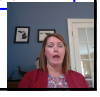
Margaret Kline
David Duewer
Steven Lund



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