LAMPIRAN

Lampiran 1. Inform Consent

PENJELASAN MENGENAI PENELITIAN

INTERFERENSI KADAR HEMOGLOBIN TERHADAP PEMERIKSAAN PROTEIN TOTAL METODE BIURET

(Informed Consent)

Responden yang terhormat, nama saya Suciyanti. Mahasiswi jurusan Analis Kesehatan Kementerian Kesehatan Bandung. Saya memerlukan serum darah responden bertujuan untuk penelitian saya yang berjudul " Interferensi Kadar Hemoglobin Terhadap Pemeriksaan Protein Total Metode Biuret".

Bantuan yang saya harapkan dari responden adalah kesediaan mendengarkan penjelasan peneliti dan bila dimengerti peneliti mohon untuk berkenan menyumbangkan darahnya. Saya meminta darah responden sebanyak 5 mL (sekitar 1 sendok makan) yang di ambil di lipatan sikut. Responden akan diberikan tindakan aseptik menggunakan kapas beralkohol 70%. Lalu dipasangkan torniquet untuk membendung vena dan dilakukan penusukan. Apabila telah didapatkan darah sebanyak 5 mL, maka responden akan diberikan kassa kering.

Pengambilan spesimen darah akan dilakukan oleh peneliti. Bila pada saat pengambilan spesimen darah akan ada sedikit rasa nyeri atau kesakitan. Apabila

keluhan berlanjut, maka responden akan diberi pertolongan pertama. Bila masih sakit responden akan diperiksakan ke dokter puskesmas terdekat dengan biaya ditanggung oleh peneliti. Besar harapan peneliti bahwa responden berkenan membantu dalam kegiatan ini. Apabila responden tidak berkenan, sewaktu-waktu dapat menolak tanpa dikenakan sanksi apapun.

Pertanyaan

Apabila ada pertanyaan yang menyangkut penelitian dari diri saudara, dapat hubungi langsung kepada Suciyanti, Jurusan Analis Kesehatan Poltekes Kemenkes Bandung, Jalan Babakan Loa Cimahi.

Telp. 085721547701

Lampiran 2. Lembar Persetujuan Setelah Penjelasan

Saya telah dibacakan dan dijelaskan seperti tercantum dalam lembar penjelasan dan telah diberi kesempatan bertanya atas apa yang tidak saya jelaskan dan dapat menolak atau mengundurkan diri sewaktu-waktu tanpa sanksi apapun. Oleh sebab itu, saya menyetujui keikutsertaan saya sebagai partisipan dalam penelitian "Interferensi Kadar hemoglobin Terhadap Pemeriksaan Protein Total Metode Biuret" yang dilakukan oleh Suciyanti dari Jurusan Analis Kesehatan Politeknik Kesehatan Kementerian Kesehatan Bandung.

Saya memahami maksud, manfaat, resiko, waktu dan prosedur penelitian ini, serta saya setuju dengan kompensasi yang akan saya terima. Saya akan membubuhkan tanda tangan saya di bawah ini dan menyatakan keikutsertaan saya dalam pelaksanaan penelitian.

Saya bertandatangan di bawah ini :

Nama : Ispi Asyipa Maulani

Alamat : Kp. Wanir Rt 01 Rw 06 Ds. Maruyung Kec. Pacet Kab. Bandung

Usia : 20 Tahun

Menyatakan bersedia untuk diambil darah sebanyak 5 mL oleh peneliti.

Saya yakin yang saya sampaikan ini terjamin kebenarannya.

Cimahi, Februari 2020

Peneliti	Responden
Suciyanti	Ispi Asyipa Maulani
P17334117013	

Lampiran 3. Data Perhitungan

Perhitungan Pengenceran *Pooled Sera*

Diketahui :

- Kadar Protein Total Pooled Sera Normal : 7,92 g/dL
- Kadar Protein Total Patologis : 4,13 g/dL

  Untuk membuat pooled sera dengan kadar protein total 4,13 g/dL sebanyak 2 mL

  V1 x N1 = V2 x N2

  2000 µl x 4,13 g/dL = V2 x 7,92 g/dL

  1043 µl = V2

  Dibuat pengenceran dengan cara dipipet 1043 µl pooled sera, diencerkan dengan 957 µl NaCl Fisiologis kemudian dihomogenkan dan diukur kadar protein totalnya

Perhitungan Pembuatan Serum Hemolisis

Diketahui :

- kadar hemolisat yang didapatkan : 17,02 g/dL

1. **Pooled sera dengan kadar haemoglobin ± 0,3 g/dL**

   V1 x N1 = V2 x N2

   500 µl x 0,3 g/dL = V2 x 17,02 g/dL

   9 µl = V2

Dipipet 9 µl hemolisat + 491 µl pooled sera, kemudian dihomogenkan dan diukur kadar hemoglobinnya

2. **Pooled sera dengan kadar haemoglobin ± 0,4 g/dL**

   V1 x N1 = V2 x N2

   500 µl x 0,4 g/dL = V2 x 17,02 g/dL

   12 µl = V2

   Dipipet 12 µl hemolisat + 488 µl pooled sera, kemudian dihomogenkan dan diukur kadar hemoglobinnya

3. **Pooled sera dengan kadar haemoglobin ± 0,5 g/dL**

   V1 x N1 = V2 x N2

   500 µl x 0,5 g/dL = V2 x 17,02 g/dL

   15 µl = V2

   Dipipet 15 µl hemolisat + 485 µl pooled sera, kemudian dihomogenkan dan diukur kadar hemoglobinnya

4. **Pooled sera dengan kadar haemoglobin ± 0,6 g/dL**

   V1 x N1 = V2 x N2

   500 µl x 0,6 g/dL = V2 x 17,02 g/dL

   18 µl = V2

   Dipipet 18 µl hemolisat + 482 µl pooled sera, kemudian dihomogenkan dan diukur kadar hemoglobinnya

Lampiran 4. Data Primer Hasil Penelitian

Hasil Pemeriksaan Kadar Protein Total Metode Biuret pada Masing-Masing Variasi Hemoglobin dalam Sampel Normal

No Sampel	Kadar Protein Total pada Masing-Masing Variasi Kadar Hemoglobin dalam Sampel Normal				
	0	0,31	0,4	0,53	0,62
	g/dL	g/dL	g/dL	g/dL	g/dL
1	7,91	8,74	8,92	9,19	9,43
2	7,94	8,74	8,95	9,18	9,45
3	7,91	8,76	8,85	9,19	9,43
4	7,90	8,75	8,92	9,2	9,46
5	7,92	8,75	8,92	9,19	9,46
Rerata	7,92	8,75	8,91	9,19	9,45

Hasil Pemeriksaan Kadar Protein Total Metode Biuret pada Masing-Masing Variasi Hemoglobin dalam Sampel Patologis

No Sampel	Kadar Protein Total pada Masing-Masing Variasi Kadar Hemoglobin dalam Serum Patologis				
	0	0,31	0,4	0,53	0,62
	g/dL	g/dL	g/dL	g/dL	g/dL
1	4,15	5,1	5,39	5,59	5,80
2	4,16	5,08	5,37	5,61	5,78
3	4,16	5,1	5,41	5,6	5,79
4	4,14	5,12	5,39	5,57	5,78
5	4,18	5,15	5,38	5,59	5,80
Rerata	4,16	5,11	5,39	5,59	5,79

Lampiran 5. Hasil Analisis Statistik

Uji Normalitas

Uji Normalitas Sampel Normal

Kadar Hemoglobin		**Shapiro-Wilk**		
		Statistic	**df**	**Sig.**
Kadar Protein Total	Tanpa Penambahan Hemolisat 0 g/dl	0,914	5	0,492
	0,31 g/dl	0,881	5	0,314
	0,4 g/dl	0,806	5	0,091
	0,53 g/dl	0,883	5	0,325
	0,62 g/dl	0,803	5	0,086

Uji Normalitas Sampel Patologis

Kadar Hemoglobin		**Shapiro-Wilk**		
		Statistic	**Df**	**Sig.**
Kadar Protein Total	Tanpa Penambahan Hemolisat (0g/dl)	0,956	5	0,777
	0,31 g/dl	0,942	5	0,679
	0,4 g/dl	0,956	5	0,777
	0,53 g/dl	0,956	5	0,777
	0,62 g/dl	0,821	5	0,119

Uji Homogenitas

Uji Homogenitas sampel normal

Levene Statistic	df1	df2	Sig.
2,174	4	20	,109

Uji Homogenitas sampel patologis

Levene Statistic	df1	df2	Sig.
1,153	4	20	,361

Uji One Way Anova

Uji One Way Anova sampel normal

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	6,786	4	1,696	4349,779	,000
Within Groups	,008	20	,000		
Total	6,793	24			

Uji One Way Anova sampel patologis

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	8,153	4	2,038	6980,664	,000
Within Groups	,006	20	,000		
Total	8,159	24			

UJI LANJUT (*Post Hoc*)

Uji post hoc sampel normal

(I) Kadar Hemoglobin	(J) Kadar Hemoglobin	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
0 g/dl	0,31 g/dl	-,83200*	,01249	,000	-,8581	-,8059
	0,4 g/dl	-,99600*	,01249	,000	-1,0221	-,9699
	0,53 g/dl	-1,27400*	,01249	,000	-1,3001	-1,2479
	0,62 g/dl	-1,53000*	,01249	,000	-1,5561	-1,5039
0,31 g/dl	0 g/dl	,83200*	,01249	,000	,8059	,8581
	0,4 g/dl	-,16400*	,01249	,000	-,1901	-,1379
	0,53 g/dl	-,44200*	,01249	,000	-,4681	-,4159
	0,62 g/dl	-,69800*	,01249	,000	-,7241	-,6719
0,4 g/dl	0 g/dl	,99600*	,01249	,000	,9699	1,0221
	0,31 g/dl	,16400*	,01249	,000	,1379	,1901
	0,53 g/dl	-,27800*	,01249	,000	-,3041	-,2519
	0,62 g/dl	-,53400*	,01249	,000	-,5601	-,5079

0,53 g/dl	0 g/dl	1,27400*	,01249	,000	1,2479	1,3001
	0,31 g/dl	,44200*	,01249	,000	,4159	,4681
	0,4 g/dl	,27800*	,01249	,000	,2519	,3041
	0,62 g/dl	-,25600*	,01249	,000	-,2821	-,2299
0,62 g/dl	0 g/dl	1,53000*	,01249	,000	1,5039	1,5561
	0,31 g/dl	,69800*	,01249	,000	,6719	,7241
	0,4 g/dl	,53400*	,01249	,000	,5079	,5601
	0,53 g/dl	,25600*	,01249	,000	,2299	,2821

*. The mean difference is significant at the 0.05 level.

Uji post hoc sampel patologis

Multiple Comparisons

Dependent Variable: Kadar Protein Total

LSD

(I) Kadar Hemoglobin	(J) Kadar Hemoglobin	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 g/dl	0,31 g/dl	-,95200*	,01081	,000	-,9745	-,9295
	0,4 g/dl	-1,23000*	,01081	,000	-1,2525	-1,2075
	0,53 g/dl	-1,43400*	,01081	,000	-1,4565	-1,4115
	0,62 g/dl	-1,63200*	,01081	,000	-1,6545	-1,6095
0,31 g/dl	0 g/dl	,95200*	,01081	,000	,9295	,9745
	0,4 g/dl	-,27800*	,01081	,000	-,3005	-,2555
	0,53 g/dl	-,48200*	,01081	,000	-,5045	-,4595
	0,62 g/dl	-,68000*	,01081	,000	-,7025	-,6575
0,4 g/dl	0 g/dl	1,23000*	,01081	,000	1,2075	1,2525
	0,31 g/dl	,27800*	,01081	,000	,2555	,3005
	0,53 g/dl	-,20400*	,01081	,000	-,2265	-,1815
	0,62 g/dl	-,40200*	,01081	,000	-,4245	-,3795
0,53 g/dl	0 g/dl	1,43400*	,01081	,000	1,4115	1,4565
	0,31 g/dl	,48200*	,01081	,000	,4595	,5045
	0,4 g/dl	,20400*	,01081	,000	,1815	,2265
	0,62 g/dl	-,19800*	,01081	,000	-,2205	-,1755
0,62 g/dl	0 g/dl	1,63200*	,01081	,000	1,6095	1,6545
	0,31 g/dl	,68000*	,01081	,000	,6575	,7025
	0,4 g/dl	,40200*	,01081	,000	,3795	,4245
	0,53 g/dl	,19800*	,01081	,000	,1755	,2205

*. The mean difference is significant at the 0.05 level.

Uji Korelasi

Uji korelasi sampel normal

Correlations

		Kadar Protein Total	Kadar Hemoglobin
Kadar Protein Total	Pearson Correlation	1	,950**
	Sig. (2-tailed)		,000
	N	25	25
Kadar Hemoglobin	Pearson Correlation	,950**	1
	Sig. (2-tailed)	,000	
	N	25	25

**. Correlation is significant at the 0.01 level (2-tailed).

Uji korelasi sampel patologis

Correlations

		Kadar Hemoglobin	Kadar Protein Total
Kadar Hemoglobin	Pearson Correlation	1	,927**
	Sig. (2-tailed)		,000
	N	25	25
Kadar Protein Total	Pearson Correlation	,927**	1
	Sig. (2-tailed)	,000	
	N	25	25

**. Correlation is significant at the 0.01 level (2-tailed).

Uji Regresi Linier

Uji regresi linier sampelnormal

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,950[a]	,903	,898	,16958

Coefficients

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	7,792	,080		97,958	,000
	Kadar Hemoglobin	,350	,024	,950	14,602	,000

a. Dependent Variable: Kadar Protein Total

Uji regresi linier sampel patologis

Model Summaryb

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,927[a]	,860	,854	,22293

Coefficientsa

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	4,084	,105		39,057	,000
	Kadar Hemoglobin	,375	,032	,927	11,882	,000

a. Dependent Variable: Kadar Protein Total

Lampiran 6. Kit Insert

BIOLABO
www.biolabo.fr
MANUFACTURER:
BIOLABO SAS,
Les Hautes Rives
02160, Maizy, France

TOTAL PROTEIN Biuret Method

Reagent for quantitative determination of total protein in human serum or plasma

REF 80016: R1 1 x 500 mL R2 1 x 500 mL
R3 1 x 5 mL

TECHNICAL SUPPORT AND ORDERS
Tel : (33) 03 23 25 15 50
Fax: (33) 03 23 256 256

CE

IVD IN VITRO DIAGNOSTIC USE

CLINICAL SIGNIFICANCE (1)

The overall composition of a patient's plasma or serum should be studied first by determining its total protein content and then its composition by electrophoresis.
Decrease in the volume of plasma water (hemoconcentration), noted in dehydratation (severe vomiting, diarrhea, Addison's disease, or diabetic acidosis), is reflected as relative hyperproteinemia. Hemodilution (increase in plasma water volume) occuring with water intoxication or salt retention syndromes, during massive intravenous infusions, and physiologically when a recombant position is assumed, is reflected as relative hypoproteinemia. Hypoproteinemia due to low levels of albumine in plasma is also common and has many causes. Mild hyperproteinemia may be caused by an increased in the concentration of specific proteins (infection). Marked hyperproteinemia may be caused by high levels of monoclonal immunoglobulins produced in multiple myelomia and other malignant paraproteinemias.

PRINCIPLE (4) (5)

Colorimetric method described by Gornall and al. The peptide bonds of proteins react with Cu^{2+} in alkaline solution to form a coloured complex which absorbance, proportional to the concentration of total protein in the specimen, is measured at 550 nm. The biuret reagent contains sodium potassium tartrate to complex cupric ions and maintains their solubility in alkaline solution.

REAGENTS

Vial R1 **SODIUM CHLORIDE**

Sodium chloride	75	mmol/L

Vial R2 **BIURET REAGENT**

Sodium hydroxide	370	mmol/L
Na-K Tartrate	10	mmo/L
Potassium iodide	3	mmol/L
Copper II sulfate	3	mmol/L

Before dilution: Corrosive, R35: Causes severe burns
Once diluted: Xi, R36/37/38: Irritating to eyes, respiratory system and skin. S36/37/39: Wear suitable protective clothing, gloves and eyes/face protection

Vial R3 **STANDARD** Bovine Albumin 6 g/dL

SAFETY CAUTIONS

BIOLABO reagents are designated for professional, in vitro diagnostic use.

- Verify the integrity of the contents before use.
- Use adequate protections (overall, gloves, glasses).
- Do not pipette by mouth.
- Contents of vial R2 remains irritating after dilution (R34: causes burns).
- In case of contact with skin and eyes, thoroughly wash affected areas with plenty of water and seek medical advice.
- Reagents contain sodium azide (concentration < 0.1%) which may react with copper and lead plumbing. Flush with plenty of water when disposing.
- Material Safety Data Sheet is available upon request.
- Waste disposal: Respect legislation in force in the country.

All specimens should be handled as potentially infectious, in accordance with good laboratory practices using appropriate precautions. Respect legislation in force in the country.

REAGENTS PREPARATION

Fill to the top of the vial with demineralised water to complete content of vial R1 (NaCl) and vial R2 (Biuret). Mix by swirling. Diluted reagents are ready for use.

STABILITY AND STORAGE

On receipt, store Standard (vial R3) at 2-8°C.

- Standard (vial R3): transfer the requested quantity, well recap the vial and store at 2-8°C.
- Stored and used as described, reagents (vial R1, R2, R3) are stable in well recapped original vial, and without contamination, upon expiry date stated on the label of the kit.
- Once opened, working reagents (ready for use) are stable stored at 18-25°C and away from light at least for 6 months.

Discard any reagent if cloudy or if the absorbance of the prediluted mixture (1V/1V) of vials R1 and R2 is > 0.050 at 550 nm.

Don't use working reagents after expiry date stated on the label of the kit.

SPECIMEN COLLECTION AND HANDLING (2)

Serum or plasma. Analyse fresh or store at 2-8°C less than 72 h.

Total protein in serum is stable for:
✓ 6 months at –20°C
✓ indefinitely at -70°C.

INTERFERENCES (3)

Tests results with Procedure n°1:

Glucose:	No interference up to 11 g/L
Ascorbic Acid:	No interference up to 250 mg/L
Total Bilirubin:	No interference up to 550 µmol/L
Haemoglobin:	Positive interference above 150 µmol/L
Lipemia:	Positive interference above 0.150 abs (measured at 600nm)

Lipemia or hemolysis may cause falsely elevated results. It is recommended to perform a specimen blank to prevent these interferences (see § **MANUAL PROCEDURE**: Procedure n°2).
For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Basic medical analysis laboratory equipment.
2. Normal and pathological control sera.

CALIBRATION (5)

- Standard (vial R3) enclosed in the Kit or BIOLABO Multicalibrator, REF 95015 traceable to SRM927d.
- Or any calibrator traceable to a reference method or material.

The calibration frequency depends on proper instrument functions and on the preservation of the reagent.

It is recommended to calibrate in the following cases:
1. When changing vial of reagent.
2. After maintenance operations on the instrument.
3. When control values are out of range, even after using a new vial of fresh serum.

QUALITY CONTROL

- BIOLABO EXATROL-N Level I REF 95010.
- BIOLABO EXATROL-P Level II REF 95011.
- Other assayed control sera referring to the same method.
- External quality control program.

It is recommended to control in the following cases:
- At least once a run.
- At least once within 24 hours.
- When changing vial of reagent.
- After maintenance operations on the instrument.

If control is out of range, apply following actions:
1. Repeat the test with the same control.
2. If control is still out of range, prepare a fresh control serum and repeat the test.
3. If control is still out of range, use a new vial of calibrator or a fresh calibrator and repeat the test.
4. If control is still out of range, calibrate with a new vial of reagent.
5. If control is still out of range, please contact BIOLABO technical support or your local Agent.

EXPECTED VALUES (2)

In serum or plasma

Total Protein	(g/dL)
In cord	4.8-8.0
Premature	3.6-6.0
Newborn	4.6-7.0
1 week	4.4-7.6
7 months-1 year	5.1-7.3
1 year-2 years	5.6-7.5
≥ 3 years	6.0-8.0
Adult, ambulatory	6.4-8.3
Adult, recombent	6.0-7.8
≥ 60 years	Lower by 0.2

Each laboratory should establish its own normal ranges for the population that it serves.

PERFORMANCES

Within run n = 20	*Low level*	*Normal level*
Mean g/dL	4.88	5.45
S.D. g/dL	0.06	0.06
C.V. %	1.2	1.2

Between run n = 20	*Low level*	*Normal level*
Mean g/dL	6.92	7.45
S.D. g/dL	0.11	0.13
C.V. %	1.6	1.7

Detection limit: approximately 0.21 g/dL.

Sensitivity for 1 g/dL: approximately 0.028 Abs. at 550 nm.

Comparison study with commercially available reagent (Biuret method)

93 specimens (sera) within 3 g/dL and 11 g/dL are assayed with 2 methods

Y (g/dL) = 0.9758 x + 0.14819 r = 0.9879

LINEARITY

The assay is linear up to 10 g/dL. Above, dilute the specimen with saline solution and reassay taking into account dilution factor to calculate the result. Linearity limit depends on specimen/reagent ratio

MANUAL PROCEDURE

Procedure n°1 (without Specimen blank)

Let stand reagents and specimens at room temperature.

Pipette into well identified test tubes:	Blank	Standard	Assay
Reagent R1	1.02 mL	1 mL	1 mL
Reagent R2	1 mL	1 mL	1 mL
Standard		20 µL	
Specimen			20 µL

Mix well. Let stand for 10 minutes at room temperature.
Record absorbance at 550 nm (530-570) against reagent blank.

Procedure n°2 (with Specimen blank)

Pipette into well identified test tubes	Blank	Specimen Blank	Standard	Assay
Reagent R1	1.02 mL	2 mL	1 mL	1 mL
Reagent R2	1 mL		1 mL	1 mL
Standard			20 µL	
Specimen		20 µL		20 µL

Mix well. Let stand for 10 minutes at room temperature.
Record absorbance at 550 nm (530-570) against reagent blank. Read specimen blank against reagent R1.

Notes:
- ✓ Specific procedures are available upon request for automated instruments. Please contact BIOLABO technical support.
- ✓ Specimen blank is recommended for cloudy, lipemic or hemolysed serum.
- ✓ Caution: Target values of control sera or multicalibrator may have been obtained with or without specimen blank.
- ✓ Bichromatic analyser: The 2nd wavelength is 600 or 700 nm.

CALCULATION

Calculate the result as follows:

Without specimen blank:

$$\text{Result} = \frac{\text{Abs (Assay)}}{\text{Abs (Standard)}} \times \text{Standard concentration}$$

With specimen blank:

$$\text{Result} = \frac{\text{Abs (Assay)} - \text{Abs (Specimen blank)}}{\text{Abs (Standard)}} \times \text{Standard concentration}$$

REFERENCES

(1) *TIETZ N.W. Text book of clinical chemistry, 2nd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders (1999) p. 477-530.*
(2) *Clinical Guide to Laboratory Test, 4th Ed., N.W. TIETZ (2000) p. 916-917.*
(3) *YOUNG D.S., Effect of Drugs on Clinical laboratory Tests, 4th Ed. (1995) p. 3-498 à 3-511*
(4) *GORNALL A. C., BARDAWILL C. J., DAVID M. M., J. Biol. Chem. 1949, 177, 751*
(5) *TIETZ N.W. Text book of clinical chemistry, 3rd Ed. C.A. Curtis, E.R. Silverman L. M., Christensen R. H. (1995) p. 523-524.*
(6) *SRM: Standard reference Material ®*

Manufacturer | Use by | In vitro diagnostic | Temperature limitation | Catalogue number | See insert | Batch number | Store away from light | sufficient for | dilute with

Made in France Latest revision : www.biolabo.fr Revision : 26/07/2011

Hemoglobin Electrophoresis Procedure

Helena's Hemoglobin Electrophoresis Procedure, using cellulose acetate in alkaline buffer, is intended for the qualitative and quantitative determination of abnormal hemoglobins.

SUMMARY

Hemoglobins (Hb) are a group of proteins whose chief functions are to transport oxygen from the lungs to the tissues and carbon dioxide in the reverse direction. They are composed of polypeptide chains called globin, and iron protoporphyrin heme groups. A specific sequence of amino acids constitutes each of four polypeptide chains. Each normal hemoglobin molecule contains one pair of alpha and one pair of non-alpha chains. In normal adult hemoglobin (HbA), the non-alpha chains are called beta. The non-alpha chains of fetal hemoglobin are called gamma. A minor (3%) hemoglobin fraction called HbA_2 contains alpha and delta chains. In a hereditary inhibition of globin chain synthesis called thalassemia, the non-alpha chains may aggregate to form HbH (β_4) or Hb Bart's (α_4).

The major hemoglobin in the erythrocytes of the normal adult is HbA and there are small amounts of HbA_2 and HbF. In addition, over 400 mutant hemoglobins are now known, some of which may cause serious clinical effects, especially in the homozygous state or in combination with another abnormal hemoglobin. Wintrobe[1] divides the abnormalities of hemoglobin synthesis into three groups:

(1) Production of an abnormal protein molecule (e.g. sickle cell anemia)
(2) Reduction in the amount of normal protein synthesis (e.g. thalassemia)
(3) Developmental anomalies (e.g. hereditary persistence of fetal hemoglobin (HPFH)

The two mutant hemoglobins most commonly seen in the United States are HbS and HbC. Hb Lepore, HbE, HbG-Philadelphia, HbD- Los Angeles, and HbO-Arab may be seen less frequently.[2]

Electrophoresis is generally considered the best method for separating and identifying hemoglobinopathies. One protocol for hemoglobin electrophoresis involves the use of two systems.[1,6] Initial electrophoresis is performed in alkaline buffers. Cellulose acetate is the major support medium used because it yields rapid separation of HbA, F, S and C and many other mutants with minimal preparation time. However, because of the electrophoretic similarity of many structurally different hemoglobins, the evaluation must be supplemented by a procedure that measures some other property. A simple procedure which confirms the identification of both HbS and HbC, as well as HbA, HbF and many other mutants, is citrate agar electrophoresis. This method is based on the complex interactions of the hemoglobin with the electrophoretic buffer (acid pH) and the agar support.

Electrophoresis is a simple procedure requiring only minute quantities of hemolysate to provide highly specific (but not absolute) confirmation of the presence of HbS, HbC and HbF as well as several other abnormal hemoglobins.

PRINCIPLE

Very small samples of hemolysates prepared from whole blood are applied to the Titan III® Cellulose Acetate Plate. The hemoglobins in the sample are separated by electrophoresis using an alkaline buffer (pH 8.2-8.6), and are stained with Ponceau S Stain. The patterns are scanned on a scanning densitometer, and the relative percent of each band determined.

REAGENTS

1. **Supre-Heme® Buffer (Cat. No. 5802)**
   Ingredients: The buffer contains Tris-EDTA and boric acid.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. NEVER PIPETTE BY MOUTH. DO NOT INGEST. Ingestion of sufficient quantities of boric acid and EDTA can be toxic.
   Preparation for Use: Dissolve one package of buffer in 980 mL deionized water. The buffer is ready for use when all material is dissolved and completely mixed.
   Storage and Stability: The packaged buffer should be stored at 15 to 30°C and is stable until the expiration date indicated on the package and box. The buffer solution is stable two months when stored at 15 to 30°C.
   Signs of Deterioration: Do not use packaged buffer if the material shows signs of dampness or discoloration. Discard the buffer solution if it shows signs of bacterial contamination.
2. **Hemolysate Reagent (Cat. No. 5125)**
   Ingredients: The reagent contains 0.005 M EDTA in deionized water with 0.07% potassium cyanide added as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT PIPETTE BY MOUTH. The reagent contains a small amount of potassium cyanide.
   Preparation for Use: The reagent is ready to use as packaged.
   Storage and Stability: The reagent should be stored at 15 to 30°C and is stable until the expiration date indicated on the bottle.
   Signs of Deterioration: The reagent should be clear and colorless.
3. **Ponceau S Stain (Cat. No. 5526)**
   Ingredients: The reconstituted stain is 0.5% (w/v) Ponceau S in an aqueous solution of 10% (w/v) sulfosalicylic acid.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST HARMFUL IF SWALLOWED.
   Preparation for Use: Dissolve one vial of stain in 1 L of deionized water.
   Storage and Stability: The stain should be stored at 15 to 30°C and is stable until the expiration date indicated on the container. It may be stored in the bottle or in a tightly closed staining dish and may be reused multiple times if properly stored.
   Signs of Deterioration: Do not use the stain solution if excessive evaporation occurs, or if large amounts of precipitate appear.
4. **Clear Aid (Cat. No. 5005)**
   Ingredients: The reagent contains polyethylene glycol.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST.
   Preparation for Use: Clear Aid is used as the clearing solution which is prepared as follows:
   30 parts glacial acetic acid
   70 parts absolute methanol
   4 parts Clear Aid
   Storage and Stability: Store the prepared clearing solution at 15 to 30°C in a tightly closed container to prevent evaporation of the methanol. When evaporation occurs, the plates may delaminate. Water contamination from over-use of the clearing solution will cause the plate to be cloudy. The reagent is stable until the expiration date indicated on the bottle.
   Signs of Deterioration: Clear Aid should be a clear, colorless liquid, although it may appear cloudy when cold. Do not use the material upon evidence of gross contamination or discoloration. Discard the prepared Clear Aid if plates appear cloudy after the clearing procedure.
5. **PermaClear Solution (Cat. No. 4950) - Optional**
   Ingredients: N-methyl pyrrolidinone and PEG.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE - IRRITANT - DO NOT PIPPETTE BY MOUTH. VAPOR HARMFUL. In case of contact, flush affected areas with copious amounts of water. Get immediate attention for eyes.
   Preparation for Use: Add 55 mL PermaClear to 45 mL deionized water and mix well.
   Storage and Stability: PermaClear should be stored at 15 to 30°C and is stable until the expiration date on the bottle.
   Signs of Deterioration: Discard the PermaClear Solution if the plates turn white and do not clear as expected.
6. **Titan III-H Plates (Cat. No. 3021, 3022)**
   Ingredients: Cellulose acetate plates.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE.
   Preparation for Use: The plates are ready for use as packaged.
   Storage and Stability: The plates should be stored at 15 to 30°C and are stable indefinitely.

INSTRUMENTS

Any high quality scanning densitometer capable of scanning a cleared cellulose acetate plate at 525 nm may be used. Recommended is the Helena Quickscan 2000.

SPECIMEN COLLECTION AND HANDLING

Specimen: Whole blood collected in tubes containing EDTA or heparin is the specimen of choice.

Specimen Preparation: Specimen hemolysates are prepared as outlined in the STEP-BY-STEP METHOD.

Specimen Storage and Stability: Whole blood samples may be stored up to one week at 2 to 6°C.

PROCEDURE

Materials Provided: The following materials needed for the procedure are available from Helena Laboratories.

Hardware	**Cat. No.**
Super Z-12 Applicator Kit (12 samples)	4093
Super Z Applicator Kit (8 samples)	4088
Microdispenser and Tubes	6008
1000 Staining Set	5122
Bufferizer	5093
Titan Plus Power Supply	1504
Consumables	
Titan® III-H Cellulose Acetate (94 mm x 76 mm)-12 samples	3021
Titan® Cellulose Acetate (76 mm x 60 mm)-8 samples	3022
Supre-Heme Buffer	5802
Hemo AFSA Control	5330
Hemo AA Control	5328
Hemo AFSC Control	5331
Hemo ASA Control	5329
Hemolysate Reagent	5125
Ponceau S	5526
Clear Aid	5005
Titan Blotter Pads	5034
Zip Zone® Prep	5090
Titan Plastic Envelopes	5052
Helena Marker	5000
Identification Labels	5006
Zip Zone® Chamber Wicks	5081
Glue Stick	5002
PermaClear	4950

Materials Needed, but not Provided:

Glacial acetic acid

Absolute methanol

5% acetic acid – Mix 5 parts of glacial acetic acid with 95 parts deionized water.

SUMMARY OF CONDITIONS

Plate	Titan® III-H
Buffer	Supre-Heme® dissolved in 980 mL deionized water
Soaking Time for Plates	5 minutes
Sample Size (hemolysate)	5 µL
Number of Applications	One (1)
Electrophoresis Time	25 minutes
Voltage	350 volts
Staining Time (total)	20 minutes
Drying Time	10 minutes at 56°C
Scanning Wavelength	525 nm

STEP BY STEP METHOD

A. Preparation of the Titan® III-H Plate

1. Dissolve one package Supre-Heme® Buffer in 980 mL deionized water.
2. Properly code the required number of Titan® III-H Plates by marking on the glossy hard side with a marker.
3. Soak the required number of plates in Supre-Heme® Buffer for 5 minutes. The plates should be soaked in the bufferizer according to the instructions provided. Alternately, the plates may be wetted by slowly and uniformly lowering a rack of plates into the buffer.

The same soaking buffer may be used for soaking up to 12 plates or for approximately one week if stored tightly closed. If used for a prolonged period, residual solvents from the plates may build up in the buffer and cause poor separation of the proteins or evaporation may cause greater buffer concentration.

B. Preparation of Zip Zone® Chamber

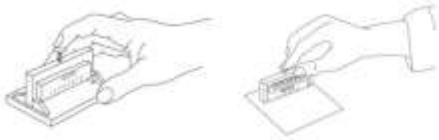
1. Pour approximately 100 mL of Supre-Heme® Buffer into each of the outer sections of the Zip Zone® Chamber.
2. Wet two chamber wicks in the buffer and drape one over each support bridge being sure it makes contact with the buffer and that there are no air bubbles under the wicks.

3. Cover the chamber to prevent buffer evaporation. Discard the buffer and wicks after use.

C. Sample Preparation and Application

1. Prepare a hemolysate of the patient samples as follows:
   a. Using whole blood: Add 1 part whole blood to 3 parts Hemolysate Reagent. Mix well and allow to stand 5 minutes.
   b. Using packed cells: Mix 1 part packed red blood cells to 6 parts Hemolysate Reagent. Mix well and allow to stand 5 minutes. NOTE: If removal of denatured hemoglobins from the sample is deemed necessary, see the **Alternate Sample Preparation Procedure.**
2. Place 5 µL of the patient hemolysates or 5 µL of the Hemo Controls into the wells of the Sample Well Plates using the Microdispenser. Do not prepare a hemolysate of the Hemo Controls.

3. To prevent evaporation, cover the Sample Well Plate with a glass slide, if the samples are not used within 2 minutes.
4. Prime the applicator by depressing the tips into the sample wells 3 or 4 times. Apply this loading to a piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load applicator again at this point, but proceed quickly to the next step.



5. Remove the wetted Titan® III Plate from the buffer with the fingertips and blot once firmly between two blotters. Place the plate in the aligning base, cellulose acetate side up, aligning the top edge of the plate with the black scribe line marked "CATHODE APPLICATION". The identification mark should be aligned with sample No. 1. Before placing the plate in the aligning base, place a drop of water or buffer on the center of the aligning base. This prevents the plate from shifting during the sample application.

6. Apply the sample to the plate by depressing the applicator tips into the sample well 3 or 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it 5 seconds.

Alternate Sample Preparation Procedure:

If removal of denatured hemoglobins from the sample is deemed necessary, perform the following steps:

a. Centrifuge the blood sample at 3500 RPM for 5 minutes.
b. Remove the plasma from the sample and wash the red blood cells in 0.85% saline (w/v) three times. After each wash, centrifuge the cells for 10 minutes at 3500 RPM.
c. Add 1 volume deionized water and 1/4 volume toluene (or carbon tetrachloride) to the washed red cells. Vortex at high speed for one minute. Centrifuge the samples at 3500 RPM for 10 minutes.
d. If toluene is used, the top layer in the tube will contain cell stroma and should be removed with a capillary pipette before proceeding to the next step. The clear middle layer contains the desired sample. If carbon tetrachloride is used, all red cell waste material will be contained in the bottom of the tube after centrifugation.
e. Filter the clear red solution through two layers of Whatman #1 filter paper.

D. Electrophoresis of Sample Plate

1. Quickly place the plate in the elecrophoresis chamber, cellulose acetate side down, such that the sample end is toward the cathodic(-) side of the chamber. Place a weight (glass slide, etc.) on the plate to insure contact with the wicks.

2. Place the cover on the chamber, and electrophorese the plate for 25 minutes at 350 volts.

E. Staining the Hemoglobin Bands

1. Remove the plates from the electrophoresis chamber and stain in Ponceau S for 5 minutes.
2. Destain in 3 successive washes of 5% acetic acid. Allow the plates to stay in each wash 2 minutes or until the background is white.
3. The plates may be dried and stored for a permanent record at this point. If a transparent background is desired for densitometry, proceed to the next step.

If using Clear Aid Solution:

4. Dehydrate, by washing the plate twice in absolute methanol, for two minutes each wash. Allow the plate to drain for 5-10 seconds before placing in the next solution.
5. Place the plate into the Clear Aid solution for 5-10 minutes.
6. Drain off excess solution. Then place the plate, acetate side up, onto a blotter, and into an I. O. D., Micro-Hood, or other drying oven at 50-60°C for 15 minutes or until dry.

If using PermaClear Solution:

4. Place the plate(s) into the diluted PermaClear clearing solution for 2 minutes.
5. Drain off excess solution by holding plate(s) vertically for 1 minute. Then place the plate, acetate side up, onto a blotter, and into an I. O. D., or other drying oven at 50-60°C for 15 minutes or until dry.

F. Evaluation of the Hemoglobin Bands

1. Qualitative evaluation: The hemoglobin plates may be inspected visually for the presence of abnormal hemoglobin bands. The Helena Hemo Controls provide a marker for band identification.
2. Quantitative evaluation: Determine the relative percent of each hemglobin band by scanning the cleared and dried plates in the densitometer using a 525 nm filter.

Stability of End Product: The dried plates are stable for an indefinite period of time, and may be stored in Titan Plastic Envelopes.

Calibration: A calibration curve is not necessary because relative concentration of the bands is the only parameter determined.

Quality Control: Four controls for hemoglobin electrophoresis are available from Helena Laboratories: AA_2 Hemo Control (Cat. No. 5328), ASA_2 Hemo Control (Cat. No. 5329), $AFSA_2$ Hemo Control (Cat. No.5330), and AFSC Hemo Control (Cat. No. 5331). The controls should be used as markers for the identification of the hemoglobin bands, and they may be quantitated for verification of the accuracy of the procedure. Refer to the package insert provided with the controls for assay values and migration patterns. Use at least one of these controls on each plate run.

RESULTS

Figures 1 illustrates how the combination of cellulose acetate and citrate agar electrophoresis can be used in tandem for the identification of hemoglobins. Figure 2 lists the relative mobilities of various hemoglobin mutants on cellulose acetate and citrate agar plates.

Calculation of Unknown: The QuickScan 2000 will automatically print the relative percent and the absolute values for each band. Alternately, the relative percent of each band can be calculated manually by referring to the Operator's Manual provided with the densitometer. The relative percent of each band is calculated by the following formula:

No. Integration Units of the Band / Total Integration of Units	x	100	=	Relative Percent of the Band

Relative Percent of the Band	x	Total Hemoglobin	=	Absolute Value of Protein per band

LIMITATIONS

Some abnormal hemoglobins have similar electrophoretic mobilities and must be differentiated by other methodologies.

Further testing required:

1. Citrate agar electrophoresis may be a necessary follow-up test for confirmation of abnormal hemoglobins detected on cellulose acetate.
2. Isoelectric focusing, high performance liquid chromatography, globin chain analysis (both acid and alkaline) and structural studies may be necessary in order to positively identify some of the more rare hemoglobins.
3. Low levels of HbF (1-10%) may be accurately quantitated using any commercially available HbF method.

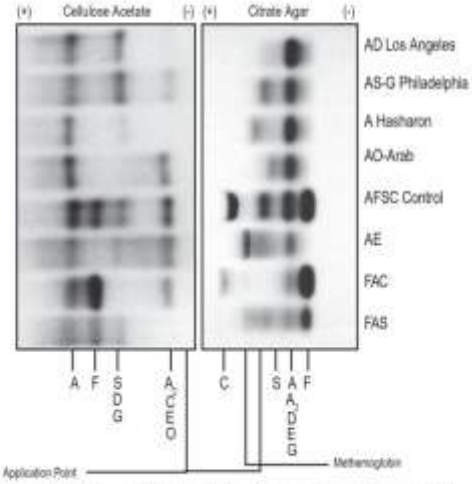
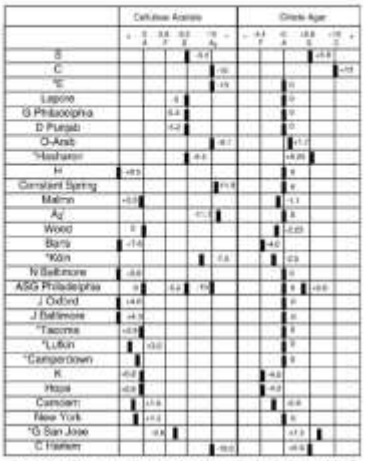


Figure 1. Electrophoretic Mobilities of Hemoglobins on Titan® III Cellulose Acetate and on Titan® IV Citrate Agar.



O Arab - Migration varies on citrate agar from Hemoglobin A through Hemoglobin S.
J Baltimore - Trait is approximately 60% of the time.
J Oxford - Trait is approximately 25% of the time.
*Unstable hemoglobin
D Los Angeles and D Punjab are the same hemoglobin.
C Harlem and Georgetown are the same hemoglobin.
Köln is broadly entrudgen on both media possibly due to instability.

Figure 2. Relative Electrophoretic Mobilities of Hemoglobins on Cellulose Acetate and Citrate Agar.[11]

REFERENCE VALUES

At birth, the majority of hemoglobin in the erythrocytes of the normal individual is fetal hemoglobin, HbF. Some of the major adult hemoglobin, HbA, and a small amount of HbA_2, are also present. At the end of the first year of life and through adulthood, the major hemoglobin present is HbA with up to 3.5% HbA_2 and less than 2% HbF.

INTERPRETATION OF RESULTS

Most hemoglobin variants cause no discernible clinical symptoms, so are of interest primarily to research scientists. Variants are clinically important when their presence leads to sickling disorders, thalassemia syndromes, life long cyanosis, hemolytic anemias or erythrocytosis, or if the heterozygote is of sufficient prevalence to warrant genetic counseling. The combinations of HbS-S, HbS-D-Los Angeles, and HbS-O Arab lead to serious sickling disorders.[7] Several variants including HbH, HbE-Fort Worth and Hb Lepore cause a thalassemic blood picture.[7] The two variant hemoglobins of greatest importance in the U.S., in terms of frequency and pathology are HbS and HbC.[2] Sickle cell anemia (HbSS) is a cruel and potentially lethal disease. It first manifests itself at about 5-6 months of age. The clinical course presents agonizing episodes of pain and temperature elevations with anemia, listlessness, lethargy, and infarct in virtually all organs of the body. The individual with homozygous HbCC suffers mild hemolytic anemia which is attributed to the precipitation or crystallization of HbC within the erythrocytes. Cases of HbSC disease are characterized by hemolytic anemia that is milder than sickle-cell anemia. The thalassemias are a group of hemoglobin disorders characterized by hypochromia andmicrocytosis due to the diminished synthesis of one globin chain (the α or β) while synthesis of the other chain proceeds normally.[8-10] This unbalanced synthesis results in unstable globin chains. These precipitate within the red cell, forming inclusion bodies that shorten the lifespan of the cell. In α-thalassemias, the α-chains are diminished or absent. In β-thalassemia, the β-chains are affected.

Another quantitative disorder of hemoglobin synthesis, hereditary persistent fetal hemoglobin (HPFH), represents a genetic failure of the mechanisms that turn off gamma chain synthesis at about four months after birth, which results in a continued high percentage of HbF. It is a more benign condition than the true thalassemias, and persons homozygous for HPFH have normal development, are asymptomatic and have no anemia.[10]

Shaded areas indicate that the text has been modified, added or deleted.

The most common hemoglobin abnormalities:

Sickle Cell Trait

This is a heterozygous state showing HbA and HbS, and a normal amount of HbA_2 on cellulose acetate. Results on citrate agar show hemoglobins in the HbA and HbS migratory positions (zones).

Sickle Cell Anemia

This is a homozygous state showing almost exclusively HbS, although a small amount of HbF may also be present.

Sickle-C Disease

This is a heterozygous state demonstrating HbS and HbC.

Sickle Cell-Thalassemia Disease

This condition shows HbA, HbF, HbS, and HbA_2. In Sickle Cell β-Thalassemia HbA is absent.

Thalassemia-C Disease

This condition shows HbA, HbF and HbC.

C Disease

This is a homozygous state showing almost exclusively HbC.

Thalassemia Major

This condition shows HbF, HbA and HbA_2.

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For Sales and Order Information, and Technical or Service Assistance, call 800-231-5663 toll free.

HELENA LABORATORIES

Pro. 15
6/17/07

Lampiran 7. Lembar Bimbingan

POLITEKNIK KESEHATAN BANDUNG

LEMBAR BIMBINGAN KARYA TULIS ILMIAH

NAMA : Sucityanti

NIM : P17331117013

NAMA PEMBIMBING : Dr. Dra. Ani Rusyani, M.Kes

NO	MATERI BIMBINGAN	WAKTU	TTD PEMBIMBING
1.	Konfirmasi judul dan bimbingan Bab 1	26 - December 2019.	
2.	Konsultasi uji Pendahuluan.	8 Januari 2020	
3.	Revisi Bab 2 dan 3	8 Januari 2020	
4	Konsultasi uji Pendahuluan.	9 Januari 2020	
5.	Revisi Bab 1, 2, dan 3.	11 Januari 2020	
6	Konsultasi UP, Revisi Bab 3.	29 Januari 2020	
7.	Konsultasi hasil UP. Diskusi hemolisis.	31 Januari 2020	
8	Revisi proposal, konsultasi Penelitian	19 Februari 2020	
9	Bimbingan Bab 4	9 Mei 2020	
10	Konsultasi Pengolahan data	10 Mei 2020	
11	Bimbingan Bab 4	6 Juli 2020	
12	Bimbingan Bab 4 dan 5	10 Juli 2020	
13	Revisi Bab 4 dan 5	18 Juli 2020	
14	Bimbingan Bab 4 dan 5	19 Juli 2020	
15	Bimbingan Bab 4 dan 5	22 Juli 2020	
16	Bimbingan Bab 4 dan 5, PPT	24 Juli 2020	

Lampiran 8. Dokumentasi

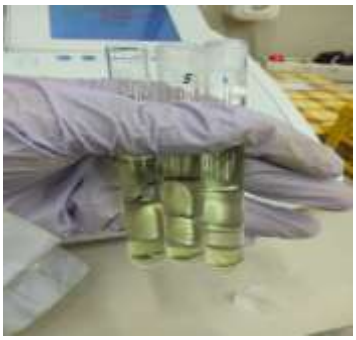
Reagen protein total

NaCl Fisiologis dan EDTA

Reagen Hemoglobin

Pooled sera

Hemolisat



Pemeriksaan Hemoglobin

Pembuatan sampel hemolisis

Pemeriksaan protein total

Pemeriksaan protein total menggunakan fotometer