PHOSPHATASES, ALKALINE, ACID, PROSTATIC ACID

(Procedure No. 104)

Revised: 2001-11

INTENDED USE

Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphatase kits are intended for use in the quantitative, colorimetric determination of alkaline, acid and prostatic acid phosphatases, respectively, in serum at 400–420 nm.

BACKGROUND AND PRINCIPLE OF TEST

In 1930, Kay demonstrated the presence of alkaline phosphatase in blood using 8-glycero-8-phosphate as substrate. Subsequently, others using this and different substrates improved the technique and broadened the test to measure both acid phosphatase and prostatic acid phosphatase in serum.

Early procedures relied on measurement of liberated phosphate from the substrate in the presence of pre-existing phosphate in serum. Thus, high blanks were frequently encountered that reduced the reliability of the phosphatase determination. To overcome this shortcoming, King and Armstrong introduced use of p-nitrophenyl phosphate as substrate and colorimetrically measured liberated phenol. However, deproteinization and reagents for color development were still required.

To eliminate time-consuming steps, Chomir and Fujita and then Bessey et al., used p-nitrophenyl phosphate as substrate for alkaline phosphatase. The p-nitrophenol liberated by phosphatase could be quantitated colorimetrically simply by addition of alkali and the need for deproteinization was eliminated. In 1947, Andersch and Szczypinski used p-nitrophenyl phosphate for serum acid phosphatase determination. In 1960, Jacobsson combined this substrate with trypsin for the assay of prostatic acid phosphatase.

The Sigma procedure for determination of alkaline phosphatase utilizes 2-amino-2-methyl-1-propanol (AMP) buffer and involves only a 15-minute incubation.

Serum acid phosphatase is derived from a number of tissues, principally the prostate, liver and spleen, as well as erythrocytes, leukocytes and platelets. A number of techniques have been devised for specifically distinguishing prostatic acid phosphatase because of its importance in the diagnosis of carcinoma of the prostate with metastasis. Since tartrate abolishes about 95% of prostatic acid phosphatase activity, it serves as a useful basis for assessing the level of this enzyme in serum.

The Sigma procedures for acid and alkaline phosphatase depend upon the hydrolysis of pH

PRECAUTIONS:

Acid and Alkaline Phosphatase reagents are for “In Vitro Diagnostic Use”. Normal precautions exercised in handling laboratory reagents should be followed. Wear suitable protective clothing, gloves and eye/face protection. Dispose of waste observing all local, state and federal laws.

Citrated Blood Solution and Tartrate Acid Buffer Solution are HIGHLY TOXIC (USA definition), TOXIC (European definition). May cause cancer. May cause heritable genetic damage. Toxic by inhalation, in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin. In case of accident or if you feel unwell, seek medical advice (show the label where possible). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves and eye/face protection. Do not breathe vapor. Target organs for Citrate Buffer: liver and kidneys. Target organs for Tartrate Acid Buffer: nerves and heart.

221 Alkaline Buffer Solution is a IRITRANT. Irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. Refer to Material Safety Data Sheets for any updated risk, hazard or safety information.

PREPARATIONS:

Stock Substrate Solution: Remove Sigma 104 Phosphatase Substrate from freezer and allow to warm to room temperature before opening package to avoid moisture pick-up. The Stock Substrate Solution is prepared according to the following table.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>WATER</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mg Capsule, Catalog No. 104-40</td>
<td>10 ml per capsule (contents only)</td>
</tr>
<tr>
<td>100 mg Capsule, Catalog No. 104-100</td>
<td>25 ml per capsule (contents only)</td>
</tr>
</tbody>
</table>

Diluted p-Nitrophenol Standard Solution is prepared by pipetting 0.5 ml p-Nitrophenol Standard Solution into a 100 ml volumetric flask. Dilute to 100 ml with 0.02 N sodium hydroxide solution. Mix thoroughly.

Recover to “Reagents Required But Not Provided” section for preparation of acid and base solutions that may be needed.

STORAGE AND STABILITY:

Store Sigma 104 Phosphatase Substrate in the freezer (below 0°C). Substrate should be off-white to yellowish and is suitable for use as long as reagent blank can be brought to zero absorbance in the instrument.

Stock Substrate Solution should be dispensed in 0.5 ml aliquots into incubation tubes, stoppered and frozen in an upright position. Stable six weeks in the freezer (below 0°C).

Store Citrate Buffer Solution, Tartrate Acid Buffer Solution and 221 Alkaline Buffer Solution in refrigerator (2–8°C). Buffers are suitable for use in the absence of microbial growth.


REAGENTS REQUIRED BUT NOT PROVIDED

NOTE: Use ACS grade chemicals throughout for reagent preparation.

HYDROCHLORIC ACID, Concentrated (For Serum Alkaline Phosphatase Procedure)

SODIUM HYDROXIDE SOLUTION, 0.1 N (For Serum Total Acid and Prostatic Acid Phosphatase Procedures) Prepare by dissolving 4.0 g sodium hydroxide in 1000 ml deionized water.

SODIUM HYDROXIDE SOLUTION, 0.05 N Prepare by dissolving 2.0 g sodium hydroxide in 1000 ml deionized water.

SODIUM HYDROXIDE SOLUTION, 0.02 N Prepare by dissolving 0.8 g sodium hydroxide in 1000 ml deionized water.

OPTIONAL REAGENTS

Reagents for preservation of total acid and prostatic acid phosphatase activity:

ACP STABILIZER, Catalog No. A 2170

Acetate buffer, 5 mmol/l, Add 0.02 ml to 1 ml serum.

PROSTATIC ACID PHOSPHATASE STABILIZER TABLETS, Catalog No. A 104-9

Tablets contain citric acid, 4 mg, and excipient. Add one tablet to 1 ml serum.

NOTE: Suitability for use can be verified by dissolving 1 tablet per ml serum and measuring pH, which should be between 5 and 6.

SPECIMEN COLLECTION AND STORAGE

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-T2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.

SERUM:

Blood should be collected without stasis or hemolysis. Ruptured red cells release acid phosphatase which is present in higher concentrations within the cell than in serum. Several authors report that prostatic massage may elevate serum total and prostatic acid phosphatase. It is recommended that 24 to 48 hours elapse after treatment before obtaining serum for acid phosphatase determination.

STABILITY OF HUMAN SERUM PHOSPHATASE:

Alkaline phosphatase in serum is stable for at least 8 days at room temperature. Several sera, for example, which initially exhibited 20 units of total acid phosphatase activity, yielded only 1.9 units of activity after storage for 2 hours at 37°C. At 25°C, the drop was less but still appreciable. Therefore, 50–90% of serum acid phosphatase activity can disappear within a few hours on a warm day.

King and Jegatheesan found acid phosphatase stable at 0°C up to 14 days, provided the serum was refrigerated immediately after separation from clot. It is suggested that promptly after collection, blood be placed in a container of crushed ice or refrigerated. Within 1 hour, centrifuge blood, separate serum and refrigerate.

If ACP Stabilizer (0.02 ml) or a Stabilizer Tablet, Catalog No. A 104-9, is added to 1 ml serum, inactivation of prostatic acid phosphatase is minimized for several days even at 37°C. This preservation is useful when serum is to be mailed.

If alkaline phosphatase is to be determined as well, divide the serum before obtaining serum for acid phosphatase determination.

PLASMA PHOSPHATASE:

Heparinized plasma may be used for alkaline phosphatase. Fluoride and anti-coagulants such as citrate, oxalate or EDTA should be avoided as these agents inhibit alkaline phosphatase activity. Plasma obtained using ACD (acid-citrate-dextrose) may be used for acid phosphatase determination. Do not use fluoride, heparin, EDTA or oxalate.

SIGMA 104® PHOSPHATASE SUBSTRATE, p-Nitrophenyl phosphate, disodium 100 mg capsule, Catalog No. 104-40

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Heparinized plasma may be used for alkaline phosphatase. Fluoride and anti-coagulants such as citrate, oxalate or EDTA should be avoided as these agents inhibit alkaline phosphatase activity. Plasma obtained using ACD (acid-citrate-dextrose) may be used for acid phosphatase determination. Do not use fluoride, heparin, EDTA or oxalate.
Tissue Phosphatase:
Prepare extract and proceed as for serum. Adjust volume assayed to the activity contained per ml.

Leukocyte Alkaline Phosphatase:
In 1970, DeChatelet and Cooper described the determination of leukocyte alkaline phosphatase using Sigma 104 Phosphatase Substrate. It was demonstrated that AMP buffer yielded adequate results.

Interfering Substances:
1. According to Sigma technique, serum alkaline phosphatase reaction mixtures are decolorized with acid after photometric measurement of the yellow alkaline p-nitrophenol color. Any residual color normally accounts for other serum chromogens and is subtracted to obtain correct alkaline phosphatase activity. Nevertheless, bilirubin and hemoglobin, if present in high concentration, can introduce error since these pigments have slightly greater absorptions in alkaline than in acid. From a practical standpoint, this error is small enough to ignore. If desired, a correction can be made by preparing a Serum blank containing 0.10 ml serum and 11.0 ml of 0.02 N NaOH.
2. A Serum Blank is routinely employed in the procedures for total acid and prostatic acid phosphatase to correct for color contributed by serum.
3. Specimens visibly hemolyzed are not suitable for test purposes as red blood cells are rich in acid phosphatase.

Instrument and Materials Required

Instrument:
Any colorimeter or spectrophotometer transmitting wavelengths between 400-420 nm can be used.

Materials:
Centrifuge
Cuvets (e.g., 19 x 100 mm)
Pipets: Conventional or automatic pipets may be used. Automatic diluters which have demonstrated acceptable reliability may also be employed. If conventional pipets are used, the following sizes will be needed: 0.1, 0.2, 0.5, 1, 2 and 10 ml serologic.

Water bath at 37°C

Procedures

Serum Alkaline Phosphatase:
1. Pipet into each of 2 tubes:
   - 0.5 ml 221 Alkaline Buffer Solution, Catalog No. 221
   - 0.5 ml Stock Substrate Solution
   Place both tubes in a 37°C water bath to equilibrate.
2. Pipet 0.1 ml water into tube labeled BLANK.
   NOTE: Only one Blank is needed for each series of tests.
3. Pipet 0.1 ml serum into tube labeled TEST. Record exact time, mix gently and replace in water bath promptly.
4. After exactly 15 minutes, add 10.0 ml 0.05 N NaOH to each tube and mix by inversion.
   NOTE: Alkali stops reaction and develops color which is stable several hours.
5. Read absorbance of TEST vs BLANK as reference at 400-420 nm. Determine alkaline phosphatase units corresponding to this reading from calibration curve.
6. Add 4 drops (0.2 ml) concentrated HCl to each tube and mix.
   NOTE: Acid removes color due to p-nitrophenol, leaving absorbance due to serum.
7. Again read absorbance of TEST using BLANK as reference. Determine alkaline phosphatase units corresponding to this reading from calibration curve.
8. Subtract the alkaline phosphatase activity of Step 7 from alkaline phosphatase activity of Step 5, yielding corrected alkaline phosphatase activity of serum.
   NOTE: With values greater than 10 Sigma Units/ml, repeat assay using a 5-minute incubation and multiply results by 2. If value is still too high, use less serum and multiply by appropriate factor.

Serum Total Acid Phosphatase:
CAUTION: Refrigerate blood immediately after drawing. Centrifuge within 1 hour. Store unhemolyzed serum in refrigerator (2-8°C). If serum cannot be refrigerated, add Prostatic Acid Phosphatase Stabilizer Tablet, Catalog No. 104-9 (1 tablet/ml) or add ACP Stabilizer (0.02 ml/ml serum) to preserve enzyme activity.

1. To each of three test tubes, labeled 1, 2, 3, add 0.5 ml Stock Substrate Solution.
2. Add as follows:
   - Tube 1: 0.5 ml Tartrate Acid Buffer, Catalog No. 104-12
   - Tube 2: 0.5 ml Citrate Buffer, Catalog No. 104-4
   - Tube 3: 0.5 ml Citrate Buffer, Catalog No. 104-4
   Place tubes in 37°C water bath for about 5 minutes.
3. To Tubes 1 and 2 only, add 0.2 ml serum and incubate exactly 30 minutes at 37°C.
4. To Tubes 1, 2 and 3, add 5.0 ml 0.1 N NaOH.
   NOTE: Alkali stops reaction and develops color which is stable several hours.
5. To Tube 3 only, add 0.2 ml serum.
6. Read each tube against water as reference (400-420 nm is satisfactory, but must be consistent with calibration). From calibration curve, determine acid phosphatase units corresponding to readings of each tube.
7. Calculations are as follows:
   Subtraction of activity of Tube 1 from that of Tube 2 yields serum prostatic acid phosphatase activity.
   Subtraction of activity of Tube 3 from that of Tube 2 yields serum total acid phosphatase activity.
   NOTE: If total acid phosphatase value is either not desired or has been determined previously, Tube 3 may be omitted.

Calibration
Calibration curves for serum alkaline phosphatase and serum acid phosphatase are prepared by diluting p-nitrophenol with alkali.

1. Pipet solutions indicated in Columns 2 and 3 of the following chart into numbered tubes:

   Tube No. | 2 | 3 | 4 | 5
   --- | --- | --- | --- | --- | ---
   | p-Nitrophenol Solution (ml) | NaOH 0.02 N (ml) | Sigma Units/ml | Serum Alkaline | Serum Acid
   1 | 1.0 | 10.0 | 1.0 | 0.28
   2 | 2.0 | 9.0 | 2.0 | 0.56
   3 | 4.0 | 7.0 | 4.0 | 1.12
   4 | 6.0 | 5.0 | 6.0 | 1.68
   5 | 8.0 | 3.0 | 8.0 | 2.24
   6 | 10.0 | 1.0 | 10.0 | 2.86

   2. Read absorbance of each of the above mixtures at 400-420 nm using 0.02 N NaOH as reference.
3. Construct calibration curves as follows:
   a. Serum Alkaline Phosphatase: Plot absorbance vs units in Column 4.
   b. Serum Acid Phosphatase: Plot absorbance vs units in Column 5.

Typical Calibration Curves:
Figures are for illustrative purposes only. Curves must be prepared by the laboratory for the instrument used.

Figure 1: Typical Alkaline Phosphatase Calibration Curve
QUALITY CONTROL:
The reliability of test results may be monitored by routine use of frozen aliquots of human serum pools or commercially available serum preparations of known alkaline and acid phosphatase activity.

Sigma offers two serum enzyme controls containing several enzymes including alkaline and total acid phosphatase. Sigma Enzyme Control 2-N, Catalog No. S 5005, contains alkaline and total acid phosphatase at normal levels. Sigma Enzyme Control 2-E, Catalog No. S 1005, contains enzymes at elevated levels. Controls do NOT contain prostatic acid phosphatase. Values are stated in Sigma Units as determined by the described procedure.

RESULTS
Results are derived as described in “Procedures” section and expressed in Sigma Units. A Sigma Unit is defined as that amount of enzyme activity that will liberate 1 μmol of p-nitrophenol per hour under the test conditions described by Bessey et al.12

EXAMPLE: Serum Alkaline Phosphatase
Absorbance of TEST (Step 5) = 0.250
Absorbance of TEST after HCl (Step 7) = 0.010

By referring to Figure 1, absorbance of TEST before and after HCl addition correspond to alkaline phosphatase activity of 2.1 and 0.1 Sigma Units/ml, respectively. By subtraction (2.1 - 0.1), the corrected serum alkaline phosphatase activity equals 2.0 Sigma Units/ml.

EXAMPLE: Serum Total Acid Phosphatase
Absorbance of TEST (Step 7) = 0.250
Absorbance of SERUM BLANK (Step 5) = 0.025

By referring to Figure 2, absorbances of TEST and SERUM BLANK correspond to total acid phosphatase activities of 0.62 and 0.05 Sigma Units/ml, respectively. By subtraction (0.62 - 0.05), the corrected serum total acid phosphatase is 0.57 Sigma Units/ml.

EXAMPLE: Serum Prostatic Acid Phosphatase
Absorbance of Tube 1, with tartrate buffer (Step 6) = 0.200
Absorbance of Tube 2, with citrate buffer (Step 6) = 0.250

By referring to Figure 2, absorbances of Tube 1 and Tube 2 correspond to serum acid phosphatase activities of 0.49 and 0.62 Sigma Units/ml, respectively. By subtraction (0.62 - 0.49), the corrected serum prostatic acid phosphatase activity is 0.14 Sigma Units/ml.

INTERNATIONAL UNITS:
A Sigma Unit of phosphatase activity is defined as that amount of enzyme activity which will liberate 1 μmol of p-nitrophenol per hour under the test conditions described by Bessey et al.12

Please note that the alkaline phosphatase procedure formerly employed glycine as buffer. The use of AMP buffer results in essentially twice the activity as that obtained using glycine. Alkaline phosphatase activity is higher in children than in adults. A rise of 2-3 times normal is observed in the third trimester of pregnancy. Decreased serum alkaline phosphatase values are infrequently encountered. Low levels are sometimes found in cases of hypothyroidism, scurvy, celiac disease, severe chronic nephritis and pernicious anemia.

SERUM PROSTATIC ACID PHOSPHATASE IN DISEASE:
Many early reports showed poor correlation between serum acid phosphatase activity and cancer of the prostate. However, the extreme instability of acid phosphatase was not recognized. Thus, normal values were probably obtained on sera originally having high acid phosphatase levels. Also, most of these reports failed to differentiate between prostatic and total acid phosphatase.

Prostatic acid phosphatase is sometimes markedly elevated even when total acid phosphatase is normal. Bonner et al.13 reported that 123 out of 125 hospitalized men over age 60 had normal prostatic acid phosphatase activity. In these cases, the high prostatic acid phosphatase was not indicative of carcinoma of the prostate. Jacobsson14 reported that 123 out of 125 hospitalized men over age 60 had normal serum acid phosphatase values when determined by the Sigma procedure, but at pH 5.5. The remaining two patients exhibited increased values and “may well have had cancer of the prostate”.

SERUM PHOSPHATASES IN DISEASE:
Serum alkaline and total acid phosphatase levels vary with the age of the patient and results should be applicable. Copeland15 suggests that each laboratory determine its own normal range. Attention should be given to the fact that certain measurements are influenced in clinically healthy individuals by diet, sex, age, diurnal variation, physical activity, menstrual cycle, pregnancy and environmental factors. Administration of certain drugs and medications has been shown to influence body levels of phosphatase activity. A comprehensive review has been prepared by Young et al.16 and should be consulted for further information.

SERUM PHOSPHATASES IN DISEASE:
Values have been compiled from several sources and may serve as a guide for interpretation of elevations encountered in various disorders.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Alkaline Phosphatase (Sigma Units/ml)</th>
<th>Total Acid Phosphatase (Sigma Units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic cirrhosis</td>
<td>2.5-10.0</td>
<td>Prostatic Cancer with Metastasis without Metastasis</td>
</tr>
<tr>
<td>Hyperparathyroidism</td>
<td>10.0-14.0</td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Generalized</td>
<td>2.7-5.5</td>
<td>Gaucher’s Disease</td>
</tr>
<tr>
<td>Senile</td>
<td>1.5-3.0</td>
<td>Hyperparathyroidism</td>
</tr>
<tr>
<td>Osteosclerosis</td>
<td>8.0-12.0</td>
<td>Metastasis to Bone</td>
</tr>
<tr>
<td>Paget’s Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>2.7-10.0</td>
<td>Myelocytic Leukemia, Acute</td>
</tr>
<tr>
<td>Polystatic</td>
<td>10.0-75.0</td>
<td>Paget’s Disease</td>
</tr>
<tr>
<td>Rickets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>15.0-90.0</td>
<td>Prostate Patiation (up to 48 h)</td>
</tr>
<tr>
<td>Healed</td>
<td>3.0-6.0</td>
<td></td>
</tr>
</tbody>
</table>

Alkaline phosphatase levels tend to parallel osteogenic activity. Therefore, serum alkaline phosphatase activity is higher in children than in adults. A rise of 2-3 times normal is observed in the third trimester of pregnancy. Decreased serum alkaline phosphatase levels are infrequently encountered. Low levels are sometimes found in cases of hypothyroidism, scurvy, celiac disease, severe chronic nephritis and pernicious anemia.

PERFORMANCE CHARACTERISTICS

REPRODUCIBILITY STUDIES:
The coefficient of variation (CV) for serum alkaline phosphatase at normal levels is 2% with a standard deviation of 0.04 Sigma Units/ml. At levels below 1.0 Sigma Units/ml, the CV is 6% and in the elevated range the CV drops to 1%. For total acid phosphatase, the reproducibility studies follow the same pattern, normal levels having a CV of 2%, values below 0.5 Sigma Units/ml a CV of 5% and elevated specimens 1%. Standard deviations for total acid phosphatase are 0.01 Sigma Units/ml throughout the range of values determined. Prostatic acid phosphatase values for 30 specimens ranging from 0.05 to 1.5 Sigma Units/ml were determined to have a CV of 5%, the standard deviation being 0.03 Sigma Units/ml.

EXPECTED VALUES

<table>
<thead>
<tr>
<th>NORMAL SERUM ALKALINE PHOSPHATASE**</th>
<th>NORMAL TOTAL SERUM ACID PHOSPHATASE**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults: 0.8-3.0 Sigma Units/ml (13-50 U/I)</td>
<td>Males: 0.13-0.63 Sigma Units/ml (2-11 U/I)</td>
</tr>
<tr>
<td>Children: 2.8-6.7 Sigma Units/ml (47-112 U/I)</td>
<td>Females: 0.01-0.56 Sigma Units/ml (0.2-10 U/I)</td>
</tr>
</tbody>
</table>
CORRELATION STUDIES:
Ten samples were analyzed for alkaline phosphatase by Sigma and SMA 12/60 procedures based on the Bessey-Lowry-Brock principle. Comparable results were obtained by both methods in the normal and elevated ranges.

Sigma 104® is a registered trademark of Sigma-Aldrich Co., St. Louis, MO

REFERENCES
19. Data obtained by Sigma Diagnostics

ORDERING INFORMATION

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