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Experimental evaluation of western blotting for serum cytokine analysis

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Abstract

Interleukin-9, or IL-9, plays a role in allergic inflammation, autoimmunity, and tumor immunity. Despite its biological relevance, measuring IL-9 in human serum is hard. The levels are usually low, and most standard protein detection techniques are not sensitive enough to detect it. This study aimed to determine the detection threshold of IL-9 using chemiluminescent Western blotting and evaluate its applicability for measuring endogenous IL-9 levels in serum samples from healthy individuals. We performed serial dilutions of recombinant IL-9 from 20 ng to 0.01 ng and analyzed them alongside serum samples (S15, S10, S5) from healthy volunteers by Western blotting. Signal intensity for each was assessed by subtracting the background signal to determine the detection limits. Membrane stripping, three-layer antibody amplification, immunoprecipitation, and prolonged exposure were trialed to enhance sensitivity as part of an optimization approach. IL-9 was effectively detected at concentrations ≥5 ng, with signal intensity dropping nattily below this threshold. Serum samples showed a negligible signal, implying that endogenous IL-9 concentrations in healthy individuals fell below the assay's detection limit. Also, signal enhancement strategies failed to improve detection at lower concentrations. Western blotting is suitable for confirming the presence of IL-9 at nanogram levels but is insufficient for detecting physiological concentrations in serum. These findings underscore the need for more sensitive platforms, such as ELISA or digital immunoassays, to accurately quantify low-abundance cytokines, such as IL-9, in clinical samples.

Keywords: Interleukin-9 (IL-9); Western Blotting; Detection Threshold; Serum Cytokines and Protein Quantification

1. Introduction

There is a growing interest in the use of biological fluids, such as blood and blood products, urine, and other easily accessible byproducts, for biomarker research. Cytokines are small, soluble proteins that play critical roles in immune regulation, inflammation, and cell signalling [1]. Their precise quantification in biological fluids such as serum is essential for understanding disease mechanisms, monitoring therapeutic responses, and developing diagnostic biomarkers [2]. However, due to their low abundance and dynamic expression, cytokine detection requires highly sensitive and specific analytical techniques [3,4]. Also, high-abundance proteins (HAPs) such as albumin tend to mask changes in low-abundance, low–molecular–weight (LMW) proteins below 30 kDa [5]. Interestingly, the most valuable biomarkers are often low-abundance LMW proteins, as they exhibit variations in quantity, post-translational modifications, and isoform expression across diseases [6].

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Western blotting, or protein blotting, is a widely used method for detecting and semi-quantifying specific proteins from crude mixtures of cell-derived proteins, valued for its ability to confirm protein identity and assess molecular weight [7,8]. However, its use in serum cytokine study remains polemic. The approach is inherently limited by low sensitivity, poor precision in complex matrices, and setbacks in analysing low-concentration targets without signal amplification [9]. These limitations are particularly pronounced when analysing serum, where cytokines are often present at picogram levels and may be masked by abundant serum proteins [9]. Consequently, in research and diagnostic testing, Western blotting is used together with other antibody-based detection platforms, such as ELISA and IHC, which offer superior sensitivity, specificity, and throughput for cytokine profiling to confirm results [10,11]. For example, in some cases, Western blotting is a necessary step in IHC to help resolve issues of antibody specificity [12]. Despite this, Western blotting is still used in some studies, raising questions about its reliability and appropriateness for detecting serum cytokines.

This study aims to critically evaluate the suitability of Western blotting for detecting and quantifying serum cytokines. By comparing its performance with more sensitive immunoassays, we seek to clarify its limitations and provide evidence-based recommendations for researchers selecting appropriate analytical tools in cytokine research.

2. Materials and Methods

2.1. Study Design

This study employed a comparative experimental design to evaluate the effectiveness of Western blotting (WB) for detecting cytokines in human serum. The primary objective was to determine the detection threshold and signal clarity of WB for low-abundance cytokines, using recombinant IL-9 as a model. Exploratory optimisation techniques were incorporated to assess whether sensitivity could be improved.

2.2. Target Cytokines

Recombinant human IL-9 (PeproTech EDK) was selected as the target cytokine due to its low serum abundance and its relevance to inflammatory signalling. The choice of IL-9 was informed by a prior multiplex assay measuring 27 cytokines, which showed significantly lower IL-9 levels in follicular lymphoma (FL) patients with CMV infection compared to those without CMV infection [13]. In that assay, IL-9 was detected in the picogram per millilitre (pg/mL) range, whereas the Western blotting experiments in this study required nanogram per millilitre (ng/mL) concentrations to achieve visible detection. This discrepancy highlights the sensitivity limitations of Western blotting for low-abundance serum cytokines and underscores the need for optimisation. Serial dilutions of IL-9 (ranging from 20 ng to 0.01 ng) were prepared and loaded in lanes 3–9 to establish the detection threshold, as shown in Table 1.

Table 1: The Serial dilutions of recombinant IL-9 for the determination of the detection threshold by Western blotting

Lane	IL-9 Concentration (ng)	Stock Volume (µL)	Laemmli buffer (µL)	Final Volume (µL)
3	20	20	10	30
4	10	10	20	30
5	5	5	25	30
6	1	1	29	30
7	0.5	0.5	29.5	30
8	0.1	10(100× dilution)	20	30
9	0.01	1(100× dilution)	29	30

Legend: Each sample was prepared to a final volume of 30 µL using Laemmli buffer. IL-9 concentrations ranged from 20 ng to 0.01 ng, enabling assessment of band visibility across a nanogram gradient. Dilutions were loaded in lanes 3–9 of the SDS-PAGE gel. Lanes 1 and 2 were excluded from the table as they contained the molecular weight ladder and a standard label placeholder, respectively, and did not represent part of the dilution series.

2.3. Sample Collection

Serum samples were obtained from healthy volunteer (HV) blood donors under protocols approved by the ethics committee. Samples were stored at −80 °C until use. Prior to analysis, samples were thawed on ice and centrifuged to remove debris. Albumin depletion was performed using a commercial kit to reduce background interference and enhance cytokine detection. For Western blotting, three dilutions of each serum sample were prepared and loaded in lanes 10–12 of the gel: 15 µL, 10 µL, and 5 µL, respectively, as shown in Table 2, each adjusted to a final volume of 30 µL with Laemmli buffer.

Table 2 Dilutions of serum samples from healthy volunteer (HV) blood donors prepared for Western blotting analysis

Lane	IL-9 Concentration (ng)	Stock Volume (µL)	Laemmli buffer (µL)	Final Volume (µL)
10	S15	15	15	30
11	S10	10	20	30
12	S5	5	25	30

Legend: Each sample was adjusted to a final volume of 30 µL using Laemmli buffer. Serum volumes of 15 µL, 10 µL, and 5 µL were loaded in lanes 10–12, respectively, to assess IL-9 detectability across varying input concentrations.

2.4. Western Blotting Procedure

2.4.1. Gel Electrophoresis

SDS-PAGE gels were cast using 7 mL of resolving gel overlaid with 300 µL of 100% isopropanol to eliminate air bubbles. After polymerization, the gel was rinsed and topped with 4 mL of 5% stacking gel. A 10-well comb was inserted and removed once set. Samples, standards, and ladder were diluted in Laemmli buffer containing β-mercaptoethanol, denatured at 95 °C for 5 minutes, cooled on ice, vortexed, and centrifuged. Electrophoresis was performed at 115 V for approximately 85 minutes.

2.4.2. Protein Transfer

Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes activated in methanol and equilibrated in cold transfer buffer (10× Tris-Glycine diluted to 1×). Membranes and gels were assembled in a transfer cassette using the following orientation: foam pad – filter paper – gel – membrane – filter paper – foam pad. The transfer was conducted in a wet tank system at 400 mA for 1 hour, with cooling and agitation to ensure uniform transfer.

2.4.3. Blocking and Antibody Incubation

Membranes were washed in TBS-T and blocked in 5% nonfat dry milk for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4 °C using rabbit anti-human IL-9 monoclonal antibody (Abcam, Cat# ab133675; 1:1000 dilution). After washing, membranes were re-blocked for 30 minutes, then incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Membranes were then washed three times for 10 minutes each in TBS-T.

2.4.4. Chemiluminescent Detection

Detection was performed using an enhanced chemiluminescence (ECL) kit. A 1:1 mixture of luminol/enhancer and peroxidase solutions was applied to the membrane for 1 minute. Membranes were wrapped and scanned using a CCD-based digital imaging system with exposure times ranging from 30 seconds to 1 minute.

2.4.5. Sensitivity Enhancement Experiments

To enhance the sensitivity of Western blotting for IL-9 detection, a series of optimization strategies was implemented. Membranes were stripped and re-probed to assess the potential for signal recovery after initial antibody exposure. A three-layer Western blotting approach was trialed, incorporating a tertiary antibody to amplify signal intensity. Immunoprecipitation was employed to concentrate IL-9 from serum samples prior to electrophoresis, thereby increasing the likelihood of detection. Additionally, prolonged exposure time during chemiluminescent imaging was used to capture faint bands that might otherwise fall below the detection threshold. These interventions formed part of the study's exploratory arm, aimed at identifying practical enhancements to WB performance.

3. Controls and Validation

To ensure the reliability of Western blotting for IL-9 detection, both positive and negative controls were incorporated. Recombinant IL-9 at concentrations ≥5 ng served as positive controls to establish detectable signal thresholds. Negative controls included blank lanes and untreated serum samples to confirm antibody specificity and rule out non-specific binding. Protein transfer and membrane integrity were verified using molecular weight ladders and Ponceau S staining, ensuring consistent loading and accurate band positioning across all lanes.

3.1. Data Analysis

Western blot data were analyzed to determine the detection threshold for IL-9, defined as the lowest concentration yielding a clearly visible band. Band presence, clarity, and migration position were qualitatively assessed across all samples and conditions. Results were compared against published detection limits and manufacturer specifications to evaluate performance. Observations were used to assess reproducibility and reliability, particularly under enhanced sensitivity protocols.

3.2. Optimization of IL-9 Detection

To determine the optimal working concentration for IL-9 detection, serial dilutions of recombinant IL-9 (1–20 ng) and serum samples were analyzed. Band intensity and visibility were assessed to determine the detection threshold and evaluate Western blot performance.

4. Results and Discussion

4.1. Signal Intensity and Detection Threshold

Quantitative analysis of Western blot signal intensity revealed a clear concentration-dependent response for recombinant IL-9. As seen in Figure 1 and Table 3, the highest signal was observed at 20 ng, followed by progressively lower intensities at 10 ng and 5 ng. Below 5 ng, signal intensity dropped sharply, with concentrations of 1 ng and lower producing minimal or undetectable bands. Serum samples (S15, S10, and S5) showed negligible signal, suggesting that endogenous IL-9 levels in healthy individuals fall below the detection threshold of this method. These findings confirm that Western blotting requires IL-9 concentrations ≥5 ng for reliable detection and highlight its limitations in assessing low-abundance cytokines in serum.

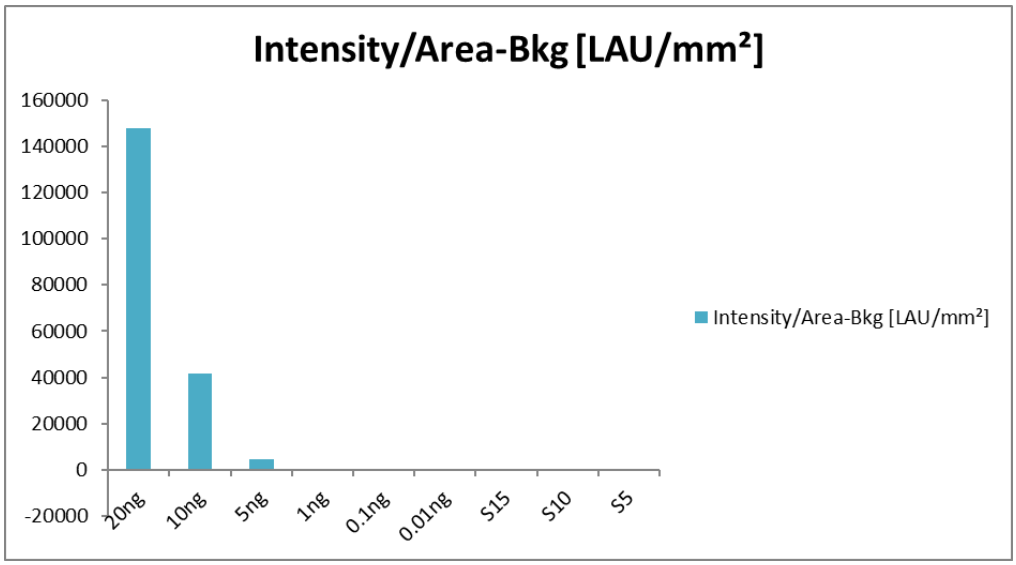

Figure 1 Western blot signal intensity for serial dilutions of recombinant IL-9 and serum samples.

Legend: Signal intensity per area minus background (LAU/mm^2) was quantified for IL-9 concentrations ranging from 20 ng to 0.01 ng, alongside serum samples S15, S10, and S5. A sharp decline in signal was observed below 5 ng, with negligible intensity detected at concentrations ≤1 ng and in serum samples, indicating a lower detection threshold for Western blotting of IL-9.

Table 3 Quantitative assessment of Western blot signal intensity for IL-9 standards and serum samples

Sample Type	Lane	IL-9 Concentration (ng)	Band Visibility	Signal Intensity (LAU/mm²)
Recombinant IL-9	3	20	Strong	~160,000
Recombinant IL-9	4	10	Moderate	~80,000
Recombinant IL-9	5	5	Faint	~40,000
Recombinant IL-9	6-9	1	Not detected	~0
Serum (S15, S10, S5)	10-12	Unknown	Not detected	~0

Legend: Western blot signal intensities for recombinant IL-9 standards and serum samples. Recombinant IL-9 at 20, 10, and 5 ng produced strong, moderate, and faint bands with signal intensities of ~160,000, ~80,000, and ~40,000 LAU/mm², respectively. At 1 ng, IL-9 was not detected in serum samples (S15, S10, S5), with signal intensities near zero.

4.2. Evaluation of Sensitivity Enhancement Strategies

Despite trailing multiple sensitivity enhancement strategies, including membrane stripping and re-probing, three-layer Western blotting, immunoprecipitation of serum samples, and prolonged exposure, none significantly improved IL-9 detection below 5 ng. Prolonged exposure marginally enhanced band visibility at higher concentrations. No detectable signal was recovered from serum samples or low-dose standards, underscoring the assay's limited sensitivity for low-abundance cytokines (unpublished).

5. Discussion

This study evaluated the detection threshold of interleukin-9 (IL-9) using Western blotting and assessed its suitability for measuring endogenous IL-9 in human serum. Our results demonstrate that IL-9 is reliably detectable at concentrations ≥5 ng, with signal intensity declining sharply below this threshold. Serum samples from healthy volunteers (S15, S10, S5) yielded no detectable signal, suggesting that physiological IL-9 levels fall below the sensitivity range of conventional Western blotting.

Western blotting remains a cornerstone method for protein detection, but its sensitivity is limited compared to modern immunoassays. Recent advances in Western blot technology, including improved antibody validation, enhanced chemiluminescent substrates, and automated imaging systems, have increased signal clarity and reproducibility [14]. Nonetheless, even with these improvements, detection thresholds typically remain in the low-nanogram range, making Western blotting unsuitable for low-abundance cytokines such as IL-9.

Attempts to enhance detection, including membrane stripping and re-probing, three-layer antibody amplification, immunoprecipitation, and prolonged exposure, did not yield significant improvements. These strategies have been shown to improve signal-to-noise ratios in some contexts, but rarely extend detection limits beyond the capabilities of the substrate and imaging system [15,16].

The absence of detectable IL-9 in serum samples is biologically plausible. IL-9 is a pleiotropic cytokine produced by Th9 cells and other immune subsets, typically expressed at low levels under homeostatic conditions and upregulated during allergic inflammation, autoimmunity, or parasitic infections [17,18]. A recent reappraisal of IL-9 in inflammation and cancer highlights its complex role in chronic disease and its tightly regulated expression in healthy tissue [19]. Healthy individuals are expected to have circulating IL-9 concentrations in the picogram range, well below the detection threshold observed in our study. This underscores the need for more sensitive platforms, such as ELISA, electrochemiluminescence, or digital immunoassays, for quantifying IL-9 in clinical samples [20,21].

6. Conclusion

Western blotting can confirm IL-9 at concentrations ≥5 ng, but is insufficient to detect physiological levels in serum. This limitation is consistent with the known sensitivity range of chemiluminescent detection and the low abundance of IL-9 under non-inflammatory conditions. Recent innovations in protein detection, including digital immunoassays capable of femtogram-level sensitivity, offer promising alternatives for cytokine quantification [20]. These findings reinforce the importance of matching detection technologies to expected analyte concentrations, particularly when studying low-expression cytokines like IL-9.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest to disclose.

Statement of ethical approval

As part of the PACIFICO trial ethics application, this study was conducted within the framework of translational research. The trial received approval from the European Union Drug Regulating Authorities Clinical Trials (EudraCT No. 2008-004759-31) and was registered under the International Standard Randomized Controlled Trial Number ISRCTN99217456. Written informed consent was obtained from all participants prior to recruitment, in accordance with International Council for Harmonization (ICH) Good Clinical Practice (GCP) guidelines.

Statement of informed consent

We declare that there are no conflicts of interest related to this manuscript. No financial, personal, or professional relationships have influenced the content or findings presented. All research was conducted independently and without external bias.

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