Cat. # MK128

For Research Use

TaKaRa

Human Gla-Osteocalcin High Sensitive EIA Kit

Product Manual

v201607Da

Table of Contents

I. Description

Osteocalcin, also known as bone γ-carboxyglutamate protein, is a vitamin K-and vitamin D-dependent, calcium-binding, non-collagenous protein. It has a molecular weight of approximately 5,900 and contains two or three γ-carboxyglutamate residues (Gla). Osteocalcin is produced only by osteoblasts and their dental counterpart, odontoblasts, and is an indicator of bone metabolism. The carboxylated form of osteocalcin, in particular, is an indicator of bone formation.

The detection antibody in this kit, like that in its predecessor, the Gla-OC EIA Kit (Cat. #MK111) specifically recognizes the γ-carboxyglutamate (Gla) at position 17, which preferentially measures the Gla-type (active type) osteocalcin that binds to the bone matrix (primarily hydroxyapatite). Further, both kits may be used to measure osteocalcin in human blood.

However, this kit is designed to precisely differentiate bovine and human osteocalcins, using a capture antibody-coated plate with a human osteocalcin-specific monoclonal antibody that recognizes the distinct difference in the amino acids at positions 3 and 4 from the N-terminus. This makes possible differential assays of human and bovine osteocalcins. (See Figure 1 for a comparison of osteocalcin amino acid sequence among different animal species.) Use of human antigen-specific antibody as a capture monoclonal antibody provides improved linearity when assaying human blood samples.

This specificity also enables direct assay of human osteocalcin in the culture supernatant of osteoblasts or differentiated osteoblasts from bone marrow or mesenchymal stem cells cultured in a bovine serum-containing medium, which has been difficult to achieve using the conventional Gla-OC EIA Kit (Cat. #MK111).

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                    10        20        30        40        50
Human     YLYQWLGAPV PYPDPLEPRR EVCELNPDCD ELADHIGFQE AYRRFYGP-V
Bovine    YLDHWLGAPA PYPDPLEPKR EVCELNPDCD ELADHIGFQE AYRRFYGP-V
Rat       YLNNGLGAPA PYPDPLEPHR EVCELNPNCD ELADHIGFQD AYKRIYGTTV
Mouse     YL----GASV PSPDPLEPTR EQCELNPACD ELSDQYGLKT AYKRIYGITI
Chicken   YAQDSGVAGA P-PNPLEAQR EVCELSPDCD ELADQIGFQE AYRRFYGP-V
Monkey    YLYQWLGAPA PYPDPLEPKR EVCELNPDCD ELADHIGFQE AYRRFYGP-V
Pig       YLDHGLGAPA PYPDPLEPRR EVCELNPDCD ELADHIGFQE AYRRFYGI-A
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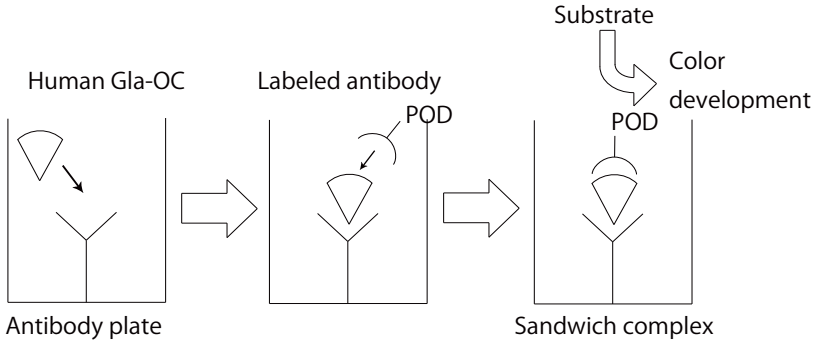
Figure 1. Primary amino acid structure of osteocalcin in various animals

II. Principle



III. Components

(1) Antibody Coated Microtiter plate Anti-Human OC Specific Monoclonal Antibody (96 well：8 wells x 12 strips)	1 plate
(2) Antibody-POD Conjugate (lyophilized) Peroxidase-labeled anti-GC monoclonal antibody	for 11 ml
(3) Standard (lyophilized) Glycocalicin-containing standard 640 ng	for 1 ml
(4) Sample Diluent 25% BlockAce-containing PBS (with preservative)	11 ml x 2
(5) Substrate Solution (TMBZ) 3,3',5,5'-Tetramethylbenzidine solution	12 ml

IV. Materials Required but not Provided

- Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)
  Contains wash solution (10X PBS, 50 ml x 5 tubes; Tween 20, 3 ml) and reaction stop solution (60 ml).
  - ＊ This product is a stop solution for peroxidase reactions without 1N sulfuric acid.
  - ＊ 1N sulfuric acid can be used as a stop solution. Handle 1N sulfuric acid with caution.
- Pipette, micropipette, and tips
- Microplate reader (capable of measuring absorbance of up to 3.5 when set to 450 nm)

V. Storage

4℃

VI. Intended Use

To assay a minute amount of Gla-osteocalcin in the supernatant of human cell cultures or in human biological samples.

VII. Protocol

1. Sample

- Suitable samples include cell culture supernatants and extracts, and human serum and ascites.
- Samples may be stored up to 12 hours at 4℃. If the assay will be performed longer than 12 hours after sample preparation, then store samples frozen at -20℃.
- Use Sample Diluent (4) for dilution if necessary.
- In the case of blood samples, it is recommended to use serum.
- Avoid using EDTA plasma.
- The recommended dilution for human serum samples is from 2- to 10-fold.

2. Preparation of Solutions

- Antibody Coated Microtiter Plate
  Allow the (1) Anti-Human OC Specific Monoclonal Antibody plate to reach room temperature unopened in its package before use.
- POD-labeled Antibody Solution
  Reconstitute (2) Antibody - POD Conjugate with 11 ml of distilled water.
  Once reconstituted, it is stable for up to 1 week at 4℃. For longer storage, freeze at -20℃, at which it is stable for up to 1 month. Once thawed, it may not be returned to frozen storage.
- Human Gla-OC Standard Solution
  Add 1 ml of distilled water to the (3) Human Gla-OC standard to reconstitute the standard (12.0 ng/ml). Dilute it with (4) Sample Diluent before use to prepare fresh serial dilutions of Standard Solution at concentrations of 12.0, 6.0, 3.0, 1.5, 0.75, 0.375, and 0.1875 ng/ml. Use Sample Diluent as the 0-concentration standard.
  The Gla-OC Standard Solution (12.0 ng/ml) is stable for up to 1 week after preparation when stored at 4℃, or for up to 1 month at -20℃.
- Substrate Solution
  Return (5) Substrate Solution (TMBZ) to room temperature before use. It is supplied ready to use. Check before use that the Substrate Solution has not developed a dark blue color. A reaction with metal ions will result in coloration; make sure it is not contaminated with any tap water.
  If the Substrate Solution will be used for several assays, divide it into aliquots of the required volume in advance.
- Stop solution
  Use the Stop solution included in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) directly.
  ＊ Because this is highly viscous, mix well using a plate mixer after its introduction.
- PBS with 0.1% Tween 20 for washing
  Dilute the 10X PBS included in Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021) 10 fold with distilled water, and then add Tween 20 to a final concentration of 0.1%.
  For 96 reactions performed with this kit, 300 ml of washing solution is required.

3. Procedure

Assay samples in duplicate.
Allow reagents in the kit and samples to return to room temperature and make sure before use that solutions are mixed uniformly without creating bubbles.

Note: Prepare reagents and samples in a separate 96 well plate in advance so that they can be added to the antibody-plate quickly (within 5 minutes) using an 8-channel pipette or similar apparatus.

1. Add prepared samples (100 μl/well) to the Antibody Coated Plate. In order to provide highly reliable results, it is recommended to place serial dilutions of the Standard Solution (100 μl/well) in the 1st and 12th rows. Perform this reaction for 2 hours at room temperature (20 - 30℃); incubation at 37℃ may compromise antigenicity. [First reaction].
2. Remove reaction mixtures from wells, discarding the liquid. Wash wells 3 times with Washing Buffer (100 μl/well). Remove excess Washing Buffer and then add 100 μl of the Labeled Antibody Solution per well using an 8-channel pipette and allow to react for 1 hour at room temperature (20 - 30℃). [Second reaction]
3. Remove reaction mixtures from wells, discarding the liquid. Wash wells 4 times with Washing Buffer (100 μl/well). Remove excess Washing Buffer and then add 100 μl of (5) Substrate Solution (TMBZ) per well using an 8-channel pipette and allow to react for 10 - 15 minutes at room temperature (20 - 30℃). [Third reaction]
4. Add 100 μl of Stop Solution to each well in the same order as for (5) Substrate Solution (TMBZ) to stop the reaction. Then mix well.
5. To make a zero adjustment, use distilled water as a blank and measure absorbance at 450 nm. The color is stable for up to 1 hour after reaction termination.
6. Plot a standard curve based on the results obtained from the Standard Solutions (with concentration as x-axis and absorbance as y-axis) and use it to determine the corresponding concentrations of Human Gla-OC based on the sample' s absorbance.

Note:

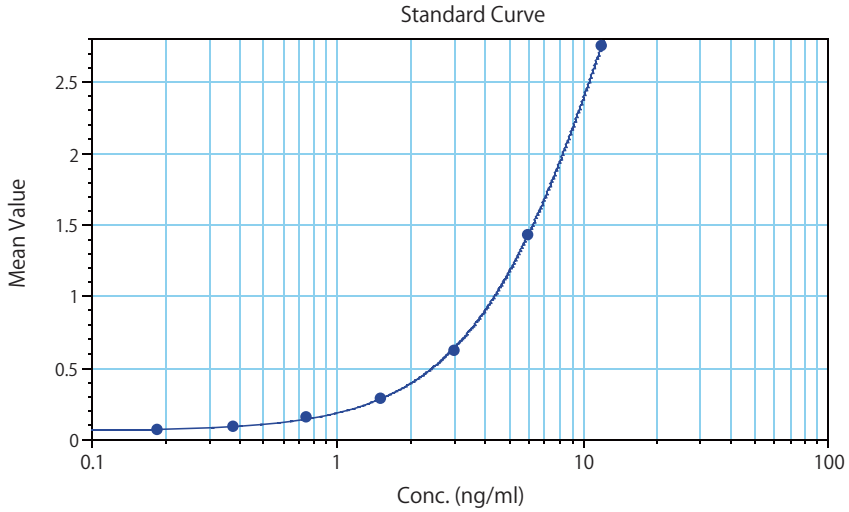
- Cover the plate with film or the like to prevent evaporation of solutions during reactions at room temperature or in an incubator.
- It is recommended that the Washing Buffer be completely discarded to get rid of the residual fluid.

VIII. Performance

1. Standard curve (Human Gla-OC High Sensitive EIA Kit)

The following shows a typical standard curve of this kit as an example.
The standard curve for calculation needs to be established in each assay.

Limit of detection: 0.2 ng/ml



4-Parameter Fit : $y = (A - D)/(1 + (x/C)^B) + D$:

A	B	C	D	R^2
0.0546	1.47	13.7	6.04	1

Human Gla-OC concentration (ng/ml)	12.0	6.0	3.0	1.5	0.75	0.375	0.187	0.0
A_{450}	2.759	1.433	0.621	0.282	0.151	0.089	0.067	0.040

Color development: 15 min

2. Reproducibility

<Intra-assay precision test (n = 16)>

A reproducibility test was performed using 3 different concentrations of control prepared from dilutions of human serum.

Sample	Mean (ng/ml)	CV (%)
Control A	7.786	2.0
Control B	4.454	2.4
Control C	2.308	3.4

<Inter-assay precision test (n = 3)>

A reproducibility test was performed by assaying 3 different concentrations of control over 3 days.

Sample	Mean (ng/ml)	CV (%)
Control A	7.282	6.2
Control B	4.344	4.7
Control C	2.417	4.2

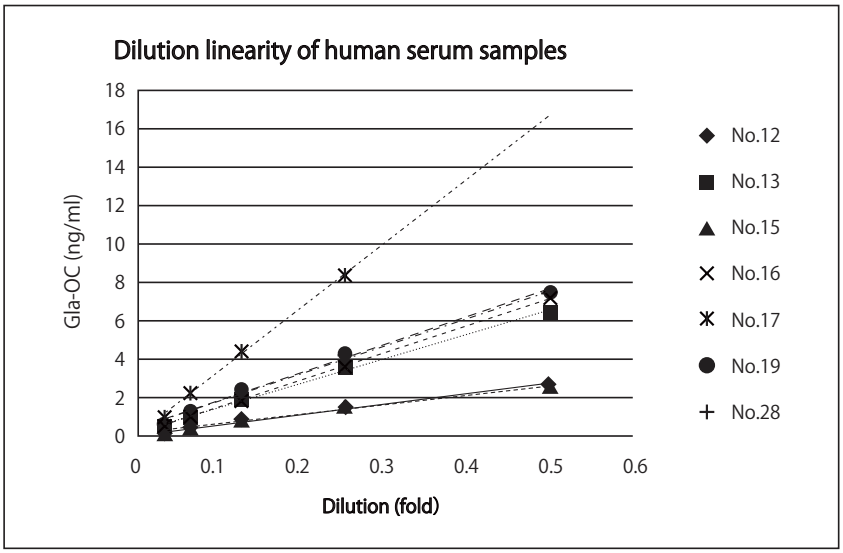
3. Recovery test

Equal volume of samples in different concentrations were combined and assayed. The assay result of each mixture was compared with the theoretical value to determine the recovery rate.

Sample A	Sample B	A + B (Assay Result)	A + B (Theoretical Value)	Recovery Rate %
6.297	3.767	5.151	5.032	102.36
6.297	2.626	4.512	4.462	101.13
6.297	2.041	4.380	4.169	105.06
6.297	1.556	4.269	3.927	108.72
6.297	0.800	4.133	3.549	116.47
3.767	2.626	3.208	3.197	100.36
3.767	2.041	2.914	2.904	100.34
3.767	1.556	2.669	2.662	100.28
2.626	1.556	2.104	2.091	100.62
2.041	2.626	2.295	2.334	98.35
2.041	1.556	1.795	1.799	99.81
2.041	0.800	1.511	1.421	106.37
1.556	0.800	1.179	1.178	100.08

(n=13, Unit: ng / ml)

4. Linearity of human serum



No.12	$y = 5.4002x + 0.1109$	$r^2 = 0.9945$
No.13	$y = 12.597x + 0.2739$	$r^2 = 0.9959$
No.15	$y = 5.0061x + 0.1529$	$r^2 = 0.9835$
No.16	$y = 13.974x + 0.1739$	$r^2 = 0.9998$
No.17	$y = 33.067x + 0.1665$	$r^2 = 0.9983$
No.19	$y = 14.349x + 0.4821$	$r^2 = 0.9932$
No.28	$y = 14.307x + 0.463$	$r^2 = 0.9961$

5. Effects of freeze-thawing on samples

We studied the effect of freeze-thaws on the concentration of Human Gla-OC by assaying concurrently human serum samples that have undergone repeated cycles of freeze-thaw and samples thawed for the first time.

Human serum	Post freeze-thaw	First thaw
No. 15, 2-fold dilution	2.552	2.404
No. 17, 4-fold dilution	7.289	7.360
No. 19, 2-fold dilution	6.429	6.633
No. 28, 2-fold dilution	6.145	6.354

(n = 2; unit: ng/ml)

Result: Freeze-thawing of samples tended to result in a slight decrease in assay results.

6. Cross reactivity with various animal serum samples

	Goose	Dove	Chicken	Turkey
2-Fold dilution	ND	ND	ND	ND
4-Fold dilution	ND	ND	ND	ND

	Bovine	Guinea pig	Rat	Pig
2-Fold dilution	ND	ND	ND	ND
4-Fold dilution	ND	ND	ND	ND

ND: not detectable

	Cynomolgus monkey		Rhesus monkey	
	Serum No. 1 (2-yr-old male)	Serum No. 2 (2-yr-old male)	Serum No. 1 (3-yr-old female)	Serum No. 2 (3-yr-old female)
2-Fold dilution	4.071	4.000	4.062	4.045
4-Fold dilution	3.838	3.970	3.831	3.850

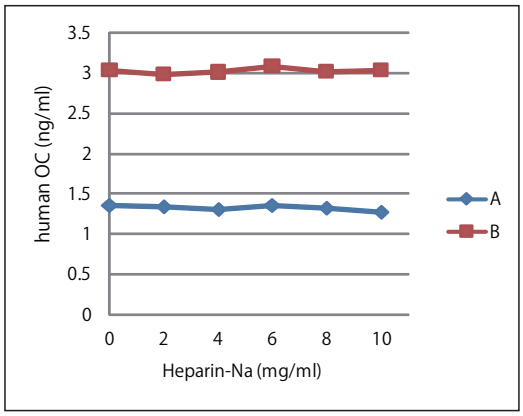
Absorbance at 450nm
ND: not detectable

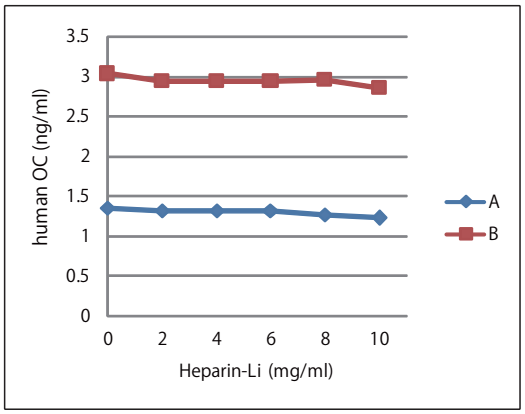
Result: This product may be used to assay monkey serum samples in addition to those of humans.

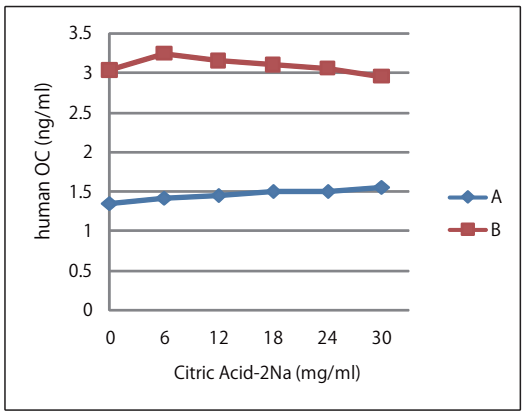
7. Effects of coexisting substances in samples

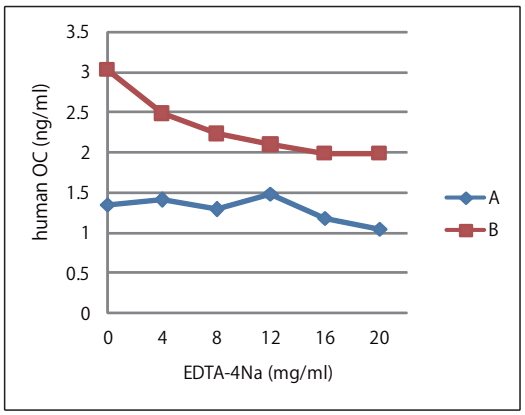
Each sample was combined with a test material at a ratio of 9 to 1 to investigate the effect on the reaction system.

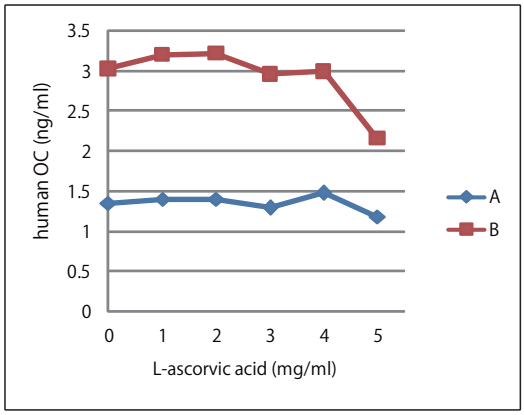
Test material concentrations shown in the graphs are the final concentration.

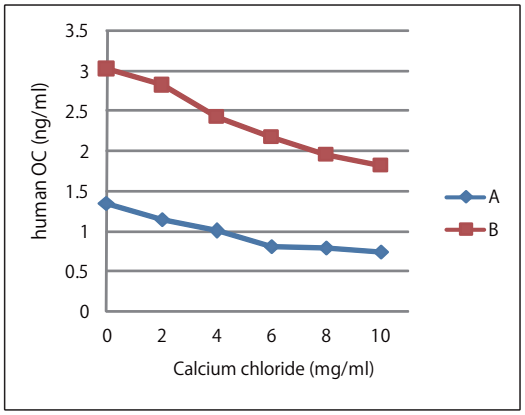






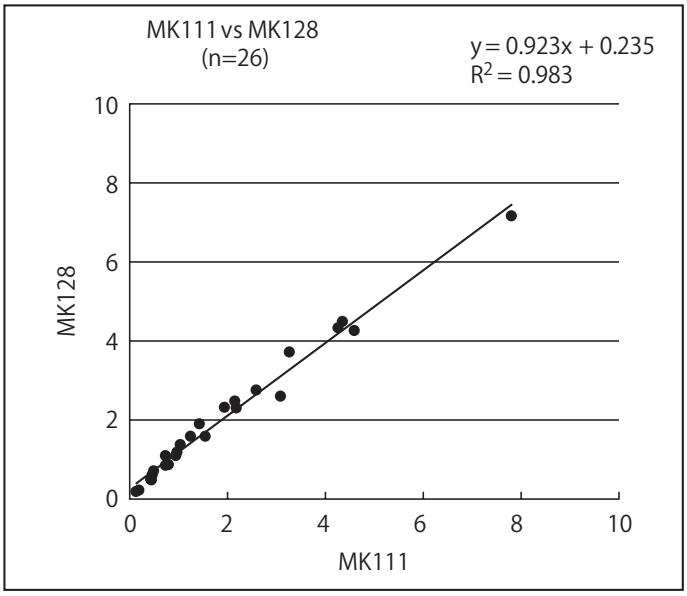






Result : EDTA 4Na and calcium chloride show an interfering tendency; care should be paid to avoid introduction of these substances into samples

8. Correlation with Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111)



Result: The results of the two kits were nearly equivalent with each other.

9. Selective use of kits when assaying Gla-osteocalcin

Based on the cross-reactivities and performances on various animal specimens, Takara Bio recommends that you select this kit or the conventional kit (Cat. #MK111) based on the assay sample.

Cat. #	Measuring range	Blood			Urine*	Human cell culture supernatant	
MK128 Human Gla-Osteocalcin High Sensitive EIA Kit	0.2 - 12 ng/ml	Human	Monkey	Bovine, rabbit, dog, sheep, goat	×	Bovine serum-containing medium	Serum-free medium
		◎	○	×		◎	○
MK111 Gla-Type Osteocalcin EIA Kit	0.5 - 16 ng/ml	Human	Monkey	Bovine, rabbit, dog, sheep, goat	×	Bovine serum-containing medium	Serum-free medium
		○	○	○		×	○

◎ : Recommended ○ : Usable × : Do not use

＊ : With the epitope regions of two antibodies located apart from each other, fragmented Gla-OC in urine samples cannot be detected.

IX. Experimental Examples

Induction of osteocalcin-production in human osteosarcoma cells

Human osteosarcoma cell line MG63 was cultured in RPMI1640 medium supplemented with 10% bovine serum and osteoblast differentiation was induced. The culture supernatant was sampled at various time points to measure the level of Gla-OC. In addition, supernatant samples from non-induced MG63 cell cultures were assayed as a control.

Days of culture	Induction	Control
1	0.055	0.049
2	0.050	0.049
3	0.050	0.049
7	0.063	0.062
10	0.146	0.101
15	0.307	0.118

Absorbance at 450 nm

Result: The specific assay of Gla-OC in the culture supernatant from bovine serum-containing medium was successfully achieved using this kit. Human osteocalcin was measured despite its presence at minute levels.
Because the conventional Gla-Type Osteocalcin (Gla-OC) EIA Kit (Cat. #MK111) also detects bovine osteocalcin, the medium used in this experiment (containing 10% bovine serum) would give an assay result of 1 to 5 ng/ml, making it difficult to assay a minute level of human osteocalcin in such a medium.

X. Precautions

1. Do not mix/use kits or reagents from different lots.
2. Do not expose reagents to strong light during storage or reactions.
3. Use pipettes free of metal when handling (5) Substrate Solution (TMBZ).
4. Exercise care to prevent (5) Substrate Solution (TMBZ) from coming into contact with hands or mucous membranes.
5. Do not use (5) Substrate Solution (TMBZ) that has developed color.
6. Each reaction varies subject to length of time and temperature. Therefore, a new standard curve must be generated for each assay.
7. Handle blood samples with great care.