A highly-sensitive sandwich enzyme-linked immunosorbent assay for detecting soluble interleukin 6 receptor (sIL-6R) in human serum

Pim van der Harst (p.van.der.harst@umcg.nl)
University of Groningen, University Medical Center Groningen, Department of Cardiology
Hilde E. Groot
University of Groningen, University Medical Center Groningen, Department of Cardiology

Method Article

Keywords: ELISA, interleukin-6, soluble interleukin-6 receptor, IL6, IL6R, sIL6R, IL6-R

Posted Date: March 17th, 2015

DOI: https://doi.org/10.1038/protex.2015.027

Abstract

A highly-sensitive sandwich enzyme-linked immunosorbent assay \(ELISA) was developed for detecting soluble interleukin 6 receptor \(sIL-6R) in human serum, a biomarker for inflammation. The aim was to set up, develop, and validate an inexpensive method to measure sIL-6R. MoAb anti-H IL-6R was used as coating and recombinant human IL-6 was added in a second step followed by detection, using PoAb anti-H IL6R biotine. The optimal concentrations of MoAb anti-H IL-6R and PoAb anti-H IL6R biotine for the ELISA were tested in serial dilutions and were 2 µg/ml MoAB anti-H IL-6R PBS and 0.3 µg/ml PoAb anti-H IL6R biotine HPE buffer. Intra- and inter-assay CV range were 4.6%, and 8.8% respectively. The developed ELISA correlated well with a commercially available kit \(R&D Systems, Spearman correlation 0.86; p<0.001). The costs of one ELISA plate was approximately €90 compared to the current list price of €560 for the commercial kit. In summary, we have developed and validated an inexpensive sIL-6R ELISA.

Introduction

Interleukin 6 receptor \(IL-6R) signalling has been suggested to play a causal role in the development of coronary heart disease \(CHD) and outcome\(1). It is thought to be involved in repair processes and scar tissue formation after myocardial infarction \(2). Circulating levels of IL-6 are correlated with impaired left ventricular systolic and diastolic function in patients with myocardial infarction \(MI) \(3). Furthermore, IL-6R signalling plays an important role in the modulation of the inflammatory response after myocardial infarction and it is associated with adverse left ventricular remodelling in animal models\(4-6). Finally, genetic evidence in humans derived from Mendelian randomization analyses have elucidated that IL-6R signalling seems to have a causal role in development of CHD \(7,8). However, to further investigate the role of IL-6R signalling, it is necessary that a validated and inexpensive enzyme-linked immuno assay \(ELISA) for sIL-6R is available.

Reagents

Coating buffer : 0.01 MB PBS, pH 7.4 Coating antibody: MoAb anti-H IL-6R \(R&D, cat. no. MAB227) Store aliquots at -20°C. Wash buffer: TBTS-buffer: 0.025M Tris.HCL, 0.15M NaCl, 0.05% Tween-20 Block buffer: 0.01M PBS + 2% BSA \(Sigma cat. no. A9647) + 0.05% Tween-20 Incubation buffer: PTG-buffer: 0.01M PBS + 0.05% Tween-20 = 0.2% gelatine. Sera: HPE-buffer 1:5 \(Sanquin, cat. no. M1940) Standard: r-hIL-6R \(R&D, cat. no. 227-SR-025), 25 micrograms. Store aliquots at -20°C. Detecting antibody: PoAb anti-H IL-6R biotine \(R&D, cat. no. BAF 227). Store aliquots at -20°C. Streptavidine-HRPO: Streptavidine poly-HRP \(Sanquin, cat no. M2032 “STREPTA-E+”). Store aliquots at -20°C. Chromogen: TMB \(3,3’,5,5’-Tetramethylbenzidine dihydrochloride) \(Sigma-Aldrich, SKU: T3405-50TAB) Reaction buffer: 0.1 acetate buffer, pH 6.0, 4°C Stop solution: 2N H_2SO_4

Equipment

-80°C freezer - 20°C freezer 2-8°C refrigerator Plates: Corning nr. 9018 \(Corning BV, Schiphol-Rijk, The Netherlands) Shaker: Heidolph unimax \(VWR International BV, Amsterdam, The Netherlands) Scanner: Emax \(Molecular Devices, Sunnyvale, USA) Software: SoftmaxPro \(Molecular Devices, Sunnyvale, USA)

Procedure

All incubation steps are at the Heidolph unimax shaker \(150rpm) at room temperature with an amount of 100ul, unless different amounts are reported. 1. **Coating**: Dilute MoAb anti-H IL-6R to 2 ug/ml. Coat the plate with MoAb anti-H IL-6R and incubate the plate for 1 hr at the shaker, and incubate the plate \(without shaker) overnight at 4°C. 2. **Washing**: Wash the plate 5 times with washing buffer \(200 ml in each well). Discard the buffer by throwing the washing buffer firmly in the sink. Wells should appear dry after throwing the washing buffer away. 3. **Blocking**: Block the MoAb anti-H IL-6R by incubation of 200 ml blocking buffer for 1 hr. 4. **Washing**: Wash the plate 5 times with washing buffer \(200 ml. in each well). Discard the buffer by throwing the washing buffer firmly in the sink. Wells should appear dry after throwing the washing buffer away. 5. **Sample incubation**: Row 1 & 2 contain the Standard samples in two-fold, start with conc.1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.625 pg/ml. Row 3 to 12 contain test samples dilutions \(diluted 1000 and 1200 times) Incubate Standard and test samples for 2 hr 6. **Washing**: Wash the plate 5 times with washing buffer \(200 ml in each well). Discard the buffer by throwing the washing buffer firmly in the sink. Wells should appear dry after throwing the washing buffer away. 7. **Detection**: Dilute PoAb anti-H IL-6R-biotine to 0.3 ug/ml and Incubate for 1,5 hr. 8. **Washing**: Wash the plate 5 times with washing buffer \(200 ml in each well). Discard the buffer by throwing the washing buffer firmly in the sink. Wells should appear dry after throwing the washing buffer away. 9. **Conjugate reaction**: Incubate the plate for 30 minutes in a dark room with Streptavidine poly-HRP 1:8000 diluted in HPE buffer. 10. **Washing**: Wash the plate 5 times with washing buffer \(200 ml in each well). Discard the buffer by throwing the washing buffer firmly in the sink. Wells should appear dry after throwing the washing buffer away. 11. **Colour reaction**: Dilute a 2 mg TMB tablet in 1 ml reaction buffer. Add 2ul 30% H2O2 before use. Let react for 15 – 20 minutes 12. **Stopping**: Stop the reaction after 15 – 20 minutes by adding 100 ul 2N H2SO4 13. **Scanning**: Scan the plate at 450 nm – 575 nm \(reference filter) in the Emax machine and calculate concentrations **Reference values**: The reference value for sIL-6R is 31 pg/ml \(Range 14 – 46 pg/ml, source: R&D Systems\(9))

Timing

Coating, 1h at the shaker and overnight at 4°C Blocking, 1h at the shaker Sample incubation, 2h at the shaker Detection, 1.5h at the shaker Conjugate reaction, 30 minutes at the shaker \(dark room) Colour reaction, 15-20 minutes

Troubleshooting

Trouble shooting advice is provided in table 1.

Anticipated Results

The standard curve was constructed by plotting the mean absorbance \(Y) of Standards against the known concentration \(X) of Standards in a logarithmic scale, using the four-parameter algorithm. Concentrations of unknown/test samples were determined using the standard curve \(Figure 1). The intra-assay coefficient of variation \(CV) was determined by replicate analysis \(n=7) of one serum sample at concentration of 110.00 ng/ml. The intra-assay CV was 4.6%. The inter-assay CV was determined by duplicate measurement of one serum sample at concentration 84.67 ng/ml in separate assays \(n=12). The inter-assay CV range was 8.8%. Comparing our ELISA with the commercial sIL-6R ELISA kit from R&D Systems \(cat. No.DR600), Spearman correlation was 0.86 \(p<0.001) \(Figure 2). The costs of one ELISA plate was approximately €90 compared to €560 of the currently commercially available kit \(R&D Systems, cat. No.DR600) \(Table 1). In total, we analysed 405 samples in duplicate using 11 plates resulting in €5214 \(85%) savings compared to the commercially available kit.

References

\(1) Danesh J, Kaptoge S, Mann AG, Sarwar N, Wood A, Angleman SB, et al. Long-term interleukin-6 levels and subsequent risk of coronary heart disease: two new prospective studies and a systematic review. PLoS Med 2008 Apr 8;5\(4):e78. \(2) Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res 2002 Jan;53\(1):31-47. \(3) Karpinski L, Plaksej R, Kosmala W, Witkowska M. Serum levels of interleukin-6, interleukin-10 and C-reactive protein in relation to left ventricular function in patients with myocardial infarction treated with primary angioplasty. Kardiol Pol 2008 Dec;66\(12):1279-1285. \(4) Kobara M, Noda K, Kitamura M, Okamoto A, Shiraishi T, Toba H, et al. Antibody against interleukin-6 receptor attenuates left ventricular remodelling after myocardial infarction in mice. Cardiovasc Res 2010 Aug 1;87\(3):424-430. \(5) Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004 Jun 25;94\(12):1543-1553. \(6) Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 1990 Apr;81\(4):1161-1172. \(7) Interleukin-6 Receptor Mendelian Randomisation Analysis \(IL6R MR) Consortium, Hingorani AD, Casas JP. The interleukin-6 receptor as a target for prevention of coronary heart disease: a mendelian randomisation analysis. Lancet 2012 Mar 31;379\(9822):1214-1224. \(8) IL6R Genetics Consortium Emerging Risk Factors Collaboration, Sarwar N, Butterworth AS, Freitag DF, Gregson J, Willeit P, et al. Interleukin-6 receptor pathways in coronary heart disease: a collaborative meta-analysis of 82 studies. Lancet 2012 Mar 31;379\(9822):1205-1213. \(9) sIL-6R ELISA protocol. Available at: http://www.rndsystems.com/pdf/dr600.pdf. \(10) R&D Systems. ELISA development guide. Available at: http://www.rndsystems.com/resources/images/5670.pdf.

Acknowledgements

We thank Janny Takens, Silke U. Oberdorf, Martin M. Dokter and Linda van Genne from the Department of Cardiology and Gerda Horst from the Department of Immunology, all at the University of Groningen,

University Medical Center Groningen \(UMCG).

Figures

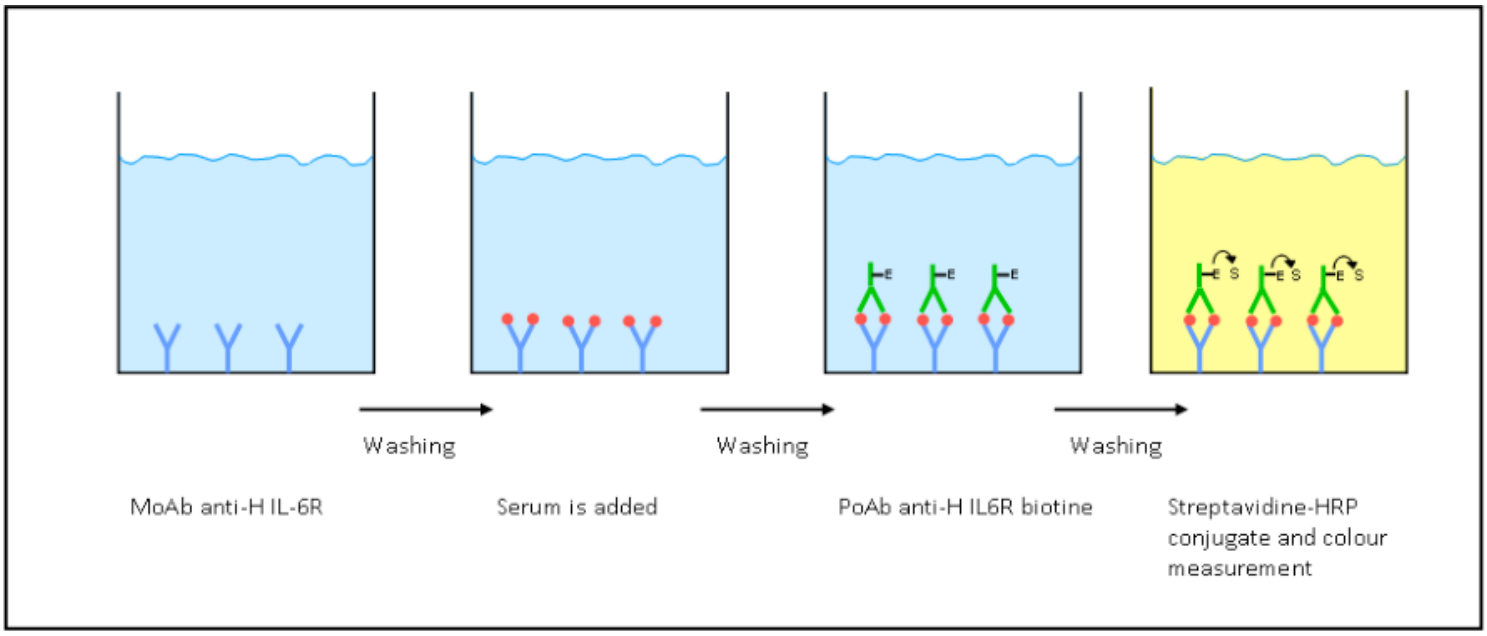

Figure 1

Consecutive steps of the ELISA (as a pdf)

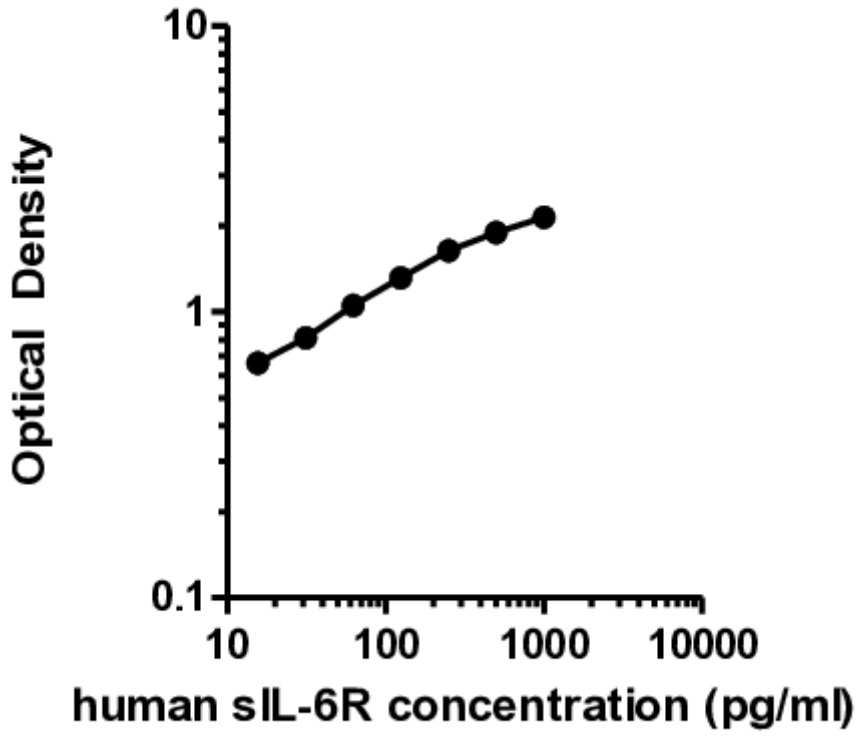

Figure 2

Typical standard curve for the human IL6R ELISA Typical standard curve for the human IL6R ELISA

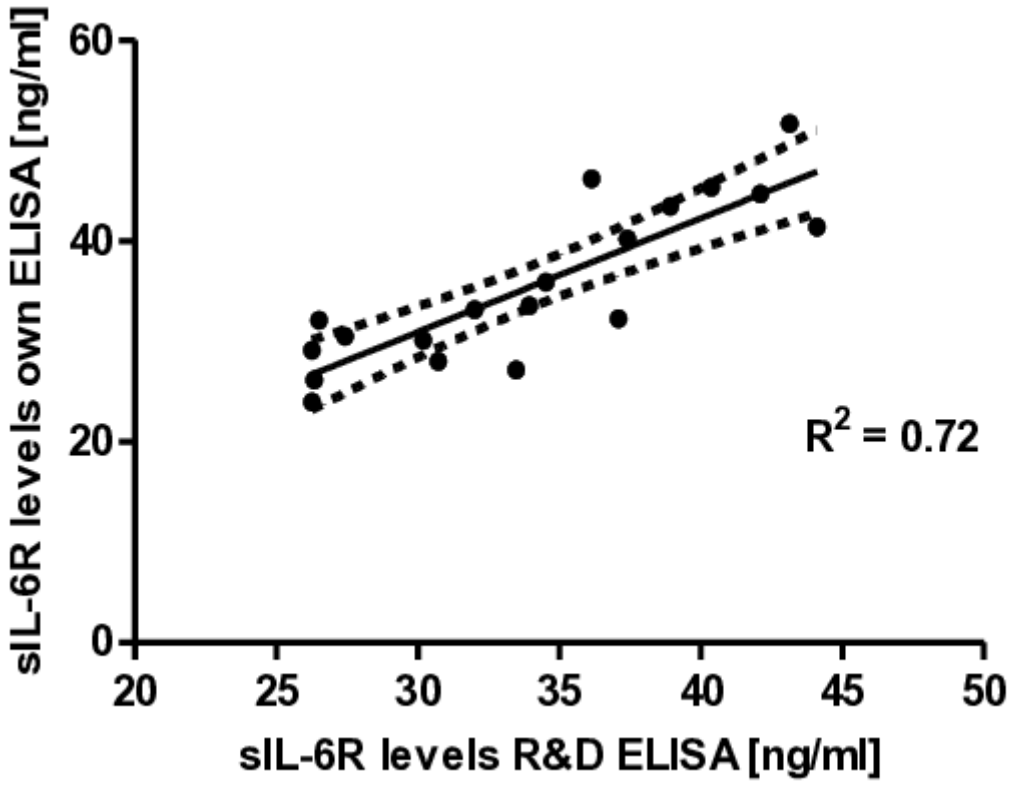

Figure 3

Correlation between commercial R&D ELISA and the developed ELISA. Correlation between commercial R&D ELISA and the developed ELISA. Dotted lines are 95 CI.

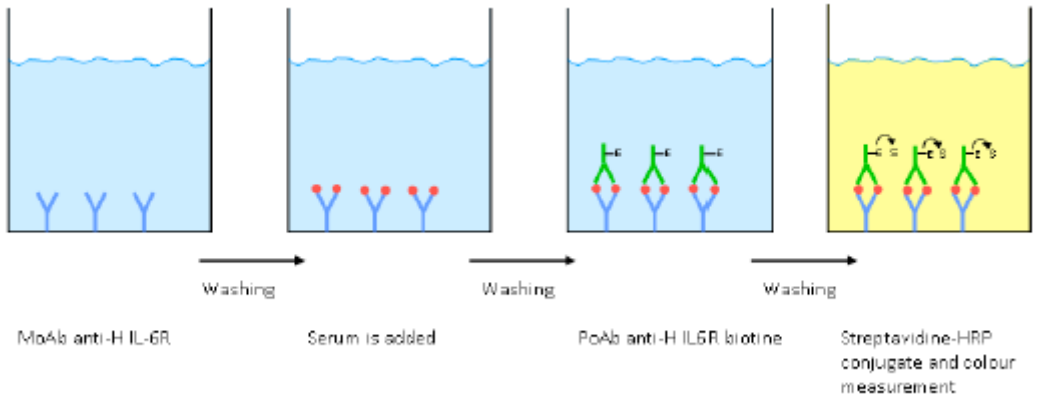

Figure 4

Figure 1 Consecutive steps of the ELISA

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.pdf
- supplement0.pdf