DiaSource®

Free 25OH Vitamin D ELISA

KARF1991

DIAsource ImmunoAssays S.A. - Rue du Bosquet, 2 - B - 1348 Louvain-la-Neuve – Belgium

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Revision date: 29/03/2024

History

Summary of change:

<table>
<tr><th>Previous Version:

230123</th><th>Current Version:

240329</th></tr>
<tr><td>V. REAGENTS PROVIDED

<table>
<tr><td>[WASH|TABLET]
PBS-Tween wash buffer tablet</td><td>2 tablets
Lyophilized</td><td>Brown</td><td>Dissolve 1 tablet in 500 ml distilled water</td></tr>
</table>
</td><td>V. REAGENTS PROVIDED

Removal of the column relative to the color code

<table>
<tr><td>[WASH SOLN|CONC]
Wash solution (TRIS-HCl)</td><td>1 vial
10 ml</td><td>Brown</td><td>Dilute 200x with distilled water. Use a magnetic stirrer.</td></tr>
</table>
</td></tr>
<tr><td>VI. SUPPLIES NOT PROVIDED</td><td>VI. SUPPLIES NOT PROVIDED

Addition of a magnetic agitator</td></tr>
<tr><td>VII. REAGENT PREPARATION

C. Working Wash Solution: according to the number of strips used, dissolve 1 wash buffer tablet in 500 ml distilled water, or 2 wash buffer tablets in 1L distilled water. Ensure that all the salt crystals are dissolved.</td><td>VIII. REAGENT PREPARATION

C. Working Wash Solution: Prepare an adequate volume of working wash solution by adding 199 volumes of distilled water to one volume of 200x concentrated Wash solution.
Use a magnetic stirrer to homogenize. Dispose of unused working wash solution at the end of the day.</td></tr>
<tr><td>VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

After reconstitution, wash buffer is stable for two weeks at room temperature

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<tr><td>Anti-Vitamin D coated plate</td><td>Maximum of 2 weeks at 2-8 °C</td></tr>
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<tr><td>Streptavidin-HRP reagent</td><td>Maximum of 2 weeks at 2-8 °C</td></tr>
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<tr><td>PBS-Tween wash buffer (reconstituted tablet)</td><td>Maximum of 2 weeks at room temperature</td></tr>
<tr><td>Chromogenic solution TMB</td><td>Maximum of 2 weeks at 2-8 °C</td></tr>
<tr><td>Stop reagent</td><td>Maximum of 2 weeks at 2-8 °C</td></tr>
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Unused working wash solution should be disposed of at the end of the day.

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<tr><td>XIII. PERFORMANCE AND LIMITATIONS

A. Limits of detection

The Limit of Blank (LoB) and the Limit of Detection (LoD) were determined in accordance with the CLSI guideline EP17-A2.
The LoB was calculated to be 1.5 pg/ml.
The LoD was calculated to be 2.4 pg/ml.

C. Precision</td><td>XIII. PERFORMANCE AND LIMITATIONS

A. Limits of detection

The Limit of Blank (LoB) and the Limit of Detection (LoD) were determined by converting mOD into pg/ml values with standard curve equation.
The LoB was calculated to be 1.86 pg/ml.
The LoD was calculated to be 2.38 pg/ml.

C. Precision</td></tr>
</table>

Intermediate Precision and repeatability was determined based on CLSI EP05-A3.

Sample	N	pg/ml		Repeatability (within run)	Intermediate Precision(Total)
Pool 1	80	5.8	SD	0.29	0.34
			CV	4.9%	5.9%
Pool 2	80	9.6	SD	0.53	0.59
			CV	5.5%	6.1%
Pool 3	80	18.4	SD	0.35	0.75
			CV	1.9%	4.0%
Pool 4	80	28.1	SD	1.26	1.76
			CV	4.5%	6.3%

SD: Standard Deviation; CV: Coefficient of Variation

D. Time delay

The time delay test between the last Calibrator and sample dispensing results is shown in the following table.

TIME DELAY				
	0 min (pg/ml)	5 min (pg/ml)	10 min (pg/ml)	15 min (pg/ml)
Sample 1	4.9	4.9	4.9	4.8
Sample 2	18.6	18.4	18.6	18.6

Assay results remain accurate even when a sample is dispensed 15 minutes after the Calibrator has been added in the coated wells.

Reproducibility and repeatability precisions were evaluated with two samples at medium and high concentrations. Their concentrations were measured in 10 different runs in duplicate for reproducibility and in a single run for repeatability precision.

Sample	N	pg/ml		Reproducibility Precision (Total runs)
Sample 1	20	8.22	SD	0.45
			CV	5.5%
Sample 2	20	29.42	SD	3.00
			CV	10.2%

SD: Standard Deviation; CV: Coefficient of Variation

Sample	N	pg/ml		Repeatability Precision (within a run)
Sample 1	12	9.02	SD	0.32
			CV	3.5%
Sample 2	12	32.38	SD	0.64
			CV	2.0%

SD: Standard Deviation; CV: Coefficient of Variation

D. Time delay

The time delay test between the last Calibrator and sample dispensing results is shown in the following table.

TIME DELAY					
	0 min (pg/ml)	5 min (pg/ml)	10 min (pg/ml)	15 min (pg/ml)	20 min (pg/ml)
Sample 1	5.60	5.68	5.70	5.46	5.71
Sample 2	13.17	14.70	13.07	14.27	13.38

Assay results remain accurate even when a sample is dispensed 20 minutes after the Calibrator has been added in the coated wells.

Read entire protocol before use.

Free 25OH Vitamin D ELISA

I. INTENDED USE

The DIAsource Free 25OH Vitamin D ELISA is a medical device intended to be used by professionals for the quantitative measurement of free 25OH Vitamin D in human serum.
For research use only. Not for use in diagnostic procedures.

II. GENERAL INFORMATION

A. Proprietary name : DIAsource Free 25OH Vitamin D ELISA Kit

B. Catalog number : KARF1991 : 96 tests

C. Manufactured by : DIAsource ImmunoAssays S.A.
Rue du Bosquet, 2 B-1348 Louvain-la Neuve, Belgium.

For technical assistance or ordering information contact :
Tel : +32 (0)10 84 99 11 Fax : +32 (0)10 84 99 90

III. BACKGROUND

Due to its hydrophobic nature 25OH Vitamin D, and other Vitamin D metabolites, circulate on binding proteins. About 90% of the total circulating 25OH Vitamin D is bound to the so-called VDBP or DBP. The remaining 10% are bound to albumin, the main protein of human blood plasma. Although the affinity of albumin toward 25OH Vitamin D is much lower than the affinity of VDBP, the high concentration of albumin compensates for this difference. A tiny fraction representing 0.04% of the total 25OH Vitamin D concentration circulates as the free form.

The conversion of 25OH Vitamin D into the biologically active $1,25(OH)_2$ Vitamin D takes place into the cells and so requires the internalization of 25OH Vitamin D from the extracellular fluid. Different transport mechanisms are likely to be involved and some of them involve the concentration of the free ligand as one of the important parameters. In these cases, the fraction of free 25OH Vitamin D relates to the biological activity of Vitamin D, and therefore may better reflect the physiological action of Vitamin D than the total concentration of 25OH Vitamin D.

IV. PRINCIPLES OF THE METHOD

The Free 25OH Vitamin D ELISA is based on a two-step immunoassay procedure performed in a microtiter plate. During the first incubation step, free 25OH Vitamin D [25(OH)Vitamin D2 and D3] is bound to the anti-Vitamin D antibody coated on the well of the microtiter plate. The in vivo equilibrium between free and bound 25OH Vitamin D is minimally disturbed. After washing, a fixed amount of biotinylated 25OH Vitamin D is added to each well. The non-bound biotinylated 25OH Vitamin D is removed by washing and a streptavidin peroxidase conjugate is added. In a next step TMB chromogenic substrate is added. Finally, the reaction is stopped by addition of stop reagent and the absorbance [A450nm] is measured using a plate spectrophotometer. The concentration of free 25OH Vitamin D (pg/ml) in the sample is inversely proportional to the absorbance in each sample well.

V. REAGENTS PROVIDED

Reagents	96 Tests Kit	Reconstitution
Anti-Vitamin D coated plate Microtiter plate coated with a mouse anti-25OH D2/D3 monoclonal antibody.	96 wells	**Ready** for use
[DIL|SPE] Sample diluent: Specimen diluent, containing a fluorosurfactant and Proclin.	1 vial 14 ml	**Ready** for use
[Ag|BIOT|CONC] 100x Biot-Vit D reagent Biotinylated 25OH Vitamin D in preservation buffer.	1 vial 250 µl	**Dilute** 100 x with Biot-Vit D dilution buffer
[SAV|HRP] Streptavidin-HRP reagent Peroxidase conjugated streptavidin, containing Proclin.	1 vial 14 ml	**Ready** for use
[CAL|N] Calibrators ; N = 1 to 6 Lyophilized 25OH Vitamin D depleted human serum containing Proclin and BND. **(see exact value on vial label)**	6 vials Lyophilized	**Add** 250 µl distilled water
[CONTROL|1] Control 1 Normal human serum containing Proclin and BND. **(see exact value on vial label)**	1 vial Lyophilized	**Add** 250 µl distilled water
[CONTROL|2] Control 2 25OH Vitamin D spiked human serum containing Proclin and BND **(see exact value on vial label)**	1 vial Lyophilized	**Add** 250 µl distilled water
[WASH SOLN|CONC] Wash solution (TRIS-HCl)	1 vial 10 ml	**Dilute 200x with distilled water** Use a magnetic stirrer
[CHROM|TMB] Chromogenic solution TMB (Tetramethylbenzydine)	1 vial 12 ml	**Ready** for use
[STOP|SOLN] Stop reagent 1M HCl.	1 vial 12 ml	**Ready** for use
[Ag|BIOT|SOLN] Biot-Vit D dilution buffer Buffer containing Proclin and BND	1 vial 14.5 ml	**Ready** for use

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:

A. Distilled water
B. Pipettes calibrated for delivery of 10 µl, 35 µl - 250 µl, 3500 µl - 5000 µl (the use of accurate pipettes with disposable plastic tips is recommended).
C. Vortex mixer
D. Magnetic agitator
E. Plate shaker incubator at 37 °C and 650 rpm
F. Washer for microplates
G. Microtiter plate reader capable of reading at 450 nm and 650 (bichromatic reading)

VII. REAGENT PREPARATION

Bring all reagents to room temperature (18-25°C) at least 30 minutes before use.

A. **Calibrators:** reconstitute the Calibrators with 250 µl distilled water and let them stand for 15 minutes at RT (18-25°C). After reconstitution, mix well and make sure that all lyophilized material has been reconstituted.
B. **Controls:** reconstitute the Controls with 250 µl distilled water. Let them stand for 15 minutes at RT (18-25°C). After reconstitution, mix well and make sure that all lyophilized material has been reconstituted.
C. **Working Wash Solution:** Prepare an adequate volume of working wash solution by adding 199 volumes of distilled water to one volume of 200x concentrated Wash solution.
Use a magnetic stirrer to homogenize. Dispose of unused working wash solution at the end of the day.
D. **Working Biot-Vit D solution:** prepare an adequate volume of working dilution of the Biot-Vit D reagent by mixing 100x concentrated Biot-Vit D reagent with Biot-Vit D dilution buffer according to the number of strips used, as indicated in the table below: for example, for 6 strips (48 wells): 60 µl 100x concentrated Biot-Vit D reagent is added to 6.0 ml Biot-Vit D dilution buffer.
Use an appropriate polypropylene container for preparation.
The preparation of working Biot-Vit D solution is not stable and must be discarded if not used.

No. Strips	100x Biot-Vit D reagent (µl)	Biot-Vit D dilution buffer (ml)
3	35	3.5
4	40	4.0
5	50	5.0
6	60	6.0
7	70	7.0
8	80	8.0
9	90	9.0
10	100	10.0
11	110	11.0
12	120	12.0

All other reagents provided are ready to use.
Do not use a plate seal during the incubation steps.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the label, if kept at 2 to 8°C.
- Protect the Streptavidin-HRP reagent and chromogenic solution TMB from light.
- Do not store diluted Biotin-Vit D reagent, dilute only the required amount.
- Once opened, reclose the foil bag containing the microtiter plate with desiccant (stable for a maximum of 2 weeks at 2-8 °C).
- After reconstitution, calibrators and controls are stable for two weeks at 2-8°C.
- Unused working wash solution should be disposed of at the end of the day.

Kit component	In use stability
Anti-Vitamin D coated plate	Maximum of 2 weeks at 2-8 °C
Sample diluent	Maximum of 2 weeks at 2-8 °C
100x Biot-Vit D reagent	Maximum of 2 weeks at 2-8 °C
Streptavidin-HRP reagent	Maximum of 2 weeks at 2-8 °C
Calibrator 1 - 6	Maximum of 2 weeks at 2-8 °C
Control 1-2	Maximum of 2 weeks at 2-8 °C
Working wash solution	1 day at room temperature.
Chromogenic solution TMB	Maximum of 2 weeks at 2-8 °C
Stop reagent	Maximum of 2 weeks at 2-8 °C
Biot-Vit D dilution buffer	Maximum of 2 weeks at 2-8 °C

IX. SPECIMEN COLLECTION AND PREPARATION

- This kit is suitable for serum samples.
- Serum samples must be kept at 2 – 8°C.
- If the test is not run within 24h, sampling and storage at -20°C is recommended.
- Avoid repeated freeze-thawing cycles. Mix samples well after thawing and before testing.

X. PROCEDURE

A. Handling notes

Do not use the kit or components beyond expiry date.
Do not mix materials from different kit lots.
Bring all the reagents to room temperature (18-25°C) prior to use.
Thoroughly mix all reagents and samples by gentle agitation or swirling.
Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
Use a clean plastic container to prepare the Working Wash Solution.
In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
For the dispensing of the Chromogenic Solution and the Stop Solution avoid pipettes with metal parts.
High precision pipettes or automated pipetting equipment will improve the precision.
Respect the incubation times.
To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section XIII paragraph D (Time delay).
Prepare a calibration curve for each run, do not use data from previous runs.
Dispense the Chromogenic Solution within 15 minutes following the washing of the microtiter plate.
During incubation with Chromogenic Solution, avoid direct sunlight on the microtiter plate.
Each well can only be used once.

B. Procedure

1. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2 – 8°C.
2. Secure the strips into the holding frame.
3. Pipette 90 µl of Sample diluent into all the wells.
4. Pipette 10 µl of each reconstituted Calibrator, Control or sample in duplicate into the appropriate wells (use a new pipette tip for each Calibrator, Control or sample).
5. Incubate for 90 minutes at 37 °C, shaking at 650 rpm.
6. Wash the plate 3 times with 350 µl working wash solution.
7. Pipette 100 µl of working Biot-Vit D solution into all wells.
8. Incubate for 30 minutes at 37 °C, shaking at 650 rpm.
9. Wash the plate 3 times with 350 µl working wash solution.
10. Pipette 100 µl of Streptavidin-HRP reagent into all the wells.
11. Incubate for 20 minutes at 37 °C, shaking at 650 rpm.
12. Wash the plate 3 times with 350 µl working wash solution.
13. Pipette 100 µl of the chromogenic solution into all the wells.
14. Incubate for 15 minutes at room temperature (18-25°C), stationary and protected from light.
15. Pipette 100 µl of the Stop reagent into all the well.
16. Read the absorbances at 450 nm within 5 minutes (reference filter 630nm or 650nm), and calculate the results as described in Section XI.

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. We recommend the use of computer assisted methods to construct the calibration curve. 4-parameter logistic function curve fitting is the preferred method. Reject obvious outliers.
4. By interpolation of the sample OD values, determine the Free 25OH Vitamin D concentrations of the samples from the calibration curve.

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

Free 25OH Vitamin D ELISA		OD units
Calibrator	0.9 pg/ml	2.16
	3.1 pg/ml	1.71
	6.5 pg/ml	1.21
	11.6 pg/ml	0.83
	23.2 pg/ml	0.45
	40.3 pg/ml	0.23

Note : 1 pg/ml = 2.5 pmol/l

XIII. PERFORMANCE AND LIMITATIONS

A. Limits of detection

The Limit of Blank (LoB) and the Limit of Detection (LoD) were determined by converting mOD into pg/ml values with standard curve equation.
The LoB was calculated to be 1.86 pg/ml.
The LoD was calculated to be 2.38 pg/ml.

B. Specificity

Cross-reactivity of the antibody used in the Free 25OH Vitamin D ELISA was determined by the supplier as depicted in the following table:

Compound and Concentration	% Cross reaction
25OH-Vitamin D_3 at 10 ng/ml	100
25OH-Vitamin D_2 at 10 ng/ml	86
1, 25 $(OH)_2$-Vitamin D_3 at 200 ng/ml	20
1, 25 $(OH)_2$-Vitamin D_2 at 690 ng/ml	1.9
Vitamin D_3 at 200 ng/ml	2.9
Vitamin D_2 at 200 ng/ml	1.3
24, 25 $(OH)_2$-Vitamin D_3 at 20 ng/ml	>100
25, 26 $(OH)_2$-Vitamin D_3 at 4 ng/ml	>100
3 epi 25OH-Vitamin D_3 at 20 µg/ml	0.1

The effect of potential interfering substances on samples tested using the DIAsource Free 25OH Vitamin D ELISA kit was evaluated according to CLSI EP07-A2. Ascorbic acid, unconjugated bilirubin, HAMA, rheumatoid factor, haemoglobin, triglycerides, biotin and cholesterol were tested in samples with different free 25OH Vitamin D concentrations. The tested substances did not affect the performance of free 25OH Vitamin D ELISA.

Substances	Concentration of interferent	Free 25OH Vitamin D (pg/mL)	Mean % Interference
Ascorbic Acid	[3mg/dl]	6.5	1%
		11.1	-1%
		16.7	0%
Unconjugated Bilirubin	[20mg/dl]	6.6	-6%
		11.2	1%
		15.7	-3%
HAMA	[2µg/ml]	6.6	-5%
		10.5	2%
		15.1	2%
Rheumatoid Factor	[600IU/ml]	6.6	-1%
		10.5	-3%
		15.1	-6%
Haemoglobin	[200mg/dl]	6.9	-8%
		11.2	-8%
		17.0	-10%
Triglycerides	[37mmol/l]	6.8	-5%
		11.1	-5%
		16.2	-5%
Biotin	[4mg/dl]	6.5	1%
		10.6	4%
		15.7	1%
Cholesterol	[13mmol/l]	6.4	0%
		10.6	-1%
		16.8	-3%

C. Precision

Reproducibility and repeatability precisions were evaluated with two samples at medium and high concentrations. Their concentrations were measured in 10 different runs in duplicate for reproducibility and in a single run for repeatability precision.

Sample	N	pg/ml		Reproducibility Precision (Total runs)
Sample 1	20	8.22	SD	0.45
			CV	5.5%
Sample 2	20	29.42	SD	3.00
			CV	10.2%

SD: Standard Deviation; CV: Coefficient of Variation

Sample	N	pg/ml		Repeatability Precision (within a run)
Sample 1	12	9.02	SD	0.32
			CV	3.5%
Sample 2	12	32.38	SD	0.64
			CV	2.0%

SD: Standard Deviation; CV: Coefficient of Variation

D. Time delay

The time delay test between the last Calibrator and sample dispensing results is shown in the following table.

TIME DELAY					
	0 min (pg/ml)	5 min (pg/ml)	10 min (pg/ml)	15 min (pg/ml)	20 min (pg/ml)
Sample 1	5.60	5.68	5.70	5.46	5.71
Sample 2	13.17	14.70	13.07	14.27	13.38

Assay results remain accurate even when a sample is dispensed 20 minutes after the Calibrator has been added in the coated wells.

E. Method comparison

The correlation between rate dialysis analysis result vs Free 25OH Vitamin D ELISA is 0.9916.

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

XV. PRECAUTIONS AND WARNINGS

Safety

For research use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains HCl. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

For more information, see Material Safety Data Sheet (MSDS)

XVI. REFERENCES

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2. SOLLID S.T. (2016), Effects of vitamin D binding protein phenotypes and vitamin D supplementation on serum total 25(OH) D and directly measured free 25(OH)D, Eur. J. Endocrinol. April 1, 174:445-452.
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6. BIKLE D. (2013), Variability in free 25(OH) vitamin D levels in clinical populations, J. Steroid Biochem. Mol. Biol., S09600760.
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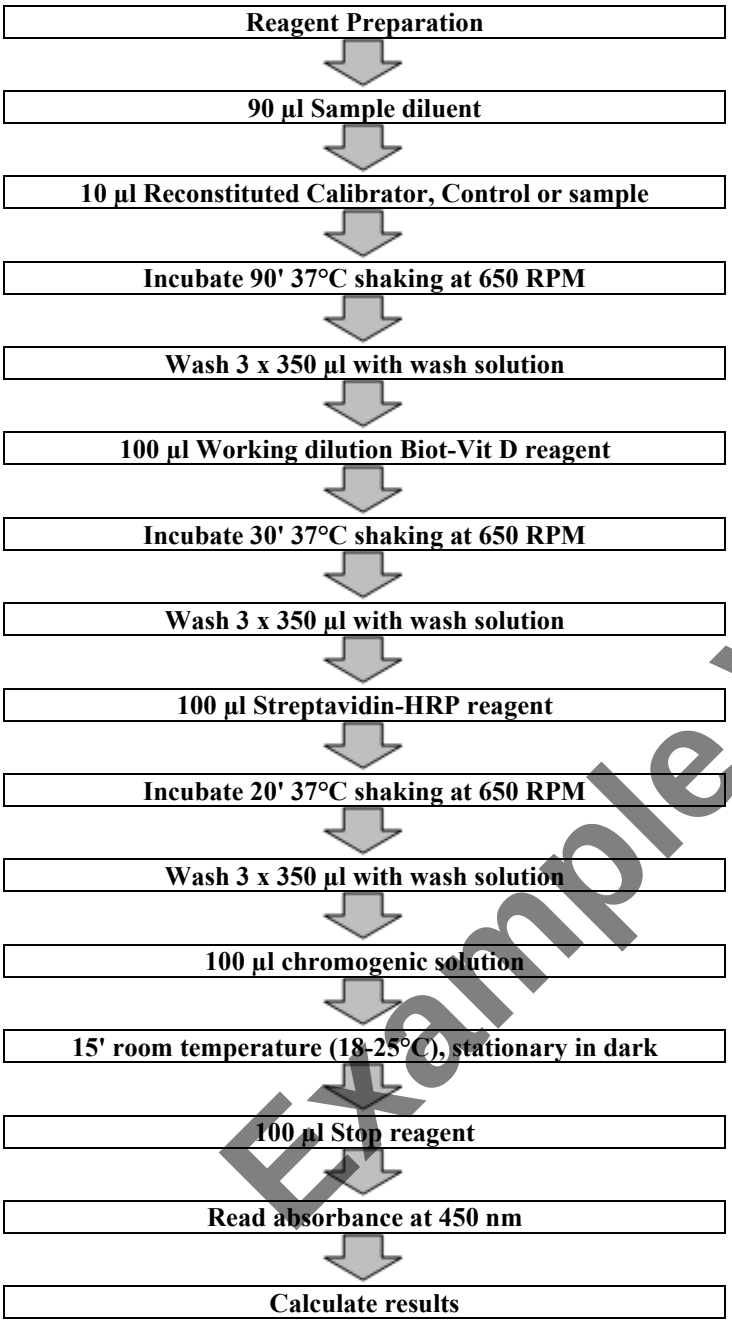
XVII. PROTOCOL SUMMARY



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