

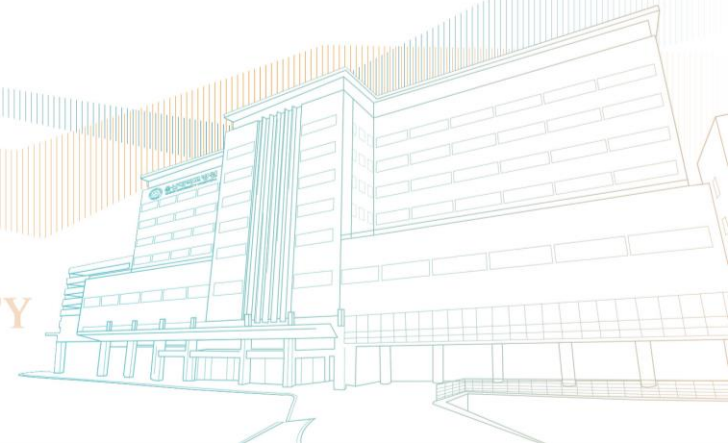
Standardizing sputum sample processing

울산대학교병원 | 호흡기내과 | **나승원**

기관지확장증 연구회 Workshop 2023.8.26

양재 aT 센터 4층 창조룸 I

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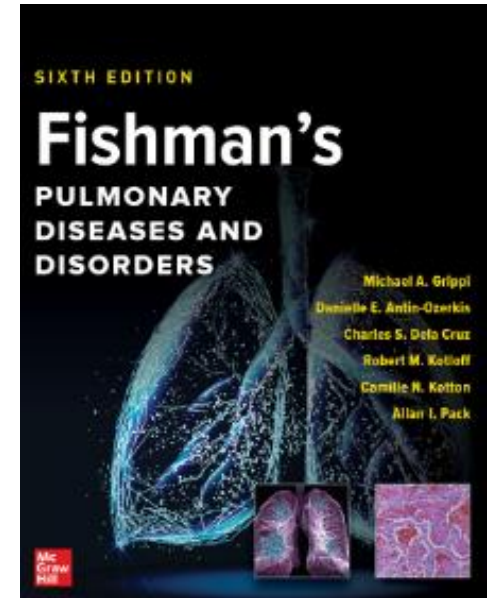
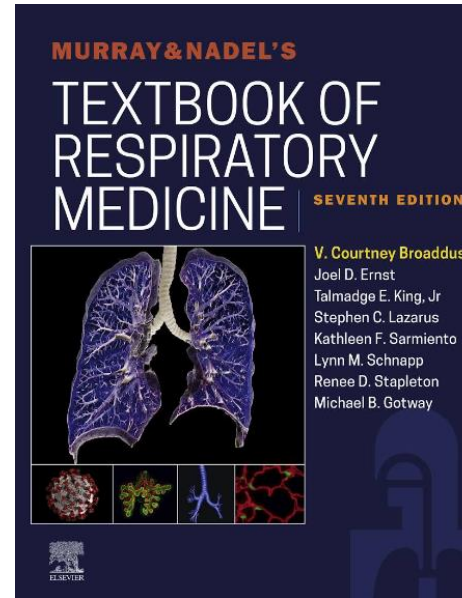
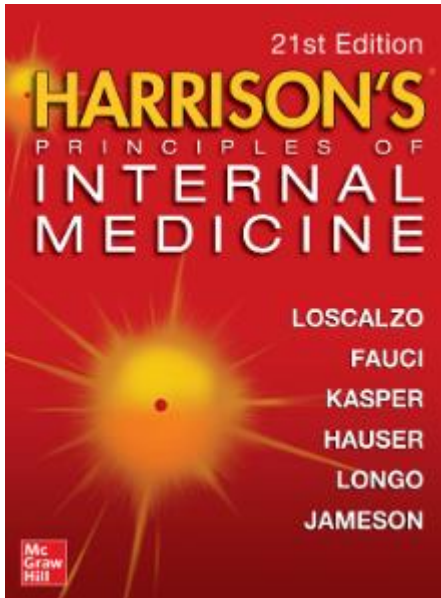


Standardizing sputum sample processing

강의 순서

- Definition of sputum
- Normal cellular profile in sputum (\approx Bronchial washing), BAL fluid
- Adequate Sputum Specimen for sputum analysis and culture
- Sputum induction and sample processing
- Standard Operating Procedures (SOP) & sputum processing WORKSHEET
- Summary

Definition of Sputum



Part 2: Cardinal Manifestations and Presentation of Diseases

Section 5: Alterations in Circulatory and Respiratory Functions

Chapter 37: Dyspnea

Chapter 38: Cough

Chapter 39: Hemoptysis

Chapter 40: Hypoxia and Cyanosis

Chapter 41: Edema

Chapter 42: Approach to the Patient with a Heart Murmur

Chapter 43: Palpitations

36. Dyspnea

37. Cough

38. Chest Pain

39. Wheezing And Stridor

40. Hemoptysis

+ DIAGNOSTIC TESTING IN THE EVALUATION OF DYSPNEA

+ COUGH

+ HEMOPTYSIS

+ CYANOSIS

+ CLUBBING

+ HYPERTROPHIC OSTEOARTHROPATHY

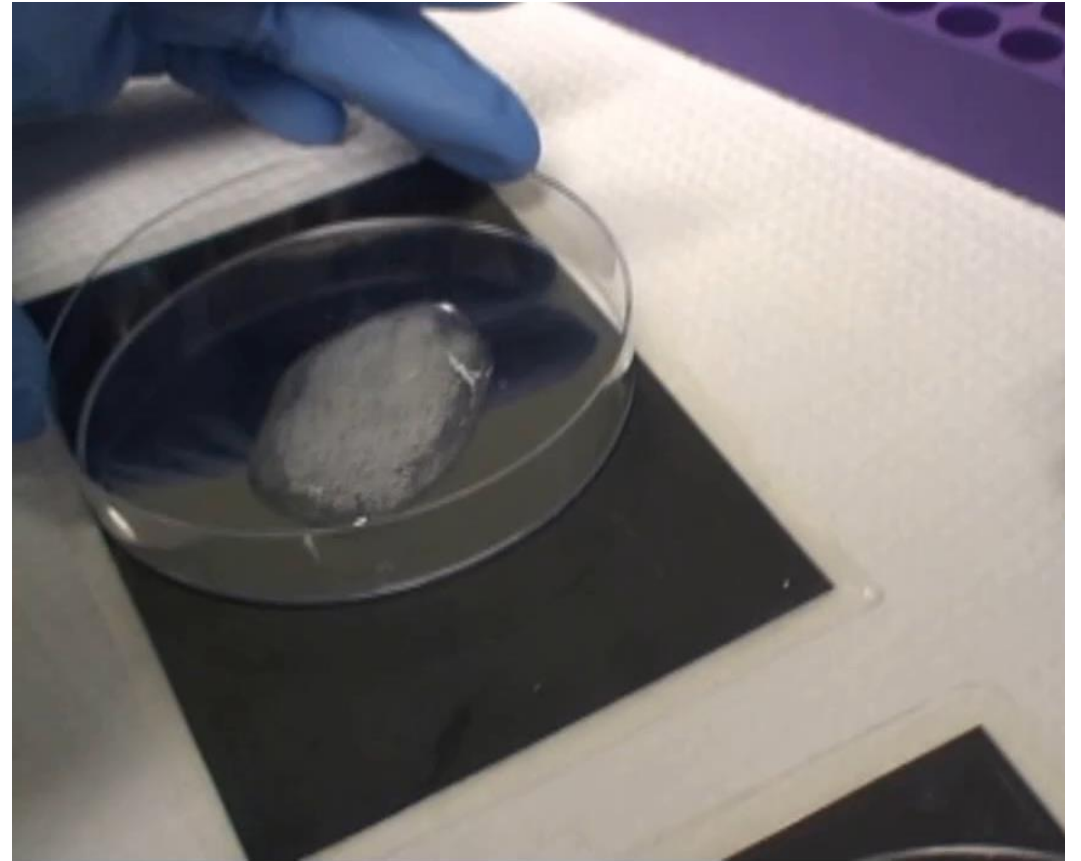
+ CHEST PAIN

+ FEVER

Definition: Sputum versus Saliva

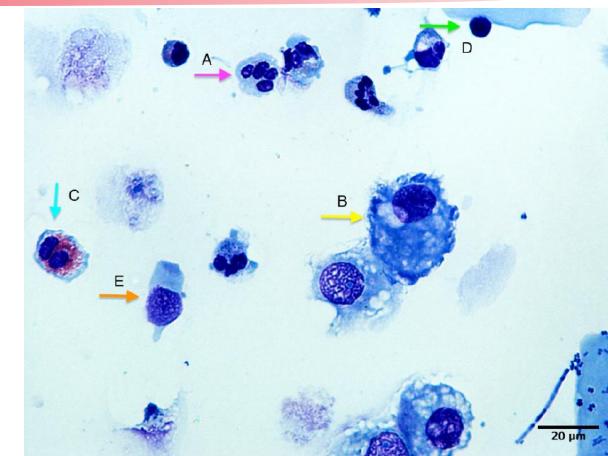
Definition of sputum

- Sputum, which is defined as expectorated lower respiratory secretions, is composed of fluid and cellular components, including macrophages, bronchial epithelial cells and inflammatory cells.
- The more viscid portions, considered to be sputum, are usually easy to recognize macroscopically when the sample is poured into a Petri dish.

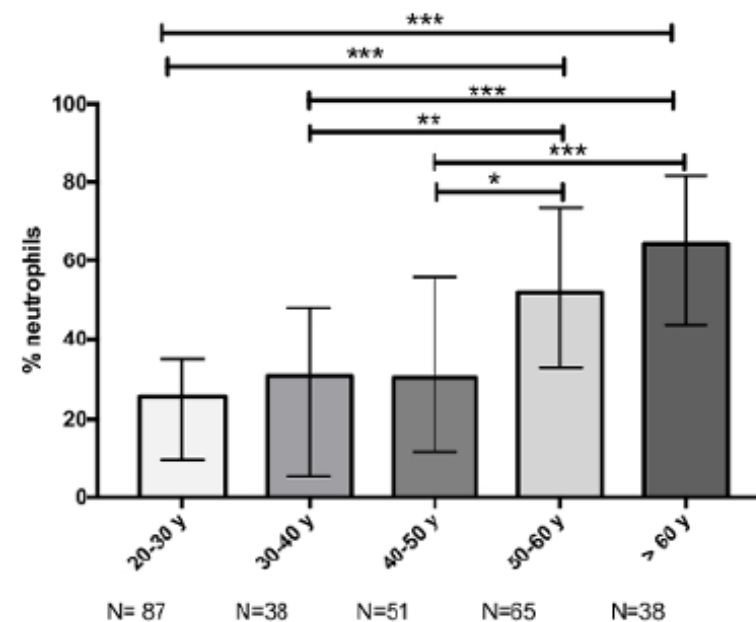
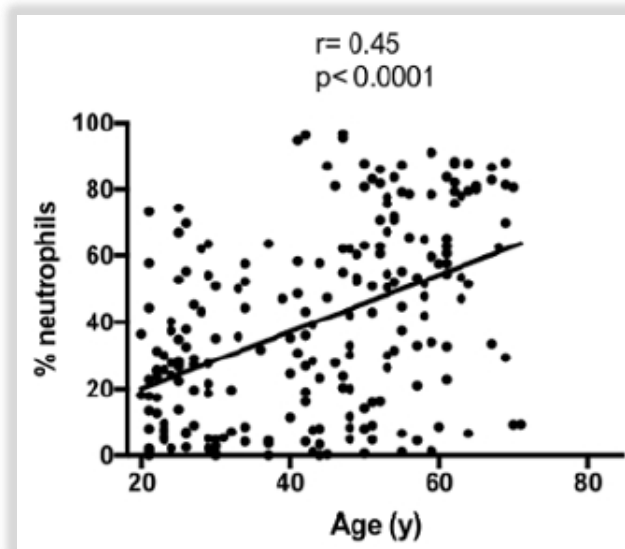
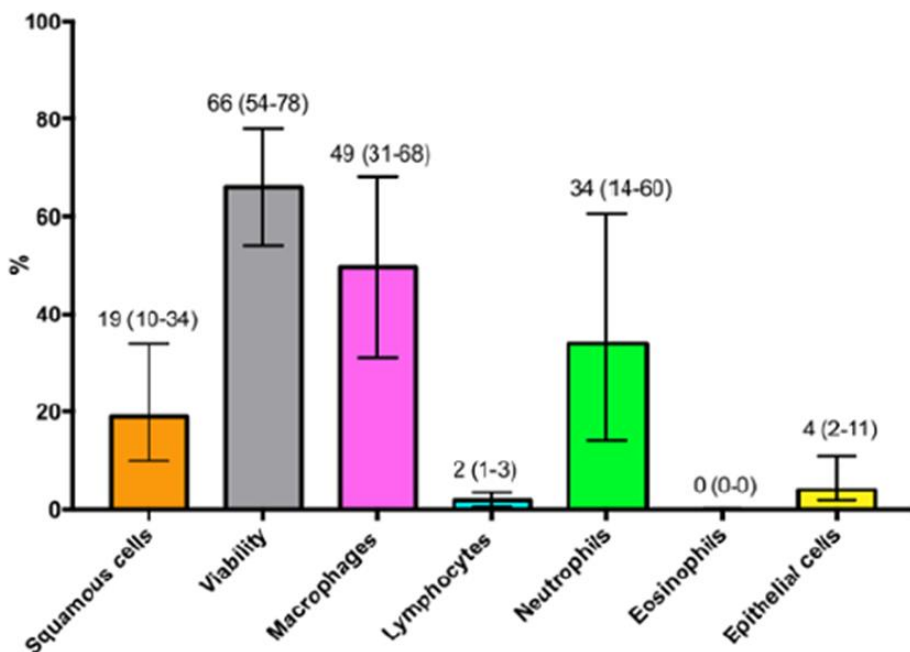


Sputum differential cell count observed in healthy subjects

Variable	Mean \pm SD	Median	Minimum	Maximum
Total cell count $\times 10^6/ml$	2.7 \pm 2.5	1.8	0.4	14.2
% Macrophages	69.2 \pm 13	69.0	40	95
% Neutrophils	27.3 \pm 13	28.5	2	49.2
% Eosinophils	0.6 \pm 0.8	0.2	0	2.4
% Lymphocytes	1.0 \pm 1.2	0.8	0	5
% Epithelial cells	1.5 \pm 1.8	1	0	8.2

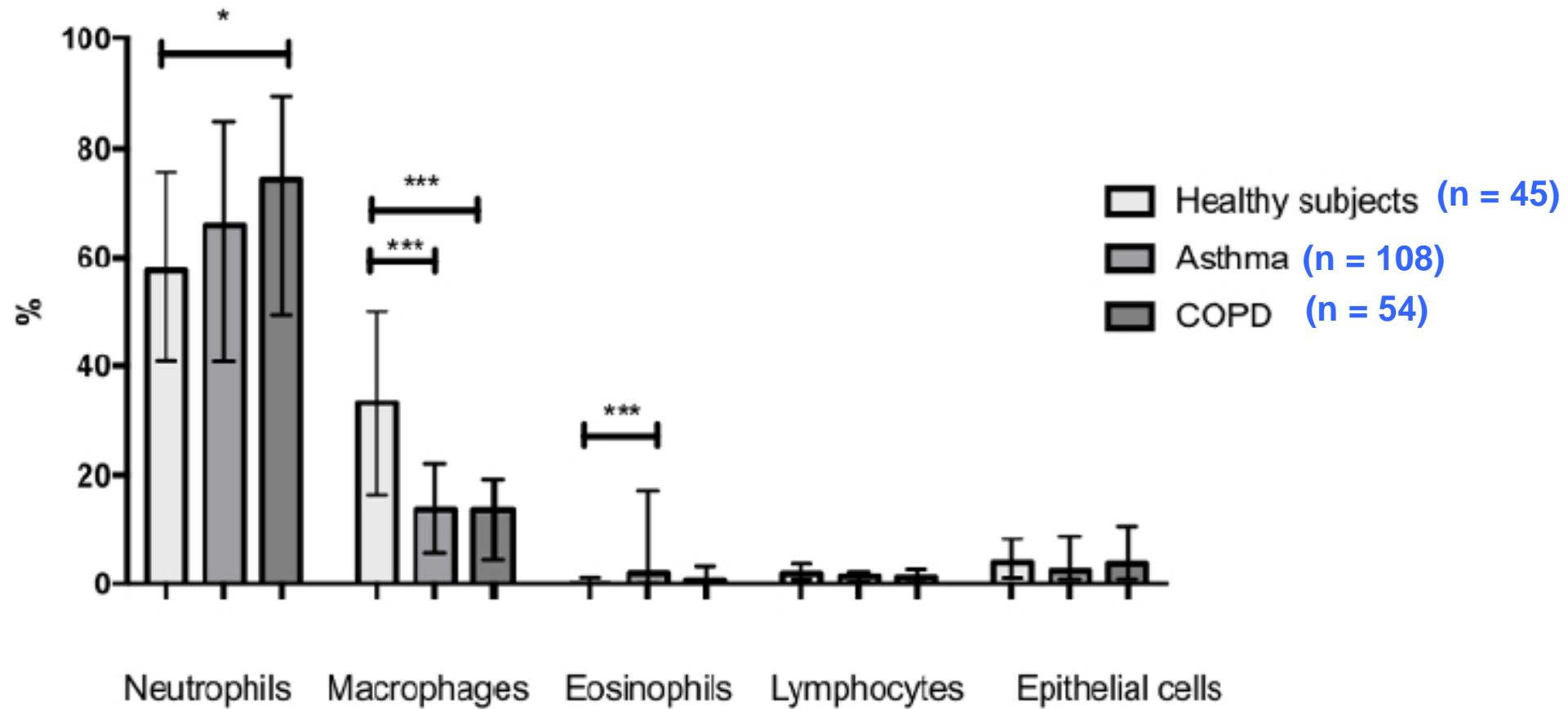


Am J Respir Crit Care Med Vol 162. pp 1172-1174, 2000



J. Vis. Exp. 2017: 130; e56612. doi:10.3791/56612

Sputum inflammatory cell profile of healthy subjects and patients suffering from airway diseases

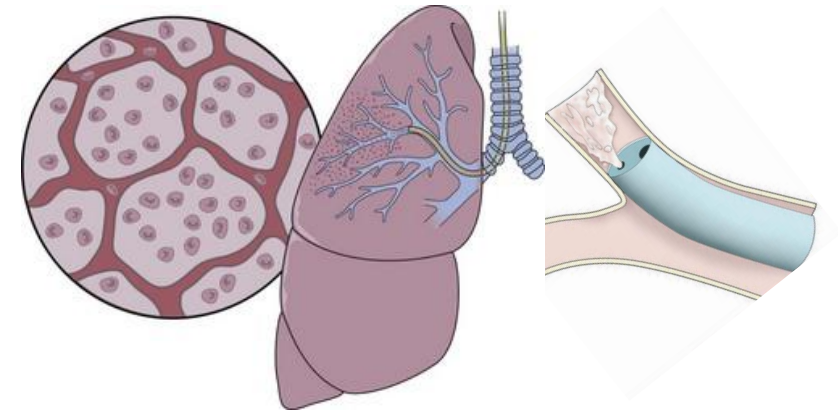


BAL cellular patterns in normal adult nonsmokers and in ILD patients

I. Normal Adults (Nonsmokers)

BAL Differential Cell Counts

Alveolar macrophages	>85%
Lymphocytes (CD4+/CD8+ = 0.9–2.5)	10–15%
Neutrophils	≅3%
Eosinophils	≅1%
Squamous epithelial*/ciliated columnar epithelial cells†	≅5%



Lymphocytic cellular pattern

Eosinophilic cellular pattern

Neutrophilic cellular pattern

>15% lymphocytes

Sarcoidosis
 Nonspecific interstitial pneumonia (NSIP)
 Hypersensitivity pneumonitis
 Drug-induced pneumonitis
 Collagen vascular diseases
 Radiation pneumonitis
 Cryptogenic organizing pneumonia (COP)
 Lymphoproliferative disorders

>1% eosinophils

Eosinophilic pneumonias
 Drug-induced pneumonitis
 Bone marrow transplant
 Asthma, bronchitis
 Churg-Strauss syndrome
 Allergic bronchopulmonary aspergillosis
 Bacterial, fungal, helminthic, *Pneumocystis* infection
 Hodgkin's disease

>3% neutrophils

Collagen vascular diseases
 Idiopathic pulmonary fibrosis
 Aspiration pneumonia
 Infection: bacterial, fungal
 Bronchitis
 Asbestosis
 Acute respiratory distress syndrome (ARDS)
 Diffuse alveolar damage (DAD)

Adequate Sputum Specimen for sputum analysis or culture

- Percentage or number of squamous epithelial cells
 - ◆ 80% before cytopsin vs. 20% after cytopsin
- Viability
 - ◆ A cell viability of < 40% may affect the Differential Cell Counts.
- Number of neutrophils
 - ◆ Grading system for assessing the quality of sputum samples for culture
- Presence of mucus plug

Grading system for assessing the quality of sputum samples for culture

Box 1-4 Bartlett's Grading System for Assessing the Quality of Sputum Samples

No. of Neutrophils Per 10 × Low-Power Field	Grade
<10	0
10-25	+1
>25	+2
Presence of mucus	+1
No. of Epithelial Cells Per 10 × Low-Power Field	
10-25	-1
>25	-2
Total*	

* Average the number of epithelial cells and neutrophils in about 20 or 30 separate 10× microscopic fields and then calculate the total. A final score of 0 or less indicates lack of active inflammation or contamination with saliva. Repeat sputum specimens should be requested.

Box 1-5 Murray and Washington's Grading System for Assessing the Quality of Sputum Samples

	Epithelial Cells Per Low-Power Field	Leukocytes Per Low-Power Field
Group 1	25	10
Group 2	25	10-25
Group 3	25	25
Group 4	10-25	25
Group 5	<10	25

TABLE I. MODIFIED BARLETT'S CRITERIA

	Criteria	Score
Neutrophils (pus cells) count (Score A)	< 10 neutrophil/10x field	0
	10-25 neutrophils/10x field	+1
	>25 neutrophils/10x field	+2
Macroscopy (Score B)	Mucoid, Mucopurelent, Purelent, or Blood stained	+1
Squamous epithelial cell count (Score C)	< 10 Squamous epithelial cell/10x field	0
	10-25 Squamous epithelial cell /10x field	-1
	>25 Squamous epithelial cell /10x field	-2

- If the total score is 1 and above, the sputum will be cultured, and the specimens will be proceeded accordingly. Whereas if the total score is 0 and below, the process of sputum will stop.

Murray PR, Washington JA. Microscopic and bacteriologic analysis of expectorated sputum. Mayo Clin Proc. 1975 Jun;50(6):339-44.

Lester K. et al. Comparison of Six Different Criteria for Judging the Acceptability of Sputum Specimens. Journal of clinical microbiology, 1982; 16(4): p. 627-631.

Inadequate sputum sample for sputum analysis



- If the percentage of squamous epithelial cells is greater than 80%, the sample is considered unsuccessful

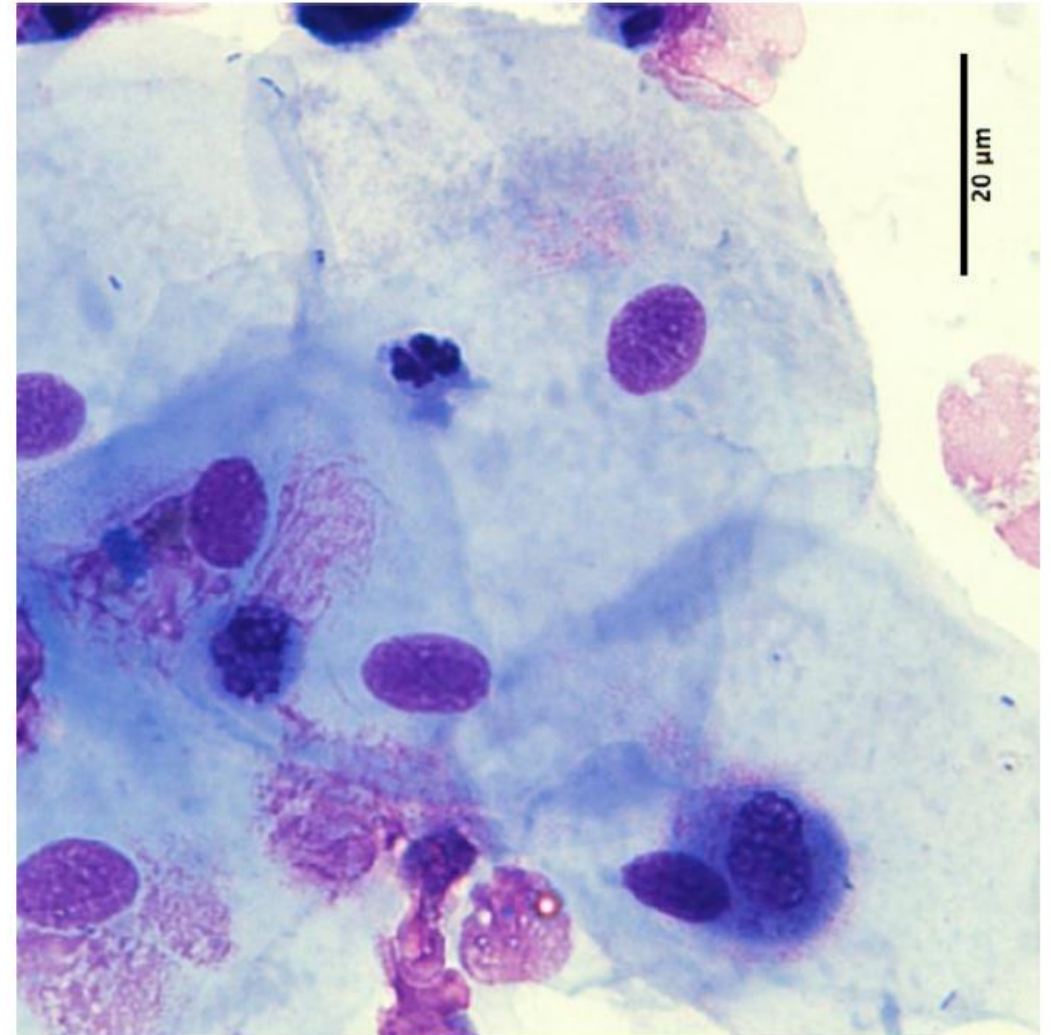


Figure 3: Example of a poor quality cytospin slide with >80% of squamous cells. Scale bar = 20 μ m.

Sputum induction and sample processing



국내 진료 현장에서 객담 세포 분석 검사 수행 현황



환자 진료 목적으로 객담 세포 분석
검사를 시행하고 있다.



연구 목적으로 객담 세포 분석 검사를
시행하고 있다.



객담세포분석 검사는 병원에 세팅/
표준화가 필요한 검사이다.

처방코드	처 방 명		
L23261	Eosinophil stain sputum		
L23262	Eosinophil stain & Diff.		
처방명	급비	1회량	수가
체액-호산구수(혈액 외)	급여	1.00	1,860 원
			1,860 원

SPUTOLYSIN® Reagent - CAS 578517 - Calbiochem

카탈로그 번호	재고 정보	패킹	포장 단위	가격(VAT 별도)
560000-10MLCN	현재 재고 없음 🚫	Glass bottle	10 ml	KRW 107,200.00 고객님의 가격을 보려면 로그인하십시오
560000-1SETCN	현재 재고 없음 🚫	Glass bottle	1 set	KRW 539,600.00 고객님의 가격을 보려면 로그인하십시오

Description

Overview

References

Hirsch, S.R., et al. 1969. *J. Lab. Clin. Med.* **74**, 346.
 Shah, R.R. and Dye, W.E. 1966. *Am. Rev. Respir. Dis.* **94**, 454.
 Dixon, J.M.S. and Miller, D.C. 1965 (November). *Lancet* p. 1046.
 Cleland, W.W. 1964. *Biochemistry* **3**, 480.
 May, J.R. 1953 (September). *Lancet* p. 535.

Concentrate of Dithiothreitol (DTT; Cat. No. 233155) in phosphate buffer, pH 6.5-7.5. Intended for the isolation of epithelial cells, pathogenic or saprophytic bacteria, fungi, and yeasts from sputum. Each vial is sufficient to make 100 ml of working solution.

Note: 1 set = 20 x 10 ml.

Catalogue Number

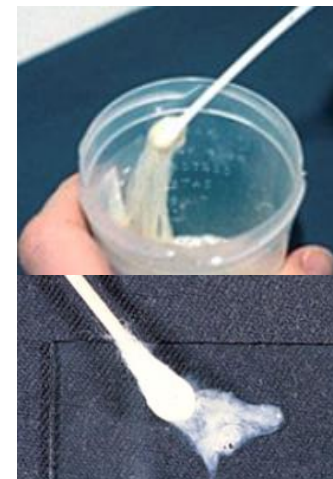
560000

Brand Family

Calbiochem®



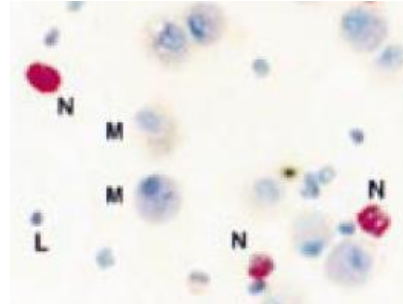
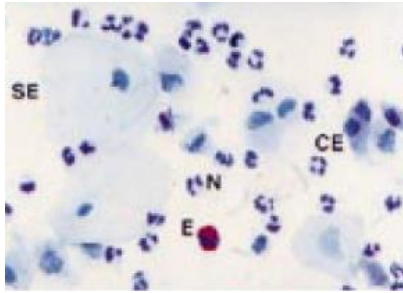
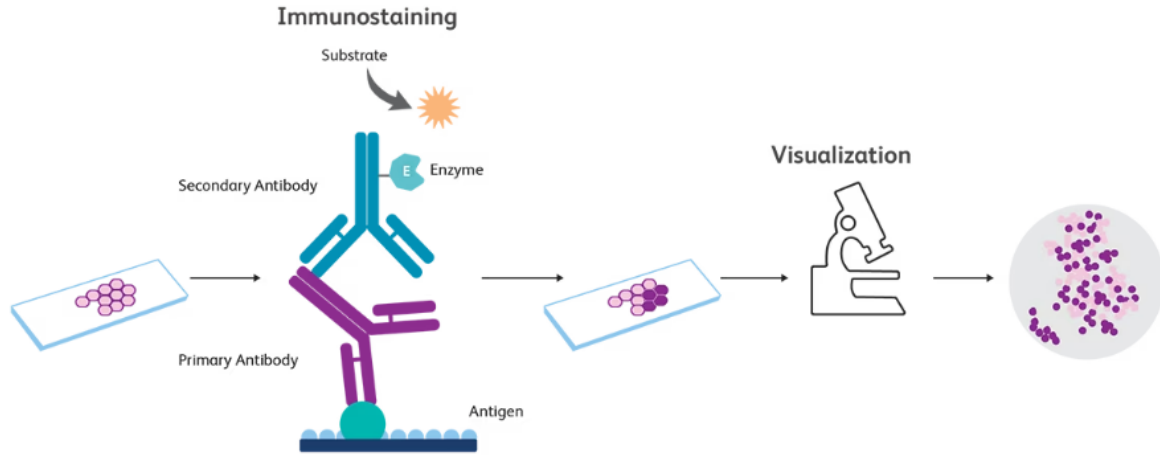
40um pore sized nylon mesh



The utility of sputum analysis

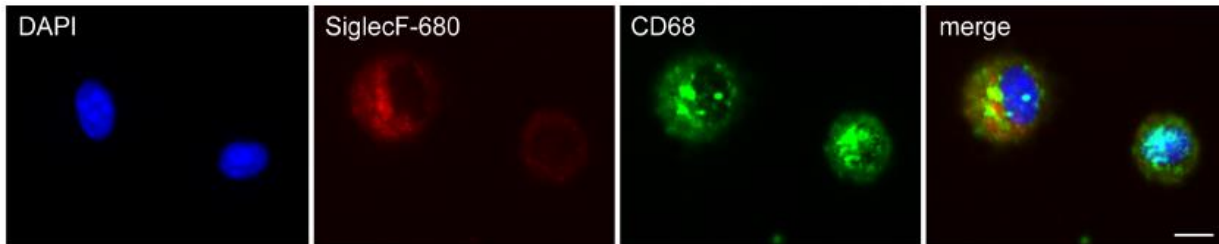
- Sputum \approx Bronchial washing, \neq BronchioloAlveolar Lavage
- Supernatant of sputum & expectorants: measure biochemical markers
- Differential count: provide the information about the inflammation status in the airway
- Flow cytometry, Genomics, transcriptomics, Proteomics
- Immunocytochemical staining, *In situ* hybridization, Microarray

Immunohistochemistry

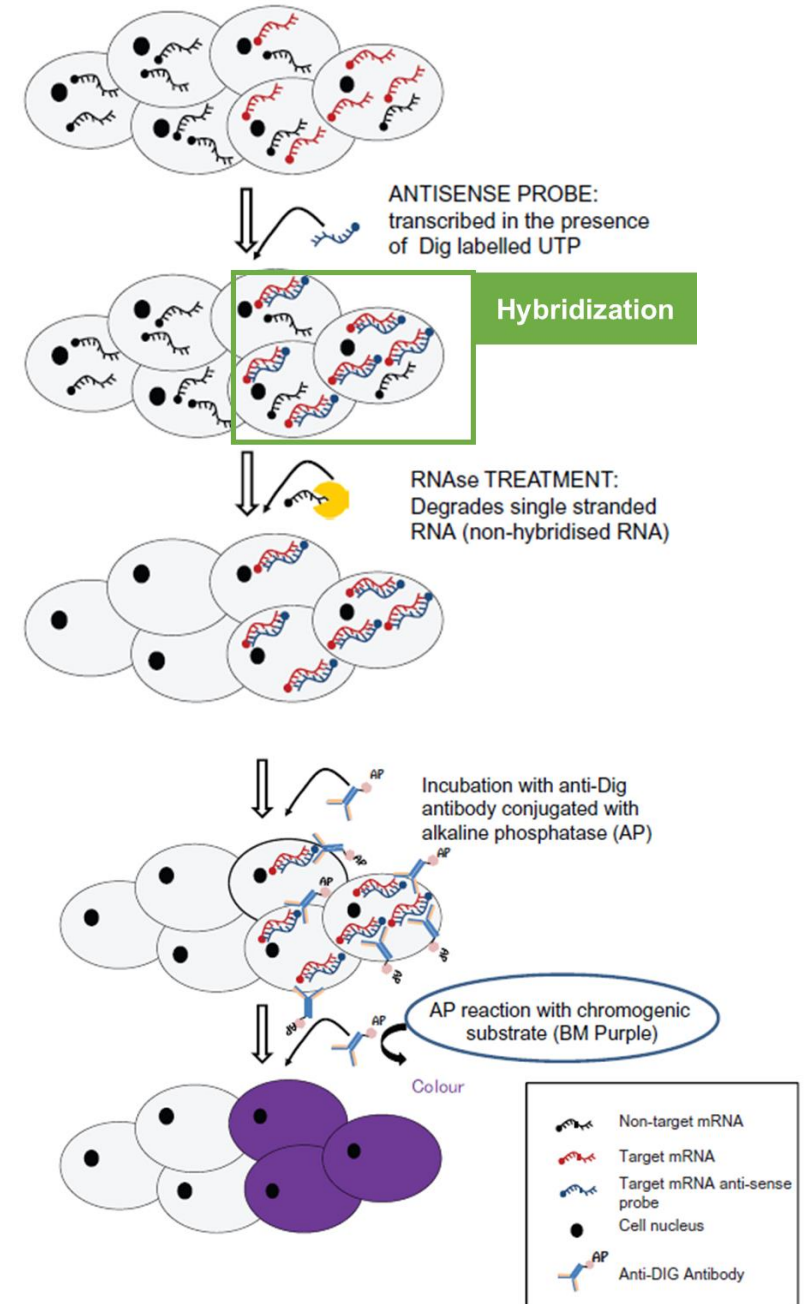


<https://www.bdbiosciences.com/ko-kr/learn/applications/immunohistochemistry#Overview>
 Thorax 2002;57:449-451

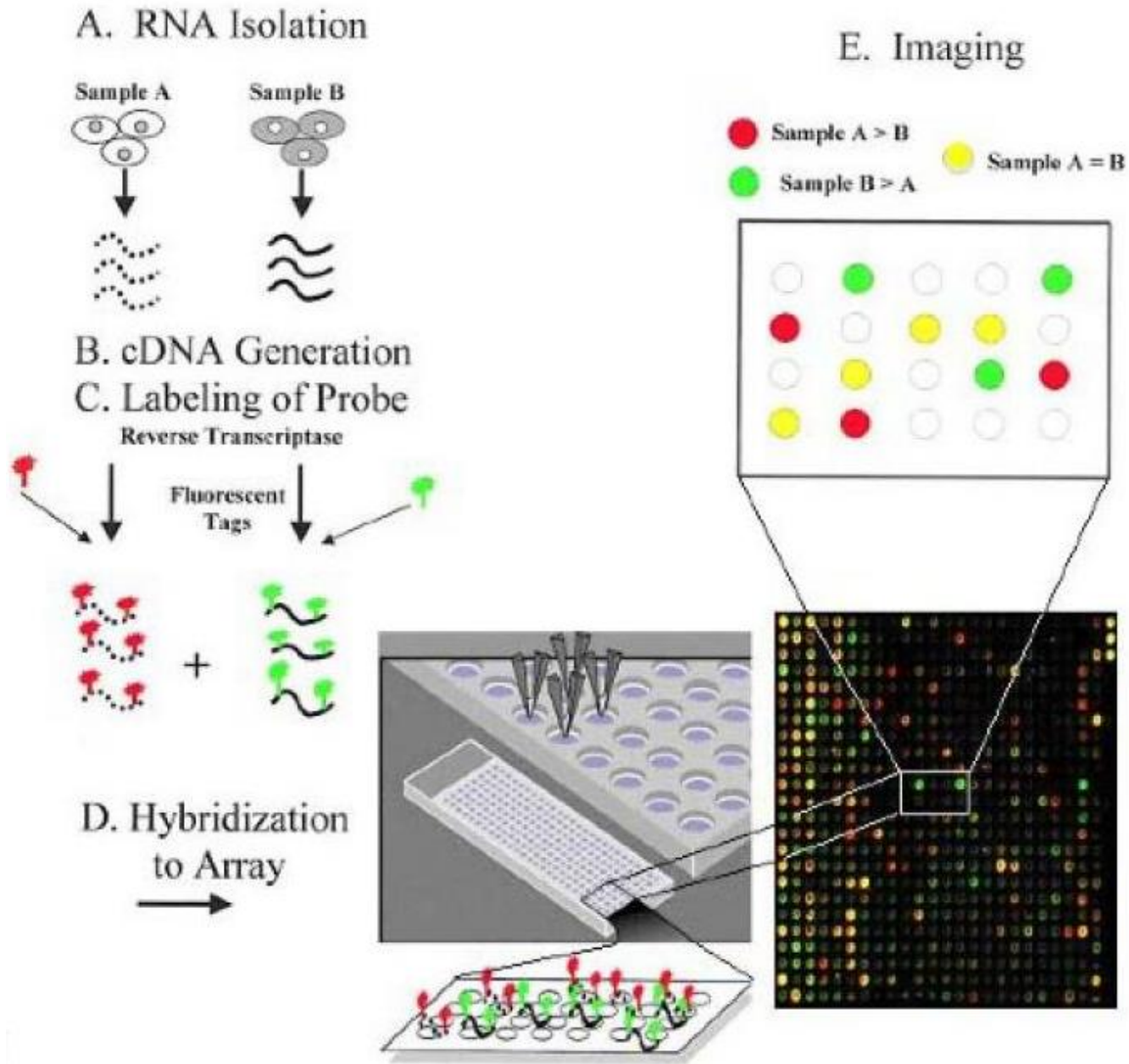
In situ hybridization with more specific RNA probe



PLoS ONE 9(2): e90017.



Microarray



Sputum processing method for: entire sputum and selected sputum



- The advantage of using the entire expectorate is that the technique is **quicker** to perform.
- 목표 squamous cell contamination: < 20% vs. < 5%
- There is conflicting data as to whether or not differential cell counts (DCCs) differ between the two methods.
 - higher eosinophils % in sputum processed by the selection method compared to the entire expectorate [AJRCCM 1998; 157: 665–668]
 - no difference between two methods [ERJ 1996; 9: 2448–2453]
- Protocols for "Analysis of fluid-phase mediators"

DTE: dithioerythritol; DTT: dithiothreitol;
TCC: total cell count; DCC: differential cell count

Sputum induction: Standard procedure

1. Explain the procedure in detail to the subject (rinse mouth before procedure, saline inhalation with tidal breathing, saliva handling during inhalation; after 5 min intervals cough and try to expectorate into the sputum cup).
2. Set nebuliser (output $\sim 1 \text{ mL}\cdot\text{min}^{-1}$), fill it with sterile saline solution (usually with concentration of 4.5%).
3. Measure baseline (pre-salbutamol) FEV₁ (or PEF).
4. Premedicate the subject with inhaled salbutamol (200 μg) and repeat FEV₁ (or PEF) measurement after 10 min.
5. Start nebulisation and ask the subject to perform tidal breathing (set the clock for 15–20 min). Ask the patient to perform inhalation for 5 min intervals followed by coughing and expectoration (the clock should be stopped at each coughing episode). Encourage the subject to cough and spit at any time during the induction if he/she feels the urge to do so.
6. After each 5 min interval carry out FEV₁ (or PEF) repeat spirometry. If FEV₁ or PEF falls more than 20% from the post-salbutamol value, stop the procedure. If induction is stopped due to an adverse effect (or for any other reason), record the total induction time.

Alternative procedure for high-risk patients: step by step

처방코드	처방명	급비	1회량	수가
HM0046	객담유도채취	급여	1.00	23,430 원
				<hr/>
				23,430 원

- 유도된 객담은 4°C에서 보관하여야 하고, **1-3시간** 내에 **객담 처리를 시작**하여야 한다.
- 유도객담검사는 호기산화질소 측정검사, 폐기능 검사, 기관지천식유발시험 등을 먼저 시행하고, 객담유도를 가장 마지막에 시행하는 것이 좋다.
- 객담유도를 2회 이상 시행하여야 하는 경우 객담 유도 간 최소 24시간 간격을 두는 것이 염증세포 결과에 있어, 재현성을 높일 수 있다.



[대한결핵 및 호흡기학회] 건강한 숨, 행복한 삶

@katrd1953 구독자 1.01만명 동영상 110개

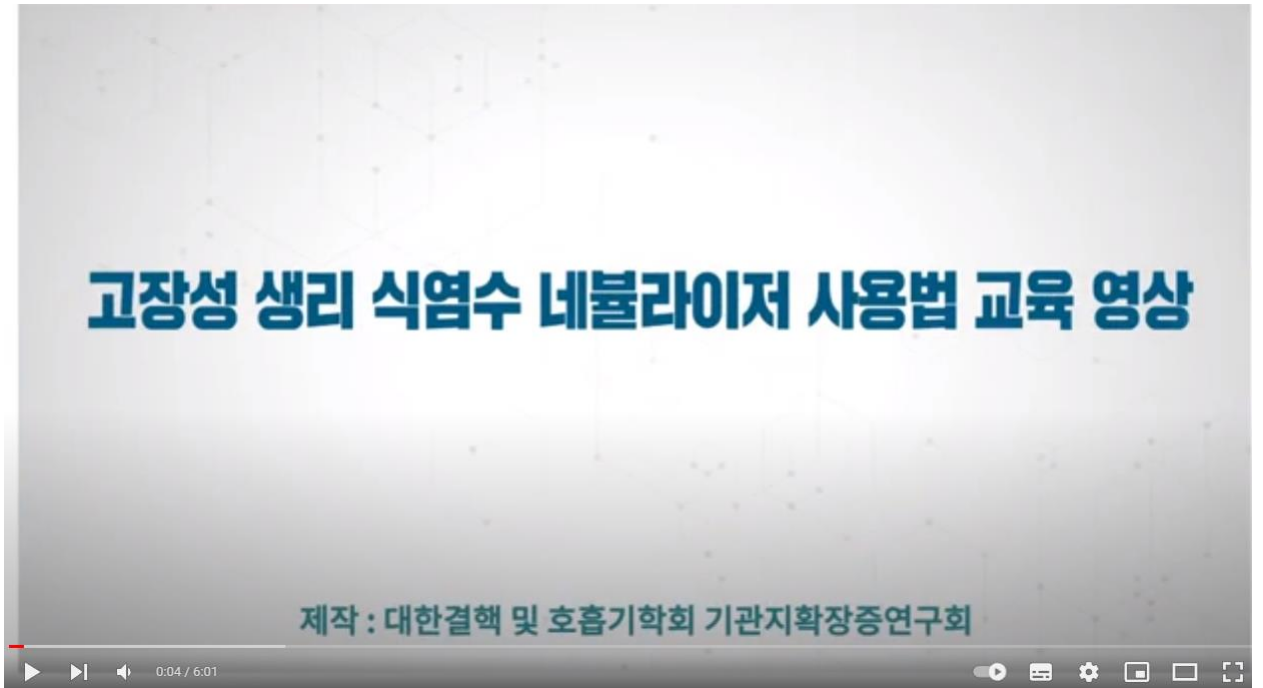
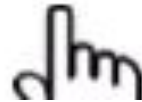
The Korean Academy of Tuberculosis and Respiratory Diseases >

- 홈
- 동영상
- SHORTS
- 라이브
- 재생목록
- 커뮤니티
- 채널
- 정보



고장성 네블라이저 사용법

조회수 533회 · 2개월 전



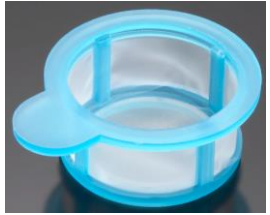
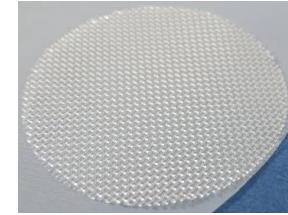
Sputum sample homogenization: entire expectorates

- 유도객담 처리는 세포 수 측정 목적의 경우 실온에서 시행 가능
- 객담 샘플의 **부피**와 무게를 기록
- 0.1%의 dithiotreitol (DTT)
 - 개봉하면 5일 이내 실온에서 사용 (UCSF protocol: 4주 이내 사용 가능)
 - 증류수로 1:9로 희석하여 사용 해야함. 10% sputolysin이라고도 하며 매일 새로 희석하여 사용
 - 1:1 같은 부피로 넣어 이때 균질화를 위해 pipetting 및 vortex를 이용
 - 실온에서 15분간 rotatory shaker (또는 rocker)로 섞어준다. (5분 간격 sputolysin 추가 가능)



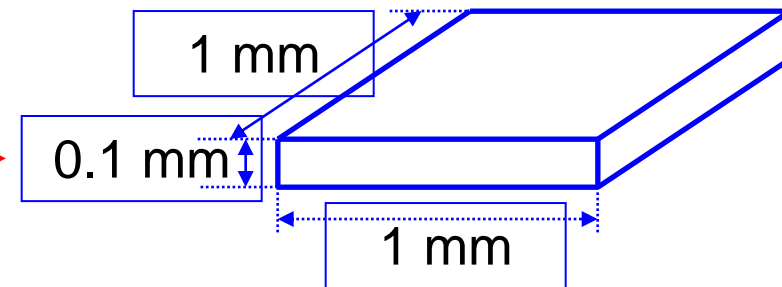
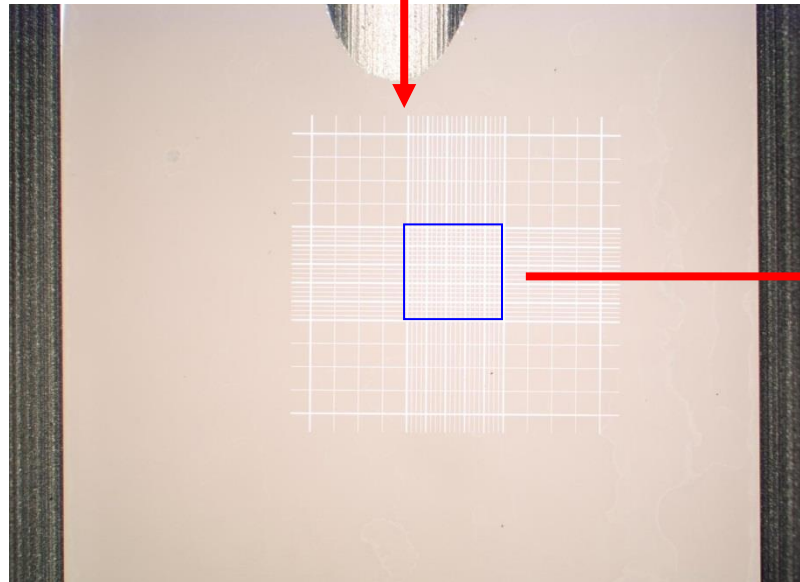
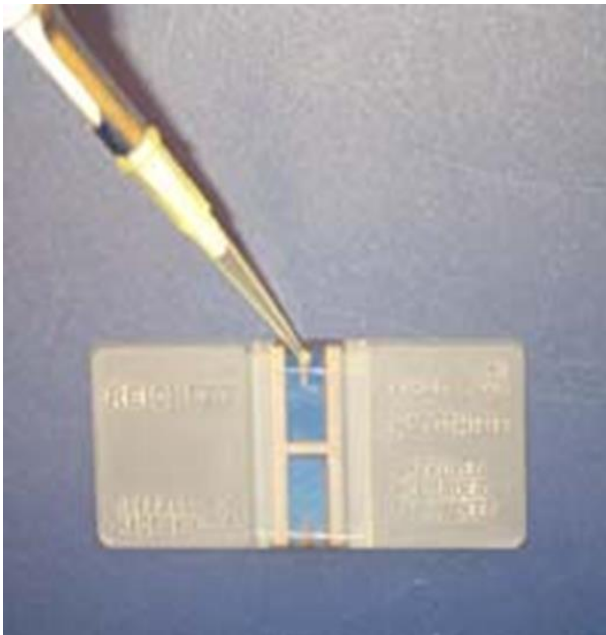
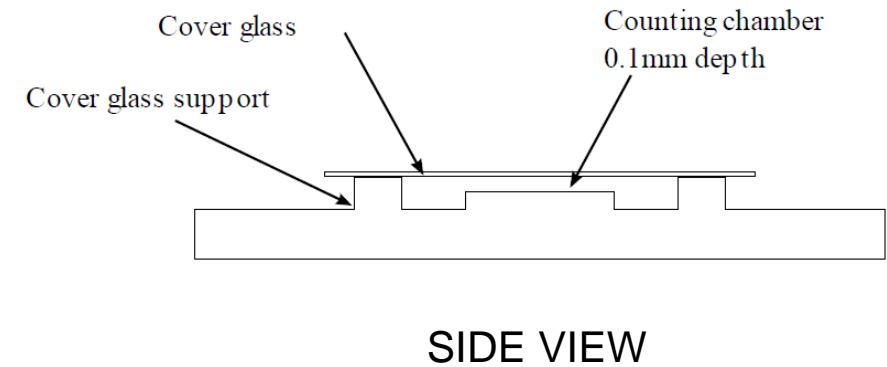
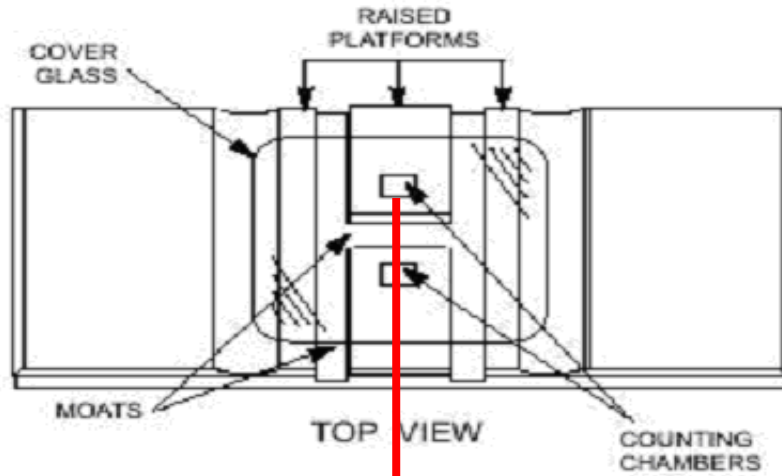
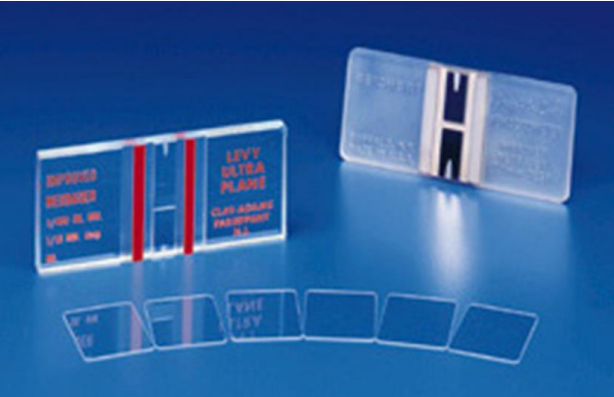
Sample filtration, Total cell count and viability

- 슬라이드 질을 높이기 위해 나일론 mesh (48 μm)에 여과
→ 국내 구입 가능한 40 μm pore sized nylon mesh 사용 가능



- 세포 생존 여부(viability)를 평가하기 위해 10 μL 의 세포 현탁액과 10 μL 의 trypan blue를 섞어서 염색을 하고 혈구계(hemocytometer counting chamber)를 이용하여 총 세포의 개수를 센다.
- 총 세포 수에 대한 생존 세포(cell viability) 수의 비율은 %로 보고한다.
→ 신뢰할 수 있는 결과를 얻기 위해 수작업으로 진행할 것을 권장

Hemocytometer and total cell counting (TCC)



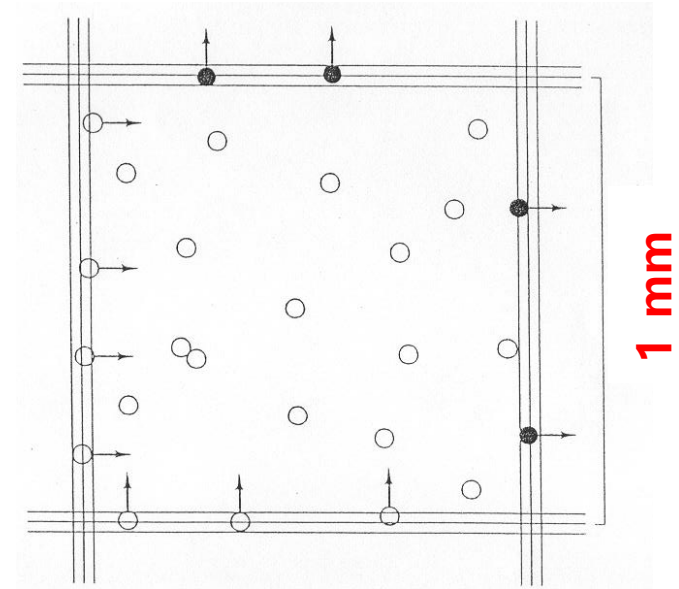
Volume : 0.1mm^3

$1\text{ ml} = 1\text{ cm}^3 = 1000\text{ mm}^3$

Example

TB : Trypan blue

○ Count
● Uncount



Dilution factor	Vol. of Cell Solution	Cell count	Total viable cells
0.1 ml CS + 0.1 ml TB (2)	20 ml	23	$20 \times 2 \times 23 \times 10^4 = 9.2 \times 10^6$ cells
0.1 ml CS + 0.3 ml TB (4)	15 ml	//	$15 \times 4 \times 23 \times 10^4 = 1.38 \times 10^7$ cells
0.1 ml CS + 0.9 ml TB (10)	10 ml	//	$10 \times 10 \times 23 \times 10^4 = 2.3 \times 10^7$ cells

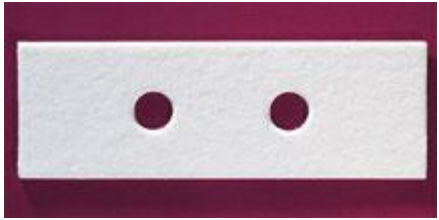
Centrifugation and storage of supernatant

- 세포와 상층액(supernatant)을 분리하기 위해 원심분리($400\times g$, 10분)를 하고, 상층액은 이후의 분석을 위해 따로 분리하여 -80°C 에서 보관할 수 있다.

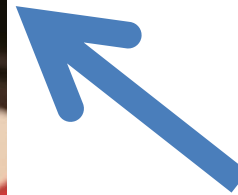
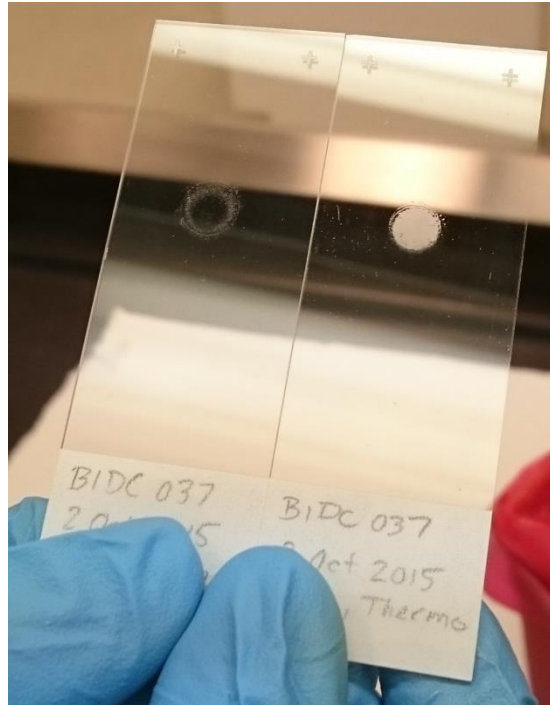
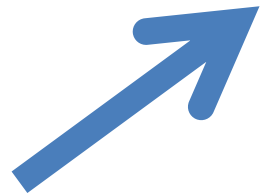
Cytospin centrifugation, staining and counts

- 여기에서 얻은 세포 침전물을 balanced salt solution (혹은 buffer)에 다시 녹이며, 이때 세포 농도는 1.0×10^6 cells/mL로 맞춘다. 이 후 각각의 cytospin에 대략 40–65 μ L의 샘플 ($450\text{--}650 \times 10^3$ cells)을 분주한다.
- cytospin 원심 속도는 $22 \times g$ 으로, 6분간 시행할 것을 권장한다.
- 이후 cytospin 슬라이드 염색을 위해 Giemsa 또는 Wright 염색을 이용할 수 있으며 다른 염색 방법도 가능하다. 감별 세포 계산(differential cell count)을 위해 400–500개의 비편평세포(nonsquamous cells)를 세면서, 이들 중 호산구, 호중구, 대식세포, 림프구, 기관지 상피세포의 분율을 퍼센트로 기록한다.
- 편평세포의 분율도 따로 기록해야 하며, 전체 세포 중 편평세포 분율이 높을 경우 (>20%), 즉 비편평세포가 80% 미만일 경우 감별 세포 계산의 재현성이 낮아지기 때문에 부적절한 검체로 생각해야 하며, 결과 해석에 주의가 필요하다.

Cytoentrifugation (Cytospin™)



200,000 cells,
Fisherbrand
filter card



200,000 cells,
Thermo Shandon
filter card

11) EDTA-DPBS Supernatants for Nucleotides and Cytokines

Supernatants	Number of aliquots	Volume stored per aliquot (µl)
Nucleotides	a1)	b1)
Cytokines	c1)	d1)
Cytokine Zymo Research RNA/DNA	e1)	f1)

*If the supernatant volume is greater than 8.6 ml, obtain 4 1000 µl aliquots for nucleotides and, 4 1000 µl aliquots for cytokines. Of the remaining sample, take 600 µl and mix it 1:1 with Zymo Research RNA/DNA shield. Apply the label called "SPU_DPBS_Zymo" to the aliquot containing the Zymo Research RNA/DNA shield.

If the sample volume is less than 8.6 ml start by getting 1 nucleotide sample between 200-500 µl and 1 cytokine sample at 200 µl. Mix one of the cytokine aliquots 1:1 with Zymo Research RNA/DNA shield and apply the label called "SPU_DPBS_Zymo" to this aliquot. If there is sample leftover after that, then continue alternating between nucleotide and cytokine aliquots (i.e., 200-500 µl for nucleotides, 200 µl for cytokines) until finished.

All supernatant samples are immediately stored in a -80° C freezer.

EDTA-DTT Supernatants

Supernatants	Volume stored (ml)
Sputolysin Sup 01	g1)
Sputolysin Sup 02	g2)

12) Cell Counts

Cell Counts:	# Dead	# Live	Total	Squamous Epithelial
a) Quadrant 1	1)	2)	3)	4)
b) Quadrant 2	1)	2)	3)	4)
c) Quadrant 3	1)	2)	3)	4)
d) Quadrant 4	1)	2)	3)	4)
e) Totals:	1)	2)	3)	4)

*Count live (clear) and dead (cell interior is blue) cells in each of the 4 corner quadrants. Include bronchial epithelial cells (BEC's), but exclude RBC's. Count squamous epithelial cells but do not include them in the total live/dead cell count. Perform total cell count and cell viability. Adjust the cell concentration of the sample to 0.5 - 1x10⁶ cells/ml and make 4 cytospin slides.

standard operating procedures

제목	Eosinophil stain & Diff.		
	검사코드	L23262	보험분류번호
			보험코드

작성자	나 승 원	작성일자	2023.8.26
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1. 검사 명: Sputum differential count or neut/eos stain
2. 검사 원리
3. 검체 채취 및 관리
4. 시약 및 장비
5. 검사과정
6. 보고 방법
7. 정도 관리: 자체 제작 표준 슬라이드
8. 임상적 의의
9. 참고문헌



지회 및 연구회
연구회

연구회 연례 사업 서식 지회 및 연구회 메뉴 매뉴얼

COPD 연구회

소개보기

ILD 연구회

소개보기

결핵 연구회

소개보기

금연 연구회

소개보기

기관지경 연구회

소개보기

기관지확장증 연구회

소개보기

기침 연구회

소개보기

분자폐암 연구회

소개보기

수면호흡장애 연구회

소개보기

집중치료 연구회

소개보기

천식 연구회

소개보기

폐혈관 연구회

소개보기

호흡기감염병 연구회

소개보기

호흡재활 연구회

소개보기

환경성폐질환 연구회

소개보기

기관지확장증 연구회

공지 객담 세포 분석 검사 프로토콜 **NEW**

메일보내기

조회수 : 3 / 추천수: 0 수정 / 삭제

23
2023.08

sputum induction_JOVE.mp4
sputum processing_JOVE.mp4

공지 2023년 11월 7일 저녁: 찰머 선생님 초청 강의 및 meet professor **NEW**

메일보내기

공지 공지: 4th World Bronchiectasis & NTM Conference - Virtual Edition

메일보내기

조회수 : 16 / 추천수: 0 수정 / 삭제

21
2020.09

공지 2020년 워크샵 강의록 다운로드

메일보내기

조회수 : 32 / 추천수: 1 수정 / 삭제

09
2020.10

공지 국내 기관지확장증 연구 출판논문 모음

메일보내기

조회수 : 37 / 추천수: 2 수정 / 삭제

18
2021.01

01 KMBARC registry protocol_bmjopen-2020.pdf
02 Population-based prevalence of BE in South Korea.pdf

공지 국내 결핵파괴폐 연구 출판논문 모음

메일보내기

조회수 : 9 / 추천수: 0 수정 / 삭제

18
2021.01

공지 2021년도 기관지확장증 연구회 집담회 강의록

메일보내기

조회수 : 12 / 추천수: 1 수정 / 삭제

02
2021.03

2021.02.17_Bronchiectasis in PID_김예진_강의록 제출.pdf
20210217 Future & direction of PCD (학회 제출).pdf

공지 심포지엄 (기관지확장증 주제) 다시보기 및 제 5차 세계 기관지확장증 conference 안내

메일보내기

조회수 : 18 / 추천수: 0 수정 / 삭제

13
2022.02

공지 연구회 연구비 공모 안내

메일보내기

조회수 : 6 / 추천수: 0 수정 / 삭제

09
2022.08

공지 2022년 우수 연구회 선정

메일보내기

조회수 : 2 / 추천수: 0 수정 / 삭제

20
2022.11

2022년도 우수연구회 표창장.jpg
2022년도 우수연구회 시상 사진.jpg

Key points: standardizing sputum sample processing

- ensure complete **homogenization** of sputum
- **filter** the sputum to remove excess mucus and debris
- perform a **manual Total Cell Count** prior to centrifugation
- count the entire **volume** of the counting chamber
- prepare cytopsins with an **optimum number of cells**
- ensure that buffers and stains are **optimized**
- perform a **400-nonsquamous** cell Differential Cell Counts
- report the **squamous cells separately**
- include positive and negative controls with special stains
- implement a regular **quality control** system
- use standard operating procedures (**SOP**) & sputum processing **WORKSHEET**

slido

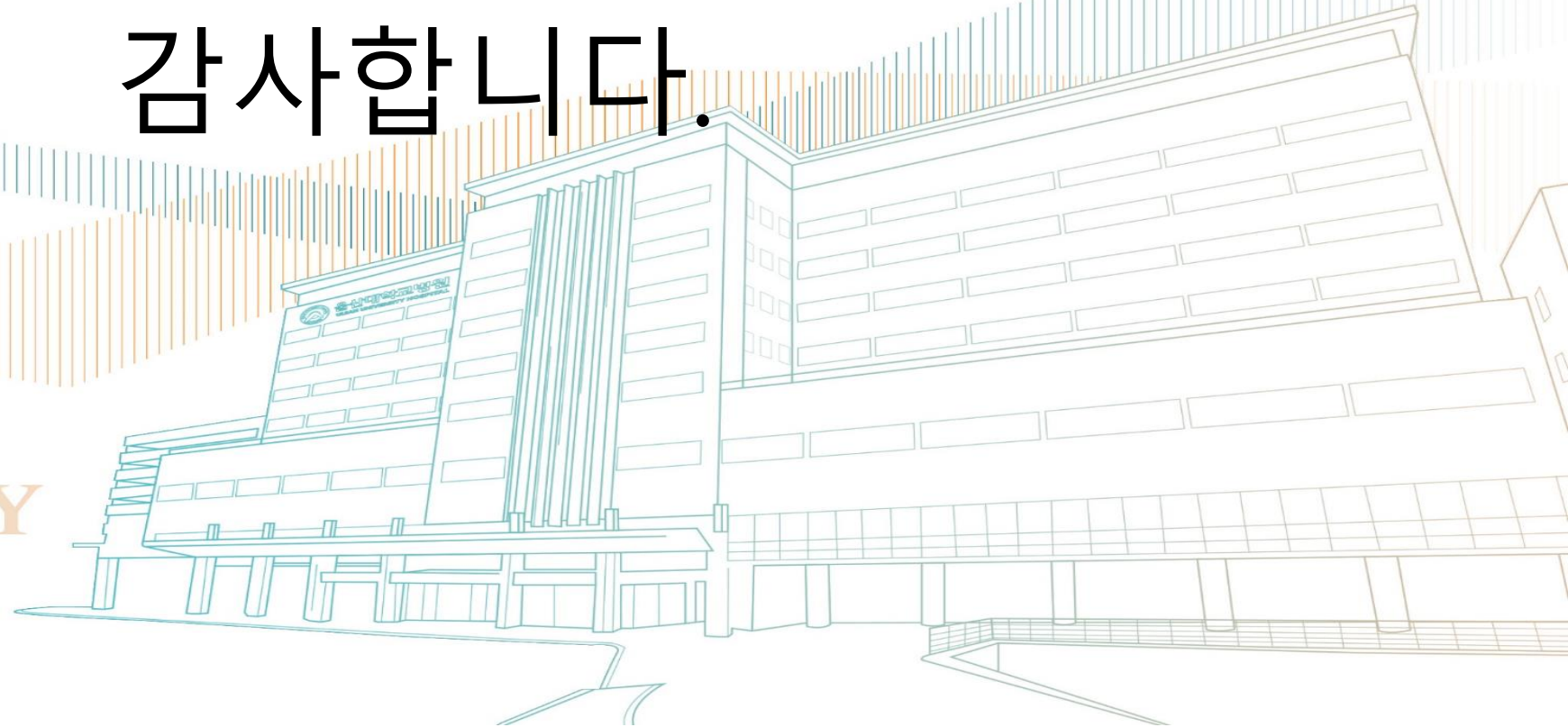


객담세포분석 검사는 병원에 세팅/
표준화가 필요한 검사이다.

① Start presenting to display the poll results on this slide.

경청해 주셔서
감사합니다.

ULSAN
UNIVERSITY
HOSPITAL



공지 사항

- 11월 7일 저녁: 찰머 선생님 초청 강의 및 meet professor (후원: 베링거) 확정
 - ◆ ROSE definition에 대한 내용과 BCO 환자에서 치료 전략 및 ICS 선별사용의 중요성
 - ◆ 국내연자 1명 (주제/연자 미정)
 - ◆ 연구회 회원분들 포함하여 많은 참석 부탁드립니다.
- 연구자료 활용 및 연구 활성화 위한 온라인 집담회
- 2024년 7월 4일(목)-6일(토) 7th World Bronchiectasis

