# Human IgM AccuSignal™ ELISA Kit – KAA9107

Rockland's Human IgM AccuSignal™ ELISA (Enzyme-Linked Immunosorbent Assay) Kit is an in vitro enzyme-linked immunosorbent assay designed for the quantitative detection of human IgM in serum, plasma, and cell culture supernatants.

## I. KIT COMPONENTS

| Human IgM AccuSignal™ ELISA Kit | |
|---|---|
| Catalog Number | **Component** |
| KAR001 | Coating Buffer |
| KAR106-07 | Capture Antibody |
| KAR002 | Wash Buffer (10X) |
| KAR003 | Assay Buffer (10x) to dilute into: Blocking buffer and Standard Reagent buffer |
| | |
| KAR005-07 | Human IgM Control Standard |
| KAR607-07 | Biotinylated Detection Antibody |
| KAR008 | Streptavidin-Peroxidase Conjugate |
| KAR011 | Streptavidin-Peroxidase Stabilizer |
| N/A | Empty Amber Vial |
| KAR009 | Substrate Solution |
| KAR010 | Stop Solution |

## II. STORAGE CONDITIONS

Store coating buffer, wash buffer, assay buffer and standard and reagent diluent at 4 ˚C for 3-6 months. Aliquot capture antibody and target protein standard contents and freeze at -20 ˚C. Store detection antibody and Streptavidin-Peroxidase conjugate vial at 4 °C prior to restoration. For extended storage aliquot contents and freeze at -20 °C or below. Avoid multiple freeze-thaw cycles. Store substrate solution at 4° C prior to opening. Protect from moisture and light.

## III. INTRODUCTION

Human IgM AccuSignal™ ELISA Kit is based on standard sandwich enzyme-linked immunosorbent assay technology. This assay employs a capture antibody specific for human IgM that can be coated onto 96-well plate. Standards and test samples are then pipetted into the wells. After sample binding, unbound proteins and molecules are washed off, and a biotinylated detection anti-human IgM antibody is added to the wells.

The wells are washed with wash buffer. After washing away unbound biotinylated antibody, Streptavidin Peroxidase Conjugate is added to the wells and unbound conjugates are washed away. Substrate solution is added to the wells to visualize HRP enzymatic reaction. The reaction produces a blue color product, which turns yellow when the reaction is terminated by the addition of stop solution. The absorbance of the yellow product at 450 nm is proportional to the amount of Human IgM present in a sample.

## IV. REQUIRED EQUIPMENT AND REAGENTS

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 96 well ELISA plate (a variety of ELISA plates can be used, e.g. Nunc MaxiSorp Plates)
- Microplate reader capable of measuring absorbance at 450nm
- Automated plate washer or a wash bottle
- Adjustable pipettes and pipette tips
- Clean tubes and Eppendorf tubes
- Distilled or deionized water
- Absorbent paper

## V. GENERAL CONSIDERATIONS

Please read the following instructions before starting the experiment. Modifications to the kit components or procedures may result in the loss of performance.

- All ELISA reagents must be at room temperature before use.
- Before using the kit, briefly spin down the vials.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Do not allow 96-well plate to dry, this will inactivate components on plate.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Ensure plates are properly sealed or covered during incubation steps.
- Do not mix or substitute reagents or materials from other kit lots or vendors.
- Do not vortex the standard during reconstitution, as this will destabilize the protein.
- Keep the standard dilutions on ice during preparation, but the ELISA procedure should be done at room temperature.
- Do not contaminate the substrate solution. If the solution is blue before use, DO NOT USE IT.
- Include a standard curve each time the assay is performed.
- For statistical reasons, we recommend both standard and samples should be assayed with a minimum of two replicates (duplicates).
- Samples generating values greater than the highest standard should be further diluted in the appropriate sample dilution buffer.

## VI. SANDWICH ELISA PROTOCOL A. Reagent Preparation

Equilibrate all reagents to room temperature (18-25 °C) prior to use.

### Blocking Buffer

Dilute 30 mL of 10X assay buffer concentrate into 70 mL deionized or distilled water to yield 100 mL of blocking buffer.

### 1X Wash Buffer

If the 10X wash buffer contains visible crystals, equilibrate to room temperature and mix gently until dissolved. Dilute 100 mL of 10X wash buffer into 900 mL deionized or distilled water to yield 1000 mL of 1X wash buffer.

### 1X Standard and Reagent Diluent

Dilute 20 mL of 10X assay buffer into 180 mL deionized or distilled water to yield 200 mL of 1X standard and reagent diluent buffer.

### Preparation of 1 µg/mL capture antibody

Briefly spin the anti-human IgM capture antibody vial before use. Prepare capture antibody working dilution (1 µg/mL) in coating buffer, pH 9.5. For example, dilute 12 µL of 1 mg/mL stock capture antibody in 11.88 mL coating buffer.

The capture antibody stock (1mg/mL) can be aliquoted and stored at -20 °C.

### Preparation of 0.75 µg/mL biotinylated detection antibody

Gently knock down any visible powder on the walls of the vial before use. Reconstitute biotinylated antibody in 100 µL of 1X standard and reagent diluent (final concentration will be 1 mg/mL). Briefly spin streptavidin-peroxidase conjugate vial before use to ensure complete recovery.

Prepare biotinylated antibody working dilution (0.75 µg/mL) in 1X standard and reagent diluent. For example, dilute 9 µL of 1 mg/mL biotinylated antibody in 11.91 mL of 1X standard and reagent diluent.

The biotinylated antibody stock concentrate (1 mg/mL) can be aliquoted and stored at -20 °C.

### Preparation of 0.1 µg/mL streptavidin-peroxidase conjugate solution

Gently knock down any visible powder on the walls of the vial before use. Reconstitute streptavidin peroxidase conjugate in 100 µL of stabilizer solution (final concentration will be 1 mg/mL). Briefly spin streptavidin-peroxidase conjugate vial before use to ensure complete recovery.

Using the empty amber vial provided, prepare streptavidin-peroxidase conjugate stock concentrate (10 µg/mL) in stabilizer. For example, dilute 8 µL of 1 mg/mL streptavidin-peroxidase conjugate in 792 µL stabilizer solution. The streptavidin-peroxidase conjugate stock concentrate can be aliquoted and stored at 4 °C (DO NOT FREEZE).

Prepare streptavidin-peroxidase conjugate working dilution (0.1 µg/mL) in 1X standard and reagent diluent. For example, dilute 120 µl of 10 µg/mL streptavidin-peroxidase conjugate in 11.88 mL of 1X standard and reagent diluent.

## B. Standard Preparation

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

### Preparation of 1µg/mL standard

Dilute 2.5 µL of target protein standard (100 µg/mL) in 247.5 µL of 1X standard and reagent diluent.

### Preparation of 100 ng/mL stock standard

Dilute 25 µL of 1 µg/mL target protein stock standard in 225 µL of 1X standard and reagent diluent.

### Preparation of 30 ng/mL stock standard

Dilute 150 µL of 100 ng/mL target stock protein standard in 350 µL of 1X standard and reagent diluent.

- Prepare **Standard #1** by adding 250 µL of the 30 ng/mL **Stock Standard**, to 250 µL of 1X standard and reagent diluent. Mix thoroughly and gently.

- Pipette 250 µL of 1X standard and reagent diluent into remaining tubes.
- Prepare **Standard #2** by adding 250 µL of Standard #1 to tube #2 and mix thoroughly.
- Prepare **Standard #3** by adding 250 of µL Standard #2 to tube #3 and mix thoroughly.
- Using the table **below** as a guide, prepare further serial dilutions.
- 1X standard and reagent diluent serves as the zero (BLANK) (0 ng/mL).

| Standard Dilution Preparation | | | | | |
|---|---|---|---|---|---|
| Standard # | Volume to Dilute (µl) | Diluent (µl) | Total Volume (µl) | Starting Concentration (ng/mL) | Final Concentration (ng/mL) |
| 1 | 250 | 250 | 500 | 30.0 | 15.0 |
| 2 | 250 | 250 | 500 | 15.0 | 7.5 |
| 3 | 250 | 250 | 500 | 7.5 | 3.75 |
| 4 | 250 | 250 | 500 | 3.75 | 1.875 |
| 5 | 250 | 250 | 500 | 1.875 | 0.938 |
| 6 | 250 | 250 | 500 | 0.938 | 0.469 |
| 7 | 250 | 250 | 500 | 0.469 | 0.234 |
| 8 | 0 | 250 | 250 | 0 | 0 |

### C. Sample Preparation

1X standard and reagent diluent should be used for dilution of serum and plasma.

The levels of the target protein may vary between different samples. Optimal dilution factors for each sample must be determined by the investigator.

Suggested dilution for normal serum/plasma: 10,000,000-fold.

### D. Assay Procedure

Samples and reagents should be mixed thoroughly and evenly after dilution. A standard curve for human IgM detection should be prepared for each experiment. For statistical reasons, it is recommended to assay all standards, controls and samples in duplicate. A suggested plate format is shown on the following page.

1. Dilute the capture antibody to a final concentration of 1µg/mL in coating buffer as described above.
2. Add 100 µL of capture antibody into appropriate wells. Cover wells and incubate overnight at room temperature.
3. Remove the cover and discard the capture antibody solution. Invert the plate and blot it against clean paper towels or other absorbent material. Do NOT let the wells dry completely at any time.
4. Add 250 µL of prepared blocking buffer to each well and incubate at room temperature for 1-3 hours.
5. Discard the blocking solution and add 100 µL of each standard (see Standard Preparations above) and sample into appropriate wells. Cover wells and incubate for 1 hour at room temperature.
6. Discard the solution and wash the plate 4 times with 1X Wash buffer. Wash by filling each well with

300 µL of wash buffer using a multi-channel pipette or auto washer. Complete removal of liquid at each step is important to good performance. (Note: for automated washing, aspirate all wells and wash four times with 1X wash buffer, overfilling the wells with wash buffer). After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels or other absorbent material.

7. Add 100 µL of biotinylated anti-Human IgM detection antibody working solution to each well, seal the plate and incubate for 1 hour at room temperature.
8. Discard the solution and repeat the wash as described in step 6.
9. Add 100 µL of streptavidin-peroxidase conjugate working solution (see preparation of streptavidin-peroxidase conjugate solution above) to each well. Incubate for 30 minutes at room temperature.
10. Discard the solution. Repeat the wash as described in step 6.
11. Add 100 µL of substrate solution to each well. Seal the plate and incubate for 30 min at room temperature in the dark. (Note: the optimal incubation time should be determined by the end user).
12. Add 100 µL of stop solution to each well.
13. Read at 450 nm in a microplate reader immediately.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | S1 | S1 | 1 | 1 | 9 | 9 | 17 | 17 | 25 | 25 | 33 | 33 |
| B | S2 | S2 | 2 | 2 | 10 | 10 | 18 | 18 | 26 | 26 | 34 | 34 |
| C | S3 | S3 | 3 | 3 | 11 | 11 | 19 | 19 | 27 | 27 | 35 | 35 |
| D | S4 | S4 | 4 | 4 | 12 | 12 | 20 | 20 | 28 | 28 | 36 | 36 |
| E | S5 | S5 | 5 | 5 | 13 | 13 | 21 | 21 | 29 | 29 | 37 | 37 |
| F | S6 | S6 | 6 | 6 | 14 | 14 | 22 | 22 | 30 | 30 | 38 | 38 |
| G | S7 | S7 | 7 | 7 | 15 | 15 | 23 | 23 | 31 | 31 | 39 | 39 |
| H | Blk | Blk | 8 | 8 | 16 | 16 | 24 | 24 | 32 | 32 | 40 | 40 |

## CALCULATIONS

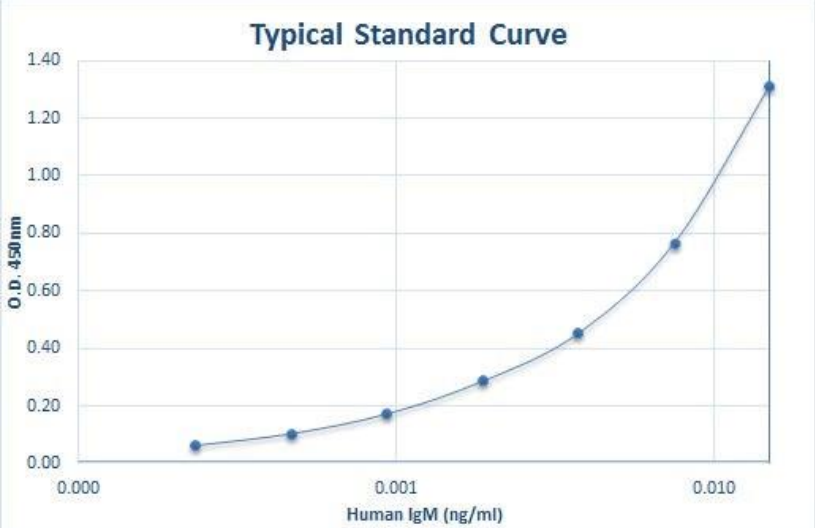
For calculations, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The Human IgM concentration of the samples can be interpolated from the standard curve.

**Note:** If the samples measured were diluted, multiply the concentrations obtained from interpolation by the dilution factor.

## VII. EXAMPLE DATA OBTAINED FROM HUMAN IgM

| **Concentration (ng/mL)** | 0 | 0.234 | 0.468 | 0.937 | 1.875 | 3.75 | 7.5 | 15 |
|---|---|---|---|---|---|---|---|---|
| **O.D** | 0 | 0.06 | 0.1 | 0.17 | 0.29 | 0.45 | 0.76 | 1.31 |

## VIII. HUMAN IgM ELISA KIT STANDARD CURVE EXAMPLE




| **Range** | 0.23 ng/mL-15.0 ng/mL |
|---|---|
| **Sensitivity** | <250 pg/mL |
| **Specificity** | Natural and recombinant Human IgM |
| **Recovery** | 100% |

## ASSAY SUMMARY

- Prepare all reagents, samples and standards as instructed.
- Add capture antibody to each well used. Incubate the plate overnight at room temperature.
- Add prepared blocking buffer to each well. Incubate for 1-3 hours at room temperature.
- Add standards or samples to each well used. Incubate the plate for 1 hour at room temperature. Wash plate four times with wash buffer.
- Add prepared biotinylated antibody to each well. Incubate the plate for 1 hour at room temperature. Wash plate four times with wash buffer.
- Add streptavidin-peroxidase conjugate working solution. Incubate the plate for 30 min at room temperature. Wash plate four times with wash buffer.
- Add substrate solution to each well. Incubate the plate in dark for 30 min or until color develops.
- Add stop solution. Read at 450nm.

## IX. TROUBLESHOOTING

### Weak or No Signal

| Possible Cause | Solution |
|---|---|
| **Problem with the standard** | Use new sample<br>Check that the standard is appropriately handled |
| **Incubation time too short** | Follow the exact guidelines for incubation time (If the problem persists, try incubating samples at 4°C overnight) |
| **Incubation temperature too low** | Ensure incubations are done at correct temperature Before proceeding |
| **Incompatible sample type** | Use sample that the assay is known to detect as a positive control (Include such control in your experiment) |
| **Target present below detection limit** | Decrease dilution factor or concentrate samples |
| **Incorrect/Insufficient/No substrate** | Check the substrate identity<br>Increase concentration or amount of substrate |
| **Antibody stored at 4°C for several weeks or subjected to repeat freeze-thaw cycles** | Use fresh aliquot of antibody that has been stored at -20°C or below |
| **Incorrect reagents added/ prepared; Missing reagents** | Check protocol, ensure correct reagents are added in proper order and prepared to correct concentrations |
| **Expired/Contaminated reagents** | Prepare fresh/uncontaminated reagents |
| **Incorrect storage of components** | Check storage conditions for the kit (Kit need to be stored at 4°C) |
| **Excessive plate washing** | Gently pipette wash buffer (manual method)<br>Ensure correct pressure (automatic wash system) |
| **Wells dry out** | Cover plate using adhesive cover at all incubation times |
| **Plate read at incorrect detection wavelength** | Use recommended wavelength/filter<br>Ensure plate reader is set correctly for substrate used |

| | |
|---|---|
| **Slow color development** | Prepare substrate immediately before use<br>Allow longer incubation time<br>Ensure stock solution is unexpired and uncontaminated |

### Saturated Signal

| Possible Cause | Solution |
|---|---|
| **High sample concentration** | Use higher sample dilutions (Determine the optimal dilutions by titration assay) |
| **Substrate color changed before use** | Prepare substrate immediately before use |
| **Incubation time too long** | Follow the exact guidelines for incubation times |
| **Excessive antibody** | Repeat the assay with lower antibody concentrations to find the optimal one for your experiment |
| **Contaminated buffers or peroxidase** | Prepare and use fresh buffers |
| **Insufficient washing** | Follow the exact guidelines<br>At the end of each washing step, flick the plate over a sink and dry the plate on a paper towel |
| **Plate adhesive cover not used or re-used** | During incubations, cover plates with adhesive cover. Use a fresh cover every time the used cover is removed from the plate |
| **Plate read at incorrect wavelength** | Use recommended wavelength/filter<br>Ensure plate reader is set correctly for the substrate used |
| **Excess time before plate reading** | Read your plate within 30 minutes after adding the stop solution |

### High Background

| Possible Cause | Solution |
|---|---|
| **Insufficient washing** | Follow the exact guidelines<br>At the end of each washing step, flick the plate over a sink and pat dry the plate on a paper towel |
| **Excessive antibody** | Repeat the assay with lower antibody concentrations to find the optimal one for your experiment |
| **Cross reactivity** | Run appropriate controls |
| **Incubation temperature too high** | Optimize incubation temperature for your assay (antibodies bind optimally at very specific temperature) |
| **Reagents were not mixed properly** | Thoroughly mix all reagents and samples before pipetting solutions into wells |
| **Blanks contaminated with samples** | Change pipette tips when switching between blanks and samples |
| **Sample contaminated with enzymes** | Test samples with substrate alone to check for contaminating enzymes |
| **Contaminated substrate solution** | Use a clean container to check that the substrate in not contaminated (TMBE substrate should be clear and colorless before adding to wells) |

| | |
|---|---|
| **Substrate exposed to light** | Carry out substrate incubation in dark |
| **Evaporation of solution from well during incubation** | Always incubate with a cover on the plate |
| **Incubation time too long** | Follow the exact guidelines for incubation times |
| **Incorrect standard curve dilutions** | Check pipetting techniques Check calculations |
| **Unstopped color development** | Use stop solution to prevent over-development |
| **Incorrect plate reading setting** | Use recommended wavelength/filter<br>Ensure plate reader is set correctly for the substrate used |

### Low Sensitivity

| Possible Cause | Solution |
|---|---|
| **Improper storage of ELISA kit** | Store all reagents as recommended |
| **Insufficient target** | Reduce sample dilution or concentrate sample |
| **Inactive substrate** | Ensure reporter enzyme has the expected activity |
| **Incompatible sample type** | Include positive control in your experiment |
| **Interfering ingredients in buffers and sample** | Check reagents for any interfering chemicals, e.g. sodium azide in antibodies inhibit HRP enzyme; EDTA used as anti-coagulant for plasma collection inhibits enzymatic reactions |
| **Mixing or substituting reagents from different kits** | Avoid mixing components from different kits |
| **Incorrect plate reading setting** | Use recommended wavelength/filter<br>Ensure plate reader is set correctly for the substrate used |

### Poor Standard Curve Generation

| Possible Cause | Solution |
|---|---|
| **Improper standard solution** | Confirm dilutions are done correctly<br>Prepare new standard curve as appropriate |
| **Standard improperly reconstituted** | Briefly spin vial before opening<br>Inspect for undissolved material after reconstituting |
| **Standard degraded** | Store and handle standard as recommended |
| **Pipetting error** | Use calibrated pipettes and proper pipetting technique |
| **Insufficient washing** | Follow the exact guidelines<br>At the end of each washing step, flick the plate over a sink and pat dry the plate on a paper towel |
| **Poorly mixed reagents** | Thoroughly mix reagents |
| **Plates stacked during incubation** | Keep plates separated if not using rotating plates |

### Poor Replicate Data

| Possible Cause | Solution |
|---|---|
| **Bubbles in wells** | Ensure no bubbles are present prior to reading the plate |
| **Insufficient washing of wells** | Carefully wash wells<br>Check that all ports of the plate washer are unobstructed |
| **Incomplete reagent mixing** | Ensure all reagents are mixed thoroughly |
| **Inconsistent pipetting** | Use calibrated pipettes and proper pipetting techniques Use a new cover every time the used cover is removed from the plate |
| **Inconsistent sample preparation or storage** | Ensure consistent sample preparation and optimal sample storage (e.g. minimize freeze/thaw cycles) |
| **Particulates in samples** | Remove the particulates by centrifugation |
| **Cross-well contamination** | Ensure plate covers and pipette tips are not contaminated with reagents |
| **Edge effect (higher or lower OD in peripheral wells than in central wells)** | Ensure plates and reagents are kept at temperatures as instructed<br>During incubation, seal the plate completely and avoid stacking plates |

### Inconsistent Assay-to-Assay Results

| Possible Cause | Solution |
|---|---|
| **Insufficient washing of wells** | Carefully wash wells<br>Check that all ports of the plate washer are unobstructed |
| **Varied incubation temperatures** | Adhere to recommended incubation temperature |
| **Variation in protocol** | Adhere to the same protocol from experiment to experiment |
| **Plate cover not used or reused** | During incubations, cover plates with plate cover<br>Use a new cover every time the used one is removed |
| **Incorrect dilutions** | Confirm dilutions are done correctly for standard solutions<br>Prepare new standard curve as appropriate |
| **Contaminated buffers** | Prepare and use fresh buffers |

### Slow Color Development

| Possible Cause | Solution |
|---|---|
| **Substrates too old, contaminated or used at incorrect pH** | Prepare fresh substrates at correct pH |
| **Expired/Contaminated solutions** | Prepare fresh reagents before use |
| **Incorrect incubation temperature** | Ensure plates and reagents are kept at temperatures as instructed<br>During incubation, seal the plate completely and avoid stacking plates |

## X. RELATED PRODUCTS

| Component | Catalog # | Size |
|---|---|---|
| 10x TBS pH 7.5 | MB-012 | 1000 mL |
| 10x TTBS pH 7.5 | MB-013 | 1000 mL |
| 10X PBS pH 7.2 | MB-008 | 1000 mL |
| 10x PBST pH 7.2 | MB-075-1000 | 1000 mL |
| 10xPBS Fish gel concentrate | MB-066-1000 | 10 Pack |