# Chapter 35
# A Scheme for the Development and Validation of Enzyme Linked Immunosorbent Assays (ELISA) for Measurement of Angiogenic Biomarkers in Human Blood

**Garry McDowell, Richard Body, Cliona Kirwan, Ged Byrne, and Mark Slevin**

## 35.1 Introduction

This handbook of methods in vascular biology reports a number of analytical methods for the quantification of angiogenesis, both *in vitro* and *in vivo*. In this chapter the authors discuss a scheme for the development and validation of ELISA for the quantification of circulating vascular biomarkers. The principles we discuss, can be applied to the majority of ELISA techniques, irrespective of the biomarker being measured. We will illustrate our method using Vascular Cell Adhesion Molecule-1 (VCAM-1) as an example.

The ELISA is an example of a non-competitive sandwich assay. The components of the ELISA consist of a capture antibody, secondary detection antibody and detection reagent. Briefly, an analyte specific capture antibody is bound to an ELISA plate, forming the solid phase. The analyte of interest in samples, or standards is then incubated with the solid phase antibody, capturing the analyte in the solid phase, due to the antibody-antigen reaction. The plate is washed to remove unbound analyte. Following the wash step, a secondary biotin-conjugated antibody is added. The secondary antibody binding to different epitopes on the antigen to the capture antibody. Following a wash step to remove unbound secondary antibody, a Streptavidin-horseradish peroxidase enzyme conjugate is added. Streptavidin binds biotin with high affinity. Again following a wash step to remove unbound enzyme

G. McDowell, Ph.D., F.R.C.Path., F.E.S.C. (✉) • M. Slevin, Ph.D., F.R.C.Path., F.A.H.A.
School of Healthcare Science, Manchester Metropolitan University, Manchester, UK
e-mail: g.mcdowell@mmu.ac.uk

R. Body, Ph.D., F.C.E.M.
Institute of Cardiovascular Science, University of Manchester, Manchester, UK

C. Kirwan, Ph.D., F.R.C.S. • G. Byrne, M.D., F.R.C.S.
Department of Academic Surgery, University of Manchester and University Hospital of South Manchester, Manchester, UK



M. Slevin, G. McDowell (eds.), *Handbook of Vascular Biology Techniques*,
DOI 10.1007/978-94-017-9716-0_35

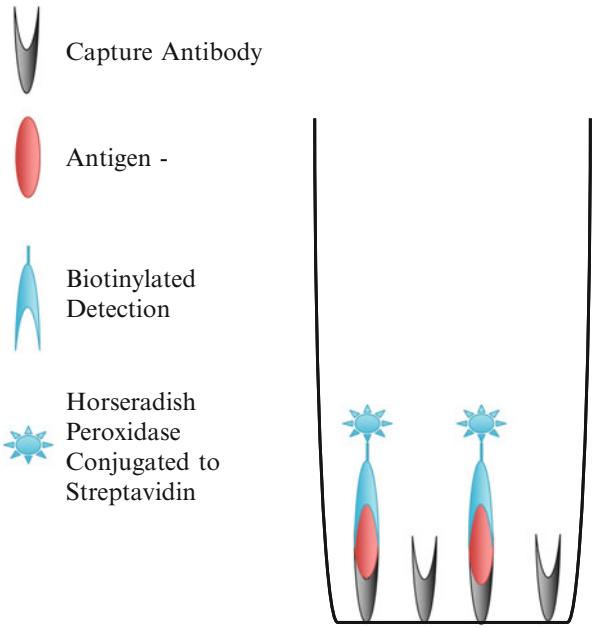


**Fig. 35.1** The analytical principle of the ELISA

conjugate a substrate solution is added, which changes colour in the presence of the enzyme. The reaction can be stopped and the colour intensity (optical density) measured. In the non-competitive format, optical density is directly proportional to concentration. Figure 35.1 illustrates the analytical principle of the ELISA.

A number of manufacturers supply readymade ELISA kits which have been fully validated providing convenience and ease of use, however, these may be impractical especially on a restricted budget. In this Chapter the authors will illustrate how it is possible to use commercially available reagents to generate and validate an in-house assay, custom designed to meet your research needs.

## 35.2 Materials and Methods

### *35.2.1 Materials and Reagents*

1. VCAM-1 DuoSet® Elisa Development System kit. (R & D systems; Abingdon, Oxford. Catalogue Number DY809). The kit includes the basic reagents required to establish the ELISA

   (a) Capture Antibody (Part 841127 1 vial) – 360 μg/ml of mouse monoclonal anti-human VCAM-1, when reconstituted with 1 ml buffer without protein. The manufacturer recommends a working concentration of 2.0 μg/ml. Note 1

(b) Detection antibody (Part 841128 1 vial) – 36 μg/ml of polyclonal biotinylated sheep anti human VCAM-1 when reconstituted. The manufacturer recommends a working concentration of 200 ng/ml. Note 1
(c) Standard solution. (Part 841129 1 vial) – 80 ng/ml of recombinant human VCAM-1, when reconstituted with 0.5 ml of reagent buffer. A upper standard concentration of 1,000 pg/ml is recommended for the standard curve.
(d) Streptavidin-HRP (Part 890803 2 vial) – Streptavidin conjugated to horse – radish peroxidase.

2. Sodium dihydrogen orthophosphate-1-hydrate ($NaH_2PO_4.H_2O$) Molecular Weight = 137.99 g. BDH Analar®, BDH Ltd, Poole, England.
3. Di-Sodium Hydrogen orthophosphate-2-hydrate ($Na_2HPO_4.2H_2O$) Molecular Weight = 177.99 g. BDH GPR™, BDH Ltd, Poole, England.
4. Albumin-Bovine (BSA): Sigma-Aldrich, Poole, Dorset. Fraction V (Initial fractionation by heat shock) Minimum 98 % (Electrophoresis).
5. Sodium azide ($NaN_3$). Molecular weight = 65.01 g. BDH GPR™, BDH Ltd, Poole, England. Warning COSHH: Very toxic if swallowed; contact with acids liberates a very toxic gas. Avoid contact with skin. After contact with skin, wash immediately with plenty of water. Heating may cause an explosion.
6. Tetramethylbenzidine (TMB): obtained as a commercial pre-prepared solution with substrate (DAKO, Cambridgeshire, UK). TMB and Hydrogen peroxide (substrate) in a 1:1 mixture for use as the Substrate-chromogen reagent.
7. 1 M Sulphuric acid – $H_2SO_4$. Sulphuric acid Analar® specific. gravity. 1.84; Molecular Weight = 98.07. BDH Ltd, Poole, England. Warning COSHH: Causes severe burns. Never add water directly to this reagent.
8. De-ionised water.

## 35.3 Buffers Required

### *35.3.1 Capture Antibody Buffer*

0.05 M Phosphate Buffer, pH 7.4 (4.83 g of $NaH_2PO_4.H_2O$ and 38.28 g of $Na_2HPO_4.2H_2O$ made up to 5 L).

### *35.3.2 Wash Buffer*

0.05 % Tween 20 in 0.05 M phosphate buffer (125 μl into 250 ml 0.05 M Phosphate Buffer, pH 7.4).

### *35.3.3 Reagent Buffer*

0.1 % BSA in 0.05 M Phosphate Buffer, pH7.4 containing 0.01 % sodium azide. Note 2.

### *35.3.4 Sample/Standard Buffer*

1 % BSA in 0.05 M phosphate buffer, pH 7.4 containing 0.1 % sodium azide.

### *35.3.5 Equipment*

1. 96 well Microtitre polystyrene base immunoassay plates.
2. Plate reader, software, and printer:
3. Sterile 30 ml universal containers.
4. Gilson eight-channel multi-pipette.
5. Plate shaker; Perkin Elmer Ltd, Milton Keynes, UK.
6. Assorted tubes to prepare dilutions and standard curve.
7. pH meter.

## 35.4 Plate Coating

1. Prepare the working dilution of capture antibody in capture antibody buffer without BSA. Note 1
2. Add 100 µl of diluted antibody to each well on a 96 well microtitre plate.
3. Seal and incubate overnight at room temperature.
4. Following overnight incubation, wash the plate with wash buffer (x3), completely filling each well using a wash bottle.
5. Remove wash solution completely between washes.
6. Following the final wash remove any residual wash buffer by blotting on clean absorbent towels.
7. Block plates by adding 300 µl of reagent buffer to each well.
8. Incubate at room temperature for 1 h.
9. Repeat washing steps 5 and 6 above.
10. The plates are now ready for use. Based on our experience plates can be dried at room temperature, sealed with an adhesive plate cover and stored in at 4 °C for up to 1 week.

## 35.5 Preparation of a Typical Standard Curve

The standard curve details the relationship between the instrument response (optical density, OD) and known concentrations of the analyte. The standard curve should contain a sufficient number of points to determine the relationship between response and concentration to enable determination of the linear range of the assay. Ideally the curve height should be >1 and preferable between 1 and 3 OD units.

1. Prepare a range of concentrations of standards using recombinant VCAM-1 as supplied.
2. Standards should be diluted in reagent buffer and an upper concentration of standard of 1,000 pg/ml is recommended.
3. Prepare a working stock standard of 1,000 pg/ml from the reconstituted standard supplied in the DuoSet ELISA kit.
4. Use the stock standard to prepare a range of standard concentrations as shown below using doubling dilutions (Table 35.1) using doubling dilutions:

**Table 35.1** Table showing the preparation of VCAM-1 standard curve by doubling dilutions method using recombinant VCAM-1 standard as supplied.

| Tube number | Dilution factor | Volume of diluted standard added (ml) | Volume of reagent buffer added | VCAM-1 [pg/ml] |
|---|---|---|---|---|
| 1 | | 1 | 0 | 1,000 |
| 2 | 1:2 | 1 | 1 | 500 |
| 3 | 1:4 | 1 | 1 | 250 |
| 4 | 1:8 | 1 | 1 | 125 |
| 5 | 1:16 | 1 | 1 | 62.5 |
| 6 | 1:32 | 1 | 1 | 31.3 |
| 7 | 1:64 | 1 | 1 | 15.6 |
| 8 | 1:128 | 1 | 1 | 7.8 |
| 9 | 1:256 | 1 | 1 | 3.9 |
| 10 | 1:512 | 1 | 1 | 1.95 |
| 11 | 1:1024 | 1 | 1 | 0.98 |
| 12 | Blank | 0 | 1 | Blank |

## 35.6 Assay Procedure

The typical assay procedure is detailed below. In the following sections the authors will describe a scheme to validate the assay to ensure optimal performance and determine the optimal performance characteristic of the assay.

1. The following procedures are carried out in duplicate.
2. Add 100 µl of standard or unknown, diluted as appropriate in reagent buffer.
3. Cover the plate with an adhesive plate cover and incubate plate for 2 h at room temperature. Note 3
4. Aspirate and wash each well with wash buffer, Using a wash bottle fill each well with wash buffer to ensure unbound VCAM-1 in standard or sample has been removed.
5. Repeat step 4 above a further two times (three washes in total).
6. Add 100 µl of detection antibody diluted in reagent buffer to each well.
7. Cover the plate with an adhesive cover and incubate for 2 h at room temperature. Note 3
8. Repeat wash steps 4 and 5 above.
9. Add 100 µl of the streptavidin-HRP conjugate to each well.
10. Cover the plate with an adhesive strip and incubate at room temperature for 20 min, avoiding exposure to direct sunlight.
11. Repeat was steps 4 and 5 above.
12. Add 100 µl of Tetramethylbenzidine (TMB): obtained as a commercial pre-prepared solution with substrate to each well.
13. Incubate the plate for 20 min at room temperature.
14. Add 50 µl of 1 M $H_2SO_4$ to each well to stop the reaction.
15. Determine the optical density of each well immediately using a microplate reader set a 450 nm. If wavelength correction is available set this to 540 nm or 570 nm. If wavelength correction is not available subtract readings at 540 nm or 570 nm from readings at 450 nm, to correct for imperfections in the microtitre plate.

## 35.7 Calculation of Results

Plot a calibration curve of optical density against concentration of standard. The optical density of the unknown can then be used to determine the concentration.

A number of software packages are available which will do this automatically, however it is always advisable to visually inspect the calibration curve to check assay range and linearity.

## 35.8 Assay Validation Methodology

Our previous validation work has utilised the Guidance for Industry: Bioanalytical Method Validation protocol as a reference guide. Please also refer to the Manufacturers ELISA Development Guide (see link below). The material that follows is in addition to the optimization that this recommended in this guide

### *35.8.1 Use of a Secondary Calibrator in Biological Matrix*

According to the Guidance for Industry: Bioanalytical Method Validation, calibrators should be prepared in the same biological matrix as the biological samples of unknown concentration. In addition, calibrators of higher concentration may facilitate an extended calibration curve.

1. Use pooled human serum from healthy volunteers and subjects suffering from pathological conditions of interest as a secondary calibrator. Note 4
2. Determine from the literature the concentrations of VCAM-1 expected.
3. Aliquot and freeze pooled serum in 1 above.
4. Prepare a standard curve of a range of dilutions of recombinant VCAM-1 as supplied in the DuoSet ELISA Development kit.
5. Prepare a range of dilutions of pooled human plasma or serum, which parallel the range of dilutions in 4 above.
6. Run an assay as detailed in Steps 1–15 under assay procedure.
7. Use the standard curve (4) to determine the concentration of VCAM-1 in the pooled human serum.
8. To assign a VCAM-1 concentration to your secondary calibrator, calculate the mean concentration from dilutions on the linear point of the calibration curve by visual inspection of curves prepared in 4 an 5 above.
9. To view the calibrations curves, plot both the standard curve of the kit standards and the pooled human serum on the same graph. They should parallel each other.

## 35.9 Establishing a Quality Control Pool

1. Use pooled human serum as described above. Note 4
2. Pool serum from healthy individuals and patients with the pathological condition of interest. This will enable the analyst to quality control the assay at normal and pathological concentrations.
3. Measure the VCAM-1 concentration across a range of dilutions and a minimum of 10 times (in duplicate) within the same assay run, and across 10 separate days (in duplicate).
4. Calculate the mean and SD for both the intra- and inter-assay results.
5. Incorporate QC material into each assay run and use to monitor assay performance over time.

## 35.10 Determine the Intra- and Inter-assay Co-efficient of Variation (CV)

The precision if an analytical method describes the closeness of individual measures of an analyte when the procedure is repeated to multiple aliquots of a single concentration in the biological matrix of interest. Precision should be measured a minimum of 5 times (10 preferred) at each concentration.

1. Within each assay, add 100 µl of pooled QC material at three separate locations across the assay plate.
2. The reminder of the assay is performed as usual using the procedure detailed above.
3. The QC material simply replaces the unknown sample.
4. Calculate the inter-assay CV from the mean and SD of the QC material included in at least five individual assays.
5. The CV = (SD/Mean)*100 and is expressed as a percentage.
6. The Intra-assay CV may be determined by repeating the process above, however the measurements of mean and SD must be determined within one assay run (i.e. within one ELISA plate).
7. The CV = (SD/Mean)*100 and is expressed as a percentage.

## 35.11 Determination of Detection Limit

This is the minimum amount of VCAM-1 that can be detected with the assay.

1. Perform the assay as described previously.
2. Set up the standard curve by adding 100 µl of calibrator in duplicate to each well.
3. Into the remaining wells of the plate, add 100 µl of blank calibrator.
4. Perform the remainder of the assay as described earlier.
5. Calculate the mean and SD of the blank readings.
6. The absorbance of the minimum detectable VCAM-1 equates to the ***mean blank absorbance reading + 2SD of the mean blank absorbance reading.***
7. The absorbance reading determined in 7 above can then be read from the standard curve to give the concentration of VCAM-1 corresponding to the minimum concentration that can be determined by the assay.

## 35.12 Final Assay Format

The format of the VCAM assay is shown in the diagrammatic representation of a 96 well plate in Fig. 35.2. This incorporates analytical standards and QC samples prepared in biological matrix and unknown samples for the determination of VCAM.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | Blank | QC Low | QC Low | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample |
| B | Std 1 | Std 1 | QC High | QC High | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample |
| C | Std 2 | Std 2 | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample |
| D | Std 3 | Std 3 | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | QC Low | QC Low | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample |
| E | Std 4 | Std 4 | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | QC High | QC High | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample |
| F | Std 5 | Std 5 | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample |
| G | Std 6 | Std 6 | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | QC Low | QC Low |
| H | Std 7 | Std 7 | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | Unknown Sample | QC High | QC High |

**Fig. 35.2** Format of ELISA plate showing position of standards, low and high quality control samples and unknown samples

## 35.13 Sample Results

In our hands, using the approach above we developed and validated an assay using the DuoSet kits with the following characteristics:

Detection Limit: 25 pg/ml.
Assay Range using Extended Matrix based Secondary Calibrator: 25–3,000 pg/ml.
Inter-assay CV: 9 % at 550 pg/ml and 12 % at 1,100 pg/ml.
Intra-assay CV: 7 % at 550 pg/ml and 9 % at 1,100 pg/ml.

## 35.14 Applications

We have used the above methodology to develop and validate ELISA's, using commercially available reagents, for determination of serum and/or plasma VCAM, P-selectin and E-selectin. The assays have been applied to our clinical investigations in rapid diagnosis of acute coronary syndromes [1] and in predicting venous thromboembolism in breast cancer [2–4].

## 35.15 Notes

**Note 1** We used the antibody concentrations specified by the manufacturer as these gave an acceptable standard curve during assay validation. Please see R&D systems ELISA Development Guide Below which contains details of how to perform a grid experiment to determine the most favourable concentrations of capture and detection antibody combination.

**Note 2** To avoid foaming when making up BSA buffer, sprinkle BSA onto the liquid surface and leave to stand for 10–15 min, until BSA dissolves. **Do not** stir or mix. We used a 0.1 % BSA buffer as on validation for our purpose this improved assay sensitivity.

**Note 3** We used manufacturers instructions for incubation time, however we validated incubation times of 1, 2 and 3 h. A 1 h incubation lowered assay sensitivity significantly, whilst 3 h increased background with minimal improvement in sensitivity.

**Note 4** Depending on National Laws and procedures for research governance, this may require approval of a Research Ethics Committee and/or other Institutional Committee overseeing research governance.

## References

1. Body R, Pemberton P, Ali F, McDowell G, Carley S, Smith A, Mackway-Jones K (2011) Low soluble P-selectin may facilitate early exclusion of acute myocardial infarction. Clin Chim Acta 412:614–618
2. McDowell G, Temple I, Li C, Kirwan CC, Bundred NJ, McCollum CN, Burton IE, Kumar S, Byrne GJ (2005) Alteration in platelet function in patients with early breast cancer. Anticancer Res 25:3963–3966
3. Byrne GJ, McDowell G, Agarawal R, Sinha G, Kumar S, Bundred NJ (2007) Serum vascular endothelial growth factor in breast cancer. Anticancer Res 27:3481–3487
4. Kirwan CC, McDowell G, McCollum CN, Kumar S, Byrne GJ (2008) Early changes in the haemostatic and procoagulant systems after chemotherapy in breast cancer. Br J Cancer 99:1000–1006

## *Helpful Websites Including Troubleshooting Guides*

DuoSet ELISA Development Systems, R&D Systems. http://www.rndsystems.com/product_detail_objectname_duoset.aspx
R&D Systems ELISA Development Guide. http://www.rndsystems.com/resources/images/5670.pdf