GENERATION OF DNA APTAMERS FOR LUNG CANCER STUDIES

By

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To God, my family, and my loving husband

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LIST OF ABBREVIATIONS

2D-PAGE	Two-dimension polyacrylamide gel electrophoresis
Ab	Antibody
ADC	Adenocarcinoma
AJCC	American Joint Committee on Cancer
ATCC	American Type Cell Culture
APC	Allophycocyanin
BB	Binding buffer
bp	base pair
CA-125	Cancer-Antigen 125
CEA	Carcinoembryonic Antigen
ChIP	Chromatin ImmunoPrecipitation
CSC	Cancer Stem Cells
CTC	Circulating Tumor Cells
CT Scans	Computerized Tomography Scans
CTSI	Clinical and Translational Science Institute
CYFRA21-1	Cytokeratin-19 Fragment
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial to Mesenchymal Transition
EGF	Epidermal growth factor
EpCAM	Epithelial Cell Adhesion Molecule
FACS	Fluorescence assisted cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FFPE	Formalin-Fixed Paraffin Embedded
FITC	Fluorescein isothiocyanate
FNA	Fine Needle Aspiration
g	g-force
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Gross Domestic Product
GPCR	G-Protein Coupled Receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IASLC	International Association for the Study of Lung Cancer
KRAS	v-Ki-ras2 Kirsten Rat Sarcoma Viral oncogene homolog
LCC	Large Cell Carcinoma
LOD	Limit of Detection
MA	Massachusetts
MID	Middle Identification Code
MNP	Magnetic Nanoparticles
MS	Mass spectrometry
NK	Natural Killer
NSE	Neuron-Specific Enolase
NSCLC	Non-Small Cell Lung Cancer
nt	nucleotide
NHS	N-Hydroxysuccinimide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Solution

PCR	Polymerase Chain Reaction
PDGF	Plateted-derived growth factor
PE	Phycoerythrin
PE-Cy5.5	Phycoerythrin-cyanine derivative 5.5
PEG	Polyethylene Glycol
PMSF	PhenylMethaneSulfonylFluoride
ProGRP	ProGgastrin-Releasing Peptide
PTK7	Protein Tyrosin Kinase 7
Qpcr	Quantitative polymerase chain reaction
RBC	Red Blood Cells
RNA	Ribonucleic Acid
rpm	revolution per minute
SCLC	Small Cell Lung Cancer
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNA
ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
SCC	Squamous Cell Carcinoma
SOP	Standard Operational Procedures
TNM	Tumor Node Metastasis
TP53	Tumor Protein p53
VALSG	Veterans' Administration Lung Study Group
VEGF	Vascular endothelial growth factor
VOC	Volatile organic compounds

WB	Washing Buffer
WBC	White Blood Cells
WHO	World Health Organization

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GENERATION OF DNA APTAMERS FOR LUNG CANCER STUDIES

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Lung cancer is a very deadly disease and the number one cause of cancer-related deaths worldwide. It is divided in two main groups, Small cell lung carcinoma (SCLC) and Non-small cell lung carcinoma (NSCLC) which is further classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Lung cancer is normally detected at stage III and stage IV with poor five-year survival rate. Due to the lack of specific symptoms, lung cancer can be misinterpreted as other respiratory infections due to an inefficient diagnosis capability. The subgroups in lung cancer are clinically differently and their response to treatments relies on the accurate classification in a particular subgroup.

To address this issue, this dissertation investigated the use of aptamers as alternatives to antibodies for the diagnosis and accurate classification of lung cancer. Aptamers are short single-stranded synthetic nucleic acids, either DNA or RNA, capable of recognizing and binding their targets with high selectivity and specificity. Using cell-based SELEX as methodology, a panel of 6 aptamers was generated for the holistic study of lung cancer. The generated aptamers possess apparent dissociation constants

in the nanomolar range, an indispensable feature in the biomedical application of these molecular probes.

Furthermore, these aptamers were validated against a pool of lung cancer cell lines corresponding to all subgroups. Aptamers HCH3, S1 and EJ4 showed affinity for the majority of cell lines tested. Simultaneously, these aptamers were probed with formalin-fixed and formalin fixed paraffin embedded cell-based model tissues. All aptamers tested were capable of recognizing and detecting their targets under these conditions. In addition, aptamers were utilized as recognition molecules on an aptamer-based strategy for the detection and enumeration of circulating tumor cells *in vitro* and *in vivo.* The developed assay was able to detect as few as ~ 50 cells in 7.5mL of blood. The concluding aptamer project was elucidating the aptamer DOV4's target. After SDS-PAGE gel and MS analysis, two proteins emerged as potential targets: Annexin A2 and desmoplakin-related proteins.

CHAPTER 1
INTRODUCTION

Cancer

Cancer is the general name for a group many diseases, in which a group of abnormal cells proliferate without control and multiplies in an autonomous fashion invading adjacent parts of the body and spreading to other organs[1]. Cancer is caused by abnormalities in the genetic material caused by a series of changes, which can be provoked by different risk factors such as: advanced age, growing older, tobacco[2], sunlight[3], ionization radiation[4], certain chemical, viruses[5], bacteria[6], hormones[7], alcohol[8], and poor diet[9]. Other genetic abnormalities are acquired during the normal process of DNA replication, and these unrepaired errors are inherited and present in every cell of the body, thereby increasing the probability of developing the disease.

Cancer has become a public health problem worldwide. Globally the overall cost of cancer was $ 895 billion in 2008, while in the United Stated alone its economic impact rose to 1.73 percent of its GDP[10]. Cancer is the leading cause of disease-related death worldwide accounting for 13% of all deaths worldwide followed by heart disease and stroke. In 2008, an estimated of 12.7 million new cases were reported, and the burden of cancer will increase to 22 million new cases by 2030. Breast, prostate, pancreatic, and lung cancers account for more than half of all cancer deaths [Figure 1-1][10]. In addition to its physical effects, cancer can emotionally impact patients and relatives with mixed feelings of fear, uncertainty, sadness and stress.[11]

Lung Cancer

Lung cancer is the leading type of cancer for both males and females and is estimated to rank second among new cases[1] [Figure 1-2]. A number of factors can

increase the risk of an individual to develop lung cancer. Smoking is by far the leading risk for lung cancer; about 90% of cancer cases result from tobacco use.[12] The disease is not selective and can affect both smokers and non-smokers equally. In fact, nearly 3,000 non-smokers die every year from lung cancer. Other factors such exposure to radon gas[13], asbestos, and occupational chemicals[14], as well as others lung diseases have been identified as lung carcinogens. Other non-variable risks include genetics, gender, age, and race.[15]

The vast majority of lung neoplasms (abnormal tissue masses) are carcinomas (malignancies that arise from epithelial cells[16]), which are divided into two classes based on morphological assessment of the stained histological samples: non-small cell lung carcinomas (NSCLC) and small cell lung carcinomas (SCLC).[17]

Non-Small Cell Lung Carcinoma

NSCLC which is the most common lung cancer type (85% of all lung cancer cases) is the slow-growing, more passive cancer type, while SCLC (15% of all lung cancer cases) is more aggressive. NSCLC are further classified into three different subtypes: adenocarcinomas (ADC), squamous cell carcinoma (SCC), and large cell carcinomas (LCL). Compared to other cancer types, lung neoplasms are highly heterogeneous, with tumors displaying more than one subtype as a common feature[18].

Adenocarcinoma

ADC is the most common subtype of lung cancer in most countries, and it accounts for 40% of NSCLC. It arises from the small bronchi, bronchioles or alveolar epithelial cells and usually is located in the periphery of the lungs with a characteristic

glandular pattern. [Figure 1-3]. These tumors tend to be found in a confined area, and therefore, they respond to chemotherapy, and tumor resection.[19]

Although ADC is the subtype least related with tobacco consumption, it is most frequently observed subtype among smokers.[20-21] Compelling evidence suggests that lung cancer in individual who have never smoke is a neoplasm with different pathologic and epidemiologic characteristics, compared to pulmonary adenocarcinomas linked to smoking.[22] Lung adenocarcinoma in never-smokers is more common in women than men.[23-24]

As the most common histological subtype, ADCs are further classified as: bronchioalvelar (BAC), papillary, acinar, or solid, according to the 2004 classification by the World Health Organization (WHO).[25] Clinically, resected specimens that display more than one histological pattern have demonstrated the urgent need for a new classification system due to the heterogeneity of mixed adenocarcinomas, in which the predominant subtype prioritizes the classification.[26- 27] Mixed subtype lung tumors usually show more aggressive clinical action than those with single histology.[28]

Squamous cell carcinoma

Lung SCC accounts for about 20-30% of NSCLC and it is the histological subtype directly associated with smoking.[29] Typically, SCC tumors are central, arising in the large bronchi. This histological subgroup responds well to chemotherapy and it is well characterize by keratinization and pearl formation[30] [Figure 1-4].

Large cell carcinoma

LCA accounts for about 10% of NSCLC and it is characterized by a poorly differentiated neoplasm. It is the “exclusion of diagnosis” subtype due to the lack of ADC, SCC, SCLC features. Typically, differentiating features include the large size of

the anaplastic cells, the lack of chromatin pattern, and the ratio of cytoplasm to nucleus[31] [Figure 1-5].

Small Cell Lung Carcinoma

Small cell lung carcinoma is the other histology subgroup in lung cancer, representing 15% of all lung cancer cases worldwide[18]. This disease is commonly present in elderly current and past heavy smokers, and its risk increases substantially with tobacco consumption13. As in 90% of all lung cancer cases, SCLC has an epithelial origin[32], consisting of small cells with fine nuclear chromatin and unnoticeable nuclei[18] [Figure 1-6].

SCLC usually starts in the bronchi close to the center of the chest. It is considered more aggressive compared to NSCLC, because of the rapid spread throughout the body at early stage.[33] Therefore, surgical removal of the tumor with surgery rarely cures the disease.

Diagnosis in Lun g Cancer

Early diagnosis of cancer can improve dramatically its prognosis. In the absence of a particular test, diagnosis relies on the primary care. As with any disease, physical examination constitutes a critical part of the diagnostic process (the majority of lung neoplasms are discovered by chance when x-ray images are used as part of the treatment of other ailments.[34] Several techniques are available for accurate diagnosis as described below.

Tissue Diagnosis

The most effective method to diagnose the presence of cancer is microscopy examination of suspected tissue. Different techniques that fall into this category are presented below.

Sputum cytology

Sputum cytology is the least invasive method to detect lung cancer[35], and involves examination of mucous collected by coughing into a container. This technology is especially useful in patients with central tumors such SCLC and SCC, and its sensitivity in detecting lung cancer depends on the number of samples.[36] However, the accuracy of this methodology has not been summarized due to the variability in the collection and analysis of the sample.

Bronchoscopy

Bronchoscopy is the most common biopsy technique when lung cancer is suspected. It involves the insertion of a bronchoscope into the large airways of the lungs. This technique is particularly useful in tumors developed in the bronchial tree and central tumors.[37]

Mediastinoscopy

Mediastinoscopy is a surgical procedure in which an endoscope is inserted through a small incision in the neck into the chest, specifically in the central area called the mediastinum. This technique plays an important role in tumor staging, especially from centrally located tumors.[38]

Transthoracic needle biopsy

Also known as fine needle aspiration (FNA) biopsy, this method is used for patients with tumors located in the peripheral area of the lung, hardly reached by bronchoscopy. During the procedure a needle is inserted into the mass tumor to extract cells for subsequent staining and visualization under the microscope.

Thoracoscopy

In thoracoscopy, another invasive medical procedure, an endoscope in inserted

into the chest. The advantage of this technique over those previously described is the possibility of obtaining pleural effusion within the sample.[39]

Thoracotomy

Thoracotomy represents the last surgical resource as diagnostic technique to sample a suspected tumor when previously described methods are unsuccessful. This procedure is performed under anesthesia, and the chest is open to expose the lungs. It is mostly employed for treatment to deep organs such as the heart, esophagus and thoracic aorta.[40]

Imaging Diagnosis

In the recent years, imaging has been the leading technology in cancer diagnosis and tumor staging. Although imaging provides information about the presence of a tumor, its malignancy is determined only after analysis under microscope. Below is a list of available imaging techniques employed in lung cancer diagnosis.

- Magnetic Resonance Imaging Scans (MRI scans)
- Computerized Tomography scans (CT Scans)
- Chest X-ray
- Position Emission Tomography (PET scans)

Treatments for Lung Cancer

The management of lung cancer depends crucially on the histological classification and the tumor staging, based on the tumor-node-metastasis staging system TNM [Figure 1-7 and Figure 1-8][41] which was updated by the American Joint Committee on Cancer (AJCC) in 2007. As previously mentioned, lung cancer is divided histologically into NSCLC (which is further divide into subtypes) and SCLC. Since the 1950's, the Veterans' Administration Lung Study Group (VALSG) has defined the

staging for SCLC as limited or extensive stage. Limited-stage SCLC refers to disease restricted to the thorax, while in extensive-stage, SCLC, the disease is distributed outside the thorax, including other pleural and pericardial conditions.[42] On the other hand, the TNM system has always defined the staging classification.

Recently, the International Association for the Study of Lung Cancer (IASLC) suggested the modification for the SCLC staging after a review of 109 SCLC patients and observation of more efficient prognostic differentiation between the two staging systems[43]. In general, the treatment of lung cancer involves a variety of approaches, including chemotherapy, surgery, and radiation therapy.

Surgery

Resection is the standard surgical procedure for NSCLC stages I & II and can be used alone or in combination with chemotherapy. Ideal candidates for surgery are considered based on the stage and the overall health condition. Lobectomy, which refers to the excision of the lobe of the lung is the main procedure performed on these patients. For stage IIIA, the option of surgery depends of the location of the tumor, and for IIIB, the option of surgery relies on the evidence of lymph node metastasis[44] [Figure 1-7].

For SCLC patients, surgery is a remote option for treatment, as it is applicable to only 10% of that population.

Chemotherapy

Chemotherapy refers to the use of drugs orally or via intravenous injection to kill cancer cells. It plays an important role for treatment of both SCLC and NSCLC. This type of treatment can be used alone as the main treatment, as adjuvant therapy simultaneously with radiation therapy and after surgery, and also as neoadjuvant

therapy before surgery to aid in reduction of the tumor. The most common chemotherapy drugs used in lung cancer treatment are: Cisplatin, Carboplatin, Doxetacel (Taxotere®), Paclitaxel (Taxol®), Gemcitabine (Gemzar®), Vinorelbine (Navelbine ®), Irinotecan (Camptosar®), Etoposide (VP-16®), Vinblastine, Pemetrexed (Alimta®).

Chemotherapy is usually administered as a combination of two or more drugs. However single drug chemotherapy is use in the elderly and in patients with compromised health status. If a combination of drugs is chosen as first line therapy, either Cisplatin or Carboplatin is predetermined in the given therapy in a combination of the other previously mentioned drugs. A second line therapy with single drug is suggested after the primary treatment effects cease[45-46]. These chemotherapy drugs target constantly dividing cells, but unfortunately healthy cells present in the intestine, mouth and hair follicles are also in constant division, thus becoming targets for those chemotherapy drugs. Therefore, side effects are expected when these drugs are used, most commonly hair loss, mouth sores, constipation, and fatigue.

Radiation Therapy

Radiation therapy employs high-energy rays or particles to kill cancer cells. It is recommended for patients with early stage lung cancer who are not suitable candidates for surgery.[47] Radiation can be used as main treatment (with or without chemotherapy), before surgery to help shrink the tumor, and after surgery to eliminate small clusters of tumor cells that were missed. There are two main types of radiation therapy: external beam and internal (Brachytherapy). Special considerations prior the beginning of treatment include dosage, frequency of the treatment, and beam angle. The side effects are similar to those developed after chemotherapy[47]. These therapies have their best

prognostic rates when administered in the earlier stages of the disease, but unfortunately lung cancer is often discovered at late stage or in the worst case when metastasis have occurred.

Metastasis

Although metastasis is the true cause of cancer-associated deaths, the process is not well understood. Generally, the term refers to the spreading of the primary tumor from one organ to another. Chaffer and Weinberg[48] suggest that this process can be divided into two main events: (i) physical translocation of a cancer cell from the primary tumor to the microenvironment of the surrounding distant organ followed by (ii) colonization. Recently, many scientific discussions relating to this topic have led to different hypothesis ranging from a genetic theory[49] to an implicit single-cell marker differentiation.[50] The process starts with the rupture of the basal lamina through the invasion of the extracellular matrix. This is followed by the intravasation event, in which the tumor cells enter the blood and lymph vessels and proceed to circulate throughout the body. Once the tumor cell has settled in a distant organ, it begins its proliferation (i.e. colonization) and forms a metastasis.

Two different classes of disseminated cells are thought to play an important role in metastasis: cancer stem cells (CSC) and circulating tumor cells (CTC). CSC theory started to gain popularity around 2003 and, since then, numerous studies have demonstrated the existence of those cells in different solid tumors.[51] CSC share similar features with normal stem cells (NSC), and have the ability to self-renew and to generate a diverse progeny. Recently, efforts have aimed to identify molecular markers that would differentiate CSC from NCSC and their potentials as therapeutic targets. The role of CSC in metastasis can be explained by the stem-like features acquired by the

tumor cells at the time of dissociation from the primary tumor, more precisely in the epithelial to mesenchymal transition (EMT).

CTCs are disseminated cells from the edges of the tumor that are carried away into the bloodstream or lymphatic system. These cells can remain as single cells in the blood stream, later clustering together and colonizing a new organ.[52] This topic will be addressed in subsequent chapters.

Biomarkers in Lung Cancer

The search for biomarkers is imperative, especially for early diagnosis and prognosis. Mutations in genes such KRAS and TP53 were extensively studied after the advent of next generation sequencing.[53] However, these studies have led to controversial results and their use as potential biomarkers has been inconsistent.[54] Other molecules studied as biomarkers include the protein present in serum (carcinoembryonic antigen (CEA), and cytokeratin-19 fragment (CYFRA) for NSCLC and neuron-specific enolase (NSE), and progestin-releasing peptide (ProGRP) for SCLC), but only a few have shown potential to be used in clinical settings because of specificity and sensitivity requirements.[55] A recent study of Non metastatic 23 protein NM23-H2 in 95 frozen tumor samples indicated that the protein was expressed selectively in lung ADC, but there is no correlation with staging or metastatic potential.[56] One limitation in the process of identifying new biomarkers is the lack of physical samples in most cases. As mentioned previously, most lung cancer cases are diagnosed at later stage when surgery is not suitable, thus restricting tissue acquisition. Recently, a new direction has focused on searching for future biomarkers in more non-invasive samples, such as the breath, sputum, and pleural effusions. In these methods volatile organic compounds (VOC) and cells directly expelled/extracted from the tumor

microenvironment of the lungs can provide useful information about their origin. Other types of molecules released into most body fluids are circulating free microRNA (miRNA), single stranded RNA only 21-25 nucleotides in length that can act as oncogenes or tumor suppressor genes. They are less susceptible to nuclease degradation than DNA, and are thus more stable in the body and its fluids. Micro RNAs are well regulated, but the lack of internal miRNA control molecules has limited their potential use as biomarkers.[57]

Membrane Proteins

Membrane proteins play a crucial role in in all cells by mediating cellular communication and nutrient transport. Comprising about 30% of all proteins, membrane proteins have become targets for the development of new therapeutic drugs. In fact, clinically most of the prescribed drugs target membrane proteins such as ion channels and G-protein coupled receptors. Despite being the most targeted of proteins, they are not well understood due to their dual hydrophobic-hydrophilic nature and their limited solubilization in aqueous solution, making them difficult to crystallize.[58]

Recent efforts in proteomic technologies have generated protein datasets commonly known as “proteomes” with representative proteins for several diseases including cancer.[59] Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) are key technologies, and multiple potential lung cancer biomarker candidates have been identified using these techniques. Comparative studies by Planque et al using cell lines for each histology group were analyzed for common proteins in lung cancer and proteins innate to each subtype. Five secreted proteins were identified and preliminarily validated in the serum of lung cancer patients as potential biomarkers.[60-61] ADAM-17, osteoprotegerin, pentraxin 3, follistatin, and tumor

necrosis factor receptor superfamily member 1A. Following the comparison principle between healthy/cancer patients, different studies have been carried out [62, 63,64,65].

Notable Membrane Proteins in Cancer.

In cancer, membrane proteins also regulate key cell processes, such as cell adhesion, migration, invasion, apoptosis, angiogenesis and tumorigenesis. The most common growth factors related to several cancers are epidermal growth factor (EGF)[66], vascular endothelial growth factor (VEGF)[67], and plateted-derived growth factor (PDGF).[68] These have become the molecular protein targets for most of the current therapies in cancer.

As previously mentioned, membrane proteins continue to serve as a focal point towards the development of new therapeutics, and the identification of new biomarkers with the potential to be translating into clinical settings in an important research area.

Aptamers

Recently, a novel type of molecules has emerged for the study of membrane proteins. About two decades ago, aptamers (meaning “to fit”) were discovered simultaneously by two independent research groups: Ellington & Szostak[69] and Tuerk & Gold[70] in 1990.

Aptamers are short single-stranded synthetic DNA or RNA probes capable of recognizing and binding to their targets with high selectivity and specificity. The molecular recognition is based on non-covalent interactions such van der Waals forces, hydrogen bonds and hydrophobic interactions.[71] Since their discovery, aptamers have been generated for a variety of targets, including, small organic molecules[72] metal ions[73], proteins[74-75], carbohydrates, toxins[76-77], and transcription factors[78], as well as whole cells,[79,80,81] viruses, [82-83] bacteria,[84-85] and as inhibitors of protein funtions.[86-87]

Aptamers were developed using the Systematic Evolution of Ligand by Exponential enrichment method. These synthetic oligonucleotides bind with specificity similar to that of antibodies and are usually referred to as their counterparts, but in contrast to antibodies, aptamers have shown no or extremely low immunogenicity.[88] This pivotal characteristic enables biological and medical applications, especially *in vivo* investigation using these probes.[89] Aptamers have been popularized as an alternative to antibodies in different fields, including imaging,[90,91] drug delivery,[92, 92] and particularly in analytical detection.[93,94,95] Aptamers are inexpensive, and their production does not require an animal model. These synthetic oligonucleotides can be easily modified to solve limitations in current biomedical applications. For example, polyethylene glycol (PEG-lated) aptamers have the ability to undergo cellular uptake and to resist rapid enzyme degradation when locked nucleic acids are used.[96] In addition, these probes are small in size and their sequences generally do not surpass 100 base pairs (bp). Consequently, aptamers demonstrated better tissue penetration compared to anitbodies.[97]

The aptamer market is a growing industry as many selected aptamers have reached clinical trials and some are already been commercialized. In 2004, Macugen, an anti-VEGF inhibitor, became the first aptamer approved by the Food and Drug Administration (FDA) for Wet-Age Related Macular Degeneration[98] (AMD), and other aptamers are currently in clinical trials.[99-100] Predictions estimated aptamer value at $10 million in 2009 and its growth is projected to reach 1.2 billion in 2014[101], making aptamers one of the fastest growing markets in therapeutics [Figure 1-9].

Sistematic Evolution of Ligands .by Exponential Enrichment (SELEX)

SELEX technology generally speaking involves an iterative process from which aptamers are generated [Figure 1-10]. The components, and critical steps involved in SELEX are:

- **Primer selection**

Perhaps this is the most important step during this process, identification of a unique set of primers to ensure proper amplification during the selection process. Generally primers are around 18bp in length and should be analyzed on nucleic acid software to eliminate primer-dimers and self-priming. When the SELEX process is carried out with living organisms such a mammalian cells, viruses and bacteria, the candidate set of primers should be screened using the program blanstn against the corresponding genome to avoid unwanted amplification of genes from the host.

- **Library generation**

Typically an aptamer library consists of 10^{14}-10^{16} random sequences generated through an automated process by solid-phase chemistry.[102] Previously, sequences utilized for selection contain around 80-100bp, but later this number has been reduced to 50bp (Unpublished data) in order to reduce costs without compromising quality. The randomized region is flanked on the 3’ and 5’ends by the set of primers that will allow amplification of the sequences.

- **Partitioning**

The partitioning step is crucial step in the entire selection process, as the randomized sequences begin to display affinity for the target. During this step the sequences with the greatest affinity (lowest dissociation constant, K_d) will be separated

from the pool (library) of sequences for further amplification. The stringency of this process increases as the SELEX process progressed.

- **Amplification by Polymerase Chain Reaction (PCR)**

During the entire selection process, approximately 270 PCR reactions will be performed, assuming 18 rounds of enrichment with 15 PCR cycles per round. However, there are some intrinsic problems with this technology. It is well known that DNA polymerases are not 100% faithful to the growing DNA strand. Therefore, it is expected that the enriched library in the final round will contain point mutations in several sequences. In addition, truncated sequences may be also present within the enriched library, as short sequences have a higher chance to be amplified.

Cell-SELEX

To select aptamers for whole cells, a negative control is usually included either as a normal cell line or different cancer cell line. Cell-SELEX begins with the binding event between the initially synthesized library and the target cells, unbound and weakly-bound sequences are washed off. Bound sequences are collected and (if negative selection is to be performed) are incubated with the negative cells. This time the, unbound sequences are collected and further PCR amplified. Then, dsDNA is converted to ssDNA and a new round starts. This process is continued until the initial library is enriched with sequences that bind to the cancer cell but no to the control cell. Once enrichment has been achieved, the pool is sequenced and analyzed using alignment programs to identify conserved sequences.[103]

Overview of dissertation

The research data presented in this dissertation demonstrate how aptamers selected against lung cancer can be utilized in clinical settings. Chapter 2 describes the

selection process of selecting for lung cancer. Chapter 3 demonstrates the potential of aptamers to be used clinically as probes for diagnostic purposes and histological subtyping. Chapter 4 demonstrates the applicability of aptamers in targeting and detection of disseminated cancer cells in blood, and chapter 5 outlines a proposed methodology that could be further exploited for the identification of the aptamers' target. The concluding chapter recapitulates the significance of cell-SELEX technology and its translation into clinical settings.

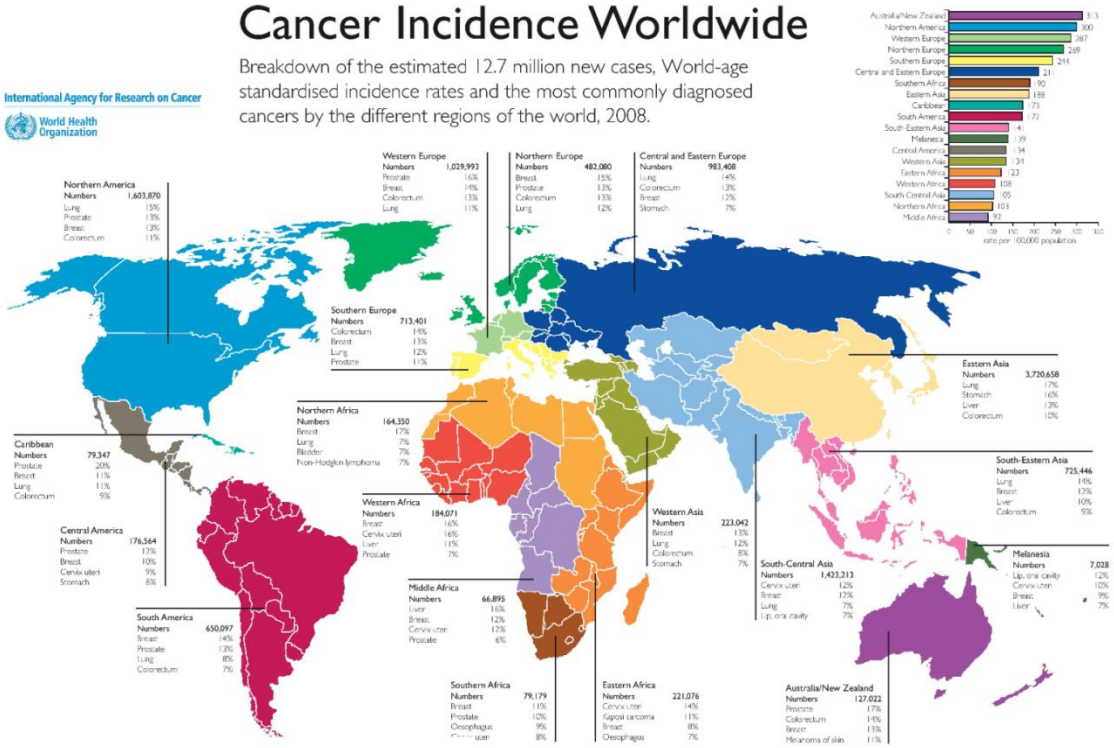


Figure 1-1. Cancer incidence and mortality worldwide in 2008. (Adapted from Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10 [Internet].Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://globocan.iarc.fr, accessed on 13/may/2013.

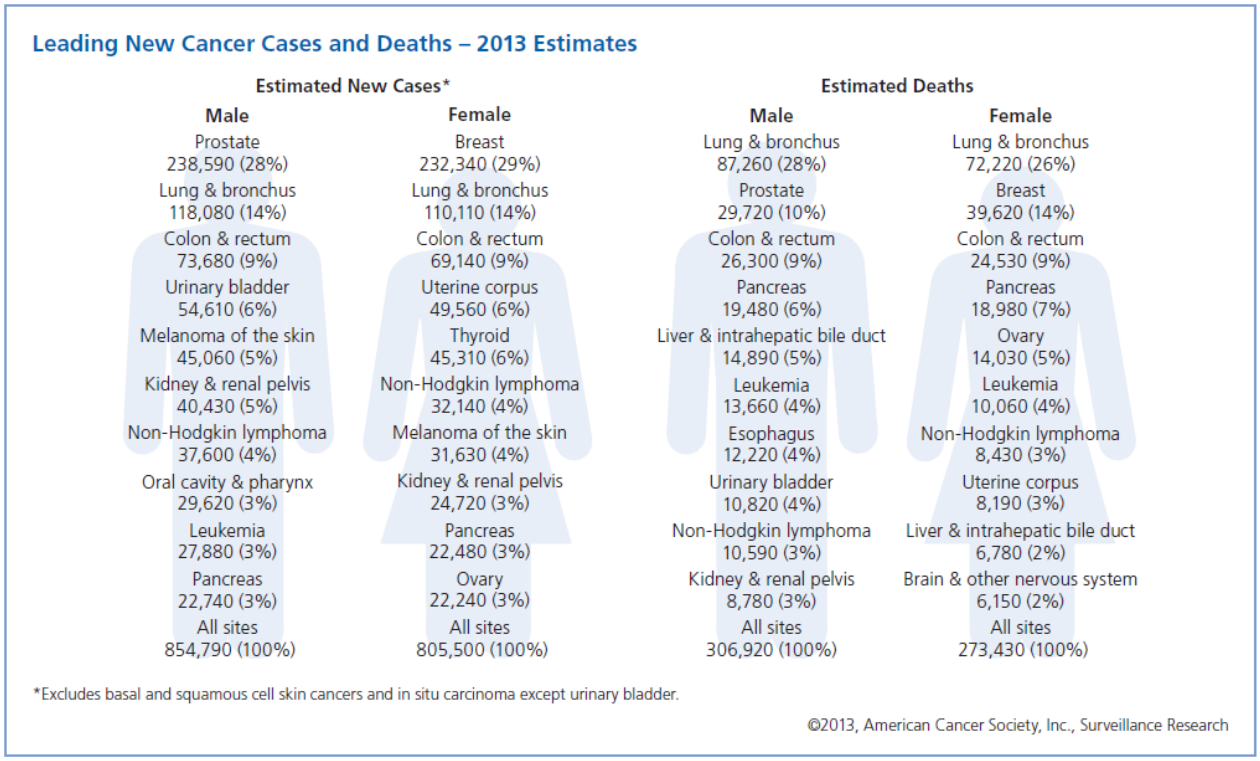


Figure 1-2. Leading New Cancer Cases and Deaths – 2013 Estimates

Figure adapted from Cancer Facts and Figures, American Cancer Society, 2013.

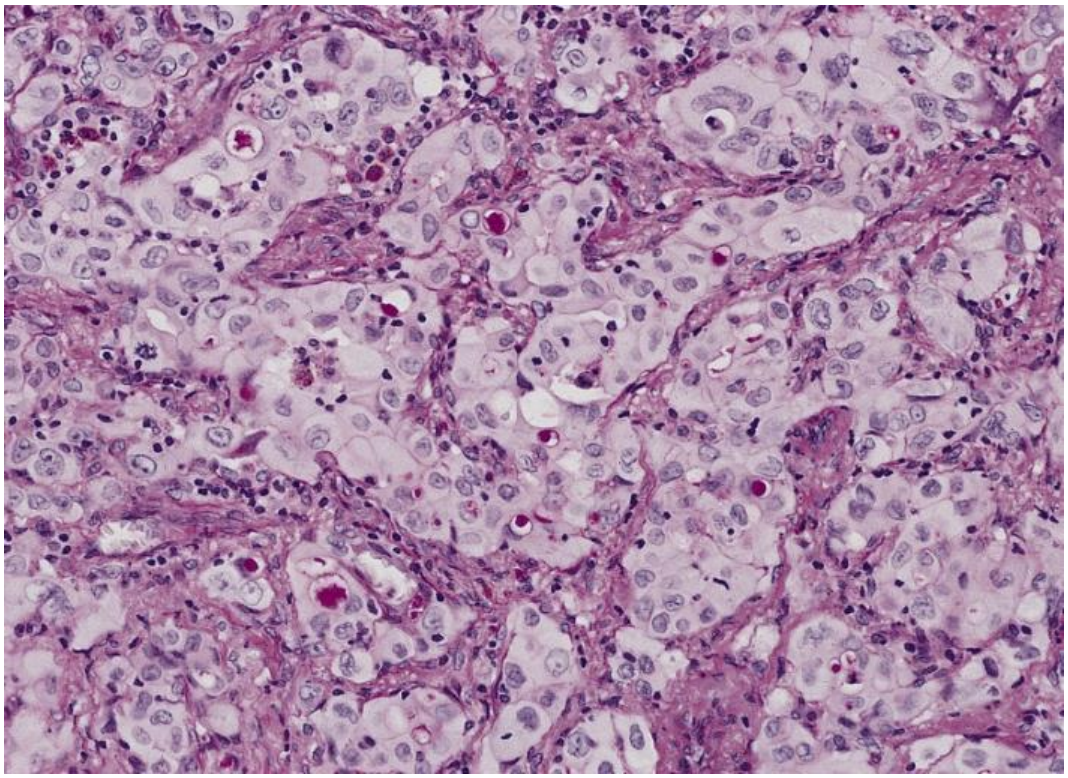


Figure 1-3. Lower respiratory tract: solid adenocarcinoma with mucin production. This poorly differentiated (solid) adenocarcinoma shows many cells with mucin vacuoles.

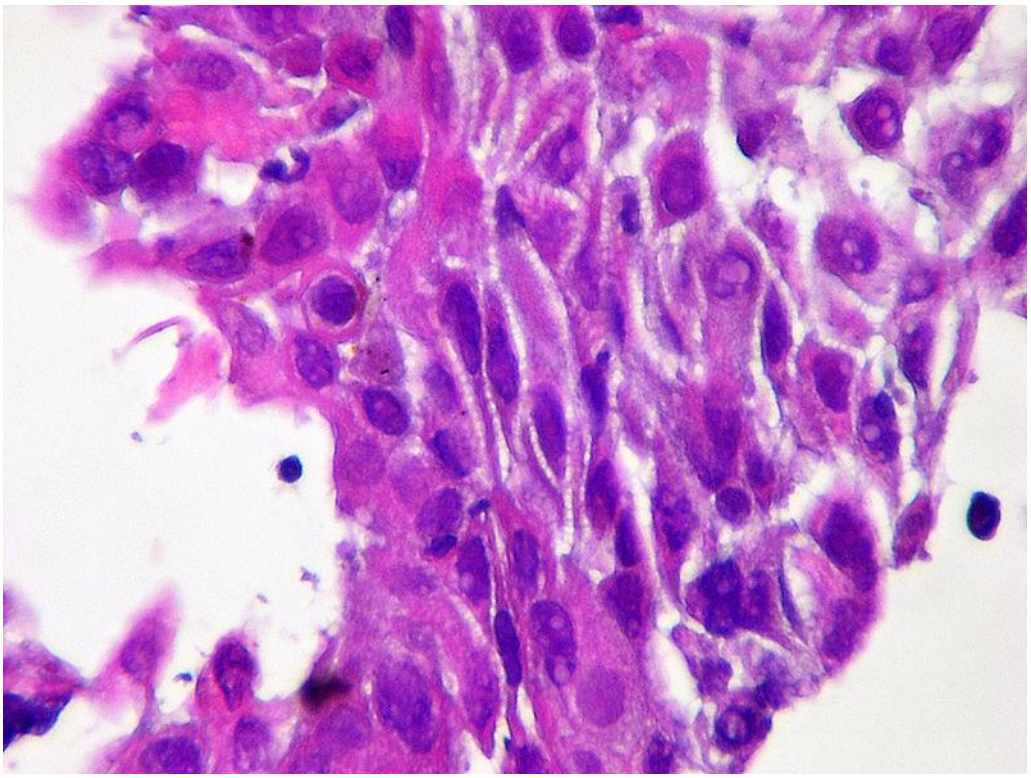


Figure 1-4. Squamous cell carcinoma of the lung. This image shows the presence of intracellular bridges which are one of the diagnostic criteria for squamous cell carcinoma. Image and description are from Yale Rosen.

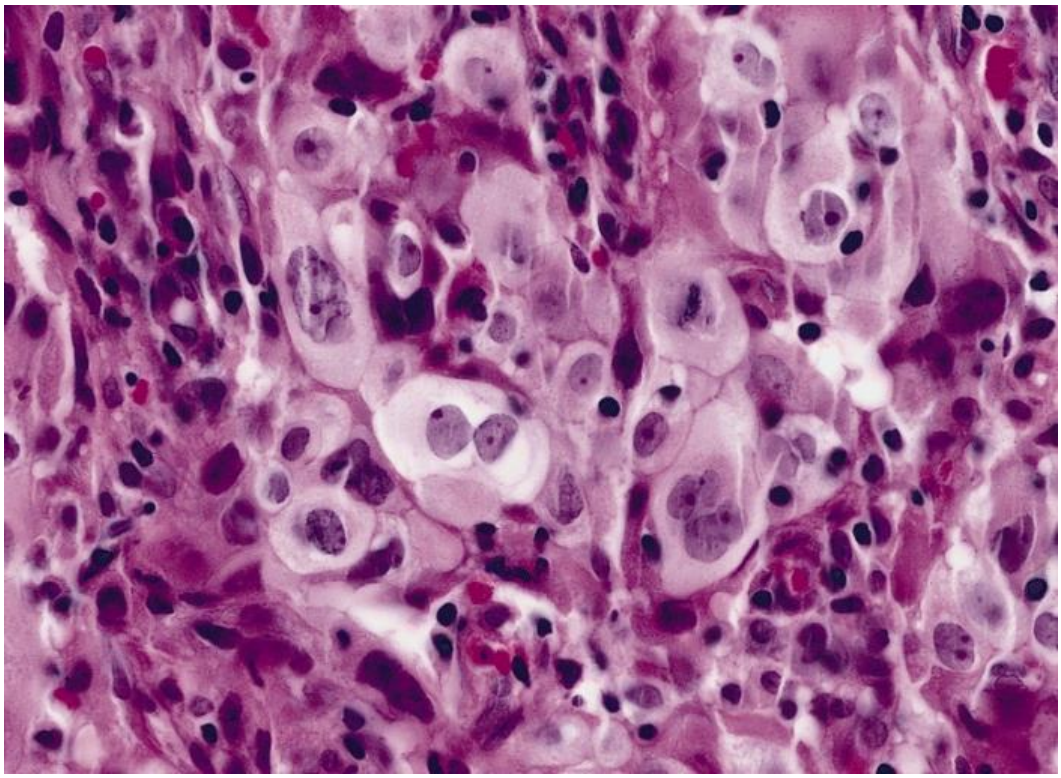


Figure 1-5. Lower respiratory tract: large cell carcinoma. A histological section shows a proliferation of atypical cells along the alveolar walls.

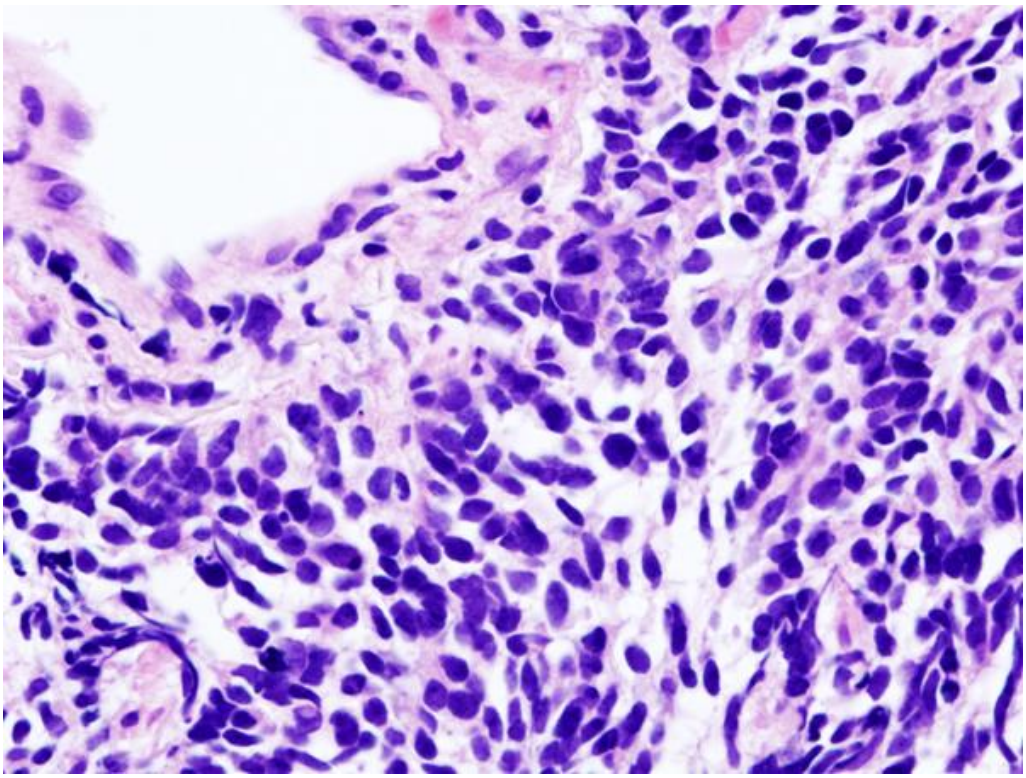


Figure 1-6. Histopathology image of small cell carcinoma of the lung. CT-guided core needle biopsy. H&E stain.

Definitions

Primary Tumor (T)

TX Primary tumor cannot be assessed, or tumor proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy

T0 No evidence of primary tumor

Tis Carcinoma in situ

T1 Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (for example, not in the main bronchus)[1]

T1a Tumor 2 cm or less in greatest dimension

T1b Tumor more than 2 cm but 3 cm or less in greatest dimension

T2 Tumor more than 3 cm but 7 cm or less or tumor with any of the following features (T2 tumors with these features are classified T2a if 5 cm or less): involves main bronchus, 2 cm or more distal to the carina; invades visceral pleura (PL1 or PL2); associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung

T2a Tumor more than 3 cm but 5 cm or less in greatest dimension

T2b Tumor more than 5 cm but 7 cm or less in greatest dimension

T3 Tumor more than 7 cm or one that directly invades any of the following: parietal pleural (PL3), chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium; or tumor in the main bronchus less than 2 cm distal to the carina[1] but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung or separate tumor nodule(s) in the same lobe

T4 Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, separate tumor nodule(s) in a different ipsilateral lobe

Distant Metastasis (M)

M0 No distant metastasis

M1 Distant metastasis

M1a Separate tumor nodule(s) in a contralateral lobe, tumor with pleural nodules or malignant pleural (or pericardial) effusion[2]

M1b Distant metastasis (in extrathoracic organs)

Notes

[1] The uncommon superficial spreading tumor of any size with its invasive component limited to the bronchial wall, which may extend proximally to the main bronchus, is also classified as T1a.

[2] Most pleural (and pericardial) effusions with lung cancer are due to tumor. In a few patients, however, multiple cytopathologic examinations of pleural (pericardial) fluid are negative for tumor, and the fluid is nonbloody and is not an exudate. Where these elements and clinical judgment dictate that the effusion is not related to the tumor, the effusion should be excluded as a staging element and the patient should be classified as M0.

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Occult Carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1a	N0	M0
	T1b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
	T1a	N1	M0
	T1b	N1	M0
	T2a	N1	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a	N2	M0
	T1b	N2	M0
	T2a	N2	M0
	T2b	N2	M0
	T3	N1	M0
	T3	N2	M0
	T4	N0	M0
	T4	N1	M0
Stage IIIB	T1a	N3	M0
	T1b	N3	M0
	T2a	N3	M0
	T2b	N3	M0
	T3	N3	M0
	T4	N2	M0
	T4	N3	M0
Stage IV	Any T	Any N	M1a
	Any T	Any N	M1b

Figure 1-7. Lung cancer staging. Primary Tumor (T) and Metastasis (M)

Figure adapted from lung cancer staging, American Joint Committee on cancer 7th edition, 2009. Reprinted with permission from AJCC.

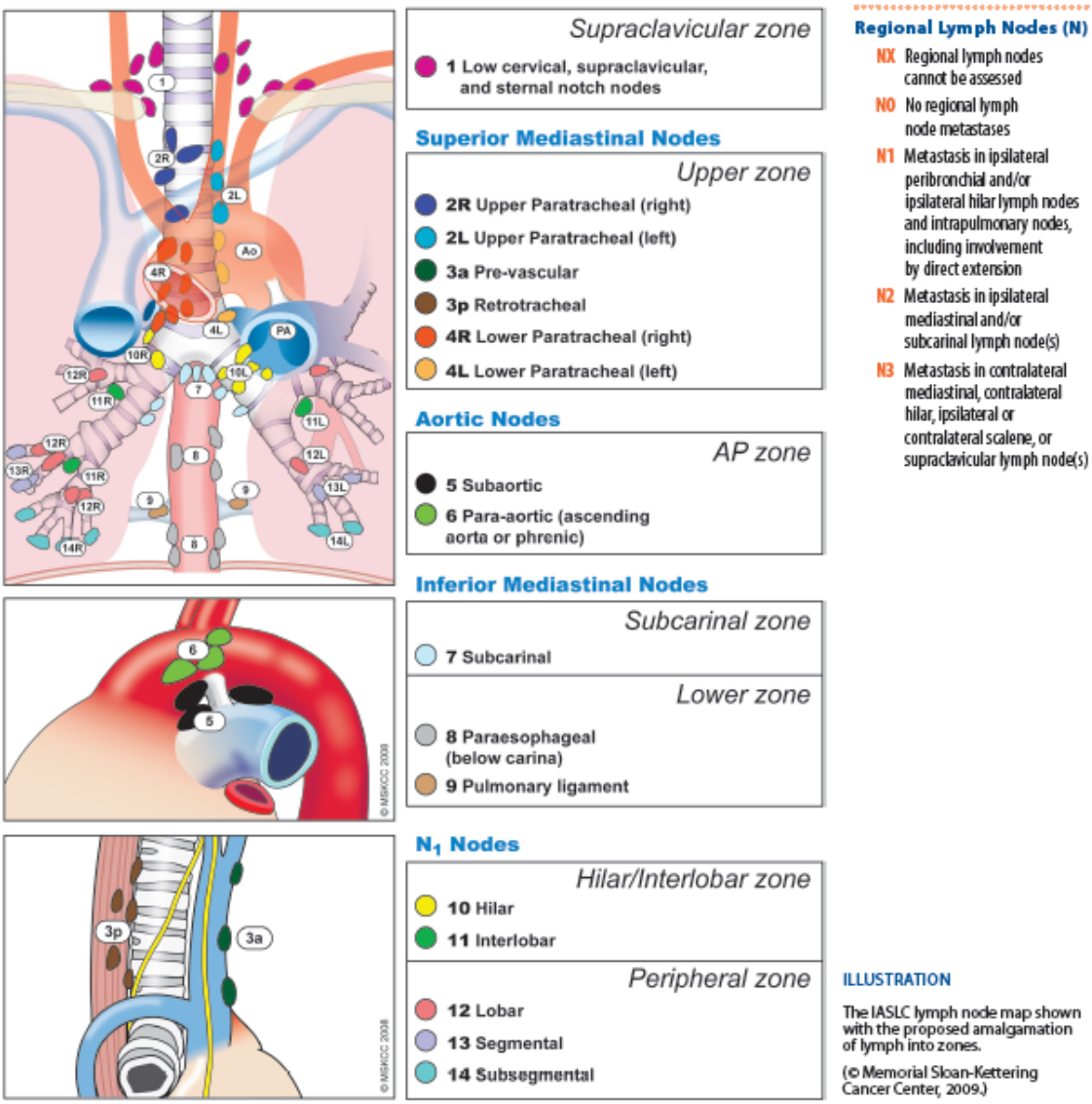


Figure 1-8 Lung cancer staging. Regional Lymph node (N)

Figure adapted from lung cancer staging, American Joint Committee on cancer 7th edition, 2009. Reprinted with permission from AJCC.

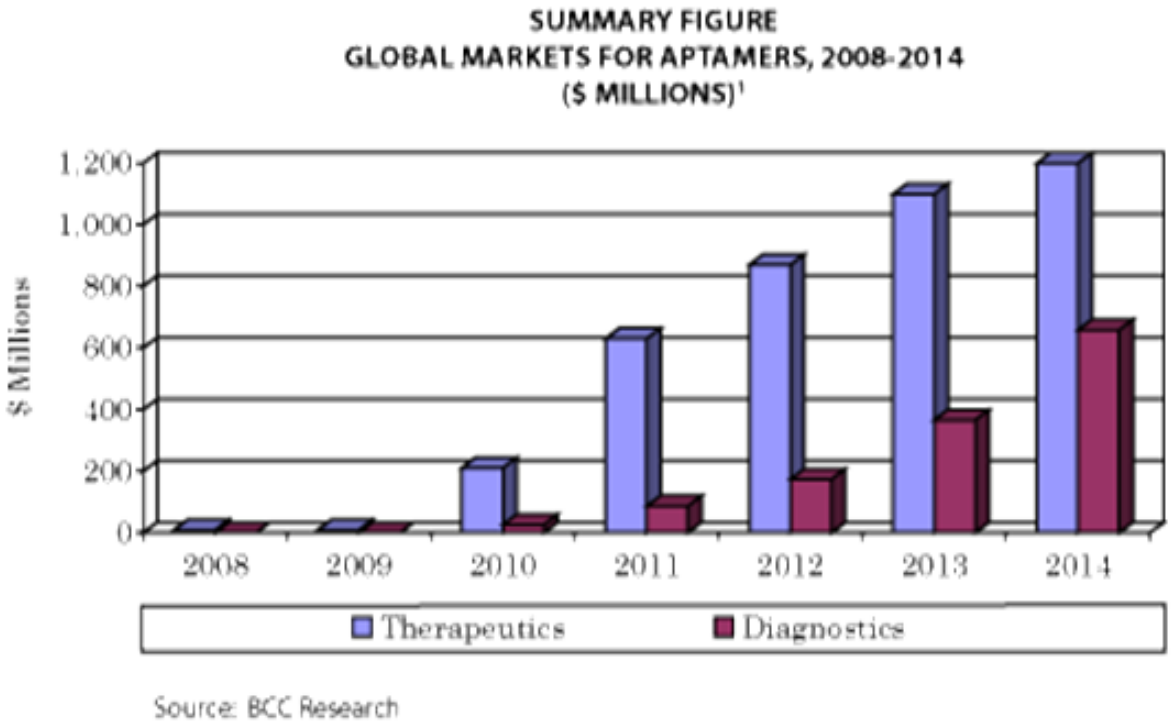


Figure 1-9. Global Market for aptamers 2008-2014.

Figure adapted from Nucleic Acid Aptamers for Diagnostics and Therapeutics: Global Markets, BCC Research, Wellesley, MA; March 2010. Reprinted with permission from BCC Research.

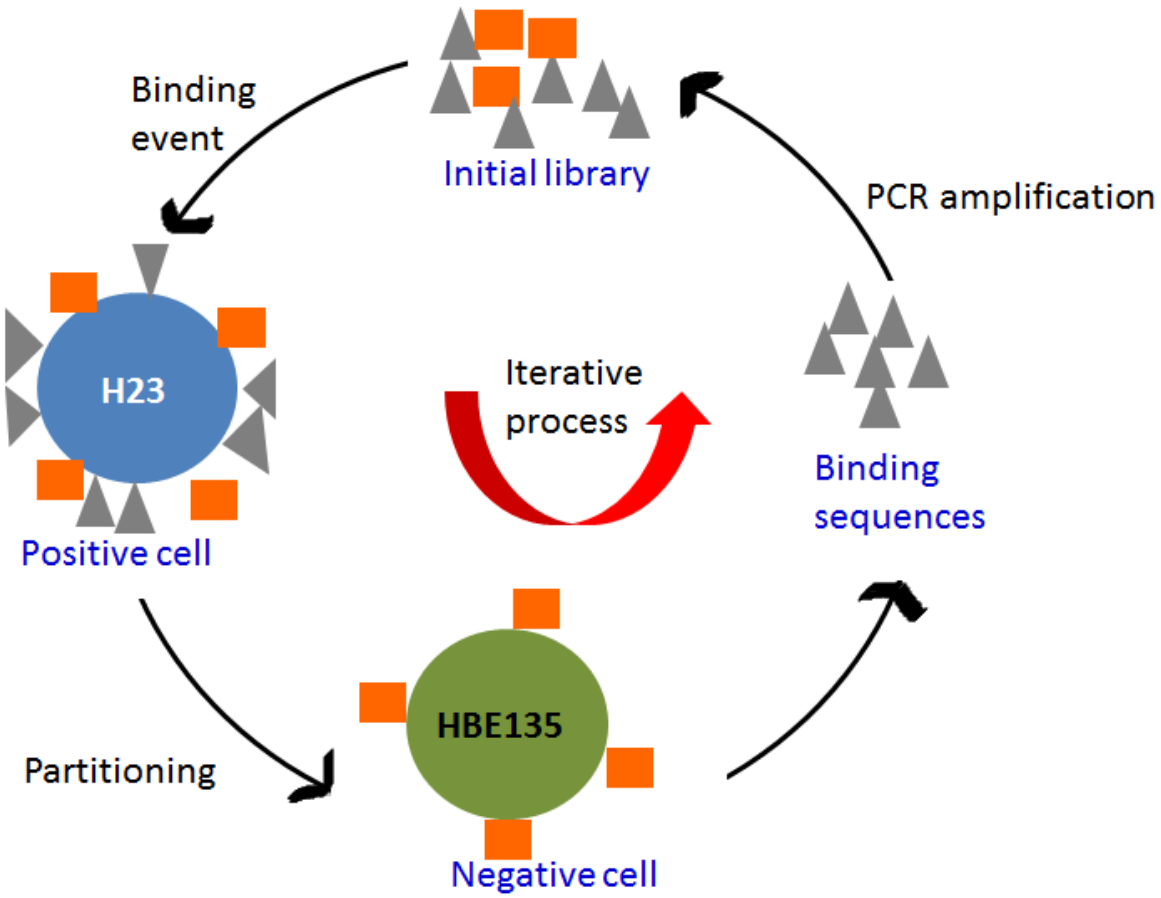


Figure 1-10. Schematic representation of cell-based SELEX methodology.

CHAPTER 2
SELECTION AND CHARACTERIZATION OF DNA APTAMERS AGAINST ADENOCARCINOMA OF THE LUNG

Introduction: Lung Adenocarcinoma

Lung adenocarcinoma is the most prevalent type of lung cancer, accounting for approximately 30% of all lung cancer cases.[1] Adenocarcinoma can occur in both smokers and non-smokers with an incidence of 30% and 70%, respectively.

Lung adenocarcinoma originates in the periphery of the lungs, and as a result, the symptoms are manifested at the late stage of its development. The growth rate is slow, and from its origination to metastatic cancer, this malignancy can be undetected. The main risk factor for adenocarcinoma is smoking with 90% of all lung cancer cases.[12] Overall, fewer than 10% of people with primary lung cancer survive five years after diagnosis. However, five-year survival rates can be as high as 35% to 40% for those who have localized lung cancer removed in its early stages.

Particular interest has arisen regarding the ADC subtype since it is the most common type of lung cancer amongst smokers and non-smokers. Incidence rates are increasing in most countries, and has exceeded all other subtypes.[10]

Accurate classification and development of probes for early detection are the current challenges in lung cancer. Thus, generating aptamers that bind specifically to certain tumor markers on the lung cancer cell membrane will expand the repertoire of probes, allowing the biology of disease to be studied at the molecular level. These probes can be utilized to detect specifically-targeted cells based on molecular differences between normal and malignant cells, thus, leading to important advances in understanding the biology of the disease and the investigation of potential cancer therapies. This chapter describes the cell-SELEX process using H23 ADC cell line as

the positive cell line and Human Bronchial Epithelial HBE 135 E6/E7 as the negative pair. The characterization of aptamers generated is also described.

Results and Discussion

Cell-based Selection for Adenocarcinoma

Since their discovery, aptamers have been generated against various targets, including proteins,[73,74] peptides,[104] and living cells.[78-80] To isolate aptamers capable of differentiating lung adenocarcinoma cells from normal lung epithelial cells, we used the cell-based SELEX strategy. H23 lung adenocarcinoma and HBE 135-E6/E7 normal epithelial lung were used as positive and negative cell lines, respectively. An initial ssDNA random library containing approximately 10^{14} different sequences of 80 nucleotides (nt) was enriched by sequential binding with the target cells, elution and subsequent amplification by PCR for 18 rounds. These DNA sequences could recognize H23 cell-surface membrane proteins which are potential markers for targeted therapy. In earlier rounds of the process, counter selection was introduced in order to remove possible sequences binding common proteins on both target and negative cell lines. This procedure was performed every other round throughout the selection.

Sequences binding to target cells were eluted and PCR-amplified, after which ssDNA was recovered and used to monitor the selection process by flow cytometry. Because the ssDNA pools were enriched with sequences specific for the target, an increase in fluorescence intensity was first noticed in round 12, indicating that those sequences showed better binding to the surface of H23 cells compared to the initial library. As the selection progressed, the fluorescence intensity of the subsequent pools gradually increased until a steady state in fluorescence intensity was observed in

rounds 17 and 18 [Figure 2-1A], indicating that maximum binding had been achieved. In contrast, such enrichment was not observed when the enriched pools were tested against normal lung epithelial cells [Figure 2-1B] implying that sequences contained in those pools were capable of distinguishing lung adenocarcinoma cells from normal lung epithelial cells. Therefore, pools 14, 16 and 18 were selected to be sequenced as aptamer candidates.

The sequencing process began with the construction of the 454 sequencing library by PCR-addition of 454-specific primers to the 3'- and 5'-ends of the enriched pools. To identify the pool for each sequence, a unique identification code (Middle Identification Code, MID) was also PCR-introduced to the 454 sequencing library. The insertion of primers in the enriched pools was confirmed by gel electrophoresis. After 454 sequencing at the UF ICBR core, around 7,000 sequences were retrieved and analyzed. They were grouped on the basis of the MID corresponding to the same pool, and primers were removed by a Perl program. The sequences containing only the random region were then aligned using the online program MAFTT 6.0[105], and six aptamer families corresponding to the most abundant sequences were chosen as aptamer candidates. They were chemically synthesized, biotin-labeled at the 3'-end, purified by HPLC, and quantified.

Characterization of Aptamers

All aptamers displayed binding with H23 lung adenocarcinoma cells, while no significant binding was observed with the control cell line HBE 135 E6/E7, normal epithelial lung cells [Figure 2-2]. These results indicate that successful negative selection had been carried out and that selected aptamers could distinguish between lung adenocarcinoma and normal lung epithelial cells, an outcome which confers these

aptamers with the potential for use in lung cancer diagnosis, monitoring tumor progression and treatment, or other relevant applications.

Dissociation Constants ($K_{d'}$s)

All the selected aptamers showed binding affinities (apparent Kd's) to the H23 cell line in the nanomolar range (45-250nM) (Table 2-1) and [Figure 2-3].

Table 2-1.Aptamer sequences and their dissociation constant (Kd_s) aptamers and pool %

Name	Sequences	K_d (nM)	% pool
ADE1	5'AGT GGT CGA ACT ACA CAT CCT TGA ACT GCG GAA TTA TCT AC 3'	70 ± 5	4,7
ADE2	5' GAG CCC TAT CTC ACA CCG CAC CCG CAA ACT ATC ATC CTACAT G 3'	208 ± 38	0,53
EJ2	5' AGT GGT CGA ACT ACA CAT CCT TGA ACT GCG GAA TTA TCT AC 3'	45 ± 5	7,3
EJ4	5' GAA GAC GAG CGG CGA GTG TTA TTA CGC TTG GAA ACA ACC CC 3'	60 ± 8	10,2
EJ5	5' TAC GGG CTG GAT CCA CTG TTA CGG CGT GTA TCC GCT ATC AA 3'	122 ± 11	0,71
EJ7	5' GAA GAC GAG CGG CGA GTG TTA TTA CGC TTG GAA ACA ACC CC 3'	55 ± 8	5,8

These results suggest that these aptamers will be widely applicable, as they tightly bind to their target. Further studies were carried out to characterize the selected aptamers. The binding was further tested against lung cancer cell lines, as well as other cell lines, including ovarian and colon cancer cell lines, as shown in Table 2-2. Aptamers EJ2, EJ4 and ADE1 displayed some recognition towards one or more of the following cell lines: TOV21G, DLD1, and H460. In the case of DLD1, a colon adenocarcinoma cell line, it was interesting to see some recognition by the selected aptamers, suggesting that a possible common cell-surface target is present in these two cell lines, since they belong to the same histological group.

Table 2-2. Binding of Adenocarcinoma aptamers with other cancer cell lines

Cell line	Name	ADE1	ADE2	EJ2	EJ4	EJ5	EJ7
H23	Lung Adenocarcinoma	+++	+++	++++	++++	++	+++
HBE 135	Normal lung bronchial cell	–	–	–	–	–	–
A549	Lung Adenocarcinoma	++	–	–	++	–	–
H460	Large cell carcinoma	–	–	++	++	–	–
H520	Squamous cell carcinoma	–	–	–	–	–	–
CAOV3	Ovary adenocarcinoma	–	–	–	–	–	–
TOV21G	Ovary clear cell carcinoma	++	–	++	++	–	–
DLD1	Colon adenocarcinoma	–	–	+++	–	–	–

In a previous study in our lab, the target protein of Sgc8 aptamer was demonstrated to be PTK7,[106] a pseudo-kinase protein present in CEM cells (the target cell line for that selection), as well as other cancers, including ovary, lung, colon, breast and some leukemia cell lines, indicating that common proteins can be present as a result of cancer.[107] Aptamers, EJ4 and ADE1 also showed particular specificity towards the lung adenocarcinoma cell line with significant increase in fluorescence intensity with respect to a random sequence, but not to normal lung cells. These results suggest that these aptamers could be used for lung cancer studies

Binding Studies at Different Temperatures

Flexible binding of aptamers at different temperatures can expand their repertoire of applications. Since the selection was performed at 4°C, we performed binding assays at 25˚C and 37˚C. As shown in this Figure 2-4, aptamers ADE1, ADE2, EJ2, EJ4 EJ5 and EJ7 conserved binding at 25°C and 37°C with fluorescence intensities similar to

those at 4˚C. This is particularly important in assays carried out under physiological conditions, such as those assessing *in vivo* applications.

Binding Studies Under Fixed Conditions

Cancer detection relies on the examination of tissue from biopsies, which are fixed under chemical conditions to preserve the integrity and antigenicity of tumor samples for later use. An important assay in cancer diagnosis is immunostaining, in which freshly dissected tissues are fixed prior to treatment with probes, such as antibodies and aptamers, specifically for tumor markers. Previous studies have shown that aptamers are capable of labeling formalin-fixed paraffin tissue (FFPE) after deparaffinization and antigen retrieval.[108] To determine the binding capability of the selected aptamers under those conditions, cells were fixed with 10% formalin before incubation with labeled aptamers. As control for these experiments, two known aptamers for leukemia, Sgc8 and TD05, and their corresponding binding cell lines were used to show that the process of fixation does not produce any fluorescence signal. Therefore any fluorescence detected should be an indication of a binding event between the aptamers and its target. As shown in Figure 2-5, both controls retained their initial binding profile, indicating that no artificial increment in fluorescence intensity occurred as a result of the fixing conditions.

Aptamers EJ2, EJ5, EJ7, ADE1, and ADE2 aptamers maintained similar binding to that displayed at 4˚C Figure 2-6 indicating that aptamers can bind to their targets even under fixed conditions. These results strongly suggest that selected aptamers have the potential to be used as recognition molecules in clinical samples.

Proteinase effect on Aptamer Binding

During cell selection, it is expected that aptamers interact specifically with the surfaces of target cells, and this behavior has been reported in several studies.[79,80,109] In our experiment, cells were treated with two enzymes, trypsin and proteinase K, for 3 and 10 minutes, washed with PBS, and then incubated with aptamers. All selected aptamers lost binding after treatment with both proteases [Figure 2-7]. For all experiments, the fluorescence intensity was significantly reduced; however, the signal corresponding to assays with proteinase decreased to background, indicating that the target protein was removed completely after treatment.

Concluding Remarks

In conclusion, we have selected a panel of aptamers capable of distinguishing lung carcinoma from normal lung epithelial cells. These aptamers showed high affinity towards the H23 cell line with apparent K_d's in the nanomolar range, but no detectable affinity for normal lung epithelial cells. Aptamers also showed binding under physiological conditions, as well as after chemical fixation, suggesting that they can be applicable for *in vivo* experiments. Proteinase treatment indicated that all aptamers in this panel bind to proteins on the target cell surface. All these results suggest broad potential applications of selected aptamers due to their specificity for cancerous tissues but not for healthy cells. These aptamers can also be used as molecular probes in clinical samples, such as freshly extracted tumors and preserved histology specimens.

Material and Methods

Library Design

The primers were designed to satisfy the following characteristics: a minimum hairpin structure, similar melting temperature (T_m) and minimal base pairing. The

primers and an 80-mer library were designed using the IDT Oligo Analyzer 3.1 software.[110] The forward primer was labeled with Fluorescein Isothiocyanate (FITC) at the 5'-end, and the reverse primer was labeled with Biotin at the 5'-end. The library consisted of a randomized 44-nt region flanked on the 5'-end by the FITC- labeled primer and flanked on the 3'-end by the complementary unlabeled strand of the reverse primer.

Instrumentation and Reagents

Libraries and primers were synthesized using the 3400 DNA synthesizer (Applied Biosystems). All reagents for DNA synthesis were purchased from Glen Research. DNA sequences were purified by reversed phase HPLC (Varian Prostar using a C18 column and acetonitrile/triethylammonium acetate as the mobile phase). PCR was performed on a Biorad Thermocycler, and all reagents were purchased from Takara. The monitoring of the selection process, binding assays, and determination of the dissociation constants for the selected aptamers were performed by flow cytometric analysis using a FACScan cytometer (BD Immunocytometry Systems).

Cell Culture and Buffers

A total of eight established cell lines was used in this project, all purchased from the American Tissue Culture Collection (ATCC). H23 (CRL-5800) adenocarcinoma NSCLC was chosen as the positive cell line, while the negative cell line chosen was HBE135-E6/E7 (CRL-2741), normal human bronchial epithelial cells. The H23 cell line was maintained in RPMI-1640 (ATCC) culture medium supplemented with 10% Fetal Bovine Serum (FBS heat-inactivated) and 1% penicillin-streptomycin. The normal bronchial lung cell line was maintained in a Keratin Serum-Free Medium supplemented with 5 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract (Invitrogen),

0.005 mg/mL insulin, and 500 ng/mL hydrocortisone (Sigma-Aldrich). Cells were incubated at 37ºC under 5% CO_2 atmosphere. Other cell lines used for the selectivity assay were the CAOV3 and TOV21G ovarian cancer cell lines, A549 lung adenocarcinoma, H520 lung squamous cell carcinoma, H460 large cell lung carcinoma, and DLD1 colon adenocarcinoma, and all were maintained according to ATCC specifications. During selection, two buffers were used: Washing Buffer (WB) (glucose 0.45% w/v and MgCl2 5mM in PBS) and Binding Buffer (BB) (1 mg/mL tRNA and 1mg/mL BSA in WB). All previous reagents were purchased from Sigma-Aldrich.

***In vitro* Selection**

Because both cell lines used during the selection, adenocarcinoma and normal human bronchial epithelial cells, are adherent cell lines, the selection was performed on cell monolayers. About 20nmol of the synthesized library was dissolved in 700 µL of binding buffer. Before the process was initiated, the DNA pool was denatured by heating at 95ºC for five minutes, followed by rapid cooling on ice. This forced the DNA sequences to adopt the most favorable secondary structures. The DNA library was then incubated with approximately $3x10^6$ target cells (H23) at 4ºC for 30 minutes. Subsequently, the cells were washed 3 times with washing buffer to remove unbound sequences. Afterwards, the bound sequences were recovered by heating at 95ºC for 10 minutes and then centrifuging at 14,000 rpm to remove cell debris.

The supernatant containing the DNA sequences was collected, and the selected pool was PCR-amplified using FITC- and biotin-labeled primers. Then, the generation of single strand DNA (ssDNA) was achieved by incubation with streptavidin-coated sepharose beads, to bind to the biotinylated strand. Counter selection was carried out after observing some enrichment with the target cells. In order to select aptamers with

high specificity and selectivity, the stringency of the washes (number of washes, wash time, and volume of washing buffer) was increased in subsequent rounds. The enrichment of the pools was monitored using flow cytometry, sequenced with 454 technology, and analyzed for aptamer candidates.

Flow Cytometric Analysis

Flow cytometry was used to monitor the enrichment of ssDNA-bound sequences within the pools during the selection process, as well as to evaluate the binding affinity and specificity of the selected aptamers. The cultured cells were washed with WB before and after incubation with the FITC ssDNA pool or selected DNA sequences. Fluorescence intensity was determined on a FACScan cytometer (BD Immunocytometry).

454 Sequencing and Analysis

After 18 rounds of selection, enriched pools 14, 16, and 18 were chosen for sequencing. The 454-specific primers and MID were PCR-amplified and added to each sequence contained in each pool, yielding a 125-bp product. The products were confirmed by gel electrophoresis, cleaned using a PCR purification kit (Qiagen), and submitted to the ICBR core at the University of Florida for analysis.

Approximately seven thousand sequences were retrieved and analyzed. First, sequences were grouped on the basis of their MID to determine the corresponding enriched pool. Second, primers and MID were removed by the Perl program in order to leave only the random portion of the sequence for homology analysis with the MAFFT 6.0 program.

Binding Assays

Different families of sequences retrieved from multiple sequence analyses were synthesized, biotin-labeled at the 3'-end, and tested for binding with the positive and negative cell line. In addition, the binding selectivity of each candidate was determined by incubating a 250nM aptamer solution with 4 x 10^5 target or counter-cells for 30 minutes at 4ºC. Cells were washed twice with WB and incubated with streptavidin PE beads for 20 minutes at 4ºC. After washing, the cells were suspended in 200µL WB. The fluorescence was determined with a FACScan cytometer by counting 3x10^4 events. A randomized 80-mer sequence was used as a control.

Binding Assays with Fixed Cells

To determine the ability of the aptamers to bind to fixed cells, procedures similar to those described above were followed, with the exception of the initial treatment of the cells. Briefly, adherent H23 cells were detached from the dish by incubation with non-enzymatic cell dissociation solution, and then fixed with 10% formalin solution for 15 min at 4°C, washed, and suspended in binding buffer.

Selectivity and Specificity Assays

To determine the specificity of these aptamers, different cell lines, including CAOV3, DLD1, A549, H520, H460, and TOV21G, were used in binding assays. Fluorescence intensity was measured in order to confirm binding. All experiments followed the flow cytometry experimental procedure described above.

Temperature Effect on Aptamer Binding

The selection methodology was performed at 4˚C.To determine if temperature would affect the binding between aptamer and the target cells, binding assays were carried out at two additional temperatures, 25˚C and 37˚C.

Determination of the Dissociation Constant

The affinity of the aptamer and its target was evaluated by saturation assay. A sample containing $5x10^5$ H23 cells was washed and incubated with different concentrations of aptamer until saturation was achieved. All binding assays were repeated three times, and the mean fluorescence intensity was calculated by subtracting the fluorescence intensity of a scrambled sequence. Data were collected, and the dissociation constant (K_d) which describes the strength of binding was obtained by fitting to a single binding site saturation model using SigmaPlot 11.2v (Jandel, San Rafael, CA).

$$y = \frac{Bmax\ X}{Kd + X}$$

Variable	Definition	Units
$X \geq 0$	Concentration of free ligand	Concentration nM
$Y \geq 0$	Specific binding (total-nonspecific)	sites/cell
$B_{max} > 0$	Maximum number of binding sites	same as Y
$K_d > 0$	Strength of binding	same as X

Trypsin and Proteinase K treatment

H23 cells were washed twice with PBS and then incubated with 3 mL of either 0.05% trypsin/0.53mM EDTA in Hank's balanced salt solution (HBSS), Cellgro, or 0.1mg/mL proteinase K in PBS at 37˚C for 3 and 10 minutes. FBS was added to quench the proteinase activity. Cells were washed with WB and subsequently used for the binding assays described above.

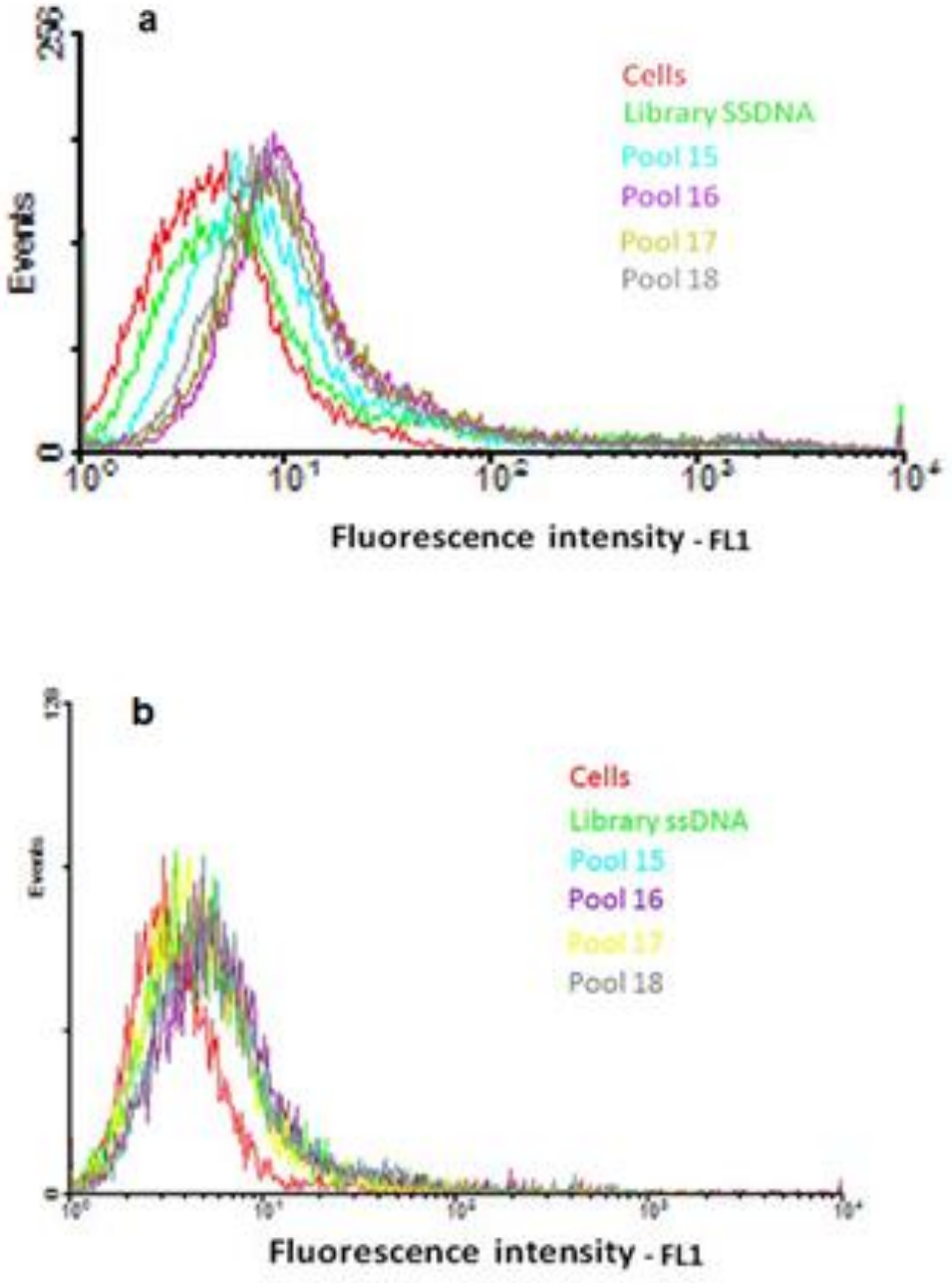


Figure 2-1. Progress of cell-SELEX methodology. Binding assay of pools 11-18 with H23 (A-B) and pools 15-18 with HBE135 E6/E7.

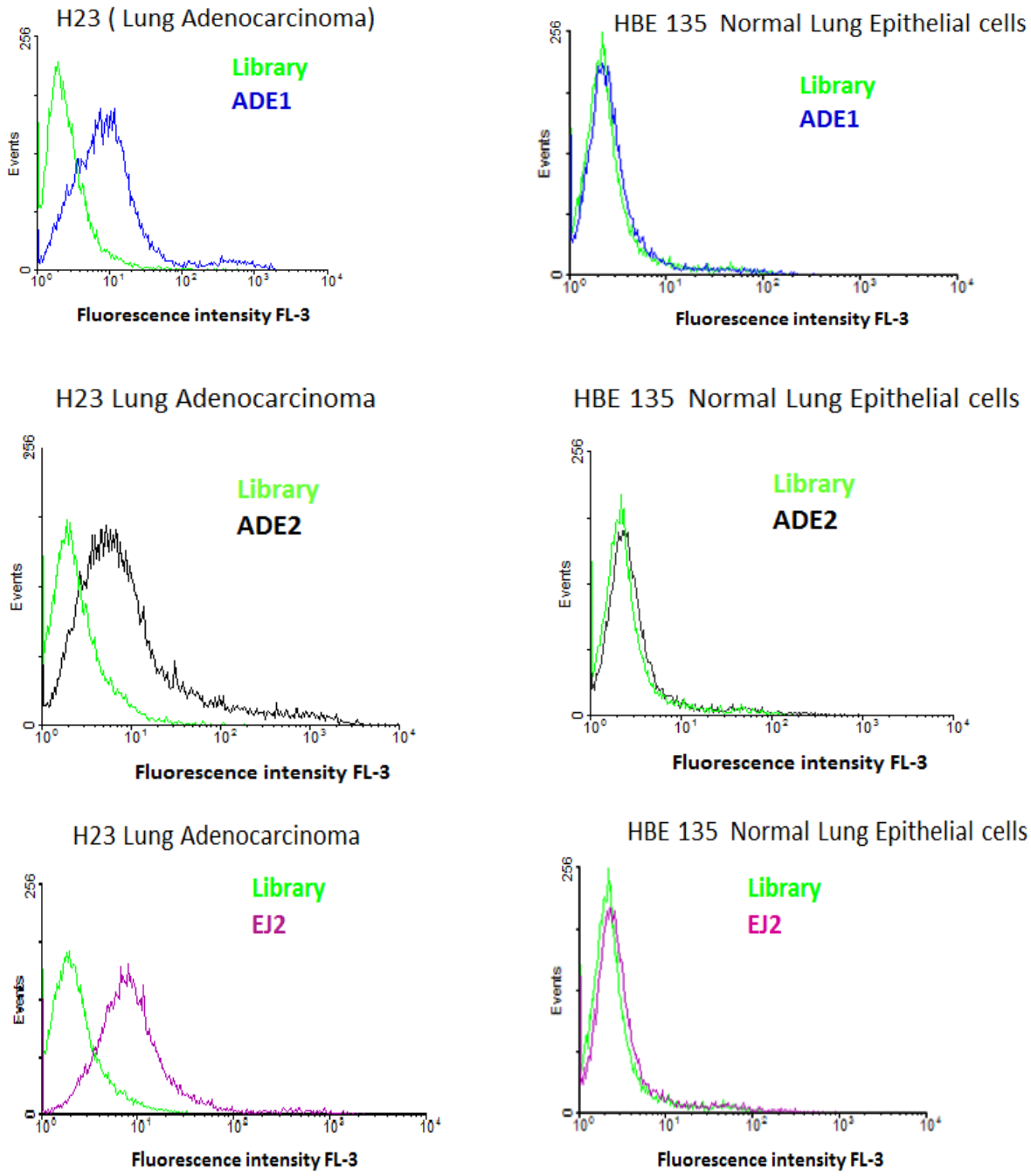


Figure 2-2. Characterization of selected aptamers. Flow cytometry assay for the binding of the aptamers ADE1, ADE2, EJ2, EJ5 and EJ7 with H23 (target cell line) and HBE135 E6/E7 (negative cell line). The green curve represents the background binding of a random sequence (library).

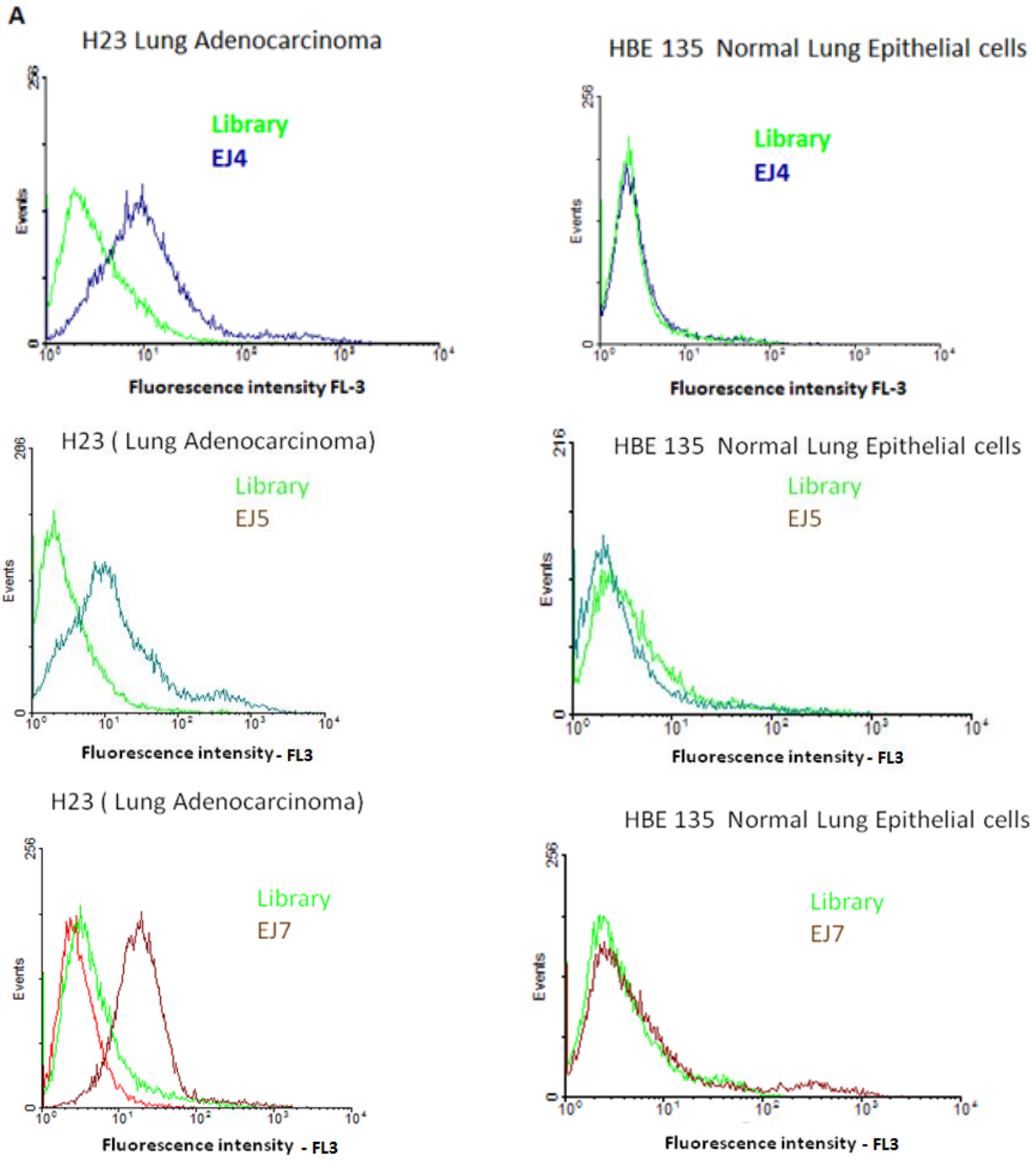


Figure 2-2. Characterization of selected aptamers. Continued.

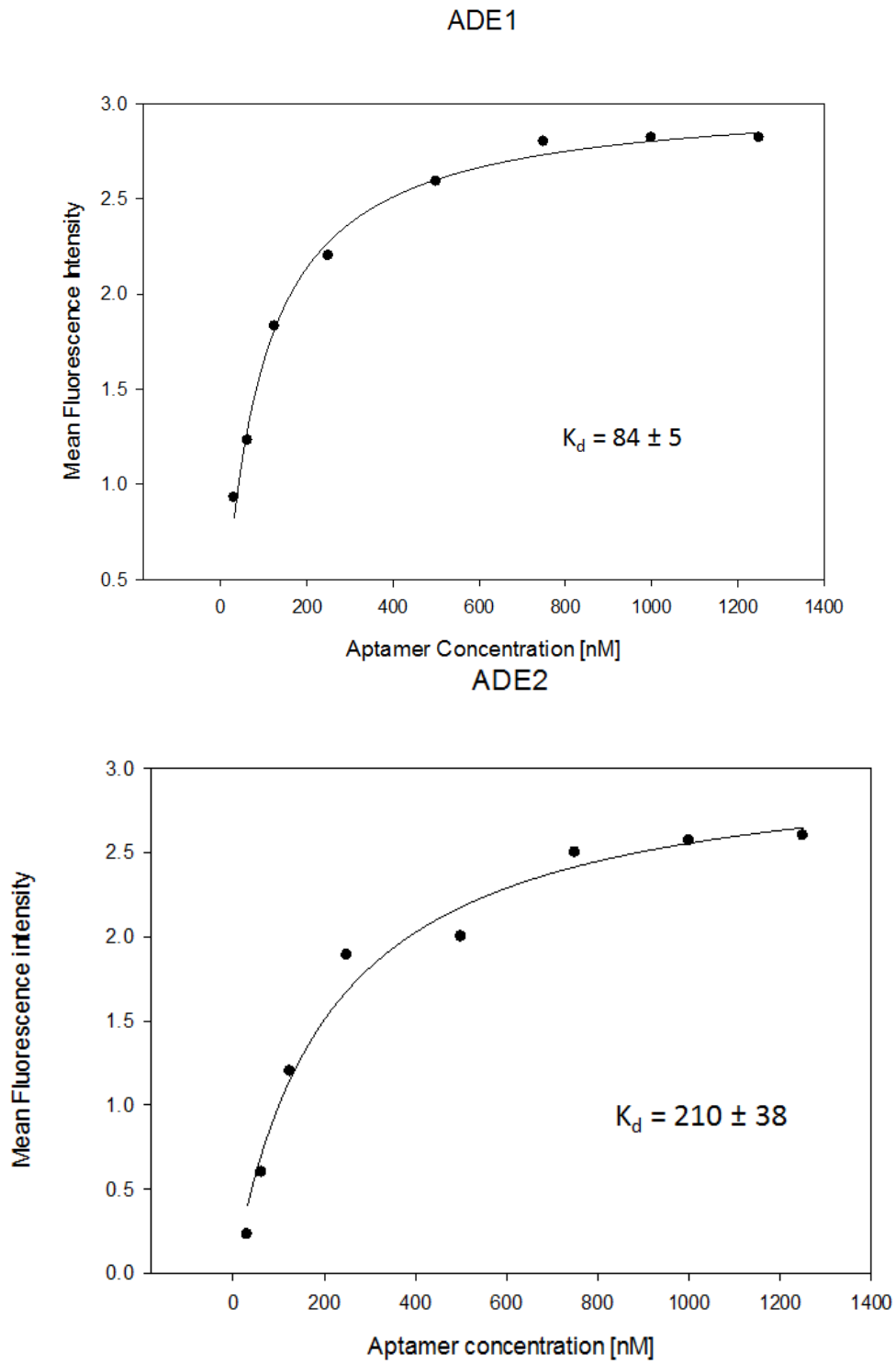
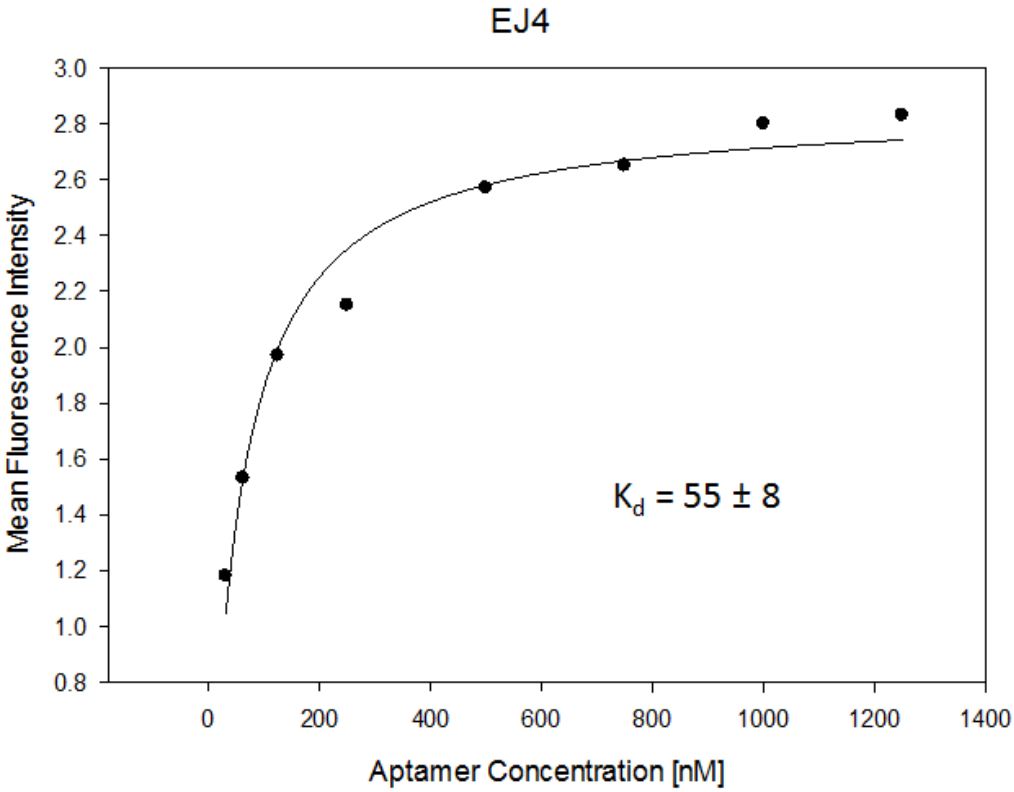


Figure 2-3. Apparent dissociation constants (kds) for selected aptamers. Saturation binding curves for selected aptamers ADE1, ADE2, EJ2, EJ4, EJ5 and EJ7. Cells were incubated with different concentrations of the aptamer in triplicate. The mean fluorescence intensity of the unselected library was subtracted from the intensity for each corresponding aptamer concentration.



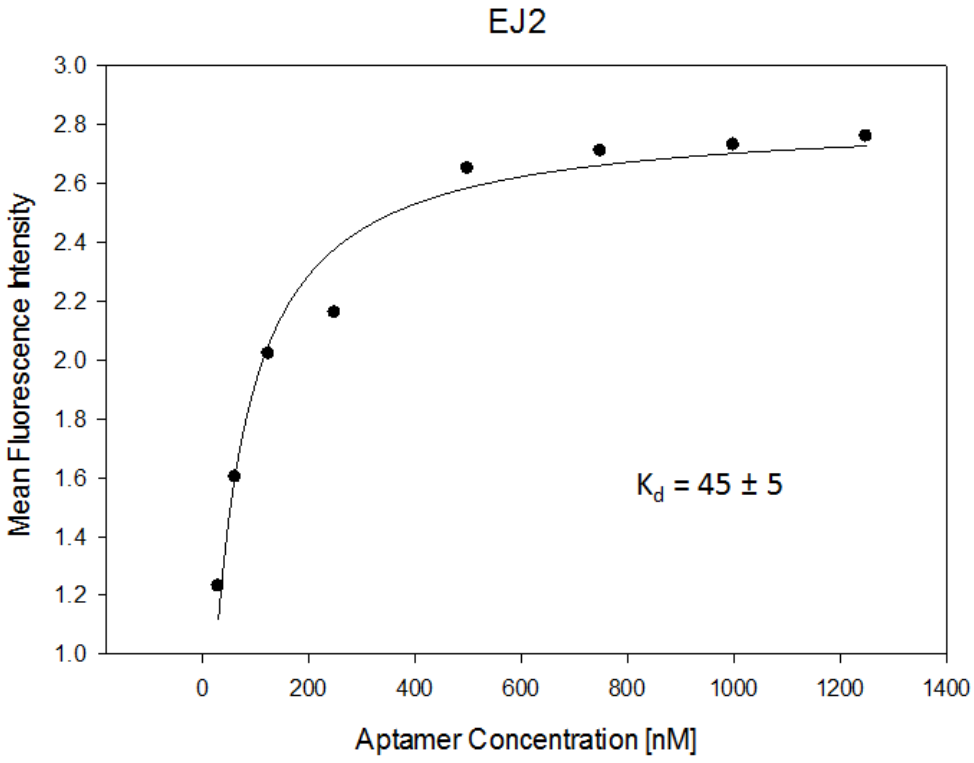


Figure 2-3. Apparent dissociation constants (kds) for selected aptamers.Continued

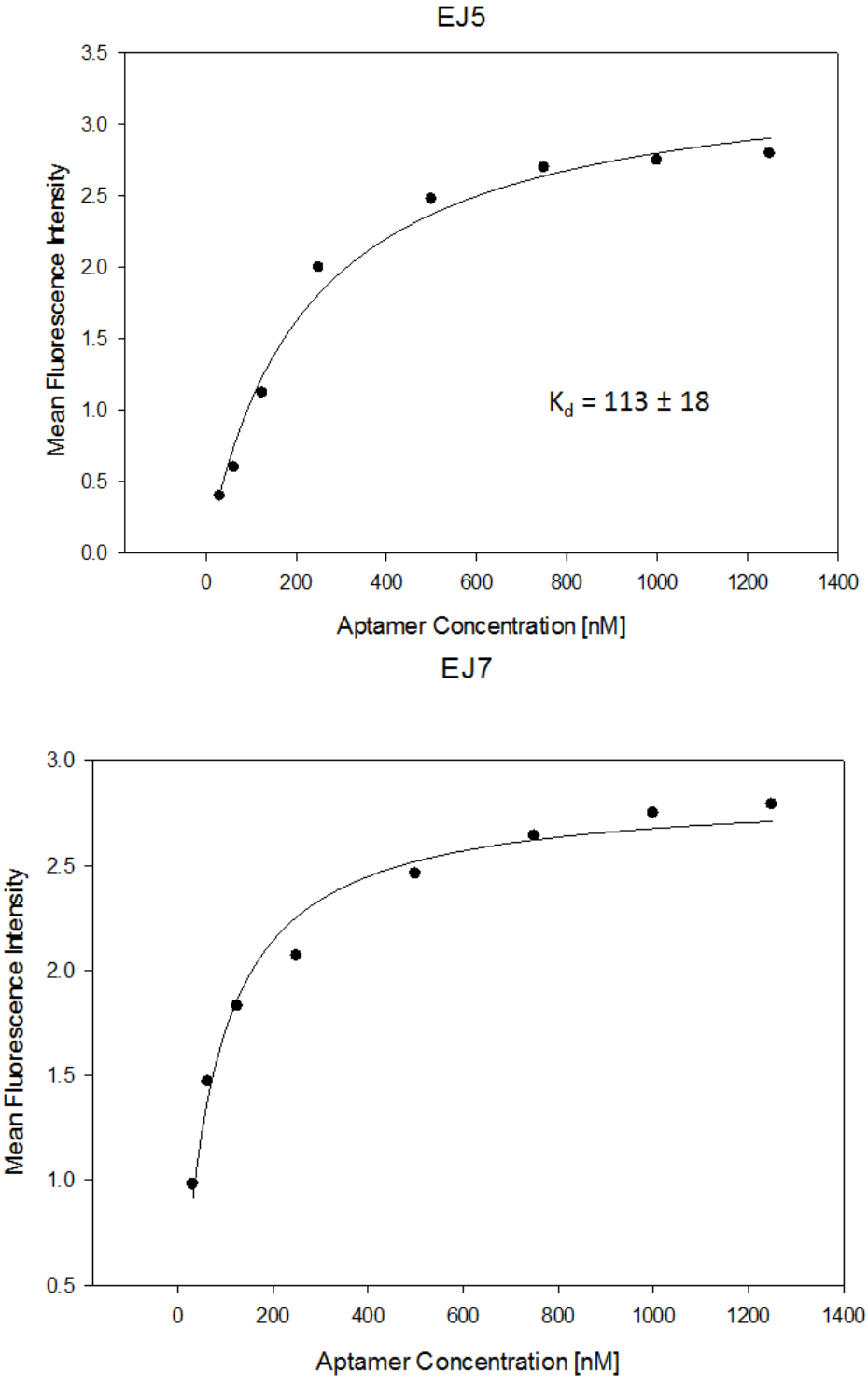


Figure 2-3. Apparent dissociation constants (kds) for selected aptamers.Continued

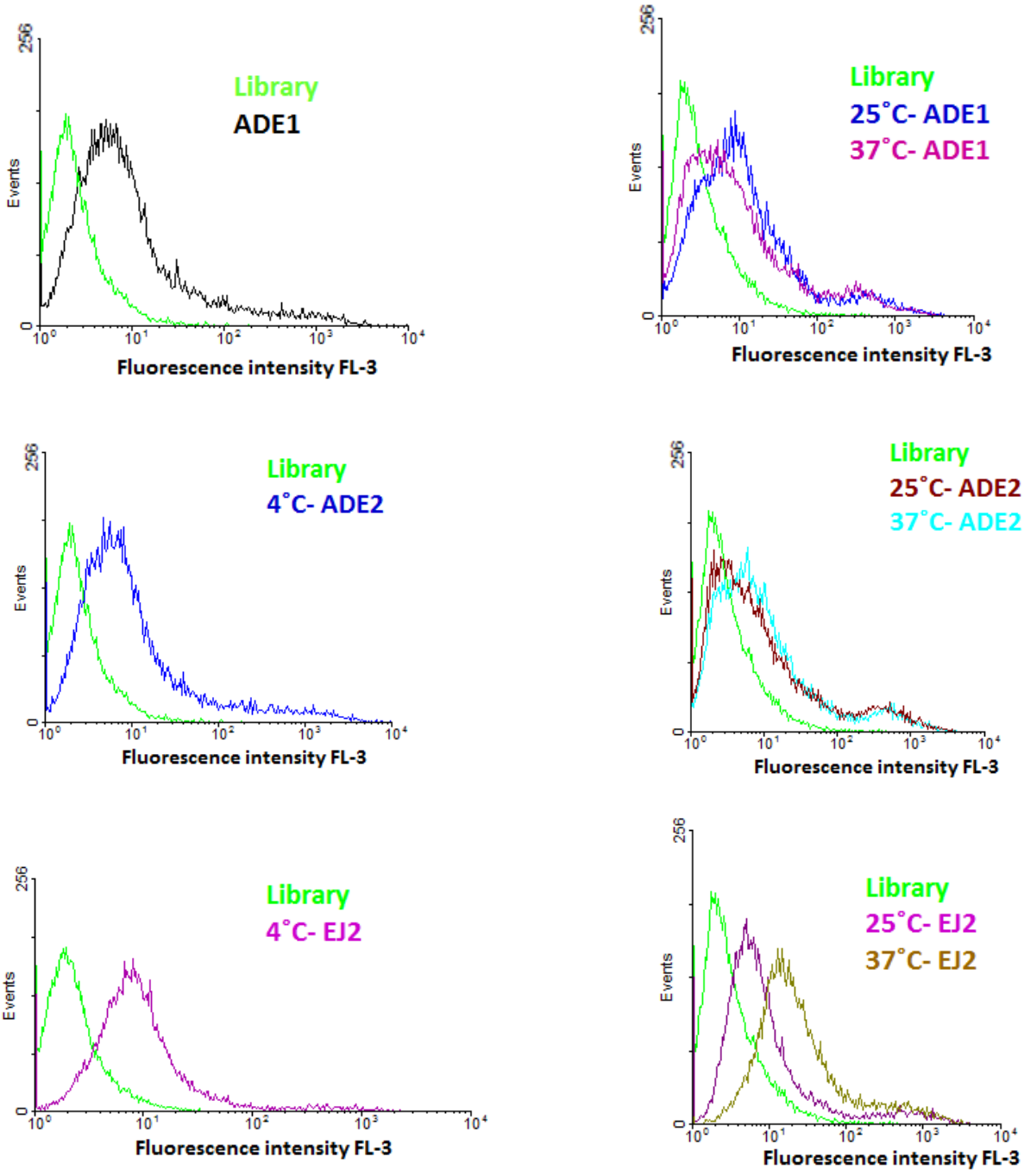


Figure 2-4. Aptamer binding at physiological conditions and room temperature. . Flow cytometry assay for the binding of the aptamers ADE1, DE2, EJ2, EJ4, EJ5, and EJ7 with H23 (target cell line) at 25°C and physiological temperature (37°C). Binding at 4°C was used as the positive control. The green curve represents the background binding of a random sequence (library).

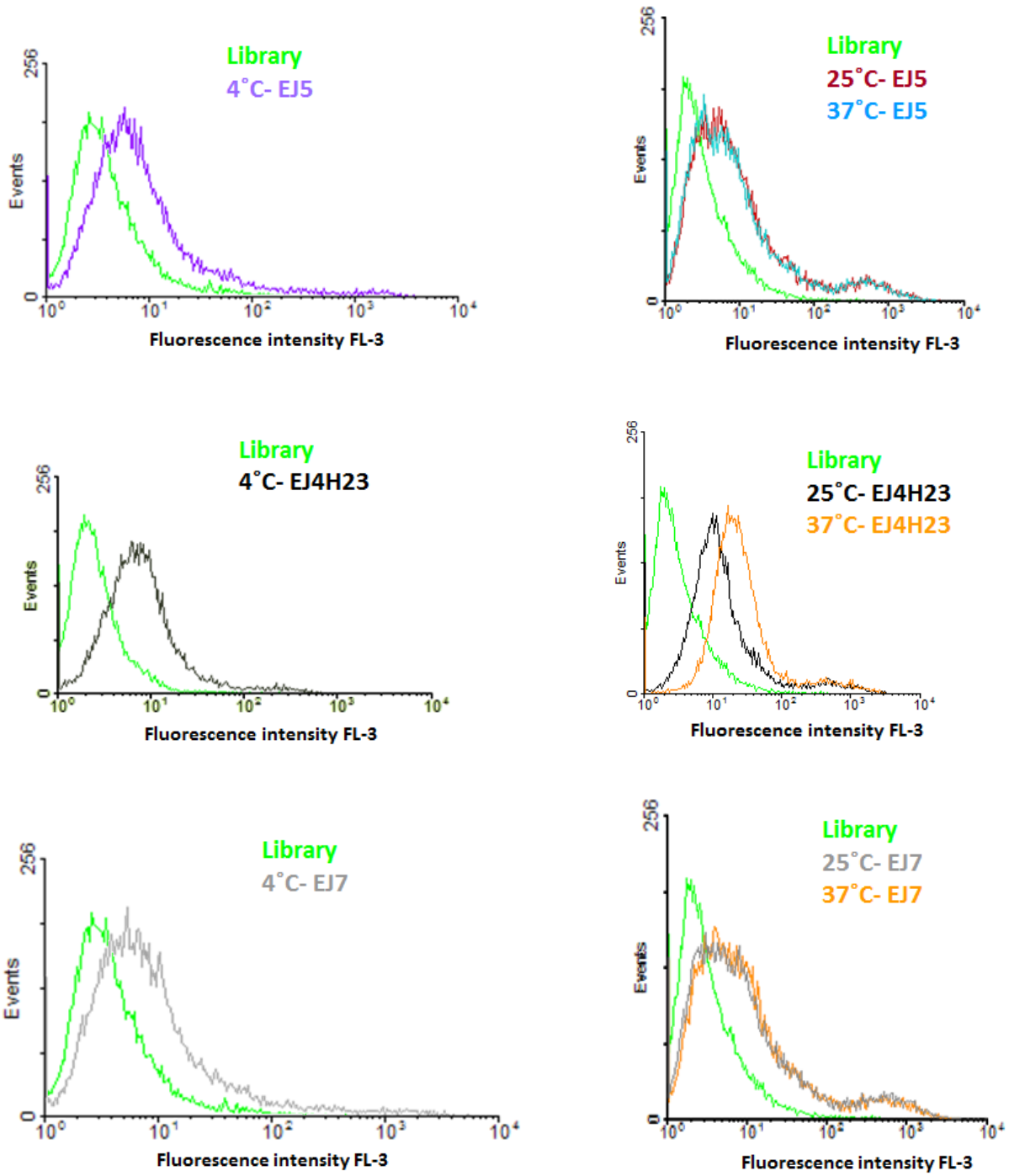


Figure 2-4. Aptamer binding at physiological conditions and room temperature. Continued

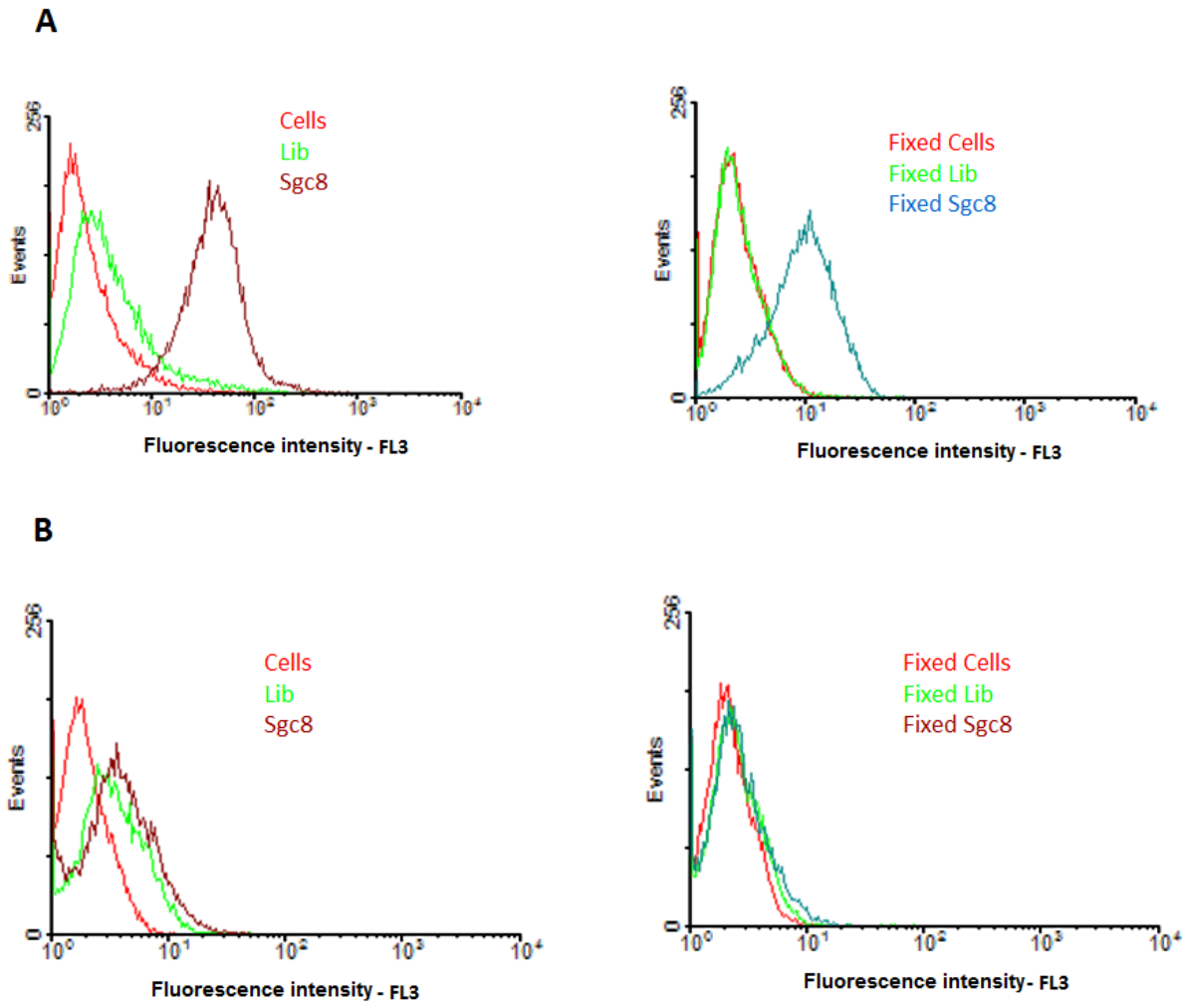


Figure 2-5. Control experiment for fixed treated cells. Binding assay of aptamer Sgc8 with (A) CEM (target) cells and (B) Ramos (control) cells before (left) and after (right) fixation, showing that no artificial fluorescence signal was produced.

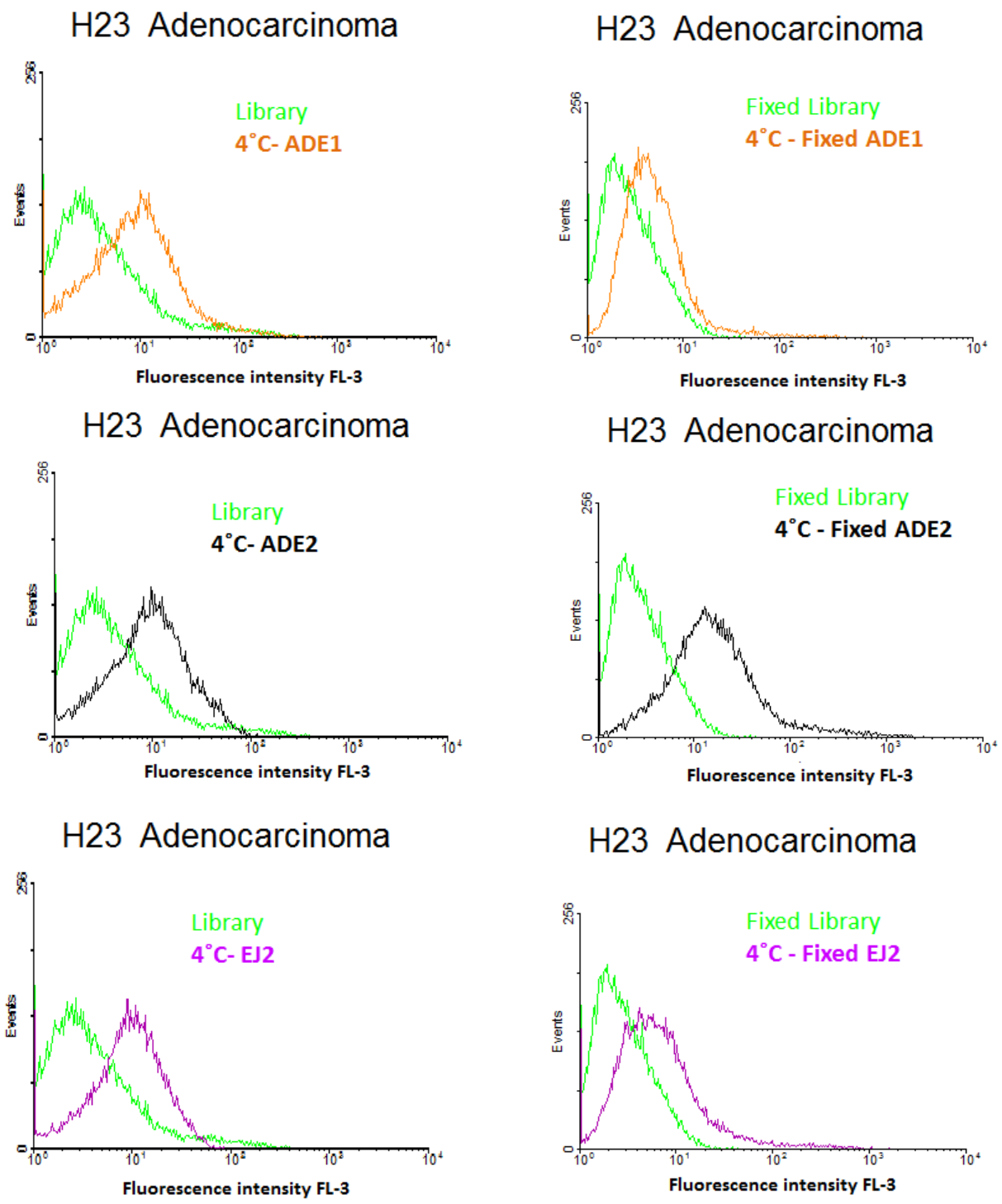


Figure 2-6. Binding assays after fixation with 10% formalin. Binding assay of aptamers with H23 (target) cells pre-fixed with 10% formalin. Left columns show the binding of aptamer ADE1, ADE2, EJ2, EJ4, EJ5 and EJ7 with untreated cells at 4°C. Right column shows the binding of the same aptamers EJ5 with fixed cells. The light green curve represents the background binding of a random sequence (library).

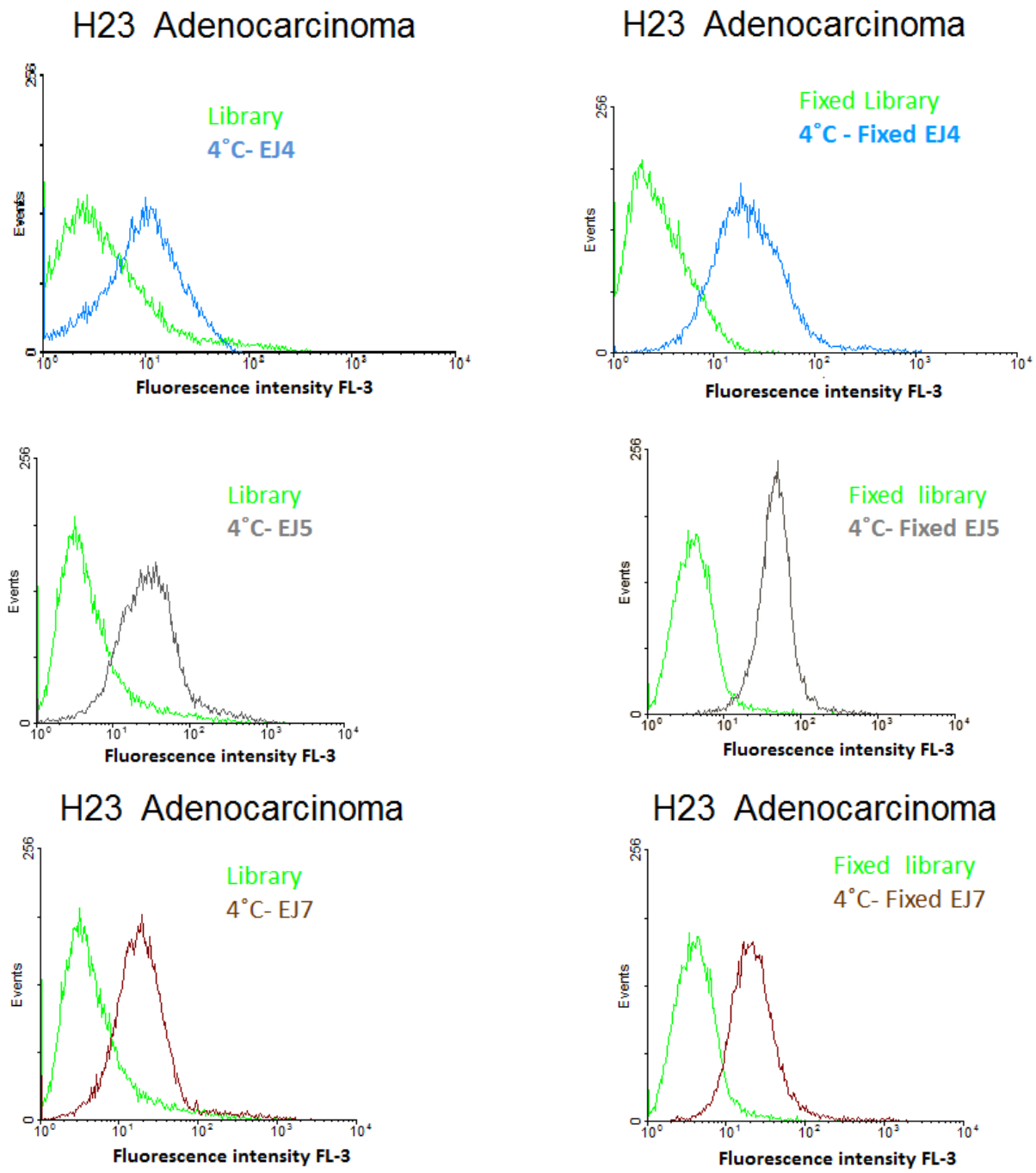


Figure 2-6. Binding assays after fixation with 10% formalin. Continued

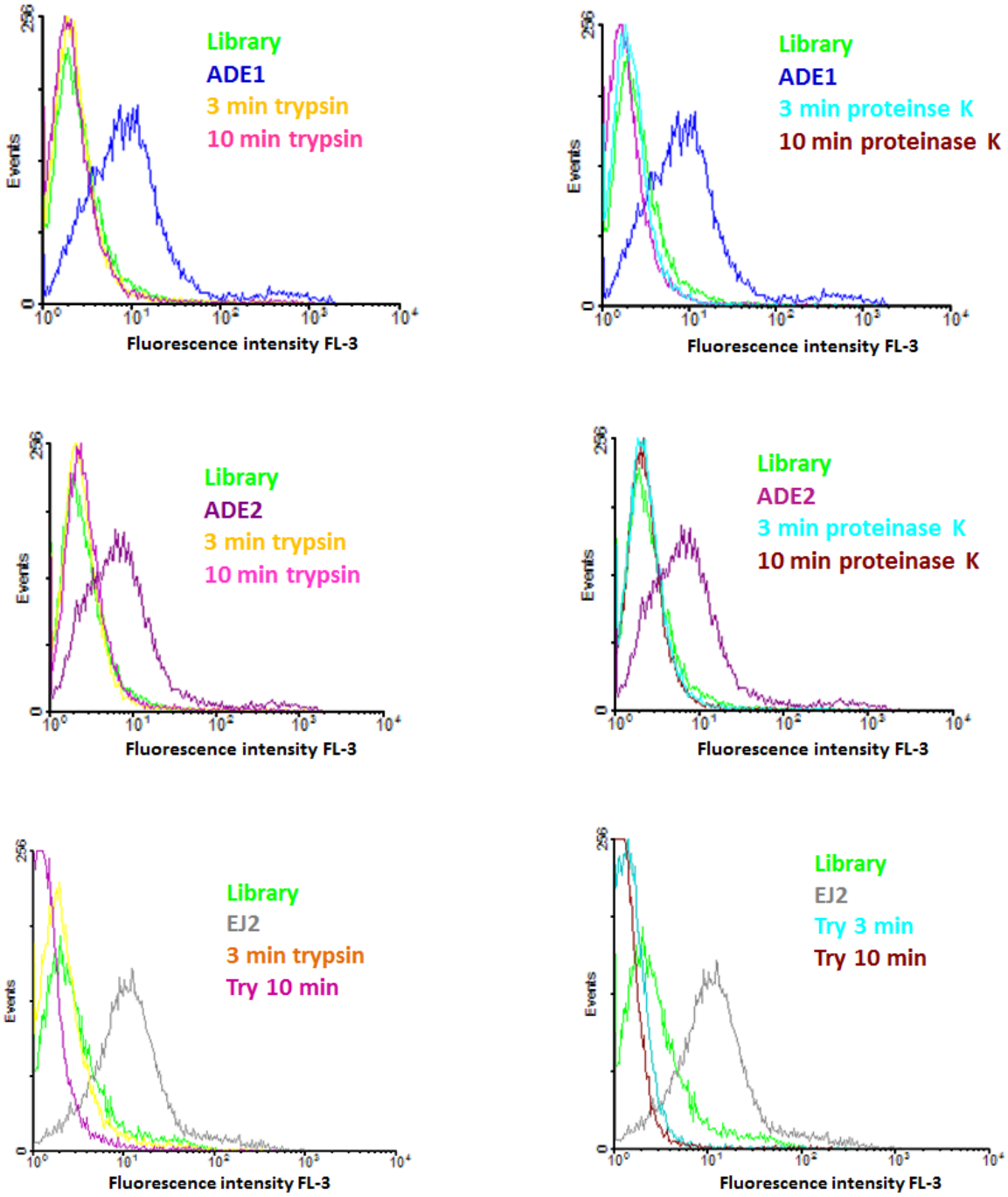


Figure 2-7. Binding assays after proteinases treatment. Flow cytometry assay for aptamers ADE1 and ADE2, EJ5 and EJ7 after treatment with proteases; untreated cells were used as positive control. Left side shows cells treated with trypsin for 3 and 10 min prior binding with aptamers ADE1 and ADE2, EJ5, and EJ7 respectively. Right side shows cells treated with proteinase K for 3 and 10 min prior binding with aptamers aptamers respectively.

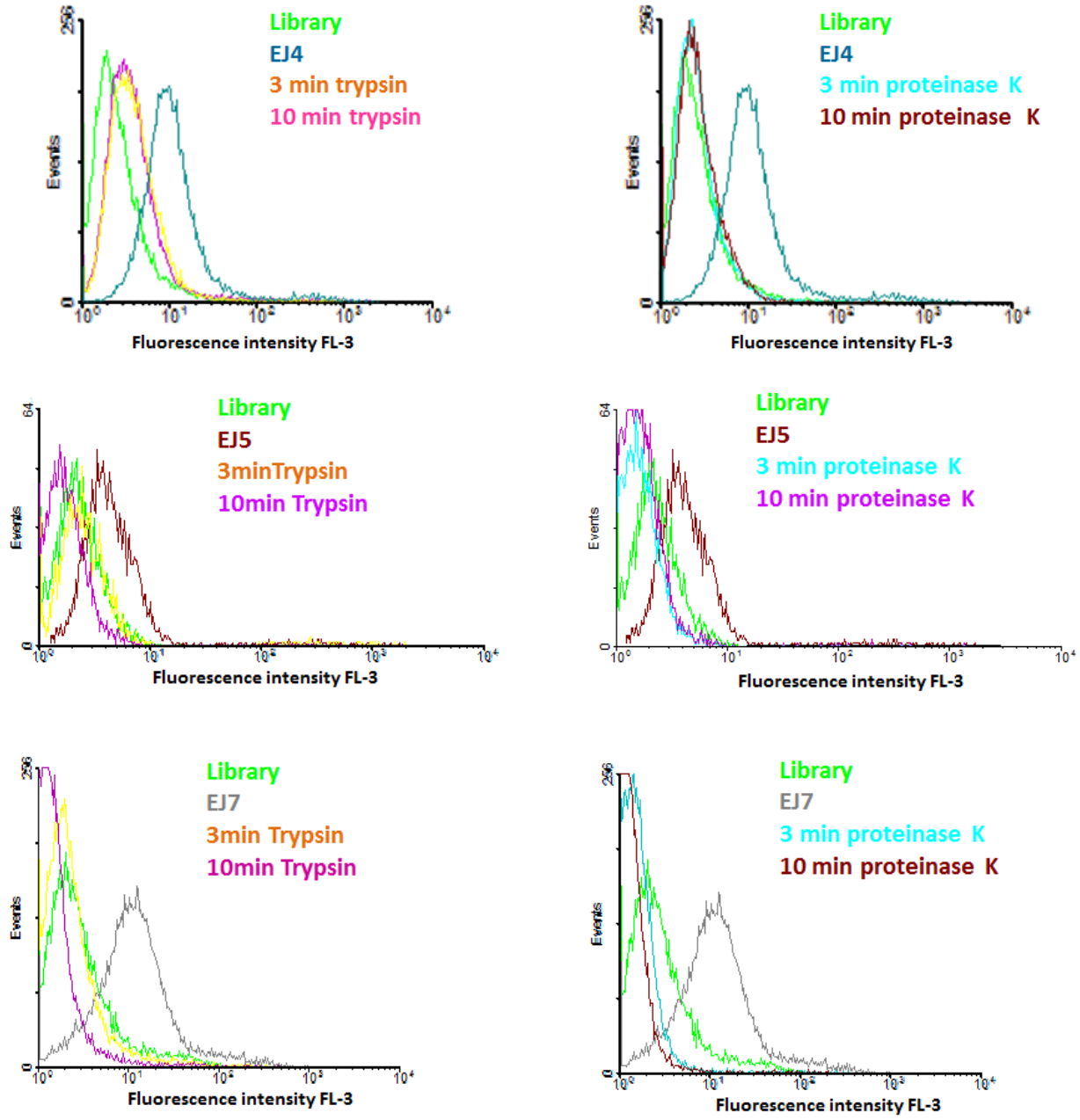


Figure 2-7. Binding assays after proteinases treatment. Continued

CHAPTER 3
VALIDATION OF LUNG CANCER APTAMERS IN LUNG CANCER CELL LINES AND CLINICAL SAMPLES

Introduction

Several types of specimens are available for conducting cancer research, including animal models,[111,112] fresh tumors or tumor-derived samples and cultured cell lines.[113,114] The animal models developed for NSCLC and SCLC have primarily been generated by genetic modification of oncogenes, allowing detailed study of initiation and progression of cancer.[115] Fresh tumors are ideal for the study of any type of cancer, but their availability to researchers is limited, and validation is required for the tumor to become an appropriate preclinical model.

Cultured cell lines have been used in past decades; and have contributed considerably to translational research in lung cancer, as well as to the discovery of new anticancer drugs. Currently, there are more than 500 lung cancer-derived cell lines available for research,[116] with possess many advantages, such as indefinite replication, clonal selection, and opportunity to assess invasion and tumorigenicity studies, to cite a few.[117] Despite much criticism as to whether these cancer cell lines resemble cancer *in vitro* due to lack of tumor architecture, genomic instability etc, their genomic similarities to their parent tumors making cultured cell lines very useful in cancer research.[118] To date, the most common probes to study cultured cell lines are antibodies. However, as discussed previously aptamers can be used as probes as well as their counterpart antibodies. Furthermore, a panel of aptamers can be used for surface marker profiling, to monitor lung cancer recurrence, and as a tool for NSCLC classification into different subgroups. Overall, the aptamer-based approach with

cultured cells will allow the understanding of different aspects of the disease, thereby offering the possibility of a holistic understanding of lung cancer from the onset.

This chapter describes profiling studies on aptamers selected for the different histological subtypes of lung cancer, as well as staining of cancer slides from cultured cell lines. Our expectations were to find aptamers that could profile specific groups, especially those for which the classification scheme is still not accurate.[119]

Results and Discussion

With only about two decades since its discovery by Ellington and Szostak[68] aptamers have become an emergent class of molecules for use in analytical, nanotechnology as capture and delivery molecules.[15] In the biomedical field, aptamers are also used as the counterpart of antibodies in diagnostics, acting basically as molecular recognition probes. However, the clinical application of aptamers is still under investigation. Previously has been demonstrated the use of aptamer for immunostaining of formalin-fixed and paraffin-embedded tissues (FFPE)[108] was described previously. This dissertation provides further support for the clinical application of aptamers selected for lung cancer by cell-SELEX in culture cell lines, artificial tissues and patient tissue samples. For this project, a panel of 12 different aptamers selected against lung cancer both, NSCLC[109,120] and SCLC,[121] were used. They were subjected to binding assays against a representative pool of lung cancer cell lines to identify the sequences with ubiquitous binding, independently of their histological subtype. As preliminary data, all aptamers were tested for binding against the normal human bronchial epithelial cell line HBE135 E6/E7 and also under formalin-fixed conditions. The purpose was to exclude probes incapable of (1) differentiating a normal lung cell line from a lung cancer cell line, and (2) binding under fixed conditions, as these two characteristics are

essential for the application of aptamers in tissue patient samples. Both [Figures 3-1 and 3-2] showed the efficiency of all aptamers in fulfilling the specificity for lung cancer cell lines and the binding affinity with 10% pre-fixed cells.

Because lung cancer is innately heterogeneous, it is difficult to find markers that can be applied to the entire field rather that to certain subtypes. To address the binding of the lung cancer aptamers, a panel of cell lines representative of all the groups and subgroups in lung cancer was utilized to determine if there is any group of aptamers that can differentiate one group from another. [Figure 3-3] shows representative ADC cell lines that were profiled with aptamers generated in three different selections. Results demonstrated that aptamers S1, S6, and EJ4 are capable of binding most of the adenocarcinoma cell lines. It is important to note that aptamer HCH03 was initially selected against H69 a SCLC cell line. Since the number of ADC and SCLC cell lines available surpasses the number of SQC and LCC cell lines tested, those will be presented at the end of this chapter Table 3-1, and 3-2 respectively.

One of the current challenges in lung cancer diagnosis is the effective classification of biopsies or any clinical sample that serve this purpose, specifically for accurate distinction between ADC and SQC.[122] Three of the most frequently used NSCLC-SQC cell lines in the literature have been used for this purpose shown in [Figure 3-4]. Unfortunately, there is no specific aptamer or set of aptamers that can specifically differentiate these two histological subtypes due to the molecular similarities in both subgroups. Therefore the search for a more specific marker continues. One approach to address this problem could be the use of a cell-based selection with an SQC cell line as target and an ADC cell line for counter selection. In this manner a

panel of several aptamers could be generated for the differentiation of these two similar histological subgroups.

The next subgroup analyzed was NSCLC-LCC; similarly two cell lines were used for this profiling, H460 and H661. Based on the molecular surface analysis, this group is distant from the NSCLC SQC and ADC, and most of the aptamers used did not recognize this group [Figure3-5]. These results suggest that the molecular signatures on the surfaces of LCC cells are different from those on ADC or SQC. The only aptamers that did not follow the above pattern was sgc8, which targets protein tyrosine kinase 7 (PTK7).

The last group analyzed was SCLC, with the majority of the cell lines being suspension cells. [Figure 3-6] shows the aptamer profiling carried out. Aptamers HCH1, HCH3, HCH7 and HCH12 selected against cell line H69 previously in the tan group the majority of SCLC cell lines, displaying specificity for this subgroup. However, aptamer S1 originally developed against A549, an ADC cell line, also displayed some affinity towards several cell lines in this group. When all lung cancer cell lines were analyzed, as a whole aptamers HCH3, S1 and EJ4 displayed binding among all of the analyzed groups and subgroups.

Another aim was to determine the binding capability of these aptamers against formalin fixed and FFPE lung cancer tissue. A tissue model was re-created by using a cell line as model. Both types of tissue samples were simulated and the results are shown in [Figures 3-7 and 3-8]. Figure 3-8 shows the immunohistochemistry results of formalin-fixed tissue using aptamers as recognition molecules. It is important to point out that no antigen retrieval was needed for the recognition of the target by the

aptamers. Aptamers were able to recognize their targets in the fixed tissue and, when compared to the negative control (random DNA) it can be clearly seen that the staining is due to specific binding. Some aptamers like sgc8 showed stronger binding when compared to EJ4 or HCH3. These observations can be explained by the amount of target expressed on the surfaces of these cells.

Figure 3-8 shows the results corresponding to the immunohistochemistry of FFPE tissues. One feature that stands out is the intracellular staining of these aptamers when compared to fixed tissue. To address this issue, samples were pre-incubated with salmon sperm DNA to saturate those DNA-binding molecules present in the nucleus of the cells. Unfortunately, after DNA blocking some nuclear staining was still observed. As the target of this aptamer is still unknown, it could be possible that the target could be also expressed intracellularly. The most notable example of this behavior is the target for nucleolin aptamer, AS1411, which expressed not only on the surface of the cell but also in the nucleus.[123]

After both methodologies were performed, one important observation was the lack of cell penetration of the aptamers during the staining of formalin-fixed tissues. This behavior could be an advantage if only extracellular staining is desired, even if the target is also expressed inside the cell.

Table 3-1. Adenocarcinoma cell lines profiled with the panel of lung cancer aptamers.

Histological group	Subtype	Cell line
NSCLC	Adenocarcinoma	H23
NSCLC	Adenocarcinoma	A549
NSCLC	Adenosquamous	H125
NSCLC	Bronchioalvelar carcinoma	H358
NSCLC	Papillary adenocarcinoma	H441
NSCLC	Adenocarcinoma	H1650
NSCLC	Adenocarcinoma	H2009
NSCLC	Adenocarcinoma	H2030
NSCLC	Adenocarcinoma	H2087
NSCLC	Adenosquamous	H2286
NSCLC	Adenocarcinoma	H738

Table 3-2. Small cell lung carcinoma cell lines profiled with the panel of lung cancer aptamers.

Histological group	Cell line
SCLC	H249
SCLC	H719
SCLC	H69
SCLC	H60
SCLC	H82
SCLC	H510
SCLC	H446
SCLC	H128
SCLC	H1672
SCLC	H128
SCLC	H620
SCLC	H446

Concluding Remarks

The validation of aptamers was carried out in this chapter by profiling the molecular surface of lung cancer cells in all classified groups. After analysis, it was determined that aptamers HCH3, S1 and EJ4 tend to recognize the majority of lung cancer cell lines. When individual groups were analyzed, other aptamers displayed a role in differentiation. Analysis of NSCLC-ADC cell lines showed aptamers S1, S6 and EJ4 as the major molecular probes in the analysis of the most abundant type of lung cancer. Similar results were encountered when the NSCLC-SQC subgroup was analyzed. Although only three cell lines were used for NSCLC-SQC analysis there seems to be no aptamer probe capable of differentiating ADC from SQC, a current challenge in lung cancer work. Large cell carcinoma proved to be the most contrastive subgroup from the NSCLC. No other aptamer than sgc8 seems to displayed affinity for this particular subtype. In contrast, this could be observed as an advantage, as any of the mentioned aptamers could be used to differentiate this subtype. Finally, for the SCLC group the previously HCH aptamer series seem to be specific for this lung cancer group. However, compared to its original H69 target cell line, stronger fluorescence intensity was observed when the HCH aptamers where profiled with other SCLC cell lines.

Material and Methods

Instrumentation and Reagents

Libraries and aptamers were synthesized using the 3400 DNA synthesizer (Applied Biosystems). All reagents for DNA synthesis were purchased from Glen Research. DNA sequences were purified by reversed phase HPLC (Varian Prostar using C18 column and acetonitrile/triethylammonium acetate as the mobile phase). The

binding assays for the selected aptamers against different lung cancer cell lines were performed by flow cytometric analysis using a FACScan cytometer (BD Immunocytometry Systems) and Accuri.

Cell Culture and Buffers

A total of twenty seven established cell lines were used in this project, corresponding to both histological groups NSCLC and SCLC. The cell lines were maintained in RPMI-1640 (ATCC) culture medium supplemented with 10% Fetal Bovine Serum (FBS heat-inactivated) and 1% penicillin-streptomycin. Cells were incubated at 37ºC under 5% CO_2 atmosphere.

Flow Cytometric Analysis

Flow cytometry was used to monitor the enrichment of ssDNA-bound sequences within the pools during the selection process, as well as to evaluate the binding affinity and specificity of the selected aptamers. The cultured cells were washed with WB before and after incubation with the FITC ssDNA pool or selected DNA sequences. Fluorescence intensity was determined on a FACScan cytometer (BD Immunocytometry).

Binding Assays

Different families of sequences retrieved from multiple sequence analyses were synthesized, biotin-labeled at the 3'-end, and tested for binding with the positive and negative cell line. In addition, the binding selectivity of each candidate was determined by incubating a 250nM aptamer solution with 4×10^5 target or counter-cells for 30 minutes at 4ºC. Cells were washed twice with WB and incubated with streptavidin PE beads for 20 minutes at 4ºC. After washing, the cells were suspended in 200µL WB.

The fluorescence was determined with a FACScan cytometer by counting $3x10^4$ events. A randomized 80-mer sequence was used as a control.

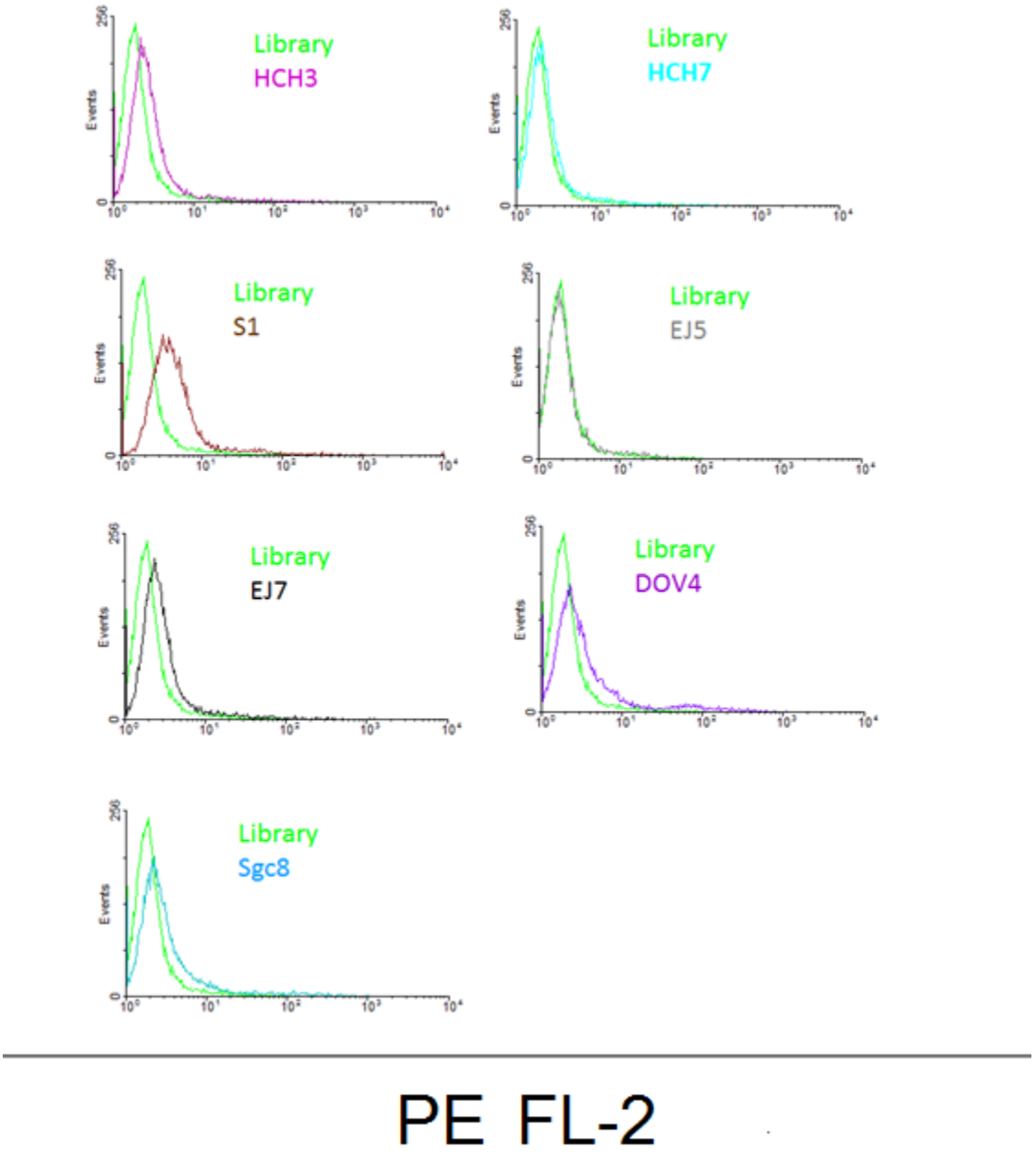


Figure 3-1.Binding assays of lung cancer aptamers with normal bronchial cell line HBE 135 E6E7. Aptamers HCH3, HCH7, EJD5, EJD7, S1, DOV4, EJ4 and Sgc8 were subjected to binding assay with normal cell line.

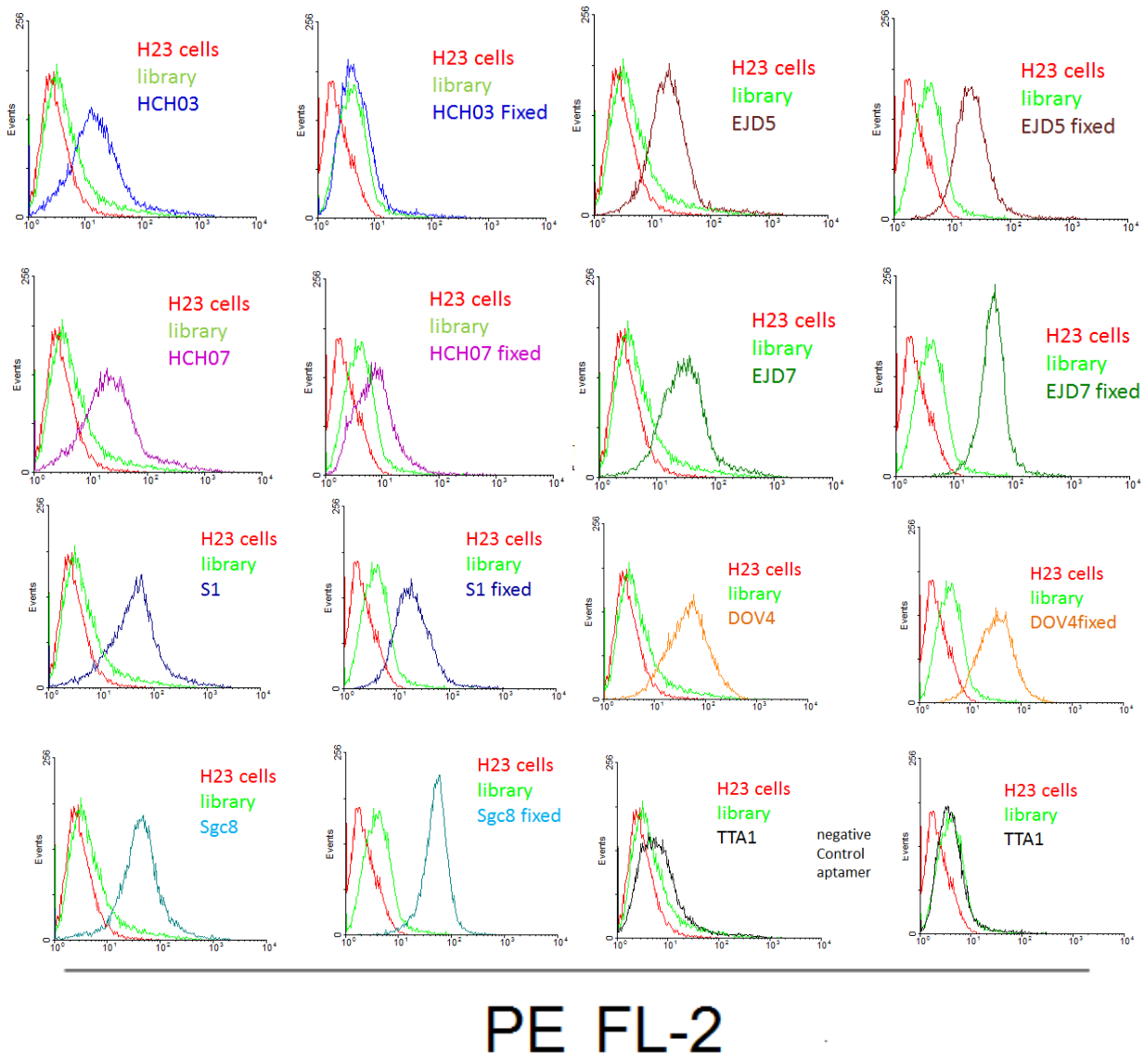


Figure 3-2.Binding of lung cancer aptamers after fixation with 4% paraformaldehyde. Aptamers HCH3, HCH7, EJD5, EJD7, S1, DOV4, and Sgc8 were subjected to binding after chemical fixing treatment. Aptamer TTA1 was used as negative control to show that fluorescence intensity does not increase due to fixation.

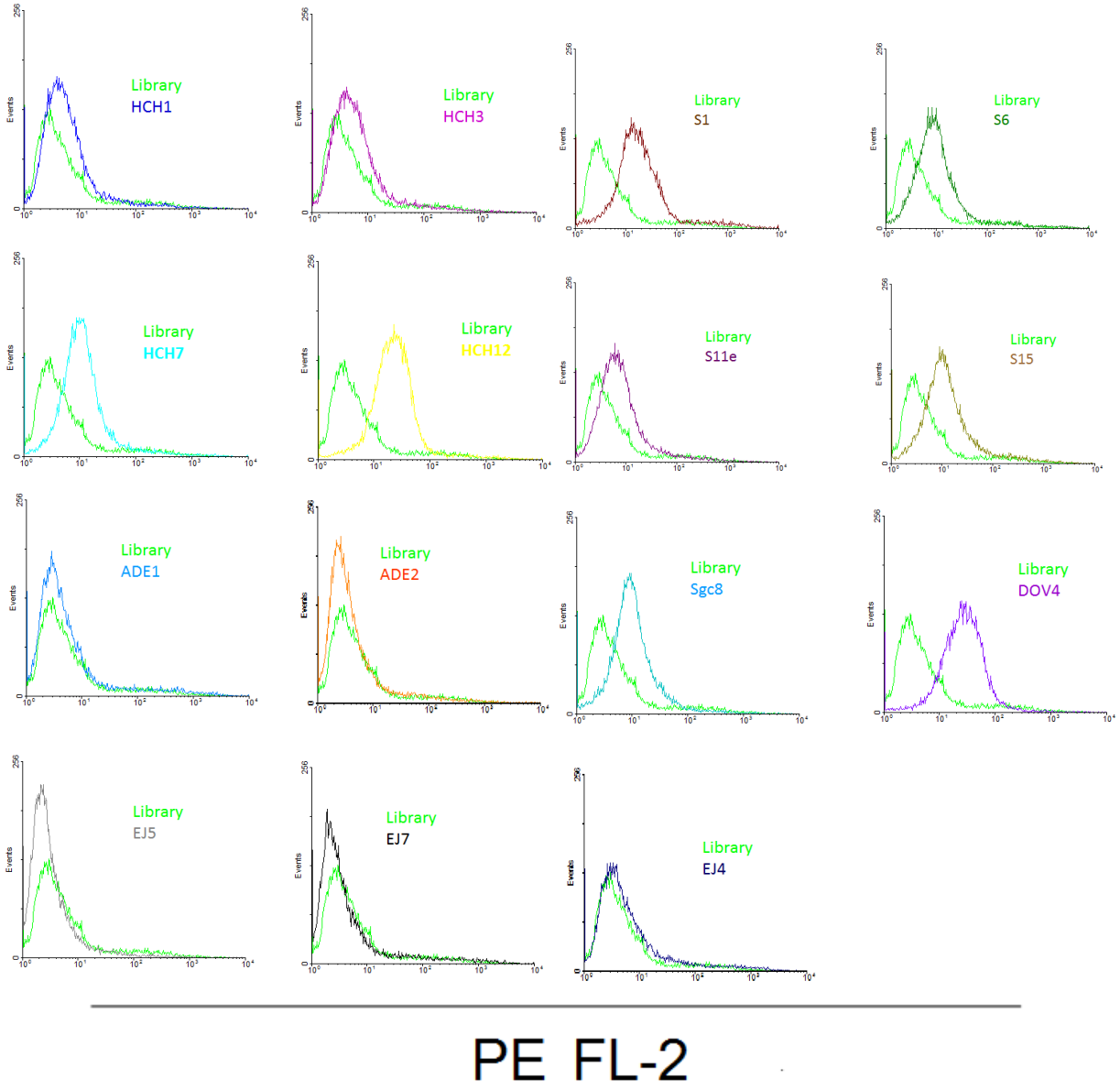


Figure 3-3. Profiling of cell line H2087 ADC with the lung cancer aptamer panel: HCH1, HCH3, HCH7, HCH12, S1, S6, S11e, S15, ADE1, ADE2, EJ5, EJ7, Sgc8, DOV4, and EJ4 were subjected to binding assays using flow cytometry.

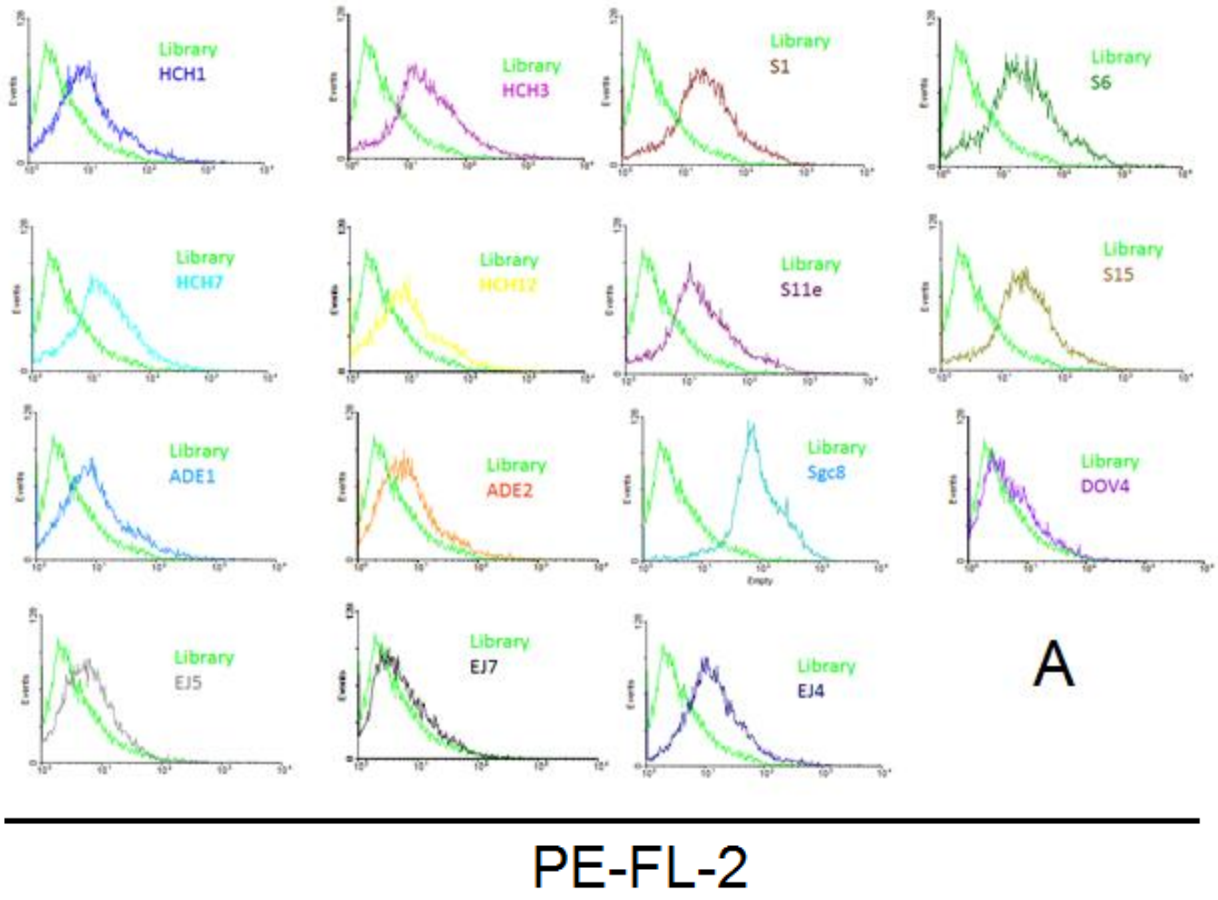


Figure 3-4. Profiling of SQC cell lines: A) H520, B) H226, and C) LUDLU-1. Aptamers: HCH1, HCH3, HCH7, HCH12, S1, S6, S11e, S15, ADE1, ADE2, EJ5, EJ7, Sgc8, DOV4, and EJ4 were subjected to binding assays using flow cytometry

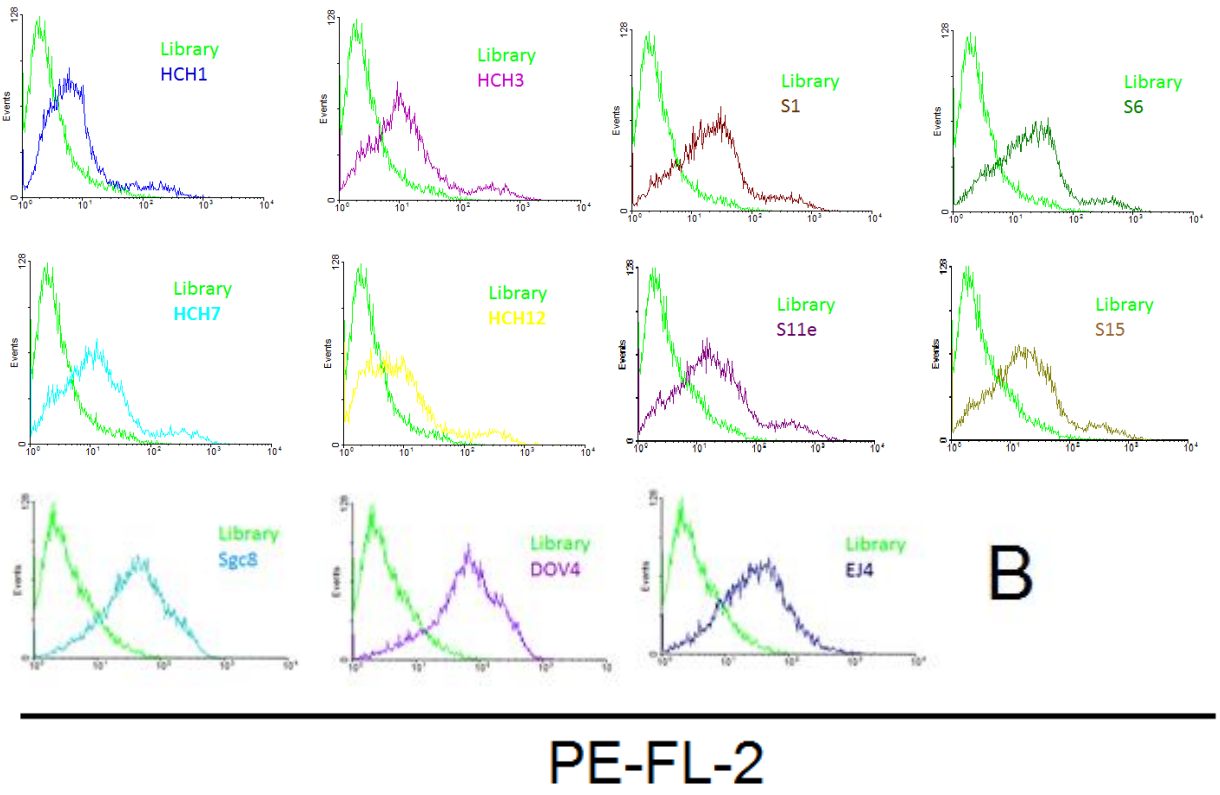


Figure 3-4. Profiling of SQC cell lines: A) H520, B) H226, and C) LUDLU-1. Continued.

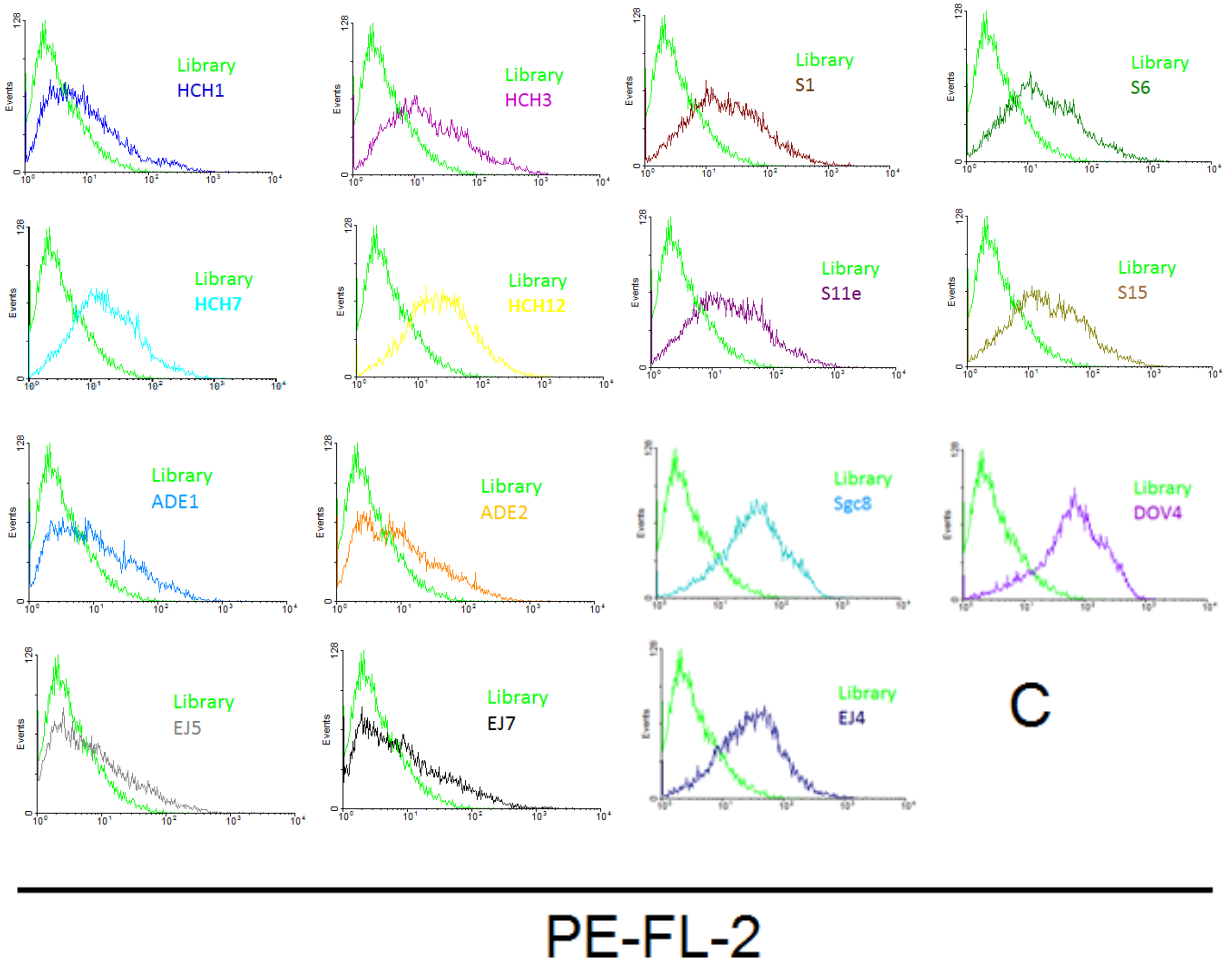


Figure 3-4. Profiling of SQC cell lines: A) H520, B) H226, and C) LUDLU-1. Continued.

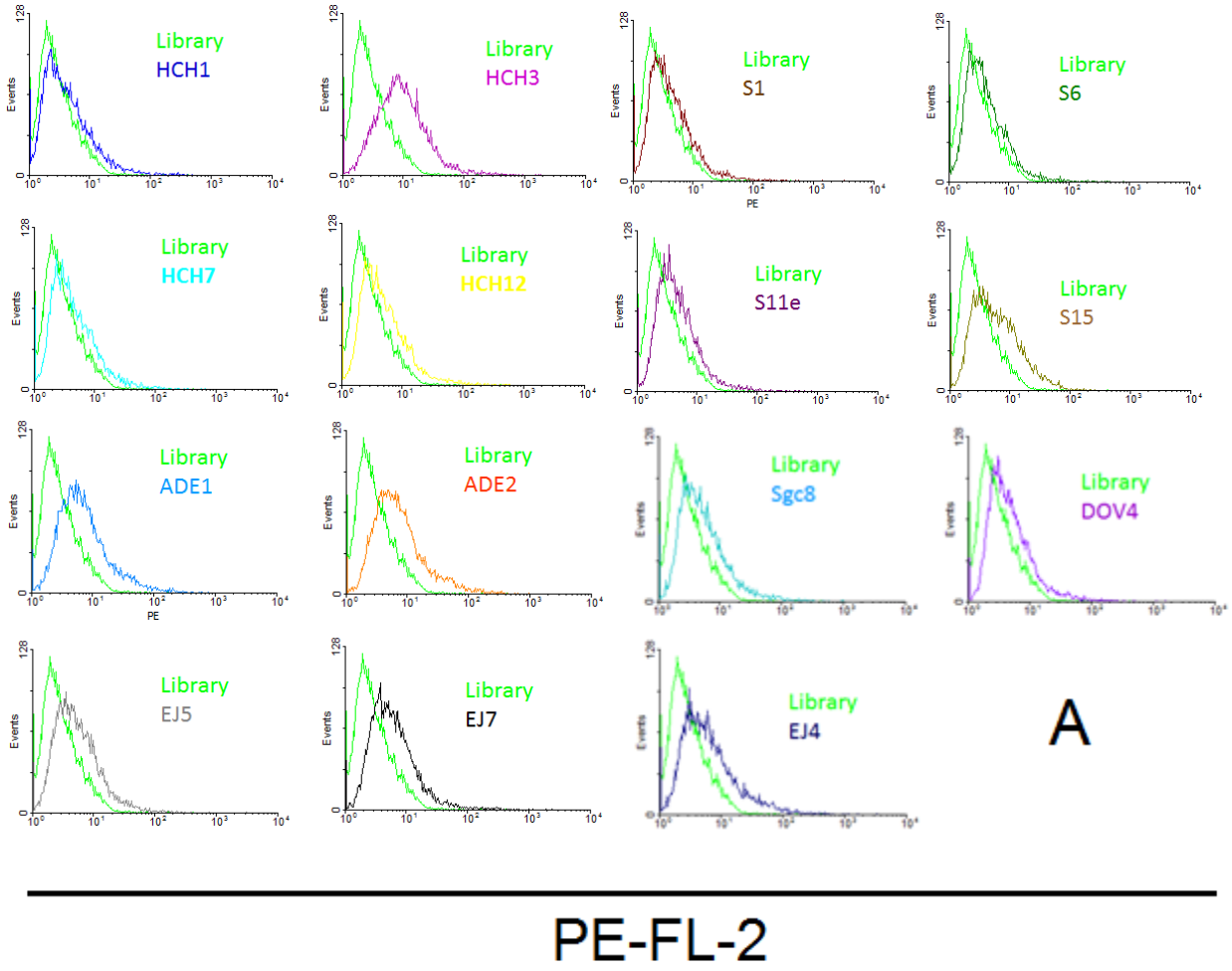


Figure 3-5. Profiling of LCC cell lines: A) H460, B) H661. Aptamers: HCH1, HCH3, HCH7, HCH12, S1, S6, S11e, S15, ADE1, ADE2, EJ5, EJ7, Sgc8, DOV4, and EJ4 were subjected to binding assays using flow cytometry

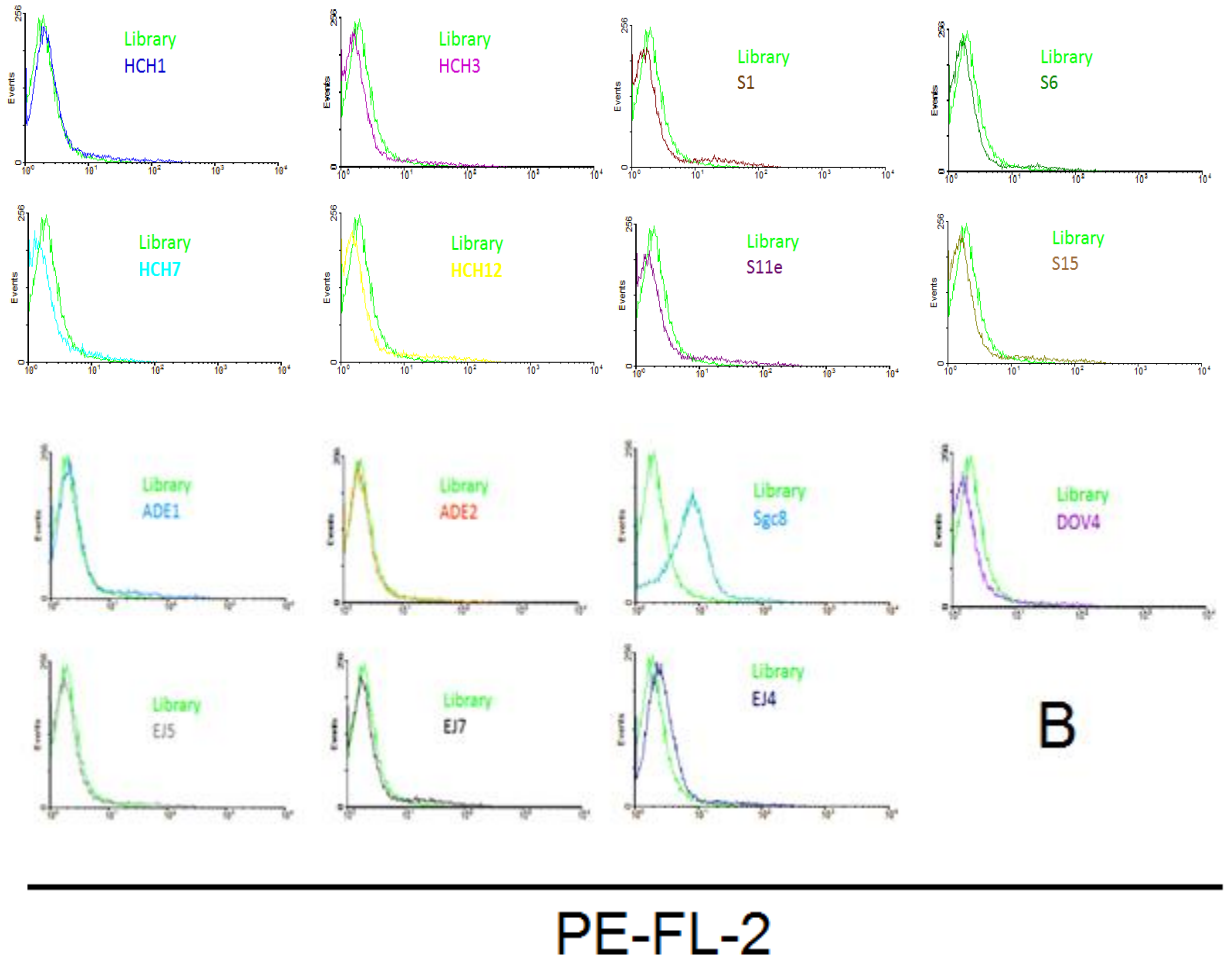


Figure 3-5. Profiling of LCC cell lines: A) H460, B) H661. Continued

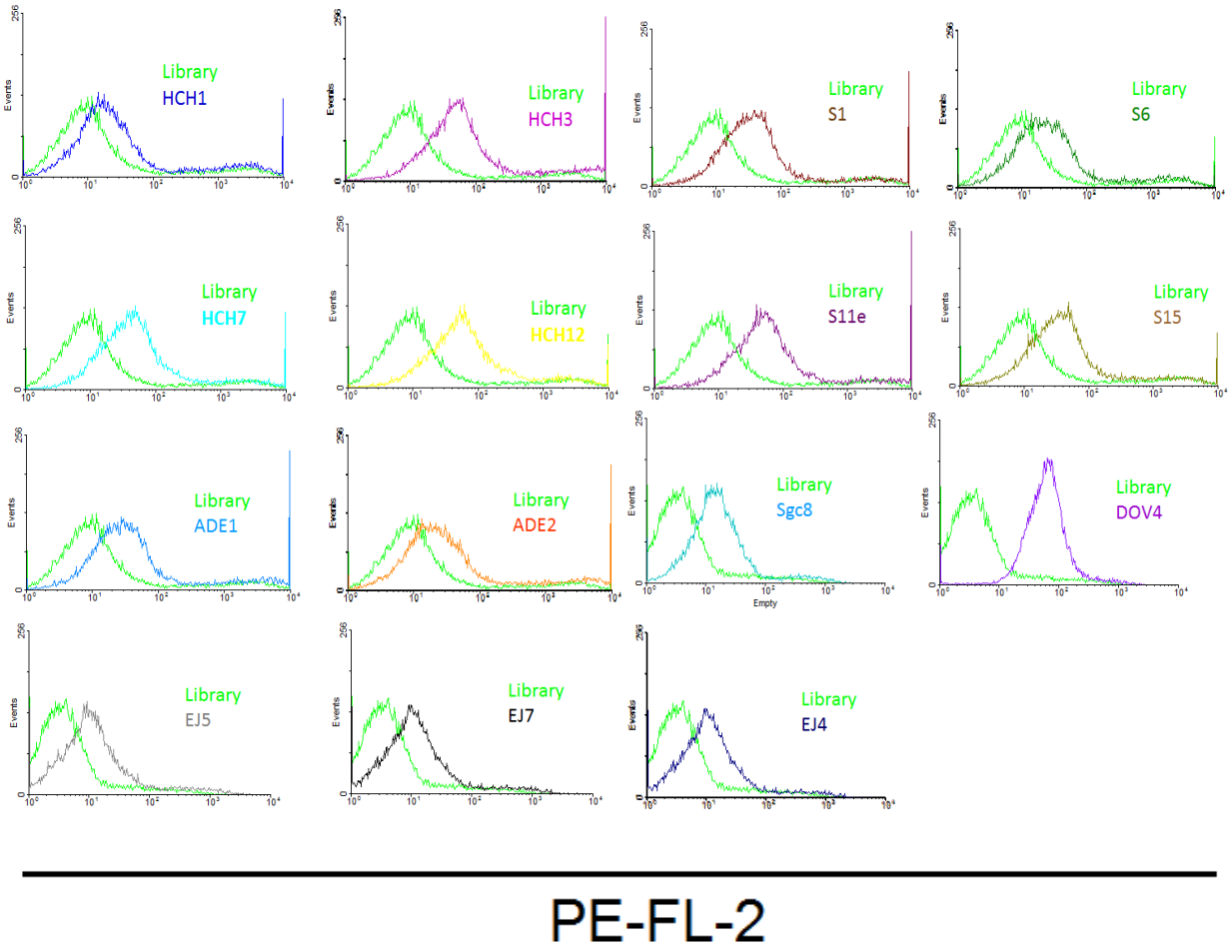
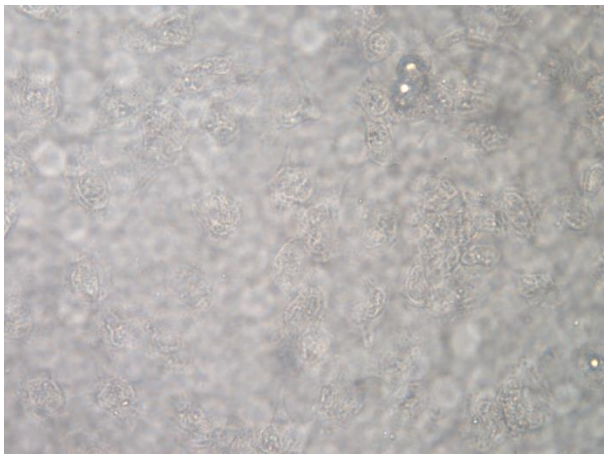
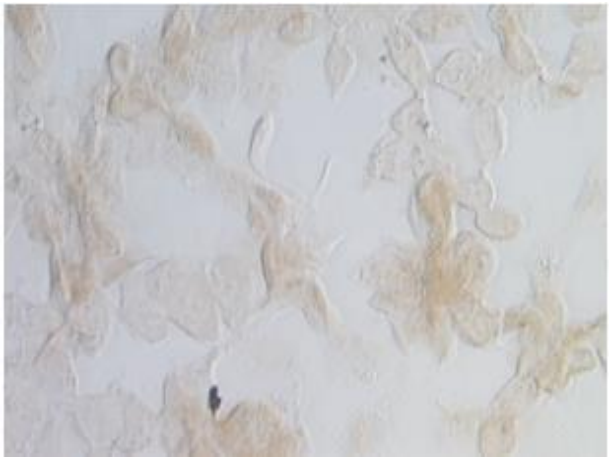


Figure 3-6. Profiling of cell line H2679 SCLC with the lung cancer aptamer panel: HCH1, HCH3, HCH7, HCH12, S1, S6, S11e, S15, ADE1, ADE2, EJ5, EJ7, Sgc8, DOV4, and EJ4 were subjected to binding assays using flow cytometry



Library

EJ4



DOV4

Figure 3-7. Immunohistochemistry of formalin-fixed artificial tissues. Aptamers EJ4, DOV4, and Sgc8 were used as recognition molecules in artificial tissues produced with NSCLC-ADC H23 cell lines.

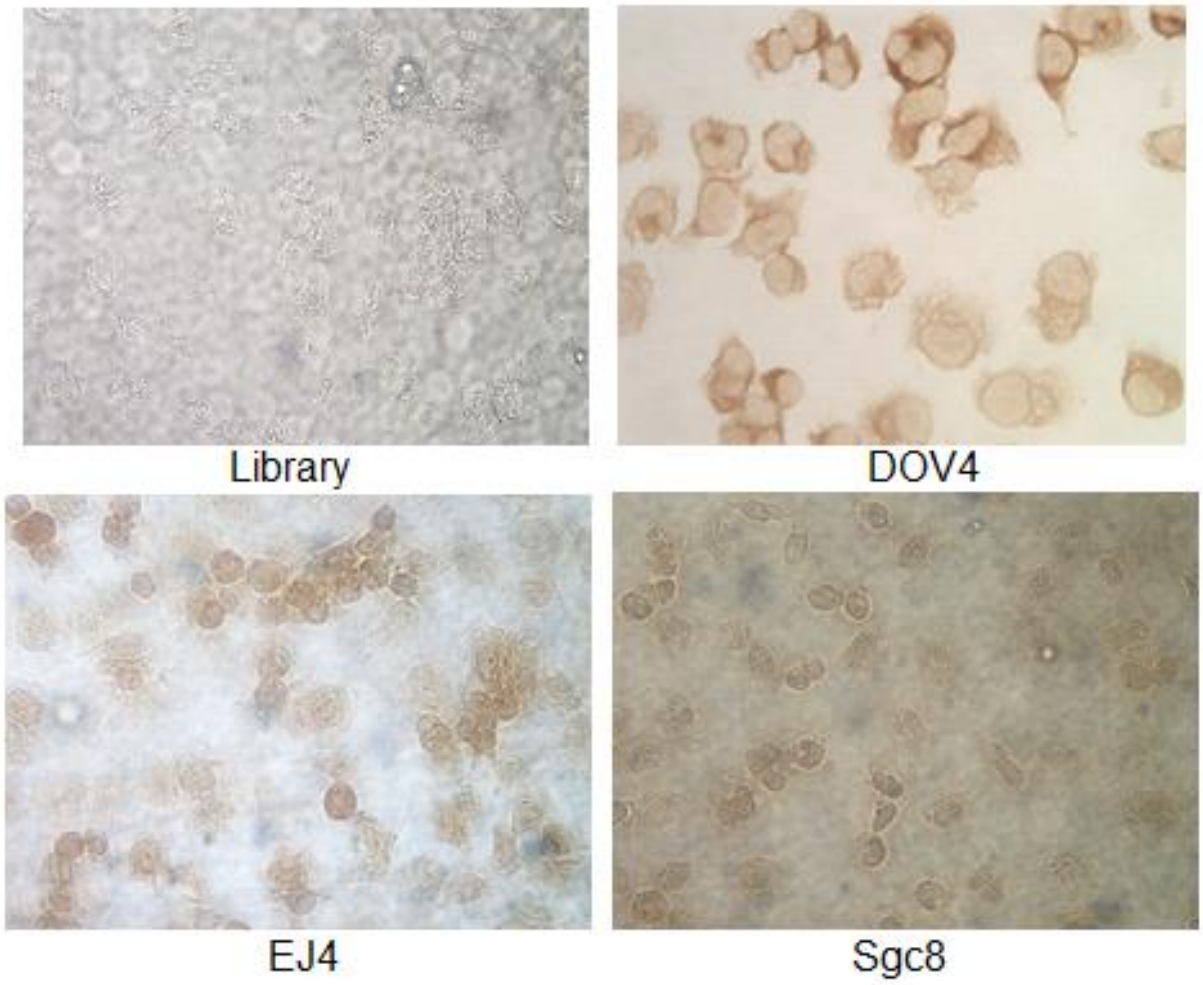


Figure 3-8. Immunohistochemistry of FFPE artificial tissues. Aptamers DOV4, EJ4, and Sgc8were used as recognition molecules in artificial tissues produced with SCLC NSCLC-ADC H23 cell lines.

CHAPTER 4
DETECTION OF CTCs USING APTAMERS IN NSCLC and SCLC

Introduction

During carcinogenesis, normal cells suffer mutations that cause them to grow indefinitely and to invade surrounding tissues. As the tumor develops, cells disseminate from the tumor can enter different biological fluids (sputum, urine, blood, etc.) through different mechanisms at the early stage of tumor development.[124] These circulating tumor cells CTCs, have significant impact on the early detection of tumor. However, due to the trace amounts of them in the biological fluids, CTCs are very difficult to target Several studies have confirmed the present of tumor cells in the peripheral blood of many cancer patients.[125,126,127] Therefore, the isolation of these cells has been proposed in order to gain more insight into the biology of cancer pathogenesis, as well as the different morphological changes during carcinogenesis. Unfortunately, due to the lack of specific markers, the identification and study of these cells have posed a great challenge.

In the blood there are millions of blood-derived cells, especially red blood cells and lymphocytes, which are the most abundant. In the early 2000's several groups started to design different strategies to capture these rare cells. Microfluidic channels,[128] magsweeper designs,[129] and magnetic separations,[130] combined with specific antibodies demonstrated that CTCs can be captured in this complex mixture. Detection of CTCs relies on features that distinguish them from blood-derive cells. For example, CTCs are generally larger in size when compared to RBC and WBC. CTCs as disseminated cells present features close to normal cells with membrane properties unlike blood cells. Finally, the molecular differences are perhaps the most exploited

features in research. Different markers such cytokeratins 19[131] and 20[132] present only in the cytoplasm of epithelial derived cells, but not in blood cells. The most widely used is Epithelial Cell Adhesion Molecule (EpCAM),[133] and this glycoprotein represents perhaps the Holy Grain of CTC detection. Several studies had been developed using this molecule as the differencing factor with blood-related cells. Despite many studies on this topic[134,135, 136] the only technology for the detection of CTC approved by the Food and Drug Administration (FDA) is CellSearch[137] for the detection of metastatic breast, colon, and colorectal cancer.

This chapter aims to generate an efficient and sensitive method for the detection of circulating tumor cells in peripheral blood samples of lung cancer patients using aptamer-magnetic nanoparticle (MNP) conjugates to detect and collect exfoliated tumor cells from the bloodstream. For CTC detection in SCLC aptamers in combination with flow cytometry will help us to detect and enumerate this population.

Results and Discussion

As previously mentioned, EpcAM is the molecule primarily used to differentiate epithelial cells from blood-cells. Therefore, the first step in our project was to profile different NSCLC and SCLC cell lines with EpCAM Antibody (Ab).

Expression of EpCAM in NSCLC Cell Lines

A total of seven cell lines were tested with EpCAM Ab. In this pool of cell lines, the four histological subgroups were included in order to provide a more general profiling within this subgroup. As shown in [Figure 4-1] EpCAM glycoprotein is not widely express in the surface membrane of NSCLC cell lines. These results were surprising, as this protein is a well-known epithelial marker and as mentioned previously, ~ 95% of lung cancers have epithelial origin. Therefore, the question that

rose was, “Is EpCAM a good molecular marker for the detection of CTCs in lung cancer?”

However, it was noticed that the histological subgroup ADC shows a more consistent expression pattern as a subtype when compared to NSCLC or lung cancer as a whole. Therefore, ADC was chosen as a model for the detection of CTC’s and compared to literature in which resected ADC tissues were tested for EpCAM.[138]

Aptamers Binding with Blood-Derived Cells

Lacking knowledge of the lung cancer aptamers’ targets, to apply our proposed methodology for the detection of CTCs in both SCLC and NSCLC, aptamers were tested with both RBCs and PBMCs. As for the Abs used in the methodology, it is well known that EpCAM is not expressed on the membrane surface of RBCs or PBMCs. However, CD56 is express on Natural Killer (NK) cells, a small percentage subpopulation of PBMCs.[139] The histograms corresponding to the binding studies of aptamers and blood-derived cells RBCs and PBMCs are shown in [Figure 4-4].

After corroboration of these results, it can be concluded that the aptamers’ targets are not present in blood derived cells. Up to this point the presented data was necessary for both methodologies in order to determine the correct cell lines, Abs and screening of cells present in blood.

H1650 or H358 as Model Cell Line for CTCs Detection in NSCLC

As this project involves the application of previously selected aptamers for lung cancer as the recognition and detection-reporting molecules, ADC and SCLC cells were profiled with previously selected aptamers as shown in Chapter 3 of this dissertation. In simple words, the cell line selected for NSCLC should be positive for both markers (EpCAM) and lung cancer aptamers. [Figure 4-3] represents the proposed methodology

for the magnetic detection of CTCs using the H1650 cell line as the lung cancer model.

Methodology for the detection of CTC in NSCLC

Indirect and direct methodologies were used. In the direct method, the biotinylated aptamers are conjugated directly to MNP prior to incubation with the buffy coat containing spiked H1650 cells. For the indirect method, the biotinylated aptamers are incubated with the BC-containing spiked H1650 cells, followed by the addition of the MNPs. In other words, in the direct method the streptavidin-biotin chemistry occurs outside and prior to contact with the buffy coat. In the indirect method, the chemistry streptavidin-biotin chemistry occurs in situ in the BC. When both methodologies were carried out to detect CTCs in blood, different outcomes were observed [Figure 4-4].

The indirect method captured approximately 30% more cells than the direct method. This behavior can be attributed to the flexible accessibility of aptamers to bind with CTCs when incubated prior to the addition of MNPs. Two situations can affect the effective detection of CTC during the direct methodology: a steric effects and aggregation can occur between MNPs in solution, reducing the number of aptamer-target binding events, thus reducing capturing efficiency. After the observation of these preliminary results, the direct methodology was employed in subsequent experiments.

Microscopy Images of Captured Spiked Cells

As previously described in the methodology scheme, pre-labeled EpCAM H1650 cells were spiked in different ratios when compared to the number of background cells present in BC. Spiked cells were isolated using an aptamer as a capturing molecule. [Figure 4-5 A & B] shows commercially available MNPs and EpCAM labeled ADC H1650 cells prior spiking into BC [Figure 4-5C] shows H1650 cells captured by aptamers. These spiked cells are completely surrounded by complex MNPs that were

non-covalently linked to aptamers via streptavidin-biotin chemistry at 4°C. This chemistry is one of the most exploited and applied in biomedical applications due to its flexibility in binding conditions, its high specificity and its non-covalent bond, which is the strongest known.[140] Also, aptamers and antibodies can be biotinylated performed to without compromising their binding ability. Because the spiked H1650 cell-aptamer-MNP are bound covalently, strong conditions are necessary to break that bond. Such conditions are harsh to cells and therefore could not be performed. Instead DNAse I treatment was able to cleave the aptamer (DNA) from the H1650 cells, thus releasing the cells without damage. This step was important, as the captured cells needed to be confirmed by confocal microscopy for EpCAM expression in order to exclude them from PBMCs.

Capturing Efficiency of Aptamer-MNP Complex

Further experiments were carried out using spiked cells in concentrations close to those reported by cell search for metastatic patients (between 1 and 500 spiked cells in 7.5mL of blood). The direct methodology was applied, and captured cells were excluded from PBMCs by EpCAM labeling using fluorescence microscopy.

[Figure 4-7] shows the capturing efficiency when different numbers of CTCs were spiked into BC. As the number of spiked cells was decreased in comparison to the number of background cells (PBMCs) the efficiency of our methodology decreased from 90% when 1 CTC was spiked in 200 PBMCs to 30% when 1 CTC was spiked into 20,000 PBMCs. These results are in concordance with similar approaches reported in the literature.[126-127] As expected, as the number of target cells decreases when

compared to background, the target efficiency is reduced, and the number of non-specific binding events decreases.

Concluding Remarks

Based on the results presented, aptamers previously selected using cell-SELEX technologies can be translated into biomedical application such detection of exfoliated cells in blood. Currently, the only FDA approved methodology is CellSearch for metastatic breast, prostate and colorectal cancer. Lung cancer is still understudied in this field, and according to the results presented in this dissertation, we hypothesize that this lack is due to the inconsistent expression of EpCAM in lung cancer in contrast to other cancers. We have overcome this problem by using aptamers as the capturing molecule, instead of EpCAM as reported in literature. EpCAM was used as a positive control marker to corroborate the identity of the captured cells.

Detection of CTCs in SCLC

EpCAM Expression in SCLC Cell Lines

Similarly to NSCLC, SCLC cell lines were profiled for EpCAM [Figure 4-8] Again its expression level was non-ubiquitous within this group. These results were also confirmed in the literature, which showed the expression was variable in neuroendocrine lung-tumors.[141] Based on these results, the search for a more universal marker within the neuroendocrine SCLC group began.

CD56 Expression in SCLC Cell Lines

After a literature search, Neural Cell Adhesion molecule (NCAM) seemed to be a good biomarker candidate.[142] To validate this, a pool of SCLC cell lines was subjected to binding studies with NCAM Ab, also known as CD56 Ab. In contrast, to the results for EpCAM, the expression was found in 95% of the cell lines tested [Figure 4-9].

H1836 as Model Cell Line for CTC Detection in SCLC

As this project involves the application of previously selected aptamers for lung cancer as the recognition and detection reporting molecule, SCLC cells lines were profiled with previously selected aptamers as shown in chapter 3. The cell line selected for SCLC needed to be positive for both markers (CD56) and lung cancer aptamers. H1836 was selected as the lung cancer model. As shown in [Figure 4-10], the chosen cell line has binding affinity with most of the aptamers against lung cancer selected in our laboratory.

Once the SCLC cell line was chosen, the methodology pictured below was carried out using H1836 cells.

Optimization of Conditions for CTC Detection

To start with the proposed methodology, a standardization procedure was carried out using a large quantity of cells. Two different spiking system were used, one before the analysis on the flow cytometer machine, and one at the very beginning of the blood preparation methodology. Two different results were observed [Figure 4.12]. The CTC pattern is clearer when cells are labeled with both Aptamer and Ab separately than when labeling occurs *in situ*, revealing high background in the FL-3 channel (aptamer labeling). In this particular case, since the CD56 Ab is FITC labeled, we chose PE.Cy5.5 dye to conjugate with the biotinylated aptamers in order to avoid compensation during analysis.

Background Optimization

To help determine the cause of high background, other dyes were used at concentrations similar to those used with PE.CY5.5. All flow cytometry results displayed

some background but not as high as the results for samples using PE.CY5.5. Allophycocyanin (APC) dye was chosen [Figure 4-13] as it is observed on channel 4 and therefore there is no signal overlap between channels and no compensation is necessary. In independent experiments carried out in the lab by other members, the same observation was noticed, suggesting that the protein albumin, which is highly present in blood, has affinity for PE.CY5.5.

After the best fluorophore was determined, the trial experiments were carried out to determine if the aptamers or the antibodies used for this methodology were feasible by subjecting them to binding assays with extracted BC. Results can be seen in [Figure 4-14] in which the percentage of background in the FITC channel and the APC channel for the aptamers and antibodies was less than 1% except for aptamers HCH3, HCH7 and DOV4 with background of approximately 4%. The later value is still considered small when compared to the entire population within the buffy coat. Thus, after these results it was determined that the level of background was acceptable for further experiments.

Detection of CTCs Using High Count Number

After most of the parameters were optimized, the first goal was to determine whether spiked cancer cells would be seen as a different population from blood-derived cells by flow cytometry. As shown in [Figure 4-15], the population of spiked H1836 can be clearly seen and differentiate from blood-derived cells in the BC. These spiked cells showed high percentage of labeling with CD56Ab. In contrast, the aptamer-cocktail labeling is not as high as with Ab. In other words the aptamer labeling is not 100% efficient. Based on these results, it was hypothesized that due to the high content

number of PBMCs in the buffy coat prevented the aptamer solution from reaching saturation during binding assays. To solve this issue, the concentration of aptamer in solution was increased from 250nM to 500nM. (It is important to note, that during regular binding assays for H1836 cells with lung cancer aptamers, the labeling efficiency was never 100%).

Detection of CTCs Using a Representative Number of Cells

As previously mentioned, SCLC is a more aggressive disease when compared to the NSCLC subgroup. Therefore a high tumor burden is expected in patients with metastatic SCLC. Based on this premise, further experiments were carried out containing 500 and 250 spiked cells in whole blood before treatment. [Figure 4-16] shows the different populations and gates that were set when trying to detect a small number of spiked cells. Figure 4-16 A and Figure 4-16B show the behavior of BC with no labeling molecules, and buffy coat labeled with CD56 Ab. As mentioned previously, some NK cells are present in PBMCs and are positive for CD56. Therefore, a small subset population is expected to shift towards the FITC channel. In that particular dot plot a threshold was set to determine intrinsic fluorescence signal expected when cells are incubated with NCAM Ab. Figures 4-17 C and D show BC containing 500 spiked H1836 cells ((CTCs) when conjugated to the respective control for Ab(C) or aptamer-cocktail (D). In figure 4-16 Isotype IGg1 and random DNA are labeled as “library”).

Spiked cells (shown in blue) did not produce a signal in either of the two channels (FITC or APC). In contrast, when the same set of cells was labeled with CD56 and aptamer cocktail, the spiked population shifted completely to the APC channel, and partially to the FITC channel. From these results we confirmed that the increase in

aptamer concentration from 250nM to 500nM solved the problem of labeling with aptamer, suggesting an optimization for the concentration of aptamers in future experiments. During Ab optimization, another issue was encountered. Several repeats showed high background of cells in APC channel population circled and arrowed [Figure 4-17].

Contribution of Dead Cells to Aptamer Background

When reviewing the methodology used and all the steps during the spiking experiments, it was noted that the background problem was not observed during optimization, and the differences in obtaining the working sample (BC) for those two set of experiments were considered. For the starting optimization, BC was obtained using freshly isolated blood from healthy donors. As for the spiking experiments, one the objectives was to mimic as much as possible the samples to be acquired from the Clinical and Translational Science Institute (CTSI) repository at the University of Florida. The CTSI blood samples were immediately processed and stored at -80°C until required for analysis. Therefore, it was hypothesize that the dead population could be cells that do not survive during the freezing and/or thawing process.

These observations were addressed when using high number of spiked cells. The population can be distinguished, but it could be due to the numbers of spiked cells. However, it was uncertain whether the background would have an effect on determined the desired population when a small number of cells were to be spiked. One quick way compatible with our methodology to prove the hypothesis was to include in the labeling with 7-Aminoactinomycin D (7-AAD) fluorescence molecule before flow cytometry analysis. [Figure 4-18] shows how 7-AAD molecule labeled some population confirming that dead cells were responsible for the background observed in previous figure.

Detection of CTC in Blood Samples

After troubleshooting with dead cells, the spiking experiments using sample mimicking of metastatic patient samples were begun. The dot plot using a high number of spiked cells served as positive control for the following experiments. [Figure 4-19] panel A & B shows no background for Ab and aptamer cocktail, suggesting that, changes in the methodology alleviate the flaws observed earlier in this chapter. Panel C shows BC with 200,000 spiked cells labeled with both Ab and aptamer and completely differentiate and isolated in gate P4 in figure 4-19.

In panel D the same reading was performed, but random DNA was used as a negative control. No cells were spotted on gate P4, confirming that the shift in fluorescence intensity for both channels FITC and APC was due to the presence of the particular targets in the membranes of spiked cancer cells, but not in the BC cells.

Limit of Detection

Further experiments were carried out to determine the limit of detection using FACS. A series of dilutions with spiked cells was analyzed. A set containing 5 samples (250, 125, 64, and 32 spiked cells in buffy coat) was tested in triplicate and lowest detected values were reported. [Figure 4-20] presents the histograms the analysis corresponding to each one of the groups tested..

As observed in [Figure 4-20] CTCs were able to be detected in each of the dilution series tested. However, the percentage of detection was not consistent throughout the analysis of the set. In panel A, when 250 cells were spiked a total of 230 cells were collected by the sorter, corresponding to 92% of the total spiked population. Panel B represents the analysis of 125 spiked cells; a total of 73 cells were recovered

corresponding to 58% of the total spiked population. For panels C & D an equal amount of cells was detected independent of the number of cells spiked; 13 cells were detected when either 64 or 32 cells were spiked in BC corresponding to 20% and 40%, respectively. These results showed ambiguities and no direct correlation between the number of spiked cells and the number of cells retrieved. Taken together, the presented results suggest that the LOD for CTCs in BC using FASC is approximately 60 cells.

To date, there is only one recent report of using aptamers for the detection of CTCs. In this work, Shen et al coated a microfluidic device through which the spiked blood traveled[143]. In our work, it was imperative to show the potential of using aptamers to detect a few spiked cells mixed with millions of PBMCs. Our results further reinforce the importance of the high specificity of aptamers. Perhaps, while numerous reports continued are published, the need to find a biomarker common to these CTCs continued to be essential to further improve cancer treatment and prognosis[144].

Detection of CTCs in Metastatic SCLC Patients

One year from the approval of IRB 634-2011 the CTSI at the University of Florida consent and start collecting blood from metastatic patients. As reminder, SCLC represents approximately 15% of all lung cancer cases. A total of 10 samples were acquired at the time of analysis. Five samples corresponded to metastatic patient samples prior chemotherapy treatment. The other five were obtained after chemotherapy treatment. After optimization and troubleshooting of the proposed methodology, it was decided the only the set of samples corresponding to the group before chemotherapy will be analyzed. Depending of the results accomplished with this group, the next set would be analyzed. [Figures 4-21 to 4-25] show the results.

As it can be observed in the analysis of the patient samples, some of the provided BC separate by the CTSI show a high number of dead cells making the application of the proposed methodology challenging.

Overall, in the five patient samples analyzed our technology was able to detect and enumerated at least 1CTCs in 7.5mL of blood from metastatic patient samples. It was expected to detect similar number of CTCs as all the patient samples corresponded to metastatic stage prior chemotherapy. In contrast, the number of CTCs detected correlated with the quality of the sample; in poor-quality samples the number of CTCs cells detected was very low compared to the samples with good-quality. Further analysis is required on those cells detected in Q1 or Q4 as the efficiency of the labeling could not have been 100% efficient, corroboration with cytokeratins and DAPI can be used to determine if any CTCs was missed during the enumeration process.

Concluding Remarks

These results showed that aptamers are molecules capable of performing tasks in the biomedical field. They enabled the specific isolation of spiked CTCs cells in blood with minimum treatment, indicating the potential of using oligonucleotides in an automated methodology that could become part of a routine cancer follow-up in the progression cancer therapies. The limit of detection was as low as 60 cells in 7.5 mL of blood. Furthermore, this principle could be applied to a less complex sample, such a liquid biopsy or sputum in which the number of background cells is decreased.

Material and Methods

Instrumentation and Reagents

Libraries and aptamers were synthesized using the 3400 DNA synthesizer (Applied Biosystems). All reagents for DNA synthesis were purchased from Glen Research. DNA sequences were purified by reversed phase HPLC (Varian Prostar using C18 column and acetonitrile/triethylammonium acetate as the mobile phase). The binding assays for the selected aptamers against different lung cancer cell lines were performed by flow cytometric analysis using a FACScan cytometer and Accuri (BD Immunocytometry Systems).

Cell Culture and Buffers

A total of two established cell lines was used in this project, H1650 and 1836, which were kindly provided by Dr. Frederick Kaye at the University of Florida. Cell lines were maintained in RPMI-1640 (ATCC) culture medium supplemented with 10% Fetal Bovine Serum (FBS heat-inactivated) and 1% penicillin-streptomycin. Cells were incubated at 37ºC under 5% CO_2 atmosphere. All previous reagents were purchased from Sigma-Aldrich. To detach cells from the culture plate, non-enzymatic cell dissociation buffer was used, and the washing steps with (WB) containing 4.5 g/L glucose and 5 mM MgCl2 in Dulbecco's phosphate buffered saline with CaCl2 and MgCl2 (PBS - Sigma). Binding buffer (BB) used for aptamer binding was prepared by adding yeast tRNA (0.1 mg/mL, Sigma) and BSA (1 mg/mL, Fisher) to the washing buffer to reduce non-specific binding. All other reagents were purchased from sigma-aldrich.

Flow Cytometric Analysis

Flow cytometry was used to monitor the enrichment of ssDNA-bound sequences within the pools during the selection process, as well as to evaluate the binding affinity and specificity of the selected aptamers. The cultured cells were washed with WB before and after incubation with the FITC ssDNA pool or selected DNA sequences. Fluorescence intensity was determined on a FACScan or Accuri (BD Immunocytometry) cytometer.

Binding Assays with Antibodies

EpCAM and CD56 FITC antibodies were purchased from BD Biosciences and tested for binding with the target cells H1650 and H1836. In addition, the binding selectivity of each Ab was determined by incubating 20µL of Ab solution with 4×10^5 target cells for 60 minutes at 4ºC. Cells were washed twice with PBS and suspended in 200µL PBS. The fluorescence was determined with a FACScan or accuri cytometer by counting $3x10^4$ events. Isotype IgG1 FITC was used as a control.

Binding Assays with Aptamers

Different families of sequences retrieved from multiple sequence analyses were synthesized, biotin-labeled at the 3'-end, and tested for binding with the target cells H1650 and H1836. In addition, the binding selectivity of each aptamer was determined by incubating a 250nM aptamer solution with 4×10^5 target or counter-cells for 30 minutes at 4ºC. Cells were washed twice with WB and incubated with streptavidin PE beads for 20 minutes at 4ºC. After washing, the cells were suspended in 200µL WB. The fluorescence was determined with a FACScan cytometer by counting $3x10^4$ events. A randomized 80-mer sequence was used as a control.

Preparation of Freshly BC from Whole Blood

Blood from healthy donors was purchased from Life South Gainesville Florida. Upon arrival, fresh blood was subjected to differential centrifugation using Histopaque1077 (sigma). A 5mL aliquot of solution histopaque was placed into a 15mL conical centrifuge, 7mL of freshly acquired blood was place onto the histopaque layer and centrifuged at 400g for 30 minutes. After centrifugation three different layers were observed: top layer containing plasma and blood-proteins, middle layer containing buffy coat (PBMC's) and bottom layer RBCs. The plasma layer was carefully removed, and the BC layer was placed on a 15mL centrifuge tube and 3mL of PBS was added to wash the extracted layer and the tube was centrifuged at 250g for 10 minutes. Supernatant was aspirated and the pellet was suspended in PBS buffer.

Spiked H1836 Cells in Blood

The same procedure was followed when different number of cells were spiked in blood before treatment. Cells were harvested and counted before each experiment. A specific number of cells was added to blood. Blood was transferred to a 15mL conical centrifuge tube and 7mL of RBCs cells, which had previously being lysed using red blood lysis buffer (Sigma) was added and incubated on ice for 10min. Then the mixture was centrifuged at 4,000rpm for 10min. The supernatant containing lysed RBCs was removed. The RBC removal process was repeated twice to ensure that the minimum number of RBCs was present in the BC. In the final step BC was suspended in PBS for future use.

Labeling of CTCs for Magnetic Separation

After the BC containing spiked cells were isolated (above procedure) they were subjected to with labeling with Ab and aptamer cocktail. BC was washed twice with WB

and incubated with either Isotype IGg1 or Ab EpCAM for 1 hour at 4°C. After incubation, cells were washed twice and incubated with either random DNA (library) or aptamer cocktail for 30min at 4°C. After the labeling steps, Streptavidin-coated magnetic beads were incubated with BC containing spiked cells for 1 hour at 4°C. Then the mixed sample was placed on a magnetic stand for 10 min and washed three times to ensure specific capture. Captured CTCs were treated with DNAse I for 30 min at 37°C. Released cells were examined using a fluorescence microscopy to corroborate EpCAM labeling.

Labeling of CTCs for its Detection Using FACS Sorter

After BC-containing spiked cells, were isolated (above procedure) they were subjected to labeling with Ab and aptamer cocktail. BC was washed twice with WB and incubated with either Isotype IGg1 or Ab CD56 for 1 hour at 4°C. After incubation, cells were washed twice and incubated with either random DNA (library) or aptamer cocktail for 30min at 4°C. Similarly, labeled BC was washed twice with WB and incubated with streptavidin-conjugated APC for 20 min at 4°C. After the labeling steps, cells were analyzed using a FACS and sorter based on dual-labeling. As a final step, 2µL of 7AAD was incubated to each sample before flow cytometry.

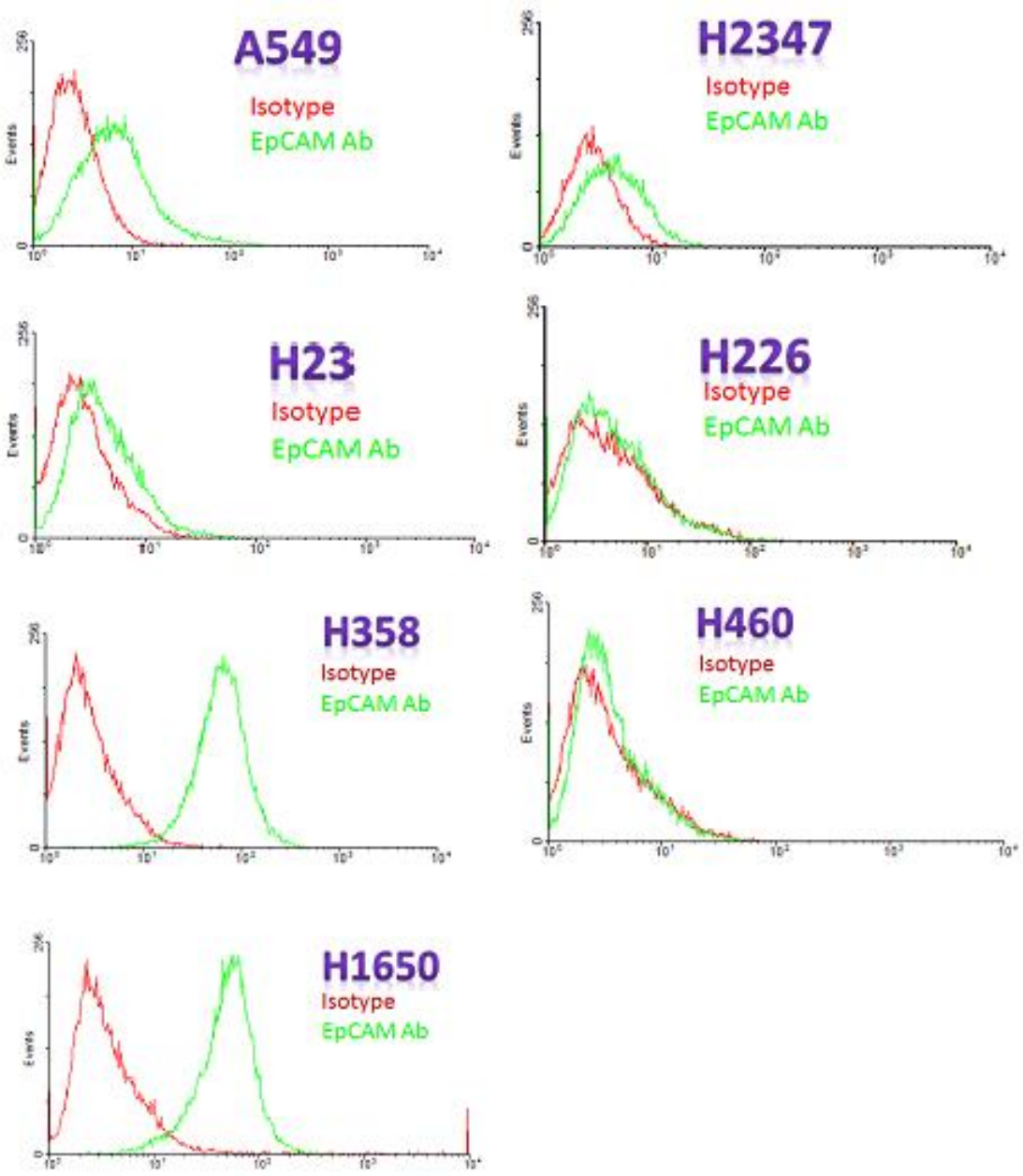


Figure 4-1. EpCAM expression in NSCLC cell lines. Cell lines from histologic subtypes ADC, SQC, and LCC were used for the binding assays. H23, A549, H1650, H358 correspond to ADC, H520 SQC and H460 LCC.

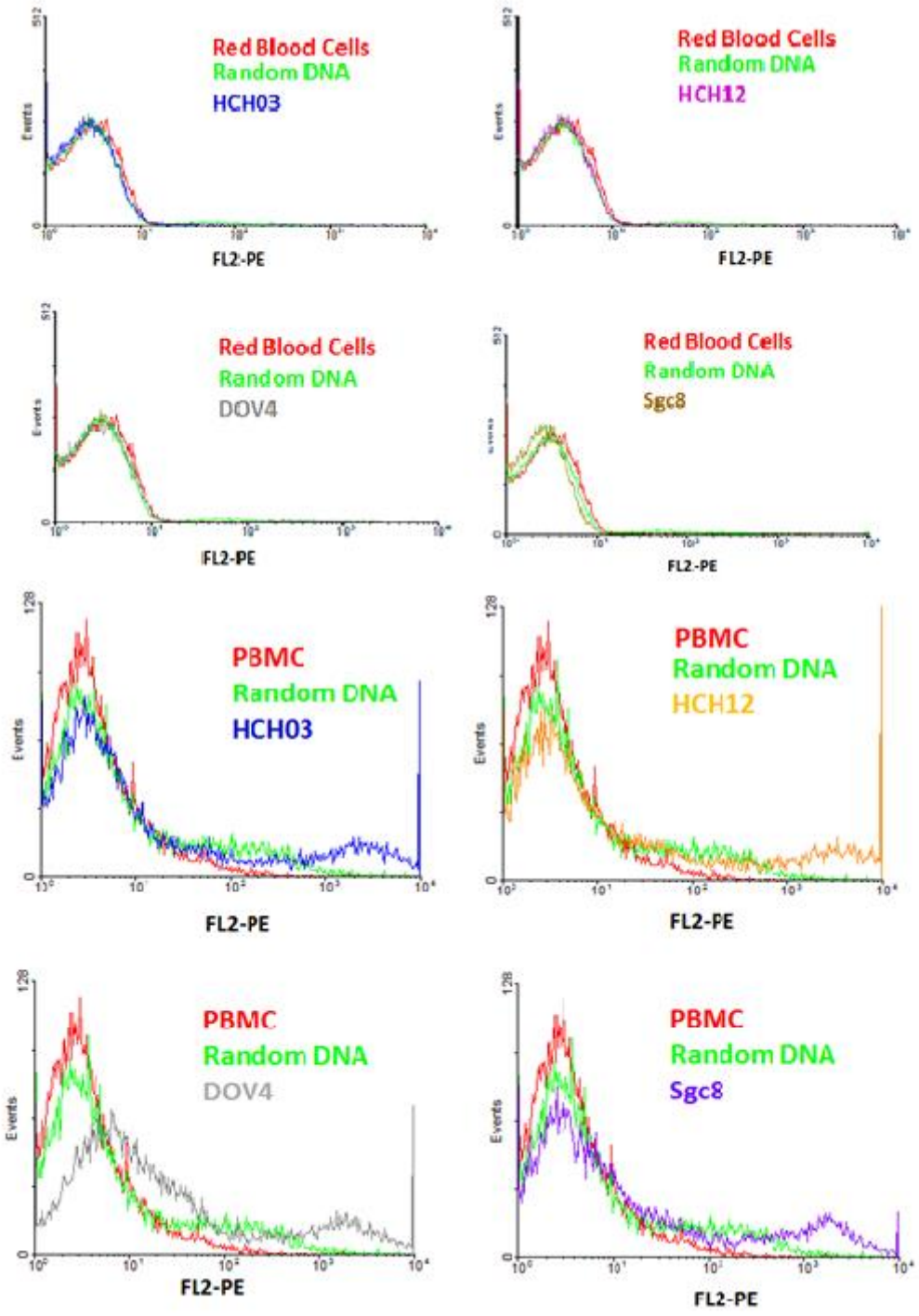


Figure 4-2. Aptamer binding with blood-derived cells. Aptamers HCH3, HCH12, sgc8 and DOV4 were tested with RBCs and PBMCs.

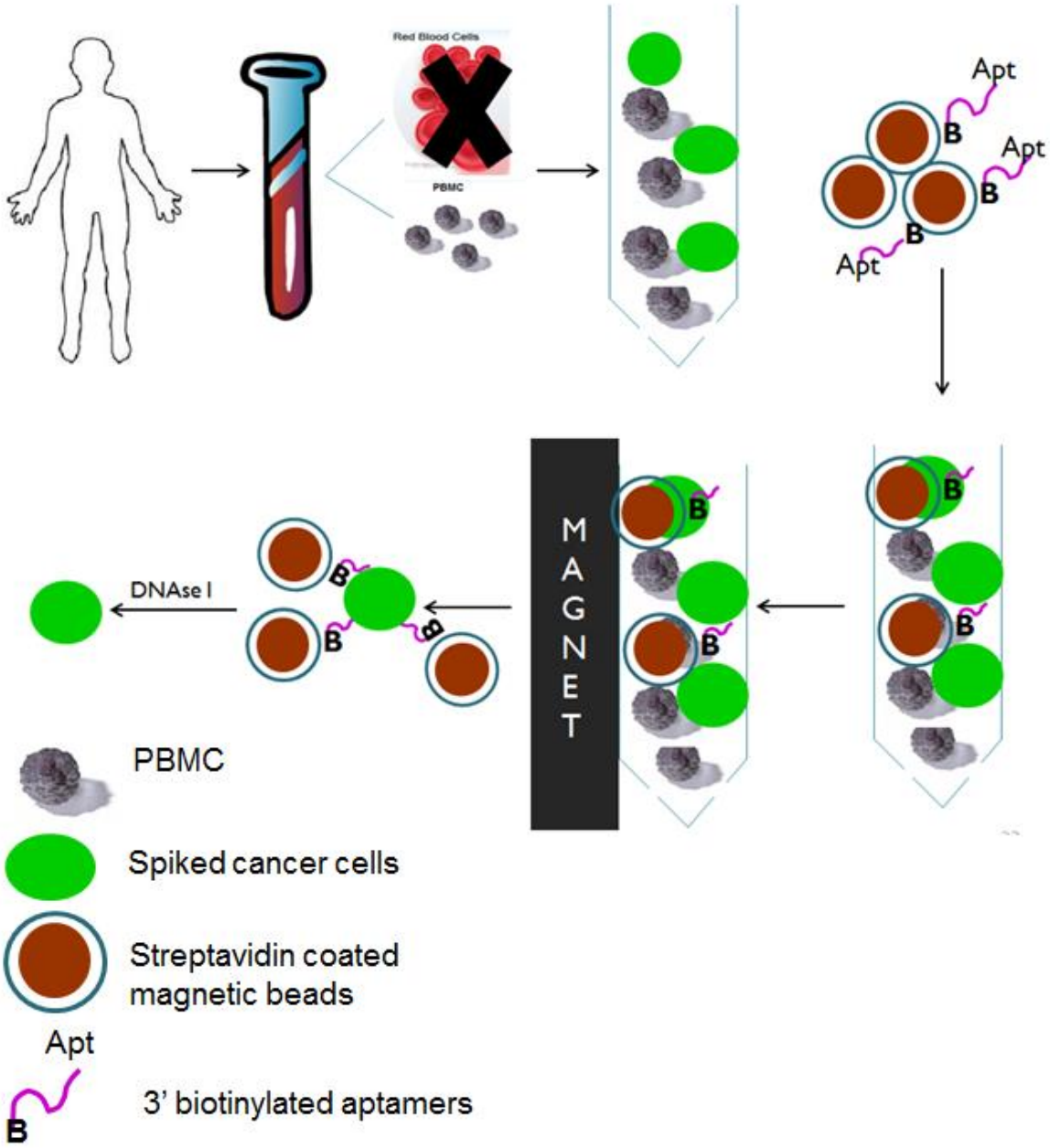


Figure 4-3. Scheme of proposed methodology for the detection of CTCs in NSCLC using blood from healthy donors as a model: H1650 cells were spiked into blood followed by the lysis of RBCs, leaving only PBMCs. This complex mixture is referred as the Buffy Coat (BC). Aptamers were conjugated separately with magnetic nanoparticles (MNP) coated with streptavidin protein via the biotin chemistry. H1650 and aptamer-MNP complex were added too the BC, and the mixture was subjected to magnetic force, in which cells attached to aptamers-MNP-spiked cells were separated from BC. To release captured CTCs treatment with DNAse I was performed.

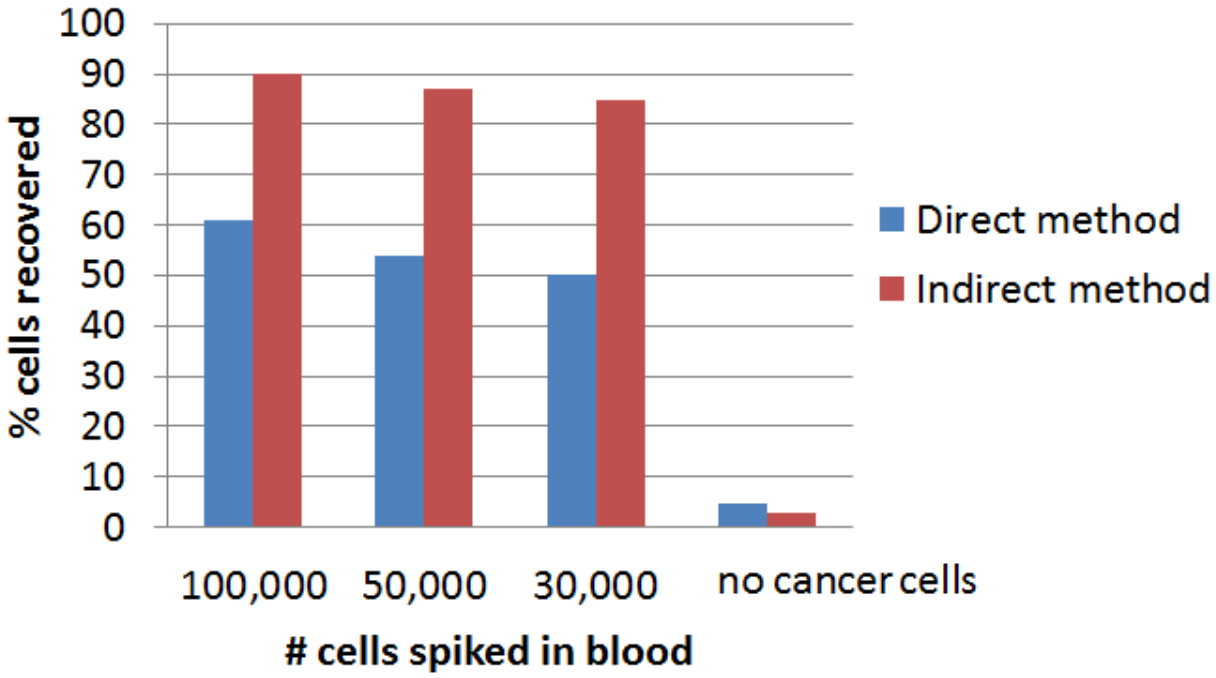


Figure 4-4. Captured CTCs by direct and indirect methodologies. Initially 30K, 50K and 100K were spiked into blood to determine the best methodology.

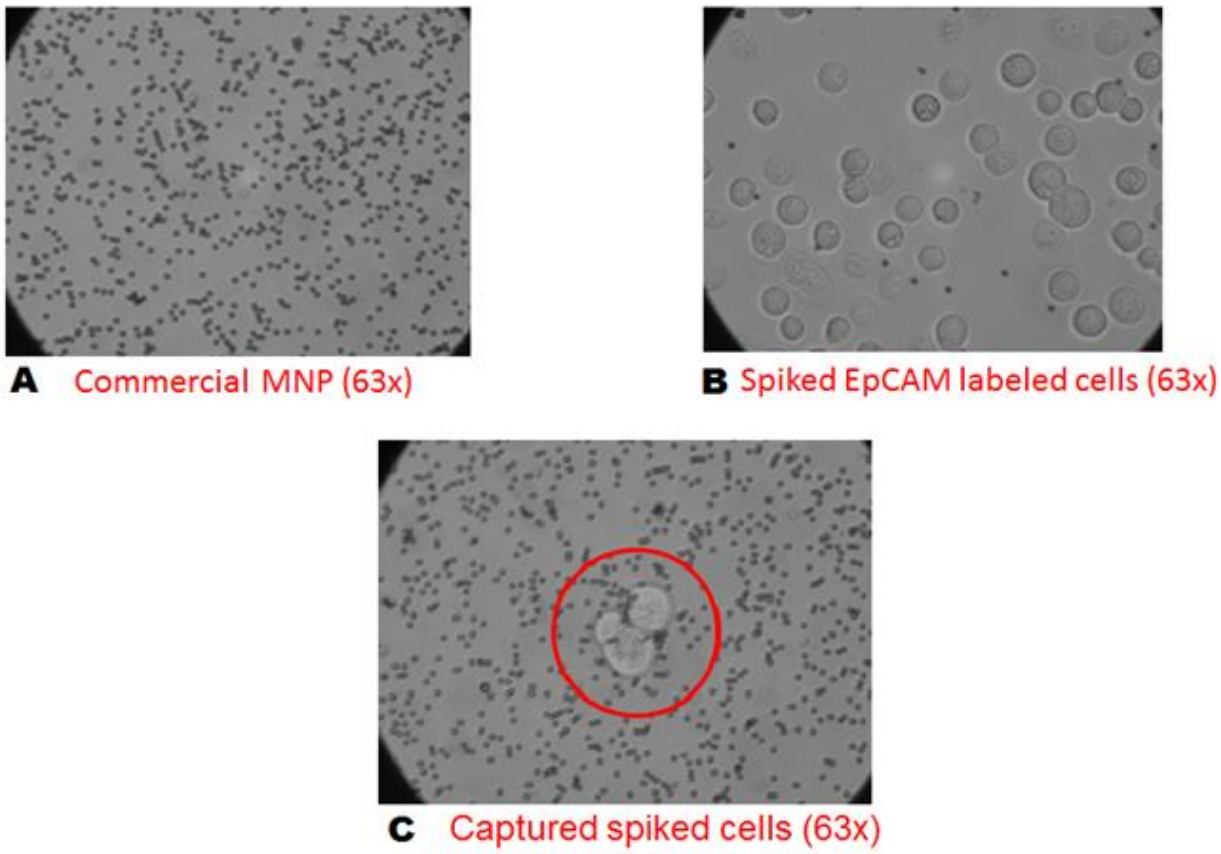


Figure 4-5. Transmittance light images of captured spiked cells. A) Image of commercial MNPs (63x). B) Image of EpCAM labeled cells prior spiking. C) Images of captured H1650 cells.

Figure 4-6. Fluorescence microscopy of captured H1650 (40x).

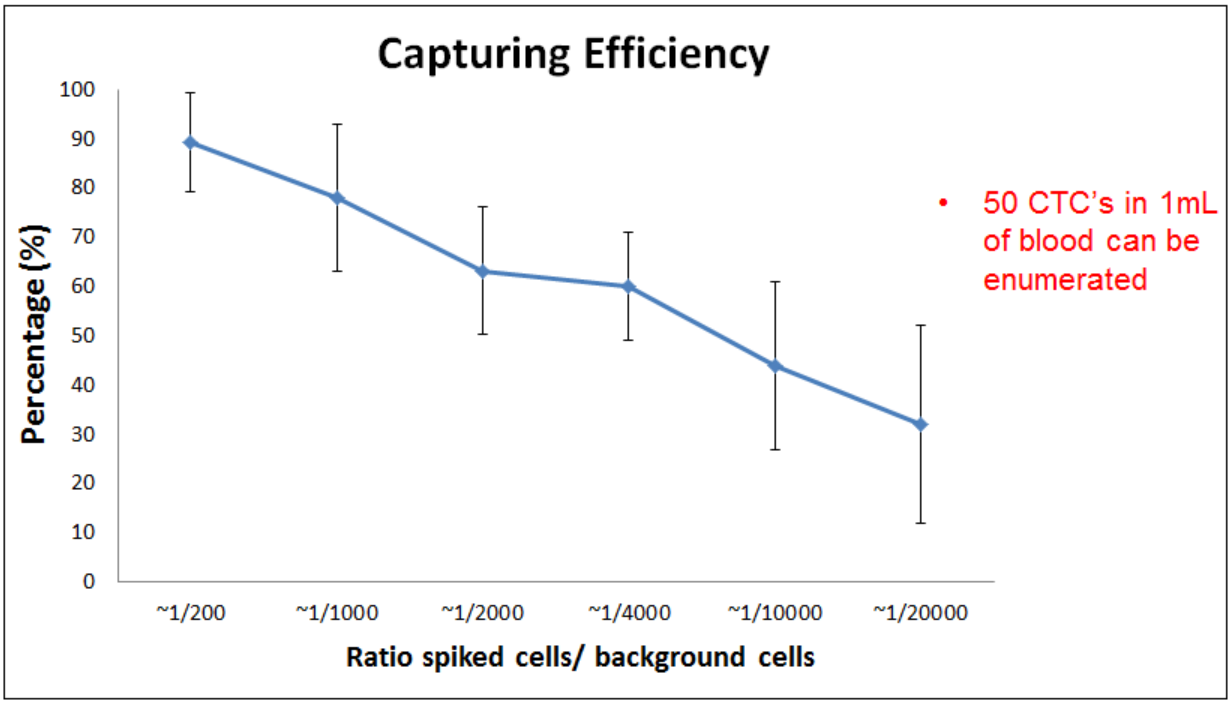


Figure 4-7. Capturing efficiency of the aptamer-MNP based approach. Different number of cancer cells was spiked into buffy coat at different concentrations with respect to the number of background cells.

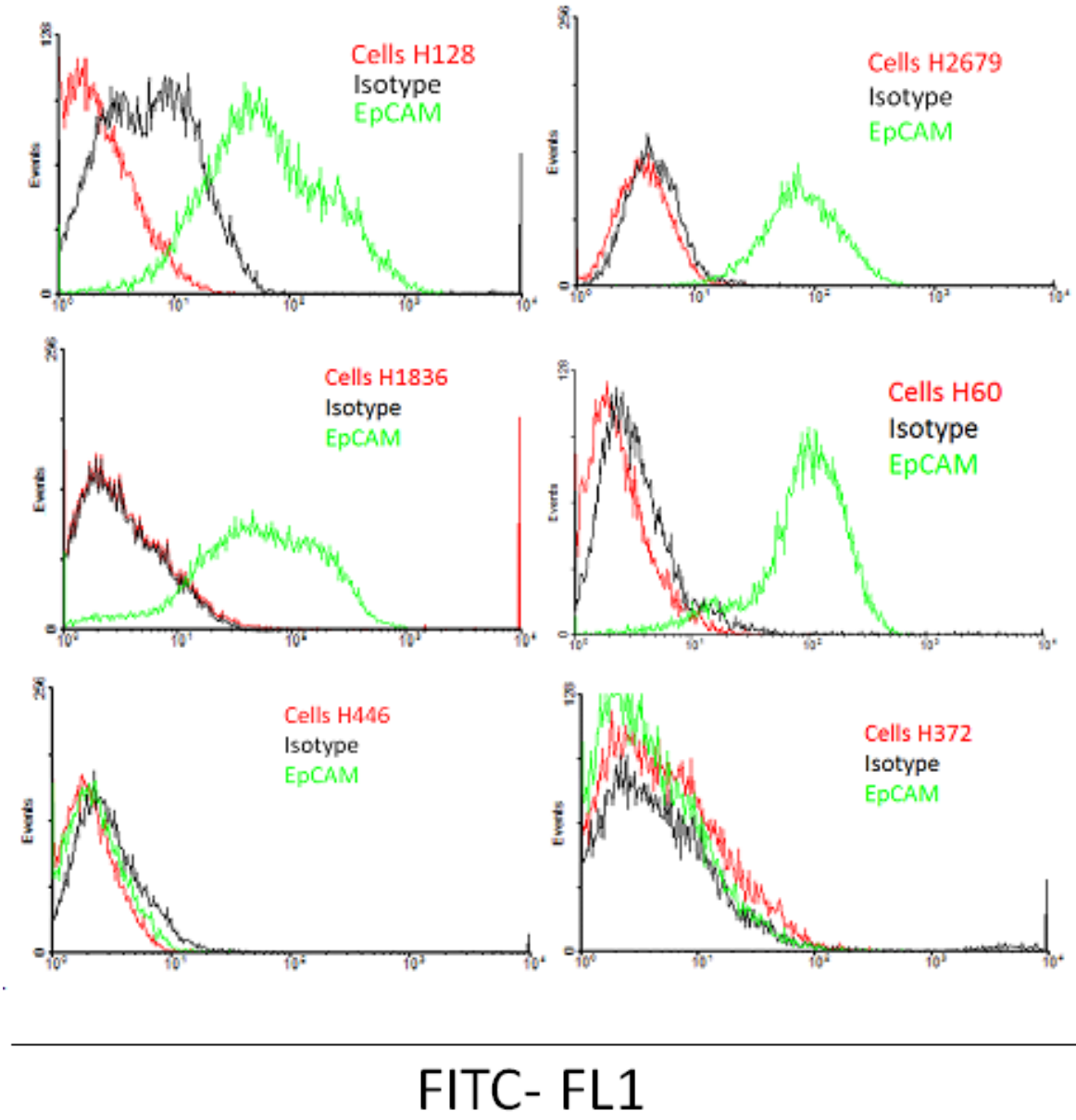


Figure 4-8. EpCAM expression in SCLC cell lines.Cell lines H446, H128, H2679, H1836, H60, H372, H1607, H1672, and H2347 from the SCLC subgroup were tested for EpCAM Ab.

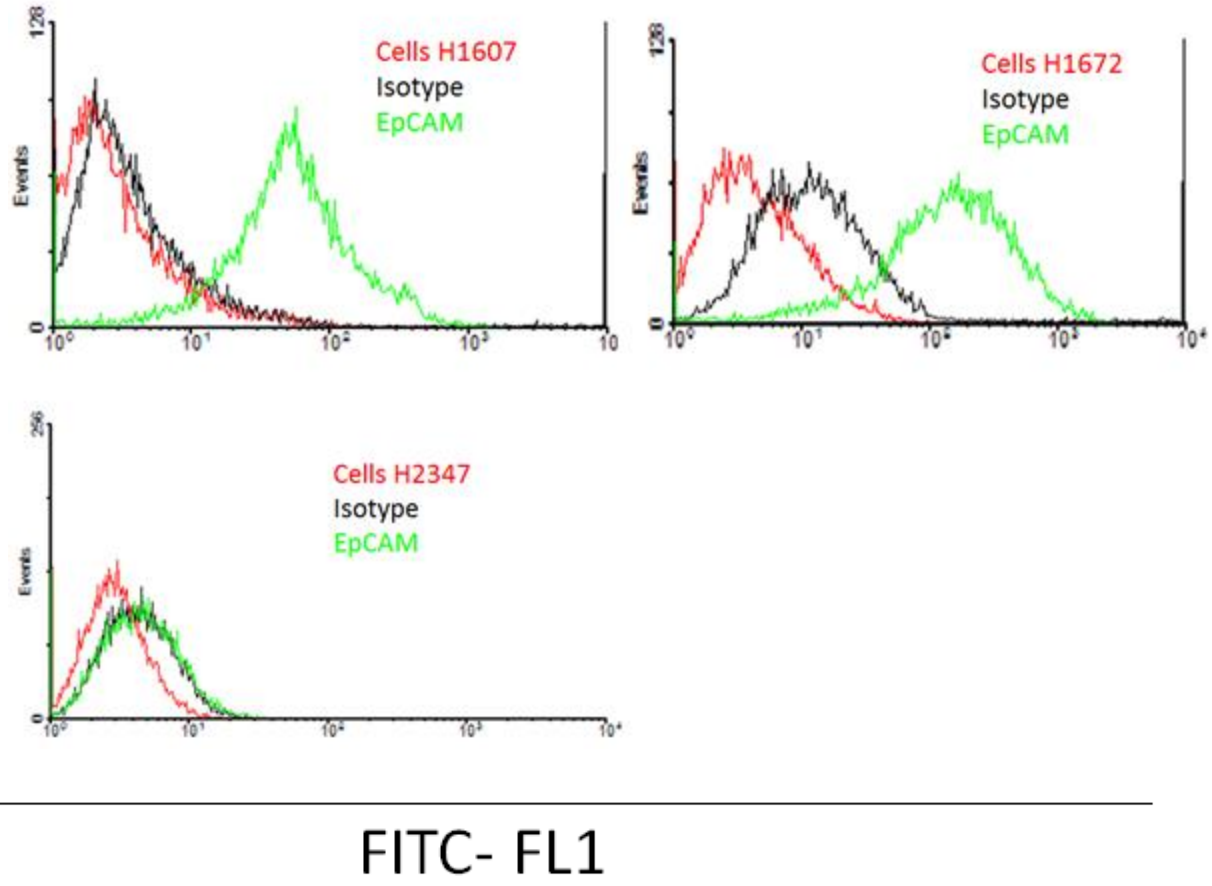


Figure 4-8. EpCAM expression in SCLC cell lines. Continued

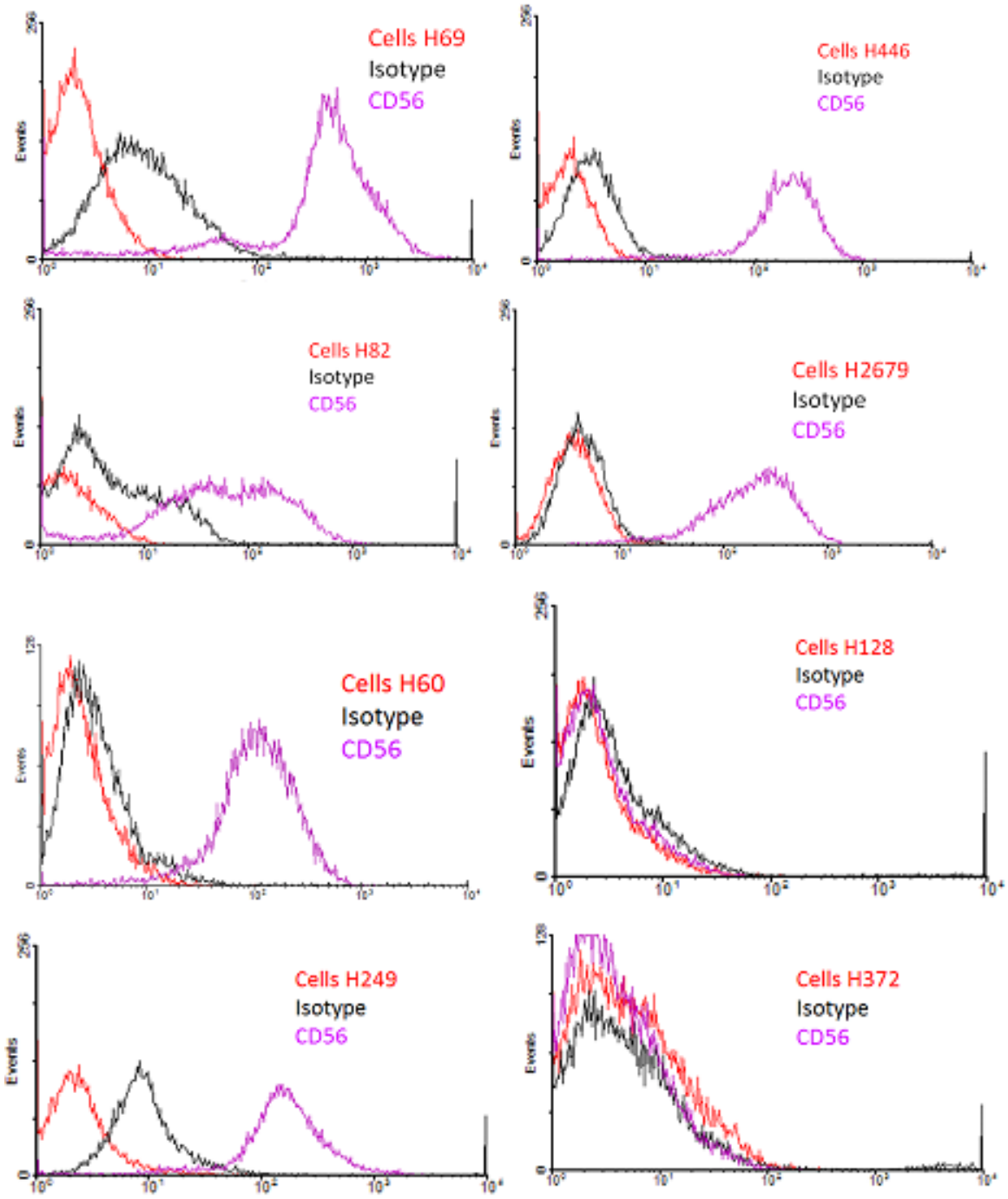


Figure 4-9. CD56 expression in SCLC cell lines. H60, H128, H249, H1836, H60, H372, H620, H1672, H738, and H446 cell lines were tested for NCAM Ab.

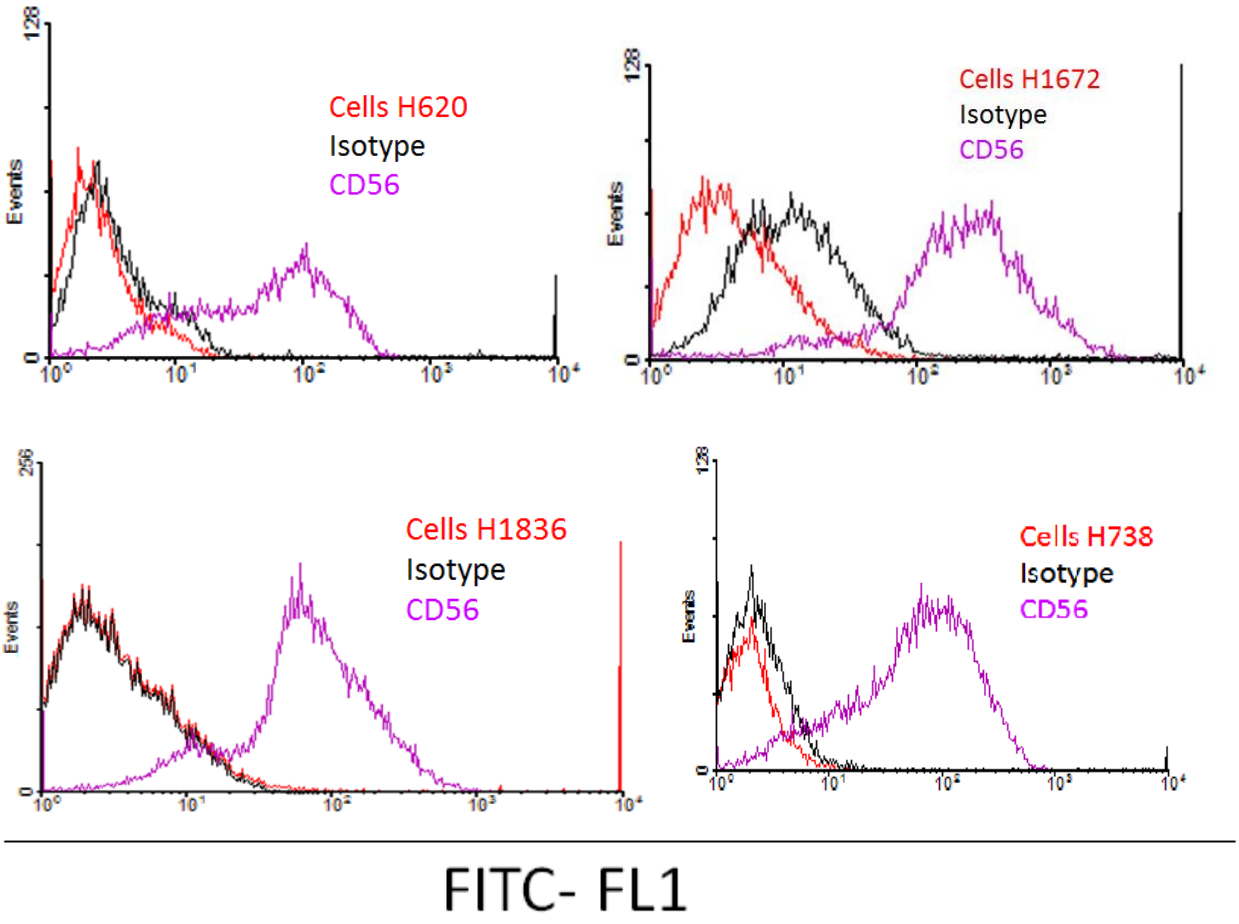


Figure 4-9. CD56 expression in SCLC cell lines. Continued

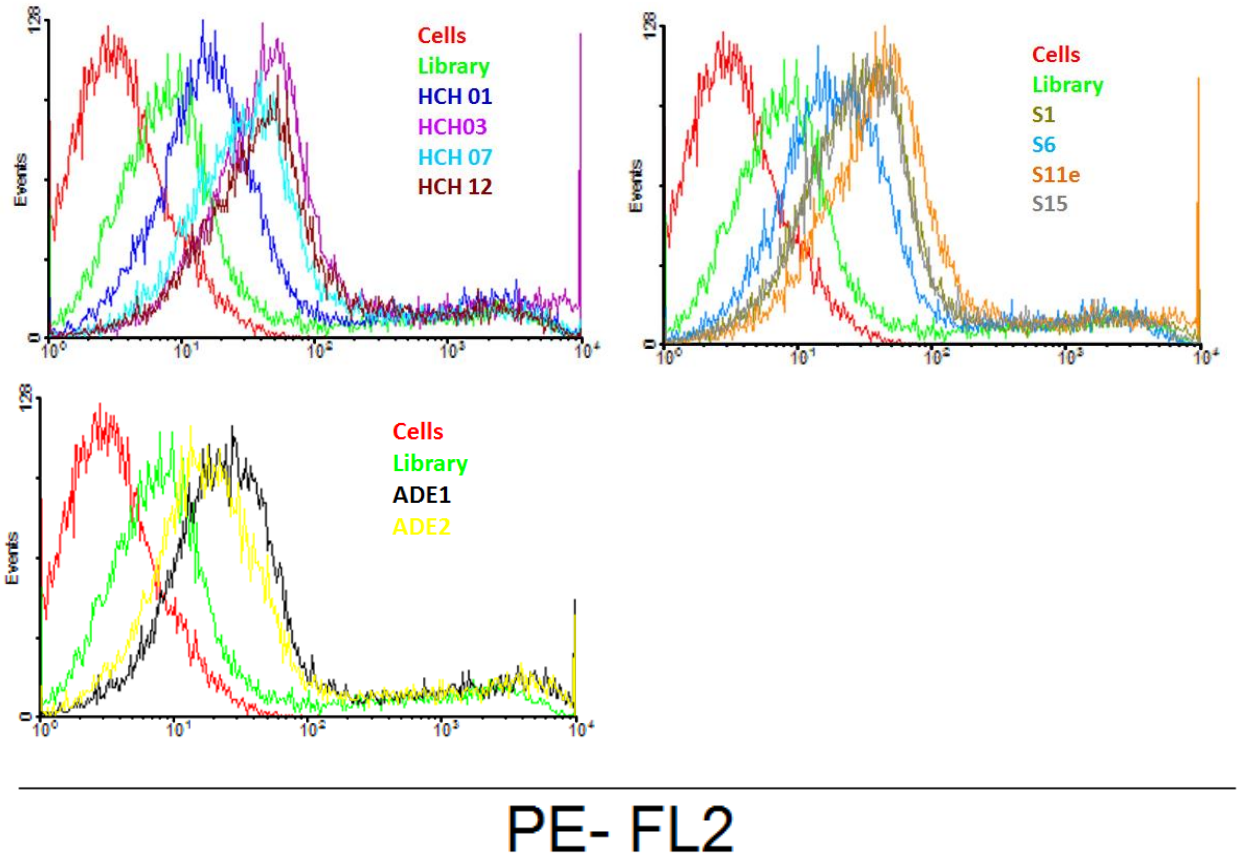


Figure 4-10. H1836 profiling with lung cancer aptamers. Cell lines H1836 was profiled with aptamers SCLC HCH1, 3, 7 & 12 and NSCLC S1, S6, S11e, S15, ADE1 & ADE2.

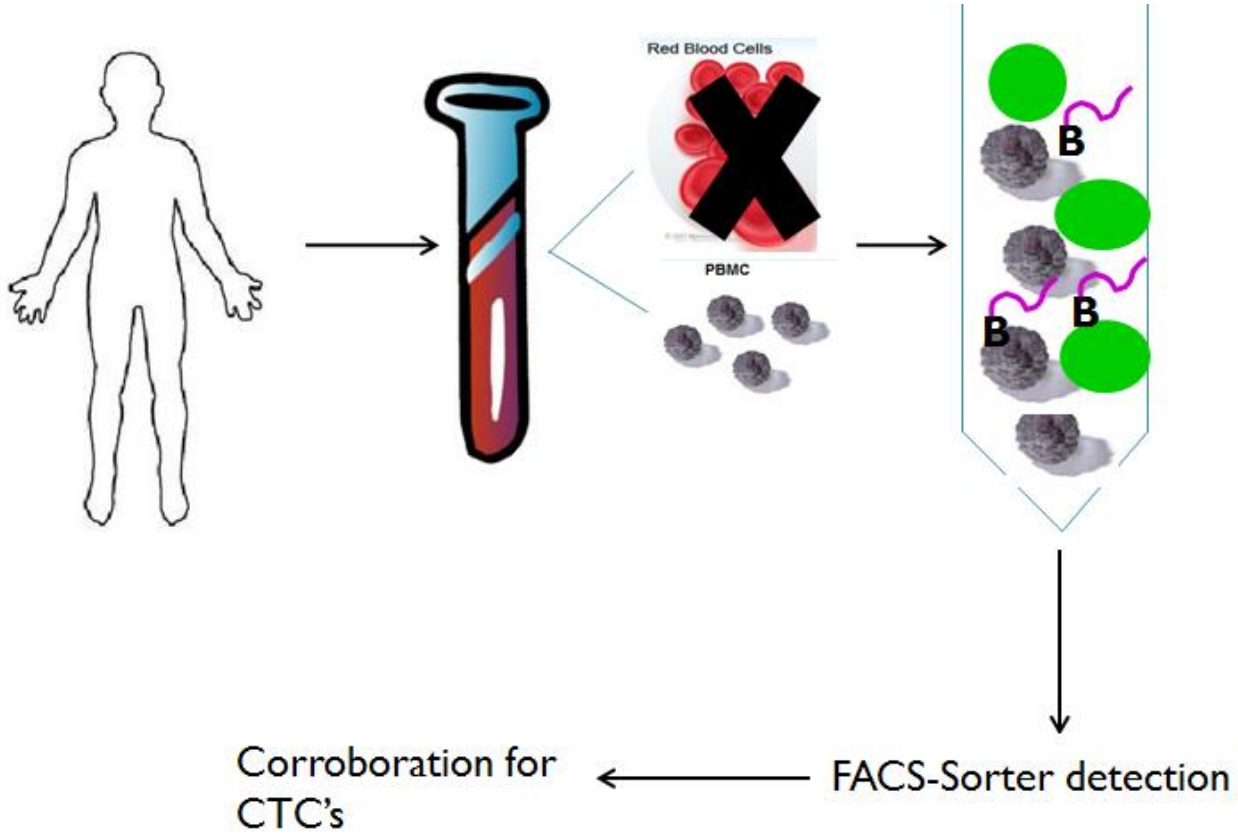


Figure 4-11. Scheme of proposed methodology for the detection of CTCs in SCLC. Blood from healthy donors will used as a model: Different amounts of H1836 cells were spiked into blood followed by the lysis of RBCs after PBMCs. This complex mixture is referred as Buffy Coat (BC). EpCAM Ab and aptamers were incubated, fluorescence for both signal molecules were detected and isolated using a FASCAN sorter machine. Isolated cells were labeled with Abs to corroborate their cancerous status.

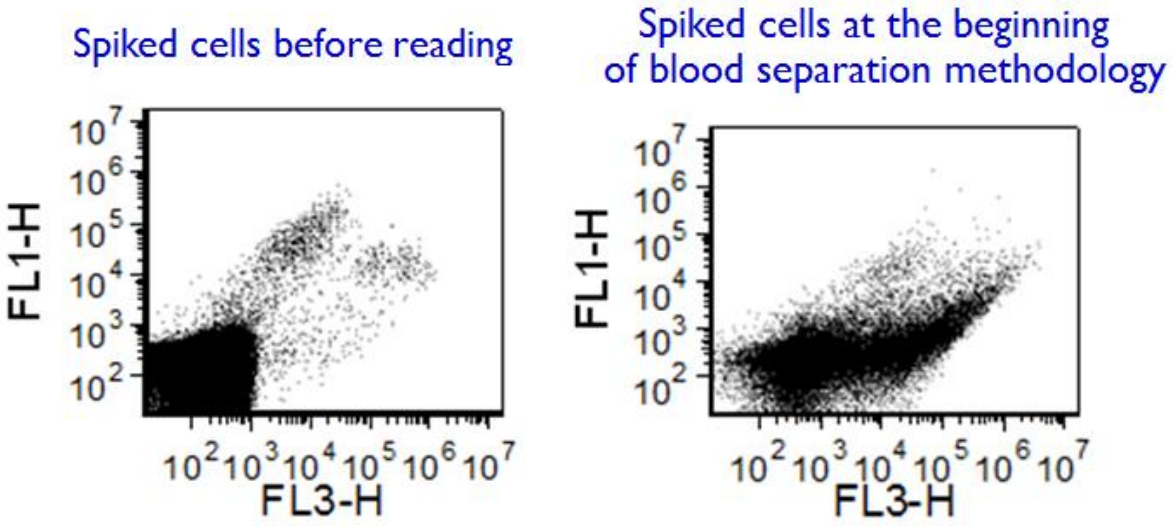


Figure 4-12. Difference in flow cytometry reading of CTCs spiked either at the beginning or the end of the methodology.

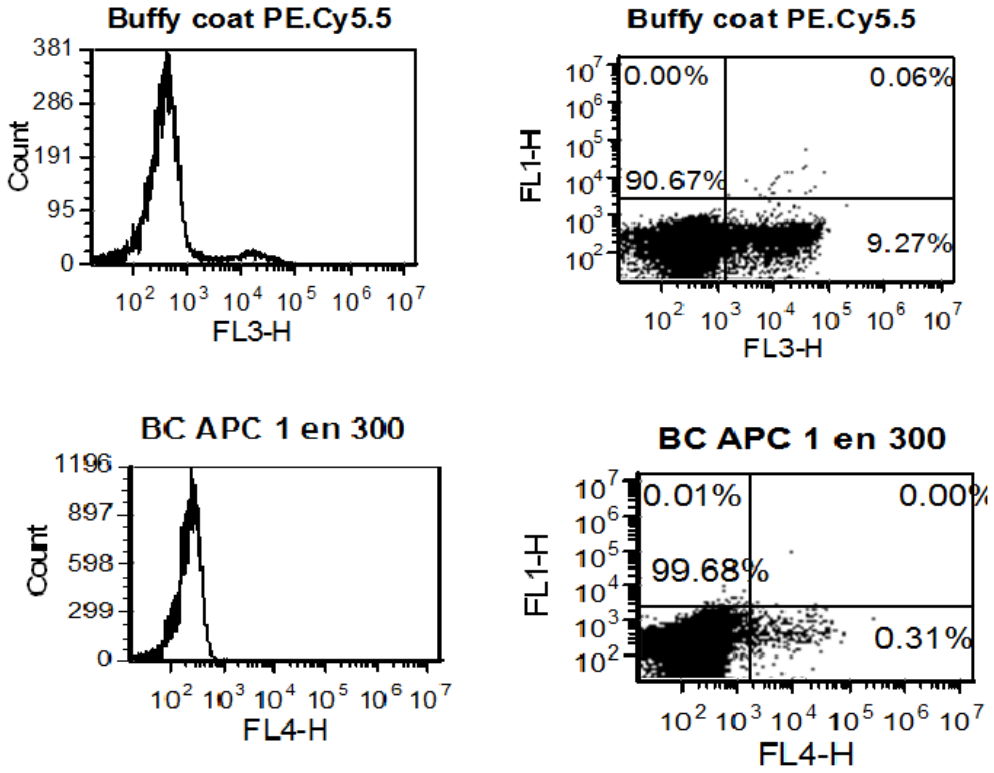


Figure 4-13. Optimization of the dye use for the conjugation to aptamers. Dyes PE.Cy5.5 and APC were tested with freshly extract buffy coat to determine their innate background.

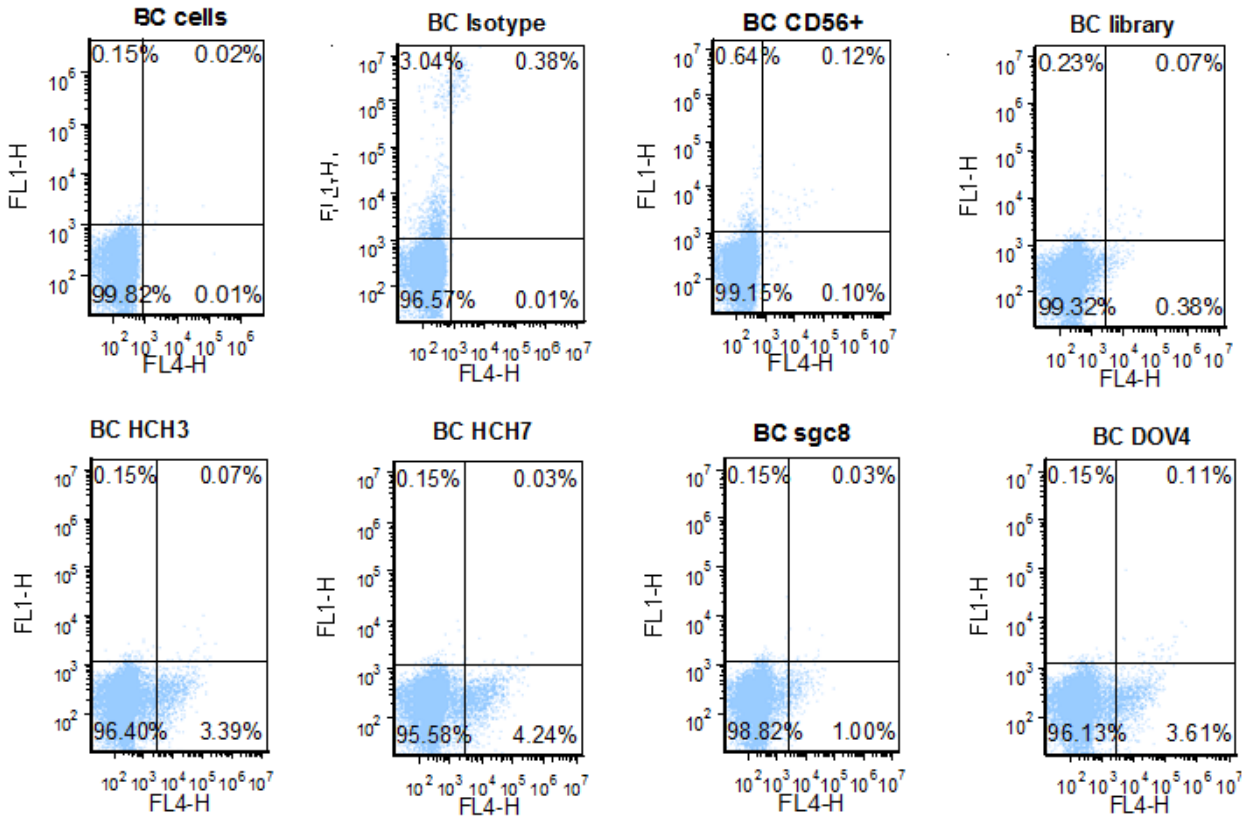


Figure 4-14. Background analysis of cocktail aptamers and Ab with freshly extracted buffy coat using flow cytometry. Isotype IGg1, Ab CD56, random library, and aptamers HCH3, HCH7, sgc8 and DOV4 were tested.

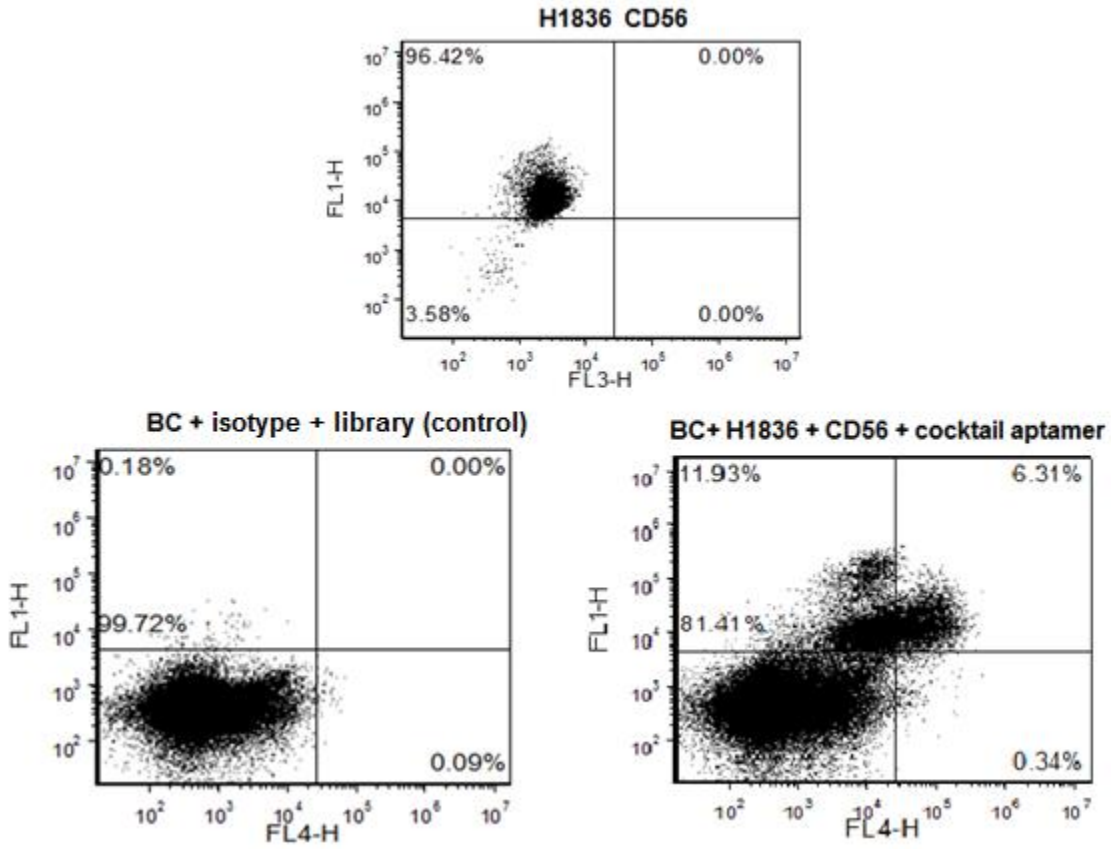


Figure 4-15. Detection of 250K spiked cells in blood. H1836 cell line was spiked in BC and stained with Ab and cocktail aptamers for subsequent flow cytometric analysis. Quadrant was set based on the fluorescence intensity of BC with CD56 and random library.

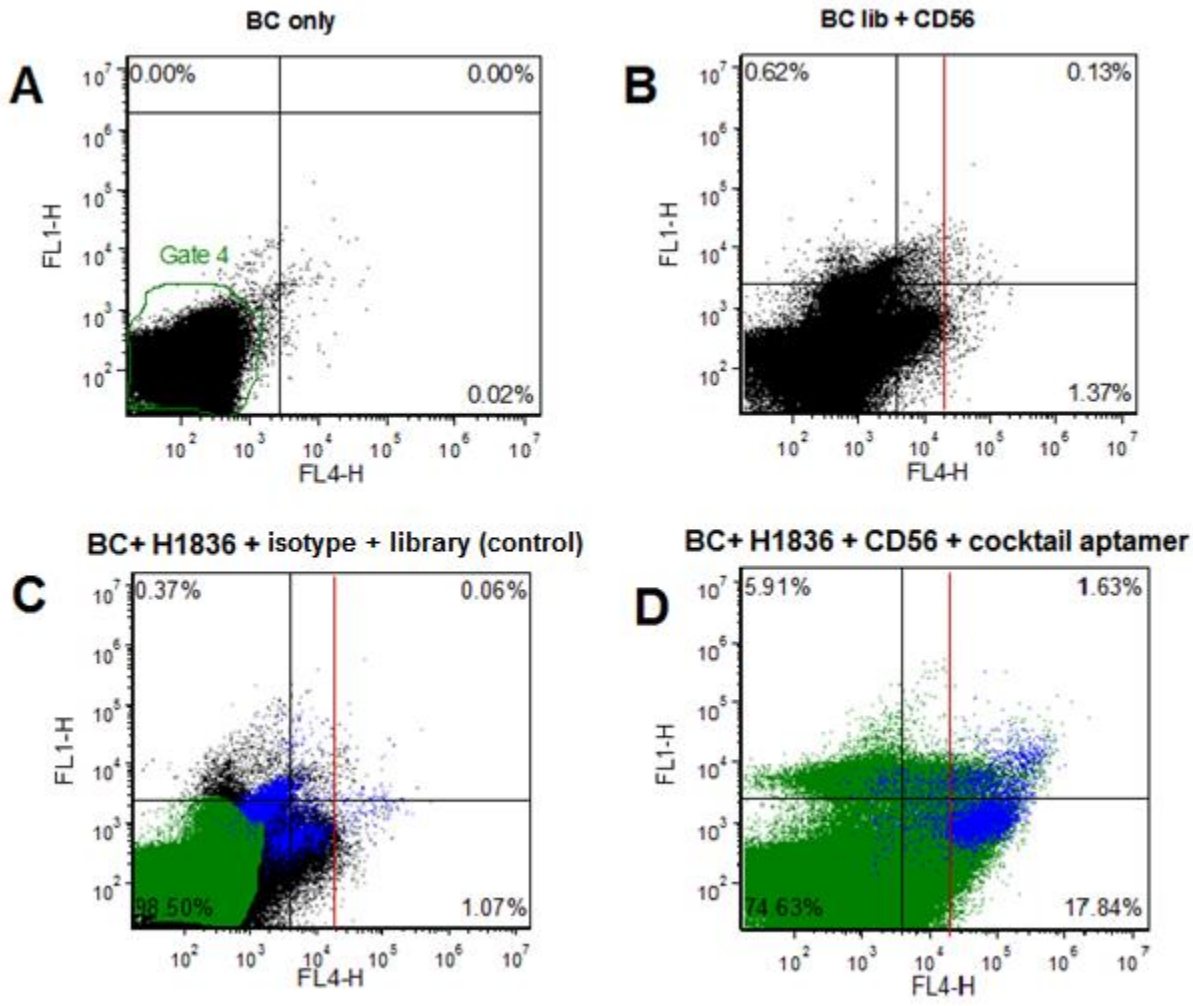


Figure 4-16. Detection of CTCs in blood. 500 cells corresponding to H1836 cell line were incubated with BC. A) Shows BC cell only. B) Shows BC cells stained with Ab CD56. C) BC containing CTCs stained with Ab CD56 and random DNA. D) BC containing CTCs with CD56 and cocktail aptamers.

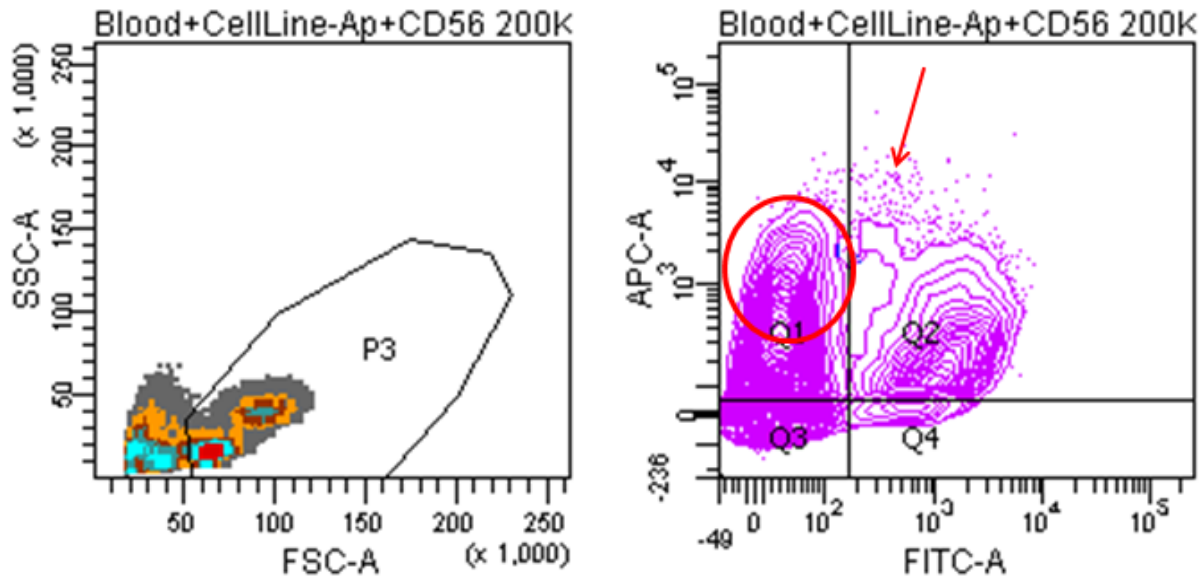


Figure 4-17. Background during CTCs detection. Dot plot on the right shows a high number of background cells in the APC channel.

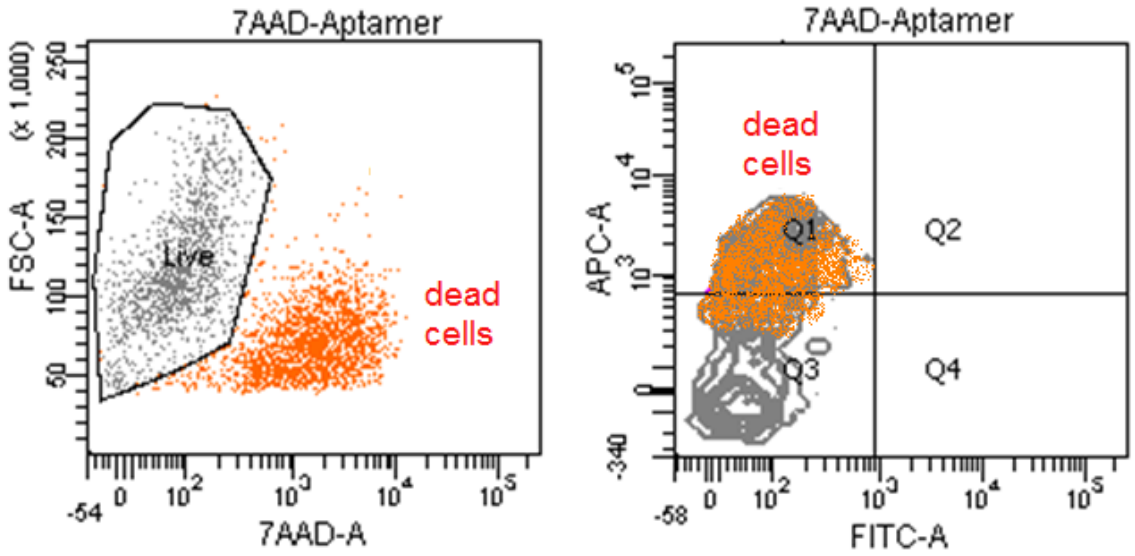


Figure 4-18. BC cells only stained with Ab CD56, aptamer cocktail and 7-AAD. Both dot plot shows live (grey population) and dead (orange population) cells present during analysis.

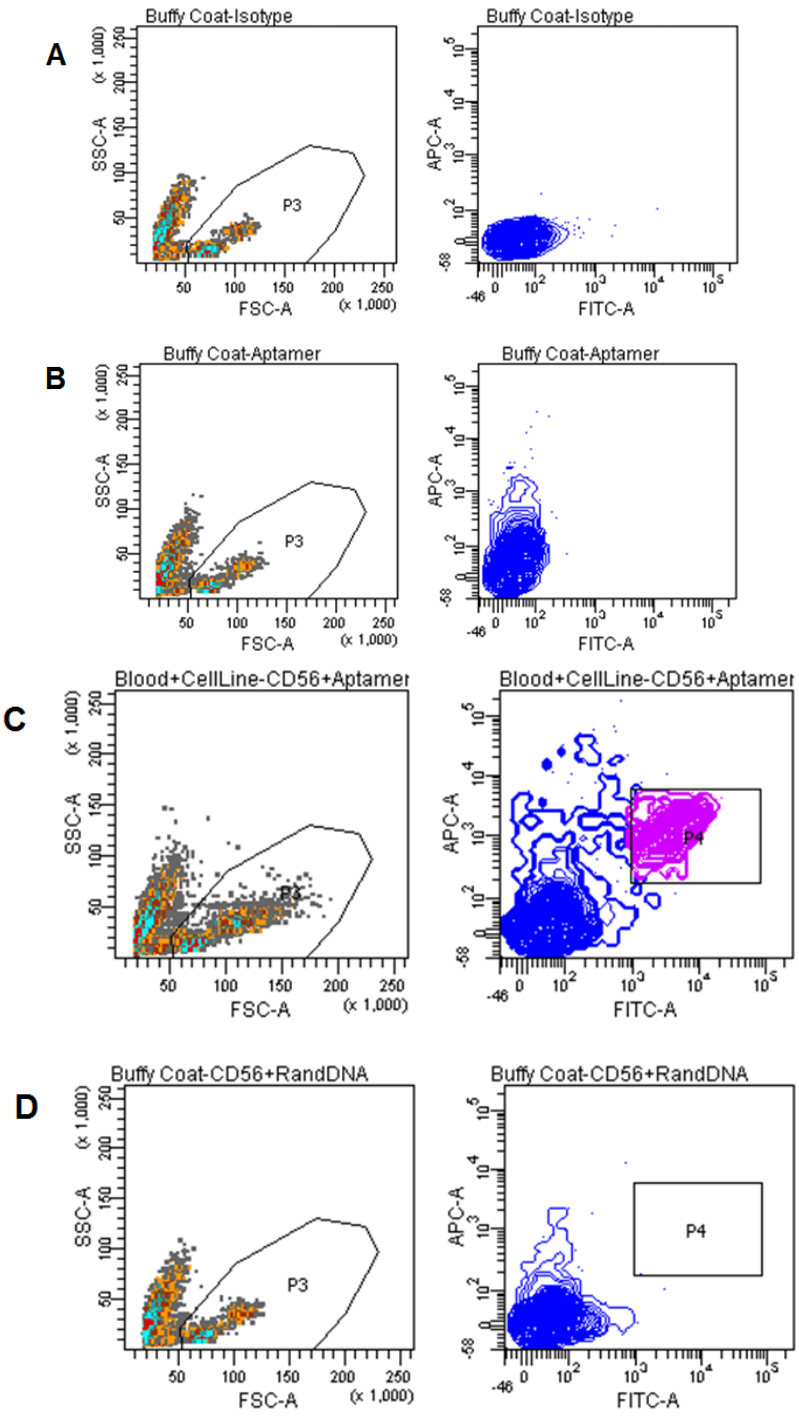


Figure 4-19. Elimination of background cells in channel APC and CTCs detection using 250k cells. A) BC cells stained with Ab CD56. B) BC cells stained with aptamer cocktail. C) BC containing CTCs aptamer cocktail. D) BC containing CTCs stained with random DNA (library).

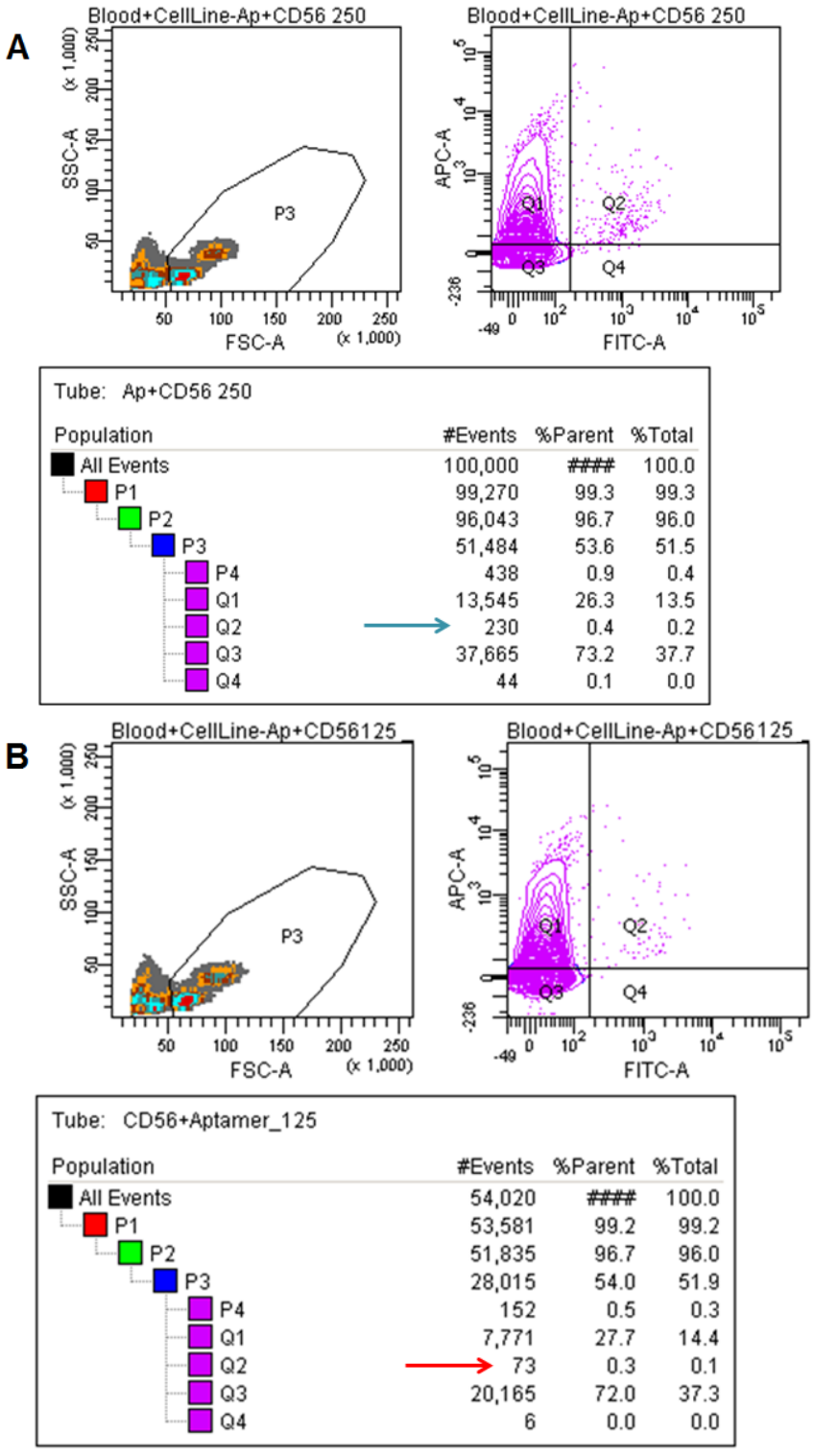


Figure 4-20. Determination of the LOD for CTC detection. A) Detection of CTCs using 250 spiked cells in BC. B) Detection of CTCs using 125 spiked cells in BC. C) Detection of CTCs using 64 spiked cells in BC. D) Detection of CTCs using 32 spiked cells in BC.

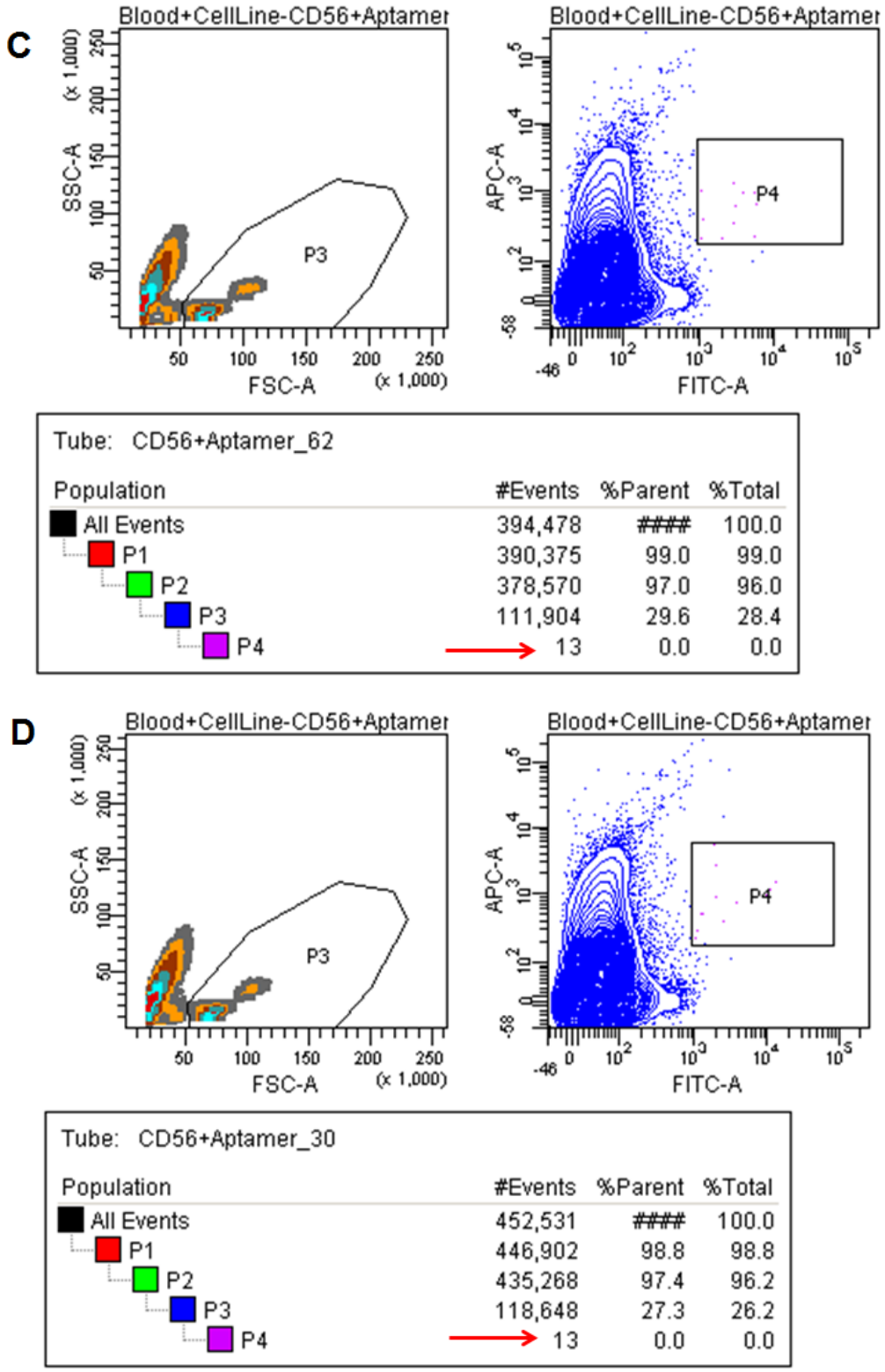


Figure 4-20. Determination of the LOD for CTC detection. Continued

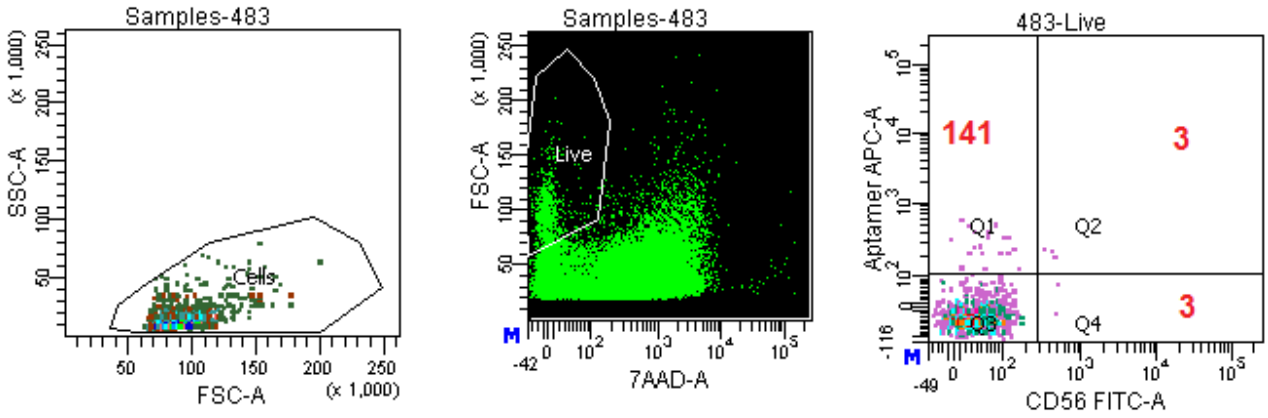


Figure 4-21. Detection of CTCs in patient sample 483. Number is red on each quadrant correspond to the number of cells red in a 7.5mL of blood.

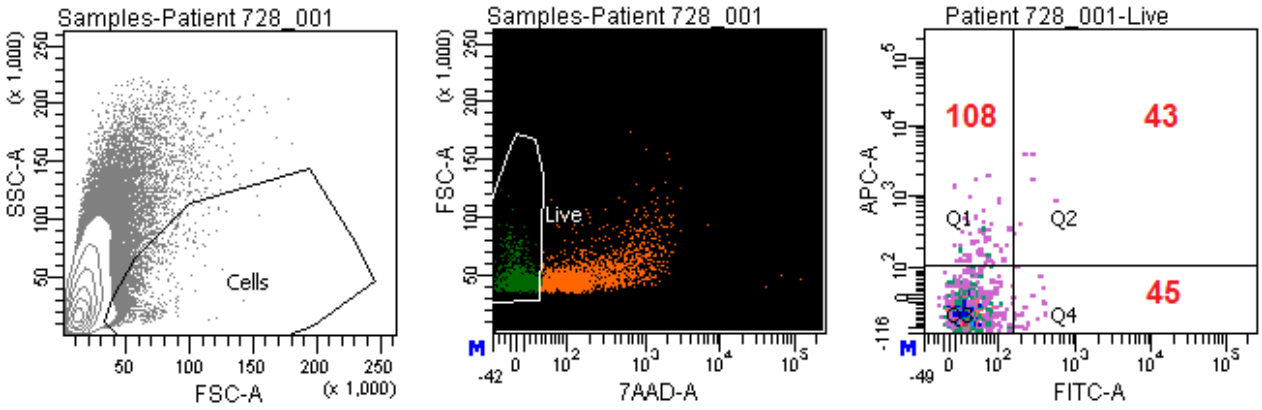


Figure 4-22. Detection of CTCs in patient sample 728. Number is red on each quadrant correspond to the number of cells red in a 7.5mL of blood.

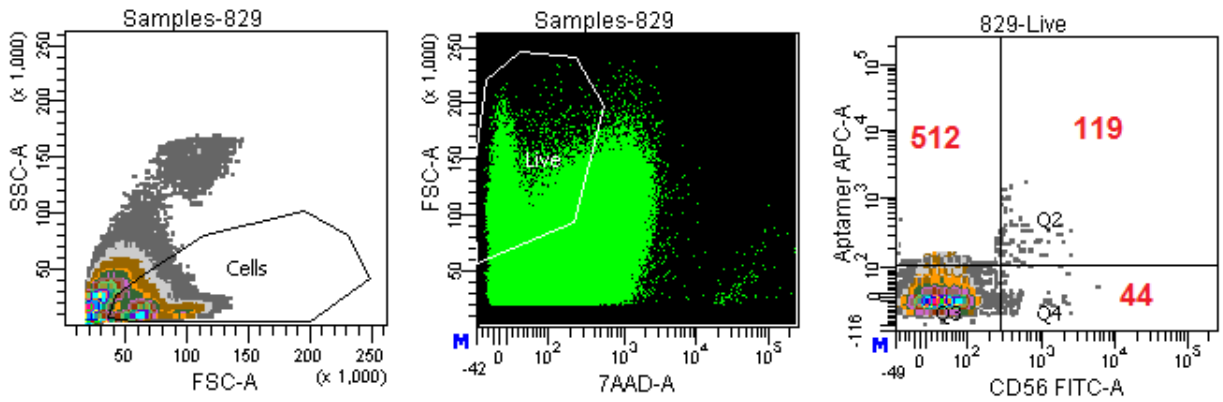


Figure 4-23. Detection of CTCs in patient sample 829. Number is red on each quadrant correspond to the number of cells red in a 7.5mL of blood.

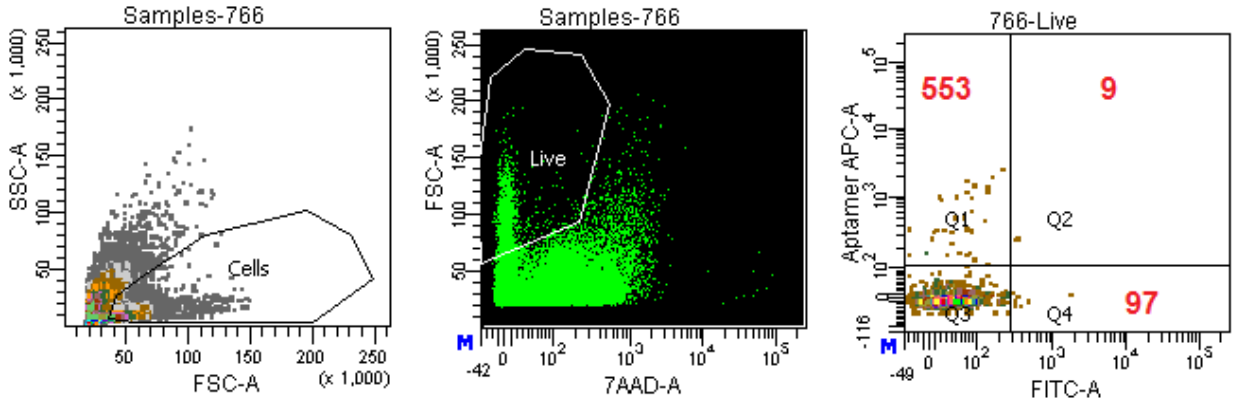


Figure 4-24. Detection of CTCs in patient sample 766. Number is red on each quadrant correspond to the number of cells red in a 7.5mL of blood.

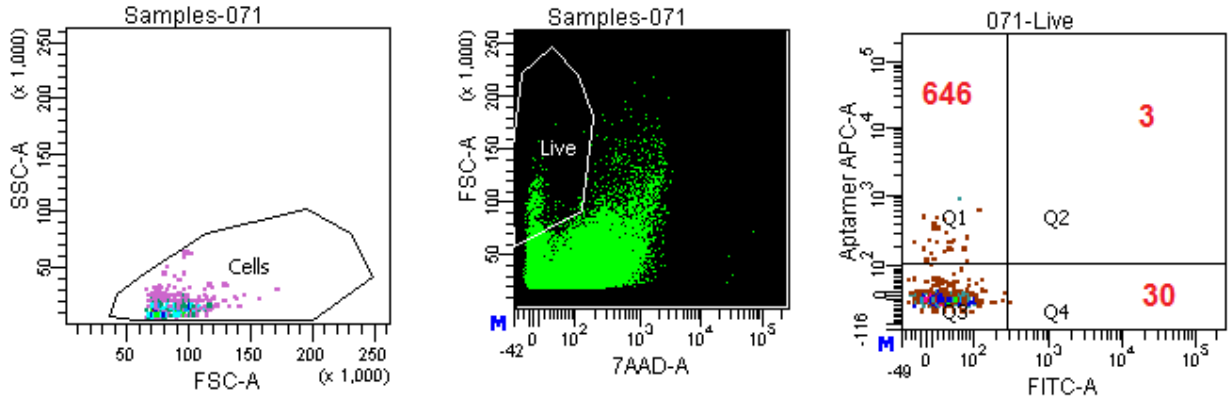


Figure 4-25. Detection of CTCs in patient sample 071. Number is red on each quadrant correspond to the number of cells red in a 7.5mL of blood.

CHAPTER 5
IDENTIFICATION OF THE APTAMER'S BINDING PROTEIN

Introduction: Biomarker

A biomarkers is defined as "a laboratory measurement that reflects the activity of a disease process.[145] "Currently, proteomics is the most widely used technique for the discovery of new proteins in cancer. The recent advancements are directly related to the improvements in mass spectrometry its complementary technique.[146]

In the tan group, cell-SELEX is a powerful technology used to study of a particular cancer type, with the ultimate goal to use those selected aptamers to identify potential biomarkers useful in cancer detection/treatment. Cell-SELEX has the distinctive advantage of not requiring previous knowledge about the aptamers' target for this application.[147] To date, the tan group has elucidated only two targets: proteinase kinase 7 (pTK7) and immunoglobuling M (IgM).[106,148] PTK7 initially was found on leukemia cells, but later it was found to be over expressed in different cancer cell lines.[149,150] In addition, sgc8 the aptamer that targets protein PTK7 is the most studied aptamer,[151,152,153] suggesting that potential biomarkers can be discovered using cell-SELEX as methodology.

As described in the introductory chapter of this dissertation, cell-SELEX has been used as the methodology for the development of aptamers against adenocarcinoma. In addition, aptamers previously selected for other lung cancer subtypes were also studied. This chapter will describe a methodology development for the elucidation of an aptamers' target using cell culture cell lines as the matrix and aptamers as the capturing-detection molecule. [Figure 5-1] outlines the proposed methodology for the elucidation of the aptamers' target.

After cell lysis, the proteins are captured by biotinylated aptamer. After washing and separation using streptavidin coated magnetic beads the mixture was separated by SDS-PAGE and submitted for mass spectral analysis.

Results and Discussion

As mentioned above the targets for only two aptamers have been identified so far in the tan group. With the urgent necessity of discovering the target proteins of aptamers obtained by cell-SELEX, a group of several students were challenged to develop methods for target elucidation. For this dissertation, aptamer DOV4 was chosen because its target is expressed on a large variety of lung cancer cell lines but not on normal bronchial lung cells.

Elucidation of the Target Protein for Aptamer DOV4

Aptamer DOV4 was originally selected using cell-SELEX technology against adenocarcinoma of the ovary CAOV3 cell line. However, based on the profiling with lung cancer cells and normal human bronchial cell lines described in chapter 3, the target of DOV4 is also expressed in several other cancer cell lines but not in normal cells, making this aptamer suitable for future applications. As shown in the first step in Figure 5-1 the biotinylated aptamer serves as the binding/capturing molecule. [Figure 5-2] shows the SDS-PAGE gel when aptamers and random DNA (library) were subjected to the methodology pictured in Figure 5-1. There is not difference between the protein captured by the library and that captured by DOV4. In other words, the aptamer lacks specificity in the task of capturing its target molecule.

These observations called for a change in the process of extracting the target proteins. In addition, a closer look at the characterization of DOV4 revealed that its target seems to be resistant to the digestion of proteases trypsin and proteinase K. The

cell-cytometry data in [Figure 5-3][154] show that the fluorescence intensity increased when cells were treated for 30min with proteinase K, indicating that the target becomes more (ie, more exposed) after digestion.

Subcellular Fractionation to Reduce Background Proteins

After these observations, it was hypothesized that the binding molecule for aptamer DOV4 could be a highly glycosylated protein as those are normally non-susceptible to proteases treatment. Therefore, a digestion step was included at the beginning of methodology to reduce the number of non-necessary proteins in the already complex mixture. In addition to the digestion by proteinase K, a subcellular fractionation was also implemented in the methodology. During this step, the proteins contained in different compartments in the cell were separated using a sucrose gradient and high centrifugation speed. After separation, only membrane proteins were retained and incubated with aptamer DOV4.

After making these alterations to the methodology and separation by SDS-PAGE, the results shown in [Figure 5-4] were observed.

The number of background proteins was highly reduced when compared to the initial SDS-PAGE gel [Figure 5-2]. However, the protein capturing by the aptamer did not show specific proteins when compared with random DNA. The amounts of proteins captured in lanes 2 and 3 of Figure 5-4 are similar, demonstrating that, even though the number of background proteins was removed, DOV-4 still did not show specificity.

Aptamer Crosslinking to its Target

To address the specificity issue, it was proposed to form a chemical crosslink the between the aptamer and its target using the well-known fixative formaldehyde.[155] This small molecule, CH_2O, molecule has been used widely in histology to preserve tumors and as cross-linker in the Chromatin Immunoprecipitation (ChIP) assay.[156] This molecule can covalently link a DNA base with an amine-residue from a protein in great proximity to the DNA, as shown in [Figure 5-5]. Figure [5-6] shows the final covalent bond between a cytosine base and a lysine residue.

To summarize the new steps included in the methodology: a protein digestion step was included to further expose the target to bind to the aptamer; (presumably a highly glycosylated protein) a subcellular fractionation using sucrose was also included in the methodology to avoid non-specific binding, especially to those DNA-binding proteins present in the nucleus of the cells; and a crosslinking chemical reaction between DNA and its target was carried out using formaldehyde. During the latter step, it was imperative to use freshly prepared methanol-free formaldehyde as it was necessary to avoid permeation of the membrane therefore internalization of the aptamer.

Troubleshooting and many optimizations were necessary to achieve the desire results. There were numerous non-specific bands corresponding to proteins binding to random library when incubated with H23 cells, with much fewer distinct bands present only on the lane corresponding to the interaction of aptamer DOV-4 and H23 cells. The important bands were finally identified after several SDS-PAGE gel separations. In addition, using this methodology a specific band was corroborated by presence in two different and separate experiments. [Figure 5-6] shows gels for two different

experiments run in parallel. The arrowed band is present in the two aptamer bands and it is absence in the negative control, thus verifying the specificity and the reproducibility of the methodology present in this chapter. The band was excised from the gel and sent for MS analysis by the Reinhold laboratory at the University of New Hampshire. Their specialty is research in glycosylated proteins and with experience in (i) development and differentiation, (ii) cell adhesion and inflammation, (iii) cancer and metastasis, and (iv) host-pathogen interactions in infection diseases.[157]

Two different methods were employed for the extraction of proteins present in the band, one with aqueous solution, the other with a 50:50 mixture aqueous/organic mixture. Both extractions were carried out in parallel, followed by trypsin digestion. The digest were examined using MS, and the results were analyzed using the protein databases Mascot and Swissprot. Only the proteins present in both analyses were considered as true hits, whereas proteins present in only one of the extractions were considered as artifacts. Both results are summarized in Table 5-1 with true hits highlighted. After the analysis performed by the Reinhold group, the results were sent to UF for further analysis.

The protein identification results after MS analysis is listed in Figure 5-8. As mentioned previously, only the highlighted proteins were considered as they appear in the analysis of both extractions. Based on the probability given by the mascot program proteins with scores greater than 56 are considered as significant ($p<0.05$).

Discrimination of Significant Proteins

As previously shown, the aptamer DOV4 used in this project targets a molecule present in the membrane of H23 cells. Therefore, our first step of discrimination is cellular localization; more specifically the protein must be present in the cell membrane.

Proteins were analyzed based on their mascot score in a descending order, as the number decreased the chances of that protein being random increased. For each protein, a basic literature search was used to determine the location and the role of the protein in the cells. It is important to note that in addition to the membrane, candidates may also might be expressed elsewhere in other parts of the cell. The most notable example of the target of nucleolin aptamer, which is present not only of the surface of the membrane, but also in the nucleous.[123]

One protein recognized by the analysis was hemoglobin, which is found primarily in the RBCs and not on epithelial cells. In this case we believe that somehow, during either the resolution of the protein by SDS-PAGE gel or the preparation for MS-analysis, there was blood-related contamination of the sample. Another protein present in the list, and directly related to blood-components is serum albumin, which is further discussed below.

Skin cells

In MS-protein analysis, some of the major contaminants are keratins, proteins present in the skin. During this analysis filaggrin was one of the proteins that could potentially be the aptamer DOV-4 target. Filaggrin is an associate protein that binds to the keratin intermediate filaments.[158]

Also caspase-14, which is a member of the caspase family of enzymes that play a pivotal role in apoptosis, was observed, but caspase-14 is limited to epidermal keratinocytes, where its role is probably cell death of keratinocytes and in skin barrier formation.[159-160]

Mytochondrial Proteins

During the analysis, the metalloprotein arginase-1 was also one of the results. Arginase-1 catalyzes the hydrolysis conversion of arginine to ornithine and urea. This enzyme has also been described as potential immonohistochemistry marker in hepatocelular tumors.[161]

Cytosolic Proteins

Only one protein present in the cytosol of cells was listed as potential target of DOV4 aptamer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which plays an important role in glycolysis. Recently, however, some functions not related to glycolysis have been studied relating to its overexpression in some types of cancer.[162]

Cytoplasmic Proteins

Also serum albumin, a protein which is temporaly present in the cyoplasm before being secreted into the plasma[163] was also found with high mascot scores (652 for extraction R1 and 531 for extraction R2). This is an example of a non-specific protein being pulled out by the aptamer or a contaminant during protein preparation prior analysis. Another cytoplasmic protein found was apolipoprotein A-I, which is in charge of lipid trafficking through the membranes of cells.[164]

Membrane Proteins

This is perhaps the most important group of candidates for the target. In this category four proteins were listed after the analysis: annexin A-2, desmoglein-I, junction plakoglobin and desmoplakin. Annexin A-2 is a calcium dependent peripheral membrane protein that plays a pivotal role in membrane organization and in endocytic pathways.[165] Several cancers have shown high levels of this protein; its roles are related to metastasis, apoptosis, angiogenesis and tumor invasion. Annexin A-2 and its binding molecule plasmin play an important role in cell invasion, migration, and cell adhesion.[166] The second group of proteins desmoglein-I, Junction plakoglobin and desmoplakin, play an important role in the formation of desmosomes and are expressed in tissue exposed to high mechanical stress. Generally these desmosomes are involved in the events of cell adhesion in epithelial cells.[167] The role of desmosomes has been suggested in carcinogenesis as they intervene with the regulation of cell proliferation and differentiation.[168] Recent studies have implicated desmosomes as potential tumor supressors in lung cancer via the Wnt/β-catenin signling pathway.[169]

Conclusions

In this chapter the major challenge was the development of a novel or improved method for the identification of the aptamer DOV-4, which is non-susceptible to protease treatment. During the optimization phase small changes were made to obtain the desired results, a more specific binding for the aptamer DOV-4 when compared to a randomized DNA oligonucleotide. Subcellular fractionation was the resource employed to eliminate non-specific binding from nuclear proteins a cellular, which include many DNA-binding molecules, to strengthen and fix the binding event between the aptamer

and its target the cross-linking molecule formaldehyde was used. The combination of these strategies aided in the identification of candidates proteins.

As mentioned previously, the combination of SDS-PAGE separation with MS-analysis demonstrated its power in the identification of potential proteins important during carcinogenesis. After MS-analysis a list of proteins was analyzed and discriminated primarily but not entirely by their cellular localizations. As expected, keratin-related proteins, which are contaminants in this type of analysis, were ruled out. Furthermore, cytoplasmic proteins, such as apolipoprotein A-I, hemoglobulin, and serum albumin were also present with high mascot scores during the analysis. Cytosolic and mitochondrial proteins were also observed in the analysis, but when compared to the basic methodology previously used in the Tan lab the percentage decreased dramatically. Finally, several membrane proteins remained as the potential targets: desmoplakin, desmoglein,and plakoglobin, all self-related proteins in the formation of desmosomes, and Annexin A-2, a peripheral membrane protein that plays a role in membrane organization.

Materials and Methods

Instrumentation and Reagents

Biotin-modified aptamer DOV4 (5’ ACTCAACGAACGCTGTGGAGGGCATCA GATTAGGATCTATAGGTTCGGACATCGTGAGGACCAGGAGAGCA- 3’ Biotin) and library (N80) were synthesized by standard phosphoroamidite chemistry using a 3400 DNA synthesizer (Applied Biosystems), and purified by reversed-phase HPLC (Varian Prostar using a C18 column and acetonitrile/triethylammonium acetate as the mobile phase). The streptavidin-coated magnetic nanoparticles (Dynabeads® M-280

Streptavidin) were obtained from Invitrogen. Transmembrane protein extraction reagent was purchased from (Five-photon Biochemicals).

Cell Culture and Buffers

The H23 cell line was purchased from American Type Cell Culture and maintained in RPMI-1640 (ATCC) culture medium supplemented with 10% Fetal Bovine Serum (FBS heat-inactivated) and 1% penicillin-streptomycin. Cells were incubated at 37ºC under 5% CO_2 atmosphere. All chemicals used in the buffers were purchased from Sigma, unless otherwise specified. To detach cells from the culture plate, non-enzymatic cell dissociation buffer was used, and the washing buffer (WB) contained 4.5 g/L glucose and 5 mM $MgCl_2$ in Dulbecco's phosphate buffered saline with $CaCl_2$ and $MgCl_2$ (PBS - Sigma). Binding buffer (BB) used for aptamer binding was prepared by adding yeast tRNA (0.1 mg/mL, Sigma) and BSA (1 mg/mL, Fisher) to the washing buffer to reduce non-specific binding. Fractionation buffer used for the separation of proteins was composed of 250mM sucrose, 20mM HEPES, and protease inhibitors at 10x general inhibitor and phenylmethanesulfonylfluoride (PMSF) at 1mM.

Aptamer Binding

H23 cells were washed twice, detached using non-enzymatic cell dissociation buffer, and incubated with a 250nM solution of biotinylated aptamer DOV4 at 4°C for 30 min.

H23 Crosslinking with its Target

Once cells were bound to aptamer DOV-4, the next step was to crosslink that protein-aptamer interaction with 4% formaldehyde methanol-free solution for 20 min at 4°C.

Extraction of H23 Proteins

The H23 cells were washed twice using WB, and incubated with transmembrane protein reagent containing 10x protease inhibitor and 200nM PSMF inhibitor. Incubation was performed at 4°C to limit endocytosis for 2 hours with mixing every 15 min to homogenize the sample. The mixture was centrifuged at maximum speed for a few seconds to pellet cell debris. The supernatant containing proteins in H23 was collected and suspended in fractionation buffer and subjected to subcellular fractionation.

Subcellular Fractionation of H23 Cells

Supernatant from the previous step was centrifuged at 4,000 rpm for 30 minutes in order to remove nuclear proteins and leave cytosolic and membrane proteins. To further separate these proteins a sucrose cushion centrifugation was performed as follows: 20% and 70% sucrose solutions were prepared and the supernatant from previous step was placed carefully on top of the sucrose cushion and centrifuged at 25,000 rpm for 1 hour at 4°C. The plasma membrane proteins appeared as a diffuse band at the interface between the two sucrose solutions. With an insulin syringe, the band was carefully removed and further centrifuged at 35,000 rpm to pellet the membrane proteins. In order to have a visual band, approximately 100 million cells were used.

Aptamer Target Purification for Protein Identification

After subcellular protein separation, the membrane proteins were incubated overnight with 200uL of streptavidin-coated magnetic beads. The beads were then washed to remove non-specific binding species and placed on a magnet for 30 min. Proteins were recovered by eluting with Laemmli sample buffer and heated at 60°C for 30min. Recovered proteins were resolved by SDS-PAGE gel.

Digestion of the Protein Band Prior MS Analysis

On the SDS-PAGE gel, the specific band present for aptamer DOV-4 was cut and sent out for MS analysis at the University of New Hampshire in Dr. Vernon Reinhold lab. Two simultaneous t extractions methods were performed, one with aqueous mixture the other with 50:50 aqueous: organic mixture to extract the proteins from the gel. Extracted proteins were subjected to trypsin digestion and processes on a HPLC-MS/MS (High resolution orbitrap MS). Analyzed proteins were processed using Mascot searching engine and Swissprot.

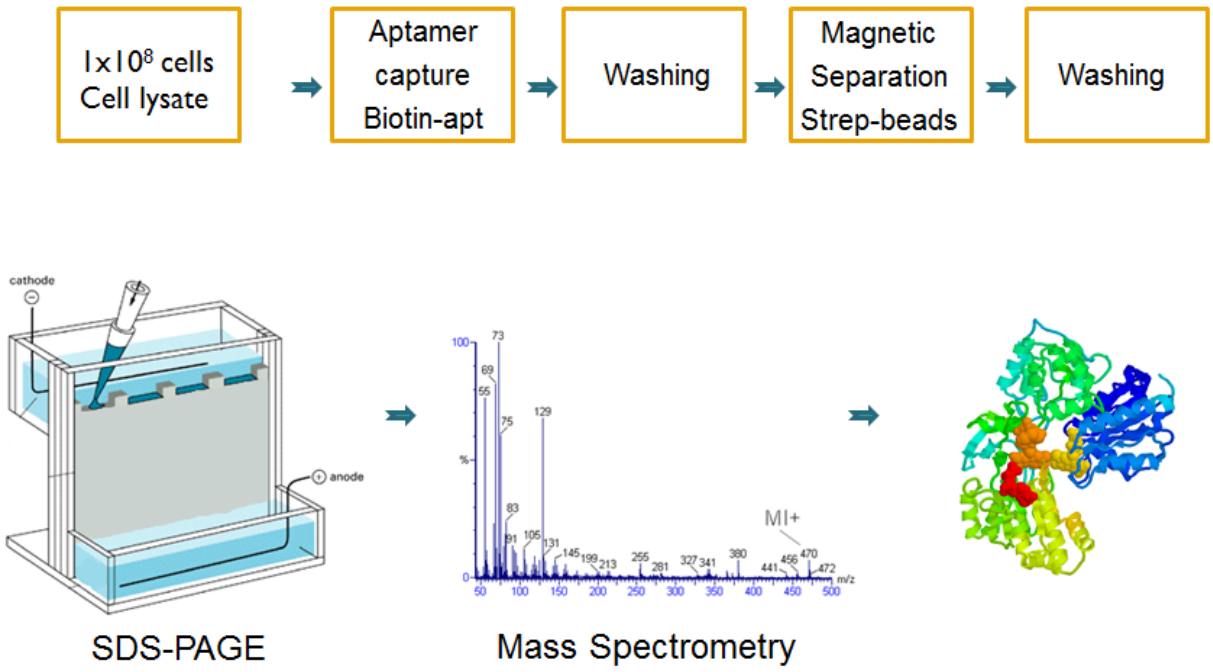


Figure 5-1. Methodology proposed for the elucidation of the aptamer DOV4.

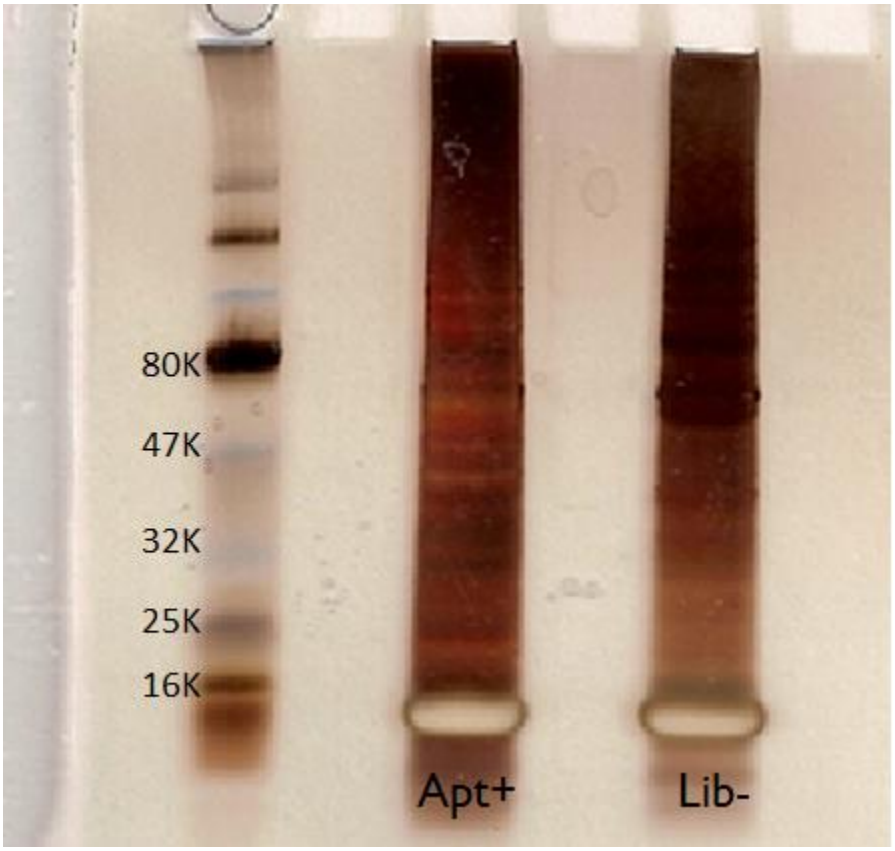


Figure 5-2. SDS-PAGE gel corresponding to the analysis of aptamer DOV4 in adenocarcinoma H23 cells. 1) Corresponds to the protein marker. 2) Proteins captured with the biotinylated aptamer DOV4. 3) Proteins captured with the biotinylated random DNA (library) negative control.

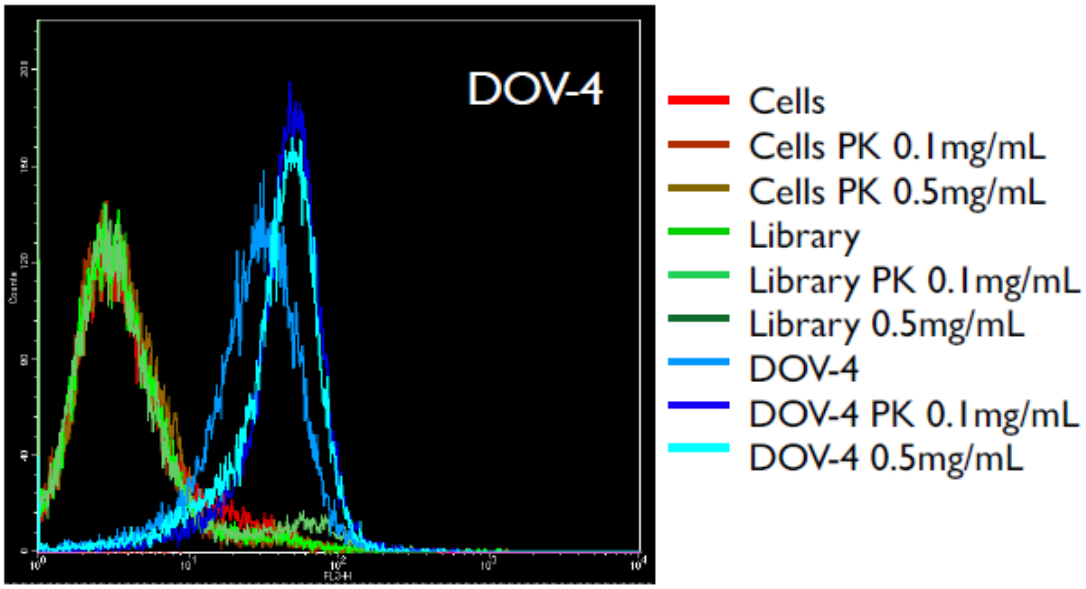


Figure 5-3. Aptamer DOV-4 binding after treatment of CAOV3 ovarian cancer cells with 0.1mg/mL and 0.5mg/mL of proteinase K.

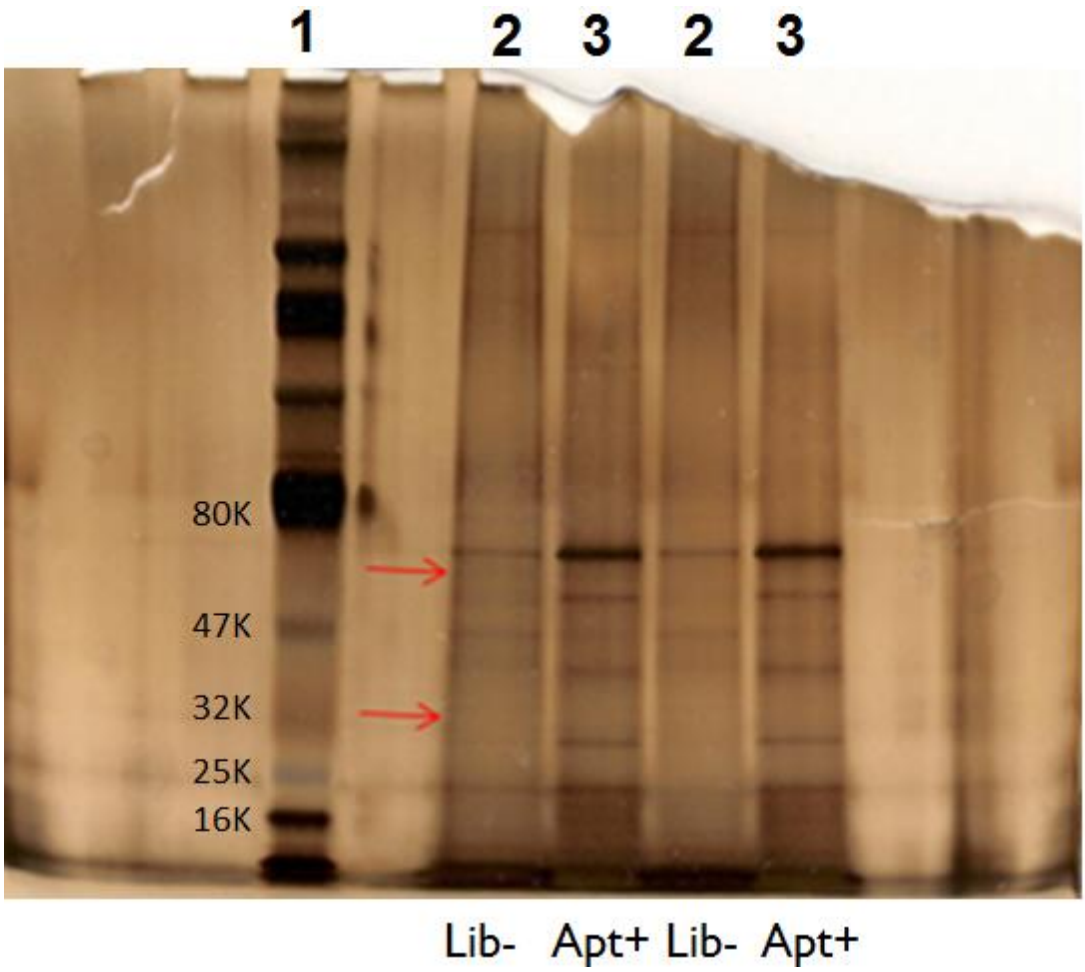


Figure 5-4. SDS-PAGE gel corresponding to the analysis of proteins captured with aptamers DOV-4 and random DNA after treatment with proteinase K. 1) protein marker. 2) Proteins captured with the biotinylated random DNA (library negative control). 3) Proteins captured with the biotinylated aptamer DOV4.

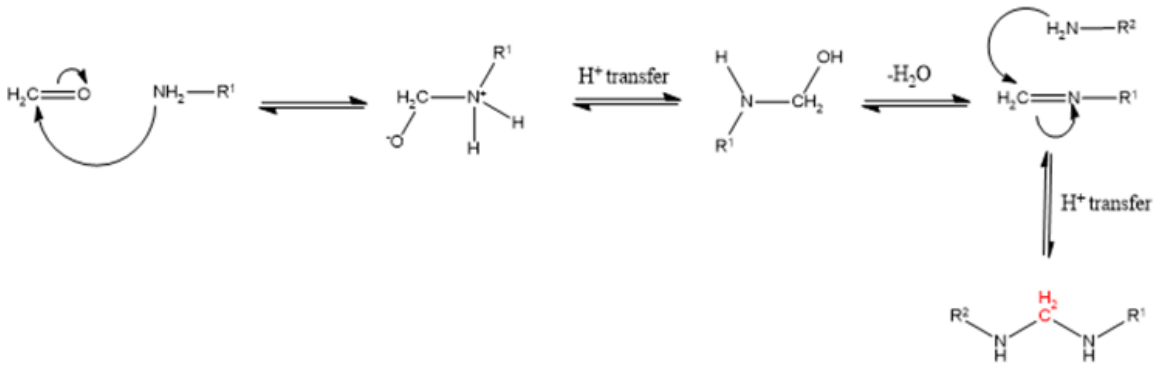


Figure 5-5. Mechanism of formaldehyde crosslinking between a DNA base and an amine residue from a nearby protein.

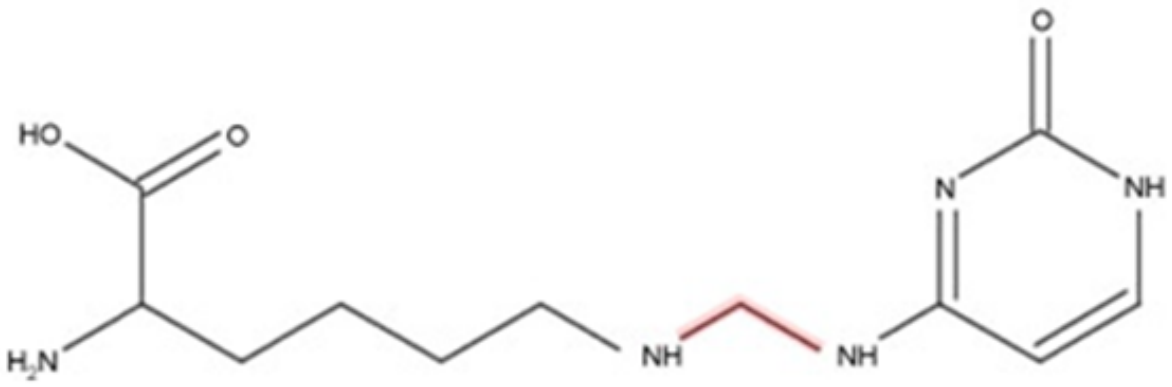


Figure 5-6. Formaldehyde induced crosslink between cytosine and lysine.

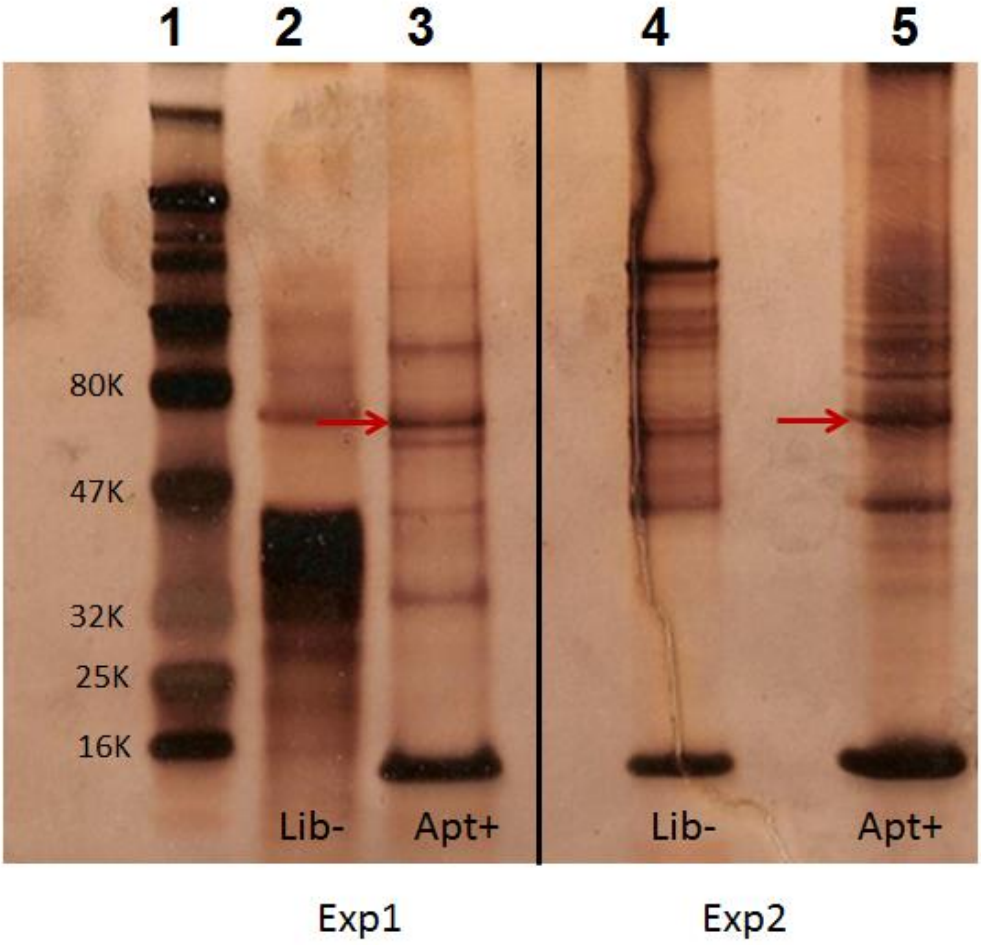


Figure 5-7. SDS-PAGE gel corresponding to the analysis of captured proteins by aptamer DOV4 in H23 cells. 1) Corresponds to the protein marker. 2) Proteins captured with the biotinylated random DNA (library) negative control. 3) Proteins captured with the biotinylated aptamer DOV4.

Swissprot Accession	Mass	Mascot Score (R1)	Mascot Score (R2)	Description
DESP_HUMAN	334063	684	548	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3
ALBU_HUMAN	71352	652	531	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
APOA1_HUMAN	30759	537	271	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1
HBB_HUMAN	16104	490	436	Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2
PLAK_HUMAN	82447	459	372	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3
DSG1_HUMAN	114720	384	307	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2
G3P_HUMAN	36204	303	225	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3
HBD_HUMAN	16161	249	172	Hemoglobin subunit delta OS=Homo sapiens GN=HBD PE=1 SV=2
CASPE_HUMAN	27952	241	210	Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2
ANXA2_HUMAN	38812	230	92	Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2
AXA2L_HUMAN	38809	230	N/A	Putative annexin A2-like protein OS=Homo sapiens GN=ANXA2P2 PE=5 SV=2
IGHG1_HUMAN	36605	220	N/A	Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1
FILA2_HUMAN	249320	208	170	Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1
HBA_HUMAN	15306	205	253	Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2
DSC1_HUMAN	101432	180	N/A	Desmocollin-1 OS=Homo sapiens GN=DSC1 PE=1 SV=2
THIO_HUMAN	12020	142	N/A	Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3
ARGI1_HUMAN	34887	142	98	Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2
S10A8_HUMAN	10886	138	N/A	Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1
TGM3_HUMAN	76932	133	96	Protein-glutamine gamma-glutamyltransferase E OS=Homo sapiens GN=TGM3 PE=1 SV=4

Figure 5-8. Mass spectral analysis of proteins captured by aptamer DOV-4 in H23 cells. Highlighted proteins are considered true hits based on MS analysis.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In this dissertation cell-SELEX methodology was utilized for the generation of a panel of aptamers capable of distinguishing the most common type of lung cancer (ADC) from normal bronchial cells. These aptamers displayed apparent dissociation constants in the nanomolar range, an important consideration in the use of aptamers in biological and biomedical applications. Furthermore these aptamers not only displayed binding to the target selection cell line, but also to other cancer types, such as colon cancer and ovarian cancer. At this point it is important to recognize the incredible value of cell-SELEX, which is a blind methodology that can generate specific aptamers towards a specific marker independently of the source tissue or cell line. In addition, some proteins involved in carcinogenesis can be common to different cancer types. Thus aptamers that bind to more than one type of cancer can be important for the identification of potential biomarkers.

One of the questions was to determine whether the selected aptamers can potentially be used in clinical samples. The selected aptamers were testing for binding in conditions similar to those present in clinical settings, such as the reaction conditions for fixation with paraformaldehyde. The selected aptamers were able to bind before and after fixation with affinity similar to that displayed in regular cell-SELEX conditions. This particular assay demonstrated one of the advantages of aptamers over antibodies, as aptamers do not need the antigen retrieval step to achieve binding to the target cells.

The tan group is a pioneer in the cell-SELEX technology, and different selections have been carried out for different types of cancer. To date, three different selections

have been performed for lung cancer, one for SCLC and two for NSCLC. The next step in this project was to profile these aptamers against a panel of lung cancer cell lines, in order to select the aptamers that would recognize the majority of lung cancer cells lines, as well as to identify aptamers that are selective for a histological subtype, in particular those that represent an immediate challenge in clinic settings.

After characterization and molecular profiling, the aptamers were use as detection and capturing molecules for circulating tumor cells in blood. In this project two different strategies were employed for the same purpose: a magnetic separation and a cell sorter. To be comparable to literature reports in the isolation of CTCs, it was initially intended to use the antibody EpCAM in both methods. However, after molecular profiling, it was determined that EpCAM was not ubiquitously expressed in lung cancer cells, but only in ~50% on the histological subtype ADC. These findings indicated the lung cancer research has been limited in the past.

To detect CTCs in NSCLC, the Ab EpCAM was used to corroborate the identity magnetic-isolated cells from blood. As detection molecules, aptamer HCH3 and S1 were used for the recognition of cancer cell in blood. Likewise, aptamers HCH3, HCH12, sgc8 and DOV4 were used as detection molecules in combination with antibody CD-56 to double-label cells for isolation and sorting by FACS. The LOD for both methodologies was similar, with ~ 50 CTCs for the magnetic separation method and ~60 CTCs using cell sorting. Also the background number of cells increased as the number of spiked cells was reduced. After optimization of the cell sorting methodology, 5 clinical blood samples from metastatic SCLC patients prior to chemotherapy were analyzed, and our methodology was able to detect CTCs in all five samples. However,

during sample processing by the CTSI at the University of Florida in, samples 843 and 076 showed a high number of dead cells. These results were due to the improper manner of storing the isolated buffy coat that I obtained 1 year previously. Also, during the first trimester of the clinical trial, we learned that the compound DMSO was not part of the standard operational procedures (SOP). As expected, the samples with high counts of live cells provided the best and anticipated results for a high CTC count in metastatic lung cancer patient samples. As SCLC is the most aggressive type of lung cancer, a high burden of cells circulating in the peripheral blood at the time of collection was expected.

In the last part of this dissertation, the aptamer DOV4 was selected for target elucidation because its target molecule is highly expressed in the majority of lung cancer cell lines with no detectable response by flow cytometric analysis in normal bronchial cells. Two major obstacles were confronted during the elucidation: the large amount of non-specific binding proteins and the poor stability of the binding event between the aptamer and its target. To address these issues, an initial step of subcellular fractionation was included to isolate only membrane proteins and to reduce the number of cytosolic and DNA binding proteins present from the nucleus. The interaction between the aptamer and its target was fixed by using the DNA-protein crosslinking molecule formaldehyde.

After optimization and MS analysis by the Reinhold group at the University of New Hampshire, a list of candidate proteins was obtained for further analysis. Proteins were discriminated based on subcellular localization and focusing on membrane proteins, because the target of aptamer DOV4 as the aptamer target is found primarily

in the membranes of lung cancer cells H23 and CAOV3, the cell line from which this aptamer was selected. Keratins and skin-related proteins were also found in the potential target list, because they are common contaminants in MS. Some cytosolic, cytoplasmic, and mitochondrial proteins were also present on the list, suggesting the subcellular fractionation step was not 100% efficient. Finally, two membrane proteins, Annexin A2 and desmoplakin-related proteins involved in the formation of desmosomes constitute the two potential targets for aptamer DOV4. These results demonstrate that cell-SELEX technology is an innovative and powerful methodology for the holistic approach and understanding of a particular disease, in this case lung cancer, without prior knowledge of a target. Initially the aptamers are selected against the target tissue cell line, and subsequently these aptamers can then be utilized as affinity and capturing molecules for the investigation of that disease. Furthermore, reducing the number of potential targets could lead to the identification of biomarkers for several cancers, as most of the proteins important in angiogenesis and tumorigenesis are conserved in several cancer types.

Future Directions

The next step in this work is to further test the selected aptamers in formalin-fixed paraffin embedded FFPE samples, which may yield valuable information for the study of human cancer. These experiments would confirm the feasibility of translation ability of the cell-based selected aptamers for clinical use. Furthermore, rather than competing with antibodies, aptamers and antibodies could be used in combination to produce a more solid determination of the histological lung cancer subtype, thereby providing knowledge for a more appropriate treatment for lung cancer patients.

In the detection of CTCs project, additional research is needed to analyze these background cells that were non-specifically captured by the aptamer. Perhaps, a size exclusion experiment could determine if some CTCs present in the clinical samples are indeed cancer cells but do not possess either one the markers used for their isolation. Some research has shown that some lung cancer patient samples lack common molecular markers. Another possible experiment could be to label the non-specifically captured cells with other common markers for SCLC that were not employed in the proposed strategy.

Finally, in the elucidation of the aptamer DOV4 target, the next step will be to obtain antibodies against those targets to perform a competition study between the aptamer and antibody. This experiment could indicate if the aptamer and antibody share the same binding site. Alternatively, 1) a cell line lacking of either one of the potential targets can be subjected to binding assay with the aptamer; or 2) a cell line can be genetically modified to express these targets and verify their binding by flow cytometric analysis.

APPENDIX A
SYNTHESIS APTAMER-RADIOACTIVE ISOMER DRUG CONJUGATE FOR SCLC TREATMENT

The problem that clinicians face when treating lung cancer is the fact that SCLC is almost always advanced beyond the point where curative therapy can be pursued. This occurs because conventional radiation therapy can be only applied to specific regions (in this case, the central chest) which are not possible when the cancer has spread beyond the confines of the chest. Intervention can improve significantly especially for advanced-staged disease if we are able to target and kill distant metastatic cells, but unfortunately there are no specific markers that can confidently been used without affecting normal cells. The flexibility of manipulating nucleic acids without significantly affecting its activity makes aptamers suitable in many diagnostic and therapeutic application platforms. For instance, aptamers have been employed in immunoprecipitation (oligonucleotide-precipitation) assays, western blot analyses, enzyme-linked immunosorbent assay (ELISA)-like formats, and flow cytometry assays. Treatment of SCLC is much more effective if the chemotherapy is combined with radiation therapy concurrently. The ability to selectively kill cancer cells without affecting the surrounding normal cells is a significant component in designing any targeted therapeutic intervention. This will require very selective molecular probes that target markers on the cancer cells but that are not found on the normal cells. Such probes should also not be sticky to cell surfaces in order to minimize the side effects. Therefore, the availability of SCLC-specific aptamers will help us design specific targeted therapies. To achieve this, we propose to conjugate SCLC specific aptamers to radioisotopes (90Y, 135I) and the drug cisplatin and simultaneously deliver this entire conjugate SCLC cells.

The goal of this aim was to develop conjugation chemistry to link SCLC aptamers with radioactive isotopes currently in nuclear medicine and cancer treatments such as Iodine-131, Phophorus-32, Lead-212, Yttrium-90[170] and/or actinium-225 and test these conjugates as possible probes for radiotherapy.

Results and Discussion

To synthesize aptamer-radioactive isomer drug, we employed carboxyl-amino chemistry for the conjugation of aptamers to DOTA. Similar procedure was utilized to conjugate random sequence to DOTA. This is shown schematically in [Figure A1]. The conjugate was purified by HPLC [Figure A2].

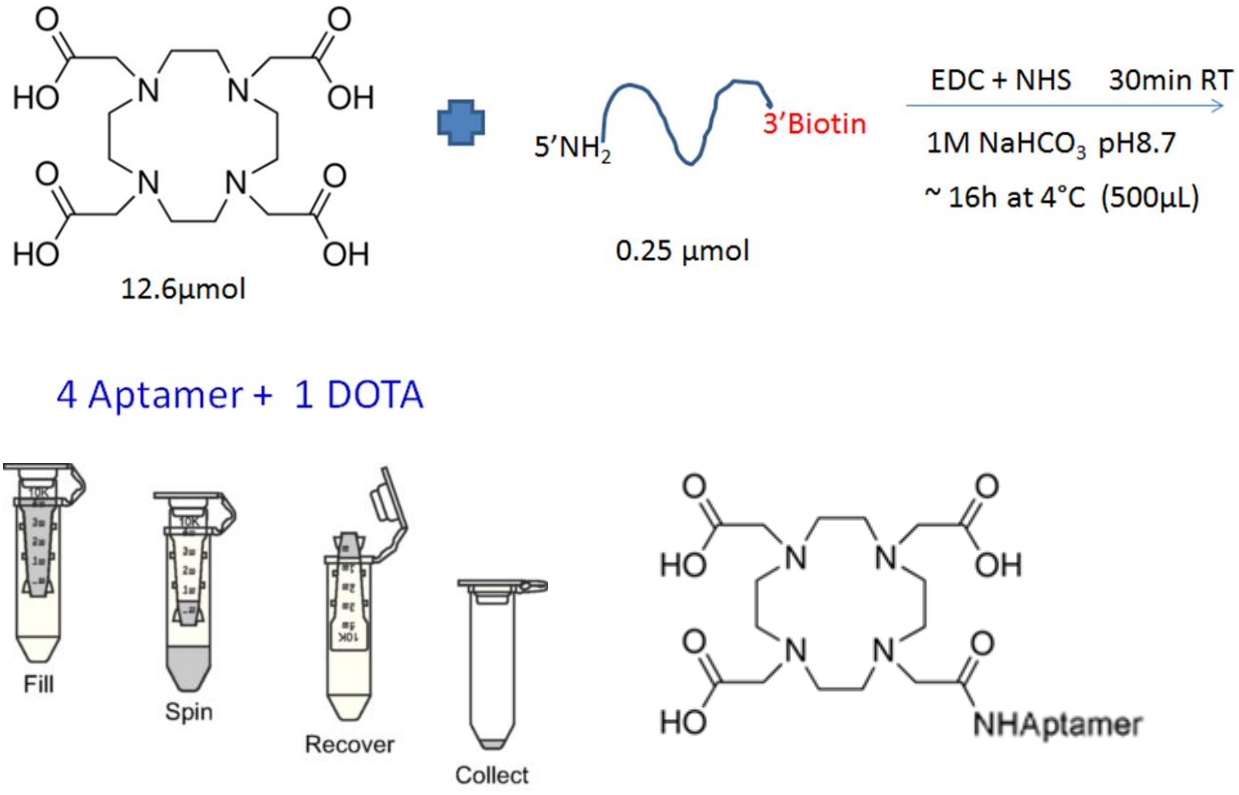


Figure A-1**.** Step-by-step synthesis of DOTA-Aptamer-conjugate. The success of the reaction was confirmed by HPLC, and compared to compound with similar structure previously reported in the literature. To purify the reaction, desalting columns with molecular weight cut-off of 30KDa was used, and buffer exchange, 1M $NaHCO_3$ to 10mM.

Once the conjugated was successfully synthesized and purified, we proceeded to test this compound with lung cancer cell line A549 and normal lung cells HBE135 E6/E7 by flow cytometry analysis (Figure A3).

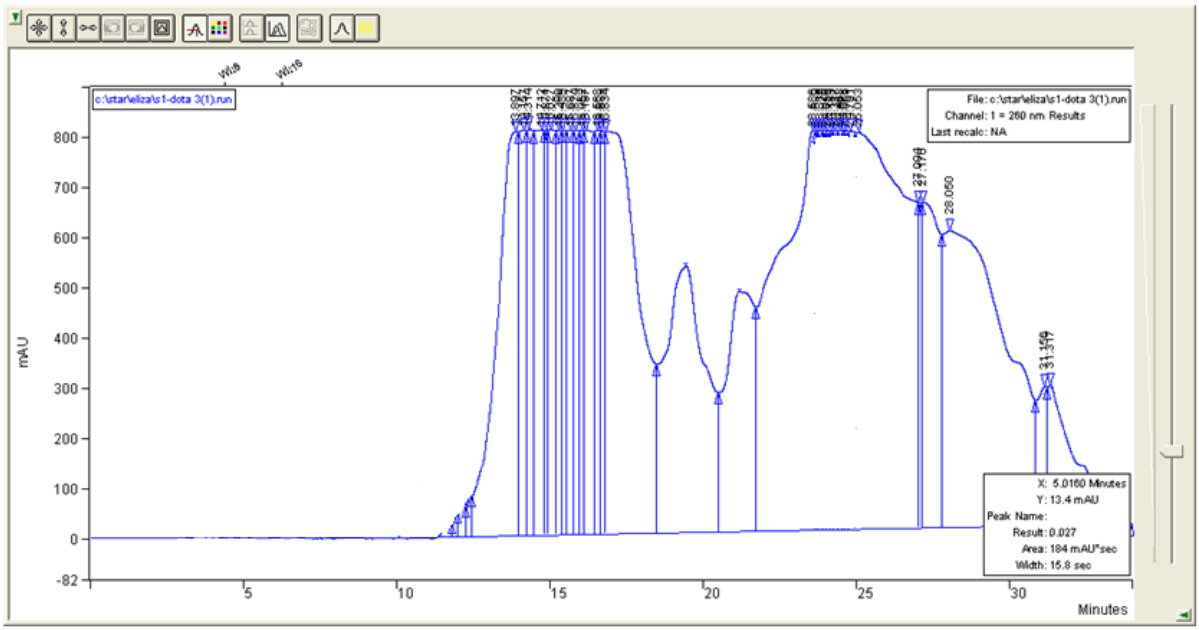


Figure A-2. Purification of DOTA-aptamer conjugate by HPLC. The peak corresponding to 12-17minutes was collected as it represents the conjugate.

Since the ultimate goal is use this conjugate to deliver radionuclide to cancer cells, we assessed if the conjugate can be internalized into cancer cells. In our previous studies, aptamers alone or in conjugate with other reagents have been demonstrated to be able to internalize into target cancer cells. The internalization of the aptamer-conjugate was analyzed by confocal microscopy as shown [Figure A4], the aptamer conjugate internalized into target cells, and a prerequisite for further use of the conjugate.

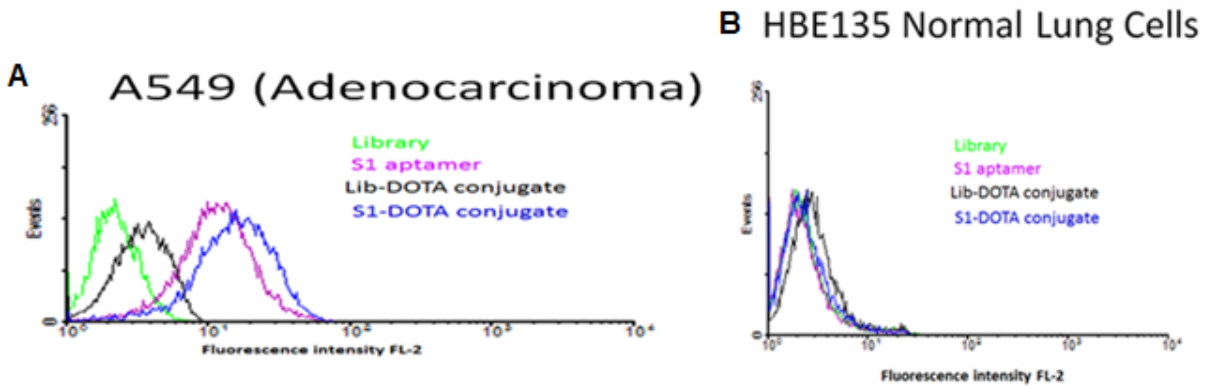


Figure A-3**.** Flow cytometry analysis of DOTA-conjugate with cell lines A549 and HBE 135. Random sequence and specific aptamers were tested along with aptamer and random sequence conjugated to DOTA compound. B. Flow cytometry analysis of DOTA-conjugate with normal lung cell line HBE135 E6/E7. Random sequence and specific aptamers were tested along with aptamer and random sequence conjugated to DOTA compound.

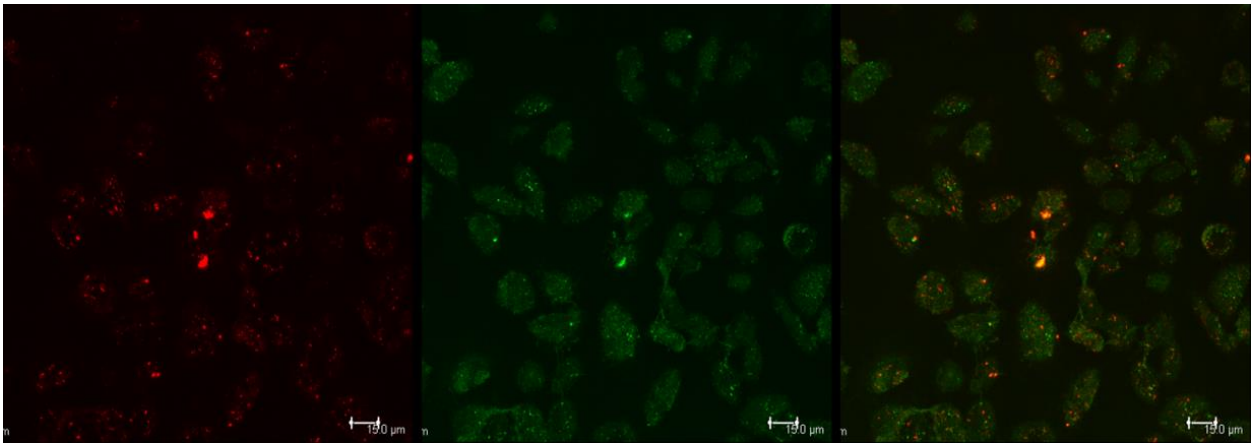


Figure A-4**.** Internalization of aptamer-conjugate in lung cancer cell line A549. Left panel (DOTA-aptamer conjugated with Cy5) middle panel (lysosome dye) right panel (merge picture of previous two panels) this indicates that the conjugate could be internalized by cell line A549 through aptamer-target mediated endocytosis

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BIOGRAPHICAL SKETCH

Elizabeth Jimenez was born in Santiago de Cali, Colombia to a working class family. She earned her Bachelor of Science degree in Chemistry at the Universidad del Valle in 2004. During her senior year she traveled to the United States for a student exchange program in the Whitney Laboratory for Marine Bioscience. She worked there three years before starting her PhD studies in Biochemistry at the University of Florida (2008) under the direction and guidance of Dr. Weihong Tan. Her graduate work focused on the development and clinical translation of aptamers in lung cancer. During summer of 2013 she completed her PhD studies and started a postdoctoral position at the University of Florida in the Department of Physiological Sciences.