# Theoretical Limitations of Quantification for Noncompetitive Sandwich Immunoassays

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**Keywords** Immunoassay · Single Molecule · Molecular shot noise · Detection limit

## Abbreviations

| | |
|---|---|
| LOD | Limit of detection |
| NSB | Non-specific binding |
| MSN | Molecular shot noise |

| | |
|---|---|
| LOQ | Limit of quantification |
| P4-P5 | Four and five parameter fitting models |

## Abstract

Immunoassays exploit the highly selective interaction between antibodies and antigens to provide a vital method for biomolecule detection at low concentrations. Developers and practitioners of immunoassays have long known that nonspecific binding often restricts immunoassay limits of quantification (LOQ). Aside from non-specific binding, most efforts by analytical chemists to reduce the LOQ for these techniques have focused on improved signal amplification methods and minimizing the limitations of the detection system. However, with detection technology now capable of sensing single fluorescent molecules, this approach is unlikely to lead to dramatic improvements in the future. Here, fundamental interactions based on the law of mass action are analytically connected to signal generation, replacing the four and five parameter fittings commercially used to approximate sigmoidal immunoassay curves and allowing quantitative consideration of non-specific binding and statistical limitations in order to understand the ultimate detection capabilities of immunoassays. The restrictions imposed on limits of quantification by instrumental noise, non-specific binding and counting statistics are discussed based on equilibrium relations for a sandwich immunoassay. Understanding the maximal capabilities of immunoassays for each of these regimes can greatly assist in the development and evaluation of immunoassay platforms. While many studies suggest that single molecule detection is possible through immunoassay techniques, here it is demonstrated that the fundamental limit of quantification (precision of 10% or better) for an immunoassay is approximately 131 molecules and this limit is based on fundamental and unavoidable statistical limitations.

## Introduction

Immunoassays are invaluable tools for the detection and quantification of important biomolecules and many other chemical compounds at low concentrations. Antibodies bind to target structures with large binding constants, which enable selective detection at low analyte concentrations. Since immunoassays were first introduced, attempts to optimize the assay process have persistently focused on improving the limit of detection (LOD).[1,2] This focus on low LOD's has been stimulated largely by the desire for earlier therapeutic intervention through the detection of diagnostic markers at lower concentrations or from smaller volumes.[3] Over the years these efforts have resulted in the shift away from radioimmunoassays to enzyme-linked

immunosorbent assays,[4,5] and in the exploration of signal amplification approaches to improve detection of antibody-antigen binding.[6-18]

The LOD is an important figure of merit for determining an immunoassay's quality and is frequently used to compare competing methods.[19,20] The term, LOD, is often used interchangeably in the immunoassay literature with the limit of quantification (LOQ), causing some confusion as to the reported capabilities of different assays.[21-23] As defined by Currie in 1968, the LOD corresponds to the presence of any detectable signal from the specific instrumental configuration that can be assigned to the target under study. The LOD is used as a demarcation of the presence or absence of an analyte (the $L_d$ term in reference) and has high quantitative uncertainty at when there are low numbers of target species in a sample (reaching 100%), undermining its use as an indicator of presence/absence. The LOQ is the level at which measurements have sufficient precision for quantitative determination.[23] The distinction between LOD and LOQ highlights that while single molecule immunoassays can detect the presence of one (or very few) putative signal generating molecules, detection at this level is highly qualitative. For immunoassays aimed at the quantification of minute amounts of proteins indicative of disease states, detection near the LOD is not adequate. Therefore, it is necessary to define the true LOQ for an immunoassay in terms of a statistical assessment and not instrumental factors.

The LOD for most immunoassays has been limited primarily by the signal-to-noise ratio provided by the instrument used to detect antibody-antigen binding or by non-specific binding (NSB).[24,25] With improving detection technology capable of routine single molecule detection, the instrumentation to detect antibody-antigen binding is no longer a fundamental factor that defines LOD's for immunoassays. It is well known that NSB often limits the LOD for immunoassays;[19,26-31] however, if an immunoassay method is optimized to reduce NSB to insignificant levels, the LOD that can be obtained with an immunoassay are then limited by antibody-antigen binding and fundamental statistical limitations.

Detection of a single molecule is an irresistible objective for analytical chemists. Recently, so-called 'single molecule immunoassay' techniques have been introduced.[26-28] Much of this work demonstrates the detection of individual signals associated with distinct putative binding events, but detection limits do not approach single molecule for the antigen.[26-28] These techniques have relied on the use of chemically-linked fluorophores to secondary (or tertiary)

antibodies with detection schemes able to sense a single fluorphore (or activity of a single enzyme). The signals from the individual counting of presumed immune complexes are averaged, summed or provided other data-processing mechanisms to generate an estimate of antigen concentration. These studies, while counting distinct signals assumed to be individual immune complexes, required the averaging of many individual signals to produce a quantitative measurement with a satisfactory coefficient of variation (<10%).[32,33] The requirement for averaging many individual signals demonstrates that while singular complexes have been, in fact, detected using immunoassay techniques, the certainty with which they are detected is not sufficient for quantification of an analyte. Therefore, the true limits of quantification lie at higher levels than a single molecule. For any analytical immunoassay measurements approaching single antigen molecule detection, the LOD is ultimately bound by 'molecular' shot noise – the absolute floor of the limit of detection, which is a statistical sampling effect that follows a Poisson distribution.[34]

In this work, relationships based on the law of mass action are used to model the theoretical limit of quantification for immunoassays. Most commercial immunoassay methods currently use P4-P5 fittings (four or five parameter mathematical fittings of resulting sigmoidal curves with no connection to fundamental interactions) to model the sigmoidal immunoassay response curves for quantitative analysis because of the difficulty of implementing theoretical models based on the law of mass action with many parameters.[35] These P4-P5 fittings are useful for practical quantitative analysis with immunoassays, but they cannot be used to explore the fundamental limit of quantification for immunoassays. The focus of this work is the ultimate limitations of immunoassays, mostly centered on molecular shot noise. However, without comparing and contrasting this limit with other effects, it cannot be put in proper context. The LOQ for immunoassays is considered using theoretical models based on the law of mass action for three situations: when the limit of quantification is defined by 1) instrumental limitations, 2) non-specific binding—the most common case, and 3) conditions where statistical sampling theory is the only limit, so-called molecular shot noise.

## Theory

### *Fundamental Relationships*

Using equations from basic immunology and describing reaction schemes according to the law of mass action, the first incubation in the sandwich-type immunoassay (Figure 1) can be described by

$$K1_{eq} = \frac{[Ab1Ag]}{[Ab1][Ag]} \qquad (1)$$

where $K1_{eq}$ is the equilibrium association constant (antibody affinity, $M^{-1}$) for the capture antibody, [*Ag*] is the concentration of free antigen (M), [*Ab1*] is the concentration of unbound capture antibody (M), and [*Ab1Ag*] is the concentration of the antibody-antigen complex formed during the reaction (M).[36] Modeling the reaction this way requires several assumptions to be made: (i) the interaction of antigen and antibody can be described using a single equilibrium constant, (ii) binding of antibody to a solid surface (or fluorophore or enzyme) doesn't affect binding characteristics, and (iii) wash steps separating bound and free antigen don't disturb the equilibrium reached.[36]

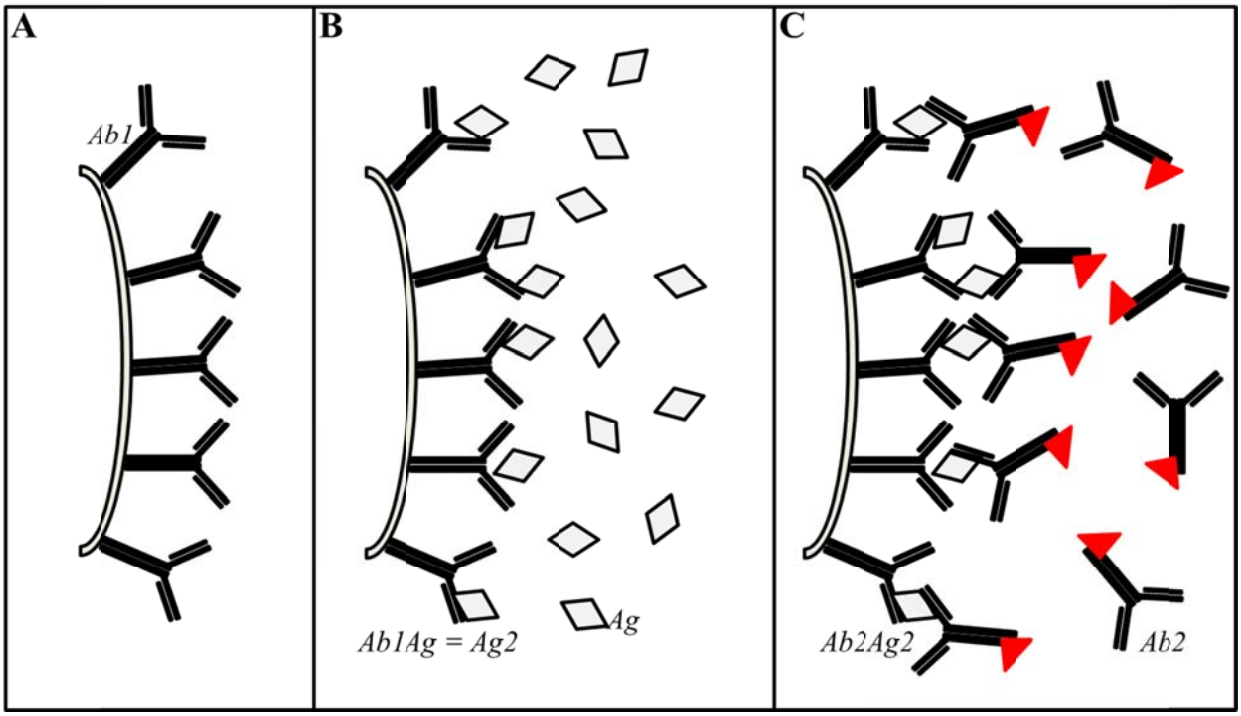



**Figure 1** Schematic of the sandwich immunoassay format. A) The primary antibody ([*Ab1*]) is bound to a solid support forming a reaction capture area. B) The target analyte ([*Ag*]*)* is incubated with the primary antibody and

captured to the surface (forming [*Ab1Ag*] which is equivalent to [*Ag2*]). After washing to remove any unbound species in the sample volume, C) incubation with the secondary detection antibody ([*Ab2])* and removal of the unbound antibody allows detection of a signal and quantification of the bound analyte ([*Ab2Ag2*]).

Given that $[Ab1_{tot}]$ is the total concentration of capture antibody, then

$$[Ab1] = [Ab1_{tot}] - [Ab1Ag] \qquad (2)$$

From Equation 2 we can write

$$[Ab1Ag] = K1_{eq}[Ag]\{[Ab1_{tot}] - [Ab1Ag]\} \qquad (3)$$

which can be simplified to:[36,37]

$$[Ab1Ag] = \frac{K1_{eq}[Ab1_{tot}][Ag]}{(1 + K1_{eq}[Ag])} \ . \qquad (4)$$

A plot of [*Ab1Ag*] with respect to log [*Ag*] is sigmoidal (Equation 4, Figure 2), with very low [*Ag*] concentrations producing linear changes in [*Ab1Ag*] (Figure 2). The shape of the curves can be trivially understood by noting that $1 + K1_{eq}[Ag] \cong 1$ when [*Ag*] is small, thus making eq. 4 a linear relationship and when [*Ag*] is very large $1 + K1_{eq}[Ag] \cong K1_{eq}[Ag]$ and the relationship becomes concentration independent.

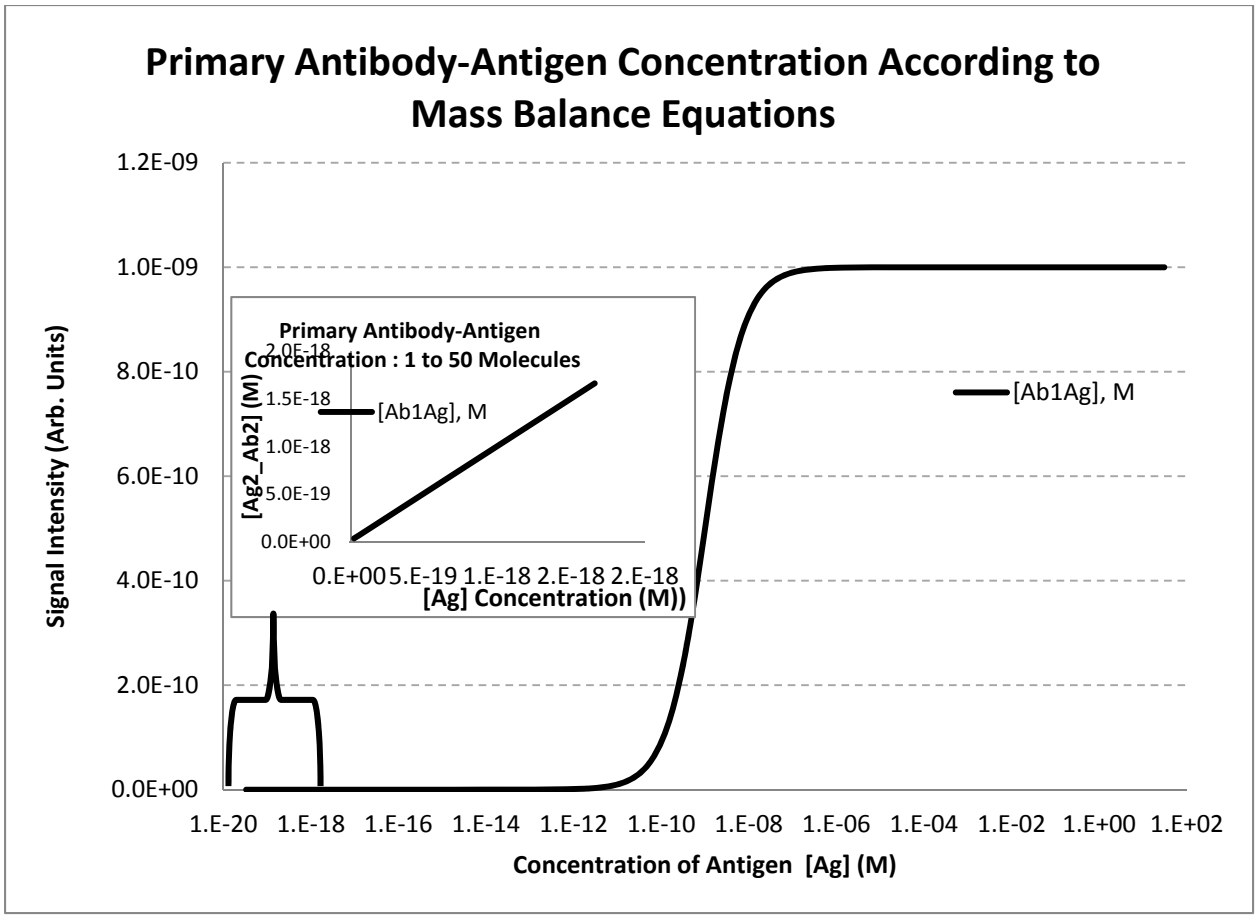



**Figure 2.** Log plot of the showing concentration of antigen (log[*Ag*]) bound ([*Ab1Ag*]) to primary antibody from a sample across approximately seventeen orders of magnitude (equation 4). This response is a typical sigmoidal-shaped response from the laws of mass action, which is commonly interpreted with four and five parameter sigmoidal fitting models (P4, P5) disconnected from core equilibrium relationships. $Ab1_{tot}$ is held at a concentration of 1 nM, $K1_{eq}$ is fixed at $1.0 \times 10^{9}\ M^{-1}$. Inset: Concentration of bound antigen ([*Ab1Ag*]) to primary antibody at low numbers of antigens ([*Ag*]), from single molecule to 600 molecules in 50 microliters (30 zeptomolar to 20 attomolar). Note that across these low concentrations the relationship is linear (inset).

*Second Equilibrium, Completion of the Sandwich Assay*

For the second step of the sandwich assay, where the detection antibody (labeled appropriately) is introduced, the final complex from step one [*Ab1Ag*] becomes the target antigen for step two. Therefore the bound antigen concentration [*Ab1Ag*] is set equal to the antigen concentration [*Ag2*] as the target of the second incubation. Maintaining the assumptions outlined during the first incubation process, and going through the same algebraic strategy (this is a straight forward manipulation, presented in the supplemental information for additional clarity) results in the concentration of the signal generating species [*Ab2Ag2*] (M) to be given by

$$[Ab2Ag2] = \frac{K2_{eq}[Ab2_{tot}]\frac{K1_{eq}[Ab1_{tot}][Ag]}{(1+K1_{eq}[Ag])}}{(1 + K2_{eq}\frac{K1_{eq}[Ab1_{tot}][Ag]}{(1+K1_{eq}[Ag])})} \qquad (5)$$

where $K2_{eq}$ is the equilibrium association constant (antibody affinity, $M^{-1}$) for the detection antibody, and $[Ab2_{tot}]$ is the total concentration of the secondary antibody (M).

*Single Molecule Detection and Fundamental Sources of Noise*

Regardless of the detection modality, issues of signal-to-noise, bias and variance can be generalized for any system that has the appropriate conditions to sense the presence of a signal-generating species consistent with a single molecule. Each system will generate a characteristic bias, variance and sensitivity (amplification). To attain 'single molecule sensitivity' ($I_{SM}$), a signal strength more than to six times the standard deviation ($\sigma$) of the noise ($I_{SM}>6\sigma_{noise}$) above the $I_{bl}$ is considered to be unequivocal evidence of distinct signal above noise, generating a 99.73% probability that it statistically represents a positive result (a single molecule is present) if a normal distribution is assumed.[38] To generalize this assessment and examine the best case scenario, the only noise considered is Johnson-Nyquist; an unavoidable fundamental source of noise for all instrumentation.[39-41] Additional fundamental sources of noise (flicker, shot, etc.) can be added to this by summing bias (as expressed by intensity, $I$, (equal to $\Sigma I_{noise}$)) and variance (variance is the square of the standard deviation ($\sigma^2$), equal to $\Sigma\sigma^2_{noise}$). Other sources of instrumental bias and variance (dark current, environmental noise, etc.) can be similarly summed, the specific values depending on the details of the specific system. The baseline intensity (bias, $I_{bl}$), in the case of Johnson-Nyquist noise, defines the variance, $\sigma_{bl}^2$, by

$$\sigma_{bl}^2 = \sqrt{2qI_{bl}\Delta f} \qquad (6)$$

Where $q$ is the charge of an electron, and $\Delta f$ is the bandwidth (in Hz). Variance also increases with signal intensity, $I_s$, with the same function, but it is added separately: $\sigma_s^2 = \sqrt{2qI_s\Delta f}$.

*Instrumental Background and Noise*

There is a broad range of conditions where the relatively high instrument intensity, $I_{bl}$ (and the resulting variance, $\sigma_{bl}^2$), or low amplification ($\xi$) defines the limit of detection. Either the amplification can be insufficient or the instrumental bias and variance may be too high. Whichever effect is the cause, the result is the same. Under these conditions the limit of detection is set at $I_s > 3\sigma_{bl}$, per standard analytical assessment.

*Non-Specific Binding*

Non-specific binding can influence the LOQ through one of two forms: those arising from the binding of antigen directly to the solid assay surface (and subsequently binding the signaling antibody) and signal antibody binding to the surface independent of the antigen. This source of noise will also have characteristic intensity (bias, $I_{NSB}$) and variance ($\sigma^2_{NSB}$) defined as in equation 6. In addition, for very low numbers of molecular interactions, this form of noise can also add molecular shot noise (a minor, rare situation—not considered further).

The sum of the sources of noise and background ($E_{system}$) in any system gives the relationship

$$E_{system} = I_{bl} + I_{NSB} + \sqrt{\sigma_{bl}^2 + \sigma_{NSB}^2 + \sigma_s^2} \qquad (7)$$

where $I_{bl}$ and $\sigma_{bl}^2$ are empirically defined functions of the instrumentation used, and $I_{NSB}$, $\sigma_{NSB}^2$, and $\sigma_s^2$ are a function of the amplification and binding properties of a system.

*Molecular Shot Noise*

In an ideal case where instrument noise is minimized and NSB is eliminated, the LOQ is set by Poisson noise (molecular shot noise). This limit arises from the fact that biomolecules are discrete entities and their binding is of a quantum nature, which produces an unavoidable source of error in any detection system, a fundamental signal-to-noise boundary for any assessment. This adds to the variance according to:[34]

$$\frac{\sigma_A}{n_A} = \frac{1}{\sqrt{n_A}} \ . \qquad (8)$$

This source of error follows a Poisson statistical distribution for a small number of targets where $\sigma_A$ is the standard deviation (variance is $\sigma_A^2$) of the number of molecular signatures detected ($n_A$). When NSB and extraneous sources of fluorescent signal are eliminated, the molecular signature is only from the actual number of antigen molecules detected. Improvement in the precision of sampling dilute targets requires the generation and averaging of multiple unique samples, since strongly fluorescent signals from non-antigen specific events may significantly impact the background sampling noise.[34] This sampling statistical effect is different from statistical fluctuations in signal (variance), and represents an intrinsic limit on detection capabilities for a liquid-phase immunoassay (Figure 3). This source of error is beyond and separate from the instrumental sources of background (bias) and noise (variance). The sum of the error in any system may be described as:

$$E_{TOT} = E_{MSN} + \frac{E_{system}}{I_{TOT}} \qquad (9)$$

where $E_{MSN}$ is error associated with molecular shot noise and $I_{TOT}$ is the total fluorescence intensity detected. Percent error (or uncertainty), $E_{percent}$, is defined:

$$E_{percent} = E_{TOT} * 100 = \left( \frac{1}{\sqrt{N_A}} + \frac{I_{bl} + \sqrt{\sigma_s^2 + \sigma_{bl}^2}}{\xi[Ag] + I_{bl} + \sqrt{\sigma_s^2 + \sigma_{bl}^2}} \right) * 100 \qquad (10)$$

**Results**

The immunoassay signal response was calculated for assays detecting between 1 and 6.7 x $10^4$ molecules (between 33 zM and 2.2 fM in a 50 µL sample volume). Equilibrium constants were fixed for both $K1_{eq}$ and $K2_{eq}$ at 1.0 x $10^9$ $M^{-1}$ to model levels typical of monoclonal antibodies.[43,44] Calculations included elements for instrument bias (background) and variance (noise), non-specific binding, and molecular shot noise. These calculations were used to examine the LODs and LOQs for the three limiting conditions for immunoassays.

To understand the limits on quantification for an immunoassay, three domains are identified, which allow for direct comparison with the ultimate MSN limits. Depending on which source of noise dominates, each imposes limitations on assay quantitation under differing conditions (Table 1).

**Table 1: Assay Limitations of Quantitation**

| Limiting Factor | Criteria | Assay Limit of Quantitation | Type of Assay |
|---|---|---|---|
| Instrument background noise | $\frac{I_{SM}}{(6\sigma_{bl} + I_{bl})} < 1$ | 11,000 molecules (0.36 fM) | Traditional laboratory assays |
| Non-specific binding noise | $I_{NSB}$ is present | ~150 molecules and up (5 aM) | Traditional laboratory assays |
| Molecular shot noise | $I_{SM} > 6\sigma_{bl} + I_{bl}$ | ~131 molecules (3.7 aM) | Optimized high-sensitivity assay |

**Table 1** shows the limitations of assays based on the type of noise that is responsible for limiting quantitation. When one source of noise is minimized or eliminated quantitation becomes more sensitive until reaching a finite statistical limitation bound by error (uncertainty) due to molecular shot noise. Concentrations are reported for a sample volume of 50 μL.

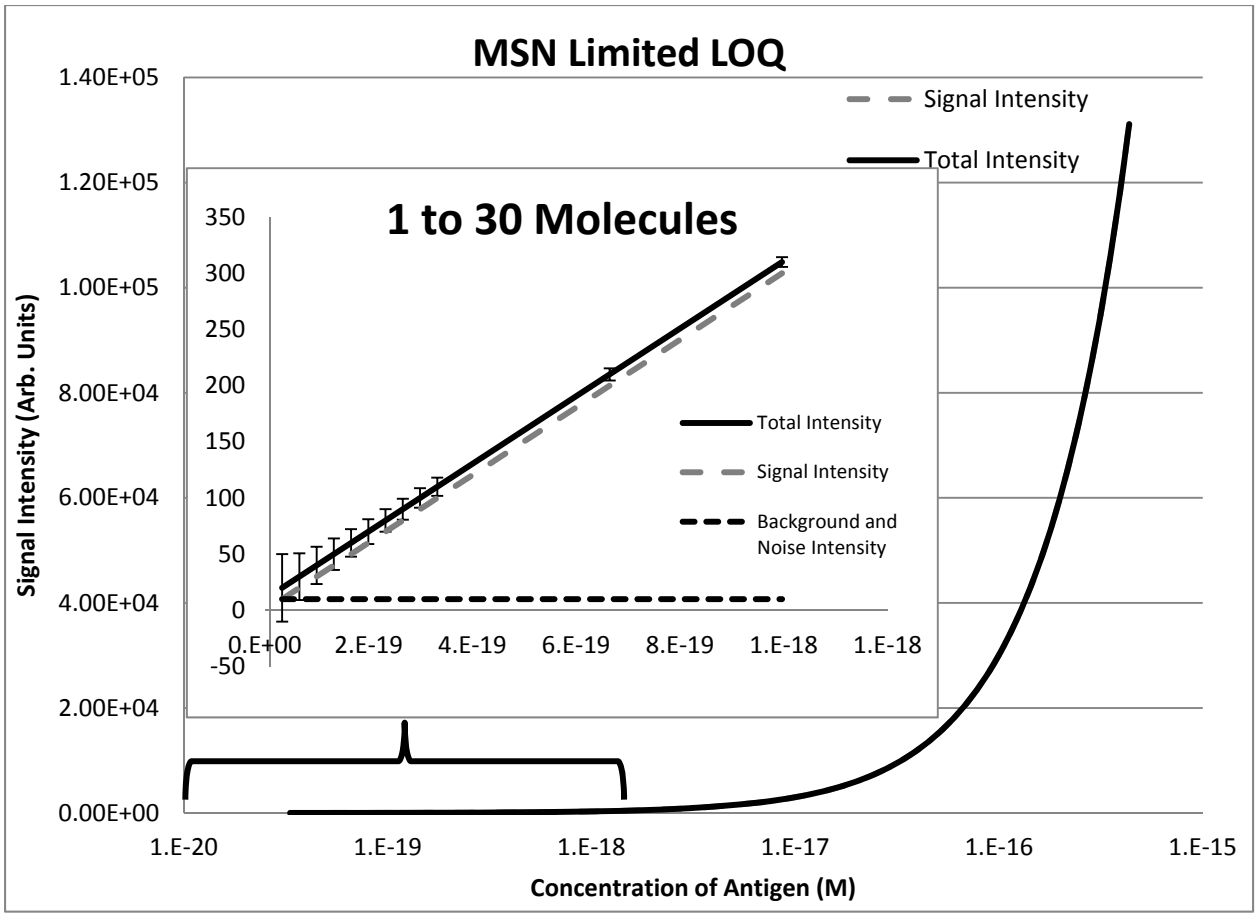
Table 1, along with equation 9 (and equation 7), demonstrates that different factors can dominate the noise observed in assays depending on experimental conditions. If insensitive or noisy detection instrumentation is used, the background from these machines will dictate the minimum amount of sample that can successfully be quantified. If those limitations are overcome with the incorporation of sensitive detection equipment, assays are typically limited to quantitation in the nano- to picomolar range by NSB effects. NSB creates a minimal background signal that exists under an assay format without sample present and limits the minimal quantitation that can take place. While it is observed that overall noise increases as signal size increases, the intensity of the noise compared to the intensity of the signal becomes proportionately smaller, resulting in a smaller coefficient of variation (CV) and a greater ability to quantitate the population of specific analytes present.

Since MSN limitations are observed under ideal assay conditions and represent a best case scenario, the impact of MSN on the LOQ is addressed first. Following this, influences from

NSB and instrumental background, sources of uncertainty that must be minimized or eliminated to observe the effects of MSN, are analyzed to illustrate further limitations on the LOQ that may be present in experimental immunoassays.

When detection systems used are capable of single molecule recognition ($I_{SM} > (6\sigma_{noise} + I_{bl})$) and NSB is eliminated, the effects of molecular shot noise are apparent (Figure 3). Plots were generated to explore conditions where assay quantification is limited only by molecular shot noise (Equation 8). Beyond defining the ultimate LOD and LOQ, operating in the regime required for single molecule sensitivity does not influence the rest of the classic sigmoidal curve, except that it may limit the dynamic range of the overall measurement due to instrumental linearity being exceeded by operating in a high amplification mode. Plotting the percent error normalized to the first data point intensity (error bars = $E_{percent}$ x total intensity (one molecule)) (Figure 3 inset) as error bars on the Total Intensity values gives an indication of the accuracy with which measurement can be made. Along with total intensity, the signal and instrument background and noise are plotted. While the detection limit is set to single molecule limit, the uncertainty (expressed as error) of the measurement still contributes finite error.

Another method to examine the same calculations is to plot contributions to percent error ($E_{percent}$) for the different sources at low molecule counts (Figure 3, bottom). The signal intensity is linear with increasing concentration, whereas the error in the measurement grows dramatically at low molecular counts, dominated by molecular shot noise with some influence from residual instrumental variance. Under these conditions, the LOD is one molecule (30 zM) and LOQ is approximately 131 molecules (3.7 aM). Note to effectively eliminate the instrumental influence, the amplification must be increased two-hundred-fold or instrument bias and variance must decrease by a thousand-fold (supplemental information).


MSN Limited LOQ
Signal Intensity
Total Intensity
1 to 30 Molecules
Total Intensity
Signal Intensity
Background and Noise Intensity
Signal Intensity (Arb. Units)
Concentration of Antigen (M)
1.40E+05
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
1.E-20
1.E-19
1.E-18
1.E-17
1.E-16
1.E-15
350
300
250
200
150
100
50
0
-50
0.E+00
2.E-19
4.E-19
6.E-19
8.E-19
1.E-18
1.E-18

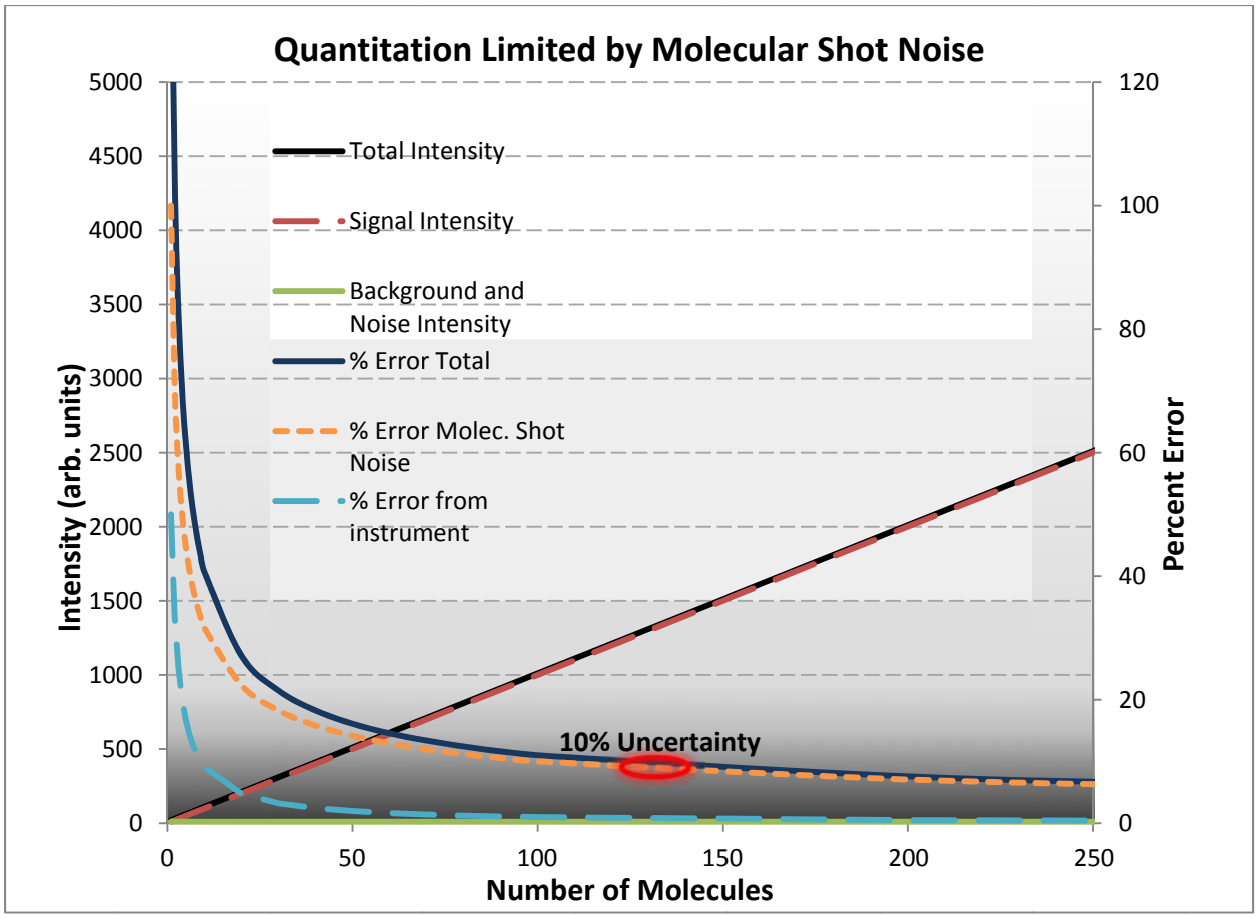



**Figure 3** Plots illustrating error from molecular shot noise and residual instrumentation effects on LOD and LOQ. Total Intensity (Equation 5 + Equation 7), Signal Intensity (Equation 5) and Background and Noise Intensity (Equation 7) were plotted. Variables set at $I_{bl}$=10, $\sigma_{bl}$=2.38x10$^{-4}$, and $\xi$=($I_{bl}$+6$\sigma_{noise}$)=10. Error bars represent percent error. Top: large range of concentration of antigen to emphasize that most of the curve is not influenced by molecular shot noise (signal intensity and total intensity lines overlap). Inset: molecular shot noise generates significant error for small numbers of molecules, error bars are equivalent to the percent error associated with the measurement multiplied by the intensity of a single molecule and reach an acceptable %CV (below 10% error) at ~131 molecules. Note instrumentation bias and variance contributes a minor, almost negligible, error (axes represent the same units as the primary graph). Bottom: Plot of the assay-specific signal intensity (Equation 5) the total intensity of the assay (Equation 5 + Equation 7) and the baseline intensity (Equation 7) (left-axis) versus the assay variance from molecular shot noise (Equation 8 * 100%) the total variance in signal intensity (Equation 10 * 100%) and the variance from the instrument (Equation 10 * 100% with MSN equal to 0; right axis). Darker background indicates increase error in the measurement. Total assay variation reaches a level of 10% error when there are fewer than ~131 molecules (approximately 3.7 attomolar in 50 µL) in a sample.

While typical conditions were used for the investigation of MSN (detection system capable of single molecule detection, equilibrium constants of monoclonal antibodies held at 1.0 x 10$^{9}$ M$^{-1}$

for both $K1_{eq}$ and $K2_{eq}$) to provide a representative analysis of immunoassay capabilities, antibody properties do influence the fundamental LOQ (Table 2). Increasing the equilibrium constant of one or both antibodies can improve the limit of quantitation to approximately 113 molecules. Increases in the equilibrium constant beyond that do not impact this fundamental limit strongly (increasing both $K1_{eq}$ and $K2_{eq}$ to 1.0 x $10^{11}$ $M^{-1}$ only lowered the limit of quantitation to 111 molecules). The effects of antibody properties are greater when antibodies with below average equilibrium constants are used. By using only one antibody with a $K_{eq}$ of 1.0 x $10^{8}$ $M^{-1}$ raised the LOQ to 277 molecules (9.2 aM in 50 µL). Based on the assumptions stated previously by modeling an immunoassay based on the laws of mass action, systems are considered to be at equilibrium.[36] An excess of both capture and detection antibody are used such that it is determined that all antigen in a system is bound and dissociation is not assumed to occur on the time scale of the experiment. If dissociation of the target compounds does occur after either wash step in a typical assay, the limit of quantitation would increase. Under optimal circumstances roughly 131 molecules must be specifically detected to afford quantitation with a CV below an acceptable threshold (<10%).

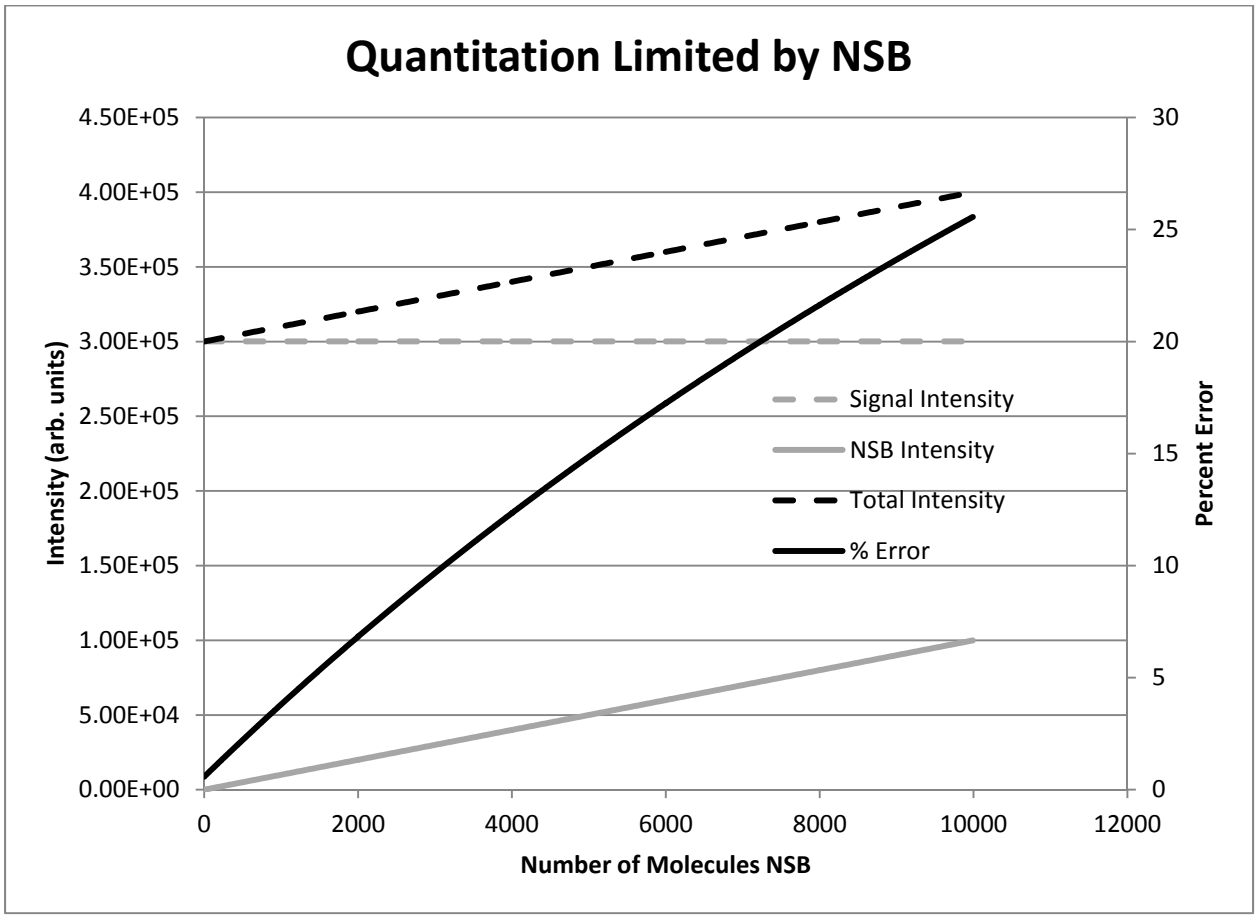
**Table 2: Impact of Antibody Equilibrium Constants on the LOQ**

| $K1_{eq}$ | $K2_{eq}$ | LOQ (number of molecules) |
|---|---|---|
| 1X | 1X | 131 |
| 10X | 1X | 113 |
| 1X | 10X | 113 |
| 10X | 10X | 112 |
| 0.1X | 1X | 277 |
| 1X | 0.1X | 277 |
| 0.1X | 0.1X | 1371 |

**Table 2** shows the influence of antibody equilibrium constants on the limit of quantitation. Here, X is defined as 1.0 x $10^{9}$ $M^{-1}$, representing the typical equilibrium constant of a monoclonal antibody.[43,44] The impact on the LOQ when this value is increased or decreased by a factor of 10 for one or both of the antibodies used in a sandwich immunoassay is demonstrated.

When NSB has not been effectively eliminated from an immunoassay system, it can limit the LOD and LOQ if the bias and variance of the signal from the NSB exceeds the instrumental bias

and variance. To examine this, amplification was placed at single molecule sensitivity ($\xi$=10). NSB can be introduced into a system at any level, and here will be considered at levels between 30 zM and 0.33 fM (1 and 10,000 molecules in 50 μL) being non-specifically adsorbed and detected (Figure 4). With a target concentration held constant at 1 fM, above the range where MSN and instrument background (capable of single molecule detection) influence impact quantitation, NSB was plotted between 30 zM and 0.33 fM. While it is observed that the intensity of the signal produced from NSB is much lower than the intensity of the specific signal, 10% uncertainty is reached with the addition of 83 aM (2500 molecules) non-specifically binding (Figure 4, top). This selection of sample concentration was an arbitrary value emphasizing that any and all NSB will decrease detection limits compared to single molecule detection, both in terms of false positive and increased molecular shot noise error. This influence can be similarly observed by comparing the influence of the number of molecules NSB on the number of specific sample targets required to achieve quantitation (Figure 4, bottom).



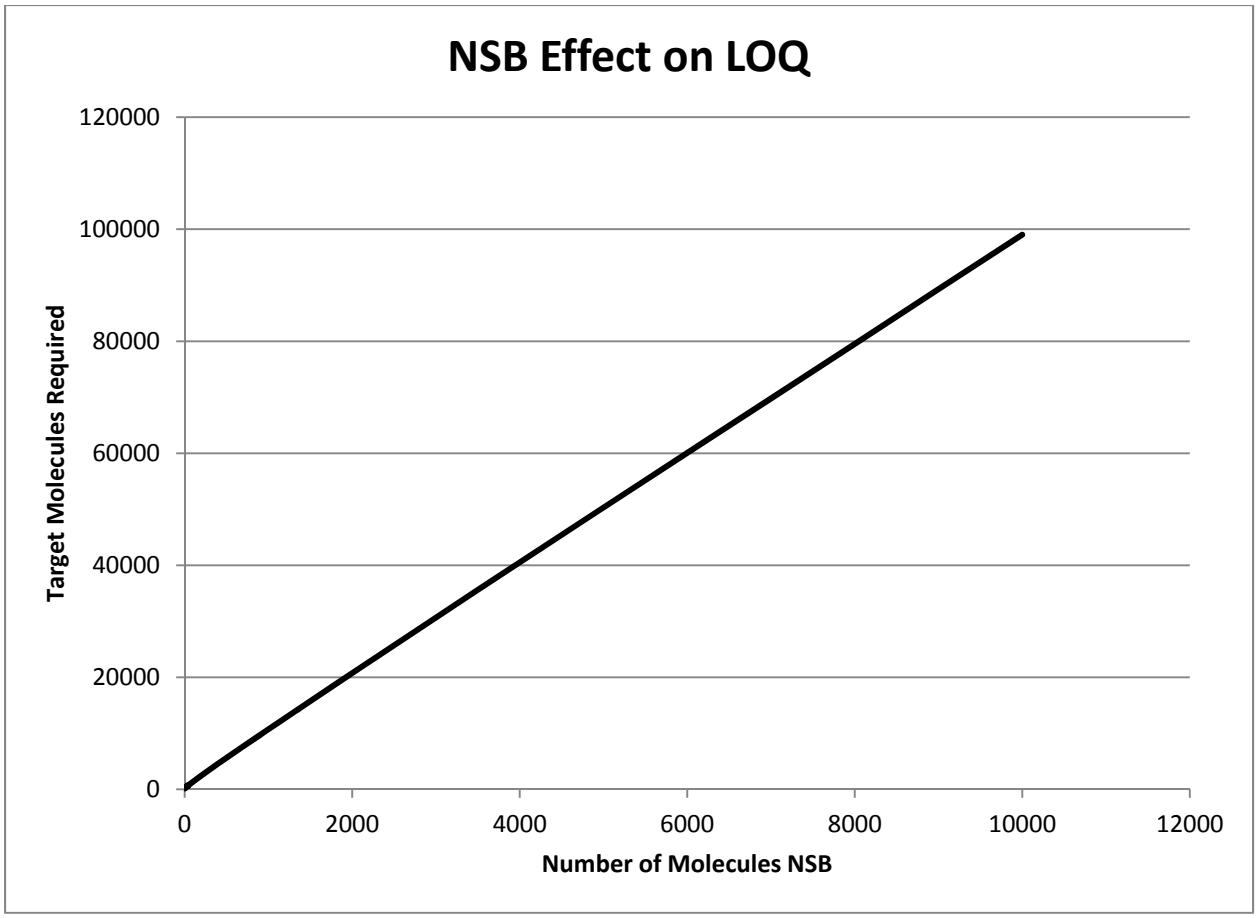



**Figure 4** Plot illustrating LOD and LOQ defined by non-specific binding properties. Variables set at $I_{bl}$=10, $\sigma_{bl}$=2.38x10$^{-4}$, and $\xi$=($I_{bl}$+6$\sigma_{noise}$)=10 and modeled for a specific target concentration of 1 fM. The intensity (bias, $I_{NSB}$) and standard deviation (noise, $\sigma_{NSB}$) were modeled for values between 0 and $10^4$ molecules NSB and included according to equation 7. Top: The Signal Intensity (Equation 5), Total Intensity (Equation 5 + Equation 7), and NSB ($I_{NSB}$) were plotted and compared to Percent Error (Equation 10). Note the signal from NSB influences quantification far beyond the number adsorbed entities, reaching the LOQ at 2500 molecules (83 aM) non-specifically absorbed. Bottom: As the number of molecules non-specifically binding increases, the concentration of target molecules required to reach the LOQ increases.

Historically, the LOD and LOQ were determined by the capabilities of the detection system, generating low amplification of signal or high instrument bias and variance (Figure 5). Reducing the amplification ($\xi$) or increasing the background ($I_{bl}$) of the instrument produced a similar impact on quantitation abilities. This relationship between signal amplification and background is modeled considering Johnson noise only to examine the best case scenario as described previously where $I_{SM} > 6\sigma_{noise}$ above $I_{bl}$ and noise is modeled according to equation 6. The

transition towards instrumentation capabilities being the limiting factor occurs when $\xi/(\frac{I_{bl}}{6}+\sigma_{bl})$ = $\xi/(\frac{I_{bl}}{6}+\sqrt[4]{2qI_{bl}\Delta f})=6$. When the ratio is less than six, the signal from a signal molecule is no longer distinguishable above noise and the system is limited by instrumental considerations. For this data, the ratio was set at 1/1000th of the transition value ($I_{bl}$=10, $\sigma_{bl}$=2.38x10$^{-4}$, $\xi$=($I_{bl}$+6$\sigma_{noise}$)/1000=0.01). Any value less than six can illustrate this point, but this value minimum that gave clear and instructive graphical information over a range of antigen concentrations. For these conditions, the LOD was ~1100 molecules (37 aM) and LOQ was 1.1x10$^4$ molecules (0.36 fM).

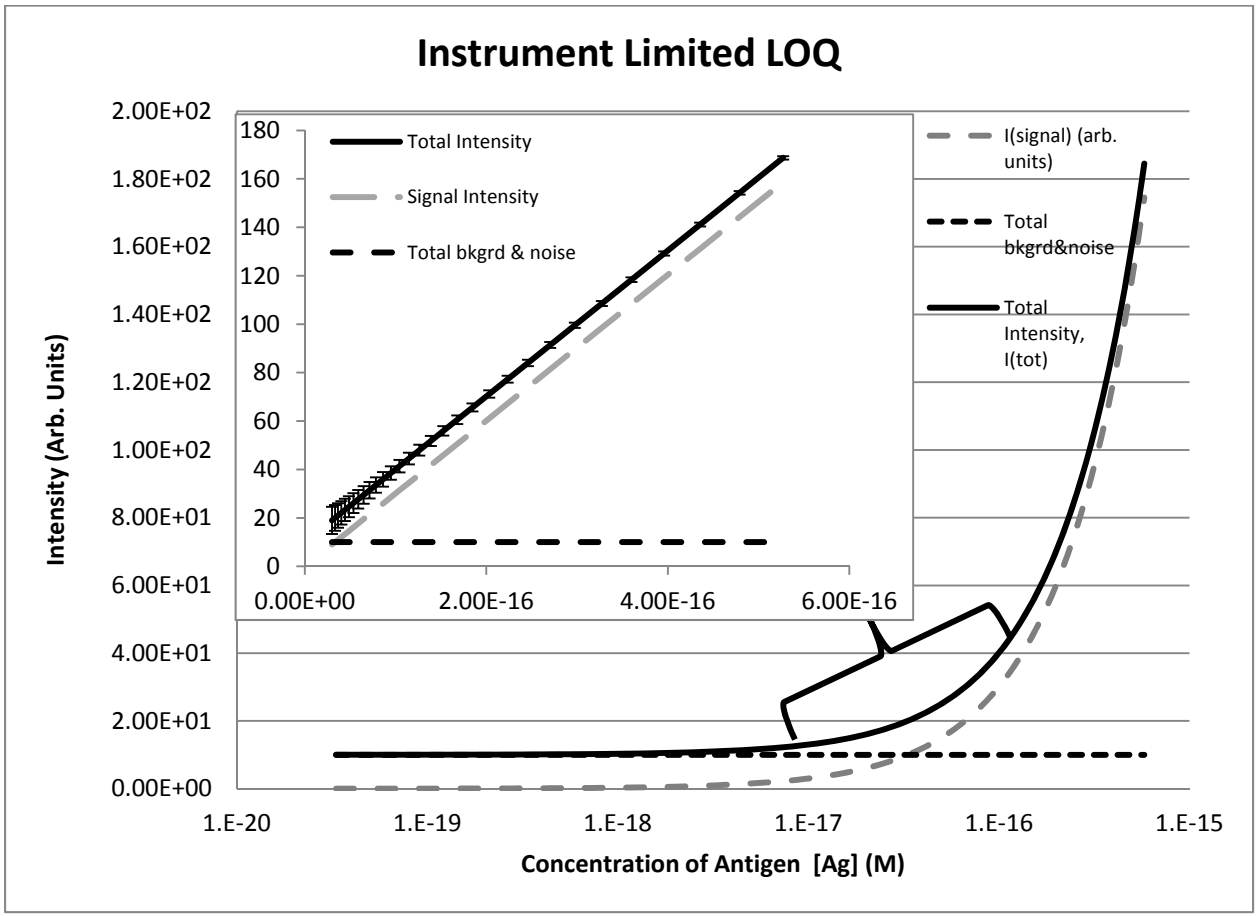



**Figure 5** Plot illustrating limited amplification or increased background bias and variance resulting in LOD and LOQ to be defined by the characteristics of instrumentation. Total Intensity (Equation 5 + Equation 7), Total Background and Noise (Equation 7), and Signal Intensity (Equation 5) were plotted versus antigen concentration [*Ag*] (M). Plot shows signal intensity versus antigen concentration according to immunoassay system of equations. Variables set at $I_{bl}$=10, $\sigma_{bl}$=2.38x10$^{-4}$, and $\xi$=($I_{bl}$+6$\sigma_{noise}$)/1000=0.01.

**Discussion**

It is commonly accepted that the sandwich-type immunoassay allows for the most sensitive detection among immunoassay formats.[30] With optimal instrumental detection capabilities, the theoretical quantification limit of the sandwich assay depends on the reaction binding constant, the percent of the reaction volume required for measurement, and the precision associated with the measurement made.[16] Precision in these measurements and the statistical limitations of certainty are important points to consider in the distinction between the LOD and LOQ. The focus on the LOQ is to ensure that the relative error in a sample measurement remains less than a logical pre-determined fraction of the total signal (<10%).[22,23,25,44]

Practically, the LOQ is affected by both constant and variable sources of noise which must be accounted for in determining finite immunoassay capabilities. The constant sources of noise, arising from detection elements, signal processing, molecular shot noise, thermal noise and Johnson noise will be present throughout any measurement at a defined intensity for a particular system. Variable noise arises predominantly from NSB, which can occur at each step in immunocomplex formation with differing effects. Although binding of the non-target species is less probable than the specific binding of an analyte, in the event that the target compound is present at a low concentration, or in a biological sample, non-specific binding will be a significant contributor to the overall signal.[25]

Plots based on the law of mass action were used here to illustrate three sets of conditions dictating the LOQ for immunoassays: a) molecular shot noise, b) non-specific binding, or c) the baseline signal intensity arising from measurement instrumentation. While the focus of this work is the determination of the LOQ based on the sources of uncertainty present in a system, and variation in signal response increases with increasing analyte concentrations,[44] above the LOQ the signal-to-noise ratio (SNR) increases and the noise factor ($\frac{total\ output\ noise\ power}{output\ noise\ power\ due\ to\ input\ source}$) decreases.[25] These findings demonstrate that while there may be more total variation in signal at higher analyte concentrations, this variation represents a smaller percentage of the overall signal than fluctuations produced at low sample concentrations. The mass action law was used in this case to gain insight into fundamental limitations of immunoassay systems. While P4 and P5 fitting models are useful in that they provide reasonable estimates of analyte concentration by back-fitting collected data without incorporating too many

parameters, their estimates of error are less reliable than using the law of mass action and they do not allow the ultimate LOQ to be determined.[1,35]

More recent models of detection statistics have relied on models built from Bayes' theorem to define the LOD as both the probability that it exceeds a signal for a zero dose and its probability density.[1,45] While the accuracy of determining the LOQ is greatest using a dose-response curve based on the law of mass action, and this analysis allows the determination of fundamental limitations that may not be surpassed, models built from Bayes' theorem provide more rigid requirements for the interpretation of data as a part of routine laboratory analysis compared with traditional P4 or P5 fittings.[1] These models not only use back-fitting, but also right-skewed probability densities to determine the extent of error both in blank calibration samples and those with target analyte present.

When the signal from instrument background is minimized and non-specific binding is effectively eliminated, molecular shot noise is responsible for establishing the fundamental statistical LOQ for immunoassay. In this case uncertainty associated with sample heterogeneity at low analyte concentrations dictates the ultimate limit of quantification (Figure 3, Equation 10). The assay plots show that regardless of sample volume, in order to establish certainty in the quantification of an analyte, there must be a minimum of ~131 molecules specifically detected. Because the error associated with molecular shot noise, coupled with a minimal variance from the instrument and baseline, below this number of molecules corresponds to a coefficient of variation greater than 10% the sample may only be potentially detected, not quantified. Establishing a fundamental limitation to the LOQ is important both in the design of immunoassay platforms and the evaluation of existing techniques. As detection instrumentation has improved to the point where sensing a single molecule is possible, many claims of single-molecule immunoassays have been made.[26-28] However, despite using instrumentation capable of sensing individual signals, the actual limits of quantitation are much higher, falling in the aM range and reaching a minimum of 800 molecules quantified.[28] While claims of single molecule detection have not yet successfully approached the fundamental LOQ imposed by MSN, it is necessary to quantitatively establish this absolute limit and assess the impact of additional noise sources that may be present in an experimental immunoassay such that new assays may be appropriately designed to meet the quantitation requirements of specific target species.

Beyond the limitations imposed by MSN, quantification can be impacted by uncertainty bias and variance introduced through non-specific binding. Non-specific binding must always be considered when discussing limit of quantitation for immunoassay. While MSN imposes a fundamental LOQ for immunoassays that cannot be improved, NSB is likely to occur to some extent in experimental immunoassay systems. It is particularly relevant in assays used for medical diagnostics, where desired targets are of relatively low concentration and present in a highly complicated sample. When present, NSB will impose a further limit on the minimal concentration quantified by a given system. This can be observed in that when even one molecule binds non-specifically the LOQ increases from 4.3 to 5.0 aM (Figure 4, bottom).

When background noise from the instrument is higher than the signal intensity arising from molecular shot noise or non-specific binding, the limit of quantification is dependent only upon what can be effectively distinguished from this baseline signal. When the LOQ is dictated by the instrumentation, the effects of MSN are of minimal importance due to the higher numbers of target analyte required for a sample to be recognized above the background noise. For a given instrumental system this background noise is a constant source and cannot be altered by adjusting experimental parameters. However, with the use of a highly sensitive detection instrument capable of sensing the presence of a single molecule, background noise can be sufficiently lowered leading to quantitation bound by the limits of the assay itself.

## Conclusions

Due to the high specificity of the interaction between antibodies and antigens, immunoassays provide a valuable tool when sensitive detection is required. While practical limitations, like non-specific binding or baseline instrumental noise, may result in a higher LOQ for experiments, due to the impact of molecular shot noise there cannot be *quantitation* with acceptable certainty if samples contain fewer than approximately 131 detected antigen target molecules. Therefore, true quantification limits lie not at the single molecule level, but in the atto- to femtomolar, or poorer, range.

The models employed here to establish the fundamental qualitative ability for noncompetitive sandwich immunoassays can be employed to assess the ability of an experimental design to quantify targets under the parameters that will be used. This provides a

valuable tool for predicting the utility of an assay for its intended application, as well as an aid in the assay design process.

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