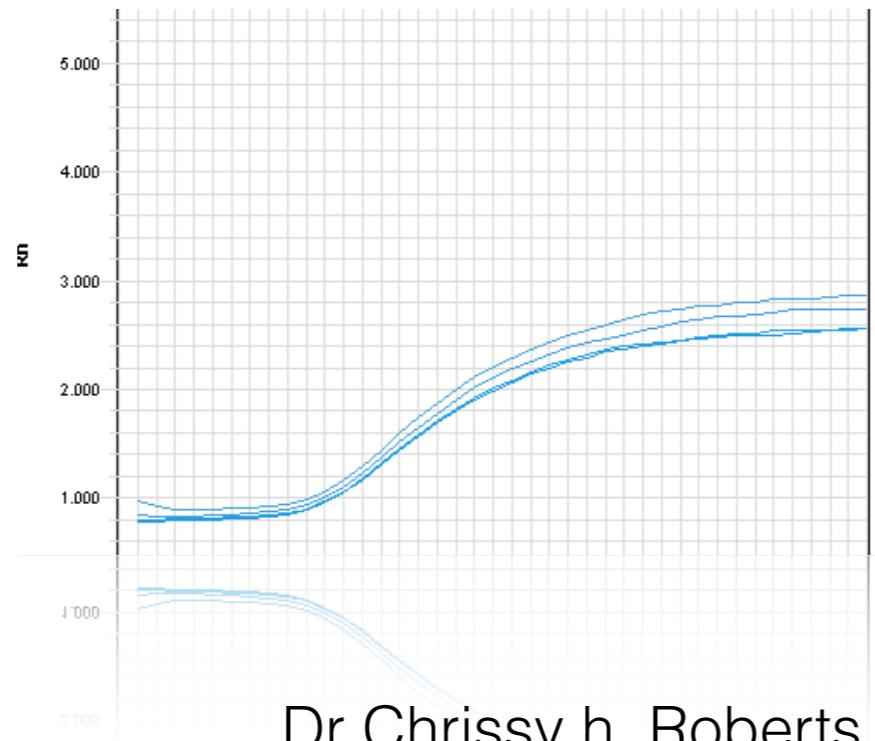
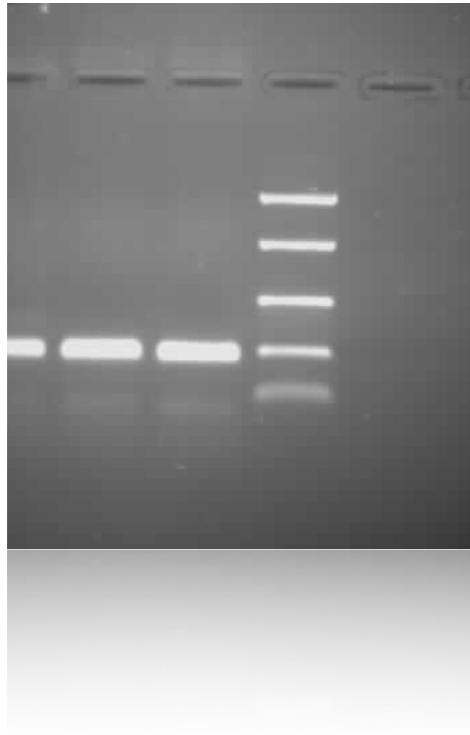
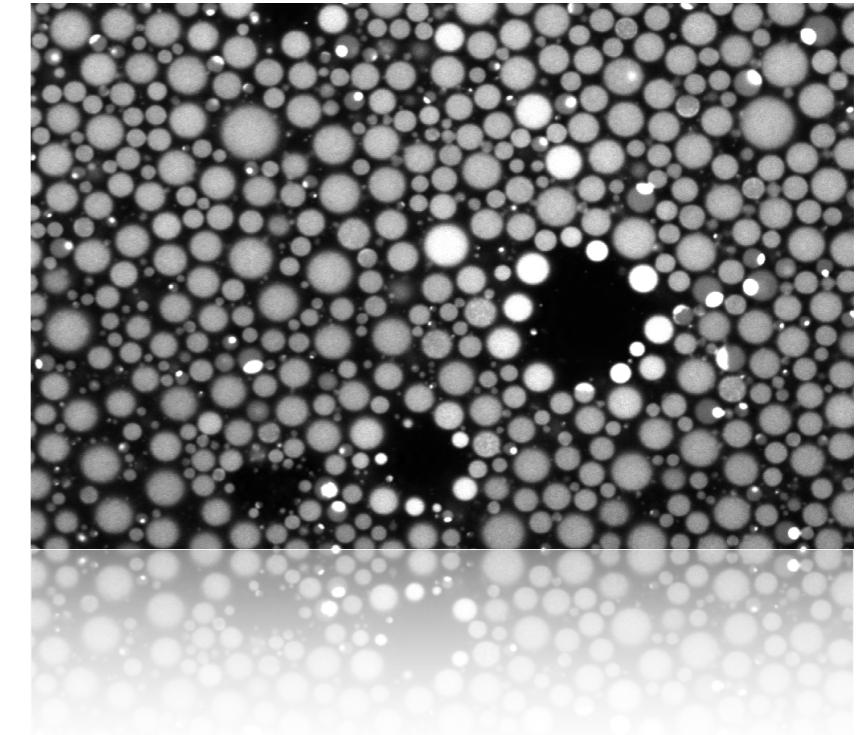


Droplet Digital PCR : Turning a cool new toy in to a validated clinical tool



Dr Chrissy h. Roberts,
Associate Professor, CRD
Room 307, KS



@LSHTM_GHA

Molecular Biology Research Progress & Applications

Aim : To provide the knowledge and experience required to **assess** and to keep up to date with rapidly **advancing research frontiers** in the molecular biology of **infectious diseases**.

This session

Aims :

To introduce an advanced molecular diagnostics platform (ddPCR)

To demonstrate the importance of rigorous diagnostic evaluations

To introduce students to methods for performing simple diagnostic evaluations



By the end of the session, you will be able to...

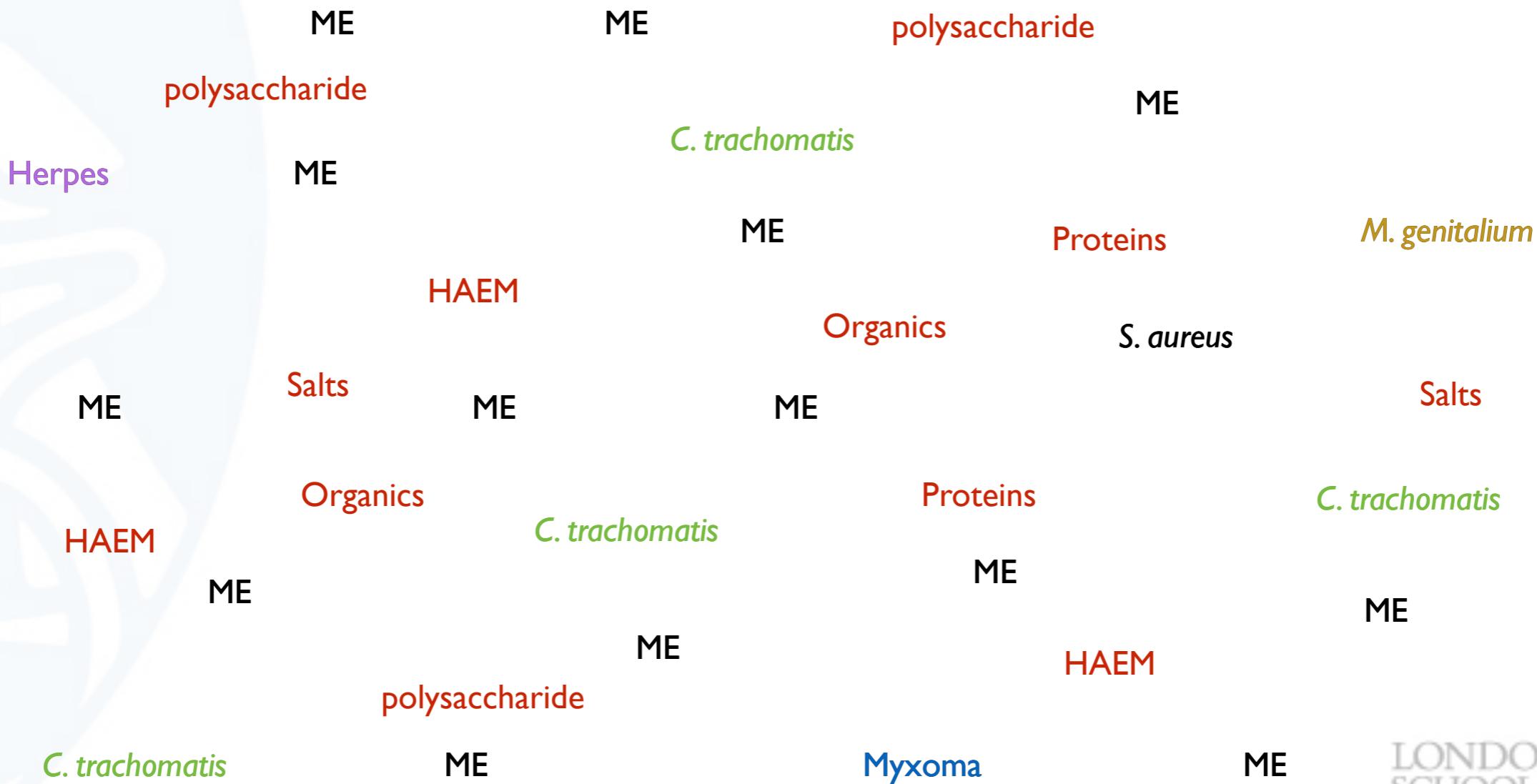
**...briefly describe how ddPCR works and explain
some of its advantages over quantitative PCR
(qPCR)**

**...understand and perform a simple diagnostic
evaluation**

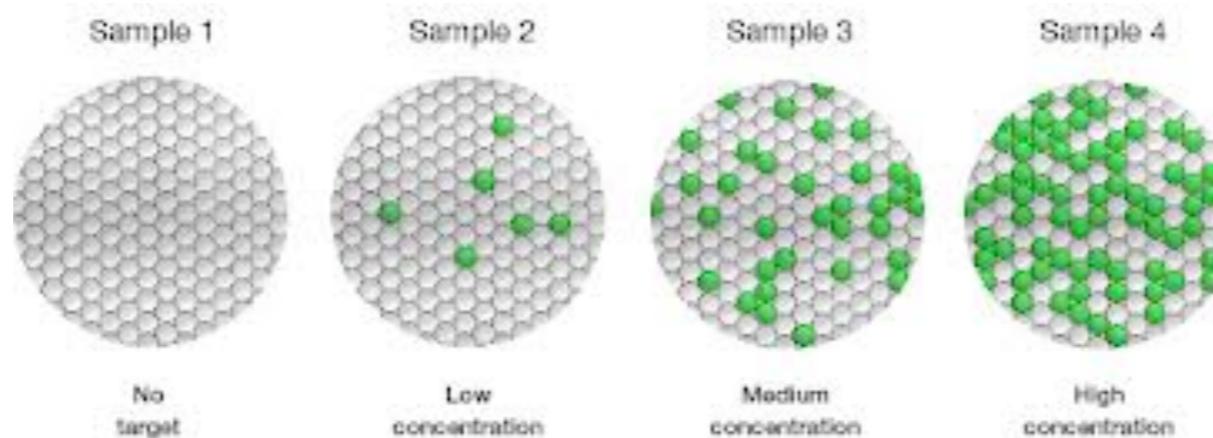


Methodological Challenge

To detect and enumerate infectious organism (*C. trachomatis*) DNA in a clinical specimen



Part One : A cool New toy

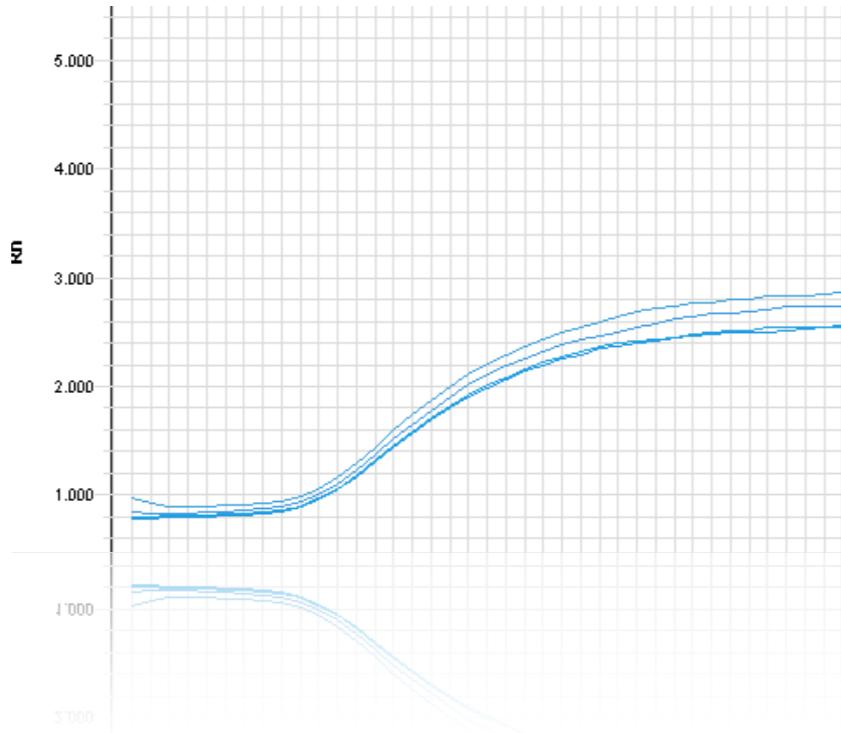


Three generations of PCR



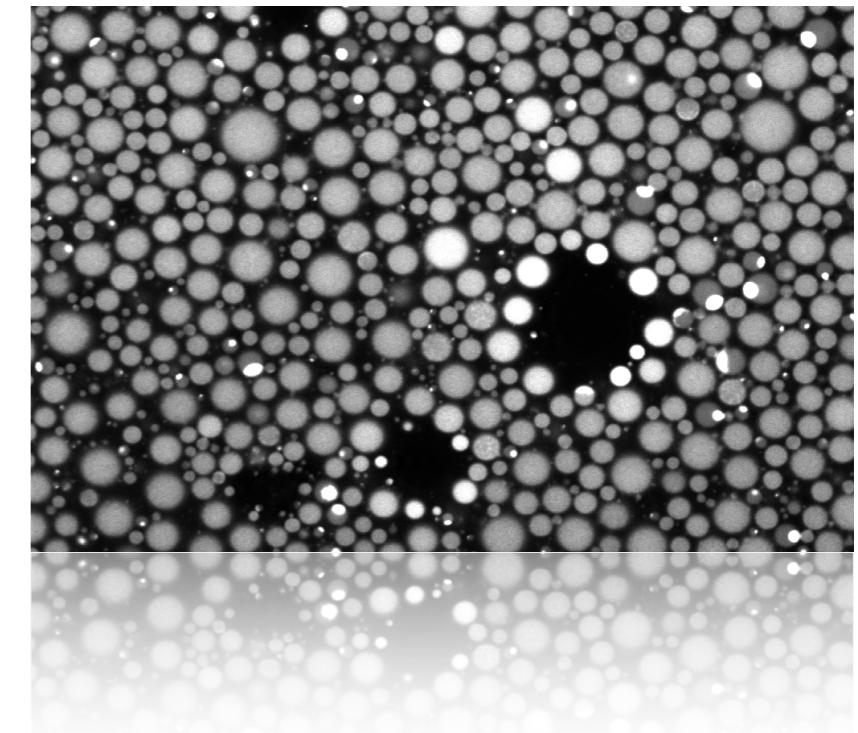
Generation One
Endpoint PCR

detect



Generation Two
quantitative PCR

enumeration
requiring external
calibration

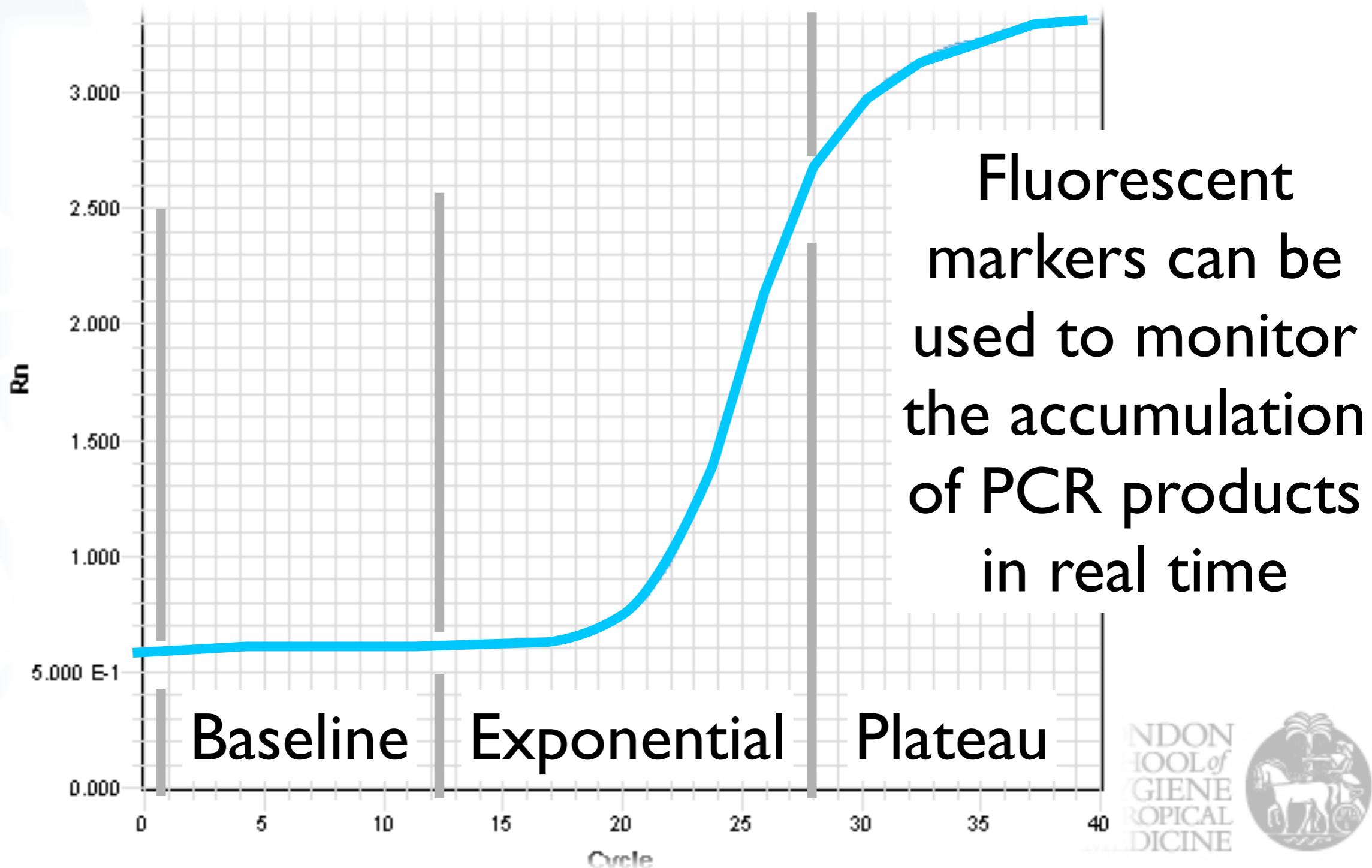


Generation Three
digital PCR

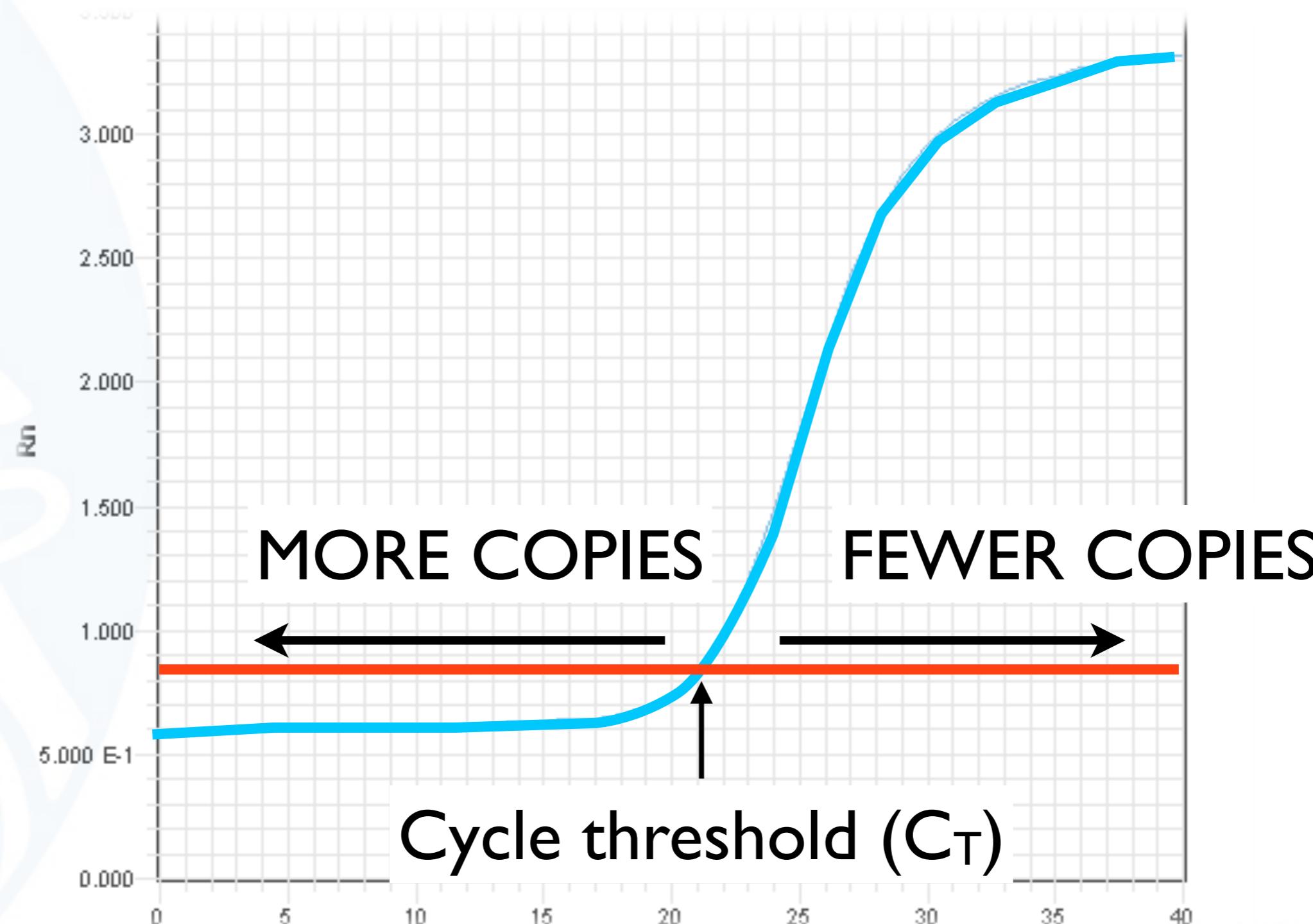
enumeration
without calibration

The diagnosis of infectious disease is a process of detection
and (sometimes) enumeration

Recall from previous learning that PCR IS A QUANTITATIVE PROCESS

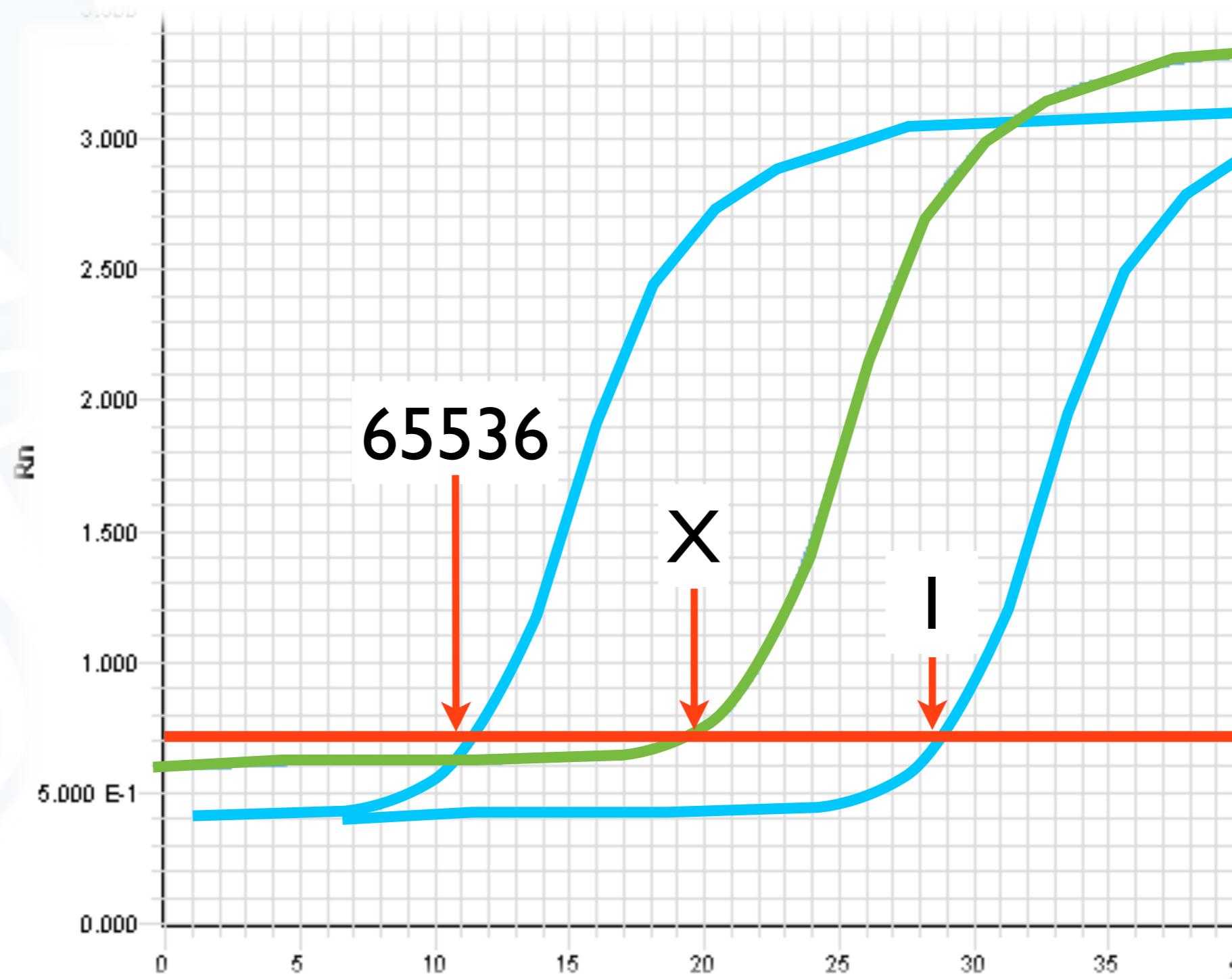


THE 'TAKEOFF POINT' OF THE EXPONENTIAL PHASE IS PROPORTIONAL TO THE STARTING NUMBER OF COPIES OF THE TEMPLATE

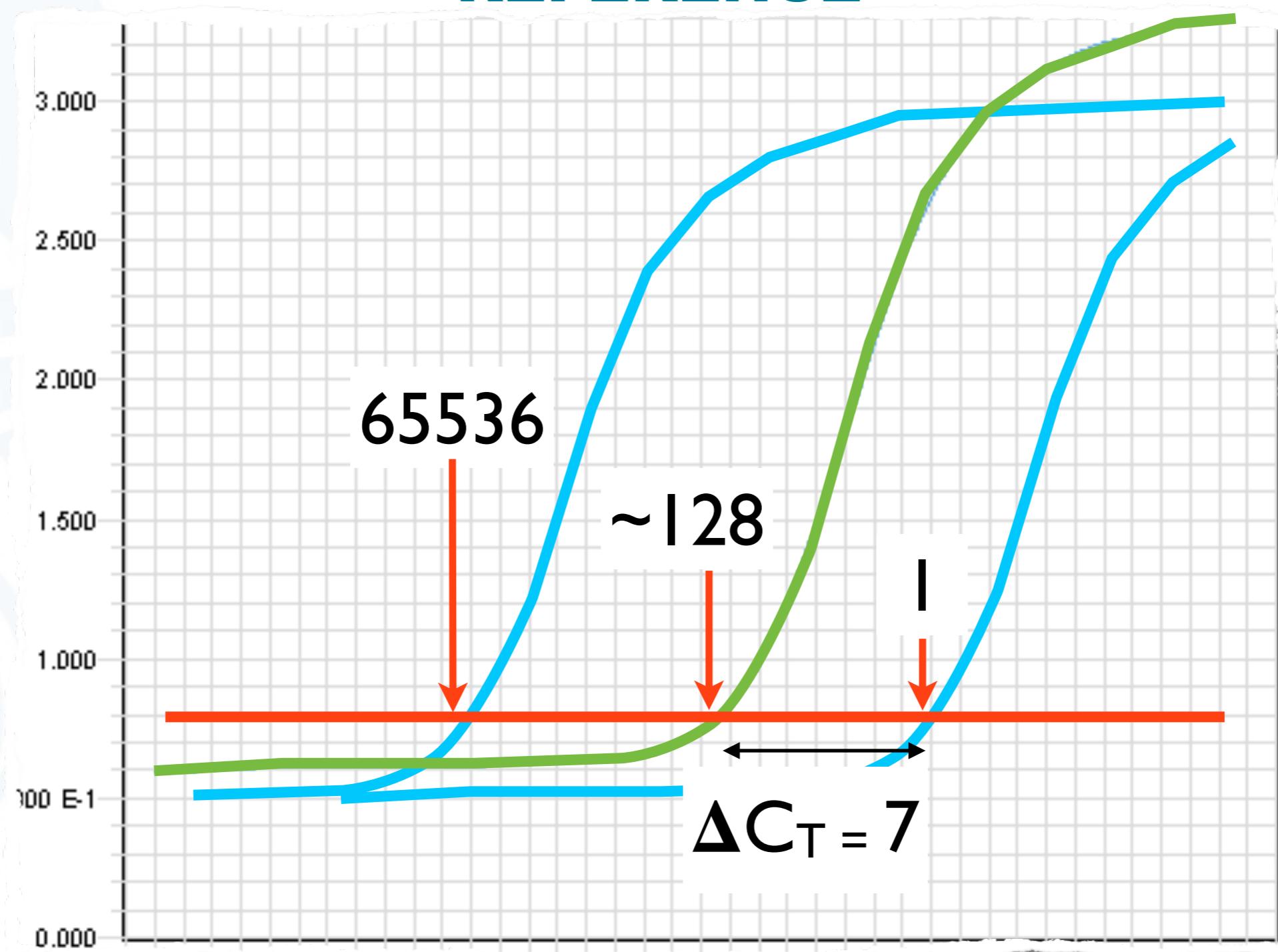


each C_T unit increase indicates a doubling in the amount of PCR product

QUANTITATIVE PCR DETERMINES THE AMOUNT OF DNA/RNA IN AN UNKNOWN SAMPLE BY COMPARISON TO A KNOWN REFERENCE

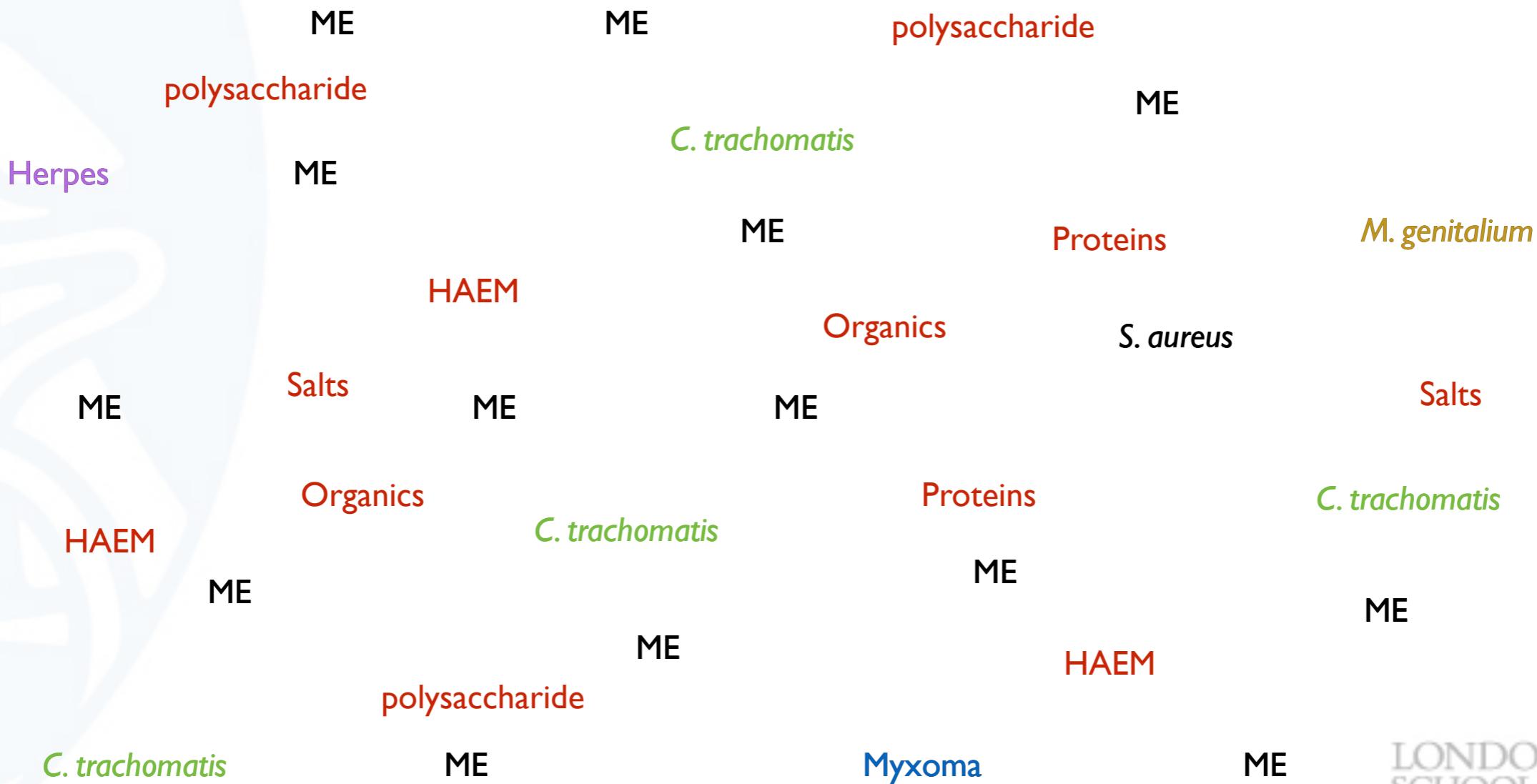


QUANTITATIVE PCR DETERMINES THE AMOUNT OF DNA/RNA IN AN UNKNOWN SAMPLE BY COMPARISON TO A KNOWN REFERENCE



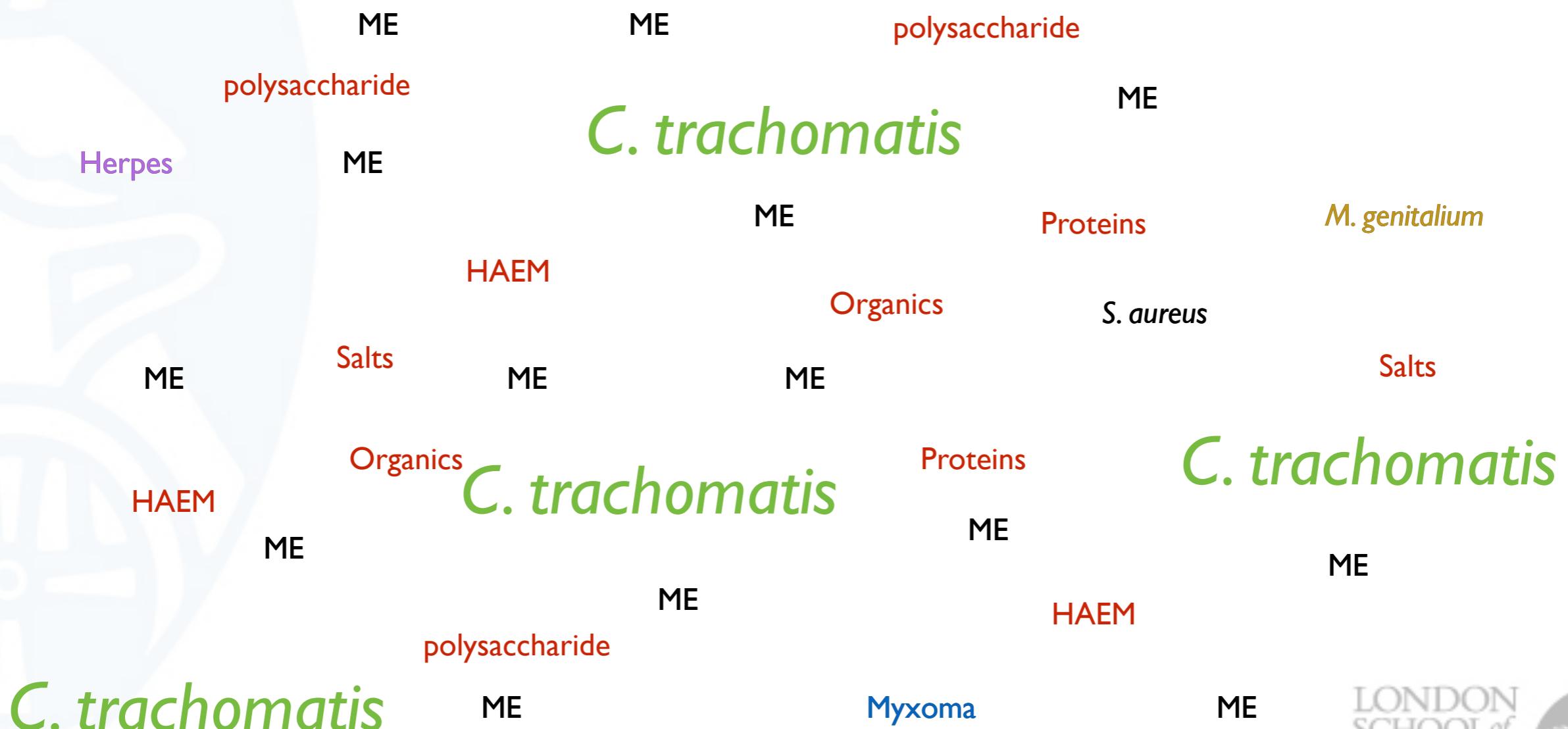
Methodological Challenge

To detect and enumerate infectious organism (*C. trachomatis*) DNA in a clinical specimen



CAN'T WE JUST COUNT THEM?

AS IN, WITHOUT HAVING TO REFER TO AN
EXTERNAL CALIBRATOR?

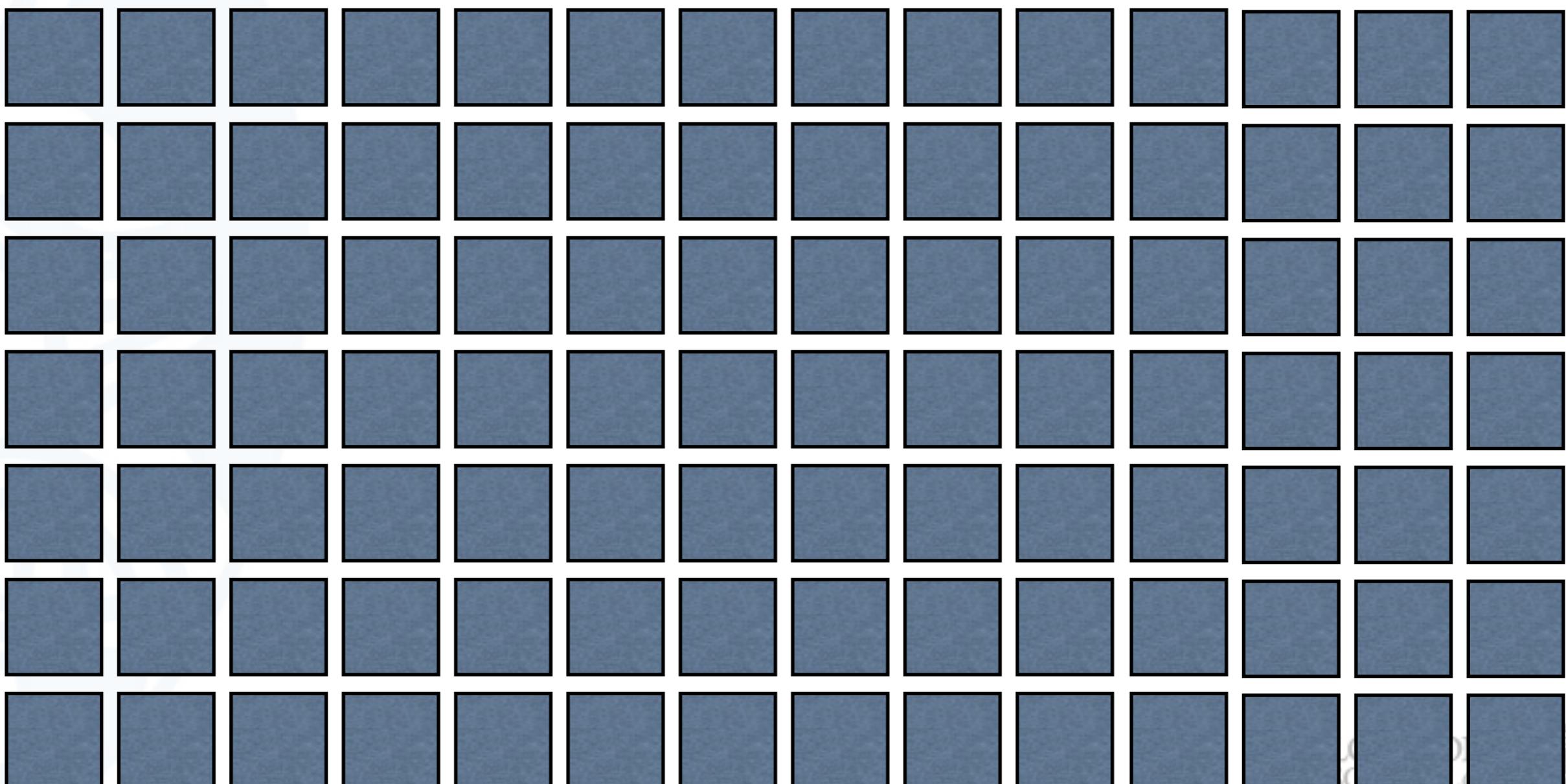


What is digital PCR

- Accurate and precise quantitative PCR
 - Unlike normal qPCR, requires no calibrator
- Three key components
 - Stochastic confinement of target molecules
 - Amplification within partitions
 - Assaying of post-amplification end-points

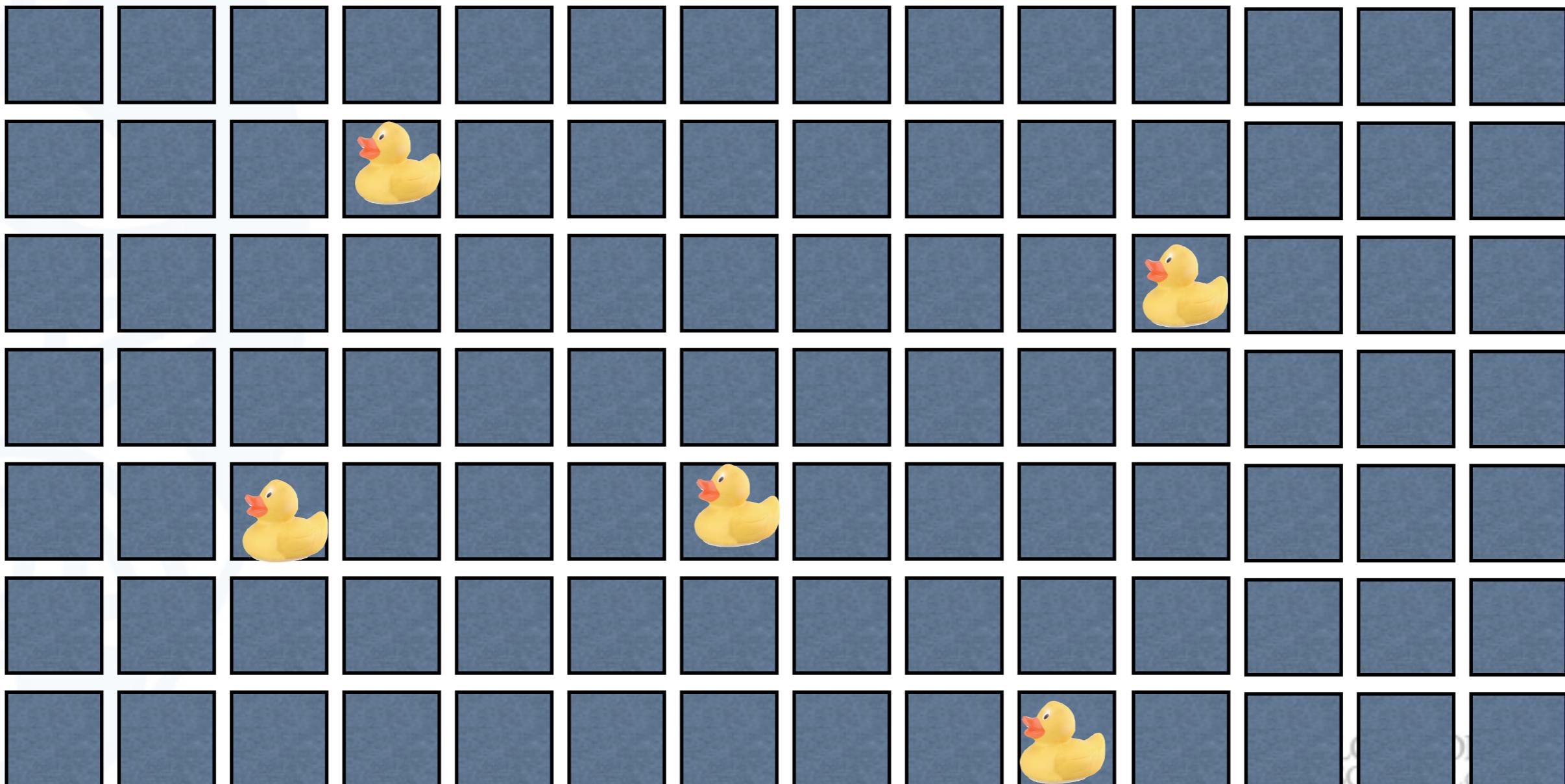
Imagine a GAME in which there are a LOAD OF BOXES AND FIVE DUCKS

You throw the ducks in the air and they fall in to the boxes



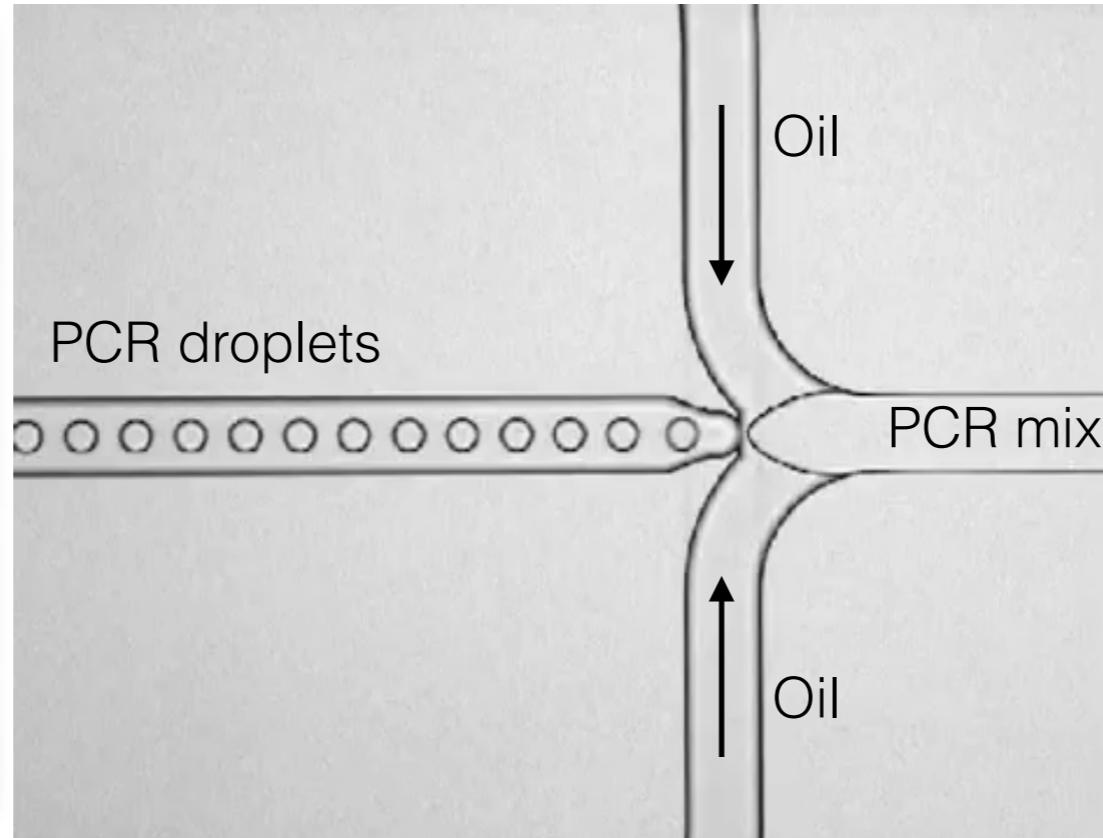
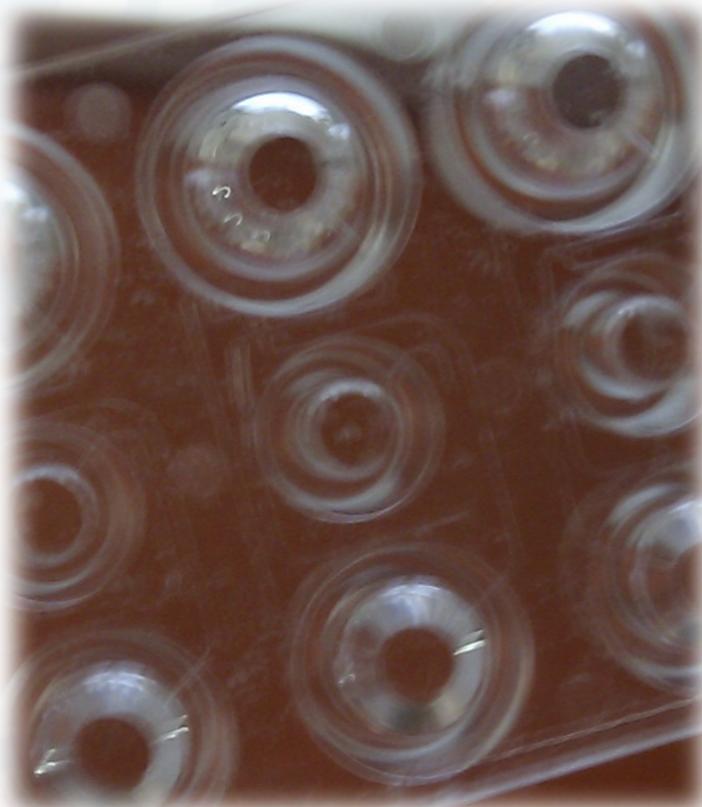
It's very unlikely that you'll get two ducks to land in the same box!

BECAUSE THERE ARE LOTS OF BOXES AND THERE ARE NOT A LOT OF DUCKS

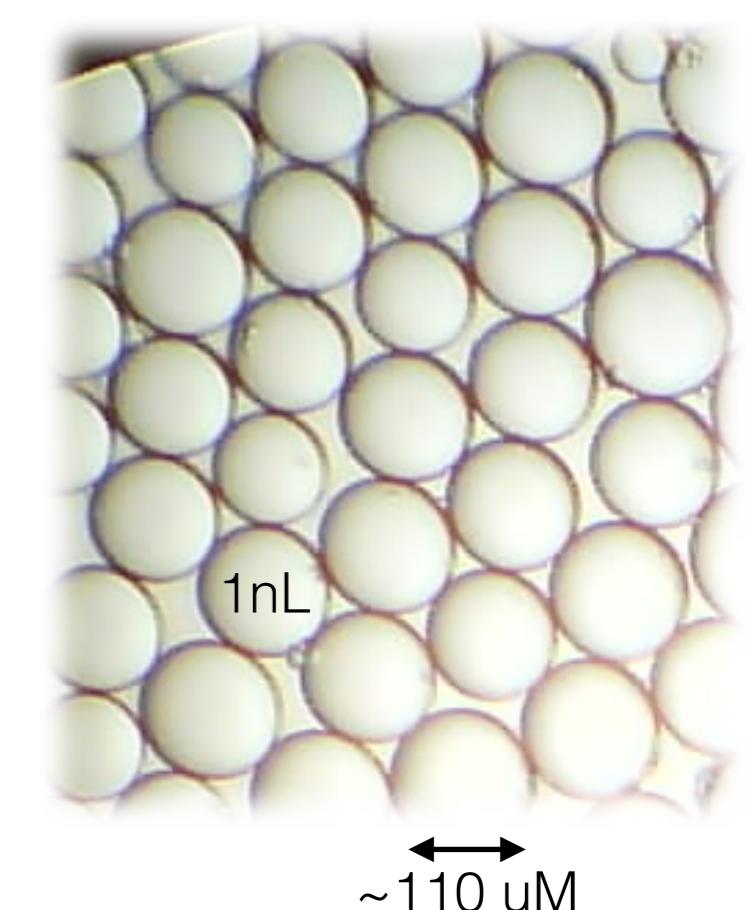


AND IF YOU COUNT UP THE NUMBER OF BOXES THAT HAVE A DUCK IN THEM AS THE NUMBER OF DUCKS THAT YOU HAD TO BEGIN WITH.

**Do the same thing with DNA instead
of ducks, and droplets of PCR mix
instead of boxes and you get**



Microfluidic droplet generator

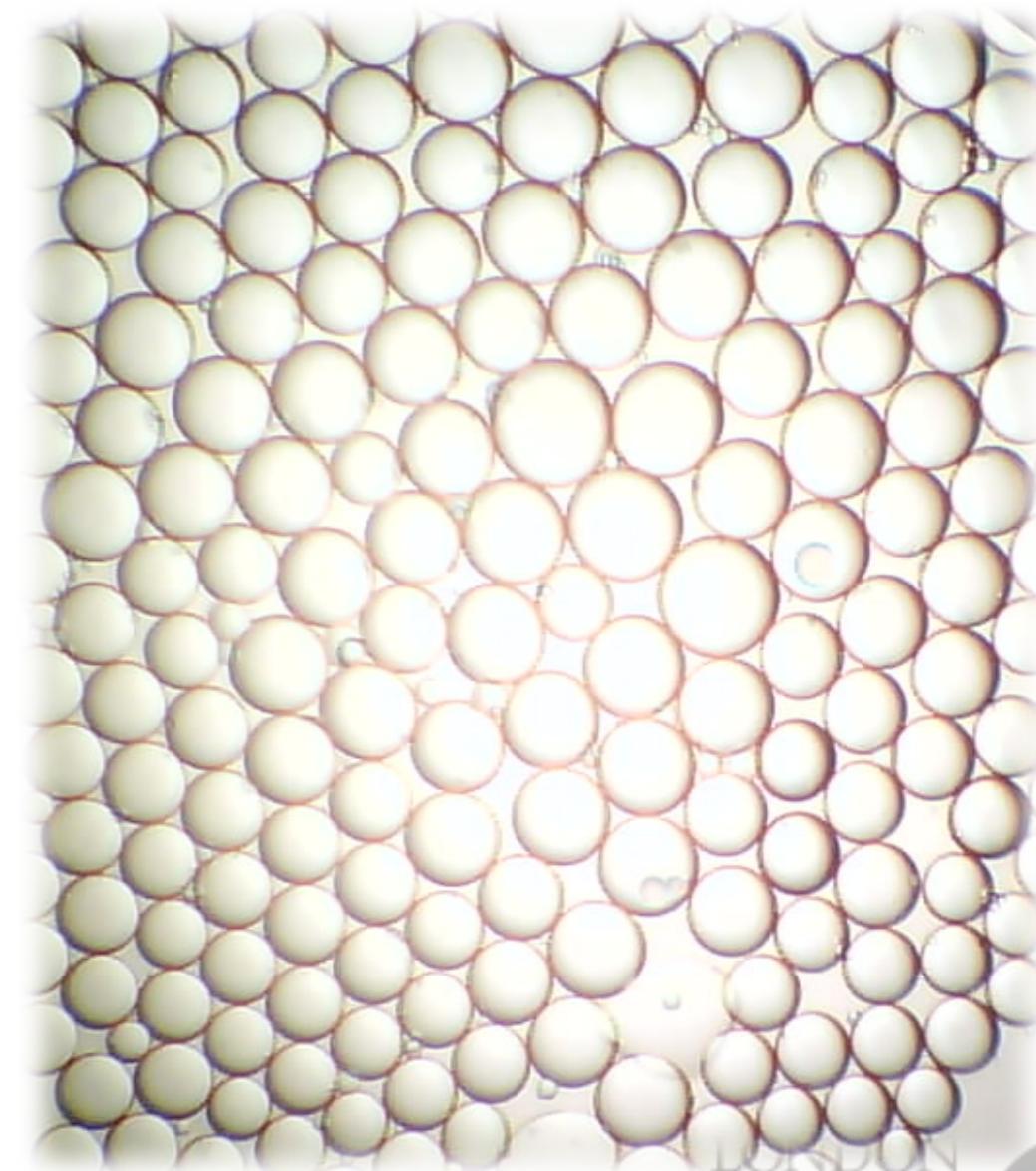


DIGITAL PCR



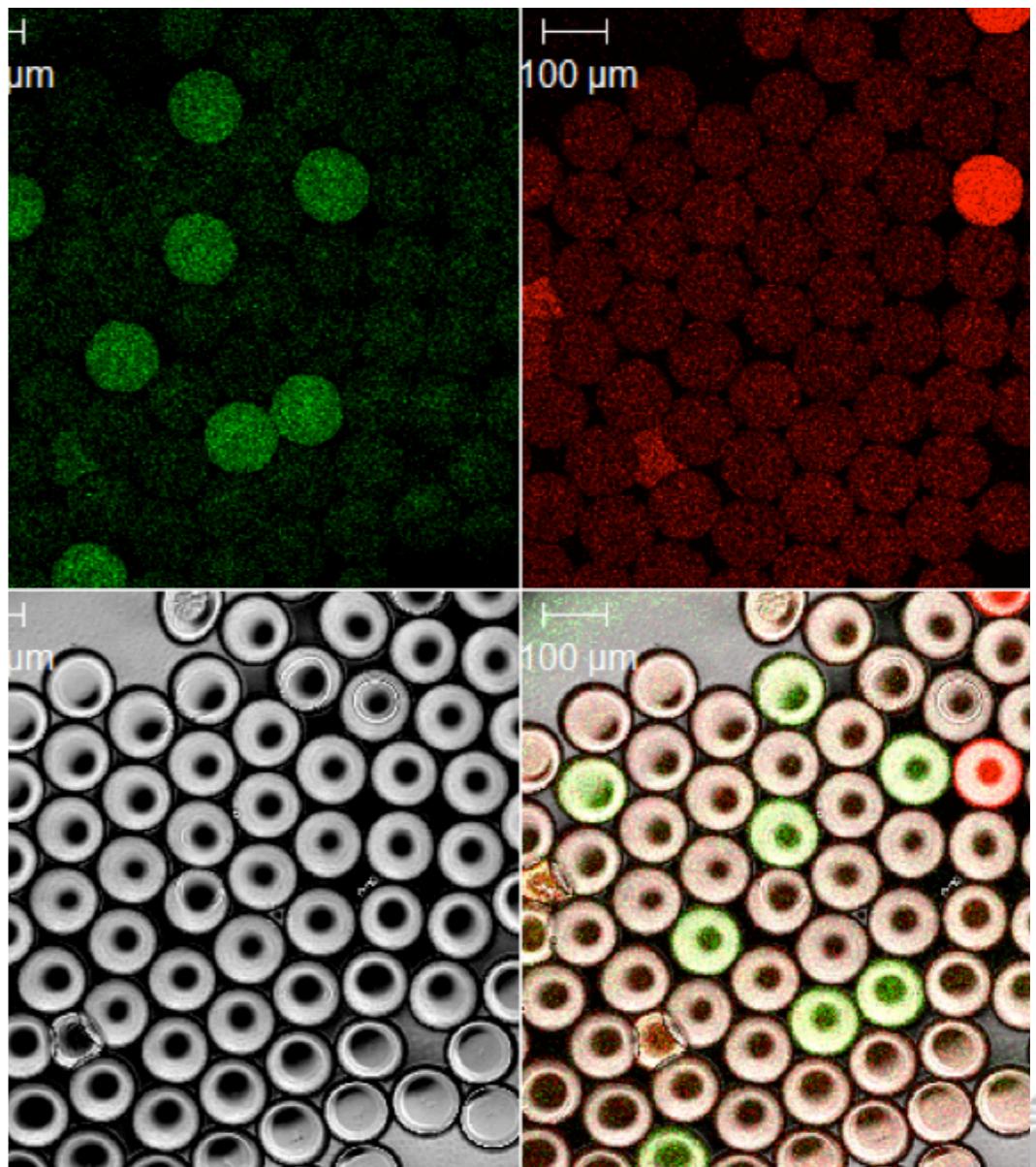
Each droplet gets zero or one template molecule

- **Because there are lots of droplets and not lots of templates**
- **most droplets get zero templates**



After PCR

- most droplets have no PCR product
- Some have a detectable PCR product
- those are the ones that had a template in them
- Count them up, just like the boxes that had ducks in them!



**Droplets are read in single file on a
modified flow cytometer with oil based
fluidics**



**IS THERE ANY PCR
PRODUCT IN THIS
DROPLET?**

**YES / NO ANSWER
(THAT'S DIGITAL)**

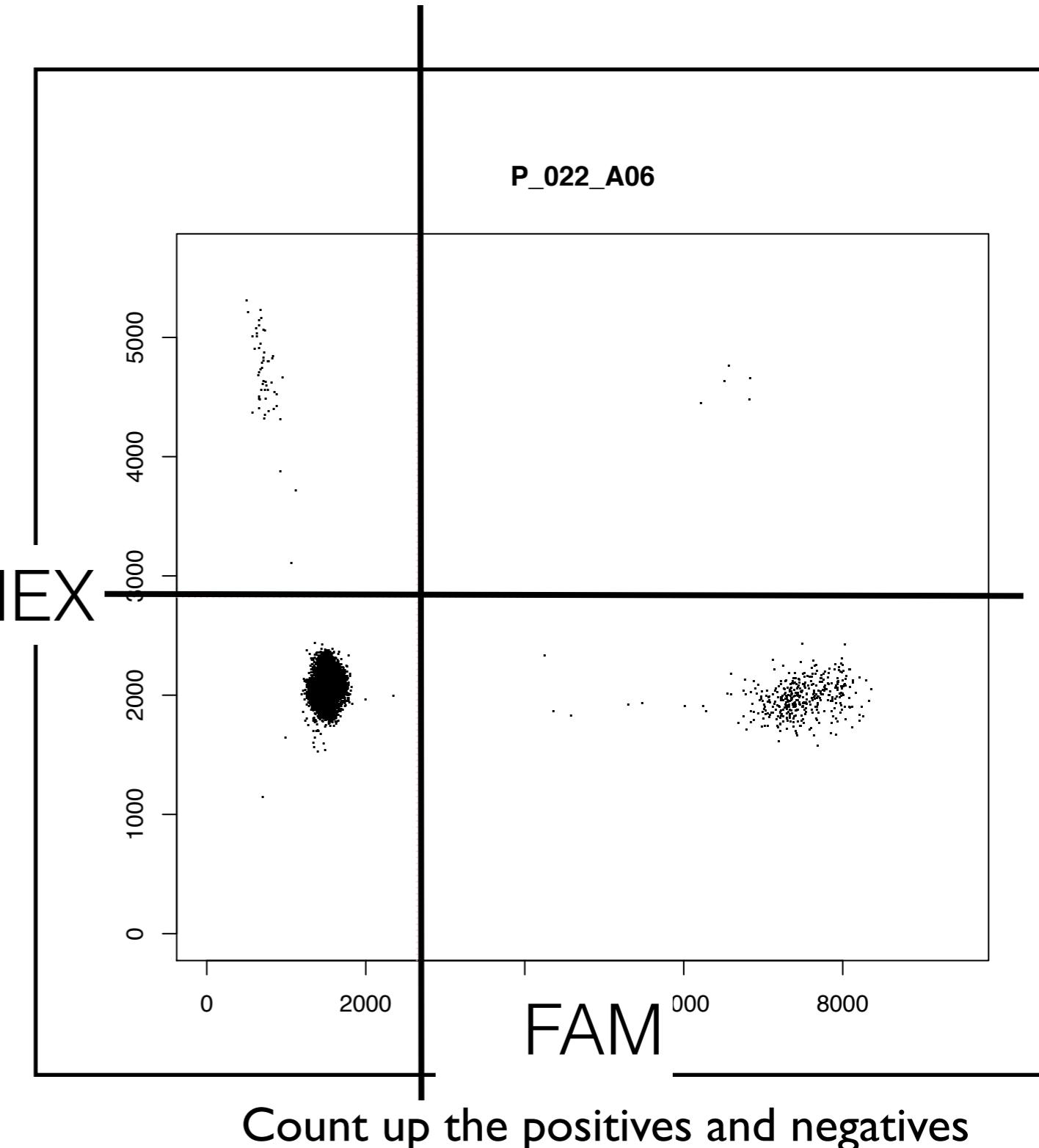
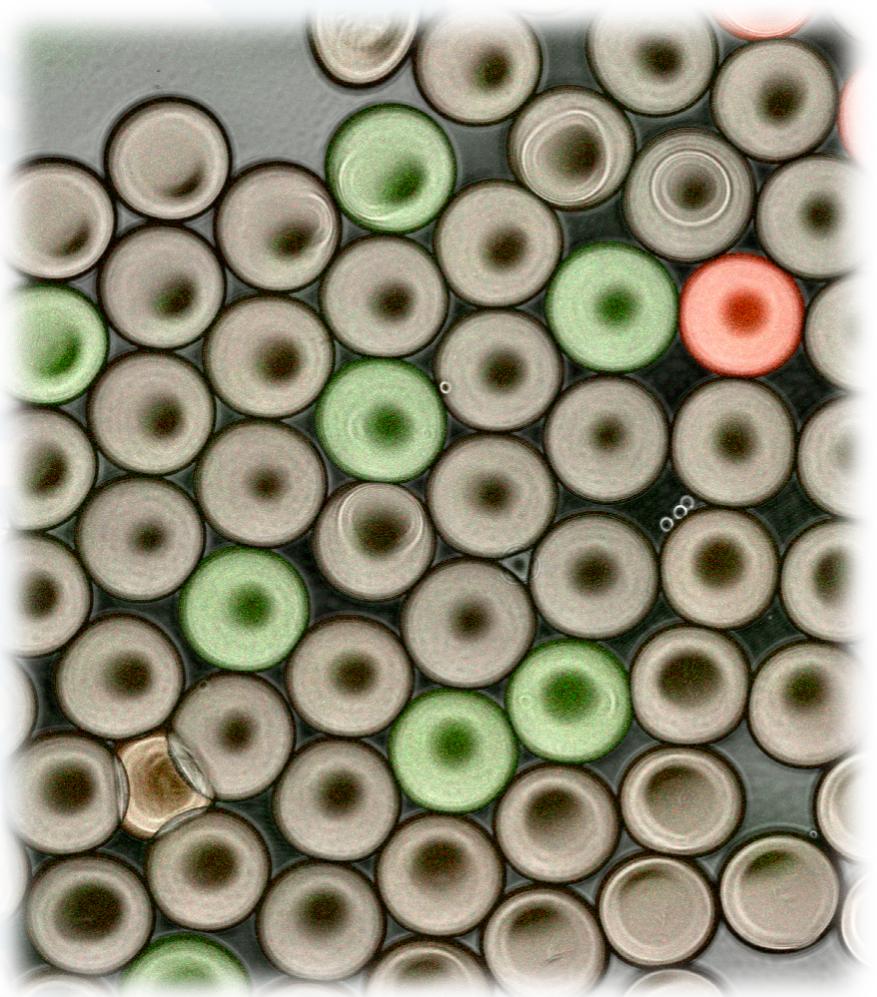
**MEASURING
ENDPOINTS
(RESISTANT TO BBW
PROBLEMS)**



droplet digital PCR example

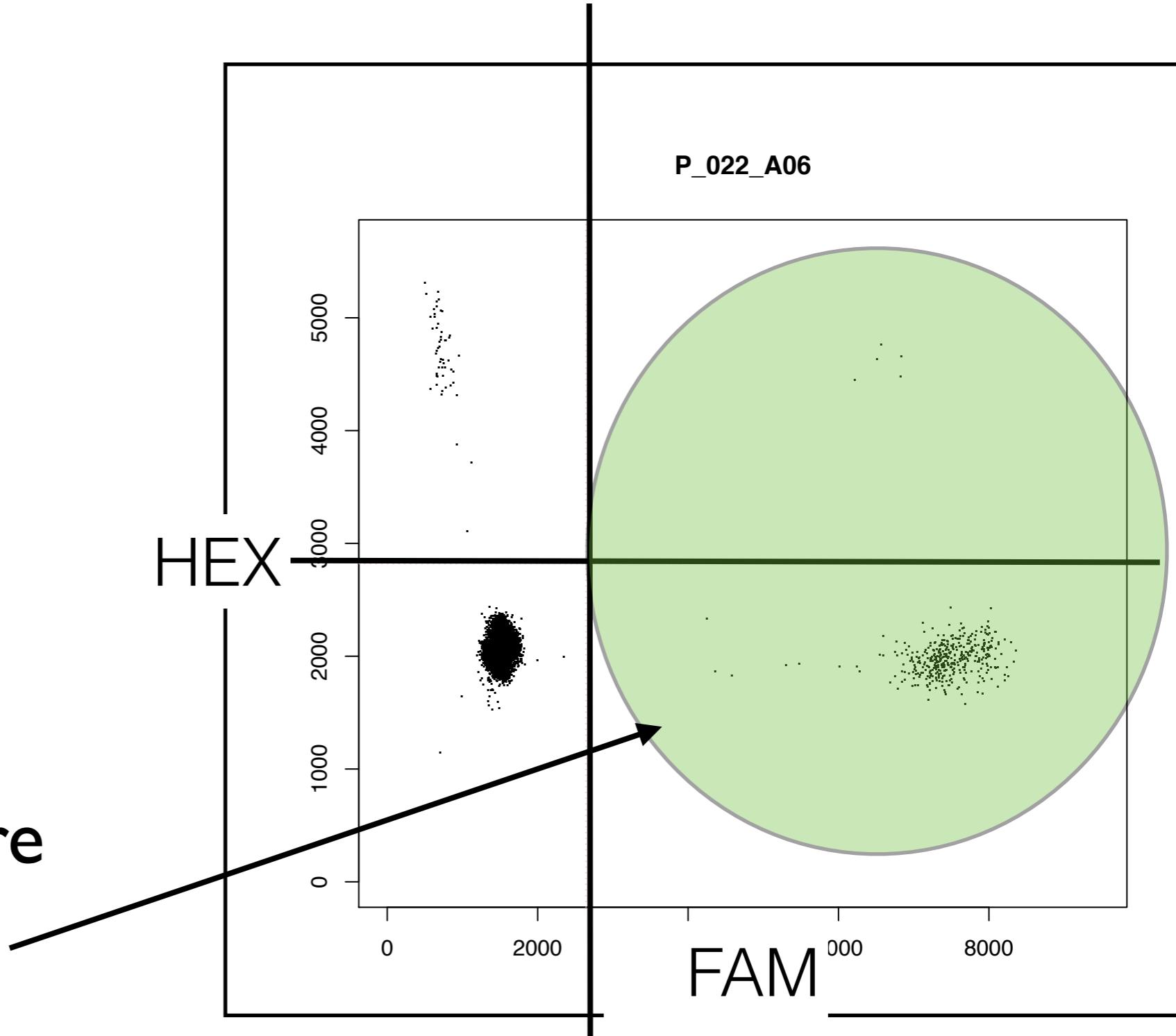
Chlamydia DNA positive droplets
fluoresce on the FAM channel (green in
figure)

Human DNA positive droplets
fluoresce on HEX channel (red)



IT WORKS!

Positive droplets here
indicate
the presence of an
infection



Great!

**I've used my cool new toy to detect and
enumerate
the infectious load of a positive sample**

but how do I know if it is accurate and precise?

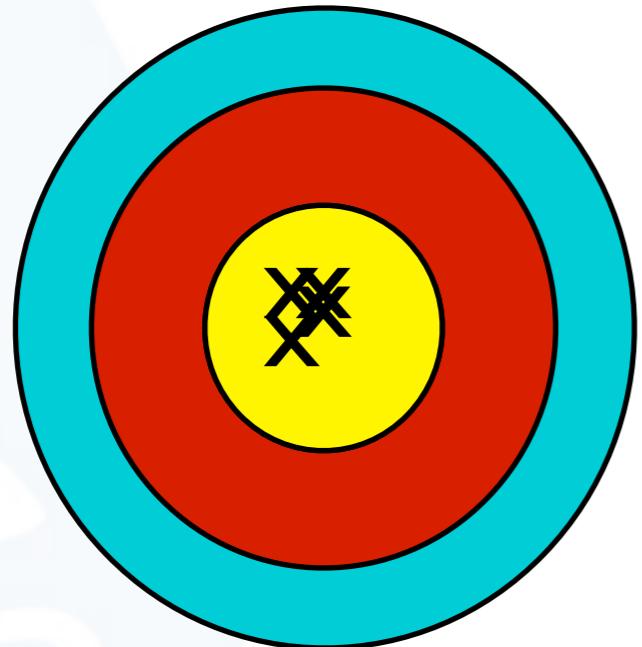


Accuracy and precision

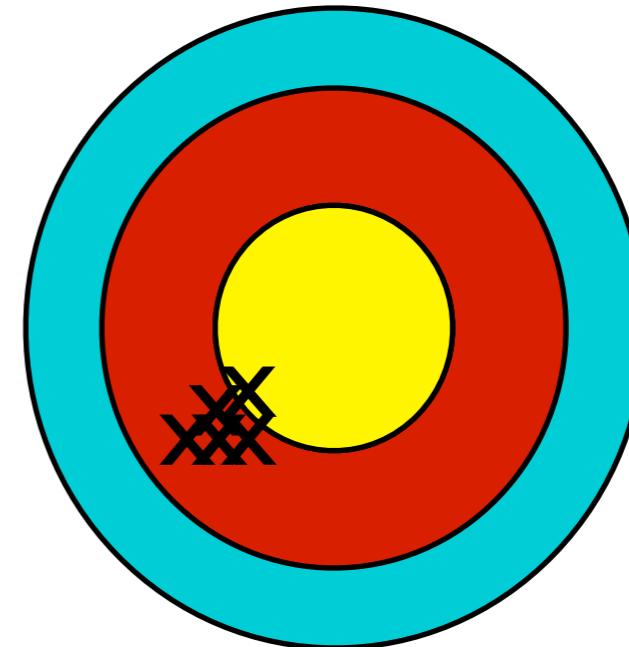


Accuracy and precision

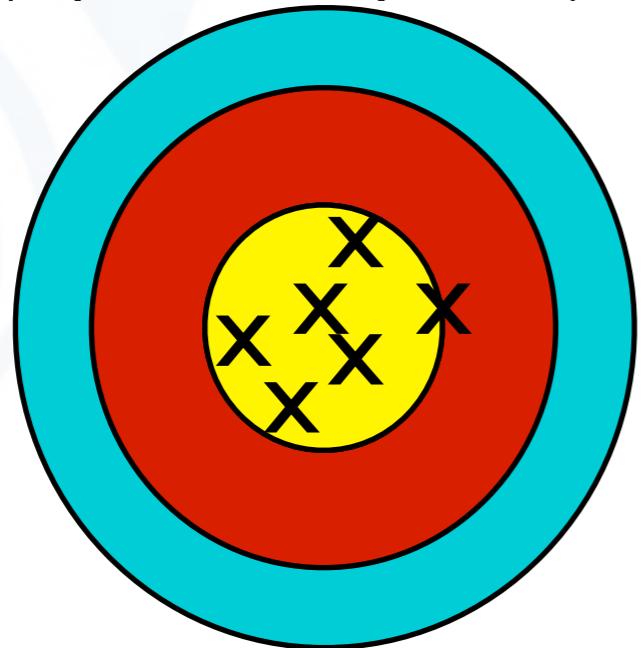
ACCURATE + PRECISE



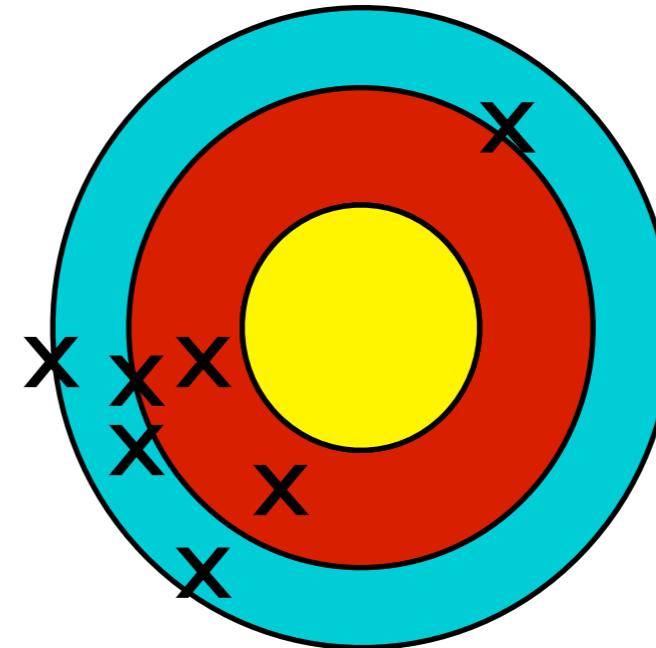
INACCURATE (systematic error) + PRECISE



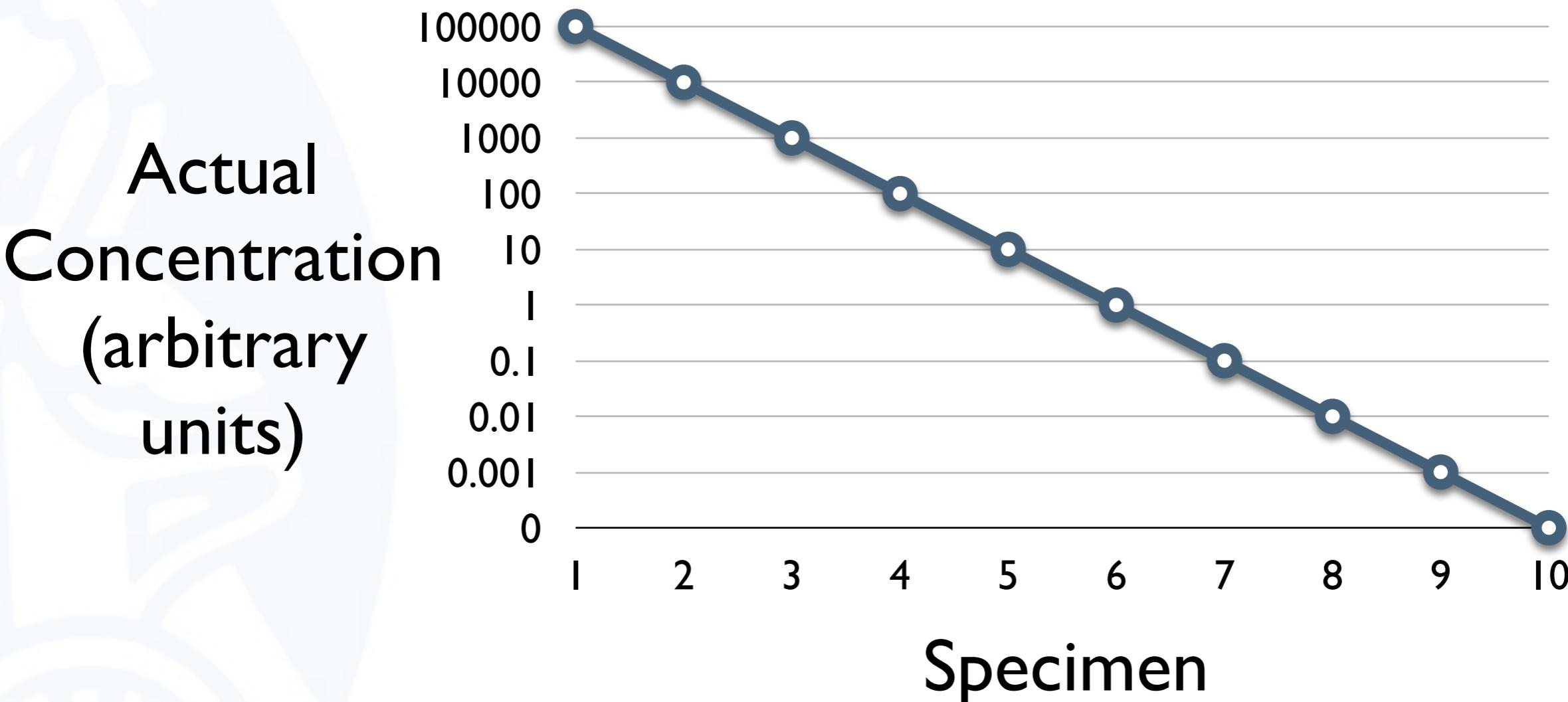
ACCURATE + IMPRECISE
(reproducibility error)



INACCURATE (systematic error)
IMPRECISE (reproducibility error)



Does the diagnostic accurately and precisely estimate the true infectious load?

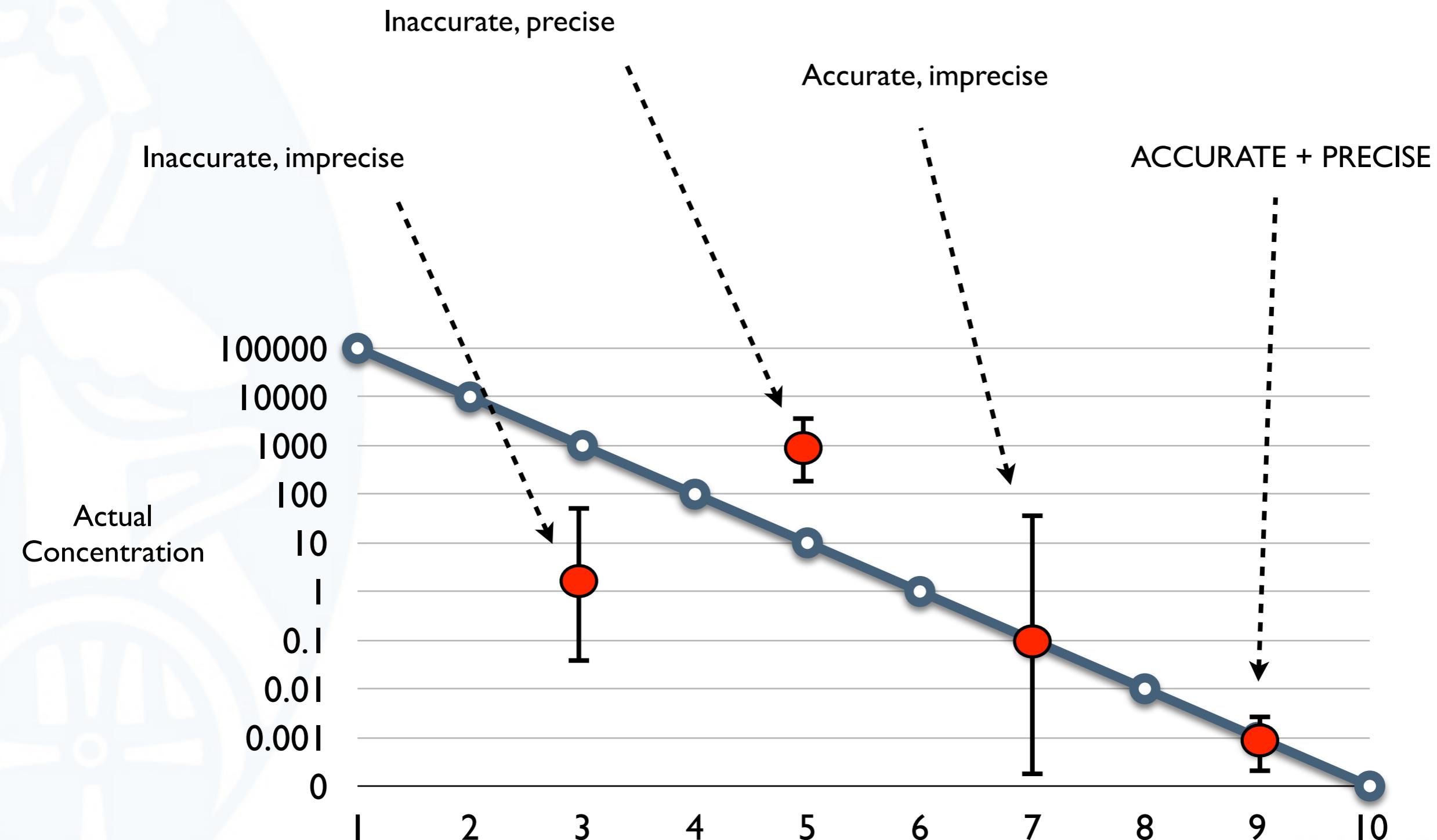


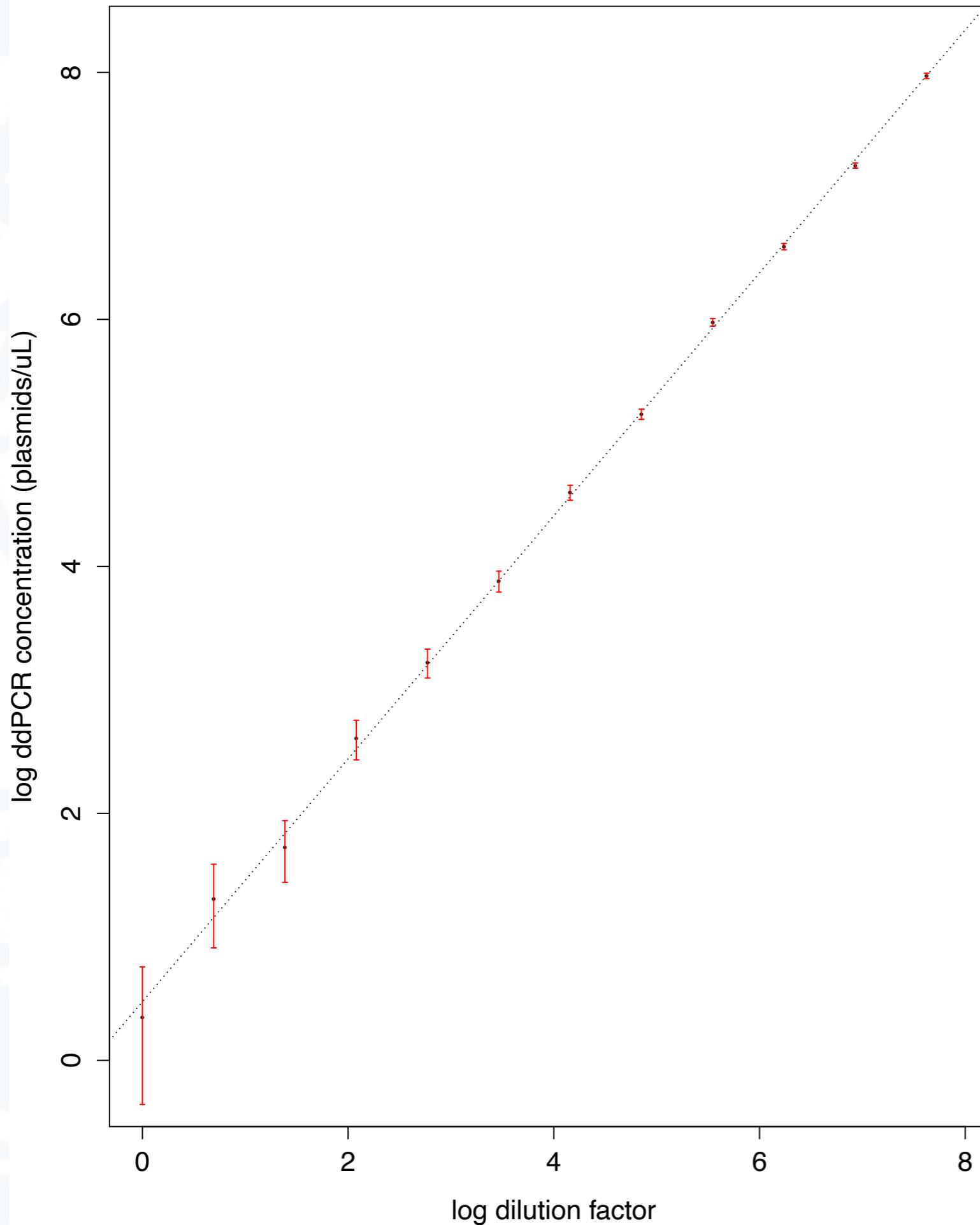
Test some specimens that have known concentrations relative to one another

A dilution series is called the
STANDARD CALIBRATION CURVE



Assuming a linear response in the test, the dilution series should fit a straight line if the test is accurate and precise





R-squared = 0.9989

1.46 ± 0.72 to 2898 ± 67 targets/L

What this means is
that the ddPCR
estimate is a very
accurate and precise
across a wide
DYNAMIC RANGE

(Note the log scale)



Coefficients of variation

The CoV is the standard deviation of a set of measurements, divided by the mean

Dimensionless number, expressed as a percentage

Inter-assay CoV : describes the plate to plate consistency of replicates

Intra-assay CoV : describes the consistency between all replicates of a test within a plate (it relates to the confidence intervals on the standard curve)

Both intra-assay CoV and inter-assay CoV indicate how the assay performs in the hands of the user.

You may wish to calculate the between user, within centre CoV and the between user, between centres CoV

Diagnostic Evaluation

A key question about a quantitative diagnostic

Enumeration :

Does the diagnostic accurately and precisely estimate the true infectious load?



Accuracy and precision have been measured against synthetic standards, but for a fair evaluation, we must test coefficients of variation using real clinical samples

Similarly, accuracy must be determined using clinical specimens and by directly comparing the concentration estimates of the new test to a reference assay, which is known to be accurate and precise
(a ‘gold standard’, more on this later)

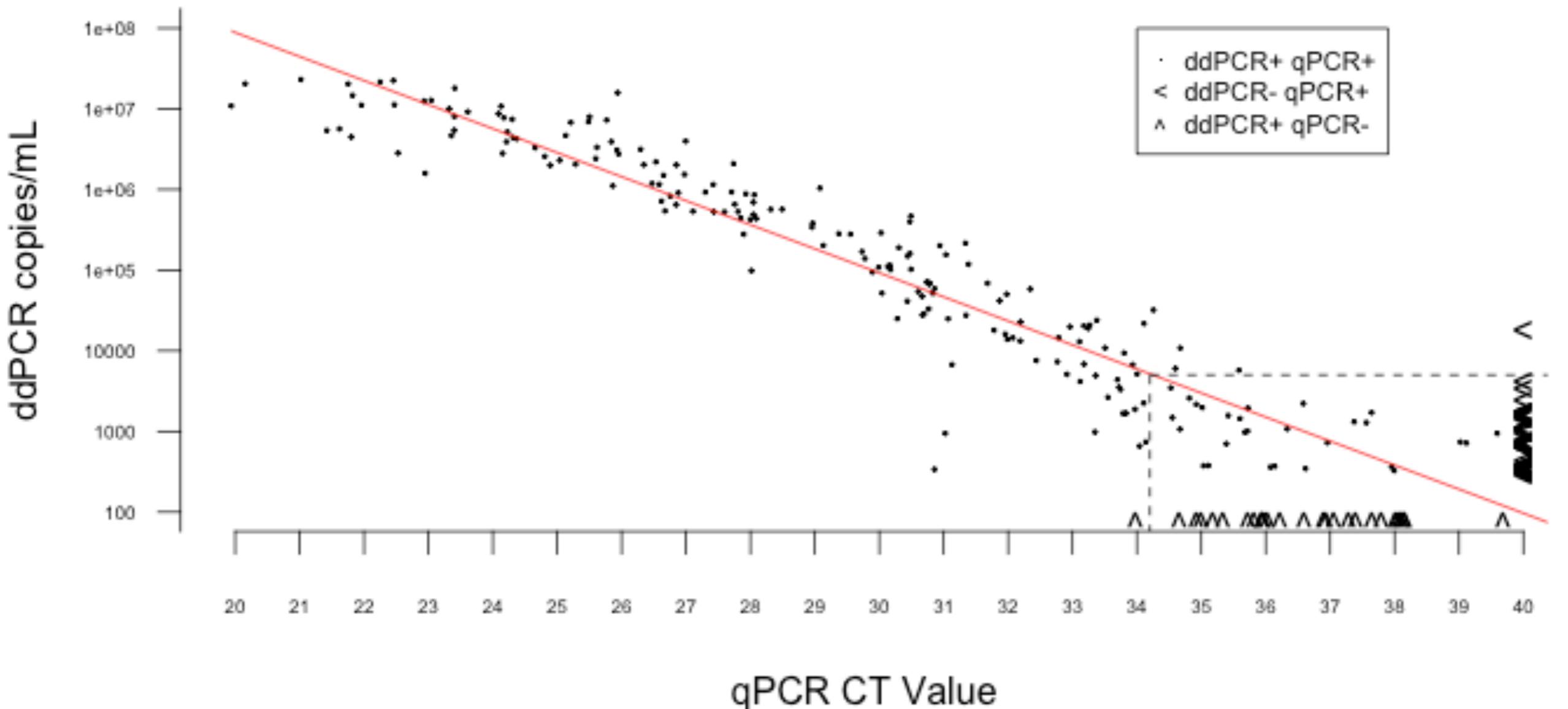


Direct comparison of quantity estimates between two assays, using clinical specimens

$R^2 = 0.90$

Correlation between the two tests is strong

Categorical discrepancies occur only at very low analyte concentrations



Part One : Recap

qPCR is a powerful technique, but specimen complexity and the requirement for external calibrators combine to introduce an unknown amount of error

By performing a plurality of nanolitre scale, endpoint PCR reactions, ddPCR can enumerate DNA molecules without this error

A dilution series can help to determine whether the assay is accurate and precise, but comparison to a standard reference test is of great importance





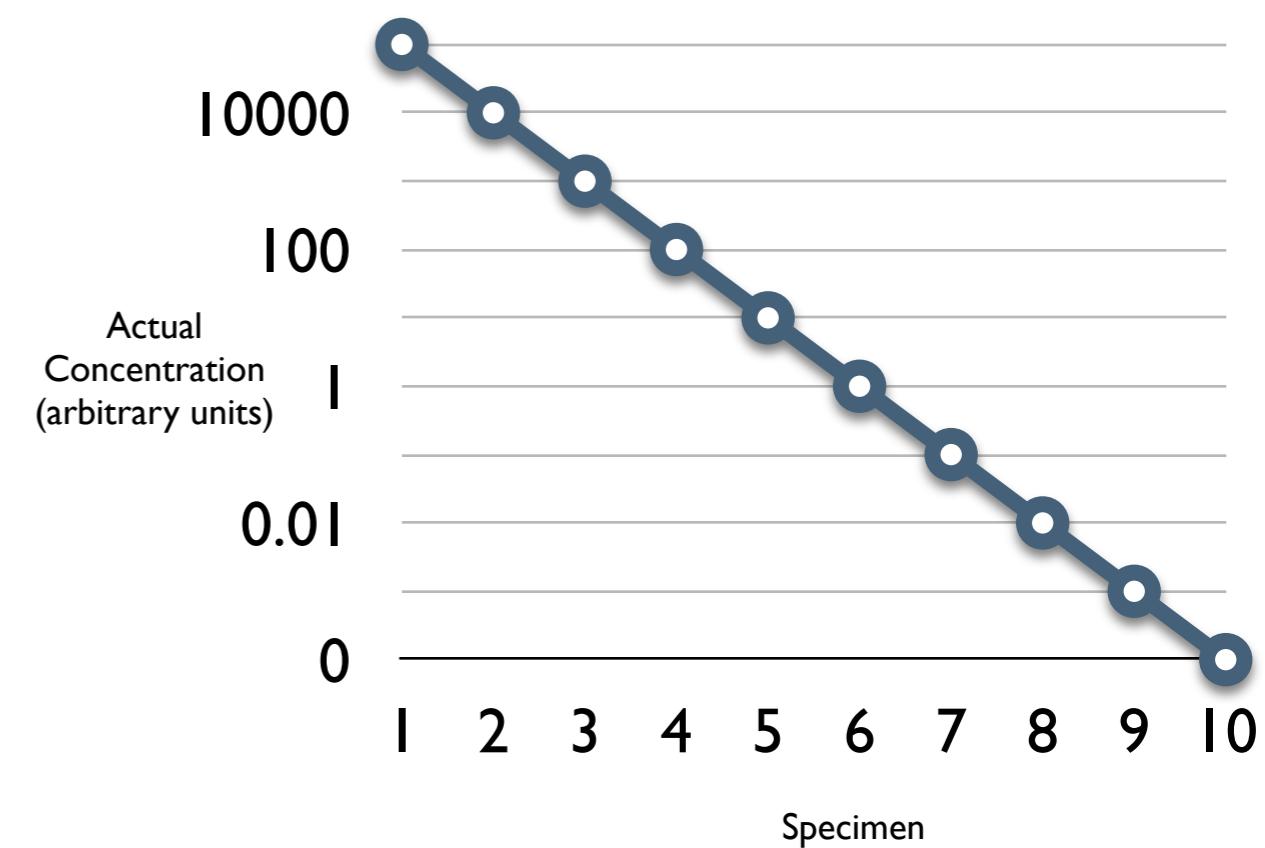
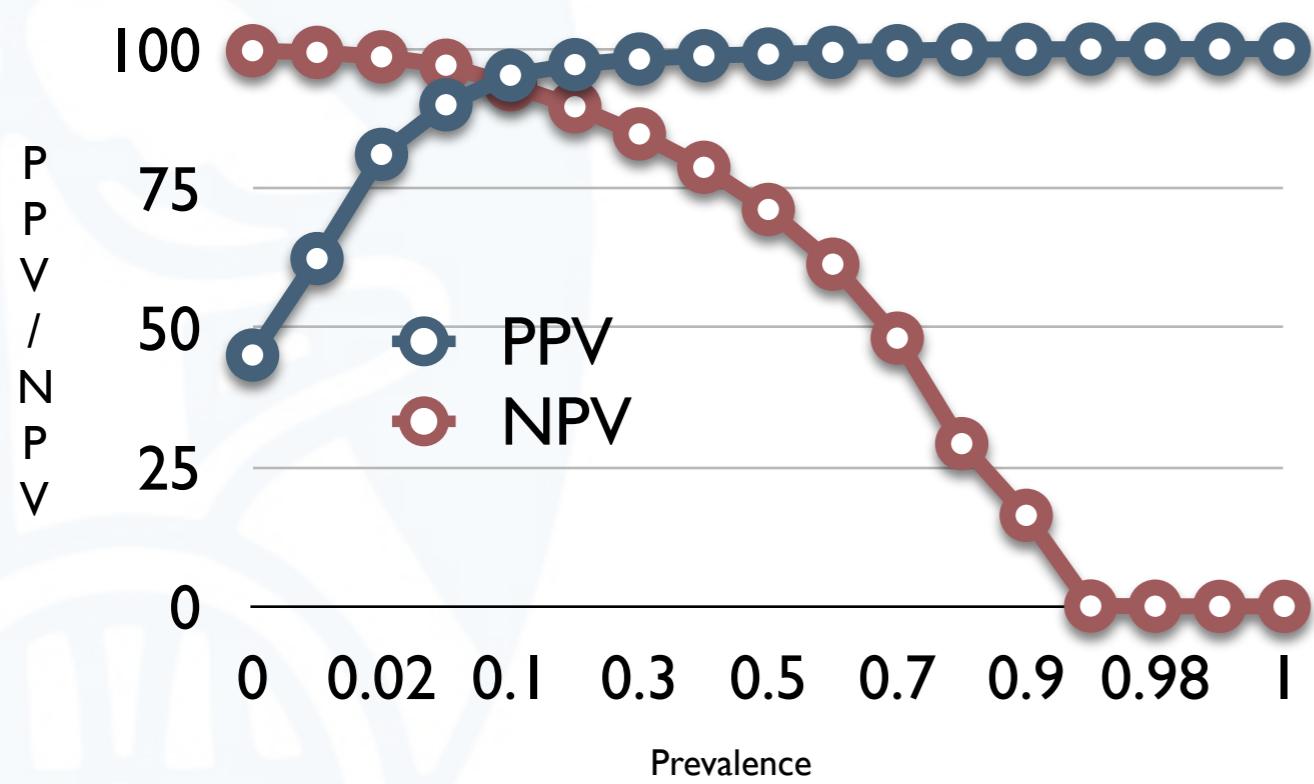
End of Part One

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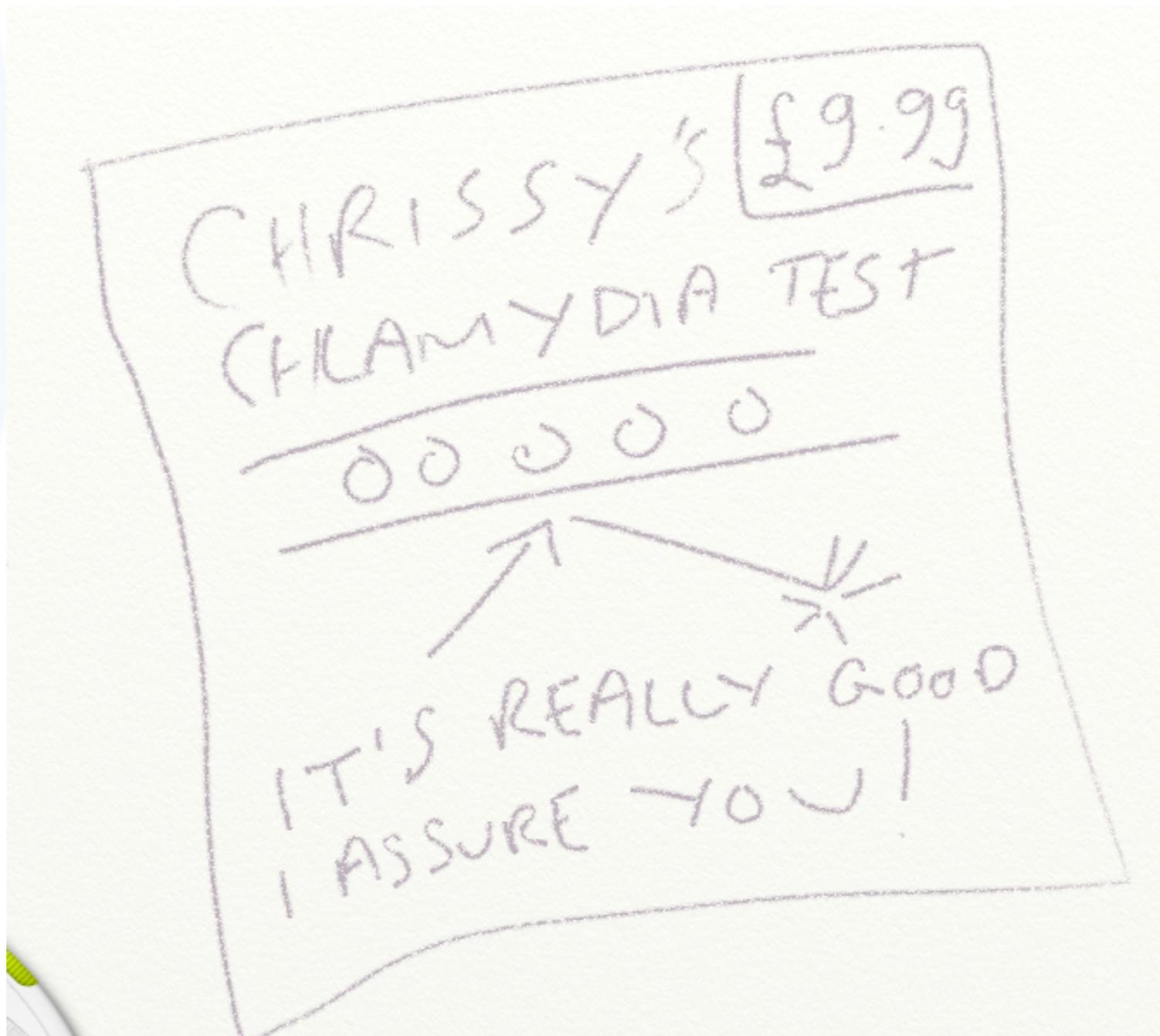


Part Two

A validated clinical tool



C. trachomatis ddPCR is accurate and precise. Let's sell it!



Oh dear....

- **How do I know if it is detecting only C. trachomatis?**
- **Are all the positives real?**
- **Have I missed any positives?**
- **If I tested the individual with another method, would they agree?**

These are issues that can only be solved by external comparisons and formal diagnostic evaluation



Diagnostic Evaluation

Where you start :

A method that CAN detect and enumerate *C. trachomatis* DNA in a clinical specimen

(hopefully it is accurate and precise, with low coefficients of variation)

Where you end :

A method that is trusted by the global community to perform *C. trachomatis* detection and enumeration in an accurate and precise manner



Diagnostic Evaluation

Two key questions about a quantitative diagnostic

Detection/Diagnosis :

Does the diagnostic accurately and precisely predict the true infectious state of the individual?

Enumeration :

Does the diagnostic accurately and precisely estimate the true infectious load?

Diagnostic accuracy

Individuals who are true positives are classified as positive by the test

Individuals who are true negatives are classified as negative by the test

	True positive	True negative
Test positive	50	0
Test negative	0	50

Accurate

	True positive	True negative
Test positive	28	22
Test negative	15	35

Inaccurate



Diagnostic precision

Replicate tests on the same specimens are classified the same way

POS

NEG

1	1
1	1

2	2
2	2

3	3
3	3

4	4
4	4

Precise

POS

NEG

1	2
1	1

2	1
2	2

3	3
3	3

3	3
4	4

Imprecise



Simplify that...

- **Accurate : There are few misclassifications**
- **Precise : If you test the same individuals again, you get the same result**



Accuracy : Misclassification

If we test some samples that we know the

TRUE CLASSIFICATION

(i.e. we know if they are positive or negative)

does the new test get the right answers?



But how do we know the TRUE CLASSIFICATION?

We need to compare the new test to a
GOLD STANDARD TEST





**The GOLD STANDARD TEST
is a perfect indicator**

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Formal diagnostic evaluation

How well does the new (index) test compare to
The GOLD STANDARD TEST ?

There are several measurable outcomes

**SENSITIVITY
SPECIFICITY**

**Positive Predictive Value
Negative Predictive Value**



Sensitivity

Sensitivity is the ‘TRUE POSITIVE’ rate

A measure of the ***proportion*** of the TRUE POSITIVES
that the test correctly identifies as such

Closely related to Type II error (false negatives)

When sensitivity is low, the false negative rate is high



Specificity

Specificity is the ‘TRUE NEGATIVE’ rate

A measure of the ***proportion*** of the TRUE NEGATIVES
that the test correctly identifies as such

Closely related to Type I error (false positives)

When specificity is low, the false positive rate is high



Positive and Negative Predictive Values

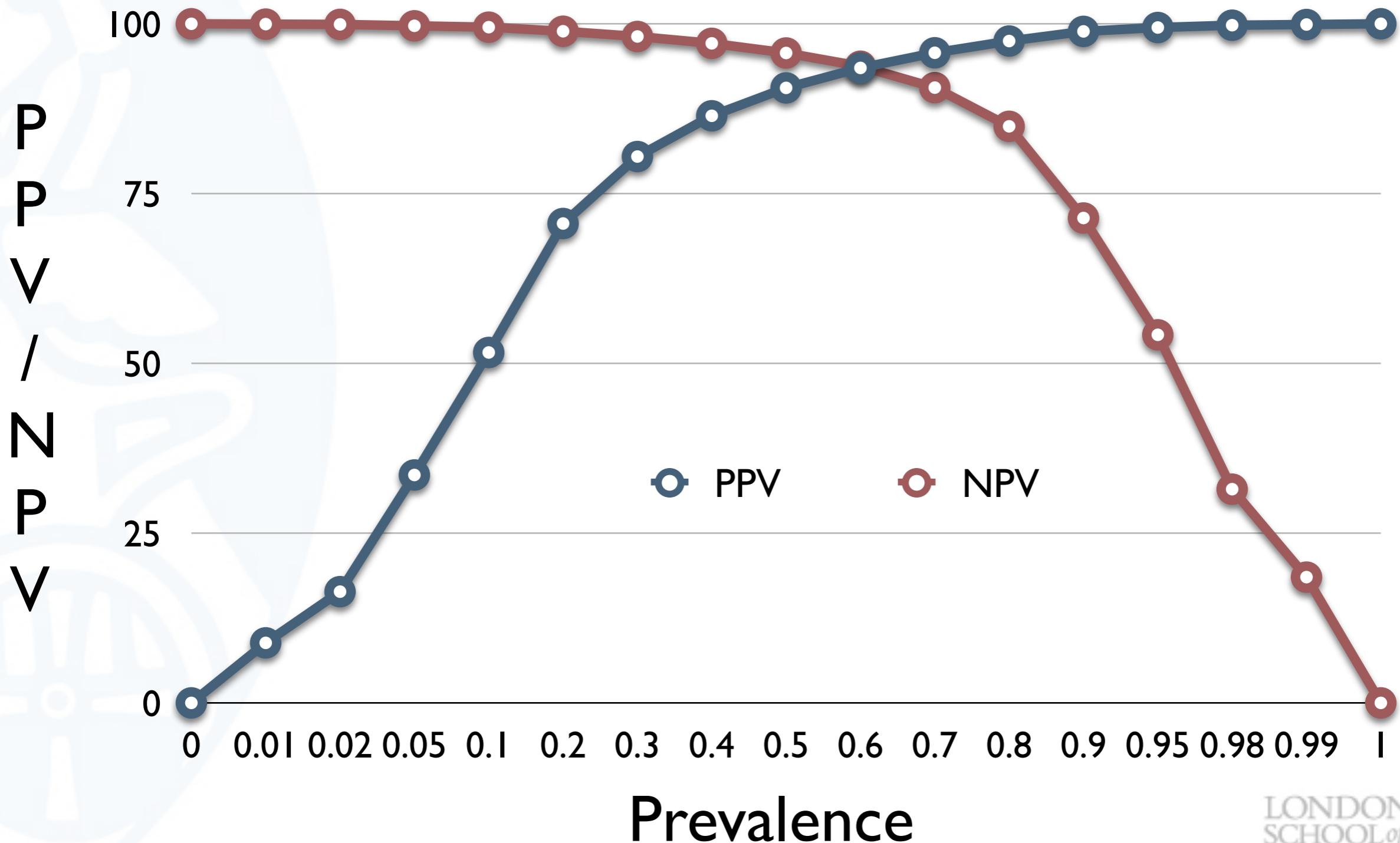
Positive Predictive Value (PPV) is the PROBABILITY that a specimen testing positive is a true positive

Negative Predictive Value (NPV) is the PROBABILITY that a specimen testing negative is a true negative

PPV and NPV depend not only on sensitivity and specificity, but also on the prevalence of the condition



Imagine a test with sensitivity 96% & specificity 90%



Test the same specimens using both tests

		GOLD STANDARD (GS) TEST		PPV TRUE + / IT +
		POSITIVE	NEGATIVE	
INDEX TEST (IT)	POSITIVE	TRUE POSITIVES	FALSE POSITIVES	NPV TRUE - / IT -
	NEGATIVE	FALSE NEGATIVES	TRUE NEGATIVES	
		SENSITIVITY TRUE + / GS +	SPECIFICITY TRUE - / GS -	



Practical Exercise

		GOLD STANDARD (GS) TEST		
		POSITIVE	NEGATIVE	
INDEX TEST (IT)	POSITIVE	TP = 189	FP = 11	PPV TP / IT +
	NEGATIVE	FN = 69	TN = 1208	NPV TRUE - / IT -
		SENSITIVITY TP / GS +	SPECIFICITY TN / GS -	

Sensitivity :

Specificity :

PPV :

NPV :



Practical Exercise

		GOLD STANDARD (GS) TEST		
		POSITIVE	NEGATIVE	
INDEX TEST (IT)	POSITIVE	TP = 189	FP = 11	PPV TP / IT +
	NEGATIVE	FN = 69	TN = 1208	NPV TRUE - / IT -
		SENSITIVITY TP / GS +	SPECIFICITY TN / GS -	

Sensitivity :

Specificity :

PPV :

NPV :



Test the same specimens using both tests

The ddPCR classifier was Zeta ≥ 0.95

		Amplicor		Totals
		Positive	Negative	
ddPCR	Positive	189	11	200
	Negative	69	1208	1281
Totals		258	1219	1477
Sensitivity 73.3% (95% CI 67.9 - 78.7%)				
Specificity 99.1% (95% CI 98.6 - 99.6%)				
PPV 94.5% (95% CI 91.3 - 97.7%)				
NPV 94.6% (95% CI 93.4 - 95.8%)				



The ddPCR test is highly specific

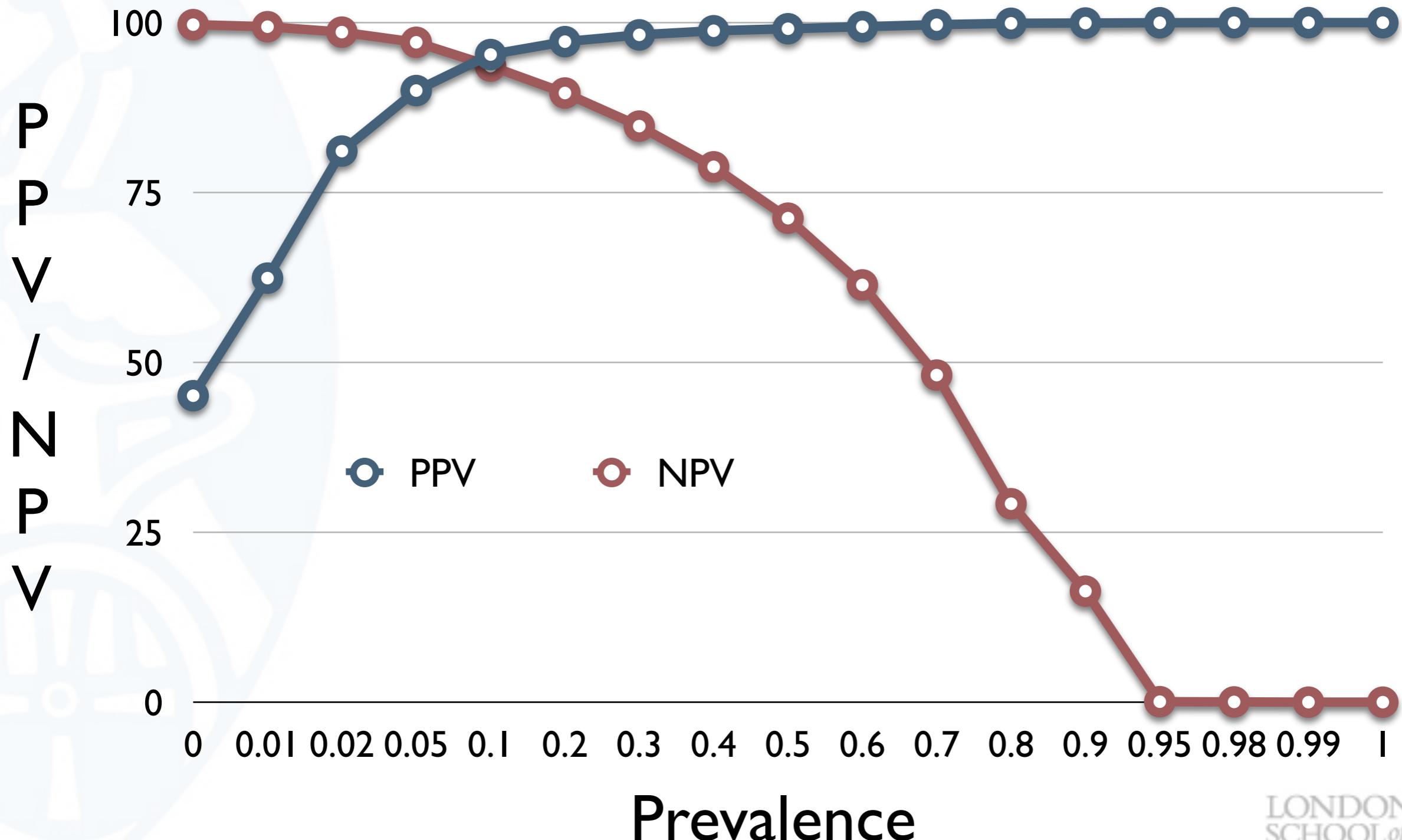
		Amplicor		Totals
		Positive	Negative	
ddPCR	Positive	189	11	200
	Negative	69	1208	1281
Totals		258	1219	1477
Sensitivity				
73.3% (95% CI 67.9 - 78.7%)				
Specificity				
99.1% (95% CI 98.6 - 99.6%)				
PPV				
94.5% (95% CI 91.3 - 97.7%)				
NPV				
94.6% (95% CI 93.4 - 95.8%)				

False positive rate is low
High false negative rate

The prevalence was 14%
and the PPV and NPV
were both around 94.5%

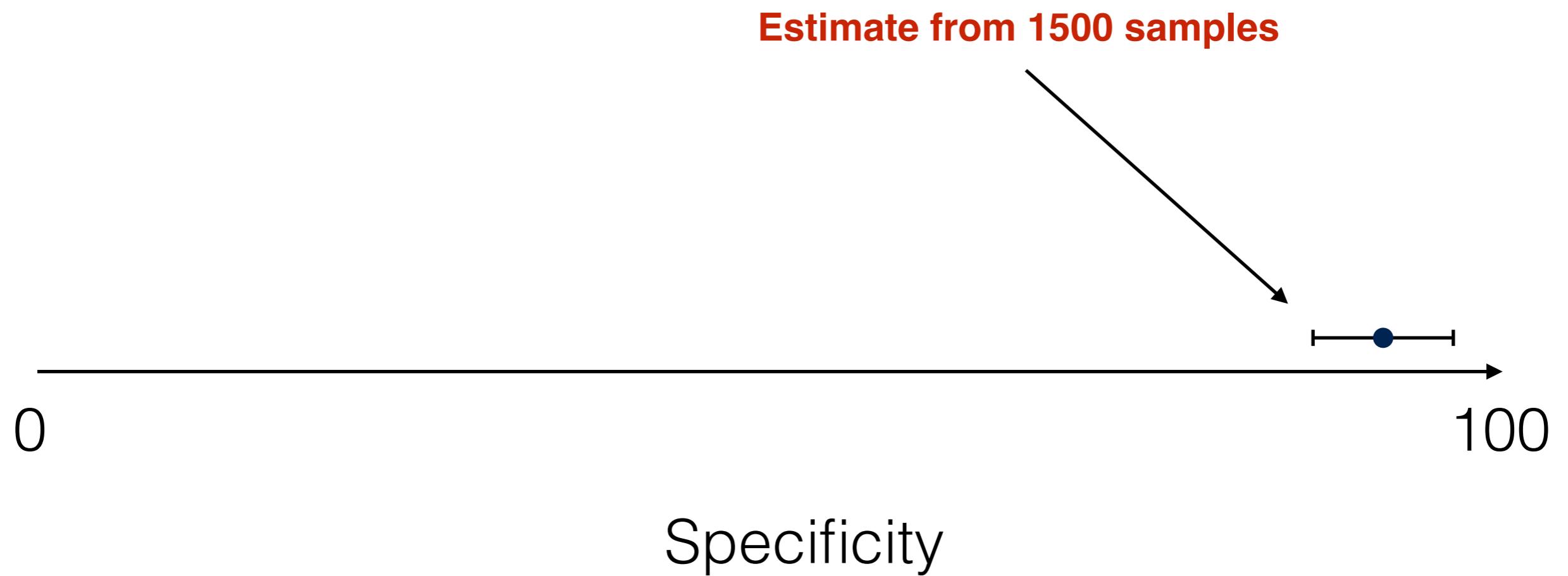


The ddPCR test has sensitivity 73.3% & specificity 99.1%



Precision of evaluation depends on experimental design

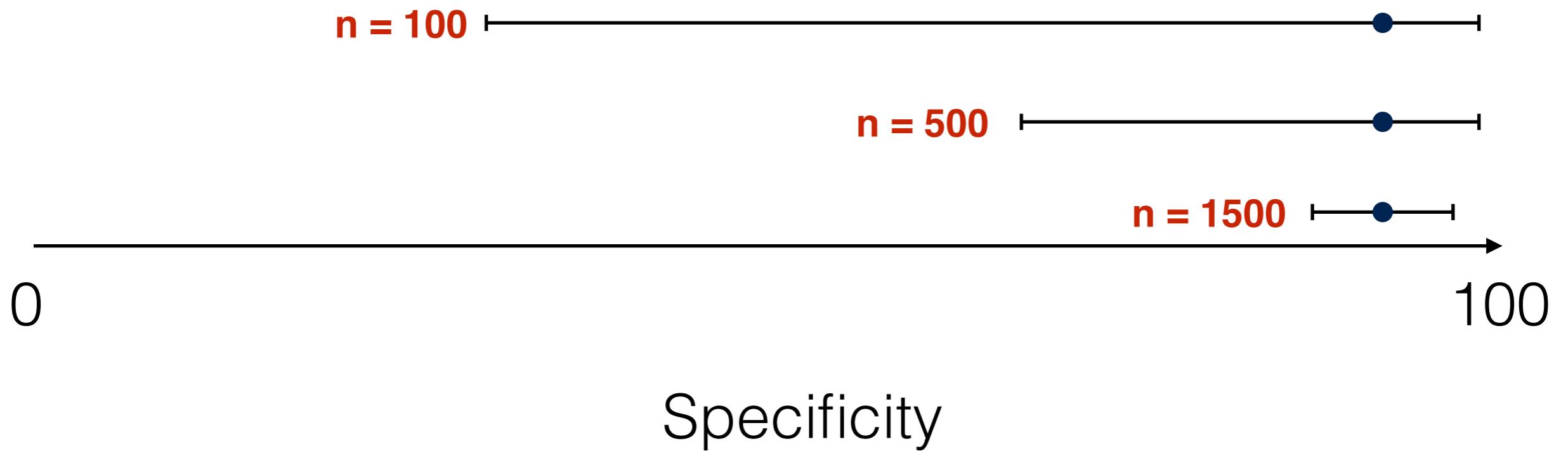
Specificity 99.1 (CI 98.6 - 99.6)



EXPERIMENTAL DESIGN

Specificity 99.1 (CI 98.6 - 99.6)

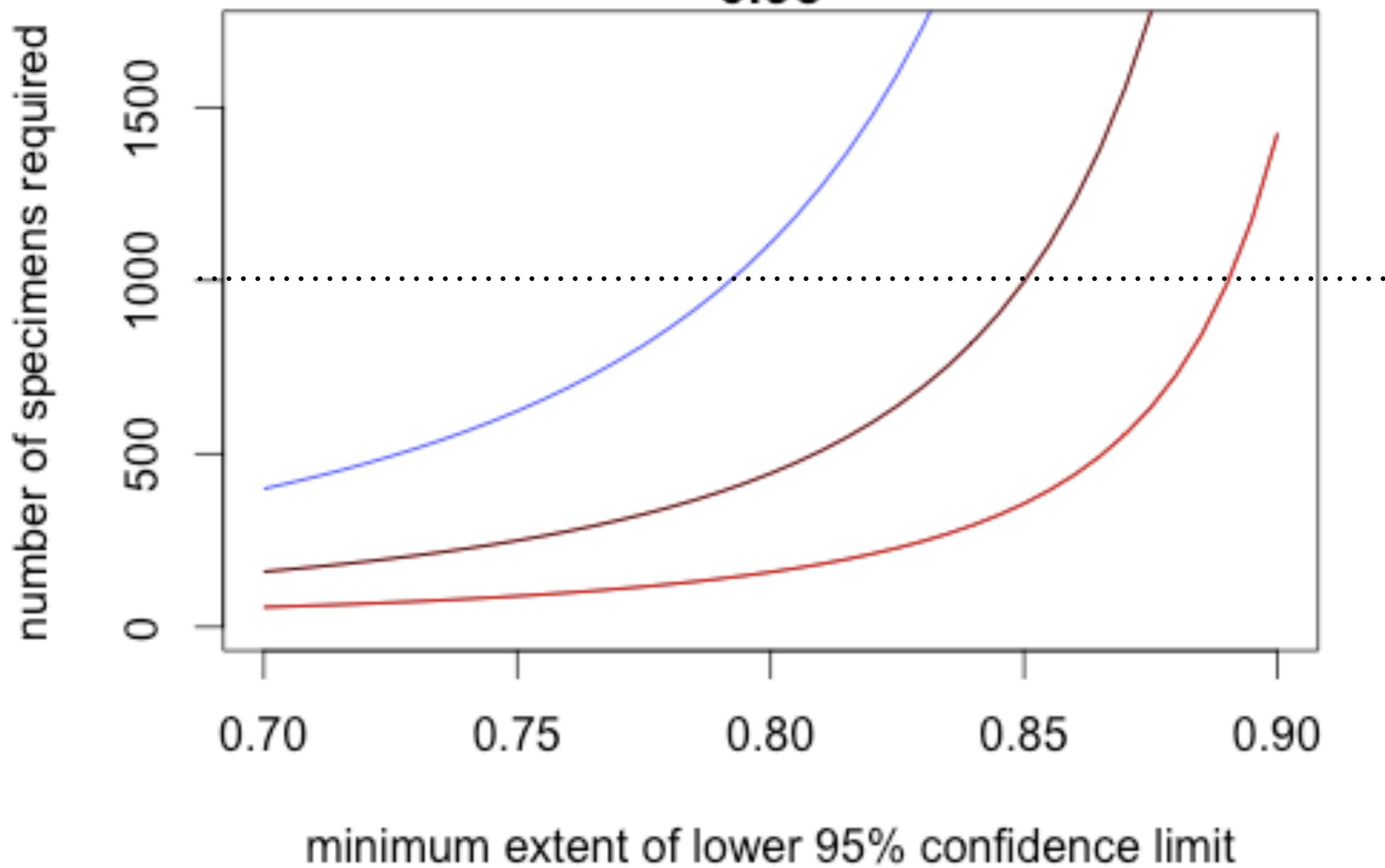
Prevalence ~ 14%



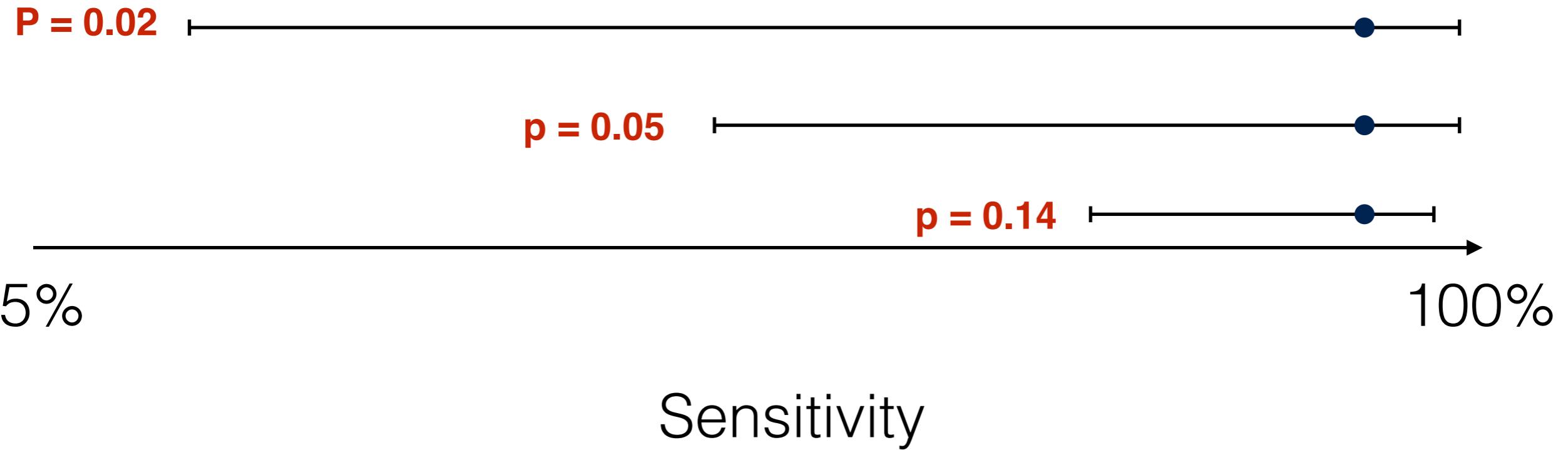
Sample size defines the lower limits of the confidence interval

Prevalence 2% (blue), 5% (brown), 14% (red)

true sensitivity :
0.95



Sample size is a crucial consideration for diagnostic evaluations (see Banoo et. al)



Low specificity diagnostic
for gallstones

Low sensitivity diagnostic
for punctured lung



2-28

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**"Rapid pulse, sweating, shallow breathing ...
According to the computer, you've
got gallstones."**

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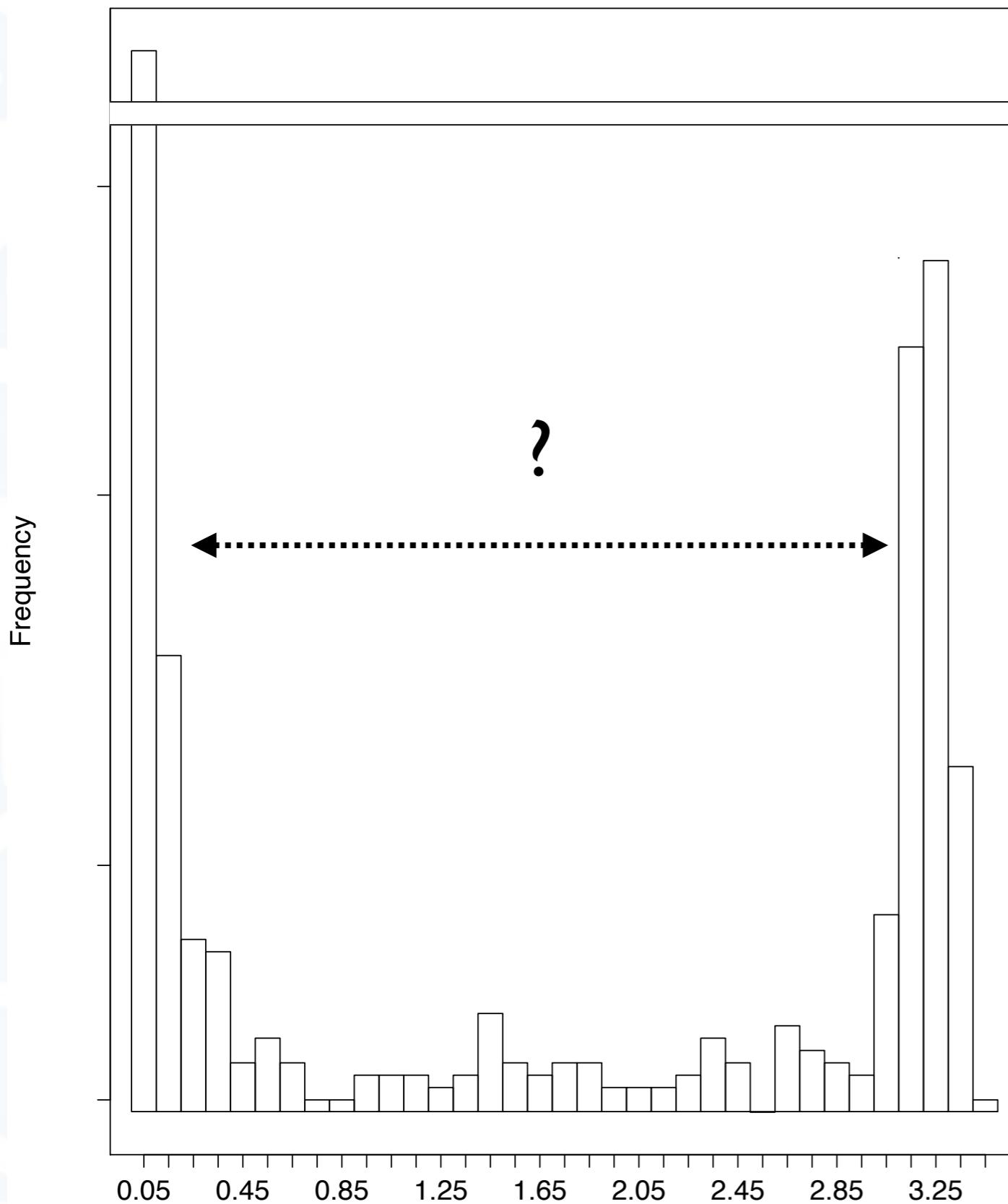


What is the classifier?

How you choose to define positives and negatives in the index test is very important as it can change the estimated sensitivity and specificity

When the output of the test is a continuous variable
(i.e. fluorescence intensity, concentration etc.)



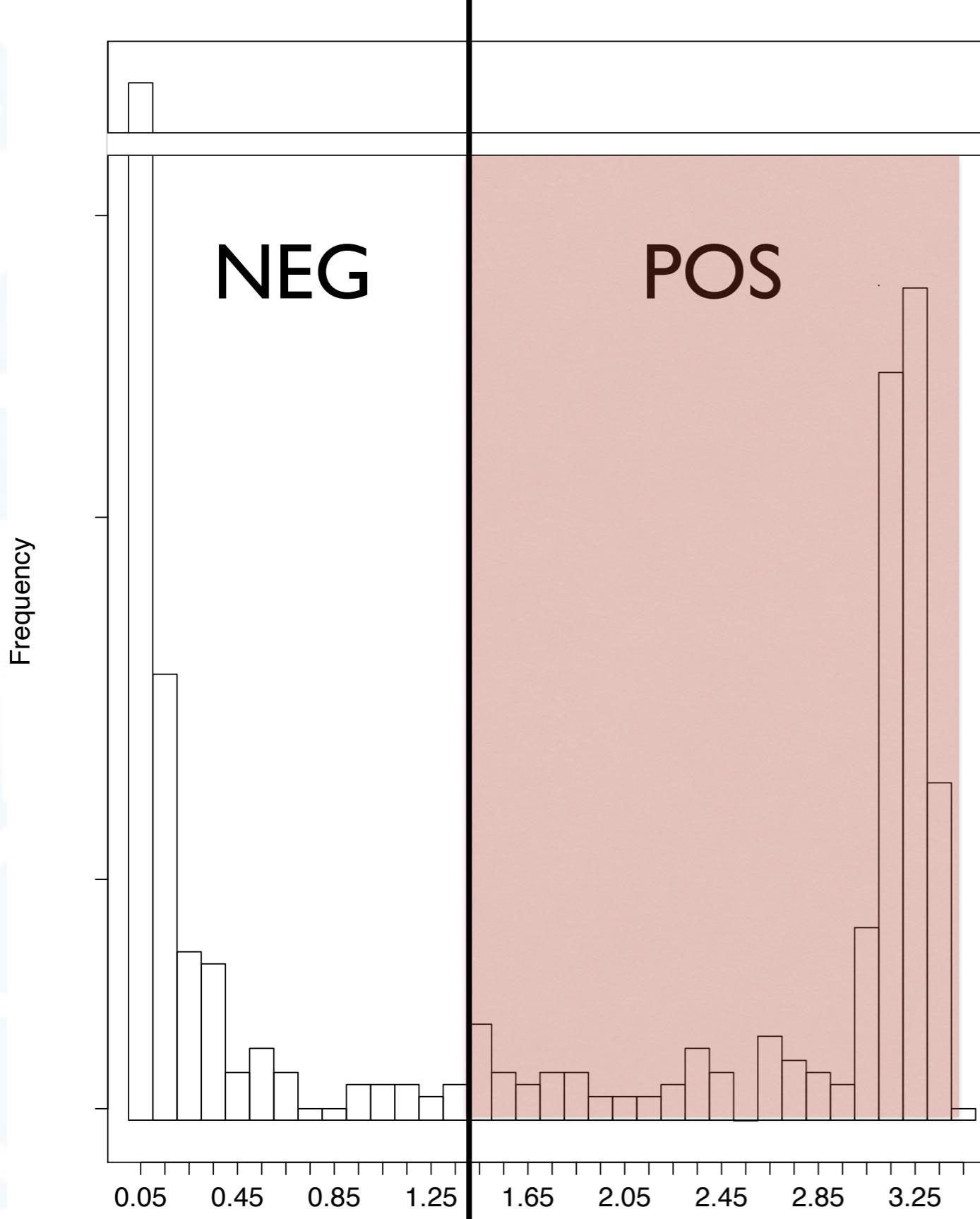


Intermediate values
have uncertain
classification

Where do you set
the threshold?



Classifier Threshold



Sens 83.3
Spec 60.0
PPV 71.4
NPV 75.0

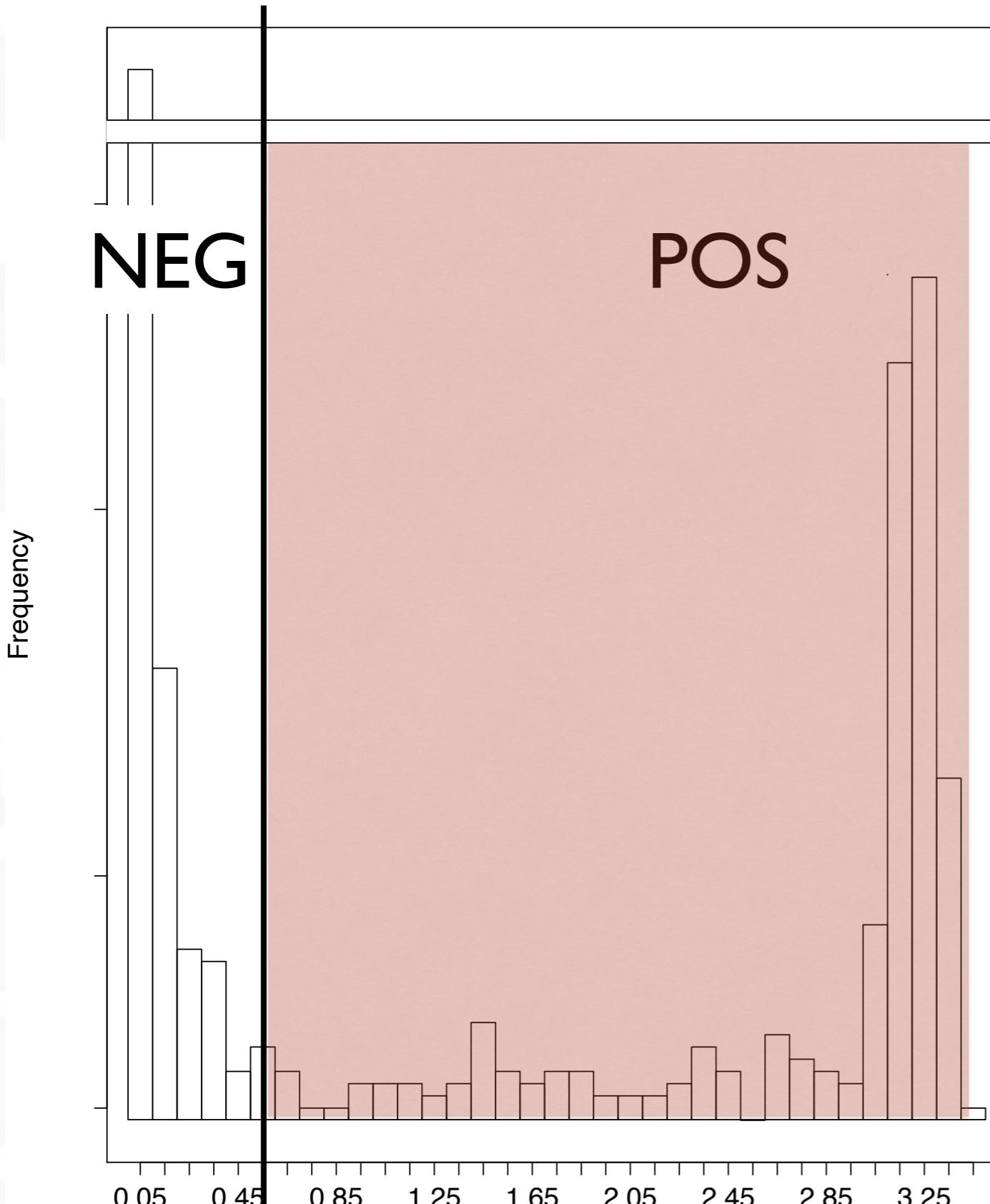
Gold standard				
	POS	NEG		
Index (IT)	POS	500	200	PPV 71.4%
	NEG	100	300	NPV 75.0%
	SENS 83.3%	SPEC 60.0%		

Index test concentration estimate

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Classifier Threshold



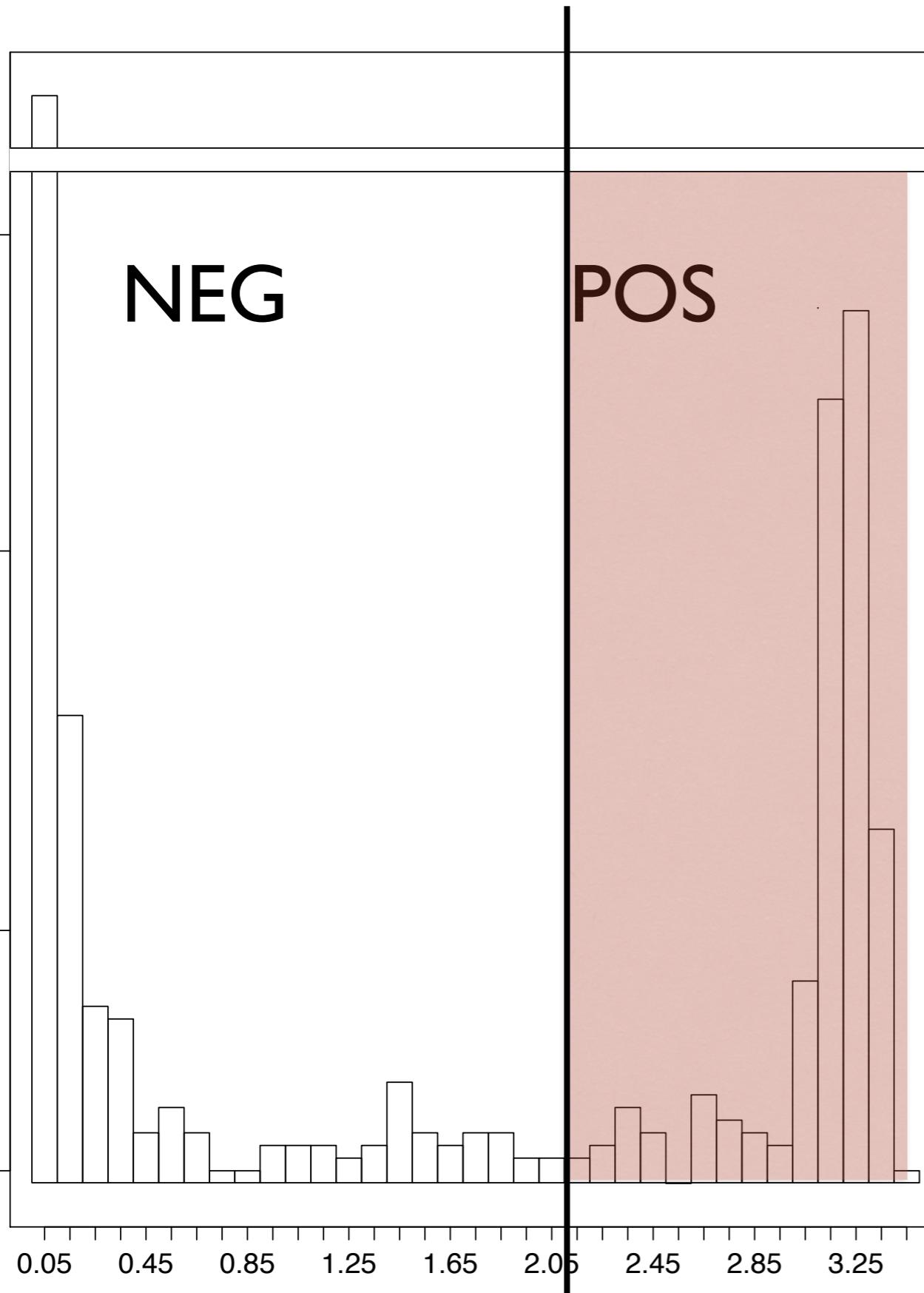
Sens	83.3	96.9
Spec	60.0	43.0
PPV	71.4	70.5
NPV	75.0	90.9

		Gold standard		PPV 70.5%
		POS	NEG	
Index (IT)	POS	620	260	NPV 90.9%
	NEG	20	200	
		SENS 96.9%	SPEC 43.0%	



Classifier Threshold

Frequency



NEG

POS

Sens	83.3	96.9	51.7
Spec	60.0	43.0	78.9
PPV	71.4	70.5	78.9
NPV	75.0	90.9	51.7

		Gold standard		PPV 78.9%
		POS	NEG	
Index (IT)	POS	300	80	NPV 51.7%
	NEG	280	300	
		SENS 51.7%	SPEC 78.9%	

Index test concentration estimate

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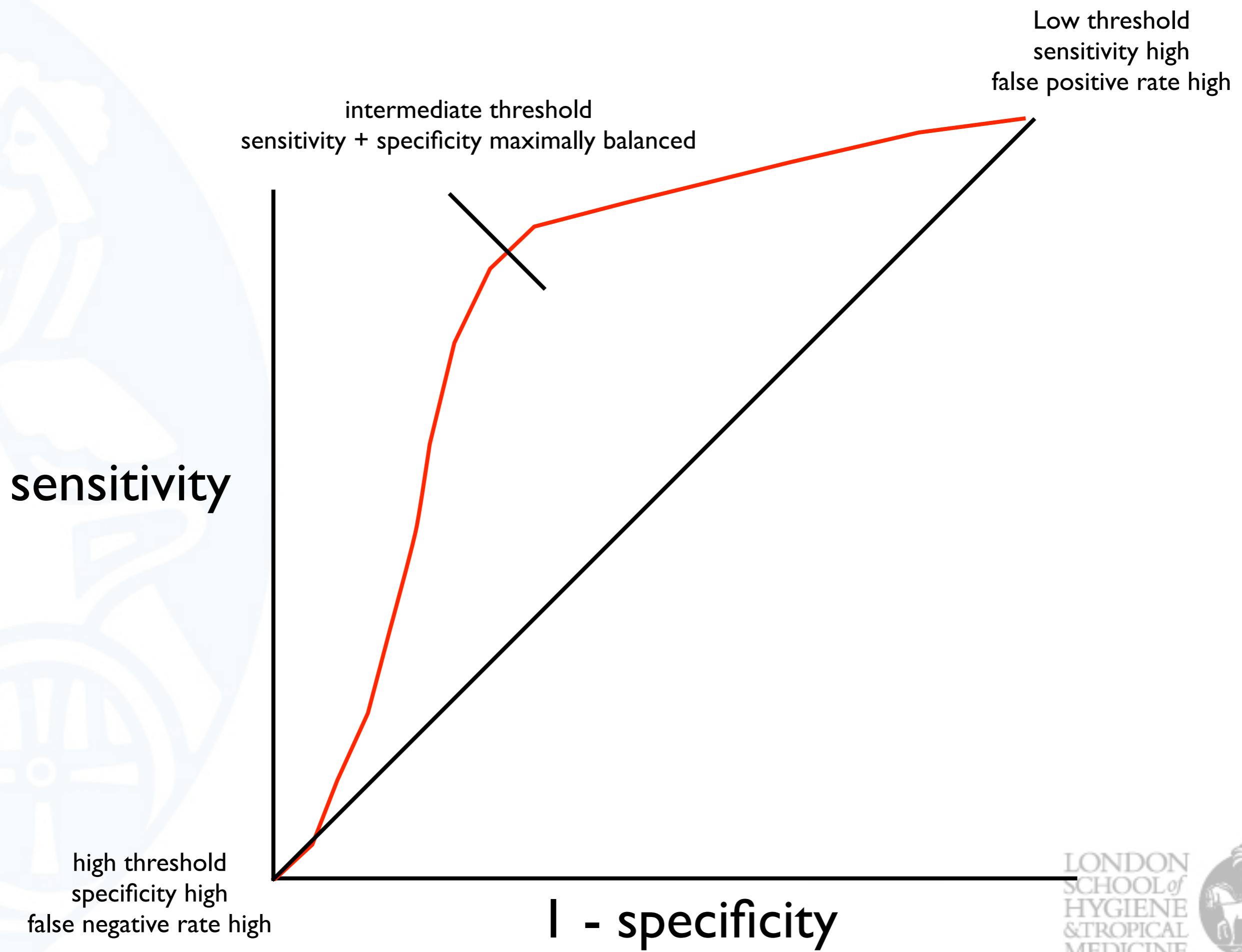


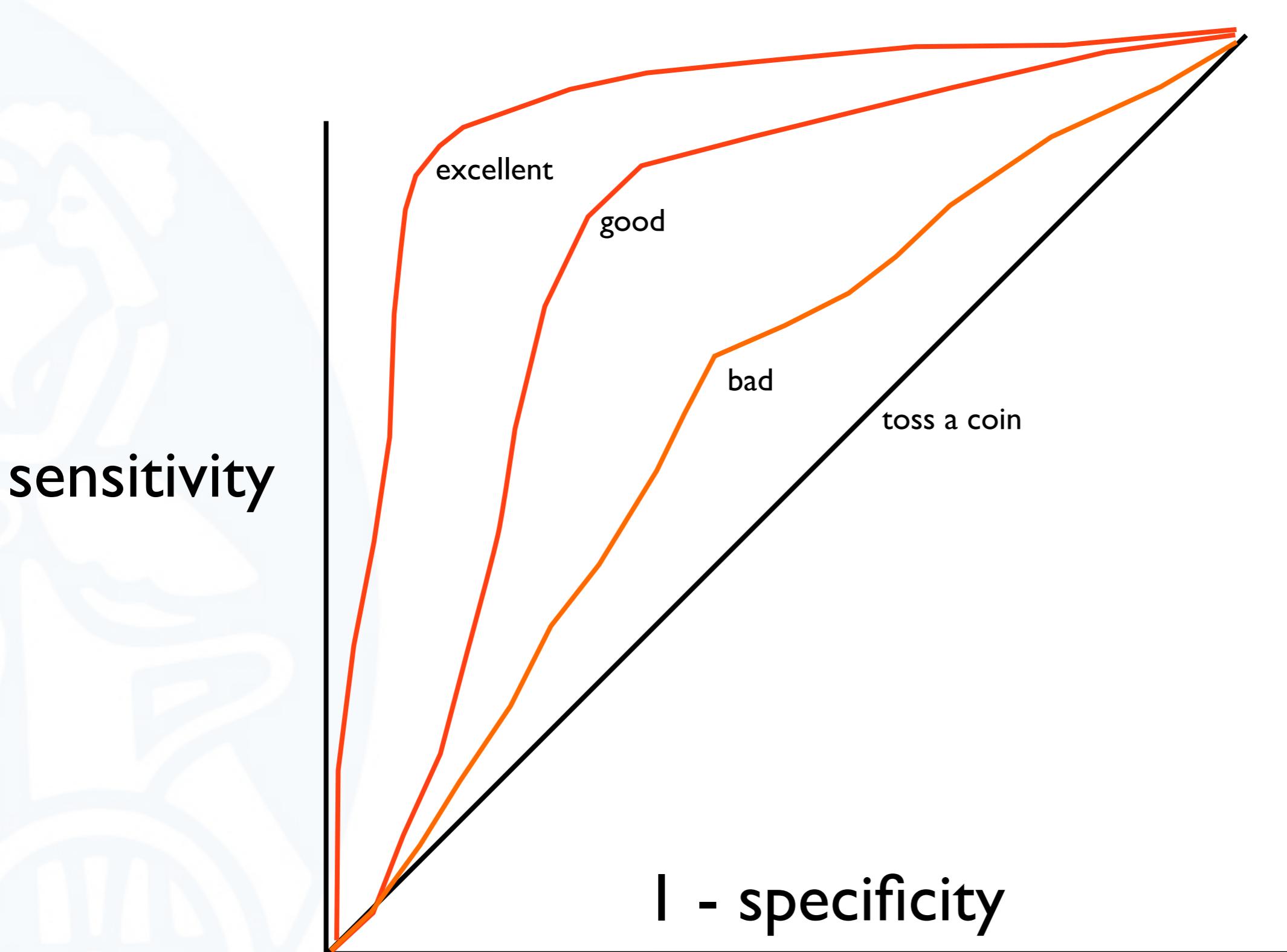
When the classifier threshold value is reduced sensitivity increases, but specificity decreases

The opposite is also true

Receiver-Operator Characteristic (ROC) curve analysis is used to assess the sensitivity and specificity at different critical **threshold values** of the classifier



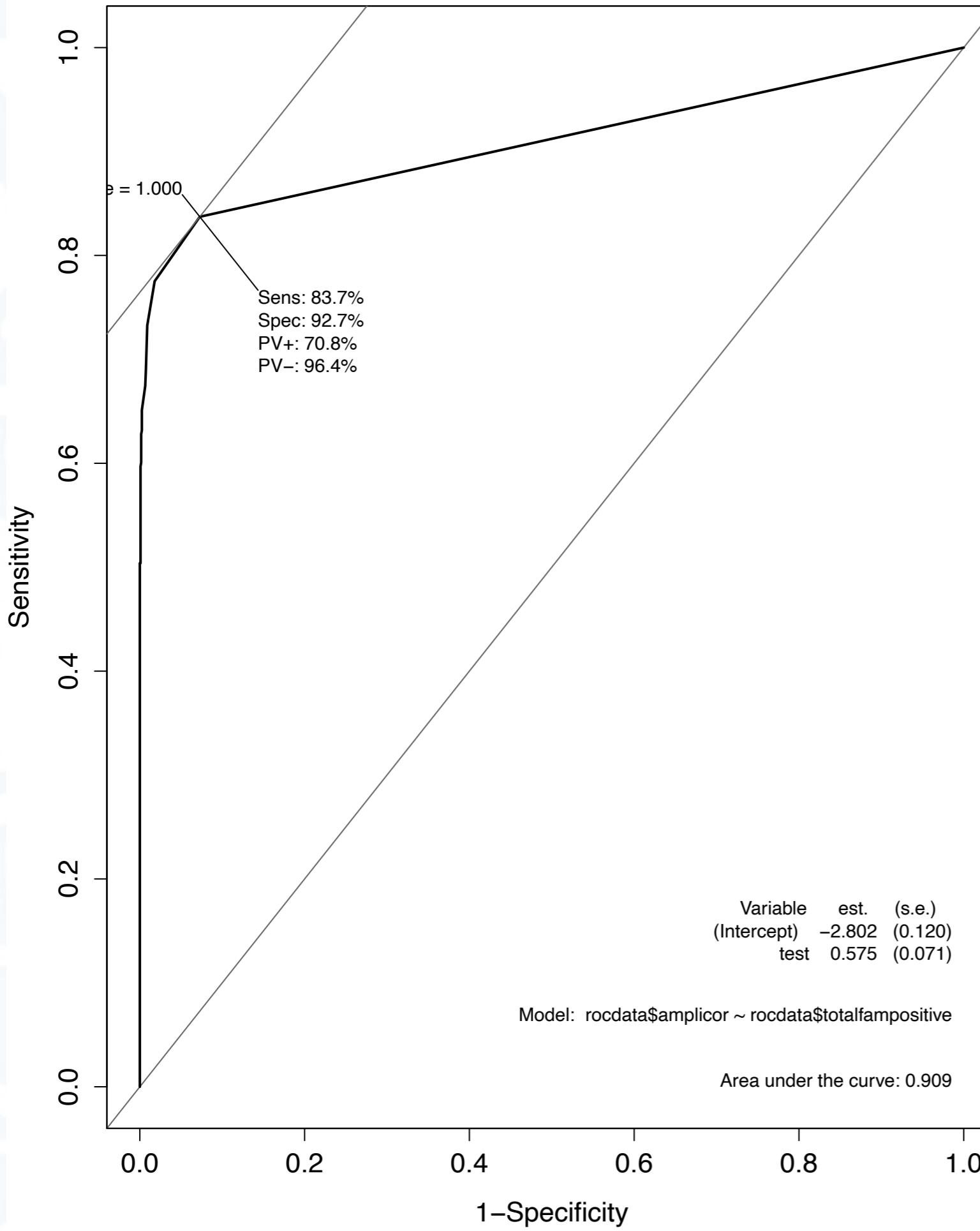




The AUC gives the probability that a randomly selected positive specimen will be ranked higher than a randomly selected negative specimen and is a general indicator of the performance or ‘predictiveness’ of the assay

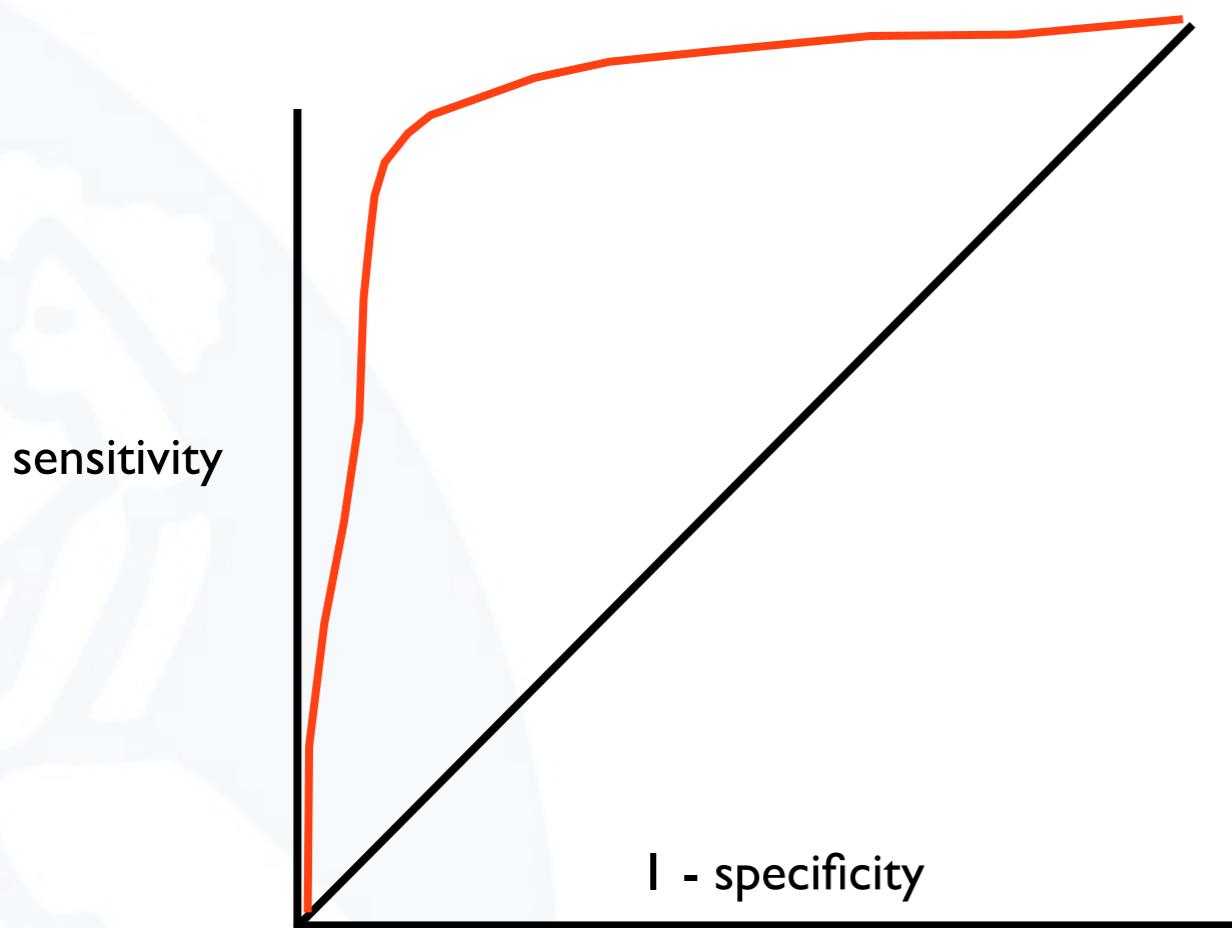


The ddPCR AUC
was 90.1%



Where we choose to set the critical classification threshold depends on the application of the test





There is always a trade-off
between sensitivity and
specificity

The choice you make depends
on what you are doing

Where would you set the threshold for...

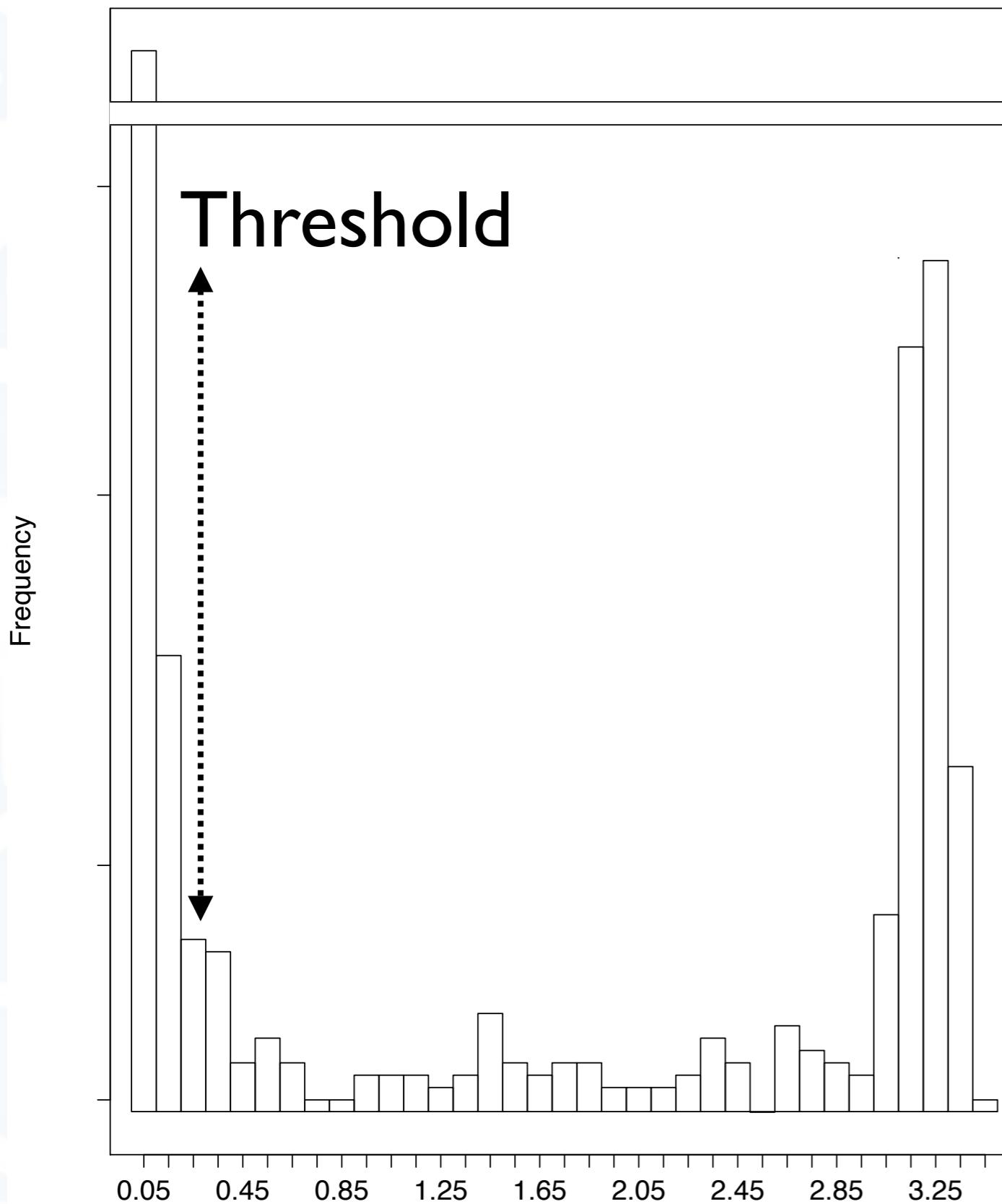
A pregnancy test

Monitoring infection after mass drug administration

Breast cancer screening



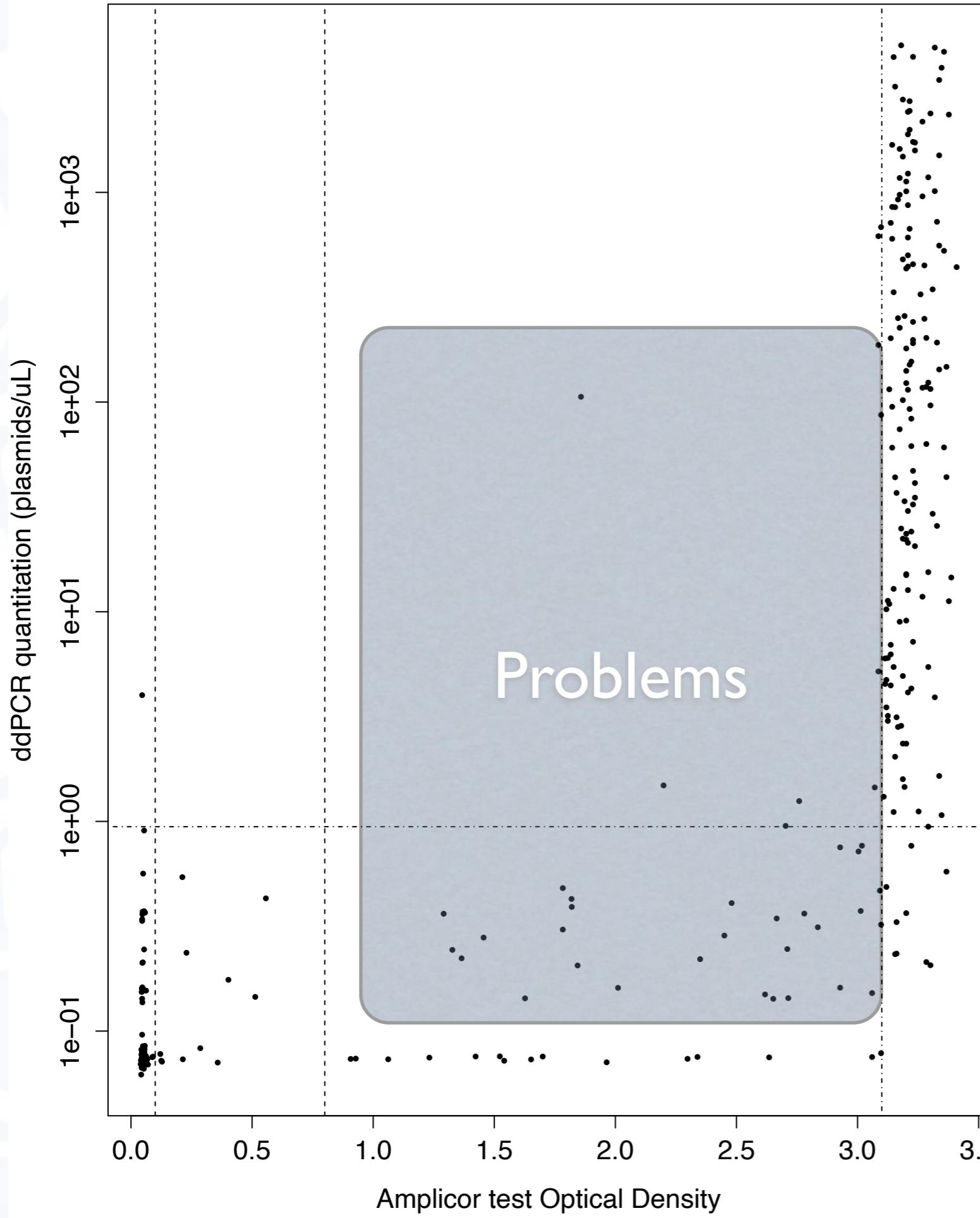
Where do you set the threshold?



The recommended threshold for this test was actually 0.2

But an earlier guideline called for re-testing of samples with intermediate values





Discrepancies between our ddPCR test and the reference occurred frequently when the critical value was between 0.2 and 3.0

Might suggest

False negatives in ddPCR

or

False positives in the reference



Is the reference test over-diagnosing Chlamydia infections?

*High sensitivity, no-one with a real infection is likely to be missed.
Infections get eliminated*

If intermediate values are definitively called negative or positive, there is less need for re-testing, making the test more cost-effective

The drugs are harmless to those without a real infection, so this seems a pragmatic choice

What about the emotional costs to those who are misdiagnosed?



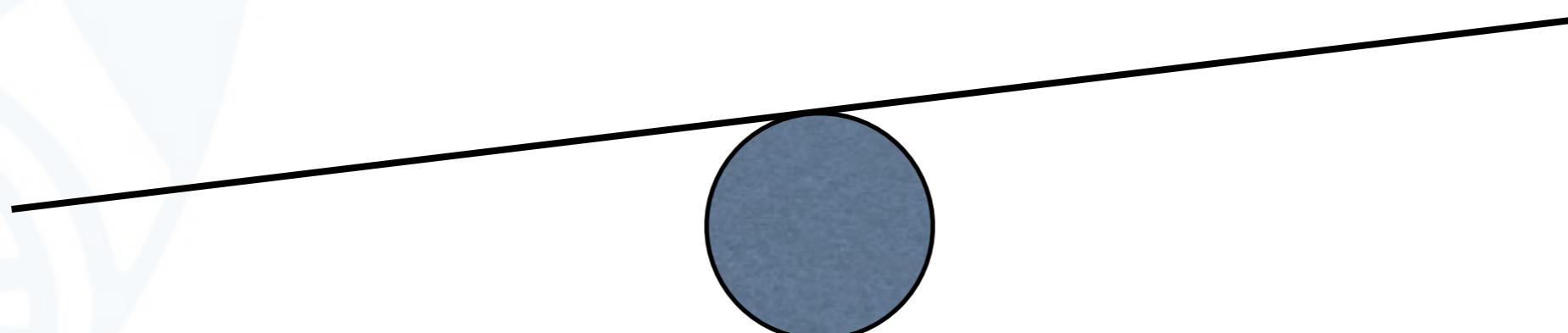
The ROC curve has an intrinsic cost-effectiveness component

What is the benefit of treating true positives

What is the detriment of treating false positives

What is the benefit of confirming a negative

What is the detriment of missing a negative





STARD

STAndards for the Reporting of Diagnostic accuracy
studies

A checklist of, among other things, the tests we've just
looked at

You have to comply with STARD in order to publish

<http://stard-statement.org>



By now you should be able to predict how your test will perform under...

...different population prevalence of the infection
...with different classifier thresholds



But you must remember that you have evaluated the whole protocol, not just the two technical assays. If you increase the amount of DNA added to the new test, sensitivity will increase as a result of the increased abundance of the analyte.

There are many procedural steps that can change the performance of your assay. If you change your protocol, you will need to re-evaluate your test.



Our ddPCR *C. trachomatis* assay is published...



Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular *Chlamydia trachomatis* Infections

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Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom^a; Department of Immunology, London School of Hygiene and Tropical Medicine, London, United Kingdom^b; Medical Research Council Unit, The Gambia^c; Programa Nacional de Saude de Visao, Ministerio de Saude Publica, Bissau, Guinea Bissau^d

Droplet digital PCR (ddPCR) is an emulsion PCR process that performs absolute quantitation of nucleic acids. We developed a ddPCR assay for *Chlamydia trachomatis* infections and found it to be accurate and precise. Using PCR mixtures containing plasmids engineered to include the PCR target sequences, we were able to quantify with a dynamic range between 0.07 and 3,160 targets/ μl ($r^2 = 0.9927$) with >95% confidence. Using 1,509 clinical conjunctival swab samples from a population in which trachoma is endemic in Guinea Bissau, we evaluated the specificity and sensitivity of the quantitative ddPCR assay in diagnosing ocular *C. trachomatis* infections by comparing the performances of ddPCR and the Roche Amplicor CT/NG test. We defined ddPCR tests as positive when we had $\geq 95\%$ confidence in a nonzero estimate of target load. The sensitivity of ddPCR against Amplicor was 73.3% (95% confidence interval [CI], 67.9 to 78.7%), and specificity was 99.1% (95% CI, 98.6 to 99.6%). Negative and positive predictive values were 94.6% (95% CI, 93.4 to 95.8%) and 94.5% (95% CI, 91.3 to 97.7%), respectively. Based on Amplicor CT/NG testing, the estimated population prevalence of *C. trachomatis* ocular infection was $\sim 17.5\%$. Receiver-operator curve analysis was used to select critical cutoff values for use in clinical settings in which a balance between higher sensitivity and specificity is required. We concluded that ddPCR is an effective diagnostic technology suitable for both research and clinical use in diagnosing ocular *C. trachomatis* infections.

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MEDICINE



But has proved controversial...

"Will droplet digital PCR become the test of choice for detecting and quantifying ocular Chlamydia trachomatis infection? Maybe Not."

Schachter J. Expert Rev Mol Diagn 2013 Nov, 13 (8):789-92

"Will droplet digital PCR become the test of choice for detecting and quantifying ocular Chlamydia trachomatis infection? Maybe."

Roberts Ch. et al. Expert Rev Mol Diagn 2014 Apr, 14(3):253-6



Key Terms to revise

Sensitivity

Specificity

Positive and Negative Predictive Values

ROC curve

Standard Calibration Curve

Coefficient of Variation

