GMP-140 (P-selectin) EIA Kit Manual

GMP-140 EIA kit

An enzyme immunoassay kit
for the quantitative determination of
granule membrane protein 140 (GMP-140)

For research use only. Not for use in diagnostic or therapeutic procedures.

Catalog Number 40-831-160005
For 96 assays

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Introduction

GMP-140, also known as CD62 or PADGEM (6), is a 140 kDa integral membrane glycoprotein functionally categorized as a member of the LEC-CAM family (lectin-epidermal growth factor-complement binding cell adhesion molecules), or the selectin family. In non-activated conditions, GMP-140 is present in the alpha-granules of platelets or in the Weibel Palade bodies of endothelial cells (1-5). However, when activated by stimulators such as thrombin or by inflammatory or hemostatic mediators during intravascular inflammation, GMP-140 is rapidly secreted to the plasma membrane to promote adhesion of cells to vascular endothelia or to neutrophils and monocytes at the site of tissue injury (10-12). Previous studies suggested that GMP-140 is indeed a useful marker of platelet and endothelium activation in experimental inflammation models, and potentially in clinical situations (5, 7-9). GMP-140 is also known to interact with leukocytes by a Ca^{2+} dependent lectin-like mechanism (13, 14). GMP-140 has recently been cloned from endothilia and is found to be highly homologous to two other LEC-CAM adhesion molecules, ELAM-1 and the Mel-14 antigen, both of which are involved in various aspects of leukocyte adhesion (3, 15). Analysis of the endothelium cDNA suggests that there are three variants of GMP-140 resulting from alternative splicing (16): two transmembrane

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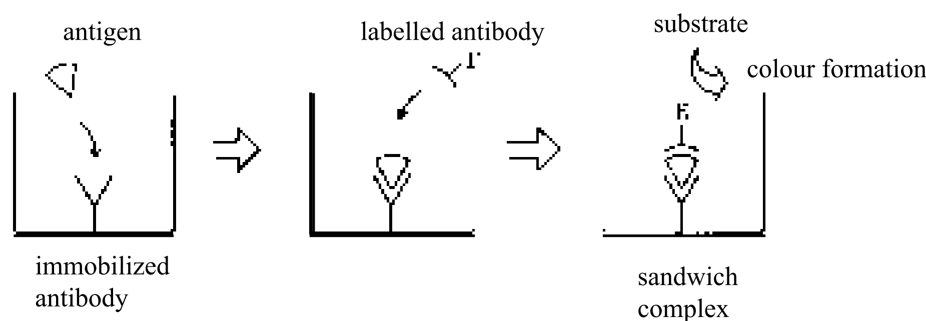
forms with different numbers of compliment-binding consensus repeats, and one putative cytoplasmic “soluble form” that lacks the hydrophobic transmembrane domain (20). Recently it was shown that GMP-140 molecules extracted by Triton X-100 from platelet membranes polymerize into soluble tetramers when returned to detergent-free conditions, and that this tetramer strongly inhibits the adhesion of activated neutrophils to endothelia. This is evidence that GMP-140-mediated adhesion works via a saturable GMP-140 receptor on the surface of myeloid cells (18). This also suggests that if free GMP-140 molecules are at all present in the circulation, they may serve to maintain the nonadhesiveness of neutrophils and prevent the development of inflammatory responses (17).
The present Enzyme Immunoassay Method (EIA) was developed for qualitative and quantitative detection of GMP-140 by using two different monoclonal antibodies (19,21). Advantages of this EIA system are speed, simplicity, and high specificity.

Intended use

The GMP-140 EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit for quantitative determination of human GMP-140 in plasma, cultured cell extracts, cell culture supernatants, and other biological fluids.
This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

Principle

The GMP-140 EIA Kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti GMP-140 antibodies to detect GMP-140 by two-step procedure. One of the mouse monoclonal anti GMP-140 has been pre-coated onto a microtiter-plate and blocked against non-specific binding. Samples and standards are incubated in microtiter-plate wells. The second step is to wash the plate and add a second anti GMP-140 labelled with peroxidase (POD). During the incubation, GMP-140 is bound to anti GMP-140 (solid phase) on one side and tagged on the other by POD-anti GMP-140. The reaction between POD and substrate (H_2O_2 and tetramethylbenzidine) results in colour development with intensities proportional to the amount of GMP-140 present in samples and standards. The amount of GMP-140 can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentrations of GMP-140 can be determined by comparing their specific absorbances with those obtained for the standards plotted on a standard curve.



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Reagents and materials

Each GMP-140 EIA Kit includes reagents sufficient for 96 wells. The expiration date for the complete kit is stated on the outer box label and the recommended storage temperature is 2 - 8°C.

A. Materials provided

Plate 1 Antibody Coated Microtiterplate - 1 plate (8 well x 12 strips)
The plate coated with murine monoclonal antibody to GMP-140.

Vial 2 Antibody-POD Conjugate - 1 vial (for 11 ml x 1)
The vial contains lyophilized horseradish peroxidase (POD) conjugated murine monoclonal antibody to GMP-140. Avoid prolonged exposure to light.

Vial 3 Standard - 1 vial (640 ng ; for 1 ml x 1)
The vial contains lyophilized GMP-140.

Vial 4 Sample Diluent - 2 vials (11 ml x 2)
Each vial contains protein in a buffered solution. Use for Zero standard, and for dilution of the Standard (vial 3) and samples which are above the calibration curve.

Vial 5 Substrate Solution - 1 vial(12 ml x 1)
Each vial contains hydrogen peroxide and tetramethylbenzidine in a buffered solution.

B. Materials required but not provided

1. Reagents
   - Washing Buffer: Phosphate-Buffered Saline (PBS)
     (Dissolve 8.0 grams of NaCl, 0.2 grams of KCl, 2.9 grams of Na_2HPO_4 $12H_2O$ and 0.2 grams of KH_2PO_4 in 1000 ml of distilled water.)
   - Stop Solution: 1N H_2SO_4

2. Materials
   - Precision pipettes with disposable tips: 20 and 100 μl micropipettes, 10 - 200 μl adjustable multiwell pipetter or 100 μl multiwell pipetter
   - Beakers, flasks, cylinders necessary for preparation of reagents
   - Disposable pipettes and test tubes
   - Microtiter plate reader for measurement of absorbance at 450 nm
   - Graph paper

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Precautions
- Do not mix reagents from different kit lots.
- Do not use reagents beyond expiration date on label.
- In order to avoid reagent contamination, use disposable pipette tips and/or pipettes.
- Sodium azide inactivates POD. Solutions containing sodium azide should not be used in this assay.
- Do not expose Substrate Solution to strong light during storage or incubation.
- Avoid contact of Substrate Solution and Stop Solution with skin or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water. Do not pipette by mouth. Do not smoke, eat, or drink in area where specimens or kit reagents are handled. All blood fluids should be considered as potentially infectious.
- Avoid contact of Substrate Solution and Stop Solution with any metal surfaces. Disposable glassware or test tubes are recommended for handling the Substrate Solution. If non-disposable glassware is used, it must be acid washed and thoroughly rinsed with distilled, deionized water.
- Do not use the Substrate Solution if its colour is changed to thick blue.

Specimen collection and handling
Cell or platelet extract is suitable for use in the assay, however, plasma or cell culture supernatant can be also used. Cell or platelet extract should be prepared by sonication in 50 mM Tris-HCl, pH8.5, containing 0.2 mM PMSF, 5 mM NEM, 10 mM EDTA and 150 mM NaCl. Platelet destruction, activation, and aggregation in specimens during the preparation of plasma or other biological fluid may affect GMP-140 levels in these samples. Venous blood samples are collected aseptically into a syringe containing citrated acid, and immediately centrifuged at 2000 g X 15 minutes. Remove the citrated plasma from the red cells soon after separation. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. All samples should be stored frozen under -20°C for optimal results. Excessive freeze-thaw cycles should be avoided. Prior to assay, frozen samples should be brought to room temperature slowly, and gently mixed by hand. Do not thaw samples in a hot bath. Do not vortex or sharply agitate.

Preparation of solutions
Note: The following solutions should be prepared directly before use.

Solution 1. Antibody-POD Conjugate Solution
Dissolve the contents of Vial 2 in 11 ml distilled water and mix gently followed by 10 minutes slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution
Rehydrate Standard (Vial 3) with 1 ml distilled water. Slowly roll for approximately 10 minutes or let vials to stand and sporadically mix gently.
The Standard Solution contains 640 ng GMP-140/ml. Prepare a dilution series of 320, 160, 80, 40, 20, 10 ng/ml by diluting the Standard Solution with Sample Diluent.

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Stability of solutions
- Solution 1 is stable for one week at 4°C.
- Solution 2. The reconstituted lyophilisate is stable for 1 week when stored at -20°C.

Procedure
Double determinations of all samples and standards should be performed.
All of the Kit's content should be brought to room temperature before use !
For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand.

Enzyme immunoassay
- *Sample incubation:* Pipette 100 µl sample and standard(Solution 2)into one well within 5 minutes. Mix, seal the microtiter plate (e.g. with a foil) and incubate 2 hours at room temperature (20 - 30°C).
- Remove sample solution and wash the wells 3 times with ca. 400 µl of PBS; between the separate washing steps empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.
- *Antibody-POD conjugate incubation*: Pipette 100 µl of Antibody-POD Conjugate Solution (Solution 1) into one well, mix, seal the microtiter plate (e.g. with a foil) and incubate 1 hour at room temperature (20 - 30°C).
- Remove sample solution by suction and wash the wells 4 times as described above (It is especially important after this step to thoroughly empty out the remaining fluid before adding the substrate).
- *Substrate incubation*: Add 100 µl Substrate Solution (vial 5) into each well and incubate at room temperature (20 - 30°C) for 15 minutes.
- Add 100 µl Stop Solution into each well in the same order as for substrate. Tap plate gently to mix.
- Measure the absorbance at 450 nm with a plate reader. The absorbance should be read as soon as possible after the completion of the assay. It may be read up to 1 hour after addition of Stop Solution if wells are protected from light at room temperature.
  Note: *It is important that Stop Solution is added to wells prior to reading at 450nm. Addition of Stop Solution causes an increase in absorbance of the Substrate Solution and shift in absorbance spectrum.*

Results
1. Standard curve
- Record the absorbance at 450 nm for each standard well.
- Average the duplicate values and record the averages.
- Plot the absorbance (vertical axis) versus the GMP-140 concentration in ng/ml (horizontal axis) for the standards using optimal fitting curve.

2. Samples
- Record the absorbance at 450 nm for each sample well.
- Average the duplicate values and record the averages.
- Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the GMP-140 concentration (ng/ml) from the horizontal axis.

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Performance characteristics

1. **Range of standard curve:** 10 - 640 ng/ml.
2. **Specificity:** This kit specifically measures GMP-140 with no detectable cross reaction with platelet Gp Ib, platelet Gp IIb, platelet Gp IIIa, von Willebrand factor, and platelet thrombospondin. This kit cannot be used to measure mouse GMP-140. The application of this kit for quantitating GMP-140 from other sources has not been tested.
3. **Assay duration:** Three and a half hours after sample incubation.
4. **Assay capacity for test samples:** If all assay wells (including standards and test samples) are run in duplicate, 40 test samples can be run in duplicate per kit.
5. **Test specimen type:** Human plasma; culture supernatants, cell extracts.
6. **Specimen volume required:** If each test sample is run in duplicate, approximately 220 μl (i.e., 100 μl per assay well plus ~10 μl for each sample transfer) is required.
7. **Limitation:** Since conditions may vary from assay to assay, a standard curve must be established for every run. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

   Thorough washing of the wells between incubations is required:
   1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
   2) Use sufficient wash solution for each wash cycle (approximately 400 μl).
   3) Do not allow wells to sit uncovered for extended periods between incubation steps.

   Only samples with absorbance values falling within the range of the standard curve should be assigned a GMP-140 concentration from the curve.

8. **Notes :** According to the assay results using control serum or urine, it could be possible to determine the concentration of antigen present in a biological. However, the measurement may be potentially disturbed by the unknown organic factors in serum, plasma or urine samples in patients with interrupted. When an antigen level in an unknown organic specimen is observed to be elevated as compared to the calibration range of the standard curve, it is recommended to dilute the specimens properly with the dilution solution included in the kit and assay them again in another run.

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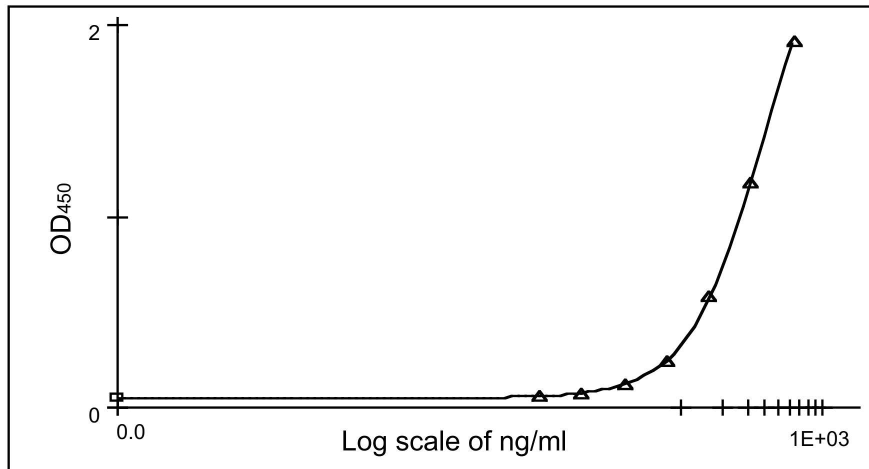
Basal data

1. **Typical standard curve**

Typical Standard Curve
(Do Not Use To Calculate Unknowns)



GMP-140 (ng/ml)	640	320	160	80	40	20	10	0
A_{450}	1.927	1.188	0.584	0.250	0.126	0.081	0.068	0.058

2. **Intra-assay precision** (n=16)

   Assay was carried out with 16 replicates of 4 samples containing different concentrations of GMP-140.

	Ave. (ng/ml)	S.D.(ng/ml)	CV(%)
Sample A	341.5	19.30	5.7
Sample B	145.1	14.08	9.7
Sample C	87.0	6.465	7.4
Sample D	64.1	4.017	6.3

Inter-assay precision (performance 3 times)

Assay to assay precision with one laboratory was evaluated in three independent experiments.

	Ave. (ng/ml)	S.D.(ng/ml)	CV(%)
Sample A	328.1	16.46	5.0
Sample B	140.7	9.555	6.8
Sample C	83.8	4.386	5.2
Sample D	60.9	3.302	5.4

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3. Recovery test

The recovery of GMP-140 was tested by adding two samples out of five different level in various matrices.

Sample A	Sample B	A+B Measured	A+B Calculated	Recovery (%)
463.3	0.0	212.5	231.6	92
463.3	463.3	455.8	463.3	98
463.3	192.2	333.5	327.7	102
463.3	100.7	276.9	282.0	98
463.3	50.4	244.9	256.8	95
463.3	24.8	205.7	244.0	84
192.2	0.0	97.5	96.1	101
192.2	192.2	212.1	192.2	110
192.2	100.7	155.3	146.4	106
192.2	50.4	129.9	121.3	107
192.2	24.8	111.6	108.5	103
100.7	0.0	47.9	50.3	95
100.7	100.7	96.2	100.7	96
100.7	50.4	72.4	75.5	96
100.7	24.8	61.7	62.7	98
50.4	0.0	26.2	25.2	104
50.4	50.4	49.2	50.4	98
50.4	24.8	36.3	37.6	97
24.8	0.0	16.9	12.4	136
24.8	24.8	23.6	24.8	95

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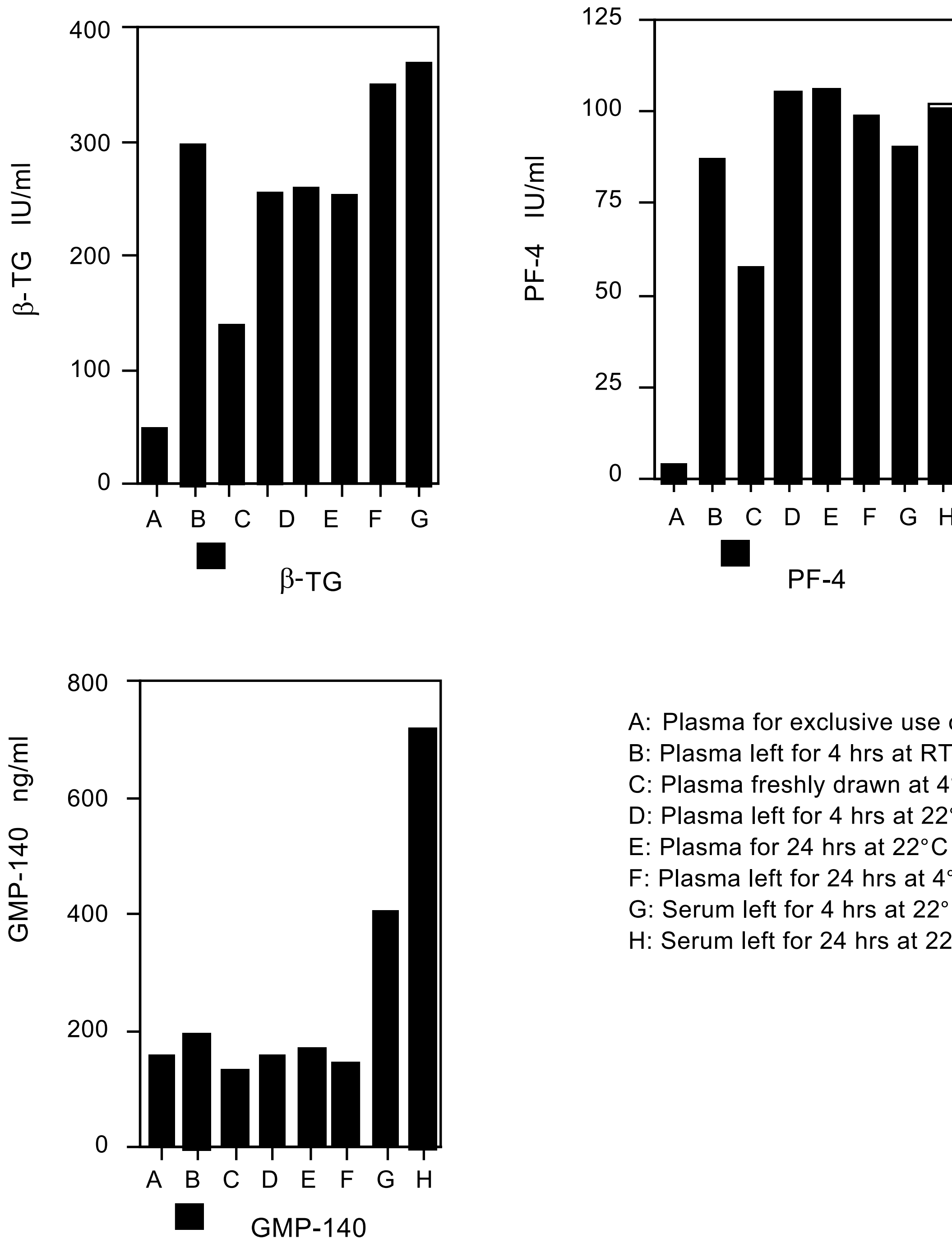
4. Study of condition of sample preparation

The change of GMP-140 concentration was observed by varing the storage temperature and period after collecting blood.
Plasma (A,B, C, D, E and F) and serum (G and H) were prepared for centrifugation.
Centrifugation condition was constant, at 3000 rpm, 4°C.
The condition C is recommended.



A: Plasma for exclusive use of β-TG test
B: Plasma left for 4 hrs at RT
C: Plasma freshly drawn at 4°C
D: Plasma left for 4 hrs at 22°C
E: Plasma for 24 hrs at 22°C
F: Plasma left for 24 hrs at 4°C
G: Serum left for 4 hrs at 22°C
H: Serum left for 24 hrs at 22°C

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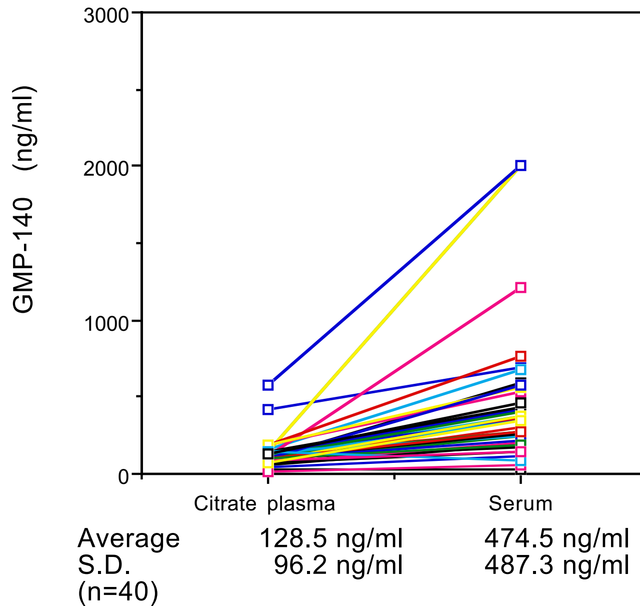
5. Correlation of plasma and serum value of GMP-140

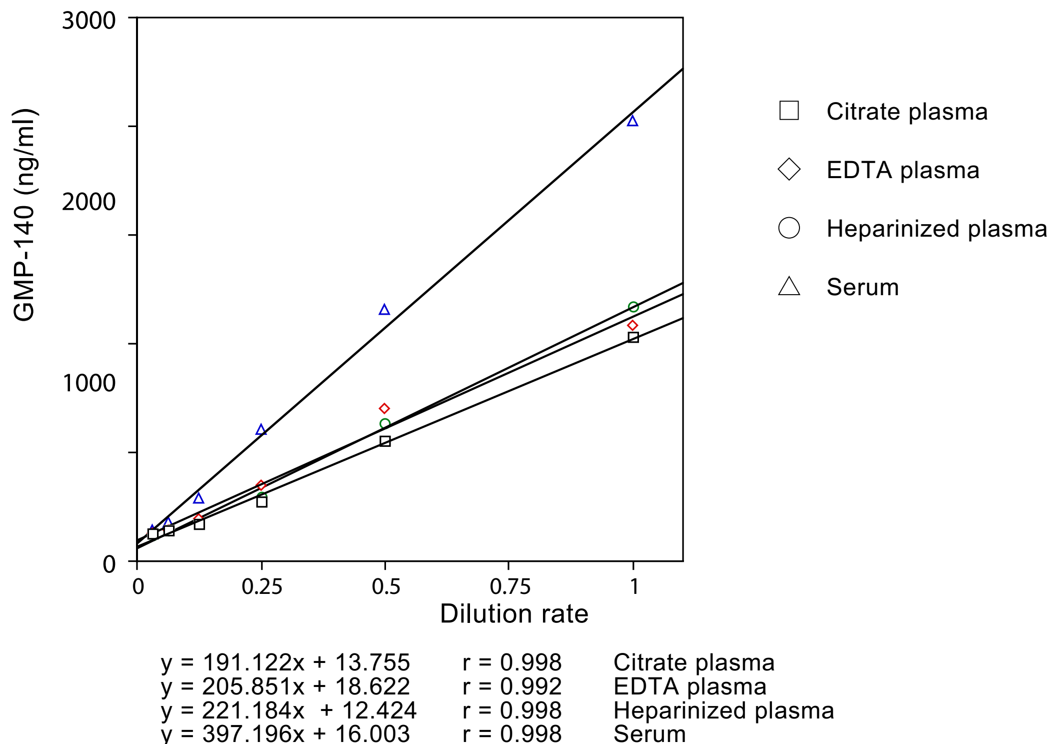
GMP-140 level in serum has a tendency to show higher level than in plasma. Increased level from serum to plasma differs in each sample.



6. **Effect of anticoagulants**

Effect of anticoagulants was examined with 3 healthy samples by comparing the dilution curve of the samples which were simultaneously treated with different anticoagulants. We recommend citrate plasma to use in this kit.

Sample 1



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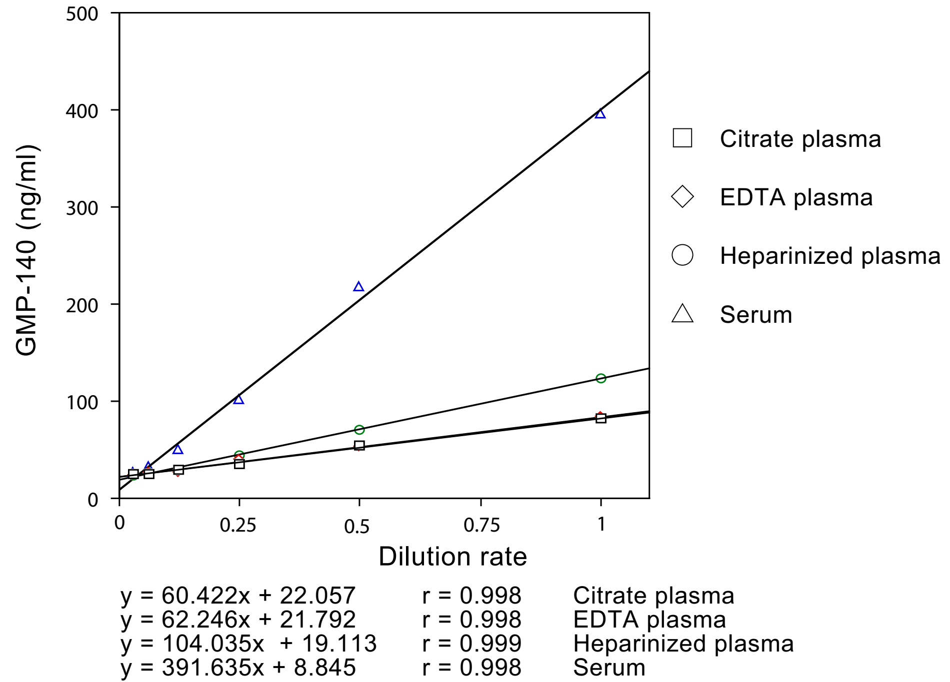
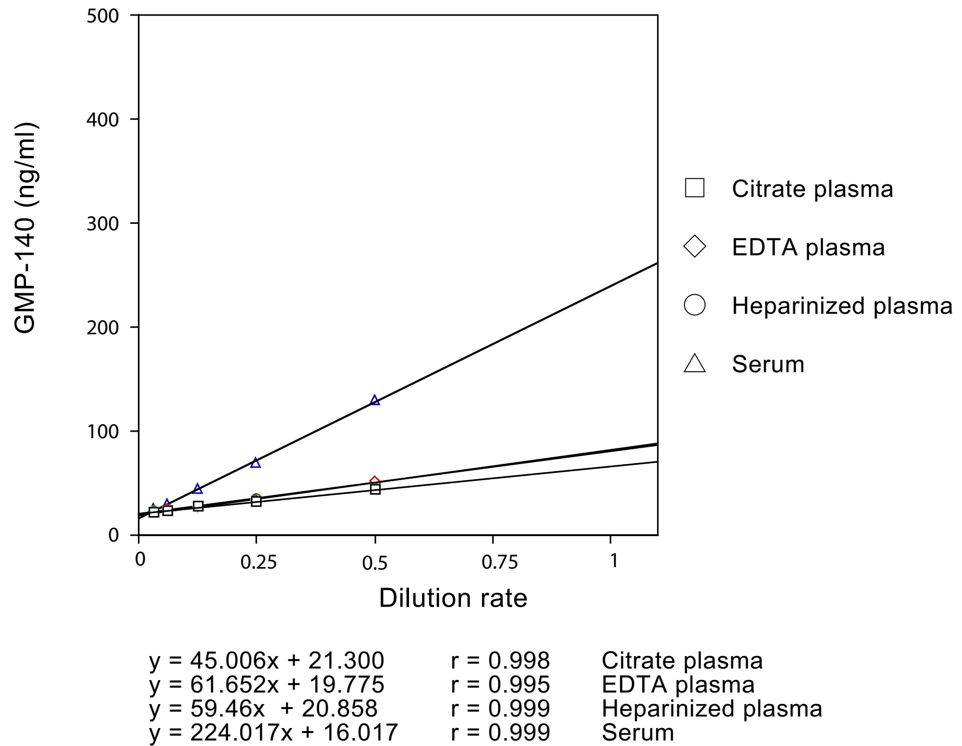
Sample 2



y = 60.422x + 22.057	r = 0.998	Citrate plasma
y = 62.246x + 21.792	r = 0.998	EDTA plasma
y = 104.035x + 19.113	r = 0.999	Heparinized plasma
y = 391.635x + 8.845	r = 0.998	Serum

Sample 3



y = 45.006x + 21.300	r = 0.998	Citrate plasma
y = 61.652x + 19.775	r = 0.995	EDTA plasma
y = 59.46x + 20.858	r = 0.999	Heparinized plasma
y = 224.017x + 16.017	r = 0.999	Serum

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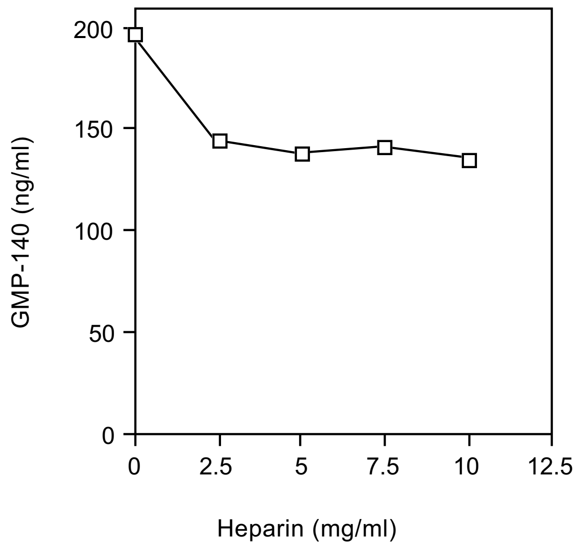
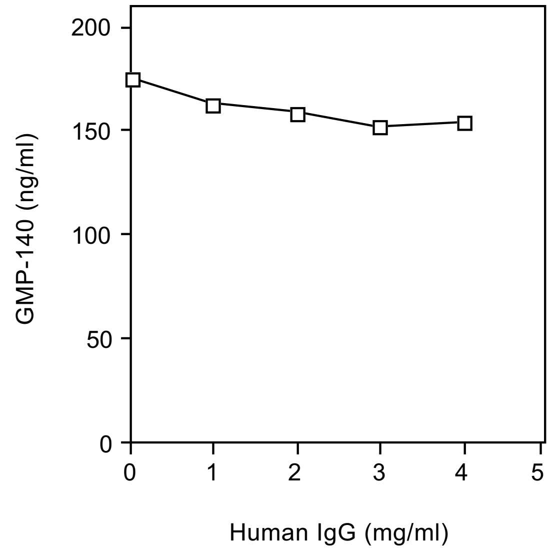
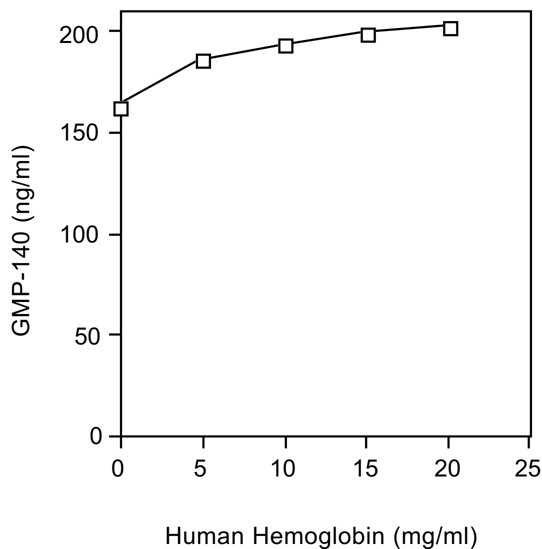
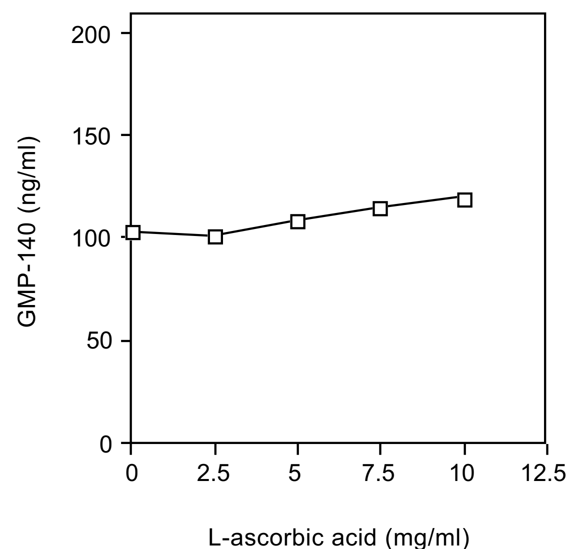
7. Influence of coexistence

The volume ratio of sample to co-existing substance is 4:1. Co-existing substance is shown in its final concentration.









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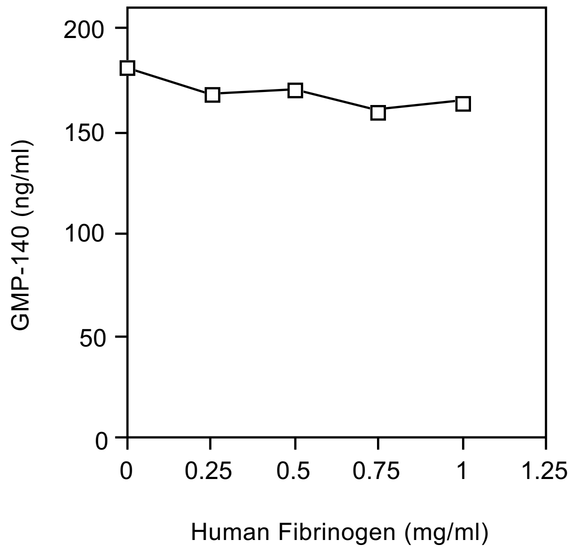

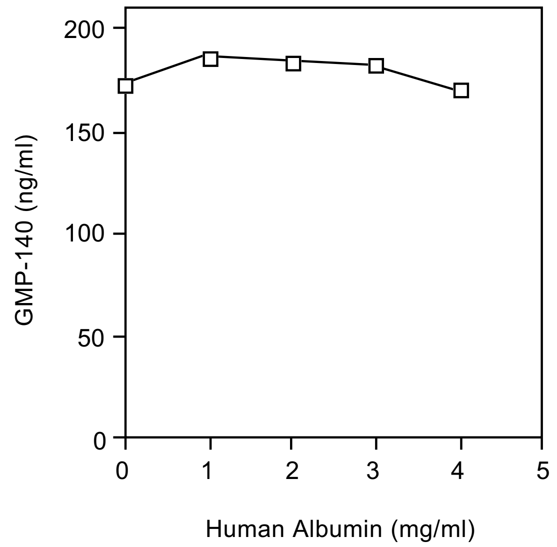

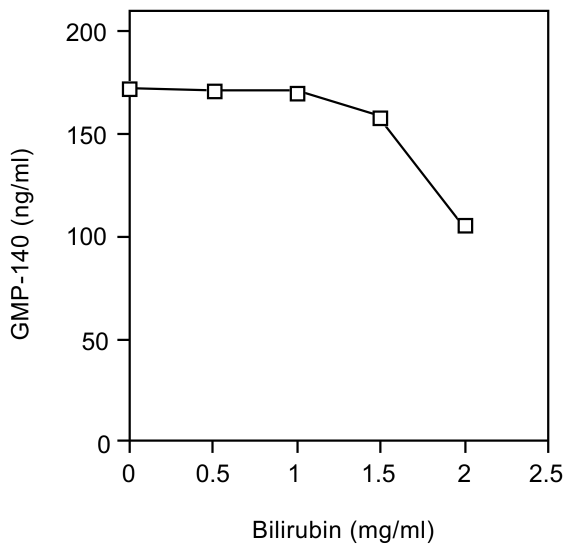

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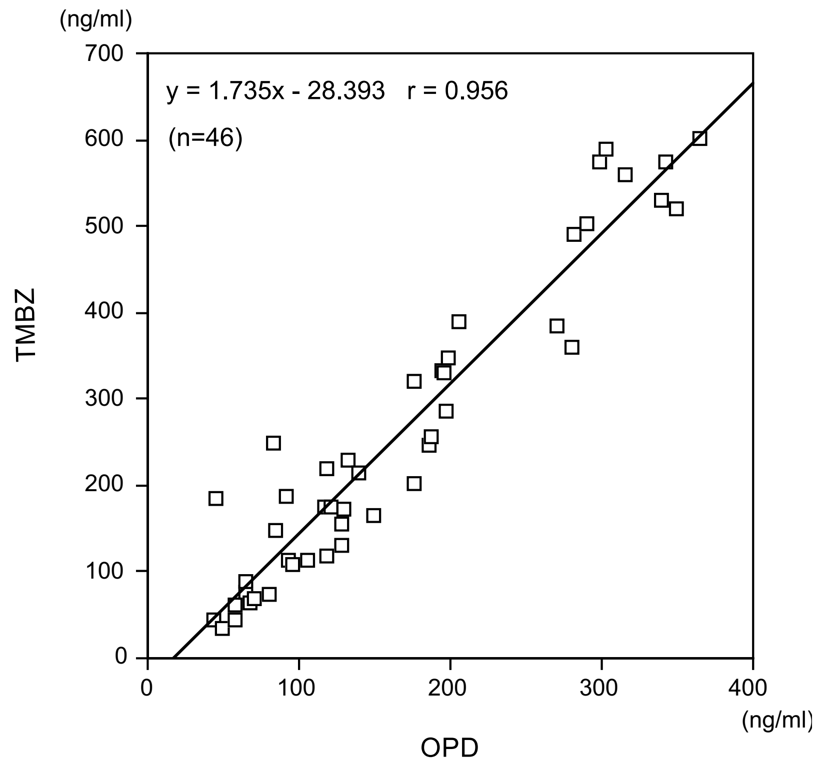
8. Correlation with the former kit

Correlation of *O*-phenylenediamine (OPD) to 3,3',5,5'-tetramethylbenzidine (TMBZ) in GMP-140 EIA in using them as the substrate.



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Storage and stability

This kit is shipped at 2 - 8°C and should be stored at 2 - 8°C if not used.
Under this condition, the kit is stable until the expiration date on label.

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Protocol summary

1. Add 100 µl of Standard or sample to appropriate wells, and incubate 2 hours at room temperature (20 - 30°C).
2. Remove sample solution and wash the wells 3 times with 400 µl of PBS.
3. Add 100 µl of antibody-POD conjugate solution into wells and incubate at room temperature for 1 hour.
4. Aspirate solution from wells. Wash 4 times with 400 µl of PBS per wells, aspirating thoroughly between washes.
5. Add 100 µl of Substrate Solution to each well. Incubate 15 minutes at room temperature.
6. Add 100 µl of Stop Solution to all wells. Mix gently.
7. Read at 450 nm as soon as possible.

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