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Early Lung Cancer Detection Project

Evaluation of 5,10,15,20 tetrakis (4-carboxyphenyl) porphine (H₂TCPP) Grant No. DE-FG02-93ER61572

Final Report

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I. Abstract:

We evaluated a synthetic porphyrin, 5,10,15,20 tetrakis (4-carboxyphenyl) porphene (H_2TCPP) as a marker of carcinogenesis. H_2TCPP was compared with two other carcinogenesis markers evaluated in our laboratory for their ability to detect exfoliated sputum cells undergoing transformation to lung cancer.

Solid tumors arise through a process (carcinogenesis) which depends on a series of genetic changes, often point mutations, which activate protooncogenes and inactivate tumor suppressor genes resulting in the expression of peptide gene products. We have evaluated the detection of these gene products as pulmonary carcinogenesis markers from exfoliated airways epithelial cells. The critical step of marker validation is accomplished through linking of marker expression on preclinical sputum epithelial cells to those on subsequent lung tumor specimens. Specimen banks of prospectively collected exfoliated airways epithelial cells and subsequent tumor have been developed (or continue in progress) during our longitudinal studies. For each of these banks, an associated prospective database includes demographic information, potential carcinogen exposure and other risk factors. The ability provided by these specimen banks to directly link marker expression on premalignant exfoliated cells with that on subsequent tumor now permits the rational selection among the many potential new markers of carcinogenesis.

We had previously reported successful recognition of two immunoperoxidaselabeled monoclonal antibodies (Mabs) which have detected tumor-associated antigens expressed on sputum epithelial cells two years in advance of clinical lung cancer (sensitivity 91%, specificity 88%) from participants in the NCI-collaborative JHLP¹. Two monoclonal antibodies originally developed against small cell and non-small cell lung cancer^{2,3}, were applied to archived specimens from the NCI-Johns Hopkins study (the JHLP)⁴ and showed that in subjects with moderate atypical metaplasia, the uptake these antibodies could be quantified by enhanced video microscopy⁵ and predict the later development of lung cancer at least two years prior to clinical recognition¹. In related collaborative research, we have shown that a novel PCR-based technique has detected identical *k-ras* and *p53* gene mutations in both preclinical sputum specimens collected 1 to 13 months prior to diagnosis and in the patients' subsequently resected lung tumor⁶.

In the present project we first established optimal conditions for cultured neoplastic and non-neoplastic (sputum) cells to take up H_2TCPP . This was accomplished using spectrofluorimetry and video-enhanced fluorescent microscopy to maximize H_2TCPP auto-fluorescence across a matrix of substrate conditions, including; reagent concentration, incubation time, temperature, and pH. The second aim was to validate H_2TCPP on clinical material obtained from subjects monitored in advance of clinical cancer and link those marker results with subsequent histologic confirmation of disease. This was accomplished by applying H_2TCPP to sputum specimens archived by the Frost Center at Johns Hopkins which maintains a record of the clinical course and long-term follow-up for the patients from whom the specimens were obtained. We have used fluorescent immunostaining and flow cytometry to compare uptake of these cytoplasmic Mabs to that of a potential new marker of carcinogenesis, 5, 10, 15, 20 tetrakis (4 carboxyphenyl) porphene (H₂TCPP). The nuclear uptake of H₂TCPP was compared to a standard quantitative fluorescent DNA marker (7-AAD). Sample preparation for flow cytometry, has required identification, enrichment and separation of exfoliated epithelial cells undergoing carcinogenesis from the heterogenous cellular and mucoid sputum. We have met this formidable challenge and have reported a technique for mucus sulfhydryl bond disruption with dithiothreitol and microfiltration which successfully separates cellular elements from mucus glycoprotein. Nevertheless, our results suggest no advantage of H₂TCPP over existing nuclear (7-AAD) or cytoplasmic (703D4) Mabs for the early detection of pulmonary epithelial cells undergoing carcinogenesis.

II. Objectives, Significance and Previous Work

A. Objective

We have evaluated a synthetic porphyrin, 5,10,15,20 tetrakis (4-carboxyphenyl) porphene (H_2TCPP) as a marker of carcinogenesis. After establishment of the optimal conditions under which neoplastic and non-neoplastic cells take up H_2TCPP , we assessed the accuracy of the porphyrin as a biomarker of carcinogenesis. We determined that H_2TCPP did not fulfill its potential as a highly sensitive detector of carcinogenesis when compared to other carcinogenesis markers evaluated in our laboratory.

B. Rationale

No current techniques employing screening radiography, bronchioalveolar cytology, or direct biopsies have proven adequate for lung cancer detection at a curable stage^{7,8}. Enhanced understanding of tumor biology has turned attention to markers of the preclinical process of carcinogenesis⁹. A focus on carcinogenesis shifts emphasis away from "early" detection of bulk clinical malignancy, which for many epithelial organs (especially lung, breast and colon) is often metastatic (incurable) at the time of diagnosis, toward detection of individual cellular markers of the pre-malignant process. New evidence indicates that sampling the epithelium at risk is the appropriate strategy for the detection of solid tissue (epithelial) carcinogenesis in colon/rectum and lung. While no blood (serum or plasma) markers have shown promise as markers of early (pre-invasive) solid tissue tumors¹⁰, markers of carcinogenesis have been recognized in stool¹¹ and in sputum cells⁶ desquamated from the epithelia at risk. Adding to our previously described immunohistochemical technique for detection of oncofetal (differentiation) and tumorassociated gene products, our collaborators recently have developed a PCR based technique which can identify one cell carrying a mutant gene among a large excess (greater than 10,000) of normal cells¹². Using this technique k-ras mutations have been

detected in DNA purified from the stool of individuals with benign and malignant colorectal tumors prior to resection¹¹, as well as in sputum samples collected prior to clinical malignancy⁶.

While detection of mutations may be the most specific class of carcinogenesis markers, the large number of potential mutations and the exacting technique required by these assays makes detection of individual mutations a poor choice for initial carcinogenesis screening. Similarly, the preparation and interpretion of immunocytological slides is intermediate in sensitivity, specificity and technical requirement. A preliminary sorting of cells by a sufficiently sensitive fluorescing porphyrin has the potential to rapidly identify those sputum specimens which should be evaluated by the more specific assays.

Neoplastic tissue has been detected by its increased fluorescence compared with surrounding normal tissue after systemic injection of the tumor-localizing porphyrin, porfimer sodium (Photofrin: Ouadra Logic Technologies, Vancouver, BC, Canada), in murine¹³ and hamster¹⁴ models and in human cancers of the colon¹⁵, bladder¹⁶, and lung¹⁷. Frankly malignant cells have been detected in the sputum specimens from lung cancer patients following hematoporphyrin derivative injection¹⁸. Pre-malignant (moderate metaplasia and severe squamous dysplasia) changes in cervix¹⁹ and in lung²⁰ also exhibit porphyrin fluorescence. In modeling the transformation from normal to malignant mucosa in the hamster cheek pouch (following exposure to 0.5% DMBA in acetone), Crean et al. injected the DMBA-exposed hamsters with 1.0 mg/kg of porfimer sodium at various stages of tumor development¹⁴. These investigators found a progressive increase in porfimer sodium uptake and fluorescence with progressive mucosal transformation as indicated by tyrosine kinase activity even prior to morphologic alteration. Fluorescence and tyrosine kinase activity showed linear increases in intensity as mucosal change progressed through dysplasia to frank malignancy. Antagonism of cancer promotion (application of bombesin antagonist or somatostatin analogue) resulted in mucosal improvement and decrease in fluorescence²¹.

Yet, while the *in-vivo* association of malignant transformation and porphyrin fluorescence has been described, no one has yet reported porphyrin fluorescence in exfoliated epithelial cells prior to the detection of frank malignancy. A sensitive, easily applied marker of carcinogenesis would be of great importance to screening exfoliated cells for epithelial cancers of all types, potentially including lung, bladder and cervix.

C. Previous Work: Tissue Banking

Three specimen banks of prospectively collected exfoliated airways epithelial cells and subsequent tumor have been developed (or continue in progress) during longitudinal studies conducted by Dr. Tockman at Johns Hopkins. For each of these specimen banks, an associated prospective database includes demographic information, potential carcinogen exposure and other risk factors. Only by using these banks to directly link marker expression on premalignant exfoliated cells with marker expression

on subsequent tumor^{1,6} are we now able to rationally select markers of carcinogenesis for validation from the myriad potential markers.

The importance of banking the carefully obtained serial pre-malignant specimens from a high risk population along with specimens of the subsequent tumors cannot be overstated. The genetic instability of cells undergoing malignant transformation leads to a plethora of mutational events. Those events which arise early in carcinogenesis and are preserved in the final tumor have the greatest potential as early detection biomarkers. If only the tumor were available to provide mutational clues, arbitrary selection of possibly late developing events could lead to misdirected efforts at screening. Similarly, if only sputum were available (as in comparisons of specimens from smokers and nonsmokers), markers of the genetic lesions/products later to be repaired during the *p53*-directed G₁ growth arrest²² would identify only smokers, not cancer risk.

These three specimen banks were drawn from populations at 3 levels of lung cancer risk. The ELC drew specimens from community dwelling, middle-aged, male cigarette smokers whose average annual incidence of lung cancer was 5 per $1,000^{23}$. The LCEDWG has collected specimens from completely resected, stage I NSCLC patients. These former patients have an average annual incidence for a second primary of 3 per 100 $(3\%)^{24}$, and represent the population at highest risk. At intermediate risk are the YTC miners, an industrially exposed, community dwelling population with an average annual lung cancer incidence of $1\%^{25}$.

The two banks of specimens from highest risk individuals (LCEDWG and YTC) are continuing to accrue specimens. These populations of highest risk individuals offer striking efficiencies for the preliminary testing of carcinogenesis markers and chemoprevention agents, requiring only 1/10 the accrual of a heavy smoking population (eg. the ELC) for a similar number of lung cancer cases.

D. Previous Work: Molecular/ Immunocytochemical/ Videomicroscopy Studies and Pilot H₂TCPP Investigations

1. DEVELOPMENT OF PCR TECHNIQUE FOR SPUTUM

Since the 1960's, the only clinical marker available to detect early pulmonary neoplastic changes was the recognition of atypical metaplasia in exfoliated epithelial cells by light microscopy²⁶. We now know that cytomorphologic criteria alone are not sufficiently sensitive for lung cancer screening. Less than 10% of lung cancers in the NCI early lung cancer detection trials were detectable only by routine sputum cell morphology. More than half of the lung cancer cases presented clinically in the interval between annual screenings. Length-biased sampling, lead-time bias and misclassification, in addition to failures of detection and of therapy contributed to the lack of improvement in mortality rates ^{23,27,28,29}.

Yet theoretically, even small early lung tumors can shed tumor cells into the airways. Presumably, these cells would be rare and degraded to some degree at

expectoration. These factors might be more pronounced if the tumor arose in the small distal airways, and may partly explain the low detection of these exfoliated epithelial cells by conventional methods. However, even degraded DNA could be amplified by the PCR a million- fold allowing detection of even a single cell. The remaining problem would be to sort out the rare cancer cell DNA from the surrounding normal cell DNA. This problem was overcome in other epithelial tumor types by our Johns Hopkins collaborators who have developed a novel PCR approach allowing identification and quantitation of mutant DNA contained within cancer cells^{6,11}. As discussed above, these gene mutations within cancer cells are an integral part of tumor development. The very mutations that allow development of these neoplastic cells could be used as highly specific markers to identify the presence of tumor cells at an otherwise undetectable clinical stage.

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].			e 1.	Table								
the arc	ELC Resected NSCLC Patients with gene mutation analysis of sputum											
sp												
sp ide fif	Sputum Mutation	Tumor Mutation	Tumor Stage	Tumor/Loc	Age/Sex	Patient						
fro	Same	Kras-12Ser	T1,N0,M0	ADN-RUL	65-M	L1						
wl	Same	Kras-12Asp	T2,N1,M0	ADN-LUL	57-M	12						
de	Same	Kras-12Val	T1,N0,M0	ADN-RUL	63-M	L3						
1	Same	p53-273His	T3,N0,M0	ADN-LUL	51-M	L4						
ad	Same	Kras-12Cys	T1,N0,M0	ADN-LUL	67-M	L5						
of	Negative	p53-281Gly	T3,N0,M0	ADN-RUL	67-M	L6						
Tł	Same	Kras-12Cys	T1,N0,M0	ADN-RUL	70-M	L7						
m	Same	Kras-12Cys	T1,N0,M0	ADN-RUL	59-M	L8						
an	Negative	Kras-12Val	T1,N0,M0	ULG-RUL	48-M	L9						
ta	Same	Kras-12Cys	T1,N0,M0	ADN-RLL	63-M	L10						
	Negative	None	T3,N1,M0	ADN-RUL	60-M	L11						
th	Negative	None	T1,N0,M0	ADN-LLL	56-M	L12						
su	Negative	None	T1,N0,M0	ADN-RUL	65-M	L13						
ph	Negative	None	T2,N0,M0	ADN-RUL	61-F	L14						
pl	Negative	None	T2,N0,M0	ADN-LUL	62-M	L15						

Using HLP ve of m mens we ified n patients that trial later loped ocarcinoma e lung. Sidransky od ifies tumor t sequences igh PCR, loning into e and ng on L-agar. Tumor

DNA is then transferred to nylon filters and the plaques are probed with mutant-specific oligo-nucleotides which recognize the specific mutations The primary lung carcinomas from 10 of these 15 patients contained either a *ras* or a p53 gene mutation (Table 1). Using this PCR-based assay, stored sputum samples obtained prior to clinical diagnosis then were examined for the presence of these same oncogene mutations. In eight out of 10 patients, the identical mutation identified in the primary tumor was also detected in at least one sputum sample. The earliest detection of a clonal population of cancer cells in sputum was found in a sample obtained more than one year prior to clinical diagnosis.

2. <u>MONOCLONAL ANTIBODY/IMMUNOCHEMICAL DETECTION OF</u> <u>BIOMARKERS OF LUNG CARCINOGENESIS</u>

Alternatively, we also have found that pre-neoplastic epithelial cells exfoliated into sputum express surface and cytoplasmic lung cancer-associated antigens. After extensive testing, we selected two monoclonal antibodies from those being developed by colleagues at the NCI for detection of antigen markers predictive of subsequent lung cancer on preserved sputum epithelial cells^{1,30} from participants in the NCI-collaborative Early Lung Cancer detection trial at Johns Hopkins (the JHLP)^{Errort Bookmark not defined.}

In our preliminary report¹, 69 preserved samples were selected from the 626 cases with atypical metaplasia. The last available sputum sample before the development of cancer (average of 26 months prior to cancer) was analyzed by staining the specimen with a biotinylated chromogen after incubation with primary antibody. Visualization of the bound monoclonal antibodies in fixed cytologic specimens was greatly enhanced by the development of a modified avidin-biotin complex immunostaining method as reported by Gupta et al³¹. Specimens from individuals who ultimately developed lung cancer stained with a sensitivity of 91%, 2 years before the earliest appearance of neoplasia, whether by cytology, chest radiograph or clinical criteria. Specificity was 88% among specimens from individuals who remained free of lung cancer for an overall clinical accuracy of 88.7% (See Table 2). In summary, it appears that these two monoclonal antibodies can, with reasonable accuracy, detect changes in sputum samples two or more years before routine clinical lung cancer detection. Of critical importance is the finding that not only squamous cell, but also adenocarcinoma, large cell carcinoma, and particularly small cell carcinoma can be detected before the onset of clinical cancer. In fact all five cases of small cell cancer included in our sample were detected by the small cell antibody.

Table 2

Development of Lung Cancer by Double-Bridge Immuno-Peroxidase Staining Applied to the Most Recent Sputum Specimen Showing Moderate (or more severe) Atypical Metaplasia Stored by the JHLP

		No	
	Can	cer Cancer	Tota
Satisfactory Specimen			
Stain (+)	20	5	25
Stain (-)	2	35	37
Subtotal	22	40	62
Unsatisfactory Specimen	4	3	7
TOTAL	26	43	69
Sensitivity = 91%	O.R.= 70		
Specificity = 88%	95%	6 C.I.= 10.4	6 - 297
Chi-square $= 35.6$	p =	< 1 X 10 ⁻⁶	
Accuracy $= 88.7\%$			

During the refinement of these biomarkers for validation in population trials, a moderate amount of overlap in binding of SCLC- and NSCLCspecific antibodies was observed. This overlap in specificities may represent in part, the common pathogenesis of the cell types of lung cancer ^{32,33,34} and may detect expression of

differentiation antigens rather than "lung cancer-specific" antigens. 624H12 has been shown to bind to a difucosylated Lewis X epitope 35 . Difucosylated Lewis X is known to be an immunodominant antigen which has enhanced expression in fetal organogenesis ³⁶. Re-expression of this carbohydrate, fetal differentiation marker may follow neoplastic transformation of airway epithelial cells, particularly to SCLC, representing an example of an oncofetal antigen. 703D4 recognizes a 31 KD protein, heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 expressed to some degree in all forms of lung cancer, although the expression is more frequent in NSCLC³⁷. The range of cell types of lung cancer which express these antigens (91% of lung cancers) exceeds that of any single gene mutation possibly making these the most sensitive markers of carcinogenesis. In summary, a search for selected mutations or tumor associated antigens may accurately identify pulmonary carcinogenesis in advance of invasive malignancy. Recent data from these prospective studies have shown that almost 70% of those with hnRNP A2/B1 upregulation in their sputum would develop lung cancer in the first year of follow-up, compared with background lung cancer risks of 2.2 and 0.9% (35- and 76-fold increase, respectively)³⁸. Future studies may show that carcinogenesis detected at this stage may be amenable to reversal and possibly cure after intervention (eg. retinoid³⁹ or endobronchial laser⁴⁰), if not surgical resection.

These observations are of potentially enormous significance. An increase in lead time of 2 or more years might be sufficient to make widespread screening feasible and warranted even if lung cancer patients without atypical metaplasia remained undetected by this technique. If our present studies demonstrate that as few as 36.9% of patients with moderate atypical metaplasia can reliably be recognized 2 years prior to sputum cell morphology or clinical evidence of cancer, and if any of the remaining patients without atypia can be diagnosed, it will be a powerful argument for re-considering formal trials of the efficacy of early detection and intervention for lung cancer mortality reduction. The availability of valid intermediate endpoints for lung cancer would be a significant resource for efficacy studies of chemo-prevention or early surgical intervention.

3. <u>CYTOMETRIC VALIDATION OF IMMUNOCHEMICAL MARKER</u> <u>DETECTION</u>

The lack of a unique chemical structure for tumor-associated antigens signifies that a **qualitative** (presence/absence) criterion of marker binding is not sufficiently specific to characterize antibody-epitope binding⁴¹. These circumstances require the development of rigorous **quantitative** criteria for marker binding based upon the number of probe adherence sites per cell and the frequency of labeled cells per specimen. We have been engaged in studies to quantify immuno-labeled cell detection by characterizing the source and magnitude of the optical/electronic probe signal compared to all other sources of variation (the noise)⁴². Noise may arise a) from technical variation in the specimen collection/preparation, b) from variation in the assay, c) from biologic variation in the host (e.g., in the degree of cytologic atypia) and d) in the quantitation of marker uptake. Careful standardization of procedures has minimized the first two sources of variability^{30,43}. By design, only sputum specimens containing epithelial cells of moderate or greater atypical metaplasia were preserved, minimizing the third source of variability. We have recently reported our procedures for quantitation of marker uptake^{5,44}.

Immunostained sputum specimens showing atypical metaplasia from JHLP patients who subsequently developed lung cancer and those who did not were similarly interrogated at 510 and 600 nm., the maxima for optical transmission of the methylene blue counterstain and DAB, respectively. Within each field, an isolated epithelial cell is defined as the region of interest. Following edge-enhancement using a LaPlace transform, a perimeter is automatically drawn around the cytoplasm. The size and shape features of the nucleus and cytoplasm are recorded. A second perimeter then excludes the nucleus, and the quantitative densitometry features of the cytoplasm are recorded. After normalization for cytoplasmic area, the ratio of the wavelength-specific cytoplasmic optical densities are recorded. Finally, indices of cytoplasmic texture are recorded. The results of these analyses are recorded in a table for each patient. Values averaged over the five fields as well as the most extreme values for each patient are entered into the discriminant function which provides the automated interpretation of immunostain uptake. This automated interpretation is entered into the patient database. Univariate, correlation, cluster, common factor and discriminant function analyses were performed using Systat software and confirmed using SPSS/PC+. Multivariate (e.g., stepwise linear discrimination analysis) statistical techniques are employed to produce discriminant functions that combine relatively independent (i.e., orthogonal) parameters. A learning set of the original JHLP slides was used to generate these discriminant functions.

Significan

t differences were

found between the moderately atypical cells from the JHLP study groups who

developed

small cell

squamous cell or

undifferentiated cancer compared to the moderately atypical cells from JHLP

Table 3

Accuracy and Goodness-of-fit Tests of the Video-Microscopy Discriminant Function

Classification Results		Video-Microscopy Algorithm Predicted Group						
		Never Ca	ancer	Cancer				
Actual Group	No. of Cases	#	%	#	%			
Never Cancer	24	21	87.5	3	12.5			
Cancer	44	6	13.6	38	86.4			

Percent correctly classified: 86.8%

Multivariate Statistic Overall Wilks _ 0.487 Hotelling-Lowly 1.052	F-test 6.783 6.783	df 9,58 9,58	p-value 0.00 0.00	
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participants who remained cancer free. The moderately atypical cells from individuals who later developed lung cancer were more dense to transmitted light at the frequency of the DAB probe (avg. gray level), showed greater difference in light transmission at the frequency of DAB compared to the counterstain (gray ratio), and were more circular (shape factor), than were those whose atypical cells did not lead to respiratory cancer. Iterative principal axis (common factor) analysis showed that three factors; average gray, gray ratio, and shape factor, together explained most of the variance. The coefficients of the canonical discriminant function were used to generate discriminant scores which demonstrate an excellent separation of cancer cases from controls. Finally, Table 3 shows the accuracy of the discriminant function in separating cells of the two groups of patients is 86.8%, comparable to the 88.7% accuracy of the original cytopathology interpretation of the same slides (Table 2). These results suggest that there was a rigorous, objective basis to immunochemical detection of carcinogenesis markers and that image analysis has potential for greatly refining the process of early lung cancer detection research.

4. <u>PILOT H₂TCPP STUDIES WITH LOS ALAMOS</u>

From 1991 through 1992, a collaboration between Dr. Dean Cole, Life Sciences Division, Los Alamos National Laboratory, and Dr. Tockman resulted in pilot studies⁴⁵ which determined that uptake and fluorescence of 5,10,15,20 tetrakis (4-carboxyphenyl) porphine (H₂TCPP) could distinguish pre-malignant exfoliated epithelial cells from clinical specimens.

Our pilot studies used the water soluble 5,10,15,20-tetrakis (4-carboxyphenyl) porphine (H₂TCPP) obtained through the Los Alamos National Laboratory (but now commercially available) to label exfoliated sputum cells. Sputum specimens were examined from 24 individuals who later developed lung cancer and from 35 individuals who did not develop cancer, maintained in Saccomanno's preservative (50% ethanol, 2% polyethylene glycol 1540) as part of the ELC specimen bank. These specimens were suspended and aliquots were removed for cytospin (800 rpm for 2 minutes) to obtain a concentration of 10⁵ cells/cc. Glass slides were coated with poly-l-lysine to improve cell adherence. Slides were stained with the Papanicolaou (Pap) technique alone, H_2TCPP alone, and combined staining with H_2TCPP followed by Pap. After 24 hour incubation with 50µg/cc H₂TCPP in a humid chamber at 37°C.under reduced ambient light, slides were rinsed with PBS x 2, mounted in glycerol and sealed with a heated mixture of equal parts of vaseline, lanolin and paraffin⁴⁶ in preliminary studies, then sealed with colorless nail enamel in later studies. These studies demonstrated that pre-malignant epithelial cells (demonstrating moderate atypical metaplasia) from individuals who ultimately developed squamous cancer of the lung, took up the porphyrin and were distinguishable from background sputum cells under epifluorescent microscopy.

III. Approach

The purpose of this research is to evaluate a synthetic porphyrin, 5,10,15,20 tetrakis (4carboxyphenyl) porphene (H₂TCPP) as a marker of carcinogenesis. After establishment of the optimal conditions under which neoplastic and non-neoplastic cells take up H₂TCPP, we will assess the accuracy of the porphyrin as a biomarker of carcinogenesis. We determined whether H₂TCPP fulfills its potential as a highly sensitive detector of carcinogenesis from its uptake by banked pre-neoplastic material when compared to other carcinogenesis markers evaluated in our laboratory. While the mechanism which underlies the selective uptake of porphyrin by neoplastic tissue remains to be described, preferential uptake of porphyrin by frankly neoplastic tissue has been recognized for four decades⁴⁷. Most earlier porphyrin studies have relied upon endoscopic installation of hematoporphyrin derivative (HPD) for localization of neoplastic invasion of bladder¹⁶ and lung⁴⁸⁴⁹. More recently, frankly malignant cells have been detected in sputum specimens from lung cancer patients following injection of HPD¹⁸. Subcellular organelle localization of porphyrin has been reported in cultured malignant cells in mitochondria, the endoplasmic reticulum and in the perinuclear tubular structures (Golgi apparatus)⁵⁰. Present studies explore the ability of H₂TCPP to detect pre-malignant epithelial cells in the process of carcinogenesis.

IV. Methods

1. Sputum Cells and cell culture

Sputum specimens induced after a 15 minute saline inhalation were obtained from Yunnan Tin Miners and preserved in Saccomanno's solution (50% EtOH, 2% polyethylene glycol [Carbowax] 1540) in the YTC Biologic Specimen Bank for Lung Cancer. Sputum cells without evidence of cancer by light microscopy were separated from mucus glycoprotein by enzymatic and mechanical techniques⁵¹. Briefly, sputum specimens were added to an equal volume liquefication buffer made of 1mM DTT (Sigma # D-9779) plus 20 Ku/ml DNAseI (Sigma # D-4263). Following a 15 min incubation at 37° C, the suspension was passed through a 40 μ nylon mesh strainer (Falcon # 2340), centrifuged, washed with PBS and resuspended. The concentration of cells was adjusted to 1x10⁶/ml prior to use.

ATCC human bronchogenic cancer cell lines H520, HTB58 (Squamous Cell Cancer), H23, Calu-3 (Adenocarcinoma) and OH3 and H345 (Undifferentiated Small Cell Cancer) are maintained in our laboratory. H520 is incubated in RPMI-1640 supplemented with l-glutamine (Gibco-BRL, #320-1875-AJ) plus 10% FBS (Gibco-BRL, #230-6140-AJ, Grand Island NY). H23, Calu-3, H345 are incubated in RPMI-1640 supplemented with l-glutamine (Gibco-BRL, #320-1875-AJ) plus 5% FBS (Gibco-BRL, #230-6140-AJ, Grand Island, NY). OH3 is incubated in RPMI-1640 supplemented with l-glutamine (Gibco-BRL, #320-1875-AJ) plus 16% FBS (Gibco-BRL, #230-6140-AJ, Grand Island NY).. HTB58 is incubated in Eagle's MEM supplemented with EBSS, lglutamine, and NEAA without sodium bicarbonate (Sigma #M-0643, St. Louis, MO) plus 10% FBS (Gibco-BRL, #230-6140-AJ, Grand Island, NY).

2. Fluorescent staining methods

Staining methods were compared for their ability to distinguish three groups of cells: normal sputum cells only, ATCC lung cancer cells only and 1:1 mixture of tumor and sputum cells.

7-amino Actinomycin-D (7-AAD)

7-AAD (Molecular Probes A-1310) is a membrane-impermeant fluorescent DNA intercalator which binds particularly well to GC-rich regions of DNA. Upon binding, 7-AAD undergoes a spectral shift resulting in an emission maximum at 655 nm after excitation at 555 nm. This chromatin marker has been used to distinguish cells held in the G_0 and G_1 phases of the cell cycle⁵². Earlier investigations have described the procedures for gentle permeabilization and fixation to simultaneously quantify DNA with 7-AAD and cell surface antigens with fluorescein-labeled antibodies⁵³. Following a PBS wash, cells are resuspended in cold PBS and fixed by addition of freshly prepared 2% phosphate-buffered paraformaldehyde (Kodak, Rochester, NY) to a final concentration of 0.25%. Fixation at 4° C for 1 hour is followed by permeabilization with 0.2% tween-20 (Sigma # P-2287) in PBS for 15 minutes. After fixation and permeabilization, DNA was stained with 7-AAD by 30-minute incubation of cells in PBS containing 25 µg/ml of 7-AAD.

5,10,15,20-tetrakis (4-carboxyphenyl) porphine (H₂TCPP) fluorescent labeling

The synthetic H2TCPP is now obtained through Porphyrin Products Co. (P.O. Box 31, Logan, UT, Cat. No. T-790, as Mesotetra(4-carbo-xyphenol)porphine crystals <u>not</u> dried in acetone. Cell spreads and cytospin preparations are fixed in 95% ETOH for 10 min., then rinsed x2 with PBS. Under reduced ambient light, H₂TCPP is applied in concentrations from 50-200 μ g/ml to the slides which are maintained in a dark humid chamber for 4 to 24 hours, at temperatures from 37 to 4°C. At the conclusion of incubation, excess porphyrin is removed by 2 quick PBS washes. Slides are then dehydrated, mounted in glycerol, cover slipped and sealed with colorless nail enamel.

Fluorescein-labeled tumor-associated monoclonal antibody

Monoclonal antibody 703D4 which binds to a cytoplasmic antigen associated with NSCLC was applied to cells which have been paraformaldehyde fixed and permeabilized as described above. Cells are suspended in a 1:50 dilution of Mab 703D4 and incubated overnight at 4° C. After washing with PBS x 2, the cells are resuspended in 1:50 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories, BA-2000), incubated for 30 min at room temperature and again washed with PBS. Cell pellets are then resuspended in a 1:50 dilution of Fluorescein avidin D (Vector Laboratories A-2001), incubated for 30 min at 4° C in the dark and washed in PBS prior to measurement of FITC fluorescence.

3. Fluorescent microscopy and quantitation

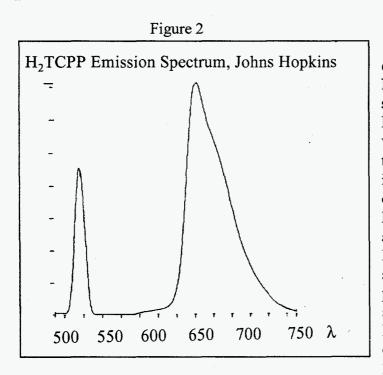
Image acquisition is performed on a Zeiss Axiophot epifluorescence and phase contrast microscope equipped with a Zeiss automatic photomicrographic system, camera control panel and with Zeiss Plan-neofluar objectives: 10x/0.30 n.a., 20x/0.50 n.a., 40x/0.75 n.a., 40x/1.3 n.a. oil, and 63x/1.25 n.a. oil (Carl Zeiss, Inc., Microscope Division, West Germany). The microscope is mounted on a Vibraplane vibration isolation table (Kinetic Systems, Inc., 20 Arboretum Rd, Roslindale, MA, Model 1201-04-11). Standard 100 W mercury vapor illumination intensity is assured by using a Multispeck multispectral fluorescence microscopy standard (Molecular Probes, Inc., H_2TCPP uptake was determined upon sputum samples from individuals who did not develop lung cancer. In each cancer patient, H_2TCPP uptake was evaluated first upon sputum samples that were collected during the time the patient was known to have cancer and progressively work backward through the pre cancerous period. Sputum specimens were counter-stained with the standard Papanicolaou procedure to assess the morphology of the cells which take up the H_2TCPP .

6. Flow Cytometry

 1×10^{6} cells suspended in 1 ml PBS were analyzed on an Elite flow cytometer. 7-AAD fluorescence was excited at 488 nm and measured through a 650 nm long pass filter. Porphyrin fluorescence was excited at 518 nm and collected through a 650 nm. long pass filter. FITC fluorescence was excited at 488 nm. and collected through a 510-530 nm narrow band filter.

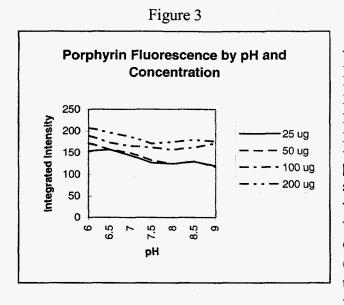
V. Results

H2TCPP FLUORESCENT EMISSION SPECTRA



copies of the emission spectrograph (Figure 1)⁵⁴.

Preliminary studies conducted at the Los Alamos National Laboratory had suggested that excitation of H₂TCPP by standard mercury vapor illumination filtered through a 495 λ fluorescein isothiocyanate (FITC) excitation filter caused the H₂TCPP to autofluoresce with a maximum emission at 560 λ . Following through after submission of the annual progress report, I have subsequently learned that the Los Alamos data showed an emission peak at 650 (not 560) λ , and was provided with



To replicate this observation, we used a Perkin-Elmer Luminescence Spectrometer (Model LS50, Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England), to excite cuvettes of H₂TCPP at concentrations of 50 μ g/ml. at the FITC wavelength and scan for the peak emission wavelength (Figure 2). Consistent with the Los Alamos data, the peak emission wavelength was 645 λ (activity range 620-710 λ). Setting the emission wavelength at 645 λ , the H₂TCPP solution was scanned

over the range 450 to 600 λ to determine the optimal excitation frequency. Optimal excitation occurred at 518 λ (activity range 490-545 λ), with subsidiary peaks at 557 and 584 λ . H₂TCPP excitation at 518 λ and emission at 645 λ is similar to the spectroscopic properties of propidium iodide, a common fluorochrome for measurement of DNA/RNA content.

Video-enhanced fluorescent microscopy was used to maximize cell-area normalized fluorescence across a matrix of substrate conditions. H₂TCPP concentration was increased (25, 50, 150 and 200 μ g/ml.) while pH was varied from 6.0 through 9.0 (Figure 3). Increase in both parameters enhanced fluorescence, however, preliminary results suggest that low concentrations and neutral pH are optimal for intracellular localization of the porphyrin.

Quantitation of fluorescence is being conducted separately for freshly harvested, Saccomanno preserved human lung cancer tissue culture cell lines. Present studies have been limited to Squamous Cell Cancer (ATCC: HTB58).

Sputum Cell Separation

Sputum specimens treated with liquefying agents 0.5mM DTT plus 10Ku/ml DNAse I and filtered by 40 micron strainer shows that this method effectively reduces background mucus and clumps of leukocytes and epithelial cells⁵¹. Sputum cells were separated well and could be evaluated by flow cytometry.

Analysis of DNA Staining

The fluorescence histograms of 7-AAD are presented in the Appendix.

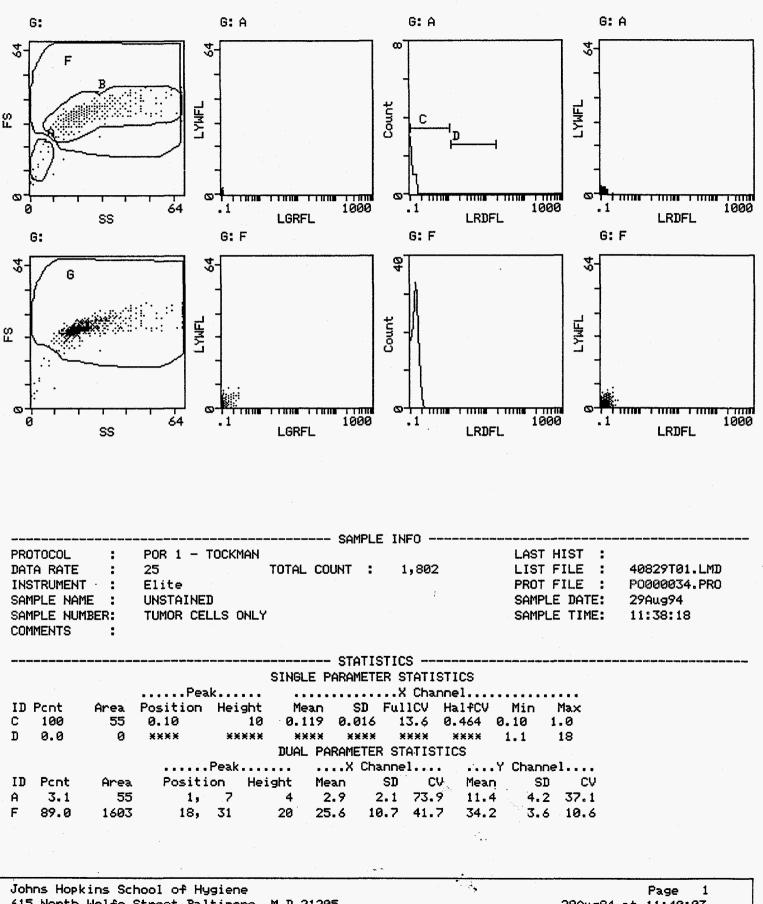
VI. Literature Cited

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		Si	ze	Granu	larity		Fluore	scence	1
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Tumor Cells									
Unstained									
Small, Non Granular	3,1	11.4	4.2	2.9	2.1	red	55	0.12	0.02
Large, More Granular	89.0	34.2	3.6	25.6	10.7				
Small, Non Granular	39.9	5.3	2.7	0.5	1.4	green			
Large, More Granular	53.5	32.8	4.4	11.6	49.5				
Sputum Cells									
Unstained									
Small, Non Granular	60.0	10.1	4.8	3.5	2.7	red	4549	0.15	0.04
Large, More Granular	29.5	29.2	7.0	32.8	15.7				
Tumor Cells									
Porphyrin Stained									
Small, Non Granular	2.1	9.7	5.2	2.5	2.8				
Large, More Granular	90.7	34.3	3.5	26.6	12.0	red	8592	77.7	30.8
Sputum Cells									
Porphyrin Stained									
Small, Non Granular	61.4	11.2	4.9		2.6				
Large, More Granular	29.5	29.5	7.0	31.3	16.0	red	3517		28.9
							732	17.9	3.24
Tumor Cells									
7-AAD Stained									
Small, Non Granular	2.2	9.8	5.4	2.9	3.1				
Large, More Granular	87.7	34.6	3.7	26.0	12.1	red	3634		7.58
							5897	19.6	1.66
Sputum Cells		· ·							
7-AAD Stained									
Small, Non Granular		10.9	4.8		2.8				
Large, More Granular	34.9	29.3	7.0	33.6	15.6	red	5118	7.2	4.8
Tumor Cells									
FITC Stained									
Small, Non Granular	8.9		5.2		3.1	-			
Large, More Granular	84.7	32.8	4.4	24.3	11.6	green	8809	0.16	0.07
Sputum Cells									
FITC Stained									
Smail, Non Granular	43.9	9.7	4.6	3.5	2.7				
Large, More Granular	42.0	29.8	7.2	32.9	14.4	green	6533	0.13	0.04

HISTOGRAM DISPLAY

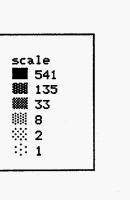
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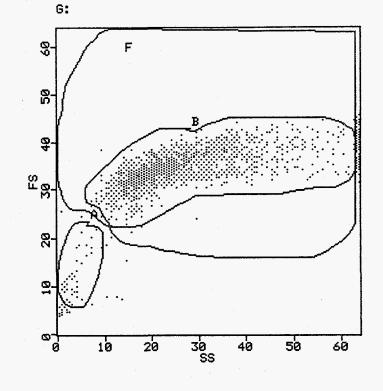


615 North Wolfe Street, Baltimore, M.D.21205

SAMPLE NAME : UNSTAINED SAMPLE NUMBER: TUMOR CELLS ONLY GATES

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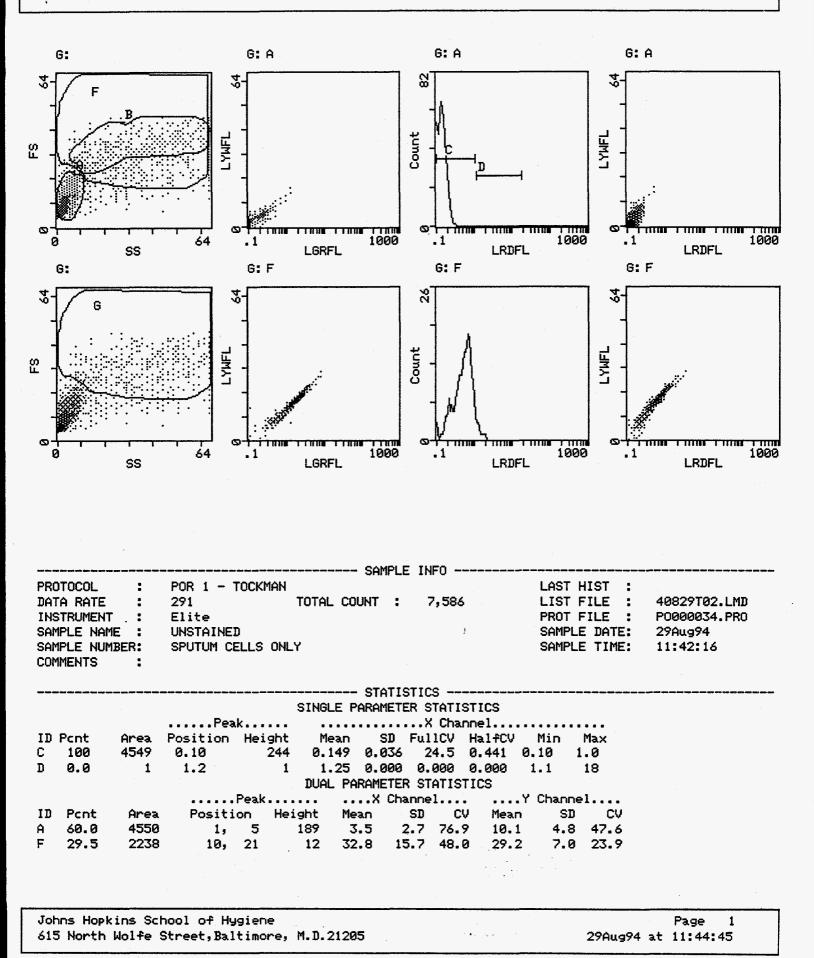
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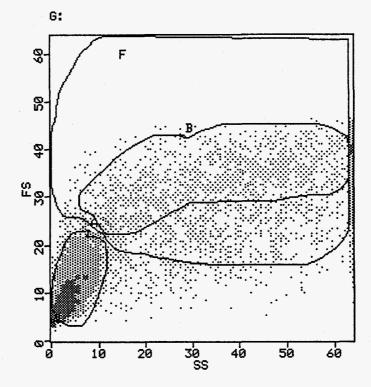
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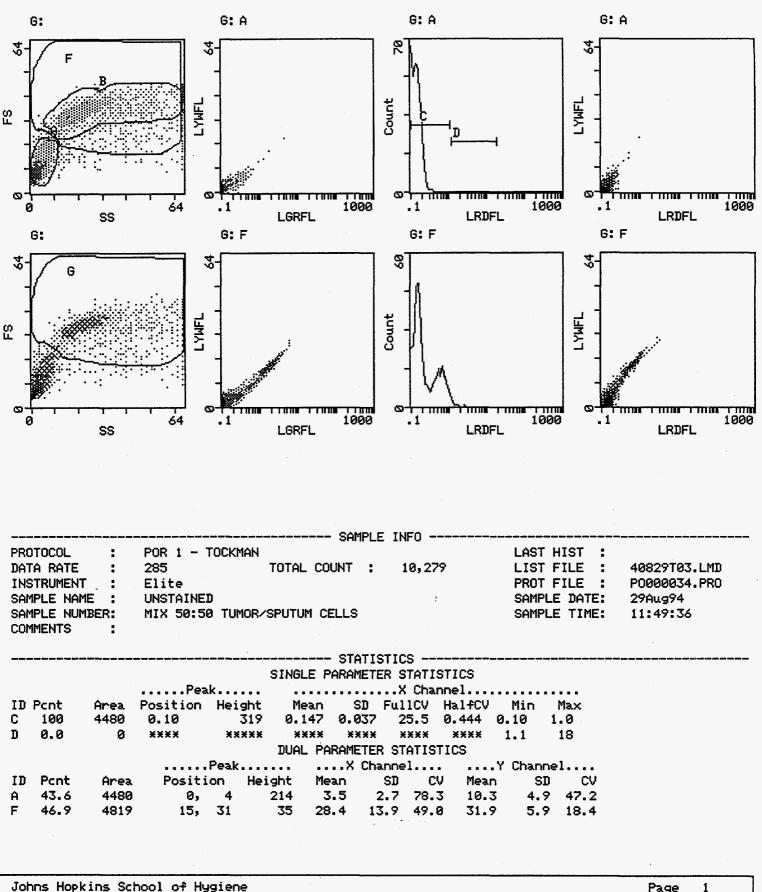
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615 North Wolfe Street, Baltimore, M.D.21205

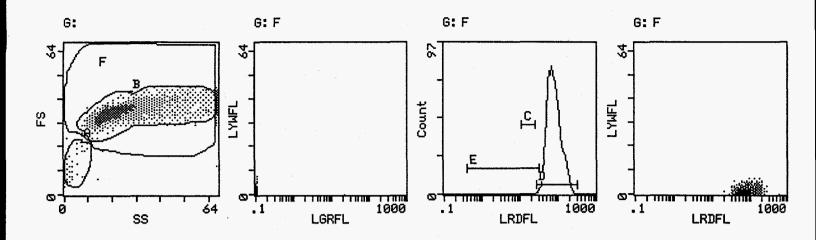
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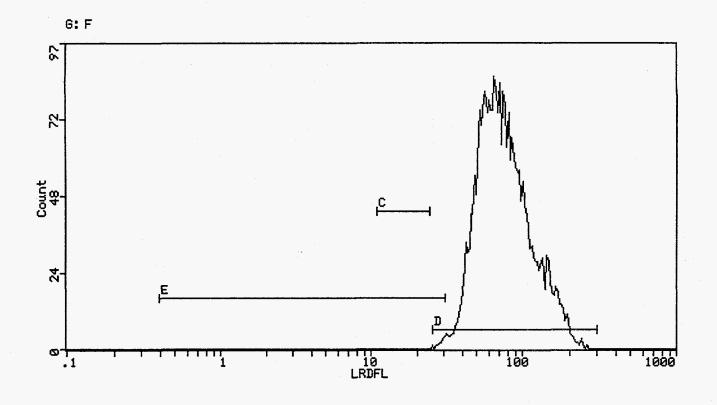


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Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205 SAMPLE NAME : STAINED PORPHIN SAMPLE NUMBER: TUMOR CELLS ONLY

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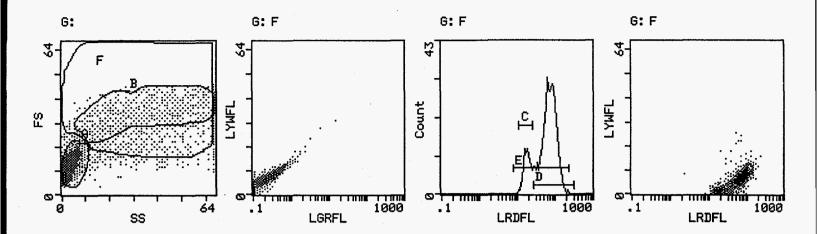
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Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205 GATES

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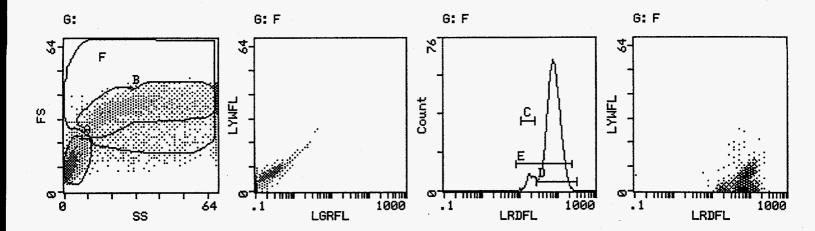
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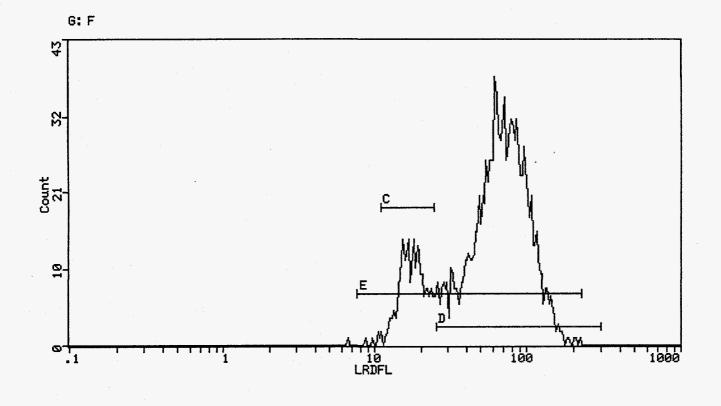


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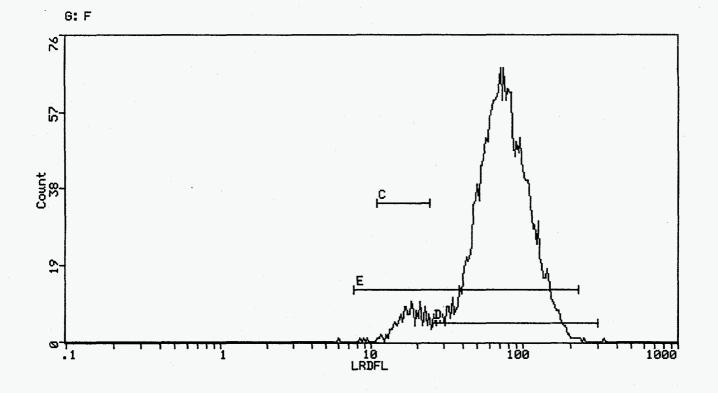


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С	Low Chan	nel =	11, High	n Channel	= 25	С				
D	Low Chan	nel =	26, High	n Channel	= 310	D				
Ε	Low Chan	nel =	7.8, High	h Channel	= 232	Е			•	

SAMPLE NAME : STAINED PORPHIN SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:20:58



GATES

من خان نال کو دی دی دی دور بختر میت خذن افت خان دی دور برو.	ب الله الله الله الله الله عنه عنه عنه الله الله الله الله الله الله عن عليه عن الله الله أليه	SA	MPLE INFO	و چې کن خت خت بنه بيه که اگر چه خت ختر وي چو چو کو که ان ختر خت ختر وي چو ه	بري بي بي من عن عل يو جد جد عن عن كار الله الله الله الله الله الله الله ال
PROTOCOL :			· · · · · · ·		
DATA RATE :	632	HIST COUNT	: 7,104	LIST FILE :	40829T09.LMD
INSTRUMENT :	Elite			PROT FILE :	P0000034.PR0
SAMPLE NAME :	STAINED PORPHIN		?	SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR	SPUTUM		SAMPLE TIME:	12:20:58
COMMENTS :					
ف نگ کا اور در دور برد دند بال نگ نگ موجه دور	ہ جوہ چیں جان اپنے ہیں ختنہ نعنہ نظار کا کہ کہ ہے ہے۔ رویہ خرب ایرن ،	ST	ATISTICS	ن خذه هي چو چيد جيد احد حلة خلن وي پريد چيد حمد حمد حلة که انته بيورد جيد خده حلة ا	بساخين خلك هو چو هجا خذا اختا قلت وو جره خلت که ای او هو او او او او او او
		SINGLE PARA	METER STATIST	TCS	
	Peak		X Chann	e1	
ID Pont Area	Position Height	Maan	ST FULLOU H	114CU Min May	

	Pcnt 6.4	Area 452	Position 22	Height 14		-	FullCV 18.4		Min 11	Max 25	
IJ	93.2	6620	73	76	75.3	29.5	39.2	21.3	26	310	
Е	99.7	7085	73	76	68.4	35.8	52.4	21.3	7.8	232	
_					05	~					
U.	Low Una	nnei =	11, Hig	n unannei	= 25	L.					
D	Low Cha	nnel =	26, Hig	h Channel	= 310	D					
F	Low Chai	nnel =	7.8. Hic	h Channel	= 232	F					

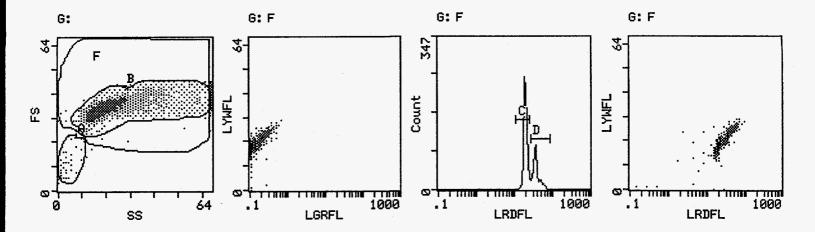
HISTOGRAM DISPLAY	
SAMPLE NAME : STAINED PORPHIN	SAMPLE DATE: 29Aug94
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM	SAMPLE TIME: 12:20:58

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C Low Channel = 11, High Channel = 25 C D Low Channel = 26, High Channel = 310 D A A AMORPHOUS F F AMORPHOUS

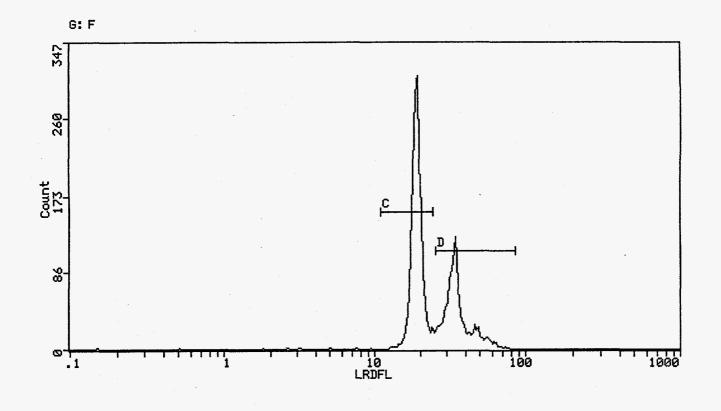
HISTOGRAM DISPLAY

SAMPLE DATE: 29Aug94 SAMPLE TIME: 11:56:46



، منبذ علما، يذلب عمل ابنی نوبه ميد وي جيه ويه.	ه سند سب هند زنند شده سب			:	SAMPLE I	NF0	د دینه است اعلیه همه مانه ساه من	م الله الله مي علم الله س	فللد فلنة سبد فلتة غقته فسا فلتد لابية ة		
PROTOCOL	:	POR 1 - TO	CKMAN					LAST I	HIST :		
DATA RATE	:	56	•	TOTAL COUN	1T :	10,991		LIST I	FILE :	40829T04.L	MD
INSTRUMENT	. :	Elite						PROT I	FILE :	P0000034.P	RO
SAMPLE NAM	Е:	STAINED 74	AD			?		SAMPLI	E DATE:	29Aug94	
SAMPLE NUM	BER:	TUMOR ONLY	•					SAMPLI	E TIME:	11:56:46	
COMMENTS	:										
									ويبيه ويريخ يعبيه فيليك فليله فليله فليته بتبيع		
				SINGLE PA							
		Peak									
ID Pent	Area	Position		Mean			alfCV		Max		
C 61.2	5897	20	347	19.6			5.00	11	.25		
D' 37.7	3634	35	138	36.7		20.7 8		26	88		
				DUAL PAR							
		P			.X Chann		• • • • ¥	Channe	1		
ID Pent	Area.	Positio	n Hei	ght Mea	n SD	CV CV	Means	SD	CV		
A 2.2	246	0,	4	20 2.9	9 3.1	107.3	9.8	5.4	55.5		
F 87.7	9640	17, 3	33	104 26.0	0 12.1	46.5	34.6	. 3.7	10.7		
									×.,		
								- -			
								• •.			
		hool of Hyg					e e tal			Page 1	
013 North	MOILE :	Street,Balt	imore,	m.D.21205			•		ZYAUGY4	at 12:01:42	

SAMPLE NAME : STAINED 7AAD SAMPLE NUMBER: TUMOR ONLY

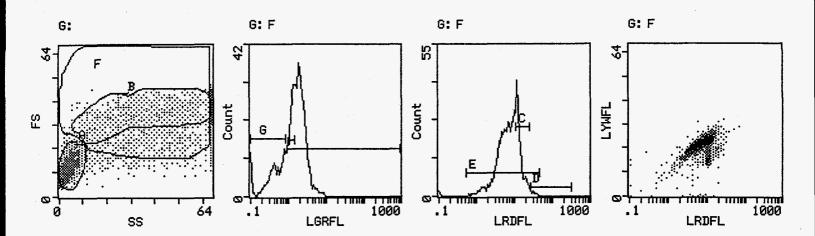


ہے جب سے بند جند بنیہ 14 جب ہور سے سے سن بندر ہے ہے ہے	هنده فشاه فيسة بنسية الثالة فكله فتشر كماة فشاه فعلا فيتين بيجه بيجه بجهد ويهد و	SA	MPLE INFO		رده من هو هو من من من من هو من من عن عن عن من من من من من من من
PROTOCOL :	POR 1 - TOCKMAN				
DATA RATE :	56	HIST COUNT	: 9,640	LIST FILE :	40829T04.LMD
INSTRUMENT :	Elite			PROT FILE :	P0000034.PR0
SAMPLE NAME :	STAINED 7AAD		;	SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR ONLY			SAMPLE TIME:	11:56:46
COMMENTS :					
وی کے این بین بین ہے جب جب کے اور کے کہ ایک بینے میں ہے	ست بیری بری بین افغا خت خت مند ست بی بین بری بی بی بی د	ST	ATISTICS		باب ماب البلد اللبة الأله على الله الله الله الله الله الله الله ال
		SINGLE PARA	METER STATISTICS		
	Peak		X Channel		
ID Pcnt Area	Position Height	Mean	SD FullCV HalfCV	Min Max	
C 61.2 5897	20 347	19.6 1	.66 8.48 6.00	11 25	
D 37.7 3634	35 138	36.7 7	.58 20.7 6.15	26 88	
	$(A_{i}, A_{i}) = (A_{i}, A_{i}) = (A_{$				
C Low Channel =	11, High Chann	el = 25 C			
B Low Channel =	26, High Chann	el = 88 D			

SAMPLE NAME : STAINED 7AAD SAMPLE.NUMBER: TUMOR ONLY	HISTO	GRAM DISPLAY	SAMPLE DATE: SAMPLE TIME:	-	
C Low Channel = 11, High Channel = D Low Channel = 26, High Channel = A A AMORPHOUS F F AMORPHOUS					

SAMPLE NAME : STAINED 7AAD SAMPLE NUMBER: SPUTUM CELLS ONLY HISTOGRAM DISPLAY

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:02:57



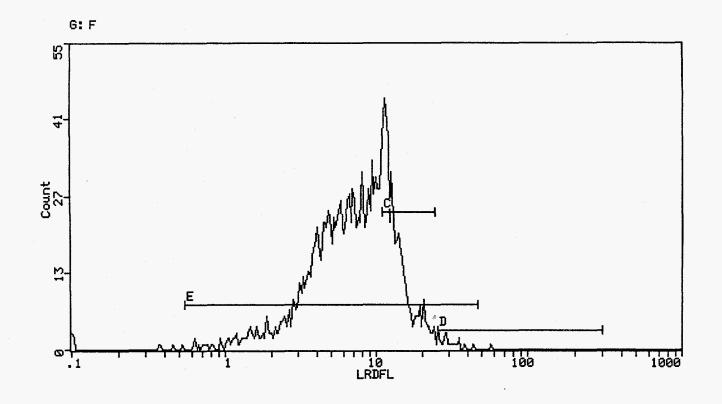
			سه سه که خل من کم رو برو برو برو		SAM	IPLE IN	1F0	مبه جد جه مدر کر منه عنه هه ه	وو خبر می وی خبر خبر الله الله		ہے۔ یہ ہے ^ا ن بین بنا ہو کا کہ اور
PROT	rocol	:	POR 1 - 1	Fockman					LAST H	HIST :	
DATA	RATE	1	527	TOT	AL COUNT	:	14,782		LIST F	FILE :	40829T05.LMD
INST	TRUMENT		Elite						PROT F	FILE :	P0000034.PR0
SAMP	PLE NAM	1E :	STAINED 7	7AAD			;		SAMPLI	E DATE:	29Aug94
SAMP	PLE NUM	IBER:	SPUTUM C	ELLS ONLY					SAMPL	E TIME:	12:02:57
COMM	1ENTS	:									
	بہ جب دے ہے۔ چید ہ	به زوی هی هی هی هی هن نوب ب	ین سے بیٹ نے نے نے نے _{کہ} ہے ہے							، بین سی جب است احب احب عبت از	چین سے اور کے چید اس مند بالیہ کیا ہیں جزنہ سار خلیے کے کہ کہ کر
			Dee		GLE PARAM						
ID F)	Area	Position		Mean S			HalfCV		Max	
C 2		1393	12	-		75 1		5.14		25	
	1.6	83	26					1.43		510	
-			20	-	al parame						
				Peak					Channe	1	
ID	Pcnt	Area	Positi	on Height		SD			SD	CV	
A	53.1	7853	0,	4 294	3.7	2.8	75.2	10.9	4.8	44.4	
F	34.9	5158	9,	22 20	33.6	15.6	46.4	29.3	7.0	23.8	

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Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205 Page 1 07Sep94 at 11:57:27 SAMPLE NAME : STAINED 7AAD SAMPLE NUMBER: SPUTUM CELLS ONLY

GATES

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:02:57



		به الله، بيبه رسو وي بسار بزنيا فحد جد الله نقل د			AMPLE	INFO -			سه عله الي بريور خين هذه كله كله ه	ی روی می وجد مرد سب شند اطار که که این بود بود جن منه سب منبا اطار
PROTOCOL	:	POR 1 - T	ockman							
DATA RATE	:	527	н	IST COUNT	•	5,158		LIST	FILE :	40829T05.LMD
INSTRUMEN	Τ. :	Elite						PROT	FILE :	P0000034.PR0
SAMPLE NA	ME :	STAINED 7	AAD			?		SAMPI	LE DATE:	29Aug94
SAMPLE NU	MBER:	SPUTUM CE	LLS ONLY					SAMPI	LE TIME:	12:02:57
COMMENTS	:									
			ور چرد برد نب خد خذ خب که ک	9	STATIST	FICS	بین وی وی من من می می می م			س بناء اسه این جی برو شنا انتا این برو بین این این این این ا
			. 9	SINGLE PAR	AMETER	R STATIS	STICS			
		Peak	<			.X Chai	nnel			
ID Pont	Area	Position	Height	Mean	SD F	FuliCV	HalfCV	Min	Max	
C 27.0	1393	12	55	13.9	2,75	19.8	5.14	11	25	
D 1.6	83	26	5	32.9	7.21	21.9	1.43	26	310	
E 99.2	5118	12	55	7.20	4.80	66.7	5.14	0.55	48	
C Low Ch	annel =	: 11. High	Channel	= 25	C ¹¹	· · ·			• •••	

D Low Channel = 26, High Channel = 310 D E Low Channel = 0.55, High Channel = 48 E

Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205 Page 1 07Sep94 at 11:57:15

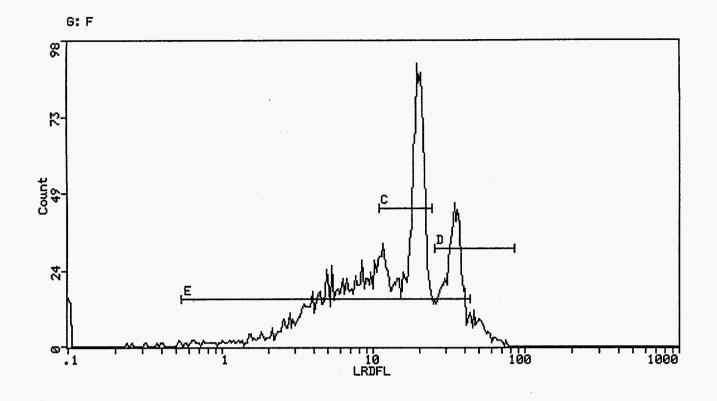
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HISTOGRAM	DISPLAY
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SAMPLE NAME : STAINED 7AAD SAMPLE NUMBER: SPUTUM CELLS ONLY SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:02:57

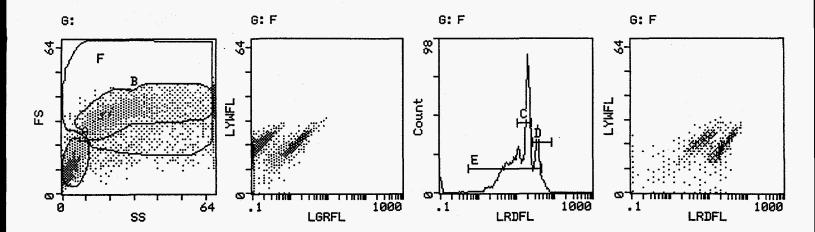
C Low Channel = 11, High Channel = 25 C D Low Channel = 26, High Channel = 310 D A A AMORPHOUS F F AMORPHOUS

Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205 Page 2 07Sep94 at 11:57:27 SAMPLE NAME : STAINED 7AAD SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM



		مینه است است جینه مینه خوان باید است. همه مینه می هم می می وجود ه		SAMPLE	INFO		، میں میں جو میں میں میں میں سے م	وهم ويد جرم عنه جند اعت اعت اينت اعت الله الله الله الله الله الله الله الل
PROTOCOL DATA RATE INSTRUMEN GAMPLE NA GAMPLE NU COMMENTS	IT : ME :	POR 1 - TOCKMA 328 Elite STAINED 7AAD MIX 50:50 TUMO	HIST COUN	т:	8,366	PROT	FILE : FILE : LE DATE: LE TIME:	40829T06.LMD P0000034.PRO 29Aug94 12:07:01
فالد زمة الإله مرك الحر ألم حمر من حف ال	سا بالد بند سد ابد بد بابد			STATIS	TICS			
					R STATISTICS			
		Peak			X Channel			
ID Pont	Area	Position Heig			FullCV Hal-		Max	
40.2	3359		98 17.8	3.96	22.2 8.5		25	
21.3	1786	35	57 36.8	7.84	21.3 7.4	1 26	88	
94.8	7928	21	98 12.6	10.8	86.2 8.5	59 0.55	45	
: Low Ch	annel =	: 11, High Cha	nnel = 25	С				
	annel =				•	- 1949 - 1949 - 1949		
		0.55, High Cha			•• [** •			
• • •			· · ·	• .	, a		€. <u>₹</u>	
		•						
			· · · ·					
		hool of Hygiene				•		Page 1
15 North	Wolfe	Street, Baltimor	e, M.D.21205				29Aug94	at 12:08:32
					· · · · · · · · · · · · · · · · · · ·	•		

HISTOGRAM DISPLAY
SAMPLE NAME : STAINED 7AAD SAMPLE DATE: 29Aug94
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM SAMPLE TIME: 12:07:01



						SAMPLE I	NFO				ی وی بری برد بی بی بی بی بی این این این این این این این این این ای
PRO	TOCOL	:	POR 1 - 1	Tockman					LAST	HIST :	
DAT	A RATE	:	328		TOTAL CO	UNT :	16,119	•	LIST	FILE :	40829T06.LMD
INS	STRUMENT	Г, :	Elite						PROT	FILE :	P0000034.PR0
SAM	IPLE NAM	1E :	STAINED	7AAD			;		SAMPL	E DATE:	29Aug94
SAM	IPLE NUM	1BER:	MIX 50:5	a TUMOR/	SPUTUM				SAMPL	E TIME:	12:07:01
COM	MENTS	:									
				سے سے میں میں میں میں ا		STATISTI	cs		د د. ده ده ده ده	و جد برب حد جد حد غنه اعنه با	بي حي قو يو بي بي بين بين حي خل نو حي حي بي بي حي جي جي بي بي
					SINGLE P	ARAMETER	STATIS	TICS			
			Pea				X Chan	nel			
ID	Pont	Area	Position	Height		SD Fu			Min	Max	
С	40.2	3359	21	[~] 98					11	25	
D	21.3	1786	35	57	36.8	7.84	21.3	7.41	26	88	
					DUAL PA	RAMETER S	TATIST	ICS			
				Peak		X Chann	el	Y	Channe	21	
ID	Pcnt	Area	Positi	on He	ight Me	an SD	CV	Mean	SD	CV	
A	36.3	5848	0,	4	206 3	.6 2.8	76.6	10.7	4.9	45.4	
F	51.9	8366	18,	33	41 28	.4 14.2	49.9	31.2	6.2	20.0	
					• • • • •						
				•.	•						

SAMPLE NAME : STAINED 7AAD SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

HISTOGRAM DISPLAY

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:07:01

C Low Channel = 11, High Channel = 25 C D Low Channel = 26, High Channel = 88 D A A AMORPHOUS F F AMORPHOUS

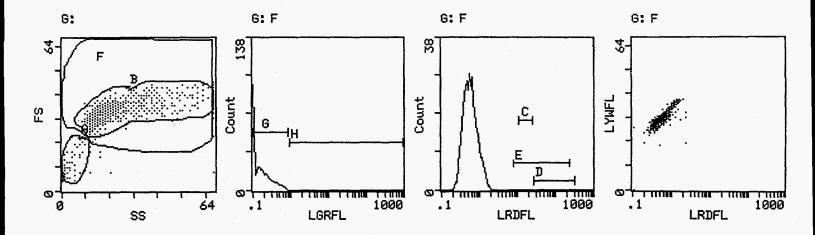
Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205

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Page 2 29Aug94 at 12:09:29

HISTOGRAM DISPLAY

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:28:13

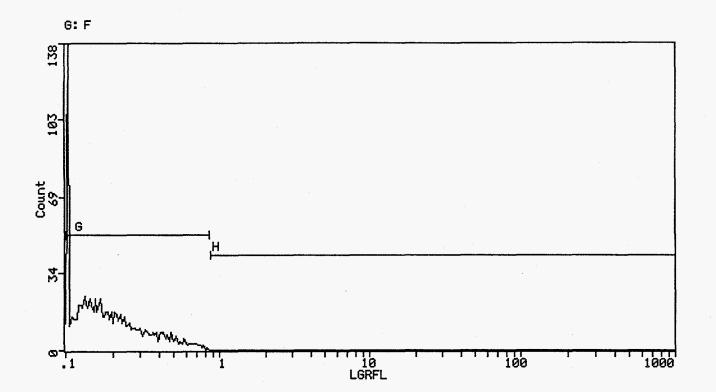


ATA RATE : 143 TOTAL COUNT : 6,009 LIST FILE : 40829T10.LMD NSTRUMENT : Elite PROT FILE : P0000034.PRO SAMPLE NAME : FITC NEG. CONTROL SAMPLE NUMBER: TUMOR CELLS ONLY SOMMENTS : SINGLE PARAMETER STATISTICS SINGLE PARAMETER STATISTICS PeakX Channel D Pcnt Area Position Height Mean SD FullCV HalfCV Min Max 0.0 0 **** ***** **** **** **** **** 11 25	ر جرب میں بران ہوتا اورد ابناء میں دی	اغة 10% چين بليت عليّا كتبر يور				- SAMPLI	E INFO -	-			، میں بین عن جب بین میں خبر خبر میں جب جب میں ہے
NSTRUMENT : Elite PROT FILE : PO000034.PRO SAMPLE NAME : FITC NEG. CONTROL SAMPLE DATE: 29Aug94 SAMPLE NUMBER: TUMOR CELLS ONLY SAMPLE TIME: 12:28:13 COMMENTS : SINGLE PARAMETER STATISTICS 	PROTOCOL	:	POR 1 - 1	TOCKMAN					LAST	HIST :	
AMPLE NAME : FITC NEG. CONTROL TUMOR CELLS ONLY SAMPLE NUMBER: TUMOR CELLS ONLY SINGLE PARAMETER STATISTICS Peak	DATA RATE		143	T	OTAL CO	UNT :	6,00	9	LIST	FILE :	40829T10.LMD
AMPLE NUMBER: TUMOR CELLS ONLY SAMPLE TIME: 12:28:13 COMMENTS : SINGLE PARAMETER STATISTICS PeakX Channel D Pont Area Position Height Mean SD FullCV HalfCV Min Max 0.0 0 **** ***** **** **** **** 11 25 0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS	INSTRUMEN	T - :	Elite						PROT	FILE :	P0000034.PR0
COMMENTS : SINGLE PARAMETER STATISTICS PeakX Channel D Pcnt Area Position Height Mean SD FullCV HalfCV Min Max 0.0 0 **** ***** **** **** **** 11 25 0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS	SAMPLE NA	ME :	FITC NEG	CONTROL				?	SAMP	LE DATE:	29Aug94
SINGLE PARAMETER STATISTICS SINGLE PARAMETER STATISTICS PeakX ChannelX Channel D Pcnt Area Position Height Mean SD FullCV HalfCV Min Max 0.0 0 **** ***** **** **** **** 11 25 0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS	SAMPLE NU	MBER:	TUMOR CE	LLS ONLY					SAMP	LE TIME:	12:28:13
SINGLE PARAMETER STATISTICS PeakX ChannelX Channel. D Pont Area Position Height Mean SD FullCV HalfCV Min Max 0.0 0 **** ***** **** **** **** 11 25 0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS	COMMENTS	:									
PeakX ChannelX Channel. D Pont Area Position Height Mean SD FullCV HalfCV Min Max 0.0 0 **** ***** **** **** **** 11 25 0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS	، مید ختن اننے ورو درو هو هو هی می پی پ	ین دی جو حد می می بی بی	نه ها کا به جه ها نظارته مرد :							19 MAR MAR (20) WAR WAR (20) (70 MAR ()	میں فروغ ہیں ہیں ایس بیرنے میں میں برب این غریب میں ہی جو میں ہے۔ میں اور
0.0 0 **** ***** **** **** **** **** 11 25 0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS			Pea	_							
0.0 1 62 1 61.9 0.000 0.000 0.000 26 310 DUAL PARAMETER STATISTICS	IB Pent	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max	
DUAL PARAMETER STATISTICS	: 0.0	0	XXXX	*****	****	****	XXXX	****	11	25	
	D 0.0	1	62	1	61.9	0.000	0.000	0.000	26	310	
PeakX ChannelY Channel					DUAL PA	RAMETE	R STATIS	STICS			
				Peak		X Ch:	annel		r Chanr	nel	

· •,

			Peak			X	Channe	21	Y Channel			
ID	Pent	Area	Positi	on	Height	Mean	SD	CV	Mean	SD	CV	
A	39.9	2399	0,	4	1119	0.5	1.4	304.7	5.3	2.7	50.3	
F	53.5	3214	16,	32	38	23.4	11.6	49.5	32.8	4.4	13.3	

		GATES	
 . 	FITC NEG. CONTROL TUMOR CELLS ONLY	SAMPLE DATE: SAMPLE TIME:	-



	ي جي جن جي ۽	و ها ها شار هو دو مو هو کا کا کا بی بی بی به نار بار بار	501		TNEO	یچ چود برده خده کی کرد برده بعد خند بلاب که کا بود برده بعد خند خند		_
PROTOCOL	:	POR 1 - TOCKMAN						
DATA RATE	:	143	HIST COUNT	:	3,214	LIST FILE :	40829T10.LMD	
INSTRUMENT ·	:	Elite				PROT FILE :	P0000034.PR0	
SAMPLE NAME	:	FITC NEG. CONTRO)L		?	SAMPLE DATE:	29Aug94	
SAMPLE NUMBE	R:	TUMOR CELLS ONLY	,			SAMPLE TIME:	12:28:13	
COMMENTS	:							

		یور میں میں میں	ہ جب جب جب نے نف ان کا ناہ کے جو ج					STATIS	STICS	ند هن ننبه دانه اود هب هب هن			و جزی چین کی برند کرتے ہیں سے جس شہ جنہ د	هند فته چه هه برد خد نده ند
						S	INGLE PA	RAMETE	ER STATI	STICS				
					Peak				X Cha	nnel				
Ι	DI	Pcnt	: Area	Positi	on ł	leight	Mean	SD	FullCV	HalfCV	Min	Max		
G	1	99.9	3212	0.11		627	0.180	0.096	53.0	0.447	0.10	0.87		
Н	I	0.1	. 2	0.89		1	1.03	0.144	14.0	0.382	0.89	1024		
G)	Low	Channel =	0.10,	High	Channel	= 0.87	G						
H		Low	Channel =	0.89,	High	Channel	= 1024	H						

· · · · · · · · · · · · · · · · · · ·		HISTOGRAM DISPLAY
SAMPLE NAME :	FITC NEG. CONTROL	
SAMPLE NUMBER:	TUMOR CELLS ONLY	

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:28:13

C Low Channel = 11, High Channel = 25 C D Low Channel = 26, High Channel = 310 D A A AMORPHOUS F F AMORPHOUS

Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205

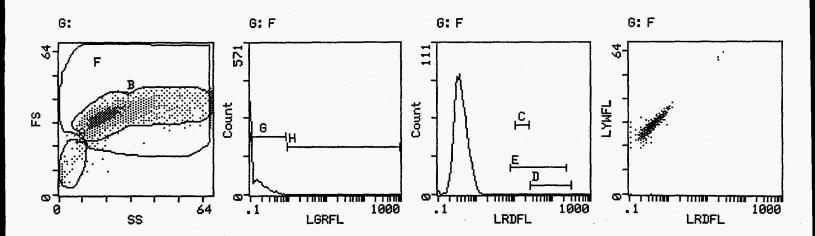
•~•

Mary Co.

1

HISTOGRAM DISPLAY SAMPLE NAME : STAINED FITC SAMPLE'NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:30:52

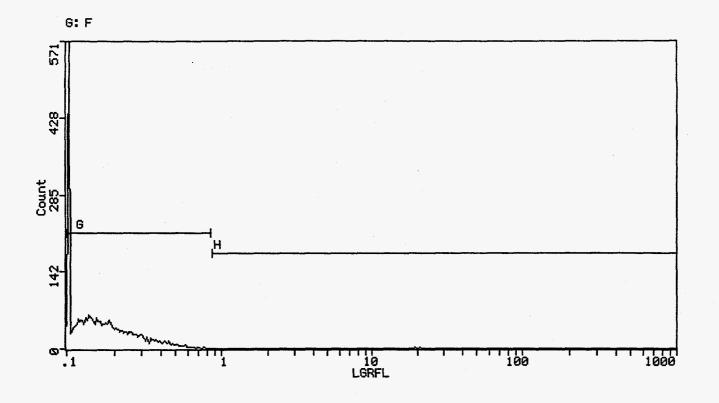


الم خدة خدة فلك بوب وجزة بثقة التي ويبة فتبد فالترجي وجو إفترة برين زورة	به المار فينه ذلك 100 منذ الحك الكرة الألب الحد الألك 100 منية وقفة الألب اليود عنيه الملك ال -	SAM	PLE INFO		وجر سه منه ليور جنه عنه فارد جرم سه فلنا جرم جره گ	چین دیک جارہ اس زید جات بھی زید جات اور جات کے جات کا ور عال کا و
PROTOCOL :	POR 1 - TOCKMAN			. Li	AST HIST :	
DATA RATE :	496	TOTAL COUNT	: 10	420 L	IST FILE :	40829T11.LMD
INSTRUMENT :	Elite			P	ROT FILE :	P0000034.PR0
SAMPLE NAME :	STAINED FITC			' Si	AMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY	,		Si	AMPLE TIME:	12:30:52
COMMENTS :						

					بروی است. حین بروی شنار بین	ST	ATIST	ICS			، مدد بیب سب سه «به سه هاه «	 	
					CTNC	LE PARA	METED	CTATT	ettee				
			Pea	. k				.X Cha	nnel				
ID	Pcnt	Area	Position	Heig	ht M	ean	SD F	ullCV	HalfCV	Min	Max		
С	0.1	8	13		1 1	8.6 3	.08	16.6	0.382	11	25		
D	0.1	5	27		1 3	9.6 1	5.4	38.9	0.382	26	310		
					DUA	l param	ETER	STATIS	TICS				
				Peak.		X	Chan	nel	Y	Chanr	el		
ID	Pont	Area	Positi	on l	leight	Mean	S	D C	V Mean	SI) CV		
A	8.9	926	0,	4	302	1.8	3.	1 171.	1 7.8	5.2	87.0		
F	84.7	8824	16,	31	74	24.3	11.	6 47.	6 32.8	4.4	13.4		

SAMPLE NAME : STAINED FITC SAMPLE NUMBER: TUMOR CELLS ONLY GATES

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:30:52



میں نہیں جاتا ہے۔ جات بارے نہیں دیکر کارا جاتی ہیں۔ ا		ک پود هند خک نالو بربه نبین فک وی بربه هده این بربو بربه نفو بربو هد ا	ند در هر در دو ر	SA	MPLE	INFO	به هه که کو نها که که برای خو که که نوع کم که که که که که که که خو خو	روی وجد ست خبن بید ست کار بیچ سه خته این بید ست کار پید تند کتا .
PROTOCOL	:	POR 1 - TOCKMAN						
DATA RATE	:	496	HIST	COUNT	:	8,824	LIST FILE :	40829T11.LMD
INSTRUMENT	:	Elite					PROT FILE :	P0000034.PR0
SAMPLE NAME	:	STAINED FITC				;	SAMPLE DATE:	29Aug94
SAMPLE NUMBE	R.	TUMOR CELLS ONLY	Y				SAMPLE TIME:	12:30:52
COMMENTS	:							

STATISTICS										
					SINGLE P	ARAMETE	ER STATI	STICS		
			Pea	k			X Cha	nnel		
ID	Pent	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
G	99.8	8809	0.11	2257	0.159	0.073	45.8	0.458	0.10	0.87
Н	0.2	15	20	2	11.3	13.6	120.7	0.382	0.89	1024

G Low Channel = 0.10, High Channel = 0.87 G H Low Channel = 0.89, High Channel = 1024 H

SAMPLE NAME : STAINED FITC SAMPLE[®] NUMBER: TUMOR CELLS ONLY

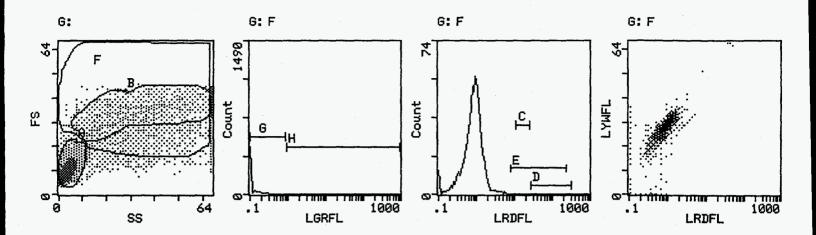
?

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:30:52

C Low Channel = 11, High Channel = 25 C D Low Channel = 26, High Channel = 310 D A A AMORPHOUS F F AMORPHOUS SAMPLE NAME : STAINED FITC SAMPLE*NUMBER: SPUTUM CELLS ONLY

HISTOGRAM DISPLAY

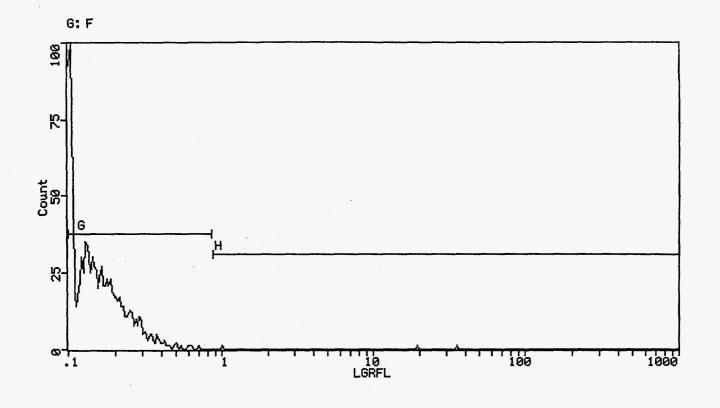
SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:32:37



	و بربید خلک اورد خلک کارد کرد ا	، برود جند الي جبة التوجيع ال	د است برین جان هیچه جان بینو چنب اینو وی جان ژوی زادار هیچه ست		· SAMPLE	INFO		به بسد هور هند ومو بعل ورو خنه بين عبد خور جه	رور چند روب مدر برید خده دی وی برند وی برند اور چند کی ست ک
PRO DAT INS SAM SAM	ITOCOL TA RATE STRUMENT IPLE NAM IPLE NUM	: : 1E:	POR 1 - TOCKM 487 Elite STAINED FITC SPUTUM CELLS	an Total Co		15,600	L1 PF Sf	AST HIST : ST FILE : ROT FILE : AMPLE DATE: AMPLE TIME:	40829T12.LMD P0000034.PR0 29Aug94 12:32:37
	د هې وند اور بده کې برد		و السو بيون براي بروه براي اليون بينه اليون بالله بور اليون براي ال					و چن میا اور غیر اور می می دو می دی اور این	ی در بر بین وارد این در در بر بر برد بین وارد بین وارد این وارد این وارد این وارد این وارد این وارد این و
				SINGLE P					
			Peak			X Channe	el		
ID	Pcnt	Area	Position Heig	ht Mean	SD F	ullCV Ha			
С	0.1	6	12	1 18.0	3.83	21.2 8	.382 11	25	
D	0.1	9	30	1 42.7	7.92	18.6 0.			
				DUAL PA	RAMETER	STATISTIC	CS		
			Peak.					annel	
ID	Pent	Area		Height Me		D CV		SD CV	
A	43.9	6850							
		_			.5 2.3			4.6 47.5	
F	42.0	6559	44, 37	17 32	.9 14.4	4 43.8	29.8	7.2 24.2	

SAMPLE NAME : STAINED FITC SAMPLE NUMBER: SPUTUM CELLS ONLY GATES

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:32:37



---- SAMPLE INFO PROTOCOL POR 1 - TOCKMAN : HIST COUNT DATA RATE 6,559 40829T12.LMD 487 : LIST FILE : : INSTRUMENT · : Elite PROT FILE : P0000034.PR0 ? SAMPLE NAME : STAINED FITC SAMPLE DATE: 29Aug94 SAMPLE NUMBER: SPUTUM CELLS ONLY SAMPLE TIME: 12:32:37 COMMENTS :

			یم هه ماد ورو هی که پرو چه داد خبر هی	يين وي المراجعة عنه وي المراجعة الي الم		STATIS	STICS	، همه این، چه هه کو جه به د	ننہ ہو، جب خت کے جب	هبه متی بیره بده غلبه ببوه جمل	فت سے ورب خدن ایے چیچ :	جد خلب روی سن خلف ویه بعب ه
				S	INGLE P	ARAMETI	ER STATI	STICS				
			Pea	k			X Cha	nnel				
ID	Pont	t Area	Position	Height	Mean	SD	FallCV	HalfCV	Min	Max		
G	99.6	6533	0.11	1490	0.129	0.043	33.2	1.01	0.10	0.87		
Н	0.4	4 26	0.96	. 1	10.1	16.3	161.2	0.382	0.89	1024		
6	Low	Channe1	= 0.10, Hig	h Channel	= 0.87	G						
Н	Low	Channel	= 0.89, Hig	h Channel	= 1024	H						

HISTOGRAM DISPLAY
SAMPLE NAME : STAINED FITC SAMPLE I
SAMPLE NUMBER: SPUTUM CELLS ONLY SAMPLE T

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:32:37

C Low Channel = 11, High Channel = 25 C D Low Channel = 26, High Channel = 310 D A A AMORPHOUS F F AMORPHOUS

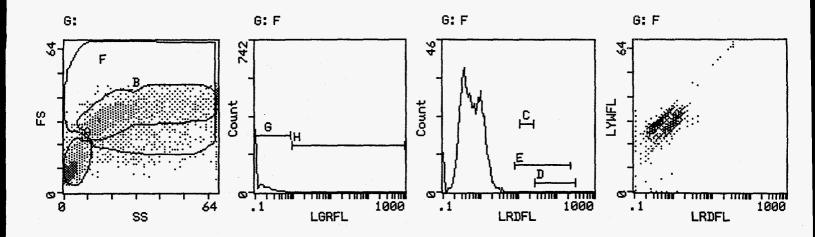
Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.D.21205 • • • • • • • •

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SAMPLE NAME : STAINED FITC SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

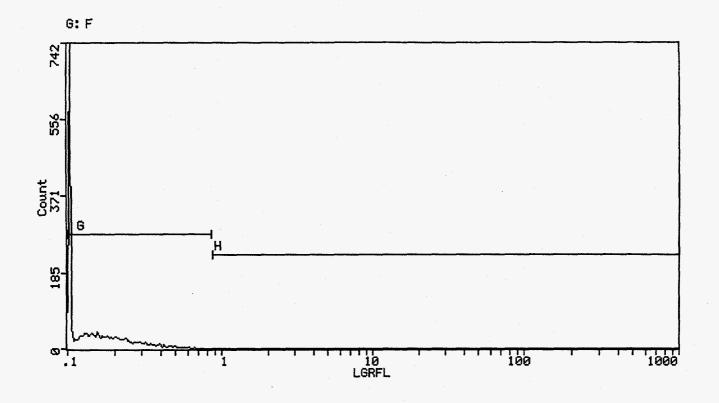
HISTOGRAM DISPLAY

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:34:59



	، بعد میں عبد بین سے بین					SAMP	LE IN	₩F0	ب من نو به مدخر بو به		و هې جمه خدې چې جده مله يوه ولو خد	، بین نظر ہے جد کہ اور جن کو بالد جو جو ہے جو بر
PRO	TOCOL	:	POR 1 -	TOCKMA	N					LAST	HIST :	•
DAT	A RATE	:	836		TOTAL	COUNT	: :	12,543	5	LIST	FILE :	40829T13.LMD
NS	TRUMEN	Т.:	Elite							PROT	FILE :	P0000034.PR0
AM	PLE NAI	ME :	STAINED	FITC				;		SAMP	LE DATE:	29Aug94
AM	PLE NUI	MBER:	MIX 50:5	OMUT 6	R/SPUTUM					SAMP	LE TIME:	12:34:59
OM:	MENTS	:										
												1
	، جنة عن حدد جنة في حد		 		ه البه حد خلا باله حو حد الله ا	STA	FISTIC	:s	بنية الله ابن منتد اللا الار زيب عنه :	ه هي جير. هذ که جي.	و چه دن کر پو دود دل چو زند نند	ر که ها، غلب نک جن ایک هی باند نکر یک جد اخذ ری جه بخذ ان
						e parame			· • • •			
			Pea	k					nel			
	Pent	Area	Position	Heigh		n Sl			HalfCV	Min	Max	
	0.2	10	11			.3 4.4			0.764	11	25	
	0.2	14	33		2 38.				0.382	26	310	
					-	PARAMET						
	-								••••Y			
D	Pent	Area			-	Mean		CV		SI		
ļ.	40.9	5133	0,	4		3.2				4.	7 49.7	
	50.0	6275	14,	31	35	27.0	14.2	52.3	31.0	· 6.1	1 19.7	
					·.					:	-11 - 11 - 11 - 11 - 11 - 11 - 11 - 11	
				•								

SAMPLE NAME : STAINED FITC SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:34:59



ک است کار ایک آلاف شد کار کار بیند ملک کار بیچا خان خان برو	که از از مناطقا باین این این این این میز خان کرد مین بعد انتراض ور مید خرد دین می جده بین می م	SAMPLE	INFO	روی مدا دی چی منظ کی برود بط کار روی بط خان وی بخت مل	ہے ہیں رائد کے میں بالک کار بین کہ خان ہیں خوا خان ہے جوا میں بین
PROTOCOL :	POR 1 - TOCKMAN				
DATA RATE :	836 HIST	COUNT :	6,275	LIST FILE :	40829T13.LMD
INSTRUMENT :	Elite			PROT FILE :	P0000034.PR0
SAMPLE NAME :	STAINED FITC		:	SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUT	UM		SAMPLE TIME:	12:34:59
COMMENTS :					

		ب ک نے برہ بند دو روہ :				STATIS	STICS	ور الله عن جود (بله اليه هي جرد ال		، بریغ جاله هی، جنه منه خان جنه هاه :	کر چین خدد سے پیس بابت کے ہیں ایجا خلک نہیں	
					SINGLE P	ARAMETI	ER STATI	STICS				
			Pea	k			X Cha	nnel				
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	- Min	Max		
G	99.5	6243	0.11	1318	0.150	0.066	44.2	0.700	0.10	0.87		
н	0.5	32	0.96	1 . 1 .	18.1	20.4	112.8	0.382	0.89	1024		
-		-								-4		

G Low Channel = 0.10, High Channel = 0.87 G H Low Channel = 0.89, High Channel = 1024 H

Johns Hopkins School of Hygiene 615 North Wolfe Street,Baltimore, M.B.21205 Page 1 29Aug94 at 12:35:49

ر محمد باند. بلایت از د

SAMPLE NAME :	STAINED FITC
SAMPLE" NUMBER:	STAINED FITC MIX 50:50 TUMOR/SPUTUM

HISTOGRAM DISPLAY

?

SAMPLE DATE: 29Aug94 SAMPLE TIME: 12:34:59

C Low Channel = 11, High Channel = 25 C B Low Channel = 26, High Channel = 310 B A A AMORPHOUS F F AMORPHOUS