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RESEARCH ARTICLE

Detection of Anti-drug Antibodies (ADAs) to an Antibody-drug Conjugate (ADC) PYX-201 in Human Plasma Using a Novel Electrochemiluminescence (ECL) Immunoassay

Feng Yin[1,*], Diana Adhikari[1], Ana Lara Rojo[2], Laura Kelly[2], Areeg Ibdah[2], Ivan Orbegoso[2], Gwen Eak[2], Chengfeng Merriman[1], Jianwen Feng[1], Kristen Trexler[2], Ann Turner Dunford[2], Roxana Ospina[2], Mike Dyszel[1], Shawn Harriman[1] and Jan Pinkas[1,*]

[1]Department of Nonclinical Research, Pyxis Oncology, Inc., Boston, USA; [2]Department of Immunochemistry, PPD Laboratory Services (A Part of ThermoFisher Scientific), Henrico, USA

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Abstract: ***Background***: PYX-201 is an Antibody-Drug Conjugate (ADC) composed of a fully human IgG1 antibody, a cleavable linker mcValCitPABC, and toxic auristatin payloads Aur0101, with a drug antibody ratio (DAR) of approximately 4. PYX-201 is a promising candidate for oncology treatment because it targets the extra domain B splice variant of fibronectin (EDB + FN), which is expressed at low levels in normal adult tissues while at moderate or high levels in various human solid tumors.

Methods: An electrochemiluminescence (ECL) immunoassay was developed and validated for the detection (screening, confirmatory, and titration) of antibodies to an ADC PYX-201 in human plasma. Anti-PYX-201 antibodies were captured by biotinylated PYX-201 (Bio-PYX-201) and detected by ruthenylated PYX-201 (Ru-PYX-201) on a Meso Sector imager S 600 or 6000 reader.

Results: The screening cut-point factor (SCPF), confirmatory cut-point (CCP), and titration cut-point factor (TCPF) were found to be 1.11, 20.7%, and 1.23, respectively. Sensitivity was determined to be 2.25 ng/mL in the screening assay and 5.34 ng/mL in the confirmatory assay for anti-PYX-201 antibodies. Sensitivity was determined to be 7.70 ng/mL in the confirmatory assay for anti-PYX-201 monoclonal antibody (mAb) antibodies. The positive controls (PCs) were set at the following levels: low positive control (LPC) at 14.0 ng/mL, medium positive control (MPC) at 100 ng/mL, and high positive control (HPC) at 5,000 ng/mL. The drug tolerance was up to 200 µg/mL at the HPC level, up to 100 µg/mL at the MPC level, and 0 µg/mL at the LPC level. The intra-assay percent coefficient of variation (%CV) was ≤ 4.5% for PCs in the screening assay and ≤ 11.5% for PCs in the confirmatory assay. The inter-assay %CV was ≤ 13.6% for PCs in the screening assay and ≤ 19.2% for PCs in the confirmatory assay. No hook effect, hemolysis effect, or lipemia effect was found in this ADA method. Anti-PYX-201 antibodies were found stable in human plasma for at least 24 hours at room temperature or after six freeze/thaw cycles.

Conclusion: Anti-PYX-201 ADA bioanalytical method validation was reported for the first time in any biological matrix. This ADA method has been successfully applied to human sample analysis to support a clinical study.

Keywords: ADA, ADC, PYX-201, extra domain B fibronectin, ECL, validation.

1. INTRODUCTION

Antibody-drug conjugates (ADCs) are precision medicines crafted to target cancer cells and tissues specifically. In recent years, ADC research has increasingly garnered attention within the pharmaceutical industry, especially following the year 2000 approval of the first ADC drug, Gemtuzumab ozogamicin with a brand name Mylotarg, for the treatment of acute myeloid leukemia (AML) by the U.S. Food and Drug Administration (FDA) [1-3]. ADC drugs have turned the concept of a "magic bullet," originally proposed by Nobel Prize laureate Paul Ehrlich over a century ago, into a reality [4]. As of now, the U.S. FDA has granted approval to 13 ADCs for the treatment of hematological malignancies and solid tumors, with more than 100 ADC candidates undergoing clinical evaluation [2, 5-8]. ADC drugs comprise three

*Address correspondence to these authors at the Department of Nonclinical Research, Pyxis Oncology, Inc., Boston, USA;
E-mails: yf118@hotmail.com; jpinkas@pyxisoncology.com

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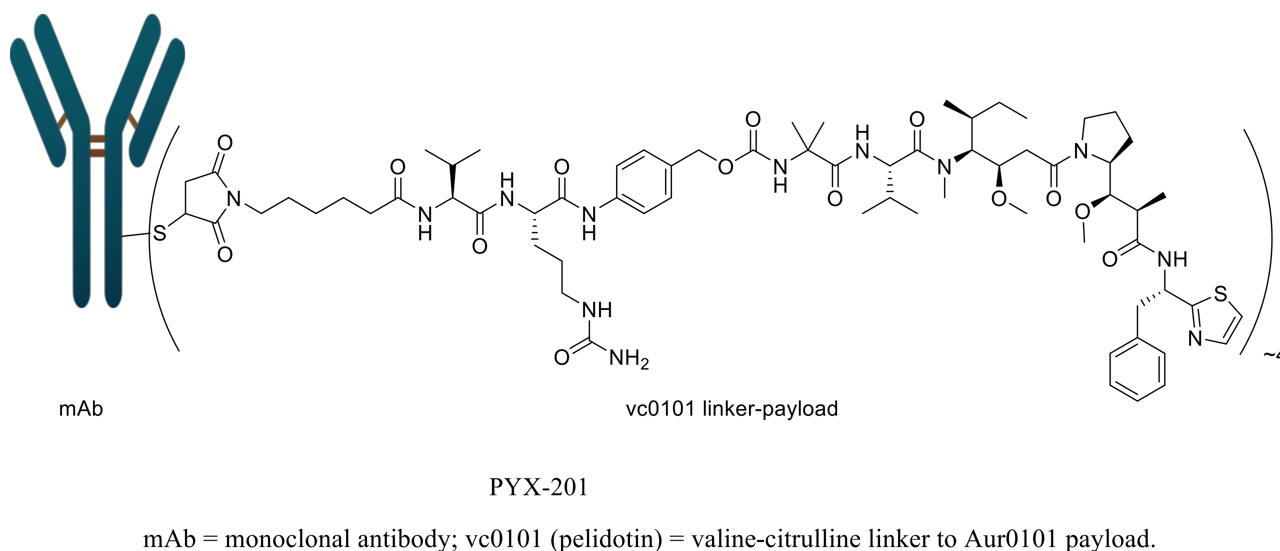
main elements: a monoclonal antibody (mAb), a chemical linker, and a cytotoxic payload (warhead). The mAb component imparts the ADC with its ability to target tumors, and an optimal mAb demonstrates specificity for the intended cancer cells, featuring a strong binding affinity to the surface antigen, while the chemical linker forms covalent chemical bonds connecting the mAb and the hydrophobic payload [9-14]. A thoughtfully engineered ADC enhances the efficient delivery of the payload to the tumor target, leading to improved effectiveness, reduced toxicity, or both [14]. The achievement of ADC drugs largely stems from the unique linker adjustments that strike a balance between the toxicity of the payload and the overall effectiveness of the ADC drug [12-14]. Typically, ADCs bind to antigens on the surface of the cancer cells and are internalized by the target cell. Once inside, the cytotoxic payload is released, allowing it to kill the designated tumor cells [7, 10, 15]. In the case of PYX-201, which is an ADC targeting a non-internalizing antigen, cell death can result from either the diffusion of the payload across the cell membrane or the bystander effect [7, 10, 15, 16]. The fusion of precise, targeted ADC delivery with the stability of ADCs during circulation helps minimize inadvertent drug exposure, diminishes off-target tissue toxicity, and enhances the effectiveness and safety of the drug [7, 10, 17, 18].

An investigational ADC drug PYX-201 (Fig. **1**) is currently undergoing a first-in-human (FIH) phase I clinical trial designed for patients with advanced solid tumors (registered under NCT05720117, www.clinicaltrials.gov, EudraCT Number: 2022-002284-30). PYX-201 consists of a fully human IgG1 antibody, a cleavable linker mcValCitPABC, and toxic auristatin payloads Aur0101, with a drug antibody ratio (DAR) of approximately 4. The backbone antibody of PYX-201 is designed to specifically target the extra domain B splice variant of fibronectin (EDB + FN). This unique feature renders PYX-201 a promising candidate for oncology treatment, as the EDB + FN target is expressed at low levels in normal adult tissues while at moderate or high levels in various human solid tumors [19-26]. Our lab has recently validated bioanalytical methods for PYX-201 ADC, TAb (total antibody), and free payload in rat, monkey, and human plasma [27-32]. Biologic drugs, including protein or antibody therapeutics, possess the capability, known as immunogenicity, to trigger the immune system, leading to the production of antibodies against the drug itself. These anti-drug antibodies (ADAs) can significantly influence drug exposure, add complexity to the interpretation of drug research outcomes, and potentially reduce the efficacy of biopharmaceuticals [33-41]. To fully understand PYX-201 immunogenicity, we developed and validated an electrochemiluminescence (ECL) immunoassay on the Meso Scale Discovery (MSD) platform for the detection of anti-PYX-201 antibodies in K_2EDTA human plasma using a three-tier approach: screening, confirmatory, and titration. The validation of this method was conducted in accordance with regulatory guidance [42, 43], and it has been effectively employed in the analysis of clinical samples.

2. MATERIAL AND METHODS

2.1. Chemicals, Reagents, and Instruments

PYX-201 was supplied by WuXi XDC (Jiangsu, China). PYX-201 mAb was obtained from WuXi Biologics (Jiangsu, China). Rabbit polyclonal anti-PYX-201 antibodies and rabbit polyclonal anti-PYX-201 mAb antibodies were manufactured at Antibody Solutions (Santa Clara, CA, USA). Bio-PYX-201 and Ru-PYX-201 were produced in PPD Laboratory Services (Henrico, VA, USA). Fibronectin/FN1 mAb was acquired from Cell Signaling Technologies (Danvers, MA, USA). 1 M Tris-HCl (pH 9.5) was bought from Boston BioProducts (Milford, MA, USA). Glacial acetic acid and 10X Dulbecco's phosphate-buffered saline (DPBS) were produced by ThermoFisher Scientific (Waltham, MA, USA). Bovine serum albumin (BSA), glycerol, L-histidine, and Tween-20 were supplied by Sigma-Aldrich (St. Louis, MO, USA). 20X Phosphate buffered saline (PBS) with 1% Tween-20 (2.7 M sodium chloride, 54 mM potassium chloride, 86 mM sodium phosphate, dibasic, 28 mM potassium



PYX-201

mAb = monoclonal antibody; vc0101 (pelidotin) = valine-citrulline linker to Aur0101 payload.

Fig. (1). Structure of PYX-201 drug substance. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

phosphate, monobasic, 1.0% Tween-20) was ordered from TekNova (Hollister, CA, USA). GOLD read buffer A was acquired from MSD (Rockville, MD, USA). Water was purified in-house on the Millipore Milli-Q IQ 7000 ultrapure lab water system (Burlington, MA, USA). Dipotassium EDTA human plasma was purchased from BioIVT (Westbury, NY, USA).

2.2. ECL-MSD System

ECL signals for complex by Bio-PYX-201, ADAs, and Ru-PYX-201 were obtained using Meso Sector imager S 600 or 6000 readers (MSD, Rockville, MD, USA).

2.3. Method Procedures

Controls/samples were thawed at room temperature. 20 µL of the controls/samples were aliquoted into a polypropylene storage plate, then added in 80 µL of 1.2 M acetic acid for a 1:5 dilution, mixed well, and incubated at room temperature (RT) on a plate shaker at 450 rpm for 10 ± 2 minutes. 30 µL of the above-diluted controls/samples was mixed well with 90 µL of the master mix (2 µg/mL of Bio-PYX-201, 2 µg/mL of Ru-PYX-201, and 40 µg/mL of Fibronectin/FN1 mAb in 1/1/3 glycerol/1X DPBS/400 mM Tris in the assay buffer (1% BSA and 0.1% Tween-20 in 1X DPBS) in the screening or titration methods; 2 µg/mL of Bio-PYX-201, 2 µg/mL of Ru-PYX-201, 10 µg/mL of PYX-201, and 40 µg/mL of Fibronectin/FN1 mAb in 1/1/3 glycerol/1X DPBS/400 mM Tris in the assay buffer in the confirmatory assay) in polypropylene cluster tubes for the final assay minimum required dilution (MRD) 20, and the mixture was incubated at RT on a plate shaker at 450 rpm for 80 ± 20 minutes. 50 µL of the above-incubated controls/samples were transferred into an MSD gold 96-well streptavidin plate, which had been incubated with the assay buffer on a plate shaker at 450 rpm at RT for one to four hours and then washed with the wash buffer (1X PBS with 0.05% Tween-20), and incubated at RT on a plate shaker at 450 rpm for approximately 60 minutes. The MSD plate was washed on a plate washer with the wash buffer, and then 150 µL of MSD GOLD reader buffer A was added. The MSD plate was mixed well and read on an MSD sector imager S 600 or 6000.

2.4. Software for Data Acquisition and Processing

MSD 600MM Methodical Mind v4.0.12.1 software (MSD, Rockville, MD, USA), Assist LIMS v7 (Version 7, PPD Laboratories Services, Richmond, VA, USA), and Microsoft Office Excel 365 (Microsoft, Redmond, WA, USA) were utilized for ECL signal data acquisition and processing from the MSD reader.

3. RESULTS AND DISCUSSION

3.1. Method Development

The assessment of clinical and nonclinical immunogenicity typically involves the identification and characterization of treatment-triggered ADA. Various analytical formats and detection methods are available for ADA detection. These methods encompass enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) or radioimmunoprecipitation assay (RIPA), surface plasmon resonance (SPR), and electrochemiluminescence (ECL) [44-72]. Each of these formats possesses its own set of advantages and disadvantages [73, 74] with ECL most used in ADA methods due to its high through-put, high tolerance to therapeutic, large dynamic range, as well as being minimally affected by matrix, *etc*. [43]. Accordingly, we employed an ECL-MSD platform in this ADA method validation.

Because there was a lack of an adequate quantity of diseased human plasma, which would have reflected the patient population, to meet the volume requirements for method development, validation, and sample analysis, a negative control (NC) pool was generated by combining K_2EDTA human plasma with low signals during screening, from 16 healthy individuals.

Soluble EDB + FN was reported to be around 1 nM in normal and diseased human plasma [25], and scavenger antibody Fibronectin/FN1 mAb was utilized in this ADA method to eliminate the false-positive results from the impact of the soluble target. Fibronectin/FN1 mAb was tried at different concentrations to optimize the ADA assays during the method development phase, and eventually its concentration was set at 40 µg/mL to ensure that excess soluble EDB + FN didn't generate a false-positive result while at the same time the addition of the scavenger antibody didn't impact the ADA assay performance.

Generally, ADA methods for ADC drugs are more complicated than antibody drugs since ADC drugs contain multiple domains, *e.g*., mAb and linker-payload. When administered to patients, a multi-domain biotherapeutic (MDB), *e.g*., an ADC drug, has the potential to induce an immune response, leading to the generation of specific ADA [42, 43, 46, 75]. As required by regulatory guidance [42, 43], domain specificity was conducted in this PYX-201 ADA method in the confirmatory assay by using PYX-201 mAb to replace the PYX-201 whole molecule.

3.2. Cut-points Determination

3.2.1. Screening Cut-point (SCP)

To determine the screening cut-point factor (SCPF) for detecting anti-PYX-201 antibodies, two analysts conducted tests on plasma samples from 50 individual drug-naïve disease-state lots over four days. Each sample was evaluated six times in 18 separate runs, after eliminating data points identified as analytical or biological outliers, a dataset of 296 remaining data points was used to calculate the SCPF, targeting a 5% false-positive rate. The calculated SCPF value was 1.11. The SCPF value served as the foundation for computing a plate-specific SCP for each screening run. This plate-specific SCP was calculated using the following formula: plate-specific SCP = 1.11 x mean ECL value of the NCs on each corresponding plate. Samples were designated as presumptive positive if their ECL values equaled or exceeded the plate-specific SCP. Following this classification, these samples underwent further testing through the confirmatory assay.

3.2.2. Confirmatory Cut-point (CCP)

A confirmatory assay was utilized to verify the specificity of presumed positive samples for anti-PYX-201 antibod-

ies against PYX-201. The CCP in plasma samples was determined by two analysts over four days, encompassing 50 different lots of diseased human plasma, both in the absence and presence of excess PYX-201 at a final concentration of 10 µg/mL. Each plasma sample underwent six tests, spread over a total of 18 separate runs. Following the removal of data points identified as analytical or biological outliers, a dataset of 289 valid data points was used to calculate the CCP. The calculated CCP, established with a 1% false-positive rate, was found to be 20.7%. During the bioanalysis phase of the study, samples were categorized as confirmed positive for ADA to PYX-201 if, in the presence of excess PYX-201, the %inhibition was ≥ 20.7%, and the ECL value of the sample without the drug was ≥ the plate-specific SCP.

3.2.3. Titration Cut-point (TCP)

The titration cut-point factor (TCPF), determined to be 1.23 using data from the SCP assessment with a 0.1% false-positive rate, played a crucial role in establishing a plate-specific TCP for each titration run. The formula for calculating the plate-specific TCP was as follows: plate-specific TCP = 1.23 x mean ECL value of the NCs on the respective plate. In the clinical analysis of ADA samples, the highest dilution of samples classified as confirmed positives would yield a positive titer result when their ECL value equaled or exceeded the plate-specific TCP. The titer control range, based on method validation results, for positive control (PC) at 50 ng/mL was set at 160 ≤ titer of ADA PC (50 ng/mL) ≤ 640.

3.3. Method Sensitivity and Low Positive Control (LPC)

The screening sensitivity was evaluated in six sets in three runs in pooled human plasma. Each sample was enriched with anti-PYX-201 antibodies PC and subjected to successive two-fold dilutions. These dilutions ranged from 50.0 ng/mL down to 0.390 ng/mL for ADA PC. The screening sensitivity for each sensitivity set was determined using Microsoft Office Excel 365 Forecast through interpolation at the SCP (5% false-positive rate). The overall screening sensitivity, as depicted in Table **1A**, was calculated as the average sensitivity across the accepted runs and was found to be 2.25 ng/mL.

The confirmatory sensitivity of anti-PYX-201 antibodies was evaluated in six sets in three runs. The confirmatory sensitivity of anti-PYX-201 mAb antibodies was evaluated in four sets in two runs in pooled human plasma. To evaluate the confirmatory sensitivity, PYX-201 or PYX-201 mAb was introduced into the diluted samples that had originally been prepared for anti-PYX-201 antibodies screening sensitivity analysis and anti-PYX-201 mAb antibodies screening sensitivity analysis, respectively. The confirmatory sensitivity for each sensitivity set was determined using Microsoft Office Excel 365 Forecast through interpolation at the CCP (1% false-positive rate). The overall confirmatory sensitivity, as detailed in Tables **1A** and **1B**, was computed as the average sensitivity across the accepted runs and was established at 5.34 ng/mL for anti-PYX-201 antibodies and 7.70 ng/mL for anti-PYX-201 mAb antibodies.

The LPC was established for both the screening and confirmatory assays by using the formula [mean sensitivity + $t_{0.99,\,df}$ × SD (sensitivity)]. Based on the sensitivity values, the LPC concentration was determined to be 6.00 ng/mL in the screening assay and 15.0 ng/mL in the confirmatory assay. For this ADA method validation, the final LPC concentration was set at 14.0 ng/mL, which was between the calculated LPC concentrations from the screening assay and the confirmatory assay while close to the calculated LPC concentration in the confirmatory assay.

Table 1A. Screening sensitivity and confirmatory sensitivity for anti-PYX-201 antibodies in pooled human plasma.

ID	Screening Sensitivity (ng/mL)	Confirmatory Sensitivity (ng/mL)
Sensitivity-1	1.15	3.26
Sensitivity-2	1.54	2.79
Sensitivity-3	2.27	4.36
Sensitivity-4	2.46	4.13
Sensitivity-5	4.37	10.3
Sensitivity-6	1.68	7.17
Overall sensitivity	2.25	5.34

Table 1B. Confirmatory sensitivity for anti-PYX-201 mAb antibodies in pooled human plasma.

ID	Confirmatory Sensitivity (ng/mL)
Sensitivity-1	6.25
Sensitivity-2	7.87
Sensitivity-3	12.9
Sensitivity-4	3.79
Overall sensitivity	7.70

Abbreviation: mAb: monoclonal antibody.

3.4. Method Selectivity

Selectivity refers to the ability of the method to detect anti-PYX-201 antibodies and anti-PYX-201 mAb antibodies in the presence of various matrix components. ADA samples, unspiked as well as spiked at LPC level at 14.0 ng/mL and HPC level at 5,000 ng/mL, were generated using 10 different lots of diseased human plasma. These samples were then subjected to screening and confirmatory assays over five separate runs. The selectivity data are presented in Tables **2A** and **2B** for anti-PYX-201 antibodies and anti-PYX-201 mAb antibodies, respectively. In cases of selectivity for anti-PYX-201 antibodies, all 10 unspiked samples (100%) produced negative results, and all 10 LPC-spiked samples and all 10 HPC-spiked samples consistently yielded positive results, confirming the absence of matrix interference when identifying anti-PYX-201 antibodies in diseased human plasma. In cases of selectivity for anti-PYX-201 mAb antibodies, all 10 unspiked samples (100%) produced negative

Table 2A. Selectivity evaluation for anti-PYX-201 antibodies in diseased human plasma.

Run ID	Sample ID	Screening Assay				Confirmatory Assay			Sample Condition
		ECL	Plate NC	Plate SCP	Condition	ECL	% Inhibition	Condition	
4 ADA Unspiked	Lot 1	89.0	87.0	96.6	< SCP	97.0	-9.0	< CCP	Negative
	Lot 2	92.5			< SCP	89.0	3.8	< CCP	Negative
5 ADA Unspiked	Lot 3	89.0	89.1	98.9	< SCP	92.5	-3.9	< CCP	Negative
	Lot 4	91.5			< SCP	96.0	-4.9	< CCP	Negative
6 ADA Unspiked	Lot 5	86.0	86.5	96.0	< SCP	87.5	-1.7	< CCP	Negative
	Lot 6	87.0			< SCP	89.5	-2.9	< CCP	Negative
7 ADA Unspiked	Lot 7	80.0	81.8	90.7	< SCP	82.0	-2.5	< CCP	Negative
	Lot 8	78.5			< SCP	82.5	-5.1	< CCP	Negative
8 ADA Unspiked	Lot 9	228	229	255	< SCP	226	1.1	< CCP	Negative
	Lot 10	237			< SCP	239	-1.1	< CCP	Negative
4 ADA LPC-spiked	Lot 1	156	87.0	96.6	> SCP	98.0	37.2	> CCP	Positive
	Lot 2	163			> SCP	93.7	42.4	> CCP	Positive
5 ADA LPC-spiked	Lot 3	157	89.1	98.9	> SCP	95.5	39.0	> CCP	Positive
	Lot 4	167			> SCP	97.0	41.7	> CCP	Positive
6 ADA LPC-spiked	Lot 5	172	86.5	96.0	> SCP	93.0	45.8	> CCP	Positive
	Lot 6	158			> SCP	94.0	40.5	> CCP	Positive
7 ADA LPC-spiked	Lot 7	161	81.8	90.7	> SCP	90.5	43.8	> CCP	Positive
	Lot 8	182			> SCP	89.5	50.8	> CCP	Positive
8 ADA LPC-spiked	Lot 9	310	229	255	> SCP	232	25.2	> CCP	Positive
	Lot 10	312			> SCP	242	22.4	> CCP	Positive
4 ADA HPC-spiked	Lot 1	27138	87.0	96.6	> SCP	2271	91.6	> CCP	Positive
	Lot 2	26821			> SCP	2376	91.1	> CCP	Positive
5 ADA HPC-spiked	Lot 3	23910	89.1	98.9	> SCP	2038	91.5	> CCP	Positive
	Lot 4	2328			> SCP	293	87.4	> CCP	Positive
6 ADA HPC-spiked	Lot 5	31995	86.5	96.0	> SCP	2189	93.2	> CCP	Positive
	Lot 6	31218			> SCP	2476	92.1	> CCP	Positive
7 ADA HPC-spiked	Lot 7	29290	81.8	90.7	> SCP	2348	92.0	> CCP	Positive
	Lot 8	36608			> SCP	2581	93.0	> CCP	Positive
8 ADA HPC-spiked	Lot 9	30735	229	255	> SCP	2675	91.3	> CCP	Positive
	Lot 10	27769			> SCP	2668	90.4	> CCP	Positive
SCPF	1.11								
CCP (%)	20.7								

Abbreviations: ADA: anti-drug antibody; CCP: confirmatory cut-point; ECL: electrochemiluminescence; LPC: low positive control; NC: negative control; SCP: screening cut-point; SCPF: screening cut-point factor.

Table 2B. Selectivity evaluation for anti-PYX-201 mAb antibodies in diseased human plasma.

Run ID	Sample ID	Screening Assay				Confirmatory Assay			Sample Condition
		ECL	Plate NC	Plate SCP	Condition	ECL	% Inhibition	Condition	
4 ADA Unspiked	Lot 1	89.0	87.0	96.6	< SCP	88.0	1.1	< CCP	Negative
	Lot 2	92.5			< SCP	85.5	7.6	< CCP	Negative
5 ADA Unspiked	Lot 3	89.0	89.1	98.9	< SCP	89.0	0.0	< CCP	Negative
	Lot 4	91.5			< SCP	81.0	11.5	< CCP	Negative
6 ADA Unspiked	Lot 5	86.0	86.5	96.0	< SCP	83.5	2.9	< CCP	Negative
	Lot 6	87.0			< SCP	87.5	-0.6	< CCP	Negative
7 ADA Unspiked	Lot 7	80.0	81.8	90.7	< SCP	80.5	-0.6	< CCP	Negative
	Lot 8	78.5			< SCP	79.5	-1.3	< CCP	Negative
8 ADA Unspiked	Lot 9	228	229	255	< SCP	243	-6.6	< CCP	Negative
	Lot 10	237			< SCP	229	3.2	< CCP	Negative
4 ADA LPC-spiked	Lot 1	156	87.0	96.6	> SCP	90.5	42.0	> CCP	Positive
	Lot 2	163			> SCP	92.0	43.4	> CCP	Positive
5 ADA LPC-spiked	Lot 3	157	89.1	98.9	> SCP	93.5	40.3	> CCP	Positive
	Lot 4	167			> SCP	91.5	45.0	> CCP	Positive
6 ADA LPC-spiked	Lot 5	172	86.5	96.0	> SCP	95.5	44.3	> CCP	Positive
	Lot 6	158			> SCP	86.5	45.3	> CCP	Positive
7 ADA LPC-spiked	Lot 7	161	81.8	90.7	> SCP	91.5	43.2	> CCP	Positive
	Lot 8	182			> SCP	84.5	53.6	> CCP	Positive
8 ADA LPC-spiked	Lot 9	310	229	255	> SCP	241	22.3	> CCP	Positive
	Lot 10	312			> SCP	248	20.5	< CCP	Negative
4 ADA HPC-spiked	Lot 1	27138	87.0	96.6	> SCP	2524	90.7	> CCP	Positive
	Lot 2	26821			> SCP	2530	90.6	> CCP	Positive
5 ADA HPC-spiked	Lot 3	23910	89.1	98.9	> SCP	2341	90.2	> CCP	Positive
	Lot 4	2328			> SCP	294	87.4	> CCP	Positive
6 ADA HPC-spiked	Lot 5	31995	86.5	96.0	> SCP	2249	93.0	> CCP	Positive
	Lot 6	31218			> SCP	2222	92.9	> CCP	Positive
7 ADA HPC-spiked	Lot 7	29290	81.8	90.7	> SCP	2583	91.2	> CCP	Positive
	Lot 8	36608			> SCP	2493	93.2	> CCP	Positive
8 ADA HPC-spiked	Lot 9	30735	229	255	> SCP	3205	89.6	> CCP	Positive
	Lot 10	27769			> SCP	3123	88.8	> CCP	Positive
SCPF	1.11								
CCP (%)	20.7								

Abbreviations: ADA: anti-drug antibody; CCP: confirmatory cut-point; ECL: electrochemiluminescence; LPC: low positive control; mAb: monoclonal antibody; NC: negative control; SCP: screening cut-point; SCPF: screening cut-point factor.

results, and 9 out of 10 LPC-spiked samples and all 10 HPC-spiked samples consistently yielded positive results, confirming the absence of matrix interference when identifying anti-PYX-201 mAb antibodies in diseased human plasma. The only negative outcome observed in the confirmatory assay for the LPC sample from lot 10 could potentially be attributed to an experimental error.

The selectivity was also tested for potential interference from hemolysis and lipemia to detect anti-PYX-201 antibodies. Three pooled human plasma samples, each containing 5% hemolyzed human blood or 300 mg/dL of triglycerides, were evaluated without any additional substance (unspiked), spiked with LPC at a concentration of 14.0 ng/mL, and spiked with HPC at a concentration of 5,000 ng/mL. In all cases, the unspiked samples were confirmed as negative, and the LPC-spiked and HPC-spiked samples consistently tested positive. These results adhered to the acceptance criteria, indicating that the presence of 5% hemolyzed blood or 300 mg/dL triglycerides in human plasma did not interfere with the accuracy of results from the method.

3.5. Assay Precision

3.5.1. Intra-assay Precision

Intra-assay precision was employed to assess the consistency within a single run of controls. This assessment involved six independent preparations of negative control (NC), LPC at 14.0 ng/mL, medium positive control (MPC) at 100 ng/mL, and HPC at 5,000 ng/mL. The results, outlined in Table **3**, demonstrate that the controls met the criteria for intra-assay precision, with ECL signal %CVs ≤ 20% for both

Table 3. Intra-assay precision of anti-PYX-201 antibodies in pooled human plasma.

Sample ID	Screening Assay			Confirmatory Assay			Screening ECL			Confirmation %Inhibition		
	ECL Mean	Plate SCP	Condition	ECL Mean	%Inhibition	Condition	Mean	SD	%CV	Mean	SD	%CV
NC	128	145	Pass	132	-3.1	Pass	133	4.45	3.4	-2.1	DNC	DNC
	135		Pass	134	0.7	Pass						
	128		Pass	137	-7.5	Pass						
	135		Pass	138	-2.2	Pass						
	138		Pass	142	-2.9	Pass						
	137		Pass	133	2.6	Pass						
LPC	186	140	Pass	135	27.4	Pass	192	8.70	4.5	30.5	3.50	11.5
	178		Pass	133	25.6	Pass						
	194		Pass	135	30.5	Pass						
	196		Pass	128	34.5	Pass						
	199		Pass	137	31.2	Pass						
	202		Pass	134	33.7	Pass						
MPC	636	140	Pass	172	72.9	Pass	614	24.3	4.0	71.5	1.29	1.8
	612		Pass	178	71.0	Pass						
	627		Pass	176	72.0	Pass						
	636		Pass	173	72.9	Pass						
	573		Pass	168	70.7	Pass						
	602		Pass	183	69.7	Pass						
HPC	28075	139	Pass	2387	91.5	Pass	27446	754	2.8	91.3	0.360	0.4
	28432		Pass	2441	91.4	Pass						
	27698		Pass	2321	91.6	Pass						
	26428		Pass	2271	91.4	Pass						
	27108		Pass	2412	91.1	Pass						
	26937		Pass	2523	90.6	Pass						
CCP (%)	20.7											

Abbreviations: CCP: confirmatory cut-point; %CV: percent coefficient of variance; DNC: data not calculated; ECL: electrochemiluminescence; HPC: high positive control; LPC: low positive control; MPC: medium positive control; NC: negative control; SCP: screening cut-point; SD: standard deviation.

screening and confirmatory assays. Furthermore, the controls adhered to specific criteria regarding the order of ECL values: NC < SCP ≤ LPC < MPC < HPC for the screening assay, and the order of %inhibition values: NC < CCP ≤ LPC, MPC and HPC for the confirmatory assay. Specifically, the intra-assay %CV for ECL was 4.5% for LPC, 4.0% for MPC, and 2.8% for HPC in the screening assay, while the intra-assay %CV for %inhibition was 11.5% for LPC, 1.8% for MPC, and 0.4% for HPC in the confirmatory assay.

3.5.2. Inter-assay Precision

Inter-assay precision assessed the between-run consistency of controls and was determined using NC, LPC at 14.0 ng/mL, MPC at 100 ng/mL, and HPC at 5,000 ng/mL across seven designated runs. As presented in Table **4**, the inter-assay coefficient of variation (%CV) for ECL in the screening assay was 12.3% for LPC, 11.1% for MPC, and 13.6% for HPC, while in the confirmatory assay, the inter-assay %CV for %inhibition was 19.2% for LPC, 4.3% for MPC, and 0.4% for HPC. Inter-assay precision successfully met the acceptance criteria for the screening assay, where ECL NC < plate-specific SCP ≤ LPC < MPC < HPC, and for the confirmatory assay, where %inhibition of NC < CCP ≤ LPC, MPC, and HPC.

3.6. Drug Tolerance

The presence of the drug PYX-201 in the samples has the potential to disrupt the detection of anti-PYX-201 antibodies. Therefore, it is crucial to evaluate the accuracy of the method in detecting ADA effectively. Drug tolerance was determined as the highest drug concentration at which varying ADA levels could still be reliably detected. To assess the potential interference caused by PYX-201, ADA positive controls were prepared at three levels: LPC 14.0 ng/mL, MPC 100 ng/mL, and HPC 5,000 ng/mL. These ADA-positive controls were intentionally mixed with different concentrations of PYX-201, specifically 0, 50.0, 100, and 200 µg/mL. As outlined in Table **5**, no interference was detected when PYX-201 was added to ADA HPC samples at concentrations of up to 200 µg/mL. Similarly, no interference was observed when PYX-201 was added to ADA MPC samples at concentrations of up to 100 µg/mL. Furthermore, no interference was detected when PYX-201 was added to ADA LPC samples at the concentration of 0 µg/mL.

3.7. Stability

To evaluate the stability of ADA samples, we examined control samples for NC, LPC, and HPC. The criteria for determining the acceptance of stability samples were defined as follows: NC < plate-specific SCP ≤ LPC < HPC in the screening assay, %CV should be ≤ 20%, and at least 66.7% (2 out of 3) of the stability samples must meet the above acceptance criteria.

3.7.1. Freeze/thaw Stability

PC samples were stored in a freezer set at -80°C for a minimum of 24 hours before the initial thawing, with an additional minimum 12-hour interval preceding each subsequent thawing. Prior to refreezing, these samples were allowed to thaw at RT. For each control (LPC and HPC), three aliquots were subjected to six freeze/thaw cycles on a plate using the screening assays. The results of the freeze/thaw stability test, as presented in Table **6**, demonstrate that anti-PYX-201 antibodies remain stable in human plasma after undergoing at least six freeze/thaw cycles, transitioning from -80°C to ambient temperature.

3.7.2. Benchtop Stability

The PC samples were maintained at a temperature of -80°C for a minimum of 24 hours before thawing. For each control (LPC and HPC), three aliquots were tested after being left on the benchtop for 24 hours, utilizing the screening assay. The results of the benchtop stability test, as presented in Table **7**, confirm that anti-PYX-201 antibodies exhibit stability in human plasma for a minimum duration of 24 hours under ambient conditions.

3.8. Hook Effect

The hook effect, also referred to as the prozone effect, is a phenomenon in which the assay signal changes from increasing to decreasing as the concentration of ADA increases. However, the hook effect is not a significant concern for ADA detection when all levels of positive antibodies consistently yield positive results. To investigate the possibility of the hook effect, a sample containing an ultra-high concentration of anti-PYX-201 antibodies, specifically 10,000 ng/mL in pooled human plasma, was employed. This sample was progressively diluted by a factor of 2 seven times in pooled human plasma. Subsequently, it was subjected to analysis using both the screening assay and the confirmatory assay. As detailed in Table **8**, all samples with an anti-PYX-201 antibody concentration of 78.1 ng/mL or higher exhibited ECL values higher than the assay SCP and %inhibition values higher than the CCP. This observation suggests the absence of a hook effect in this ADA method.

3.9. Method Application

The validated ADA bioanalytical method has been effectively employed for the analysis of human plasma samples in support of the clinical trial PYX-201-101 (A first-in-human, open-label, multicenter, phase 1 clinical study to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics, and preliminary efficacy of PYX-201 in participants with advanced solid tumors) (EudraCT No. 2022-002284-30). The results of ADA data analysis, in conjunction with pharmacokinetic (PK) profiles, will be presented in a separate manuscript in the future.

CONCLUSION

We conducted a comprehensive validation of a bioanalytical method for the detection of anti-PYX-201 antibodies in human plasma. This method utilized an ECL immunoassay on the MSD platform, employing a three-tier approach encompassing screening, confirmatory, and titration assays. We rigorously assessed various parameters, including assay cut-points, sensitivity, selectivity, intra-assay and inter-assay precision, drug tolerance, stability, and hook effect, *etc*., in accordance with the 2019 U.S. FDA guidance [42] and the 2017 European Medicines Agency (EMA) guideline [43].

Table 4. Inter-assay precision of anti-PYX-201 antibodies in pooled human plasma.

Run ID	NC		LPC			MPC			HPC		
	Uninhibited (ECL)	Inhibited (ECL)	Uninhibited (ECL)	Inhibited (ECL)	% Inhibition	Uninhibited (ECL)	Inhibited (ECL)	% Inhibition	Uninhibited (ECL)	Inhibited (ECL)	% Inhibition
1	132	134	201	148	33.0	669	181	75.4	28217	2559	92.0
	131	N/A	240	N/A		802	N/A		35389	N/A	
	130	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	132	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
2	121	128	185	137	32.3	580	172	73.6	22653	2190	91.7
	125	N/A	220	N/A		722	N/A		30242	N/A	
	127	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	132	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
3	124	125	200	142	30.9	627	176	73.8	27332	2300	92.1
	123	N/A	210	N/A		713	N/A		30920	N/A	
	129	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	126	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
4	88.5	97.0	166	93.0	44.1	622	132	79.6	26213	2254	91.9
	89.5	N/A	168	N/A		673	N/A		29784	N/A	
	84.0	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	86.0	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
5	88.5	89.0	156	96.0	42.3	560	135	78.3	24043	2159	92.0
	88.5	N/A	177	N/A		686	N/A		29619	N/A	
	89.0	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	90.5	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
6	87.0	91.5	172	100.0	46.2	643	142	80.1	25722	2321	92.2
	89.0	N/A	201	N/A		786	N/A		33556	N/A	
	83.5	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	86.5	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
7	84.0	85.0	187	90.5	50.0	789	137	82.1	35910	2425	92.9
	80.0	N/A	175	N/A		736	N/A		32027	N/A	
	81.0	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
	82.0	N/A	N/A	N/A		N/A	N/A		N/A	N/A	
N	28	7	14	7	7	14	7	7	14	7	7
Mean	104	107	190	115	39.8	686	153	77.6	29402	2315	92.1
SD	21.1	20.8	23.3	25.5	7.67	76.2	21.6	3.33	4010	139	0.364
%CV	20.4	19.5	12.3	22.2	19.2	11.1	14.1	4.3	13.6	6.0	0.4
Plate SCP: 143 in run 1, 137 in run 2, 137 in run 3, 94.8 in run 4, 97.1 in run 5, 94.3 in run 6, and 89.1 in run 7.											
CCP: 20.7%											

Abbreviations: CCP: confirmatory cut-point; %CV: percent coefficient of variance; ECL: electrochemiluminescence; HPC: high positive control; LPC: low positive control; MPC: medium positive control; NA: not available; NC: negative control; SCP: screening cut-point; SD: standard deviation.

Table 5. Drug tolerance: interference of PYX-201 with the detection of anti-PYX-201 antibodies in pooled human plasma.

PYX-201 Drug Concentration (µg/mL)	ADA LPC (14.0 ng/mL)				ADA MPC (100 ng/mL)				ADA HPC (5,000 ng/mL)			
	ECL	Inhibited ECL	% Inhibition	Result	ECL	Inhibited ECL	% Inhibition	Result	ECL	Inhibited ECL	% Inhibition	Result
200	96.0	89.0	7.3	Neg	121	107	11.6	Neg	1253	605	51.7	Pos
100	109	97.0	11.0	Neg	157	116	26.1	Pos	3249	966	70.3	Pos
50.0	112	94.0	15.7	Neg	214	118	45.1	Pos	6021	1247	79.3	Pos
0	171	105	38.6	Pos	627	137	78.1	Pos	30167	2118	93.0	Pos
Plate SCP	102											
CCP (%)	20.7											
Drug tolerance (µg/mL)	0				100				> 200			

Abbreviations: ADA: anti-drug antibody; CCP: confirmatory cut-point; ECL: electrochemiluminescence; HPC: high positive control; LPC: low positive control; MPC: medium positive control; Neg: negative; Pos: positive; SCP: screening cut-point.

Table 6. Freeze/thaw stability (six cycles from -80°C to ambient temperature) of anti-PYX-201 antibodies in pooled human plasma.

Sample ID	Screening Assay			
	ECL Mean	Plate NC	Plate SCP	Condition
FT-LPC 1	173	89.9	99.8	Pass
FT-LPC 2	172			Pass
FT-LPC 3	182			Pass
FT-HPC 1	32085			Pass
FT-HPC 2	31057			Pass
FT-HPC 3	29556			Pass

Abbreviations: ECL: electrochemiluminescence; FT: freeze/thaw; HPC: high positive control; LPC: low positive control; NC: negative control; SCP: screening cut-point.

Table 7. Benchtop stability (24 hours at ambient temperature) of anti-PYX-201 antibodies in pooled human plasma.

Sample ID	Screening Assay			
	ECL Mean	Plate NC	Plate SCP	Condition
BT-LPC 1	174	89.9	99.8	Pass
BT-LPC 2	183			Pass
BT-LPC 3	191			Pass
BT-HPC 1	36355			Pass
BT-HPC 2	31289			Pass
BT-HPC 3	34696			Pass

Abbreviations: BT: benchtop; ECL: electrochemiluminescence; HPC: high positive control; LPC: low positive control; NC: negative control; SCP: screening cut-point.

Table 8. Hook effect of anti-PYX-201 antibodies in pooled human plasma.

Dilution Factor	ADA Concentration (ng/mL)	ECL	% Inhibition	Condition
1	10,000	104317	94.0	Positive
2	5,000	45131	93.4	Positive
4	2,500	18435	91.9	Positive
8	1,250	8205	90.3	Positive
16	625	4383	88.9	Positive
32	313	2170	87.4	Positive
64	156	1089	83.4	Positive
128	78.1	546	75.6	Positive
Plate SCP	95.6			
CCP (%)	20.7			

Abbreviations: ADA: anti-drug antibody; CCP: confirmatory cut-point; ECL: electrochemiluminescence; SCP: screening cut-point.

In this method, a complex was formed by anti-PYX-201 antibodies, the biotinylated capture antibody Bio-PYX-201, and the ruthenylated detection antibody Ru-PYX-201 within MSD gold streptavidin plates. Measurement was performed using the Meso Sector imager S 600 or 6000. This method is selective in human plasma, with a sensitivity of 2.25 ng/mL in the screening assay and 5.34 ng/mL in the confirmatory assay. The SCPF, CCP, and TCPF values were calculated to be 1.11, 20.7%, and 1.23, respectively.

Furthermore, the method demonstrated robust drug tolerance, accommodating concentrations up to 200 µg/mL at the HPC level (5,000 ng/mL), up to 100 µg/mL at the 100 ng/mL level, and 0 µg/mL at the LPC level (14.0 ng/mL).

The intra-assay %CV was consistently ≤ 4.5% for PCs in the screening assay and ≤ 11.5% for PCs in the confirmatory assay. Additionally, the inter-assay %CV remained within acceptable limits, with values ≤ 13.6% for PCs in the screening assay and ≤ 19.2% for PCs in the confirmatory assay. Importantly, anti-PYX 201 antibodies were found to be stable in human plasma after storage at room temperature for 24 hours, as well as after undergoing six freeze/thaw cycles.

This thoroughly established and validated method has been successfully applied in the analysis of clinical human plasma samples, contributing to the ongoing support of a clinical trial with the registration number EudraCT No. 2022-002284-30.

AUTHORS' CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: Writing the paper: FY, Validation; DA, ALR, LK, AI, IO, GE; KT, ATD; Investigation: CM, JF, RO; Writing - Reviewing and Editing: MD, SH; Methodology: JP. All authors reviewed the results and approved the final version of the manuscript.

LIST OF ABBREVIATIONS

ADC	=	Antibody-drug Conjugate
BSA	=	Bovine Serum Albumin
CCP	=	Confirmatory Cut-point
DAR	=	Drug Antibody Ratio
DPBS	=	Dulbecco's Phosphate-buffered Saline
ECL	=	Electrochemiluminescence
ELISA	=	Enzyme-linked Immunosorbent Assay
FDA	=	Food and Drug Administration
HPC	=	High Positive Control
LPC	=	Low Positive Control
mAb	=	Monoclonal Antibody
MPC	=	Medium Positive Control
MRD	=	Minimum Required Dilution
MSD	=	Meso Scale Discovery
NC	=	Negative Control
PBS	=	Phosphate Buffered Saline
PCs	=	Positive Controls
RIA	=	Radioimmunoassay
RT	=	Room Temperature
SCPF	=	Screening Cut-point Factor
SPR	=	Surface Plasmon Resonance

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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