Instruction Manual

HGF Extraction Reagent
for use with IMMUNIS® HGF EIA

- Thoroughly read this instruction manual before use of this kit.
- This kit is intended for research use only.

I. General description

This kit is developed for extraction of human hepatocyte growth factor (hHGF) from the human tissues for determination with **IMMUNIS® HGF EIA** available as a separate kit. This kit is for research purpose only and should not be used for diagnostic applications.

II. Components

1. Extraction buffer ..... 30 mL x 5 vials
2. Sample diluent H..... 15 mL x 1 vial
3. HGF standard solution 1 (0 ng/mL) ..... 0.5 mL x 1 vial
   HGF standard solution 2 (0.3 ng/mL) ..... 0.5 mL x 1 vial
   HGF standard solution 3 (1 ng/mL) ..... 0.5 mL x 1 vial
   HGF standard solution 4 (3 ng/mL) ..... 0.5 mL x 1 vial
   HGF standard solution 5 (6 ng/mL) ..... 0.5 mL x 1 vial
4. 20x concentrated washing solution ..... 25 mL x 1 vial
5. Plate seal ..... 1 sheet

III. Components of IMMUNIS® HGF EIA required for determination of samples extracted from human tissues

1. Anti-HGF monoclonal antibody coated microplate (8 wells/strip x 12) .. 1 plate
2. Sample diluent ..... 15 mL x 1 vial
3. Enzyme labeled monoclonal antibody..... 10 mL x 1 vial
4. Enzyme substrate ..... 30 mL x 1 vial
5. Color developer..... 4 tablets
6. Reaction stopper ..... 6 mL x 1 vial
7. 20x concentrated washing solution ..... 25 mL x 2 vials
8. Plate seal ..... 3 sheets

IV. Reagents not provided but needed

1. Phenylmethanesulfonyl fluoride (PMSF)
2. Isopropyl alcohol

V. Preparation of extraction reagents

1. Preparation of 100 mM PMSF solution

1) Dissolve 17.4 mg of PMSF in 1 mL of isopropyl alcohol.

2. Addition of 100mM PMSF solution

1) Add 0.3mL of 100 mM PMSF into 30 mL of Extraction buffer and mix. (final concentration of PMSF should be 1 mM)
2) Add 0.15 mL of 100 mM PMSF into 15 mL of Sample diluent H and mix. (final concentration of PMSF should be 1 mM)

Note: Prepare a sufficient volume of those reagents shown above before use.

VI. Preparation of a tissue sample for extraction of hHGF

1. Sample weight measurement

1) Place a sample tissue section on a sheet of non-absorbent material such as aluminum foil and weigh. Use a micro balance when the sample is small in size and weighs several milligrams only.
2) Transfer the sample to a polypropylene or polyethylene vessel.

Note: Do not use a glass vessel as HGF adsorbs easily on the glass surface.

3) Cut tissue samples into as much small pieces as possible when they weigh more than 20 mg. (Use scissors to cut samples. Wash tissue samples and tissue exudates adhered to the scissors into Extraction buffer, when the buffer is added to the sample.)

2. Addition of Extraction buffer

1) Add to samples 5 ~ 100 volumes of Extraction buffer per tissue sample weight.
   (1 volume: 1 µL of Extraction buffer / 1 mg of a tissue sample.)
2) Adjust the volume of Extraction buffer in the range of 5 to 100 volumes depending on the size of tissue samples. 5 to 100 volumes assure the same extraction efficiency.
   Refer to the table **[Examples of extraction]** below.

3. Extraction.

1) Homogenize by a homogenizer the mixture of a tissue sample and Extraction buffer prepared above. Select the optimum extraction method for the size of a sample.

[Examples of the extraction method]

- Tissue samples weighing less than 5 mg: Homogenizer
  Note: Do not use a homogenizer made of glass. (Combination of an Eppendorf micro test tube and a micro pestle will serve the purpose.)
- Tissue samples weighing 5 mg or over: Sonicator (with a micro tip and on ice).
- Tissue samples weighing 1 g or over: Polytron (on ice).

Note: HGF is thermally unstable. When a Sonicator or Polytron is used, homogenize on ice.

2) Centrifuge the extracted mixture at 15,000 rpm for 30 min. (A table top centrifuge will do.)
   It is desirable to centrifuge the sample at 4°C. If this is not possible, centrifuge it at the room temperature. (When the tissue sample weighs less than 10 mg, it can be centrifuged at 15,000 rpm for 5 min as the volume of the residual tissue is small.)

4. Recovering of hHGF

1) Recover the intermediate layer without mixing it with the top lipid layer or the bottom tissue layer.

Note: Do not use a glass vessel for recovery of the sample. Use a polypropylene or polyethylene vessel.

[Examples of extraction]

Tissue weight	Extraction buffer vol.	Examples		
		Tissue weight	Extraction buffer vol.	
1 ~ 5 mg (Biopsy sample weight)	100 ~ 200 volume	1 mg 5 mg	200 µL 500 µL	Homogenizer or Sonicator (with micro tip and on ice)
5 ~ 50 mg	10 ~ 100 volume	5 mg 50 mg	500 µL 500 µL	Sonicator (with micro tip and on ice)
50 ~ 100 mg	5 ~ 10 volume	50 mg 100 mg	500 µL 500 µL	Sonicator (with micro tip and on ice)
100 mg or over	5 volume	100 mg 1 g	500 µL 5 mL	Sonicator (on ice) Polytron (on ice)

(1) For determination of HGF in crude extract solution, 50 µL (for n=1 determination) or 100 µL (for n=2 determination) is required.
(2) 500 µL is the optimum volume for extraction by a homogenizer or sonicator (with a micro tip).
(3) The HGF concentration of 1 mg of an tissue sample extracted in 100 µL of Extraction buffer is roughly 1 ng/mL for a normal liver and the same to several ten times higher than a normal liver level for livers with disorder.
(4) Taking into consideration the above factors, select the volume of Extraction buffer to be added.

Note: When HGF is extracted from a large tissue sample, contact us for our recommendation.

VII. Operation

1. Preparation of reagents (for the component of **IMMUNIS® HGF EIA**)

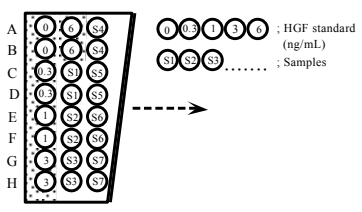
1) Enzyme substrate solution (containing the color developer)
   Add one tablet of Color developer into 3 mL of Enzyme substrate and leave the solution in a dark place. Thoroughly mix the solution after the Color developer is dissloved completely. Prepare a sufficient volume of this solution to cover all wells to be used for the test. This solution must be prepared 15 minutes before use and used up within 60 min.
2) Washing solution
   Dilute 20x concentrated washing solution 20 times with purified water. Keep this solution at 2 ~ 10°C.

2. Materials required but not provided

1) Micropipettes, 50 µL and 100 µL
2) A measuring pipette, 10 mL
3) A measuring cylinder, 1 L
4) A microplate shaker
5) An aspirator and a polypropylene washing bottle, or a microplate washer
6) A dark box (A light tight cupboard or drawer will do.)
7) A microplate reader (main wavelength 492 nm or 490 nm, sub wavelength 620 nm or longer)

3. Assay procedure (Use **IMMUNIS® HGF EIA**)

It is desirable to test the same sample twice. Bring all kit reagents to 20 ~ 30°C before use.
For each test, determine the HGF standard solution (2 wells x 5 concentrations) as illustrated.



1) Preliminary dilution of samples
   Dilute as required the extracted samples with Extraction buffer. When HGF in 1 mg of a tissue sample is extracted in 100 µL of Extraction buffer, HGF concentration is roughly as follows.
   - Normal liver: 1 ng/mL
   - Liver with disorder: Same as a normal liver to several ten times higher than a normal liver.
2) Pre-treatment of the anti-HGF coated microplate
   Pipette 200 µL each of Extraction buffer in the wells for determination of the HGF standard solution and samples and cover them with the plate seal provided. Set the microplate on a microplate shaker and shake it for 10 min at 20 ~ 30°C.

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3) Washing
Remove the plate seal and suck out the well contents by an aspirator. Wash all wells 5 times with the washing solution prepared under **"2) Washing solution"** in **"1. Preparation of reagents"**, using polyethylene washing bottle, or a microplate washer. Then tap the microplate upside down on a clean paper towel. Then tap the microplate upside down on a clean paper towel.

Note: Exert extreme care not to dry inside the wells. Follow the next step immediately after washing is completed.

4) Dispensation of samples and the HGF standard solutions

	HGF standard solution (of **HGF Extraction Reagent**)	Extracted samples	Diluent	
			Sample diluent (of **IMMUNIS® HGF EIA**)	Sample diluent H (of **HGF Extraction Reagent**)
Determination of the standard solution	50 µL	--	--	50 µL
Determination of samples	--	50 µL	50 µL	--

HGF Standard solution: Use 5 standard concentrations of 0, 0.3, 1, 3 and 6 ng/mL in this reagent.

After the primary reaction, follow the instructions described in the instruction manual of the **IMMUNIS® HGF EIA**.

5) Primary reaction
Cover the microplate with the plate seal provided and shake the microplate on a microplate shaker at 200 ~ 700 rpm for 1 hr at 20 ~ 30°C.

6) Washing
Repeat the procedure **3)** above to wash the microplate wells.

7) Addition of Enzyme labeled monoclonal antibody.
Pipette 100 µL of Enzyme labeled monoclonal antibody to each well.

8) Secondary reaction
Cover the microplate with the plate seal and shake the microplate on a microplate shaker for 1 hr at 20 ~ 30°C.

9) Preparation of Enzyme substrate solution
Approximately 15 min before the end of the secondary reaction, prepare the enzyme substrate solution (containing the color developer) according to the instructions given in **1)** under **"1. Preparation of reagents"**.

10) Washing
Repeat the procedure **3)** above to wash the microplate wells.

11) Addition of the enzyme substrate solution
Pipette 100 µL of the enzyme substrate solution (containing the color developer) prepared in **9)** above to each well.

12) Enzyme reaction
Cover the microplate with new plate seal and leave the microplate at 20 ~ 30°C in the dark for 30 min.

13) Addition of Reaction stopper
Remove the plate seal and stop color development by adding 50 µL of Reaction stopper in all wells, and mix sufficiently.

14) Absorbance measurement
Measure absorbance of each well at 492 nm or 490 nm. When a dual wavelength microplate reader is used, set the reference wavelength at 620 nm or longer. Abosrbance must be measured within 2 hrs after stopping the enzyme reaction.

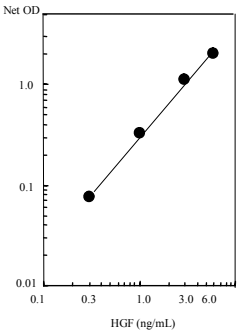
VIII. Determination

1. Validation of assay and calculation of the specific absorbance (Net OD).

1) Make sure that absorbance of the HGF standard solution 1 (0 ng/mL) is less than 0.050.

2) Calculate the mean value of the 2 absorbance readings of the HGF standard solution 1. If either of the reading is 0.050 or more, disregard that reading and use the other reading. When both readings are 0.050 or more, assay is void and should be repeated again.

3) Subtract the mean absorbance reading (or one reading) of the HGF standard solution 1 from the reading of each well to calculate the Net OD.



2. Preparation of the calibration curve by HGF standard solution

1) Using the logarithmic scales, prepare a calibration curve by plotting concentration of the standard solution on the X axis and Net OD on the Y axis.

3. Calculation of HGF concentration

1) Apply the Net OD of each sample to the calibration curve and read HGF concentration.

2) When absorbance of samples exceeds 6 ng/mL (the highest concentration of the HGF standard solution), dilute samples and determine again.

4. Calculation of the amount of HGF per tissue weight

$$\text{Amount of HGF/organ weight (ng/mg)} = \frac{\text{HGF concentration (ng/mL)} \times \text{Preliminary sample dilution folds} \times \text{Amount of added Extraction buffer (mL)}}{\text{Organ weight (mg)}}$$

IX. Warnings and precautions

This kit must be used according to the instructions and for the purpose described in this manual. No result is guaranteed in any use or for any purpose other than those described in this manual.

1. General precautions

1) Do not use expired reagents.
2) Do not use reagents of different production lots.
3) Extraction buffer and Sample diluent H of this kit are not identical to those of **HGF Extraction buffer for Mouse & Rat HGF**, **Rat HGF EIA**, or **Mouse HGF EIA**, and should not be used with those reagents.
4) Vials and tools provided should not be used for other purposes.

2. Operational precautions

1) Check accuracy of tools and properly use them according to their instructions.
2) Do not make react with any other enzyme immuno assay system using color developers contain organic sorbent, such as Tetramethylbenzidine. The reaction of OPD may be inhibited and it may affect the results.

3. Handling precautions

1) All samples should be handled as if they were capable of transmitting HBV, HCV and/or HIV and all tools must be immersed in and sterilized with 0.1% sodium hypochlorite.
2) Avoid contact of reagents. If they contact skin, wash with plenty of water. Get medical care if necessary.
3) Sample diluent H and HGF standard solution contain sodium azide and should be washed down with a sufficient volume of water to prevent formation of explosive metal azide.
4) When reagents or tools are discarded, it should be handled and disposed accroding to the instraction manual of **IMMUNIS® HGF EIA**.

X. Storage and shelf life

Store the kit at 2 ~ 10°C and avoid freezing. This kit is stable for 1 year after the date of manufacture. Validity of kit is shown in the package.

XI. Package

1 kit for 96 tests Code No. 1Z72

XII. Reference

1) Nakamura T, Nawa K, Ichihara A：Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun **122**：1450-1459, 1984.
2) Nakamura T, Nishizawa T, Hagiya M, et al：Molecular cloning and expression of human hepatocyte growth factor. Nature **342**：440-443, 1989.
3) Nakamura T：Structure and function of hepatocyte growth factor. Prog Growth Factor Res **3**：67-85, 1991.
4) Yamada A, Matsumoto K, Iwanari H, et al：Rapid and sensitive enzyme-linked immunosorbent assay for measurement of HGF in rat and human tissues. Biomed Res **16**：105-114, 1995.

Assay procedure and well arrangement

Well Arrangement		Reagents	HGF standard 1A - 1H, 2A - 2B	Samples 2C - 12H
1	Preliminary dilution of samples	Extraction buffer	--	Rough HGF concentration in extracted samples Normal : 1 ng/mL Disorder: 1 ng/mL to ten times
2	Pre-treatment of the anti-HGF coated microplate	Extraction buffer	200 µL for 10 min at 20 ~ 30°C, shaking	
3	Washing	5 times		
4	Addition of HGF standard solution and samples	HGF standard Sample Sample diluent of **Immunis** Sample diluent H	50 µL -- -- 50 µL	-- 50 µL 50 µL --
5	Primary reaction	for 1 hr at 20 ~ 30°C, shaking		
6	Washing	5 times		
7	Addition of Enzyme labeled antibody	Enzyme labeled monoclonal antibody	100 µL	100 µL
8	Secondary reaction	for 1 hr at 20 ~ 30°C, shaking		
10	Washing	5 times		
11	Addition of Enzyme substrate solution	Enzyme substrate solution (containing the color developer)	100 µL	100 µL
12	Enzyme reaction	for 30 min at 20 ~ 30°C in the dark		
13	Addition of Reaction stopper	Reaction stopper	50 µL	50 µL
14	Absorbance measurement	492 nm or 490 nm		

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