EarlyFound® MMP-1 ELISA KIT

For in vitro diagnostic use only

Specimen type saliva
Specimen volume 50 µL

Intended Use

The device is an in vitro diagnostic device that quantitatively measures, by immunochemical techniques, the concentration of human Matrix metalloproteinase-1 (MMP-1) in human saliva specimen from individuals. A positive result indicates salivary MMP-1 higher than the preset cut-off value (i.e. 232.7 pg/mL). The device is intended for use on individuals 30~90 years as an aid in diagnosis of oral squamous cell carcinoma (OSCC) at oral cavity, including cheek mucosa, floor of mouth, gum, retromolar area, tongue (excluding base of tongue), and multiple sites at the oral cavity, in conjunction with other laboratory findings, imaging studies, and clinical assessment. The intended user is laboratory professional user.

Introduction

Human Matrix metalloproteinase-1 (MMP-1) is the earliest-identified MMP family member involved in extracellular matrix (ECM) remodeling and is closely associated with metastasis, angiogenesis, and inflammation in tumorigenesis. Upregulated expression of MMP-1 has been reported in several types of cancer, including oral cancer. Elevated mRNA and protein levels of MMP-1 have been confirmed in both tissue and saliva specimens from oral squamous cell carcinoma (OSCC) patients[1]. The level of salivary MMP-1 was dramatically elevated in patient with OSCC but only slightly increased in patient with Oral Potentially Malignant Disease (OPMD)[2]. Salivary MMP-1 measurement will aid in diagnosis of oral mucosa malignant disorders.

The test result does NOT replace an oral cancer screening or an oral cancer diagnosis. If the level of salivary MMP-1 changes or increases, it may be caused by oral mucosa malignant disorders. The subjects need to go to a doctor for further oral examination such as visual inspection, dental radiology exam and biopsy for diagnosis. A negative result does NOT rule out the possibility of OSCC.

[1] ref: CEBP 2011, 20(12), 2628; doi: 10.1158/1055-9965.EPI-11-0503.

[2] ref: Cancers 2020, 12, 2273; doi: 10.3390/cancers12082273.

Principle of the Assay

This assay employs the enzyme-linked immunosorbent assay (ELISA) technique. A monoclonal antibody (Ab) specific to human MMP-1 has been pre-coated on a strip microplate. Standards

and specimens are added into the wells, and MMP-1 is bound by the immobilized Ab on microplate. After washing away unbound substances, a horseradish peroxidase (HRP)-conjugated monoclonal Ab specific to human MMP-1 is added to the wells. Following wash to remove unbound HRP-Ab, a substrate solution (TMB) is added to the wells and the color develops in proportion to the amount of MMP-1. The color development is stopped and the intensity of the color is measured by Spectrophotometer. The level of MMP-1 in specimen is relatively quantified according to the standard curve.

Product Content

Catalogue number: STBP01E1-1P

No.	PART	DESCRIPTION	Amount
1.	Microplate	96 well strip microplate pre-coated with Ab specific to human MMP-1. In vacuum bag. Ready to use.	12 strips of 8 wells
2.	Standard	100 ng/mL human MMP-1 recombinant protein in a buffered protein base with preservatives. To be prepared using Assay Diluent before use.	110 µL/vial*2
3.	Detection Ab	100-fold concentrated MMP-1 specific Ab conjugated with HRP in a buffered protein base with preservatives. To be prepared using Assay Diluent before use.	150 µL/vial*1
4.	Assay Diluent	Diluent for standard, specimens, and detection Ab. Ready to use.	31 mL/bottle*1
5.	15x Wash buffer	15-fold concentrated buffered solution. To be prepared using ddH_2O before use.	21 mL/bottle*1
6.	TMB	TMB (3, 3', 5, 5'-Tetramethylbenzidine). Ready to use.	12 mL/bottle*1
7.	Stop Solution	1N sulfuric acid. Ready to use.	6 mL/bottle*1
8.	Plate Sealer	Adhesive strips. Use to seal the microplate.	transparent sealer*3 foil sealer*1
9	Instructions for Use	Package insert	*1

NOTE：

1. Please read the instructions for use before use.
2. After first opening, keep the stock solution of Standard, Detection Ab, and TMB at 2-8°C, and such solutions should be prepared only before use.
3. After first opening the microplate bag, tightly re-seal the bag to minimize exposure to moisture for unused wells and keep at 2-8°C for storage.
4. If crystals have formed in 15x Wash Buffer, warm it to room temperature and mix gently until the crystals have completely dissolved before dilution.
5. Do NOT reuse the strip microplate and reagents.
6. Do NOT mix or substitute reagents with those from other lots or sources.

Storage and Stability

- Storage condition for whole kit: 2-8 °C.
- Shelf-life: 12 months.
- In-Use Lifetime: 1 month.
- Do NOT use beyond the expiration date.

Materials Required but Not Provided

- Sample collection cup/tube.
- Horizontal orbital microplate shaker capable of maintaining a speed of 800±50 rpm for incubation.
- Manifold dispenser, or automate microplate washer.
- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm, 570 nm or 620 nm.
- Deionized or distilled water.
- General equipment, including: vortex mixer, mini-centrifuge, timer, pipettes, pipette tips, and microtubes.
- Disposable gloves, protective clothing, and biohazard disposal container.

Precautions

- Do NOT use the device beyond the expiration date or if its package has been damaged.
- Read the instructions for use carefully before performing the test.
- Follow standard Lab procedure and biosafety guidelines for handling and disposal of potentially infectious material. All specimens and reagents should be considered potential hazardous.
- The stop solution provided is an acid solution. Treat it with caution.

- Some components contain ProClin™ which may cause an allergic skin reaction. Avoid breathing mist.
- Wear protective gloves, clothing, and goggles for eye and face protection during operation. Wash hands thoroughly after handling.

Saliva Specimen Collection and Storage

Donors should NOT eat food, drink, and smoke in 60 minutes before collection. Rinse mouth 5 times with drink water (Never Use Mouthwash), then move tongue in oral cavity to stimulate saliva secretion. Spit out saliva into collection cup/tube until a sufficient amount (at least 5 mL) is collected. Store the crude saliva on ice or at 4°C before centrifugation. Centrifuge the specimen at 3000 x g for 15 minutes to collect supernatant for assay. Perform the assay immediately or aliquot and store the specimen at -80°C freezer. Cryopreserved specimen should avoid repeated freeze-thaw cycles.

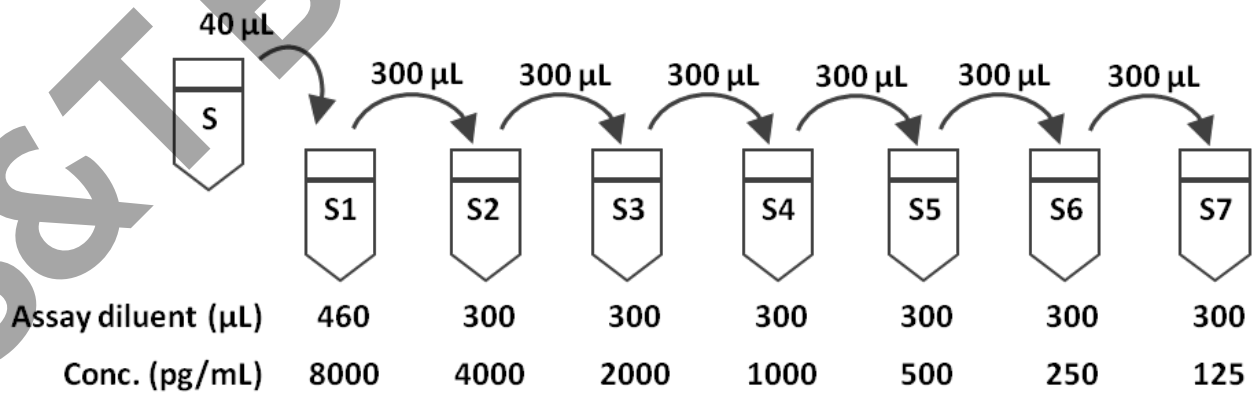
NOTE：

1. Saliva collection should be avoided under bleeding condition. Do NOT use saliva that contains blood.
2. Rinse mouth with drinking water (Never Use Mouthwash).

Reagent Preparation

Wash buffer - if crystals have formed in the 15x concentrate, warm it to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of 15x Wash Buffer Concentrate to deionized or distilled water to prepare 300 mL of Wash Buffer.

Standard curve - the concentration of Standard is 100 ng/mL. Prepare 12.5-fold dilution with Assay Diluent as below to generate S1 and then 2-fold serial dilution to generate standard curve S2~S7. Use Assay Diluent as the Blank. Prepare it only before use and keep it on ice or at 4°C before adding it to wells.



Detection Ab working solution - the Detection Ab solution is 100-fold concentrate. Prepare the

required amount by 100-fold dilution with Assay Diluent. Prepare it only before use and keep it on ice or at 4°C before adding it to wells.

Assay Procedure

1. Bring Wash buffer and Assay diluent to room temperature (RT) before use.
2. Open 96-well microplate, remove excess microplate strips from the plate frame, place back the unused strips to the foil bag containing the desiccant pack, and reseal.
3. Prepare specimens and standard curve (S1~S7 & Blank) as mentioned above.
4. Add 50 µL Assay diluent to each well.
5. Add 50 µL Standard or specimen per well. Cover with the transparent sealer provided. Incubate for 1 hour at RT on a horizontal orbital microplate shaker set at 800±50 rpm.
6. Aspirate each well and wash with 300 µL Wash buffer for total four times. After the last wash, remove any remaining liquid by invert the plate and blot it with clean paper towels.
   NOTE: Complete removal of liquid at each step is essential to good performance.
7. Add 100 µL Detection Ab working solution to each well. Cover with a new transparent sealer and incubate for 40 minutes at RT on the shaker.
8. Repeat the aspiration/wash step as step 6.
9. Add 100 µL TMB solution to each well. Cover with the foil sealer and incubate for 20 minutes at RT on the shaker.
10. Add 50 µL Stop solution to each well. Incubate for 2 minutes on the shaker to mix thoroughly. The color in the well should change from blue to yellow.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm and set to 540 nm, 570 nm or 620 nm as reference wavelength.
12. Calculation of results :
    (i) Average the duplicate readings of each standard and specimen and subtract the average zero standard (Blank) optical density (O.D.)
    (ii) Construct the standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. Create a linear or cubic regression curve-fit standard curve using software of ELISA reader.
    NOTE:
    - For specimens with high O.D. value (higher than O.D. value of S1), a 2-fold to 5-fold dilution of specimen is recommended for measurement again.
    - If specimens have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.

Technical Hints

- When mixing or reconstituting protein solution, always avoid foaming.
- To avoid cross-contamination, change pipette tips between each well when add standards, specimens, and reagents and seal plates with sealer when incubation.
- Keep TMB solution (substrate) protected from light. TMB solution should remain colorless until added to the plate and change from colorless to gradient of blue.
- Stop solution should be added to the plate in the same order as the TMB solution and mixed thoroughly. The color developed in the wells will turn from blue to yellow upon addition of the Stop solution.

Limitation of the Procedure

- The test result does NOT replace an oral cancer screening or an oral cancer diagnosis.
- If the saliva specimen is so sticky, it may affect the test performance and/or produce invalid results.
- Do NOT test the saliva specimen under bleeding condition as it may cause a false positive (inaccurate) result.
- Oral medicine/spray may affect test performance and/or produce invalid results.
- Variation in saliva collection, processing, and storage may cause specimen value differences.
- The device is based on an immunochemical reaction. Patients receiving monoclonal antibody treatment may produce HAMA (human anti-mouse antibody), which may cause an invalid result.

Performance Characteristics

Sensitivity

The LoB (limit of blank) is 57.395 pg/mL; LoD (limit of detection) is 117.02 pg/mL; LoQ (limit of quantification) is 158.06 pg/mL

Five specimens pre-treated with immuno-precipitation by MMP-1 specific antibody were tested to estimate the LoB. Another five specimens at a concentration around 1~5 folds of LoB were tested to estimate the LoD. Another ten specimens at a concentration around LoD were tested to estimate the LoQ using functional sensitivity evaluation method.

Linearity

The linearity is 140.807~8000 pg/mL. The best polynomial regression model is Cubic regression.

Due to the broad range of MMP-1 concentration that can be found in clinical specimens, 0~60000 pg/mL, three sets of specimens (saliva specimen), which have concentration ranges overlapping with each other, were used to evaluate the linearity. The criterion for repeatability and non-linearity is less or equal to 5%.

Precision

According to the Multisite Precision Evaluation Study (3 sites x 5 days x 5 replicates), the %CV of the repeatability based on six samples (range of mean concentration: 181.58~2734.37 pg/mL) is 3.10~5.80%, with a mean of 4.22%; the %CV of within-laboratory precision is 7.40~9.20%, with a mean of 8.25%; the %CV of reproducibility is 7.70~9.70%, with a mean of 8.80%.

For low concentration sample, one sample (the mean concentration is 127.24 pg/mL, which is lower than LoQ) is evaluated following the same approach and shows %CV of the repeatability, within-laboratory precision, and reproducibility is 8.20%, 10.90%, and 11.50%, respectively.

Cross-Reactivity

No cross-reactivity of the MMP-1 ELISA assay with MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-13.

The commercial MMP-family recombinant proteins were tested at concentrations of 200 pg/mL, 1000 pg/mL, 5000 pg/mL, 25 ng/mL, and 1 µg/mL. All of the MMP-1 measuring levels were lower than the LoD (117.02 pg/mL) of the MMP-1 ELISA assay.

Interference

- Risk factors of oral cancer: According to the clinical study, risk factors of OSCC, including personal habits of betel nut chewing (former or current) and smoking (former or current), showed no interference with the detection of MMP-1[ref].

  * ref: Cancers 2020, 12, 2273; doi: 10.3390/cancers12082273.

- Common components in saliva: The MMP-1 levels were only correlated with total protein levels (Pearson's r = 0.361, p < 0.001) but not levels of IgA (Pearson's r = 0.029, p = 0.698) or alpha-amylase (Pearson's r = 0.021, p = 0.756), suggesting that the total salivary protein concentration might be an endogenous interfering factor, and the concentration of a single protein (amylase and immunoglobulin A) in it should not affect the detection of MMP-1 by this device. Differential total protein levels in saliva were due to individual differences, and this interference couldn't be avoided. When abnormal results occur in the test, whether it is affected by this factor can be considered.
- Specimen condition: The interference of blood was evaluated by hemoglobin (Hb) spike-in analysis using CLSI EP07-A2 as guideline. The dose effect evaluation indicated 6.86~22.77% increased MMP-1 under Hb interference. Blood contained in specimen is an interference which may cause a false positive result. BE SURE to collect saliva specimens following the instruction "Saliva collection should be avoided under bleeding condition. Do NOT use saliva that contains blood."
- External source substances: Dietary Substances (including protein residuals and common drinks), Mouthwash, and Oral medicine/spray (listed below) were added for testing. Each interfering substance was tested with 2 added concentrations, respectively added to 3 samples (blank sample, low concentration sample (~190 pg/mL), and high concentration sample (~1900 pg/mL)) and tested in duplication. The ratio (substances added/non-added)*100 is used to facilitate the interpretation. If the ratio is between 90~110, then it will be interpreted as "Non-affected". When the ratio is beyond 90~110, it will be interpreted as "Decrease" (<90), "Increase" (>110), or "Inconsistent" (when the ratio in duplication is inconsistent). Some dietary substances, mouthwash, and oral medicine caused inaccurate results (10% of the interfering substances were added) but the interference could be decreased or eliminated by additional dilution (0.1% of the interfering substances added). When the concentrations of the interfering substances were reduced to 0.01%, the test results were unaffected (with a dose-response), suggesting that these interferences could be avoided. In other words, sufficient mouth wash with drink water could decrease external source interference. BE SURE to collect saliva specimens following the instruction "Donors should NOT eat food, drink, and smoke in 60 minutes before collection. Rinse mouth 5 times with drink water (Never Use Mouthwash)". When the test results near the preset cut-off value,

careful confirmation must be made that the saliva sample was collected following the instructions.

No.	Interfering substances	10% of the interfering substances added	0.1% of the interfering substances added
01	Whole milk	Decrease at low conc.	Inconsistent at low conc.
02	Soy milk, sugar free	Decrease	Decrease at low conc./ Inconsistent at high conc.
03	Coffee, sugar free	Non-affected	Non-affected
04	Black tea, sugar free	Decrease	Decrease at low conc.
05	Juice (100% orange juice)	Decrease	Decrease at low conc.
06	Carbonated drink (sugary Coke)	Decrease	Decrease
07	Parmason Gargle Solution, green	Decrease	Increase at high conc.
08	LISTERINE Total care Mouthwash, crystal violet	Decrease	Inconsistent at low conc.
09	LISTERINE Original Mouthwash, caramel	Decrease	Non-affected
10	YADRAN Mouthwash, blue	Non-affected	Non-affected
11	Eudesmol, 0.092% w/v.	Decrease at low conc.	Non-affected
12	Xylitol, 0.09 g/mL	Inconsistent at low conc.	Non-affected
13	BECLOMET NASAL AQUA, including beclomethasone, 1.11 mg/mL.	Non-affected	Non-affected

14	CUFFLAM ANTI-INFLAMMATORY SPRAY, including Benzydamine, 1.5 mg/mL.	Increase	Non-affected
15	BETADINE® Throat Spray, including povidone-iodine, 0.45% w/v.	Decrease	Non-affected
16	TCHHTHUSUI, including Benzocaine, 0.067 gm/mL.	Decrease	Non-affected
17	Togiam, including polycresolsulfonate, 50%.	Decrease	Decrease
18	MUNDISAL GEL, including choline salicylate, 0.02 g/mL.	Non-affected	Non-affected
19	Oralog Orabase 1mg/g "Purzer", including triamcinolone acetonide, 0.01 g/mL.	Non-affected	Non-affected
20	Koulening Oralbase, including dexamethasone acetate, 0.02 g/mL.	Non-affected	Non-affected
21	CARBOXE ORABASE 20MG (CARBENOXOLONE) "SHITEH", including carbenoxolone, 0.01 g/mL.	Inconsistent	Non-affected
22	STREPSILS LOZENGE, including 2,4-dichlorobenzyl alcohol and amylmetacresol, 1 Tablet/5 mL.	Non-affected	Non-affected
23	Watermelon frost, including herbal extract mixture, 0.02 g/mL.	Decrease	Inconsistent at low conc./ Decrease at high conc.

Clinical validation

- **The clinical performance of EarlyFound® MMP-1 ELISA Kit was validated in 1031 subjects, including 327 OSCC patients and 704 OPMD patients.**

Disease condition / Test result	OSCC	OPMD	Sub-total
Test Positive MMP-1 ≥ 232.727 pg/mL	280	150	430
Test Negative MMP-1 < 232.727 pg/mL	47	554	601
Sub-total	327	704	1031 (Total)

Sensitivity: 85.6% (95% CI: 81.4%~89.0%)

Specificity: 78.7% (95% CI: 75.5%~81.6%)

PPV (Positive Predictive Value): 65.1% (95% CI: 60.4%~69.6%)

NPV (Negative Predictive Value): 92.2% (95% CI: 89.7%~94.2%)

Accuracy: 80.9% (95%CI: 78.4%~83.2%)

- **The sensitivity for patient population groups that be diagnosis with different OSCC stages were analyzed and listed below:**

Compare with OPMD (case number: 704), Cut-off: 232.727 pg/mL					
Group	case number	AUC ROC curve	95%CI of AUC	Sensitivity	Specificity
Stage I	59	0.715	0.636~0.794	61.0%	78.7%
Stage II	67	0.884	0.842~0.925	82.1%	78.7%
Stage III	43	0.941	0.906~0.976	90.7%	78.7%
Stage IV	138	0.948	0.925~0.971	95.7%	78.7%
Stage II-IV	248	0.93	0.910~0.949	91.1%	78.7%
Stage I-II	126	0.805	0.758~0.851	72.2%	78.7%
Stage III-IV	181	0.946	0.926~0.967	94.5%	78.7%

Symbols

IVD	In vitro diagnostic medical device	Σ	SUFFICIENT FOR<n>TESTS		DO NOT REUSE
	DO NOT USE IF PACKAGE IS DAMAGED		CAUTION		CONSULT INSTRUCTIONS FOR USE
REF	CATALOGUE NUMBER	LOT	BATCH CODE		USE BY DATE
	TEMPERATURE LIMITATION		MANUFACTURER		

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