WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 16, 966-977. Research Article ISSN 2277–7105

SICKLE HEMOGLOBIN (EFFECTIVE) DIAGNOSIS INTERFERED BY FALSE POSITIVE AND NEGATIVE RESULTS, DURING FIELD SCREENING BY DTT TEST, CAN BE IMPROVED BY FINDING THE OPTIMUM CONCENTRATION OF SAPONIN AND NA-DITHIONITE

Jignisha S. Patel* and Dr. Jigna P. Naik

Arts, Science & Commerce College, Department of Microbiology (PGDMLT), Kholwad, Surat, Gujarat, India.

Article Received on
15 Nov. 2017,
Revised on 21 Nov. 2017
Accepted on 27 Nov. 2017
DOI: 10.20959/wjpr201716-10249

***Corresponding Author**
Jignisha S. Patel
Arts, Science & Commerce College, Department of Microbiology (PGDMLT), Kholwad, Surat, Gujarat, India.

ABSTRACT

For field screening of sickle hemoglobin, the first method of choice is Dithionite Tube Test (DTT). DTT test is not 100% sensitive and specific, but its user friendly features make it the first choice for mass screening as well as for routine laboratory testing. For DTT test the buffer solution used contains KH_2PO_4, K_2HPO_4, Saponin and Na-dithionite. KH_2PO_4 and K_2HPO_4 provide buffering effect. Saponin (a mild detergent) act as a lysing agent for RBC and releases hemoglobin which is reduced by a reducing agent Na-dithionite if the released hemoglobin is sickle hemoglobin (HbS). Thus Saponin and Na-dithionite play significant role in DTT buffer. In this experiment, five different concentration of saponin (0.1, 0.2, 0.3, 0.4, and 0.5 gm) and six different concentration of Na-dithionite (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 gm) were used along with fixed concentration of KH_2PO_4 (16.9 gm) and K_2HPO_4 (21.5 gm) for 100 mL of DTT buffer solution. As a result, total thirty different DTT buffer solutions were prepared. This experiment was performed to establish the optimum concentration of saponin and Na-dithionite in DTT buffer to increase the sensitivity and specificity of test results. Out of 30 different DTT buffer compositions, one composition gave the most desired results and was recommended to be used for mass screening of HbS for effective diagnosis.

KEYWORDS: Sickle hemoglobin (HbS), Screening Test, Dithionite Tube (DTT) Test, DTT Buffer, Saponin, Na-Dithionite.

INTRODUCTION

Sickle cell disease is a lifelong, inherited, genetic disorder of haemoglobinopathies characterized by the presence of abnormal sickle hemoglobin (HbS) in RBC, from both parents or HbS gene from one parent and a gene for an abnormal hemoglobin or β-thalassaemia from the other parent.[1-4] For primary diagnosis or screening of sickle hemoglobin, other than DTT there are various methods available which includes, sickling test, peripheral blood smear test, cellulose acetate electrophoresis, HPLC methods, Iso-electric focusing etc.[2, 4, 5-6] But all these techniques are costly, time consuming, require skilled technician to perform and interpret the test result. Moreover they also require sophisticated instruments. In contrast to this DTT test does not require any machine. It is rapid, easy to perform, and results can be read visually with naked eye. Thus DTT is a user friendly test. As DTT test fulfils all the essential parameters for being a primary screening technique, it can be used for mass field screening of HbS.[4, 7-8]

But the main disadvantage of DTT test is that, it is not 100% sensitive and specific. Many factors affect the test results like high protein and lipid concentration, volume of test sample, volume of DTT buffer, concentration of Na-dithionite etc.[9-12] Thus DTT test may give false positive and false negative results. Hemoglobin concentration also plays a vital role in output of the test result.[12-13] In addition to this there is no standard test procedure and DTT buffer composition available for field screening of HbS.[4, 12] So the present study was carried out to reduce the false results of DTT test by optimizing the concentration of Saponin and Na-dithionite.

DTT buffer solution is composed of KH_2PO_4, K_2HPO_4, Saponin and Na-dithionite. KH_2PO_4, K_2HPO_4 provides buffering effect, saponin (a mild detergent) acts as a lysing agent for RBC and releases hemoglobin which is reduced by a reducing agent Na-dithionite if the released hemoglobin is sickle hemoglobin (HbS).[4, 14] Saponin and Na-dithionite play significant role in DTT buffer reaction. In this experiment, five different concentration of saponin (0.1, 0.2, 0.3, 0.4, and 0.5 gm) and six different concentration of Na-dithionite (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 gm) were used along with fixed concentration of KH_2PO_4 (16.9 gm) and K_2HPO_4 (21.5 gm) for 100 mL of DTT buffer solution. As a result, total thirty (30) different DTT buffer solutions were prepared and tested with different experiments to find out the most suitable DTT buffer composition for mass field screening of HbS.

MATERIALS AND METHODS

This study was carried out at Valsad Raktdan Kendra (VRK), a Regional Blood Bank and Haematological Research Centre, Sickle Cell Department, Valsad, Gujarat, India.

Test sample: In this study total 145 EDTA anti-coagulated whole blood samples were used and characterized as Normal/ Negative (AA), Sickle cell trait (AS), Sickle cell anaemia (SS), Sickle β - Thalassaemia (S.Thal), β - Thalassaemia Trait (BTT) by following the procedures as mentioned below. Different types and numbers of samples used in this study for optimization of saponin and Na-dithionite concentration and for validation of optimized DTT test were shown in table 1.

METHODOLOGY

Characterization of clinical samples: All the test samples were well characterized by performing the below mentioned test procedure.

Complete hemogram: Calibrated Sysmex Poch-100*i* particle counter was used for getting complete hemogram of EDTA whole blood samples and hemoglobin concentration was noted. This test was performed as soon as the samples arrived to the laboratory. When not in use, all the samples were stored at 2-8°C.

Hemoglobin electrophoresis: For determining the hemoglobin pattern (AA, AS, SS) of test samples, Hb electrophoresis was performed by using the cellulose acetate membrane with TEB buffer (pH 8.4).[4-5]

High Performance Liquid Chromatography (HPLC): For finalizing the sickle status of test samples, HPLC test - based on the principles of cation-exchange high –performance liquid chromatography (VARIANT β thalassaemia short program from BioRad laboratories) was used as a gold standard method.

Table 1: Different types and numbers of well characterized EDTA whole blood samples used in this study

Sr. No.	Types / characteristics of samples	Number of samples used for optimization of DTT buffer	Number of samples used for validation of optimized DTT test
1	Normal / Negative (AA)	10	30
2	Sickle Cell Trait (AS)	16	37
3	Sickle Cell Anaemia (SS)	13	25
4	Sickle β - Thalassaemia (S.Thal)	05	07
5	β - Thalassaemia Trait (BTT)	01	01
	Total number of samples:	**45**	**100**

Test procedure for performing DTT test / Solubility test

The common test procedure was adopted and finalized from different references.[4] All the reagents and test samples were allowed to attain room temperature. 2.0 mL of working DTT buffer and 20 µL of whole blood specimen were added to 12 x 75 mm test tube. After mixing properly, tube was left at room temperature for 15 minutes. Visual result interpretations of the test results were made.[4]

This DTT test is based on the principle that if sickle hemoglobin is present in the test sample, get reduced in the presence of reducing agent-sodium dithionite and form turbidity in hypertonic organic buffer solution. This is because the solubility of sickle hemoglobin is less as compared to other hemoglobins in presence of reducing agent. Thus the normal hemoglobin - HbA produces no turbidity and the buffer solution remains clear.[4] Finally, the visual result interpretations of the test results were made by mixing well the contents of the test tube. Results were noted by keeping the tubes in front of white paper having dark black lines. The clear visibility and invisibility of lines through the test tube indicated the absence and presence of sickle hemoglobin, respectively.[4]

Preparation of thirty different DTT buffer solutions

By using combination of five different concentration of saponin, six different concentration of Na-dithionite and fixed concentration of KH_2PO_4 (16.9 gm) and K_2HPO_4 (21.5 gm) for 100 mL of DTT buffer solution following thirty (30) different types of DTT buffer compositions (table 2) were prepared to establish the effect of saponin and Na-dithionite with different types of clinical samples. Fresh working DTT buffers were prepared before performing the test.

Table 2: Different Compositions of DTT Buffers

Sr. No. of Different DTT Buffer Compositions ↓	Reagents → Composition no ↓	Saponin gm% concentration	Na-Dithionite gm% concentration
1	C1-a	0.1	0.5
2	C1-b	0.1	1.0
3	**C1-c**	**0.1**	**1.5**
4	C1-d	0.1	2.0
5	C1-e	0.1	2.5
6	C1-f	0.1	3.0
7	C2-a	0.2	0.5
8	C2-b	0.2	1.0
9	C2-c	0.2	1.5
10	C2-d	0.2	2.0
11	C2-e	0.2	2.5
12	C2-f	0.2	3.0
13	**C3-a**	**0.3**	**0.5**
14	C3-b	0.3	1.0
15	C3-c	0.3	1.5
16	C3-d	0.3	2.0
17	C3-e	0.3	2.5
18	C3-f	0.3	3.0
19	C4-a	0.4	0.5
20	C4-b	0.4	1.0
21	C4-c	0.4	1.5
22	C4-d	0.4	2.0
23	C4-e	0.4	2.5
24	C4-f	0.4	3.0
25	C5-a	0.5	0.5
26	C5-b	0.5	1.0
27	C5-c	0.5	1.5
28	C5-d	0.5	2.0
29	C5-e	0.5	2.5
30	C5-f	0.5	3.0

Optimization of thirty (30) different DTT buffer compositions

To find out the most reliable DTT buffer composition from the above mentioned thirty (30) different compositions (table 2), total forty five different well characterized EDTA anti-coagulated whole blood samples (table 1) were used.

Following parameters were investigated while selecting the most reliable DTT buffer composition out of thirty.

- Number of false positive reaction
- Number of false negative reaction

- Formation of red clumps with true sickle positive and true sickle negative samples
- Formation of coarse flocculation and turbidity with normal (Hb AA) samples

Validation of optimized DTT buffer composition

➢ Out of thirty (30) different buffer compositions, the most reliable DTT buffer composition based on above mentioned experiment: *i.e.*, C1-c formulation (table 2) was selected. This test reagent formulation was evaluated and validated by using 100 various types of well characterized clinical samples (table 1) with optimum test sample volume and buffer volume.[12, 15]

RESULTS AND DISCUSSION

Optimization of thirty (30) different DTT buffer compositions

To find out the most reliable DTT buffer composition from thirty (30) different DTT buffers, we have focused on different parameters like, number of false positive and false negative results, formation of small red clumps and coarse flocculation, and finally the quantity of turbidity with sickle positive test samples.

Results of thirty (30) different DTT buffers with above mentioned parameters are shown in table 3.

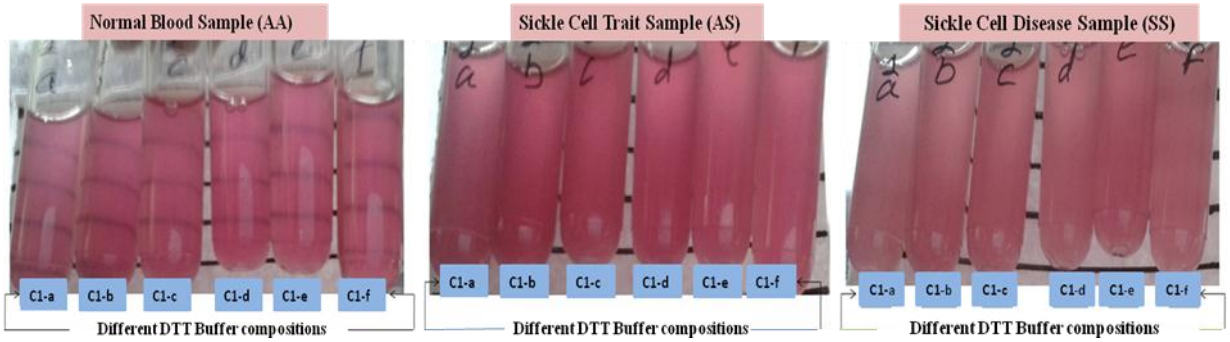
In this experiment we did not find any false positive as well as false negative results. But the most important parameter *i.e.*, ease of visual result interpretation of DTT test was different with various compositions.

Results of this experiment demonstrated that as the concentration of Saponin and Na-dithionite were increased, the amount of small red clumps in the test solution was also increased. Small red clumps were observed in maximum number of samples with compositions C4 and C5 (a to f) (table 3).

Table 3: Results of thirty (30) different DTT buffer compositions with forty five (45) well characterized clinical samples

DTT buffer composition No.	Parameters for evaluating the most reliable performance of DTT buffer having different concentration of saponin and Na-dithionite				
	No. of false positive reaction	No. of false negative reaction	Red clumps observed in number of samples	Coarse flocculation observed in number of samples	Turbid solution with negative (AA) blood samples
C1-a	0	0	**3**	0	0
C1-b	0	0	**3**	0	0
C1-c	0	0	**1**	0	0
C1-d	0	0	**4**	0	0
C1-e	0	0	**4**	0	0
C1-f	0	0	**9**	0	0
C2-a	0	0	**6**	0	0
C2-b	0	0	**4**	0	0
C2-c	0	0	**3**	**1**	0
C2-d	0	0	**4**	0	0
C2-e	0	0	**4**	0	0
C2-f	0	0	**37**	**1**	**2**
C3-a	0	0	**2**	0	0
C3-b	0	0	**9**	0	0
C3-c	0	0	**9**	0	0
C3-d	0	0	**8**	0	0
C3-e	0	0	**10**	**1**	0
C3-f	0	0	**12**	**1**	0
C4-a	0	0	**26**	0	0
C4-b	0	0	**27**	0	0
C4-c	0	0	**27**	0	0
C4-d	0	0	**29**	0	0
C4-e	0	0	**31**	0	0
C4-f	0	0	**33**	**1**	**5**
C5-a	0	0	**25**	0	0
C5-b	0	0	**24**	0	0
C5-c	0	0	**21**	0	0
C5-d	0	0	**22**	0	0
C5-e	0	0	**22**	0	0
C5-f	0	0	**24**	0	0

From thirty different compositions, we found that Composition C1-c and C3-a were giving the superior results in terms of clarity of DTT buffer solution with normal AA type of blood samples (Figure 1 a) and amount or quantity of turbidity formed in case of sickle positive samples (Figure 1 b & 1 c). Visual result interpretation was very easy and user-friendly with composition C1-c and C3-a (table 3).



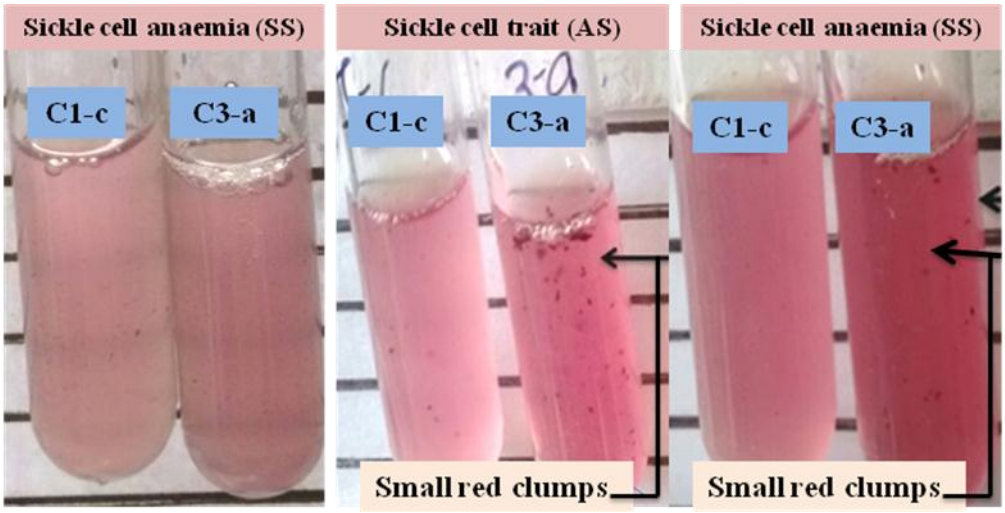
a **b** **c**

Tube no.1- buffer composition C1-a; Tube no.2- buffer composition C1-b; Tube no.3- buffer composition C1-c; Tube no.4- buffer composition C1-d; Tube no.5- buffer composition C1-e; Tube no.6- buffer composition C1-f respectively.

Figure 1: Results of a. Normal (AA), b. Sickle Cell Trait (AS), and c. Sickle Cell Disease (SS) blood samples with different DTT buffer compositions C1-a; C1-b; C1-c; C1-d; C1-e; C1-f respectively.

Whereas, the remaining 28 compositions gave more or less hazy / turbid solutions, and coarse flocculation along with presence of small red clumps in case of normal AA type of blood samples. In addition to these, small to large red clumps and less turbidity with sickle positive samples were also observed with different DTT buffer compositions (table 3).

Based on the results of this experiment, we have finalized composition C1-c and composition C3-a as the most reliable compositions giving satisfactory results. One such results of composition C1-c and composition C3-a with sickle cell anaemia (SS) sample is shown in Figure 2 a. We did not find any false positive or false negative results with composition C1-c and composition C3-a.



a **b** **c**

Figure 2: a. Results given by composition C1-c and composition C3-a with sickle cell anaemia (SS) sample. b. Formation of small red clumps in composition C3-a with sickle cell trait (AS) sample. c. Formation of small red clumps in composition C3-a with sickle cell anaemia (SS) sample.

But after considering the importance of ease of visual result interpretation of DTT test, we found that composition C1-c gave superior results in terms of clarity of DTT buffer solution with normal AA type of samples (Figure 3). In addition to this with composition C1-c, the quality and quantity of turbidity formed in case of sickle positive samples were also satisfactory. In contrary, composition C3-a also gave 100% sensitive and specific results, the visual result interpretation quality with composition C3-a was little bit poorer than composition C1-c., *e.g.*, composition C3-a gave small red clumps with one sickle cell trait (AS) and one sickle cell anaemia (SS) samples out of total forty five (45) sample panel (Figure 2 b and Figure 2 c).

Thus composition C1-c was selected as a most reliable composition and was further validated and clinically evaluated by using hundred (100) well characterized clinical samples along with the different other parameters like blood sample volume (20 µL) and buffer volume (2 mL) finalized in other experiments.

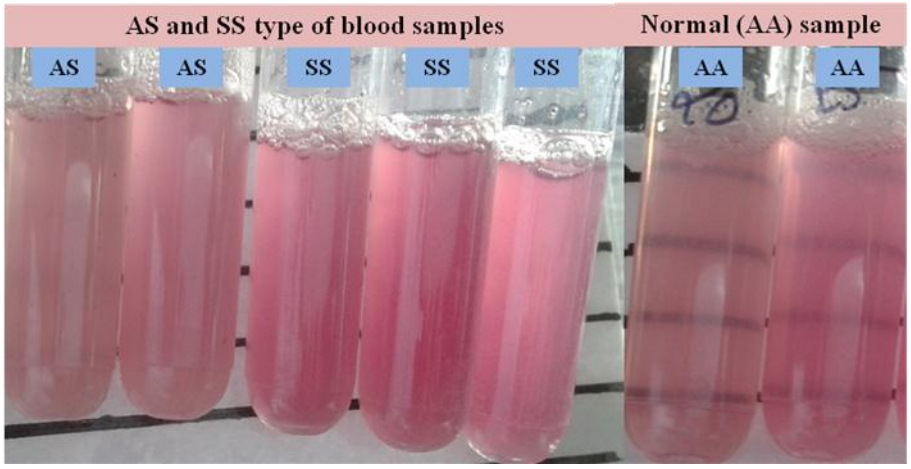
Validation of optimized DTT buffer composition *i.e.*, C1-c

Out of 100 well characterized sample panel used, two samples having lowest hemoglobin (5.4 gm/dL) observed in sickle cell trait (AS) and normal (AA) sample. But the amount of turbidity formed with this sickle cell trait (AS) sample was enough to interpret the test result

correctly. From sample panel used the highest hemoglobin concentration was 15.7 gm/dL, found in normal (AA) sample. However the result interpretation of this test sample with composition C1-c was very user friendly. No small red clump was observed. Moreover no dark red coloration of solution obtained with composition C1-c. Thus composition C1-c gave satisfactory results with both of these types of samples having low and high hemoglobin concentration.

In sample panel total seven double heterozygous (Sickle -β Thalassaemia) samples were used in which the highest Hb concentration was 10.4 gm/dL, whereas the lowest Hb concentration was 7.4 gm/dL. All these samples gave satisfactory results with composition C1-c. Based on the results of experiments, including homozygous (SS) and double heterozygous (Sickle- β Thalassaemia) gave specific results with composition C1-c and there was no significant effect of hemoglobin concentration on result interpretation.

Out of 100 different types of sample panel, we did not find any false positive or false negative results with composition C1-c. Composition C1-c also gave superior results in terms of clarity of DTT buffer solution with normal AA type of samples (Figure 3 b). In addition to this quality and quantity of turbidity formed in case of sickle positive samples was also excellent (Figure 3 a).



a **b**

Figure 3: a. Quality and quantity of turbidity formed in composition C1-c with sickle positive AS & SS type of blood samples. b. Clarity of buffer solution with normal AA type of blood sample.

The result interpretation with composition C1-c was very user friendly. Composition C1-c gave 100% sensitive and specific results with 20 µL of test sample volume and 2 mL of buffer volume.

By adopting these parameters we found that the DTT buffer prepared by using formulation of composition C1-c and combination of 20 µL of blood sample volume and 2 mL of buffer volume were giving the most reliable results with incubation time of 15 minutes at room temperature.

CONCLUSION

In summary, the studies communicated indicates from the result of this experiment, it is highly recommended that the use of composition C1-c (KH_2PO_4 – 16.9 gm, K_2HPO_4 – 21.5 gm, Saponin- 0.1 gm and Na-Dithionite – 1.5 gm for 100 mL of buffer volume) with the test procedure used in this research work can very effectively reduces the chance of false positive and false negative results in addition to best visual result interpretation without compromising the ease of use of the test for mass field screening of sickle hemoglobin.

REFERENCES

1. Patki V, Zade V, Gawande V, Chede S, Dabhadkar D, Thakare V. Study of sickle cell disease phenotypes and sickle cell gene frequency in some tribals belonging to Tipeshwar forest region, Yavatmal district, Maharashtra, India. International Res J of Biolog Sci, 2013; 2(6): 57-59.
2. Wajcman H, Moradkhani K. Abnormal haemoglobins: detection & characterization. Indian J of Med Res, 2011; 134: 538-546.
3. Doshi N, Choudhari S, Shah N, Joshi T, Shah M, Singh U. Prevalence of sickle cell disorder in rural Pipalwada, Gujarat. National J of Community Medicine, 2011; 2(2): 284-288.
4. Rajput JH. Naik JP, Italia YM. Clinical evaluation and enhancement of Dithionite Tube Turbidity (DTT) test reagents used for field screening of Sickle Haemoglobin (HbS). International J of Pharmacy & Life Sci, 2015; 6(2): 4280-4287.
5. Clarke GM, Higgins TN. Laboratory investigation of haemoglobinopathies and thalassaemias: Review and update. Clinical Chemistry, 2000; 46:8(B): 1284-1290.
6. Kaur M, Dangi CBS, Singh M. An overview on sickle cell disease profile. Asian J of Pharma Clin Res, 2013; 6(1): 25-37.

7. Rupani MP, Vasava BC, Mallick KH, Gharat VV, Bansal R. Reaching community through school going children for sickle cell disease in Zankhvav village of Surat district, Western India. Online J Health Allied Scs, 2012; 11(2): 1-4.
8. Dhumne UL, Jawade AA. Sickle cell anaemia and mortality in rural population of Chandrapur district, Maharashtra, India. Anthropologist, 2011; 13(1): 61-63.
9. Okwi AL, Byarugaba W, Parkes A, Ocaido M. The reliability of sickling and solubility tests and peripheral blood film method for sickle cell disease screening at district health centers in Uganda. Clin Mother Child Health, 2010; 7(1): 1205-1210.
10. Robert M, Schmidt MD, Sylvia MW. Standardization in detection of abnormal hemoglobins. Solubility tests for hemoglobin S. JAMA, 1973; 225(10): 1225–1230.
11. Loh WP. Evaluation of a rapid test tube turbidity test for the detection of sickle cell hemoglobin. American J Clin Pat, 1971; 55: 55-57.
12. Rajput JH, Naik JP. Optimization of blood sample volume in Dithionite Tube Turbidity (DTT) Test with respect to hemoglobin concentration used for field screening of sickle hemoglobin (HbS). Int J Pure App Biosci, 2015; 3(2): 325-330.
13. British Committee for Standards in Haematology. Guideline-The laboratory diagnosis of haemoglobinopathies. British J Haematology, 1998; 101: 783-792.
14. Health and Family Welfare Department, Government of Gujarat. Sickle cell anaemia control program manual, http://www.nrhm.gujarat.gov.in/Images/pdf/SickleCellAnemiaManual.pdf
15. Patel JS, Naik JP. Optimization of DTT buffer volume in Dithionite Tube Test (DTT) with respect to hemoglobin concentration used for field screening of sickle hemoglobin (HbS). Indian J App Res, 2016; 6(2): 63-66.