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(54) Title: METHOD FOR DETECTION OF NEUTRALIZING ANTI-RITUXIMAB ANTIBODIES

(74) [Continued on next page]

Figure 1

(57) Abstract: This invention is related to a method of detecting neutralizing activity of anti rituximab antibodies in a rheumatoid factor positive rheumatoid arthritis patient's sample, wherein the method retrieves anti-rituximab antibodies using an acidic buffer compatible with cell based CDC assay. In addition, the method employs use of 5 magnetic beads coupled rituximab for the retrieval of ARIA. The said method is able to measure the neutralizing anti-rituximab antibodies in presence of higher amounts of free rituximab and RF.
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METHOD FOR DETECTION OF NEUTRALIZING ANTI-RITUXIMAB ANTIBODIES

RELATED APPLICATION

This application is related to and takes priority from Indian Provisional Application 1187/CHE/2014 filed 07th March, 2014 and is herein incorporated in its entirety.

BACKGROUND

Protein based therapeutics, in particular, monoclonal antibodies are highly successful for treatment of various diseases including oncological disorders. However, when administered for treatment, monoclonal antibody drugs often induce undesirable immune response in patients. One such common drug induced adverse response is the production of anti-drug antibodies against the monoclonal antibody in use.

Anti-drug antibodies generally form immune complexes by binding to the Fab and/or the Fc portions of the administered antibody thereby neutralizing the activity of the antibody and blocking its therapeutic activity. Such neutralizing antibodies (NAb) may also obstruct the normal functions of the endogenous counterpart of the administered antibody. Consequently, presence of NAb impacts the bioavailability, efficacy and safety of the therapeutic antibody. Hence screening and detection of NAb becomes highly important, and is mandated by the regulatory agencies when assessing the safety/immunogenicity of a therapeutic drug. (http://www.fda.gov/downloads/Drugs/.../Guidances/UCM192750.pdf)

Detection of NAb can be performed either using cell based biological assays or a non-cell based competitive ligand-binding assays. However, cell based assays are preferable and recommended as they directly assess the biological activity/potency of the therapeutic molecule and are more reflective of the in vivo situation.

Complement Dependent Cytotoxicity (CDC) is a widely used cell based assay for detecting the neutralizing activity of anti-drug antibodies or NAb. CDC assay involves the measurement of extent of binding and lysis of target cells by rituximab in
the presence of NAb and complement. In a typical CDC assay, the sample
presumed to contain NAb is first incubated with the therapeutic antibody followed by
addition of complement and target cells. NAb, if present, is expected to neutralize the
activity of the therapeutic antibody resulting in decrease in cytotoxicity of the cells,
and in turn an increase in the number of live cells, this being proportional to the
amount of NAb.

However, performance of cell based assays are largely influenced by various
factors such as presence of excess amount of therapeutic molecule and interfering
components of serum/plasma sample, assay conditions and reagents, including the
buffers employed in the assay. Particularly, detection of NAb involves several
critical steps that include retrieval of NAb from the serum/plasma sample,
preparation of the retrieved NAb for the bioassay, performance of the functional
bioassay. Hence the choice of buffer employed in the steps may influence the
characteristic of both the NAb and the target cell in use. For example, properties of
the buffer such as ionic strength and pH greatly influence the conformation of an
antibody (or NAb) and hence its retrieval process. Further, frequent change in buffer
conditions increase the process time, resource and expense, and in addition may
readily introduce contamination in a relatively sensitive cell based assay system.
Hence, it is essential to have a buffer that is compatible with all the steps of a cell
based assay.

Further, detection of NAb is generally complicated by the presence of
‘interfering factors’ which may affect the sensitivity and performance of the CDC
assay. These interfering factors are specific to drug and disease condition to be
treated. For example, monoclonal antibody drugs administered for therapeutic
purpose are generally at higher doses and have relatively longer half-lives. This
results in the presence of higher levels of monoclonal antibodies in blood which may
form complexes with neutralizing antibodies leading to interferences in the detection
of NAb. In addition, rituximab, a chimeric anti-CD20 monoclonal antibody, when
administered to individuals for the treatment of Rheumatoid Arthritis (apart from NHL
and CLL) may result in the production of neutralizing anti-rituximab antibodies
impacting the efficacy and safety of the antibody drug, thus making its detection
important. However, samples from RA patients often contain high concentrations of
rheumatoid factors (RF) which are antibodies with high affinity to the Fc region of IgG. These RF may form complexes with neutralizing anti-rituximab antibodies blocking the later’s ability to neutralize rituximab resulting in interference in the detection of NAb by the CDC assay described above.

Thus any assay employed to detect NAb of rituximab requires customization and optimization for reducing such interfering factors. More specifically, methods to measure NAb is challenging as it involves a cell-based assay which is generally limited by rapid loss in assay performance/sensitivity relative to a non-cell based ligand binding assay.

Hence the primary objective of the invention is to develop a method for detection of neutralizing anti-rituximab antibodies wherein the buffer, optimized and used for the retrieval and preparation process of anti-rituximab antibodies, is compatible with the cell based CDC assay. Another objective of the invention is to develop the method such that it is sensitive in detecting the NAb without interferences from RF or excess free rituximab.

SUMMARY

The present invention discloses a method for detecting neutralizing anti-rituximab antibodies in a rheumatoid factor positive rheumatoid arthritis patient’s sample, wherein an acidic buffer comprising phosphoric acid is used for the retrieval of anti-rituximab antibodies. The said acidic buffer is also compatible with the cell based CDC assay, since the performance/sensitivity of the assay is not influenced by the buffer conditions.

Further, the method discloses the use of magnetic bead coupled rituximab for the retrieval of anti-rituximab antibodies, which significantly removes interferences from free rituximab and/or rheumatoid factors.

The method proposed in the present invention is highly sensitive as it is able to detect neutralizing anti-rituximab antibodies of about 300 ng/ml of sample, in presence of excess of rituximab (as high as 80 µg/ml), in turn indicating the high drug tolerance of the assay.
In addition, the method mitigates the RF interference remarkably to a level such that the performance of the assay in RA patient’s plasma sample is comparable to that the assay performed with normal plasma sample.

**BRIEF DESCRIPTION OF THE DRAWINGS**

5  **Figure 1:** Illustrates a comparison of % cytotoxicity of samples (spiked with rituximab) in presence of phosphoric acid and assay medium.

**Figure 2:** Illustrates a dose response curve of rituximab in assay media. The concentration of rituximab (125 ng/ml) highlighted in circle in the figure was selected for the incubation of retrieved anti-rituximab antibodies prior to the subjecting of sample for CDC assay.

**DETAILED DESCRIPTION**

Detection of neutralizing anti-rituximab antibodies in a rheumatoid factor positive rheumatoid arthritis patient’s sample by a cell based CDC assay is complicated both by the presence of high levels of rituximab and/or RF as well as the choice of buffer used for pre-treatment of the sample.

The present invention discloses a method wherein magnetic beads coupled rituximab is used to retrieve anti-rituximab antibodies. Magnetic beads covalently coupled to rituximab used in the current invention, significantly removes any RF and free rituximab that would cause interference in the CDC assay. Since, covalent coupling of the beads to rituximab prevents any leaching of the antibody and also exhibits low background binding.

In addition, the phosphoric acid buffer used for retrieving anti-rituximab antibodies prevents formation of immune complexes. Further, the retrieved anti-rituximab antibodies can be maintained in the same buffer for its use in the CDC assay. The results demonstrate that the percent cytotoxicity of the target cell in phosphoric acid buffer is similar to that of the value obtained with the assay medium that is used for maintenance of the target cell. This indicates the compatibility of the buffer with the CDC assay, alleviates the need of using a different buffer, which in turn decreases the possibility of contamination and improves the process time, resource and use.
The results show that the method is very sensitive in detecting neutralizing anti-rituximab antibodies even in presence of high concentration of rituximab (80 µg/ml) in the plasma sample. In other words, excess free rituximab present in the sample does not interfere in the performance/sensitivity of the assay, indicating the high drug tolerance of the method.

The results obtained show that the performance of the assay in RA patients sample comprising RF units as high as 650 units/ml is similar and comparable to that of the assay performed with samples of normal healthy individuals, indicating significant mitigation of RF interferences in the assay.

Various embodiments of the invention discloses in detail a method of detecting neutralizing anti-rituximab antibodies in a rheumatoid factor positive rheumatoid arthritis patient’s sample.

In an embodiment the claimed invention discloses a method for detecting neutralizing anti-rituximab antibodies in a rheumatoid factor positive rheumatoid arthritis patient’s sample comprising the steps of:

a) pre-treatment of the sample with an acidic buffer to dissociate immune complexes present in the sample

b) applying the sample comprising anti-rituximab antibodies to magnetic beads coupled rituximab matrix

c) retrieving the anti-rituximab antibodies from the said matrix using a buffer comprising phosphoric acid

d) incubating the retrieved anti-rituximab antibodies with rituximab

e) subjecting the sample as obtained from step d) to a cell based CDC assay involving a target cell and,

f) detecting the neutralizing anti-rituximab antibodies by measuring the cytotoxicity of the target cell.

In another embodiment of the invention, the said buffer comprising phosphoric acid is at a pH value of about 2.0-2.5.
In one embodiment of the invention, the acidic buffer used for dissociating the immune complexes is a glycine buffer with a pH value of about 2.0-2.5.

The method is able to detect neutralizing anti-rituximab antibodies in presence of excess free rituximab and, wherein the method mitigates RF interference significantly.

In addition, the disclosed method can detect about 300 ng/ml of neutralizing anti-rituximab antibodies in presence of 80 μg of free rituximab/ml of the sample. Further, the method is able to detect neutralizing anti-rituximab antibodies in presence of high RF levels such as 650 units/ml.

The term ‘sample’ as used herein the invention refers to an RF positive plasma or serum sample obtained from patients who are not treated with rituximab and, in which case, the sample obtained is spiked with anti-rituximab antibodies and/or rituximab. However, the method as disclosed in the invention can be used for the detection of neutralizing anti-rituximab antibodies in RF positive plasma/serum sample obtained from patients treated with rituximab.

The term used here in “anti-rituximab antibody (ARiA)” refers to an antibody that is raised/form ed/produced against rituximab in animals including humans.

“P/N ratio” is a ratio between mean absorbance of positive control to mean absorbance of negative control. The ratio helps in normalization of data as it dissociates the signal from the background noise. This ratio is also known as highest signal to noise ratio.

“Positive control” as used here in the invention, is a sample (plasma / serum from RF positive RA patients or plasma/serum from healthy volunteers) spiked with neutralizing anti-rituximab antibody. Some of the positive controls are spiked with rituximab to mimic the physiological condition of rituximab treated patient’s sample and/or to evaluate drug tolerance. The term ‘drug tolerance’ as is defined as the maximal amount of free drug i.e., rituximab, present in the sample that still results in detectable neutralizing anti-rituximab antibody signal. Hence, to determine the drug tolerance, samples from RF positive patients (not treated with rituximab) are spiked with high concentrations of rituximab and neutralizing anti-rituximab antibodies.
“Negative quality control (NQC)” is an unspiked sample (pooled plasma / serum from healthy volunteers) in which no anti-rituximab antibodies and rituximab have been added, whereas “Negative control (NC)” is an unspiked sample obtained from individual RF positive rheumatoid arthritis patient, wherein no anti-rituximab antibodies and rituximab have been added.

“Sensitivity” of the assay is defined as the lowest concentration of positive control antibody preparation which consistently provides signal in the assay.

“RFPHP” is the rheumatoid factor positive individual human plasma samples obtained from rheumatoid arthritis patients.

“NPHP” is normal pooled human plasma samples from healthy volunteers obtained from authorized blood banks.

“Cut point” is defined as the level of response at or below which a sample is defined to be positive and above which the sample is termed as negative for neutralizing activity of anti drug antibodies towards the drug product and it is determined in terms of % cytotoxicity of NC to % cytotoxicity of NQC (NC/NQC). NC/NQC ratio of 1 indicates no neutralization while presence of NAb would result in NC/NQC ratio of < 1.

Certain specific aspects and embodiments of the invention are more fully described by reference to the following examples. However, these examples should not be construed as limiting the scope of the invention in any manner.

EXAMPLES

During the development of the assay for detection of neutralizing anti-rituximab antibodies (ARiA) in a rheumatoid factor positive rheumatoid arthritis patient’s sample, various parameters were tried and optimized to check the compatibility of such parameters on performance of CDC assay. One such critical parameter was the choice of buffer and, phosphoric acid buffer was selected for the retrieval of anti-rituximab antibodies and the same buffer was checked for its compatibility with the cell based CDC assay.
In addition, another parameter i.e., fixed concentration of rituximab to be used in the assay was selected by performing the dose response curve (DRC). The concentration is selected such that it lies on the linear part of the curve and is able to detect the neutralizing activity of anti-rituximab antibodies.

Example 1: Sample preparation

Plasma samples obtained from various rheumatoid factor positive individual RA patients were spiked with rat-anti-rituximab antibodies (positive control – rheumatoid factor positive individual human plasma samples from RA patients (RFPHP)). To compare the study with normal samples, plasma samples were collected from normal healthy individuals, pooled, and spiked with rat-anti-rituximab antibodies (positive control – Normal Pooled Human Plasma (NPHP)). Further, some of the positive controls were spiked with rituximab to mimic the physiological condition of rituximab treated (drug administered) RA patients and to determine the drug tolerance level of the assay. The spiked samples were incubated at 37 °C for 1h under shaking conditions (500 rpm) to allow the formation of immune complexes. For a comparative analysis, the unspiked samples were also subjected to identical experimental conditions. The samples were diluted in glycine buffer in 1: 5 ratio and incubated at 37 °C for 30 ± 10 mins with shaking at 500 rpm for dissociation of immune complexes.

Example 2: Retrieval of anti-rituximab antibodies

20 mM phosphoric acid buffer, pH 2.0 to 2.2, was selected to be used as an elution buffer for the retrieval of ARiA and hence checked for its compatibility with WIL2-S cells and in turn the CDC assay, prior to its employment in the retrieval process.

CDC assay with WIL2-S cells was performed with 20 mM phosphoric acid buffer and assay media (RPMI 1640 medium, 10% Fetal Bovine Serum (FBS), 1mM Sodium pyruvate, 25 mM HEPES and penicillin and streptomycin) separately. Two set of samples were prepared by mixing varying concentrations of rituximab separately in assay media and in phosphoric acid buffer (neutralized to pH 7.2 to 7.4 with 0.2 M disodium hydrogen phosphate). The samples comprising rituximab were incubated for 2 h ± 30 mins in a humidified CO₂ incubator in the respective buffers. Aliquot (50 µl) of pre-incubated rituximab samples were transferred to separate wells
of a 96 well micro titer plate and 50 μl of WIL2-S cell suspension (at a cell density of 0.2×10^6 cells/ml) were added to each well. Followed by which, 50 μl of 1:8 diluted baby rabbit complement (dilution were done in assay medium) was added immediately to each well and the plates were incubated for 120 minutes at 37 °C in 5% CO₂ incubator. After incubation, 50 μl of Alamar blue® (Invitrogen) was added to each well and incubated for 18 ± 2 h in a humidified CO₂ incubator maintained at 37 °C with 5% CO₂. Viability of cells were then measured in terms of relative fluorescence units with an excitation max at 530 nm and emission max at 590 nm using a spectrofluorometer. Results of the assay, represented in table 1, were calculated in terms percentage of cytotoxicity based on the following formula:

\[
\text{Percentage cytotoxicity} = \frac{\text{Mean Relative Fluorescence Units (RFU) of Cell + lysis}}{\text{mean RFU of Drug Control/Mean RFU of Cell + lysis}} \times 100
\]

Table 1: Percentage cytotoxicity of rituximab in presence of phosphoric acid buffer and assay media.

<table>
<thead>
<tr>
<th>Rituximab (ng/ml) Drug Control (DC)</th>
<th>Assay Media</th>
<th>Phosphoric acid buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>500</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>250</td>
<td>61</td>
<td>66</td>
</tr>
<tr>
<td>125</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>62.5</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Cell+ lysis (complement)</td>
<td>39</td>
<td>42</td>
</tr>
</tbody>
</table>

From the above results (also plotted as Figure 1), it was evident that % cytotoxicity obtained with the use of phosphoric acid buffer is similar to that of the assay medium. Hence, phosphoric acid buffer was selected for elution of the anti-rituximab antibodies and for its continuous usage in the CDC assay. Thus in the proposed invention, the methodology used does not involve a buffer exchange step. Dissociated immune complexes comprising ARiA were retrieved using commercially available magnetic beads (Dynabeads® M-280 Tosylactivated, Invitrogen) that are covalently coupled to rituximab. As a first step, rituximab was coupled to the magnetic beads by incubating rituximab with the magnetic beads as instructed by the product insert. Followed by which, the rituximab coupled magnetic beads were
transferred to an eppendorf, washed with phosphate buffer saline (PBS, pH 7.2-7.4) solution to equilibrate the beads and 1M tris buffer was added to each tube and the tube was placed on a magnet. The eppendorf containing beads were removed from the magnet and dissociated immune complexes comprising ARiA sample was added to magnetic beads coupled rituximab. The content was mixed thoroughly by pipetting and the mixture was incubated at 25 °C for 1 h with end to end mixing.

Post incubation, the tubes containing magnetic beads coupled rituximab and anti-rituximab antibodies were placed on a magnet, until the beads were migrated to the side of the tube and unbound supematant was removed. The beads were then suspended in phosphate buffer saline solution and the tube was placed on a magnet until the beads migrated to the side of the tube and the supematant was pipetted off. The above wash step was repeated two times to remove unbound material. Followed by which, 500 µl of elution buffer containing phosphoric acid (pH 2.0-2.2) was added to the tubes containing magnetic beads coupled rituximab for the elution of bound ARiA and the tubes were placed on a magnet till the beads were migrated to the sides of tube. Supernatant was collected and transferred to a fresh tube containing 250 µl of disodium hydrogen phosphate buffer and 8 µl of 2.5% BSA.

Two such elutes were collected from magnetic beads coupled rituximab by adding 500 µl of 20 mM phosphoric acid buffer and such elutes were transferred to the tubes comprising disodium phosphate and 2.5% BSA. Finally, the retrieved ARiA was concentrated by using Amicon® (ultra 0.5 ml, Millipore) 30 kDa filters.

Example 3: CDC assay methodology for detection of NAb

1) Sample preparation for CDC assay:

To prepare a sample for CDC assay, equal volume of retrieved and concentrated ARiA was incubated with an equal volume of fixed concentration of rituximab (125 ng/ml), that was selected using DRC.

A fixed concentration of rituximab, different concentrations of rituximab from 3.96 ng/ml to 2000 ng/ml, was prepared by diluting it in assay medium. 50 µl of different concentrations of rituximab was transferred to separate wells of 96 wells micro titer plate and 50 µl of WIL2-S cell suspension (at a cell density of 0.2×10^6 cells/ml) were added to each well. Followed by which, 50 µl of 1:8 diluted baby rabbit complement (dilution were done in assay medium) was added immediately to each
well and the plates were incubated for 120 minutes at 37 °C in 5% CO₂ incubator. After incubation, 50 µl of Alamar blue® (Invitrogen) was added to each well and incubated for 18 ± 2 h in a humidified CO₂ incubator maintained at 37 °C with 5% CO₂. Viability of cells were then measured in terms of relative fluorescence units with an excitation max at 530 nm and emission max at 590 nm using a spectrofluorometer. Results were represented in figure 1.

From the above results (also plotted as Figure 2), 125 ng/ml of fixed concentration of rituximab was selected from the linear part of the DRC for incubating it with anti-rituximab antibodies. Hence, equal volume of 100 µl of concentrated anti-rituximab antibody was added to 100 µl of rituximab (125 ng/ml) and the mixture was incubated for 2 h ± 30 min.

Various controls were maintained in CDC assay to know the background noise and details were given below table 2.

Table 2: Description of various controls used in CDC assay

<table>
<thead>
<tr>
<th>Controls</th>
<th>Description</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC (Cell Control)</td>
<td>Indicates total number of cells added to the plate</td>
<td>Cells only</td>
</tr>
<tr>
<td>C+ Lysis (Complement control)</td>
<td>Indicates background lysis due to complement alone</td>
<td>Cells + Complement</td>
</tr>
<tr>
<td>DC (Drug Control)</td>
<td>Indicates combined cytotoxicity induced by drug and complement</td>
<td>Cells + Complement + rituximab</td>
</tr>
<tr>
<td>PC-DC (Positive Control)</td>
<td>Indicates cytotoxicity of drug in presence of Nab</td>
<td>Cells + Complement + Drug (rituximab) + rat-ARiA (Nab)</td>
</tr>
<tr>
<td>PC-C+ Lysis (Background)</td>
<td>Indicates background lysis due to complement alone in presence of NAb</td>
<td>Cells + Complement + rat-ARiA</td>
</tr>
</tbody>
</table>

2) CDC assay methodology:

WIL2-S cells were employed in CDC assay to assess the neutralizing or non-neutralizing nature of anti-rituximab antibodies, in the presence of complement component and rituximab. WIL2-S cells were cultured in growth medium (RPMI 1640 medium, 10% FBS, 1mM sodium pyruvate, 25 mM HEPES and penicillin and
streptomycin) at 37 °C in a humidified CO₂ incubator and in presence of 5% CO₂ for required period of time.

Aliquot of 50 µl of pre incubated rituximab-anti-rituximab antibody reaction mixture was taken from the sample which was prepared by incubation of equal volume of ARiA and rituximab as described in example 4 and was transferred to each well of 96 micro titer plate. 50 µl of WIL2-S cell suspension (at a cell density of 0.2×10⁶ cells/ml) were added to each well. Followed by which, 50 µl of 1:8 diluted baby rabbit complement (dilution were done in assay medium) was added immediately to each well and the plates were incubated for 120 minutes at 37 °C in 5% CO₂ incubator. After incubation, 50 µl of Alamar blue® (Invitrogen) was added to each well and incubated for 18 ± 2 h in a humidified CO₂ incubator maintained at 37 °C with 5% CO₂. Viability of cells were then measured in terms of relative fluorescence units with an excitation max at 530 nm and emission max at 590 nm using a spectrofluorometer.

Results were calculated in terms of percentage cytotoxicity, percentage of neutralization and P/N ratio as per the below given formulas.

\[
\text{Percentage cytotoxicity of positive control} = \frac{\text{Mean RFU of PC} - C + \text{lysis} - \text{mean RFU of PC} - DC''}{\text{Mean RFU of PC} - C + \text{lysis}} \times 100
\]

\[
\text{Percentage cytotoxicity of negative control} = \frac{\text{Mean RFU of C} + \text{lysis} - \text{mean RFU DC}}{\text{Mean RFU of C} + \text{lysis}} \times 100
\]

\[
\text{Percentage neutralization} = \left[ \frac{\text{cytotoxicity of negative control} - \text{% cytotoxicity of positive control}}{\text{cytotoxicity of negative control}} \right] \times 100
\]

\[
\frac{P}{N} \text{ ratio} = \% \text{ cytotoxicity of positive control} + \% \text{ cytotoxicity of negative control}
\]

3) Determining the sensitivity of the assay in detection of NAAb

Sensitivity and high drug tolerance of the assay was determined by spiking various concentrations of rat-anti-rituximab antibodies from 200 ng/ml to 700 ng/ml in
NPHP sample containing 80 μg/ml of fixed concentration of rituximab and all the samples were prepared in duplicates.

These prepared samples were incubated for 1 h at 37 °C with shaking at 500 rpm to form the immune complexes and glycine buffer was added to the sample containing immune complexes in 1:5 ratio for the dissociation of immune complexes and the mixture was incubated at 37 °C for 30 ±10 minutes with shaking at 500 rpm.

Anti-rituximab antibody was retrieved and concentrated using magnetic beads coupled rituximab as described in example 2. Then, 100 μl of concentrated ARiA was incubated with 100 μl of 125 ng/ml of rituximab for 2 h at 37 °C in a humidified CO₂ incubator.

Then, 50 μl of aliquot of the above mixture was subjected for CDC analysis as described above (subsection 2) to assess the neutralizing/non neutralizing nature of the ARiA towards rituximab. Results of the assay were measured in terms of P/N ratio which is given in table 3. The assay cut point (NC/NQC) was determined to be 0.96.

Table 3: P/N ratios of samples containing varying concentrations of ARiA for the calculation of sensitivity of the assay.

<table>
<thead>
<tr>
<th>Positive control Samples</th>
<th>P/N ratio</th>
<th>Cut point</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRC1</td>
<td>DRC2</td>
<td></td>
</tr>
<tr>
<td>PC1 (0.7 μg/ml of rat-ARiA+ 80 μg/ml of rituximab)</td>
<td>-0.04</td>
<td>-0.07</td>
<td>Positive-</td>
</tr>
<tr>
<td>PC2 (0.6 μg/ml of rat-ARiA+ 80 μg/ml of rituximab)</td>
<td>0.21</td>
<td>0.29</td>
<td>Positive</td>
</tr>
<tr>
<td>PC2 (0.5 μg/ml of rat-ARiA+ 80 μg/ml of rituximab)</td>
<td>0.59</td>
<td>0.74</td>
<td>Positive</td>
</tr>
<tr>
<td>PC2 (0.4 μg/ml of rat-ARiA+ 80</td>
<td>0.85</td>
<td>0.80</td>
<td>Positive</td>
</tr>
</tbody>
</table>
From the above results, it was evident that, the disclosed method is able to detect 300 ng/ml of anti-rituximab neutralizing antibodies in presence of high amounts of rituximab (80 µg/ml).

5 Example 4 Assessment of RF tolerance:

The RF interference in the NAb assay was tested using plasma samples that were obtained from 5 individual rheumatoid arthritis donors with RF in the range of (25 to 646 units/ml). Samples were spiked with 80 µg/ml of rituximab antibodies and few samples were spiked with anti-rituximab antibodies to assess their neutralizing and non-neutralizing nature on rituximab activity. In addition, samples were prepared in NPHP (which contained ≤ 20 RF units/ml of sample) in a similar way as that of the samples prepared for RF positive individual plasma samples to compare and assess the mitigation of RF interference.

The spiked and unspiked samples were incubated to allow the formation of immune complexes and dissociated the formed immune complexes as per the conditions mentioned in example 1. Followed by, the retrieval of ARiA from the samples containing dissociated ARiA by the procedure described in example 2 and similar procedure was performed for the samples which were not spiked with ARiA to remove any drug and/or RF interferences. Then, aliquot of 100 µl retrieved and concentrated samples were incubated with 100 µl of 125 ng/ml of rituximab for 2 h ± 30 mins in a humidified CO₂ incubator. Followed by, the performance of CDC assay by the procedure as described in example 4. Results were calculated in terms of % cytotoxicity and % recovery of ARiA by below given formulas and were also represented in table 4 and 5 respectively.
% recovery = \left\{ \frac{\% \text{ Neutralization in RA individual plasma}}{\% \text{ Neutralization in normal pooled human plasma}} \right\}

Table 4: % Cytotoxicity of rituximab spiked individual RA patient’s sample and spiked NPHP

<table>
<thead>
<tr>
<th>Plasma details</th>
<th>RF (units/ml)</th>
<th>Average % cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma containing 80 µg/ml free rituximab</td>
</tr>
<tr>
<td>NPHP</td>
<td>&lt; 20</td>
<td>55±9</td>
</tr>
<tr>
<td>RA-IP3</td>
<td>25.3</td>
<td>54±5</td>
</tr>
<tr>
<td>RA-IP2</td>
<td>70.0</td>
<td>58±5</td>
</tr>
<tr>
<td>RA-IP5</td>
<td>131.5</td>
<td>55±5</td>
</tr>
<tr>
<td>RA-IP4</td>
<td>354.9</td>
<td>54±5</td>
</tr>
<tr>
<td>RA-IP1</td>
<td>646.0</td>
<td>57±8</td>
</tr>
</tbody>
</table>

Table 4 represents comparison of % cytotoxicity of unspiked samples at 125 ng/ml rituximab and Table 5 shows recovery of neutralizing activity in individual RA plasma samples spiked with 400 ng/ml ARiA.

Table 5: Recoveries of NAb in RA individual plasma samples

<table>
<thead>
<tr>
<th>Plasma details</th>
<th>RF factor (units/ml)</th>
<th>% Neutralization of CDC activity with 400 ng/ml ARiA complexed with 80 µg/ml rituximab</th>
<th>% Recovery</th>
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<td>646.0</td>
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From the above results, it is evident that, comparable cytotoxicity and neutralization was observed in RA individual plasma samples with respect to normal pooled plasma samples which indicates that there is no considerable RF interference in the assay. Further, the % recovery of ARiA is in the range from 100-120%.
CLAIMS

1. A method for detecting neutralizing anti-rituximab antibodies in a rheumatoid factor positive sample comprising the steps of;

   a) pre-treatment of the sample with an acidic buffer to dissociate immune complexes present in the sample

   b) applying the sample from step (a) comprising anti-rituximab antibodies to magnetic beads coupled rituximab matrix

   c) retrieving the anti-rituximab antibodies from the said matrix using a buffer comprising phosphoric acid

   d) incubating the retrieved anti-rituximab antibodies with rituximab

   e) subjecting the sample as obtained from step (d) to a cell based CDC assay comprising a target cell and,

   f) detecting the neutralizing anti-rituximab antibodies by measuring the cytotoxicity of the target cell

   wherein the said method mitigates RF interference.

2. A method according to claim 1, wherein the acidic buffer used for dissociating the immune complexes is a glycine buffer with a pH value of about 2.0-2.5.

3. A method according to claim 1, wherein said buffer comprising phosphoric acid is at a pH value of about 2.0-2.5.

4. A method for detecting neutralizing anti-drug antibodies in a rheumatoid factor positive sample comprising the steps of;

   a) pre-treatment of the sample with an acidic buffer to dissociate immune complexes present in the sample

   b) applying the sample from step (a) comprising anti-drug antibodies to magnetic beads coupled drug matrix

   c) retrieving the anti-drug antibodies from the said matrix using a buffer comprising phosphoric acid
d) incubating the retrieved anti-drug antibodies with fixed concentration of therapeutic antibody

   e) subjecting the sample as obtained from step (d) to a cell based CDC assay comprising a target cell and,

   f) detecting the neutralizing anti-drug antibodies by measuring the cytotoxicity of the target cell

wherein the said method, mitigate RF interference significantly.

5. A method according to claim 4, wherein the said drug is a therapeutic antibody.

6. A method according to claim 5, wherein the therapeutic antibody includes chimeric or humanized or human antibody.
Figure 2
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/53, G01N33/68 Version=2015.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

IPO-INTERNAL, PATSEER

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
  “A” document defining the general state of the art which is not considered to be of particular relevance
  “E” earlier application or patent but published on or after the international filing date
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  “O” document referring to an oral disclosure, use, exhibition or other means
  “P” document published prior to the international filing date but later than the priority date claimed
  “T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  “X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  “Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  “&” document member of the same patent family

Date of the actual completion of the international search
17-06-2015

Date of mailing of the international search report
17-06-2015

Name and mailing address of the ISA/Indian Patent Office
Plot No.32, Sector 14, Dwarka, New Delhi-110075

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Telephone No. +91-1125300200

Form PCT/ISA/210 (second sheet) (January 2015)
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