Biodiagnostika
Oligonukleotide
Markierte Biomoleküle

BIOTEZ

ELISA MMP13 (Collagenase 3)

REF Cat. No.:30 518 101

For quantitative determination of human activated Collagenase 3

96 Determinations

2°C – 8 °C

For research use only
Not for diagnostic use

Contents

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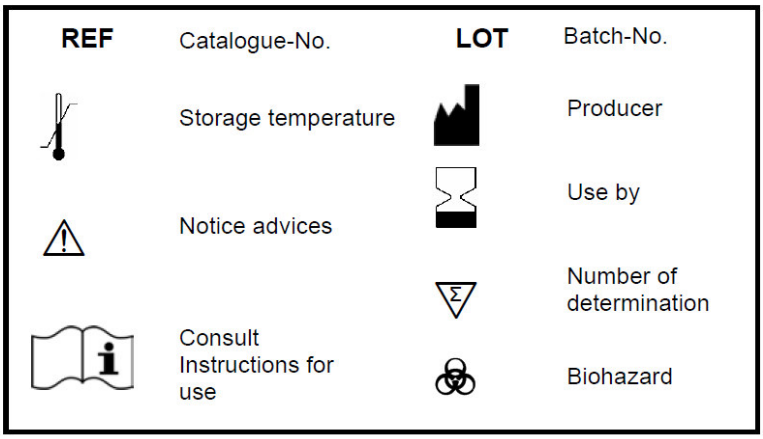
Short Review

Matrix Metalloproteinases (MMPs) comprise a family of secreted and membrane-bound endopeptidases which hydrolyse extracellular matrix proteins (1). Based on their preferred substrates and on structural features MMPs can be divided into collagenases, gelatinases, stromelysins and membrane-type matrix metalloproteinases. Their activity is tightly regulated by inhibitors (2).

Collagenase 3 (MMP-13) is a secreted 452 residue protein which is released from cells as an inactive zymogen and activated extracellularly by removal of its propeptide. Cleavage of the 84 residue propeptide can be catalysed by other MMPs such as MMP-2 and MMP-14, or by other proteases like plasmin (3).

Under normal conditions, MMP-13 is expressed during embryogenesis (fetal bone development) and is present only at very low levels in adult tissue. However, this enzyme is reported to be involved in the development and metastasis of breast and lung carcinomas, chondrosarcomas and osteosarcomas, head and neck carcinomas and some forms of skin cancer (4). Additionally, this enzyme plays an important role in degenerative bone diseases including osteoarthritis (5) and rheumatoid arthritis (6).

Our MMP-13 test kit enables the user to measure the concentration of this putative marker enzyme in body fluids such as serum and synovial fluid with high sensitivity. The ELISA MMP-13 is specific for the activated enzyme.



(1): Nagase, H. and Woessner, F. Jr. (1999) J. Biol. Chem. 274, 21491-21494.
(2): Brew, K. et al. (2000) Biochim Biophys. Acta 1477, 267.283.
(3): Knauper, V. et al. (1996) J. Biol. Chem. 271, 17124-17131.
(4): Pendas, AM. et al (2000) Clin. Chim. Acta 291, 137-155.
(5): Bluteau, G. et al. (2001) Biochim. Biophys. Acta 1526, 147-158
(6): Westhoff, CS. et al. (1999) Arthritis Rheum. 42, 1517-1527.

Intended Use

The **assay** system from BioTeZ Berlin-Buch GmbH provides a highly sensitive and specific quantitative determination of activated human MMP-13 in serum, synovial fluid, and cell culture supernatants.

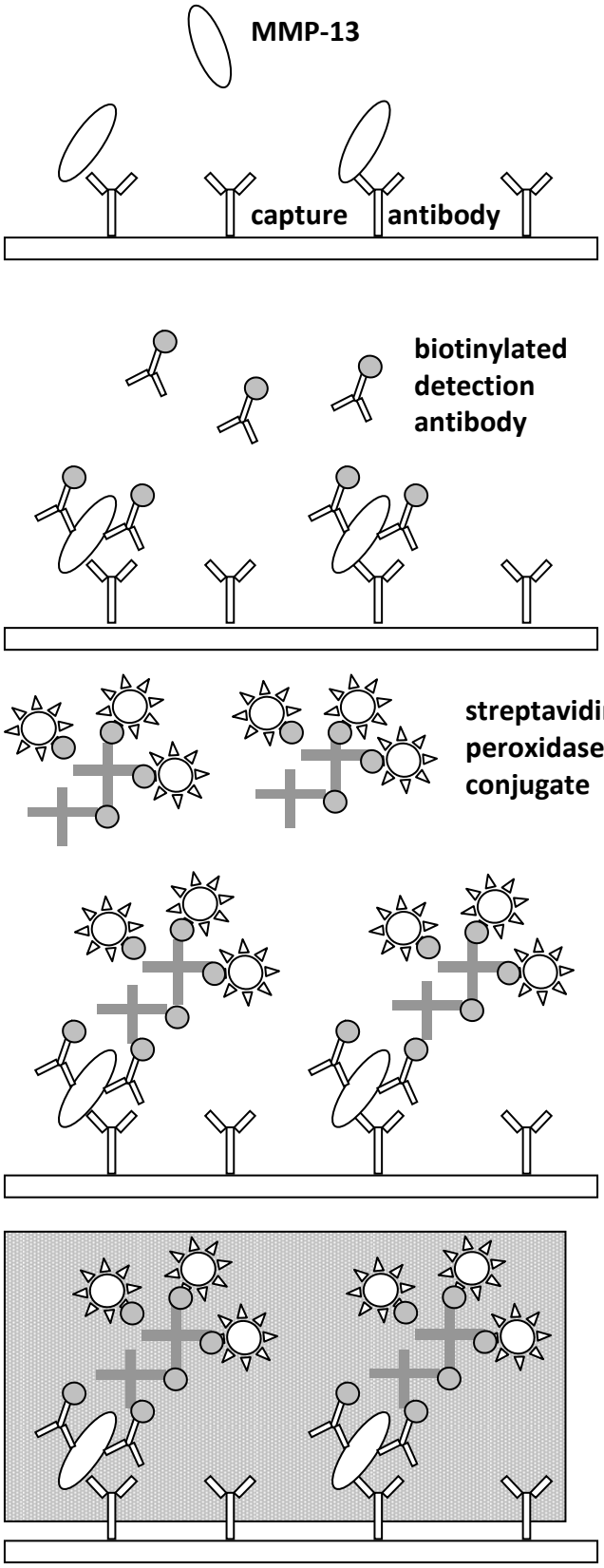
The calibration curve covers the range from 32 pg/ml to 2000 pg/ml. The sensitivity of the assay is 7 pg/ml.

This quantitative assay is based on a two site sandwich format. A highly specific monoclonal antibody for the activated form of MMP-13 is immobilised on the plate. MMP-13 will be bound to the wells, other components of the sample are removed by aspiration and washing of the plate. The analyte is detected in two steps using a secondary biotin-labeled antibody and a highly polymerised streptavidin-peroxidase conjugate. Any excess is removed by aspiration and washing after each detection step. The amount of peroxidase bound to each well is determined by the addition of TMB Substrate. The reaction is stopped by adding the Stop Solution, and the resultant colour is read in a microtiter plate reader at 450 nm. The concentration of activated MMP-13 in a sample is determined by interpolation from the standard curve.

One kit contains reagents for 96 determinations, thus allowing the measurement of one standard curve and 40 samples in duplicate.

For use in diagnostic procedures!

Principle of the assay



Step 1: Incubation of standard or sample on the microtiter plate. Specific binding of activated MMP-13
(duration: 120 minutes)

Step 2: Detection of bound MMP-13 with biotinylated antibody
(duration: 90 minutes)

Step 3: Addition of streptavidin-peroxidase conjugate
(duration: 30 minutes)

Step 4: Color development after addition of TMB Substrate
(duration: 15 minutes)

Fig. 1: Scheme of the assay procedure

Safety warning and precautions

Warning: Stop Solution contains 0.25 M sulphuric acid. Wear eye, hand, face, and clothing protection when using this material.
The Serum Standard Diluent contains human serum that has been tested negatively for HAV, HBV, HCV and HIV. Wear suitable protective clothing, such as laboratory overalls and gloves and observe caution when working with this material.
All chemicals should be considered as being 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 (P 280). Avoid contact with skin and eyes (H315, H319). In case of skin and eyes contact, wash immediately with water (P280, P302+P352, P305+P351+P338).

Storage

All components of the kit can be stored in the refrigerator (4-8 °C).
Once reconstituted, the standard solution should be used immediately or stored at -20 °C. Diluted biotinylated detection antibody and diluted conjugate solution should be prepared freshly directly before use. When running a partial plate, only suitable aliquots of these solutions should be made.

Components of the assay system

Microtiter plate: The plate contains 6 x 16 strips coated with anti-MMP-13.
Assay Buffer: Bottle contains 25 ml of a phosphate buffer with additives.
Wash Buffer Concentrate: Two bottles containing each 25 ml of a buffer concentrate.
Standard: The vial contains 20 ng of lyophilized act-MMP-13 in Assay Buffer. It has to be reconstituted with 1 ml of distilled water prior to use to get a 20 ng/ml stock solution.
Serum Standard Diluent: This solution (20 ml) contains certified human serum in a buffer with additives. Ready to use. **Warning :** Component contains human serum! Wear eye, hand, face, and clothing protection when using this material!
Detection Buffer: Solution (25 ml) consists of phosphate buffer containing additives.
Biotinylated Antibody: Solution (150 µl) contains biotinylated monoclonal anti-procollagenase 3 antibody in buffer with additives. The antibody has to be diluted 100-fold with Detection Buffer before use.
Conjugate Solution: This solution (300 µl) contains a highly polymeric streptavidin-peroxidase conjugate with preservatives. It has to be diluted 40-fold with Detection Buffer prior to use.
TMB Substrate: Bottle contains 12 ml of a TMB solution ready to use.
Stop Solution: Bottle contains 12 ml 0.25 M H_2SO_4. Ready to use. **Warning**: Stop Solution contains 0.25 M sulphuric acid. Wear eye, hand, face, and clothing protection when using this material!

EQUIPMENTS REQUIRED BUT NOT PROVIDED

- Pipettes with disposable tips (100 µl, 500 µl and 1 ml), a multi-channel pipette (100 - 200 µl) would be appropriate
- Distilled or deionised water
- Horizontal orbital microplate shaker
- Microplate reader capable of measuring at 450 nm
- Squirt bottle or automated microplate washer
- 500 ml graduated cylinder.

Sample preparation and storage

Serum:

- Serum samples may be stored at -80°C. When stored at -80°C, it is absolutely necessary to mix the samples thoroughly prior to measuring. Avoid freeze-thaw cycles.
- Dilute the serum samples 1:10 with Assay Buffer, depending on the possible concentration of the analyte. For measuring these dilutions use the **standards prepared with Serum Standard Diluent**!
- If you need samples with a dilution of more than 1:10, prepare first a 1:10 dilution with Assay Buffer and do further dilutions using Serum Standard Diluent. This is necessary to maintain a serum concentration of 10 % in the sample!

Synovial fluid:

- Centrifuge the collected synovial fluid for 15 min at 4,000 x g or higher. The supernatant can be stored at -80°C. When stored at -80°C, it is absolutely necessary to mix the samples thoroughly prior to measuring. Avoid freeze-thaw cycles.
- Dilute the samples 1:10 or more with Assay Buffer, depending on the possible concentration of the analyte. For measuring these samples, use the **standards prepared with Serum Standard Diluent!**
- If you need samples with a dilution of more than 1:10, prepare first a 1:10 dilution with Assay Buffer and do further dilutions using Serum Standard Diluent.
- Keep in mind that recovery of MMP 13 in synovial fluid is only 50 % or less (see Table 5)

Tissue culture supernatant

- Centrifuge the samples to remove any particles. The supernatants can be stored at -80°C. Avoid freeze-thaw cycles.
- Dilute the samples 1:3 or more with Assay Buffer, depending on the possible concentration of the analyte. For measuring these samples, use the **standards prepared with Assay Buffer**.

Please note: Fetal or neonatal calf serum may contain Collagenase 3! Always measure your culture media as background controls!

Limitation of the procedure

- Allow samples and all reagents to equilibrate to room temperature (20°C - 30°C) prior to performing the assay. This is especially a prerequisite for the TMB Substrate!
- For the highly sensitive determination of collagenase 3 in serum or synovial fluid samples, use the Serum Standard Diluent to prepare your standard curve.
- It is absolutely important that all wells are washed thoroughly and uniformly. When washing is done by hand, use a squeeze bottle and ensure that all wells are completely filled and emptied at each step.
- Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.
- A separate standard curve must be run on each plate.
- Mix all reagents thoroughly prior to use, but avoid foaming!
- Keep the wells sealed with the foil except when adding reagents and during reading.
- Any variation in the protocol can cause variation in binding!
- The kit should not be used beyond the expiration date on the kit label.

- The values obtained by the samples should be within the standard range. If this is not the case, dilute the sample and repeat the assay.
- We take great care to ensure that this product is suitable for all validated sample types, as designated in this manual However, it is possible that in some cases, high levels of interfering substances may cause unusual results.
- Take care that your samples do not contain EDTA or other chelating reagents. EDTA inhibits the assay!

Preparation of reagents

Please note: To prevent margin effects it is absolutely necessary to equilibrate all reagents **to room temperature (1 h incubation on the bench)** prior to use. For the reconstitution of the standard and for the dilution of the wash buffer concentrate use either distilled or deionised water. Always seal the plates with the provided foil during incubation!

Assay Buffer: After equilibration to room temperature, the buffer is ready to use.
Wash Buffer: Dilute the contents of a 25 ml bottle to 500 ml with distilled or deionised water. Make sure that the whole content of the bottle is used by repeated washing.
Standard: Add 1 ml distilled or deionised water to the standard tube (yellow lid) and allow the contents to dissolve for 30 minutes. Gently mix, but avoid foaming of the reagent!
Serum Standard Diluent: After equilibration to room temperature, the reagent is ready to use.
Detection Buffer: After equilibration to room temperature, the reagent is ready to use.
Biotinylated Antibody: Dilute Biotinylated Antibody 100-fold with Detection Buffer. For a whole plate add 120 µl from the antibody solution tube (red lid) to 12 ml Detection Buffer. When running half a plate, add 60 µl antibody solution to 6 ml Detection Buffer.
Conjugate Solution: Dilute the provided Conjugate Solution 40-fold with Detection Buffer.
For the whole plate, add 300 µl of the conjugate tube (blue lid) to 12 ml Detection Buffer. When running half a plate, add 150 µl of the conjugate to 6 ml of Detection Buffer.
Substrate and Stop Solution: After equilibration to room temperature, the reagents are ready to use.

Preparation of standards with Assay Buffer

(For tissue culture supernatants)

1. Label 7 tubes with 32, 63, 125, 250, 500, 1000, and 2000 pg/ml.
2. Pipette 900 µl of Assay Buffer into the 2000 pg/ml tube, in the remaining tubes pipette 500 µl of Assay Buffer.
3. Pipette 100 µl of the stock standard (20 ng/ml) into the 2000 pg/ml tube and mix thoroughly.
4. Pipette 500 µl of the 2000 pg/ml standard into the tube labelled with 1000 pg/ml and mix thoroughly.
5. Repeat this dilution procedure with the other standard tubes.
6. The blank value (0 pg/ml) is obtained by using simple Assay Buffer.
7. The stock solution is not part of the standard curve and can be stored at -20°C

Preparation of standards with Serum Standard Diluent

(For the highly sensitive measurement of serum and synovial fluid samples)

1. Label 7 tubes with 32, 63, 125, 250, 500, 1000, and 2000 pg/ml.
2. Pipette 900 µl of Serum Standard Diluent into the 2000 pg/ml tube, in the remaining tubes pipette 500 µl of Serum Standard Diluent.
3. Pipette 100 µl of the stock standard (20 ng/ml) into the 2000 pg/ml tube and mix thoroughly.

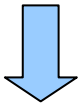
4. Pipette 500 µl of the 2000 pg/ml standard into the tube labelled with 1000 pg/ml and mix thoroughly.
5. Repeat this dilution procedure with the other standard tubes.
6. The blank value (0 pg/ml) is obtained by using Serum Standard Diluent.
7. The stock solution is not part of the standard curve and can be stored at -20°C.

Assay protocol

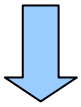
1. Prepare reagents and standards as described in the sections above. For assessing serum samples, prepare the standard using the Serum Standard Diluent. **Remind that it is necessary to equilibrate the reagents to room temperature before use**.
2. Prepare the unknown samples as described above by appropriate dilution with Assay Buffer.
3. Prepare the Microtiter plate by inserting the required amount of wells into the frame. **Note that you need 16 wells for the standard curve**.
4. Pipette 100 µl of the reconstituted standards in duplicate in the wells using a clean pipette tip for each standard. Assay Buffer serves as zero blank.
5. Pipette 100 µl of the prepared unknown samples in duplicate into the wells.
6. Seal the plate with the provided foil and incubate on a shaker at room temperature for exactly 120 minutes.
7. Aspirate and wash the wells 4 times with Wash Buffer. Take care that all wells are completely filled and emptied at each wash.
8. Blot the plate on paper towels to remove residual fluid from the plate.
9. Add 100 µl of diluted Biotinylated Antibody into each well.
10. Seal the plate and incubate on a shaker at room temperature for exactly 90 minutes.
11. Aspirate and wash the wells 4 times with Wash Buffer. Take care that all wells are completely filled and emptied at each wash.
12. Blot the plate on paper towels to remove residual fluid from the plate.
13. Add 100 µl of diluted Conjugate Solution into each well.
14. Seal the plate with the provided foil and incubate on a shaker at room temperature for exactly 30 minutes.
15. Aspirate and wash the wells 6 times with Wash Buffer. Take care that all wells are completely filled and emptied at each wash.
16. Blot the plate on paper towels to remove residual fluid from the plate.
17. Add 100 µl of Substrate Solution to each well.
18. Seal the plate with foil provided and incubate in the dark at room temperature for exactly 15 minutes.
19. Stop the reaction by adding 100 µl of Stop Solution to each well.
20. Read the plate at 450 nm (540, 570 or 620 nm reference filter) within 30 minutes. Reading of the plate without reference may yield higher absorbances and thus may be less accurate.

Protocol summary

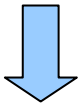
Prepare reagents, standards and samples as described, equilibrate reagents to **room temperature.**
For assessing human serum samples, prepare a
Serum Standard!



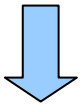
Pipette 100 µl standard or sample in duplicate into the wells. Incubate 120 minutes at room temperature on a shaker



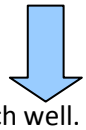
Aspirate and wash 4 times



Add 100 µl of diluted Biotinylated Antibody to each well. Incubate 90 minutes at room temperature on a shaker.

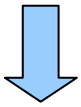


Aspirate and wash 4 times.

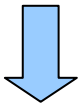


Add 100 µl of diluted Conjugate Solution to each well. Incubate for 30 minutes at room temperature on a shaker.

Aspirate and wash 6 times.



Add 100 µl of Substrate Solution to each well. Incubate for 15 minutes at room temperature **in the dark** without shaking!



Add 100 µl Stop Solution to each well. Read at 450 nm within 30 minutes (when possible with reference filter at 620 nm).

Data processing

Calculation of results

The calculation is illustrated using representative data: the assay data should be similar to that shown in table 1.

1. Calculate the average absorbance for each set of standard wells.
2. A standard curve is generated by plotting the mean absorbance (y axis) against pg/ml standard (x-axis, fig. 2).
3. The pg/ml values of the samples can be read directly from the graph or calculated by the regression coefficients.
4. Multiply the calculated pg/ml values by the dilution factor of the samples.

Standard (pg/ml)	Standard curve in Assay Buffer	
	Absorbance (450 nm)	Average
0	0.095 / 0.080	0.088
32	0.130 / 0.126	0.128
63	0.169 / 0.166	0.168
125	0.247 / 0.290	0.269
250	0.406 / 0.460	0.433
500	0.781 / 0.746	0.764
1000	1.419 / 1.373	1.396
2000	2.491 / 2.398	2.445

Table 1: Typical assay data

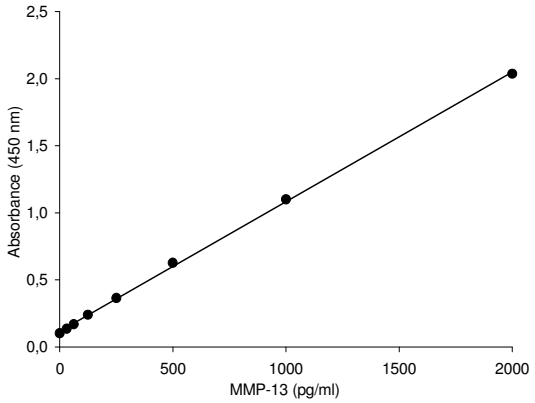


Fig. 2: Typical standard curve (prepared with Assay Buffer).

Performance characteristics

Specificity

The assay recognizes the activated form of collagenase 3. It does not cross-react with latent or activated forms of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and the catalytic domains of MT-1, MT-2, MT-3, MT-4, and MT-5 MMP. High concentrations of latent MMP 13 may contribute to OD-values measured with the assay. When present at equal concentrations latent MMP 13 yields about 1/10 the OD-value of active MMP 13.

Reproducibility

Within assay precision

The within assay precision was measured by assaying four control samples twenty times on one plate.

Control	Mean (pg/ml)	Standard deviation	CV (%)	N
1	1017	32	3.1	20
2	516	19	3.6	20
3	254	13	5.0	20
4	63	4.7	7.5	20

Table 2 A: Within assay precision in Assay Buffer (Precision in Serum Standard Diluent is similar).

Between assay precision

The between assay variation was measured by assaying four control samples 16 times on different plates.

Control	Mean (pg/ml)	Standard deviation	CV (%)	N
1	1000	48	4.8	16
2	490	25	5.1	16
3	242	16	6.6	16
4	68	4.7	7.0	16

Table 3: Between assay precision in Assay Buffer (Precision in Serum Standard Diluent is similar)

Precision profile

The precision profile was calculated as % CV from the mean and standard deviation of absorbance for each standard. The results for the determination in Assay Buffer are shown in table 4.

Standard (pg/ml)	% CV (Assay Buffer)	%CV (Serum Standard Diluent)	N
0	2.5	5.3	12
32	3.5	5.7	12
63	3.3	3.4	12
125	3.6	3.5	12
250	4.0	2.8	12
500	3.6	3.0	12
1000	1.3	2.3	12
2000	1.2	1.2	12

Table 4: Precision profile

Recovery

The recovery of MMP-13 standard spiked to levels throughout the range of the assay in various matrix types diluted down to 1:10 is shown in table 5.

Dilution factor	Measured (pg/ml)	Expected (pg/ml)	Measured:expected (%)
Serum (Standards in Serum Standard Diluent)			
1:10	351	--	--
1:20	165	176	94
1:40	85	88	97
1:80	42	44	95
1:160	26	22	118
Synovial fluid (Standards in Serum Standard Diluent)			
1:10	1449	--	--
1:20	863	725	119
1:40	474	363	131
1:80	237	182	130
1:160	127	91	140
Tissue culture media (Standards in Assay Buffer)			
1:3	479	--	--
1:6	226	240	94
1:12	113	120	94
1:24	61	60	102
1:48	38	30	127

Table 5: Recovery

Linearity

The following samples below were measured after dilution with Assay Buffer to assess linearity of the assay

Sample type	Added concentration (pg/ml)	Measured (pg/ml)	Recovery (%)
Serum	1000	939	94
	500	509	102
	250	243	97
	100	88	88
Synovial fluid	1000	382	38
	500	204	41
	250	124	50
	100	47	47
Tissue culture media	1000	939	94
	500	538	108
	250	236	94
	100	116	116

Table 6: Linearity

Sensitivity

The sensitivity, defined as two standard deviations above the concentration mean of the calculated concentrations of 48 blank replicates was determined. The sensitivity was determined as 7 pg/ml.

Troubleshooting

Problem	Potential cause	Recommendation
Low absorbance	• Wrong wavelength • Enzyme conjugate out of date/reagents improperly stored • Improper incubation time and temperature • Reagents not equilibrated to RT • Reagents not correctly prepared	• Check reader wavelength • Control the expiration date/storage conditions • Control the incubation time and temperature • Check equilibration of reagents to RT • Check preparation of reagents
High absorbance/ high zero standard value (>0.15 OD)	• Incomplete washing • Improper removing of residual fluid • Improper incubation time and temperature • Reagents not equilibrated to RT • Reagents not correctly prepared	• Ensure that every well is completely filled/emptied during each washing step • Check that plates are blotted on tissue paper after each washing step • Control the incubation time and temperature • Check equilibration of reagents to RT • Check preparation of reagents
Flat curve/poor reproducibility	• Wrong wavelength • Enzyme conjugate out of date/reagents improperly stored • Improper preparation of working standards • Pipette errors • Contamination of components by use of unclean reservoirs/used pipette tips • Margin effects by using of cold substrate solution • Washing incomplete	• Check wavelength • Control the expiration date/storage conditions • Check preparation of standards • Check pipette calibration • Use separate reservoirs and always new pipette tips • Equilibrate substrate to room temperature • Ensure sufficient washing procedure

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