AssayLite™
Human IgA, IgG, and IgM Multiplex EFSIA Kit
(Blue, Red, and Orange Fluorescent Probe)

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This product is manufactured under patent pending technology by Assaypro LLC

For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

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Assay Summary

Step 1. Add 100 µl of Standard or Sample per well.
Incubate 2 hours at 37°C.

Step 2. Wash, then add 100 µl of Human IgA, IgG, and IgM Fluorescent Probe Mixture per well.
Incubate 1 hour at 37°C.

Step 3. Wash, then add 50 µl of Stabilizing Solution per well.

Step 4. Read immediately.
IgA: Read at EX 590/10 and EM 660/10
IgG: Read at EX 485/20 and EM 575/15
IgM: Read at EX 485/20 and EM 680/30

Symbol Key

Consult instructions for use.

Assay Mechanism

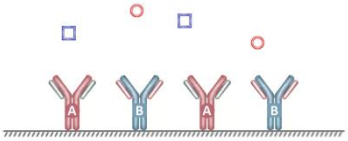


Figure 1. Standard and samples are added to wells and incubated.

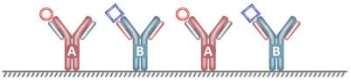


Figure 2. The Antigen A in the standard and samples binds to the immobilized Antibody A. The Antigen B in the standard and samples binds to the immobilized Antibody B. All unbound materials are washed away.

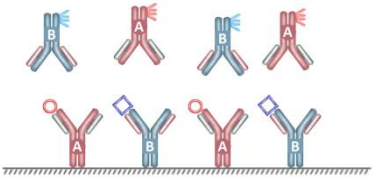


Figure 3. The Antibody A and B Fluorescent Probes are added to the wells. The Antibody A Fluorescent Probe binds to the Antigen A. The Antibody B Fluorescent Probe binds to the Antigen B.

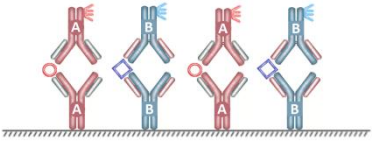
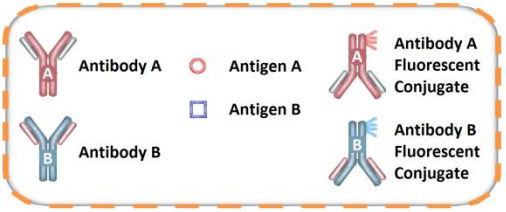


Figure 4. The microplate is washed and the endpoint fluorescence is measured. The fluorescence intensity is proportional to the concentration of the Antigens A and B in the Standard and samples.



Assay Template

	A	B	C	D	E	F	G	H
1								
2								
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Human IgA, IgG, and IgM Multiplex EFSIA Kit

Catalog No. FI700161M3

Sample insert for reference use only

Introduction

Human Immunoglobulin A (IgA) is the most abundant antibody isotype in mucosal secretions and exists in two subclasses IgA1 and IgA2 (1). While circulating serum IgA1 occurs mainly in the monomeric 160 kDa form (2), mucosal secretary IgA2 is in dimeric form and serves as the first line of defense against microorganisms through immune exclusion (3). Selective IgA deficiency is the most common primary immunodeficiency observed by a maturation defect in B cells to produce IgA (4).

Human immunoglobulin G (IgG), the most abundant antibody in serum, constitutes 75% of serum immunoglobulins. IgG is synthesized and secreted by plasma B cells and contains two heavy chains and two light chains. IgG has four subclasses IgG1, IgG2, IgG3, and IgG4 and is involved in the secondary immune response. As it is the only isotype that can pass through the human placenta, maternal IgG provides the defense against infection for the first few weeks of a neonate (5).

Human immunoglobulin M (IgM) is a large mushroom-shaped antibody against A and B antigens on red blood cells and is produced by B cells (6). It forms a pentamer or a hexamer in serum and also a monomer on B cell surface. Each of the five monomers has a molecular mass of 180 kDa, consists of two light and two heavy chains, and a joining J chain required for the synthesis of the pentamer (7, 8). Upon an exposure to an acute infection, IgM is the predominant antibody produced to fight the foreign red blood cell antigen. It activates complement and agglutinates red blood cells. IgM is the first immunoglobulin made by the fetus and by B cells when stimulated by antigens (9, 10). It does not pass across the human placenta due to its large size (11-13).

Principle of the Assay

The AssayLite Human IgA, IgG, and IgM Multiplex EFSIA (Endpoint Fluorescent Sandwich Immunoassay) Kit employs a quantitative **sandwich fluorescent probe technique** that measures IgA, IgG, and IgM in human **plasma, serum, and cell culture samples** in less than 4 hours. A polyclonal antibody specific for human IgA, a polyclonal antibody specific for human IgG, and a polyclonal antibody specific for human IgM have been pre-coated onto a 96-well opaque microplate with removable strips. IgA, IgG, and IgM in standards and samples

are sandwiched by the immobilized antibody and a polyclonal antibody specific for IgA, IgG or IgM conjugated to a fluorescent probe. All unbound material is washed away before the endpoint fluorescence is measured.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (working diluent buffer, wash buffer, standard, and fluorescent probe) as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- The kit should not be used beyond the expiration date.
- Avoid direct light exposure to the assay.
- Store the fluorescent probe in a dark place. Do not freeze.

Reagents

- **Human IgA/IgG/IgM Microplate:** A 96-well black polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human IgA, a polyclonal antibody against human IgG, and a polyclonal antibody against human IgM.
- **Sealing Films:** Each kit contains 3 precut, pressure sensitive aluminum sealing films that can be cut to fit the format of the individual assay.
- **Human IgA/IgG/IgM Standard:** Human IgA/IgG/IgM in a buffered protein base (IgA: 300 ng, IgG: 500 ng, IgM: 250 ng, lyophilized).
- **Blue Fluorescent Human IgA Probe:** 6 vials, lyophilized.
- **Red Fluorescent Human IgG Probe:** 6 vials, lyophilized.
- **Orange Fluorescent Human IgM Probe:** 6 vials, lyophilized.
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml).
- **Stabilizing Solution:** A ready-to-use solution to stabilize the fluorescent component (8 ml).

Storage Conditions

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Microplate, Diluent Concentrate (10x), Stabilizing Solution, and Wash Buffer at 2-8°C.

- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.
- Store the Standard at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.
- Store the Fluorescent Probe in a dark place at 2-8°C before and after reconstituting with Diluent. Do not freeze.

Other Supplies Required

- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel)
- Deionized or distilled reagent grade water
- Incubator (37°C)
- Fluorescent Microplate Reader

Fluorescent Microplate Reader (BioTek® Synergy H1F, filter-based reader; available for order from Assaypro)

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10 minutes. Dilute samples 1:50000 into EIA Diluent, or within the range of 20000x – 200000x, and assay (*suggested dilution factor only; user should determine optimal dilution factor*). The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes, and remove serum. Dilute samples 1:50000 into EIA Diluent, or within the range of 20000x – 200000x, and assay (*suggested dilution factor only; user should determine optimal dilution factor*). The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x *g* for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Refer to Sample Dilution Guidelines below for further instruction.

Guidelines for Dilutions of 1:100 or Greater (*for reference only; please follow the protocol for specific dilution suggested)*	
1:100	**1:10000**
A) 4 ul sample: 396 µl buffer = 100 fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000 fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1:1000	**1:100000**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000 fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000 fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.
- **Standard Curve:** Reconstitute the Human IgA, IgG, and IgM Standard (IgA: 300 ng, IgG: 500 ng, IgM: 250 ng) with 1 ml of EIA Diluent to generate a standard stock solution (IgA: 300 ng/ml, IgG: 500 ng/ml, IgM: 250 ng/ml). Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard stock solution (IgA: 300 ng/ml, IgG: 500 ng/ml, IgM: 250 ng/ml) 1:2 with EIA Diluent to generate a standard curve that measures all IgA, IgG, and IgM proteins simultaneously. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

Standard Point	Dilution	[IgA] (ng/ml)	[IgG] (ng/ml)	[IgM] (ng/ml)
P1	1 part Standard	300.0	500.0	250.0
P2	1 part P1 + 1 part EIA Diluent	150.0	250.0	125.0
P3	1 part P2 + 1 part EIA Diluent	75.0	125.0	62.5
P4	1 part P3 + 1 part EIA Diluent	37.5	62.5	31.25
P5	1 part P4 + 1 part EIA Diluent	18.75	31.25	15.63
P6	1 part P5 + 1 part EIA Diluent	9.375	15.63	7.813
P7	1 part P6 + 1 part EIA Diluent	4.688	7.813	3.906
P8	EIA Diluent	0.000	0.000	0.000

- **Blue Fluorescent Human IgA Probe (1x):** Reconstitute the Fluorescent Probe with 0.9 ml EIA Diluent to produce a working solution. Allow the probe to sit for 10 minutes with gentle agitation prior to use. Any remaining solution should be refrigerated at 2-8°C and used within 30 days. Do not freeze.
- **Red Fluorescent Human IgG Probe (1x):** Reconstitute the Fluorescent Probe with 0.9 ml EIA Diluent to produce a working solution. Allow the probe to sit for 10 minutes with gentle agitation prior to use. Any remaining solution should be refrigerated at 2-8°C and used within 30 days. Do not freeze.
- **Orange Fluorescent Human IgM Probe (1x):** Reconstitute the Fluorescent Probe with 0.9 ml EIA Diluent to produce a working solution. Allow the probe to sit for 10 minutes with gentle agitation prior to use. Any remaining solution should be refrigerated at 2-8°C and used within 30 days. Do not freeze.

    - Once Fluorescent Blue, Red, and Orange Probes are reconstituted and ready, combine equal parts of all probes to the desired volume to generate the **Human IgA/IgG/IgM Fluorescent Probe Mixture**.

      Example:
      Combine the following reagents according to the number of wells in the assay (n) plus one.

      1633 µl Blue Fluorescent Human IgA Probe
      1633 µl Red Fluorescent Human IgG Probe
      1633 µl Orange Fluorescent Human IgM Probe
      Assuming the needed volume is less than or equal to 4900 µl (48 wells + 1)

- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is incubated at 37°C.
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 100 µl of Human IgA/IgG/IgM Standard or sample per well. Cover wells with a sealing film and incubate for 2 hours at 37°C. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Add 100 µl of Human IgA/IgG/IgM Fluorescent Probe Mixture per well. Cover wells with a sealing film and incubate for 1 hour at 37°C. Start the timer after the last addition.
- Wash the microplate as described above.
- Immediately add 50 µl of Stabilizing Solution to each well.
- **IgA:** Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 590/10 nm and emission wavelength of 660/10 nm **immediately** for best results.
  IgG: Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 485/20 nm and emission wavelength of 575/15 nm **immediately** for best results.
  IgM: Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 485/20 nm and emission wavelength of 680/30 nm **immediately** for best results.
- **IgA:** For the Synergy H1F, a gain of 90 is suggested. However, the user should determine the optional gain/ amplification.
  IgG: For the Synergy H1F, a gain of 75 is suggested. However, the user should determine the optional gain/ amplification.
  IgM: For the Synergy H1F, a gain of 90 is suggested. However, the user should determine the optional gain/ amplification.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean emitted

fluorescence on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.

- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Typical Data

The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

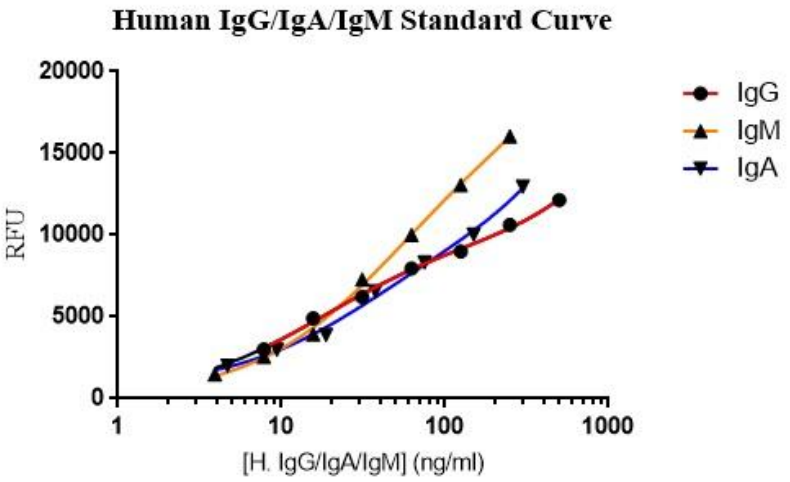
Reader	Standard Point	ng/ml	Average Blue IgA RFU
BioTek – Synergy H1F	P1	300.0	12959
	P2	150.0	10031
	P3	75.0	8331
	P4	37.5	6552
	P5	18.75	3895
	P6	9.375	2983
	P7	4.688	2011
	P8	0.000	1062
Sample: Sodium Citrate Plasma (50000x)			6684

Reader	Standard Point	ng/ml	Average Red IgG RFU
BioTek – Synergy H1F	P1	500.0	12147
	P2	250.0	10628
	P3	125.0	8998
	P4	62.5	7964
	P5	31.25	6207
	P6	15.63	4913
	P7	7.813	3014
	P8	0.000	1123
Sample: Sodium Citrate Plasma (50000x)			9386

Reader	Standard Point	ng/ml	Average Orange IgM RFU
BioTek – Synergy H1F	P1	250.0	16044
	P2	125.0	13065
	P3	62.5	10005
	P4	31.25	7291
	P5	15.63	3920
	P6	7.813	2550
	P7	3.906	1476
	P8	0.000	631
Sample: Sodium Citrate Plasma (50000x)			4305

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Note: Standard curve reading with Biotek® Synergy H1F

Reference Value

- Normal human IgA plasma levels range from 0.5 to 4 mg/ml.
- Normal human IgG plasma levels range from 6 to 18 mg/ml.
- Normal human IgM plasma levels range from 0.3 to 2 mg/ml.

Sample	n	IgA Average Value (µg/ml)	IgG Average Value (µg/ml)	IgM Average Value (µg/ml)
Normal Plasma and Serum Samples	30	2058	10324	1109

Performance Characteristics

- The minimum detectable dose of IgA as calculated by 2SD from the mean of a zero standard was established to be 3.7 ng/ml.
- The minimum detectable dose of IgG as calculated by 2SD from the mean of a zero standard was established to be 6.9 ng/ml.
- The minimum detectable dose of IgM as calculated by 2SD from the mean of a zero standard was established to be 3.3 ng/ml.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay. Average intra-assay coefficient of variation was 5.2%.
- Inter-assay precision was determined by testing three plasma samples in twenty assays. Average inter-assay coefficient of variation was 10.1%.

Recovery

	IgA	IgG	IgM
Standard Added Value	9.4 – 150 ng/ml	15.6 – 250 ng/ml	7.8 – 125 ng/ml
Recovery %	88 – 109%	93 – 111%	92 – 113%
Average Recovery %	99%	95%	96%

Linearity

- Plasma and serum samples were serially-diluted to test for linearity.

Average Percentage of Expected Value (%)						
Sample Dilution	Plasma			Serum		
	IgA	IgG	IgM	IgA	IgG	IgM
1:25000	95%	92%	94%	95%	92%	92%
1:50000	99%	99%	100%	99%	99%	99%
1:100000	101%	106%	96%	106%	102%	94%

Cross-Reactivity

Species	Cross Reactivity (%)
Bovine	None
Canine	None
Mouse	None
Monkey	<5%
Rat	None
Rabbit	None
Swine	None
Human	100%
Proteins	**Cross Reactivity (%)**
IgM	100%
IgA1	100%
IgA2	100%
IgG1	<50%
IgG2	<5%
IgG3	<30%
IgG4	<50%
IgD	2%
IgE	<1%

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of expired components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are dry after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.

	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the Standard and Fluorescent Probe after reconstitution. • Thoroughly mix dilutions.
	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Wash step was skipped	• Consult the provided procedure for all wash steps.
	Improper wash step	• Check that the correct wash buffer is being used.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.
	Prolonged exposure of assay or Fluorescent Probe to light	• Overexposure can affect the stability of the Fluorescent Probe, store in a dark location. • Cover and cap all reagents when not in use. • Cover assay with aluminum sealing film immediately after loading.
	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporated	• Verify that the aluminum sealing film is firmly in place before placing the assay in the incubator.
	Used filters with an overlapping bandpass	• As an example, do not use a filter combination of 620/20 EX and 660/40 EM, use a 660/20 filter instead.
Deficient Standard Curve Fit	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the Standard and Fluorescent Probe after reconstitution. • Thoroughly mix dilutions.

References

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Version 1.1

Fluorescent Microplate Reader

- BioTek® Synergy H1F, Filter-Based Reader
- Gen5 Software included
- Includes three filters designed specifically for AssayLite® Multiplex Assays. Additional filters are available for purchase
- Available for order from Assaypro for $19,950