Antibody-Chip for Multianalyte Immunoassay of Thyroid Hormones

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(Bharti Jain**)**

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

(Bharti Jain)

List of Publications arising from the thesis

a. Published:

1. A sensitive solid-phase micro-spot radio-tracer assay for the simultaneous estimation of thyroid stimulating hormone and thyroglobulin. Bharti Jain, Savita Kulkarni, C.Gholve, J.Kumarasamy, M.G.R.Rajan in Indian Journal of Applied Research, 2015, 5(9), 269-271.Available online DOI : 10.15373/2249555X.

b. Accepted:

1. A microarray immunoassay for serum thyrotropin and thyroglobulin using antibodies immobilized on track-etched membranes. Bharti Jain, J.Kumarasamy, C.Gholve, Savita Kulkarni, M.G.R.Rajan in Indian Journal of Clinical Biochemistry. Published online DOI: 10.1007/s12291-016-0589-2.
2. A Multi-analyte Immunoassay for Thyroid Related Analytes. Bharti Jain, J.Kumarasamy, C.Gholve, Savita Kulkarni, M.G.R.Rajan in Journal of Immunoassay and Immunochemistry. Published online DOI: 10.1080/15321819.2016.1250771

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Dedicated

To my

Father

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Bharti Jain.

TABLE OF CONTENTS

SYNOPSIS

Antibody-Chip for Multianalyte Immunoassay of Thyroid Hormones.

Introduction

Immunoassays play a prominent role in the analysis of many analytes of clinical importance in blood, CSF and other body fluids in patients, since they were first reported simultaneously in the early 1960s by Yalow and Berson from the USA and Ekins from the UK [1-3]. Immunoassays have been widely in use for the past half a century and have revolutionized clinical endocrinology [4-6]. However, immunoassays, are currently performed for a single analyte at a time and, hence, are low through-put assays and consume relatively large amount of expensive antibodies and patients' sample. Further, many clinical conditions require information on more than one analyte for an unequivocal clinical diagnosis or a differential diagnosis. The need for rapid and reliable measurement of multiple analytes in clinical samples has encouraged the development of multi-analyte immunoassays (MAIA), the concept of which was put forth by Ekins in the 1980s, in his "ambient analyte theory" [7, 8]. MAIA consists of "antibody chip" which contains several antibodies, each specific to a different analyte, spotted in minute quantities on spatially isolated and predetermined sites on an inert solid support. When the sample is incubated on an antibody chip, all analytes of interest will bind to their specific antibodies and as a result, all of them can be estimated simultaneously. Substantial savings could be made in terms of assay cost, assayist time and sample volume by using MAIA [9]. In spite of several advantages, there are some of the critical issues that have to date prevented MAIA from emerging as the gold standard in immunoassay industry. These involve selection of suitable solid support, maintenance of antibody activity on the chosen solid supports, the ability to achieve low detection limits,

minimize cross-reactivity and the capacity to maintain MAIA performance over a broad dynamic range [10, 11]. Moreover, some analytes are small, thus not amenable to sandwich immunoassays and others are large. Some are present in lower concentrations than others and therefore require assays of greater sensitivity. It is a challenge to produce a single assay, accommodating all these differences that can provide individual values for each analyte.

Detection of thyroid disorders utilizes several analytes whose levels are measured as an indication of the presence and type of disorder. Triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) are important in hyperthyroidism and hypothyroidism. In autoimmune thyroid disorders, anti-thyroglobulin (anti-Tg) antibodies and anti-thyroid peroxidase (anti-TPO) antibodies are elevated. Thyroglobulin is used as a marker for the thyroid cancer. Thus, to diagnose a thyroid disorder by serum analyses, detection of four to five different analytes is required [12, 13]. Rather than measuring these analytes individually, using present immunoassay methods, all the analytes can be detected simultaneously using MAIA, in a single assay. Benefits of doing so include low costs, reduction in time involved in analysis and reporting and less probability of errors. Therefore, the aims and objectives of the thesis are:

- Identifying a suitable support and standardizing the surface chemistry for the immobilization of the antibodies to make an ‘antibody-chip’
- Standardizing the assay conditions for development of the microarray immunoassay (MI) for individual analytes (T4, TSH and Tg) and its validation and comparison with routinely done RIA and IRMA for these analytes.
- Standardizing the assay protocol and validation of MAIA for different analyte panels. (T4 and TSH, TSH and Tg and T4,TSH and Tg panel)
- Detection and quantification of analytes.

The above work is presented in the thesis in the following chapters:

CHAPTER 1 will give an introduction to the concept of immunoassays and the published information available in literature to the present work and its objectives. It will give general introduction to thyroid and its diseases, conventional techniques like immunoassays, concepts of MAIA and the techniques used in its development. Also presented in this chapter are the parameters in designing an antibody-chip, vis-à-vis the choice of solid-supports that can be used, the chemistry used to treat the solid-support for immobilization of antibodies, the immunoassay procedures adopted and the end-point detection methods. The chapter will also describe the various critical factors and challenges faced in the development of MAIA.

CHAPTER 2 will describe experimental work done and results obtained while testing a large number of solid-supports and the selection of the suitable support and standardizing surface chemistry for immobilization of antibodies.

We explored, for the first time, the use of polycarbonate (PC) and polyethyleneterepthalate (PET) track-etched membranes (TEM) as a novel support for the development of MAIA. PC-TEM and PET-TEM were compared with glass, PVDF and nitrocellulose to evaluate its utility as a suitable support for MAIA. Both hydrophobic PC and PET-TEM and hydrophobic PC-TEM (provided by Dr. R. H. Iyer, WMD, BARC) as well as hydrophilic PC-TEM (Millipore) were tested. Hydrophobic PC-TEMs of different pore densities was tested to the optimum membrane for immobilization of antibodies. Glass was extensively cleaned, silanized using γ-aminopropyltriethoxysilane and activated with 2.5 % glutaraldehyde according to the method of Bhatia et al [14]. All other membranes and TEMs used were activated using 2.5% glutaraldehyde.

All the immobilization studies were done using anti-T4 antibodies. Using micropipette, 0.5 µl (1mg/ml) of anti-T4 antibodies were spotted on various supports. Nonspecific binding sites

were blocked using 4% BSA followed by reaction with ^{125}I-T4. The spot intensity and morphology was viewed using autoradiography. The autoradiograms were scanned and densitometric analysis of autoradiograms provided mean spot intensity of each spot. Supports were judged based on spot morphology, signal intensity and background obtained on each surface. Of all the supports tested, highest signal to noise ratio (SNR) with good spot morphology was obtained with both hydrophobic PC- and PET-TEM followed by glass and hydrophilic PC-TEM. This was followed by hydrophilic PET-TEM. Although glass proved to be a good substrate giving good spot morphology and low background, but extensive pre-treatment required and fragility of glass was a serious limitation, since the antibody chips have to be made in large batches and stored. Nitrocellulose membranes gave lowest SNR due to low signal and high background obtained. PVDF membrane was very hydrophobic and, hence, gave very small spots with SNR comparable to hydrophilic PET-TEM. It was found that the signal intensity increases as the pore density increases. A hydrophobic PC-TEM with pore density of 10^8 pores/cm^2 was found to be better than the ones with lower pore densities, the former was used for assembling antibody-chips for MAIA. Since hydrophobic PC- and PET-TEM was not continuously available from Dr. Iyer, commercially available hydrophilic PC-TEM from Millipore, USA, was selected as a substitute. Immobilization studies using different concentration of the antibodies, for different time periods, showed that antibodies spotted at the concentration of 1mg/ml, and incubated for two hours gave optimum results.

CHAPTER 3 will show the experimental results of the proof of concept of the validity of MI for quantification of three individual analytes (T4, TSH and Tg). The chapter will describe the use of radiotracers and auto-radiography on X-ray films to quantify the antibody occupancy by the labeled analyte or labeled second antibody for competitive and non-competitive assays respectively. With the availability of a Phosphor-Imager, it was found to

be better than X-ray films/gamma counter and, hence, used for detection and quantitation of analytes.

A competitive radio-immunoassay was standardized for T4 and non-competitive immunoradiometric assay was standardized for TSH and Tg. Polyclonal anti-T4 antibodies, monoclonal anti-TSH and polyclonal anti-Tg antibodies were spotted (0.5 µl) (1mg/ml) individually, on separate PC-TEMs. Parameters, such as concentration of tracer (^{125}I-T4/^{125}I-anti-TSH monoclonal antibodies/^{125}I-anti-Tg monoclonal antibodies) and time of incubation were optimized for each analyte. The optimum tracer concentration was 60,000cpm/100 µl for T4 was and 100,000cpm/100 µl for TSH and Tg. The optimum tracer incubation time was found to be two hours for T4 and TSH and longer incubation was required for Tg. The developed MI was validated by studying the sensitivity and range of the assay, precision studies, linearity and recovery, comparison with the established techniques. MI were optimized with high sensitivity (0.32 µg/dl for T4, 0.01 µIU/ml for TSH and 0.05 ng/ml for Tg) and wide working range (0.32-20 µg/dl for T4, 0.01-100 µIU/ml for TSH and 0.05-250 ng/ml for Tg) comparable to conventional immunoassays. Precision studies done using three control serum for T4 and two control serum for TSH and Tg showed both intra-assay and inter-assay %CV was less than 20% for all the three analytes. Linearity was determined in serum sample containing high concentration of analyte which were serially diluted in standard matrix. Observed to expected ratio (O/E) obtained for linearity studies varied from 80% to 102% for T4, 85.5% to 112 % for TSH and 72.5% to 104 % for Tg. Recovery was determined by mixing the standards in samples in 1:1 ratio. Recovery, expressed as a percentage of the expected values, was determined in four serum samples and ranged from 74.6% to 113.6% for T4, 93.3% to 126.9% for TSH and 69.8% to 102.6% for Tg. One hundred and forty (140) human serum samples were analyzed for T4, TSH and Tg by the standardized MI and values obtained were correlated to conventional immunoassays. Good

correlation coefficient was found between the two techniques (r =0.94, p<0.001 for T4, r=0.98, p<0.001 for TSH and r=0.97, p<0.001 for Tg, n=140 for all). The regression equation obtained was [MI = 0.98*RIA+0.03 for T4, MI=1.01*IRMA-0.36 for TSH and MI = 0.93 IRMA-2.6 for Tg]. Thus, the standardized MI fulfills all the validation criterias. The next proposed application of the technology is in simultaneous detection of different analytes for thyroid disorders.

CHAPTER 4 will show the experimental results obtained with the optimization and validation of the MAIA for a panel of T4 and TSH useful for testing thyroid function and panel of TSH and Tg important in thyroid cancer patients.

T4 and TSH MAIA

Competitive immunoassay was developed for T4 and sandwich immunoassay was developed for TSH. PC-TEMs were spotted with 0.5 µl of 1 mg/ml polyclonal anti-T4 and monoclonal anti-TSH antibodies. Assay optimization revealed, tracer (mixture of ^{125}I-T4 and ^{125}I-monoclonal TSH antibodies) concentration of 60,000 cpm/100 µl for T4 and 100,000 cpm/100µl for TSH incubated with PC-TEM for two hours was optimum. Typical standard curves for T4 and TSH were generated using 50µl of serum sample with analytical sensitivity of 0.12 µg/dl for T4 and 0.03 µIU/ml for TSH. The upper limit of assay was 20 µg/dl for T4 and 50 µIU/ml for TSH. Reproducibility of MAIA was good with both intra and inter-assay % CV for two serum pools (n=10) being less than 20%. Recovery determined in three serum samples spiked with two standards varied from 80.3% to 103.7%. O/E for linearity was found to be varying from 77.5% to 141.6%. Thirty-four human serum samples measured by MAIA for T4 and TSH. The results obtained correlated well with the T4 results obtained by conventional RIA (MAIA = 0.86RIA−0.04, r = 0.92, p < 0.001). Results of the TSH MAIA

also showed a good correlation with conventional TSH IRMA (MAIA = 1.01 IRMA−0.70, r = 0.995, $p < 0.001$).

TSH and Tg MAIA

Sandwich immunoassays were developed for both TSH and Tg. PC-TEMs were spotted with 0.5 µl (1mg/ml) of monoclonal anti-TSH and polyclonal anti-Tg antibodies. Spotted membranes were reacted with 50 µl of standard/sample along with optimized concentration of tracer containing 100,000 cpm/100 µl of ^{125}I-monoclonal TSH antibodies and 100,000 cpm/100 µl of ^{125}I-monoclonal Tg antibodies for overnight. Typical standard curves for both TSH and Tg were generated. High analytical sensitivity (0.03 µIU/ml for TSH and 0.1 ng/ml Tg) and satisfactory working range (0.03-50 µIU/ml for TSH and 0.1-250 ng/ml for Tg) was obtained for both analytes. Precision of the assay was acceptable with both intra and inter-assay %CVs for ten replicates of two samples was less than 20%.O/E for recovery determined in three serum samples varied from 76% and 111.2%. Thirty four human serum samples were measured by TSH and Tg MAIA and results obtained correlated well with the TSH results obtained using conventional IRMA. (MAIA=0.95IRMA+0.32, r = 0.98, $p < 0.001$). Results of the Tg MAIA also showed a good correlation with conventional Tg IRMA (MAIA=1.03 IRMA+10.1, r = 0.91, $p < 0.001$). The results described, demonstrate the applicability of MAIA to monitor simultaneously more than one analyte per sample using small amount of sample and reagents.

CHAPTER 5 will describe the optimization and validation of the MAIA for T4, TSH and Tg, where all three antibodies: anti-T4, anti-TSH and anti-Tg antibodies were spotted on PC-TEM. Cross reactivity, among the three analytes was determined by reacting all the three immobilized capture antibodies with either individual tracer or tracer cocktail along with a

standard-mixure where all three analytes were present at high concentration of all three analytes. No noticeable cross-reactivity was seen among the antibodies for the analytes tested.

Antibody-spotted membranes were incubated with 50 µl of standard-mixture cocktail mixed with tracer cocktail (mixture of ^{125}I-T4, ^{125}I-TSH antibodies and ^{125}I-Tg antibodies) containing optimum concentration of tracer at 60,00 cpm /100 µl for T4, 100,000 cpm/100 µl for TSH and 1,50,000 cpm/100 µl for Tg for overnight. Standard curves with high analytical sensitivity (0.11 µg/dl for T4, 0.07µIU/ml for TSH and 0.03 ng/ml for Tg) wide working range (0.11-20 µg/dl for T4, 0.07-50 µIU/ml for TSH and 0.03-250 ng/ml for Tg) suitable for clinical use was obtained. Precision of the MAIA was acceptable with both intra-assay and inter-assay %CV less than 20%. O/E for both recovery and linearity falls between expected ranges of 100± 20% showing that matrix used for preparation of standards is compatible with serum samples. Fifty four human serum samples were measured by T4, TSH and Tg MAIA and values obtained were compared to conventional assays. MAIA showed good correlation with conventional assays [(MAIA=1.1RIA-0.47, r=0.91, p<0.001 for T4), (MAIA=0.95 IRMA+0.51, r = 0.98, p < 0.001 for TSH) and (MAIA = 0.99IRMA+2.08 r = 0.89, p < 0.001 for Tg). The validation study demonstrated MAIA to be both quantitative and reproducible at assay working ranges. In conclusion, a MAIA has been developed that compares favorably with the standard RIA/IRMA assay in regards to sensitivity and specificity utilizing only 50 µl of serum sample. The technique is simple to perform and will save on assayist time and costly reagents.

CHAPTER 6 will be a discussion and conclusion on the results obtained and a general discussion on the application of anti-body chip and MAIA in the clinical laboratory context.

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PUBLICATIONS:

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LIST OF FIGURES

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Abbreviations

2-D - 2 dimensional
3-D - 3 dimensional
Ab - Antibody
AFM - Atomic force microscopy
Ag - Antigen
ANS - 8-Anilinonaphthalene-1-sulfonic acid
AP - Alkaline phosphatase
a.u. – Arbitrary units
BRIT - Board of Radiation and Isotope Technology
BSA - Bovine serum albumin
CCD - Charge coupled device
cpm - Counts per minute
CT- Calcitonin
CV - Coefficient of variation
DAB - Diaminobenzidine
DIT - Diiodotyrosine
dpm - Disintegration per minute
DTC - Differentiated thyroid carcinoma
ELISA - Enzyme Linked Immunosorbent Assay
FDA - Food and Drug Administration
FSH - Follicle stimulating hormone
fT4 - Free thyroxine
GATS - γ-aminopropyltriethoxysilane
GH - Growth hormone
hCG – Human chorionic gonadotropin
HFS - Hormone free serum
HHFS - Horse hormone free serum
HRP - Horseradish peroxidase
IRMA - Immunoradiometric assay
LH - Luteinizing hormone
LOD - Limit of detection.
MAIA - Multianalyte immunoassay

MI - Microarray immunoassay

MIT - Monoiodotyrosine

NRCC - National Research Center on Camel

NSB - Non-specific binding

PBS - Phosphate Buffer Saline

PC - Polycarbonate

PET - Polyethyleneterepthalate

PMT - Photomultiplier tube

PVDF - Polyvinylidene fluoride

QC – Quality control

RCA - Rolling circle amplification

RIA - Radioimmunoassay

RT - Room temperature

SD - Standard deviation

SHFS - Synthetic hormone free serum

SNR - Signal to noise ratio

SPR - Surface plasmon resonance

T3 - Triiodothyronine

T4 - Thyroxine

TEM - Track-etched membrane

TgAb - Tg antibodies

Tg - Thyroglobulin

TMB - 3,3´,5,5´-tetramethylbenzidine

TPOAb - TPO antibodies

TPO - Thyroid peroxidase

TRH - Thyrotropin releasing hormone

TSA - Tyramide signal amplification

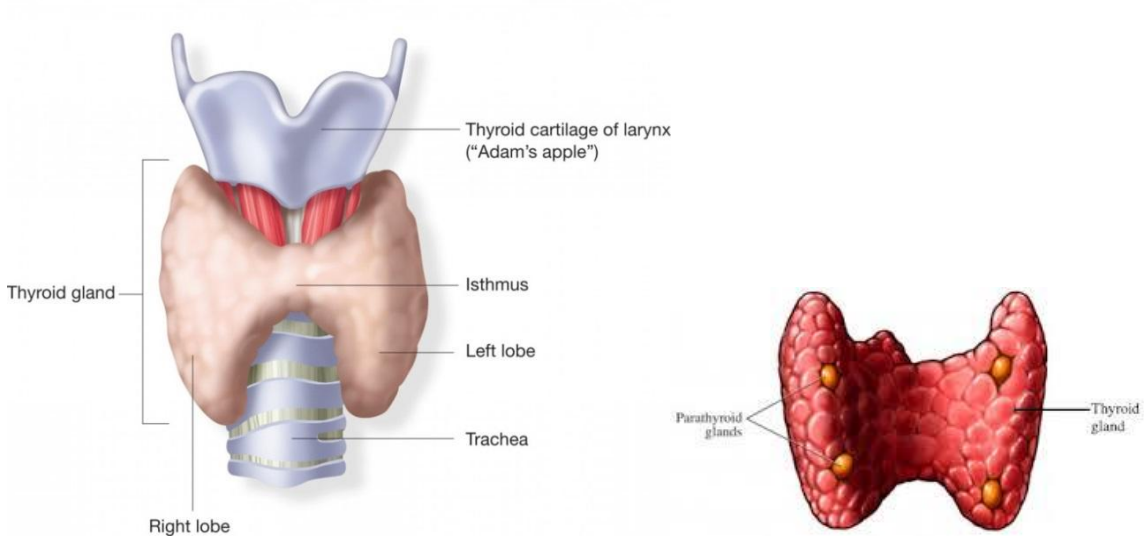
TSH - Thyroid Stimulating Hormone

ud - Undetectable

CHAPTER 1
Introduction

1.1. The thyroid gland

The thyroid gland (Greek: meaning “shield gland”) is a ‘butterfly-shaped’ gland located at the base of neck. It wraps itself about and becomes firmly fixed to the anterior and lateral parts of the larynx and trachea. It is made up of two lobes, joined together by a narrow band of thyroid tissue, known as the isthmus. The thyroid hormones regulate both anabolism and catabolism, which determine the basal metabolic rate as well as growth and maturation of the human body. The thyroid performs these processes by producing thyroid hormones, principally thyroxine (T4) and triiodothyronine (T3) and constantly releasing them in blood stream. The parathyroid glands located in the thyroid produces the hormone calcitonin (CT), which plays a role in calcium homeostasis [1].



(a) (b)

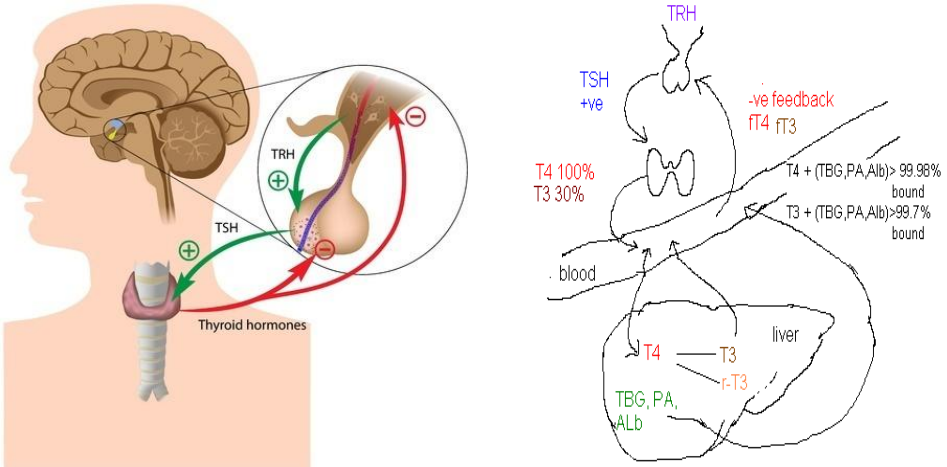
Figure 1.1 (a) Structure of thyroid gland (b) Back view of thyroid gland showing location of parathyroid glands.

The adult thyroid is composed of follicles and may be considered, from both the structural and functional points of view, as the primary or secretory, units of the organ. Follicles are roughly spherical enclosing lumen which is filled with colloid. The wall of follicle consists of thyrocytes, which are the makers of hormone; the lumen is the storage depot. Iodine is an essential component of both T3 and T4. Thyroid synthesizes T4 and T3 by actively concentrating the iodide across the basolateral plasma membrane of thyrocytes (thyroid epithelial cells) by the sodium/iodide symporter (NIS). Intracellular iodide is then transported in the lumen of thyroid follicles. Meanwhile, the thyrocytes synthesizes two key proteins, thyroid peroxidase (TPO) and thyroglobulin (Tg). Tg is a 660kDa glycoprotein secreted into the lumen of follicles. The tyrosine residues in the Tg are the substrate for iodination and hormone formation. TPO sits at the apical plasma membrane, where it reduces H_2O_2, elevating the oxidation state of iodide to an iodinating species (I^+), and attaches the iodine to the phenolic ring (ortho to the –OH) of the tyrosine in Tg. H_2O_2 is generated at the apex of the thyrocyte by dual oxidase (Duox), a NADPH oxidase. Mono-iodination of tyrosine produces monoiodotyrosine (MIT) and with further iodination producing diiodotyrosine (DIT). Coupling of two residues of DIT produces T4, whereas coupling of MIT and DIT produces T3. When thyroid hormones are needed, Tg is internalized at the apical pole of thyrocytes, conveyed to endosomes and lysosomes and digested by proteases. After Tg digestion, T4 and T3 are released into the circulation [2]. If there is sufficient iodine available for the iodination of all tyrosine residues in Tg, then one Tg molecule can be the source of three T4 and one T3.

Hormones that are secreted from the gland are about 90% T4 and about 10% T3. T4 is also considered to be the prohormone for the more metabolically active T3. Deiodinase enzymes present in the liver and to a lesser extent in the kidneys convert T4 to T3. Although T4 is present at a higher concentration (~ 50 X) than T3, it is several times less biologically active

than T3. T4 circulates approximately 99.97% bound to the plasma proteins: thyroxine binding globulin (60-75%), thyroxine binding pre-albumin (15 -30%) and albumin (~10%). In contrast, approximately 99.7% of T3 is protein-bound, primarily to thyroid binding globulin (80%). Only a minute fraction of T4 (0.03%) and T3 (0.3%) are unbound, in free form. The total concentration of thyroid hormone in the blood is therefore dependent on the concentration of specific binding proteins as well as the functioning of the thyroid gland. The concentration of free T4, (or T4 if free-T4 is not available) in serum or plasma is an important indicator of thyroid status [2].

The thyroid is controlled by the pituitary, which in turn is controlled by the hypothalamus. The production of T4 and T3 is regulated by thyroid stimulating hormone (TSH), released by the anterior pituitary. TSH is released as a result of thyrotropin releasing hormone (TRH) released by the hypothalamus. TSH production is suppressed when the T4 levels are high through a negative feedback mechanism, and vice versa. TSH production is blunted by somatostatin, rising levels of glucocorticoids and sex hormones (estrogen and testosterone), and excessively high blood iodide concentration. Measurement of serum TSH is a valuable test in the diagnosis of thyroid disorders. An imbalance in TSH can be caused by a variety of disease states. TSH is the best indicator for hypothyroidism but has also been suggested to be a sensitive indicator for hyperthyroidism [3].



(a) (b)

Figure 1.2 (a) Anatomical locations of the hypothalamus, pituitary and thyroid. (b) Interactions between them, peripheral deiodination, and plasma T4/T3-binding proteins that regulates thyroid hormone production.(Fig 1.2 (b)-courtesy Dr. M. G. R. Rajan)

1.2. Disorders of thyroid gland

The thyroid gland is prone to a number of diseases that can alter its function and structure. These diseases frequently have wide-ranging systemic effects because thyroid hormones regulate the metabolism of almost every organ in the body. In infancy and childhood, thyroid hormone is essential for normal growth including physical and mental development. In the adult, thyroid hormones control protein synthesis, oxygen consumption, heat generation and overall metabolic activity. Therefore an imbalance in these hormones can have a detrimental effect and can be identified in several disease states. Hyperthyroidism (overactive thyroid) and hypothyroidism (underactive thyroid) are the most common problems of the thyroid gland.[3]

Thyroid diseases are among the commonest endocrine disorders reported in India and are seen worldwide too. According to a projection from various studies on thyroid disease, published in 2011, it has been estimated that about 42 million people in India suffer from

thyroid diseases [4]. The profile of thyroid disorders encountered in pediatric and adolescent age groups in India is similar to that seen in most parts of the world except for the prevalence of iodine deficiency disorders in certain endemic regions of this country. Clinical presentation is most commonly for hypothyroidism and goiters and infrequently for hyperthyroidism [5].

In hypothyroidism, thyroid hormone levels are lower than normal. Hypothyroidism is by far the most common thyroid disorder in the adult population and is more common in older women. Worldwide, too little iodine in the diet is the most common cause of hypothyroidism. In countries with enough dietary iodine, the most common cause of hypothyroidism is Hashimoto's thyroiditis, an autoimmune condition where the body makes antibodies that destroy parts of the thyroid gland. Other causes of hypothyroidism include pituitary problems, hypothalamus problems and previous treatment with radioactive iodine or previous thyroid surgery. Some babies are born with hypothyroidism - this is called congenital hypothyroidism [6].

In hyperthyroidism, thyroid hormone levels are higher than normal; *Graves' disease* is the most common cause of hyperthyroidism. This condition occurs when the immune system produces an antibody that stimulates the thyroid gland; this leads to over activity and higher levels of thyroid hormones. Another form of hyperthyroidism is called *toxic nodular goiter* or *toxic thyroid adenoma*. Adenomas, abnormal nodules of tissue in the thyroid, constantly produce thyroid hormones even when they are not needed. Secondary hyperthyroidism is caused when the pituitary gland makes too much TSH, leading to constant stimulation of the thyroid gland. A pituitary tumor may cause TSH levels to rise. More rarely, the pituitary gland becomes insensitive to thyroid hormones, no longer responding to high levels and no feedback inhibition is seen [6].

Another possible cause of hyperthyroidism is a condition called *thyroiditis*. This condition occurs when the thyroid gland becomes inflamed. Depending on the type of thyroiditis, this may lead to temporary hyperthyroidism that might be followed by hypothyroidism.

Thyroid cancer varies between slow-growing tumors with a good prognosis, to aggressive tumors with limited treatment options. There are four types of thyroid cancers reported: *papillary*, *follicular*, *anaplastic*, and *medullary* cancer. These are associated with radiation treatment to the head, neck, or chest. In other cases, a genetic mutation might be associated with thyroid cancer, either alone or in conjunction with other types of cancers (e.g., multiple endocrine neoplasias, BRAF gene mutations). Less commonly, other cancers might metastasize to the thyroid. Tg as well as the measurement of CT in serum have become important tumor markers for managing patients with differentiated and medullary thyroid carcinomas, respectively [6].

Clinical manifestations of hyperthyroidism or hypothyroidism are so diverse that diagnosis based on clinical features lacks sensitivity and specificity. Physicians need quality laboratory testing support for the accurate diagnosis of thyroid disorders. However, overt or subtle thyroid disease symptoms may be, an open collaboration between the physicians and clinical laboratory scientists is essential for optimal, cost-effective management of the patients with thyroid diseases. Hence, reliance is placed on measurement of circulating thyroid hormones and TSH, to confirm or rule out thyroid dysfunction by immunoassays [7].

1.2.1. Reference Ranges

For diagnostic testing, thyroid test results are reported together with a "normal" reference interval that reflects inter-individual variability. This reference range provides for a benchmark for case finding. Although reference ranges for analytes vary to some extent

depending on the methods employed, the current reference ranges according to American Thyroid Association (ATA) guidelines are as follows [7, 8]:

Test	Abbreviation	Typical Ranges
Serum thyroxine	T4	4.0-12.5 µg/dl
Free Thyroxine	fT4	0.9-1.6 ng/dl
Serum Triiodothyronine	T3	75-175 ng/dl
Thyroid stimulating hormone	TSH	0.4- 4.5 µIU/ml
Serum Thyroglobulin	Tg	Undetectable (ud) -30 ng/ml

Table 1.1 Normal reference ranges for thyroid hormones and related substances

1.3. Review of laboratory tests of thyroid function.

Studies evaluating the thyroid function have changed, together with the progress of diagnostic methods. It evolved from classic studies, such as an assessment of basic metabolism, protein-bound iodine and serum cholesterol level, through isotopic studies *in vivo* (determination of radioiodine uptake, test of suppression of iodine uptake by the thyroid gland, and bovine TSH stimulation test) to isotope tests *in vitro* or modern non-isotope methods to miniaturized multianalyte immunoassays (MAIA). The isotopic in-vitro test takes the form of radio-immunoassays. Immunoassays are sensitive analytical tests that harness the unique properties of antibodies. They proved to be one of the most productive technological contributions to the medicine and fundamental life science research in the 20^{th} century. In the 1950s, only one serum-based thyroid test was available - an indirect estimate of the total (free + protein-bound) T4 concentration, using the protein bound iodide technique [9]. Since 1970, the development of competitive immunoassays [10, 11], non-competitive immunometric assay methods [12] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodologies [13, 14] have progressively improved the specificity and sensitivity of thyroid hormone testing. Since 1996, serum-based tests are available for measuring the concentration of both the total T4 and T3 as well as free-T4 and free-T3 in the circulation [15, 16]. In

addition, measurements of the thyroid hormone binding plasma proteins, thyroxine binding globulin, transthyretin/prealbumin and albumin are available [17]. Improvements in the sensitivity of assays to measure the TSH allows TSH to be used for detecting both hyper- and hypothyroidism [18]. Furthermore, measurement of Tg as well CT in serum have become important tumor markers for managing patients with differentiated and medullary thyroid carcinomas, respectively [19]. The recognition that autoimmunity is a major cause of thyroid dysfunction has led to the development of more sensitive and specific tests for autoantibodies to TPO, Tg and the TSH receptor [20]. Thyroid tests are usually performed on serum by either manual or automated methods that employ specific antibodies [21]. Methodology continues to evolve as performance standards are established and new technology and instrumentation are developed [22, 23].

1.4. Immunoassays

Immunoassays have provided us with biochemical toolbox, which can be applied to investigate and manipulate minute concentrations of complex molecules. Radioimmunoassay (RIA) was first described by Yalow and Berson in 1959, a discovery for which they won the 1977 Nobel Prize in Physiology or Medicine [24]. In search for alternative labels to replace radioactive isotopes, Enzyme-Linked ImmunoSorbent Assay (ELISA) was introduced in the 1970s [25]. During the past several decades, various other formats of immunoassays such fluorescence immunoassays, chemiluminescence immunoassays, electrochemical immunoassays have been developed for clinic diagnosis. Immunoassays are highly robust methods that can be easily standardized and automated. They are widely used in fundamental life science research, clinical evaluation and medical diagnostics [3].

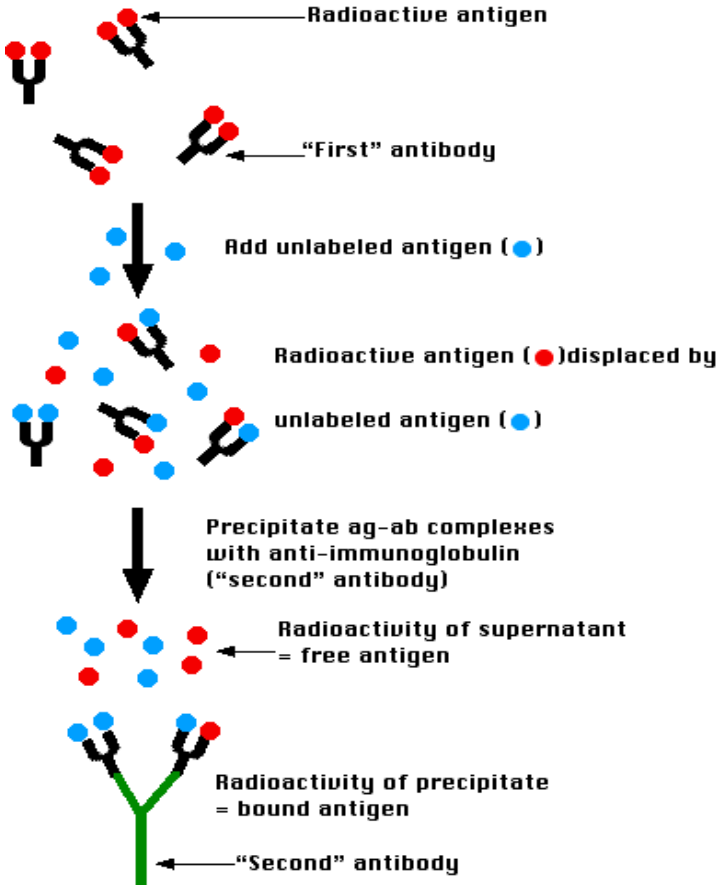
1.4.1. Principle of immunoassays

1.4.1.1. RIA

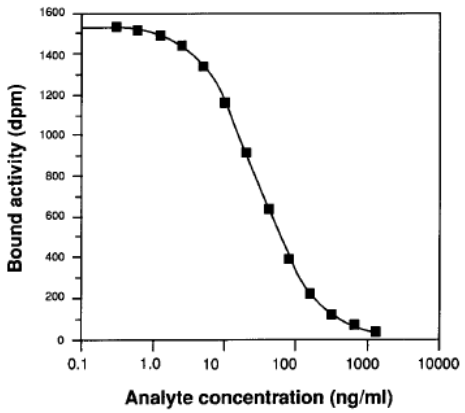
RIA was the first of many immunoassay techniques developed to analyze nanomolar and picomolar concentrations of the hormones in biological fluids. Solomon A. Berson and Rosalyn S.Yalow of the Bronx Veterans Administration Hospital initially described the method to determine insulin levels in human plasma in early sixties [24].

PRINCIPLE:

RIA is a competitive binding assay in which fixed amounts of antibody and radiolabelled antigen (Ag) react in presence of unlabelled Ag. The labeled and unlabelled Ag competes for the limited binding sites on the antibody. The competition is determined by the level of the unlabelled Ag present in the reacting system. After the reaction the Ag is separated into free and bound fractions and their radioactive counts measured. A calibration or standard curve set up with increasing amounts of known Ag, and from this curve the amount of Ag in the unknown samples can be calculated [24].



(a)



(b)

Figure 1.3 (a) Schematic illustration of principle of RIA (b) Sample standard curve

1.4.1.2. Immunoradiometric assay (IRMA)

IRMA was first proposed by Miles and Hales in the 1960s. IRMA is an improvement of the RIA principle and is more sensitive and specific technique as compared to RIA. In IRMA, the Ag (either from standard or from sample) is incubated with an excess of radiolabeled antibody (Ab^*).

$$Ag + Ab^* \longrightarrow Ag\text{-}Ab^* + Ab^*$$

At the end of reaction, Ag bound and free Ab^* are separated and Ag bound fraction is counted for radioactivity. Activity with this fraction is directly proportional to concentration of Ag and hence concentration of unknown Ag can be read from standard curve.

Labeling of antibodies (Ab) and non-availability of separation system were the problem associated with the development of IRMA. To solve this problem, IRMA technique was further modified by Addison and Hales. Two-site IRMA technique, a further modification of the IRMA technique, uses two antibodies for sandwiching the Ag of which one of the antibodies is labeled with radioisotope. Two site IRMAs have better specificity than RIA. Two-site IRMA technique uses two antibodies (Ab_1 & Ab_2), specific to two different epitopes on the same Ag, of which an excess of antibody (Ab_1) is used, bound to a solid phase. The standard/sample is added to the solid-phase Ab_1 and since there is an excess of solid phase antibody, all the Ag, present in the standard/sample, reacts with the solid phase antibody. This is followed by the addition of an excess of radiolabeled antibody Ab_2* which binds only to the second epitope on the antigen molecules which have been extracted on Ab_1. The unreacted labeled antibody is removed by decanting the solution followed by rinsing with buffer .The solid phase containing Ab_1-Ag-Ab_2* is counted for radioactivity. The amount

radioactivity associated with the solid phase fraction is directly proportional to the concentration of the Ag [26].

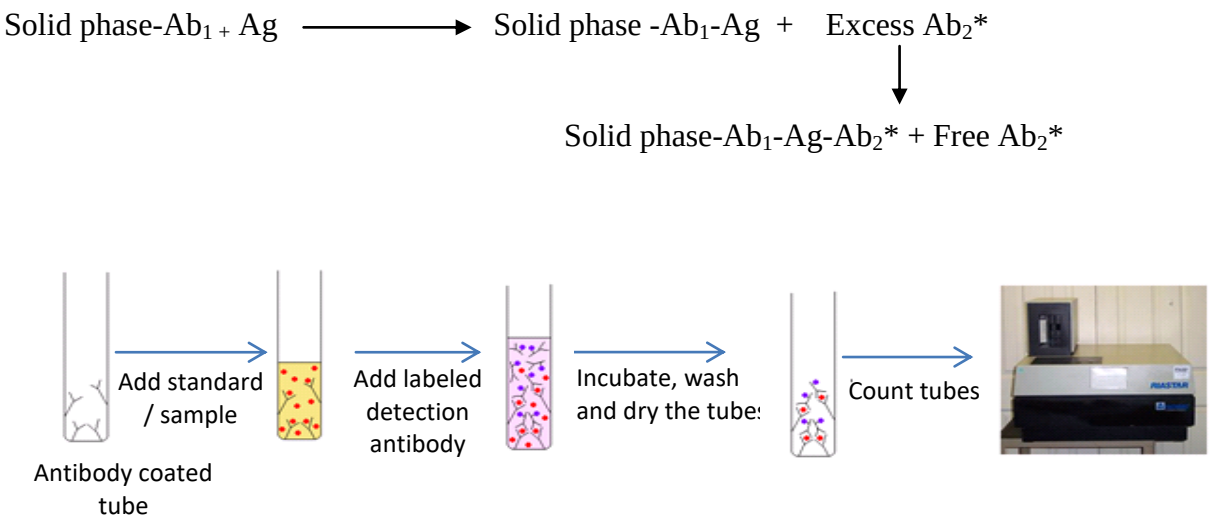


Figure 1.4 Schematic illustration of principle of IRMA.

1.5. Arrays as a tool for multianalyte immunoassays: Technology Review and advantages

Antibody-based immunoassays have been the workhorse of protein measurement for more than half a century, with hundreds of assays available on the diagnostic market [27]. The need to determine "analyte panels" in blood and other biological fluids has become increasingly evident in many fields such as endocrinology, cancer, infectious diseases, autoimmune or allergy disorders. In all these conditions, resolution of a particular diagnostic problem may require determination of the concentrations of several analytes. Immunoassays are the most commonly used assay format, but can be laborious and expensive. They also require relatively large amounts of the patient's sample and permit the measurement of only one analyte at a time. This shortcoming of the conventional immunoassays limits the ability of immunoassays to meet investigators' needs to include multiple analytes. These needs to determine multiple analytes has stimulated the development of array based miniaturized assays or multianalyte immunoassays (MAIA)/multiplexed immunoassays that provide

multiple, parallel analyte measurements on the same sample. A miniaturization of immunoassays for diagnostic purposes started already in the early sixties [28, 29]. More than two decades later, the fundamental principles of MAIA was described in the 1980's by Roger Ekins in his "ambient analyte theory" [30, 31] explaining the potential of MAIA. His microspot assays can be seen as the ancestor of contemporary microarrays. Ekins's ambient analyte theory explained why high sensitivity can be achieved with MAIA. Also due to the fact that multiple analytes can be detected in a single experiment, Ekins was convinced that MAIA had enormous potential for diagnostic applications. Historically these findings have influenced the trajectory of MAIA development.

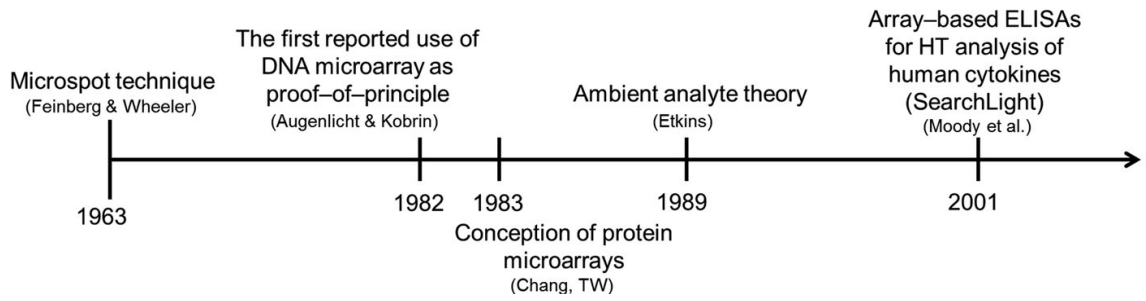


Figure 1.5 Timelines charting the theoretical and practical milestones in the development of MAIA [32] .

Measuring several disease-associated analytes in a sample may lead to a more accurate prognosis and/or diagnosis [33]. Compared with single-analyte immunoassay methods or conventional immunoassays, MAIA offers some remarkable advantages, such as high sample throughput, short assay time, low sample and reagents consumption which reduces overall cost per assay and is economical on assayist time. For example, to diagnose a thyroid disorder by serum analyses, the physician needs to detect the levels of three to five different analytes. Using an individual procedure for each analyte can be an expensive undertaking in terms of materials and labor. By contrast, if one can detect all of the analytes in a single test,

the cost would be less, the probability of error would be significantly decreased, and the need for a repeat test would be lessened. In addition, the time involved in diagnosis may be substantially reduced.

MAIA is an advanced version of traditional solid-phase immunoassays, enabling parallel analysis of analytes. At the core of MAIA is the “antibody-chip” or “antibody microarray” which consists of several antibodies (capture antibodies), each specific to different analyte, spotted in minute quantities on spatially isolated predetermined sites on an inert solid support. When the sample is incubated on such an antibody chip, the analytes of interest will bind to their specific capture antibodies and, as a result, all of them can be estimated simultaneously. Unbound analytes are removed by washing and captured analytes are usually detected by using detection antibodies labeled with various reporter molecules. After quantification of the detection label, signal intensities are either converted to mass units using calibration curves or evaluated qualitatively. The schematic illustration of the MAIA is given below.

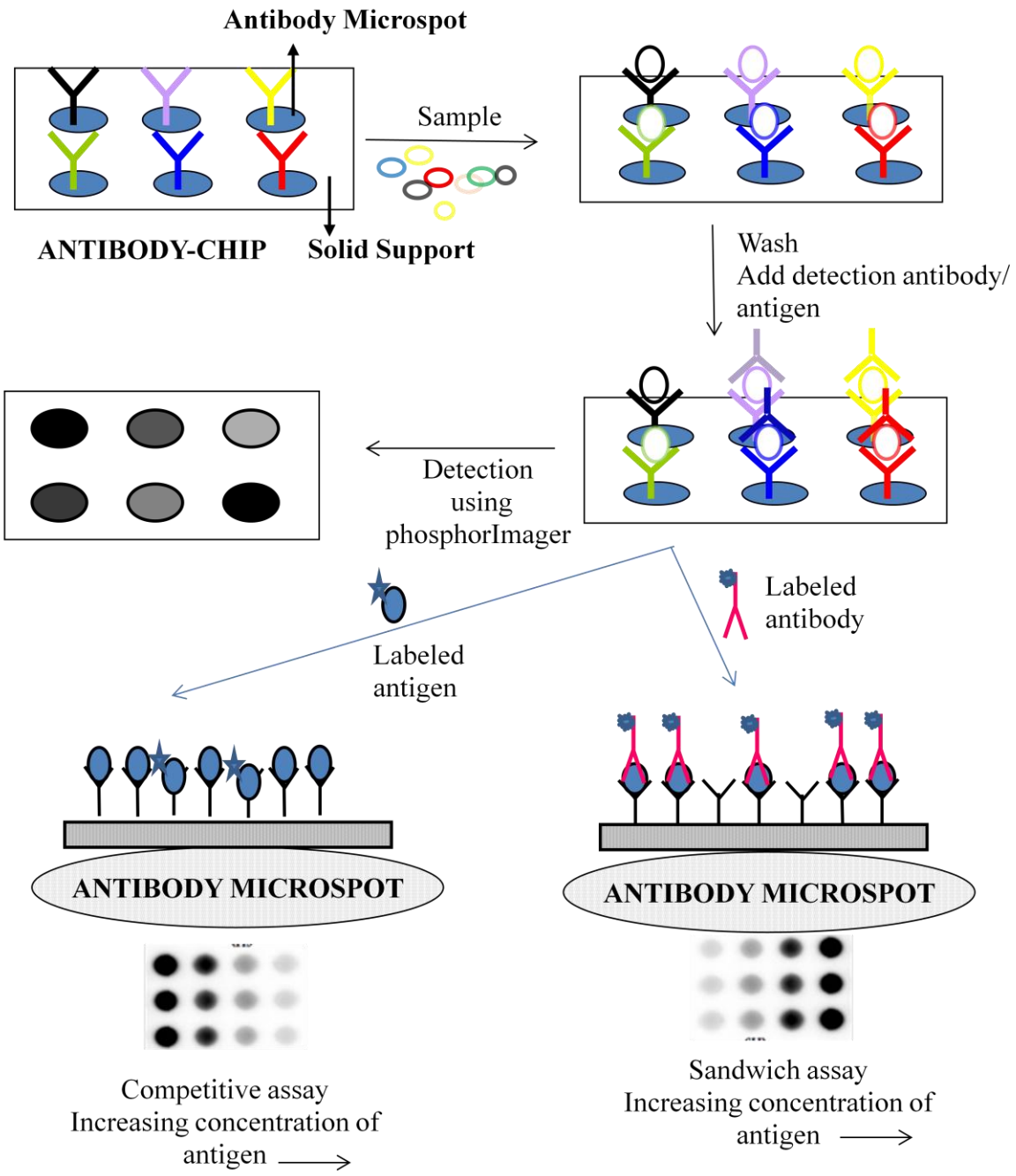


Figure 1.6 Schematic illustration of principle of MAIA

To be of use for diagnostic purposes, MAIA should have statistically similar performance (specificity and sensitivity) as corresponding conventional assays. When this criterion is fulfilled, the MAIA offers rapid and accurate qualitative or quantitative detection methods for many different analytes simultaneously from one sample.

1.5.1. Array based assay formats

Arrays can be distinguished on the basis of the solid support (planar or suspension array), molecule immobilized (e.g., DNA or protein array) and detection method (label or label-free). Many different molecules can be attached as discrete spots to a solid support, and these molecules interact with the intended target molecules in sample and produce either qualitative or quantitative data [34].

1.5.1.1. Planar and suspension arrays

An array format can be defined as planar or suspension, based on the choice of support [35]. In two-dimensional planar array, molecules (proteins, antibodies, DNA, RNA) are printed in rows and columns at known locations onto the same support. In suspension-based arrays, the molecules are coupled to the beads, which are randomly arranged to their final locations (Fig.1.7). In planar arrays, the position within the array allows to identify individual immobilized molecules, whereas bead-based assay systems differentiate between individual bead types either by means of an internal color code or by size. E.g. the Luminex xMAP reader can differentiate between 100 bead types which are dyed with different amounts of two fluorophores [36]. On each bead type, an antibody against a certain analyte is immobilized. Analytes that are captured on the bead surface are then detected using an appropriate reporter molecule. The bead suspension assays are performed using standard laboratory equipment, i.e. using microtiter plates, and can therefore easily be automated.

Several MAIA systems are available commercially. Planar format includes platforms such as Mesoscale Discovery Technology Platform (MSD R), Q–Plex^{TM} array (Quansys Biosciences) and Biochip Array from Randox. The suspension format includes platforms such as Luminex^{TM}, Cytometric Bead Arrays and Bio-PlexPro^{TM} [32].

The thyroid MAIA panel (T3, T4 and TSH) and (free-T3, free-T4, and TSH) is available only from Randox which is a planar array. Suspension array for thyroid panel is available from Millipore. (Rat thyroid three plex for T3, T4 and TSH and Rat thyroid two plex for T3 and T4).

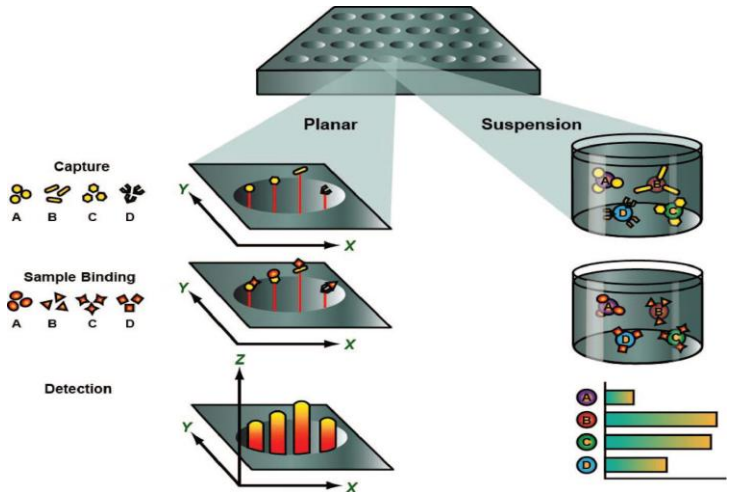


Figure 1.7 Planar and suspension MAIA formats [35] .

1.5.1.2. Protein and DNA array

Protein array technology, derived from DNA array technology, features a large number of protein immobilized at discrete location within a small area. Protein-arrays are fast, high-throughput methods that can be divided into three groups:

- Functional arrays.
- Reverse phase arrays.
- Analytical arrays

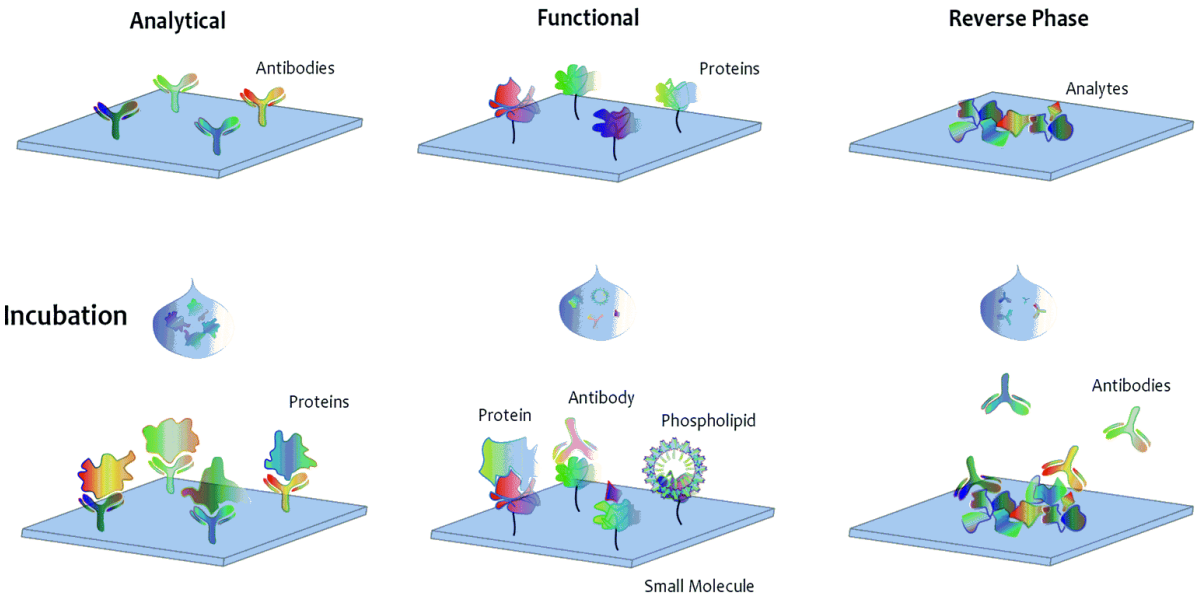


Figure 1.8 Types of protein microarrays [37].

Functional arrays are used to study protein-protein interactions (e.g., protein-protein, enzyme-substrate, ligand-receptor, protein-DNA), protein function, and enzymatic activities [34, 38, 39]. These arrays are prepared by immobilizing on a solid surface, a selection of capture proteins (e.g., antibodies, antigens, lysates, peptides, or aptamers), which then bind the target analyte in the sample [40].

In reverse phase arrays (RPA), the sample (e.g., cultured cells, tissue lysates, blood samples, or other) is immobilized onto an array support surface, which is then reacted with antibodies against the protein of interest. RPAs are used to detect altered proteins from patient samples (e.g., phosphorylated proteins), which may be indicative of diseases. In diagnostic applications, RPA can be used to help to profile a particular signaling pathway that may be dysfunctional in the cell, aiding to recognize and treat the disease of interest [41, 42].

Analytical arrays can be used to recognize and quantify target proteins in a specimen, and to profile disease-related proteins and is classified as antigen and antibody microarray.

1.5.1.3. Antigen and antibody microarray

Analytical arrays can also be classified as antigen- or antibody-based arrays, the latter of which represent a powerful approach for the large-scale characterization of antigens in specimens.

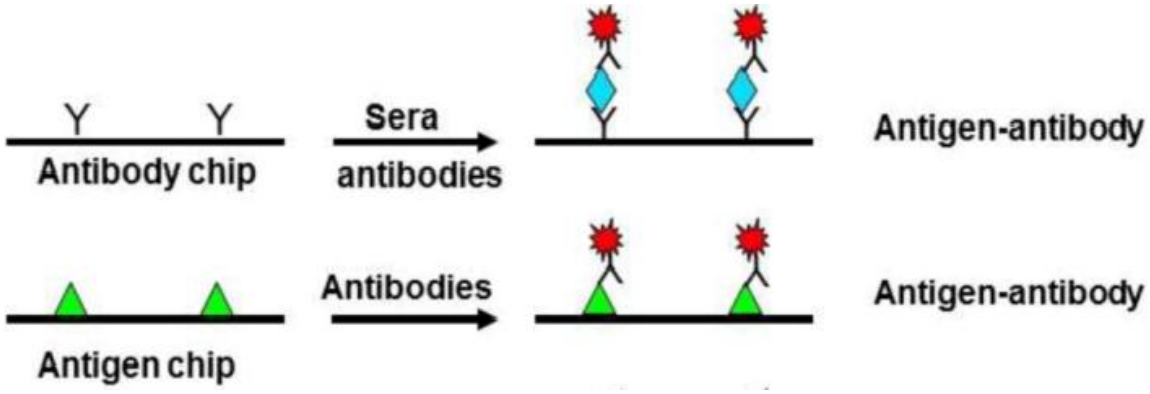


Figure 1.9 Types of analytical protein microarray [43].

1.5.1.3.1. Antigen microarray

A special type of protein microarray is the antigen microarray for the detection of antibodies. Here, antigens are immobilized to test serum for the presence of antibodies towards these antigens, and bound antibodies are detected with a species-specific antibody. Antigen arrays are used for detection of antibodies against microbes or viruses [44-46], for allergy testing [47-49] or detection of auto-antibodies that are involved in autoimmune diseases. Joos et al. used complex antigen microarrays to detect up to 18 different rheumatic disease-specific auto-antibodies in human sera, achieving sensitivities and specificities that were similar to established ELISA methods [50]. Sharp et al. suggested the application of auto-antibody profiling to improve diagnosis and prediction of disease onset and severity [51]. These studies demonstrate the potential of antigen microarrays, which can compete with ELISA

tests in terms of sensitivity and robustness. Antigen microarrays are excellent tools for these types of applications and can support health personnel in diagnosis and prognosis.

1.5.1.3.2. Antibody microarray based MAIA- Concept and basic principles

Antibody microarray approach is based on miniaturized parallelized sandwich immunoassays with the purpose of measuring multiple antigens in the same sample at the same time. The concept of antibody microarray based MAIA was put forth by Ekins in eighties [31, 52]. MAIA relies on high specific activity labeled reagents and small amount of antibodies, each specific to a different antigen, located at high surface density on a suitable support at spatially isolated and pre-determined sites. Ekins introduced the "ambient analyte" theory showing the feasibility of highly sensitive MAIA [31]. According to this theory, miniaturization itself leads to an increase in detection sensitivity. Considering the law of mass action for the dissociation of a complex between an antibody (Ab) and an antigen (Ag) at equilibrium state, the dissociation constant K_D can be written as:

$$K_D = \frac{([Ab_0]-[AbAg])([Ag_0]-[AbAg])}{[AbAg]}$$

In the case of MAIA, only a minute amount of capture antibodies [Ab_0] is immobilized in a microspot. Therefore, according to the mass action law, only a tiny fraction of the initial amount of antigen [Ag_0] is captured onto the microspot. According to Ekins, "ambient analyte" conditions prevail, when the total concentration of capture antibodies [Ab_0] is approximately 100-fold below the value of the dissociation constant K_D. The consequence is that under ambient analyte conditions, the initial concentration of antigen [Ag_0] in the sample is not changed significantly even though the capture antibodies is a high affinity binder and the antigen concentration is low. This type of miniature immunoassay or MAIA is concentration dependent: i.e. the antigen molecules captured in the spot directly reflect the

antigen concentration in the sample. Due to the unaltered antigen concentration, the signal becomes independent of the sample volume. High sensitivity is achieved because the measurement always takes place at the highest antigen concentration possible. Thus, for a given antigen concentration, it is possible that the detection sensitivity in a microspot is higher than in a macrospot, since the antigen is contained in a small area and only signal density (i.e. signal per area unit) not the total signal strength, is relevant for signal-to-noise ratios (SNR), defining the lower limit of detection (LOD) (Figure 1.10). Saviranta et al. [53] confirmed the "ambient analyte" theory for sandwich type assays. Titration curves for 24 mouse serum proteins were obtained for assay volumes of 20, 40 and 80 µl and no significant depletion of antigens was observed for the different assay volumes.

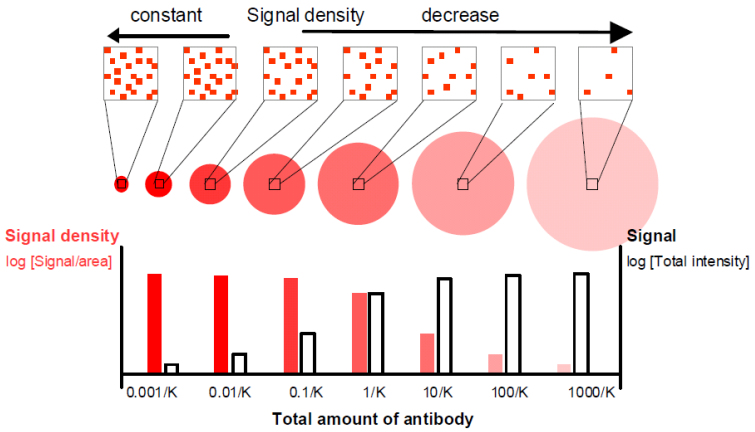


Figure 1.10 Signal and signal density in microspots [54].

All immunoassays essentially depend on measurement of the 'fractional occupancy' of the capture antibody after its reaction with antigen. Techniques relying on the measurement of unoccupied antibody binding sites (from which antibody occupancy is implicitly deduced by subtraction) necessitate for attainment of maximal sensitivity – the use of capture antibody concentrations tending to zero; these assays are therefore categorized as "competitive."

Conversely techniques in which occupied sites are directly measured permit (in principle) the use of relatively high concentrations of capture antibody and may be described as "noncompetitive".

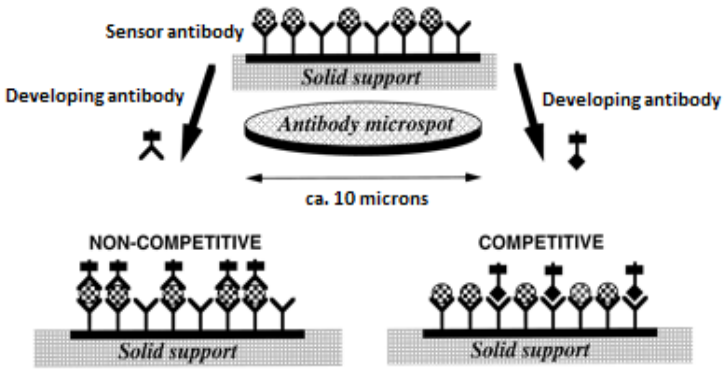


Figure 1.11 Competitive and non-competitive immunoassay schemes [30].

"Capture"/ "sensor" and "developing"/ "detection" antibodies may be labeled with a pair of either radio-isotopic, enzymatic (chromogenic), chemiluminescent or fluorescent markers. Assays relying on non-isotopic labels are gaining importance due to environmental, legal, economic and logistic problems associated with the use of radioactive materials [30].

1.6. Critical factors and challenges in MAIA.

In the development of MAIA, many technical and analytical challenges are encountered. One of the most critical factors involves the selection and immobilization of antibodies onto the solid support. It is crucial that antibodies are stable and remain functional once immobilized [43, 55-57]. Another major challenge concerns the wide range of analyte concentration to be detected. Analytes of interest may exist in a broad dynamic range (up to a concentration of factor 10^{10}), especially in body fluids [58]. Consequently, the assay may need to

simultaneously detect analytes present at very different concentrations on a single microarray. It is, therefore, important to identify antibodies which are highly specific for the analyte of interest, with an affinity sufficient to effectively capture analytes at various concentrations [59]. Another critical factor is the careful selection of antibodies to avoid cross-reactivity. The antibodies in MAIA must specifically recognize a single analyte in a complex mixture like serum or plasma and not cross-react with any other molecule [43, 57, 59]. Among the other technical factors which are critical for the successful development of MAIA are preparation of antibody microarray/chip, assay matrix, signal generated and signal detection [43, 55-57].

1.6.1. Antibodies

A key step for development of robust MAIA is availability of highly specific antibodies that bind tightly to target analytes [56]. Monoclonal antibodies produced by classical hybridoma techniques are commonly used. Highly specific monoclonal antibodies can be generated by mouse immunization and continuous culture of hybridoma cells [60]. Importantly, this represents a potentially unlimited supply of uniform and pure binding molecules. Polyclonal antibodies on the other hand usually contain multiple epitope specificities and are limited in quantity to the amount of serum that can be obtained from the immunized animal. Moreover, their performance in MAIA format may be impaired by a decreased density of specific binding sites due to the presence of antibodies that do not recognize the analyte of interest. Polyclonal antibodies may have higher background and lower specificity and detection limit than monoclonal antibodies. Thus, monoclonal antibodies are preferred as capture agents for MAIA. However, due to the lengthy, labour intensive and thus expensive nature of monoclonal antibody production, efforts have been undertaken to develop alternative technologies [59]. One attractive approach is to use phage display techniques combined with

highly diverse synthetic libraries. These libraries can be used to isolate antibody fragments against target analytes in a significantly shorter time frame than it is possible with immunization-based methods [61]. Phage-display libraries of antibody fragments, therefore, offer the potential for antibody production in a large scale.

Capture molecule	Reviewed in
Monoclonal Antibody	[62, 63]
Polyclonal sera	[64, 65]
Antibody fragments (single chain variable region fragment (scFv)/ Antigen binding fragment (Fab)	[66, 67]

Table 1.2 Antibodies for antibody microarray.

Since antibodies cannot be manufactured with known affinity and specificity, it is necessary to validate the specificity and affinity of each antibody before using for antibody arrays.

1.6.2. Support for immobilization of antibodies

One of the most important factors that determine the performance of MAIA is the solid support on which the antibodies are immobilized. An optimal solid support for MAIA should have the following characteristics: high binding capacity, preservation of antibody functionality after immobilization, low background, ease of manufacture, high reproducibility, high SNR and reasonably easy to manipulate [68-70]. Seurynck-Servoss et al [70] have shown that immobilization parameters that influence robust immunoassay performance include spot size and morphology, total antibody binding capacity, background signal, LOD, and spotting reproducibility within and across antibody microarrays. They have shown the importance of evaluating these parameters and illustrated it by the generation of calibration curves for 23 assays across 17 commercially available slide chemistries; these

curves showed that slide surface properties affect immobilized antibody activity and subsequent data quality

1.6.2.1. Typical supports and surface chemistry

Previously, filterable membranes, such as polyvinylidene fluoride (PVDF) and cellulose nitrate membranes were chosen as the support for high-density antigen/antibody microarrays. Benefits include their low-price, easy-preparation, and direct immobilization of large amount of protein without surface modification [71]. Hydrophobic plastics such as polystyrene are commonly used surfaces where proteins adsorb physically by van der Waals, hydrophobic and electrostatic interactions [72]. Physical adsorption is the simplest process of protein binding, although it is rather uncontrollable. Close proximity between the adsorptive surface and the reactive site of protein/antibody could have unfavorable effects on the biological activity towards its analytes [73]. Besides, the surface of the solid support may also be susceptible to exchanging adsorbed protein due to the surrounding solution and non-specific adsorption could be also a problem. Similar effects are observed on other surfaces used for non-covalent protein adsorption, such as poly-lysine coated glass which is hydrophilic and positively charged [55], or hydrophobic nitrocellulose. Therefore, filter membranes have been gradually replaced by other solid supports due to their too many uncontrolled parameters.

Glass is a popular material as solid support for antibody microarray, primarily due to its low fluorescence, transparency, low cost and resistance to high temperature [74]. Glass surfaces can be modified by silane chemistry introducing specific functional group such as amino, epoxide, carboxylic acid and aldehyde groups which can directly react with antibodies by chemisorption [38, 68, 75]. The advantages of covalent binding is its stability and that the antibodies are immobilized at very high densities [76]. This directly translates to highly

sensitive detection. A drawback, however, is the possibility of antibody denaturation. In addition, gold film deposited on the solid support is commonly employed as the protein microarray support with the SPR detection [77].

1.6.2.2. Oriented immobilization

Both adsorption and covalent binding approaches, however, attach antibodies to the surface in a random fashion. As a consequence, this may make the antibodies inaccessible to the analytes [43]. An alternative approach is the utilization of site-oriented immobilization methodologies [54, 78]. Oriented immobilization of the antibodies can increase the accessibility of the antibodies for analytes. Different biologically active fusion proteins and fusion tags, e.g., cutinase (a serine-esterase), glutathione-S-transferase, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) peptide, and hexa-histidine tag (His6-tag) can be used for the oriented attachment of proteins to solid surfaces [79-81]. A bio-affinity approach using the high binding affinity ($K = 10^{15}\ M^{-1}$) between biotin and streptavidin is also commonly utilized in protein immobilization. Peluso et al. found that 90% of Fab fragments attached to a streptavidin coated surface through biotinylation of the thiol group of antibody Fab fragment are active, while randomly attached Fab have up to sixfold lower activity [75]. Alternatively, specific affinity, such as protein A or G is used for site specific antibody immobilization. They bind specifically to the heavy-chain constant (Fc) region of the antibody, and the antibody binding sites located on the Fab variable region remains accessible for the target analyte [82]. Protein A is found in the cell wall of *Staphylococcus aureus* and protein G is found on the surface of streptococcal cell. Both proteins interacted mainly with the Fc part of IgGs. However, the protein G is able to bind both the Fab and Fc portions of IgG and protein A is able to bind the Fab fragment of human IgM [82]. The benefit of orienting full-length antibodies is, however, by far not as pronounced. This is reflected in the

fact that a wide variety of supports have been used successfully for antibody microarray applications [83]. The disadvantage of the oriented immobilization is the possible loss of the antibody functionality.

Oriented covalent protein immobilization can be achieved if only one thiol (-SH) group in the cysteine amino acid side chain is used for the attachment. The thiol group selectively reacts with α-haloacetyl- and maleimide-modified surfaces forming a stable thioether bond [84]. Free thiol groups are present at selected locations on antibodies, but they can also be generated by chemically reducing the disulfide bridges.

DNA-directed protein immobilization is another site-specific protein attachment bioaffinity strategy. In this approach, proteins are coupled with single-stranded DNA moieties through direct covalent attachment, bifunctional linkers, streptavidin-biotin interaction or expressed protein ligation [85].

1.6.2.3. Three dimensional supports

From the viewpoint of physical structure, the immobilization support includes 2-dimension (2D, flat) or 3-dimension (3D), with microstructure such as microwell on the surface. The flat (2D) slides are commonly used as the solid support for manufacturing antibody microarray due to the low cost and easy treatment. An important development is to move from planar to microporous supports [86, 87]. Rather than immobilize the antibody only in two-dimensions available on a planar surface, the addition of third dimension provided by the pore wells yields superior signal response because of the additional capture molecules that can be immobilized within the pore wells but accessible to reagents. One major category of microarray slide that exist is gel-coated surfaces, such as polyacrylamide [88], or agarose [89] coated slides. Although gel coated slides providing high immobilization efficiency,

wetting property and enhanced signal output as a result of molecules immobilization just not on the surface but throughout the 3-D matrix, this kind of combination supports are difficult to fabricate and the spotted solution spreads on the hydrophilic support. It is difficult to equilibrate with different buffers, if different pH is required during immobilization and assay steps using gel coated slides. Hence, the introduction of micropores in glass can increase the surface area available for immobilization resulting in higher antibody densities [90]. Glycidyl methacrylate-modified polyethylene terephthalate (PET) plastic, which can introduce high density of epoxy groups to PET surface by grafting glycidyl methacrylate photopolymer, was described as good solid support to manufacture high performance protein microarrays [91]. Organic polymer supports such as polymethylmethacrylate [92] polystyrene [93] and polyurethane [94] are also employed for DNA or protein microarrays. Polycarbonate (PC) is an inexpensive plastic material with desirable physicochemical properties. In the work reported in this thesis, we have used thin films (10 μm) of track-etched membranes (TEM) as a novel immobilization support. TEM are highly micro porous membranes having pore density of 10^5-10^8 pores/cm^2. Up to date, TEM has not been used to fabricate antibody microarrays. TEMs are having discrete pores that are formed through a combination of charged particle bombardment in a particle accelerator followed by strong alkali-treatment.

1.6.3. 'Antibody-Chip' manufacturing process

One of the main technical challenges of antibody microarray is the production of the antibody-chip itself. In order to carry out reproducible and reliable assays on a microarray, it is necessary to immobilize the antibodies in a way that results in efficient deposition of functional antibodies. Commercially available arrayers, provides different types of printing methods, can be used for the printing of microarrays in a variety of configurations [95]. Each printing method has its advantages and disadvantages depending on the type of sample to be

printed, sample volume and microarray density. Printing technologies currently exist in two forms: contact and non-contact arrayers.

1.6.3.1. Contact arrayers

Contact printing is accomplished by direct contact between a metal pin head and the solid surface. Contact printing devices come in three main formats, solid pin, quill, and pin and ring.

A solid pin printing assembly is composed of a solid pin with a flat end. Submerging the pin in a liquid sample transfers a sub-nanolitre volume of sample to the tip of the pin. The pin diameter and fluid properties determine the volume of fluid deposited, and thus the spot size. The solid pin format does not lend itself to duplicate or triplicate printing due to the necessity of the pin to be re-submerged in the sample for each spot printing [58, 95].

A quill type printing assembly consists of a flat pin head with a defined hollow bore, similar to quill-style writing instruments. Sample fluid wicks into the hollow space and is deposited on the solid surface when the pin head touches the surface. The quill style formats allow multiple spot printing from each sample [95].

Pin and ring assemblies are a combination of a ring that holds microliter quantities of sample and a flat head pin. The pin travels through the fluid retained in the ring and deposits the sample on the solid surface. The pin and ring assembly is capable of replicate spot printing [95].

The main disadvantage of contact printing is the danger that proteins might by denatured while the pin touches the surface. The protein can lose its functionality because of direct contact with the metal pin during printing. Moreover, proteins might remain attached to the

pin heads following washing leading to cross-contamination of samples. Lastly contact printing might result in less homogenous protein spotting causing assay variability.

1.6.3.2. Non-contact arrayers

Non-contact printing devices utilize a sensor for depositing solution above the surface [58]. This sensor may be either a piezoelectric crystal or a solenoid. Piezoelectric devices consist of a glass capillary tube surrounded by a deformable piezoelectric material [58, 95]. Piezoelectric material is typically a ceramic that changes form in the presence of an electrical charge. The deformation induced by electric charge of the piezoelectric material provides pressure on the glass capillary containing the sample, causing fluid to be dispensed from the tip of the glass capillary. Picoliter quantities of fluid may be dispensed with a piezoelectric tip. Typical sample delivery volumes for these devices are 0.1–0.3 nl. Syringe solenoid systems utilize pressure supplied by a syringe to aspirate fluid into a sample tip. Opening the solenoid valve allows droplets of fluid to be ejected from the tip. The dispensed droplet volume is 4–8 nl [95].

Non-contact printing is generally believed to yield the lowest spot to spot variability in the amount of sample deposited. In addition, non-contact printing could cause less harm to the protein structure because the capillary does not touch the surface, but the shear forces produced during droplet delivery may have a negative effect on protein structure [73].

1.6.4. Cross-reactivity

MAIA was proposed in 1989 by Ekins et al. a few years before the introduction of DNA microarrays [31]. Since then, multiplexing has progressed from 4 to ~50 targets for MAIA, while DNA microarrays were scaled from 400 to 6.4 million targets within a decade. Vulnerability to cross-reactivity can account for the difficulty of scaling up MAIA and for the

lack of reproducibility. Cross-reactivity between antibodies and nonspecific analytes limits the number of analytes that can be used in a given MAIA. Cross-reactivity is a crucial analytical parameter regarding specificity and reliability of MAIA [96]. Some analyte combinations are not possible because nonspecific binding may produce a large background signal, thereby decreasing assay sensitivity. For example, the sensitivity of an 11-plex planar assay was decreased by factors of 1.7–5.0, compared with ELISAs, because of higher background signals in the MAIA [97]. Antibody cross-reactivity may limit measurements to 30–50 analytes [98].

Cross-reactivity can be evaluated in either assay format with three experiments that measure the signal produced when (1) single analyte is incubated with complete detection antibody cocktail; (2) complete analyte mixture is incubated with single detection antibodies; and (3) antibody cocktails with one antibody removed are incubated with complete analyte mixtures to detect cross-reactivity between detection antibodies and specific analyte [99].

Assay diluents are also a source of assay interference. Diluents and assay buffers must interact effectively with all reagents and proteins included in a multiplex under common assay condition. This presents a challenge because proteins require specific conditioning to maintain conformation; differences in electrical charge, hydrophobicity, and post-translational modifications may alter requirements. Minor changes in buffer pH and ionic strength may irreversibly modulate structure, impairing assay performance. For example, comparison of commercially available diluents demonstrated that diluent selection significantly suppressed signal intensity and assay sensitivity in a 19-plex suspension assay [100].

1.6.5. Wide range of concentration and molecular weight of analytes

Approximately 7 % of the human body weight is blood and roughly 45 % of it consists of cellular components. The cell-free component of blood (the plasma) contains, (apart from the hormones and other analytes) the proteins and other low molecular weight compounds, e.g. electrolytes, glucose etc. The total protein amount in plasma is between 60 and 80 mg/ml and the plasma proteome is the most complex human-derived proteome [101]. However, the dynamic range of the plasma proteome extends over more than 10 orders of magnitude. Approximately two thirds of the total protein amount in plasma belongs to albumin while there are low abundant proteins, such as Interleukin 2, which is present at concentrations below 1 pg/ml. It is a very challenging task to quantify or even detect a single species of protein in plasma among the other matrix components. First, an analytical detector must have the sensitivity in terms of detection limit. By using mass spectrometry in combination with enrichment strategies, it is possible to detect proteins down to concentrations of a few ng/ml [102, 103], while sandwich immunoassays allow a detection down to 1 pg/ml [104]. A second problem arises when multiplex detection is performed and the difference of two protein concentrations is larger than the measurement range of the detector. For example, if one would be interested to quantify the total immunoglobulin G (IgG) concentration and Interleukin 2 in one assay, a detector with a measurement range of 10 orders of magnitude would be required. Consequently, the assay may need to simultaneously detect proteins present at very different concentrations on a single antibody-chip. It is, therefore, important to identify antibodies which are highly specific for the protein of interest, with an affinity sufficient to effectively capture proteins at various concentrations [59]. Proteins have wide molecular variability and concentration range in different samples. In MAIA, the challenge is to detect low and high abundance proteins simultaneously in a complex biological sample material [55]. While each analyte can be detected by an immunoassay of some kind, the chemistries of the immunoassay differ from one analyte to the next and different reagents are

added at different times. It is indeed a challenge to accommodate these differences and produce an assay that can provide individual values for each of the analyte and yet be performed in a single reaction mixture.

A greater challenge is the wide range of molecular weight. Small molecules with less than 1000 Da in molecular weight are not considered amenable to sandwich immunoassays due to their difficulty of simultaneous recognition by two antibodies. In this case, competitive assays have to be used. Parro et al. and Calvo et al. described the development of protein microarray technologies for automatic in situ detection and identification combining sandwich and competitive immunoassays [105, 106].The assay was developed to analyze liquid and solid samples from extraterrestrial origin, ranging from small molecules and proteins to whole cells and spores. Although the direct immobilization of analytes on the MAIA support for the competitive assay was successfully performed, this is not always the case. There are small molecules whose structure does not have enough functional groups for immobilization or are not available in the required amounts [107]. In this case, the immobilization may affect the antibody recognition or does not provide concentrated spots [108]. Additionally, the direct immobilization of molecules on the support may require previous coupling to other larger molecules (e.g. albumin) or different chemical functionalities on the support, which increases the work and cost of production. Thus, it is a challenge to accommodate both competitive and sandwich immunoassay on the same platform.

1.6.6. Signal generation and signal detection

The binding between antibodies and analyte can be monitored by quantifying the signal generated from each pair using various detection techniques [43, 109]. Current detection techniques for microarray can be typically divided into two groups [110].

1.6.6.1. Labelled detection molecule methods

Detection of captured analyte can be accomplished by radioactivity, fluorescence, chromogenic, or chemiluminescent labeling [111]. The bottlenecks of labeled detection molecule methods are the production of antibodies/antigens and the quantitative labeling of antibodies/antigens. Existing collections of analyte specific antibodies cover a limited fraction of the proteome. It is necessary that the detection method provides high sensitivity, with high SNR and high throughput for MAIA due to the miniaturized format.

1.6.6.1.1. Radioisotopic Labels

A number of radioisotopes are used in immunoassays to generate the signal required to quantify the analyte. The most common is ^{125}I with a half-life of 60 days. Tritium (^{3}H), ^{57}Co and ^{14}C are also occasionally used. Methods for labeling have been developed that substitutes the radiolabel onto the aromatic rings of the amino acids tyrosine or histidine of the protein. Radioisotopes are successfully used for conventional assays and also for protein microarrays. The ability to incorporate ^{32}P in protein, DNA and RNA enabled the production of a universal array for the detection of protein–protein, protein–DNA, protein–RNA and protein–ligand interactions [112]. Purified proteins immobilized on a nitrocellulose membrane were probed sequentially with a ^{32}P-labeled protein probe, a ^{32}P-labeled DNA probe, a ^{32}P-labeled SV40 pre-mRNA probe, as well as a ^{125}I triiodothyronine probe. Signal detection was visualized by autoradiography and quantified with a densitometer.

Prior to their success with fluorescently labeled probes, Zhu et al. [78] analyzed the yeast proteome for in vitro kinase activity with ^{32}Pγ-ATP. Recombinant proteins were immobilized on microarrays made with the silicone-elasotomer polydimethylsiloxane. Seventeen different kinase assays were prepared and quantified using high-resolution phosphorimaging. Their

high-throughput system allowed comparison of the protein kinase functional relationships with one another.

1.6.6.1.2. Chemiluminescence

In chemiluminescence detection, the signal is generated with secondary antibodies conjugated enzymes for instance to alkaline phosphatase (AP) or horseradish peroxidase (HRP). The enzymatic oxidation of a substrate, such as luminol, produces a prolonged emission of light, which is captured with X-ray film or a charge coupled device (CCD) camera. The sensitivity of chemiluminescence can also be increased by performing the oxidation of luminol by HRP in the presence of chemical enhancers such as phenols. This has the effect of enhancing the light output by ~1000-fold and extending the time of light emission, consequently increasing the sensitivity. Chemiluminescence detection has been used to detect proteins on membranes [50], glass arrays [88] and 96-well plates [72, 113]. Chemiluminescence, although highly sensitive, has drawbacks in terms of the potential for low feature resolution due to signal bleeding and limited dynamic range. Amplification strategies such as the biotinyl–tyramide can be coupled to chemiluminescent detection [114]. Fall et. al., in 2009, [115] demonstrated the utility of chemiluminescent detection in an IgE allergen array. Purified and recombinant allergens were immobilized on slides coated with 1% (3-glycidyloxypropyl) trimethoxysilane. A 25-µl sample of serum was used to screen 24 different allergens. Detection with streptavidin-HRP reagents produced results with an intraslide standard deviation of 2.6–7.6% and chemiluminescent detection methods have the ability to create a permanent record of results with film-based detection, high sensitivity and speed.

1.6.6.1.3. Fluorescence

Ekins et al. described fluorescence applications to microarrays in 1990 [116]. Today, fluorescence is the most commonly used method to detect proteins on microarray formats [54]. This popularity is mainly for reasons of high sensitivity, simplicity, stability and availability of fluorescent scanners tailored for microarray use. Fluorescent molecules or fluorophores absorb photons of light energy from an external light source causing an excitation of electrons within the molecule and an emission of light at a different wavelength than the incident light. Fluorescein, rhodamine, phycobiliproteins, acridines, cyanines and Bodipy compounds are commonly used for protein labeling. Selection of fluorophores for use with microarrays depends on sample type, support used for microarray fabrication and excitation and emission characteristics of the dye. However, photobleaching and quenching of fluorophores that may decrease the total signal observed on a microarray. The Cy3 and Cy5 dyes are commonly used for fluorescent detection for overcoming the effects of photobleaching and quenching [117]. Fluorescent molecules can be directly labeled onto proteins [55] or conjugated onto other detection molecule such as streptavidin. Since there is low auto-fluorescence in glass slides, protein arrays are normally constructed on glass when using fluorescence detection. However, the sensitivity of fluorescence detection is usually lower than chemiluminescenceand may be insufficient in some cases to measure proteins present at very low concentrations. To address this limitation, a powerful signal-enhancement methods (e.g. rolling circles amplification (RCA) [118] and tyramide signal amplification (TSA) [119] have been developed. In RCA, an oligonucleotide-conjugated antibody binds to a hapten (such as biotin) common on all antigen-specific secondary antibodies. A circular DNA molecule then hybridizes to the oligonucleotide and replicates using DNA polymerase, thereby amplifying the signal. Tyramide signal amplification (TSA) uses HRP to catalyze biotin accumulation from biotinyl–tyramide, an HRP substrate. The "amplified" biotin

localized at the reaction site can be detected using strepatvidin-HRP in conjunction with an HRP substrate that produces a fluorescent or chemiluminescent product. The signal obtained on spots is detected by scanning the array, using either a scanning confocal laser or CCD camera-based reader [120]. Once the array image has been generated, sophisticated software is used to obtain signal intensity values for each of the spots.

1.6.6.1.4. Colorimetry

Chromogens are molecules that serve as a substrate for an enzymatic reaction that generates a colored product. For MAIA application detection molecule is labeled with an enzyme. The enzyme acts on a colorless substrate generating a colored precipitate. Commonly used enzymes for the chromogenic reactions are HRP and AP. Diaminobenzidine (DAB) is a commonly used chromogen with HRP and is applicable to microarray detection with femtomolar sensitivity [41, 121]. The DAB precipitate is stable and produces an intense signal with relatively low background. The signal may be further intensified with nickel, copper, silver, gold or cobalt to enhance the staining [122]. A disadvantage of DAB is the potential mutagneicity of the substrate and the need for proper disposal of chromogens. Staining reactions using the substrate 3,3´,5,5´-tetramethylbenzidine (TMB) [123], a non-carcinogenic, non-mutagenic derivative of benzidine, are more sensitive than DAB reactions, although increased washing and blocking may be required due to higher background staining.

1.6.6.2. Label-free detection

The label-free detection methods are promising tools to characterize binding events on surface of solid support and there is no need for labeling of detection molecules that may affect their activity. However, they are generally based on sophisticated equipment not easily available in all clinical and research laboratories.

Label-free methods are mass spectrometry [124-126], surface plasmon resonance (SPR) imaging [127], and atomic force microscopy (AFM) [128]. Mass spectrometry microarrays utilize a protein-selective surface, such as hydrophobic, ionic, or biological surfaces for immobilization of a complex protein solution. Ions liberated from the surface by laser desorption/ionization fly to a detector and are classified based on their mass/charge ratio. Genetic algorithms and neural network data analysis are used for data mining and disease/non-disease data clustering analyses. SPR detectors are optical biosensors for monitoring biomolecular interactions. A molecule of interest is immobilized on a thin metal film, typically gold or silver. Incident light is directed at a sharp angle to the side of the metal film opposite of the molecule. The light is reflected from the film at a certain angle. Changes to the molecule on the film, such as binding of a cognate analyte, causes a change in the electrons in the metal film, causing the angle of the reflected light to vary from the original angle. Measurement of the angle of reflectance indicates a binding event between the immobilized molecule and the capture molecule.

AFM applications for protein microarrays capitalize on the change in height of an immobilized antibody upon binding of its complimentary antigen [129, 130]. Label-free methods generally require sophisticated equipment not available in all laboratories or clinics and it is usually not as sensitive as label-based detection.

1.7. Aim and objectives of thesis

In view of limitations of the traditional immunoassays, there is a need to develop cost effective and sample economical MAIA for quantitative detection of analytes so that all relevant analytes to clinical condition (to rule out or confirm) can be measured in one assay.

The aim of the thesis was to develop antibody-chip for performing MAIA for thyroid function.

Thus, one major aim of this thesis was the selection of suitable support and immobilization chemistry for immobilizing the antibodies. We tested various supports (2D and 3D) and evaluated their ability to retain maximal biological activity of the immobilized antibody. The immobilization capacity of each surface was evaluated using T4 antibodies reacted with ^{125}I-T4. TEM were selected as a suitable support. TEM were critically evaluated to determine their utility for MAIA.

The other aim of the studies carried out for this thesis was standardizing the assay conditions for development of the microarray immunoassay (MI) for individual analytes (T4, TSH and Tg), as a proof of concept, of development of MAIA. Parameters such as the concentration of antibody, incubation conditions, blocking conditions as well as the detection antibody/analyte concentration were optimized for each analyte. The MI was validated and compared with RIA and IRMA as done presently.

In continuation of the above aim, studies were carried out to develop MAIA for three different analyte panels for assessment of thyroid disorders and their validation. The developed analytes panels were:

- T4 and TSH
- TSH and Tg
- T4, TSH and Tg

Parameters such as cross-reactivity, incubation conditions and detection antibody concentration, were optimized for each panel. Developed MAIA was validated using several

parameters such as sensitivity, range, precision, linearity and with real sample measurements in patients having thyroid disorders.

CHAPTER 2

Selection of Suitable Support and Optimization of Surface Chemistry for Antibody Immobilization

2.1. Introduction

Immobilization of proteins on solid supports, is to date, an extensively studied subject, but yet remains a significant challenge in the field of protein microarray. Unlike nucleic acids or peptides, the tertiary structure and reactivity of a given protein is different from any other one, leading to complexity in the immobilization process. Preserving the tertiary structure of proteins is essential for their biological activity. Thus, the suitable support and the interface between the support and the protein, e.g. surface chemistry, is a key point to efficiently immobilize proteins and to retain their biological activities [75, 131, 132]. Therefore, to prepare antibody-chip for MAIA, one important aim is the selection of suitable support and optimizing the surface chemistry, which could provide a suitable environment for solid-phasing of the antibodies, preventing loss of their biological activity and nonspecific adsorption.

The wide variety of supports available and advantages and disadvantages of both 2-D and 3-D supports are illustrated in Chapter1 in Section 1.6.2. Despite the variety of the supports being available, having a wide range of properties, none seems to meet all the demands required of a support for making an antibody-chip. Hence, there is a need for research to develop a novel support suitable for making an antibody-chip. In this context, we have explored the use of microporous track-etched membranes (TEM) as a novel solid support for

immobilization of the antibodies. TEMs are very thin membranes, with tightly controlled pore sizes. They have traditionally been used for high specification filtration in many laboratory applications. These thin films have discrete pores that are formed through a combination of charged particle bombardment followed by strong alkali-treatment. High energy krypton or Si^{+7} and Cl^{+8} ions from an accelerator are bombarded on the membrane, that pierce it all the way through leaving fine holes called 'tracks'. These tracks in the membranes are different from the rest of the membrane in terms of the physical and chemical properties. Holes of cylindrical shapes form in position of tracks when etching the film in strong alkaline solution. Diameter of these holes may vary in the range from 0.05 to 3 µ (micron) depending on the conditions of etching. The particle accelerator used for the mass production of TEMs has beam currents, which produces up to 10^6 - 10^8 ions/cm^2 that allows producing TEMs with pores in the range of 10^5-10^9 pores/cm^2 [133]. The porosity of such membranes constitutes 10-15% of the total membrane-area. TEMs are available from commercial sources like from Millipore, which have a defined pore size with intra-lot variation in pore size of 2-3%. They show excellent chemical resistance with good thermal stability and are non-hygroscopic. They are biologically inert and the possibilities of assay interference are remote. Though a variety of membranes can be used for making TEM, we have limited our use to polyethyleneterepthalate (PET) and polycarbonate (PC) TEM in our studies.

(a) *(b)*

Figure 2.1 (a) Polycarbonate track-etched membranes from Millipore (HTTP02500).(b) Single TEM.
They are 10 µm thick membranes with a diameter of 25mm. Pore density of TEM is 10^8 pores/cm^2 and pore diameter is 0.4 micron.

We investigated the use of microporous TEMs as a support for MAIA, since 3-D surfaces provides larger surface area for antibody immobilization. The performance of TEM was compared with planar supports like glass and flexible supports like filter membranes [Nitrocellulose and Polyvinylidene Fluoride (PVDF)]. Finally, various parameters of TEM (pore density and hydrophobicity) were studied to select the optimal membrane for antibody immobilization for making antibody-chip. Anti-T4 antibody reacted with ^{125}I-T4 was chosen as a model system for support selection and studying the immobilization parameters. After selecting the suitable support, the aim of this study was to identify the main parameters influencing antibody immobilization. Antibody concentration and time of immobilization were varied in order to define the best conditions for immobilization of the antibodies on TEM. Results should allow fixing the best support and the immobilization conditions for antibodies.

2.2. Materials

2.2.1. General reagents

All chemicals were of analytical grade or commercial-grade with the highest quality. (γ-aminopropyl)trimethoxysilane (96 %) was from Fluka, 25 % glutaraldehyde was purchased from Merck, bovine serum albumin (BSA), Tween-20 and L-thyroxine-sodium salt was purchased from Sigma, USA. ^{125}I was procured from BRIT. Sephadex G-25 was from Pharmacia Fine Chemicals. All other chemicals (sulphuric acid (H_2SO_4), 37 % hydrochloric acid (HCl), methanol, toluene, ammonium sulphate, Chloramine-T, sodium metabisulphite, developer powder and fixer for developing of X-ray film were purchased locally.

2.2.2. Buffers

The following buffers required for experiments were prepared as and when required:

1. Phosphate buffer

   0.5 M phosphate (pH 7.4)

   0.1M phosphate (pH 7.4)
2. PBS (Spotting buffer)

   0.025 M phosphate (pH 7.4)

   0.15 M sodium chloride

   0.01% sodium azide.
3. Wash buffer (PBS-T)

   0.05 M PBS with 0.1 % v/v Tween 20 at pH 7.4
4. Blocking solution (PBS-BSA)

   4% w/v BSA in 0.05 M PBS.
5. ANS buffer (PBS-ANS)

   PBS (0.025 M) with 0.5% w/v BSA and 0.25% w/v ANS.

2.2.3. Laboratory equipment

Orbital shaker

X-ray film from Kodak

X-ray film cassette from Kodak.

2.2.4. Solid supports for making antibody-chip

Plain standard glass coverslips No.1 (13-mm diameter and 0.18 mm thick) was purchased locally. Silanized glass slides were purchased from Sigma, USA. Samples of hydrophobic PET and PC-TEM and hydrophilic PET-TEMs with different pore densities (10^5-10^8 pores/cm^2) prepared elsewhere, were provided by Dr. R.H.Iyer, Emeritus Scientist, Waste Management Division, Bhabha Atomic Research Centre. Track-etched hydrophilic PC membranes (K04CP 02500) having pore diameter of 0.4 μm was purchased from GE Osmonics (Poretics polycarbonate, Minnetonka, MN). Hydrophilic PC TEM (HTTP 02500) having pore diameter of 0.4 micron were also purchased from Millipore, USA. Nitrocellulose (Nitrobind) with pore size of 0.45 μm was purchased from GE Osmonics and PVDF (HVHP, 04700) was purchased from Millipore. We have also etched Mylar membranes bombarded with ^{28}Si and ^{19}F and PC membrane bombarded with ^{35}Cl in our laboratory, provided by Mr. J.P. Nair of the Pelletron Acceletor Facility, BARC, Mumbai.

2.2.5. Softwares

Densitoquant software available with Gel documentation system (Biovis) was used for measuring the density of the exposed X-ray film.

2.3. Methods

2.3.1. Antibody production

Polyclonal antibodies against T4, were produced using standard immunizing procedures in rabbits from our animal house and approval from the BARC Institutional Animal Ethics Committee was obtained for this purpose. The total Ig component of the antiserum was precipitated by 50% ammonium sulphate and extensively dialyzed in PBS. The Ig concentration was measured spectrophotometrically at 280 nm and diluted with PBS to give a 1 mg/ml final working solution.

2.3.2. Labeling of T4 with ^{125}I

T4 was labeled with ^{125}I using Chloramine-T method at a specific activity >1000 µCi/µg as follows: 1.5 µg of T3 was mixed with 2 mCi of ^{125}I in 50 µl of 0.5M phosphate buffer. 10µl of (4 mg/ml) Chloramine-T was added and the reaction was allowed to proceed for 90 sec after which it was terminated by adding 50 µl (2mg/ml) of sodium metabisulphite. Twenty five µl of 2% KI solution was added as a carrier. The mixture was loaded on Sephadex G-25 column (25 X 1 cm) equilibrated with 0.02 M NaOH and eluted using 0.02M NaOH at a flow rate of 6-10 drops per minute. One ml fractions were collected. The ^{125}I-T4 eluted after free ^{125}I and ^{125}I-T3. The ^{125}I-T4 fractions were pooled, neutralized with sufficient 0.1 M HCl and mixed with an equal volume of propylene glycol and stored at 4°C. Immunoreactivity of the tracer was checked with anti-T4 antibodies coupled to magnetic particles.^{125}I-T4 preparations which gave more than 90% binding of the tracer with excess antibodies were used in this study. Prepared tracer was diluted in PBS-ANS to contain ^{125}I-T4 at desired concentration.

2.3.3. Manufacturing of antibody-chip

For developing a reliable antibody-chip, various supports including glass, both hydrophobic and hydrophilic PC and PET-TEM, PVDF and nitrocellulose membrane were assessed. Finally, PC- TEM of various pore densities was tested to select the optimal membrane for antibody immobilization for antibody microarray.

First, we choose glass slide as it is a widely used support for protein microarrays since it is cheap, transparent and exhibit low auto-fluroresence. As with most solid supports, it is not easy to graft proteins directly on the surface and keep the proteins stable. As a consequence, it is necessary to introduce new chemical reactive groups on the glass-surface using bifunctional cross-linker, which possesses one reacting group towards the support and a second reacting group for protein to be immobilized. Silanes are often used to functionalize glass slide for covalent protein immobilization or further coupling with other chemistries to attach proteins [134]. Furthermore, commercial silanes bearing various chemical functional groups are readily available for different requirements. The silanes are normally composed of two distinct reactive moieties: the silyl head group and the organic reactive group carried at the end of an aliphatic chain. The silyl head group undergoes reaction with surface hydroxyl groups leading to a robust tethering of the silane molecules to glass. Then, organic functions (carboxyl, amino, epoxy etc,) permit the immobilization of proteins via covalent or electrostatic interaction [134-136]

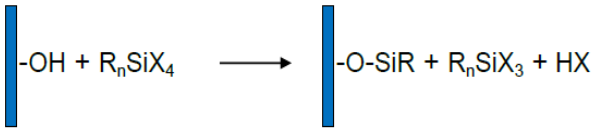


Figure 2.2 General equation for silanization of glass surface

In our work, γ-aminopropyltriethoxysilane (GATS) was used that introduced amino groups on the glass surface. The antibodies were immobilized on the silanized glass surface using glutaraldehyde as a bifunctional agent according to method of Bhatia et al. [137]. The schematic diagram for activation of glass is given below.

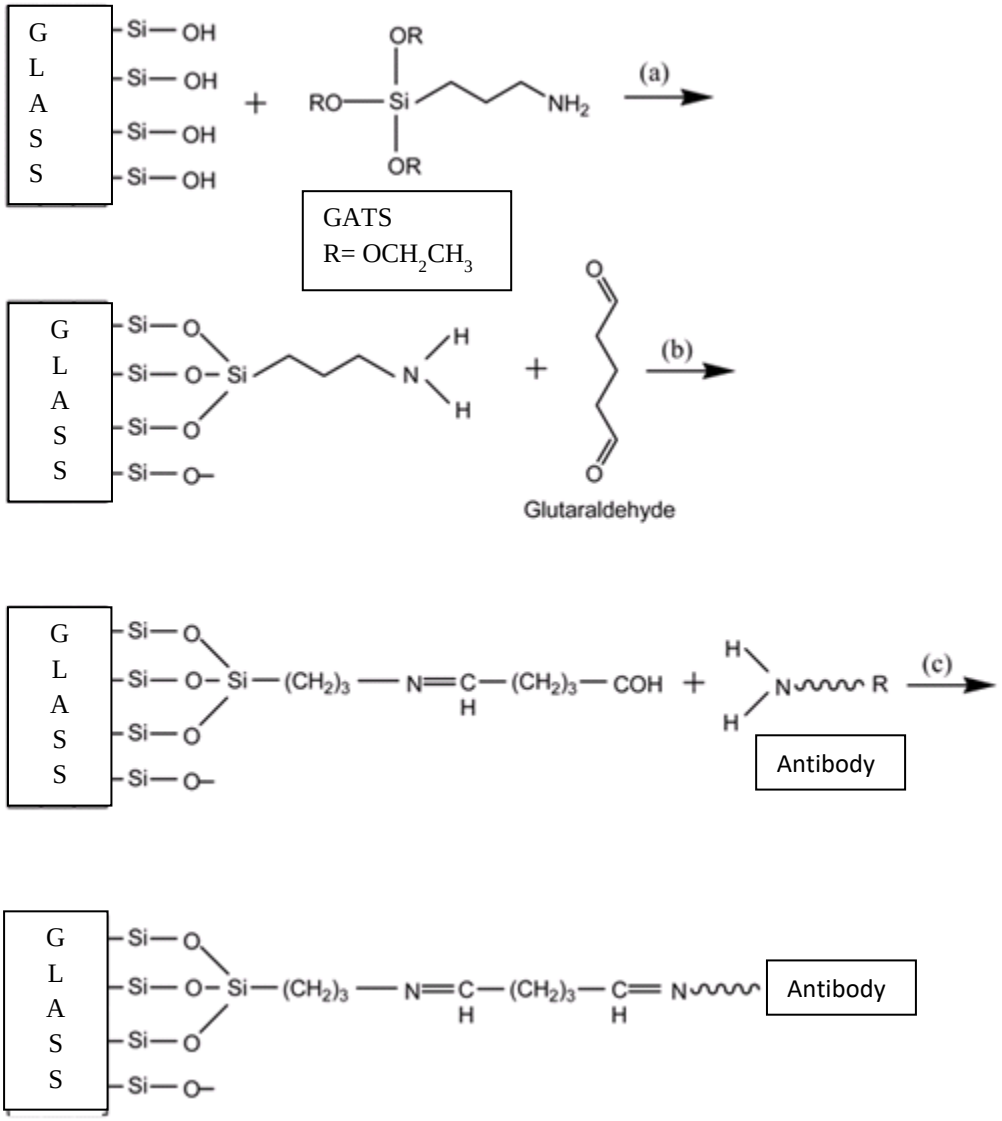


Figure 2.3 Schematic illustration of activation of glass with GATS followed by glutaraldehyde activation for immobilization of antibodies.

2.3.4. Cleaning of glass

The glass discs were cleaned by immersing in 1:1 HCl-methanol mixture for 20 min and rinsed thoroughly with water. Next, they were treated with concentrated H_2SO_4 for 30 min, rinsed with water and immersed in boiling water for a few min and air-dried.

2.3.4.1. Silanization and activation of glass-surface

The dry discs were silanized using 2% GATS in dry toluene for 2 hr. Activation was accomplished by immersing amino modified discs in 2.5% glutaraldehyde in PBS for 2 hr followed by washing using PBS. Commercially available silanized glass slides were also activated by immersing in 2.5% glutaraldehyde in PBS for 2 hr.

2.3.4.2. Activation of TEM and filter membranes

All the membranes were cut to appropriate size required. All PET and PC-TEMs, PVDF and nitrocellulose were activated using 2.5% glutaraldehyde for 2 hr at room temperature (RT) on orbital shaker. The effect of glutaraldehyde-activation on TEM was seen by immobilizing the antibodies on activated membranes and compared to unactivated ones. TEMs were activated using 2.5% glutaraldehyde for different time periods to optimize the time of activation.

2.3.4.3. Immobilization of antibodies

Anti-T4 antibodies (0.5 µl) in spotting buffer at concentration of 1mg/ml were spotted manually on various supports with the help of micropipette. After spotting, supports were incubated for 2 hr at RT. The supports were rinsed with PBS to remove excess unbound antibodies.

2.3.4.4. Blocking

The nonspecific binding (NSB) sites were blocked using PBS-BSA for 2 hr at RT with gentle agitation and rinsed thoroughly with PBS. Since BSA is a relatively inert protein, it is used often as a blocking agent in microarray based application to prevent NSB.

2.3.5. Detection and quantification of spots

^{125}I-T4 at 60,000cpm/100µl was applied to supports for 2 hr with constant agitation. Subsequently, the supports were washed thrice with PBS-T to remove unbound reactants. Spot-intensity and morphology was viewed using autoradiography. The supports were kept in close contact to X-ray films and exposed for 48 hr and films were developed manually. The autoradiograms were scanned and densitometric analysis of each spot was done with help of image analysis software (DensitoQuant) by scanning a fixed circle diameter where the antibody spot was located. The software provided average signal intensity per unit area on a gray scale in arbitrary units (a.u.). Signal intensity and spot diameter was highly influenced by the surface properties. To compare the signal for different types of surface we averaged the overall signal intensity per unit area from all the spots on each support. Local background was subtracted from the average signal intensity.

2.4. Results

2.4.1. Selection of suitable support

For developing a reliable antibody-chip, many supports including glass, hydrophobic and hydrophilic PC and PET-TEMs, PVDF and nitrocellulose membrane were assessed. The support suitability was judged by antibody binding properties based upon spot-morphology, signal intensity, background and reproducibility.

As can be seen from Fig. 2.4, visual inspection of autoradiogram revealed hydrophobic PET and PC-TEM produced dark, distinct, uniform, good morphology spots with clear background. Hydrophilic PET and PC produced larger and less distinct spots but with a clear background. The spotted antibody-solution spread a bit on the membrane leading to some diffusion and blurring around the spots. The spot-morphology was inversely dependent on the wetting ability of the support. A low wetting support will result in more confined spots (smaller size). Although glass discs silanized in-house proved to be good support giving good spot- morphology and low background, but the signal intensity from the spots was lower than TEM indicating that glass had a lower binding capacity. Further, glass required extensive pretreatment and was fragile. The spots on silanized glass slides from Sigma Chemical Co., which were activated using glutaraldehyde, were hardly visible. Nitrocellulose membranes were unsuitable as it gave poor signal and comparatively higher background. PVDF membrane was very hydrophobic and hence gave very small spots with weak signal, though it had good spot-morphology and low background comparable with PET and PC-TEMs. Spot diameter was similar on all the supports except nitrocellulose and PVDF. Of the TEMs etched in-house, the Mylar membrane bombarded with ^{28}Si gave the best signal intensity and spot-morphology while the ^{19}F bombarded Mylar membrane and PC membrane bombarded with ^{35}Cl were inferior. The wetting property of the in-house etched PC and PET TEM was lower than that of commercially available TEM (Fig. 2.4).Of all the supports tested, hydrophobic PC and PET-TEM showed highest immobilization followed by hydrophilic TEMs (Table 2.1 and Fig. 2.5).

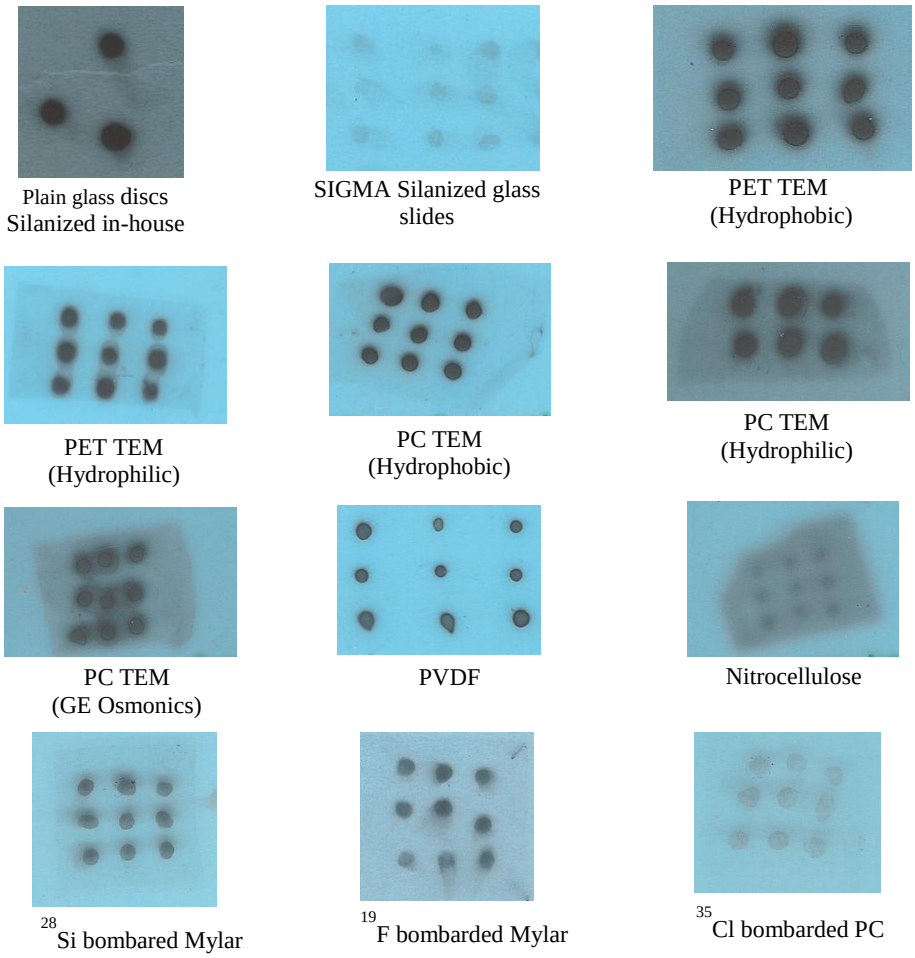


Figure 2.4 Autoradiogram showing comparison of signal intensities and spot morphologies on various supports.
On each surface, identical 3× 3 array (except for glass), composed of anti-T4 antibodies, was incubated with ^{125}I-T4.

Support used	Average signal intensity per unit area (arbitrary units) (a.u.)	Average background per unit area (a.u.)	Average signal-background per unit area (a.u.)
Plain glass discs Silanized in-house	186.3±3.0	96±1.4	90.4±3.0
PET TEM (Hydrophobic)	201.7±8.6	87±6.1	115.1±8.6
PET TEM (Hydrophilic)	181.4±10.5	112±15.8	69.5±10.5
PC TEM (Hydrophobic)	199.7±7.4	86±4.2	113.7±7.4
PC TEM (Hydrophilic)	186.7±3.1	96.9±4.2	89.8±3.1
PC TEM (GE Osmonics)	182.7±2.6	116±3.5	66.7±2.6
Nitrocellulose	119.4±2.7	104±1.1	15.4±2.7
PVDF	151.0±8.0	93±3.3	55±8.0
^{35}Cl bombarded PC	111±4.6	87.5±3.6	23.5±4.6
^{19}F bombarded Mylar	159.6±6.4	96.1±1.6	63.4±6.4
^{28}Si bombarded Mylar	175±9.1	89.8±3.5	85.2±9.1
Sigma silanized glass slide	105.5±4.8	99.6±1.3	5.9±4.8

Table 2.1 Comparison of signal intensity on various supports spotted with anti-T4 antibodies.

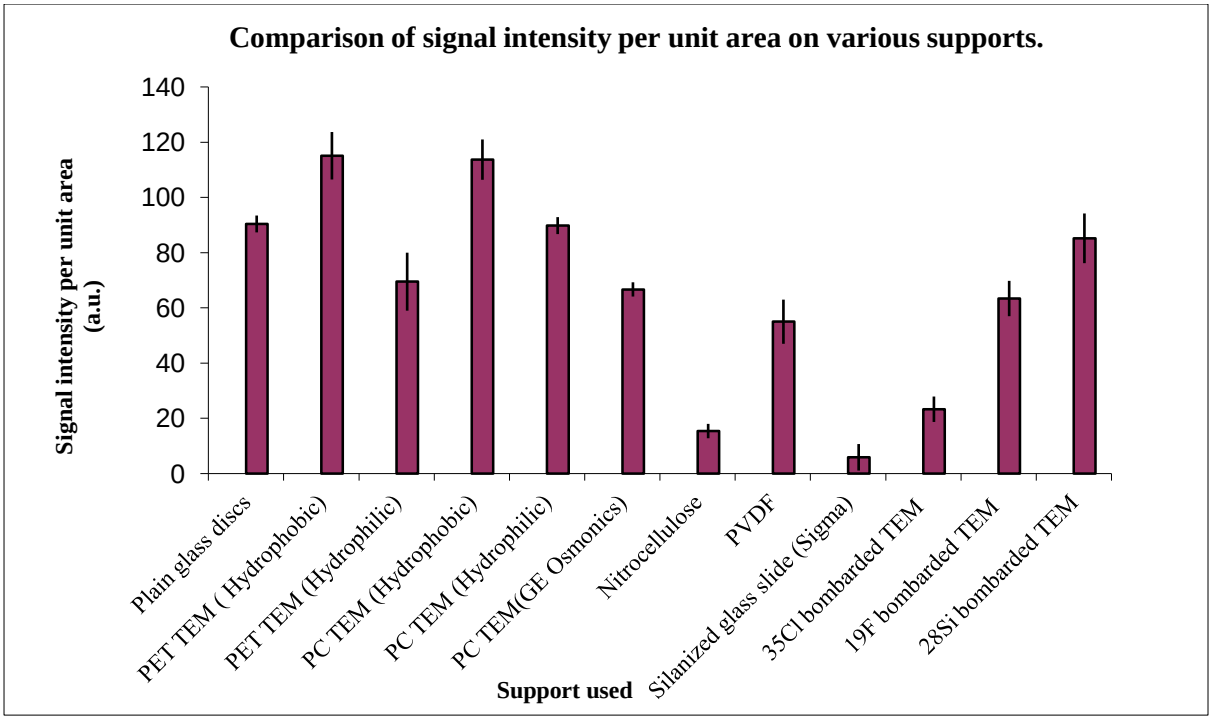


Figure 2.5 Graph showing comparison of signal intensities on various supports.
On each surface, identical 3× 3 array (except for glass), composed of an anti-T4 antibodies was incubated with ^{125}I-T4.

2.4.2. Optimization of immobilization parameters

2.4.2.1. Effect of glutaraldehyde activation

Glutaraldehyde activated TEMs show darker spots and there is an enormous increase in spot-intensity as compared to unactivated TEMs as seen from the autoradiogram and graph in Fig. 2.6. Glutaraldehyde activated PC-TEM retains antibodies by covalent interaction thereby resulting in better immobilization of the antibodies as compared to unactivated TEMs on which antibodies gets passively adsorbed. As seen from Fig. 2.7, spot-intensity increases with increasing time of activation but beyond 2 hr there is an increase in membrane background resulting in lower spot-intensity. Thus, the optimum time of glutaraldehyde activation was found to be 2 hr.

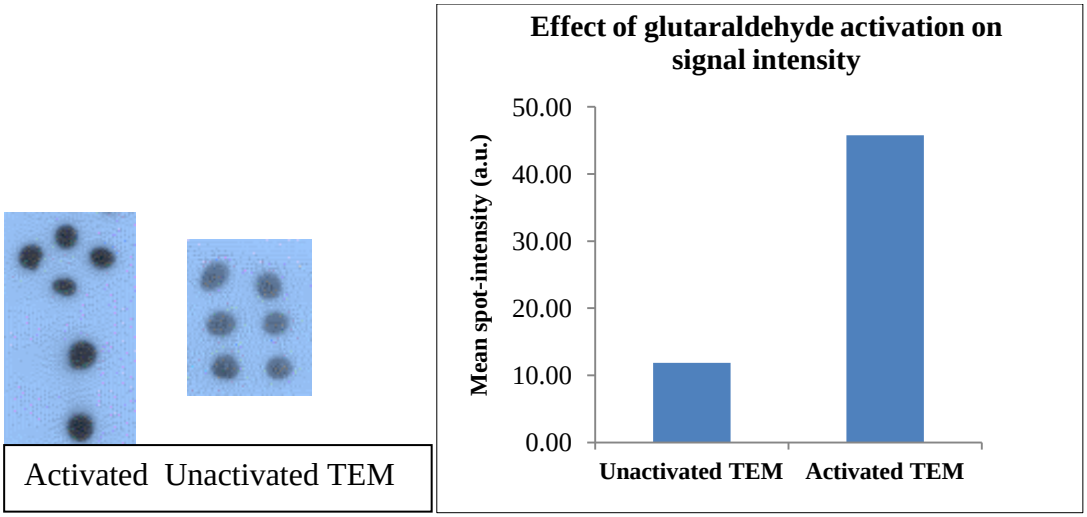


Figure 2.6 Autoradiogram and graph showing the effect of glutaraldehyde activation on spot-intensity.

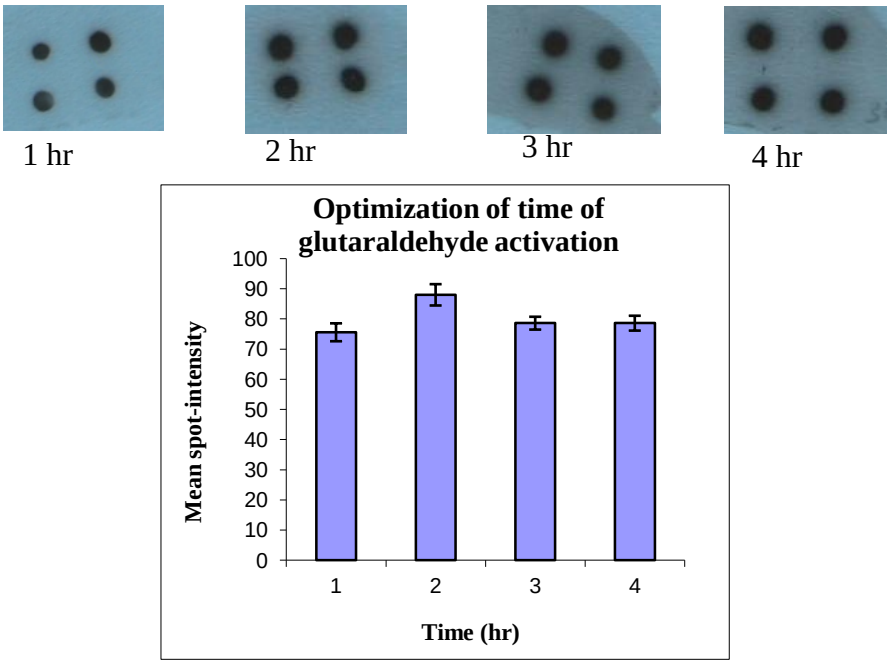
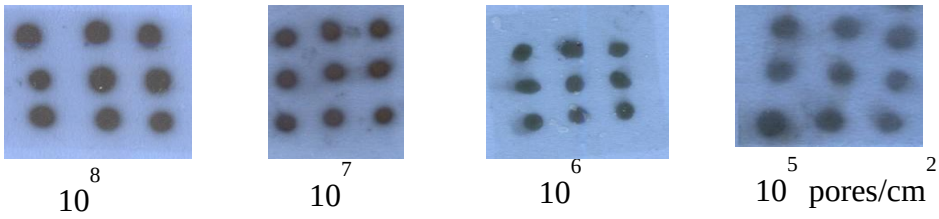


Figure 2.7 Autoradiogram and graph showing the optimization of time of glutaraldehyde activation for antibody immobilization on PC-TEM.

2.4.2.2. Pore-density of TEMs

Increasing the pore-density of TEM provides larger surface area for immobilization of the antibodies. The effect of pore-density on immobilization was seen by immobilizing antibodies on TEMs of different pore densities. TEMs having pore-density of 10^5,10^6,10^7 and

10^8 pores/cm^2 were selected for the immobilization of anti-T4 antibodies. Fig.2.8 indicates that the spot-intensity and morphology is highly dependent on the pore-density. Higher the pore-density, more the spot-intensity and better the spot-morphology. TEMs with higher pore-density shows dark, uniform and distinct spots as compared to TEMs with lower pore-density. Graph in Fig. 2.8 indicates that the signal intensity increases as the pore-density is increasing.



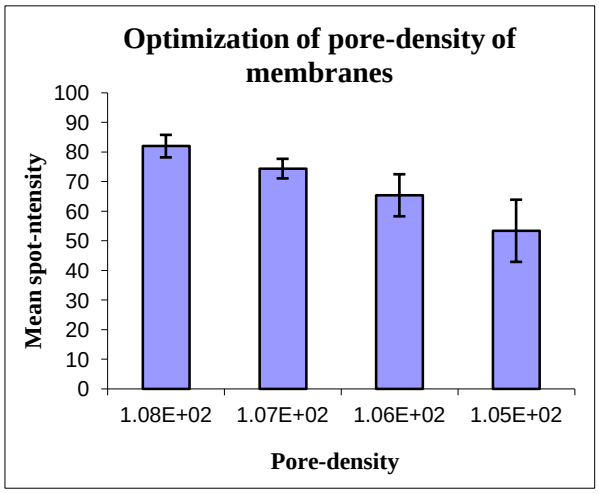


Figure 2.8 Autoradiogram and graph showing the effect of pore density of PC-TEM on spot-morphology and intensity respectively.

2.4.2.3. Time of immobilization

Anti-T4 antibodies were spotted on TEM for the various time periods:- 0.5 hr, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr and overnight. The spot-intensity increased as the time of the incubation was increased from 0.5 to 2hr. After that there was no significant increase in spot-intensity. The optimum time was found to be 2 hr. The amount of immobilized protein reached a plateau for a reaction time longer than 2 hr as indicated in graph in Fig.2.9.

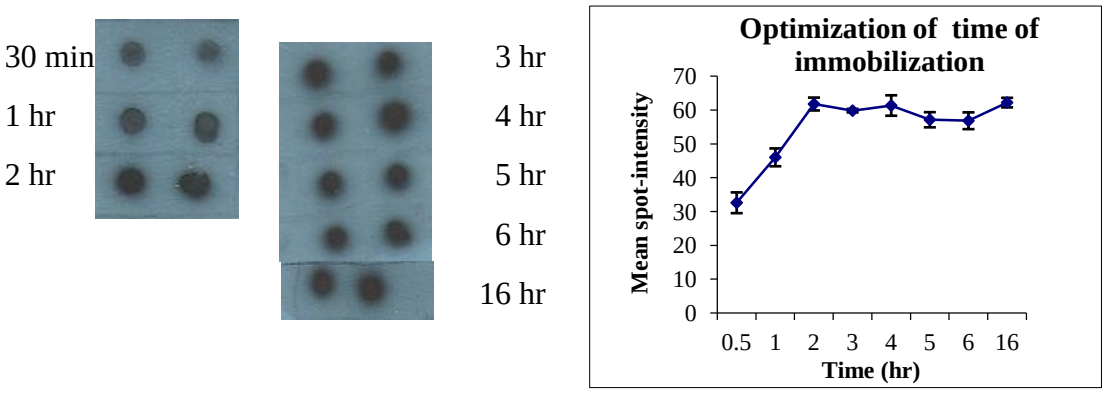
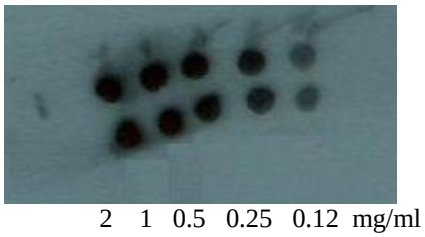


Figure 2.9 Autoradiogram and graph showing optimization of time of antibody immobilization on TEM.

2.4.2.4. Antibody concentration

Among the various parameters that influence antibody-chip performances, the concentration of antibody, used for spotting, has to be initially optimized. Indeed, antibody density on the surface should be optimal to reach maximal signal intensity. Different concentrations of antibody were tested in order to select the concentration which gave highest intensity. Anti-T4 antibodies were spotted at different concentrations (2, 1, 0.5, 0.25, 0.12 mg/ml). The TEMs were blocked with 4% BSA and reacted with ^{125}I-T4 (60,000 cpm/100 µl) .The mean signal intensity increased with the increasing concentration of the antibodies upto 1 mg/ml beyond which no increase in signal intensity was observed (Fig. 2.10).



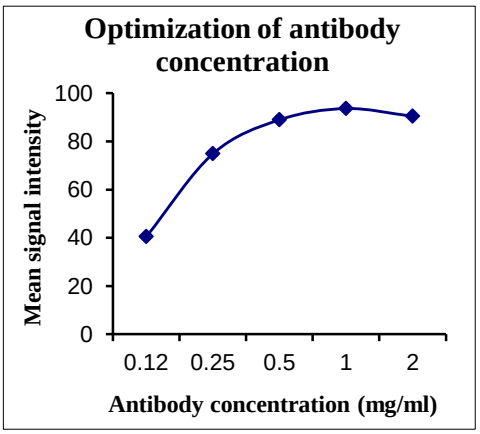


Figure 2.10 Autoradiogram and graph showing mean signal intensity obtained by immobilizing increasing concentration of anti-T4 antibody on TEM.

2.5. Discussion

The selection of a suitable support for the 'antibody-chip' is an essential prerequisite for its fabrication. As given in Chapter-1, although several supports are available, none of them were able to meet all the requirements for the fabrication of antibody-chip in functional form. For antibody microarray, an improvement in the efficiency of antibody immobilization, in terms of functional conservation and the amount immobilized is required. Since the antibody binding capacity is highly dependent on the surface area available, we have tested the thin microporous TEM as a novel support. Our data indicates that such membranes can provide a solid support for making antibody-chip. Although glass appeared as a promising support for protein immobilization in general, it required extensive pretreatment during its activation and thin glass discs are fragile. We compared microporous PET and PC-TEM with existing surfaces like modified glass and filter membranes like nitrocellulose and PVDF. Parameters such as spot-morphology, signal intensity, background were evaluated on each surface. High signal per unit area, good spot-morphology and low background was achieved with hydrophobic PET and PC-TEMs indicating their high protein binding capacity. Because of the hydrophobicity, the spotted antibody solution remains confined to its spotted location resulting in well-defined spots. Hydrophilic TEMs caused some spreading of antibody

solution causing smudged spots and higher local background than hydrophobic membranes, producing lower signal per unit area. In our experience, hydrophobic surfaces tend to produce small, uniform spots, whereas most hydrophilic surfaces yield homogeneous spots, which, however, are often of irregular shape. Nitrocellulose showed low signal intensity because of high background on nitrocellulose. Immobilization is dependent both on pore size as well as density. On comparing TEMs of different pore densities, a membrane with pore density of 10^8 pores/cm^2 is appropriate for making antibody-chip for MAIA.

Attaching antibodies via physical adsorption can lead to leaching of antibodies from the support under experimental conditions. Glutaraldehyde activated PET and PC-TEMs retain antibodies by covalent interaction, thereby resulting in better immobilization of the antibodies. Moreover, the chemistry used for activating the TEM for immobilization of antibodies is simple and rapid as compared to other covalent or affinity linkage used for coupling of antibodies to glass.The schematic diagram of the activation is given below:

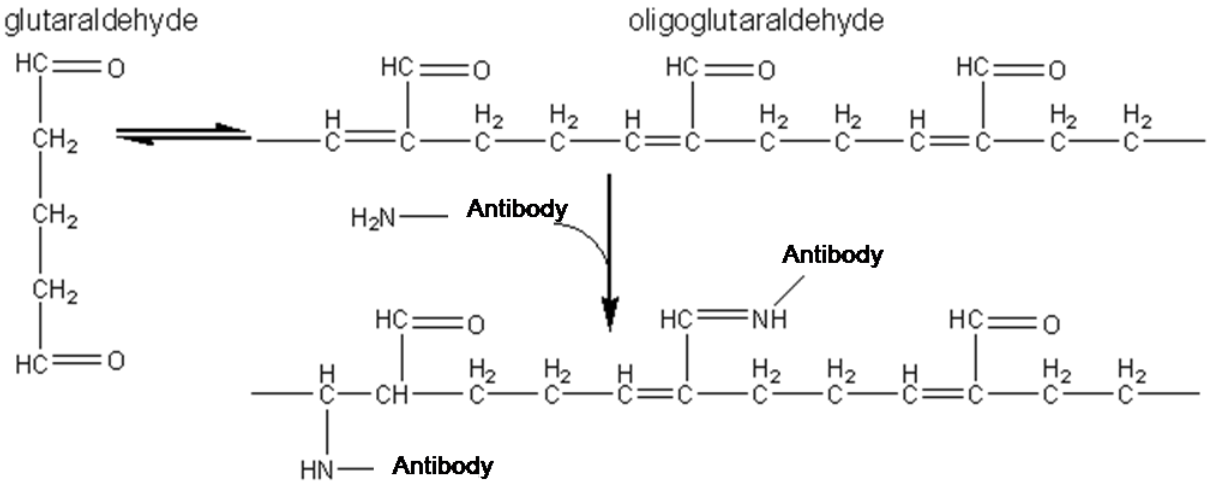


Figure 2.11 General mechanism of activation of hydrophobic surfaces by glutaraldehyde for immobilization of proteins.

The method of antibody immobilization on TEM using glutaraldehyde was found to be excellent and highly convenient with the preservation of biological activity. TEM provided

flat smooth surface with good spot-morphology, high protein binding capacity, low background, and physically and chemically robust support for MAIA. The results clearly demonstrated an enhanced performance of hydrophobic PET and PC-TEM as compared with that of other supports. Although nitrocellulose coated slides are used as a material for the production of antibody chip, they suffer from high non-specific adsorption and thereby a high background. Our study revealed a novel MAIA support of superior characteristics and single step activation method for coupling of antibodies for making antibody-chips for MAIA. Due to the unavailability of hydrophobic PET and PC-TEM commercially, we used PC-TEM from Millipore as a solid support for making antibody-chip.

In this Chapter, we studied several solid supports for antibody-immobilization, their chemical activation, the effect of antibody concentration and time of immobilization. We found that glutaraldehyde activated PC-TEM was the most suitable and that antibodies at 1mg/ml when spotted and incubated for 2 hr on 2.5% activated PC-TEM gave best results for antibody immobilization.

These results showed that immobilization efficiency depends on surface properties (chemical groups, wetting properties) and spotting conditions (concentration and time of immobilization). In conclusion, this study allowed defining the best immobilization conditions of antibodies on TEM.

CHAPTER 3

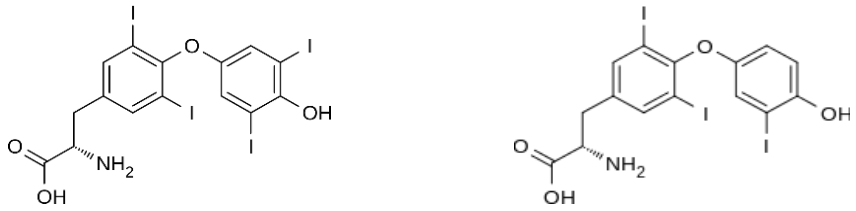
Standardization and Validation of Single Analyte Microarray Immunoassay.

3.1. Introduction

Antibody microarrays have played a significant role in the detection and quantification of analytes in complex biological samples as a high throughput technique. The ability of antibodies to bind (capture) its antigen (analyte) with high specificity makes this approach particularly well suited for detecting low concentration-analytes in highly heterogeneous mixtures, like in serum [138-140]. However, as presented in the previous Chapters, many parameters such as solid support for immobilization of antibodies, surface chemistry, blocking procedures, antibody concentration, time of immobilization etc., influence antibody immobilization. These parameters may significantly influence analytical performances (sensitivity, specificity, limit of detection) of antibody microarray [75, 132].

Thus, this Chapter attempts to show the proof of concept of the validity of microarray immunoassay (MI) for three individual analytes viz. T4, TSH and Tg.

As described in Chapter 1, the thyroid hormones, T3 and T4 are tyrosine-based hormones produced by the thyroid gland. They are primarily responsible for regulation of metabolism and are essential for proper development and differentiation of all cells of the human body. T4 is normally present in the serum within the range of 71.5–158 nmol/l [7].



(a) *(b)*

Figure 3.1 Structure of (a) T4 and (b) T3.

Thyroid-stimulating hormone (TSH, thyrotropin) is a glycoprotein hormone secreted by the pituitary with a molecular weight of ~28,000 Da. TSH is a heterodimer consisting of α and β subunits tightly, but non-covalently, bound. α subunit is common to TSH, follicle stimulating hormone (FSH), luteinizing hormone (LH), and chorionic gonadotropin (CG). The β subunit confers specificity to the molecule since it interacts with the thyroid cell TSH receptor and is rate-limiting in the formation of the mature heterodimeric protein. TSH stimulates the synthesis of thyroid hormones from thyroid, which in turn has a negative regulatory feedback effect on the production of TSH. Normal serum TSH concentrations range from 0.4-4.5 μIU/ml [7].

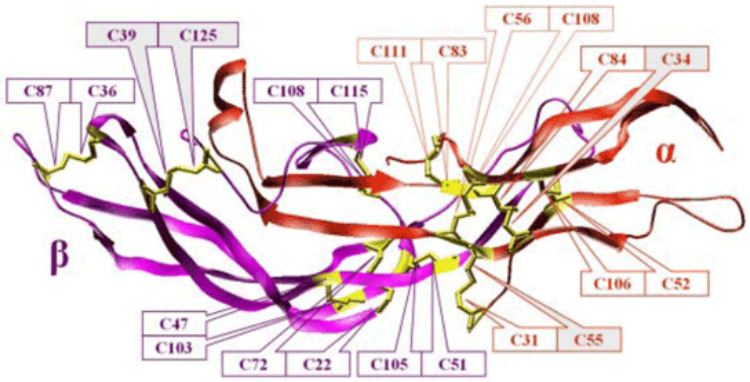


Figure 3.2 Structure of human TSH. Adapted from www.thyroid manager.com

Thyroglobulin is a glycoprotein of high molecular weight (660kDa) localized within the colloid of the thyroid follicle. It plays an essential role in the storage of iodine and acts as substratum for the synthesis of iodinated T4 and T3. Thyroglobulin also plays a central role in a wide variety of pathophysiologic conditions affecting the thyroid gland. For example, Tg

has been implicated as a possible autoantigen involved in the production of thyroid autoimmune diseases [141, 142]. Genetic defects in Tg biosynthesis have been shown to result in congenital hypothyroidism. The recognition of the tissue-specific origin of the circulating Tg concentration has led to serum Tg measurement becoming firmly established as the primary tumor-marker for monitor patients with differentiated thyroid carcinoma (DTC).

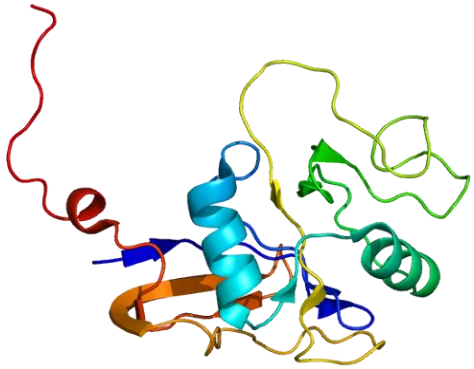


Figure 3.3 Structure of human Tg. Adapted from www.thyroidmanager.com.

Different components of MI development such as immobilization conditions, assay optimization, and detection system were investigated. Herein, the selected capture-antibodies (anti-T4, anti-TSH, anti-Tg antibodies) were immobilized on PC-TEM individually. Detection was performed using ^{125}I-labeled antibodies or antigens as tracer. Both the tracer concentration and time of incubation were optimized to estimate low concentration (ng/ml range) of analytes in patient's sera. Finally MI was validated using various parameters.

3.1.1. Validation

It is very important for the immunoassay to be accurate and precise. Accuracy is defined as the closeness of the concentration value obtained by the method to the known true concentration of analyte. Precision is the closeness of individual measures of an analyte when the method is applied repeatedly to multiple aliquots of the same biological sample. These

key characteristics of any bioanalytical method are investigated during the process of validation. Validation of an analytical method identifies the sources and quantifies the potential errors in the method [143, 144]. Classical immunoassays have to be validated in line with the FDA directives (Food and Drug Administration 2001) [143]. Such regulations do not exist for MAIA. Therefore, in order to validate MI/MAIA, FDA guidelines for pharmacokinetic immunoassays validation were followed. The validation covered the assessment of the method robustness and reproducibility.

This evaluation includes:

- Limit of detection (defined as the lowest concentration or quantity of an analyte that can be detected with a stated reasonable uncertainty for a given analytical procedure). It is the concentration corresponding to a signal, two standard deviations (SD) above the mean for a calibrator that is free of analyte.
- Range of linearity
- Imprecision (within- and between-batch concentrations using specimens that are in an appropriate biological matrix)
- Recovery of pure analyte spiked into the test matrix (e.g., serum, plasma)
- Comparability of the results obtained by the method being validated with those obtained by a reference quality method from patients selected to include a range of values for the analyte likely to be encountered in routine application.

The analytical performance of MI was performed by evaluating parameters such as sensitivity, range, precision, linearity and recovery studies for matrix evaluation. Finally, under the optimal conditions, MI was validated by evaluation of sera from patients referred to RMC for thyroid evaluation. Sera obtained from patients were analyzed both by RIA/IRMA and MI. The results obtained by both methods were compared using linear regression analysis.

3.2. Materials

3.2.1. Chemicals and biological products

Glutaraldehyde (25 % w/v) was purchased from Merck. Bovine serum albumin (BSA) and L-thyroxine-sodium salt were procured from Sigma, USA. Bovine immunoglobulin was purchased from HiMedia Laboratories Pvt. Ltd.**,** Mumbai, India. TSH standards and ^{125}I-monoclonal anti-TSH antibodies (TSH tracer) were obtained were obtained from Board of Radiation and Isotope technology (BRIT), Mumbai, India. The TSH standards were calibrated against the reference TSH preparation IRP 80/558. Tg standards and ^{125}I labeled monoclonal anti-Tg antibodies (Tg tracer) were purchased from Izotop, Budapest, Hungary. The Tg standards were calibrated against the reference Tg preparation BCR-CRM 457. PC-TEM was procured from Millipore, USA. Monoclonal antibodies to TSH, used as capture-antibodies, were purchased from Biodesign International, USA. Polyclonal antibodies against T4 were from our animal house and produced using standard immunizing procedures in rabbits, with approval from BARC Animal Ethics Committee. Polyclonal antibodies against Tg were also produced in camel in collaboration with National Research Center on Camel (NRCC), Bikaner. T4 was labeled with ^{125}I using Chloramine -T method at a specific activity >1000µCi/µg as described in Chapter 2 (Section 2.3.2). Synthetic hormone free serum (SHFS) was prepared by dissolving 4.5% BSA and 2% bovine immunoglobulin in sterile PBS (pH 7.4, 0.05 M) along with 0.2% sodium azide and 20mg/l of gentamycin as preservatives. All other chemicals and reagents required for this study were purchased locally and were of analytical or equivalent grade. Low conductivity deionised water was used wherever required.

3.2.2. Buffers

1. Antibody spotting buffer (PBS) - PBS 0.025 M (pH 7.4)

2. Washing buffer (PBS-T) - PBS with 0.1 % v/v Tween 20 (PBS-T)
3. Blocking solution (PBS-BSA) - 4% w/v BSA in PBS.
4. ANS buffer (PBS-ANS) - PBS with 0.5% w/v BSA and 0.25% w/v ANS.

3.2.3. Equipments

1. X-ray film from Kodak.
2. γ-radiation counter (gamma counter) from Stratec, Germany.
3. PhosphorImager -Typhoon trio^{+} from GE Healthcare

3.2.4. Softwares used

1. ImageJ (http://rsbweb.nih.gov/ij/) for quantification of the spots obtained using phosphorimager.
2. Densitoquant software for quantification of the spots on X-ray films.
3. Mgamma in-vitro test (available with Stractec Gamma Counter) for plotting of the standard curve and quantification of the spot intensity.

3.3. Methods

3.3.1. Immunoassays

3.3.1.1. RIA

RIA was performed for T4 using T4 RIA kits. The kit components were

- T4 standards - 0, 2.5, 5, 10 and 20 µg/dl.
- Quality control samples
- T4 tracer – ~70,000 cpm/100 µl.
- Anti-T4 polyclonal antibodies coupled to magnetic particles.

Protocol for T4 RIA is summarized below.

- A 10 µl of serum sample/standard was pipetted into RIA tubes (Plain polystyrene tubes - 75mm X 12mm).
- 100 µl of T4 tracer was pipetted in all tubes.
- 0.5 ml of magnetic particles was added to all tubes.
- The tubes were vortexed and incubated for two hour on orbital shaker.
- The tubes were placed on magnetic rack for 15 min after incubation.
- The tubes were decanted and counted in gamma counter.

3.3.1.2. IRMA

3.3.1.2.1. TSH

TSH concentrations were estimated using human TSH IRMA kits (BRIT, Mumbai, India). Following are the kit components.

- Anti-TSH antibody coated tubes
- Standards – 0, 0.15, 0.5, 1.5, 5, 15, 50, 100 µIU/ml
- Tracer- ~ 100,000 cpm/100 µl
- Quality control samples
- TSH wash buffer.

Protocol for TSH IRMA is described below

- 100 µl of TSH standards/ serum samples/ QC samples were pipette in coated tubes followed by addition of 100 µl of tracer.
- Tubes were incubated for two hours on orbital shaker.
- Tubes were washed thrice with 1 ml of TSH wash buffer
- Tubes were counted for two minutes in gamma counter.

3.3.1.2.2. Tg

Tg concentrations in serum samples were estimated using human Tg IRMA kits (Izotop, Hungary). Following are the kit components.

- Coated tubes
- Standards – 0, 0.3, 1, 4, 20, 100, 250 ng/ml
- Tracer- ~ 150,000 cpm/100 µl
- Quality control samples

The protocol for Tg IRMA is given below,

- 100 µl of Tg standards / serum samples / QC samples were pipette in coated tubes followed by addition of 200 µl of tracer.
- Tubes were incubated for two 18 hours on orbital shaker.
- Tubes were washed thrice with 1 ml of wash buffer and counted for two minutes in gamma counter.

3.3.2. Microarray immunoassays with individual analytes

A competitive MI was standardized for the estimation of T4 and sandwich MI was standardized for estimation of TSH and Tg in human serum samples.

3.3.2.1. Preparation of antibody-chip

Antibody-chip was produced by spotting 0.5 µl each of anti-T4, anti-TSH or anti-Tg antibodies, individually at concentration of 1mg/ml on glutaraldehyde activated PC-TEM, with the help of micropipette. PC-TEMs were incubated for 2 hr at RT. Duplicate spots were made for each sample/standard/quality control (QC). TEMs were washed thrice with 1 ml PBS and dried by keeping between sheets of Whatman filter paper.

TEMs were blocked with PBS-BSA for 2 hr at RT on orbital shaker to prevent NSB and washed thrice with 1 ml of PBS. TEMs were cut into 0.5 cm X 1.0 cm pieces and placed individually in wells of 24 well plate for carrying out immunoassays.

3.3.2.2. Immunoassays

3.3.2.2.1. T4

A five point standard curve was established for estimation of T4 in serum samples. The standards were prepared by mixing the required known amount of the L-thyroxine-sodium salt in SHFS. Five standards (S1- S5) were prepared having T4 concentration of 0, 2.5, 5.0, 10.0 and 20.0 µg/dl.

Different concentrations of ^{125}I-T4 were dissolved in PBS-ANS to prepare T4 tracer. TEMs were reacted with 10 µl of standard/sample/QC and 100 µl of tracer having different concentration of ^{125}I-T4. Reaction was carried out at RT, for different time periods, with continuous shaking and rinsed thrice with PBS-T and air-dried.

3.3.2.2.2. TSH and Tg

A seven point standard curve was established for estimation of TSH and Tg in human serum samples. The concentration of the standards (S1 –S7), used were 0, 0.15, 0.5, 1.5, 5, 15, 50 µIU/ml for TSH and 0, 0.3, 1, 4, 20, 100, 250 ng/ml for Tg. The TEMs were reacted with 100 µl of corresponding standard, QC or test-sample and 100 µl of corresponding tracer at different concentrations. Incubation was carried out for different time periods on an orbital shaker and washed thrice with PBS-T.

3.3.2.3. Detection and quantification

The binding between antibodies and analyte can be monitored by quantifying the signal generated from each spot using various detection techniques as described in Chapter 1

(Section 1.6.6). We have used radioactivity for detection and quantification of the spots. The signal from ^{125}I labeled antigens/antibodies was visualized using autoradiography. Quantification was done either by directly counting the spots in gamma counter, by scanning of the X-ray films followed by quantification of the spots or by obtaining the digital autoradiograms using PhosphorImager and quantifying the spot signal intensity. Quantification of X-ray films was done using Densitoquant software as described in Chapter 2 (Section 2.3.5). Average spot intensities presented in the graphs and tables are calculated from two adjacent microarray spots. Because of the limited range and sensitivity of the X-ray film, the standard curve obtained had a narrow range. Hence, to determine the performance of the MI, PhosphorImager was used to image the membranes. The images were generated using ImageQuant TL software provided by GE Healthcare Biosciences. The intensity of each spot was quantified by open source image analysis software, ImageJ (http://rsbweb.nih.gov/ij/). The intensity of each spot was measured and averaged across its circular area generating mean spot intensities. Local background was determined in the same manner and subtracted from the mean spot intensities. Average spot intensities presented in the graphs and tables are calculated from two adjacent microarray spots. RIA analysis software was used for standard curve plotting and extrapolating the concentration of the sample.

3.4. Results

For implementation of MI, many parameters such as immobilization conditions, concentration of the tracer, incubation time and detection system was optimized.

3.4.1. Selection of quantification method

Three different quantification methods were evaluated for quantification of spot signal intensity. For quantification of the spot intensity, initially the autoradiography of TEMs was

done. Because of limited dynamic range of X-ray film, the film got saturated and the expected standard curve was not obtained. This was proved when the individual spots were cut and counted in gamma counter. Finally the TEMs were scanned using PhosphorImager and the intensity of each spot was quantified. The results obtained were compared with results using gamma counter. Fig.3.4 shows the comparison of results obtained using X-ray film and gamma counter. Fig.3.5 shows the comparison of results obtained using gamma counter and PhosphorImager.

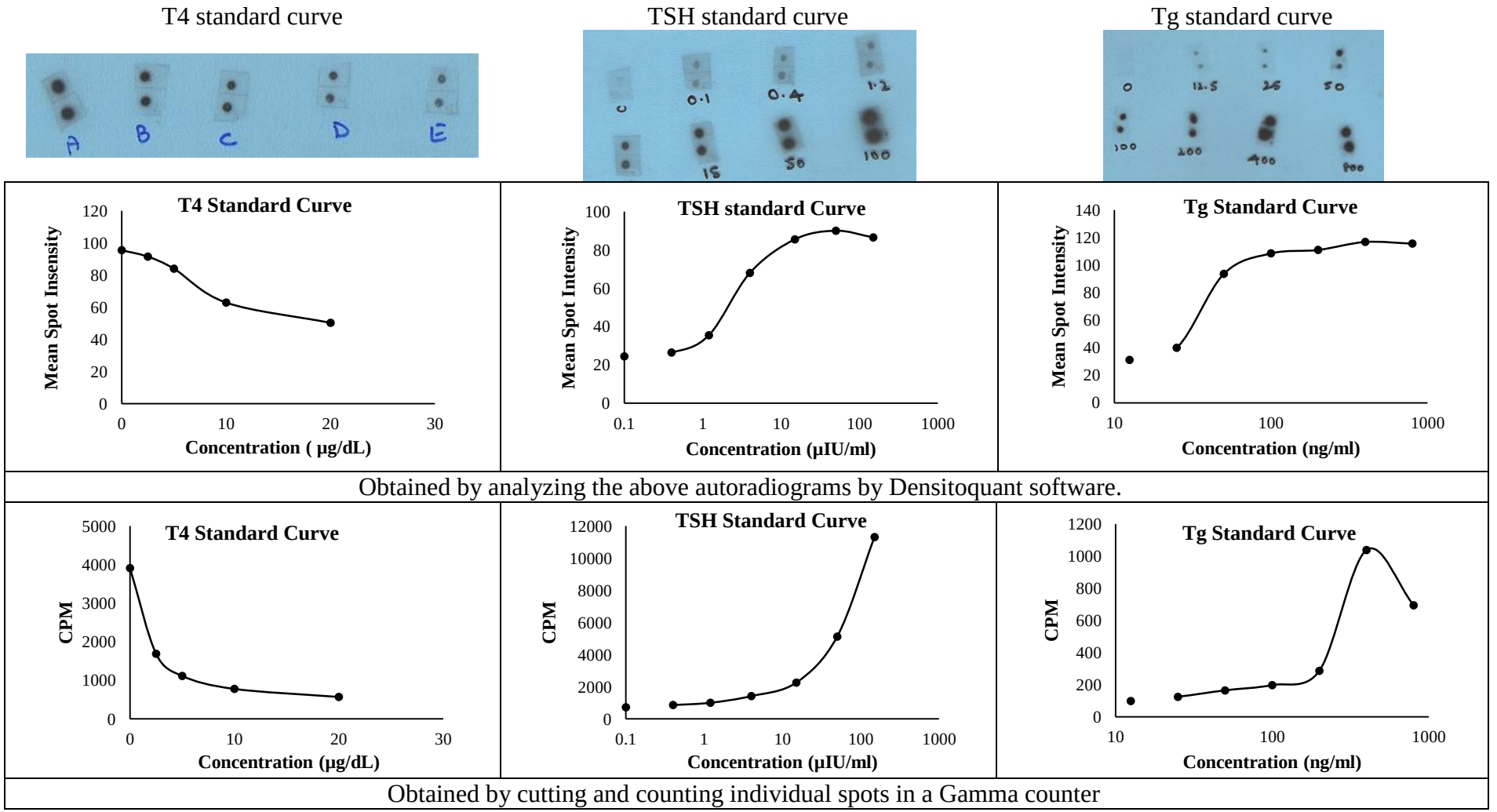


Figure 3.4 Autoradiogram and graphs showing comparison of results obtained using X-ray film and gamma counter. X-ray films were scanned and quantified using Densitoquant software.

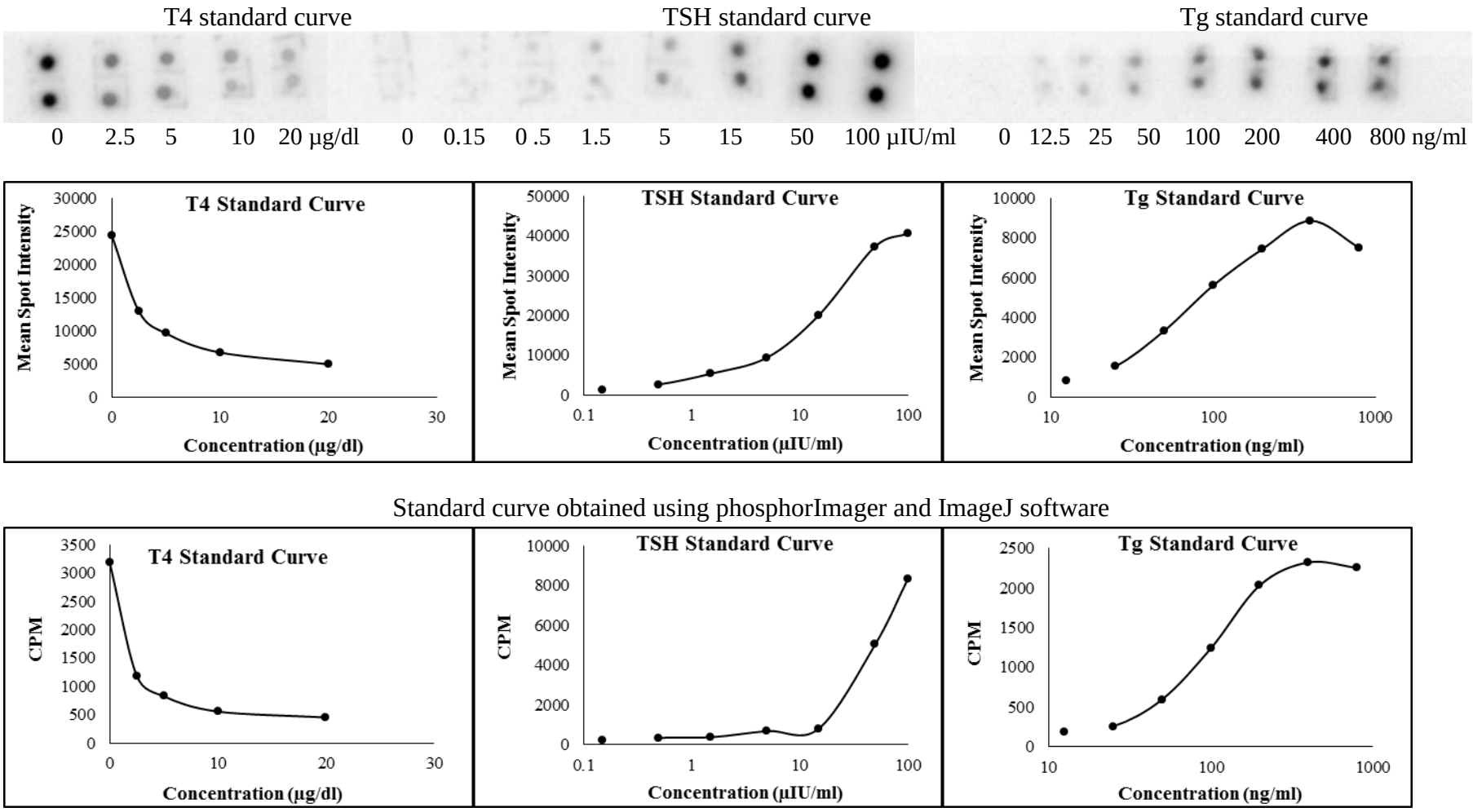


Figure 3.5 Autoradiogram and graphs showing comparison of results obtained using PhosphorImager and gamma counter.

3.4.2. MI optimization

For developing any immunoassay, samples of known analyte concentration are tested according to an optimized procedure and a calibration curve (or dose-response curve) is usually plotted of the signal response as a function of the analyte concentration. The concentration of analyte in the unknown sample may then be interpolated from the calibration curve. An assay's performance can be gauged by its ability to accurately detect low concentrations of analyte and a wide range of analyte concentrations based on the linearity of the calibration curve. Optimal assay conditions and reagent concentrations must be experimentally determined to provide the desired analytical information. We had already optimized the solid-phase capture antibody concentration and the concentration and incubation time of the blocking agent. The optimization of detection antibody concentration and time of incubation is described later in the Chapter. The signal generation scheme and quantification method was described earlier in this Chapter.

3.4.2.1. Optimization of tracer concentration

3.4.2.1.1. T4

Different concentrations of ^{125}I-T4 were tested in order to select the concentration which gave the highest signal-to-noise ratio (SNR). Anti-T4 antibodies (0.5 µl) at 1000 µg/ml were immobilized as spots on the PC-TEM. T4-tracer having approximately 20,000, 40,000, 60,000, 80,000 and 100,000 cpm/100 µl was prepared. The signal generated for each concentration of ^{125}I-T4 was divided by the corresponding background at each standard concentration. The highest SNR was obtained with 60,000 cpm/100 µl of ^{125}I-T4. Fig.3.6 shows the corresponding graph and autoradiogram.

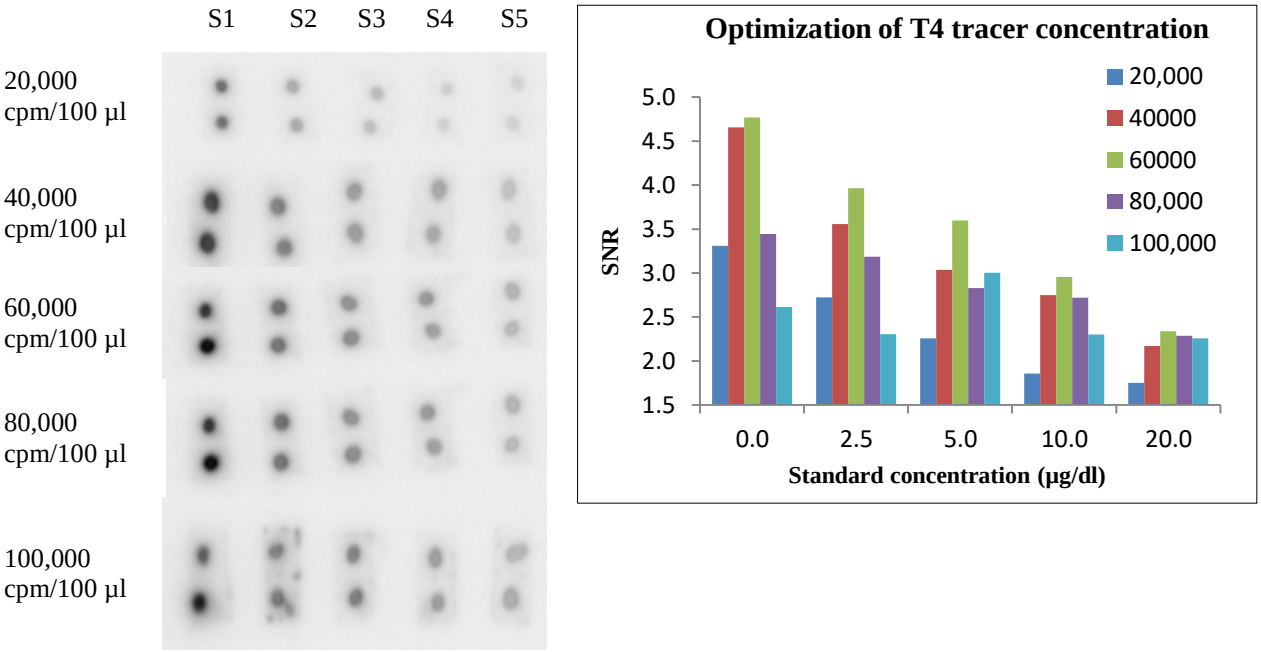


Figure 3.6 Autoradiogram and graph showing the optimization of T4 tracer concentration.

3.4.2.1.2. TSH

For TSH, 0.5 µl (1000 µg/ml) capture antibodies were immobilized as spots on the PC-TEM. ^{125}I labeled anti-TSH antibody at concentration of ~ 60,000, 80,000, 100,000 and 120,000 cpm /100 µl was tested for all standard concentrations. The highest SNR was obtained with 100,000 cpm/100 µl of TSH tracer. Fig.3.7 shows the corresponding graph and autoradiogram.

3.4.2.1.3. Tg

For Tg, capture antibodies were immobilized as spots by placing 0.5µl (1000 µg/ml) of them on PC-TEM.^{125}I-labeled anti-Tg monoclonal antibodies having 50,000, 100,000 and 150,000 cpm /100 µl was tested and highest SNR was obtained with 100,000 cpm/100 µl of Tg tracer. Fig.3.8 shows the corresponding graph and autoradiogram.

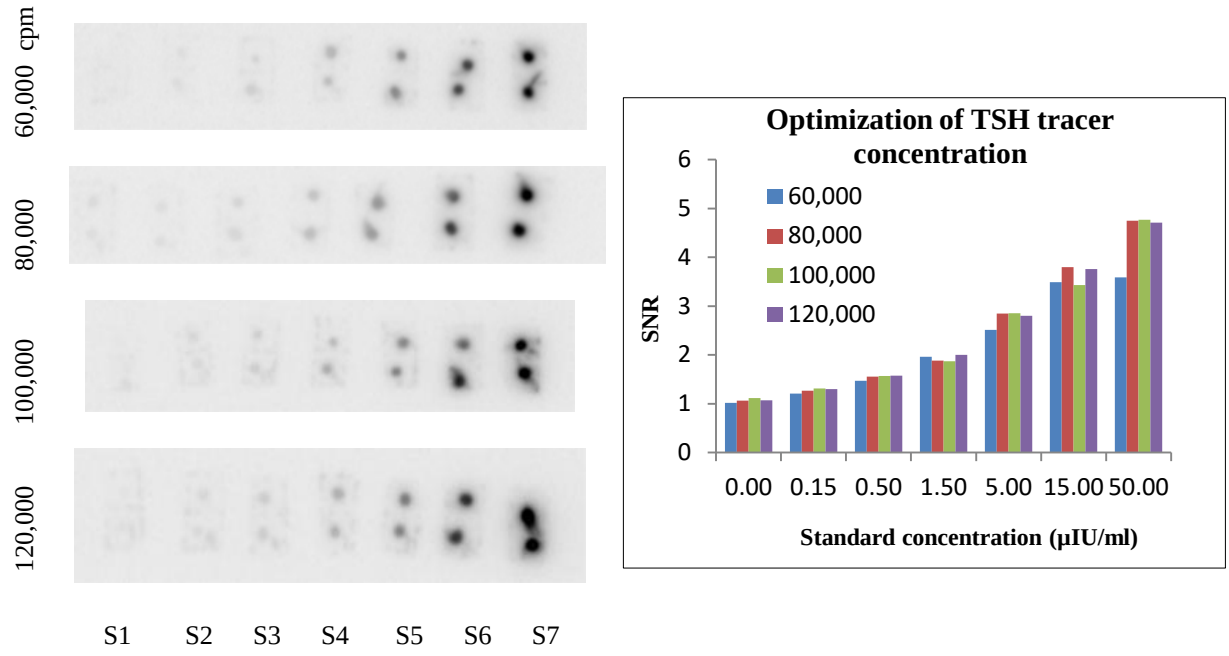


Figure 3.7 Autoradiogram and graph showing the optimization of the tracer concentration for TSH.

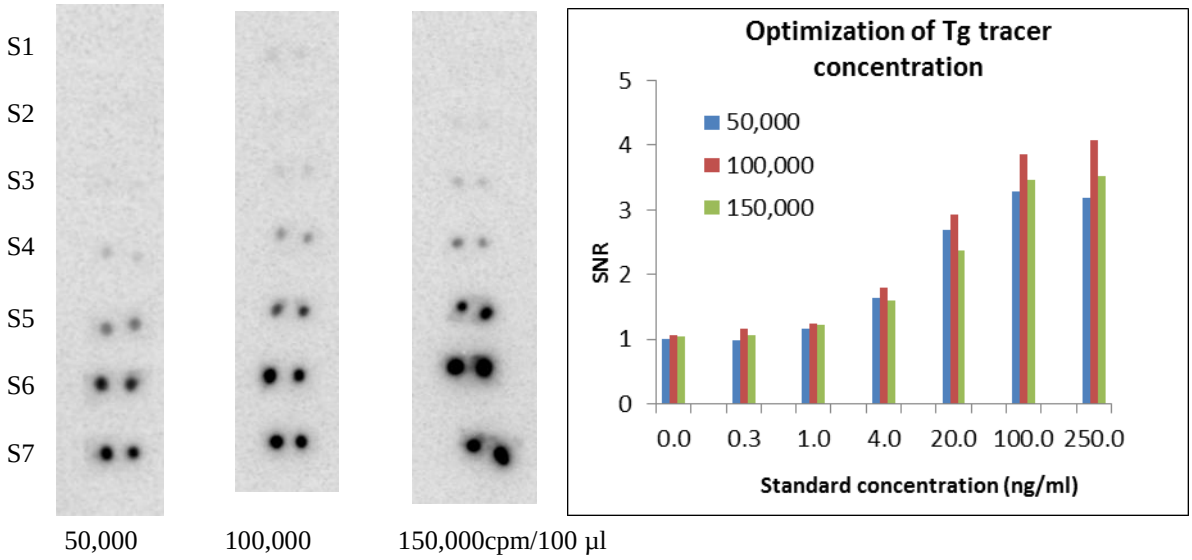


Figure 3.8 Autoradiogram and graph showing the optimization of the tracer concentration for Tg

3.4.2.2. Optimization of time of incubation

3.4.2.2.1. T4

After selection of the optimum concentration of the tracer, TEMs were incubated with tracer along with standards for different time period in order to select the time of incubation which gave highest SNR. TEM immobilized with anti-T4 antibodies were reacted with 10µl of T4 standard along with 100 µl of ^{125}I-T4 (60,000 cpm /100 µl) for 1, 2, 3 and 4 hr. The spot signal intensity was divided by local background. The highest SNR was obtained at 2 hr of incubation. Fig.3.9 shows the corresponding graph and autoradiogram.

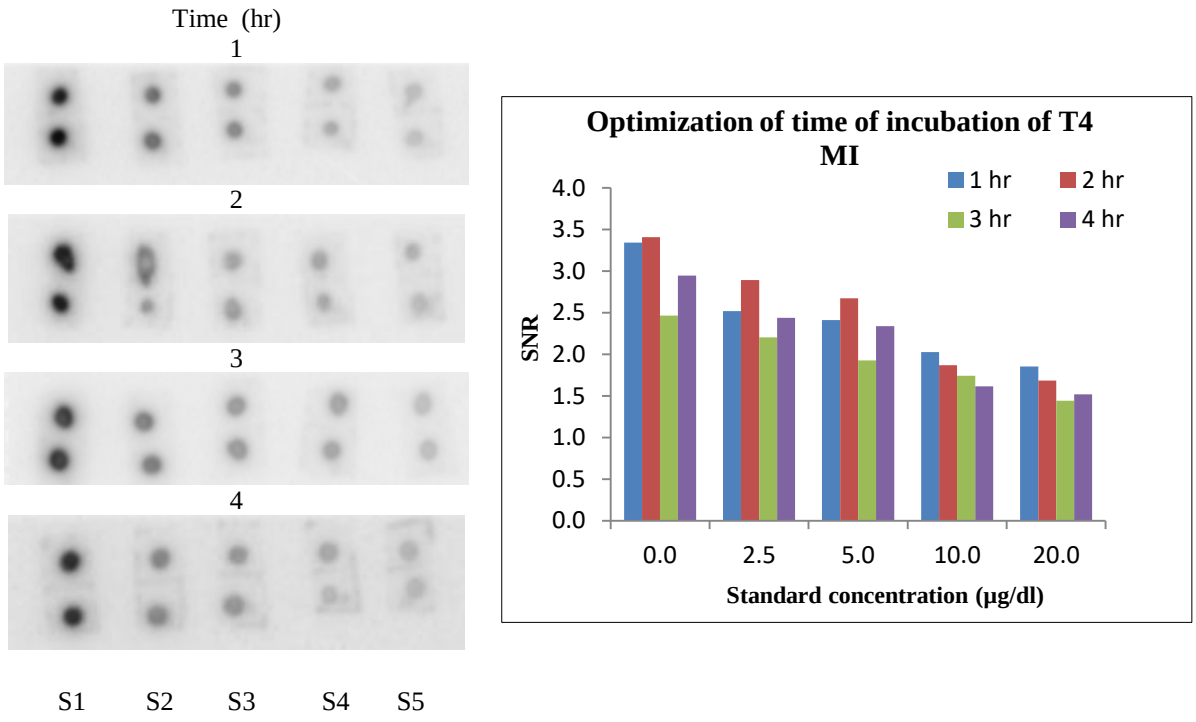


Figure 3.9 Autoradiogram and graph showing the optimization of time of incubation with tracer for T4

3.4.2.2.2. TSH

Anti-TSH antibody immobilized TEMs were reacted with 100 µl of standard along with ^{125}I-anti-TSH monoclonal antibodies (100,000 cpm/100 µl) for 1, 2, 3 and 4 hr. The highest SNR

was obtained with 2 hr of incubation. Fig. 3.10 shows the corresponding graph and autoradiogram.

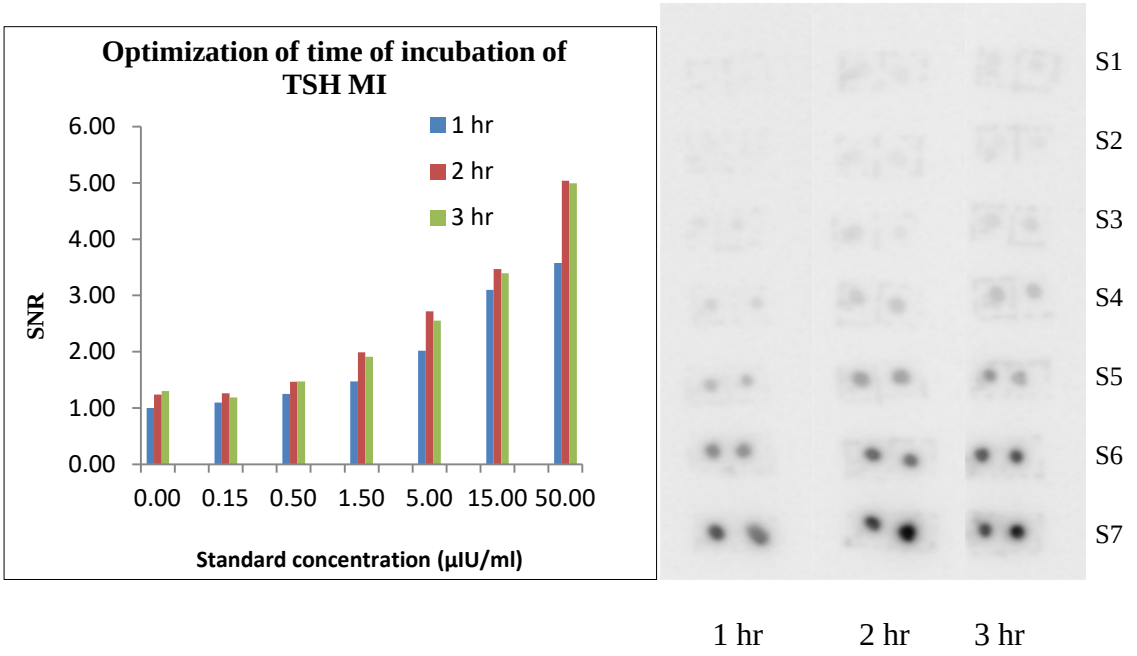


Figure 3.10 Graph and autoradiogram showing the optimization of time of incubation with tracer for TSH

3.4.2.2.3. Tg

TEMs immobilized with anti-Tg antibodies were reacted with 100 µl of standard along with ^{125}I-anti-Tg antibody tracer at 100,000 cpm/100 µl for 2 hr, 4 hr and overnight. The highest SNR was obtained when TEMs were incubated with standard and tracer for overnight. Fig. 3.11 shows the corresponding graph and autoradiogram.

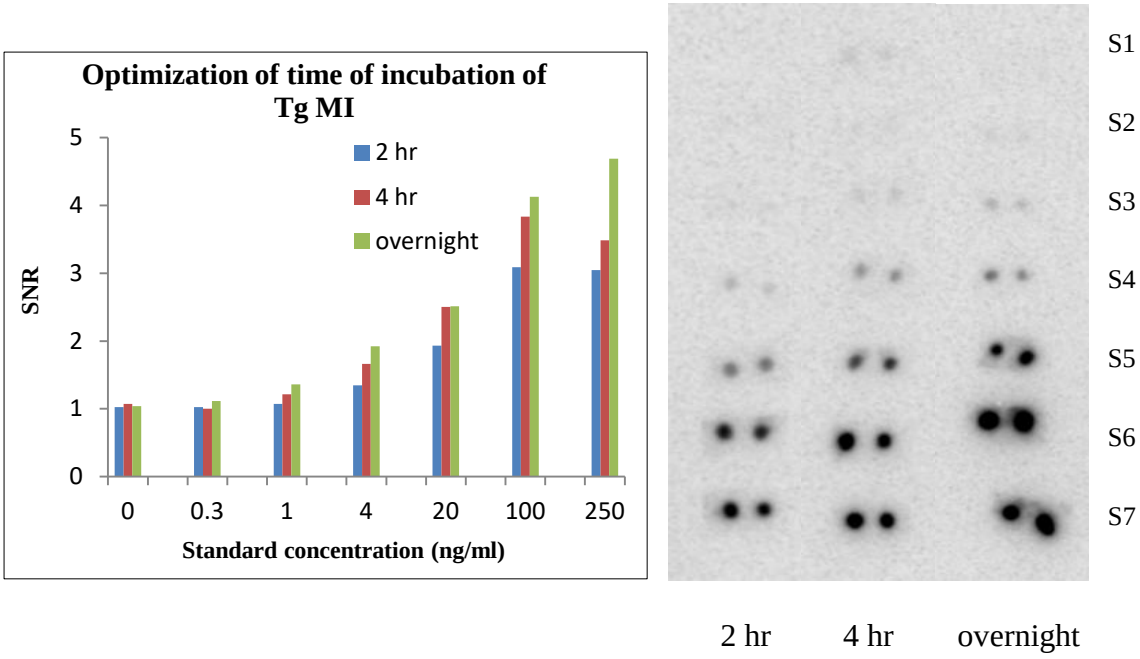


Figure 3.11 Graph and autoradiogram showing the optimization of time of incubation with tracer for Tg

3.4.3. MI performance

3.4.3.1. T4

Taking into account the results presented above, a standard curve for T4 MI was established. Antibody-chip prepared by spotting 0.5 µl of 1 mg/ml polyclonal anti-T4 antibodies, were incubated with 10 µl of T4 standards mixed with ^{125}I-T4 (60,000 cpm/100 µl) for 2 hr. Typical standard curves for T4 was generated using MI as shown in Fig.3.12. Analytical sensitivity or limit of detection (LOD) of the assays was characterized by determining the bound counts, or spot intensity, for ten replicates of zero calibrator. The mean bound counts, or spot intensity, is calculated and the concentration corresponding to the mean-2SD of counts, or spot intensity, was interpolated from the standard displacement curve. The analytical sensitivity of the assay was 0.32 µg/dl and the upper assay limit was 20 µg/dl.

3.4.3.2. TSH and Tg

Similarly, standard curves for TSH and Tg were generated. Antibody-chips were prepared on TEM as described above. Antibody chips were incubated with 100 µl of corresponding standards mixed with 100 µl of corresponding tracer (100,000 cpm /100 µl) for 2 hr. Standard curves for TSH and Tg were generated as shown in Fig.3.12. Analytical sensitivity of MI was determined as described for T4 from 10 replicates of zero standards and calculating the concentration corresponding to mean+2SD. The analytical sensitivity of TSH was 0.01µIU/ml with assay range upto 100 µIU/ml. The analytical sensitivity of Tg was 0.05 ng/ml and upper limit of the assay was 250 ng/ml.

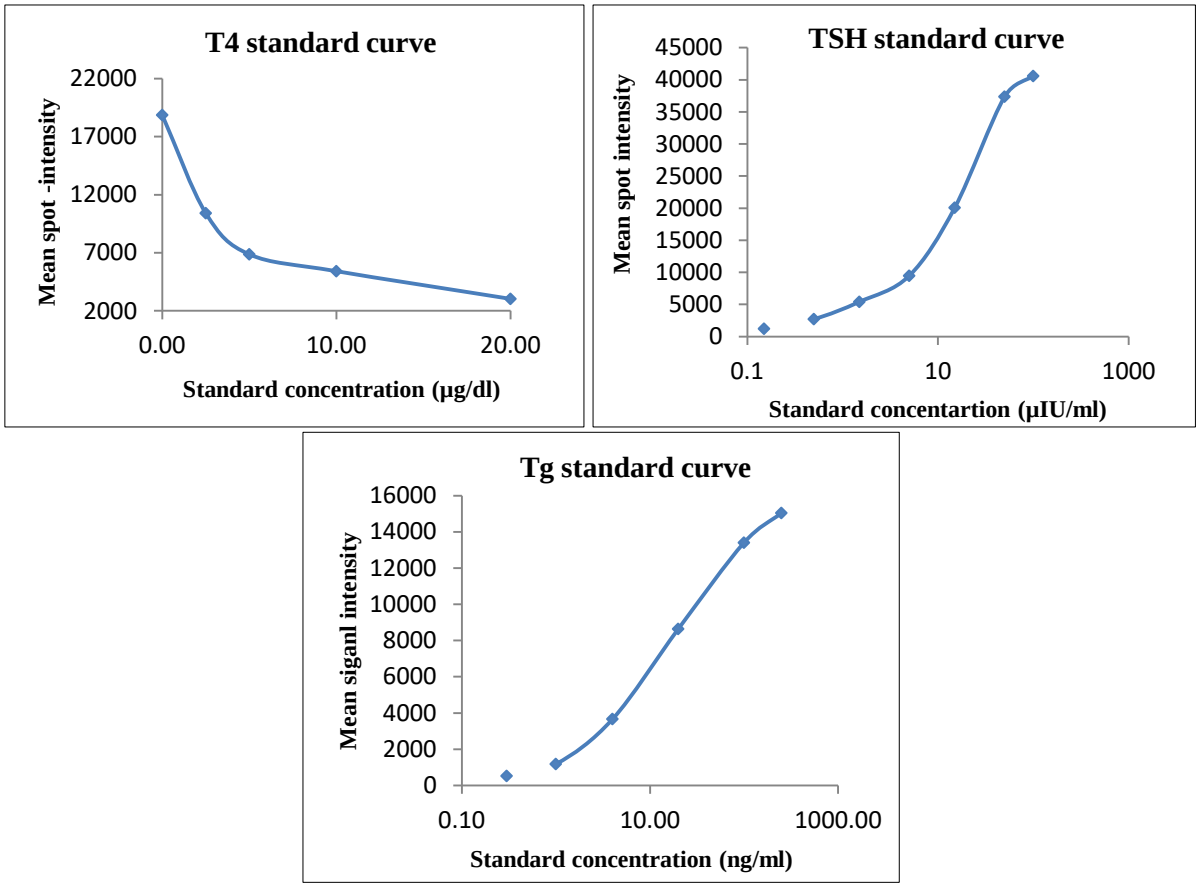


Figure 3.12 Standard curve obtained for MI of three individual analytes

3.4.4. Influence of incubation conditions and hydrophobicity of TEM on MI.

Spotting conditions should be optimized to achieve a non-denaturing environment, minimize non-specific interactions, and to provide good spot morphology, homogeneity and spot-to-spot reproducibility. It is known that spotting buffer acts in conjugation with the support (hydrophobic or hydrophilic) and environmental conditions (temperature and humidity) to determine the final spot diameter, spot uniformity and spot-to-spot reproducibility. We had performed MI on human serum samples for all the three analytes on both hydrophobic and hydrophilic PC-TEM under dry and humid conditions. The results obtained by MI were compared to RIA/IRMA as applicable.

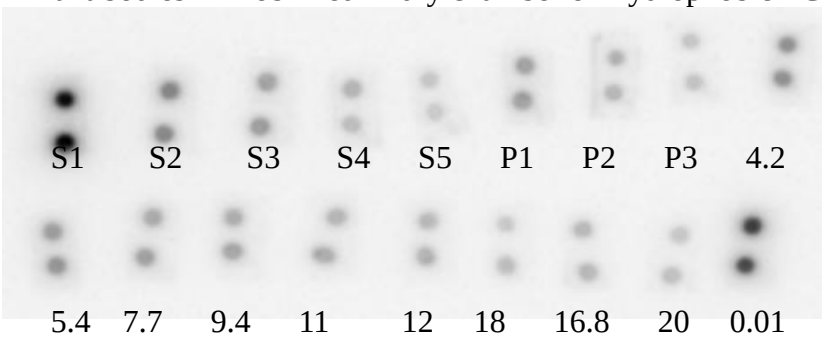
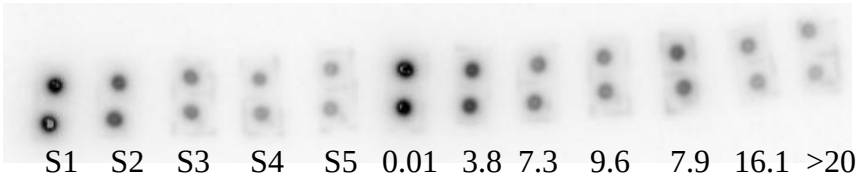
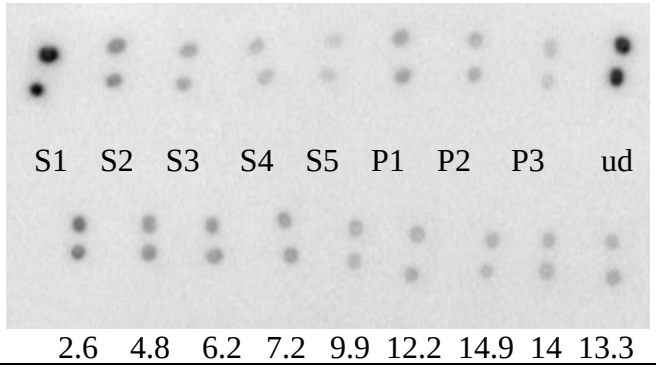
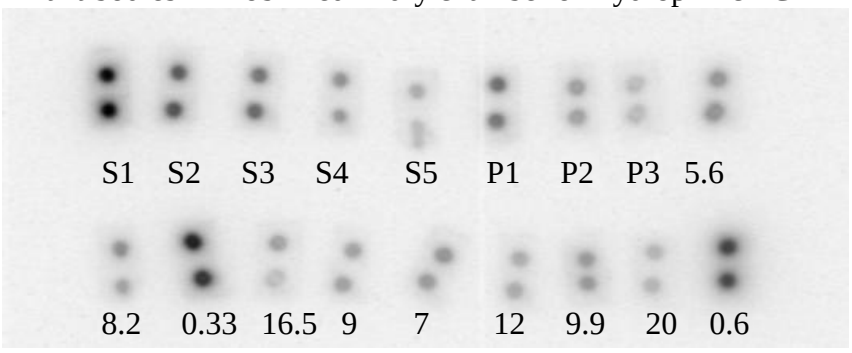
T4 antibodies immobilized in dry chamber on hydrophobic PC-TEM  	*r=0.51, r^2=0.26 MI=0.67 RIA+2.1 n=29 p<0.001 <u>Mean value of QC samples</u> P1 - 5.2, P2 - 8.7, P3 - 16 µg/dl
T4 antibodies immobilized in humid chamber on hydrophobic PC-TEM  	*r=0.95, r^2=0.90 MI=0.95 RIA+0.13 n =30 p<0.001
T4 antibodies immobilized in humid chamber on hydrophilic PC-TEM  	*r=0.97, r^2=0.94 MI=0.56 RIA+0.13 n=25 p<0.001
T4 antibodies immobilized in dry chamber on hydrophilic PC-TEM  	*r=0.82, r^2=0.67 MI=0.75 RIA+1.1 n=23 p<0.001

Figure 3.13 Autoradiogram showing the influence of humidity and hydrophobicity of TEM on T4 MI.

**The correlation coefficient and regression equation for larger number of serum samples under given condition is shown. S1-S5: Standards, P1-P3: QC samples and numbers in autoradiogram represent the concentration of T4 in serum samples estimated by RIA.*

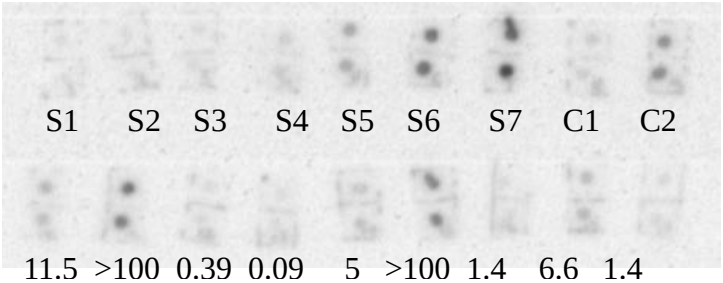
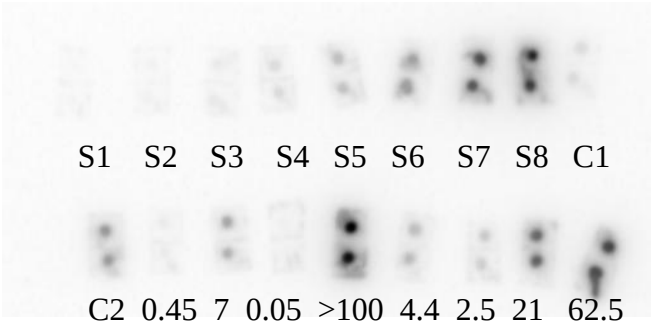
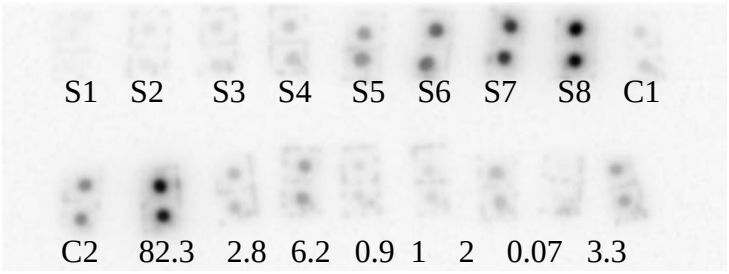
TSH antibodies immobilized in dry chamber on hydrophobic PC-TEM  	*r =0.41, r^2=0.168 MI=0.35 IRMA+6.9 n=37 p<0.001 <u>Mean value of QC samples</u> C1 - 1.39 C2 - 14.5 µIU/ml
TSH antibodies immobilized in humid chamber on hydrophobic PC-TEM  	*r=0.96, r^2=0.92 MI=1.01 IRMA+0.95 n=20 p<0.001
TSH antibodies immobilized in humid chamber on hydrophilic PC-TEM  	*r=0.999, r^2=0.998 MI=0.75 IRMA+0.17 n=30 p<0.001
TSH antibodies immobilized PBS in dry chamber on hydrophilic PC-TEM  	*r=0.98, r^2=0.96 MI=1.03 IRMA-0.73 n=30 p<0.001

Figure 3.14 Autoradiogram showing the influence of humidity and hydrophobicity of TEMs on TSH MI.
**The correlation coefficient and regression equation for larger number of serum samples for given condition is shown. S1-S8: Standards (S8-100 µIU/ml), C1 and C2: QC samples and numbers in autoradiogram represent the concentration of TSH in serum samples estimated by IRMA.*

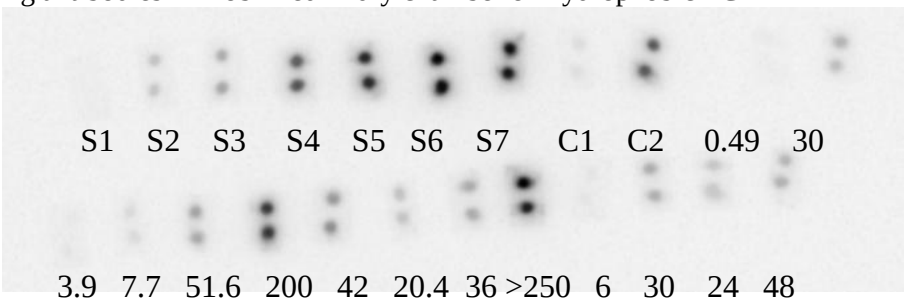
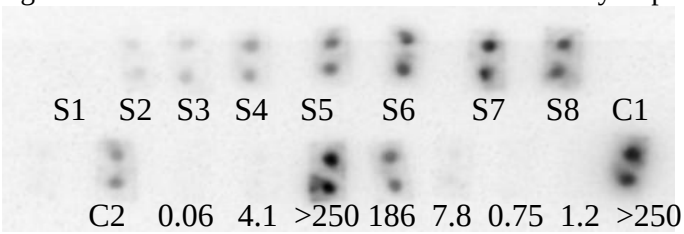
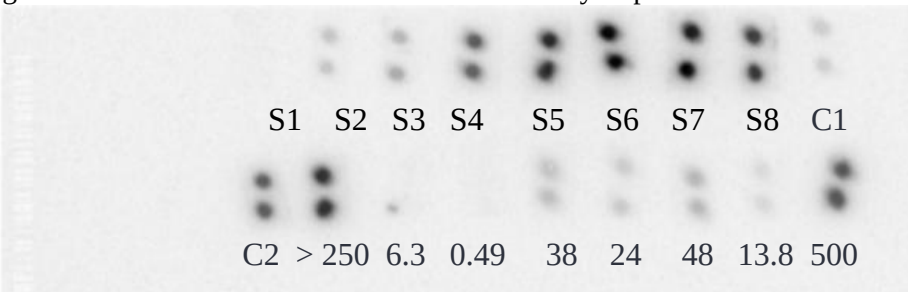
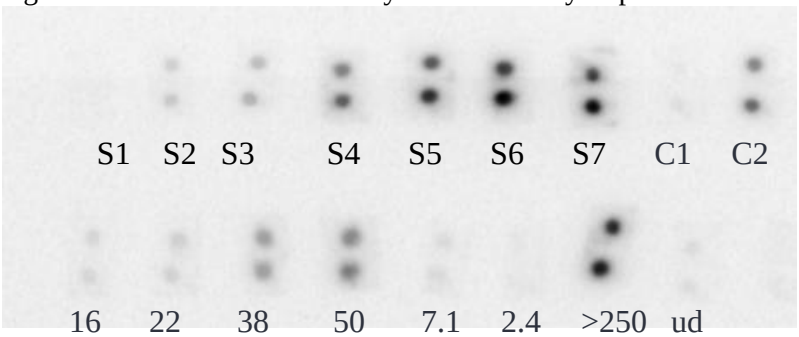
Tg antibodies immobilized in dry chamber on hydrophobic PC TEM  	*r=0.78, r^2=0.61 MI=0.52 IRMA+5.4 n=22 p<0.001 <u>Mean value of QC samples</u> C1 - 2.5 C2 - 58 ng/ml.
Tg antibodies immobilized in humid chamber on hydrophobic PC TEM  	*r=0.94, r^2=0.884 MI=0.98IRMA +8.2 n =25 p<0.001
Tg antibodies immobilized in humid chamber on hydrophilic PC TEM  	*r=0.95, r^2=0.90 MI=0.61IRMA+0.97 n=36 p<0.001
Tg antibodies immobilized in dry chamber on hydrophilic PC TEM  	*r=0.91,r^2=0.83 MI=0.90IRMA-15 n=19 p<0.001

Figure 3.15 Autoradiogram showing the influence of humidity and hydrophobicity of the membranes on Tg MI.

**The correlation coefficient and regression equation for larger number of serum samples for a given condition is shown. S1-S8: Standards (0, 12.5, 25, 50, 100, 200, 400, 800 ng/ml), C1 and C2: QC samples and numbers in autoradiogram represent the concentration of Tg in serum sample estimated by IRMA.*

It is seen that the correlation coefficients for all the three antigens, though statistically significant was always poorer on hydrophobic TEM in dry chamber. This can be seen from the much lower r^2 values, which shows the coefficient of determination for the association. Further, very significant correlation with RIA/IRMA was obtained when the antibodies against all analytes were immobilized on hydrophobic TEM in humid chamber (Fig.3.13, 3.14 and 3.15). Similarly, it is seen that the correlation coefficient for all the three antigens is poorer on hydrophilic TEM in humid chamber as compared to hydrophilic TEM in dry chamber. This is confirmed by the good correlation with RIA/IRMA was obtained when the antibodies were immobilized on hydrophilic TEM in dry chamber (Fig.3.13, 3.14 and 3.15).

3.4.5. Comparison with established assays

After fixing of the immobilization conditions, 150 patients' samples were assayed for T4 by MI. The values were compared to RIA. The correlation coefficient between the two techniques was 0.94 ($p < 0.001$). The regression equation was MI = 0.98*RIA+0.03 (Fig.3.16). Similarly, 140 samples were quantified for their TSH content by MI and correlation coefficient between IRMA and MI was 0.98 ($p<0.001$). The regression equation was MI=1.01*IRMA-0.36 (Fig.3.16). Also, 140 samples were quantified for their Tg content by MI and compared with IRMA. The correlation coefficient was found to be 0.97 ($p<0.001$) with regression equation MI= 0.93*IRMA-2.6 (Fig.3.16). The values of quality controls samples analyzed during the assay for all the three analytes were within the reference range.

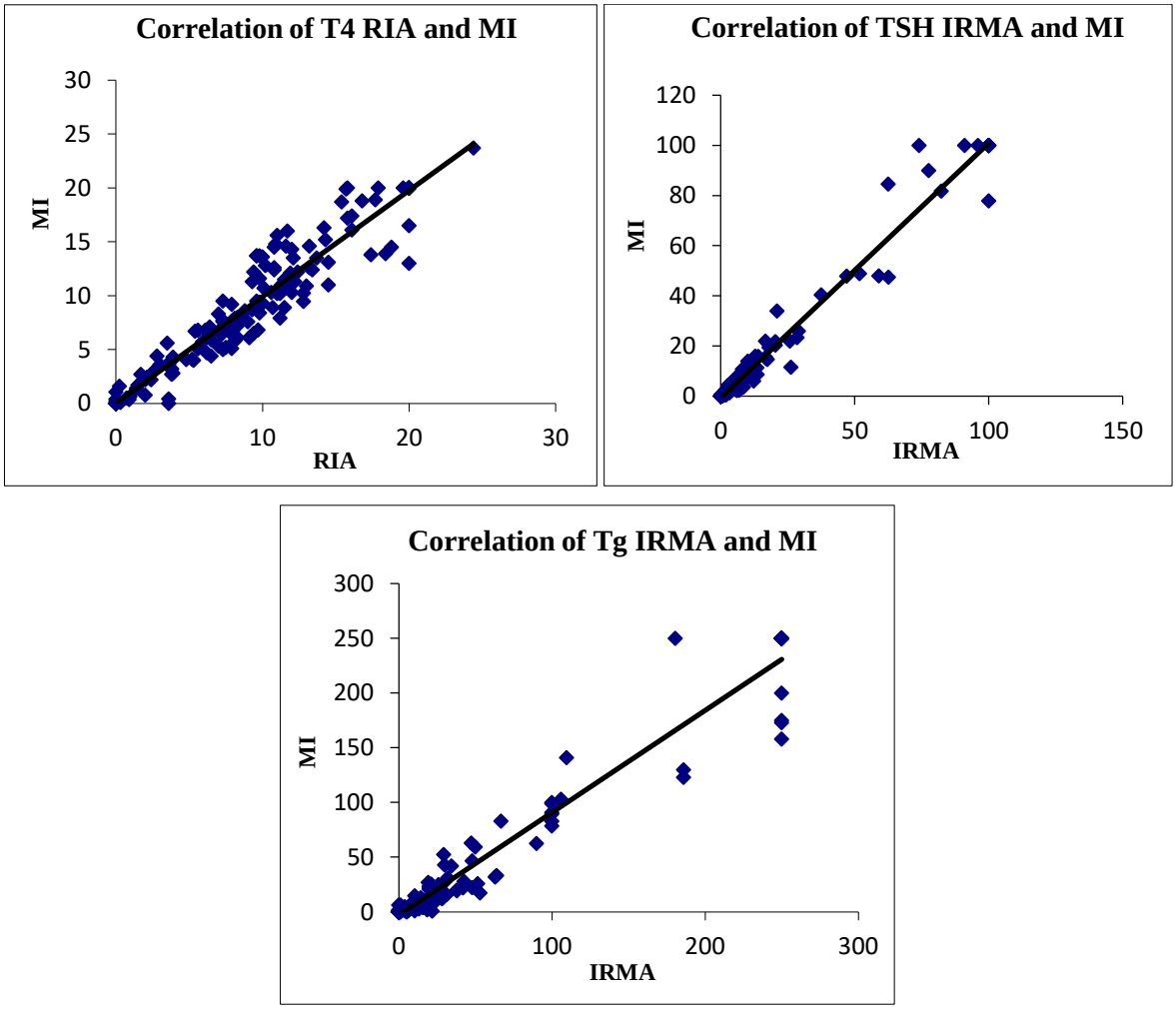


Figure 3.16 Graphs showing the correlation of serum samples analyzed by MI and RIA/IRMA.

3.4.6. Precision

The precision of any immunoassay is important because it enables an assessment to be made of the probability that a given concentration differs from a specified value. It also describes the repeatability of the test. An immunoassay's repeatability is usually measured as its imprecision and often expressed as the percent coefficient of variation (%CV) at a particular analyte level as shown below:

%CV = 100 × (Standard deviation of replicate test values) / (Mean of replicate test values)

The within-run precision, or intra-assay coefficient of variation, is defined as the precision of the same sample run on several occasions within the same assay. The between-run precision, or inter-assay coefficient of variation, is a measure of the assay to reproduce the same result on the same sample, from run to run and from day to day. The intra-assay CV was determined by putting ten replicates of control serum samples. Controls were run in 10 consecutive assays to determine the inter-assay variations. Table 3.1 shows the intra and inter-assay %CV obtained for all the three analytes.

Analyte	Intra-assay variation (n=10)			Inter-assay variation (n=10)		
	Control	Mean ± SD	%CV	Control	Mean ± SD	%CV
T4	P1	4.7±0.82	17.1	P1	5.0±0.85	16.8
	P2	8.1±1.06	13.1	P2	9.6±1.7	18
	P3	16.5±0.94	5.6	P3	16.7±2.7	16.5
TSH	C1	1.4±0.28	19.8	C1	1.1±0.25	20
	C2	16.6±1.9	12	C2	15.3±2.32	15.2
Tg	C1	2.5±0.31	12.5	C1	2.9±1.4	48.3
	C2	55.7±16.2	29.1	C2	57.8±14.2	24.7

Table 3.1 Intra-assay CVs and inter-assay CVs for 10 replicates of control samples. Mean concentration of control samples T4: P1=5.2, P2=8.7, P3=16 µg/dl, TSH: C1=1.39 and C2=14.5 µIU/ml,Tg: C1=2.5 and C2= 58 ng/ml.

3.4.7. Matrix evaluation

One of the primary conditions in any immunoassay is that the standard and the sample should be chemically identical and their reaction matrix should be same. If either of the two requirements is not satisfied, the results obtained become inaccurate. Standard curve should be prepared in the same matrix as the analyzed samples. Alternatively, other species matrixes or analyte free buffer can be used. We have prepared standards for T4 in SHFS. TSH standards obtained from TSH IRMA kit were prepared in horse hormone free serum (HHFS). The Tg standards obtained from Tg IRMA kits were prepared in human HFS. The matrix was evaluated using linearity and recovery studies.

Linearity was determined in sample containing high concentration of analytes which were serially diluted in standard matrix. Observed to expected ratio (O/E) for linearity studies should be 100 ± 20%. Linearity was found to be varying from 80%-102% for T4, from 85.5% -112 % for TSH and 72.5% -104 % for Tg. The autoradiograms are shown in Fig.3.17 and results are summarized in Table 3.2.

Recovery test in immunoassays is done to find out the errors arising out of differences in the matrix between the standard and sample. For estimating recovery, a sample is spiked with known amount of standard and re-assayed. Recovery tests are done by taking both the standards and sample in half the specified volume and should lie between 100 ± 20%. Abnormal recoveries indicate that standard and sample are not identical in all respects. Recovery, expressed as a percentage of the expected values, was determined in three serum samples. The percentage of recovery for T4 ranged from 74.6% to 113.6%, for TSH recovery ranged from 93.3% to 126.9% and for Tg recovery ranged from 69.8% to 102.6%. The results are shown in Table 3.3. Fig.3.18 shows the corresponding image.

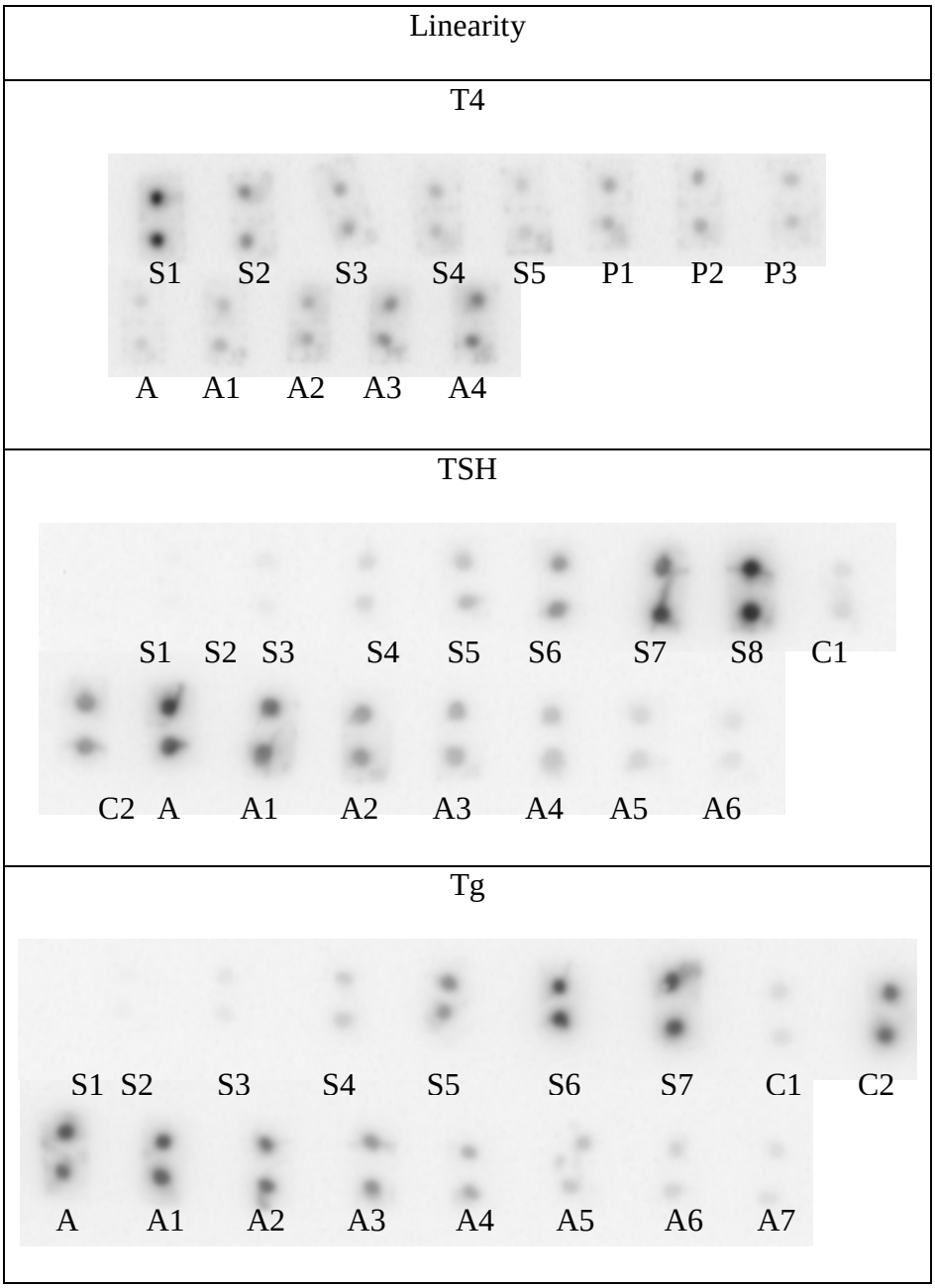


Figure 3.17 Autoradiogram showing the linearity of serum sample serially diluted in hormone free serum.
S1- S8: Respective standards, P1-P3: QC samples for T4, C1 and C2: Respective QC samples. For T4, A= sample having T4 =20µg/dl, for TSH A= sample having TSH =60 µIU/ml and for Tg A=sample having Tg =160 ng/ml. A1 –A8 serial dilutions of sample A from 1:1 – 1:128 in HFS.

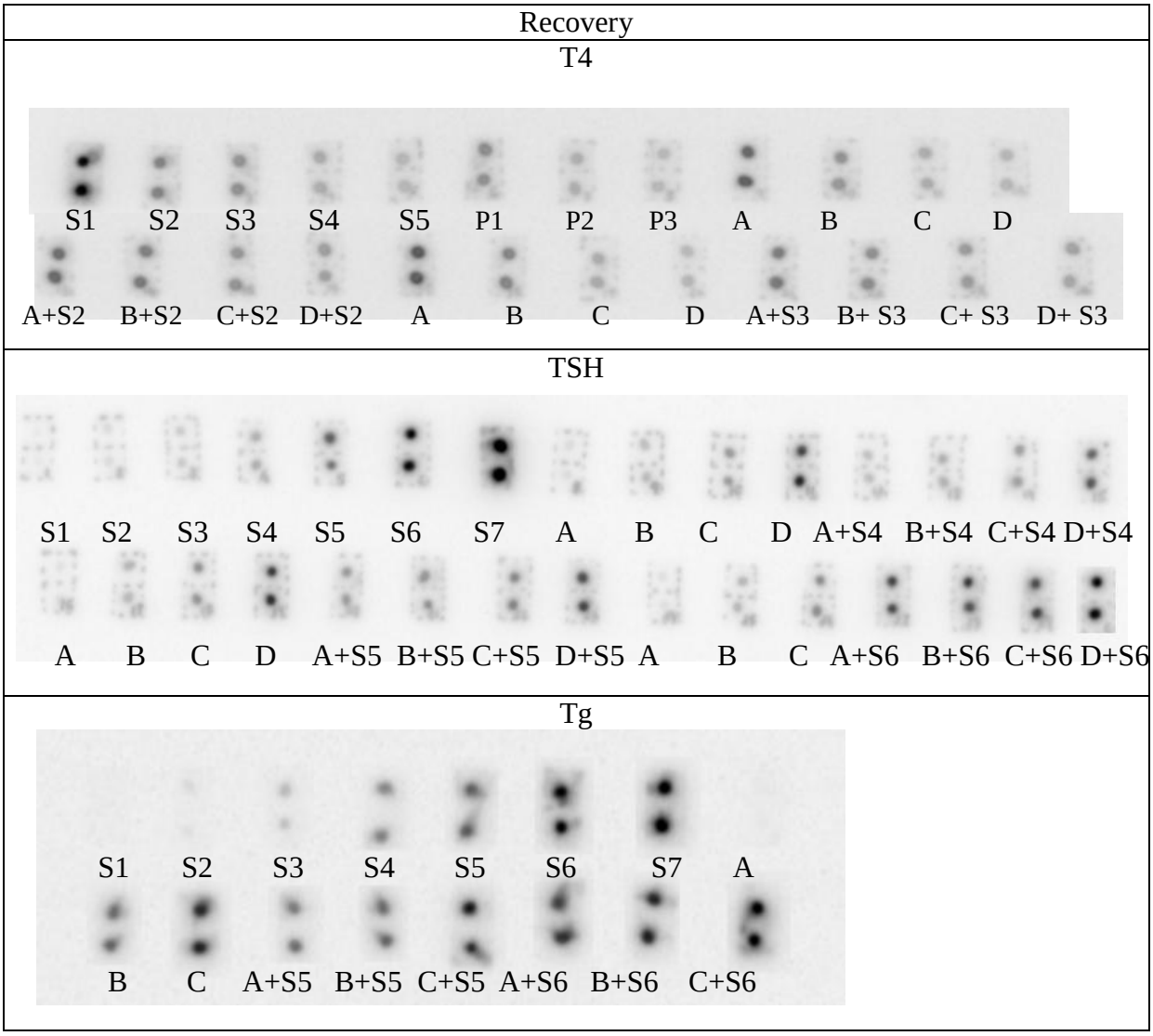


Figure 3.18 Autoradiogram showing the recovery of serum samples spiked with calibrators. S1- S7:Respective standards. For T4 A, B, C and D are serum samples having T4 concentration of 2, 5.4, 9.9 and 15.6 µg/dl. For TSH, A, B, C and D are serum samples having TSH concentration of 0.22, 1.6, 4.4 and 13.9 µIU/ml. For Tg, A, B, and C are serum samples having Tg concentration of 0.01, 15.3 and 58.2 ng/ml. The serum samples were spiked with standards in 1:1 ratio.

Linearity of serum samples diluted in hormone free serum								
T4			TSH			Tg		
Expected Concentration (E)(µg/dl)	Observed concentration (O) (µg/dl)	%O/E	E µIU/ml	O µIU/ml	%O/E	E ng/ml	O ng/ml	%O/E
20	20	100	60	59	98	160	116	72.5
10	8	80	30	33	110	80	77.7	97
5	5.1	102	15	14.7	98	40	40	100
2.5	2	80	7.5	8.4	112	20	20.1	100
1.2	1.1	91	3.75	4.1	109	10	8.4	84
			1.87	1.6	85.5	5	5.2	104
			0.93	0.95	102	2.5	1.8	72
						1.2	1.2	100

Table 3.2 Linearity of serum samples serially diluted in HFS.

Recovery of serum samples spiked with calibrators					
TSH					
1:1 dilution with calibrator (mIU/L)	Sample A	Sample B	Sample C	Sample D	Mean
1.5	117.65	93.33	96.67	112.99	105.1
5	126.92	96.97	102.13	116.36	110.6
15	125.00	124.10	117.35	96.50	115.7
T4					
1:1 dilution with calibrator (µg/dl)	Sample A	Sample B	Sample C	Sample D	Mean
2.5	113.64	87.50	88.71	81.11	92.7
5	108.57	84.62	74.67	81.55	87.3
Tg					
1:1 dilution with calibrator (ng/ml)	Sample A	Sample B	Sample C	Mean	
20	79.00	69.89	81.07	76.6	
100	87.40	101.22	102.65	97.0	

Table 3.3 Recovery of three serum samples spiked with calibrators.

3.5. Discussion

This Chapter provides experimental data from MI for T4, TSH and Tg to demonstrate proof of concept of the validity of MI for quantification of three individual analytes. Assessment covered the optimization of immobilization parameters, reagents titration and signal detection.

Spotting buffer acts in conjugation with the support properties (hydrophobic or hydrophilic) along with antibody immobilization conditions (temperature and humidity) to determine the final spot diameter, uniformity and spot-to-spot reproducibility. Hydrophobic surfaces tend to produce smaller spots. To prevent the complete drying of the spot on hydrophobic surfaces which leads to non-uniform spots and spot-to-spot variation, the antibodies are immobilized on hydrophobic TEMs in a humid chamber. Hydrophilic surfaces tend to produce larger spots. Spots tend to spread on hydrophilic TEMs when incubated in humid chamber which impairs quantification. On hydrophilic surfaces, antibody immobilization should be done in dry chamber. Environmental conditions (temperature and humidity) should be optimized so that spot neither dries completely nor spreads on a given surface. Best correlation between RIA/IRMA and MI could be obtained on hydrophilic TEMs in dry chamber and hydrophobic TEMs in humid chamber.

During optimization of tracer concentration, SNR initially increased with increasing tracer concentration for all the three analytes. After reaching the optimum tracer concentration, any further increase in tracer concentration, leads to decreased SNR. Increasing the amount of tracer, however, also increases the amount of background noise. Eventually a point is reached where the level of background increases faster that the rate of specific binding and the SNR begins to drop. For all the three analytes, increasing tracer concentration leads to increase in the noise, and a fall in SNR. Thus, a balance must be obtained during optimization procedure

so that the highest SNR is obtained. This was achieved when the tracer concentration was 60,000cpm/100 µl for T4 and 100,000 cpm/100 µl for both TSH and Tg. Also, during the optimization of time of incubation, initially SNR increased with increasing time of incubation. In case of T4 and TSH, after 2 hr SNR becomes constant with slight decrease in SNR with increasing time. In case of Tg, maximum SNR is obtained at overnight incubation. Tg being a large molecule, reaction kinetics is poor and requires overnight incubation to achieve required sensitivity.

The range of calibration curve of T4 was assigned according to the possible range of T4 inside human body. For T4 MI, the measuring range is between 0.32-20 µg/dl, with inter- and intra-assay CV < 20%.

For TSH MI, there was no hook effect up to the concentration tested (100 µIU/ml), when the spots were counted or detected using phosphorImager, but was seen when quantified from autoradiographs by densitometry. The measuring range is between 0.01-100 µIU/ml of TSH, with inter- and intra-assay CV < 20%. Also, for clinical use, ultra-sensitive TSH assays are required to distinguish hyper-thyroid patients (in whom TSH is suppressed) from normal individuals. TSH is used in the diagnosis of thyroid gland disorders (e.g., primary and secondary hypo- and hyperthyroidism) [145]. Sensitive TSH assays are also needed for screening of neonatal hypothyroidism, monitoring thyroid hormone therapy, evaluating thyroid dysfunction in non-thyroidal illnesses and screening for thyroid dysfunction [146, 147]. A very sensitive spot-based immunoassay for TSH was developed using a site specifically biotinylated recombinant Fab fragment spotted (spot diameter 2.5 mm) on the bottom of streptavidin-coated 96-well microtiter plate [148]. Here, we have achieved a sensitive MI using TEM and ^{125}I- labeled detection antibodies utilizing 50 µl of sample. TEM being highly microporous provides high immobilization capacity, and thereby high

sensitivity. The MI, we have described here, can be used for the estimation of very low TSH in human serum samples.

For Tg, the hook effect was seen after 250 ng/ml at which point saturation of the capture antibody by the analyte occurs. This result suggested that maximum binding capacity of biologically active immobilized antibodies was reached. Measuring range for Tg is between 0.05-250 ng/ml. The inter- and intra-assay CV >20%. This can be attributed to spotting and background variability observed between and within the antibody-chips. High intra-assay imprecision - ranging from 4.2% to 67.0%, and 9.4% to 56.0% for inter-assay imprecision have been reported by workers using MAIA. They have attributed this to spotting and background variability [149, 150].

To verify the utility of the MI, analytes measured in human serum by MI were compared to RIA/IRMA. Similar analyte concentrations were measured by both methods as given earlier in Section 3.4.5 ($r = 0.94$, $p<0.001$ for T4, $r=0.98$, $p<0.001$ for TSH and $r =0.97$, $p<0.001$ for Tg).

Detection is another key step in MI. The analyte molecules bound to each antibody at each location must be quantified across the entire antibody-chip. We have used radioisotopes for detection and quantification. Radiolabeling has several advantages over fluorescence for MI including no interference from dust, no photo bleaching effects, no autofluorescence, and no pollution of the sample emission signal. Although, initially autoradiography was used for viewing the spot morphology and signal intensity, the X-ray film has limited dynamic range and standard curve obtained has narrow range. Response of gamma counter to radioactivity is much more linear as compared to X-ray film since the former is based on use NaI(Tl) scintillator with quite low dead time losses.. Although, standard curves obtained using gamma counter has wide working range, cutting and counting the spots is tedious and time

consuming process and there is no provision of subtracting the background. PhosphorImager, based on use of PMT as a detector, provides high resolution images and accurate quantification from radioisotopes. It provides higher sensitivity (10 times more than X-ray film), wide linear dynamic range (~1000 times greater than film), and takes lesser time (10% of time as required by film), easier to use than X-ray film autoradiography, provides high resolution images than X-ray film autoradiography and is equivalent to counting for radioactivity. The wide, linear dynamic range (~ 1000 times greater than film) of the PhosphorImager system yields useful data at every exposure intensity, enabling visualization and quantitation of data for both weak and strong spots at the same exposure level [151, 152]. As seen from the results in Fig. 3.4, X-ray film gets saturated at an analyte concentration which gives ~2000 cpm per spot. This means that TSH concentrations of >15 µIU/ml and Tg concentrations of >50 ng/ml cannot be measured reliably. Similarly sensitivity obtained for T4 was poor (3.2 µg/dl), as the X-ray film saturated at ~2.5 µg/dl. Use of phosphorImager provided standard curves with wide working range (100 µIU/ml for TSH and 250 ng/ml for Tg and high sensitivity for T4 (0.32 µg/dl). Moreover, the time taken for exposure on phosphorImager screen was two hours instead of 48 hours on X-ray film, saving assay time. Use of phosphorImager is also convenient as use of dark rooms and chemicals are not required. Use of radioisotopes along with phosphorImager for detection and quantification of the antibody spots, provides standard curve with wide working range and high sensitivity.

In this Chapter MI has been standardized for detection of individual analytes, and the subsequent Chapters will address the development of MAIA for simultaneous estimation of all three analytes.

CHAPTER 4

Development and Validation of MAIA for Two Analytes.

4.1. Introduction

Experimental evidence for using the 'Antibody Chip' or MI for the quantitative detection of T4, TSH and Tg, individually was shown in Chapter 3. MI has been optimized with good sensitivity and analytical range for each of these analytes and validated by comparison to RIA and IRMA as applicable. In this Chapter, two panels of microarrays for the simultaneous estimation of two analytes will be presented. These are:

(i) T4 and TSH microarray

This microarray will be useful for detecting thyroid diseases, notable hypothyroidism and hyperthyroidism, which are most common endocrine disorders caused by disturbances in the function of pituitary and thyroid glands. Although measurement of serum TSH has been recommended as a first-line test for screening thyroid disorders [153, 154], measurement of T4 is required to give a more comprehensive picture of the patient's condition. For example in patients developing hypothyroidism due to Hashimoto Thyroiditis or with a limited ability to synthesize thyroid hormones because of prior thyroid surgery, radioiodine treatment or severe iodine deficiency may have TSH concentration above normal range and T4 and T3 level within normal range. Transient hypothyroidism with elevated serum TSH may occur in

infants during early neonatal period and probably does not require treatment. Patients on treatment with replacement doses of T4, may have normal levels of T4 before high TSH level has reached to normal range. Conversely, serum TSH concentration may remain low or normal for up to five weeks after withdrawal of thyroid hormone replacement when serum level of T4 have declined to values well below the lower range of normal. Although TSH is recommended as the first-line test for thyroid disorders, a valid estimate of serum T4 is required for definitive assessment of thyroid dysfunction [155]. Thus, T4 and TSH form an important panel of analytes in patients suffering from hypothyroidism and hyperthyroidism.

(ii) TSH and Tg microarray

This microarray will be useful in follow up of patients with DTC. After thyroid ablation, Tg estimation plays a very important role for detection of recurrence and metastasis. The higher the basal serum Tg seen post-operatively (while on L-T4 suppression therapy), the greater the likelihood that the patient will have persistent or recurrent disease present whereas a declining Tg levels suggests the absence or regression of disease [156]. Hence, it is difficult to interpret Tg values without the knowledge of corresponding TSH values. Thus, TSH and Tg forms an important panel of analytes in patients of DTC [157].

Both microarrays were developed on PC TEM using ^{125}I labeled monoclonal antibodies and antigens for the detection and quantification of analytes. Various parameters such as tracer concentration and time of incubation were optimized. Finally MAIA was validated using various parameters.

4.2. Materials

The materials, chemicals and equipments were used for experiments in this Chapter are the same as described in Chapter 3 (Section 3.2).

4.3. Methods

4.3.1. Preparation of antibody-chip

4.3.1.1. Activation of TEM

Activation of TEM was accomplished in the same manner as described in Section 2.3.4.2 in Chapter 2.

4.3.1.2. Antibody immobilization

4.3.1.2.1. T4 and TSH

For T4 and TSH panel, 0.5 µl of anti-T4 and TSH antibodies in spotting buffer at concentration of 1mg/ml were spotted together on activated PC-TEM for 2 hr. Two replicate spots were made so that each standard/QC/sample can be assayed in duplicate.

4.3.1.2.2. TSH and Tg

For TSH and Tg panel, 0.5 µl (1 mg/ml) of anti-TSH and Tg antibodies were spotted on activated PC-TEM for 2 hr with two replicate spots of each antibody were made for each standard/QC/ sample. The TEMs were rinsed thrice with PBS and dried.

4.3.1.3. Blocking

The NSB sites were blocked for 2 hr at RT with PBS-BSA and rinsed thoroughly with PBS. The TEMs were cut into 5mm X 20 mm pieces and placed in 12 well plate for performing the immunoassays.

4.3.2. Preparation of combined standards.

4.3.2.1. T4 and TSH

Standards for calibration were prepared by spiking T4 and TSH at required concentrations directly into SHFS. Seven standards (S1 - S7) were prepared having T4 concentration of 0, 0, 0, 2.5, 5, 10, 20 µg/dl and TSH concentration of 0, 0.15, 0.5, 1.5, 5, 15 and 50µIU/ml. The standards were calibrated against reference preparation of TSH IRP 80/558. Commercially available T4 RIA kit was used to measure the concentration of T4 in prepared standards.

4.3.2.2. TSH and Tg

Standards were prepared by spiking human recombinant TSH procured from Genzyme Corporation, USA, in Tg standards from Izotop at required concentrations. Prepared standards were calibrated against the reference TSH preparation, IRP 80/558. Seven standards namely S1 to S7 were prepared having TSH concentration of 0, 0.15, 0.5, 1.5, 5, 15 and 50µIU/ml and Tg concentration of 0, 0.3, 1 ,4, 20, 100 and 250 ng/ml respectively.

4.3.3. Preparation of tracer cocktail

4.3.3.1. T4 and TSH

T4 was labeled with ^{125}I as described in Chapter 2 (Section 2.3.2). The labeled T4 was diluted in PBS-ANS buffer to contain ^{125}I-T4 at the desired concentration. To this tracer, required amount of ^{125}I-anti-TSH antibodies were added.

4.3.3.2. TSH and Tg

To the required concentration of ^{125}I-TSH monoclonal antibodies, required amount of ^{125}I-anti-Tg monoclonal antibodies were added.

4.3.4. Immunoassays

Antibody-chips prepared for T4 and TSH panel were placed in 12 well microtiter plate for performing the immunoassays. An aliquot of 50 µl of standard cocktail or serum sample and 200 µl of tracer cocktail was added to each antibody-chip. The microtiter plate was covered and incubated at RT for different time periods with gentle agitation on orbital shaker. After the reaction, the TEMs were washed thrice with PBS-T to remove unbound reactants and dried. Similar protocol was followed for TSH and Tg panel by reacting the antibody-chip prepared for TSH and Tg panel with 50 µl of standard cocktail or serum sample along with 200 µl of tracer cocktail.

4.3.4.1. Detection and quantification

TEMs were imaged using PhosphorImager and analysis was done in same manner as described earlier in Chapter 3 (Section 3.3.1.3). Average spot intensities presented in the graphs and tables were calculated from duplicate spots spotted on TEMs. RIA analysis

software was used for standard curve plotting and interpolating the concentration of the sample.

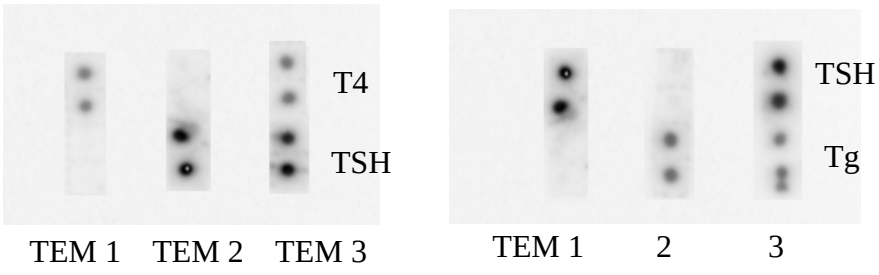
4.3.5. Cross-reactivity

The assay specificity is a very important issue that should always be evaluated when developing MAIA [99]. In MAIA, cross-reactivity between antibodies may cause major problems [54, 96]. Antibodies validated for single analyte immunoassays may display cross-reactivity in MAIA formats. Cross-reactivity reduces assay specificity and sensitivity leading to false interpretations of the positive and negative results. In this study, the cross-reactivity were tested, when antibody-chip prepared for T4 and TSH panel was reacted with either with individual tracer or tracer cocktail along with standard cocktail containing zero concentration of T4 and 50µIU/ml of TSH. For TSH and Tg panel, cross reactivity was tested by reacting the TEMs with either individual tracer or tracer cocktail along with standard cocktail S7, containing high concentration of TSH and Tg.

4.4. Results

4.4.1. Cross-reactivity

For both the panels, when reacted with the individual tracers, only the spots of the respective antibodies showed a signal and no signal was obtained in other antibody spots showing non-cross-reactivity among them. The results of cross-reactivity are summarized in Fig.4.1.



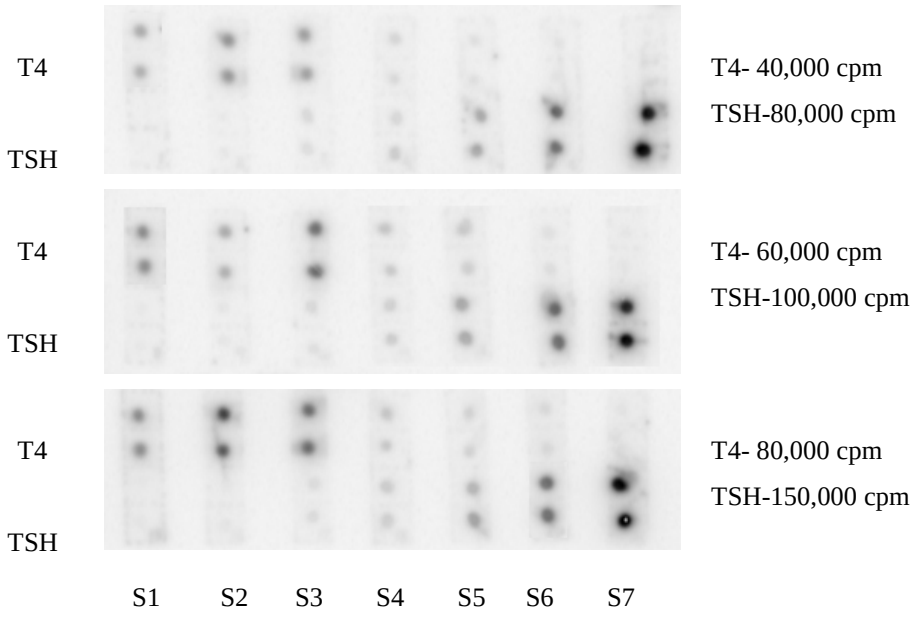
(a) *(b)*

Figure 4.1 (a) Image showing the cross-reactivity of T4 and TSH MAIA.
Each TEM was reacted with standard cocktail containing no T4 and high concentration of TSH. TEM1 was reacted with ^{125}I-T4. TEM 2 was reacted with ^{125}I-TSH detection antibodies. TEM 3 was reacted with tracer cocktail of ^{125}I-T4 and ^{125}I-TSH detection antibodies.
(b)Image showing the cross-reactivity of TSH and Tg MAIA.
Each TEM was reacted with standard cocktail, S7. TEM1 was reacted with ^{125}I-TSH detection antibodies. TEM 2 was reacted with ^{125}I-Tg detection antibodies. TEM 3 was reacted with tracer cocktail of ^{125}I-TSH and ^{125}I-Tg detection antibodies.

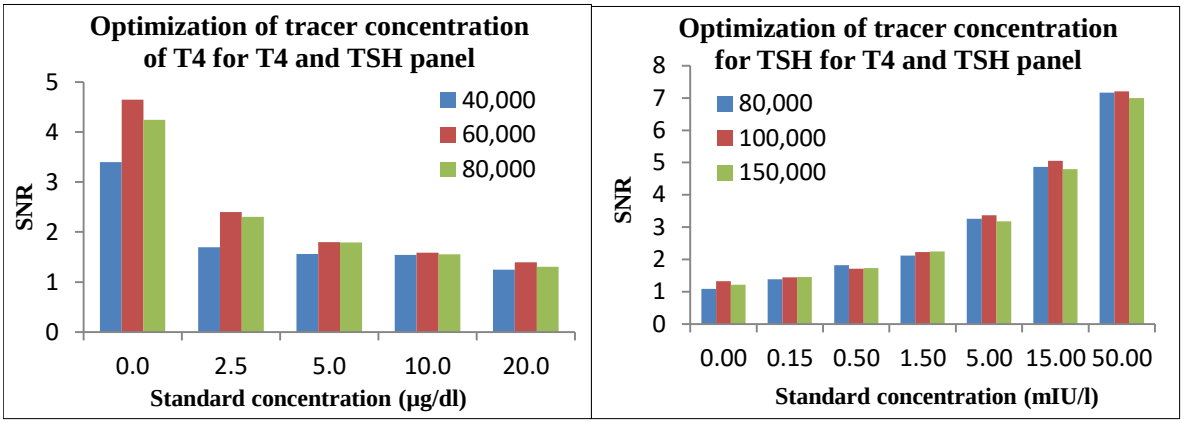
4.4.2. Optimization of tracer concentration

4.4.2.1. T4 and TSH

Tracer cocktail having different concentrations of ^{125}I-T4 and ^{125}I-TSH monoclonal antibodies was tested in order to select the concentration which gave the optimal SNR. Three different tracer cocktails was prepared having ^{125}I-T4 giving 40,000, 60,000 and 80,000 cpm/100 µl and ^{125}I-TSH monoclonal antibodies giving 80,000, 100,000 and 150,000 cpm/100 µl respectively. The signal generated for each concentration of tracer cocktail was divided by the corresponding background. The highest SNR was obtained with tracer containing 60,000 cpm/100 µl of ^{125}I-T4 and 100,000 cpm /100 µl of ^{125}I- TSH monoclonal antibodies Fig.4.2 shows the corresponding autoradiogram and graph.



(a)



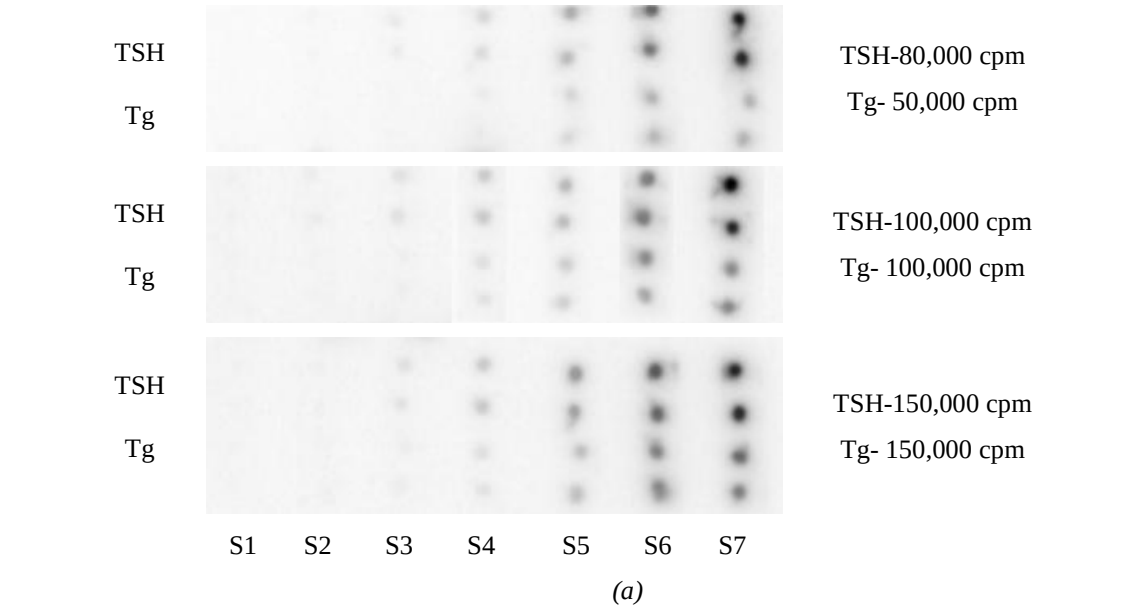
(b)

Figure 4.2 Autoradiogram (a) and graphs (b) showing the optimization of tracer concentration for T4 and TSH panel.

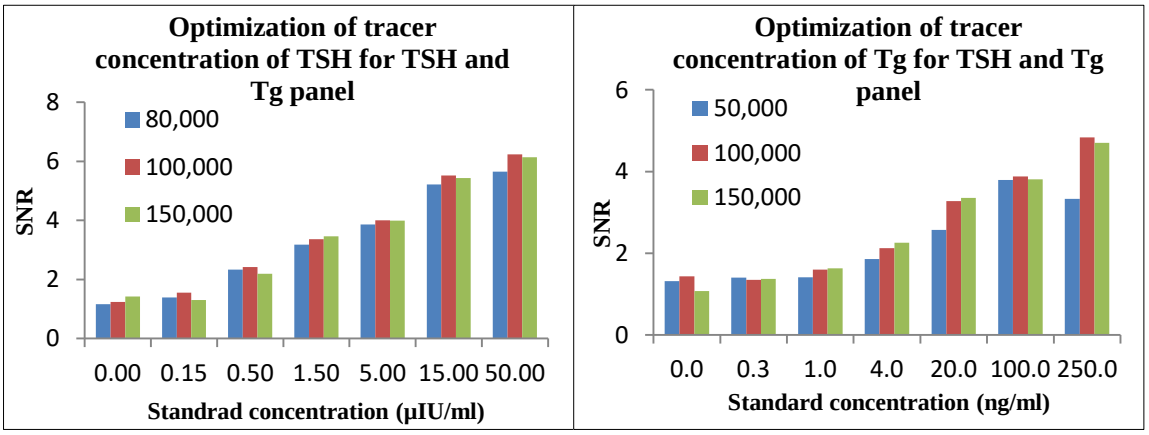
4.4.2.2. TSH and Tg

Tracer cocktail having different concentrations of ^{125}I-TSH and ^{125}I-Tg monoclonal antibodies was tested in order to select the concentration which gave the optimal SNR. Three different tracer cocktails was prepared having ^{125}I-TSH monoclonal antibodies giving 80,000,

100,000 and 150,000 cpm/100 µl and ^{125}I-Tg monoclonal antibodies giving 50,000, 100,000, and 150,000 cpm /100 µl. The highest SNR was obtained with 100,000 cpm /100 µl of ^{125}I-TSH monoclonal antibodies and 100,000 cpm/100 µl of ^{125}I-Tg monoclonal antibodies. Fig.4.3 shows the corresponding autoradiogram and graph.



(a)



(b)

Figure 4.3 Autoradiogram (a) and graph (b) showing the optimization of tracer concentration for TSH and Tg panel.

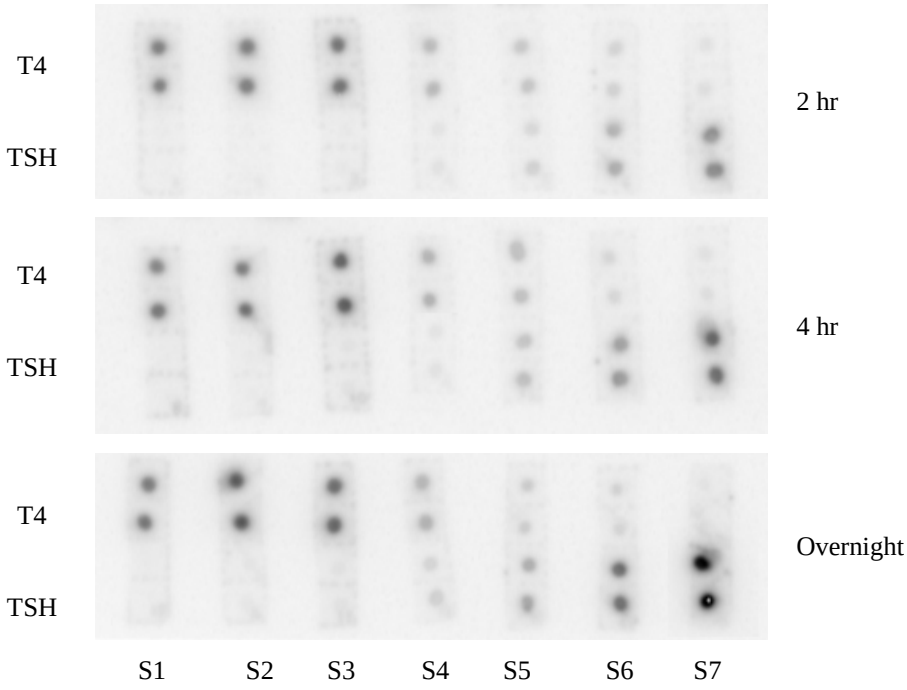
4.4.3. Optimization of time of incubation

4.4.3.1. T4 and TSH

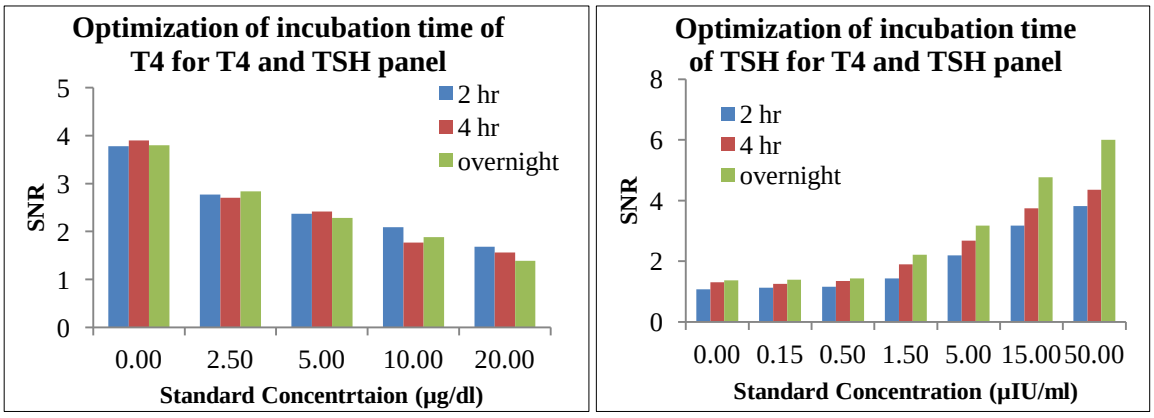
After selection of optimum tracer concentration, TEMs were incubated with optimized tracer for different time periods in order to select the time of incubation which gave highest SNR. TEMs were reacted with 50µl of standard cocktail along with tracer cocktail for 2 hr, 4 hr and overnight. The spot signal intensity was divided by corresponding background. The highest SNR for T4 was obtained at 2 hr but highest SNR for TSH was obtained at overnight incubation. Fig.4.4 shows the corresponding autoradiograms and graph.

4.4.3.2. TSH and Tg

Antibody-chip prepared for TSH and Tg panel was reacted with 50µl of standard cocktail along with optimized tracer cocktail for 2 hr, 4 hr and overnight. Highest SNR was obtained with overnight incubation. Fig.4.5 shows the corresponding autoradiogram and graph

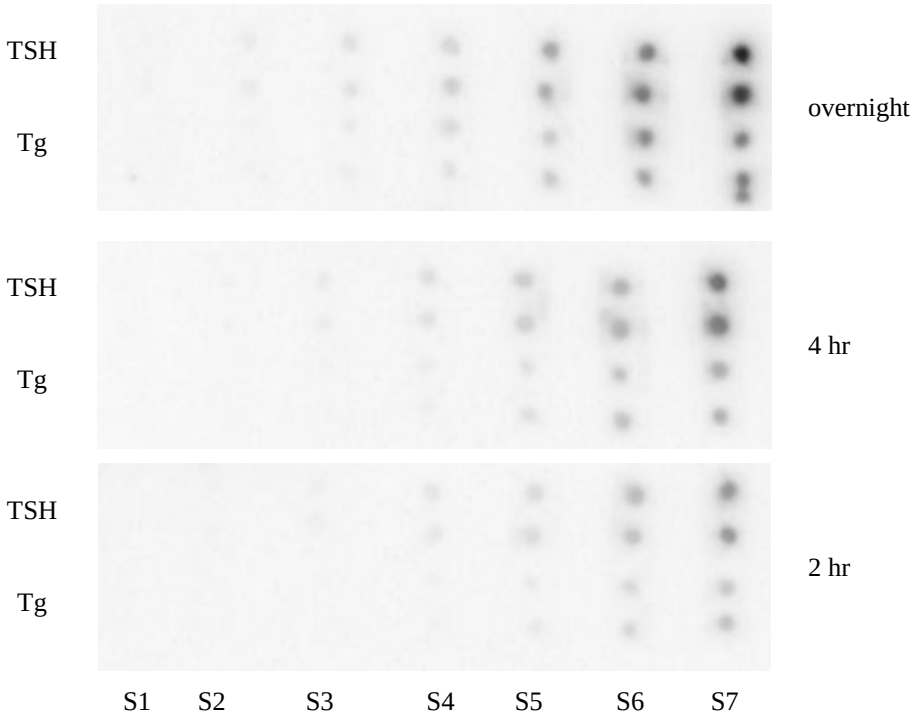


(a)

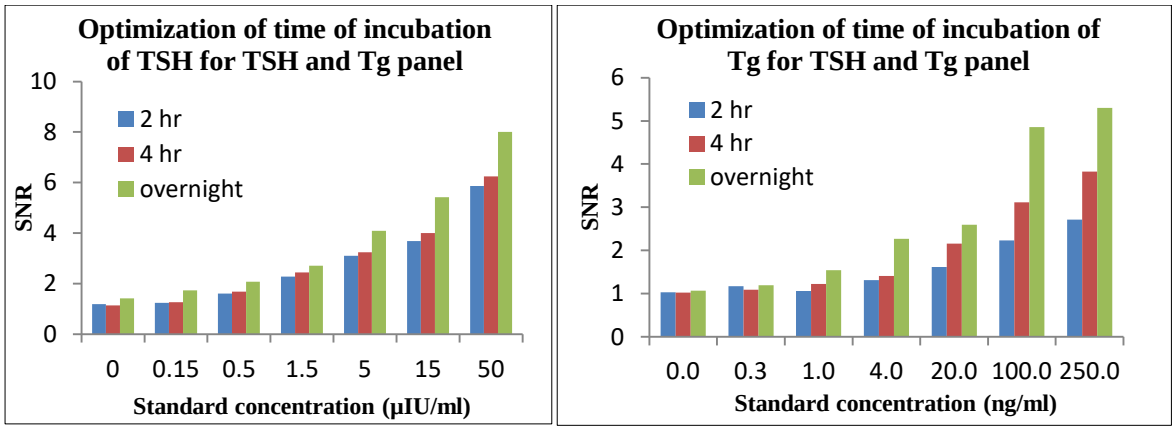


(b)

Figure 4.4 Autoradiogram (a) and graph (b) showing the optimization of time of incubation for T4 and TSH panel.



(a)



(b)

Figure 4.5 Autoradiogram (a) and graph (b) showing the optimization of time of incubation for TSH and Tg panel.

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4.4.4. Assay performance

4.4.4.1. T4 and TSH

Taking into account the results presented above, standard curves for T4 and TSH MAIA were generated. Antibody-chip prepared for T4 and TSH panel were incubated with 50 µl standard cocktail mixed with optimized tracer cocktail containing 60,000 cpm/100 µl for T4 and 100,000 cpm/100µl for TSH for 2 hr. Typical standard curves for T4 and TSH was generated using MAIA as shown in Fig.4.6.

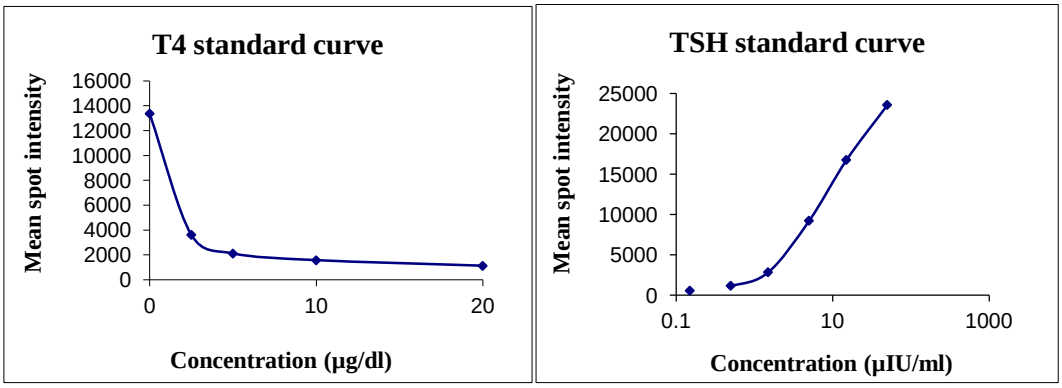


Figure 4.6 Calibration curve for simultaneous estimation of T4 and TSH by MAIA.
The standard curves were generated by plotting the mean of spot-intensity of the duplicate spots against each dilution of standards.

4.4.4.2. TSH and Tg

Similarly, standard curves for TSH and Tg MAIA was generated as shown in Fig.4.7. Antibody-chip was incubated with 50 µl of standard cocktail mixed with optimized tracer cocktail containing 100,000 cpm/100 µl each for TSH and Tg for overnight.

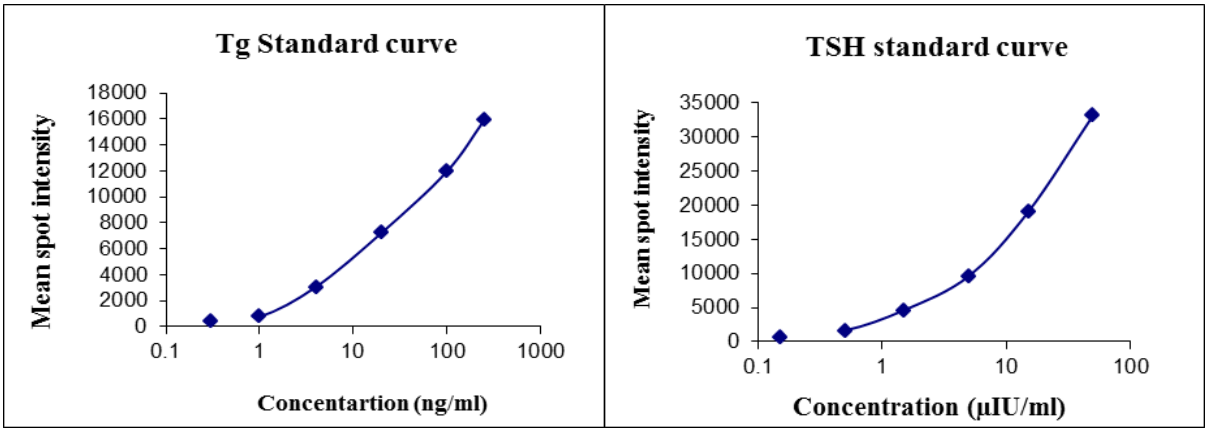


Figure 4.7 Calibration curve for simultaneous estimation of TSH and Tg by MAIA.
The standard curves were generated by plotting the mean of spot intensity of the duplicate spots against each dilution of standards.

4.4.5. Sensitivity and working range

4.4.5.1. T4 and TSH panel

The analytical sensitivity of the T4 assay was 0.12 µg/dl and upper limit of the assay was 20 µg/dl. The analytical sensitivity of the TSH assay was 0.03 µIU/ml with upper limit of the assay of 50 µIU/ml. Fig.4.8 shows the corresponding autoradiogram.

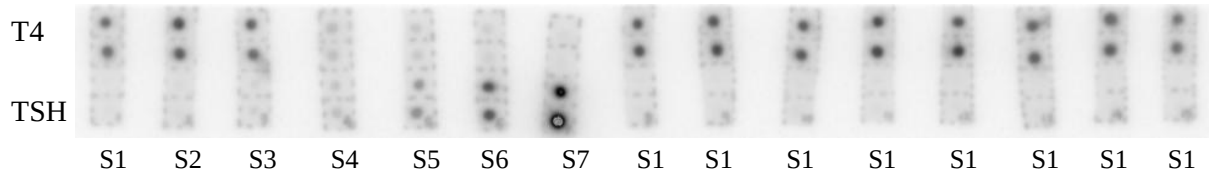


Figure 4.8 Autoradiogram showing the sensitivity of T4 and TSH MAIA.
TEMs were reacted with standard cocktail (S1-S7) along with tracer cocktail for 2 hr.

4.4.5.2. TSH and Tg panel

The analytical sensitivity of the TSH assay was 0.03 µIU/ml. The upper limit of the assay was 50 µIU/ml. The analytical sensitivity of the Tg assay was 0.1 ng/ml. The upper limit of the linear range of the assay was 250 ng/ml. Fig.4.9 shows the corresponding autoradiogram.

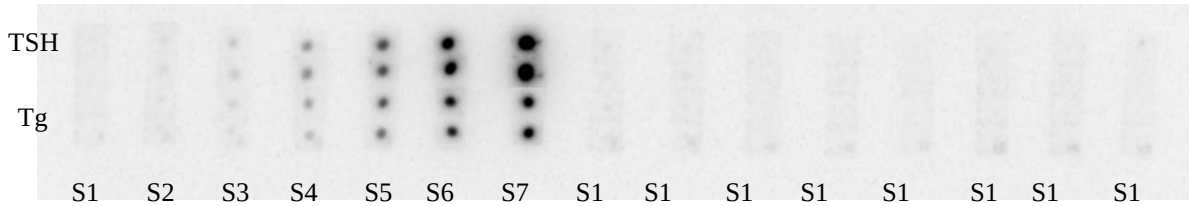


Figure 4.9 Autoradiogram showing the sensitivity of TSH and Tg MAIA.
TEMs were reacted with standard cocktail (S1-S7) along with mixture of ^{125}I labeled detection antibodies.

4.4.6. Precision

The intra-assay CV for both the panels was determined by analyzing ten replicates of two serum samples. Another three serum samples were determined in ten consecutive assays and the mean concentration of duplicates in each run were used to calculate the inter-assay CV.

4.4.6.1. T4 and TSH

The autoradiogram depicting the intra-assay variation for T4 and TSH panel is represented in Fig.4.10. Table 4.1 summarizes the %CV obtained.

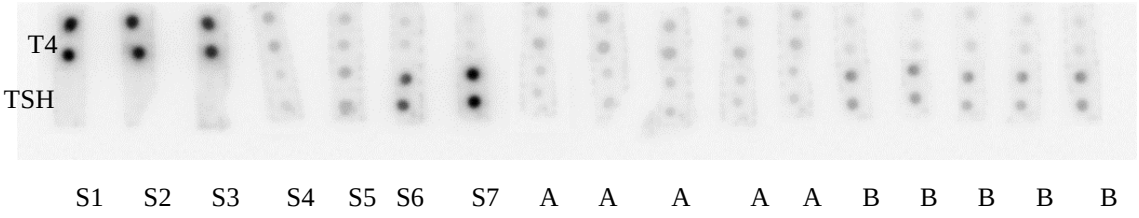


Figure 4.10 Autoradiogram showing the intra-assay variation of T4 and TSH MAIA.
S1-S7- standard cocktail. A=serum sample having T4 concentration of 3.5µg/dl and TSH concentration of 2.2µIU/ml. B=serum sample having T4 concentration of 12.1µg/dl and TSH concentration of 8µIU/ml.

	T4			TSH		
	Intra-assay variation					
	Concentration (µg/dl)	Mean ± SD	% CV	Concentration (µIU/ml)	Mean ± SD	% CV
Sample A	3.5	3 ± 0.25	8.1	2.2	1.8 ± 0.19	10.5
Sample B	12.1	11.8 ± 1.0	9	8	7.3 ± 0.52	7
	Inter-assay variation					
Sample A	8	7.8 ± 1.3	17.2	1.6	1.8 ± 0.14	7.8
Sample B	4.5	3.9 ± 0.86	21.7	3.4	3.7 ± 0.49	13.3
Sample C	11.5	11.3 ± 1.4	12.4	12	12.1 ± 1.8	15.4

Table 4.1 Intra and inter assay variation of T4 and TSH MAIA.

4.4.6.2. TSH and Tg

The autoradiogram depicting the intra-assay variation for TSH and Tg panel is represented in Fig.4.11. Table 4.2 summarizes the %CV obtain.

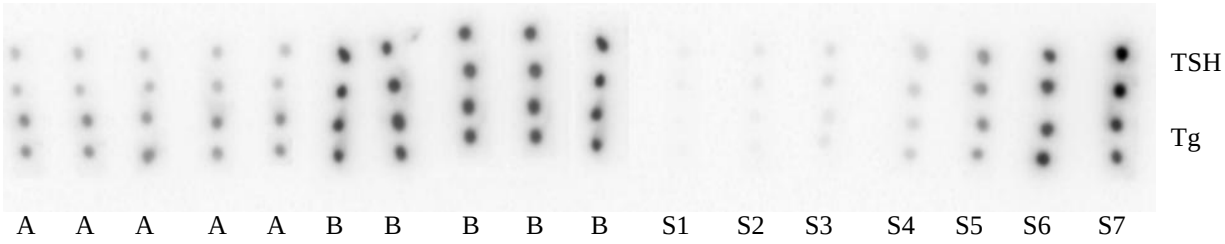


Figure 4.11 Autoradiogram showing the intra-assay variation of TSH and Tg MAIA. S1-S7:standard cocktail. A= TSH concentration of 2µIU/ml and Tg concentration of 20ng/ml. B= TSH concentration of 16.8µIU/m and Tg concentration of 67ng/ml.

	TSH			Tg		
	Intra-assay variation					
	Concentration (µIU/ml)	Mean±SD	%CV	Concentration (ng/ml)	Mean±SD	%CV
Sample A	2	1.6±0.15	9.1	20	21.1±2.1	9.9
Sample B	16.8	17.2±1.79	10.3	67	65.2±7.6	11.6
	Inter- assay variation					
Sample A	1.8	1.64±0.32	20	2	2.42±0.43	18
Sample B	14	14.3±1.75	12	56	58.4±7.56	13
Sample C	4	4.02±0.20	5	19.2	19.38±3.84	20

Table 4.2 Intra and Inter assay variation of TSH and Tg MAIA.

4.4.7. Recovery

Recovery was determined by spiking standards in samples. Three different samples were spiked with two standards (S4 and S5) in 1:1 ratio. Recovery was expressed as a percentage of the expected values.

4.4.7.1. T4 and TSH

Fig.4.12 shows the autoradiogram of T4 and TSH panel showing the recovery of three serum samples spiked with calibrators. Table 4.3 summarizes the % recovery obtained for T4 and TSH panel.

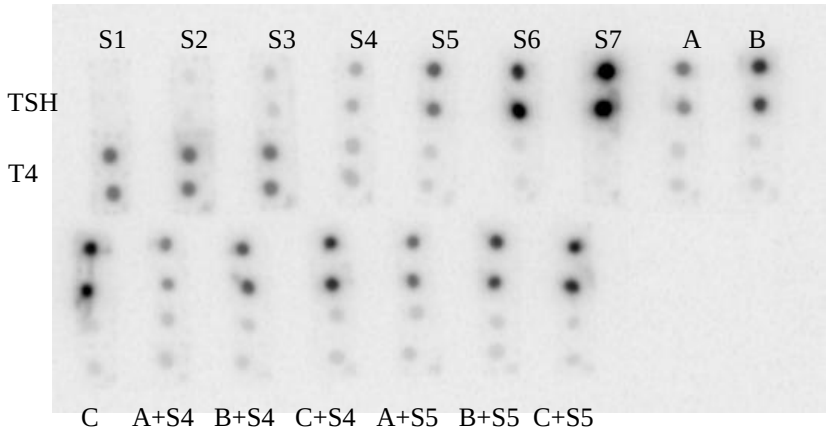


Figure 4.12 Image showing the recovery of three serum samples spiked with calibrators for T4 and TSH MAIA.
S1-S7= standard cocktail. A, B and C are serum samples having T4 concentration of 4, 10.2, 18.5 µg/dl and TSH concentration of 5.1, 8.5 and 9.7 µIU/ml. The serum samples were spiked with standards S4 and S5 in 1:1 ratio.

Recovery of serum samples spiked with calibrators					
1:1 dilution with calibrator		Recovery %			
	µg/dl	Sample A	Sample B	Sample C	Mean
T4	2.5	97.3	94.5	95.0	95.6
	5	94	81.4	99.3	91.6
	µIU/ml				
TSH	1.5	103.7	94.8	81	93.1
	5	93.3	89.4	80.3	87.7

Table 4.3 Recovery of serum sample spiked with calibrators for T4 and TSH MAIA.
A, B and C are serum samples having T4 concentration of 4, 10.2, 18.5 µg/dl and TSH concentration of 5.1, 8.5 and 9.7 µIU/ml. The serum samples were spiked with standards S4 and S5 in 1:1 ratio

4.4.7.2. TSH and Tg

Fig. 4.13 shows the autoradiogram of TSH and Tg MAIA showing the recovery of three serum samples spiked with calibrators. Table 4.4 shows the % recovery obtained for TSH and Tg panel.

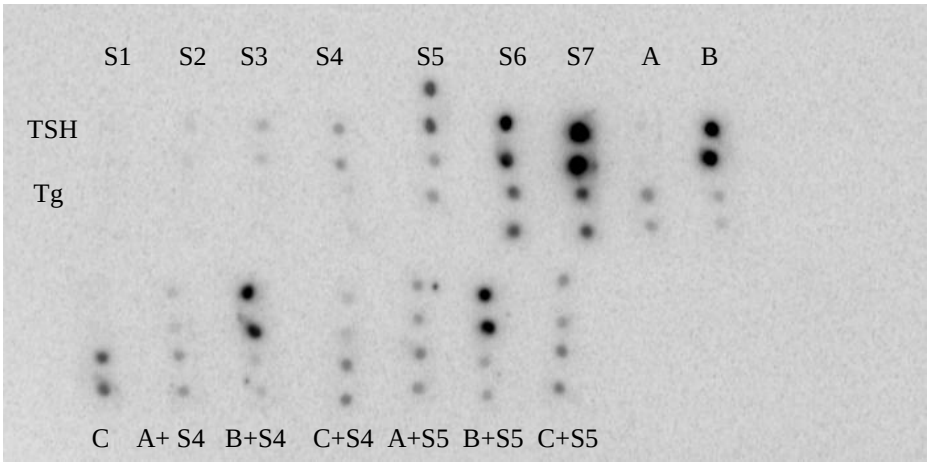


Figure 4.13 Autoradiogram showing the recovery of three serum samples spiked with calibrators for TSH and Tg panel.
S1-S7= standard cocktail. A, B and C are serum samples having TSH concentration of 0.03, 19.5, 0.09 µIU/ml and Tg concentration of 33, 10.2 and 58.8 ng/ml. The serum samples were spiked with standards S4 and S5 in 1:1 ratio.
-

Recovery of serum samples spiked with calibrators					
1:1 dilution with calibrator		Recovery %			
TSH	(IU/L)	Sample A	Sample B	Sample C	Mean
	1.5	78.57	100.95	83.75	87.75
	5	80	110.65	76	88.8
Tg	ng/ml				
	4	89.18	111.26	97.13	99.19
	20	86.79	78.80	82.99	82.86

Table 4.4 Recovery of serum sample spiked with calibrators for TSH and Tg panel.
A, B and C are serum samples having TSH concentration of 0.03, 19.5, 0.09 µIU/ml and Tg concentration of 33, 10.2 and 58.8 ng/ml. The serum samples were spiked with standards S4 and S5 in 1:1 ratio.

4.4.8. Linearity

Linearity or dilution test in immunoassays is done to find out the errors arising out of differences in the matrix between the standard and sample. Linearity was determined in sample containing high concentration of analytes which were serially diluted in standard matrix.

4.4.8.1. T4 and TSH

Fig.4.14 shows the autoradiogram obtained for linearity studies of T4 and TSH MAIA. Table 4.5 represents the % O/E obtained for the same.

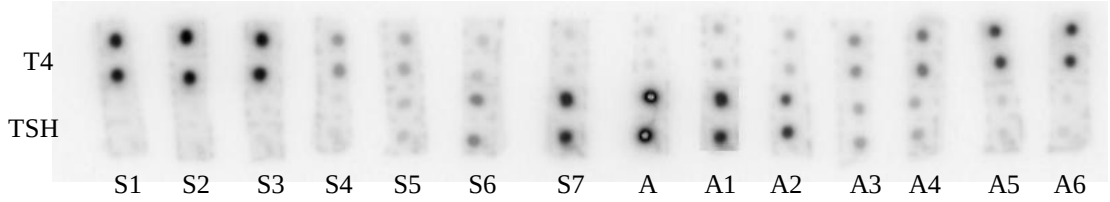


Figure 4.14 Autoradiogram showing the linearity of serum sample serially diluted in SHFS for T4 and TSH MAIA.
S1-S7 = standard cocktail. A= sample having T4 =16µg/dl and TSH =100 µIU/ml. A1-A6 serial dilutions of sample A in hormone free serum.

Linearity of serum samples diluted in hormone free serum		
Analyte	Dilution factor	Observed/Expected %
T4	2	77.5
	4	110
	8	140
	16	140
	32	132
	64	141.6
TSH	2	116
	4	96.8
	8	80
	16	114.5
	32	126.6
	64	120

Table 4.5 Linearity of serum sample serially diluted in hormone free serum.

4.4.8.2. TSH and Tg

Fig.4.15 shows the autoradiogram obtained for linearity studies of TSH and Tg panel. Table 4.6 summarizes the % O/E obtained for the same panel.

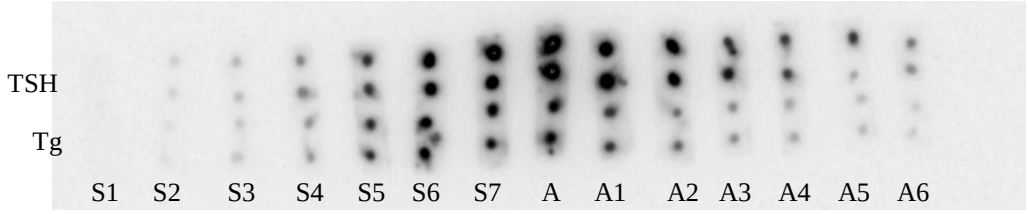


Figure 4.15 Image showing the linearity of serum sample serially diluted in human HFS for TSH and Tg panel.
S1-S7 = standard cocktail. A= sample having TSH =100µIU/ml and Tg =50 ng/ml.A1 –A6 serial dilutions of sample A in hormone free serum.

Linearity of serum samples diluted in hormone free serum		
Analyte	Dilution factor	Measured/Expected %
TSH	2	82.6
	4	106.8
	8	88.1
	16	79.2
	32	116
	64	73.3
Tg	2	79.6
	4	84.2
	8	69.3
	16	87.1
	32	100
	64	105.3

Table 4.6 Linearity of serum sample serially diluted in HFS.
Sample having TSH =100µIU/ml and Tg =50 ng/ml was serially diluted in hormone free serum.

4.4.9. Comparison to established assays

For T4 and TSH MAIA, T4 results were compared to T4 RIA used routinely at our centre. TSH results were compared to human TSH IRMA kit from BRIT. Fig.4.16 shows the corresponding graphs obtained. The results obtained correlated well with the T4 results obtained by the RIA (MAIA = 0.86*RIA−0.04, r = 0.92, $p < 0.001$, n = 34). Results of the TSH MAIA also showed a good correlation with TSH IRMA (MAIA = 1.03*IRMA−0.70, r = 0.995, $p < 0.001$, n = 34).

For TSH and Tg MAIA, TSH results were compared to those obtained from human TSH IRMA kit and Tg results were compared to human Tg IRMA kit from Izotop. Fig.4.17 shows the corresponding graphs obtained. Significant correlation was found between the TSH

results obtained by MAIA and IRMA. (MAIA=0.95*IRMA+0.32, r = 0.98, p < 0.001, n = 41). Results of the Tg MAIA also showed a good correlation with Tg IRMA (MAIA=1.03*IRMA+10.1, r = 0.91, p < 0.001, n = 41)

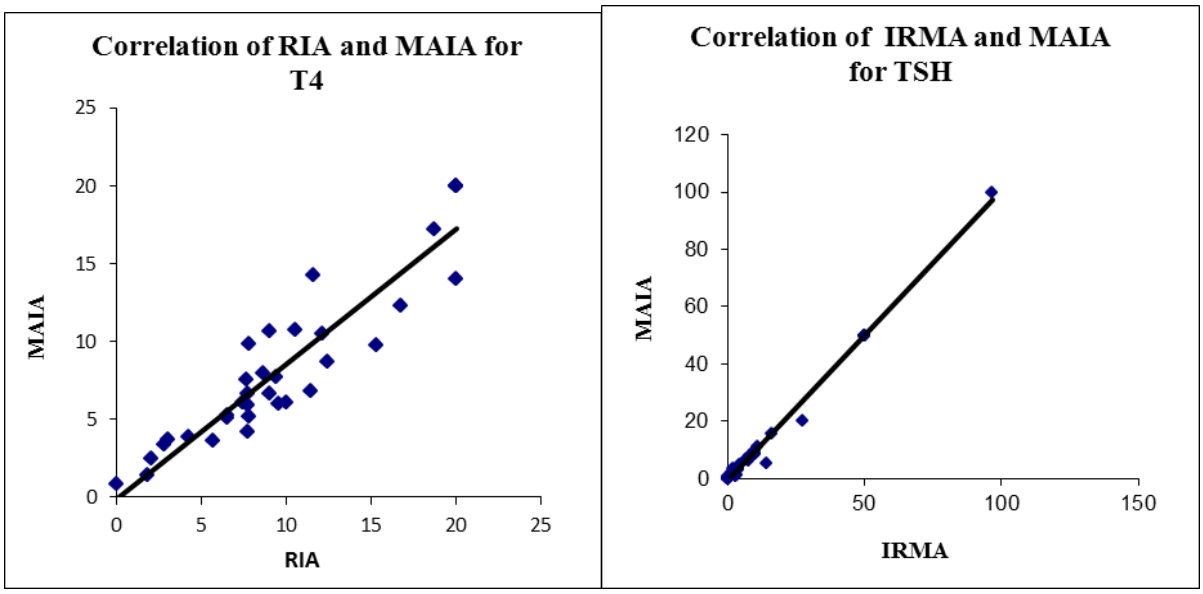


Figure 4.16 Graph showing the comparison of T4 and TSH concentration in 34 human serum samples measured by developed MAIA and RIA for T4 IRMA for TSH .

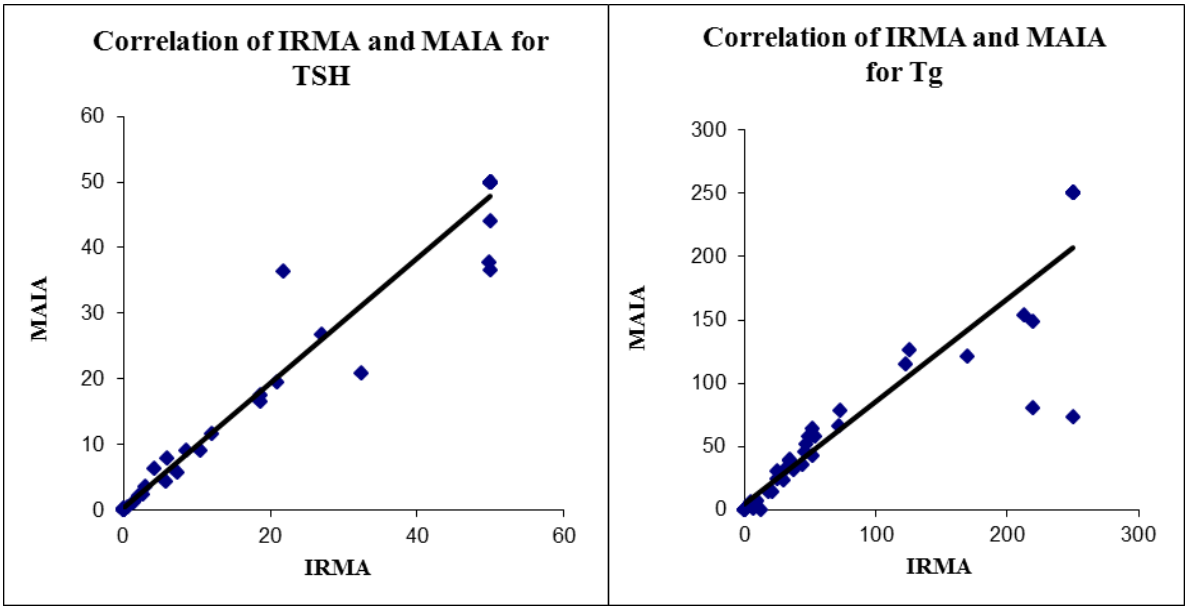


Figure 4.17 Graph showing the comparison of TSH and Tg concentration in 41 human serum samples measured by MAIA and IRMA.

4.5. Protocol for developed MAIA

4.5.1. T4 and TSH MAIA

General reagents

Support - PC-TEM

Spotting buffer - PBS (0.025 M, pH-7.4)

Matrix for standard preparation - SHFS

Detection - PhosphorImager

Software - ImageJ

Assay Protocol

Preparation of antibody-chip

- 1000 µg/ml of antibody
- 0.5 µl/ spot
- 2 hr incubation

in humid chamber on hydrophobic membranes.

in dry chamber on hydrophilic membranes.

- Washing - 3 X 1000µl in PBS
- Drying- between sheets of Whatman paper.

Blocking

- 1000 µl of PBS-BSA /well on shaking platform for 2 hr
- Washing - 3 X 1000µl in PBS

Sample and tracer incubation

- 50 µl/well of sample
- 200 µl/well of cocktail of ^{125}I-TSH antibodies and ^{125}I-T4.
- 2 hr incubation at RT on shaking platform
- Washing- 3 X 1000 µl in PBS-T
- Drying- between sheets of Whatman paper

Exposure to phosphor screen

- 2 hr

Imaging

- PhosphorImager
- Images digitized and saved as 16-bit tiff files

- Signal quantification – ImageJ

- Plotting of standard curve and quantification of unknown analyte - Mgamma in-vitro test software.

4.5.2. TSH and Tg MAIA

General reagents

Support - PC TEM

Spotting buffer - PBS (0.025 M pH-7.4)

Matrix for standard preparation - Human HFS.

Detection - PhosphorImager

Software - ImageJ

Assay Protocol

Preparation of antibody-chip

- 1000 µg/ml of antibody
- 0.5 µl/ spot
- 2 hr incubation
  - in humid chamber on hydrophobic membranes.
  - in dry chamber on hydrophilic membranes.
- Washing - 3 X 1000µl in PBS
- Drying - between sheets of Whatman paper.

Blocking

- 1000 µl of PBS-BSA/well on shaking platform for 2 hr
- Washing - 3 X 1000 µl in PBS

Sample and tracer incubation

- 50 µl/well of sample
- 200 µl/well of cocktail of ^{125}I- TSH antibodies and ^{125}I-Tg antibodies.
- Overnight incubation at RT on shaking platform
- Washing - 3 X 1000µl in PBS-T
- Drying - between sheets of Whatman paper

Exposure to phosphor screen

- 2 hr

Imaging

- PhosphorImager
- Images digitized and saved as 16-bit tiff files
- Signal quantification – ImageJ
- Plotting of standard curve and quantification of unknown analyte- Mgamma software.

4.6. Discussion

The objective for this Chapter was to develop MAIA for a panel of two analytes. The main components, steps and conditions that make up MAIA were evaluated.

Optimization of the tracer concentration and time of incubation was done. For, T4 and TSH MAIA, the tracer cocktail that gave highest SNR was found to be consisting of ^{125}I-T4 giving 60,000 cpm /100 µl and ^{125}I-TSH monoclonal antibodies giving 100,000 cpm /100 µl. For T4, it was seen that there was an increase in SNR when the tracer concentration was increased from 40,000 cpm to 60,000 cpm /100 µl, after which there was a minor decrease in SNR, because background increases much more in comparison to spot signal intensity. For TSH, that there was a minor increase in SNR when the tracer concentration was increased from 80,000 cpm to 100,000 cpm, after which there was not much change in SNR. Thus the tracer concentration giving 100,000 cpm /100 µl to 150,000 cpm /100 µl can be used although we have selected the 100,000 cpm /100 µl for the assay to obtain minimum background.

The optimum time of incubation for T4 and TSH MAIA was found to be 2 hr. For T4, as the time of incubation was increased from 2 hr to 4 hr, there was no significant change in SNR. As the time of incubation is further increased, there was decrease in SNR as the background increases much more as compared to signal. For TSH, throughout the standard curve, SNR

goes on increasing with the increasing time from 2 hr to 4 hr and maximum SNR is achieved with overnight incubation. Although the SNR increases with the increasing time for TSH, 2 hr was selected as an optimum time for TSH, since sufficiently high sensitivity is obtained at 2 hr of incubation. At 2 hr, sensitivity was found to be 0.03 µIU/ml. The normal range recommended for TSH in human serum is 0.4- 4 µIU/ml [7]. The developed assay is clearly sensitive enough to cover the diagnostic cut-off levels at 2 hr of incubation. It is not required to increase the time of incubation till overnight which is technically inconvenient and will increase the reporting time.

For TSH and Tg panel, the tracer cocktail that gave highest SNR was found to be consisting of ^{125}I-TSH antibodies giving 100,000 cpm/100 µl and ^{125}I-Tg monoclonal antibodies giving 100,000 cpm /100 µl. For TSH, there was an increase in SNR when the tracer concentration was increased from 80,000 cpm to 100,000 cpm after which there was a minor decrease in SNR. Thus the tracer concentration giving 100,000 cpm/100 µl was selected for the assay. For Tg, increasing SNR was seen when the tracer concentration was increased from 50,000 cpm to 100,000 cpm/100 µl and when the tracer concentration is further increased to 150,000 cpm/100 µl there was a minor decrease in SNR. Thus the tracer concentration giving 100,000 cpm/100 µl was selected for the assay.

The optimum time of incubation for TSH and Tg panel was found to be overnight. For TSH as the time of incubation was increased from 2 hr to 4 hr to overnight, increasing SNR was seen throughout the standard curve. For Tg, SNR goes on increasing with time and maximum SNR is achieved with overnight incubation. For Tg, overnight incubation is essential as clinically useful sensitivity of assay can be achieved only with overnight incubation. The

optimum time of incubation for TSH and Tg panel was overnight to obtain good sensitivity and high SNR.

In MAIA, antibody specificity is more critical compared to individual immunoassay. The anti-TSH monoclonal used was very specific, with no detectable cross-reaction to FSH, LH, hCG, GH and prolactin. For T4, we used polyclonal antiserum which shows 10% cross-reactivity to T3. However, as the concentration of T3 present ordinarily in the serum is ~50 times less than T4, this cross-reactivity is of no consequence in the assay. In this study, the possible cross-reactivities were tested when the antibody-chip was incubated with standard cocktail along with individual tracers. There was no cross-reactivity detected between the antibodies for both the panel of analytes.

In some cases, MAIA may lack the adequate assay sensitivity compared to ELISAs [158]. However, assays that have a high analytical performance by reaching an excellent assay sensitivity and wide linear range have been reported [159, 160]. Sensitive assays were developed for both the analyte panels. Sensitivity for T4 and TSH MAIA was comparable with the existing RIA for T4 (0.1 µg/dl) and IRMA for TSH (0.025 µIU/ml). Since the lower cut-off values of T4 and TSH in clinical diagnosis are 4.0 µg/dl and 0.2-0.4µIU/ml, respectively [7], the sensitivity and linear ranges of T4 and TSH panel is satisfactory for clinical applications. For TSH and Tg panel, the analytical sensitivity of the TSH was 0.03 µIU/ml and that of Tg was 0.1 ng/ml. The upper limit of the linear range of the assay was 50 µIU/ml for TSH and 250 ng/ml for Tg. Sensitivity of TSH in TSH and Tg panel was comparable to current IRMA for TSH (0.025 µIU/m) and Tg (0.02 ng/ml). TSH assays are clearly sensitive enough to cover the diagnostic cut off levels of 0.2 µIU/ml of TSH that are

used to identify the patient as hyperthyroid or normal and 2 ng/ml for Tg which identifies the patient with an elevated risk of DTC [161].

The reproducibility of MAIA was assessed by measuring the intra- and inter-assay %CVs. Precision obtained for all samples for both the analytes panels fulfilled the acceptance criteria. Both intra- and inter-assay CVs was less than 20%, thus, the precision and reproducibility of MAIA is acceptable. Linearity and dilution experiments suggest that the antigen in the sample is behaving similar to the native standard.

Immunoassays are standard methods that are widely used for the detection of thyroid disorders. In this chapter, performance of MAIA is compared with RIA/IRMA as applicable. The concentrations of analytes were chosen in the manner to cover the entire standard curve used for analyte level determination. Results obtained with both methods were compared using linear regression analysis. Results of MAIA for the both analyte panels showed a good correlation with RIA/IRMA. Good correlation between MAIA and RIA/IRMA shows its potential to replace single-analyte immunoassays as a cost-effective method. The results described, demonstrate the applicability of MAIA to monitor simultaneously more than one analyte per sample.

CHAPTER 5

Development and Validation of MAIA for a Thyroid Panel of three Analytes

5.1. Introduction

Following the development and validation of MAIA for two analytes viz.,(i) T4 and TSH (ii) TSH and Tg as described in Chapter 4, in continuation of that, this Chapter describes experiments and presents the data from them for the MAIA for a thyroid panel that is expected to be very useful in a thyroid clinic: the simultaneous estimation of T4, TSH and Tg for detecting thyroid dysfunction.

Measuring several disease-associated analytes in a sample may lead to a more accurate prognosis and/or diagnosis. Definitive assessment of thyroid disorders may require detection of thyroid related hormones, viz., TSH, free-T4 (or T4) and free-T3 (or T3) and other analytes in serum such Tg, anti-Tg antibodies, anti-thyroid peroxidase antibodies. While the former panel provides information on thyroid function (biochemical function) *per se*, the latter panel provides information on the structural integrity of the gland. The thyroid hormone binding proteins in circulation would form another panel since these proteins determine the concentration of the free hormones in plasma. It would take a lot of time and effort to combine all the above analytes in one 'antibody-chip' and validate it. Further, thyroidologists seek TSH, free-T4, free-T3 as the first line of tests to rule out or confirm the presence of

thyroid dysfunction. In the experience at our centre, clinicians request Tg levels too, since auto-immune thyroiditis is present in a significant number of patients. Serum Tg levels are also useful in patients coming for follow-up after thyroid cancer surgery and ^{131}I-ablation. With this in mind, we decided on a panel of T4, TSH and Tg as the essential tests for the laboratory diagnosis of thyroid disorders. Free-T3 (or T3), is the least informative of the tests for thyroid gland function, since only about 10% of the T3 in circulation is from the thyroid and rest by peripheral de-iodination. In fact, T3 levels are a measure of peripheral de-iodination since it has a half-life of 1-day in circulation. Free-T4 (or T4) provides the same information vis-à-vis thyroid function, except in the case of T3-toxicosis, which can be identified normal T4 and suppressed TSH seen with a sensitive TSH assay.

It is expected that when MAIA is available on a commercial platform, it will reduce the assay costs since; estimating each analyte individually can be expensive in terms of materials, time and labour.

This chapter describes the development of MAIA for a panel of three analytes (T4, TSH and Tg) on PC TEM using ^{125}I-labeled analyte or antibodies as the tracer molecules. Various parameters such as tracer concentration and time of incubation were optimized. Finally the developed assay was validated using several parameters viz. sensitivity, specificity, precision, linearity and recovery that are performed for any bio-analytical method.

5.2. Materials

The materials, chemicals and equipments required for the experiments described in this Chapter are the same as described in Chapter 3 under Section 3.2.

5.3. Methods

5.3.1. Preparation of antibody-chip

5.3.1.1. Activation of PC-TEM

Activation was accomplished in same manner as described in Chapter 2 in Section 2.3.4.2.

5.3.1.2. Antibody immobilization and blocking

Anti-T4, TSH and Tg antibodies (0.5 µl) at the concentration of 1mg/ml in spotting buffer were spotted manually on PC-TEM for 2 hr at RT using micropipette. TEMs were rinsed with PBS to remove excess unbound antibodies. The NSB sites were blocked using PBS-BSA for 2 hr at RT with gentle agitation and rinsed thoroughly with PBS.

5.3.2. Preparation of combined standards

The standards were prepared by adding the required amount of the T4, TSH (Genzyme Corporation, USA) and Tg in the SHFS. Seven standards namely S1 to S7 were prepared having T4 concentration of 0, 0, 0, 2.5, 5, 10, 20 µg/dl, TSH concentration of 0, 0.15, 0.5, 1.5, 5, 15 and 50 µIU/ml and Tg concentration of 0, 0.3, 1, 4, 20, 100 and 250 ng/ml respectively. Zero concentrations are repeated for T4 standards so that the number of standards is the same for all the analytes. The TSH standards used were calibrated against the reference TSH preparation IRP 80/558, while the Tg standards used were calibrated against Tg reference preparation BCR-CRM 457. Commercially available T4 RIA kit was used to measure the concentration of T4 in prepared standards.

5.3.3. Preparation of tracer cocktail

T4 was labeled with ^{125}I as described in Chapter 2 (Section 2.3.2) .The ^{125}I-T4 was diluted in PBS-ANS buffer so as to contain ^{125}I-T4 at the desired concentration. ^{125}I-anti-Tg monoclonal antibodies and ^{125}I-anti-TSH monoclonal antibodies, at the desired concentrations were added to the same buffer.

5.3.4. Immunoassays

TEMs were cut into 15mm X 10 mm pieces and placed in 12 well plate (one per well) for performing the immunoassays. 50 µl of each of the combined standards or sample and 300 µl of tracer cocktail having varying concentration of tracer was applied to TEMs and incubated for different time periods with gentle agitation on a rotary shaker. After the reaction, the TEMs were washed thrice with PBS to remove unbound reactants and dried on Whatman filter paper.

5.3.5. Detection and Quantification

Digital autoradiograms of TEMs were obtained using PhosphorImager. Image analysis, spot detection and quantification were performed with ImageJ analysis software. The analysis was done in same manner as described earlier in Chapter 3. Average spot intensities presented in the graphs and tables are calculated from duplicate spots spotted on PC-TEM.

5.3.6. Cross-reactivity

Possible cross-reactivity, if any, was looked between the antibodies used in the MAIA for T4, TSH and Tg. This was checked by reacting the antibody-chip with individual tracer or tracer

cocktail along with a combined standard containing 0 µg/dl of T4, 50µIU/ml of TSH and 250 ng/ml of Tg.

5.4. Results

5.4.1. Cross-reactivity

When reacted with the individual tracers, only the spots of antibodies corresponding to the tracer added showed binding, and no signal was obtained at the other antibody spots showing non-cross-reactivity among them. The results of cross-reactivity are shown in Fig.5.1.

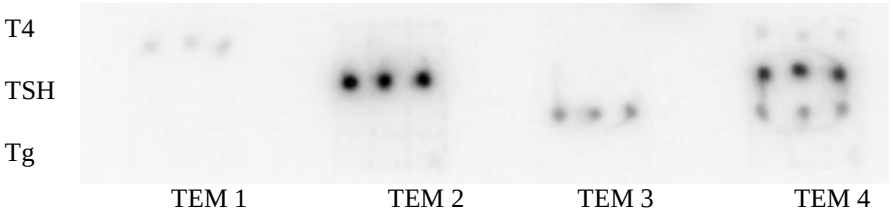


Figure 5.1 Image showing the cross-reactivity of T4, TSH and Tg MAIA.
Each TEM was reacted with a combined standard containing '0' T4 and high concentration of TSH and Tg. TEM 1 was reacted with ^{125}I-T4. TEM 2 was reacted with ^{125}I-anti-TSH antibodies. TEM 3 was reacted with ^{125}I-anti-Tg antibodies. TEM 4 was reacted with tracer cocktail of ^{125}I-T4, ^{125}I-anti-TSH and ^{125}I-anti-Tg antibodies.

5.4.2. Optimization of tracer concentration

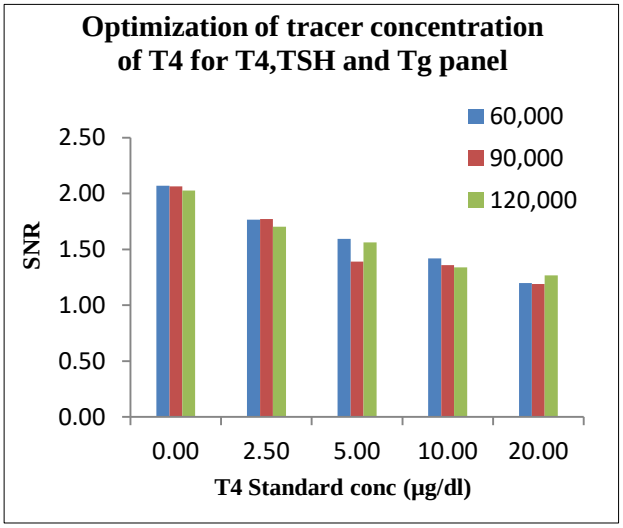
Tracer cocktail having different concentrations of ^{125}I-T4, ^{125}I-anti-TSH monoclonal antibodies and ^{125}I-anti-Tg monoclonal antibodies was tested. Three different tracer cocktails was prepared having ^{125}I-T4 giving 60,000, 90,000 and 120,000 cpm/100 µl, ^{125}I-anti-TSH antibodies giving 80,000, 100,000 cpm and 150,000 cpm /100 µl / respectively, and ^{125}I-Tg monoclonal antibodies giving 50,000, 100,000 cpm/100 µl and 150,000 cpm respectively. The signal generated for each concentration of tracer cocktail was divided by the

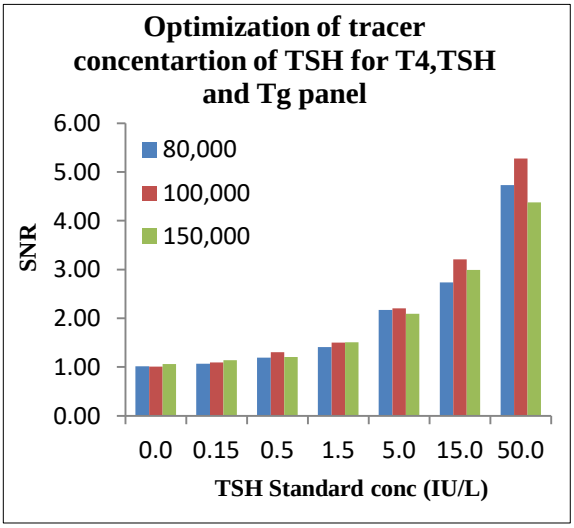
corresponding background. The highest SNR was obtained with 60,000 cpm of ^{125}I-T4, 100,000 cpm/100 µl of ^{125}I-anti-TSH antibodies and 150,000 cpm/100 µl of ^{125}I-anti-Tg antibodies. Fig.5.2 shows the corresponding autoradiogram and graph.

5.4.3. Optimization of time of incubation

After selection of the optimum tracer concentration, TEMs were incubated with optimized tracer cocktail and each of the combined-standards cocktail for different time periods in order to select the time of incubation which gave highest SNR. The TEMs were reacted with 50µl of each combined-standard along with 300 µl of tracer cocktail for 4hr, 6hr and overnight. The spot signal intensity obtained at different time periods was divided by corresponding background. Fig.5.3 shows the corresponding autoradiogram and graph.

cpm/100µl	Optimization of tracer concentration for T4, TSH and Tg panel
T4: 60,000 TSH: 80,000 Tg: 50,000	S1 S2 S3 S4 S5 S6 S7 T4 TSH Tg
T4: 90,000 TSH: 100,000 Tg: 100,000	
T4: 120,000 TSH: 150,000 Tg: 150,000	





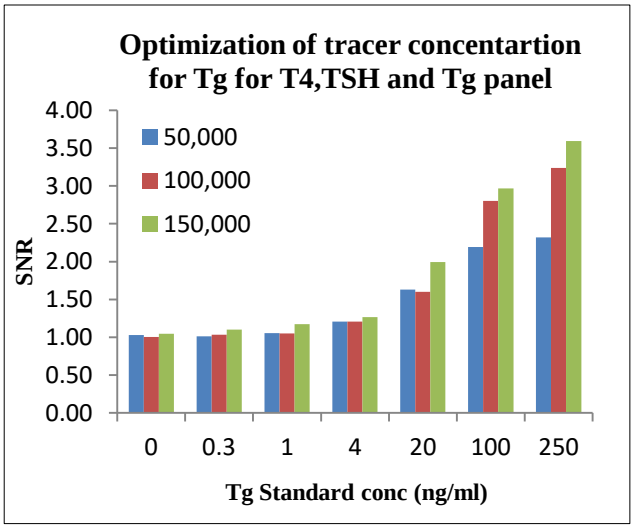


Figure 5.2 Autoradiogram and graph showing the optimization of the tracer concentration for T4, TSH and Tg panel.

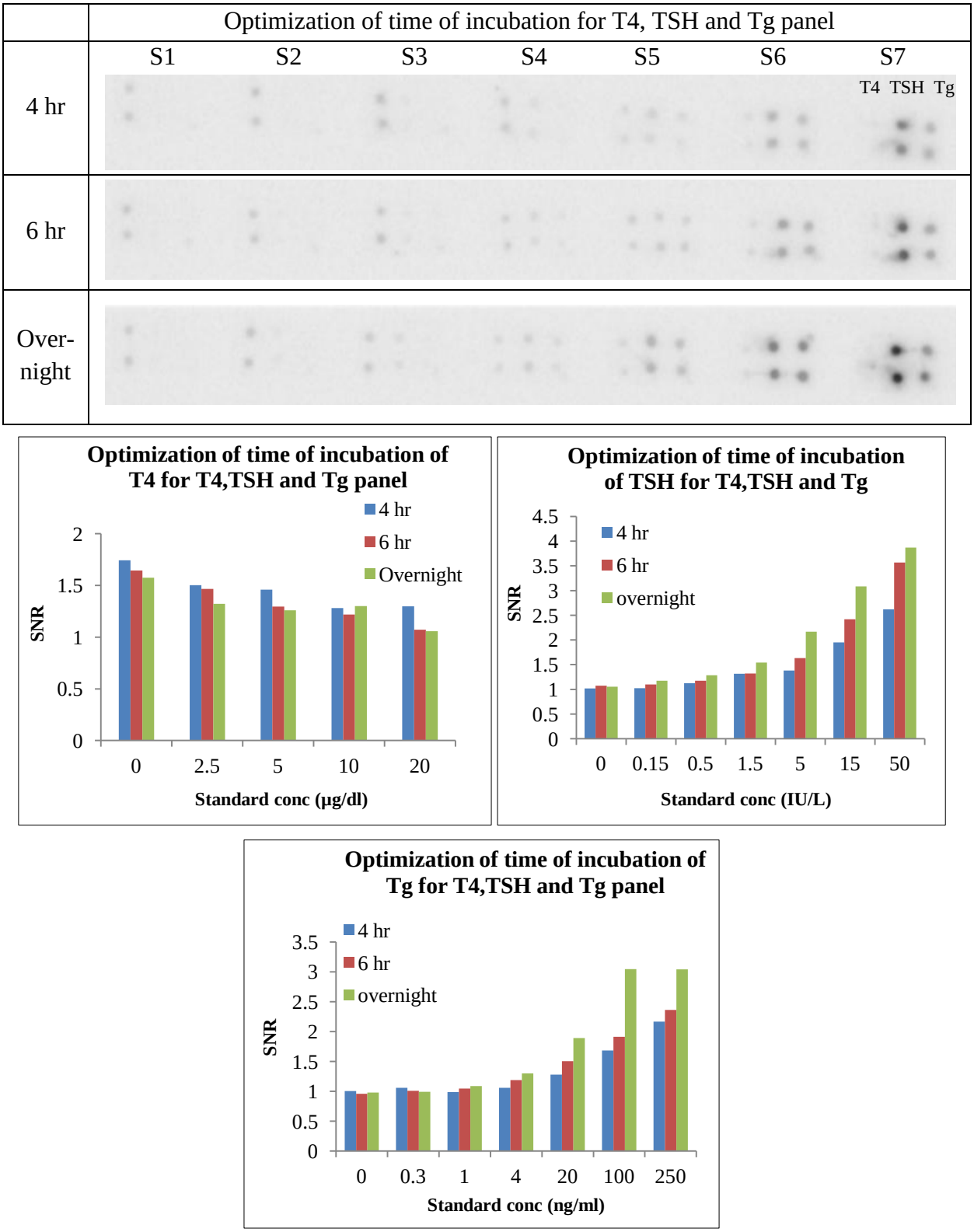


Figure 5.3 Autoradiogram and graph showing the optimization of time of incubation for T4, TSH and Tg panel.

5.4.4. Assay performance

Considering the results shown above, standard curves for T4, TSH and Tg was generated as shown in Fig.5.4. Antibody-chip was prepared by spotting 0.5 µl of 1 mg/ml polyclonal anti-T4, monoclonal anti-TSH and polyclonal anti-Tg antibodies. Two replicate spots were made for each standard. Antibody-chip was incubated with 50 µl combined-standards and with 300 µl of tracer cocktail having optimized concentration of T4 at 60,000 cpm/100 µl, TSH at 100,000 cpm/100 µl and Tg at 150,000 cpm/100 µl for overnight.

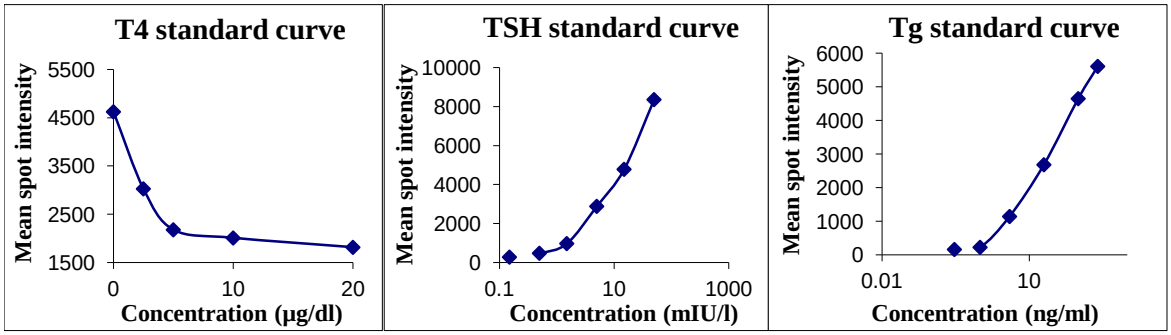


Figure 5.4 Standard curve for simultaneous estimation of T4, TSH and Tg by MAIA.
The standard curves were generated by plotting the mean of spot intensity of the duplicate spots against each dilution of standards.

5.4.5. Sensitivity and assay range

The sensitivity and assay range obtained for all the three analytes is summarized in Table 5.1. Assay range and sensitivity obtained for MAIA were comparable to RIA/IRMA. Fig.5.5 shows the corresponding autoradiogram.

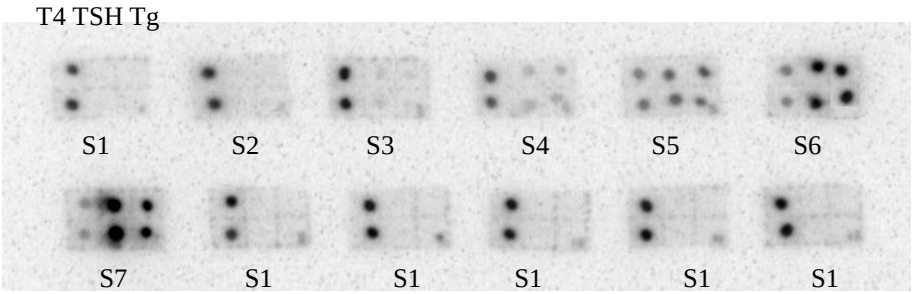


Figure 5.5 Image showing the sensitivity of T4, TSH and Tg in the MAIA.
Two replicate spots for each of the antibodies are made. TEMs were reacted with combined standards (S1-S7) along with cocktail containing ^{125}I labeled antibodies and antigen.

	T4 (µg/dl)	TSH (µIU/ml)	Tg (ng/ml)
Sensitivity	0.11	0.07	0.03
Range	0.11-20	0.07-50	0.03-250

Table 5.1 Sensitivity and working range of T4, TSH and Tg MAIA.

5.4.6. Precision

The precision of the developed T4, TSH and Tg MAIA panel was measured by intra- and inter-assay CV measurements as shown in Table 5.2. Fig. 5.6 shows the corresponding autoradiogram.

Intra-assay variation (n=10)									
	T4			TSH			Tg		
Sample	Concentration (C) (µg/dl)	Mean ±SD	%CV	C µIU/ml	Mean ±SD	%CV	C ng/ml	Mean ±SD	%CV
S	5	4.8±0.71	14.8	8.7	9.1 ± .90	9.9	31	30 ±6.1	20.3
Inter-assay variation (n=10)									
S1	2	2.1±0.35	16.7	16	21.4±3.4	16.2	18	19.9±1.5	7.6
S2	10	9.4±0.99	10.5	1.8	2.4±0.26	10.6	47	54.4±4.5	8.3
S3	5	4.4±0.74	16.7	10	10.4±1.9	18.2	35	34.5±4.5	13.2

Table 5.2. Intra- and Inter-assay %CV of MAIA
A serum sample with a known concentration was assayed ten times in a single assay to calculate intra-assay variation. Three serum samples with a range of concentrations were assayed in ten different assays to calculate inter-assay variation.

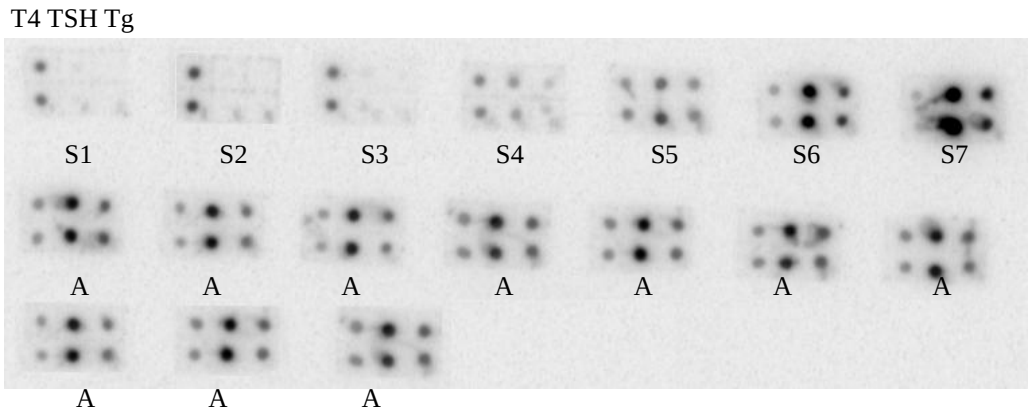


Figure 5.6 Autoradiogram showing the intra-assay variation of T4, TSH and Tg MAIA. S1-S7- combined Standards. A=serum sample having T4 concentration of 5 µg/dl, TSH concentration of 8.7µIU/ml and Tg concentration of 30ng/ml.

5.4.7. Matrix evaluation

5.4.7.1. Recovery

Recovery was determined by spiking the standards in samples. Three different samples were spiked with two standards (S4 and S5) in 1:1 ratio. Recovery was expressed as a percentage of the expected values. Recovery determined in three serum samples was found to be varying between 76% and 119%. The results are summarized in Table 5.3. Fig.5.7 shows the corresponding image.

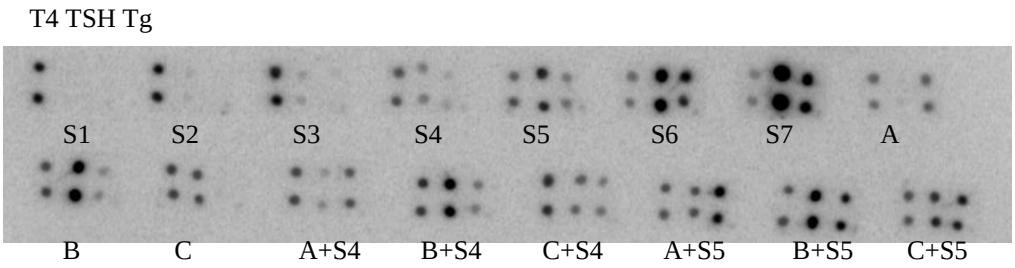


Figure 5.7 Autoradiogram showing the recovery of three serum samples spiked with calibrators for T4, TSH and Tg MAIA.
S1-S7= combined standards. A, B and C are serum samples having T4 concentration of 11.6, 2.0 and 6.8µg/dl, TSH concentration of 0.03, 19.5 and 3µIU/ml and Tg concentration of 33, 10.2 and undetectable ng/ml. The serum samples were spiked with standards S4 and S5 in 1:1 ratio.

Recovery of serum samples spiked with calibrators					
1:1 dilution with calibrator		Recovery %			
		Sample A	Sample B	Sample C	Mean
T4	µg/dl				
	2.5	82.8	81	84.7	82.8
	5	106	88.5	76	90.1
TSH	(µIU/ml)				
	1.5	90	92	100	94
	5	92	93.3	82.5	89.2
Tg	ng/ml				
	20	116	115	119	116.6
	100	115	106	114	111.6

Table 5.3 Recovery of three serum samples spiked with calibrators.
A, B and C are serum samples having T4 concentration of 11.6, 2.0 and 6.8 µg/dl, TSH concentration of 0.03, 19.5 and 3 µIU/ml and Tg concentration of 33, 10.2 and undetectable ng/ml. The serum samples were spiked with standards, S4 and S5, in 1:1 ratio.

5.4.7.2. Linearity

Linearity was determined in sample containing high concentration of T4, TSH and Tg which were serially diluted in standard matrix. Linearity was between 73% to 130% except for T4 which showed linearity of 42% and 33% at very low concentrations of 0.6 and 0.3µg/dl

respectively. The results are summarized in Table 5.4. Fig.5.8 shows the corresponding image.

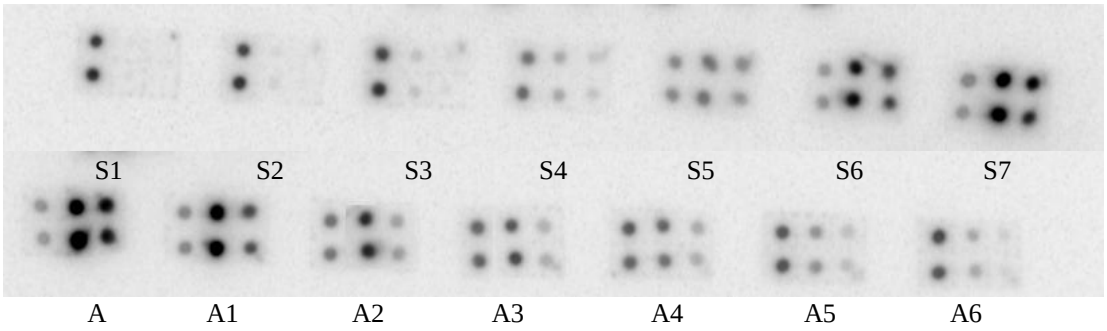


Figure 5.8 Autoradiogram showing the linearity of serum sample serially diluted in SHFS for T4, TSH and Tg MAIA.
S1-S7 =combined standards. A= sample having T4 concentration of 20 µg/dl, TSH =74µIU/ml and Tg =129ng/ml. A1 –A6 serial dilutions of sample A in SHFS.

Analyte	T4	TSH	Tg
Dilution factor	Observed/ Expected	Observed/ Expected	Observed/ Expected
No dilution	0.73	1.05	1.07
2	0.85	1.19	1.30
4	1.12	0.86	0.77
8	0.84	1.10	0.82
16	0.83	1.24	1.09
32	0.42	1.15	1.13
64	0.33	1.23	1.15

Table 5.4 Linearity of serum sample serially diluted in SHFS for T4, TSH and Tg MAIA. A= sample having T4 concentration of 20 µg/dl, TSH =74µIU/ml and Tg =129ng/ml which was serially diluted in SHFS.

5.4.8. Comparison to established RIA and IRMA

A statistical comparison of results from MAIA for 54 human serum samples with results from individual RIA for T4, and IRMAs for TSH and Tg showed significant correlation coefficients. T4 showed good correlation with RIA (MAIA=1.1*RIA-0.47, r=0.91, p<0.001, n=54). Similarly, significant correlation was seen with the TSH results obtained by IRMA (MAIA=0.95*IRMA+0.51, r = 0.98, p < 0.001, n = 54). Results of Tg MAIA also showed a good correlation with IRMA (MAIA=0.99*IRMA+2.08 r = 0.89, p < 0.001, n = 54). Fig.5.9 shows the corresponding graphs.

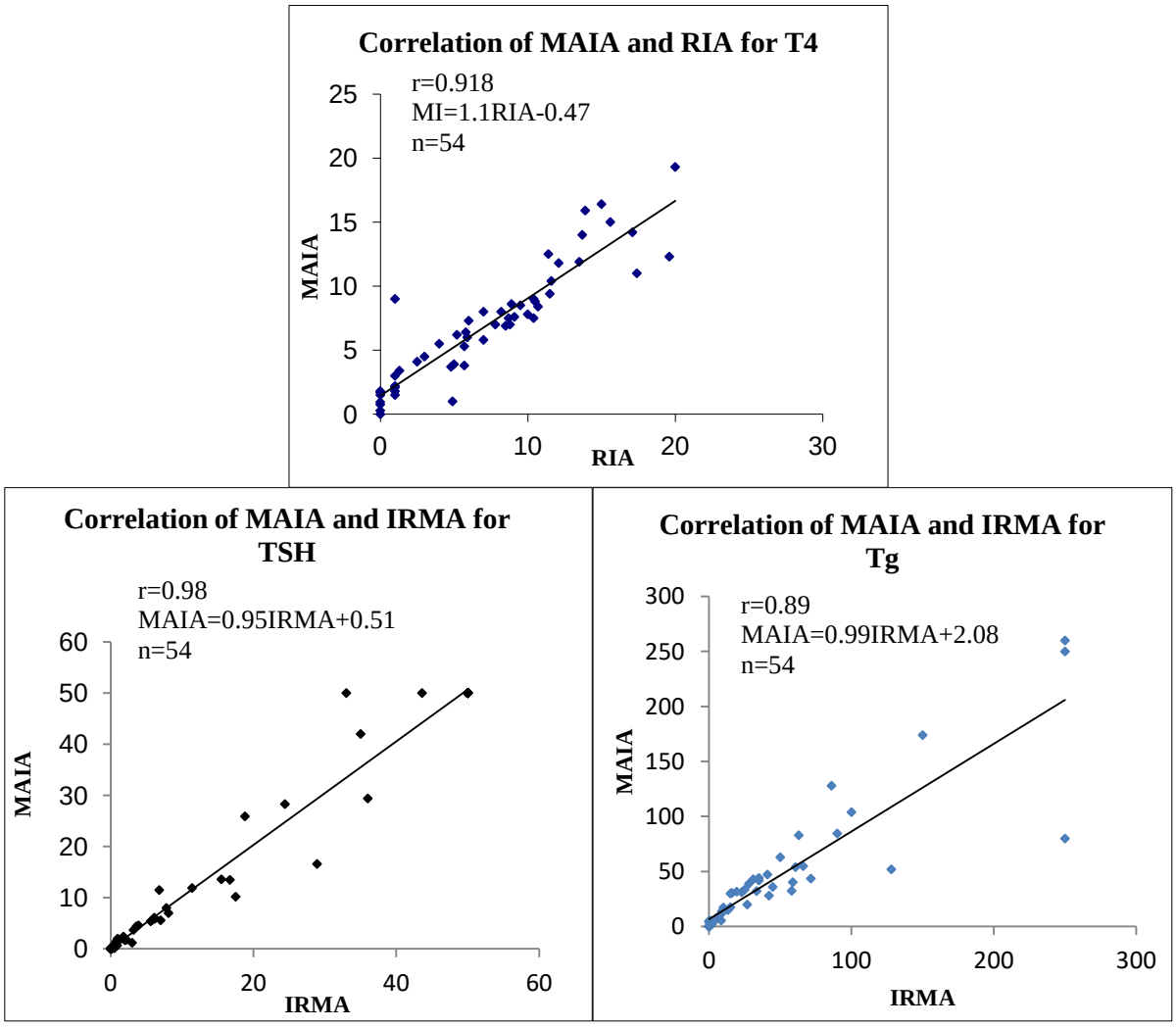


Figure 5.9 Graph showing the comparison of T4, TSH and Tg concentration in fifty-four human serum samples measured by developed MAIA and RIA/IRMA.

5.4.9. Protocol for T4, TSH and Tg MAIA

General reagents

Support - PC-TEM

Spotting buffer- PBS (0.025 M pH-7.4)

Matrix for standard preparation - SHFS

Detection - PhosphorImager

Software - ImageJ software.

Assay Protocol

Preparation of antibody-chip

- 1000 µg/ml of polyclonal anti-T4, monoclonal anti-TSH and monoclonal anti-Tg antibodies.
- 0.5 µl / spot
- 2 hr incubation
  - in humid chamber on hydrophobic membranes.
  - in dry chamber on hydrophilic membranes.
- Washing - 3 X 1000µl in PBS.
- Drying- between sheets of Whatman paper.

Blocking

-1000 µl of PBS-BSA / well on shaking platform for 2hr.

-Washing - 3 X 1000µl in PBS.

Sample and tracer incubation

- 50 µl / well of sample
- 300 µl / well of cocktail of ^{125}I-anti-TSH monoclonal antibody and anti-Tg monoclonal antibody and ^{125}I-T4.
- Overnight incubation at RT on shaking platform
- Washing - 3 X 1000µl in PBS-T
- Drying - between sheets of Whatman paper

Exposure to phosphor screen

-2 hr

Imaging

- PhosphorImager
- Images digitized and saved as 16-bit tiff files
- Signal quantification - ImageJ
- Plotting of standard curve and quantification of unknown analyte - Mgamma software.

5.5. Discussion

In this Chapter, parameters such as concentration of the detection antibodies and time of incubation were optimized for implementation of MAIA for T4, TSH and Tg.

Three different tracer cocktail containing different concentration of ^{125}I-T4, ^{125}I-anti-TSH antibodies and ^{125}I-anti-Tg antibodies were tested. ^{125}I-T4 was tested at 60,000 cpm, 90,000 cpm and 120,000 cpm /100 µl, ^{125}I-TSH monoclonal antibodies was tested at 80,000 cpm, 100,000 cpm and 150,000 cpm/100 µl and ^{125}I-Tg detection antibody was tested at 50,000 cpm, 100,000 cpm and 150,000 cpm/100 µl.

As shown in Fig.5.2, for T4 there was no obvious differences in SNR value obtained at three different concentrations used, indicating that the ^{125}I-T4 concentration has insignificant effects on the signal obtained. Therefore, ^{125}I-T4 concentration was set at 60,000 cpm/100 µl to obtain lowest background. For TSH, highest SNR was obtained at 100,000 cpm/100 µl. Any further increase in detection antibody concentration leads to decrease in SNR due to increasing background. For Tg, there was minor increase in SNR as the tracer concentration was increased from 50,000 to 100,000 cpm/100 µl. There was further increase in SNR when the tracer concentration was raised to 150,000 cpm /100 µl. Therefore, Tg tracer concentration was set at 150,000 cpm/100 µl to obtain highest SNR.

At the selected tracer concentration, three different time period of incubations were tested. For T4, maximum SNR is achieved at 4 hr of incubation, a further increase in time of incubation leads to decrease in SNR since background increased much more as compared to signal. For TSH, SNR goes on increasing with increasing time of incubation, a maximum SNR is achieved after overnight incubation. For Tg, similar trend was observed and maximum SNR was achieved at overnight incubation. Thus the optimum time of incubation for T4, TSH and Tg MAIA was selected as overnight.

The analytical performance of developed MAIA was judged by evaluating the sensitivity, range, precision, recovery and linearity of the assay.

The LOD was calculated from the standard curves as the analyte concentrations corresponding to a signal mean -2SD of the '0' standard assayed in 10 replicates. The sensitivity was found to be 0.11 µg/dl for T4, 0.07µIU/ml for TSH and 0.03 ng/ml for Tg. All the assays were sensitive enough to cover the diagnostically important ranges. The calibration curves had comparable dynamic range as the standard immunoassays (RIA and IRMAs respectively). The dynamic range of MAIA was 0.11-20 µg/dl for T4, 0.07-50 µIU/ml for TSH and 0.03-250 ng/ml for Tg. The dynamic ranges of T4, TSH and Tg achieved in the panel meet the requirements of possible range of T4, TSH and Tg concentrations in human blood for prediction of thyroid disorders. Precision of the MAIA was acceptable with both inter- and intra-assay CV less than 20%.We have not seen any cross-reactivity among the detection antibodies when reacted with individual tracer. Recovery and linearity results show that matrix used for preparation of standards is compatible for performing the MAIA.

Performance of MAIA thyroid panel of T4, TSH and Tg was studied by measurement of analytes in clinical samples. We had quantified the concentration of all the three analytes in 54 serum samples obtained from our routine thyroid clinic, and compared with the MAIA assay results to RIA and IRMA, which are clinically established. Good correlation between both the techniques indicates the potential of MAIA be useful as a quantitative method in the clinical laboratory.

CHAPTER 6

Discussion and Conclusion

6.1. General discussion

MAIA is valuable analytical approach for the clinical laboratory because of its various advantages. Firstly, it can provide more information from a single investigation and improve the diagnostic performance with a single assay [162, 163]. Secondly, MAIA can achieve work simplification, which includes shorter assay time, minimal reagents and sample volume, reduced numbers of pipetting and washing steps. Thirdly, MAIA allows robotic automation for running the tests, thereby achieving a higher throughput. Finally, MAIA can reduce the overall cost per test as it is time saving, less labor intensive and able to provide a high-throughput assay.

This thesis describes the process of antibody-chip development and its application for MAIA for the estimation of analytes related to thyroid disorders in human serum samples. Selection of appropriate solid support and immobilization procedure is a key challenge faced in the antibody-chip production as described in Chapter 1.The binding of antibody to their target analyte depends not only on primary sequence, but also on tertiary structure, which is highly vulnerable to degradation [164]. During the dispensing and immobilization process, the antibodies must maintain the integrity of their 3-D structures. Selection of support should ensure that the antibodies remain in functionally active form. Another important requirement

for manufacturing of antibody-chip is that the solid support used should provide low NSB, which is one of the severe problems in MAIA. The achievement of a low degree of NSB and background noise is extremely difficult when the analyte is present in a complex mixture of a large number of molecules such as serum or plasma. TEM was used as a novel solid support for MAIA and it was found to provide high immobilization capacity, good spot quality and low background with the preservation of functional activity of the antibody.

The solid support also plays an important role in determining the concentration of the antibody immobilized. Increasing the concentration of capture antibodies on the spot increases the sensitivity of assay, which in our case has been achieved by using highly microporous TEM. TEMs have high surface area and provide better immobilization as compared to glass or other planar supports because of the additional capture molecules that can be immobilized within the depth of the support. Careful titration of the antibody-analyte-antibody interactions allowed us to achieve the same level of sensitivity as the RIA and IRMAs used in the laboratory.

The proof-of-principle of MAIA in microarray format was carried out, initially, using individual estimation of T4, TSH and Tg as described in Chapter 3. Having standardized the required procedures for antibody immobilization and detection, the process was continued to develop a MAIA for the simultaneous estimation of two analytes (T4, TSH) and (TSH and Tg), which too was developed as described in Chapter 4. Finally, a MAIA panel was developed for T4, TSH and Tg, for their simultaneous measurement as described in Chapter 5.

Although, several MAIA have been developed for diagnostic applications and commercially available [32] but those are mainly for cytokines or immunoglobulins [44, 139, 165-167]. In

contrast, very few such assays have been developed for the measurement of steroid or other small hormones, which in contrast to the larger peptide hormones, require a competitive assay format involving labeled hormone as a tracer. Although competitive immunoassays using immobilized analyte derivative is the common format for the detection of multiple small molecules (e.g. pesticides, pharmaceuticals, small toxins) [168] in environmental monitoring only few has been described for clinical diagnostics [169]. Moreover development of sensitive MAIA based on competitive immunoassay format is a challenging work because, according to the principle of competitive immunoassays, the amount of the specific antibody available for binding is limited and there is a limitation on the amount of reporter molecule that can be attached during the labeling process to preserve the immunological reactivity of tracer. Both these aspects provide problems for signal generation in the MAIA format.

This work describes the development of MAIA for three different analyte panels: (1) T4 and TSH (2) TSH and Tg (3) T4, TSH and Tg. Both TSH and Tg are large molecules having molecular weights of 28kDa and 660 kDa respectively and development of TSH and Tg MAIA is based on non-competitive sandwich immunoassays. However, T4 is a small molecule with molecular weight of 0.77 kDa (<1 kDa), and it cannot be detected using sandwich immunoassays because it is too small for simultaneous binding by two different antibodies. Based on this need, the thesis work describes the use of ^{125}I-T4 along with ^{125}I-anti-TSH antibodies for T4 and TSH MAIA panel to combine both competitive and non-competitive immunoassay principles in one assay. Similarly, ^{125}I-T4 along with mixture of anti-^{125}I-TSH antibodies and anti-^{125}I-Tg monoclonal antibodies was used for T4, TSH and Tg MAIA. For T4 estimation, a competition between labeled T4 and unlabeled T4 takes place

for the limited amount of antibody immobilized on the TEM. TSH and Tg was detected in sandwich immunoassay format where labeled antibodies bind to the antigen and the antibody-antigen pairs are captured by the immobilized antibodies. This strategy avoids the need for immobilization of analyte on the solid support which may require coupling of analyte to a large molecule or different surface chemistry for immobilization. Some reports show the development of MAIA for simultaneous detection of small and large molecules combining both competitive and sandwich immunoassays on one platform either by immobilizing anti-idiotypic antibodies or modified antigen on chip [105, 170]. Here, we report the simultaneous detection of analytes varying widely in their molecular weights (a key challenge faced during development of MAIA) in human serum using labeled antigen to compete with unlabeled antigen using immobilized capture antibody.

In MAIA, it is more difficult to optimize the assay range for all the analytes, because of the fixed sample volume that has to be used. Thus, the large sample volume can improve the detection limit of one analyte but restrict the dynamic range of other analytes that are present in serum at higher concentrations. Using a 50 µl of sample volume, we could achieve sensitive as well as biologically targeted assays for all the three analytes, where T4 is present at much higher concentration (4.5-12.5 µg/dl, ~ 0.1 micromolar) as compared to TSH (0.2-4 µIU/ml, ~ 0.3 femto molar) and Tg (1.5 – 38 ng/ml, ~ 3 nanomolar).

During the development of MAIA, issues commonly faced for the development of any immunoassay, viz., matrix for standard preparation, cross-reactivity and choice of detection system were investigated. Existing guidelines for pharmacokinetic immunoassays recommend preparing standard curve for analyte measurement in the matrix of the same origin as the analyzed samples [143]. This approach is quite often problematic during MAIA

development because the presence of endogenous molecules of interest, which may interfere with measurements. In addition, other endogenous molecules such as soluble receptors and heterophilic antibodies bind to the assay reagents leading to false positive or false negative results [171]. To avoid endogenous molecule interference, commercially available ELISA kits use analyte of interest free buffers for standard curve preparation. We have prepared the combined standards in SHFS - made up of immunoassay grade BSA and bovine immunoglobulin for T4 and TSH panel, and for T4, TSH and Tg panel, since human HFS, free of required analytes, was not available. Standards for TSH and Tg panel was prepared in human HFS, which was easy to obtain. In order to explore the matrix effect, linearity and recovery tests were done. Observed/Expected ratio, for both recovery and linearity studies, for all analyte panels were within the expected range of 100±20%.

Multiplex assays, by their very nature, involve interactions between multiple antibodies, and different analytes in the sample. Hence, one cannot assume that a multiplex assay is a sum of several uniplex assays. Therefore, QC parameters, like cross reactivity, for multiplex assays have to be looked for critically. Non-reactivity to all other antibodies must first be established. Multiplexing requires testing for cross-reactivity between different antibodies and antigen targets. Although in theory, unlimited number of analytes can be assayed by MAIA, several practical problems exist. For MAIA to be successful, the capture antibodies must be very specific, otherwise cross-reacting moieties will be immobilized leading to negative or positive errors. This problem is more pronounced as compared to single analyte assays since more than one detection antibody is present in reaction mixture. Thus, cross-reactivity is a critical analytical parameter regarding specificity and reliability of MAIA.

Generally, antibodies produced against T4 can show cross-reactivity to T3 because of structural similarity of T4 and T3. The T4 antibodies used in MAIA showed 10% cross-reactivity to T3. However, as the concentration of T3 present ordinarily in the serum is ~50 times less than T4 and, hence, this cross-reactivity is of no consequence in MAIA. The specificity of an immunoassay depends on the ability of the antibody reagent to discriminate flawlessly between the analyte and structurally related ligands. TSH antibodies are more likely to be affected by such cross-reactivity problems than thyroid hormones where chemically pure iodothyronine preparations are available for selecting antibody specificity. The anti-TSH monoclonal antibody used in MAIA was very specific, with no detectable cross-reaction to FSH, LH, hCG, GH and prolactin. Cameline anti-Tg antibodies did not show any cross-reactivity to T4. We have not seen any cross-reactivity among the detection antibodies when reacted with individual tracer.

The effects of interfering substances (hemoglobin, chylomicrons) have not been specifically looked for in the MAIA studied here. Hemolysis, lipemia, and hyperbilirubinemia do not produce significant interference in RIA/IRMA in general [172]. The inherent properties of solid-phase antibodies, micro-sample volumes, and adequate washing ensure that the problems caused by these interfering substances are negligible.

With regard to the choice of detection system, although fluorescence and chemiluminescence are most common means of detection for MAIA, we have used isotopic labels for our experiments. Not all the supports are compatible with fluorescence detection strategies due to inherent autofluorescence of the material. Autofluorescence can cause significant reduction in SNR. Nitrocellulose-coated slides cause light scatter and higher background as compared to glass with laser scanner detection methods [173], limiting the use of nitrocellulose support for fluorescent detection methods. PC-TEM used in our study showed autofluorescence higher

than glass. In addition to autofluorescence of the support, the sample may have components that interfere with a selected fluorophore. Examples are flavins and flavoproteins found in liver and kidney tissue which autofluorescence and emit light in the same region as fluorescein, limiting fluorescein use in samples rich in flavoproteins [117]. However, no interference from any extraneous substance in serum is possible in radioactive detection and the amount of radioactivity used in the assay is too small to cause any significant radiation hazard. Although, fluorescence and chemiluminescence are shown to provide higher sensitivity compared to radioactivity, sensitivity of the developed MAIA is not compromised by using radiotracer. All the assays for all analyte panels are clearly sensitive enough to cover the diagnostic cut-off levels. The assays with radioisotope are as sensitive as with non-isotopic labels, though the counting times are higher due to the long half-life of ^{125}I. In case of chemiluminescence based competitive assay for small analytes, enzyme is used for labeling the small analyte. Enzymes are relatively larger proteins and when they are used to label the small analyte, the labeled analyte may lose its reactivity to be captured by antibodies. Presence of very small amount of chemicals can inhibit the enzyme action in chemiluminescence based MAIA whereas radioactive measurement is not influenced by any extraneous material. In addition, radiotracers have convenient and precise end-point detection, because of the long half-life of ^{125}I (60 days). Further, colour from enzyme labels and light from chemiluminescent labels fade soon and cannot be re-measured in case of error. Radioactive measurement provides several advantages over fluorescence for MAIA including no interference of signal generation due to autofluorescence of support or dust and no photo-bleaching of fluorescent probes.

Each panel of MAIA developed was finally validated with real samples. Immunoassays have been widely used for protein quantification in a sample and are often treated as a reference

standard when investigating the performance of MAIA [44, 119, 174, 175]. The MAIA developed and validated in this thesis was also compared with RIA/IRMA. Correlation coefficients between the two techniques were calculated for each analyte in the serum samples assayed. Very significant correlation coefficients were obtained for all the developed MAIA panels. Our validation study demonstrated that MAIA is both quantitative and reproducible at assay working ranges covering the relevant physiological concentrations of the analytes, and assay precision less than 20%.

Immunoassay allows for the analysis of one analyte per assay, thus requiring more than one assay for multiple analytes. This increases the cost per test and sample volume when compared to a MAIA approach. This is critical in samples that may come from infants, small experimental animals, biopsy samples or very sick individuals. The developed MAIA is economical allowing the quantitative measurement of three analytes using only 50 µl of sample, whereas, 500 µl will be required if all the three analytes were estimated, in duplicates, using RIA/IRMA as per protocol described in Chapter 3 (Section 3.3.1). Moreover, there will be considerable saving in assysist time as current MAIA assay can be completed in about one-third of the time required for the completion of three separate RIA/IRMA assays. For example, each of the RIA/IRMA will require 2.5 hours of technician time including pipetting of reagents, washing of tubes, cpm measurements and data reduction – so a total of 7.5 hours of technician time is required for three assays. MAIA will also lead to work simplification as there are reduced numbers of washing and pipetting steps and will be less error prone as the risk of error is directly proportional to number of assay steps performed. Further, MAIAs are designed for automation and, hence, not labour intensive.

Although MAIA is a powerful research tool, one major disadvantage is the high cost for its development. However, the cost of a commercial systems range from 25,000 to 220,000 USD [176], which is similar to that for a commercial immunoassay analyser used in large clinical chemistry laboratories. Until recently, MAIA has been developed exclusively with fluorescence. The level of training and expense of required sophisticated laser scanners limited their application in routine clinical diagnostics. The colorimetric and chemiluminescent detection has been applied to antibody microarrays as a low-cost alternative [177]. However, the sensitivity remains high compared to fluorescence. This has expanded the number of clinical diagnostic applications and dramatically increased the number of potential service providers eligible to afford and offer testing services utilizing this technology. We developed simple, low-cost and sensitive MAIA using radioisotopes suitable for quantitative detection of three analytes.

6.2. Conclusion

In summary, we had developed and demonstrated an antibody-chip for performing MAIA for T4, TSH and Tg, the most useful panel for the detection of thyroid disorders. The MAIA uses PC-TEM as a novel solid support. Here we present a simple method for fabrication of antibody-chip on microporous PC-TEM based on single step activation method using glutaraldehyde. Being microporous, TEM provides high immobilization capacity and consequently high sensitivity of the assay. Radioisotope was used for the detection and quantification of the signal from the spots and was found to provide high sensitivity to cover the diagnostic cut-off levels of all the three analytes. By combining the high antibody loading capacity of PC-TEM with the sensitivity of radioactivity detection we could develop a MAIA, and as proof of the concept showed the convenient detection of T4, TSH and Tg

simultaneously with high sensitivity. It should be noted that the three analytes vary widely in their molecular weight and concentrations in serum. MAIA also shows acceptable accuracy and reproducibility, which make it a promising approach to the clinical laboratory diagnosis and detection of thyroid disorders.

We have demonstrated development of MAIA, where all steps have been performed manually using radio-isotopes. However, sensitivity and precision of the MAIA can be increased further by automation of anti-body chip production, assay steps and quantification methods and by using non-isotopic detection methods to match the precision and accuracy obtained using current single analyte third generation immunoassays. Non-isotopic tracers that can give a higher ratio of 'counts/number of labeled molecules used' without environmental interference and spurious counts is definitely desirable and will help in the automation of MAIA for laboratory and commercial use.

In conclusion, MAIA has been developed that compares favorably with the standard RIA/IRMA assay in regards to sensitivity and specificity.

6.3. Future outlook

In this section, some recommended future work is included in order to further intensify and utilize the advantages of MAIA.

6.3.1. Combination of more analytes in panels

We have developed MAIA for simultaneous estimation of T4, TSH and Tg. Since there is no single pathological model that can truly reveal the situation of a patient referred to a laboratory, more the analytes investigated for diagnosis or prognosis of a disease, more the

information that can be provided to the clinicians. For example, a most serious technical problem that limits the clinical value of Tg determinations is interference that is caused by endogenous Tg antibodies (TgAb). These are seen in autoimmune thyroid diseases including Grave's and Hashimoto's thyroiditis. TgAb is more common in patients with sporadic goiter, multinodular goiter and DTC patients than in the general population [178]. The extent and type of interference that is caused by these autoantibodies depends on the specific Tg assay method that is used by the clinical laboratory. Underestimation of the total Tg concentration is characteristic of noncompetitive immunometric assays (IMA), and overestimation of Tg is typical of most competitive immunoassays that are capable of measuring free and TgAb-bound Tg, although underestimation may also be observed [178]. Hence, in TgAb positive sera while IMA methods generally underestimate the Tg value, RIA methods could yield either a false high or false low values. A false negative result may cause a delay in detecting and treating recurrent or metastatic disease. A false positive result can lead to further clinical studies or therapy and unnecessary patient anxiety.

Exogenous Tg recovery studies have been used in an attempt to identify TgAb interference. Serum can be prescreened for TgAb and the use of faster IMA methods can be restricted to specimens without TgAb. Therefore, it would be more preferable for any panel including Tg to include test for TgAb to further strengthen the test accuracy for Tg.

Tests for antibodies against other thyroid-specific antigens such as TPO (TPOAb) and TSH receptors (TRAb) are used in the diagnosis of autoimmune thyroid disorders. Autoantibodies against Tg are encountered in autoimmune thyroid conditions, usually in association with TPOAb [6].An autoimmune panel for testing antibodies against thyroid specific antigen can be formed.

A T3 assay can be added to T4 and TSH panel to detect T3 thryotoxicosis. However, T3 is the least useful analyte for assessing thyroid function as it has a short biological half-life of just one day, and between 30 - 50 % of T3 is from peripheral de-iodination of T4. Further, except in T3-toxicosis and acute non-thyroid illness, there is a very good correlation between T4 and T3. This makes the measurement of T3 unnecessary. The T4 component of MAIA can be easily converted to fT4 one by choosing an antibody with very high affinity and avoiding ANS in the tracer. A panel consisting of fT4, TSH and Tg can be considered as high frequency of binding protein abnormalities are encountered in clinical practice, especially the high TBG state of pregnancy. Since fT4 is responsible for biologic activity at the cellular level [17], its measurements will reflect the physiological effects of thyroid hormones better than total hormone concentrations when binding proteins are abnormal [17].

Among the thyroid hormone binding proteins, TBG binds ~70% of T4 and ~80% of T3 in circulation. Hence, TBG assay can be added to T4 and TSH panel to identify binding protein abnormalities under various conditions (pregnancy or estrogen therapy and genetic abnormalities in TBG).

6.3.1.1. Detection of other category of analytes

Along with thyroid disorders, MAIA can be used for evaluation of organ function in myocardial, renal, brain and endocrine disorders. For example, MAIA can be developed to quantify serum levels of Creatine-Kinase muscle brain, heart type fatty acid binding protein, myoglobin and troponin I to assist with evaluation of chest pain. Albumin, B2-microglobulin, clusterin, cystatin C, osteopontin can be combined in one assay for detection of kidney

toxicity. MAIA for estradiol, follicle stimulating hormone, luteinising hormone, progesterone, prolactin, and testosterone will be useful detection for fertility disorders.

Other applications of MAIA includes the detection of antibodies directed against antigens implicated in allergy [47, 48], autoimmune disease [50, 179], and parasitic/viral infections [44]. In the serodiagnosis of autoimmune and infectious diseases, IgG and IgM immunoglobulins are the antibody responses determined in patient sera. Determination of these circulating antibodies is made directly, using labeled anti-IgG and anti-IgM antibodies. Allergy is a state of immune dysregulation that results in an overproduction of IgE [180]. Identification of the causative agents that provoke allergies in individuals is a challenging task owing to the large numbers of such agents. Immunoassays do not address the full spectrum of allergens because of the quantities of blood required and the expense of the kits. MAIA the possibility of determining and monitoring the IgE reactivity profile of allergy sufferers to very large numbers of disease-causing allergens simultaneously and use minute volumes of serum.

Another application of MAIA is determine the concentration of tumor markers. Common tumor markers include IL-6, IL-8, AFP, CA125, CA15-3, CA19-9, CEA, Leptin, Osteopontin, Prolactin, PSA (free), PSA (total), TRAIL.

Cytokines are small to medium-sized proteins and glycoproteins that play important roles in cell-to-cell communication and immunoregulation; they have been implicated in inflammatory, neoplastic, and infectious disease processes [181]. They form intricate regulatory and homeostatic networks that serve to regulate production of each other and to modulate a multitude of cellular functions. Deeper understanding of the normal operation of these networks in health and disease is needed to unravel how cytokines mediate their diverse effects on biological systems. Such knowledge will also be important for developing and

understanding the action of therapeutic agents. For these reasons, MAIA for cytokines expressed within biological fluids is of greater value for research in various disease states than analysis of a single cytokine.

6.3.2. Automation

Although in this thesis work, antibody-chip was produced by manual spotting of antibodies but manual spotting is a labor-intensive and error prone process. A robotic arrayer can provide minute and more uniform spots resulting in good quality, repeatable and accurate spotting which in turn facilitates the subsequent evaluation of data. In this context, we have developed an antibody-chip arrayer in collaboration with instrumentation and automation experts at Division of Remote Handling and Robotics (DRHR), BARC. Antibody-chip arrayer is a robotic system which picks up given solutions from a source plate and deposits them at specified locations on a support in small quantities (nl to pl). The developed arrayer is a non-contact type arrayer that uses piezoelectrically driven print head having 50 micron nozzle diameter to deposit antibody solution on thin microporous TEM. The system is capable of depositing thousands of extremely small droplets of antibodies, one droplet at a time, with each droplet containing a different antibody. The important considerations while designing this precise 3-axes robotic system are high speed, micron level accuracy and minimum vibration.

For holding TEM, a novel antibody-chip cassette was designed and developed in which PC-TEM is mounted by auto-stretching technology. It provides uniform surface without any wrinkle. Each cassette has two 20 mm diameter and 10 micron thick PC TEM. The thin TEM in the cassette allows accessibility to different reagents during assay procedure and provides

easy and efficient washing method by using vacuum. A multi-plate shaker to improve the reaction kinetics has been designed and developed. The shaker is attached to a vacuum pump to allow easy washing of the chip.

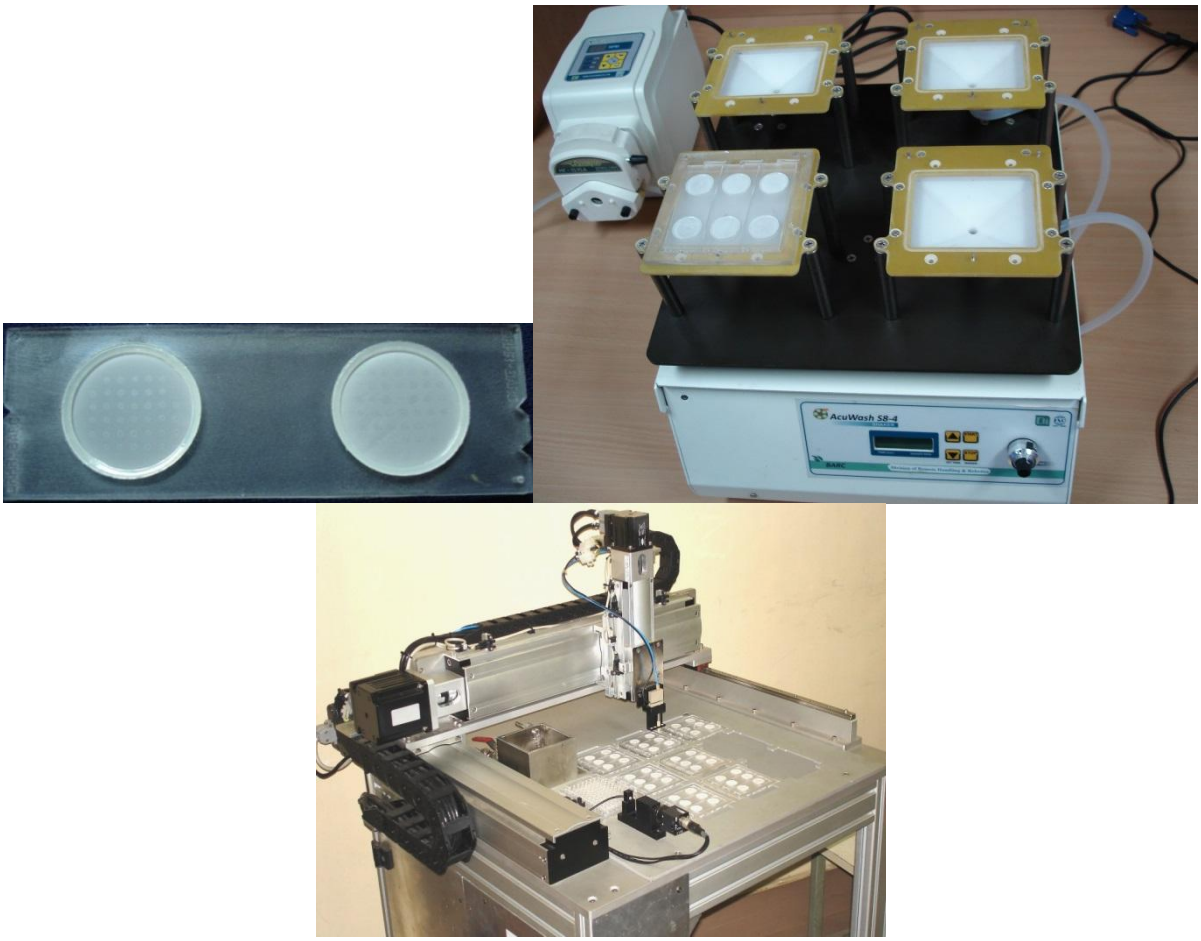


Figure 6.1 Antibody-chip Cassette, multiplate shaker and arrayer with imaging System

Arrayer Specifications	
Positional accuracy	10 microns
Dispensing volume	50pl-10µl
Cassette size	3” x 1” x 0.04”
Cassette format	Cassette containing membrane , 2D glass slide
Well plate	96/384 wells
Drives	Servomotors

Table 6.1 Specification of developed antibody-chip arrayer

Arrayer Features

- Piezoelectric based pin head for non-contact spotting of solution on the porous TEM.
- Real time pressure control and electronics circuit for voltage and pulse width for the piezo-dispenser.
- Real time machine vision system to monitor the dispenser performance and quantify the falling protein antibodies parameters.
- Precise x-y-z robotic system to automate and streamline different processes with excellent reproducibility.
- User friendly control software.
- Membrane based biochip cassette similar to standard glass slide.

Our future plan is automate the entire procedure. Arrayer will be used for spotting of the antibodies on TEM held in antibody-chip cassette for making antibody-chip. Assay will be carried out on a multiplate shaker to improve the reaction kinetics. The shaker is attached to a vacuum pump to allow easy washing of the chip. After the assay, the slides can be imaged

using PhosphorImager for isotopic detection or standard microarray scanner for fluorescence or CCD camera for chemiluminescence based detection system.

Appendix-I

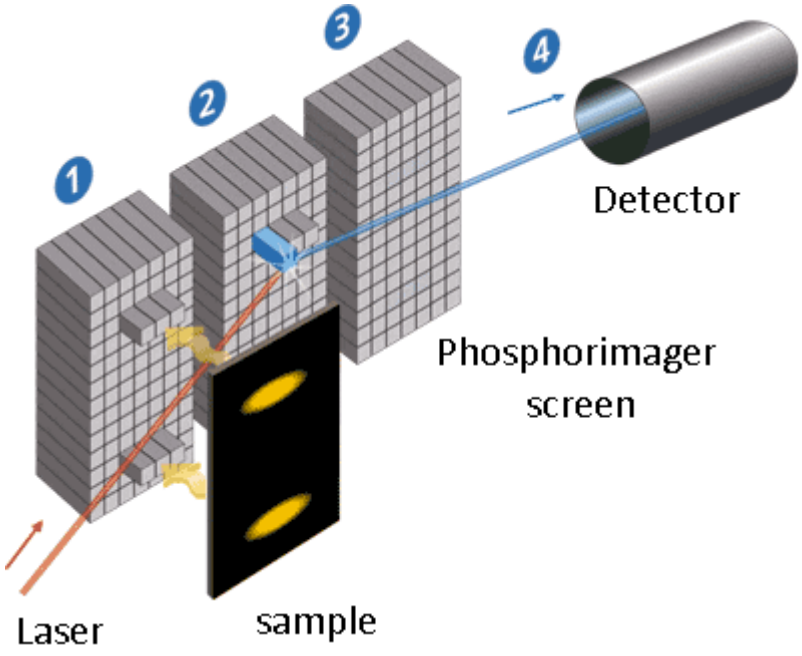
Principles of Autoradiography and Phosphor-imaging used in Chapter-3

Autoradiography is a procedure for localizing and recording a radiolabeled compound within a solid sample, which involves the production of an image on a photographic emulsion. The photographic emulsions consist of silver halide crystals in suspension in a clear gelatinous phase. Following passage through the emulsion of a β-particle / γ-ray emitted by a radionuclide, the Ag^{+} ions are converted to Ag atoms. The resulting latent image can then be converted to a visible image once the image is developed, an amplification process in which entire silver halide crystals are reduced to give metallic silver. The fixing process results in removal of any unexposed silver halide crystals, giving an autoradiographic image which provides a two-dimensional representation of the distribution of the radiolabel in the original samples having different optical densities or shades of gray. The darkness or density of the film increases as the exposure is increased. An image contains areas with different densities that are viewed as various shades of gray. The optical density of film is assigned numerical values related to the amount of light that penetrates the film.

Phosphors are compounds that absorb energy at one wavelength and reemit at another. Storage phosphor release stored energy only when stimulated by light of particular wavelength. Phosphor screen is made up of fine crystals of $BaFBr:Eu^{+2}$.When phosphor screen placed against radioactive sample –high energy radiation from sample excites Eu^{+2} electrons – move freely and become trapped in "F –centres" , empty sites in $BaFBr^{-}$ crystals. During this process, Eu^{2+} oxidized to Eu^{3+} and remains oxidized even when screen is removed. Phosphor stores the energy from ionizing radiation. Excited $BaFBr^{-}$ complexes absorb light in 600 nm range. In phosphorImager, a He-Ne laser emitting red light at 633 nm

is used to scan the screen. When the energy from the laser is absorbed, electrons are again freed and reduce Eu^{+3} to Eu^{+2*}. As Eu^{+2*} returns to ground state, the released energy is emitted as blue light, which is collected and converted to an electrical signal by a photomultiplier .The electrical signal is digitized to permit image display and analysis. Light is emitted from the storage phosphor screen in proportion to the amount of radioactivity in the sample upon laser-induced stimulation. The image obtained is a false color image, where the different colors represent different amount of radioactivity, from the lowest (yellow) to the highest (black).This technique has several advantages over standard autoradiography, but the most important is that it is much more accurate in quantifying the amount of radioactivity in a substance. This is because its response to radioactivity is far more linear than that of an X-ray film. With standard autoradiography, a band with 50,000 radioactive disintegrations per minute (dpm) may look no darker than one with 10,000 dpm because the emulsion in the film is already saturated at 10,000 dpm. But the PhosphorImager collects radioactive emissions and analyzes them electronically, so the difference between 10,000 dpm and 50,000 dpm would be obvious**.** Moreover, storage phosphor screen exposure takes 10% of the time required for an equivalent exposure to conventional film.

(a)



(b)

Figure AI. 1 (a) Typhoon trio^{+} variable mode imager; (b) working principle of PhophorImager;
http://www.mchem.btinternet.co.uk/SciImage/gel_blot_storm_storage_phosphor.htm

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