Instructions for Use

MMP-9 (human) ELISA

RUO

REF EIA-4861

96

DRG Instruments GmbH, Germany
Frauenbergstraße. 18, 35039 Marburg
Phone: +49 (0)6421-1700 0, Fax: +49 (0)6421-1700 50
Website: www.drg-diagnostics.de
E-mail: drg@drg-diagnostics.de

Distributed by:

DRG International, Inc., USA
841 Mountain Ave., Springfield, NJ 07081
Phone: (973) 564-7555, Fax: (973) 564-7556
Website: www.drg-international.com
E-mail: corp@drg-international.com

(20.11.14 (24))

Please use only the valid version of the Instructions for Use provided with the kit.
Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Gebrauchsanweisung.
Si prega di usare la versione valida delle istruzioni per l'uso a disposizione con il kit.
Por favor, se usa solo la version valida de la metodico técnico incluido aqui en el kit.
Utilisez seulement la version valide des Instructions d'utilisation fournies avec le kit.

Table of Contents / Tabella die Contenuti / Tabla de Contenidos

1 INTENDED USE

The human MMP-9 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human MMP-9.
The human MMP-9 ELISA is for research use only. Not for diagnostic or therapeutic procedures.

2 SUMMARY

Matrix metalloproteinases (MMPs) form a family of enzymes with major actions in the remodelling of extracellular matrix (ECM) components.

MMP-9, also called Gelatinase B, is the most complex family member in terms of domain structure and regulation of its activity. MMP-9 activity is under strict control at various levels: transcription of the gene by cytokines and cellular interactions; activation of the pro-enzyme by a cascade of enzymes comprising serine proteases and other MMPs; and regulation by specific tissue inhibitors of MMPs (TIMPs) or unspecific inhibitors. Further glycosylation has a limited effect on the net activity of Gelatinase B and chemotactic factors are another level of control of activity.

The main function of MMPs is degradation of the extracellular physiologic function including wound healing, bone resorption and mammary involution. MMPs also contribute to pathological conditions such as rheumatoid arthritis, coronary artery disease and cancer. They are thought to promote the growth of these tumor cells once they have metastasized. MMP-9 plays an important role in tumor invasion, angiogenesis and metastasis.

Increased levels of MMP-9 have been demonstrated in colorectal cancer, in acute leukemia, breast cancer, human melanoma and bladder cancer. MMP-9 potentiates pulmonary metastasis formation and glioma invasion.

Apart from its function in tumor formation and spread, MMP-9 with its destructive effects is in close association with lung diseases and asthma.

Leukocyte recruitment to the central nervous system MMP-9 means an involvement in the pathogenesis of multiple sclerosis. MMP-9 has a crucial role in reproductive endocrinology and shows a changed expression pattern after myocardial infarction.

For literature update please contact drg@drg-diagnostics.de.

3 PRINCIPLES OF THE TEST

An anti-human MMP-9 coating antibody is adsorbed onto microwells.

Human MMP-9 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human MMP-9 antibody is added and binds to human MMP-9 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-human MMP-9 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human MMP-9 antibody.

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of human MMP-9 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human MMP-9 standard dilutions and human MMP-9 sample concentration determined.

4 REAGENTS PROVIDED

Reagents for human MMP-9 ELISA (96 tests)

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human MMP-9
- 1 vial (70 µL) **Biotin-Conjugate** anti-human MMP-9 polyclonal antibody
- 1 vial (150 µL) **Streptavidin-HRP**
- 2 vials human MMP-9 **Standard** lyophilized, 30 ng/mL upon reconstitution
- 1 vial (5 mL) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 mL) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
- 1 vial (15 mL) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 mL) **Stop Solution** (1M Phosphoric acid)
- 4 **Adhesive Films**

5 STORAGE INSTRUCTIONS – ELISA KIT

Store kit reagents between 2 °C and 8 °C. Immediately after use remaining reagents should be returned to cold storage (2 °C to 8 °C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6 SPECIMEN COLLECTION AND STORAGE INSTRUCTIONS

Cell culture supernatant, serum* and plasma (citrate, heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Pay attention to a possible "**Hook Effect**" due to high sample concentrations (see chapter 11).

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20 °C to avoid loss of bioactive human MMP-9. If samples are to be run within 24 hours, they may be stored at 2 °C to 8 °C (for sample stability refer to 13.5).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

** Pay attention to a possibly elevated serum level of human MMP-9 due to MMP-9 release by platelets during platelet activation (sampling process).*

7 MATERIALS REQUIRED BUT NOT PROVIDED

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8 PRECAUTIONS FOR USE

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5 °C.

- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9 PREPARATION OF REAGENTS

Buffer concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the **Buffer concentrates**, warm it gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2 °C to 25 °C.

Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 mL) of the **Assay Buffer Concentrate** (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.

Store at 2 °C to 8 °C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

9.5 Human MMP-9 Standard

Reconstitute **human MMP-9 Standard** by addition of distilled water.

Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilisation (concentration of reconstituted standard = 30 ng/mL).

Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate (see 10.d) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:

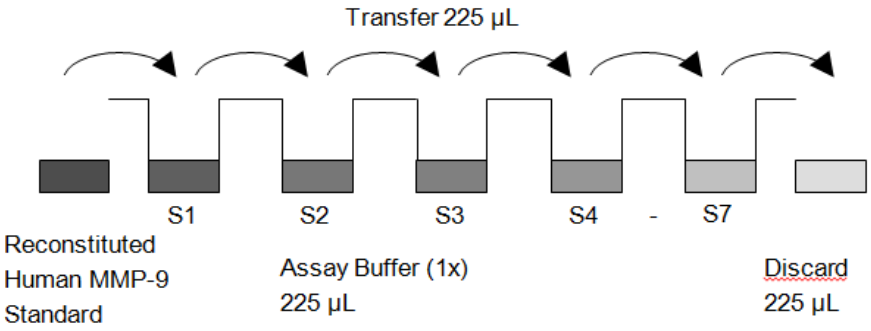
Pipette 225 µL of Assay Buffer (1x) into each tube.

Pipette 225 µL of reconstituted standard (concentration of standard = 30 ng/mL) into the first tube, labelled S1, and mix (concentration of standard 1= 15 ng/mL).
Pipette 225 µL of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.

Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 1).

Assay Buffer (1x) serves as blank.

Figure 1



10 TEST PROTOCOL

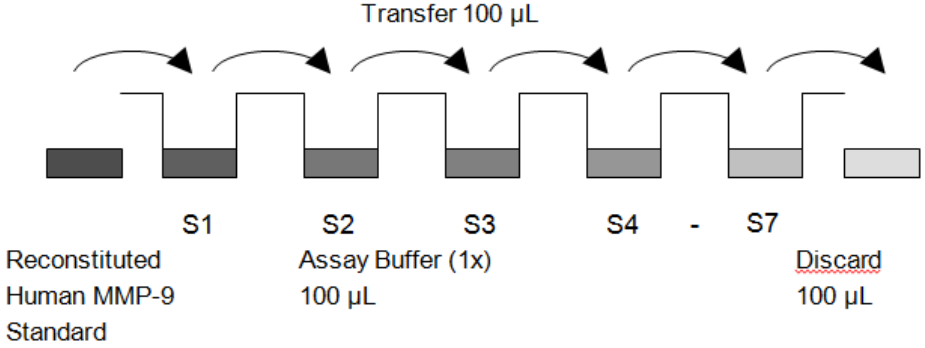
a. Samples from apparently healthy donors will not need external predilution (see 13.7 Expected Values). For pathological samples with expected higher values of MMP-9 predilute your samples 1:10 to 1:25 with Assay Buffer (1x) before starting with the test procedure according to one of the following schemes:
1:10 dilution: 25 µL sample + 225 µL Assay Buffer (1x)
1:25 dilution: 10 µL sample + 240 µL Assay Buffer (1x)

b. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard and blank should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2 °C - 8 °C sealed tightly.

c. Wash the microwell strips twice with approximately 400 µL **Wash** Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**.

d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1.):
Add 100 µL of Assay Buffer (1x) in duplicate to all **standard wells**. Pipette 100 µL of prepared **standard** (see Preparation of Standard 9.5, concentration = 30 ng/mL), in duplicate, into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 15 ng/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 2). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human MMP-9 standard dilutions ranging from 15.0 to 0.23 ng/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

Figure 2



In case of an **external standard dilution** (see 9.5.1) pipette 100 µL of these standard dilutions (S1-S7) in the standard wells according to Table 1.

Table 1:
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (15.0 ng/mL)	Standard 1 (15.0 ng/mL)	Sample 1	Sample 1
B	Standard 2 (7.5 ng/mL)	Standard 2 (7.5 ng/mL)	Sample 2	Sample 2
C	Standard 3 (3.75 ng/mL)	Standard 3 (3.75 ng/mL)	Sample 3	Sample 3
D	Standard 4 (1.88 ng/mL)	Standard 4 (1.88 ng/mL)	Sample 4	Sample 4
E	Standard 5 (0.94 ng/mL)	Standard 5 (0.94 ng/mL)	Sample 5	Sample 5
F	Standard 6 (0.47 ng/mL)	Standard 6 (0.47 ng/mL)	Sample 6	Sample 6
G	Standard 7 (0.23 ng/mL)	Standard 7 (0.23 ng/mL)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

e. Add 100 µL of **Assay Buffer (1x)** in duplicate to the **blank wells**.

f. Add 90 µL of **Assay Buffer (1x)** to the **sample wells**.

g. Add 10 µL of each **sample** in duplicate to the **sample wells**.

h. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).

i. Add 50 µL of **Biotin-Conjugate** to all wells.

j. Cover with an adhesive film and incubate at room temperature (18 °C to 25 °C) for 2 hours, if available on a microplate shaker set at 400 rpm.

k. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).

l. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point c. of the test protocol. Proceed immediately to the next step.

m. Add 100 µL of diluted **Streptavidin-HRP** to all wells, including the blank wells.

n. Cover with an adhesive film and incubate at room temperature (18 °C to 25 °C) for 1 hour, if available on a microplate shaker set at 400 rpm.

o. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point c. of the test protocol. Proceed immediately to the next step.

p. Pipette 100 µL of **TMB Substrate Solution** to all wells.

q. Incubate the microwell strips at room temperature (18 °C to 25 °C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable.
Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

r. Stop the enzyme reaction by quickly pipetting 100 µL of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 °C - 8 °C in the dark.

s. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11 CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human MMP-9 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human MMP-9 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human MMP-9 concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:10 (10 µL sample + 90 µL Assay Buffer (1x)) on the plate, the concentration read from the standard curve must be multiplied by the final dilution factor (depending on the predilution factor), for example:**
  Not prediluted samples: x 10
  1:10 prediluted samples: x 100
  1:25 prediluted samples: x 250
- **Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low human MMP-9 levels (Hook Effect). Such samples require further external predilution according to expected human MMP-9 values with Assay Buffer (1x) in order to precisely quantitate the actual human MMP-9 level.**
- It is suggested that each testing facility establishes a control sample of known human MMP-9 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3:
Representative standard curve for human MMP-9 ELISA. Human MMP-9 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

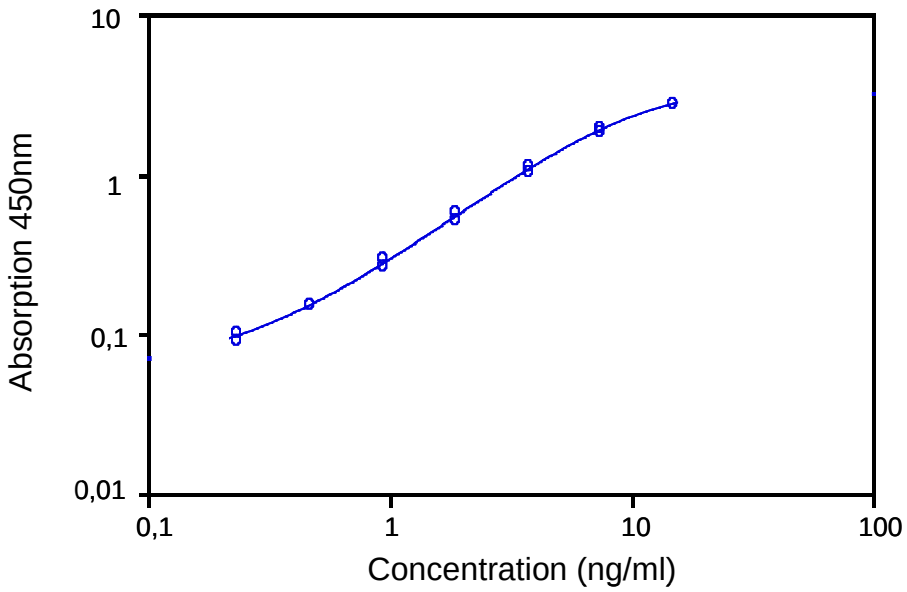


Table 2: Typical data using the human MMP-9 ELISA

Measuring wavelength: 450 nm, Reference wavelength: 620 nm

Standard	Human MMP-9 Concentration (ng/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	15.00	2.69 2.76	2.72	1.2
2	7.50	1.81 1.95	1.88	3.9
3	3.75	1.01 1.10	1.06	3.7
4	1.88	0.51 0.57	0.54	4.9
5	0.94	0.26 0.29	0.28	4.7
6	0.47	0.15 0.15	0.15	0.1
7	0.23	0.09 0.10	0.10	3.2
Blank	0	0.04 0.03	0.04	14.3

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The limit of detection of human MMP-9 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.05 ng/mL (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 4 independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of human MMP-9. 2 standard curves were run on each plate. Data below show the mean human MMP-9 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 7.3%.

Table 3:
The mean human MMP-9 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human MMP-9 Concentration (ng/mL)	Coefficient of Variation (%)
1	1	1424.8	4.4
	2	1314.8	1.3
	3	1496.3	5.8
	4	1517.1	2.6
2	1	1324.7	16.9
	2	1231.5	17.5
	3	1297.7	17.6
	4	1412.3	16.9
3	1	105.1	5.5
	2	101.7	6.2
	3	103.8	5.0
	4	130.2	7.7
4	1	1709.0	2.1
	2	1747.7	3.2
	3	1752.8	2.4
	4	1889.9	8.2
5	1	94.1	12.9
	2	87.2	7.4
	3	98.4	2.8
	4	115.9	8.2
6	1	67.3	5.8
	2	68.7	5.0
	3	69.1	6.8
	4	86.4	6.5
7	1	95.9	7.6
	2	91.3	3.5
	3	96.3	10.0
	4	131.9	4.5

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of human MMP-9. 2 standard curves were run on each plate. Data below show the mean human MMP-9 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 10.2%.

Table 4:
The mean human MMP-9 concentration and the coefficient of variation of each sample

Sample	Mean Human MMP-9 Concentration (ng/mL)	Coefficient of Variation (%)
1	1438.2	6.3
2	1316.5	5.7
3	110.2	12.2
4	1774.9	4.5
5	98.9	12.4
6	72.9	12.4
7	103.9	18.1

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human MMP-9 into serum. The amount of endogenous human MMP-9 in unspiked serum was subtracted from the spike values.

Recoveries were determined in 2 independent experiments with 4 replicates each. The overall mean recovery was 103.1%.

13.4 Dilution Parallelism

Serum samples with different levels of human MMP-9 were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 103.2% to 114,0% with an overall recovery of 107.4% (see Table 5).

Table 5

Sample	Dilution	Expected Human MMP-9 Concentration (ng/mL)	Observed Human MMP-9 Concentration (ng/mL)	Recovery of Expected human MMP-9 Concentration (%)
1	1:250		1419.0	
	1:500	709.5	809.1	114.0
	1:1000	354.7	372.1	104.9
	1:2000	177.4	186.8	105.3
2	1:250		778.6	
	1:500	389.3	401.6	103.2
	1:1000	194.6	207.7	106.7
	1:2000	97.3	107.3	110.3

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20 °C and thawed 5 times, and the human MMP-9 levels determined. There was no significant loss of human MMP-9 immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20 °C, 2 °C - 8 °C, room temperature (RT) and at 37 °C, and the human MMP-9 level determined after 24 h. There was no significant loss of human MMP-9 immunoreactivity detected under above conditions.

13.6 Specificity

The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human MMP-9 positive sample.

There was no cross reactivity or interference detected.

13.7 Expected Values

Serum as well as plasma samples from apparently healthy donors were tested for human MMP-9. The detected human MMP-9 levels ranged between 2.0 and 139.4 ng/mL for serum, between 9.6 and 87.3 ng/mL for plasma (citrate) and between 9.5 and 80.2 ng/mL for plasma (heparin). Elevated human MMP-9 levels depend on the type of immunological disorder.

14 REAGENT PREPARATION SUMMARY

14.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

14.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

14.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

14.4 Streptavidin-HRP

Make a 1:200 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

14.5 Human MMP-9 Standard

Reconstitute lyophilized human MMP-9 standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 TEST PROTOCOL SUMMARY

1. Predilute pathological samples with Assay Buffer (1x) 1:10 – 1:25. Samples from apparently healthy donors do not require predilution.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µL Assay Buffer (1x) in duplicate, to all standard wells. Pipette 100 µL prepared standard into the first wells and create standard dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells.
   Alternatively external standard dilution in tubes (see 9.5.1): Pipette 100 µL of these standard dilutions in the microwell strips.
5. Add 100 µL Assay Buffer (1x) in duplicate, to the blank wells.
6. Add 90 µL Assay Buffer (1x) to sample wells.
7. Add 10 µL sample in duplicate, to designated sample wells.
8. Prepare Biotin-Conjugate.
9. Add 50 µL Biotin-Conjugate to all wells.
10. Cover microwell strips and incubate 2 hours at room temperature (18 °C to 25 °C).
11. Prepare Streptavidin-HRP.
12. Empty and wash microwell strips 4 times with Wash Buffer.
13. Add 100 µL diluted Streptavidin-HRP to all wells.
14. Cover microwell strips and incubate 1 hour at room temperature (18 °C to 25 °C).
15. Empty and wash microwell strips 4 times with Wash Buffer.
16. Add 100 µL of TMB Substrate Solution to all wells.
17. Incubate the microwell strips for about 10 minutes at room temperature (18 °C to 25 °C).
18. Add 100 µL Stop Solution to all wells.
19. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:10 (10 µL sample + 90 µL Assay Buffer (1x)) on the plate, the concentration read from the standard curve must be multiplied by the final dilution factor (depending on the predilution factor), for example:

Not prediluted samples: x 10

1:10 prediluted samples: x 100

1:25 prediluted samples: x 250

1 REAGENTI FORNITI

Reagenti Forniti per human MMP-9 ELISA (96 test)

1 busta d´alluminio con una **Piastra Micropozzetti rivestita** con anticorpo monoclonale anti human MMP-9

1 flaconcino (70 µL) di anticorpo **Biotina Coniugato** (anticorpo policlonale human MMP-9)

1 flaconcino (150 µL) di **Streptavidina-HRP**

2 flaconcini human MMP-9 **Standard** liofilizzato, 30 ng/mL previa ricostituzione

1 flaconcino (5 mL) con **Tampone del Saggio concentrata** 20x
(PBS con 1% Tween 20 e 10% BSA)

1 bottiglia (50 mL) con **Tampone di Lavaggio concentrato** 20x
(PBS con 1% Tween 20)

1 flaconcino (15 mL) di **Soluzione Substrato** (tetrametilbenzidina)

1 flaconcino (15 mL) di **Soluzione bloccante** (acido fosforico 1M)

4 **Copripiastra** adesivi

2 ISTRUZIONI DI CONSERVAZIONE

Conservare i reagenti del kit a 2 °C - 8 °C. Subito dopo l'uso riporre i reagenti nel luogo di conservazione a 2 °C - 8 °C. La scadenza del kit e dei reagenti è indicata sulle etichette.

La data di scadenza dei componenti del kit può essere garantita solo se questi sono conservati correttamente e, in caso di uso ripetuto di un componente, il reagente non è stato contaminato durante la prima manipolazione.

3 PRECAUZIONI PER L'USO

- Tutti i prodotti chimici vanno considerati come potenzialmente pericolosi. Raccomandiamo, perciò, l'utilizzo di questo prodotto solo da personale addestrato alle tecniche di laboratorio e che siano avvezze alle comuni pratiche di laboratorio. Indossare abbigliamento idoneo come camici, guanti ed occhiali. Attenzione ad evitare contatto con la pelle e gli occhi. Nel caso di contatto con pelle o occhi, immediatamente lavare con acqua. Consultare la scheda di sicurezza del prodotto per specifici consigli.
- I reagenti sono **solo per uso di ricerca** e non sono destinati all'uso in diagnostica o terapia.
- Non mischiare tra loro reagenti di diversi lotti o provenienza.
- Non usare i kit dopo la data di scadenza.
- Non esporre i reagenti del kit, durante la conservazione e incubazione a forti fonti di luce.
- Non pipettare utilizzando la bocca.
- Non mangiare o fumare nell'area dove sono utilizzati i reagenti dei kit o i campioni.
- Evitare il contatto dei reagenti o campioni con la pelle o le mucose.
- Guanti di gomma o lattice dovrebbero essere sempre indossati quando si usano reagenti e campioni.
- Evitare il contatto tra il substrato del kit e agenti ossidanti e metallo.
- Evitare schizzi o produzione di aereosol.
- Per evitare contaminazione microbica o cross-contaminazione dei reagenti o dei campioni che invaliderebbero il test, usare sempre pipette e puntali mono-uso.
- Usare vaschette pulite e dedicate per la dispensare il reagente substrato.
- L'esposizione agli acidi inattiva il coniugato.
- Acqua distillata o de-ionizzata deve essere utilizzata per la preparazione dei reagenti.
- La soluzione di substrato deve essere portata a temperatura ambiente prima dell'utilizzo.
- Decontaminare ed eliminare i campioni e tutto il materiale potenzialmente contaminante perchè potrebbero contenere agenti infettanti. Il metodo preferito per la decontaminazione è l'autoclavaggio per minimo 1 ora a 121.5 °C.
- Gli scarti liquidi, non contenenti acido e gli scarti neutralizzati possono essere mischiati con sodio ipoclorido in un volume finale di 1.0%. Lasciare minimo 30 minuti per l'effettiva decontaminazione. Scarti liquidi contenenti acido devono essere neutralizzati prima dell'aggiunta di sodio ipoclorido.

4 PREPARAZIONE DEI REAGENTI

Prima di cominciare con le procedure del test **i concentrati** dei tamponi devono essere portati a temperatura ambientale e diluiti alle concentrazioni adeguate. Se i **concentrati dei tampone** presenta cristalli in sospensione, riscaldare lievemente i tamponi fino a ottenere la completa dissoluzione dei cristalli.

4.1 Tampone di Lavaggio (1x)

Versare l'intero contenuto (50 mL) del **tampone di lavaggio concentrato** (20x) in un cilindro graduato pulito da 1000 mL. Portare il volume finale a 1000 mL utilizzando acqua distillata o acqua deionizzata. Mescolare delicatamente per evitare la formazione di schiuma.

Trasferire il prodotto in una bottiglia pulita e conservare a temperature comprese fra 2 °C e 25 °C. Il tampone di lavaggio è stabile per 30 giorni.

Se necessario, è possibile preparare il tampone di lavaggio secondo la tabella seguente:

Numero di strip	Tampone di lavaggio (20x) (ml)	Acqua distillata (ml)
1 - 6	25	475
1 - 12	50	950

4.2 Tampone del Saggio (1x)

Versare l'intero contenuto (5 mL) del **tampone del saggio concentrato** (20x) in un cilindro graduato pulito da 100 mL. Portare il volume finale a 100 mL utilizzando acqua distillata o acqua deionizzata. Mescolare delicatamente per evitare la formazione di schiuma.

Conservare a temperatura compresa fra 2°C e 8°C. La soluzione tampone diluita è stabile per 30 giorni.

Se necessario, è possibile preparare la soluzione tampone secondo la tabella seguente:

Numero di strip	Tampone del Saggio (20x) (ml)	Acqua distillata (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

4.3 Biotina Coniugato

Il biotina coniugato deve essere utilizzato entro 30 minuti dalla diluizione.

Il biotina coniugato deve essere diluito 1:100 con Tampone del Saggio (1x) in una provetta di plastica pulita secondo la tabella seguente:

Numero di strip	Biotina coniugato (ml)	Tampone del Saggio (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

4.4 Streptavidina-HRP

Il Streptavidina-HRP deve essere utilizzato entro 30 minuti dalla diluizione.

Il Streptavidina-HRP deve essere diluito 1:200 con Tampone del Saggio (1x) in una provetta di plastica pulita secondo la tabella seguente:

Numero di strip	Streptavidina-HRP (ml)	Tampone del Saggio (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

4.5 Human MMP-9 Standard

Ricostituire lo **human MMP-9 standard** aggiungendo con acqua distillata. Il volume di ricostituzione è indicato sull'etichetta della flaconcino. Girare o mescolare gentilmente per garantire la completa ed omogenea solubilizzazione (concentrazione dello standard ricostituito = 30 ng/mL).

Permettere allo standard ricostituito di riposare per 10-30 minuti. Prima di fare le diluizione mescolare bene.

Dopo l'uso, lo standard rimanente non può essere riutilizzato e deve essere buttato.

La diluizione dello standard può essere fatto direttamente nella piastra (vedi d).) oppure nei tubi (vedi 4.5.1).

4.5.1 Diluizione degli Standard esterni

Etichettare 7 tubi, uno per ogni punto dello standard.

S1, S2, S3, S4, S5, S6, S7

Preparare diluizione seriali 1:2 per lo standard nel seguente modo:

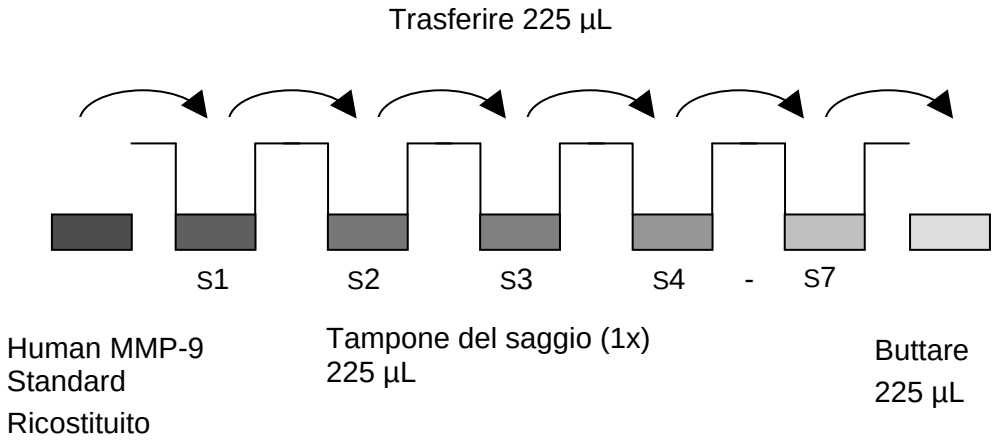
Pipettare 225 µL di Tampone del Saggio (1x) nei tutti tubi.

Pipettare 225 µL di standard ricostituito (concentrazione dello standard = 30 ng/mL) nel primo tubo, etichettato S1, e mescolare (concentrazione dello standard 1 = 15 ng/mL).

Pipettare 225 µL di questa diluizione nel secondo tubo, etichettato S2 mischiare accuratamente prima del successivo trasferimento. Ripetere le 5 diluizioni seriali in modo da creare i punti della curva di calibrazione (vedere Figura 1)

Tampone del Saggio (1x) serve come bianco.

Figura 1



5 PROCEDURA DEL TEST

a) Campioni patologici prediluti con Tampone del Saggio (1x) 1:10 - 1:25. I campioni prelevati da donatori apparentemente sani non richiedono prediluizione.
Prediluizione 1:10: 25 µL campione + 225 µL Tampone del Saggio (1x)
Prediluizione 1:25: 10 µL campione + 240 µL Tampone del Saggio (1x)

b) Stabilire il numero di strip dei micropozzetti necessarie per analizzare la quantità desiderata di campioni più le strip per i bianchi e gli standard. Tutti i campioni, gli standardi e il bianco devono essere processati in duplicato. Rimuovere dal supporto le strip micropozzetti non utilizzate e conservarle nella bustina metallica contenente la polvere essiccante, mantenendole a 2 °C - 8 °C e perfettamente sigillate.

c) Lavare due volte le strip micropozzetti utilizzando circa 400 µL di **tampone di lavaggio** per pozzetto, aspirando accuratamente il contenuto dei micropozzetti tra un lavaggio e l'altro. Permettere al tampone di lavaggio di rimanere, nei pozzetti, circa **10-15 secondi** prima dell'aspirazione. Evitare di scalfire la superficie dei micropozzetti.
Dopo l'ultimo lavaggio, asciugare le strip micropozzetti con un tampone o carta assorbente per rimuovere il tampone di lavaggio in eccesso. Utilizzare le strip subito dopo il lavaggio o sistemarle capovolte su carta assorbente umida per non più di 15 min. **Non lasciar asciugare i pozzetti.**

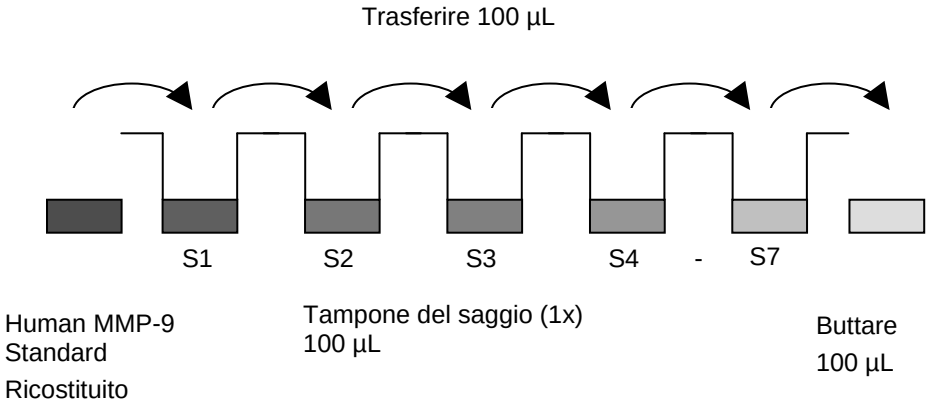
d) **Diluizione dello standard in micropozetti** (alternativamente la diluizione dello standard può avvenire in tubi – vedi 4.5.1)
Aggiungere 100 µL di Tampone del Saggio (1x) in duplicato a tutti i **pozzetti dello standard**. Pipettare 100 µL **standard** preparato (vedi preparazione dello standard 4.5.1, concentrazione = 30.00 ng/mL) in duplicato nei pozzetti A1 e A2 (vedi

e) Tabella *1*). Mescolare il contenuto dei pozzetti A1 e A2 attraverso ripetute aspirazione ed iniezioni (concentrazione dello standard 1, S1 = 15.00 ng/mL) e trasferire 100 µL, rispettivamente, ai pozzetti B1 e B2 (vedere Figura 2). Fare attenzione a non graffiare la parte interna dei pozzetti. Continuare questa procedura per 5 volte, creando due colonne di standard in diluizione con concentrazione da 15.00 a 0.23 ng/mL.
Buttare 100 µL del contenuto degli ultimi pozzetti (G1 e G2).

In caso di **diluizione esterna dello standard** (vedi 4.5.1) pipettare 100 µL di queste diluizioni standard (S1 – S7) nei pozzetti degli standard come da

f) Tabella *1*.

Figura 2



g) Dispensare 100 µL di Tampone del Saggio (1x) in duplicato ai pozzetti de bianco.

h) Dispensare 90 µL di Tampone del Saggio (1x) in duplicato ai pozzetti dei campioni.

i) Dispensare 10 µL di campione in duplicato ai pozzetti dei campioni.

Tabella 1: Tabella rappresenta un esempio dell'organizzazione dei bianchi, standardi e campioni nei pozzetti:

	1	2	3	4
A	Standard 1 (15.0 ng/mL)	Standard 1 (15.0 ng/mL)	Campione 1	Campione 1
B	Standard 2 (7.5 ng/mL)	Standard 2 (7.5 ng/mL)	Campione 2	Campione 2
C	Standard 3 (3.75 ng/mL)	Standard 3 (3.75 ng/mL)	Campione 3	Campione 3
D	Standard 4 (1.88 ng/mL)	Standard 4 (1.88 ng/mL)	Campione 4	Campione 4
E	Standard 5 (0.94 ng/mL)	Standard 5 (0.94 ng/mL)	Campione 5	Campione 5
F	Standard 6 (0.47 ng/mL)	Standard 6 (0.47 ng/mL)	Campione 6	Campione 6
G	Standard 7 (0.23 ng/mL)	Standard 7 (0.23 ng/mL)	Campione 7	Campione 7
H	Bianco	Bianco	Campione 8	Campione 8

j) Preparare la **biotina coniugato** (consultare la sezione biotina coniugato 4.3 sulla preparazione dei reagenti).

k) Dispensare 50 µL di **biotina coniugato** a ciascun pozzetto.

l) Coprire con un copripiastra e incubare a temperatura ambiente (18 °C - 25 °C) per 2 ore utilizzando, se disponibile, un vortex a 400 rpm.

m) Preparare la **streptavidina-HRP** (consultare la sezione streptavidina-HRP 4.4 sulla preparazione dei reagenti).

n) Rimuovere il copripiastra e svuotare i pozzetti. **Lavare** le strip della pozzeti 4 volte come descritto in punto 5.c). del protocollo. Procedere immediatamente al punto successivo.

o) Dispensare 100 µL di **streptavidina-HRP** a ciascun pozzetto.

p) Coprire con un copripiastra e incubare a temperatura ambiente (18 °C - 25 °C) per 1 ora utilizzando, se disponibile, un vortex a 400 rpm.

q) Rimuovere il copripiastra e svuotare i pozzetti. **Lavare** le strip della pozzeti 4 volte come descritto in punto 5.c). del protocollo. Procedere immediatamente al punto successivo.

r) Pipettare 100 µL di **soluzione substrato TMB** in tutti i pozzetti, inclusi quelli del blank.

s) Incubare le strip a temperatura ambiente (18 °C - 25 °C) per circa 10 minuti. Evitare l'esposizione diretta a luci intense.
È necessario monitorare i valori O.D. a livello della piastra e interrompere la reazione del substrato (vedi il punto prossimo del protocollo) prima che i pozzetti positivi cessino di essere appropriatamente registrabili. La determinazione del tempo necessario per lo sviluppo del colore dev'essere fatto per ogni singolo parametro.
Si raccomanda di aggiungere la soluzione di stop quando lo standard più elevato ha sviluppato un colore blu scuro. Alternativamente lo sviluppo del colore può essere monitorato con un lettore ELISA a 620 nm. La reazione del substrato deve essere bloccata non appena viene misurato un valore delle OD di 0.9 - 0.95.

t) Interrompere la reazione enzimatica pipettando rapidamente 100 µL di **soluzione bloccante** in ciascun pozzetto, inclusi i pozzetti del bianco. È importante che la soluzione bloccante si diffonda rapidamente e uniformemente attraverso i micropozzetti per inattivare completamente l'enzima. I risultati devono essere letti immediatamente dopo l'aggiunta della soluzione bloccante o entro 1 ora se le strip sono conservate in un luogo buio a 2 °C - 8 °C.

u) Leggere l'assorbanza di ciascun micropozzetto su uno spettrofotometro che utilizza 450 nm come lunghezza d'onda primaria (620 nm come lunghezza d'onda di riferimento alternativa; valori da 610 nm a 650 nm sono accettabili). Azzerare il lettore della piastra secondo le istruzioni del produttore e utilizzando i pozzetti del bianco. Determinare l'assorbanza sia dei campioni, sia degli standard di human MMP-9.

I campioni sono stati diluiti 1: 10 nella piastra, la concentrazione dalla curva standard risultante deve essere moltiplicata per il fattore di diluizione e per il fattore di prediluizione:

Senza prediluizione: x 10,

Prediluizione 1:10: x 100,

Prediluizione 1:25: x 250;

Annotazione: In caso di incubazione senza agitazione i valori di densità ottica (O.D.) potranno essere più bassi di quanto indicato sotto. Tuttavia i risultati saranno da ritenersi validi.

1 REACTIVOS SUMINISTRADOS

Reactivos Suministrados para human MMP-9 ELISA (96 tests)

1 bolsa de aluminio con **una placa de micropocillos recubiertos** con anticuerpos monoclonales anti-human MMP-9

1 vial (70 µL) con **conjugado de biotina** (anticuerpos policlonales anti-human MMP-9)

1 vial (150 µL) con **estreptavidina-HRP**

2 viales con **Estándar** human MMP-9 liofilizado, 30 ng/mL tras la reconstitución

1 vial (5 mL) de **concentrado de tampón de ensayo** 20x
(PBS con Tween 20 al 1% y BSA al 10%)

1 frasco (50 mL) de **concentrado de tampón de lavado** 20x
(PBS con Tween 20 al 1%)

1 vial (15 mL) de **solución de sustrato** (tetrametil-bencidina)

1 vial (15 mL) de **solución de parada** (ácido fosfórico 1M)

4 **tapas para placas**, adhesives

2 INSTRUCCIONES DE CONSERVACIÓN

Conservar los reactivos del kit a una temperatura comprendida entre 2 °C y 8 °C. Inmediatamente después de utilizarlos deberá volver a conservar los reactivos a dicha temperature (2 °C y 8 °C). En las etiquetas figuran las fechas de caducidad del kit y de los reactivos.

Sólo se podrá garantizar la fecha de caducidad de los componentes del kit si se conservan adecuadamente y, en caso de uso reiterado de un mismo componente, si el reactivo no queda contaminado en la primera manipulación.

3 PRECAUCIONES DE USO

- Todos los productos químicos deben considerarse potencialmente peligrosos. Por tanto, recomendamos que este producto sea manipulado únicamente por aquellas personas que hayan sido entrenadas en técnicas de laboratorio y que sea usado de acuerdo con los principios de buenas prácticas de laboratorio. Se debe llevar ropa de protección apropiada como puedan ser las batas de laboratorio, gafas de seguridad y guantes. Se debe trabajar con cuidado para evitar cualquier contacto con piel y ojos. En el caso de que tenga lugar un contacto con piel u ojos, proceder de forma inmediata a lavar la parte afectada con abundante agua. Véase la(s) hoja(s) de seguridad y/o declaraciones de seguridad para recomendaciones específicas.
- Los reactivos están destinados **exclusivo para uso de investigación** y no se deben usar en procedimientos diagnósticos o terapia.
- No mezclar o sustituir los reactivos por los equivalentes de otros lotes u otras fuentes.
- No usar reactivos caducados.
- No exponer los reactivos del kit a una luz intensa durante su almacenamiento o incubación.
- No pipetear con la boca.
- No se recomienda comer o fumar en las zonas donde se manipulen muestras o reactivos.
- Evitar el contacto de los reactivos del kit o de las muestras con piel o mucosas.
- Se recomienda el uso de guantes desechables de goma o látex durante la manipulación de las muestras y reactivos.
- Evitar el contacto de la solución de sustrato con agentes oxidantes y metales.
- Evitar salpicaduras y la generación de aerosoles.
- Con el propósito de evitar una contaminación microbiológica o contaminaciones cruzadas de reactivos y muestras que puedan invalidar el test se recomienda el uso de pipetas y/o puntas de pipetas de un solo uso.
- Usar recipientes limpios y específicos de reactivos para la dispensación de reactivos de sustrato.
- La exposición a los ácidos inactiva el conjugado.
- Se debe usar agua destilada o desionizada en la preparación de los reactivos.
- La solución de sustrato debe de estar a temperatura ambiente antes de su uso.
- Descontaminar y disponer las muestras y todos los materiales potencialmente contaminados como si pudieran contener agentes infecciosos. El método preferente de descontaminación es un autoclavado durante un mínimo de 1 hora a 121.5 °C.
- Los residuos líquidos que no contengan ácido y los residuos neutralizados pueden ser mezclados con hipoclorito sódico en volúmenes tales que la mezcla final contenga 1.0% de hipoclorito sódico. Dejar actuar durante 30 minutos para una efectiva descontaminación. Los residuos líquidos que contengan ácido deben ser neutralizados previamente a la adición de hipoclorito sódico.

4 PREPARACIÓN DE LOS REACTIVOS

Los **tampónes concentrados** debe de alcanzar la temperatura ambiente y ser diluidos antes de iniciar el procedimiento del test. Si en el concentrado de **tampónes concentrados** se han formado cristales, caliente suavemente hasta su completa disolución.

4.1 Tampón de Lavado (1x)

Vierta todo el contenido (50 mL) del **concentrado de tampón de lavado** (20x) en un matraz aforado de 1000 mL limpio. Enrase en matraz con agua destilada o desionizada. Mezcle suavemente para evitar la formación de espuma.

Transfiera la solución a un frasco de lavado limpio y consérvela a una temperatura entre 2 °C y 25 °C. El tampón de lavado permanece estable durante 30 días.
En función de la cantidad que vaya a necesitar, prepare el tampón de lavado de acuerdo a la siguiente tabla:

Número de tiras	Tampón de lavado (20x) (mL)	Agua destilada (mL)
1 – 6	25	475
1 - 12	50	950

4.2 Tampón de Ensayo (1x)

Vierta todo el contenido (5 mL) del **concentrado de tampón de ensayo** (20x) en un matraz aforado de 100 mL limpio. Enrase en matraz con agua destilada o desionizada. Mezcle suavemente para evitar la formación de espuma.

Conserve la solución a una temperatura de entre 2 °C y 8 °C. El tampón de trabajo permanece estable durante 30 días.

En función de la cantidad que vaya a necesitar, prepare el tampón de ensayo de acuerdo a la siguiente tabla:

Número de tiras	Tampón de ensayo (20x) (mL)	Agua destilada (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

4.3 Conjugado de biotiona

Se utilizará el conjugado de biotiona antes de transcurridos 30 minutos desde su dilución.

Justo antes de utilizar el **conjugado de biotina**, se debe diluirlo con Tampón de ensayo (1x) en un tubo de ensayo de plástico limpio, en una proporción de 1:100.

En función de la cantidad que vaya a necesitar, prepare el conjugado de biotina de acuerdo a la siguiente tabla:

Número de tiras	Conjugado de biotiona (mL)	Tampón de ensayo (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

4.4 Estreptavidina-HRP

Se utilizará el estreptavidina-HRP antes de transcurridos 30 minutos desde su dilución.

Se debe diluirlo la **estreptavidina-HRP** con Tampón de ensayo (1x) en un tubo de ensayo de plástico limpio, en una proporción de 1:200.

En función de la cantidad que vaya a necesitar, prepare la estreptavidina-HRP de acuerdo a la siguiente tabla:

Número de tiras	Estreptavidina-HRP (mL)	Tampón de ensayo (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

4.5 Dilución estándar human MMP-9

Reconstituir el **estándar human MMP-9** la adición de agua destilada.

El volumen de reconstitución está indicado en la etiqueta del vial del estándar. Girar o mezclar cuidadosamente para garantizar una completa y homogénea solubilización (concentración del estándar reconstituido = 30 ng/mL).

Permitir que el estándar reconstituido se asiente durante 10-30 minutos. Mezclar bien previamente a realizar las diluciones.

Tras su uso los restos del estándar no pueden ser almacenados y deben ser descartados.

Las **diluciones estándar** pueden ser preparadas directamente en la placa multipocillo (véase 5.d) o alternativamente en tubos (véase 4.5.1).

4.5.1 Dilución Estándar Externa

Rotular 7 tubos, uno para cada punto de la curva estándar.
S1, S2, S3, S4, S5, S6, S7.

Acto seguido, preparar diluciones seriadas 1:2 para la curva estándar como se indica a continuación:

Pipetear 225 µL de Tampón de ensayo (1x) a todos los tubos.

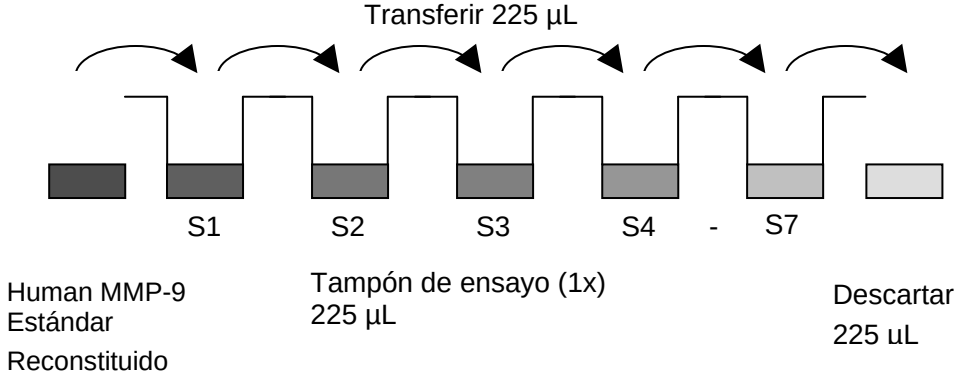
Pipetear 225 µL de estándar reconstituido (concentración del estándar = 30 ng/mL) en el primer tubo, etiquetado como S1, y mezclar (concentración del estándar 1 = 15 ng/mL).

Pipetear 225 µL de esta dilución en el segundo tubo, etiquetado como S2, y mezclar completamente antes de la siguiente transferencia.

Repetir la serie de diluciones 5 veces más de manera que se obtengan los diferentes puntos de la curva estándar (véase Figura 1).

Tampón de ensayo (1x) sirve como blanco.

Figura 1



5 PROTOCOLO DE ENSAYO

a. Prediluir las muestras patológicas con Tampón de ensayo (1x) 1:10 - 1:25. No es necesario prediluir las muestras de donantes aparentemente sanos:
Dilution 1:10: 25 µL de muestra + 225 µL de Tampón de ensayo (1x)
Dilution 1:25: 10 µL de muestra + 240 µL de Tampón de ensayo (1x)

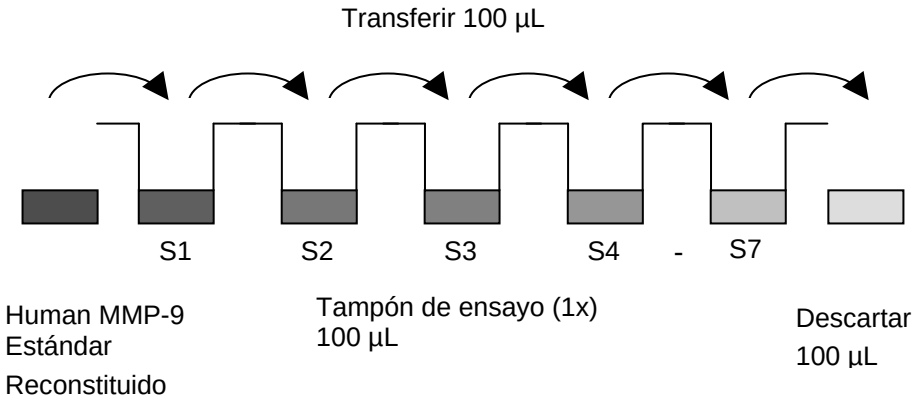
b. Determine el número de tiras necesarias para analizar el número deseado de muestras y además añada las tiras para blancos y patrones (de color). Todas las muestras, estándares y blancos deben ser analizadas por duplicado. Retire del soporte las tiras sobrantes y consérvelas, junto con el desecante suministrado en una bolsa metalizada y cerrada herméticamente, a una temperatura de 2 °C - 8 °C. Coloque las tiras que contienen la curva de valoración en las posiciones A1/A2 a H1/H2 (véase la Tabla).

c. Lave 2 veces las tiras con aproximadamente 400 µL de **tampón de lavado** por cada pocillo, aspirando completamente el contenido de los pocillos entre cada lavado. Permitir que el tampón de lavado permanezca en los pocillos durante **10-15 segundos** antes de su aspiración. Evite rayar la superficie de los pocillos.
Tras el último lavado, golpee suavemente las tiras contra un papel absorbente o una toallita de papel para eliminar el exceso de tampón de lavado. Utilice las tiras inmediatamente después de lavadas o bien colóquelas boca abajo sobre un papel absorbente húmedo durante como máximo 15 minutos. **No deje secar los pocillos.**

d. **Dilución de los Estándars en la placa multipocillo** (Alternativamente, la dilución de los estándars puede ser preparada en tubos – véase 4.5.1)
Añadir 100 µL de Tampón de ensayo (1x) a todos los **pocillos estándar**. Pipetear 100 µL de **estándar** preparado (véase Preparación del Estándar 0 , concentración = 30.00 ng/mL) por duplicado en los pocillos A1 y A2 (véase Tabla). Mezclar el contenido de los pocillos A1 y A2 por repetidas aspiraciones y expulsiones del contenido con la pipeta (concentración del estándar 1, S1 =15.00 ng/mL), y transferir 100 µL a los pocillos B1 y B2, respectivamente (véase Figura 2). Levar cuidado de no rascar la superficie interior de los micropocillos con la punta de la pipeta.

Continuar este procedimiento 5 veces, formando dos filas de diluciones estándar del human MMP-9 ordenadas des de 15.00 a 0.23 ng/mL. Descartar 100 µL de los contenidos de los últimos micropocillos (G1, G2) usados.

Figura 2



En caso de **una dilución estándar externa** (véase 4.5.1), pipetear 100 µL de estas diluciones estándar (S1 - S7) en los pocillos correspondientes al estándar de acuerdo con la Tabla.

e. Añada 100 µL **Tampón de ensayo (1x)** a los **pocillos del blanco**, por duplicado.

f. Añada 90 µL de **Tampón de ensayo (1x)** a los **pocillos con muestras**.

g. Por duplicado, añada 10 µL de cada **muestra** a **los pocillos designados**.

Tabla 1: Tabla que describe un ejemplo de la disposición de los blancos, estándars y muestras en los micropocillos de las tiras:

	1	2	3	4
A	Estándar 1 (15.00 ng/mL)	Estándar 1 (15.00 ng/mL)	Muestra 1	Muestra 1
B	Estándar 2 (7.50 ng/mL)	Estándar 2 (7.50 ng/mL)	Muestra 2	Muestra 2
C	Estándar 3 (3.75 ng/mL)	Estándar 3 (3.75 ng/mL)	Muestra 3	Muestra 3
D	Estándar 4 (1.88 ng/mL)	Estándar 4 (1.88 ng/mL)	Muestra 4	Muestra 4
E	Estándar 5 (0.94 ng/mL)	Estándar 5 (0.94 ng/mL)	Muestra 5	Muestra 5
F	Estándar 6 (0.47 ng/mL)	Estándar 6 (0.47 ng/mL)	Muestra 6	Muestra 6
G	Estándar 7 (0.23 ng/mL)	Estándar 7 (0.23 ng/mL)	Muestra 7	Muestra 7
H	Blanco	Blanco	Muestra 8	Muestra 8

h. Prepare la **conjugado de biotiona** (véase la preparación de la conjugado de biotiona 4.3.)

i. Añada 50 µL la **conjugado de biotiona** a todos los pocillos.

j. Cubra la placa con una tapa e incúbela a temperatura ambiente (18 °C - 25 °C) durante 2 horas (en un agitador mecánico a 400 rpm, si es posible).

k. Prepare **estreptavidina-HRP** (véase la preparación de estreptavidina-HRP 4.4).

l. Retire la tapa y vacíe los pocillos. **Lavar** los micropocillos de las tiras 4 veces de acuerdo al punto c del protocolo del test. Proseguir inmediatamente después al próximo paso.

m. Añada 100 µL **estreptavidina-HRP** a todos los pocillos.

n. Cubra la placa con una tapa e incúbela a temperatura ambiente (18 °C - 25 °C) durante 1 hora (en un agitador mecánico a 400 rpm, si es posible).

o. Retire la tapa y vacíe los pocillos. **Lavar** los micropocillos de las tiras 4 veces de acuerdo al punto c del protocolo del test. Proseguir inmediatamente después al próximo paso.

p. Pipetee 100 µL de **solución de sustrato TMB** y viértalos en todos los pocillos, incluidos los del blanco.

q. Incube las tiras a temperatura ambiente (18 °C - 25 °C) durante aproximadamente 10 minutos. Evite la exposición directa a la luz intensa.

Deben monitorizarse los valores DO de la placa para detener la reacción del sustrato (véase el siguiente punto de este protocolo) antes de que deje de ser posible registrar correctamente los pocillos positivos. La determinación del tiempo adecuado para el desarrollo del color, debe realizarse de forma individual para cada ensayo.

Se recomienda añadir la solución de parada cuando el estándar más alto presente un color azul oscuro. Alternativamente el desarrollo de color puede ser monitorizado con un lector de placas de ELISA a 620 nm. La reacción del substrato debería ser parada cuando este estándard alcanze una DO entre 0.9 y 0.95.

r. Detenga la reacción enzimática pipeteando rápidamente 100 µL de **solución de parada** en cada pocillo, incluidos los del blanco. Es importante dispensar la solución de parada de forma rápida y uniforme en todos los pocillos para inactivar totalmente la enzima. Los resultados deben leerse inmediatamente después de añadir la solución de parada o, como máximo, en el plazo de 1 hora si las tiras se conservan a una temperatura entre 2 °C - 8 °C en un lugar oscuro.

s. Lea la absorbancia de cada pocillo en un espectrofotómetro utilizando 450 nm como longitud de onda principal (opcionalmente 620 nm como longitud de onda de referencia; los valores comprendidos entre 610 nm y 650 nm son aceptables). Utilizando los pocillos de blanco, haga el blanco del lector de placas de acuerdo con las instrucciones del fabricante. Determine la absorbancia de las muestras y de los human MMP-9.

Las muestras han sido diluidas 1:10 en la placa. La concentración leída a partir de la curva estándar debe ser multiplicada por el factor de dilución y el factor de predilución:

Sin predilución: x 10

Predilución 1:10: x 100

Predilución 1:25: x 250

Nota: En caso de incubar sin agitar, los valores de D.O. pueden ser inferiores a los indicados más abajo. De todas formas los resultados siguen siendo válidos.

SYMBOLS USED

Symbol	English	Deutsch	Italiano	Español	Français
CE	European Conformity	CE-Konformitäts-kennzeichnung	Conformità europea	Conformidad europea	Conformité normes européennes
	Consult instructions for use *	Gebrauchsanweisung beachten *	Consultare le istruzioni per l'uso	Consulte las instrucciones de uso	Consulter les instructions d'utilisation
IVD	*In vitro* diagnostic medical device *	*In-vitro*-Diagnostikum *	Dispositivo medico-diagnostico in vitro	Producto sanitario para diagnóstico In vitro	Dispositif médical de diagnostic in vitro
REF	Catalogue number *	Artikelnummer *	Numero di Catalogo	Nûmero de catálogo	Référence de catalogue
LOT	Batch code *	Chargencode *	Codice del lotto	Codigo de lote	Numéro de lot
	Contains sufficient for <n> tests *	Ausreichend für <n> Prüfungen *	Contenuto sufficiente per "n" saggi	Contenido suficiente para <n> ensayos	Contenu suffisant pour "n" tests
	Temperature limit *	Temperaturbegrenzung *	Temperatura di conservazione	Temperatura de conservacion	Température de conservation
	Use-by date *	Verwendbar bis *	Utilizzare prima del	Establa hasta	Utiliser jusque
	Manufacturer *	Hersteller *	Fabbricante	Fabricante	Fabricant
	Caution *	Achtung *			
RUO	For research use only	Nur für Forschungszwecke	Solo a scopo di ricerca	Sólo para uso en investigación	Seulement dans le cadre de recherches
Distributed by	Distributed by	Vertreiber	Distributore	Distribuidor	Distributeur
Content	Content	Inhalt	Contenuto	Contenido	Contenu
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantità	Volumen/Número	Volume/Quantité