Developing an Ultrasensitive PK Assay Using SIMOA: Step-by-Step to the Target

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INTRODUCTION

Single Molecule Array (SIMOA) is a powerful ultrasensitive immunoassay technology that enables the quantification of analytes at very low concentrations. In addition to biomarker research, there is an increasing need for ultrasensitive pharmacokinetic (PK) assays. These homebrew PK assays do not rely on ready-made kits and can require extensive optimization during method development.

Here we describe a step by step-approach for developing an ultrasensitive homebrew SIMOA PK assay with the semi-automated SR-X detection system. We also provide our recommendations for assay optimization.

SIMOA 3-STEP ASSAY PRINCIPLE

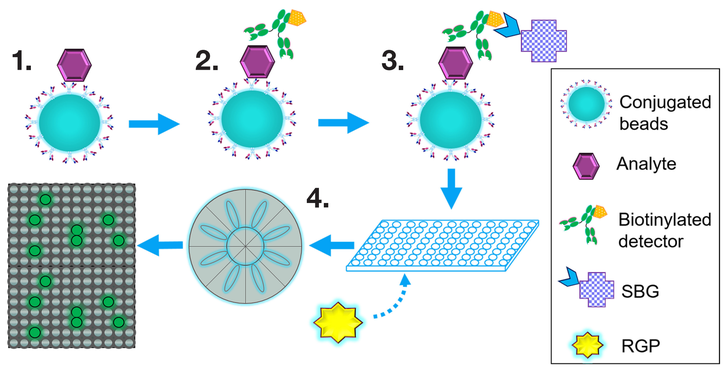


Figure 1. Assay principle. Schematic illustration of assay steps.

1: Paramagnetic beads conjugated with capture reagent are incubated with the sample and washed to remove unbound molecules. **2:** Biotinylated detector is added, followed by a second wash step. **3:** Streptavidin Beta Galactosidase enzyme (SBG) is added, followed by a third wash. **4:** Inside the SR-X analyzer, Resorufin-D-galactopyranoside (RGP) substrate is added and the immunocomplex-containing single beads are isolated into micro-array wells on SIMOA discs. Fluorescent signal is detected in wells containing a labeled bead. The assay can be also shortened to a 2-step format (steps 1 and 2 combined).

ASSAY DEVELOPMENT STRATEGY

1 Capture and detection reagent pairs	• Screen at least 3 different reagents • Change orientation
2 Critical reagent preparation and titration	• Optimize SIMOA bead coupling conditions • Optimize biotinylation conditions • Titration of detector reagent
3 Assay diluent and MRD evaluation	• Include matrix (several individuals) early in method development • Perform selectivity experiments • Screen different diluents
4 Additional assay parameters	• Compare 3-step vs. 2-step • Optimize incubation times • Test helper beads • Adjust sample volume

Figure 2. Assay development strategy. List of different parameters to be evaluated during method development.

When starting assay development, our default strategy is to start with the 3-step approach, as illustrated in Figure 1. The different optimization parameters are listed in Figure 2.

1. Capture and detection reagent pairs

Capture and detection reagent pairs: Recommendations

- Ensure reagent availability throughout the study (preferably using the same lot)
- Evaluate at least 3 pairs ⟶ up to 9 pair combinations
- Check different orientations
- Aim to have S/N-ratio of ≥ 3 at LLOQ
- Ab purification method can have an impact on assay performance

Choosing the best reagent pair (mAbs, pAbs, FAbs and/or target molecules) with the optimal orientation plays a critical role to reach the desired sensitivity, typically in pg/mL range. Ideally, at least three pairs should be evaluated and signal-to-noise (S/N)-ratio of at least 3 at the LLOQ should be targeted. To minimize variability, it is advisable to use one reagent lot throughout the study. Importantly, when using Abs, their purification method (e.g. Protein G and/or affinity purified) can impact assay performance. Suitability of the selected pair should be confirmed in a selectivity experiment to evaluate potential matrix effects.

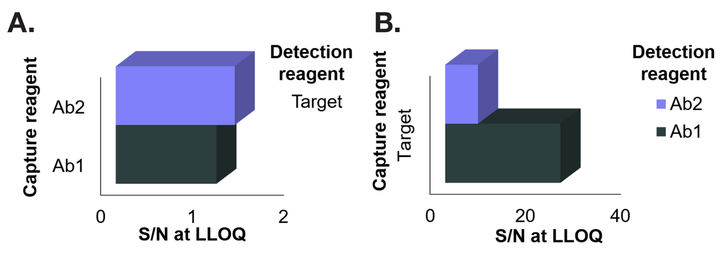


Figure 3. Finding an optimal reagent pair. A. Drug target as a detection reagent. B. Drug target as a capture reagent.

No sufficient S/N-ratio was obtained when a drug target molecule was used as a detector in the example shown in Figure 3A. However, by changing the orientation using target as a capture reagent, S/N-ratios were significantly improved (Figure 3B).

2. Critical reagent preparation and titration

Bead conjugation: Recommendations

- Compare EDC vs. EDC/Sulfo-NHS protocols
- Adjust capture reagent conc.
- Adjust EDC conc.
- Adjust buffer pH
- Reaction volume / bead batch max. 500 µL ⟶ consider pooling

Detection reagent biotinylation and titration: Recommendations

- Test different biotin types (consider spacer length)
- Test different molar excesses (typically 10–60)
- Titrate the detector (0.1–0.4 µg/mL)
- Detector has a high impact on assay sensitivity!

After selecting the optimal reagent pairs, preparation of these critical reagents should be optimized. To improve SIMOA bead conjugation efficiency, and potentially assay sensitivity, different bead activation procedures with (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) or EDC combined with Sulfo-NHS, changing the EDC and capture reagent concentrations and/or testing different pH of the conjugation buffer could be tested. The effect on assay sensitivity is highly dependent on the capture reagent. Based on our experience, changing bead conjugation parameters have minimal impact on assay sensitivity. The reaction volume per bead batch is typically kept at max. 500 µL. For bigger studies, each batch can be individually tested and pooled at the end.

A.

Biotin type	Detector (µg/mL)	ME	S/N at LLOQ
Sulfo-NHS	0.1	20	7.0
		40	5.5
		60	4.6

B.

Biotin type	Spacer arm length	Detector (µg/mL)	S/N at LLOQ
Sulfo-NHS	Medium (22.4 Å)	0.1	7.0
PEG4-NHS	Long (29.0 Å)	0.1	14.1

Table 1. Detector biotinylation. A. Impact of molar excess (ME) on sensitivity. B. Impact of biotin type on sensitivity

For detector biotinylation, different MEs and biotin types can be tested. For the initial tests, it is also advised to use a fixed concentration of the detector. In the first experimental example, a lower ME resulted in higher S/N-ratios at the desired LLOQ (Table 1A). In the second example, highest S/N-ratios were obtained using biotin with long spacer arm (PEG4-NHS biotin), suggesting that minimizing steric hindrance was beneficial (Table 1B).

We also recommend to optimize the final detector concentration. In our example, higher biotin concentration resulted in higher S/N-ratios and improved assay sensitivity (Figure 4).

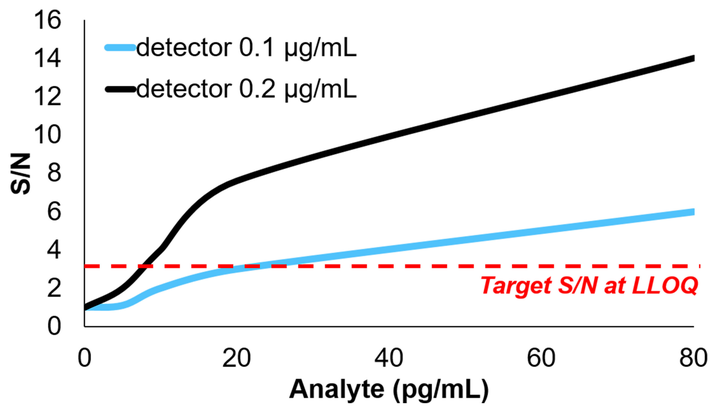


Figure 4. Detector titration. Comparison of two concentrations of biotinylated detector.

3. Assay diluent and MRD evaluation

Assay diluent: Recommendations

- Test at least 3 diluents
- Add detergents (0.05–1%)
- Add blockers (5–20%)

MRD evaluation: Recommendations

- Check buffer vs. matrix
- Assess selectivity
- Aim to have S/N-ratio of ≥ 3 at LLOQ

Choosing an optimal sample and detector diluent can improve assay performance. To optimize assay performance, addition of blocking agents or detergents (e.g. Tween 20) to the diluents could be evaluated.

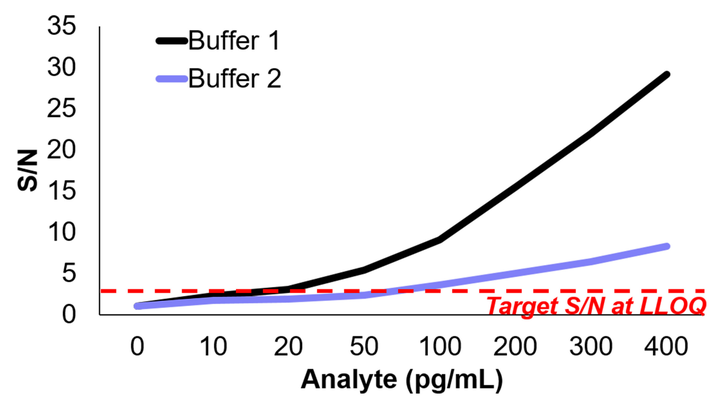


Figure 5. Impact of diluent on assay sensitivity. Comparison of two different diluent buffers.

In an optimization experiment shown in Figure 5, two buffers were compared. Improved S/N-ratios even at low analyte concentrations, were observed using Buffer 1.

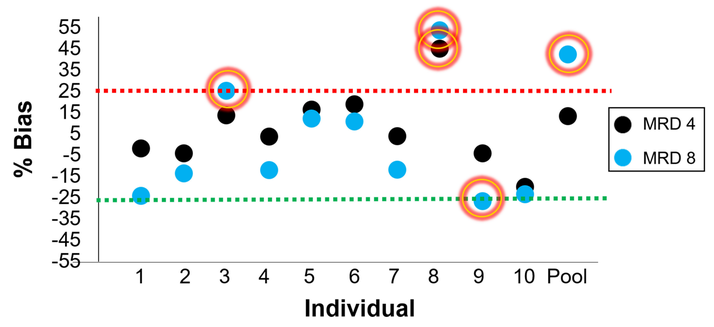


Figure 6. MRD optimization. Evaluation of % bias of different individuals using 2 different MRDs.

Since immunoassays are prone to matrix interference, we highly recommend to test different MRDs using matrix samples (including different individuals) very early in method development. Selectivity experiment should be included to confirm the target LLOQ can be reached. As depicted in Figure 6, 10 individual matrices and a matrix pool were spiked at LLOQ and analyzed using two different MRDs. In this example, the majority of individuals (9/10) passed acceptance criteria with MRD 4 (≤ 25% bias at LLOQ), while only 7/10 individuals passed with MRD 8, suggesting that MRD4 was the optimal MRD allowing to reach the targeted LLOQ.

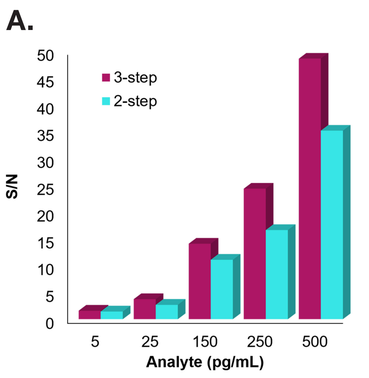
4. Additional assay parameters

Assay protocol: Recommendations

- Compare 2-step and 3-step assay formats
- Optimize incubation times

Additional parameters: Recommendations

- Test different assay bead and helper bead-ratios
- Adjust sample volume



Incubation time (min)			
Assay type	Sample	Biotin	SBG
Short	30	10	10
Long	60	60	30

Figure 7. Assay protocol optimization. A. Comparison of assay formats. B. Comparison of incubation times.

Additional SIMOA assay parameters, such as different formats and incubation times can be evaluated during method optimization. 3-Step assay format and longer incubation times often result in improved assay sensitivity (Figure 7A and 7B). In our experience, helper bead utilization (to maximize analyte-to-bead ratio) or increase in sample volume have less effect on assay sensitivity.

TROUBLESHOOTING

Problem	Possible solution
Changes in assay background / signal	• Test buffers to exclude contamination, consider buffer aliquoting • Control temperature of the shaker • Ensure each sample has a unique ID in the plate layout of the SR-X software
Bead fill issues	• Test detergents in assay diluent • Washer maintenance
High %CVs, bead aggregation	• Ensure appropriate bead mixing • Consider preparing a new bead batch if the beads tend to aggregate over time

Table 2. Troubleshooting. Typical problems and possible solutions

Typical problems encountered during SIMOA assay development include, among others, background and bead fill issues, bead aggregation and variation in assay signal. Possible solutions are listed in Table 2.

DISCUSSION & CONCLUSIONS

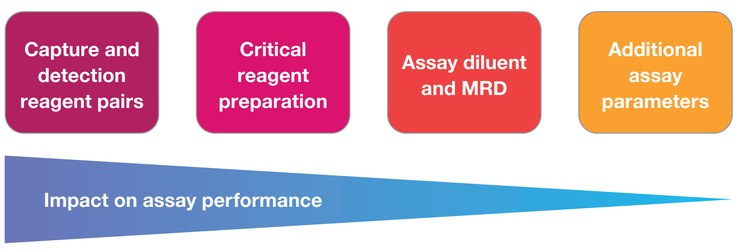


Figure 8. Impact assessment. The effect of optimization steps on assay performance.

The successful establishment of a robust and ultrasensitive SIMOA PK assay is a sum of various parameters: optimized capture and detection reagent pairs being among all the most critical ones (Figure 8). Since the assay is often prone to matrix effects, tests with several individual matrices and different diluents during early method development is very important. In addition, other optimization steps can be tested to reach the targeted sensitivity and get an optimal in-house developed SIMOA PK assay.

REFERENCES

- ICH guideline M10 on bioanalytical method validation and study sample analysis, Step 5, 25 July 2022.
- Simoa Bead-Based Homebrew Assay Development Guide, 06 Jan 2022, Quanterix.