The **ADHESION SLIDE** according to Dr. Bross

Instructions for the

Immunoperoxidase (PAP) Slide Assay

and for the APAAP Slide Assay

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Characteristics of the Adhesion Slide

The Dr. Bross Adhesion Slide opens up new possiblities for carrying out tests on individual cells.
Living cells may be anchored on a specially prepared glass surface on 12 bordered reaction fields. The firmly adhered cells can be tested using various methods.
The adhesion slide (Fig. 1) has two functional units:

a) the reaction fields (RF)
on whose glass surfaces the cells are anchored.

b) the hydrophobic coating
the remaining glass surface which borders the reaction fields and allows separate processing on 12 clearly separated fields.

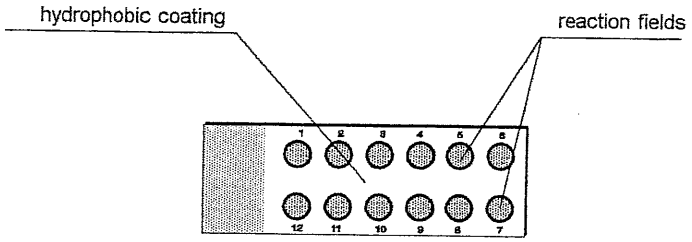

Fig.1: Adhesion Slide

Advantages of the Adhesion Slide

Cells may be studied in suspensions, on smears, or after cytocentrifugation. Compared to these methods the Dr. Bross Adhesion Slide is:

1. **space saving**
Some 12 tests may be carried out in the separate reaction fields on one single slide.

2. **material saving**
A small number of cells is required for tests on the reaction fields. The quantity of reagents required is correspondingly small (maximum 20 µl).

3. **time saving**
Incubation and washing can be carried out on the slide. This means that time consuming centrifugation and cell losses can be avoided.

4. **versatile**
Depending on the goal of the test, the anchored cells can be treated in various ways, including:
a) directly as living cells
b) fixed, eg. with glutaraldehyde for testing surface structures and antigens
c) dried and fixed for intracellular evidencing of antigens, cell morphology, immunocytochemistry

The use of adhesion slides is thus rational and cost saving.

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Application possibilities for the Adhesion Slide

Various **techniques** may be used with this slide:

a) Immunoperoxidase PAP test or comparable immunoenzyme tests
b) Immunofluorescence methods or other comparable methods for the staining of the cell surface
c) Dyeing the cells using the Pappenheim method (morphology)
d) Intracellular antigen evidencing
e) Immuncytochemical tests

For c), d) and e) the reaction fields are dried using a **cold air blower** to dry them quickly after the adhesion of the cells.
Drying medium: NKH - 0.2% BSA (9).

These instructions provide help in the evidencing of cell surface antigens and give a description of the immunoperoxidase (PAP) slide test.
Evidencing cell surface antigens is required for the following **diagnoses:**

a) immune defects (T4/T8 quotient)
b) non-Hodgkin lymphoma (monoclonality, arrangement in B or T cell lines)
c) acute and chronic leukemias
d) autoimmune system diseases
e) Hodgkin's disease
f) carcinomas

The simple principle of the slide facilitates the application and typing of **cells from various body fluids:**

a) peripheral blood
b) bone marrow
c) cerebrospinal fluid
d) lymph node aspirate
e) lymph node or tumor biopsy
f) effusion, such as pleura, ascites and pericard
g) bronchoalveolar lavages
h) cultured cells

The preparation of the Adhesion Slide

The green stain of the reaction fields (RF) is first to be dissolved under tap water, rinsed and then **cleared of any residue** with an isotonic buffer (NKH).

An invisible film is left on the reaction fields whose positive charge causes electrostatic adhesion of negatively charged cells. For this reason the reaction fields must be neither scratched with pipette tips nor allowed to dry out.
To prevent the twelve reaction fields to dry out, the processing is carried out in a **wet chamber**.
The electrostatic adhesion of the cells on the slide is so stable that the reaction fields may be washed in the cuvette or carefully with a rinsing bottle without risking any cell loss.

Visible cell adhesion is ensured:
if the cells are **vital**, i.e. undamaged,
if the washed cells are applied using a **protein-free** physiological buffer.

The hydrophobic coating of the adhesive slide around the reaction fields prevents the solutions being mixed among the different reaction fields even if the slide is shaken on a Vortex or Superior micro-mixer.

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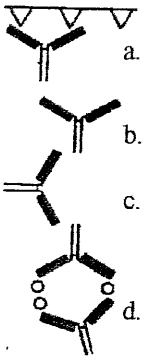
The PAP Slide Test

Sternberger Peroxidase - Anti-Peroxidase Method

The PAP test allows surface antigens to be evidenced for the identification and characterization of cells.



1. Cell isolation
2. Cell anchoring on the reaction fields
3. Fixation
4. Preincubation
5. Incubation with antisera
   a. monoclonal antibodies (mouse)
   b. Rabbit anti-mouse immunoglobulin
   c. Swine anti-rabbit immunoglobulin
   d. peroxidase - anti-peroxidase (PAP) (from rabbit)
6. Staining with diaminobenzidine (DAB)
7. Fixation with OsO_4
8. Mounting
9. Light microscopic evaluation

All steps are to be carried out at room temperature.
To prevent temperature shocks, all refrigerated solutions are to be warmed up to room temperature before starting work.
The slides are to be kept in a wet chamber.

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I. Cell isolation

A. Cell isolation from peripheral blood
The following is required for this:
10 ml EDTA blood,
Leukocyte count,
Differential count.

Direct Ficoll Hypaque method for T4/T8 determination

The following is put into a 10 ml centrifuge test tube:

1) 3 ml blood with
3 ml NKH buffer (1) diluted and underlayed with
3 ml Ficoll Hypaque. (Fig. 3)

2) Centrifugation **without brake**
2 minutes - 400 g
turn up 5 minutes - 1.500 g

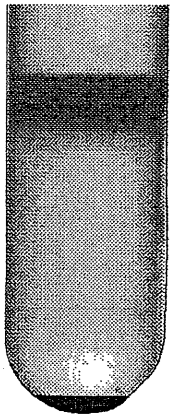


Fig. 2: Interphase after Ficoll Hypaque density centrifugation in 10 ml centrifuge test tube

3) Pipette the **interphase** (Fig. 2) (mononuclear cells) into a 2 ml Eppendorf tube

4) Wash once with NKH - 0.2% BSA (9)
Centrifugation 2 minutes - 400 g
(latex phagocytosis, see next page)

B. Cell preparations for lymphomas and leukoses

1. Allow the EDTA blood to settle for 1 to 2 hours.

2. Remove the plasma with leukocytes and thrombocytes and centrifuge it for 2 minutes at 400 g.

3. Make a smear of the cell pellet.

4. Wash once with NKH - 0.2% BSA (9) in a 2 ml Eppendorf tube
Centrifuge for 2 minutes at 400 g.

Ficoll Hypaque centrifugation

5) 1 ml cell suspension in NKH - 0.2% BSA (Fig. 3)
Underlay with 1 ml Ficoll

6) Centrifugation **without brake**
2 minutes at 400 g
turn up 5 minutes at 1.500 g.

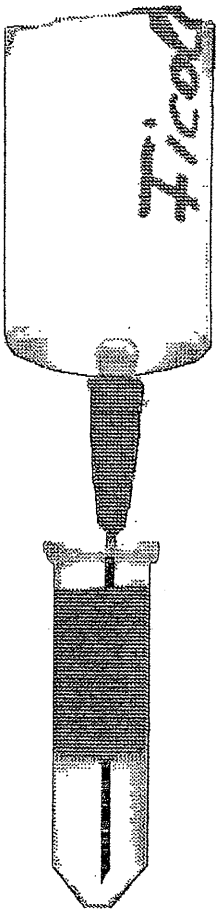


Fig. 3: Underlaying with Ficoll in 2 ml Eppendorf test tube.

7) Pipette the **interphase** (mononuclear cells) in a 2 ml Eppendorf tube.

8) Wash once with NKH-0.2% BSA (9)

9) Wash once with NKH buffer (or latex phagocytosis, see next page).

Latex phagocytosis

Monocytes may be identified by latex phagocytosis.This may be helpful for the evaluation of blood mononuclear cells.

Preincubation of the Latex beads:

1) 1 ml culture medium (RPMI or MEM)
   50 µl 22% BSA
   5 µl Latex beads
   Incubation 15 minutes in a 37°C water bath

2) Put the Latex suspension on the cell sediment, mix well and incubate for 30 minutes at 37°C.
   Wash three times with NKH buffer (see I,4)

II. Cell anchoring on the reaction field of the slide

1) Suspend the cells in approx. 0.5 ml of the NKH buffer in cases of normal leukocyte and normal lymphocyte counts in the peripheral blood or:
   adjust number of cells to 5 x 10^3 µl after cell count in a Neubauer chamber.

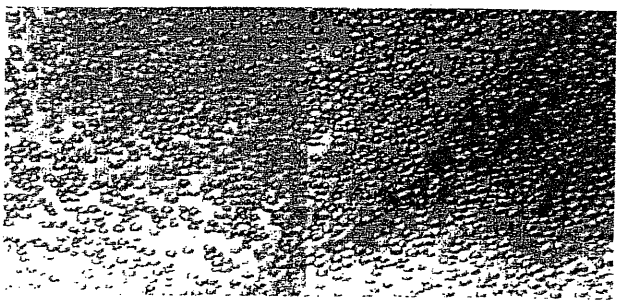
2) Apply 10 µl of cells to each reaction field of the prepared slide.

3) Check the cell density under the microscope (if available use an inversion microscope) (Fig. 4).

4) Wash off the non-adhering cells with the NKH buffer after approx. 5 minutes using a rinsing bottle.

5) Tap off the superfluous buffer.

Fig. 4: Cells seen through an inversion microscope



III. Fixation

Fixation ensures the following:

a) good preservation of the cell surface structure
b) improved adhesion of the cells
c) blockage of Fc receptors of the cell membrane
d) storage for several days at 4°C in a wet chamber

1) Apply the fixing solution 1 (6) on each reaction field. Incubation: 5 minutes.
2) Rinse the fixing solution 1 with NKH buffer (syringe)
3) Tap off as under II,5)

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IV. Preincubation

To avoid unspecific protein bindings on the glass surface and on the cells.

1) Apply the NAG medium (2) onto the reaction field (Fig. 5)

2) Shake on the vortex mixer for approx. 15 seconds (Fig. 6)

3) Incubate for at least 15-30 minutes on a wobbler or in a refrigerator overnight.

V. Incubation with antisera

Step 1: Monoclonal antibodies (Moab)

The manufacturer recommends the Moab concentration or it is found by testing (various dilutions). The sera are diluted in NAG medium (2) and kept in Mikrodel drip bottles at a temperature of 4°C (can be kept for approx. 4 weeks).

1) Draw off the buffer and drip the Moab onto each reaction field (Fig. 7)

2) Shake and incubate for 15 to 30 minutes on the wobbler.

3) Rinse with NKH buffer.

4) Dip the slides approx. 10 times in 2 cuvettes containing NKH buffer.

5) Tap off as under II,5)

Step 2: **Rabbit anti mouse immunoglobulin (RaM)**

Prepare stock solution at least one day before use (17a) (can be kept at 4°C for up to 4 weeks), test dilution: 1:500 in NAG.

1) Apply the RaM onto the reaction field

2) Shake and incubate for 5 minutes on the wobbler.

3) Rinse, see above.

Step 3: **Swine anti rabbit immunoglobulin (SaR)**

Prepare stock solution at least one day before use (17b) (can be kept for approx. 1 week at 4°C), test dilution: 1:20 in NAG.

1) Apply the SaR onto the reaction field.

2) Shake and incubate for 5 minutes on the wobbler.

3) Rinse, see above.

Step 4: **Peroxidase - anti-peroxidase (PAP)**

Make fresh dilution each day (17c), test dilution 1:20 in NAG.

1) Apply the PAP onto the reaction area.

2) Shake and incubate for 5 minutes on the wobbler.

3) Rinse, see above.

Steps 3 and 4 may be repeated
if a stronger reaction is desired.

The RaM, SaR and PAP sera are to be centrifuged thoroughly before use.
Incubation of Moab can also be carried out overnight at 4°C.

VI. Incubation with 3'3 diaminobenzidine hydrochloride (DAB)

Centrifuge the DAB thoroughly before use.
The working solution can be stored for just one day in darkness at 4°C.

1) Apply the DAB utility solution (16b)

2) Shake and incubate for 10 minutes under a vent.

3) Rinse, see above.

4) Wash in a cuvette with buffer for 5 minutes (Fig. 8).

VII. Postfixation with osmium (OsO_4)

1) Apply 10 µl OsO_4 onto each reaction field (fixation solution 2) (8) under a vent.

2) Incubate on ice for 10 minutes or longer.

3) Rinse with destilled water.

4) Tap off.

As a result of the strong blackening characteristics of osmium and its vaporisation, OsO_4 must be kept in well sealed bottles in darkness at a temperature of 4°C.
Disposable pipettes are to be used.

VIII. Mounting the slide

1) Apply 10 µl of mounting medium to each reaction field.

2) Cover the slide with a coverslip (60x24 mm).

3) Press out the air bubbles under the coverslip with a pipette tip (Fig. 9).
Avoid pushing the coverslip out of place.

4) Press the glass down with a paper towel and remove the excess mounting medium with water.

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IX. Controls

To control the reaction process both negative and positive controls are carried out:

1) Negative control: Step 1 (Incubation with Moab) is left out. Instead the process is begun with incubation step 2 (RaM).
2) Positive control: Anti-HLA.

X. Microscopic evaluation

1) Assessment of reaction strength

The assessment of the reaction is carried out at 400 or 1000 times magnification with oil immersion. A positive reaction can be seen quite clearly as a granular, brown-black staining on the cell membrane, in the case of a strong reaction rings are formed.

The reaction strength can be documented using symbols:

-	negative
(+)	weak, but definitely positive
+	strongly positive, rings
++	very strongly positive, rings, dark cells.

Since the cells have retained their three-dimensional structure the viewer first sees a rather unusual picture of individual cells, but he will soon become familiar with this. The three-dimensional form with the possibility of assessing the membrane structure, particularly with positive staining, facilitates and allows individual cell identification without counter staining (which is possible but not necessary).

2) Criteria for the identification of individual blood cells

Lymphocytes

T-lymphocytes are generally compact with a low tendency to spread on the glass surface. Their surface is covered with fine, dense and regular spiculae.
B-lymphocytes are more variable in shape, they generally spread over the glass surface, the hair-shaped membrane offshoots are a little longer and more irregular.

Monocytes

Their identification is important for many aspects of lymphocyte separation. They possess endogenous peroxidase activity which appears as intracellular granulation as a result of the DAB reaction.

Monocytes generally spread widely over the surface of the glass. The membrane offshoots are clearly plumper, longer and more irregular than those of lymphocytes. Their characteristic of phagocyting latex particles can be used as an additional means of identification.

Granulocytes

These are shown by means of their strong endogenous peroxidase reaction. They spread quickly across the glass surface.

Thrombocytes

These can be recognized by their small size. They also have a strong tendency to spread. Their contents of cell preparations should thus be as low as possible since they otherwise spread beyond the borders of the field.

Erythrocytes

These are easy to recognize as a result of their pseudo peroxidase reaction. They are homogeneously dark brown.

Dead cells

The recognition of dead cells is important to prevent any confusion with positively reacting cells. By the penetration of antibodies they are stained diffusely brown without the typical membrane staining. Identification can thus also be carried out using the trypan blue test.

3) Percentage counting of positive cells

Eg. for the T4/T8 quotient.

T4 and T8 antibody reaction: Some 100 (or 200) lymphocytes are counted and the share of positive cells differentiated (relative percentage).

The absolute numbers for T4 and T8 lymphocytes are calculated from the leukocyte count and the percentage of lymphocytes in the differential count.

Normal values: T4: approx. 1000 lymphocytes per µl
T8: approx. 600 lymphocytes per µl

Preparation of cells from various body fluids

"buffy coat" method

Some 10 ml EDTA blood are centrifuged in a 10 ml centrifuge test tube at room temperature for 5 minutes at 400 g.
The buffy coat, the cell layer over the erythrocytes, is carefully lifted up (approx. 0.5 ml) and transferred to a 2 ml Eppendorf test tube. The cell suspension is washed three times with NKH-0.2% BSA (9) and centrifuged at 400 g for 3 minutes.

1 ml cell suspension in NKH-0.2% BSA (9) is underlaid with 1 ml of Ficoll Hypaque.

Centrifugation - see PAP test, page 4.
The interphase cells are collected and washed as follows:
1st wash: 400 g, 3 minutes with NKH-0.2% BSA (9)
2nd wash: 400 g, 3 minutes with NKH-0.2% BSA (9)
3rd wash: 400 g, 3 minutes with NKH buffer (1)

Suspend the cells in NKH buffer (1).
Set the cell concentration to approx. 5000 per µl.

Preparation of cells from bone marrow

1 ml bone marrow blood is aspirated into a 20 ml syringe with 1 ml NKH-0.1% EDTA (10) and mixed immediately. The further preparation is as for blood cells:
Addition of NKH-0.1% EDTA (10) ad 10 ml
Centrifugation: 400 g, 5 minutes, room temperature
Washing with 10 ml NKH-0.2% BSA (9)
Ficoll Hypaque centrifugation: 3 ml suspended bone marrow cells are underlaid with 3 ml Ficoll Hypaque.
Centrifugation: see PAP test, page 4

The interphase cells are collected and washed in 2 ml Eppendorf tubes with NKH buffer.

Cell preparation of cerebrospinal fluid

Centrifuge the cerebrospinal fluid at 150 g for 5 minutes at room temperature.
Remove the excess as thoroughly as possible.
Suspend the cells in NKH buffer (1).
Set the cell concentration, generally a few hundred per reaction field is sufficient.
If the liquor has a high protein content it is recommended that the cells be washed with NKH buffer (1).

Cell preparation of lymph node aspirates

Aspiration is carried out using a suction gun containing a 20 ml syringe. The syringe is filled with 2 ml NKH-0.2% BSA -0.1%EDTA (15). The lymph node is punctured using a 1 mm needle. While under suction the needle is pushed into the lymph node in several directions and then withdrawn.
The fluid with the aspirated cells in the syringe is then transferred to a 2 ml Eppendorf test tube.

Centrifugation: 400 g, 3 minutes, room temperature
Washing: suspend cell sediment in 2 ml NKH-0.2% BSA (9)
Centrifugation: 400 g, 3 minutes
Ficoll-Hypaque centrifugation:
1 ml cell suspension in NKH-0.2% BSA (9) is underlaid with 1 ml FicollHypaque

Centrifugation: see PAP test, page 4.

Cell preparation of lymph node or tumor biopsies

The biopsy tissue is reduced to as small pieces as possible using a fine pair of scissors, placed in a Petri dish with NKH-0.2% BSA (9) and suspended several times using a plastic pipette.
After the large particles have settled the cell suspension is transferred to a centrifuge test tube and processed further as described in "Cell preparation of lymph node aspirates".
Please note:
The cells are applied immediately after washing. Dead cells in the suspension must be removed since their decay products block the cell adhesion.
Dead cells are eliminated by Ficoll Hypaque centrifugation.
Tumor cells often adhere badly to the reaction fields. After the cells have adhered, all further mechanical influence (eg. by washing) should thus be avoided.
The cells are therefore fixed after adhesion without washing, the fluid on the reaction fields first being tapped off gently. Cell adhesion is considerably improved by fixation.

Cell preparation of effusions

Effusion of pleura, pericard, ascites etc. The effusion is collected in EDTA centrifuge test tubes. Between 10 and 40 ml is required depending on the cell content and number of tests.

Centrifugation of the effusion at 400 g for 5 minutes.
Remove the excess, suspend the cell sediment in the excess fluid (volume 1 plus 1) and prepare smears for cytology.

Washing: Suspend cells in 2 ml NKH-0.2% BSA (9) and centrifuge at 400 g for 5 minutes.
Repeat the washing process once.
Ficoll Hypaque centrifugation:
1 ml cell suspension (9) is underlaid with 1 ml Ficoll Hypaque.
Centrifugation: see PAP test, page 4.

Interphase cells are collected and washed in 2 ml Eppendorf test tubes.

Cell preparations of bronchoalveolar lavage (BAL)

Bronchoalveolar lavage is carried out with 0.9% NaCl.
The fluid thus produced is placed on ice (max. 1 hour) and processed further as quickly as possible.

1. The BAL fluid is filtered through gauze.

2. Centrifugation: 400 g, 5 minutes.

3. Wash the cells with NKH-0.2% BSA-0.1% EDTA. Centrifugation: 400 g, 3 minutes.

4. Repeat cell washing.

5. Suspend cells in NKH buffer, set the cell concentration to approx. 2000 per µl.

Preparation of cultured cells

Wash the cultured cells three times with NKH buffer (1).

Ficoll Hypaque centrifugation is recommended to remove dead cells. The trypan blue test can be used to establish whether the dead cells have been removed.

The trypan blue test is to be carried out as follows:

50 µl suspended cells in NKH
10 µl trypan blue (14)
Place the cell suspension in a Neubauer counting chamber.
Dead cells will be stained blue.

With some culture cells adhesion to the adhesive slide is weaker.
After the cells have adhered, all further mechanical influence (eg. by washing) should thus be avoided.
Therefore, without being washed, the cells have to be fixed after adhesion and having carefully removed the fluid on the reaction fields. Cell adhesion is considerably improved by fixation.

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Trouble shooting

Cells do not adhere on the reaction fields

Unsatisfactory adhesion and the loss of cells may be caused by various factors.

1. The blue stain on the reaction field has not been removed properly

   Rinse completely with water and then rinse with NKH buffer. Do not allow the reaction fields to dry out.

2. Mechanical damage to the coating of the reaction field

   Mechanical influences on the reaction field, eg. by pipette tips or wiping, are to be avoided since they prevent cell adhesion.

3. Cell suspension was not completely free of protein

   The coating of the reaction field is neutralised by soluble proteins. The cells are thus to be washed thoroughly in NKH buffer without any added protein.
   Dead cells which give off proteins must be removed (e.g. by Ficoll Hypaque centrifugation).
   Cells are to be applied immediately after isolation and washing.

4. The cells are damaged

   Cell damage can be caused by long storage, non-physiological buffer or temperature shock by cold media.
   Damaged cells adhere badly.
   When destroyed they can give off substances which prevent the adhesion of other cells.
   Certain cell types are very sensitive e.g. tumor cells isolated from a cell group and some culture cells.
   Dead cells are to be removed (e.g. Ficoll Hypaque centrifugation).

5. Cell types which adhere badly

   Some cultured cells and tumor cells adhere badly to the reaction field, probably because of their altered membrane characteristics. After cell sedimentation the fixation solution is to be added without washing. Fixation improves adhesion.

Wide spreading of cells

Monocytes, thrombocytes, granulocytes and hair cells generally spread rapidly across the glass surface. To prevent this the cells are to be fixed immediately after sedimentation.

Large numbers of thrombocytes

Thrombocytes spread rapidly on the glass surface and thus make the assessment of other cells more difficult. Thrombocytes are thus to be removed during cell isolation by means of repeated washing at a low g number.

No reaction after test

1. The order of the secondary antibodies was incorrect.

2. The primary antibody or a secondary antibody was forgotten.

3. H_2O_2 addition to the DAB substrate was forgotten.

4. Primary antibody or secondary antibody was too old (mostly PAP)

DAB sample: 0.5 ml DAB H_2O_2 solution
+ 10 µl PAP
= black-brown staining after a few seconds.

The reaction only occurred in patches

Mixing during the different incubation processes was insufficient: After applying the antisera mix thoroughly for approx. 15 seconds (vortex).

Cells appear diffusely brown

The cell membrane was damaged and allowed antisera to penetrate. Damage to the cell membrane can occur as follows:

a) temperature shock
b) non-physiological pH or ionic strength
c) drying of the cells
d) toxic impurities of antisera or buffer.

Non-specific staining (background)

a) Glass surface

Immunoglobulins bind non-specifically to glass. This effect is exaggerated by the special coating. This binding may be prevented by neutral proteins, such as gelatine and albumin.

b) Background reactions on cells

These are mainly caused by non-specific cross reactions of the secondary antisera and may be prevented by absorbing the latter.
Undesired binding by Fc receptors may be prevented by glutaraldehyde fixation.

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APPENDIX

1. Material and equipment

Adhesion slides

Coverslips (60 x 24 mm)

Nail polish

Wet chamber

Eppendorf pipettes and tips

Multidispensor (10 µl)

Water bath, 37°C

Staining jar

Microdel bottle

Rinsing bottle

Suction device

Centrifuge with swinging bucket rotor

Light-optical microscope (objectives: 10x, 40x plan oil or 60x plan oil, 100x plan oil) eventually inverted light-optical microscope

Refrigerator

Deep freeze -20°C (-80°C)

Centrifuge tubes 10 ml, 2 ml

Vortex Mixer or Superior Micro-Mixer

Wobbler

Stop-watch

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2. Solutions

The durability of the solutions is limited. Therefore, do not prepare more than the quantities you need for the tests.

(1) NKH-Buffer	8.0 g	NaCl (Merck 6404)	
	0.4 g	KCL (Merck 4936)	
	2.0 ml	Hepes-buffer 1 M, ph 7.4	
	ad 1000.0 ml	aqua bidest	prepare freshly!
(2) NAG-Medium	50.0 ml	NKH	
	0.5 ml	NaN3 10% (Merck 822335) in aqua bidest	
	2.0 ml	Hepes 1 M, pH 8.0	
	2.0 ml	Gelatine 5%, warmed up	
	0,5 ml	bovine serum albumin 22 % (Behring)	1 - 2 weeks durable
(3) Hepes 1 M	23.83 g	Hepes (Merck 10 110)	
	approx. 80.0 ml	aqua bidest, adjust pH 7.4 / 8.0 with concentrated NaOH	
	ad 100.0 ml	aqua bidest, filtration with Sartorius SM 56 (preliminary filter for filtration of serum)	
(4) Gelatine 5 %	5.0 g	Gelatine (Merck 4070) dissolved in	
	80.0 ml	aqua bidest at 50°C, adjust pH with NaOH to pH 7.4	
	1.0 ml	NaN_3 10 % (in H_2O)	
	ad 100.0 ml	aqua bidest	
	Store in 1.0 ml	fractions in refrigerator, warm before using	
(5) PBS		phosphate buffered saline see page A.22	
(6) Fixation solution 1	1.0 ml	Hepes-buffer 1 M, pH 8.0	
	0.2 ml	Glutaraldehyde 25 % (Merck 12179)	
	2.5 ml	Glucose solution 40 %	
	ad 100.0 ml	PBS	durable for 1 month when stored in refrigerator!
(7) Mounting medium			
	a) 80.0 ml	Glycerin (Merck 4095)	
	15.0 ml	Phosphate buffer 0.1 M, pH 7.4	
	5.0 ml	Glutaraldehyde 25 % (Merck)	or
	b)	Glycergel (Dakopatts C 563),	warm before using!
(8) OsO_4 - Fixation solution 2			
	50.0 ml	PBS	
	1.0 g	OsO_4 (Sigma No. 0-5500)	durable for months when stored in leakproof **amber** glass bottle in refrigerator at 4°C!
9) NKH-0.2 % BSA	99.0 ml	NKH	
	1.0 ml	Bovine serum albumin 22 %	(Behring ORHN 44/45)
(10) NKH-0.1 % EDTA			
	99.0 ml	NKH	
	0.1 g	EDTA (Merck 8418)	
		pH 7.4, adjusted with NaOH	prepare freshly!
(11) MEM (10x)		Gibco 042-1435	

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(12) MEM working solution

5.0 ml	MEM (10x)
2.0 ml	Hepes 1 M pH 8.0 (3)
ad 50.0 ml	aqua bidest

prepare freshly!

(13) Latex stock solution — Sigma LB-8

(14) Trypan blue — Gibco 630-5250

(15) NKH-0.2% BSA-0.1% EDTA

98.5 ml	NKH
1.0 ml	BSA, Bovine serum albumin 22 % (Behring)
0.1 g	EDTA (Merck 8418)

prepare freshly!

(16) Peroxidase-substrate solution (DAB)

a. Stock solution

40.0 mg	DAB Sigma D-5637
2.5 ml	Hepes pH 8.0
1.0 ml	Gelatine 5 % (4)
ad 50.0 ml	NKH

Freeze in 5 ml fractions at -20°C — durable for ca. 2 months.

b. Working solution

5 ml	stock solution
5 µl	H_2O_2, 30 % (Merck 7210)

Strongly centrifuge before use for 10 min. !
stable for 1 day when stored in darkness at 4°C.

Buffer solutions may be stored in appropriate quantities at -20°C.

(17) Secondary Antisera

a. RaM — Rabbit anti mouse

Stock solution 1:50 :

40 µl	RaM, Dakopatts Z 259
100 µl	AB Serum Behring RVD 14
2000 µl	NAG

durable for approx. 2 weeks.

Working solution 1:500:

200 µl	Stock solution
2000 µl	NAG

centrifugation before use!
As this solution is only stable for 1 week prepare small quantities as far as possible!

b. SwaR — Swine anti rabbit

100 µl	SwaR, Dakopatts Z 196
Stock solution 200 µl	AB Serum Behring

pre-incubate at least 1 day at 4°C! — stable for 4 weeks.

Working solution 300 µl	Stock solution
2000 µl	NAG (2)

centrifuge before use! — stable for 1 week.

c. PAP

50 µl	PAP, Dakopatts Z 113
1000 µl	NAG (2)

dilute every day freshly!

(18) AB-Serum — Behring RVD 14

(19) BSA — Bovine serum albumin 22 % Behring ORHN 44/45

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3. Table of conventional primary antibodies

Ortho Diagnostic Systems GmbH, 69151 Neckargmünd, Karl-Landsteiner-Str. 1

		Code-No.
OKT 3	Identification of human T lymphocytes expressing the 19.000 dalton cell surface antigen; complement fixing immunoglobulin; IgG_{2a} subclass	960 811
OKT 4	Identification of human T lymphocytes expressing the 60.000 dalton cell surface antigen, including T cells exhibiting inducer/helper functions; complement fixing immunoglobulin; IgG_{2b} subclass	960 821
OKT 6	Identification of human thymocytes expressing the 12.000 dalton and 49.000 dalton cell surface antigen complex; IgG_1 subclass	960 831
OKT 8	Identification of human T lymphocytes expressing the 31.000 dalton cell surface antigen, including T cells exhibiting suppressor/cytotoxic functions; complement fixing immunoglobulin; IgG_{2a} subclass	960 841
OKT 9	Identification of the 94.000 dalton transferrin receptor on activated and/or proliferating cells; IgG_1 subclass	960 842
OKT 10	Identification of the 12.000 dalton and 46.000 dalton cell surface antigen complex on activated lymphocytes and precursor cells; IgG_1 subclass	960 843
OKT 11	Identification of human T lymphocytes expressing the 45.000 dalton cell surface antigen responsible for binding sheep erythrocytes; complement fixing immunoglobulin; IgG_{2a} subclass	960 844
OKT 16	Identification of human null cells and immature T cells	960 950
OKT 17	Identification of human activated helper T lymphocytes	960 952
OKT 26a	Identification of human cells with Interleukin-2 receptors	960 954
OKM 1	Identification of the 170.000 dalton cell surface antigen expressed by monocytes, granulocytes, and null (NK) cells; complement fixing immunoglobulin; IgG_{2b} subclass	960 861
OKM 5	Identification of the 88.000 dalton cell surface antigen expressed by monocytes and platelets; IgG_1 subclass	960 865
OKB 2	Identification of B cells, granulocytes, pre-B and B cell leukemias and cAlla-positive cells; IgG_1 subclass	960 981
OKB 7	Identification of human B cells expressing the 175.000 dalton cell surface antigen; complement fixing immunoglobulin; IgG_{2a} subclass	960 985
OKI a1	Identification of the 29.000 dalton and 34.000 dalton human DR antigen complex expressed on B cells, activated T cells, and monocytes; complement fixing immunoglobulin; IgG_2 subclass	960 851

OKDR	Identification of cells expressing the human DR antigen; the SC-2 clone; complement fixing immunoglobulin; IgG_{2a} subclass	960 992
OKB-cAlla	Identification of the 100.000 dalton cell surface antigen expressed by non-T, non-B acute lymphoblastic leukemia cells; complement fixing immunoglobulin; IgG_{2a} subclass	960 990
OK-CLL	Identification of the 67.000 dalton cell surface antigen expressed by >90% of the sheep erythrocyte binding lymphocytes, by T cell leukemias, and by B cell chronic lymphocytic leukemias; the SC-1 clone; complement fixing immunoglobulin; IgG_{2a} subclass; equivalent to OKT1	960 996
OK-NK	Identification of human Killer/Natural Killer cells and neutrophils	960 870

Dako Diagnostika GmbH, 22047 Hamburg, Am Stadtrand 52

Code-No.

DAKO-C3bR CD35 M 710

DAKO-C3bR labels B cell follicles in cryostat sections of human lymphoid tissue. The most strongly labelled elements are dendritic reticulum cells, but mantle zone lymphoid cells show labelling to a lesser degree.
DAKO-C3bR gives strong staining of C3b receptor on the epithelial cells of renal glomeruli, and stains macrophages and histiocytes to a variable degree. The antibody labels paraffin-embedded tissue less strongly than cryostat sections. Approximately 15 to 20 per cent of peripheral blood mononuclear cells, representing monocytes and B cells, is labelled by DAKO-C3bR. It reacts with the antigen designated CD35.

DAKO-C3bi-R CD11b M 741

DAKO-C3bi-R reacts specifically with a leucocyte surface receptor for the C3bi complement fragment. It labels most granulocytes and monocytes, as well as a subpopulation of "null cell" peripheral lymphocytes which contains most of the circulating natural killer cells. It also reacts with macrophages in many tissues. DAKO-C3bi-R reacts with neoplastic cells in myelomonocytic and monocytic leukaemia and, less frequently, in acute myeloid leukaemia. It is useful for labelling granulocytes and macrophages in cryostat sections and cell smears. Dako-C3bi-R reacts with the antigen designated CD11b.

DAKO-CALLA CD10 M 727

DAKO-CALLA labels the neoplastic cells in acute lymphoblastic leukaemia of "common" type. In conjunction with a panel of other monoclonal antibodies, DAKO-CALLA enables classification of most acute leukaemias. DAKO-CALLA should be used on cryostat sections or cell smears. DAKO-CALLA reacts with the antigen designated CD10.

DAKO-M1 CD15 M 733

DAKO-M1 reacts with an antigen, termed X hapten or CD 15 antigen, found on mature granulocytes and on Hodgkin and Reed-Sternberg cells. DAKO-M1 is of value in confirming the diagnosis in most cases of Hodgkin's disease. DAKO-M1 works very well on formalin fixed paraffin-embedded sections in contrast to DAKO-RSC1, monoclonal anti-Reed-Sternberg cell.

DAKO-IL2-R CD25 M 731

DAKO-IL2-R is of value for revealing activated lymphoid cells in normal and pathological lymphoid tissue. DAKO-IL2-R labels a varying proportion of lymphoid cells in the T zones and in the light zones of the germinal centres. It labels macrophages of the germinal centres weakly, and those present in the sinuses more strongly. DAKO-IL2-R reacts with the antigen designated CD25.

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Code-No.

DAKO-Gplb CDw42 M 719

DAKO-Gplb labels glycoprotein Ib on megakaryocytes and platelets in cryostat sections and cell smears. It is useful for the detection of leukaemias of megakaryoblastic type, and for diagnosis of the Bernard Soulier disease. DAKO-Gplb reacts with the antigen designated CDw42.

DAKO-p150, 95 CD11c M 732

DAKO-p150, 95 reacts with a macrophage and hairy cell associated protein. It labels macrophages in tissue, including germinal centre macrophages, alveolar macrophages, and splenic red pulp-macrophages and is of value in the identification of hairy cell leukaemia. DAKO-p150,95 reacts with the antigen designated CD11c.

DAKO-T11 CD2 M 720 / F 767

DAKO-T11 reacts with the E-rosette receptor on T-cells. It is a valuable pan-T antibody, detecting normal T cells in cryostat sections of human tissue and in cell smears. DAKO-T11 reacts with the antigen designated CD2. F 767 is well-suited for immunofluorescence staining with FACS analysis or fluorescence microscopy.

DAKO-T4 CD4 M 716 / F 766

DAKO-T4 is well suited for identification of the helper/inducer subpopulation of T cells in cryostat sections or blood smears. It reacts with the antigen designated CD4. F 766 is well-suited for immunofluorescence staining with FACS analysis or fluorescence microscopy.

DAKO-CD22 CD22 M 738

DAKO-CD22 is a pan-B antibody which enables B cells (both normal and neoplastic) to be detected in cryostat sections or cell smears. It reacts with the antigen designated CD22.

DAKO-CD19 CD19 M 740 / F 768

DAKO-CD19 reacts with the antigen designated CD19 which appears early during B cell maturation, probably prior to pre-B cell stage, and is lost shortly before the terminal plasma cell stage. F 768 is well-suited for immunofluorescence staining with FACS analysis or fluorescence microscopy.

DAKO T8 CD8 M 707 / F 765

DAKO-T8 is well-suited for detection of the suppressor/cytotoxic subpopulation of T cells in cryostat sections. It reacts with the antigen designated CD8. F 765 is well-suited for immunofluorescence staining with FACS analysis or fluorescence microscopy.

DAKO-T3 CD3 M 756

DAKO-T3 is a pan-T marker for detecting normal and neoplastic T cells. It can be used for enumerating normal T cells in perpheral blood, for detecting reactive T cells in tissue lymphoid infiltrates, and for diagnosing T cell neoplasms. DAKO-T3 reacts with the antigen designated CD3.

DAKO-T1 CD 5 M 705

DAKO-T1 is well-suited for detecting normal and neoplastic T cells and a range of neoplastic B cells (e.g.chronic lymphocytic leukaemia and centrocytic lymphoma) in cryostat sections. DAKO-T1 is analogous to antibodies reacting with antigens designated CD5.

DAKO-T2 CD 7 M 706

DAKO-T2 is of particular value in detecting acute leukaemia of T cell type. The antibody should be used on cryostat sections. DAKO-T2 reacts with the antigen designated CD7.

DAKO-UCHL1 M 742

DAKO-UCHL1 labels most thymocytes, a subpopulation of resting T cells within both CD4 and CD8 subsets, and mature, activated T cells. It is effective on formalin-fixed and paraffin-embedded tissue, and is, therefore, a reliable marker of T cell neoplasms and reactive T cells when only routinely processed material is available.

SUPERIOR MARIENFELD

Code-No.

DAKO-RSC1 CD30 M 723

DAKO-RSC1 is of value for the detection of Reed-Sternberg cells and mononuclear Hodgkin's cells which may be difficult to identify with conventional staining procedures. It also allows large cells to be distinguished from the histiocytic malignancies and lymphomas derived from resting and precursor lymphoid cells. DAKO-RSC1 as well as the monoclonal antibody DAKO-Ber-H2 (anti-Ki-1 antigen, CD30) react with the antigen designated CD30; however, DAKO-Ber-H2 is effective on formalin fixed tissues, while DAKO-RSC1 can be used only on cryostat sections.

DAKO-T6 CD1 M 721

DAKO-T6 is a marker of Langerhans cells in normal, dysplastic, and neoplastic epithelium. It may be of help in distinguishing anaplastic carcinomas from large cell lymphoma. The antibody should be used on cryostat sections. DAKO-T6 reacts with the antigen designated CD1.

DAKO-HLA-ABC M 736

DAKO-HLA-ABC is directed against a monomorphic epitope on the 45 kD polypeptide products of the HLA-A,B, and C loci. It is valuable as a research reagent for analysing variations in HLA-ABC expression in different disease states.

DAKO-HLA-DR M 704 / F 764

DAKO-HLA-DR is well-suited for the detection of HLA-DR on a variety of human cells (e.g.B lymphocytes, macrophages, leukaemic myeloblasts, Langerhans cells, "dendritic" cells, etc.). Staining reactions are considerably stronger in cryostat sections than in paraffin-embedded tissues. However, after fixation in Bouin's fixative satisfactory staining is often achieved in paraffin sections. F 764 is well-suited for immunofluorescence staining with FACS analysis or fluorescence microscopy.

DAKO-IgD M 703

DAKO-IgD labels surface IgD on lymphoid cells in cryostat tissue sections.

DAKO-IgM M 702

DAKO-IgM labels surface IgM on B cells in cryostat sections and also intracytoplasmic IgM in paraffin-embedded tissue.

DAKO-Kappa M 730

DAKO-Kappa labels B cell follicles of human lymphoid tissue in cryostat sections. In paraffin-embedded tissue it gives a strong labelling of kappa-positive plasma cells and cells which have absorbed exogenous immunoglobulins (e.g.Reed-Sternberg cells.)

DAKO-Lambda M 614

In cryostat sections, DAKO-Lamda labels B cell follicles of human lymphoid tissue. In paraffin-embedded tissues it gives strong labelling of lamda-positive plasma cells and cells which have absorbed exogenous immunoglobulin (e.g.Reed-Sternberg cells).

DAKO-DRC1 M 709

Dendritic reticulum cells form a network within B cell follicles. Labelling of cryostat sections with DAKO-DRC1 enables follicular structures to be identified, even when the follicles are partially destroyed by pathological processes.

DAKO-EMA M 613

DAKO-EMA labels normal and neoplastic epithelium in cryostat sections as well as paraffin-embedded material. It demonstrates the epithelial origin of an anaplastic tumour, and in conjunction with antibody DAKO-LC, it enables an objectie distinction to be drawn between lymphomas and carcinomas.

DAKO-LC CD45 M 701

DAKO-LC enables tumours of white cell origin to be distinguished from non-haematopoietic neoplasms (e.g.carcinomas) using either cryostat sections or paraffin-embedded material. It reacts with the antigen designated CD45.

DAKO-Macrophage M 718

DAKO-Macrophage labels human macrophages in cryostat sections. It can be used for identifying a population of cells as being of mononuclear phagocyte origin, for demonstrating the macrophage origin of giant cells, and for assessing the number of macrophages infiltrating a neoplasm.

Code-No.

DAKO-MPO M 748

DAKO-MPO reacts with neutrophil granulocytes and monocytes in blood and with cells of the granulocyte series in the bone marrow. It is valuable for phenotyping acute leukaemias since it detects myeloperoxidase in the great majority of cases of acute myeloid leukaemia.

DAKO-Ber-H2 CD30 M 751

DAKO-Ber-H2 strongly labels (mononuclear) Hodgkin and (multinucleated) Reed-Sternberg cells, most anaplastic large cell lymphomas and all cases of Lymphomatoid papulosis. It labels routinely processed paraffin sections following treatment with pronase or trypsin. The staining in paraffin sections is most intense on the cell surface membrane but in addition dot-like labelling in the Golgi region is often seen.

DAKO-L26 M 755

DAKO-L26 reacts with the majority of B cells present in peripheral blood and lymphoid tissue. It reacts with most B cell lymphomas in formalin fixed material.

T Cell (T12), DAKO-CD6 CD6 M 739

DAKO-CD6 reacts strongly with the great majority of T cells in peripheral lymphoid tissue. The antibody is useful for the immunophenotyping of non-Hodgkin lymphomas in conjunction with other DAKO monoclonal antibodies against lymphoid tissue antigens.

DAKO-Elastase M 752

DAKO-Elastase reacts with human neutrophil elastase, a neutral protease which is found principally in the primary (azurophilic) granules of human neutrophils.
The antibody is of value to the haematologist in detacting the enzyme in leukemias of myeloid origin. As a few cases of acute myeloid leukemia lack elastase, it is therefore recommanded that staining for myeloperoxidase (using DAKO-MPO) or an enzyme cytochemical reaction is also performed. DAKO-Elastase works on paraffin sections.

Biotest AG, 63303 Dreieich, P.O.Box 1340

		Code-No.
Clonab T CD6	Pan T, mature T cells	812 130
Clonab T4 CD4	T helper, T inducer cells	812 140
Clonab T8 CD8	T suppressor, T cytotoxic cells	812 180
Clonab IL-2R CD25	Interleukin-2-receptor, Tac	812 190
Clonab DR/DP	B cells, activated T cells	812 120
Clonab DQ	B cells, activated T cells	812 125

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Becton Dickinson, P.O. Box 10 16 29, 69126 Heidelberg

			Code-No.
Anti-Leu-1	CD5	pan-T lymphocytes	6300
Anti-Leu-2a	CD 8	suppressor/cytotoxic T lymphocytes (mouse IgG_1 Isotype)	6310
Anti-Leu-2b	CD 8	suppressor/cytotoxic T lymphocytes (mouse IgG_{2a} Isotype)	7350
Anti-Leu-3a	CD 4	helper/inducer T lymphocytes	6320
Multi-Clone™	CD 4	helper/inducer T lymphocytes	7410
Anti-Leu-4	CD 3	pan T lymphocytes (mitogen)	7340
Anti-Leu-5b	CD 2	pan T lymphocytes, E-rosette receptor	7590
Anti-Leu-6	CD 1	thymocytes	7430
Anti-Leu-8		T lymphocytes, B lymphocytes, neutrophils, monocytes	7440
Anti-Leu-9	CD 7	pan T lymphocytes, NK cellls	7480
Anti-Leu-15/CR_3	CD11	T suppressor-lymphocytes, NK cells, monocytes, granulocytes (C3bi receptor)	7550
Anti-Leu-18		suppressor/inducer, T lymphocytes, B lymphocytes NK cells (2H4-equivalent)	7720
Anti-Leu-7		T lymphocytes-, NK cell subgroups	7390
Anti-Leu-11b	CD16	NK cells	7530
Anti-Leu-19		NK cells, subgroups of cytotoxic T lymphocytes	7740
Anti-Leu-12	CD19	pan B lymphocytes	7540
Anti-Leu-16	CD20	pan B lymphocytes (B1-equivalent)	7670
Anti-Leu-M1	CD15	monocytes/granulocytes	7420
Anti-Leu-M2		monocyte subgroups, thrombocytes	7400
Anti-Leu-M3		monocytes/makrophages	7490
Anti-Leu-M5		monocytes/makrophages, histiocytic lymphomas, hairy cell and acute myeloid leukemias	7630

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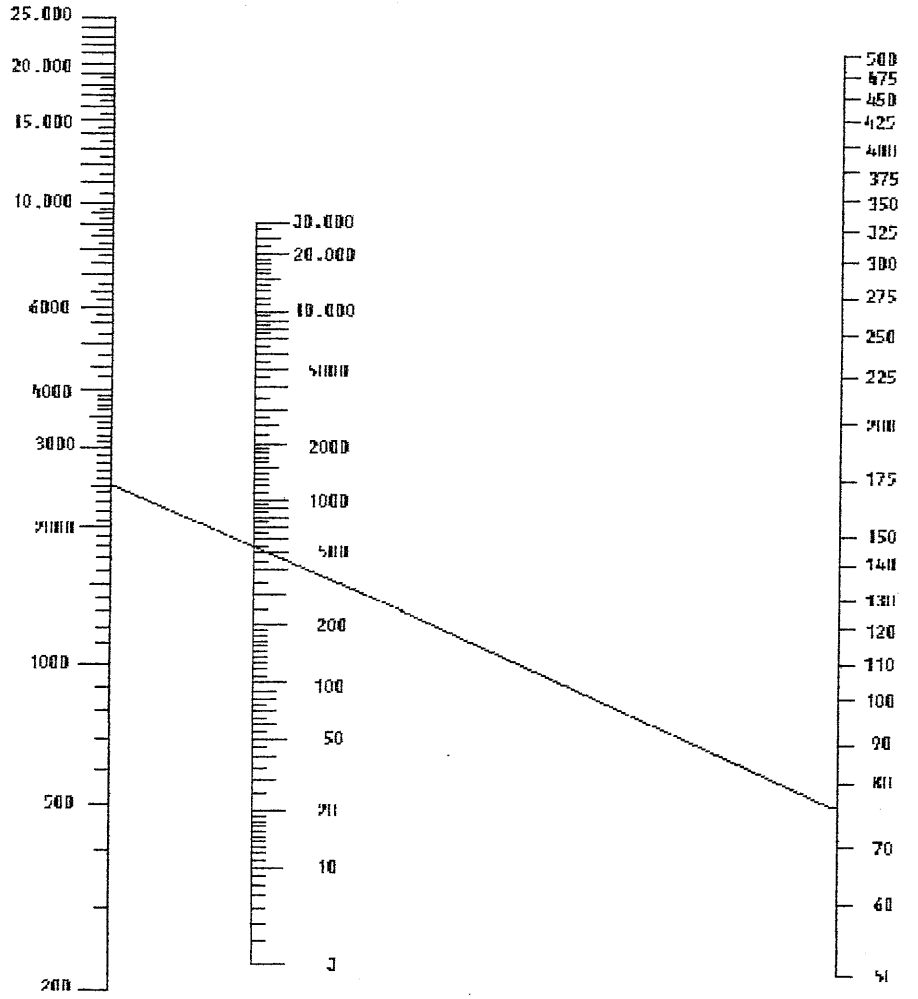
4. Centrifuge Nomogram



revolutions per minute (r.p.m.)

centrifugal force (x g)

radius (in mm) distance between centre of centrifuge and bottom of tubes

Diagram for calculation of speed

To calculate revolutions per minute (r.p.m.) measure radius in mm from the centre of the centrifuge to the bottom of the centrifuge tubes. Draw a straight line from the radius (on the right scale) through the known point of the centrifugal force (scale in the middle) to the point of intersection (on the left scale).

SUPERIOR MARIENFELD

5. Basic Programs for Clinical Diagnosis

with conventional primary antibodies

Indications in cases of suspected clinical diagnoses:

1. Immunodeficiency
2. Acute leukemia:
   - acute lymphoid leukemia
   - acute myeloid leukemia
   - chronic myeloid leukemia, blast crisis
3. Malignant lymphoma
   - B-cell Non Hodgkin lymphome
   - T-cell Non Hodgkin lymphome
   - Hodgkin's disease
4. Malignant effusion

Controls for each test:

1. Positive control: anti HLA - AB
2. Negative control: Rabbit anti mouse immunoglobulin (without monoclonal antibody)

Antisera for identification of not relevant cells:

3. Anti-monocyte (e.g. Leu M3)
4. Anti-granulocyte (e.g. DAKO M1)

In our laboratories the above mentioned antisera in brackets proved to be well suited for the immunoperoxidase slide test.

SUPERIOR MARIENFELD

1. Immunodeficiency:

Material to be tested: blood

1. CD3	(DAKO-T3, OKT3)
2. CD4	(DAKO-T4, Leu-3a)
3. CD8	(DAKO-T8, OKT8)
4. CD20	(B1)
5. HLA-DR	(Clonab DR/DP)

2. Acute lymphoid leukemia

Material to be tested: blood, bone marrow, cerebrospinal fluid

1. CD19 2. CD20 3. CD10 4. CD24 5. IgM 6. HLA-DR	(Coulter, B4) (Coulter, B1) (DAKO - CALLA) (Hybritech, BA-1) (DAKO, Miles) (Clonab, DAKO)	(Prae) - B - ALL
7. CD7 8. CD2 9. CD5 10. CD1 11. CD3 12. CD4 13. CD8	(DAKO-T2, Leu-9) (DAKO-T11, OKT11) (DAKO-T1, Leu-1) (OKT6, DAKO-T6) (DAKO-T3, OKT3) (DAKO-T4, OKT4a) (DAKO-T8, OKT8)	(Prae) - T- ALL
14. CD13 15. CD14 16. CD15	(Coulter, My7) (Coulter, My4) (DAKO-M1, Leu-M1, Behring, BMA-210)	AML AMOL
17. Glycophorin 18. Gp IIIa	(Dianova) (DAKO)	Erythroleukemia Megakaryocytic Leukemia

SUPERIOR MARIENFELD

3. Malignant lymphoma

Material to be tested: blood, bone marrow, lymph node needle aspiration
single cell suspension after biopsy, cerebrospinal fluid

1.	CD19	(Coulter B4, DAKO - CD19)	
2.	CD20	(Coulter B1)	
3.	CD10	(DAKO - CALLA)	B-cell lymphoma
4.	CD24	(Hybritech BA-1)	
5.	CD5	(DAKO-T1, Leu-1)	
6.	Kappa	(DAKO, Biotest)	
7.	Lambda	(DAKO, Biotest)	
8.	IgM	(Miles, DAKO)	
9.	IgD	(DAKO)	
10.	HLA-DR	(Clonab DR/DP)	
11.	CD7	(DAKO-T2, Leu-9)	
12.	CD2	(DAKO-T11, OKT11)	T-cell lymphoma
13.	CD5	(DAKO-T1, Leu-1)	
14.	CD1	(OKT6)	
15.	CD3	(DAKO-T3, OKT3)	
16.	CD4	(DAKO-T4, OKT4a)	
17.	CD8	(DAKO-T8, OKT8)	
18.	LeuM5	(B/D)	hairy cell leukemia
19.	Interleukin-2--Receptor	(DAKO, Biotest)	"
20.	OKT10	(Ortho)	Plasma cells . . .
21.	CD30	(DAKO-Ber-H2)	Hodgkin cells . . .
22.	CD15	(Leu-M1, DAKO-M1)	"
23.	CD45	(DAKO-LC)	hematopoetic cells

4. Malignant effusion

Material to be tested: pleural and pericandial effusion, ascites

1.	CEA	(Hybritech)
2.	EMA	(DAKO)
3.	HEA-125	(Progen Biotechnik)
4.	BMA-120	(Behring)
5	CD45	(DAKO, B/D)
6.	HLA-AB	(DAKO)

SUPERIOR MARIENFELD

Suggestion for a Diagnostic Protocol:

name:	date of birth:	test date:	No.
service:			
diagnosis:			
sample:			

Cell preparation:

buffy coat: _____ Leukocyte count: _____

prewash: _____ Lympho (%): _____

Ficoll separation: _____ Mono (%): _____

Latexphagocytosis: _____ blast cells (%): _____

Antisera	Percentage of positive cells % +	Lymph	Mono	Gran	Blasts	
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						
11.						
12.						

Remarks:	Conclusion:
Morphology:	
Reaction strength:	
Background:	
further studies needed:	

5. LITERATURE

1. Bross KJ, Pangalis GA, Staatz C
Demonstration of cell surface antigens and their antibodies by the peroxidase-antiperoxidase method
Transplantation 25: 331-334 (1978)

2.. Bross KJ, Schmidt GM, Blume KG, Spruce WE, Farbstein MJ
Confirmation ob bone marrow engraftment by demonstration of blood group antigens on red blood cell gosts
Transplantation 28: 257-259 (1979)

3. Fauser AA, Neumann HA, Bross KJ, Kanz L, Löhr GW
Cytotoxic T-cell clones derived from pluripotent stem cells (CFU-GEMM) of patients with Hodgkin's lymphoma
Blood 60: 1317-1320 (1982)

4. Costabel U, Bross KJ, Matthys H
Sonderdruck aus Verhandlungen der Deutschen Gesellschaft für Innere Medizin 88. Band: 422-426 (1982)
Lymphozytensubpopulationen in der bronchoalveolären Lavage bei Sarkoidose
J.F. Bergmann Verlag, München 1982

5. Morich FJ, Momburg F, Moldenhauer G, Hartmann KU, Bross KJ
Immunperoxidase slide assay (IPSA) - A new screening method for Hybridoma supernatants directed against cell surface antigens compared to other binding assays
Immunbiologie 164: 192-202 (1983)

6. Costabel U, Bross KJ, Matthys H
Pulmonary sarcoidosis: Assessment of disease activity by lung lymphocyte subpopulations
Klin. Wochenschr. 61: 349-356 (1983)

7. Costabel U, Bross KJ, Fischer J, Guzman J, Matthys H
Die Bedeutung der Helfer-T-Lymphozyten in der bronchoalveolären Lavage für die Aktivitäts-beurteilung der pulmonalen Sarkoidose
Prax. Klin. Pneumol. 37: 574-577 (1983)

8. Andreesen R, Osterholz J, Löhr GW, Bross KJ
A Hodgkin cell-specific antigen is expressed on a subset of auto- and alloactivated T (helper) Lymphoblasts
Blood 63: 1299-1302 (1984)

9. Schneider H, Jobke A, Bross KJ, Künzer W
Combined autoimmune neutro- and thrombocytopenia
Eur. J. Pediatr. 142: 216-219 (1984)

10. Costabel U, Bross KJ, Marxen J, Matthys H
T-lymphocytosis in bronchoalveolar lavage fluid of hypersensitivity pneumonitits. Changes in profile of T-cell subsets during the course of disease.
CHEST 85: 514-518 (1984)

11. Andreesen R, Osterholz J, Bodemann H, Bross KJ, Costabel U
Expression of transferrin receptors and intracellular ferritin during terminal differentation of human monocytes
Blut 49: 195-202 (1984)

12. Costabel U, Bross KJ, Rühle KH, Löhr GW, Matthys H
Ia-like antigens on T-cells and their subpopulations in pulmonary sarcoidosis and in hypersensitivity pneumonitis. Analysis of bronchoalveolar and blood lymphocytes
Am. Rev. Respir. Dis. 131: 337-342 (1985)

13. Costabel U, Huck E, Bross KJ, Rühle KH, Matthys H
Verschiebungen der Lymphozytensubpopulationen in der bronchoalveolären Lavage (BAL) bei Pneumokoniosen
Verh. d. Dtsch. Ges. f. Innere Medizin, 1124-1129 (1984)

14. Schneider H, Vogt A, Bross KJ
Identification of proliferating lymphocyte subpopulations in microcultures by surface marker and autoradiography
Immunological Communications 13: 553-561 (1984)

15. Kranz BR, Thiel E, Bross KJ, Thierfelder S
Immunzytochemische Liquorzellenuntersuchung auf Poly-L-Lysin-beschichteten Objektträgern:
In: Aktuelle Aspekte der Tumorimmunologie, ed. Urbanitz, Haubeck Springer-Verlag: Berlin, Heidelberg 1985)

16. Frickhofen N, Bross KJ, Heit W, Heimpel H
Modified immunocytochemical slide technique for demonstrating surface antigens on viable cells
J. Clin. Pathol. 38: 671-676 (1985)

17. Costabel U, Bross KJ, Matthys H
Bronchoalveoläre Lavage: Klinische Bedeutung zytologischer und immunozytologischer Befunde
Prax. Klin. Pneumol. 39: 343-355 (1985)

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The **ADHESION SLIDE** according to DR. BROSS

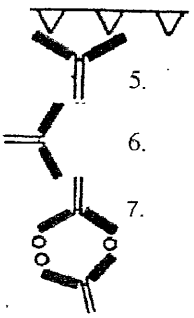
Instructions for the

PAP slide test using the

Universal DAKO mouse PAP-kit$_{TM}$ K550

SUPERIOR MARIENFELD

The P A P - slide test using the DAKO PAP-Kit for immunocytological staining



1. Cell isolation
2. Cell anchoring on the reaction fields
3. Fixation
4. Preincubation
5. Incubation with primary antibody (monoclonal antibody)
6. Incubation with rabbit anti-mouse immunoglobulin
7. Incubation with peroxidase- anti-peroxidase (PAP)
8. Incubation with substrate
9. Mounting
10. Light microscopic evaluation

DAKO PAP-Kit™ System 40 K550 Mouse and
supplementary reagents and solutions, see page A.23

Trouble shooting, see page 12f

All steps are to be carried out at room temperature.
To prevent temperature shocks, all refrigerated solutions are to be warmed up to room temperature before starting work.
The slides are to be kept in a wet chamber.

SUPERIOR MARIENFELD

1. Cell isolation

The cells to be investigated are to be prepared as usual. It is important that cells are undamaged and suspended in a completely **protein-free** buffer.

The following describes the isolation of mononuclear cells from peripheral blood:

1) In a 10 ml centrifuge tube
   3 ml EDTA-blood are diluted with
   3 ml buffer (PBS) and underlayed with
   3 ml Ficoll-Hypaque.

2) Centrifugation: **without brake**
   400 g - 2 minutes
   1500 g - 5 minutes

3) Pipette the **interphase** into 2 ml Eppendorf vessels.

4) Wash with PBS (2)
   Centrifugation: 400 g, 2 minutes

5) **Latexphagocytosis** (to identify monocytes) optional:
   1 ml minimal essential medium (MEM)
   50 µl Bovine serum albumin (BSA), 22% (Behring)
   5 µl Latexparticles (Sigma LB-6)
   Incubation 15 minutes in a 37°C water bath.
   Add the Latex suspension to the cell sediment, mix well and incubate for 30 minutes at 37°C.

6) Wash cells three times with PBS buffer (2):
   Centrifugation: 400 g, 2 minutes

7) Suspend cells in PBS buffer (2):
   Adjust cells to 5000 per µl.

2. Cell anchoring on the reaction field of the adhesion slide

1) Preparing the slide:
   Thoroughly wash away the green coating of the reaction area under running tap water until it is completely dissolved. Then rinse with PBS buffer (2). Avoid touching the reaction fields. Store slide in a moist chamber and never allow the reaction wells to become dry.

2) Applying cells:
   Apply 10 µl of the PBS cell supension to each reaction field.

3) Sedimentation of cells:
   Incubate slide in moist chamber for approx. 5-10 minutes until cells have settled and anchored to the glass surface.
   Check the cell density under the microscope (if available use an inversion microscope).

4) Carefully wash the reaction areas with PBS buffer (2) with a wash bottle or in a staining jar. Tap off the superfluous buffer.

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3. Fixation

1) Add one drop of fixation solution to each reaction field.

2) Incubate for 5 minutes (in moist chamber).

3) Rinse fixation solution with PBS buffer (2)

4) Tap off superfluous buffer.

4. Preincubation

To avoid unspecific compound of protein to the glass surface or the cells

1) Drip on PAG medium (4) (Fig.5).

2) Shake well holding the slide against the vortex mixer for approx. 15 seconds (Fig.6).

3) Incubate 15 minutes at room temperature or over night in refirgerator

5. Incubation with primary antibody

1) Tap off the buffer.

2) To each reaction field apply always a primary monoclonal antiboy of the mouse in appropriate dilution.

3) Shake well holding the slide against the vortex mixer for approx. 15 seconds. Avoid mixing the solutions between the reaction fields.

4) Incubate 20 minutes at room temperature or over night at 4°C.

5) Carefully rinse each reaction field with PBS buffer (2).

6. Incubation with link antibodies

1) Tap off the buffer.

2) Add the reagent from the yellow bottle (No.4 rabbit anti-mouse immunoglobulin) to the reaction areas.

3) Shake well holding the slide against the vortex mixer for approx. 15 seconds.

4) Incubate 20 minutes at room temperature.

5) Carefully rinse with PBS buffer.

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7. Incubation with Peroxidase - Anti-peroxidase

1) Tap off the buffer.

2) Add the reagent from the red bottle (No.5, peroxidase- anti-peroxidase, PAP) to the reaction fields

3) Shake well holding the slide against the vortex mixer for approx. 15 seconds.

4) Incubate 20 minutes at room temperature.

5) Carefully rinse with PBS buffer.

8. Incubation with substrate

1) Prepare the substrate (during incubation with PAP):

   a. Pour reagent from bottle 7 (Substrat-Puffer) into the attached graduated test tube. The quantity needed depends on the number of tests. 2ml are sufficient for 6 slides.

   b. To each 2 ml buffer add 1 drop of reagent from bottle No.6 (AEC). Immediately mix well.

   c. To each 2 ml add 1 drop from bottle No.8 (hydrogen peroxide). Immediately mix well. Press attached filter column into the substrate test tube.

2) Tap off the buffer from the slide.

3) Apply 1 drop of substrate to each reaction field. Shake.

4) Incubate 10-15 minutes

5) Carefully rinse with aqua dest.

Note: The prepared substrate is only stable for 2 hours. After use clean test tube, pipette, and filter column with aqua dest.

9. Mounting of the slides

1) Tap off aqua dest from the slide

2) Add 1 drop of Glycergel (pre-warmed) to each reaction field

3) Place cover slip (60 x 24 mm) and be careful pressing down with a pipette tip. Remove the excess Glycergel with absorbent paper and water. Avoid pushing cover slip out of place.

10. Microscopic evaluation: see page 8

Trouble shooting: see page 12

Material and equipment: see page A.1

SUPERIOR MARIENFELD

SOLUTIONS

(1) DAKO PAP Kit System 40 Dakopatts K 550 Mouse (see A.23)

(2) PBS

Phosphate buffered saline
substitute for NKH buffer

PBS (10x), without Calcium or Magnesium
Gibco, Cat. No. 042-04200 H or
Sigma, Cat. No. 1000-3
Dilute with aqua bidest before using

(3) PBS - 0.2 % BSA

9.9 ml	PBS (1x)
0.1 ml	Bovine serum albumin 22% (Behring ORHN 44/45)

Prepare freshly!

(4) PAG-Medium

(substitute for NAG-Medium, page A.2 (2))

47.0 ml	PBS (1x)
2.0 ml	Gelatine 5% (solution 4)
0.5 ml	Bovine serum albumin 22%
0.5 ml	NaN_3 10% in aqua bidest. approx. 2 weeks durable at 4°C

(5) Fixation solution

1 ml	Hepes1 M, pH 8.0
0.2 ml	Glutaraldehyde 25% (Merck 12179)
2.5 ml	Glucose solution 40%
ad 100.0 ml	PBS, store in refrigerator approx. 2 weeks durable

(6) For Latexphagocytosis

1 ml	MEM 10 x (Minimum Essential Medium with 25 mm Hepes buffer, diluted 1:10 with aqua bidest, Gibco, 042-1435)
50 µl	BSA (Bovine serum albumin 22%)
5 µl	Latex stock solution (Sigma LB-6)

can be stored in 1 ml fractions at -20°C

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DAKO PAP Kit$_{TM}$ System 40 K550 Mouse (stable for 1 year)

Contents

No.	Description	Quantity	colour code
1.	H_2O_2 (3%) (not used)	15 ml	grey
2.	Normal rabbit serum	15 ml	violet
3.			
4.	link antibody (RaM)	15 ml	yellow
5.	Peroxidase - Anti-peroxidase (PAP)	15 ml	red
6.	3-amino-9-ethylcarbazole	1 ml	violet
7.	Acetate buffer	2 x 15 ml	violet
8.	H_2O_2 (0,3%)	1 ml	violet

Supplementary:

1. Primary antibodies (monoclonal)
2. Phosphate buffered saline (PBS)
3. 15 ml Mounting medium (Glycergel, DAKO Code-Nr. C 563)
4. 15 ml Fixation solution (Glutaraldehyde 0,05%)
5. Cover slips (60 x 24 mm)
6. Adhesion slides

Reagents are sufficient for at least **50 slides = 600 Tests**

Substrate is sufficient for **15 preparations** (15 x 2 ml); 5 slides per preparation

SUPERIOR MARIENFELD

Instructions for the APAAP slide test
using APAAP-Kit™ K670

1. Cell isolation and

2. Anchoring the cells on the reaction areas

as described on page A.19.

3. Fixation

1) Apply 1 drop of fixation solution to each reaction area

2) Incubate for 5 min. (in moist chamber, at room temperature)

3) Rinse fixation solution with TBS or PBS.
(Each of both buffers can be used alternatively during all following steps)

4. Incubation with primary antibody

1) Tap off the buffer

2) To each reaction area apply always a primary monoclonal antibody of the mouse in appropriate dilution

3) Shake well holding the slide against the Vortex mixer or placing it on a SUPERIOR micro mixer for approx. 15 sec. Don't let the solutions of different reaction areas mix with each other.

4) Incubate 30 minutes at room temperature

5) Rinse each reaction area with PBS and wash for 1 min. in a Coplin jar

5. Incubation with bridge antibodies

1) Tap off the buffer

2) To each reaction area apply 1 drop of rabbit-anti-mouse bridge antisera in appropriate dilution, (e.g. DAKO Code Nr Z259, diluted 1:25 with PBS).

3) Incubate for 30 min. at room temperature in moist chamber on a wobbler

4) Rinse each reaction area with PBS and wash for 1 min. in a Coplin jar.

6. Incubation with APAAP complex

1) Tap off the buffer

2) To each reaction area apply 1 drop of APAAP solution (z.B. DAKO Code Nr D651, diluted 1:50 in PBS)

3) Incubate 30 min. at room temperature in moist chamber on a wobbler

4) Rinse each reaction area with PBS buffer and wash for 1 min. in a Coplin jar

To increase the sensitivity repeate steps **5** and **6** once or twice. The incubations may be shortened to 10 min..