DEMO ELISA

5991DEMO[1]02.24

A sandwich enzyme immunoassay
for training and testing

EUROPROXIMA DEMO ELISA

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TABLE OF CONTENTS

The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

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1. INTRODUCTION

DEMO ELISA is a sandwich enzyme immunoassay that can be used for training purposes and quality control of automatic ELISA platforms. DEMO ELISA is a typical sandwich ELISA that is commonly used in clinical diagnostics for the detection of proteins, bacteria, viruses in patient's samples and also in the food and feed diagnostics for the detection of allergens such as milk residues, nuts or gluten. All reagents required to perform the test are contained in the test kit. The test kit is sufficient for 96 determinations. The kit is composed of a set of standards to prepare the calibration curve, two quality control samples (QC samples) and one spiking solution, from which several samples with different concentration can be prepared. Some examples for the experiments are presented in chapter 11. The test can be performed manually on an automated ELISA platform such as ThunderBolt®. The validated ThunderBolt® method file is available upon request from R-Biopharm Nederland

2. PRINCIPLE OF DEMO ELISA

DEMO ELISA is a sandwich enzyme linked immunosorbent assay. The wells of the microtiter strips are coated with specific antibodies against the analyte. After adding the standard solutions and samples containing the analyte to the wells, the analyte binds to the immobilized antibodies. After an incubation time of 20 min, unbound reagents are removed in a washing step. Then, the second antibody (against the analyte), conjugated to horseradish peroxidase enzyme, is added to each well and binds to the antibody-analyte complex to form antibody-analyte-antibody sandwich. After 20 min incubation step, any unbound conjugate is removed in a washing step. The amount of bound conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound conjugate transforms the colorless chromogen into a coloured product. The substrate reaction is stopped after 20 min by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The absorbance is proportional to the analyte concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The test is specific for the detection of the analyte present in the standard solutions, QC samples and spiking solution. No other analytes were tested.

4. HANDLING AND STORAGE

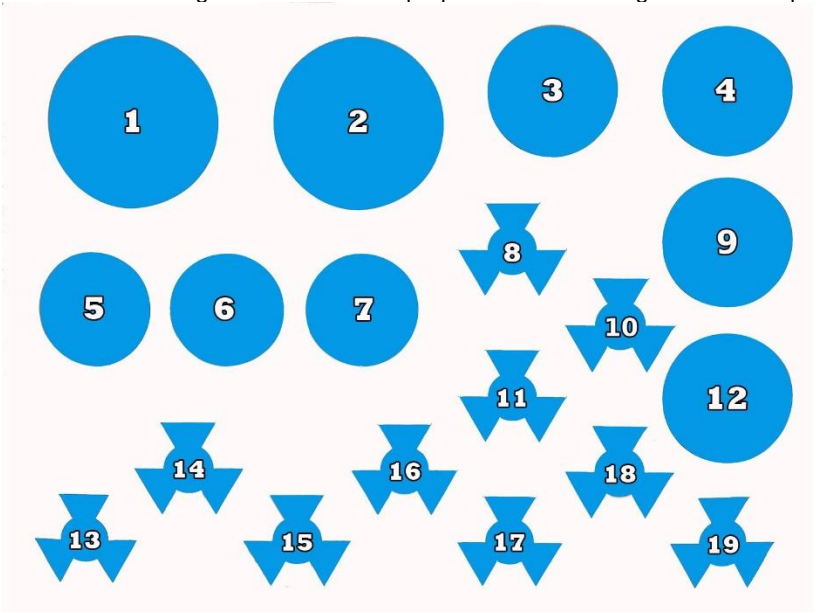
- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies directed against the analyte (ready-to-use).

Position of the reagents in the kit. For preparation of the reagents see Chapter 8.



1. **Dilution buffer** (22 ml, ready-to-use)
2. **Rinsing Buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (12 ml, ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Spiking solution** (3 ml, ready-to-use) **4000 ng/ml** blue cap
9. **Conjugate solution** (12 ml, ready-to-use) red cap
10. **QC sample 1** (3 ml, ready-to-use) **20 ng/ml** blue cap
11. **QC sample 2** (3 ml, ready-to-use) **150 ng/ml** blue cap
12. not in use
13. **Standard solution 1** (3 ml, ready-to-use) **0 ng/ml** white cap
14. **Standard solution 2** (3 ml, ready-to-use) **12.5 ng/ml** white cap
15. **Standard solution 3** (3 ml, ready-to-use) **25 ng/ml** white cap
16. **Standard solution 4** (3 ml, ready-to-use) **50 ng/ml** white cap
17. **Standard solution 5** (3 ml, ready-to-use) **100 ng/ml** white cap
18. **Standard solution 6** (3 ml, ready-to-use) **200 ng/ml** white cap
19. not in use.

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Gloves
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml or 5 ml combitips
- Aluminium foil or parafilm
- Deionised or distilled water
- Microtiter plate spectrophotometer (450 nm)
- Optional: RIDASOFT® Win. NET Software.
  This software program is available from R-Biopharm Nederland B.V. upon request.

7. SAFETY PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. PREPARATION OF REAGENTS

Before starting the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2°C to +8°C. Prepare reagents fresh before use.

Microtiter plate
Return unused strips into the resealable zip lock bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
The substrate solution (ready-to-use) precipitates at 4°C. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

Dilution buffer
The dilution buffer is ready-to-use.

Conjugate solution
The conjugate solution is ready-to-use. Store the unused conjugate immediately in the dark at +2°C to + 8°C.

Standard solutions
The standards are ready-to-use.

QC Samples
The QC samples are ready-to-use.

Spiking solution
Spiking solution 4000 ng/ml should be diluted in the dilution buffer to obtain concentrations within the measuring range of the test. Please see some examples in chapter 11.

9. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, between each immunological incubation step, un-bound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results. Basically, manual rinsing or rinsing with automatic plate wash equipment can be done as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out for at least 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate washing equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is correctly dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare reagents according to Chapter 8.

2. Pipette 100 µl of each of the standard solutions in duplicate (wells A1,2 to F1,2) i.e. 0, 12.5, 25, 50, 100 and 200 ng/ml.

4. Pipette 100 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

5. Incubate for 20 minutes in the dark at room temperature (20ºC – 25ºC).

6. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

7. Pipette 100 µl of conjugate solution into each well.

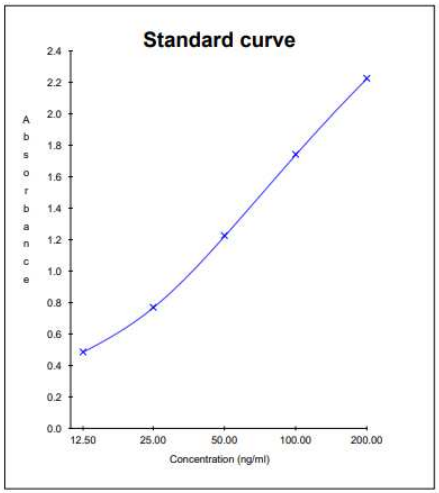
8. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

9. Incubate for 20 minutes in the dark at room temperature (20ºC – 25ºC).
10. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
11. Pipette 100 µl of substrate solution into each well.
12. Incubate for 20 minutes in the dark at room temperature (20ºC – 25ºC).
13. Add 100 µl stop solution into each well.
14. Read the absorbance values immediately at 450 nm.

10. INTERPRETATION OF RESULTS

Calibration curve

The absorbance values are plotted (on the Y-axis) versus the standard concentration (ng/ml) on a logarithmic X-axis. Any software with 5-parameter, 4-parametr or cubic spline fit functions can be used.
Special software RIDASOFT® Win.NET is also available for evaluation.



Standards

Conc.(ng/ml) / A(Mean)

12.50 0.485 (Coeff. of Var.: 2.2%)

25.00 0.770 (Coeff. of Var.: 1.4%)

50.00 1.226 (Coeff. of Var.: 0.3%)

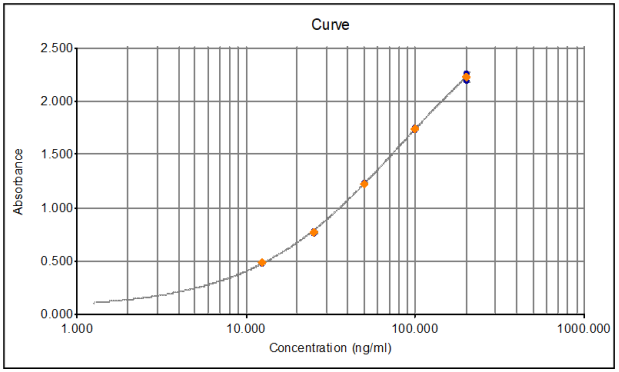
100.00 1.743 (Coeff. of Var.: 0.8%)

200.00 2.226 (Coeff. of Var.: 2.0%)

Standards

Ser. No.	Concentration ng/ml	Absorbance (Mean)	(CV)	B/Bmax (%)
1	0.00	0.055E	3.9	2.5
2	12.50	0.485E	2.2	21.8
3	25.00	0.770E	1.4	34.6
4	50.00	1.226E	0.3	55.1
5	100.00	1.743E	0.8	78.3
6	200.00	2.226E	2.0	100.0

Fig 1. Example standard curve prepared using RIDASOFT® Win.NET (cubic spline fit function).



Concentration (ng/ml)	Absorbance 1	Absorbance 2	Mean Abs.	Mean %
0	0.053	0.056	0.055	2
12.5	0.492	0.477	0.485	22
25	0.778	0.763	0.771	35
50	1.229	1.223	1.226	55
100	1.753	1.733	1.743	78
200	2.257	2.194	2.226	100

Fig 2. Example standard curve prepared using Gen5 software (5-parameter fit function).

Alternatively, the absorbance values can be normalized. The absorbance (Abs) values of the six standards and the samples (mean values of the duplicates) are divided by the mean Abs value of the 200 ng/ml standard (highest concentration, wells F1 and F2) and multiplied by 100. The 200 ng/ml standard is thus made equal to 100% (maximal absorbance) and the other Abs values are quoted in percentages of the maximal absorbance.

$$\frac{Abs\ standard\ (or\ sample)}{Abs\ 200\ ng/ml\ standard} \times 100 = percentage\ maximal\ absorbance$$

The values (percentage maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

The standard curve performance is shown in the QC Certificate enclosed in the test kit.

A further dilution and new testing of the samples is recommended for absorbance values ≥ standard 6 (200 ng/ml).

Details about automated assay procedures and further product information are available on request from your local distributor or R-Biopharm Nederland B.V.

11. EXAMPLE EXPERIMENTS

11.1. Determination of recovery for the QC samples

For the determination of the recovery the standard curve should be run together with the provided QC samples.
Run the assay and calculate the recovery for the QC samples.

$$Recovery\ (\%) = \frac{Measured\ concentration}{Theoretical\ concentration} \times 100$$

For example, if the measured concentration is 140 ng/ml for the QC sample 150 ng/ml, the recovery is 93.3%:

$$Recovery\ (\%) = \frac{140\ ng/ml}{150\ ng/ml} \times 100 = 93.3\%$$

11.2. Determination of recovery for the samples prepared from the spiking solution

Additional samples at different concentrations can be prepared from the spiking solution 4000 ng/ml, for example at 120, 60 or 30 ng/ml.
The following equation can be used for calculations:

$$\frac{volume\ needed\ x\ required\ concentration}{4000\ ng/ml\ spiking} = \ldots\ldots\ldots ml\ spiking\ 4000\ ng/ml$$

For example to obtain 1 ml of the solution at 120 ng/ml:

$$\frac{1\ ml\ x\ 120\ ng/ml}{4000\ ng/ml} = 0.03\ ml = 30\ \mu l$$

To prepare 1 ml of 120 ng/ml solution add 30 µl of the spiking solution 4000 ng/ml to 970 µl of the dilution buffer (1 ml – 0.03 ml = 0.97 ml = 970 µl).

For the preparation of lower concentrations it is recommended to first dilute the spiking solution 4000 ng/ml 10 times in the dilution buffer to obtain intermediate concentration of 400 ng/ml. For example:

$$\frac{1\ ml\ x\ 400\ ng/ml}{4000\ ng/ml} = 0.1\ ml = 100\ \mu l$$

To prepare 1 ml of 400 ng/ml solution add 100 µl of spiking solution 4000 ng/ml to 900 µl of the dilution buffer (1 ml – 0.1 ml = 0.9 ml = 900 µl).
Then use 400 ng/ml intermediate solution to prepare other concentrations. For example to prepare 60 ng/ml:

$$\frac{1\ ml\ x\ 60\ ng/ml}{400\ ng/ml} = 0.15\ ml\ std\ 400\ ng/ml = 150\ \mu l$$

To prepare 1 ml of 60 ng/ml solution add 150 µl of intermediate solution 400 ng/ml to 850 µl of the dilution buffer (1 ml – 0.15 ml = 0.85 ml = 850 µl).

Run the assay and calculate the recovery for the samples prepared from the spiking solution.

$$Recovery\ (\%) = \frac{Measured\ concentration}{Theoretical\ concentration} \times 100$$

11.3. Determination of pipetting precision

Prepare at least 12 ml of the solution at a selected concentration from the spiking solution 4000 ng/ml. The selected concentration should yield an absorbance value around 1.5-2.0, therefore samples at 200 ng/ml, 150 ng/ml or 100 ng/ml are recommended.
For example, to prepare 12 ml of 150 ng/ml solution:

$$\frac{12\ ml\ x\ 150\ ng/ml}{4000\ ng/ml} = 0.45\ ml = 450\ \mu l$$

To prepare 12 ml of 150 ng/ml solution add 450 µl of spiking solution 4000 ng/ml to 11.55 ml of the dilution buffer (12 ml – 0.45 ml = 11.55 ml).

Pipette the prepared standard solution over the whole plate (96 wells) and follow the assay procedure.
Calculate the mean absorbance and standard deviation (SD). Calculate CV (%):

$$CV\ (\%) = \frac{SD\ Abs}{Mean\ Abs} \times 100$$

11.4. Determination of repeatability

Analyze two QC samples in 6 replicates (2 wells/sample) over 3 different days (n=18 in total). Calculate mean concentration and standard variation (SD) for each sample. The repeatability CV (%) for each sample can be calculated as:

$$CV\ (\%) = \frac{SD\ measured\ conc.}{Mean\ measured\ conc.} \times 100$$

11.5. Summary of the quality control criteria

The following guidelines can be used to determine whether the test has been performed to a satisfactory level:

Standard (ng/ml)	% maximal absorbance (B/B0*100%)
0	0 – 8
12.5	12 – 24
25	23 – 39
50	41 – 59
100	66 – 84
200	100

Parameter	Typical results
Recovery for QC samples	70-130%
Pipetting/dispensing precision CV	< 6%
Repeatability CV	<12%

12. ORDERING INFORMATION

For ordering the DEMO ELISA kit please use cat. Code 5991DEMO.

13. REVISION HISTORY

Not applicable.

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