APhL ELISA® IgG and IgM HRP Kit

REF	LAPL-K-HRP-00GM

For the Measurement of IgG and IgM Antiphospholipid Antibodies

IVD

For *In Vitro* Diagnostic Use

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INSTRUCTIONS BOOKLET

APhL ELISA® IgG and IgM HRP Kit

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1 – INTENDED USE

The *APhL ELISA® IgG and IgM HRP Kit* is a semi-quantitative enzyme linked immunosorbent assay (ELISA) for use as an aid in diagnosing the Antiphospholipid Syndrome (APS) in patients presenting with thrombosis, pregnancy losses and/or thrombocytopenia. It enables measurement of IgG and IgM Antiphospholipid antibody levels in human serum or plasma.

2 – EXPLANATION OF THE TEST

The anticardiolipin test (1) was devised for diagnosis of patients with the Antiphospholipid Syndrome (2). The Antiphospholipid Syndrome is a disorder of recurrent venous thrombosis, pregnancy losses, and thrombocytopenia associated with positive anticardiolipin and/or lupus anticoagulant tests (3). Both the anticardiolipin and lupus anticoagulant tests detect antibodies which bind phospholipids (4, 5). These antibodies are heterogeneous, and the two tests do not necessarily identify the same antibodies (6-8). Hence, both tests should be performed in individuals suspected of having the Antiphospholipid Syndrome.

One of the major drawbacks of the anticardiolipin ELISA test has been false positive results. *Nonspecific* binding of sera from patients with a variety of diseases, other than the Antiphospholipid Syndrome is frequent (9-12). Through extensive testing of single and mixtures of phospholipids, a combination of phosphatidylserine, phosphatidic acid, and β_2Glycoprotein I appears to enable the best distinction between Antiphospholipid Syndrome sera and sera from patients with other disorders, in particular syphilis. This antigen has been designated the *APhL ELISA® Phospholipid Antigen* and this has been used to coat ELISA plates in *APhL ELISA® IgG and IgM HRP Kit.* Other than use of this antigen, the principle of the *APhL ELISA® IgG and IgM HRP Kit* is the same as the anticardiolipin test (13, 14).

The *APhL ELISA® IgG and IgM HRP Kit* is calibrated using standard anticardiolipin units (GPL and MPL units) (15-20) and can be used to detect IgG and IgM isotypes. In addition to six *APhL ELISA® HRP IgG Calibrators* and six *APhL ELISA® HRP IgM Calibrators*, an *APhL ELISA® HRP IgG Positive Control* and an *APhL ELISA® HRP IgG Positive Control* (with a defined value and error range) and an *APhL ELISA® HRP Negative Control* are included as **in-house controls** so that operators can determine whether a particular run is acceptable.

3 – PRINCIPLE

A standard indirect enzyme linked immunoassay (ELISA) technique has been employed in this assay. Calibrators, controls and sera or plasmas are incubated in polystyrene microwell strips coated with the *APhL ELISA® Phospholipid Antigen*. This process allows IgG and/or IgM antiphospholipid antibodies in patient sera or plasmas to react with the *APhL ELISA® Phospholipid Antigen*. Washing removes any unbound protein. Antibodies specific for anti-human IgG and IgM labeled with peroxidase conjugate are added. After an additional washing, a measurable color reaction ensues with the addition of a TMB peroxidase substrate.

4 – COMPONENTS

4.1 Contents of the *APhL ELISA® IgG and IgM HRP Kit*

Inspect all contents of the *APhL ELISA® IgG and IgM HRP Kit* against the list below.

24 - *APhL ELISA® Phospholipid Antigen*-coated polystyrene microwell strips, 1 x 8 wells

2 - 55 ml bottle *APhL ELISA® Sample Diluent* ready to use. *

1 - 1.0 ml vial *APhL ELISA® HRP IgG Calibrator #1* (200 GPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgG Calibrator #2* (100 GPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgG Calibrator #3* (50 GPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgG Calibrator #4* (25 GPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgG Calibrator #5* (12.5 GPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgG Calibrator #6* (6.25 GPL) ready to use.

1 - 1.0 ml vial *APhL ELISA® HRP IgM Calibrator #1* (200 MPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgM Calibrator #2* (100 MPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgM Calibrator #3* (50 MPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgM Calibrator #4* (25 MPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgM Calibrator #5* (12.5 MPL) ready to use.
1 - 1.0 ml vial *APhL ELISA® HRP IgM Calibrator #6* (6.25 MPL) ready to use.

1 - 15 ml bottle *APhL ELISA® HRP IgG Conjugate* ready to use.
1 - 15 ml bottle *APhL ELISA® HRP IgM Conjugate* ready to use.

2 - 15 ml bottle *APhL ELISA® TMB Substrate* ready to use.

2 - 15 ml bottle *APhL ELISA® HRP Stopping Solution* ready to use.

1 - 15 ml bottle de *APhL ELISA® HRP PBS Concentrate* to be diluted in 1000 ml of dH2O.

1 - 200 µl vial *APhL ELISA® HRP Negative Control* to be diluted in sample diluent. *.

1 - 200 µl vial *APhL ELISA® HRP IgG Positive Control* to be diluted in sample diluent. *.

1 - 200 µl vial *APhL ELISA® HRP IgG Positive Control* to be diluted in sample diluent. *.

*** Contains 0.2% Sodium Azide as preservative**

4.2 Warnings

- This product should only be used by appropriately trained personnel.
- The use of automated systems to run the assays, to dilute samples, or to wash plates, should be validated and compared with the manual system by the user.
- Materials of human origin included in the *APhL ELISA® IgG and IgM HRP Kit* have tested negative for HIV-I antibodies and Hepatitis B surface antigen. However, these materials and other sera to be tested should be handled as if they are infectious.
- Sodium azide under acidic conditions yields hydrazoic acid, a very toxic compound. Azide compounds have been classified, under the directives of the European Community (CEE) as **Xn (Harmful)** and should be discarded with running water to avoid deposit in the piping system
- Avoid contact of any component of the kit with skin or mucous membranes. If an accident occurs, rinse the area affected immediately with water and consult a physician.
- The Stopping Solution contains a dilute acid. Use with care to avoid contact with skin and eyes. Avoid exposure to bases, metals, and other compounds which may react with acids. Spills should be cleaned up immediately.

R20 Harmful if inhaled.
R21 Avoid contact with skin.
R22 Harmful if swallowed.
R32 Contact with acids liberates very toxic gas.
S2 Keep out of the reach of children.
S13 Keep away from food, drink, and animal feeding stuffs.
S36 Wear suitable protective clothing.
S37 Use gloves.
S46 If swallowed, seek medical advice immediately and show this container or label.

R36: Irritating to eyes.
R38: Irritating to skin.

4.3 Required materials to run the test but not provided

- Micropipette/Multichannel pipette to deliver 5-1000 µl
- 1-liter cylinder
- Test tubes and racks
- Distilled water.
- ELISA plate reader with a 450 nm filter
- Automatic or semiautomatic ELISA plate washer (optional)
- Magnetic stirrer
- Vortex mixer

4.4 Storage and Stability

It is recommended that the *APhL ELISA® IgG and IgM HRP Kit* be stored at 2-8 °C until expiration date, either unopened or the unused components after opening it.

- Do not freeze any of the components in the *APhL ELISA® IgG and IgM HRP Kit.*
- Do not mix reagents between separate lots.
- Do not change any component. Substitutions will result in unreliability
- Do not use reagents beyond the expiration date.

5 – SPECIMEN COLLECTION AND STORAGE

Testing can be performed using human serum or plasma. Heat-inactivated samples (56°C for 30 minutes or more) should be avoided. Samples that are hemolyzed, lipemic or grossly contaminated should also be avoided.
Following collection, serum or plasma samples should be separated from the whole blood as soon as possible. Once separated, samples may be kept at room temperature no longer than 48 hours or 14 days refrigerated at 2-8°C or 365 days at -20°C or lower. Frozen samples must be mixed well after thawing and prior to testing. Do not freeze and thaw repeatedly.

6 – INSTRUCTIONS TO USE THE KIT

6.1 Procedural Precautions

- Read instruction booklet in its entirety and review prior to testing.
- Bring all reagents and samples to room temperature before use.
- Store all unused samples in the refrigerator as soon as possible after use.
- The *APhL ELISA® HRP IgG Calibrators* and the *APhL ELISA® HRP IgM Calibrators* should only be used in the *APhL ELISA® IgG and IgM HRP Kit.*

- Monitor incubation times carefully.
- Start the incubation time immediately after adding the last reagent.
- Use clean tips for each sample and reagent used.
- Pour reagents into the appropriately labeled reservoirs NOT provided with the *APhL ELISA® IgG and IgM HRP Kit*.
- Do not use Tween or other detergents, and ensure glassware is free of this agent.
- Substrate and stopping solutions must be handled carefully. Avoid contact of these solutions with skin and mucous surfaces
- Estimate the volume needed of each reagent for the run before starting. Make estimate according to the number of samples to be tested.

6.2 Detailed Procedure

a. Phosphate Buffered Saline (PBS).
   - Remove the powder from the bottle labeled *APhL ELISA® HRP PBS Concentrate* and add to a 1-liter cylinder.
   - Add distilled water to complete 1 liter.
   - Stir with a magnetic stirrer until *APhL ELISA® HRP PBS Concentrate* powder is completely dissolved.
   - Pour required amount into a reservoir labeled PBS and keep at room temperature until ready for use.
   - Store excess in the refrigerator.

b. Plates
   - Remove the plate(s) from the pouch at least 10 minutes before use
   - If the whole plate will not be used, select the strips to be used and cut the plastic cover with a sharp blade
   - Separate and return unused strips to the pouch and place them in the refrigerator.
     - After finishing the test, take out the used strips from the frame and discard.
     - Clean and dry the frame.
     - Reattach the unused strips to the frame. Put the frame back into the pouch and seal the pouch with tape. Store the pouch in the refrigerator.

c. Dilution of Calibrators, Samples and Controls
   - Calibrators (6) are ready to use and do not need to be further diluted.
   - For the Positive Control, the Negative Control and unknown samples make 1/50 dilutions as follows:
     - Dilute 10 µl of *APhL ELISA® HRP IgG Positive Control* or 10 µl of *APhL ELISA® HRP IgM Positive Control* in 490 µl of *APhL ELISA® Sample Diluent.*

     - Dilute 10 µl of *APhL ELISA® HRP Negative Control* in 490 µl of *APhL ELISA® Sample Diluent.*
     - For each sample, dilute 10 µl of the sample in 490 µl of *APhL ELISA® Sample Diluent.*
   - Vortex after each dilution is made.

d. Addition of pre-diluted calibrators, controls and samples to ELISA plates
   - **All test samples, calibrators and controls should be run in duplicate.**
   - Add 100 µl of *APhL ELISA® Sample Diluent* to blank wells.
   - Add 100 µl of the *APhL ELISA® HRP IgG calibrators* or 100 µl of the *APhL ELISA® HRP IgM calibrators* to wells.
   - Add 100 µl of the diluted *APhL ELISA® HRP IgG Positive Control or APhL ELISA® HRP IgM Positive Control* to wells
   - Add 100 µl of the diluted *APhL ELISA® HRP Negative Control* to duplicate wells.
   - Add 100 µl of the diluted patient samples to wells.
   - After addition, tap the plate gently once or twice to ensure even distribution.
   - Incubate plates for 30 minutes at room temperature

e. Washing Plates
   - After incubation period, wash plates x 3 with *APhL ELISA® HRP PBS.*
   - This can be performed with an automatic or semiautomatic plate washer or using a multichannel pipette.
   - Add 200 µl of *APhL ELISA® HRP PBS* to each well for each wash.
   - A reservoir should be labeled *APhL ELISA® HRP PBS* for operators using a multichannel pipette. *APhL ELISA® HRP PBS* can be added to this reservoir and required amounts removed as necessary.
   - After each addition of *APhL ELISA® HRP PBS*, tap plates gently, then discard *APhL ELISA® HRP PBS.*
   - ***Make sure strips remain in place***.
   - At the end of the third wash, invert plates and gently tap by turning face down on a flat area covered with blotting paper.

f. Addition of *APhL ELISA® HRP IgG Conjugate* or *APhL ELISA® HRP IgM Conjugate*
   - Carefully remove the estimated necessary amount of solution from the bottle labeled *APhL ELISA® HRP IgG Conjugate* or *APhL ELISA® HRP IgM Conjugate* and put it into a reservoir for pipetting
   - Remove 100 µl aliquots of *APhL ELISA® IgG HRP Conjugate* or *APhL ELISA® HRP IgM Conjugate* in groups of 8 (using a multichannel pipette) and add to consecutive columns of the plates.
   - After addition of the conjugate, incubate the covered plates for 30 minutes at room temperature.

g. Addition of Substrate *APhL ELISA® TMB Substrate*
   - After the plates have been incubated with *APhL ELISA® HRP IgG Conjugate,* or *APhL ELISA® HRP IgM Conjugate* wash the plate x 3 with PBS as described above.
   - Add 100 µl of *APhL ELISA® TMB Substrate* (substrate solution) per well, in groups of 8 or 12 using a multichannel pipette, until complete the plate.
   - Incubate the plates exactly 30 minutes covered at room temperature.

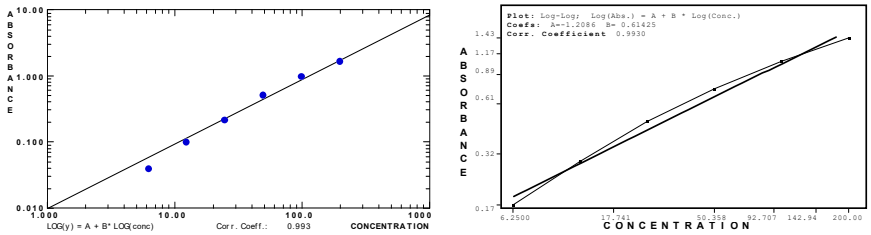
h. Stopping of the color reaction
   - Stop the color reaction by adding 100 µl of *APhL ELISA® HRP Stopping Solution* to each well, in groups of 8 or 12 using a multichannel pipette.
   - Read the plate(s) in a microplate reader at 450 nm.
   - Use the data obtained to establish a calibration curve.

7 – RESULTS

7.1 Elaboration of the calibration curve

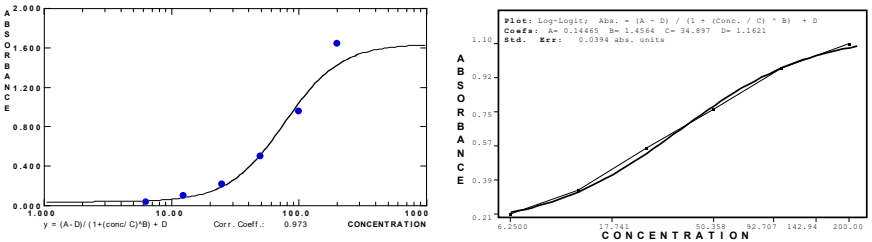
- A calibration curve should be constructed every time for each isotype (IgG or IgM).
- Determine mean optical density (O.D.) reading of the calibrators (C1 to C6), positive control (P) and reagent blank (B).
- Subtract the mean O.D. readings of reagent blank (B) from all mean readings.
- Plot mean O.D. of *C1 - C6* against appropriate concentration using a log-log (Figure 1) or a log-logit (Figure 2) calibration plot.
- This is best done using a computer with appropriate software.
- The concentration of **each calibrator** is listed on each calibrator label.

Figure 1:
Example of a calibration curve for (a). IgG aPL antibodies and (b). IgM aPL antibodies using a log-log plot.



a. IgG aPL b. IgM aPL

Figure 2:
Example of a calibration curve for (a). IgG aPL antibodies and (b). IgM aPL antibodies using a log-logit plot.



a. IgG aPL b. IgM aPL

7.2 Example of a calibration curve.

- The O.D. values obtained from a typical run are found in Table 1 for IgG and Table 2 for IgM
- Do not use these values to construct a calibration curve.
- **This is an example only.**
- **O.D. values obtained for the given run should be used.**

Table 1: Calibration Curve Typical Values for IgG

	Typical Curve *IgG APhL ELISA®*	
Calibrator	**O. D.**	**GPL**
APhL ELISA® HRP IgG Calibrator 1	1.802	200
APhL ELISA® HRP IgG Calibrator 2	1.058	100
APhL ELISA® HRP IgG Calibrator 3	0.676	50
APhL ELISA® HRP IgG Calibrator 4	0.314	25
APhL ELISA® HRP IgG Calibrator 5	0.147	12.5
APhL ELISA® HRP IgG Calibrator 6	0.035	6.25

GPL: 1 GPL unit is the phospholipid binding activity of 1µg/ml of an affinity purified IgG antibody.

Table 2: Calibration Curve Typical Values for IgM

	Typical Curve *IgM APhL ELISA®*	
Calibrator	**O. D.**	**MPL**
APhL ELISA® HRP IgM Calibrator 1	1.608	200
APhL ELISA® HRP IgM Calibrator 2	1.070	100
APhL ELISA® HRP IgM Calibrator 3	0.841	50
APhL ELISA® HRP IgM Calibrator 4	0.481	25
APhL ELISA® HRP IgM Calibrator 5	0.294	12.5
APhL ELISA® HRP IgM Calibrator 6	0.154	6.25

MPL: 1 MPL unit is the phospholipid binding activity of 1µg/ml of an affinity purified IgM antibody.

7.3 Expected results

- The range within which the *APhL ELISA® HRP IgG Positive Control* and *APhL ELISA® HRP IgM Positive Control* should fall is indicated in the label of each vial.
- If the *APhL ELISA® HRP IgG Positive Control* and/or *APhL ELISA® HRP IgM Positive Control* falls outside the range indicated in the label, the operator should review the calculations and procedure for errors. If there are no apparent errors, the assay should be repeated.
- The *APhL ELISA® HRP Negative Control* should give values lower than the suggested cut-off points of 15 GPL units or 15 MPL units.
- Values lower than 27 GPL and 38 MPL and above the cut-off point for IgG and IgM respectively are considered "indeterminate (Grey Zone)". Samples falling in this category should be retested to confirm positivity at a later date. (21)
- If a patient sample has a higher O.D. reading than calibrator C1, the sample could be serially diluted and tested again and the values obtained in GPL or MPL units should be multiplied by the appropriate dilution factor(s), or the value reported as >200 GPL or MPL.

8 – QUALITY CONTROL

- The *APhL ELISA® HRP IgG Positive Control,* the *APhL ELISA® HRP IgM Positive Control,* and the *APhL ELISA® HRP Negative Control* have been provided to help ensure that the assay is performing correctly.
- The *APhL ELISA® HRP IgG Positive Control* has a defined IgG antiphospholipid level. Its range is indicated in the label of the vial.
- The *APhL ELISA® HRP IgM Positive Control* has a defined IgM antiphospholipid level. Its range is indicated in the label of the vial.
- The assay should be considered to be performing correctly when the IgG antiphospholipid level of the *APhL ELISA® HRP IgG Positive Control* and the IgM antiphospholipid level of the *APhL ELISA® HRP IgM Positive Control* fall within the defined range.
- The *APhL ELISA® HRP Negative Control* should give values lower than the suggested cut-off points of 15 GPL units or 15 MPL units.
- The net O.D. of the highest calibrator should be ≥ 1.0
- The mean O.D. of reagent blank should be less than 0.2.

9 – LIMITATIONS

- Diagnosis of the Antiphospholipid Syndrome cannot be based solely on a positive antiphospholipid antibody test.
- Criteria for this diagnosis include a history of one of the following clinical features: thrombosis, pregnancy loss or thrombocytopenia, combined with a positive antiphospholipid ELISA test and/or positive lupus anticoagulant test.
- Patients may have positive lupus anticoagulant but negative antiphospholipid tests; hence, both tests should be performed in patients suspected of having the Antiphospholipid Syndrome.
- Although the *APhL ELISA® IgG and IgM HRP Kit* substantially reduces the frequency of syphilis samples giving positive tests, it is still possible that some samples may yield positive results.
- In addition, a variety of infectious states (including HIV positive patients) and drug-induced disorders may yield false positive tests.

10 – CHARACTERISTICS OF THE ASSAY

10.1 Specificity

Normal

Samples from 50 normal healthy donors were tested in the *APhL ELISA® IgG and IgM HRP Kit.* A cut-off value of 15 GPL units and 15 MPL units was determined based on 99% percentile.

Disease

Positive samples for IgG and/or IgM from different diseases were tested in the *APhL ELISA® IgG and IgM HRP Kit.* The values obtained are listed in the following Table 3 for IgG and Table 4 for IgM.

Table 3: Positive samples for IgG from different diseases

Sample	Number of samples tested.	Number of positive samples for IgG *
APS	43	43
Syphilis +	16	1
Other autoimmune diseases	16	1

* Positive is defined as greater than 15 GPL units for IgG aPL

Table 4: Positive samples for IgM from different diseases

Sample	Number of samples tested.	Number of positive samples for IgM *
APS	39	39
Syphilis +	16	1
Other autoimmune diseases	16	1

* Positive is defined as greater than 15 MPL units for IgM aPL

10.2 Sensitivity

- Sera from 43 patients defined with the Antiphospholipid Syndrome were tested using the *APhL ELISA® IgG and IgM HRP Kit.*
- All 43 patients tested positive for IgG antiphospholipid antibodies.

- Sera from 39 patients defined with the Antiphospholipid Syndrome were tested using the *APhL ELISA® IgG and IgM HRP Kit.*
- All 39 patients tested positive for IgM antiphospholipid antibodies.

10.3 Precision

Intra-assay Variations

Intra-assay variations were determined by running 3 samples for IgG aPL antibodies and 3 samples for IgM aPL antibodies in the *APhL ELISA® IgG and IgM HRP Kit* 10 times in the same plate.
Statistics were calculated and are shown in the following Table 5 for IgG and Table 6 for IgM.

Table 5: IgG Samples

Sample	Mean	Standard deviation	% Coefficient of Variation
A	85.2	4.5	5.3
B	24.0	2.1	8.7
C	10.9	1.1	9.6

Table 6: IgM Samples

Sample	Mean	Standard deviation	% Coefficient of Variation
A	181.6	12.8	7.0
B	44.9	3.8	8.5
C	30.8	2.5	8.0

10.4 Reproducibility

Inter-assay Variations

Inter-assay variations were determined by testing 3 positive samples (high, medium and low) samples for IgG aPL antibodies and 3 positive samples (high, medium and low) samples for IgM aPL antibodies and on the *APhL ELISA® IgG and IgM HRP Kit* 10 different runs.
Statistics were calculated and are shown in the following Table 7 for IgG and Table 8 for IgM.

Table 7: IgG Samples

Sample	Mean	Standard deviation	% Coefficient of Variation
A	86.5	8.5	9.8
B	21.5	2.5	11.6
C	11.2	1.4	12.5

Table 8: IgM Samples

Sample	Mean	Standard deviation	% Coefficient of Variation
A	178.5	6.5	3.6
B	45.6	4.4	9.6
C	21.2	2.0	9.3

10.5 Recovery

- The *APhL ELISA® HRP IgG Calibrator 1* was diluted with normal serum as indicated in the table and the diluted samples run in the *APhL ELISA® IgG and IgM HRP Kit*.
- The expected values, in GPL units, were calculated by dividing the concentration of the *APhL ELISA® HRP IgG Calibrator* 1 by the dilution factor.
- The observed values, in GPL units, were determined from the calibration curve.

Table 9: Recovery of *APhL ELISA® HRP IgG Calibrator 1*

Calibrator Dilution	Observed GPL	Expected GPL	% of Recovery
Neat	212.0	200.0	106
1:2	92.0	100.0	92
1:4	48.0	50.0	96
1:8	22.0	25.0	88
1:16	14.6	12.5	116
1:32	5.2	6.25	83

- The *APhL ELISA® HRP IgM Calibrator 1* was diluted with normal serum as indicated in the table and the diluted samples run in the *APhL ELISA® IgG and IgM HRP Kit*.
- The expected values, in MPL units, were calculated by dividing the concentration of the *APhL ELISA® HRP IgM Calibrator* 1 by the dilution factor.
- The observed values, in MPL units, were determined from the calibration curve.

Table 10: Recovery of *APhL ELISA® HRP IgM Calibrator 1*

Calibrator Dilution	Observed MPL	Expected MPL	% of Recovery
Neat	200.7	200.0	100
1:2	86.8	100.0	87
1:4	53.6	50.0	106
1:8	19.2	25.0	77
1:16	12.6	12.5	100
1:32	7.9	6.25	126

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