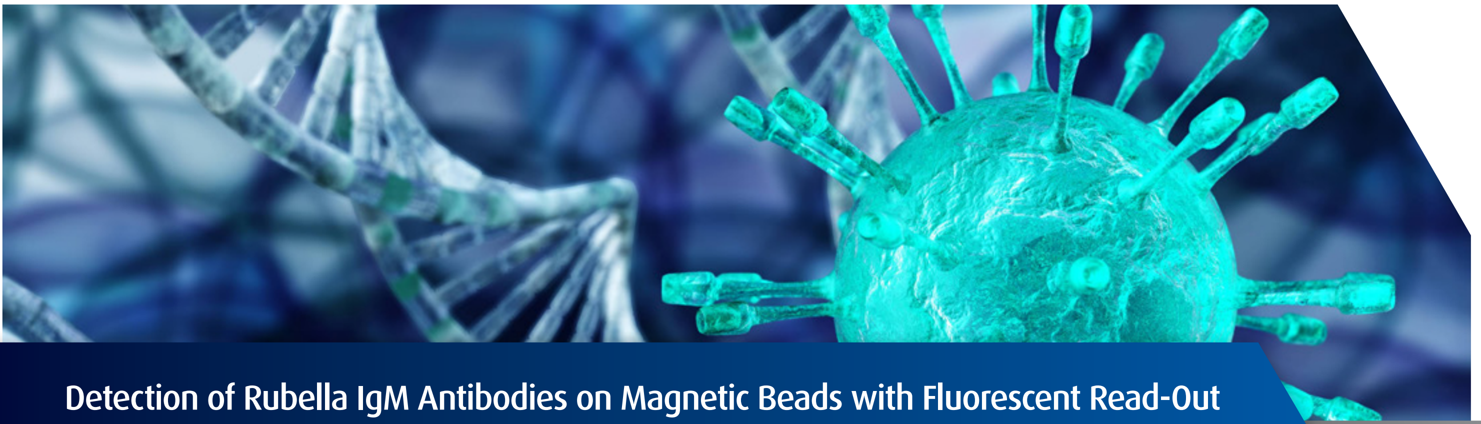


Detection of Rubella IgM Antibodies on Magnetic Beads with Fluorescent Read-Out

First Recombinant Rubella Spike Ectodomain (E1-E2) Antigen: Performance Comparison with Established IVD Rubella IgM Assays

*The work presented was carried out in collaboration with MilliporeSigma (is a subsidiary of Merck KGaA, Darmstadt, Germany)

Introduction

Serology: antibody detection via immobilized antigen

Serological diagnosis of infectious diseases is accomplished by detection of antibodies against antigens from pathogenic viruses, bacteria, parasites or fungi. These antigens are typically immobilized on a solid support. Upon application of serum samples, analyte antibodies may bind to the immobilized antigen and can subsequently be detected indirectly in a second incubation step with labelled detection antibodies. A very common in vitro diagnostic (IVD) approach is the enzyme-linked immunosorbent assay (ELISA) which is performed in microtiter plate format. This is, however, increasingly being replaced by bead-based methods.

Magnetic beads: automated coupling & assay workflow

Magnetic beads typically offer higher analytical sensitivity than immobilization on ELISA plates and they allow straightforward incorporation in automated IVD solutions, as the technology is already widely established in chemiluminescent immunoassays (CLIA). However, not even during the assay workflow but already during the coupling process of antigens one can take advantage of magnetic beads. Since coupling antigens on beads is a very crucial step in assay development and production, automated methods based on magnetic beads can contribute to more robust and reproducible processes.

New recombinant Rubella antigen

Rubella is a contagious viral infection typically occurring in childhood and mostly causing mild symptoms only. Rubella virus infection during pregnancy, however, can cause congenital rubella syndrome with serious damage to unborn babies. The global incidence of Rubella is approximately 100,000 cases per year, manifesting mainly in Southeastern Asia, India, China and Africa. Consequently, the diagnosis of Rubella virus infection, especially before and during pregnancy, is of considerable importance. Detection of Rubella IgM specific antibodies is used for monitoring infectious diseases associated with Rubella virus[1-3]. Here, a new recombinant Rubella antigen based on spike E1-E2 ectodomain (Patent disclosure notice WO 2021/005461) is used for the detection of Rubella IgM antibodies[4]. The E1-E2 heterodimers on the viral surface are the major target for neutralizing antibodies during infection.

Fluorescent readout

This technical note presents semi-automated processes, both for coupling Rubella antigen on magnetic beads and for performing a Rubella IgM assay workflow. Detection of Rubella IgM antibodies from human serum samples is carried out by fluorescent readout in a flow cytometer. Performance of the magnetic bead-assay is compared to established Rubella IgM assays.

Material and Methods

Estapor® Carboxylated Microspheres (-COOH) Ref. EM1-100/40, Cat. No 23 710 087

Lot	COOH groups density	COOH groups density / µeq/g	Mean diameter / µm	Magnetic pigment
M8965/12	Low	29	1.10	40.1 %
M9202/3	Medium	50	1.03	42.5 %
M8861/7	High	93	0.96	46.2 %

Rubella antigen

The new Rubella Spike Ectodomain (E1-E2) antigen is a capsid-free, highly pure recombinant antigen produced in insect cells. The sequence of the recombinant protein is derived from the Rubella vaccine strain HPV-77 and combines the ectodomains of glycoproteins E1 and E2, which are the major immunological targets of Rubella virus. The sequence and the eukaryotic expression system were carefully chosen to provide a reliable product for development of highly specific IgG and IgM detection assays.

Code	Description	Packaging
BA129R01	Rubella Spike Ectodomain (E1-E2) Antigen, recombinant	1 mg

Equipment

A magnetic particle processor (Thermo Scientific, KingFisher Flex), intended for automated transfer and processing of magnetic particles in a microplate format, was used for coupling of antigen onto magnetic beads and for running the Rubella IgM assay.

A flow cytometer (Beckman Coulter, Cytoflex S) was used for quantitative read-out of fluorescent assay signals.

Reagents for coupling

The carboxylated microspheres were activated with EDC (Thermo Scientific, 22980) and Sulfo-NHS (Thermo Scientific, 24510) in order to covalently immobilize the Rubella antigen.

After coupling of Rubella antigen, the beads were blocked with proteolytic degraded gelatin (Blocking Reagent for ELISA, Roche, 11112589001).

Blocked Rubella antigen beads were stored in StabilCoat® Plus Stabilizer (Surmodics, SC02-0050).

Reagents for assay

Serum samples from patients with suspected infections (n = 20), from an in-house inventory (n = 8), from healthy blood donors (n = 31) and pregnant women (n = 23) were tested. All the samples had been previously tested with Rubella IgM tests from Siemens (Enzygnost® Anti-Rubella-Virus/IgM, OWBO15) and Medac (Rubella-IgM-ELA Test PKS medac, 135-PKS). Prior to use, 5 µL serum were added to 500 µL dilution buffer (Virion\Serion, B431) and 100 µL rheumatoid factor absorbent buffer (Virion\Serion, Z200) and incubated for a minimum of 15 minutes.

Bound analyte was detected with R-Phycoerythrin-conjugated AffiniPure $F(ab')_2$ Fragment Donkey Anti-Human IgM, $Fc_{5\mu}$ Fragment Specific (Jackson Immunoresearch, 709-116-073) detection antibody. The antibody had been prepared 1:2000 in 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 4 mg/mL bovine serum albumin, 0.09% sodium azide, 0.02% Tween 20, pH 7.4 dilution buffer.

Assay scheme

Rubella antigen is immobilized covalently on magnetic beads. After incubation of the beads with human serum samples, a fluorescently labelled anti-human IgM detection antibody is added. The fluorescent signal is measured in a flow cytometer.

Coupling procedure

A procedure for coupling of antigen to magnetic particles was optimized by testing particles with different COOH-group densities and different concentrations of antigen in order to determine saturation level of the beads for Rubella antigen. The whole coupling procedure was performed in an automated fashion by a magnetic particle processor capable of transferring magnetic particles between 96-well microplates:

1st plate: preparation of beads – For an individual coupling per well, 50 x 10^7 magnetic beads were prepared in 100 µL 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 0.02% Tween 20, pH 7.4 dilution buffer.

2nd plate: activation – 100 µL EDC and NHS were prepared each 50 mg/mL in 10 mM $NaH_2PO_4 \cdot H_2O$ pH 6.0 activation buffer and incubated with beads for 20 minutes at room temperature.

3rd plate: washing step – 100 µL 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 0.02% Tween 20, pH 7.4 washing buffer was added per well.

4th plate: coupling – 100 µL antigen was prepared with different concentrations in 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, pH 7.4 coupling buffer and incubated with activated beads for 60 minutes at room temperature.

5th plate: washing step – 100 µL 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 0.02% Tween 20, pH 7.4 washing buffer was added per well.

6th plate: blocking – 100 µL 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 8 mg/mL gelatin, 0.09% sodium azide, 0.02% Tween 20, pH 8.4 blocking buffer was added per well and incubated with beads for 30 minutes at room temperature.

7th plate: washing step – 100 µL 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 0.02% Tween 20, pH 7.4 washing buffer was added per well.

8th plate: storage – 100 µL storage buffer was added per well.

Assay procedure

Like the coupling procedure, the assay was performed in an automated fashion by the magnetic particle processor. All reagents and samples were prepared in 96-well microplates:

1st plate: preparation of Rubella antigen beads – Beads were diluted in storage buffer to a concentration of 10^7 beads/mL (10 µg/mL) and 100 µL of bead suspension were added to each well.

2nd plate: sample incubation – 50 µL of diluted sample and 70 µL of storage buffer were added to each well and incubated with beads for 20 minutes at 37 °C.

3rd plate: washing step – 200 µL ELISA *classic* washing buffer (Virion\Serion, B232) was added per well.

4th plate: 50 µL of diluted detection antibody was added per well and incubated with beads for 20 minutes at 37 °C.

5th plate: washing step – 200 µL ELISA *classic* washing buffer (Virion\Serion, B232) was added per well.

6th plate: read-out – 150 µL 50 mM $NaH_2PO_4 \cdot H_2O$, 150 mM NaCl, 0.02% Tween 20, pH 7.4 read-out buffer were added per well.

Finally, the fluorescent signal was measured in a flow cytometer.

Statistical data analysis

Data obtained from flow cytometry (as FCS-files) were analyzed with FlowJo (v10.6.2) software. Median fluorescence intensity (MFI) from a minimum of 100 beads was used for further analysis. Method comparison was done using receiver operating characteristic (ROC) analysis by help of Analyse-it for Microsoft Excel 4.97 software.

Results

Coupling

A procedure for coupling of antigen to magnetic particles was optimized by testing particles with different COOH-group densities (low, medium and high) and different coupling concentrations of antigen (308 µg/mL – 4.81 µg/mL) in order to determine saturation level of the beads for Rubella antigen. Potential unspecific adsorption of analyte or IgM antibodies in general to the bead surface was tested by mock-coupling without antigen. The whole coupling procedure was performed in an automated fashion and all couplings were done in parallel.

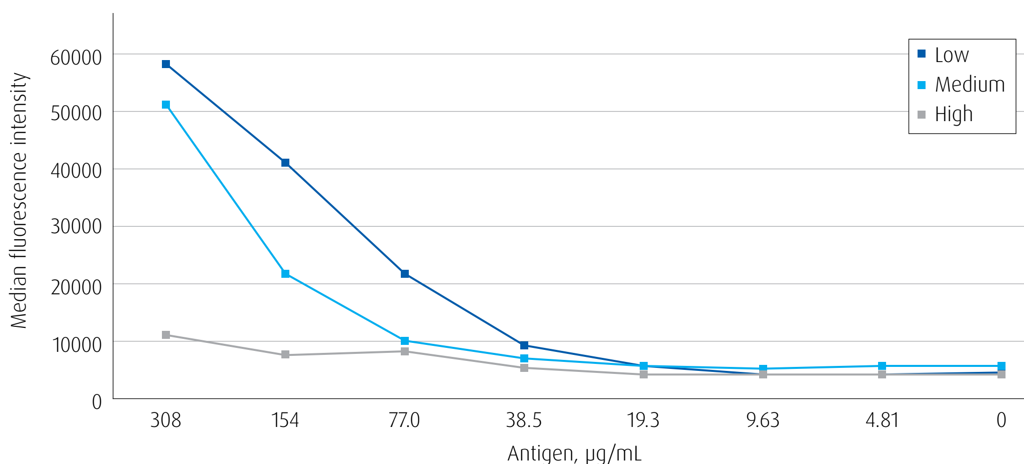


Figure 1: Magnetic beads with different COOH groups densities: titration of Rubella antigen.

The highest signals were achieved by incubation of Rubella IgM positive serum and antigen coated beads with the lowest COOH group density (Figure 1). Using the highest coupling concentration of Rubella antigen, the signal of beads with low COOH group density was more than 5 times higher than observed for beads with high COOH group density. The same behavior has been observed in the past and with other immunoassays for Estapor® Carboxylated Microspheres[5, 6].

In the experiments described here, a saturation level for antigen coating has not been reached. Due to limiting antigen availability at the time of testing, higher coupling concentrations have not been tested. It should be noted, that lowering the bead concentration or increasing the volume of antigen might also push the antigen coating towards saturation, but these conditions have not been tested here, too.

For the further method comparison study, coated beads with low COOH group density and highest Rubella antigen coupling concentration (308 µg/mL) were used.

Method comparison

In total, 82 human serum samples from patients with suspected infections, from an in-house inventory, from healthy blood donors and pregnant women were tested. All the samples had been previously tested with two commercial IVD tests from Medac and Siemens. Equivocal results of these two tests were excluded from method comparison. For the Medac test, 17 of the remaining samples were indicated as positive and 64 as negative. For the Siemens test, 16 of the remaining samples tested positive and 63 negative. Figure 2 displays the observed MFI measured with the Rubella IgM bead assay in comparison to the results of the two reference assays.

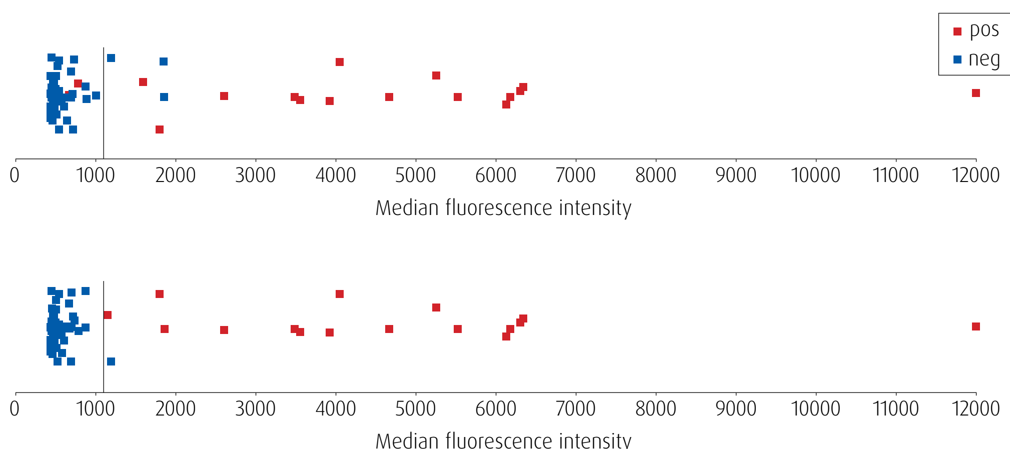
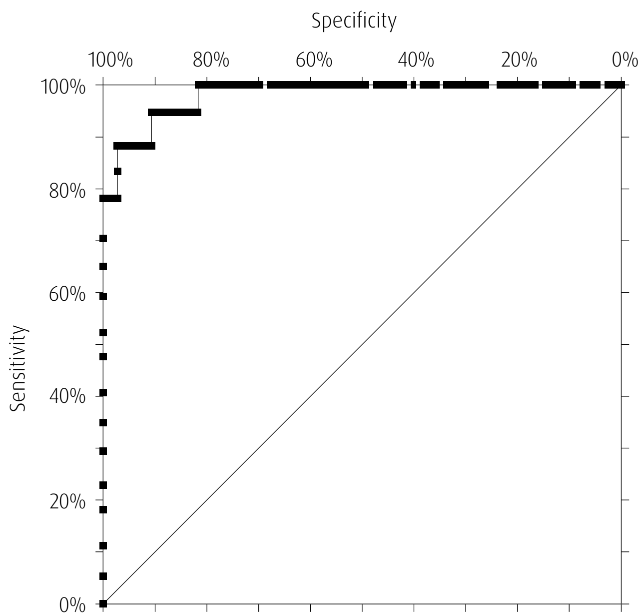


Figure 2: Distribution of MFI with human serum samples measured by Rubella bead assay and interpretation as positive or negative results as indicated by IVD tests from Medac (top) and Siemens (below). Single data points are arbitrarily distributed along y-axis for a better resolution.

A ROC analysis was performed in the next stage. Comparison with the Medac test revealed an Area under the Curve (AUC) of 0.981 based on a 95% confidence interval from 0.956 to 1.006. When comparing to the Siemens test an AUC of 0.999 was determined based on a 95% confidence interval from 0.996 to 1.002 (Figure 3).



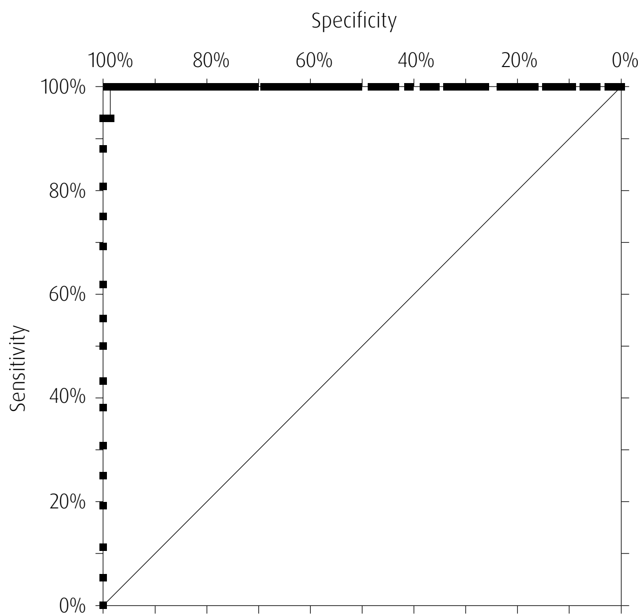


Figure 3: ROC analysis for method comparison with Medac test (top) and Siemens test (bottom).

A first cut-off value for the Rubella bead assay was set at 1100 MFI (vertical line in Figure 2) in order to minimize the number of false positive and false negative results in comparison to the reference tests. Using the Medac test as reference, 3 false positive and 2 false negative results were determined. In comparison to the Siemens test, however, 1 false positive and no false negative results were observed.

		Rubella Bead Assay		
		pos	neg	total
Rubella-IgM-ELA Medac	pos	15	2	17
	neg	3	61	64
	total	18	63	81

		Rubella Bead Assay		
		pos	neg	total
Siemens-Anti-Rubella-Virus/IgM	pos	16	0	16
	neg	1	62	63
	total	17	62	79

In comparison with the Medac test a sensitivity of 88.2% based on a 95% confidence interval from 65.7% to 96.7% and specificity of 95.3% based on a 95% confidence interval from 87.1% to 98.4% was achieved. In comparison with the Siemens test the sensitivity was 100.0% based on a 95% confidence interval from 80.6% to 100.0% and a specificity was 98.4% based on a 95% confidence interval from 91.5% to 99.7%. It should be noted here that a distinct cut-off was used for the Rubella bead assay. Introducing an equivocal zone around this cut-off would effectively result in smaller false-positive and false-negative numbers. Furthermore, comparison with the Medac test, which was a little poorer, may be impaired by the fact that this test uses a µ-capture protocol and the assay principles differ to some degree.

Conclusion

Although saturation of Rubella antigen on the magnetic beads had not been reached and their diameter is relatively small for effective use in flow cytometry, the observed results indicate impressively that Estapor® Carboxylated Microspheres are very well suited for detection of Rubella IgM antibodies.

Furthermore, the magnetic characteristics of Estapor® Carboxylated Microspheres facilitate easy setups for automated coupling processes and assay workflows.

The method comparison with Rubella IgM tests from Siemens and Medac revealed an excellent diagnostic potential of the presented bead-based Rubella IgM assay. In particular, the consensus with the Siemens test was found to be excellent.

In summary, it has been shown that the presented new recombinant Rubella Spike Ectodomain (E1-E2) antigen has proven to be a very promising candidate for replacement of the traditional native antigens used in the reference tests.

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For inquiries regarding Rubella Antigen please contact
Stefan Papadileris, International Sales Manager
Phone +49 931 3045 561 Mobile +49 931 3045 053
s.papadileris@virion-serion.de

For inquiries regarding **Estapor®** Carboxylated Microspheres
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