

Instruction Manual for RAT HGF EIA

Rat HGF Determination EIA Kit for Research Purpose

General Description

This kit is developed for determination of rat Hepatocyte Growth Factor (rat HGF) for research purposes. Rat HGF can be determined quantitatively using the rat HGF standard solution supplied with this kit.

This kit shows the same reactivity with mouse HGF and can be used for the determination of mouse HGF. When mouse HGF is determined by this kit, however, the results should be expressed as “rat HGF”. This kit also cross reacts with human HGF but its cross reactivity is about 4%.

Kit Composition

1. Solid phase microplate (8 wells/strip × 12)1 ea.
(Coated with anti-rat HGF monoclonal antibody)
2. Rat HGF standard solution.....6 vials
(1 vial each of 0, 0.4, 1, 3, 10 and 25 ng/mL standard
concentration: 0.5 mL/vial)
3. Sample diluent L, low salt concentration..... 15 mL × 1 vial
(Fetal calf serum)
4. Sample diluent H, high salt concentration..... 15 mL × 1 vial
(Fetal calf serum)
5. Anti-rat HGF rabbit antibody..... 10 mL × 1 vial
(Anti-rat HGF rabbit polyclonal antibody)
6. Enzyme labeled antibody..... 10 mL × 1 vial
(Peroxidase labeled anti-rabbit goat immunoglobulin)
7. Enzyme substrate solution..... 30 mL × 1 vial
(Sodium perborate, tetrahydrate)
8. Color developer.....4 tablets
(o-phenylenediamine, dihydrochloride)
9. Reaction stopper solution.....5 mL × 1 vial
(2 mol/L sulfuric acid)
10. Washing solution (20 times concentrated).....25 mL × 3 vials
(Sodium chloride)
11. Plate seal.....3 ea.

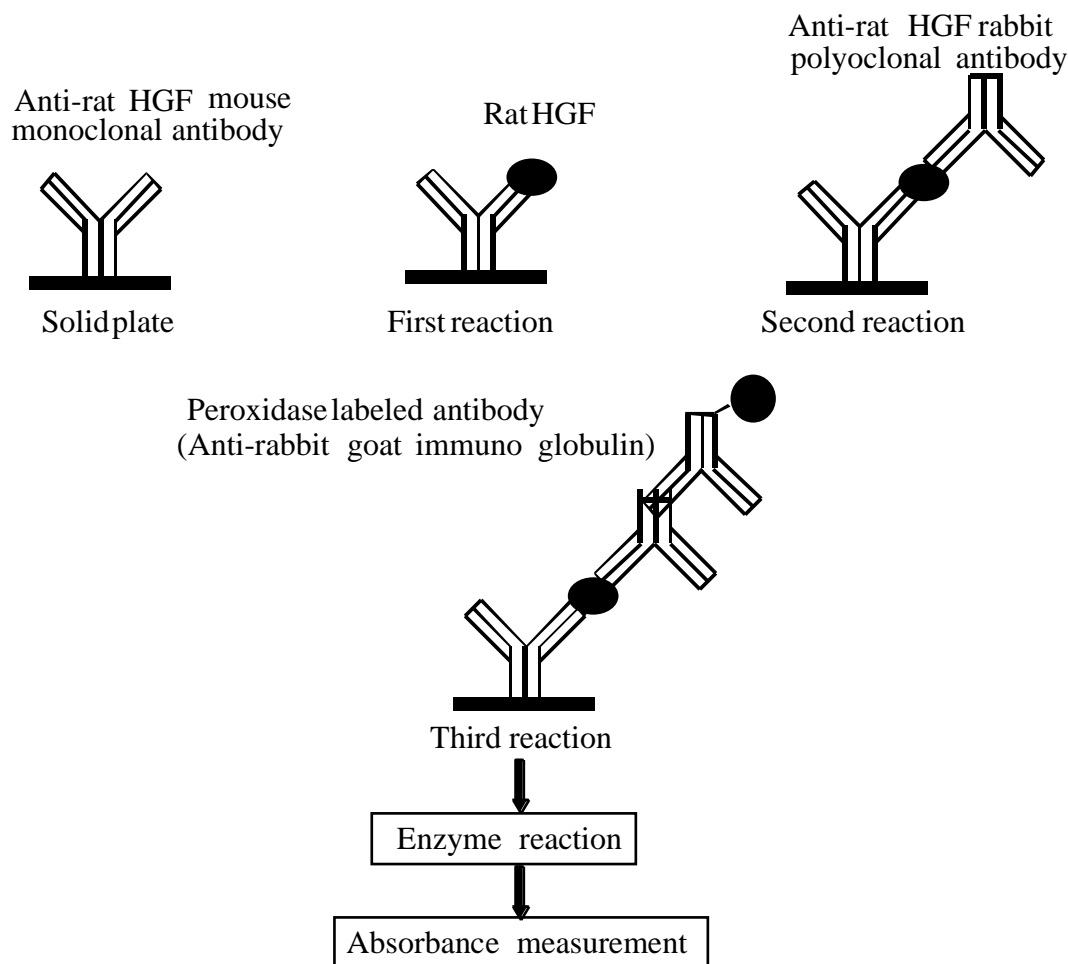
Application

For determination of rat hepatocyte growth factor (rat HGF).

Assay Principle

The detection system of this kit is of a sandwich configuration based on Enzyme Immuno Assay consisting of three steps of antigen-antibody reaction between a monoclonal antibody raised against genetically engineered human HGF and most cross-reactive with rat HGF (anti-rat HGF mouse monoclonal antibody), rat HGF in samples, anti-rat HGF rabbit antibody and anti-rabbit goat

immunoglobulin, followed by color development by enzyme reaction. The 1st reaction takes place between the anti-rat HGF mouse monoclonal antibody immobilized on a solid phase microplate and rat HGF in samples; the 2nd reaction between rat HGF in samples bound to the solid plate antibody and rat HGF rabbit antibody, and the third reaction between the enzyme labeled antibody (peroxidase labeled anti-rabbit goat immuno globulin) and the anti-rat HGF rabbit antibody. After the third reaction, color proportional to the rat HGF concentration is developed by enzyme reaction and its absorbance is measured for the determination of rat HGF on a working curve prepared using the Rat HGF standard solution.



Operation

1. Preparation of reagents.

1) Enzyme substrate solution (containing color developer)

Dissolve one tablet of the color developer per 3 mL of the enzyme substrate solution and leave it to stand in the dark. After bubbles are gone, thoroughly mix the solution. Prepare a sufficient volume (100 μ L/well) of this solution to fill all wells to be used. This solution should be prepared 15 min before use and used up within 60 min after preparation.

2) Washing solution

Dilute the washing solution 20 times with purified water. Keep this solution at 2 - 8°C after use.

2. Materials required for assay but not supplied with this kit.
 - 1) One each of micro pipettes, 50 μ L and 100 μ L.
 - 2) A measuring pipette, 10 mL.
 - 3) A measuring cylinder, 1 L.
 - 4) An aspirator and a polyethylene washing bottle, or a microplate washer.
 - 5) A dark box. (A light tight cupboard or a drawer will do.)
 - 6) A microplate reader capable of reading wavelength at 492 nm or 490 nm.

3. Operation.

For the determination of samples extracted from organs or samples partially purified, follow the instruction for high salt concentration samples (Method A) and for plasma and culture samples, follow the instruction for low salt concentration samples (Method B).

Return the kit to room temperature before use.

As illustrated in Figs. 1 and 2, using 2 wells for each standard concentration, measure absorbance of the rat HGF standard solution in each assay (2 wells \times 5 different standard concentration).

- Keep samples in plastic vials such as polyethylene and polypropylene. Do not use glass vials.
- Platelet is rich with Rat HGF and can be easily damaged during isolation of plasma. To prevent mixture with platelet derived Rat HGF, isolate plasma with absolute care to minimize damage of platelet.

Fig. 1 Method A

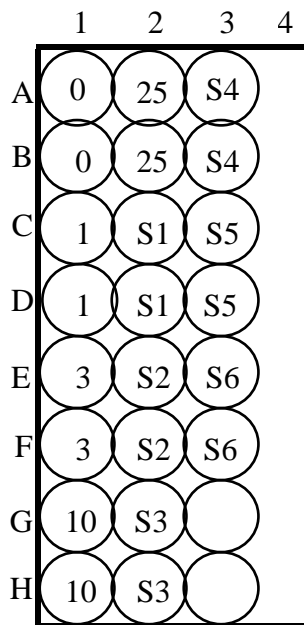
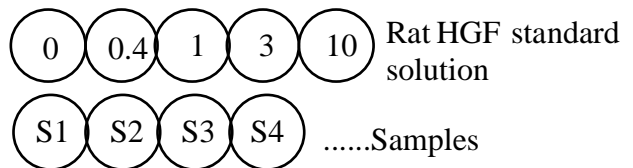
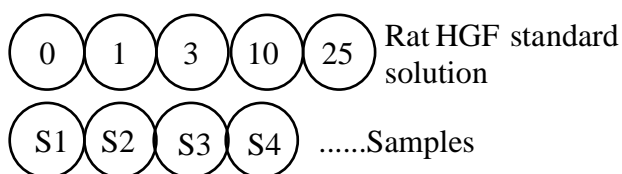
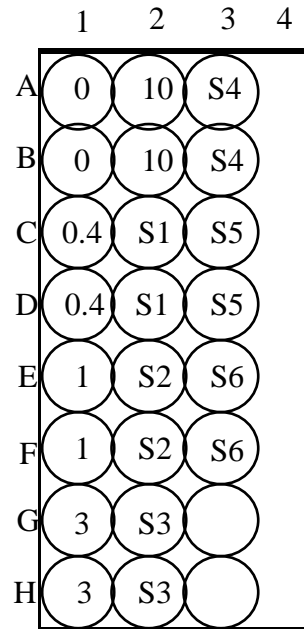


Fig. 2 Method B



1) Adding samples

(1) High salt concentration samples. (Method A).

i) Sample preparation. (Refer to Appendices 1 and 2)

For the determination of HGF in rat and mouse organs, remove by perfusion as much blood as possible before their excision. Weigh each organ and homogenize with a homogenizer like Polytron after supplementing each organ with the HGF extraction buffer at the ratio of W/V = 1/4. Centrifuge the solution at 15,000 rpm at 4°C for 30 min to separate it in 3 layers and recover the middle layer for the HGF determination.

For the sake of better accuracy, concentrate and partially purify HGF according to the following procedures. Most of the physiological fluid samples can be tested for HGF either by the following procedures or by their modification.

For extraction and partial purification of samples, their salt concentration is adjusted to 0.5M with the diluent or by dialysis and they are applied to a 1 mL gel heparin column (Pharmacia). After washing the column with 10 mL of the adsorbance buffer to equilibrate, apply 5 mL of the eluent and discard the first 0.5 mL of the eluent and as partially purified samples, collect next 1.5 mL of the eluent which contains major portion of HGF. (To confirm binding of HGF to the column and its elution in the partially purified samples, it is recommended to collect fractions before and after the partially purified samples and to determine HGF concentration.)

ii) Addition of samples and the standard solution.

Add 50 µL each of the sample diluent H in the wells for the standard solution (10 wells) and add 50 µL of each rat HGF standard solution (concentration 0, 1, 3, 10 and 25 ng/mL, 2 wells for each concentration). Add 50 µL each of the sample diluent L in sample wells and add 50 µL each of samples (extracted samples or partially purified physiological fluid samples), 2 wells for each sample. If sample dilution is required, dilute with the sample diluent H before assay.

(2) Low salt concentration samples. (Method B)

Add 50 µL each of the sample diluent L in all standard solution and samples wells. Add 50 µL each of the rat HGF standard solution (concentration 0, 0.4, 1, 3 and 10 ng/mL) and samples in respective wells, 2 wells for each standard solution and samples. If sample dilution is required, dilute with the sample diluent L before assay.

Sample dilution.

- Some types of samples may affect the 1st reaction time. Check by a dilution test and dilute samples with the sample diluent before assay, if required.
- Samples which may show absorbance beyond the working curve should be diluted with the sample diluent before assay.

Note:

- The solid phase microplate consists of 12 detachable 8-well strips. Repack in the aluminum pouch unused strips together with desiccant and seal it for storage at 2 - 8°C.
- While in use, the rat HGF standard solution should be maintained at the ice cold water temperature and stored at 2 - 8°C after use. Samples should be stored frozen.

2) 1st reaction.

Cover the microplate surface with the plate seal supplied and leave the microplate to stand at 15 - 30°C for 20 hrs (16 - 24 hrs).

3) Washing.

Remove the plate seal and suck out the well content with an aspirator.

Using a polyethylene washing bottle, fill wells with the washing solution prepared in 2) Washing solution of 1) Preparation of reagents. Hold the microplate upside down and vigorously shake out the washing solution. After repeating this washing 5 times, gently tap the plate surface on paper towel to remove the washing solution.

Note: While washing the microplate wells, do not dry the inner wall of wells.

As soon as washing is over, immediately follow the next steps.

4) Addition of the rat HGF rabbit antibody.

Add 100 μ L each of the rat HGF rabbit antibody in all wells.

5) 2nd reaction.

Cover the microplate surface with the plate seal supplied. Leave the microplate to stand at 15 - 30°C for 2 hrs.

6) Washing.

Repeat the procedure 3) above to wash the microplate wells.

7) Addition of the enzyme labeled antibody.

Add 100 μ L each of the enzyme labeled antibody in all wells.

8) Third reaction.

Cover the microplate surface with the plate seal supplied. Leave it to stand at 15 - 30°C for 2 hrs.

9) Preparation of the enzyme substrate solution (containing color developer).

15 min before the end of the third reaction, prepare the enzyme substrate solution (containing color developer) according to the instruction in 1) Enzyme substrate solution (containing color developer) of 1. Preparation of reagents.

10) Washing.

Repeat the procedure 3) above to wash the microplate wells.

11) Addition of the enzyme substrate solution (containing color developer).

Add 100 μ L each of the enzyme substrate solution (containing color developer) prepared in 9) above in all wells.

12) Enzyme reaction.

Leave the microplate to stand at 15 - 30°C in the dark for 30 min.

13) Addition of the reaction stopper solution.

Add 50 μ L each of the reaction stopper in all wells and thoroughly mix.

14) Absorbance measurement.

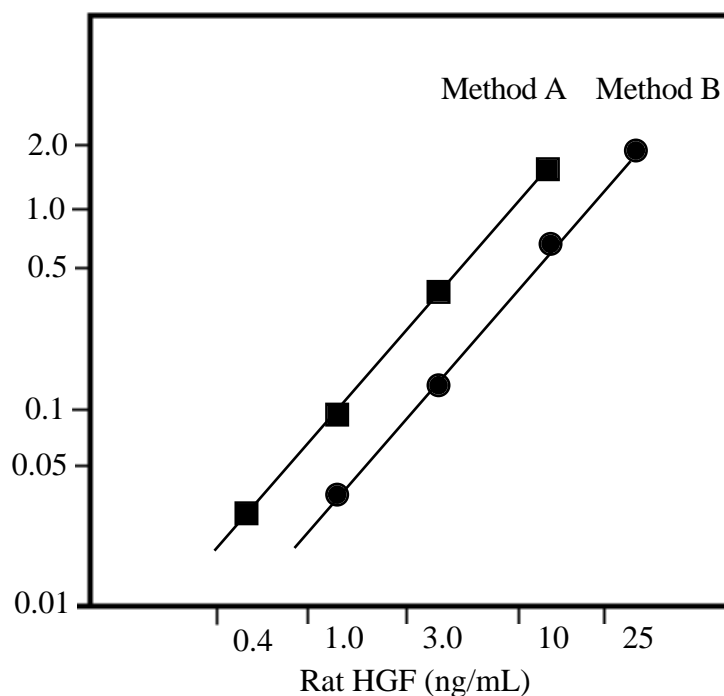
Measure on a microplate reader absorbance of each well (wavelength 492 nm or 490 nm). When a dual wavelength microplate reader is used, set the reference wavelength at 620 nm

or longer. Absorbance must be measured within 2 hrs after stopping the enzyme reaction.

Determination

1. Preparation of the working curve by the rat HGF standard solution.
 - 1) Calculate (mean absorbance of the respective concentration of the rat HGF standard solution/mean absorbance of 0 ng/mL of the rat HGF standard solution). (Net OD).
 - 2) Plot on logarithmic scales Net OD of the respective concentration.

Net OD



2. Calculation of the rat HGF in samples.
 - 1) When absorbance of samples exceeds that of 25 ng/mL concentration of the rat HGF standard solution, dilute samples and repeat the assay again.
 - 2) Calculate (mean absorbance of samples/mean absorbance of 0 ng/mL concentration of the rat HGF standard solution) (Net OD).
 - 3) Apply the values calculated in 2) above to the working curve and determine rat HGF concentration of samples.

Handling and operational precautions

1. General precautions.
 - 1) Make sure to return the kit to the room temperature before use.
 - 2) Do not mix up kit components of different production lots. (The rat HGF standard solution carries a different lot number from other components in the kit.)
 - 3) Assay strictly as instructed.
 - 4) Avoid contamination of the kit with microorganisms.
 - 5) Thoroughly wash equipment used for the assay and rinse them with distilled water.
 - 6) Replace micropipette tips for each sample and reagent.

2. Operational precautions.

- 1) Measure absorbance of the rat HGF standard solution for each assay.
- 2) Once assay is started, complete it within the prescribed time and allow the same length of reaction time for the rat HGF standard solution and samples.
- 3) Make sure that all reactions take place at 15 - 30°C.
- 4) The enzyme substrate solution (containing color developer) should be prepared 15 min before use and used up within 60 min after preparation.
- 5) Measure absorbance within 2 hrs after stopping the enzyme reaction.
- 6) Do not scrape the microplate or touch the bottom of wells. Do not dry up the inner wall of the wells during operation.

3. Kit handling precautions.

- 1) Avoid contact with skin of the enzyme substrate, color developer and reaction stopper. (They are toxic and irritable and may cause a burn.)
- 2) When discarding the sample diluent, run sufficient volume of tap water as it contains 0.1% sodium azide.

Storage and shelf life

Store the kit including the rat HGF standard solution at 2 - 10°C but do not freeze. This kit is stable for 12 months after the date of manufacture.

Package

1 kit for 96 tests.

Reference

N. Nakayama, et al: Prediction of prognosis in LEC rats with fulminant hepatitis. ACTA HEPATOLOGICA JAPONICA 31: 363 - 369, 1994

A. YAMADA, et al: Biomedical Research, 16 (2): 105 - 114, 1995

Inquiry

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Assay Procedures and Well Arrangement

Method A - High salt concentration samples

		HGF Standard	Samples
1	Well Arrangement	1A - 1H, 2A - 2B	2C - 12H
2	Addition of the HGF Standard and samples		
	HGF standard solution	50 μ L	--
	Samples*	--	50 μ L
	Sample diluent L	--	50 μ L
	Sample diluent H	50 μ L	--
3	1st reaction	20 hrs at 15 - 30°C	
4	Washing	5 times	
5	Addition of the anti-rat HGF rabbit antibody	100 μ L	100 μ L
6	2nd reaction	2 hrs at 15 - 30°C	
7	Washing	5 times	
8	Addition of the enzyme labeled antibody	100 μ L	100 μ L
9	Third reaction	2 hrs at 15 - 30°C	
10	Washing	5 times	
11	Addition of the enzyme substrate (containing color developer)	100 μ L	100 μ L
12	Enzyme reaction	30 min in the dark at 15 - 30°C	
13	Addition of the reaction stopper	50 μ L	50 μ L
14	Absorbance measurement	492 nm or 490 nm	
15	Determination		

*Samples or samples diluted with the sample diluent H.

Assay Procedures and Well Arrangement

Method B - Low salt concentration samples

		HGF Standard	Samples
1	Well Arrangement	1A - 1H, 2A - 2B	2C - 12H
2	Addition of the HGF Standard and samples		
	HGF standard solution	50 μ L	--
	Samples*	--	50 μ L
	Sample diluent L	50 μ L	50 μ L
3	1st reaction	20 hrs at 15 - 30°C	
4	Washing	5 times	
5	Addition of the anti-rat HGF rabbit antibody	100 μ L	100 μ L
6	2nd reaction	2 hrs at 15 - 30°C	
7	Washing	5 times	
8	Addition of the enzyme labeled antibody	100 μ L	100 μ L
9	Third reaction	2 hrs at 15 - 30°C	
10	Washing	5 times	
11	Addition of the enzyme substrate (containing color developer)	100 μ L	100 μ L
12	Enzyme reaction	30 min in the dark at 15 - 30°C	
13	Addition of the reaction stopper	50 μ L	50 μ L
14	Absorbance measurement	492 nm or 490 nm	
15	Determination		

*Samples or samples diluted with the sample diluent L.

Appendix 1

Extraction of samples from the rat (mouse) organs for the determination of HGF

1. Rat (mouse): Perfuse a rat (mouse) with physiological saline to substitute blood.
2. Collect organs (liver, kidney and lung) and weigh each of them after thoroughly wiping off water.
3. Transfer each organ to respective vessels and cut it with scissors to pieces as small as possible.
4. Add the extraction buffer* 4 times as much as the weight of each organ (w/v).

Example

	Liver		Kidney (pair)		Lung	
	Weight (g)	Buffer (mL)	Weight (g)	Buffer (mL)	Weight (g)	Buffer (mL)
Rat	10	40	2.2	8.8	1.5	6
Mouse	1.3	5.2	0.4	1.6	0.15	0.6

5. Homogenize each mixture at the ice cold temperature. (Polytron: 24,000 rpm × 1 min)
6. Spin the above mixture at 15,000 rpm for 30 min (or to reach the centrifugal force equivalent to 19,000 g × 30 min) at 4°C.
7. After centrifugation, the mixture is separated in 3 layers. Collect the middle layer as a sample for the determination of HGF.

Top layer: Lipid. Suck out by an aspirator.

Middle layer: Sample for the determination of HGF.

Precipitate: Consisting of three layers of, from the bottom, residue, red blood cells and membrane components. When collecting the middle layer as a sample, do not disturb the precipitate layer and mix it up with the middle layer.

8. For long term storage, keep frozen at -20°C or below the samples extracted.

* Extraction buffer (available at option):

20mM Tris-HCl buffer (pH 7.5)

2M NaCl, 0.1% Tween-80, 1mM PMSF supplemented with 1mM EDTA.

Calculation of HGF volume per organ:

HGF concentration (ng/mL) in a sample × volume (mL) of the buffer supplemented.

It is assumed that water on the organs is thoroughly wiped off and they contain no water.

Appendix II

Purification of the samples extracted from the rat (mouse) organs for the determination of HGF

1. Samples extracted from the organs (NaCl concentration: 2M).
2. Dilute each sample extracted from the organs 4 times with the sample diluent* (not supplemented with NaCl). (NaCl concentration after dilution: 0.5M).
3. Purification through heparin column.
 - Column: HiTrap Heparin column, bed volume 1 mL. (Pharmacia)
 - Equilibrate the column with 10 mL of the adsorbance buffer** (NaCl concentration: 0.5M). (10 mL = 10 beds).
 - Apply to the column 6 mL (4 times diluted and equivalent to 1.5 mL each of extracted sample) of a sample.
 - Collect the sample fraction passing through the column. Then, flash the column with 10 mL of the adsorbance buffer and collect the buffer fraction. (10 mL = 10 beds). Determine HGF concentration in each fraction and confirm that there is no HGF contained in the fraction.
 - Apply about 5 mL of the eluent buffer.*** Collect 0.5 mL - 2 mL (total 1.5 mL) fraction and determine its HGF concentration. (Empirically, 80 - 90% of the total HGF is contained in this fraction). HGF collected in this fraction corresponds to that in a sample extracted from each organ. (It is, however, recommended to collect fractions before 0.5 mL and after 2 mL and confirm they contain no or very small amount of HGF until your own protocol is established.)
 - Apply 5 mL of the eluent buffer to flash the column. (5 mL = 5 beds).
 - Apply 10 mL of the adsorbance buffer to equilibrate the column. (10 mL = 10 beds).
 - Apply next sample.

* Extraction buffer:	20mM Tris-HCl buffer (pH 7.5) Supplemented with 0.1% Tween-80, 1mM PMSF and 1mM EDTA <u>but not with NaCl</u> .
** Adsorbance buffer:	20mM Tris-HCl buffer (pH 7.5) Supplemented with <u>0.5M NaCl</u> , 0.1% Tween-80, 1mM PMSF and 1mM EDTA.
*** Eluent buffer:	20mM Tris-HCl buffer (pH 7.5) Supplemented with 2M NaCl, 0.1% Tween-80, 1mM PMSF and 1mM EDTA.