SpearBio

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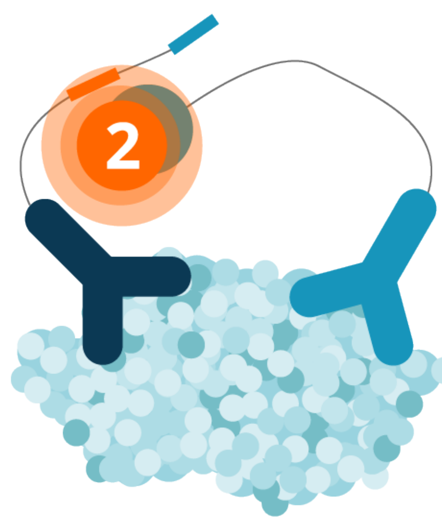
TECHNICAL NOTE

Successive Proximity Extension Amplification Reaction (SPEAR) and its two-factor authentication mechanism

Introduction

Protein detection has historically been less sensitive compared to nucleic acid detection, primarily due to the lack of intrinsic signal amplification in conventional immunoassays. The first introduction of proximity-based immuno-PCR suffered from high non-specific background from amplification of transient interactions of DNA oligos. Advancements in sensitivity for immunoassays and immuno-PCR (for example: ECLIA, digital ELISA and NULISA) followed but included tradeoffs such as reliance on high-affinity antibodies, complex specialized instrumentation, and imperfect antibody immobilization.

Successive Proximity Extension Amplification Reaction (SPEAR) is a novel, patented assay technology overcoming the sensitivity limitation of conventional proximity-based methods utilizing nucleic acid amplification with a unique two factor authentication. The result is the first ultra-sensitive assay for protein detection that fully realizes the specificity and simplicity advantages of a wash-free homogeneous format.



SPEAR Two-Factor Authentication Mechanism

SPEAR utilizes two target-specific antibodies, each conjugated to a unique DNA device. One DNA device is primarily double-stranded, containing two toeholds, while the other device is single-stranded, containing a sequence domain complementary to the toehold of the double stranded device. When both antibodies bind the target, their proximity enables hybridization of this toehold region. DNA polymerase-driven extension then adds a sequence complementary to the second toehold. As the binding to the target maintains the colocalization of the probes for a sustained duration, it triggers a second toehold hybridization and polymerase extension to create a complete, amplifiable DNA sequence (Figure 1A-F). Therefore, qPCR signal will only be generated if the antibody pair remains in proximity to each other for an extended duration to complete the second step extension.

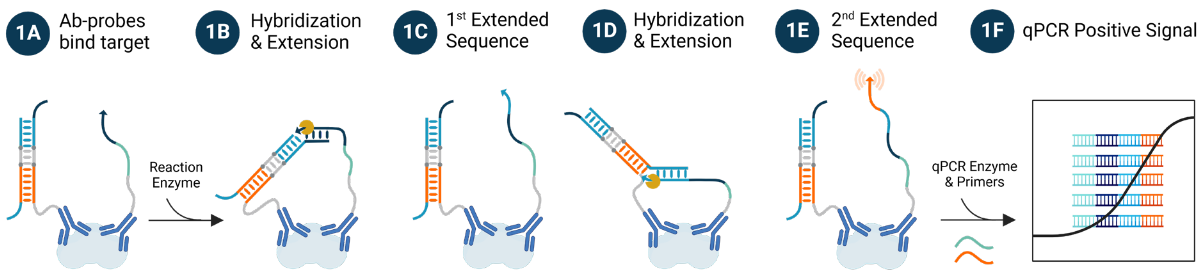


Figure 1. A) Ab-probes bind target, **B)** hybridization and first sequence extension, **C)** displacement and probe dissociation, **D)** hybridization and second sequence extension, **E)** displacement and probe dissociation, and **F)** amplification of double extended sequences in the presence of qPCR enzyme and primers.

This two-step authentication mechanism dramatically reduces the background signal generated from random associations of probes in solution and ensures specificity to the target (Figure 2A-E). The final product uses standard qPCR instrumentation with data analysis using **SPEARview,** proprietary software converting qPCR results into quantifiable results.

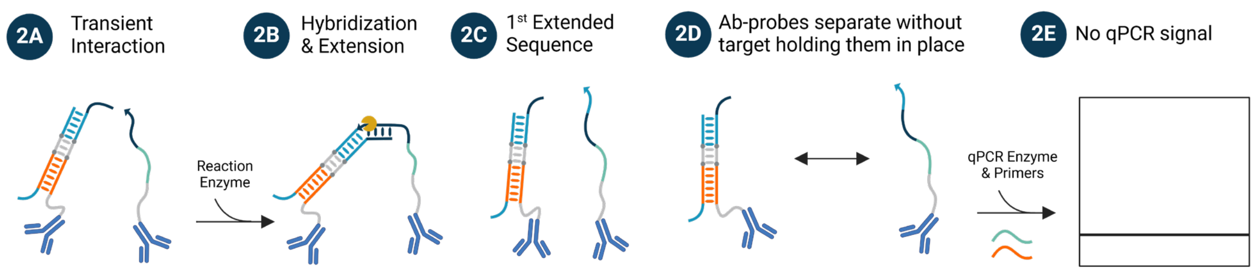


Figure 2. A) Ab-probes bind target, **B)** hybridization and first sequence extension, **C)** displacement and probe dissociation, **D)** hybridization and second sequence extension, **E)** displacement and probe dissociation, and **F)** amplification of double extended sequences in the presence of qPCR enzyme and primers.

Data from any qPCR model is imported into SPEARview analysis software with an algorithm that identifies and matches the Ct results to a customizable plate map set by the user. DNA copy number is calculated for the calibration curve and samples, with 4PL or 5PL curve fit interpolating sample concentrations.

Theory and Proof behind SPEAR's ultra-sensitive detection

Assuming the same probe concentration, antibody affinity, reaction time and reaction rate constant, the sum of target-specific signal (Pw*) and background in solution (Pu*) were simulated for a single and double extension assay (Figure 3A-B). While single extension, "1-step", is unable to discern 100 fM target concentration from 0 fM, "2-step" is capable to differentiate down to 0.01 fM, four orders of magnitude improvement.

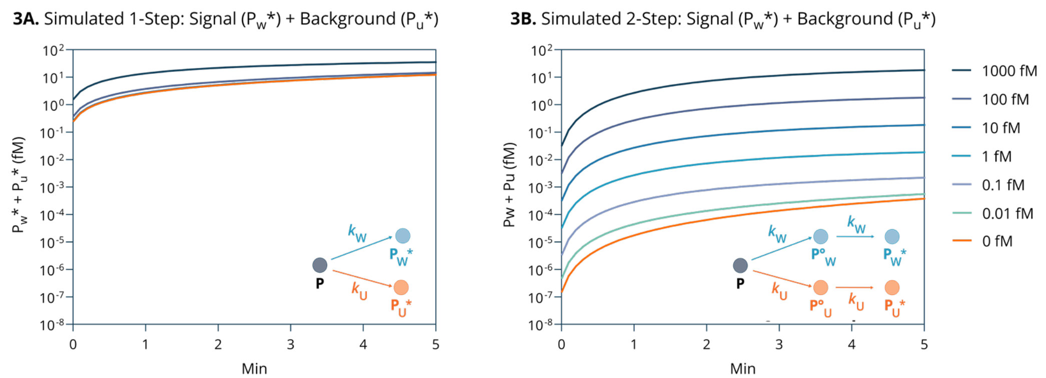


Figure 3. Simulated dose response for the sum of target-specific signal (Pw*) and background (Pu*) in solution for **A)** 1-step proximity extension, and **B)** 2-step proximity extension. Same Ab-probe concentration, reaction time and rate were assumed for simulation.

In conventional proximity binding assays, the nature of transient interactions between probes in solution also limits the capability to optimize the assay with higher antibody/probe concentrations. Experimentally the theory of double extension applied at various Ab-probe concentrations was tested using proof of concept assays designed for 1 and 2 extensions. The data at the lowest (50 pM) and highest (1000 pM) Ab-probe concentrations tested (Figure 4A-C) demonstrated proof that double extension significantly reduces background signal while maintaining target-specific signal, resulting in orders of magnitude increase in signal to background ratio over single extension. The use of double extension in a proximity binding assay not only improves sensitivity at low Ab-probe concentrations but also allows for Ab-probe concentrations to be increased for better binding yield, ensuring the best performance on sensitivity.

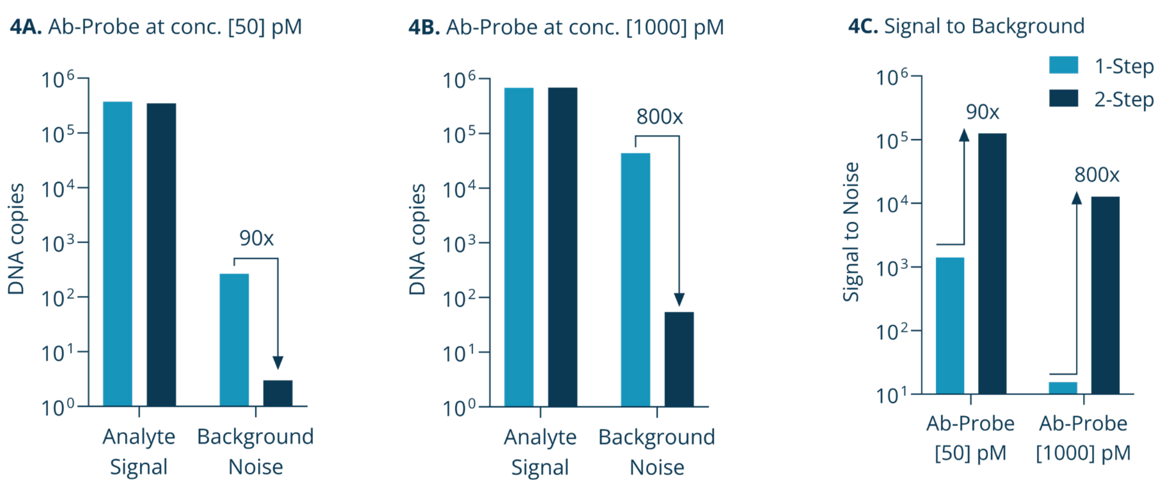


Figure 4. 1-step (light blue) and 2-step (dark blue) results for **A)** Analyte and background DNA copies for Ab-probe concentration [50] pM, **B)** Ab-probe concentration [1000] pM, and **C)** respective signal noise at each Ab-probe concentration.

Exploring the sensitivity of SPEAR experimentally, a proof-of-concept assay was developed using biotin labeled SPEAR probes bound to streptavidin. The results demonstrated SPEAR technology capable of detecting as few as six copies of streptavidin from a one microliter sample (Figure 5).

Unique advantages of adopting SPEAR Technology for biomarker research

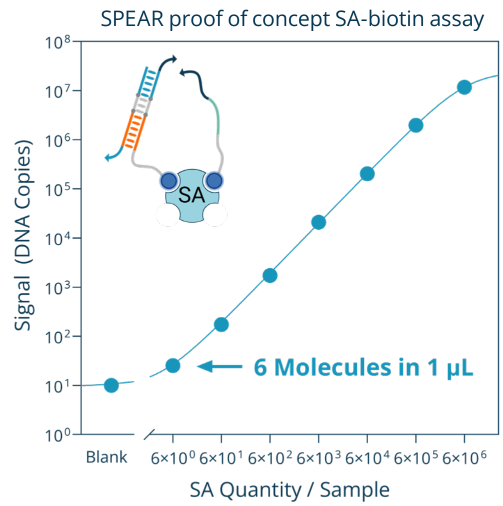


Figure 5. Dose response curve for streptavidin (SA) assay with biotin-labeled SPEAR probes. Lowest level of detectable SA above the background demonstrated at 6 molecules of SA in 1 μL of sample.

- Attomolar Sensitivity: achieving protein quantification at the attomolar level from 1 μL of sample, equivalent to dozens of protein molecules in a single sample.
- High Specificity: homogeneous format without antibody immobilization to a solid surface enables free-binding interactions between antibody epitope and target, and eliminates non-specific binding associated with imperfect surface chemistry.
- Enhanced Precision: wash-free and separation-free assay preserves the integrity of the immunocomplex, minimizing variability associated with workflow.
- Low Sample Volume Requirement: 1 μL of diluted sample is used in the assay with 10 μL or less sample volume required for liquid handling automation.
- Scalability and Automation: hands-on time under 20 minutes with Formulatrix® F.A.S.T™ and accessibility with standard qPCR readout enabling high throughput and adoptability in most labs.

Clinical and Research Applications

Combining the simplicity and scalability of nucleic acid testing workflow with the unique two-factor authentication mechanism, SPEAR pushes the boundaries of sensitivity and simplicity for low abundant protein biomarker measurement and makes it a high potential tool for clinical and research applications.

SPEAR has demonstrated its utility in multiple applications, particularly in neurodegenerative disease biomarker detection. For example, the SPEAR UltraDetect™ pTau 217 plasma assay has shown superior sensitivity and specificity in detecting amyloid pathology related to Alzheimer's disease, eliminating the need for costly PET scans. Additionally, SPEAR UltraDetect™ Neurofilament light chain (NfL) enables precise monitoring of neurodegeneration in diseases such as ALS and MS.

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