Lecture 5
Determining serostatus and estimating seroprevalence

May 22, 2025

Seroanalytics Training
Blantyre, Malawi

Lecture outline

- Selecting controls
- How to choose a cutoff method
- Sensitivity and specificity
- Apply different methods for cutoffs
  - ROC curves
  - Mean + SD of negative controls
  - Mixture model

Controls

- If we don’t have a correlate of protection (e.g., **Part 4**), we can use well-characterized control samples to determine cutoffs for seropositivity.
  - Ideally, we would have both positive and negative controls.
  - Often, we only have negative controls, and sometimes we don’t have positive or negative controls.
- The controls we use to establish cutoffs can affect our interpretation of seroprevalence.

Non-target antigen controls

- We also have controls with non-target antigens (SNAP in our dataset; other common ones are GST and Vero cell).
- These controls can ensure that the plate ran correctly, whether values are similar between plates, and high values can indicate non-specific binding.
- The non-target antigen MFI values should ideally not differ between samples with high and low antigen MFI values.

ID	SNAP MFI
1	80
2	74
3	214
4	1495
5	226
6	189
7	2211
8	228
9	67
10	915
11	50

Positive controls

- Often, positive controls are based on antibody responses from people who are known to have had symptomatic disease.
- **How might positive controls differ from positives in samples?**

Positive controls

- Often, positive controls are based on antibody responses from people who are known to have had symptomatic disease.
- **How might positive controls differ from positives in samples?**
    - Vaccination vs. natural infection
    - Intensity of infection – severe, symptomatic, asymptomatic – (controls may be more likely to have clinical or severe infection)
    - Timing since infection (controls likely were taken from acute infection phase)

Negative controls

- **What populations might be best to get negative controls from?**

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  - Very young presumed unexposed (likely to be from target population)

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Negative controls

- **What populations might be best to get negative controls from?**
  - Non-endemic area (likely to be adults)
  - Very young presumed unexposed (likely to be from target population)
- **Why would you choose to get - or not get - controls from each of these populations?**
  - Controls from non-endemic area may differ from target population in many ways (geographic, socioeconomic, disease history)
  - Very young immune systems vs. adult immune systems

Controls

You may not have ideal controls.

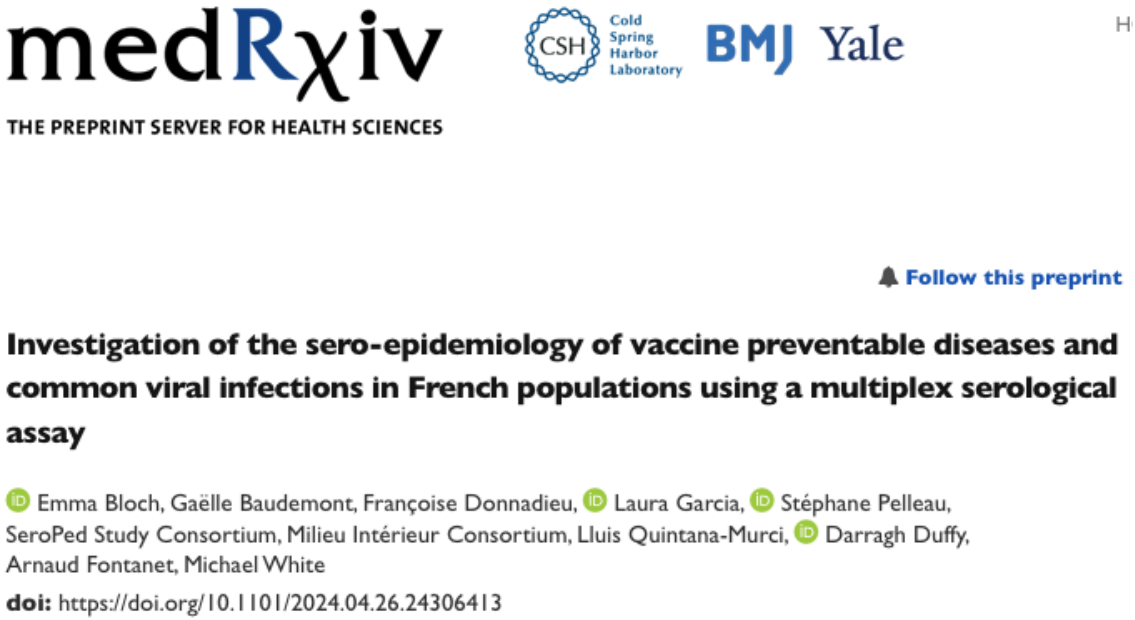
You should understand the assumptions/biases of your controls!

It’s important to understand the controls you use, and think about how these may affect your inference.

Cutoff methods

- There isn't always a clear answer for how to chose cutoff methods
- Today we will go through 4 methods for determining a cutoff:
  - Externally-defined correlate of protection (covered in **Part 4**)
  - Receiver Operating Characteristic curve (ROC, based on sensitivity and specificity, using both positive and negative controls)
  - Unexposed population (negative controls)
  - Finite mixture model (FMM; only based on sample results)

Examples of choosing cutoff methods



Serosurveillance of viral and vaccine-preventable diseases using 47-plex assay

Examples of choosing cutoff methods

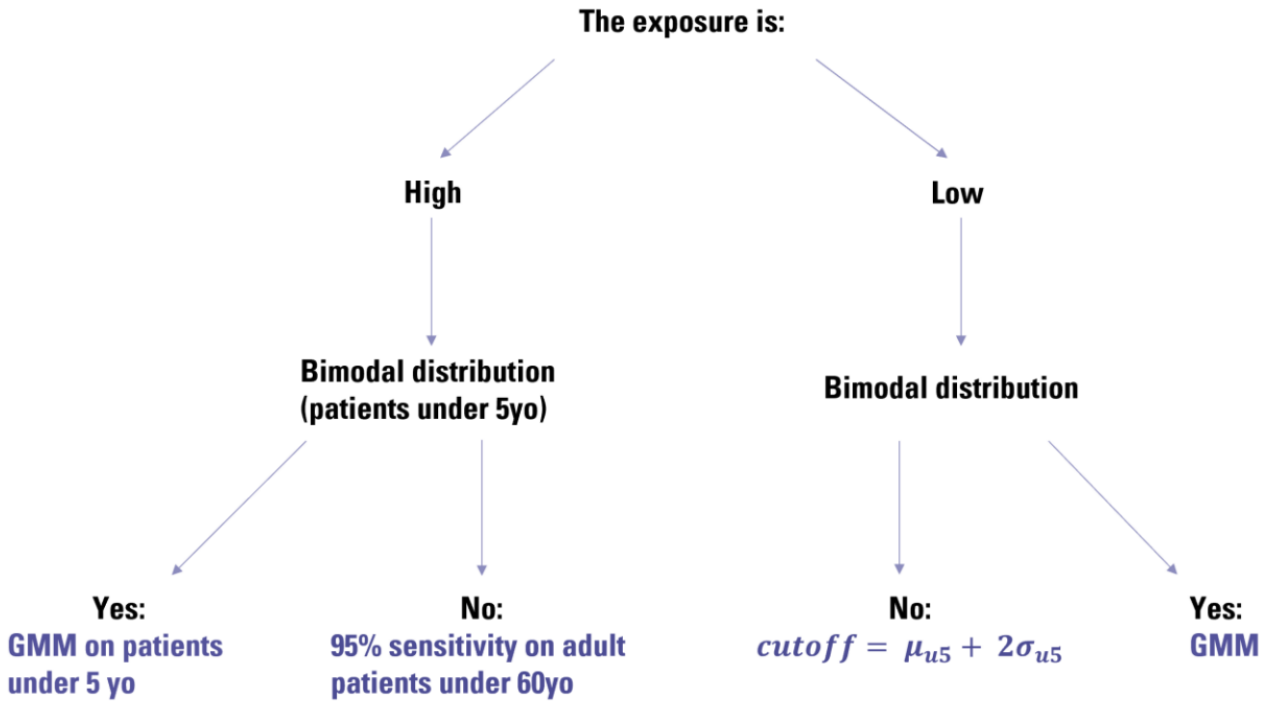
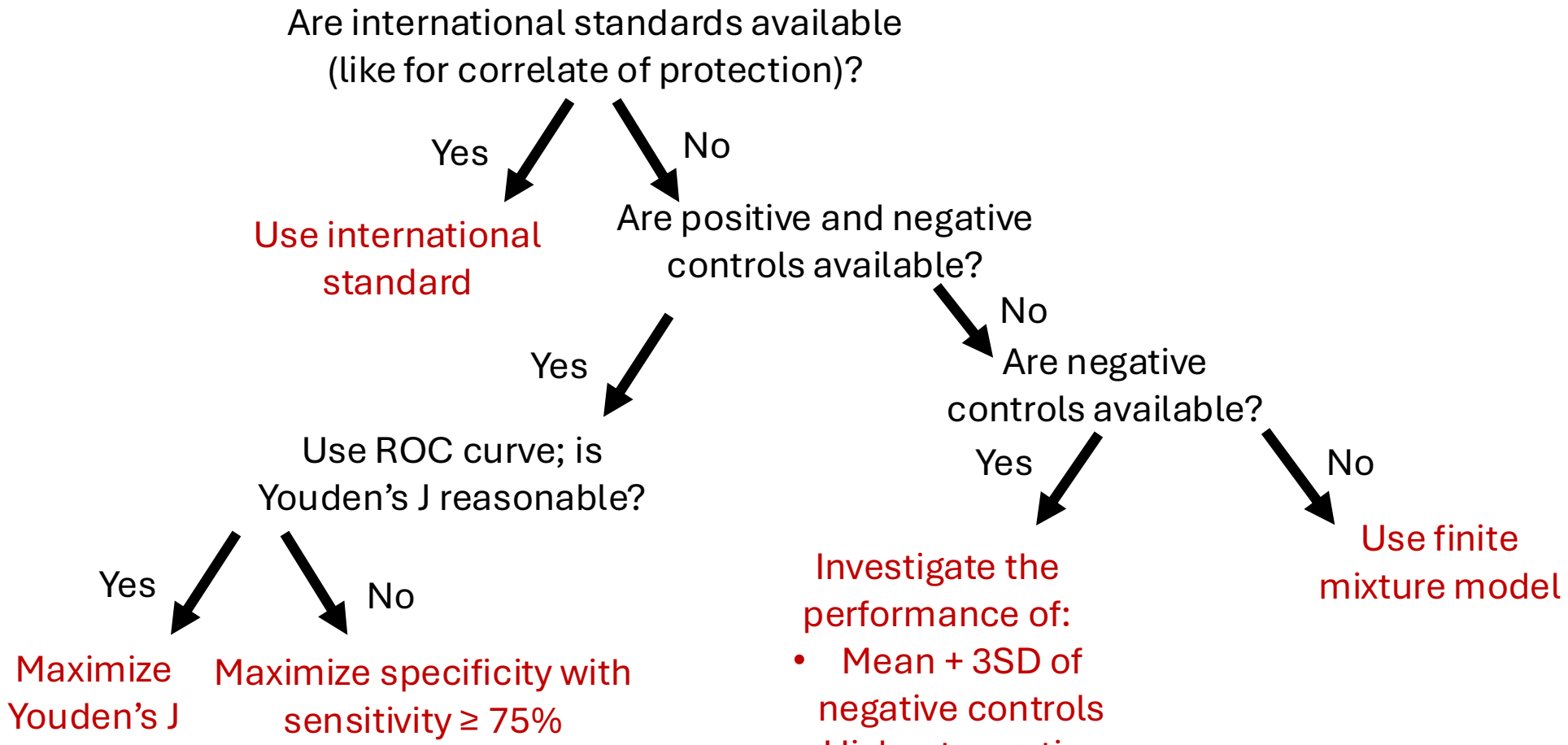


Figure 1: Decision tree used to choose between the different methods available to determine the cut-off discriminating between the seropositive and seronegative patients.

Bloch et al. Investigation of the sero-epidemiology of vaccine preventable diseases and common viral infections in French populations using a multiplex serological assay. 2024. medRxiv.

Examples of choosing cutoff methods



Example from Zambezia, Mozambique panel; Carcelen et al.

Other considerations:

- You may want to set up a way to decide cutoffs based on:
  - Previous methods used on the panel
  - Sample and/or control distributions
  - Available information about controls
  - Whether to prioritize minimizing false negatives vs. false positives
  - How you want to interpret seropositivity

Classifying controls in serological testing

We can define controls into the following categories:

- **True positives (TP):** Test positive, are positive
- **False positives (FP):** Test positive, are negative
- **True negatives (TN):** Test negative, are negative
- **False negatives (FN):** Test negative, are positive

We need to have positive and negative controls to know which samples are **truly positive and truly negative.**

Applying cutoffs to ZIKA antigen controls

Antigen	Control type	MFI	Cutoff	Serostatus
ZIKA	negative	402	500	0
ZIKA	negative	499	500	0
ZIKA	negative	551	500	1
ZIKA	negative	573	500	1
ZIKA	negative	645	500	1
ZIKA	positive	421	500	0
ZIKA	positive	431	500	0
ZIKA	positive	684	500	1
ZIKA	positive	848	500	1
ZIKA	positive	1094	500	1

← Based on cutoff

← True negatives

← False positives

← False negatives

← True positives

2X2 table for ZIKA

- A 2x2 table is a way to summarize TPs, FPs, TNs, and TPs

		True disease status	
		Positive	Negative
Serostatus based on cutoff	Seropositive	28	4
	Seronegative	2	26

True positives

False positives

True negatives

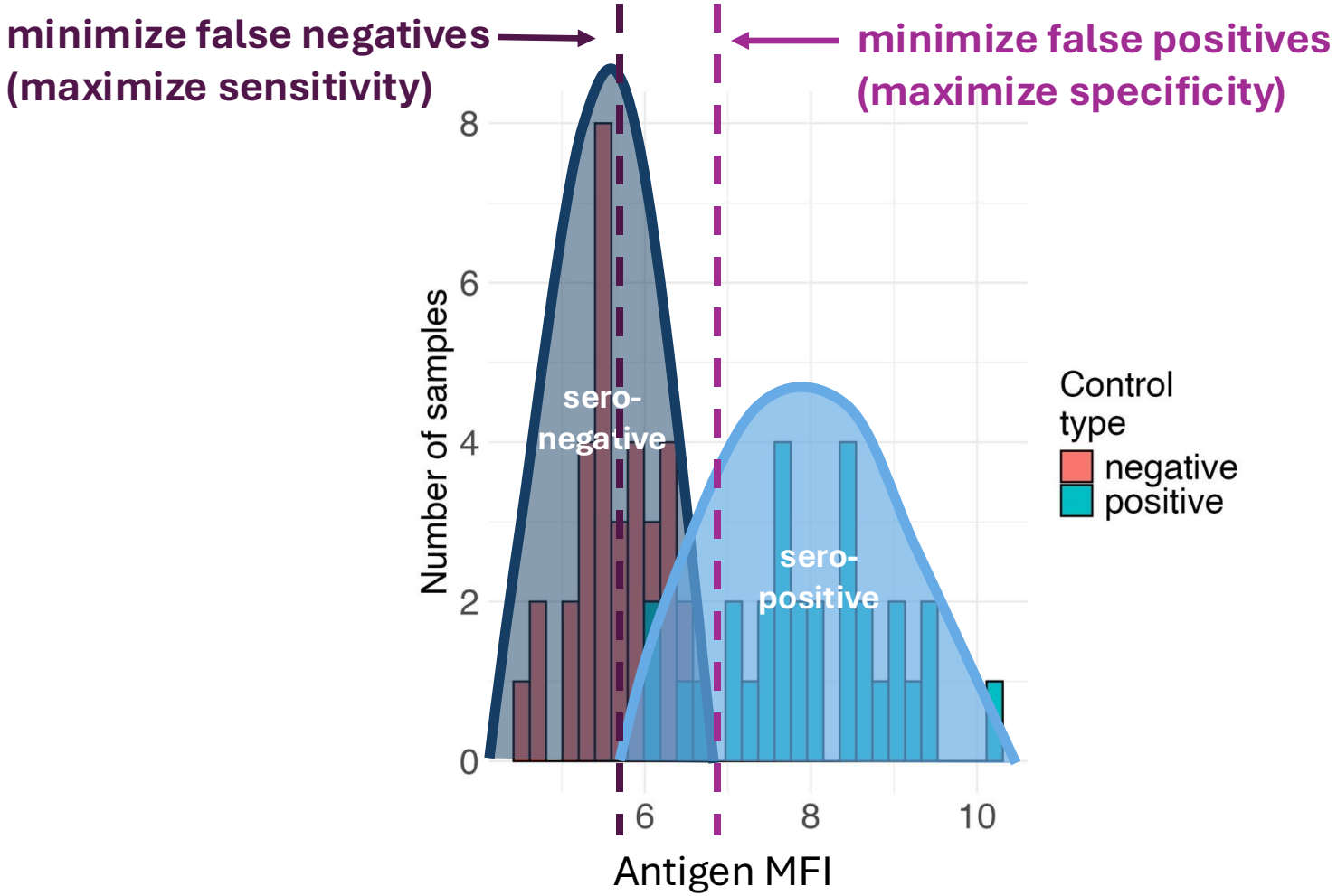
False negatives

False positives and false negatives: which are “worse”?

- When would we want to **minimize false positives**?

- When would we want to **minimize false negatives**?

One way to think about this…



False positives and false negatives: which are “worse”?

- When would we want to **minimize false positives**?
  - Do not want to over-estimate exposure or immunity
  - Minimize unnecessary interventions
- When would we want to **minimize false negatives**?
  - Do not want to miss any cases (risk of further transmission)
    - E.g., diseases near elimination or in early outbreak
  - Under-estimate disease burden

False positives and false negatives

- In other cases, false positives and false negatives may be equally non-desirable.
- Then, we can choose to minimize the total number of false positives and false negatives (balanced approach).

Sensitivity and specificity

- Sensitivity and specificity are measures of test accuracy that estimate how well true positives and true negatives are being captured.
- In serosurveillance, sensitivity and specificity measure how well a cutoff accurately classifies people in the population as seronegative or seropositive.

Sensitivity and specificity

Sensitivity is a measure of how many samples that should test positive actually do test positive. The **numerator** is the total number of positive controls that are seropositive based on a selected cutoff. The **denominator** is the total number of positive controls.

$$Sensitivity = \frac{True\ Positives\ (TP)}{True\ Positives\ (TP) + False\ Negatives\ (FN)}$$

Specificity is a measure of how many samples that should test negative actually do test negative. The **numerator** is the total number of negative controls that are seronegative based on a selected cutoff. The **denominator** is the total number of negative controls.

$$Specificity = \frac{True\ Negatives\ (TN)}{True\ Negatives\ (TN) + False\ Positives\ (FP)}$$

Sensitivity of ZIKA antigen, using MFI cutoff = 500

For ZIKA antigen, using a cutoff of 500, we have:

- 26 true negatives
- 2 false negatives
- 28 true positives
- 4 false positives

$$Sensitivity = \frac{True\ Positives\ (TP)}{True\ Positives\ (TP) + False\ Negatives\ (FN)}$$

$$Sensitivity = \frac{28}{28 + 2} = \frac{28}{30} = 0.933$$

$$Sensitivity = 93.3\%$$

Sensitivity of ZIKA antigen, using MFI cutoff = 500

		True disease status	
		Positive	Negative
Serostatus based on cutoff	Seropositive	28	4
	Seronegative	2	26

True positives

False negatives

Specificity of ZIKA antigen, using MFI cutoff = 500

For ZIKA antigen, using a cutoff of 500, we have:

- 26 true negatives
- 2 false negatives
- 28 true positives
- 4 false positives

$$Specificity = \frac{True\ Negatives\ (TN)}{True\ Negatives\ (TN) + False\ Positives\ (FP)}$$

$$Specificity = \frac{26}{26 + 4} = \frac{26}{30} = 0.867$$

$$Specificity = 86.7\%$$

Specificity of ZIKA antigen, using MFI cutoff = 500

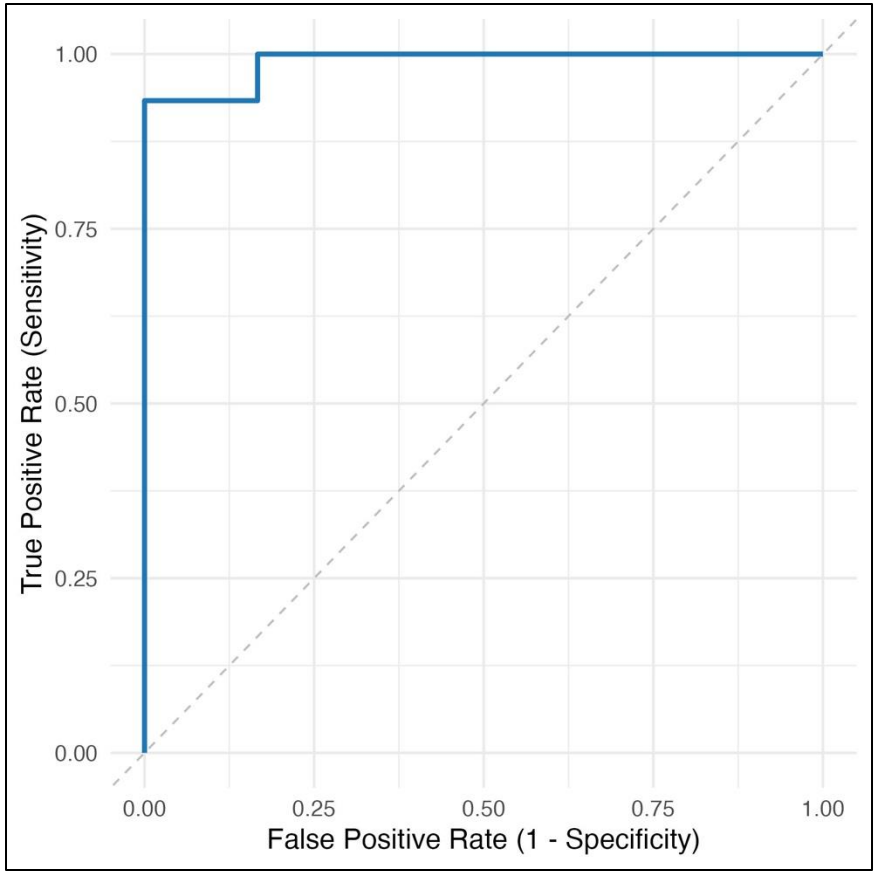
		True disease status		
		Positive	Negative	
Serostatus based on cutoff	Seropositive	28	4	← False positives
	Seronegative	2	26	← True negatives

Receiver Operating Characteristic (ROC) Curve

- If we have positive and negative controls, we can compare sensitivity and specificity to help determine a cutoff.
- The **ROC curve uses known positive and negative controls** to compare sensitivity vs. specificity.
- We can use the ROC curve to choose a cutoff value that minimizes false positives and false negatives.

ROC Curves

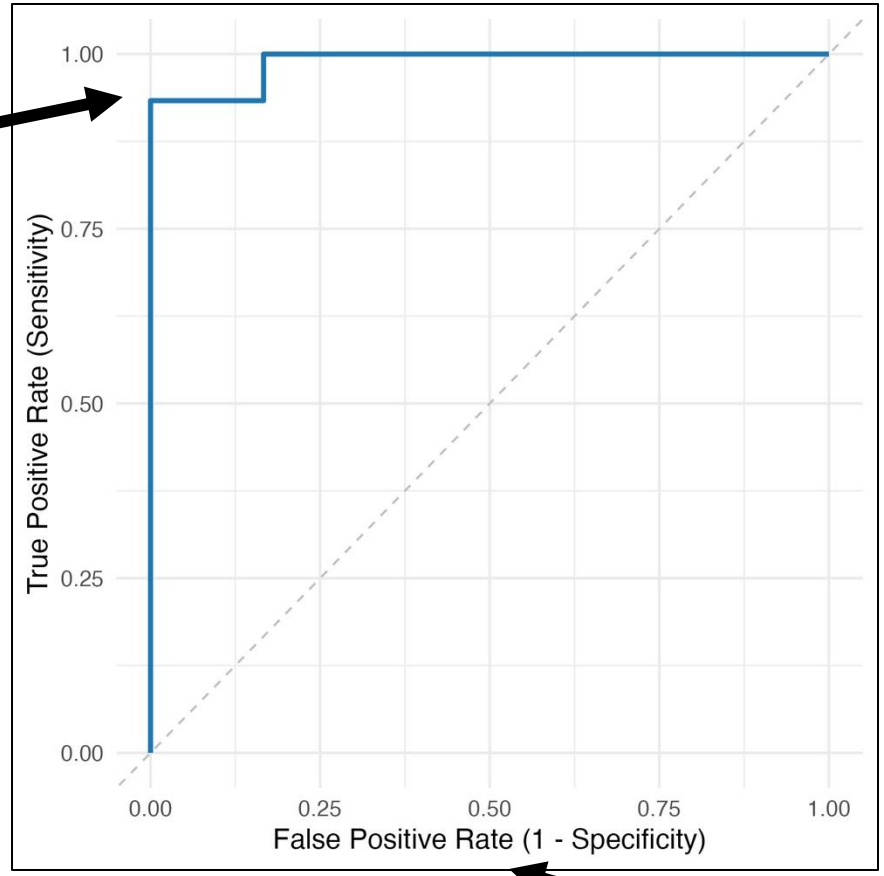
- The ROC curve shows *sensitivity* (true positive rate) and *1 – specificity* (false positive rate) calculated at many different cutoff values.



ROC Curves

If the curve is close to the top left corner, then we can identify a cutoff with high sensitivity and specificity

Y-axis is sensitivity, equal to true positive rate



X-axis is 1-Specificity, equal to false positive rate

Choosing a cutoff from ROC Curve

The ROC curve shows us how sensitivity and specificity change as we adjust the cutoff value. It does not give a specific cutoff value.

How to chose what cutoff to chose:

Consider: **Is it worse to have false negatives or false positives?**

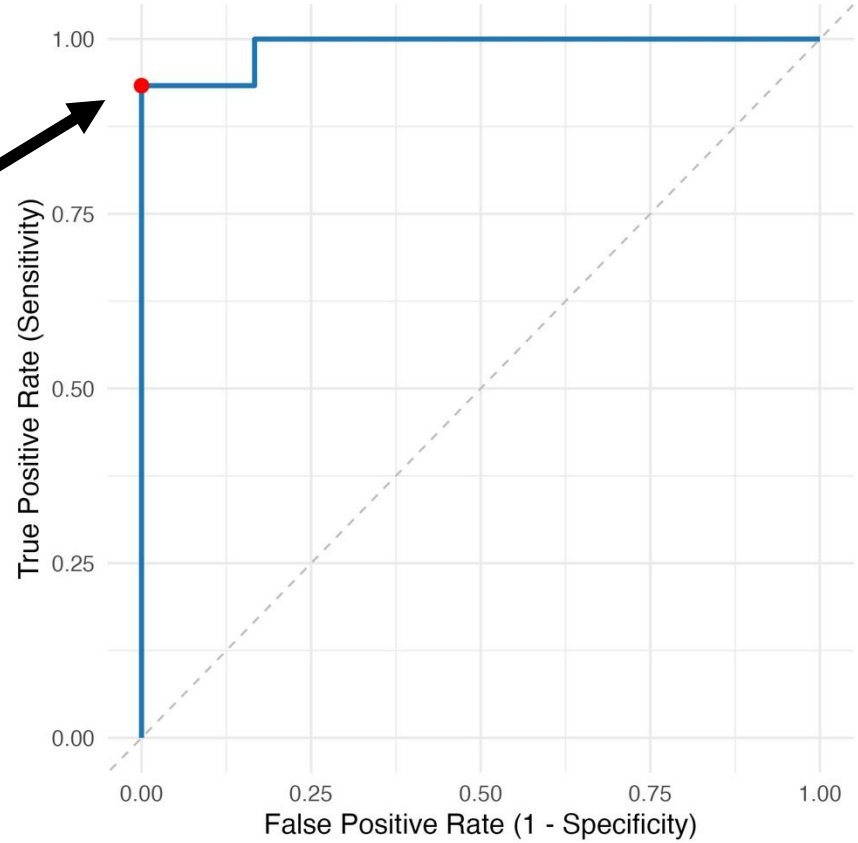
- Is it worse to have false positives (labeling negative people as positive)? For example, for a rare disease?
- Is it worse to have false negatives (missing seropositive people)?
- Or are false positives and false negatives equally bad?

Cutoffs from ROC curve – Youden's Index

- One common method to choose a cutoff from a ROC curve is to use **Youden's Index**
- This method assumes **false positive and false negatives are equally bad**
- This method finds the cutoff that **maximizes Sensitivity + Specificity – 1**

Cutoffs from ROC curve – Youden's Index

When we maximize Sensitivity + Specificity – 1 for this ROC curve:



Cutoff based on Youden's index:

TPR: 0.933

FPR: 0

Cutoff: 664.5

Cutoffs from ROC curve

- Can also adjust the cutoff if we primarily want to minimize false positives or false negatives, for example:
  - If we are concerned about too many false positives, we could set a minimum value for specificity (e.g., find cutoff that provides maximum sensitivity while maintaining >80% specificity)

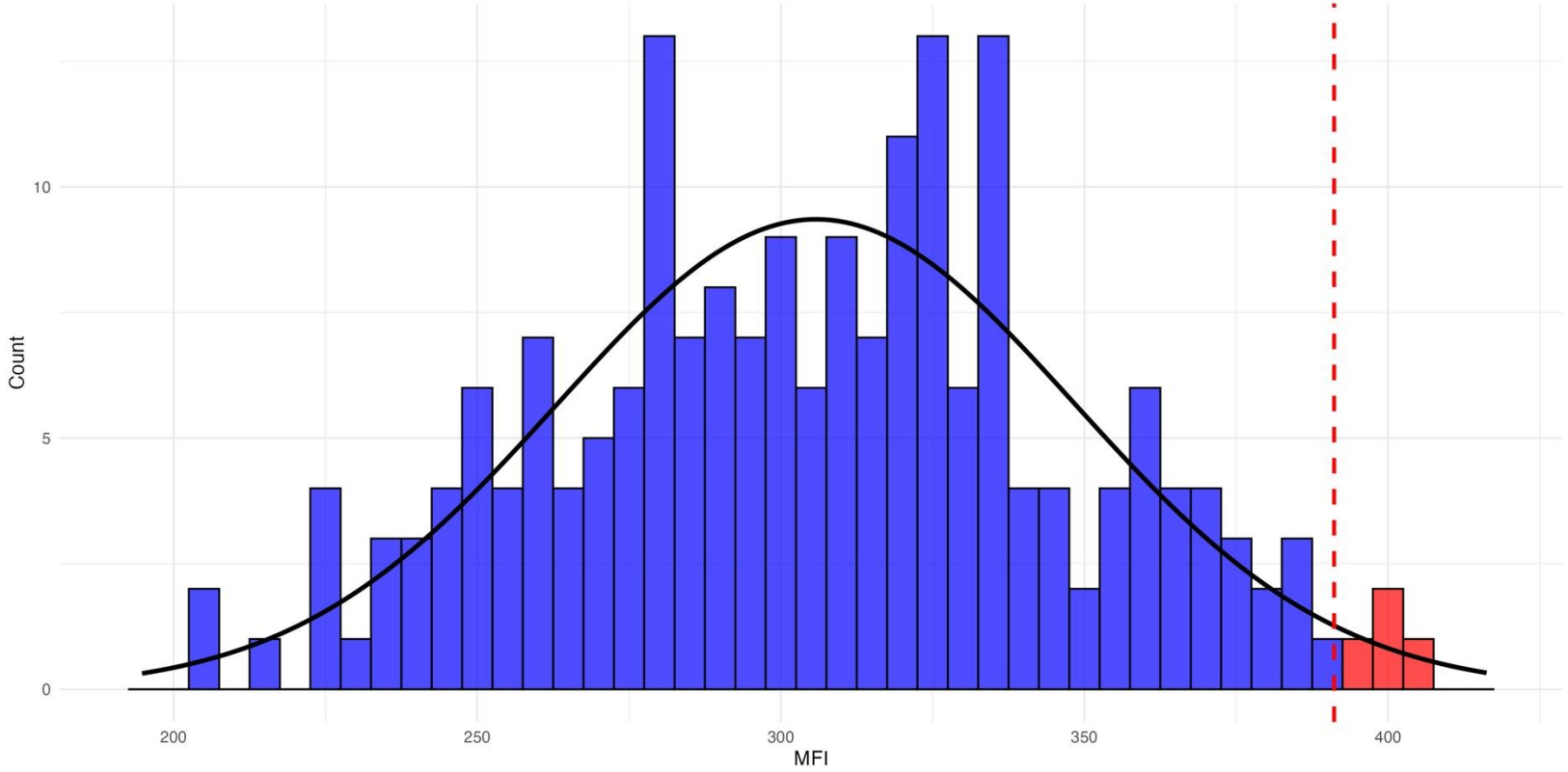
ROC curve summary

- Advantages:
  - Flexibility to prioritize sensitivity or specificity
- Disadvantages:
  - Requires positive and negative controls
  - Controls may differ from sample population

Mean + Standard Deviation

- This method uses negative controls: for example, from a non-endemic region or from young individuals who are presumed to be unexposed
- Cutoff = mean + 2 or 3 standard deviations
  - If negative controls are normally distributed:
    - Only ~2.5% of negatives are expected to be above mean + 2SDs
    - Only ~0.14% of negatives are expected to be above mean + 3SDs

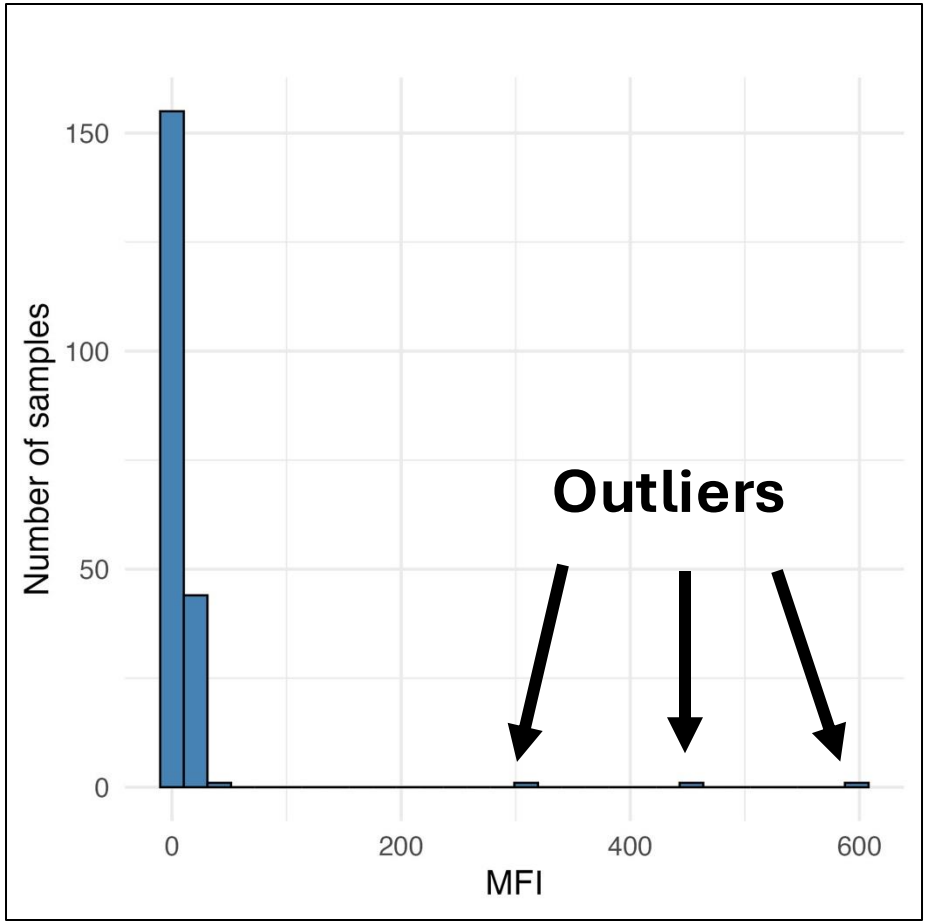
Unexposed controls



If controls are normally distributed: For mean + 2 SDs, we expect 2.5% of negative samples to be greater than cutoff (i.e., assay has 97.5% specificity).

Unexposed controls

- There can sometimes be outliers in unexposed controls (high-responders)
- This could be a reason for a non-normal distribution
- A more conservative approach is using highest negative control (equals 100% specificity)
- It’s also worth investigating the outlier samples to try to understand if they were truly unexposed

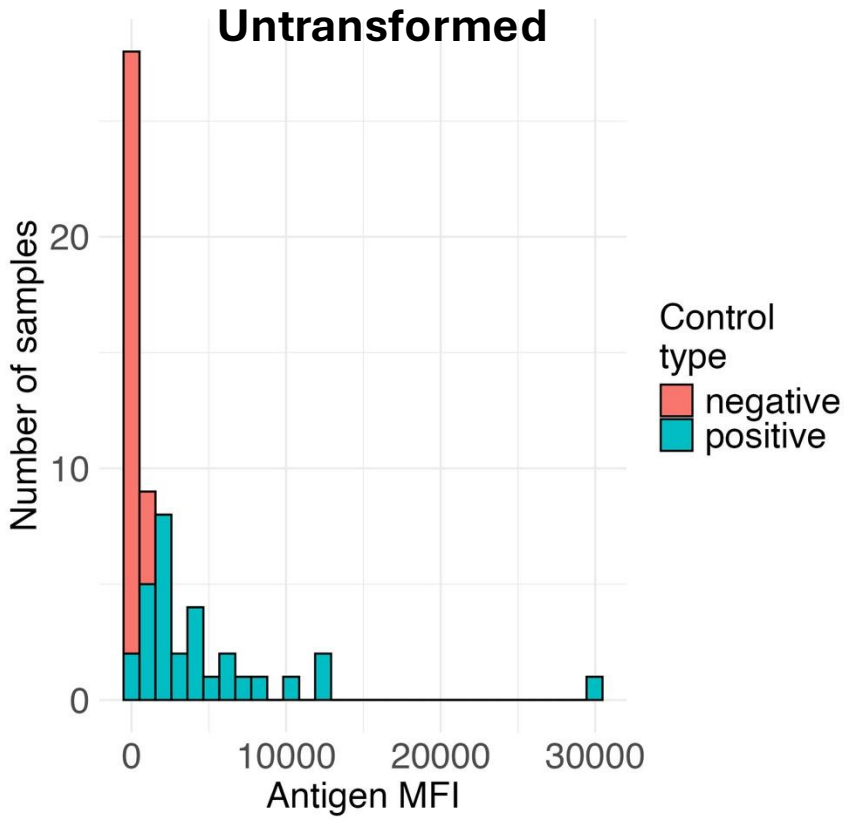


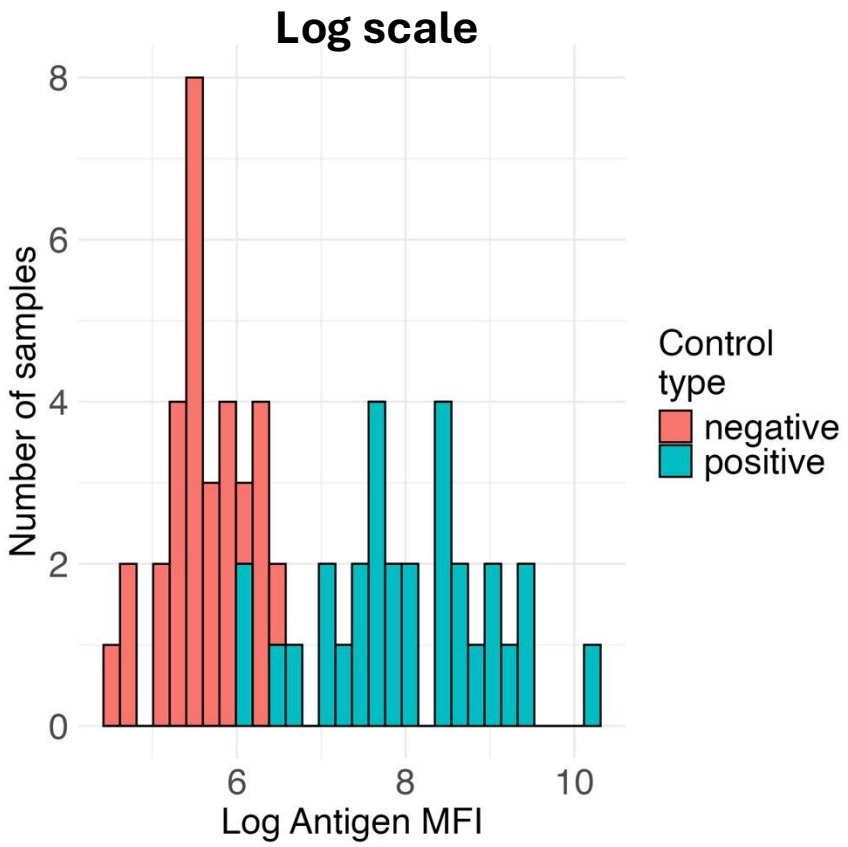
Unexposed controls

- Advantages:
  - Simple to implement
  - Doesn’t require positive controls (often harder to find, depending on pathogen)
- Disadvantages:
  - Negative controls should be comparable to true negative samples in population
  - Specificity may be challenging to estimate depending on the number and distribution of negative controls
  - Can’t estimate sensitivity or numbers of false negatives (if no positive controls)

Distributions of controls

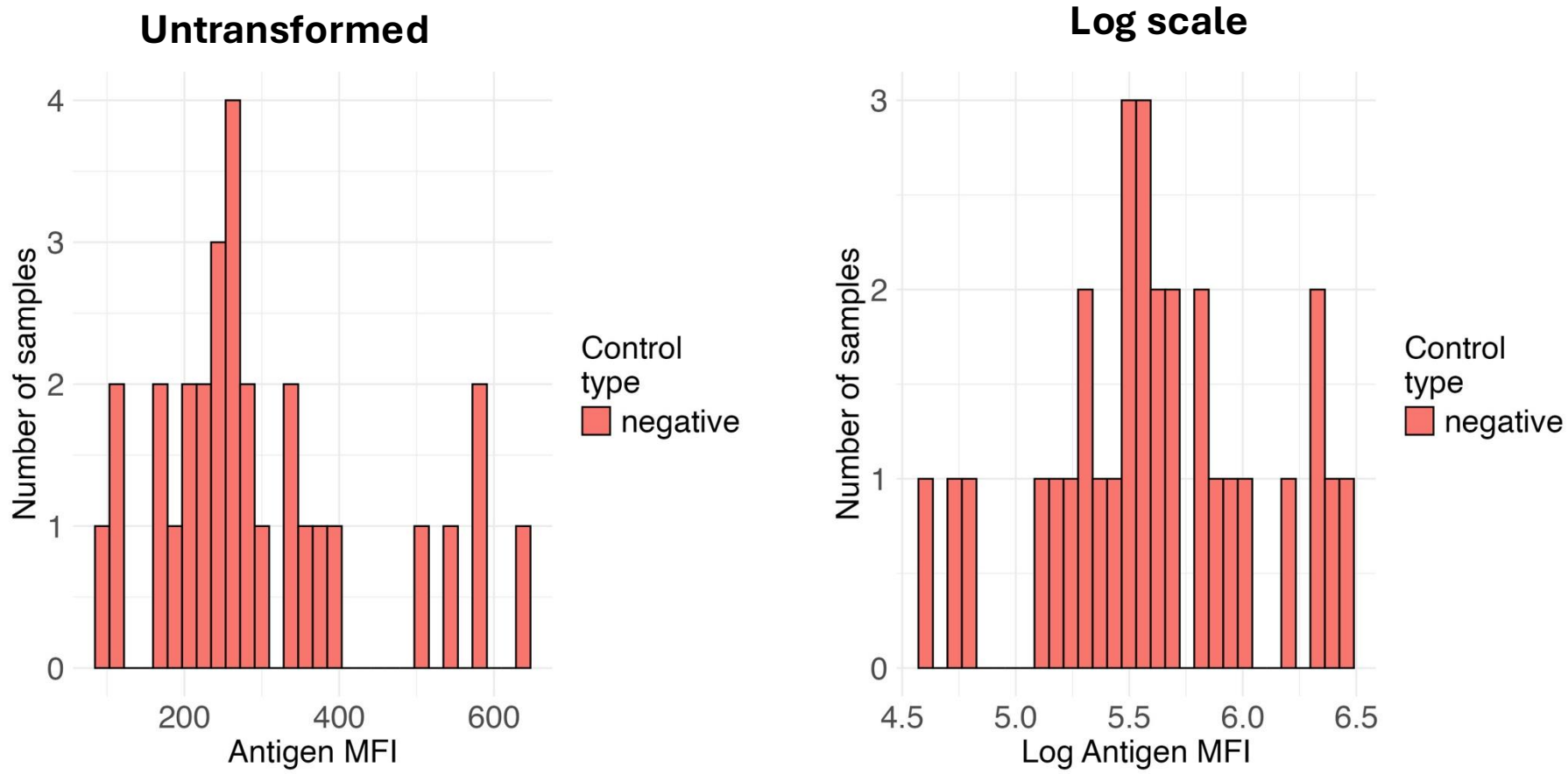
For ZIKA antigen: Overlap between distributions of true positives and negatives





Distribution of negative controls

ZIKA antigen negative controls



Cutoffs using ZIKA negative controls:

- Mean log(ZIKA negative controls) = 5.600
- SD log(ZIKA negative controls) = 0.473

- Cutoff = mean + 2 SD of negative controls
- Cutoff = exp(5.600 + 2 * 0.473) = exp(6.545) = **695.9**

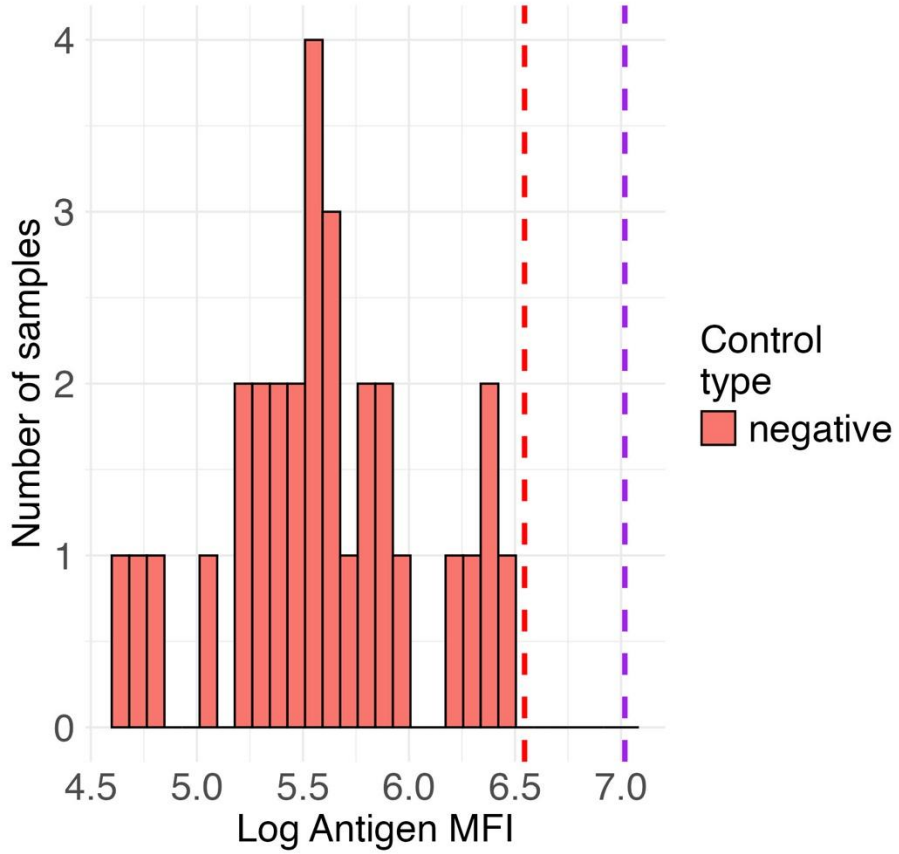
- Cutoff = mean + 3 SD of negative controls
- Cutoff = exp(5.600 + 3 * 0.473) = exp(7.018) = **1116.5**

Cutoffs using ZIKA negative controls:

Histogram of **negative controls with cutoffs:**

In red: **Mean + 2SD = 695.9**

In purple: **Mean + 3SD = 1116.5**



Specificity of ZIKA antigen

- **For a cutoff = 695.9:**
- If no positive controls available:

		True disease status	
		Positive	Negative
Serostatus based on cutoff	Seropositive	-	0
	Seronegative	-	30

Specificity of ZIKA antigen

- **For a cutoff = 695.9:**

$$Specificity = \frac{True\ Negatives\ (TN)}{True\ Negatives\ (TN) + False\ Positives\ (FP)}$$

$$Specificity = \frac{30}{30 + 0} = \frac{30}{30} = 1.00$$

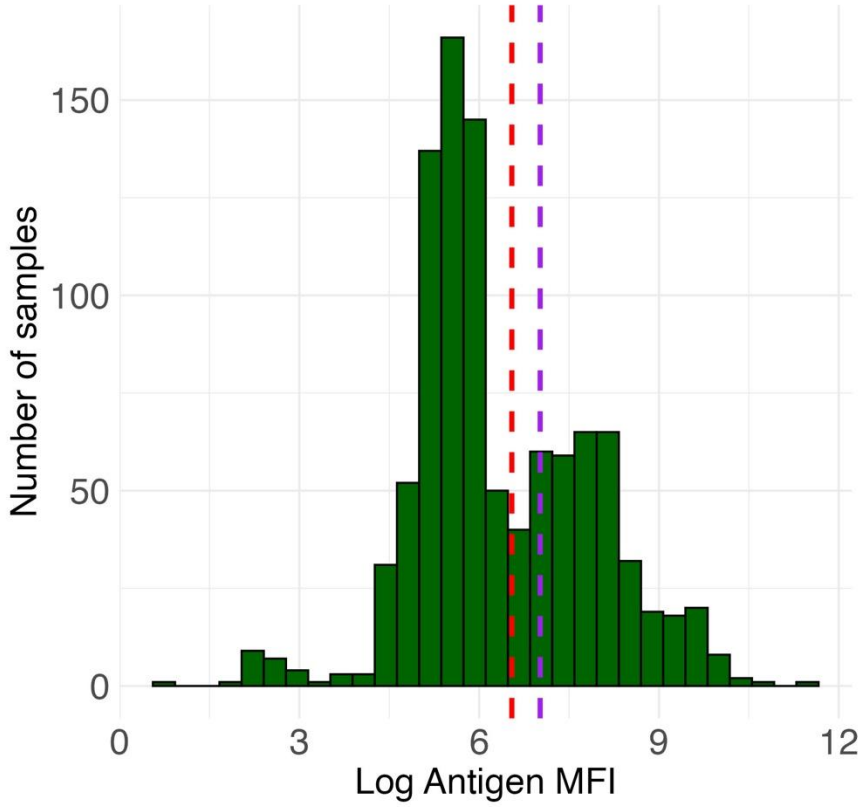
$$Specificity = 100\%$$

Cutoffs using ZIKA negative controls:

Histogram of **samples**
with cutoffs:

In red: **Mean + 2SD = 695.9**

In purple: **Mean + 3SD = 1116.5**



Mixture models

- Mixture models are the only method to estimate (1) the probability that a sample is seropositive and (2) seroprevalence directly from the sample distribution
  - Does not require positive or negative controls

Mixture models

- Advantage:
  - Doesn’t require positive or negative controls
- Disadvantage:
  - Only works if we can identify distributions representing exposed and unexposed individuals (ideally with minimal overlap)

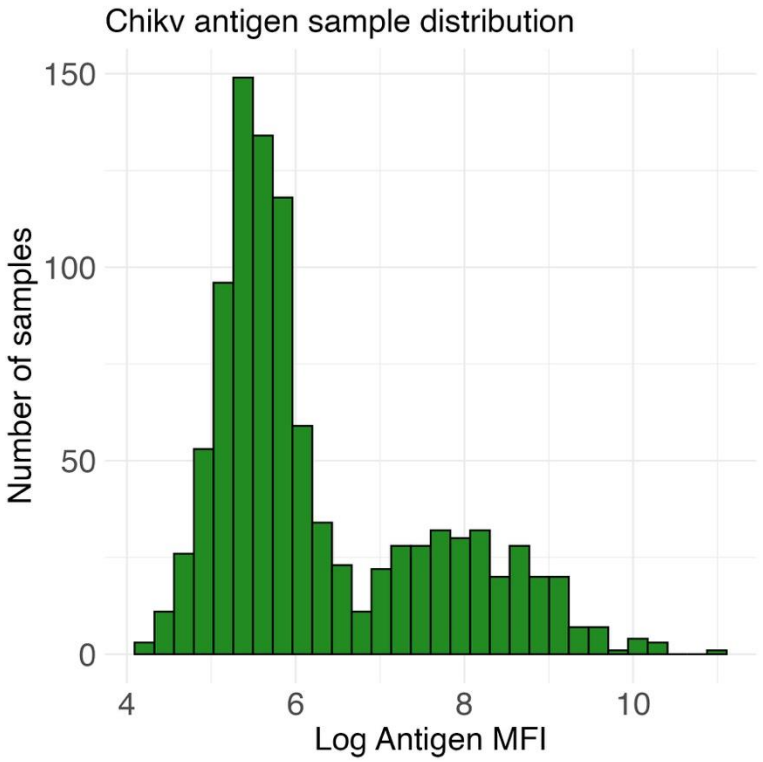
Mixture models

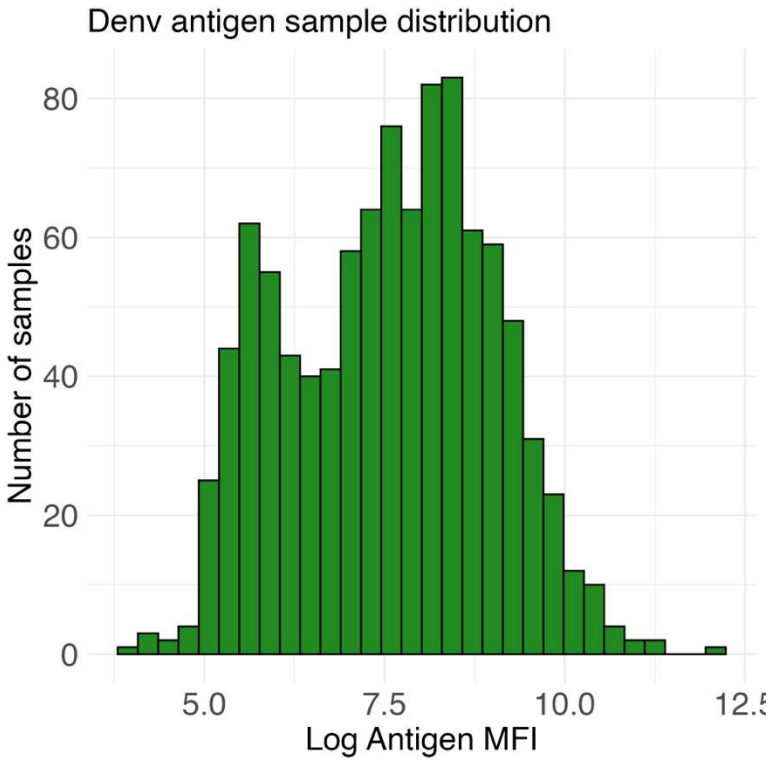
Why may we not see two distinct distributions for exposed and unexposed individuals in a population (e.g., either 1 or 3+ distributions)?

- Almost all / almost no individuals exposed
- Waning antibodies over time
- Cross-reactive antibodies
- Magnitude of antibody response could vary with intensity of infection
- Natural infection vs. vaccination

Mixture models

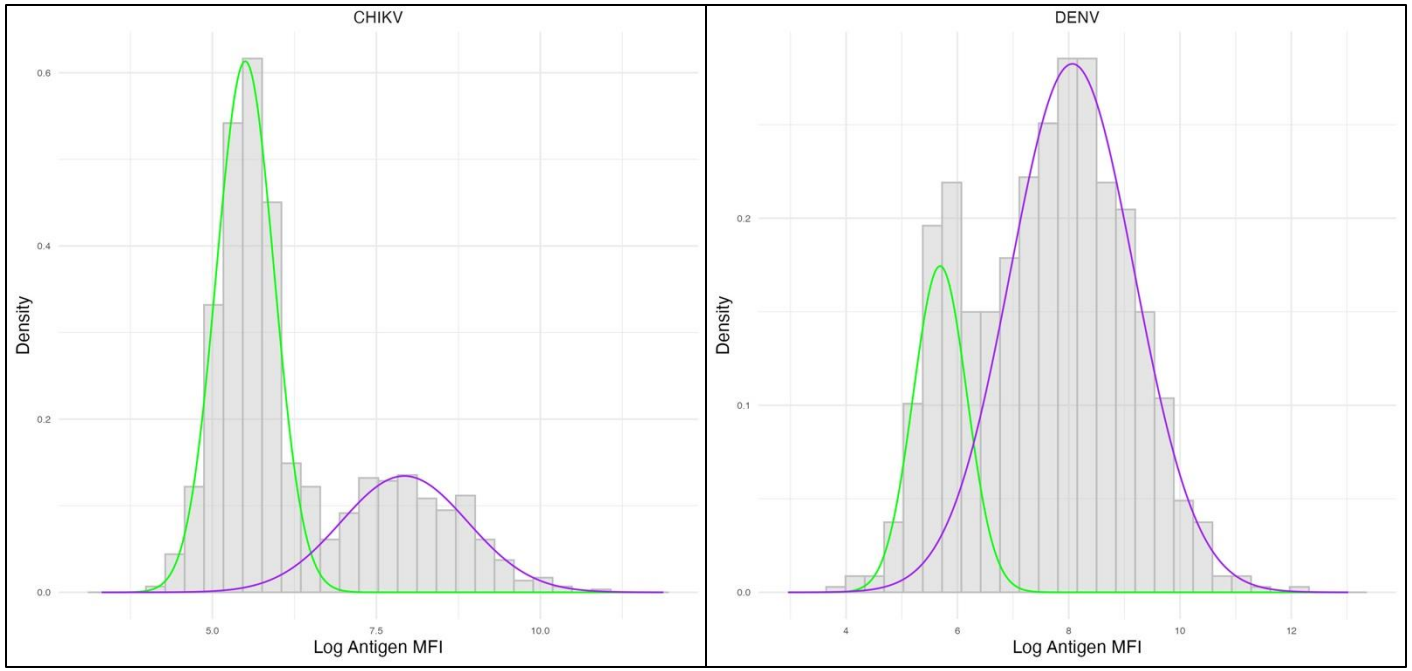
Chikungunya and Dengue could be appropriate distributions for fitting a mixture model (possibly two components per antigen)





Mixture models

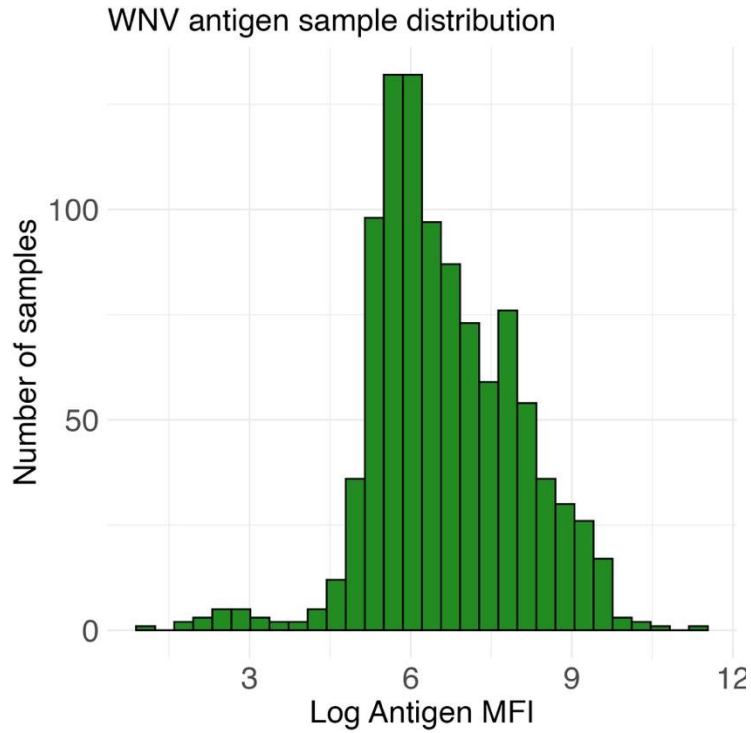
- Statistics also show that 2 components fit these distributions better than 1 component

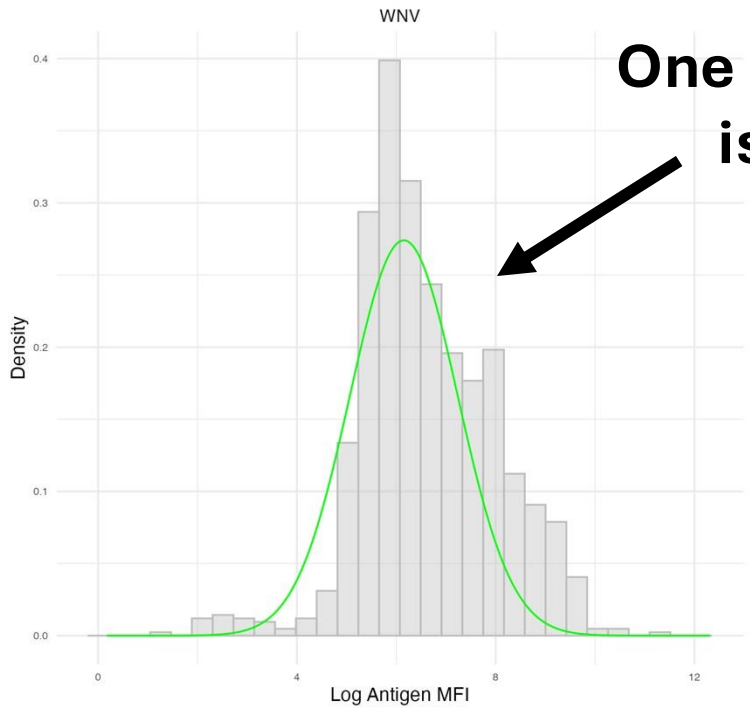


Antigen	Likelihood_1	Likelihood_2	BIC_1	BIC_2	Pval_2
CHIKV	-1691.179	-1388.954	-3396.173	-2812.446	1.093050e-130
DENV	-1757.501	-1711.558	-3528.818	-3457.655	8.617921e-20

Mixture models

Some antigen distributions like West Nile Virus are not as good:





One component is best fit

Mixture models

- Unlike other methods, this method doesn't calculate a cutoff
- Instead, it calculates the probability of each sample being seropositive
- Seroprevalence equals the sum of the individual-level probabilities of being seropositive

Antigen	MFI	log_MFI	Prob_positive
WNV	191	5.252273	0.0171
WNV	265	5.579730	0.0286
WNV	345	5.843544	0.0430
WNV	440	6.086775	0.0624
WNV	605	6.405228	0.1001
WNV	915	6.818924	0.1780
WNV	1475	7.296413	0.3186
WNV	2595	7.861342	0.5375
WNV	5125	8.541886	0.7767

$$Seroprevalence = \frac{\sum probablities}{number\ of\ samples} = \frac{253}{1000} = 25.3\%$$

Conclusion

- Methods to determine **seropositivity** come with different **assumptions**, which are important to understand.
- The method you choose will depend on **your study** and the **particular antigen(s)**, in addition to **controls** available, and sample and control **distributions**.