Development and Validation of the Anti-Rituximab Antibody Detection Methods for Rituximab Biosimilar Program

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INTRODUCTION

Rituximab is a genetically engineered chimeric monoclonal antibody that targets CD20 on either normal or malignant B-lymphocytes surfaces. The drug has been successfully used to treat solid tumor (ST) of lymphoid cells and rheumatoid arthritis (RA). Due to these therapeutic values, it has being actively developed as a biosimilar (BSI) product. Here we report a study that we developed and validated two immunoassays for the detection of either anti-Rituximab innovator EU or anti-Rituximab BSI antibodies in human solid tumor or RA serums to support ongoing clinical studies.

MATERIALS AND METHODS

The anti-Rituximab antibody detection assays utilized the bridging format with the MSD ECL technology. The Rituximab innovator EU and biosimilar drugs were labeled with either biotin or ruthenium. Two independent assays were developed and validated.

- Intra-Assay and Inter-Assay Precision: Three different positive control (PC) antibodies were evaluated: rat anti-Rituximab monoclonal antibody (AbD Serotec), rabbit anti-Rituximab polyclonal antibody (Pfizer, Inc.) and mouse anti-PF-05280586 (Rituximab BSI) monoclonal antibody (Pfizer, Inc.).
- Screening and Confirmatory Cut Point Determination for Targeted Population: The parameters needed to calculate the screening cut point value were established by running 50 lots of Solid Tumor and 75 lots of RA human serum samples. The confirmatory cut point value were obtained from 28 lots of Solid Tumor and 28 lots of RA human serum samples. The individual samples were analyzed over 3 separate days.
- Drug Tolerance Test: Positive controls (50 and 400 ng/mL) were pre-incubated with different concentrations of Rituximab or PF-05280586, i.e. 0, 0.8, 1.6, 3.2, 6.4, 12.5, 25, 50 and 100 µg/mL.
- Matrix Specificity (Recovery): The test was performed by analyzing negative control and 10 individual lots of ST or RA human serum spiked with and without positive control antibodies at 50 and 800 ng/mL.
- Mass-based Assay Sensitivity: Determined from all three positive control antibodies.
- Stability Assessments: Room temperature and freeze/thaw stability of the PC.

VALIDATION RESULTS

The intra-assay precision was determined from 2 plates performed each day for 3 days by 2 analysts. The inter-assay precision was calculated from all accepted plates (Table 1).

Table 1. Intra-Assay and Inter-Assay Precision

Assay	Negative Control		Rat Anti-Rituximab mAb (Primary PC)		Rabbit Anti-Rituximab polyAb		Mouse Anti-PF-05280586 mAb	
	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay
Innovator EU	1.4-4.1%	12.2%	0.4-3.9%	3.3%	0.3-6.6%	4.1%	0.1-2.0%	3.4%
Rituximab-BSI	1.1-5.5%	10.1%	0.5-7.3%	4.0%	1.0-4.8%	6.0%	0.6-2.3%	3.8%

The screening cut point were determined from the individual samples analyzed each at 3 separate days. Individual male and female serum samples were included on each plate. A floating screening cut point factor was adapted to calculate the plate specific cut point. The confirmatory cut point was determined by analyzing individual samples with and without the presence of excess innovator drug or PF-05280586 (Table 2).

Table 2. Screening an Confirmatory Cut Point in Anti-Rituximab ADA Assays

Drug	Screening Cut Point Factor		Confirmatory Cut Point	
	Rheumatoid Arthritis	Solid Tumor	Rheumatoid Arthritis	Solid Tumor
MabThera	1.19	1.12	18.0%	17.0%
Rituxan			26.0%	16.0%
PF-05280586	1.21	1.07	19.0%	18.5%

The positive control antibodies (50 and 400 ng/mL) were pre-incubated with innovator drug or PF-05280586 for at least one hour at room temperature before being analyzed. The last reading above the plate cut point was highlighted (Table 3).

Table 3. Drug Tolerance Test in Anti-Rituximab ADA Assays

Drug (µg/mL)	MabThera		Rituxan		PF-05280596	
	PC 50	PC 400	PC 50	PC 400	PC 50	PC 400
0.0	1144.0	5698.5	1055.0	6130.0	1014.5	5691.5
0.8	395.5	4545.0	224.0	709.0	283.5	953.0
1.6	262.0	2728.5	219.0	738.5	273.5	951.5
3.2	258.5	905.5	196.0	648.0	252.0	855.0
6.4	238.0	656.5	159.5	464.0	215.0	668.0
12.5	**157.0**	358.5	**136.5**	309.5	177.0	448.5
25.0	134.0	228.5	126.5	201.0	**148.0**	280.5
50.0	123.5	**160.0**	115.5	**141.0**	131.0	**185.5**
100.0	116.5	129.0	108.5	115.5	121.0	140.0
Plate Cut Point	142.9		132.6		140.8	

Matrix specificity (recovery) was demonstrated by the analysis of ten individual lots of human serum, neat or spiked with rat anti-Rituximab antibodies at 50 and 800 ng/mL. Eight (8) out of 10 ST individual lots (Table 4A) and 8 out of 10 RA individual lots (Table 4B) had recovery between 75 to 125%.

Table 4A. Matrix Specificity (Recovery) in ST individual Lots

Solid Tumor HUSE	Response Units (RU)		PC 50 Recovery (%)	Response Units (RU)		PC 800 Recovery (%)
	Unspiked	PC 50 Spiked		Unspiked	PC 800 Spiked	
Matrix 01	109.5	1350.5	184.4	102.0	12437.0	137.8
Matrix 02	110.0	767.0	97.6	103.5	8429.5	93.0
Matrix 03	114.0	672.5	83.0	109.0	7116.0	78.3
Matrix 04	110.0	678.0	84.4	119.5	7727.5	85.0
Matrix 05	112.0	648.0	79.6	109.0	7166.0	78.9
Matrix 06	105.5	635.0	78.7	99.0	7039.5	77.6
Matrix 07	105.5	762.0	97.5	106.0	8110.5	89.5
Matrix 08	110.5	758.0	96.2	101.0	8440.0	93.2
Matrix 09	111.0	587.0	70.7	110.0	7138.0	78.5
Matrix 10	108.0	1396.5	191.4	111.0	14331.0	158.9

Table 4B. Matrix Specificity (Recovery) in RA individual Lots

Rheumatoid Arthritis HUSE	Response Units (RU)		PC 50 Recovery (%)	Response Units (RU)		PC 800 Recovery (%)
	Unspiked	PC 50 Spiked		Unspiked	PC 800 Spiked	
Matrix 11	122.0	719.0	82.8	122.5	7958.0	82.5
Matrix 12	120.5	1326.0	167.3	132.0	10048.0	104.4
Matrix 13	119.0	682.5	78.2	128.5	7397.0	76.5
Matrix 14	123.0	703.0	80.5	128.0	7318.0	75.7
Matrix 15	117.0	688.0	79.2	117.5	8260.0	85.7
Matrix 16	121.5	857.5	102.1	121.0	12242.5	127.6
Matrix 17	115.0	657.0	75.2	120.5	8266.5	85.7
Matrix 18	121.0	692.0	79.2	123.0	10985.0	114.3
Matrix 19	122.0	721.0	83.1	151.0	8335.5	86.2
Matrix 20	120.0	613.0	68.4	127.0	6194.0	63.9

Assay sensitivity was determined by the concentration at which the PC produced a response equal to the cut point determined for the assay. It was reported as the 95% confidence interval value from all acceptable validation runs (Table 5).

Mean PC concentration = PC working stock concentration/End Point Titer

Assay detection limit = mean PC concentration + (1.645*SD)

Assay sensitivity = MRD x assay detection limit

Table 5. Mass-based Assay Sensitivity in Anti-Rituximab ADA Assays

Assay	*Rat Anti-Rituximab mAb*	*Rabbit Anti-Rituximab pAb*	*Mouse Anti- Rituximab biosimilar mAb*
Innovator EU	1.8 ng/mL	15.5 ng/mL	1.5 ng/mL
PF-05280586	2.0 ng/mL	26.8 ng/mL	2.1 ng/mL

The stability of the rat anti-rituximab antibody was assessed. It was found that the antibody was stable for at 24 hours at room temperature and over at least 5 freeze/thaw cycles (Data not shown).

CONCLUSIONS

- Two independent immunoassays to detect anti-Rituximab innovator and anti-Rituximab biosimilar (PF-05280586) antibodies were validated in human ST as well as RA matrices to support ongoing clinical studies.
- Screening cut point factors and confirmatory cut points were determined for both ST and RA populations.
- Two anti-Rituximab innovator antibodies (one monoclonal, one polyclonal) and one anti-PF-05280586 monoclonal antibody were evaluated in both innovator EU and biosimilar ADA assays for cross reactivity and sensitivity.
- The two assays were found to have comparable precision, drug tolerance and assay sensitivity.

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