

# DEVELOPMENT, VALIDATION AND APPLICATION OF ADA ISOTYPE SPECIFIC ASSAYS

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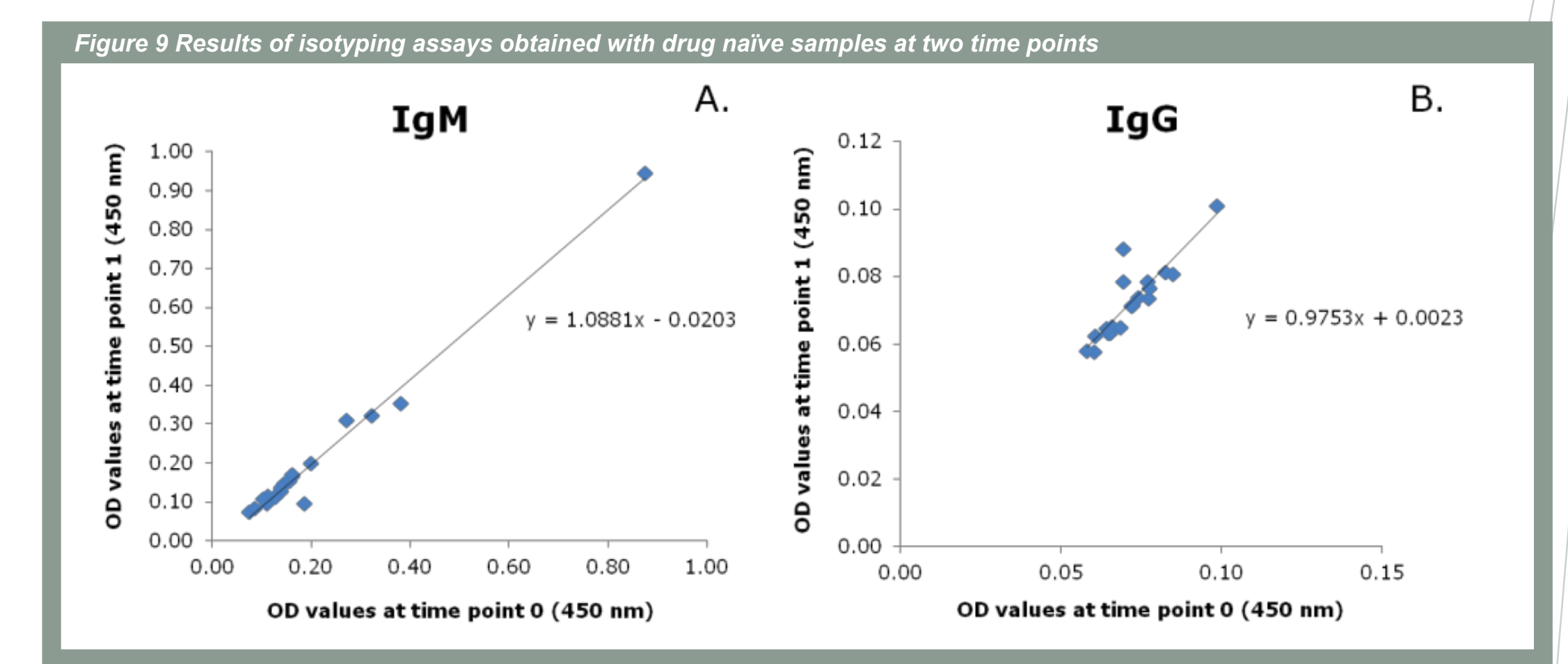
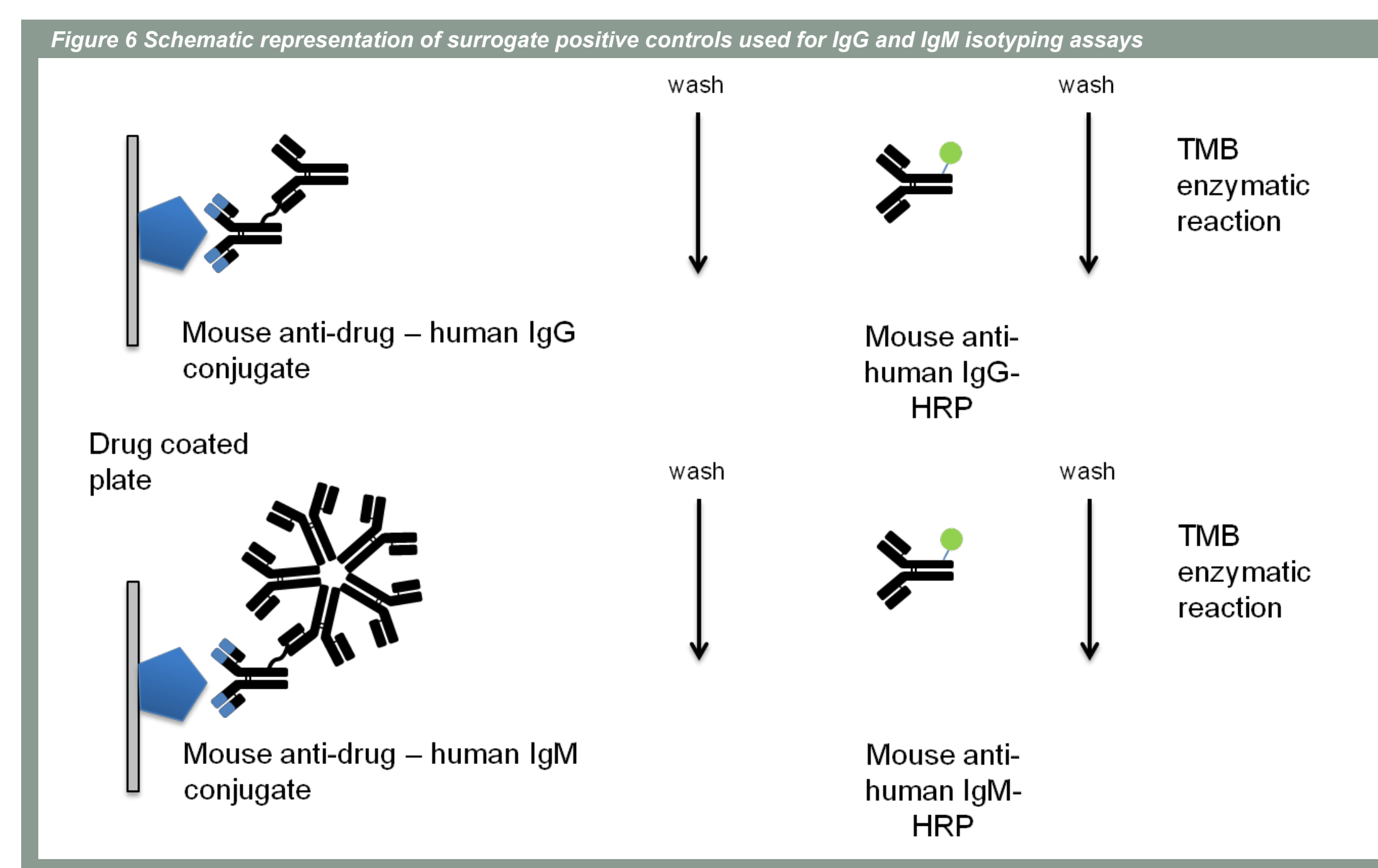


## INTRODUCTION

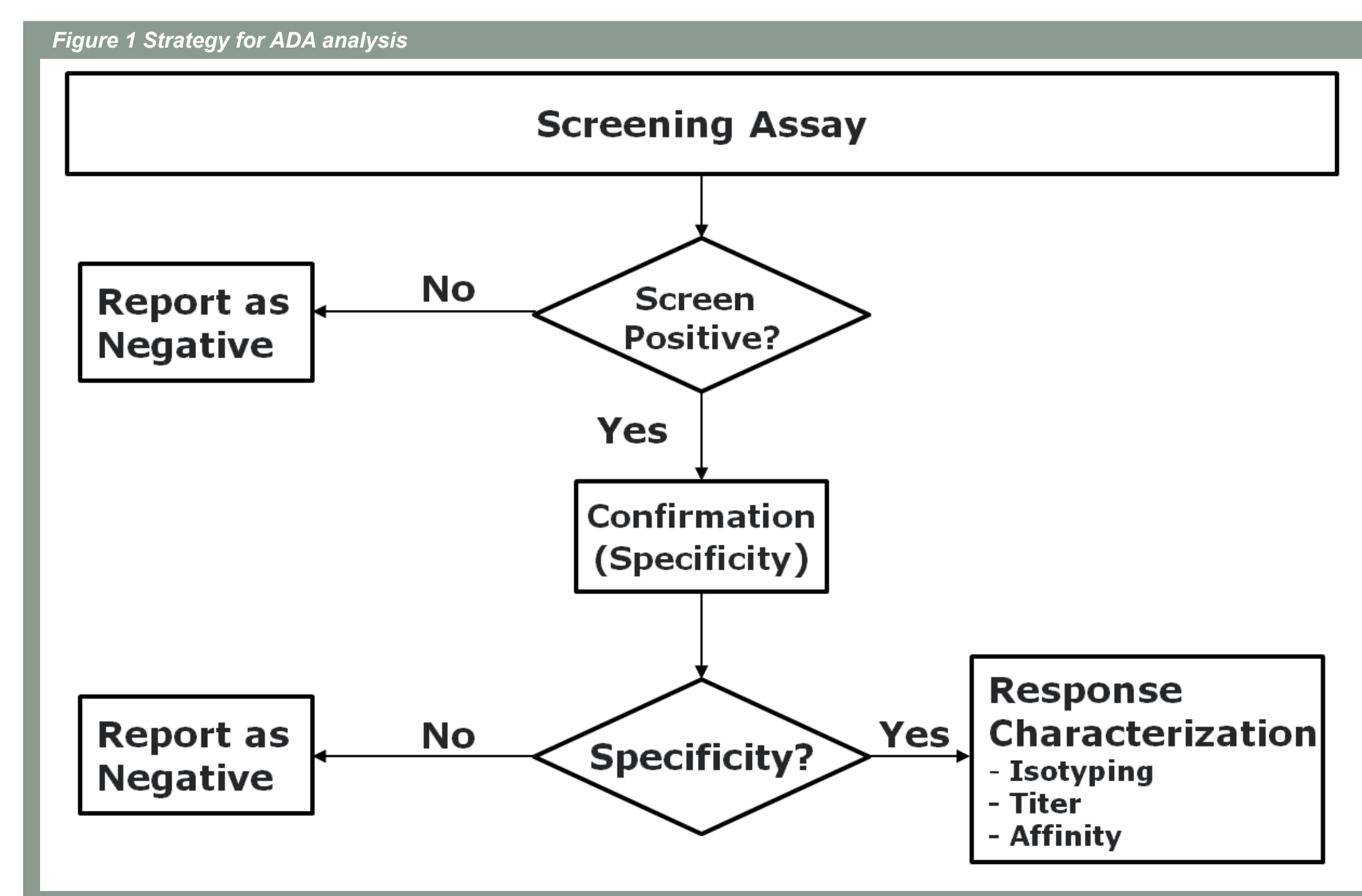
Immunogenicity of biopharmaceutical products is a major concern in both pre-clinical and clinical studies. Reliable validated assays for detection and characterization of anti-drug antibodies (ADA) are required. Beside screening and confirmatory assays, ADA-isotyping provides additional information that is important for the understanding of a particular immune response. In order to characterize immunogenicity of a drug (a serine protease, MW ca 50 KDa) in human, specific assays for both IgG and IgM ADAs were developed in our laboratory. For comparison of relative IgG and IgM responses, ADA-positive human plasma samples were subjected to both assays.

## ANALYTICAL METHODS

The immunogenicity-assessment strategy adopted in our laboratory included a screening assay, followed by specificity confirmation in a confirmatory assay. Positive samples were subjected to a titer assay for relative quantification. Further characterization included IgG, and IgM isotypes detection and titration (Fig 1). IgE ADA detection and ADA relative affinity determination were also performed but are not presented here.



Intra and inter run precisions were determined at 4 control levels. Selectivity was measured on 10 individual plasma spiked at 3 levels. Results of validations are summarized in Fig 10.



## DEVELOPMENT OF ISOTYPING ASSAYS

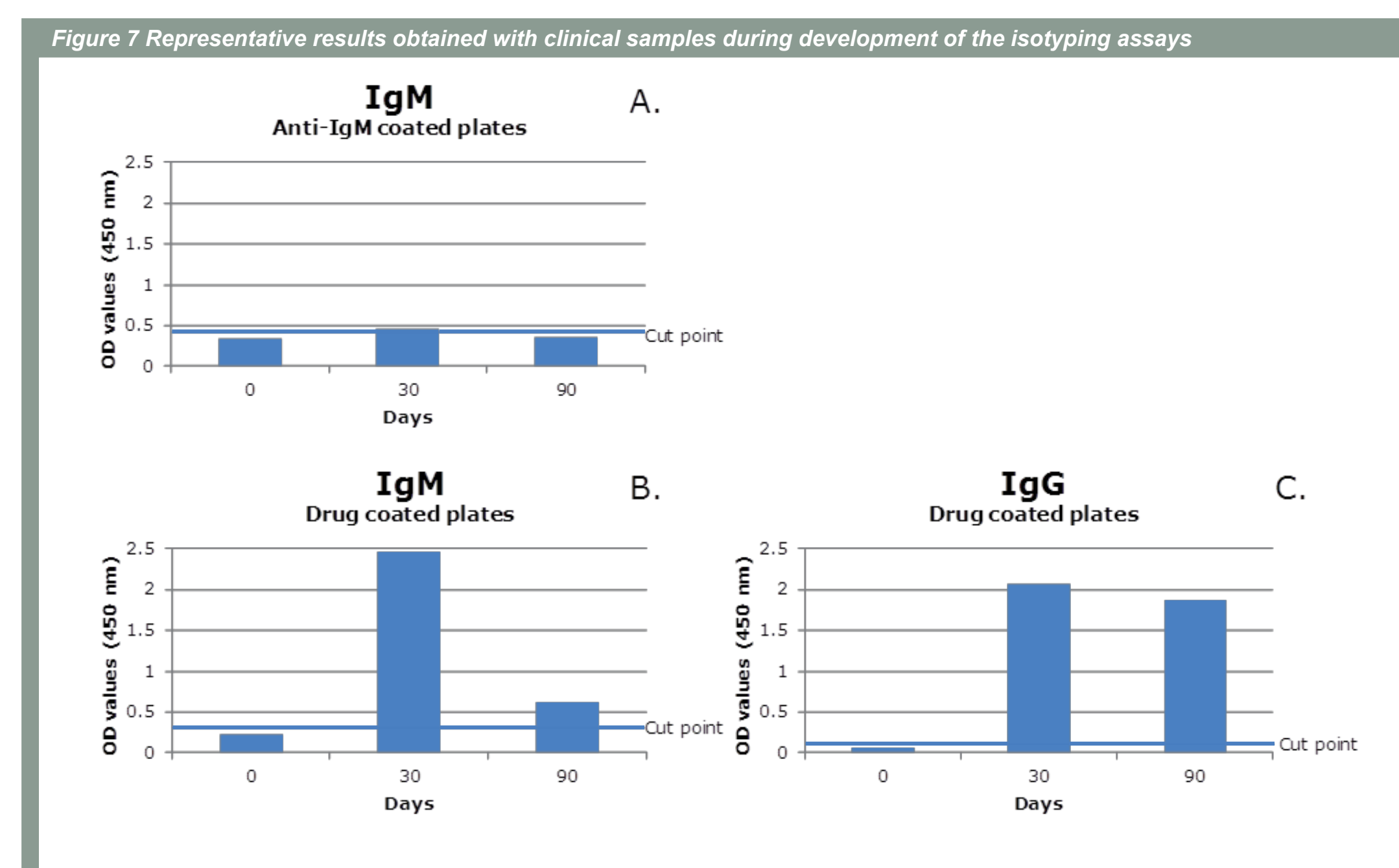
The critical steps of assay development included:

- Determination of best conditions for reducing unspecific binding
- Determination of the minimum required dilution (MRD)
- Adaptation of final concentrations of detection antibodies
- Detection of IgM ADA in presence of high titers of IgG
- Strategy for cut point calculation adapted to variation between individuals.

High background was a challenge successfully addressed by choosing suitable blocking conditions. Best results were obtained with 2% Casein blocking buffer. Best sensitivity and reduction of matrix effects were achieved with an MRD of 1:50 for both assays. Dilutions of detection antibodies were optimized for each test (1:8'000 and 1:100'000 for IgM and IgG, respectively). The ability to detect specific IgM ADA in presence of high titers of IgG was investigated in 2 assay formats. Clinical samples were included in method development and tested with either drug or anti-human IgM capturing format. Samples taken at three time points (0, 30 and 90 days post treatment) from patients subjected to a single dosage were used (Fig 7). A clear IgG interference was not detected. However, the format with drug as capture reagent showed better sensitivity and was chosen (Fig 7A and 7B). Therefore, this assay format was used for validation and sample analysis.

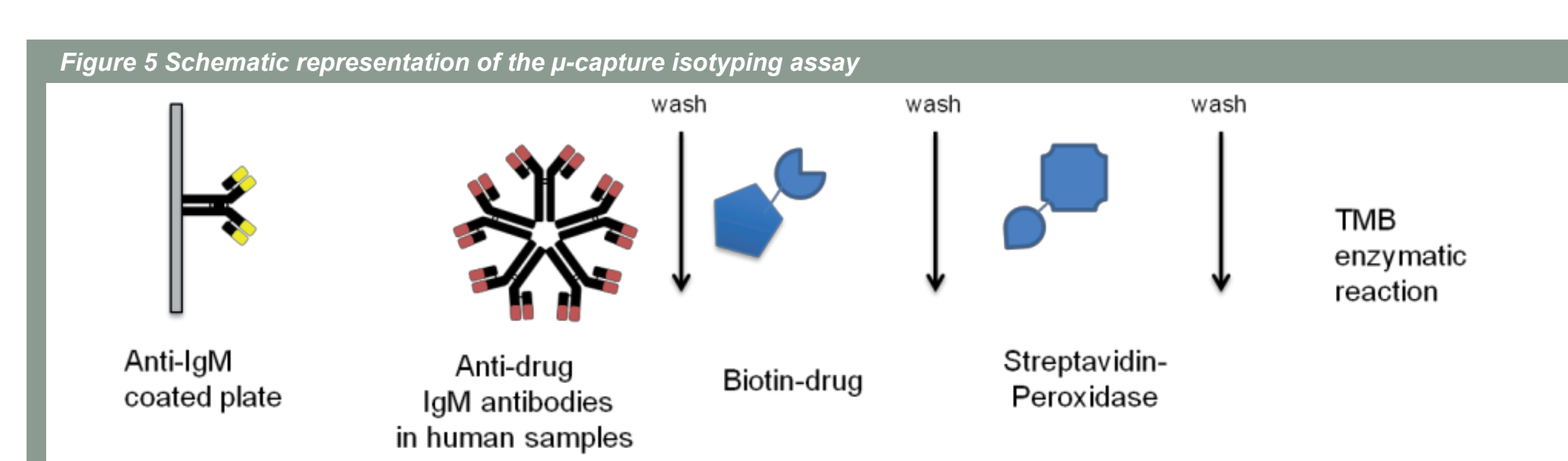
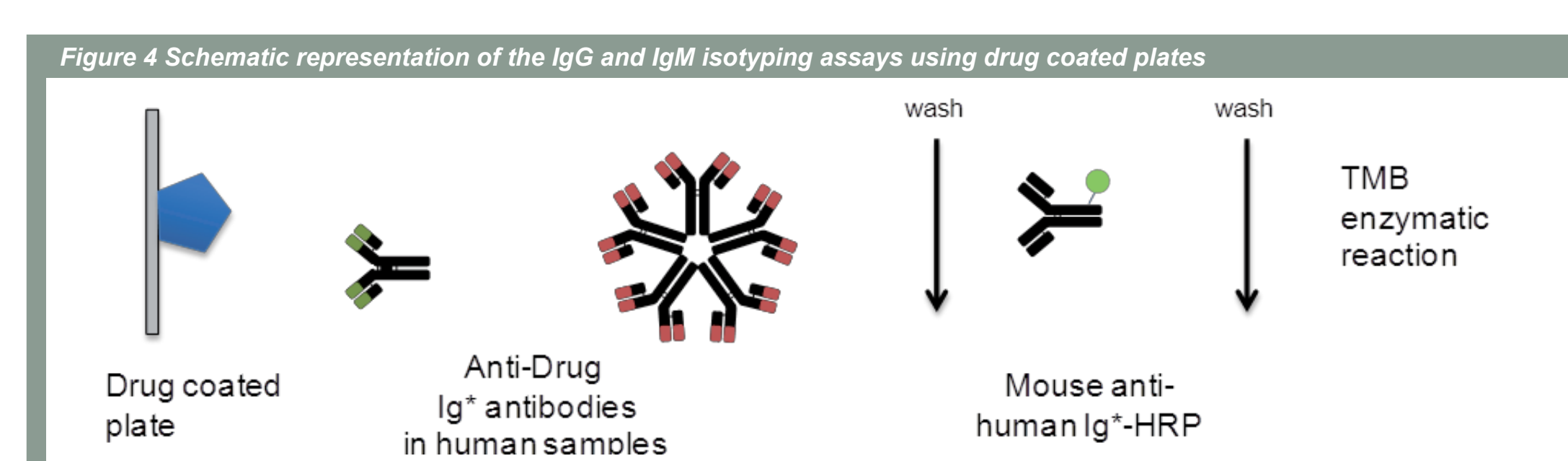
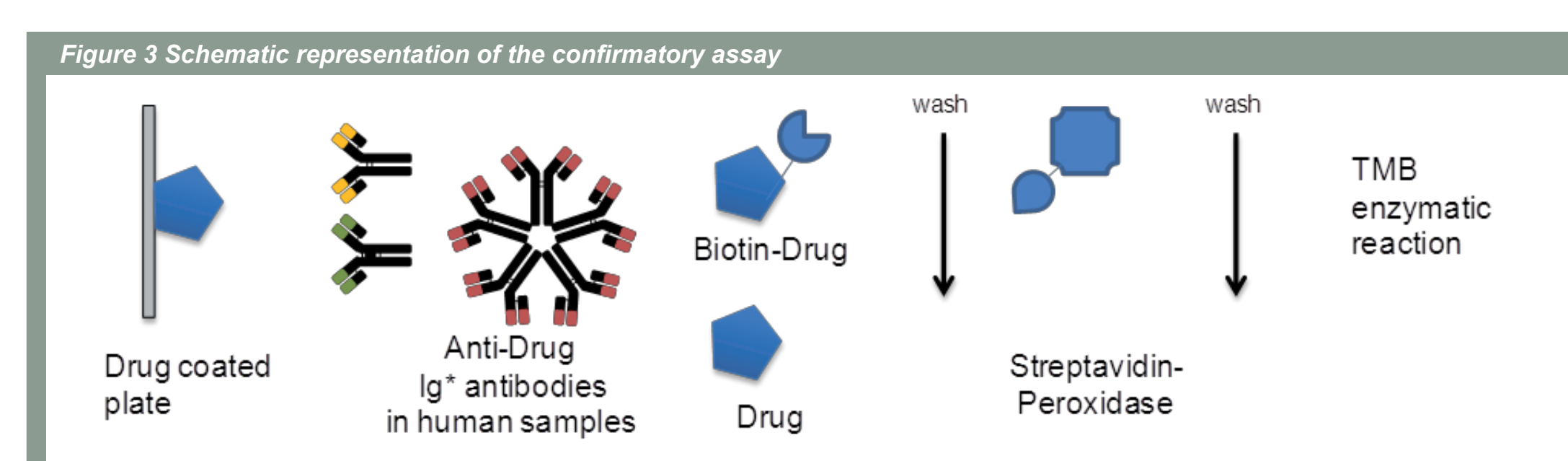
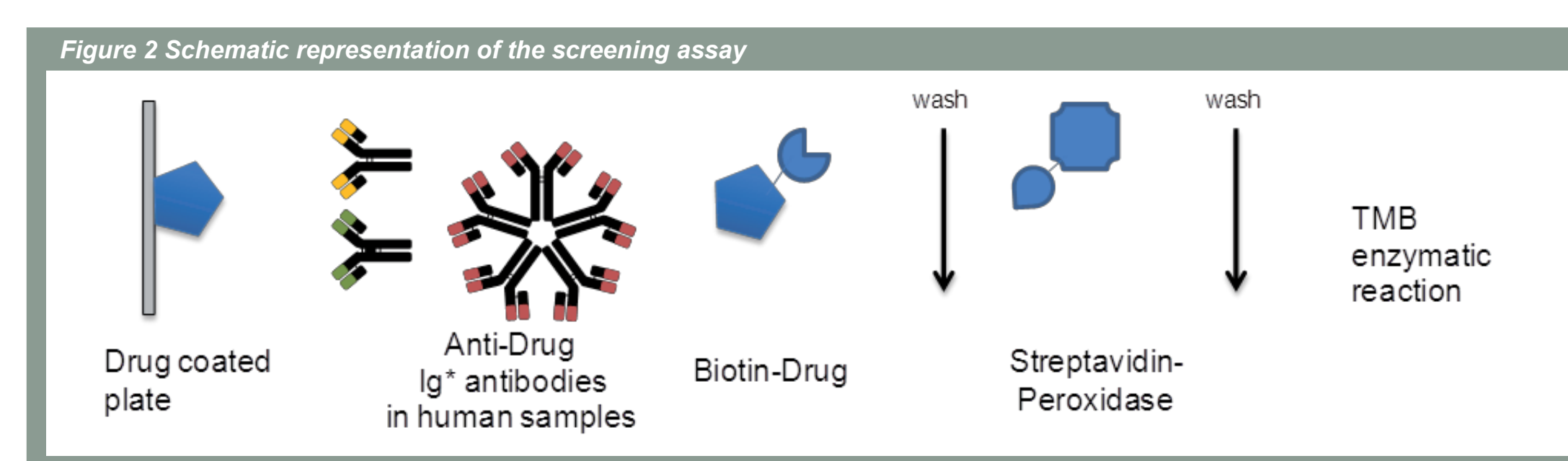
Parameter	IgM Assay	IgG Assay
Validation Cut Point (OD)	0.2494	0.0805
1% false positive targeted		
CF	1.30541	1.17523
Subject-specific cut point	OD pre-dose sample x CF	
Intra Run Precision (%CV) N=12 (NC, LPC, MPC, HPC)	1.9 - 3.9	2.6 - 5.6
Intra Run Precision (%CV) N=24 (NC, LPC, MPC, HPC)	8.1 - 11.5	13.3 - 28.6
Sensitivity (at 99% consistency level) N=6	7.450 µg/ml	1.367 µg/ml
Selectivity/Recovery N=10 (SD)	84.3 % (14.9)	109.7 % (9.2)
Stability 1h RT N=6 (SD)	HPC 93 % (1) LPC 88 % (4)	HPC 94 % (1) LPC 93 % (4)

For screening a bridging assay format was used, consisting of ADA capture on drug-coated microplates and detection by biotin-labeled drug and streptavidin peroxidase. (Fig 2). For specificity confirmation an excess of free drug was added to compete with labeled drug for plate-bound ADAs (Fig 3). Isotyping assays were either based on drug (for IgG and IgM assays) or anti-ADA (only for IgM assay) as capture reagent (Fig 4 and 5, respectively). The second format was investigated since high titers of IgG ADA in a sample could prevent binding of IgM ADA to the drug-coated plate. This IgG interference is less likely to influence an assay with anti-human IgM as capture reagent. The format of the ADA affinity assay was similar to the screening assay but included modifications of the coating conditions.



## CASE STUDY

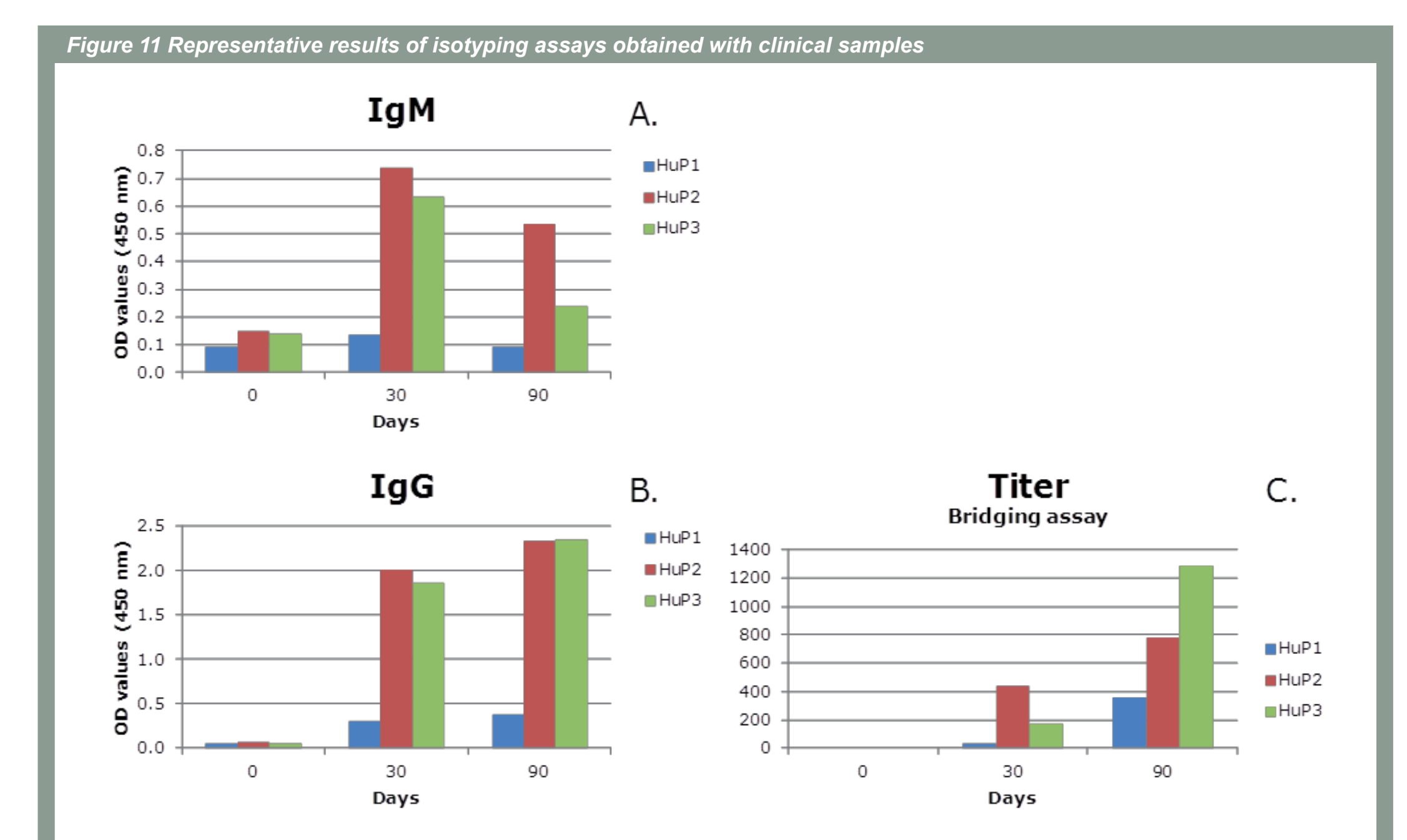
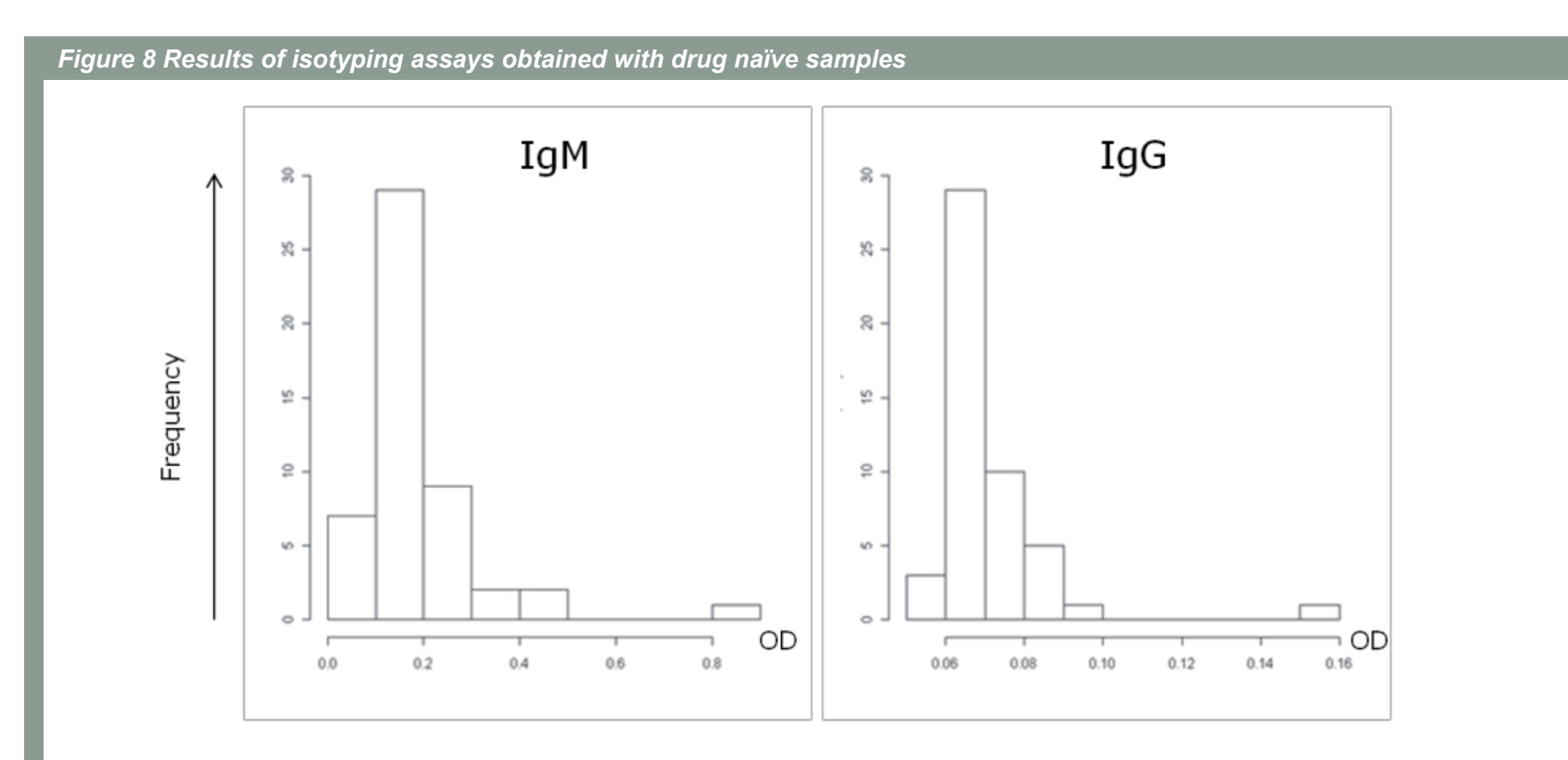
Patients were submitted to a single dosage and samples were taken at three time points (0, 30 and 90 days post treatment). Clinical samples shown to be ADA positive were titrated and further tested for both isotypes. Finally, isotype positive samples were titrated for each isotype specifically. Representative results are illustrated in Fig 11. A clear increase of both ADA isotypes was observed within the fixed time frame with specific kinetics for IgM and IgG ADA. IgM peaked at Day30 and were shown to decrease at Day90 (Fig 11A), whereas IgG were still highly present at Day90 (Fig 11B). Samples which showed OD values higher than HPC OD values in the isotyping assays were diluted before isotype titrations.



Sample specific cut points were calculated to take into consideration the observed variations between individuals. The adequacy of this approach was confirmed during validation.

## VALIDATION OF ISOTYPING ASSAYS

IgG and IgM assays were successfully validated based on current ADA validation standards. Fifty individual samples from healthy subjects were repeatedly tested to determine validation cut point and correction factor (Fig 8).



## DISCUSSION AND CONCLUSION

The design of surrogate positive control fusions permitted the development and validation of ADA isotyping assays to be used for analysis of human samples. The adequacy of the sample specific cut point approach was confirmed during validation. The isotyping assays presented here were shown to be sensitive, precise, specific and robust. It was demonstrated that low levels of IgM ADA could be detected even in the presence of high titers of IgG ADA.

## REFERENCES

- Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. Committee for Medical Products for Human Use. Adopted 13 Dec 2007. [www.ema.europa.eu/pdfs/human/biosimilar/1432706enfin.pdf](http://www.ema.europa.eu/pdfs/human/biosimilar/1432706enfin.pdf)

Controls were obtained by covalently linking an affinity purified mouse anti-drug IgG to human IgG or IgM (Fig 6). This procedure generated a conjugate specific for drug (mediated by mouse anti drug antibody) which could be detected by anti-human IgG or IgM, respectively (mediated by human IgG or IgM). The use of these fusion surrogate controls allows the analysis of human samples under the same assay system.

As seen of Fig 8, a variation between samples was observed. Therefore, it was decided to use sample specific cut points, each subject being its own control. The suitability of the sample specific cut point approach was confirmed by measuring drug naive samples collected at 2 time points from 20 patients within 10 days. Although the values obtained with IgM assay were more widely spread than for IgG, a high consistency was observed between the 2 time points (Fig 9). Thus, sample specific cut point calculation was indicated to take into consideration the observed variations between individuals. One sample was found to be false positive with the IgG assay.