CHAPTER 4
METHODS AND MATERIAL

The study was further pursued with respect to the broad and specific objectives and creating a study design for all four phases of the present study. Methods and material were specifically and carefully chosen to optimize the data collection process. We made our sincere efforts to accurately collect and analyze study data.

Under this chapter all methodology and experimental design (Consort) that were used are mentioned and discussed in details to accomplish the objectives of the study divided in four phases.

STUDY FUNDING:

This study was partially funded by Department of Biotechnology (DBT), Government of India, Ministry of Science and Technology, Department of Biotechnology, Block 2, 6-8th Floors, CGO Complex, Lodi Road, New Delhi – 110003.

STAUTORY CLEARANCE FROM ETHICAL COMMITTEE:

Before practically implementing the study, it was submitted and presented before the ethical committee for approval.

Approval was obtained from the Institutional Ethics Committee for Human Research (IECHR) of Department of Foods and Nutrition, Faculty of Family and Community Sciences, The M.S. University of Baroda and its reference number for medical ethics approval is **# IECHR/2012/13.**

Further, this study was also submitted to Clinical Trial Registry of India (CTRI) for approval and was registered under reference number **# CTRI/2018/03/012514.**

Since there was no drug involved in this trail, approval from Drug Controller General of India (DCGI) was not needed.

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PHASE I – SNAP-SHOTING THE PRESENCE OF OBESITY IN YOUNG BANKS EMPLOYEES OF URBAN VADODARA

4.1.1: Sample size calculation for finite population (Freedman, 1997)

Present study was conducted on private bank employees and hence the population of private bank employees is finite. To calculate the sample size for the screening phase, formula for finite population was used. The sample size estimates were based upon two sided confidence level of 95%, confidence interval of 4 and a power of 90% Sample size was calculated using formula for finite population, where the population is less than 50,000. This formula was selected as the population of bank employees working in private sector banks in Vadodara city is limited to 1500 employees.

Sample Size – Infinite Population (Where the population is more than 50,000)

$$SS = \frac{Z2 \times [P] \times [1- P]}{C2}$$

Where, SS = Sample size

Z = Z value [e.g. 1.96 for 95 % Confidence level]

P = Percentage of population picking a choice, expressed as decimal

C = Confidence Interval expressed as decimal

Sample Size – Finite Population (where the population is less than 50,000)

$$\text{New SS} = \frac{SS}{[\,1 + \frac{(SS - 1)}{Pop}]}$$

Where, SS = Sample size calculated using infinite population

Pop = Population (1500 for private bank employees of Vadodara city)

Using the formula for infinite population a figure of 600 subjects was arrived. This figure of 600 was put in the formula of finite population and the minimum sample size required

for screening of bank employees was calculated to be 428 subjects for screening. However, as bank employees were interested in basic health screening, total of 650 employees participated in our health check-up camp and screening data was collected and analyzed for the same (650 employees).

4.1.2: Selection of banks

Website of Indian Banks Association provides list of all categories of banks in India. On hovering through the tabs on website searching for the list of private banks in city of Vadodara we found total 24 banks. Out of listed 24 private banks 18 banks existed in Vadodara city. Out of these 18 private banks, six banks were conveniently selected based on the permission obtained. These banks had around 20 -30 branches in all different zones and areas of Vadodara city. Senior professionals of bank like Cluster head and Regional head were approached for seeking permission to organize health screening camp. Based on the centralized permission obtained from cluster heads, permission was also sought individually from the concerned branch manager for their convenient date and time with a purpose of smooth functioning of the daily banking procedures and importantly to obtain maximum participation of the bank employees in health screening camp.

Screening was performed on all bank employees irrespective of their age and gender. Total six hundred and fifty (650) bank employees participated in health screening and were assessed for their anthropometric measurements, body fat percentage, basal metabolic rate (BMR), blood pressure values and random blood sugar. In the present study, all anthropometric measurements were made using the guidelines adopted at the NIH sponsored Arlie Conference (Lohman et al., 1988)

4.1.3: Statistical Analysis

The data was entered into Microsoft excel spread sheets and then subjected to appropriate statistical analysis. Frequency and percentages were calculated for background information. Microsoft Excel data analysis package was used to express results as mean

and standard deviation. Paired t test was used to calculate the difference between the means. ANOVA was performed to determine the significant differences in the mean scores. Percent increase and decrease was also determined by calculations to compare. Student "t" test of unequal variance was used to observe the difference that exists between the values. The significance levels were set at 5% by two sided tests. Student "t" test was performed for the comparison between two groups. Epi-info 7, Version 7.2.0.1 was used to calculate chi-square values, odds ratio, relative risk and absolute risk reduction. Pearson's correlation was used to determine the relevant association between the parameters. Statistical Package for Social Sciences (SPSS 21.0v) software was used for obtaining results for Regression analysis.

PHASE II– COMPARISON BETWEEN BASELINE PARAMETERS OF NON-OBESE AND OBESE BANK EMPLOYEES WITH REGARDS TO:

4.2.1: Selection and distribution of bank employees in Non-obese and Obese group

Out of 650 subjects screened, 150 Non-obese (BMI 18.5–22.9kg/m2) and 150 obese subjects (BMI 25-30 kg/m2) between the ages of 25 – 35 year and middle income group were voluntarily recruited from the private banks of urban Vadodara. All subjects had a stable body weight for ≥ 3mo before the study. Subjects who had clinically significant cardiovascular abnormalities, liver or pancreatic disease, diabetes, major gastrointestinal surgeries, were pregnant or lactating, exhibited alcohol or drug dependence, were on drugs influencing appetite, were following a diet or exercise regimen designed for weight loss, or chronically used antacids or bulk laxatives were excluded from study. All subjects completed a health and lifestyle questionnaire to determine eligibility. Subjects were encouraged to maintain their regular lifestyle, and not to consciously try to gain or lose weight throughout the study. Verbal and written informed consent was obtained from each one of the subjects who participated in this study (Appendix I).

4.2.2: Inclusion and Exclusion criteria for subject selection

Inclusion Criteria	Exclusion Criteria
• BMI (kg/m$^{2)}$ • Normal :18.5 – 22.9 • Obese : 25 - 30 • Age 25 - 35 years old • Middle income group • Willing to participate with signed written informed consent	• Hypertension • Diabetes mellitus • Cardiovascular Disorder • Thyroid Hormone Disorder • Valve Replacement Surgery • Gastric surgery or Perforation • Renal Disorder • Cancer / AIDS • Psychological disorder • Heavy physical activity • Weight loss regime • Subjects Consuming Alcohol for analysis of fasting plasma Gut - hormones

Figure 4.1: Inclusion – Exclusion criteria for selection of Non-obese and Obese bank employees

Employees were inquired about their weight pattern whether their weight was stable in last 3 months or were doing any efforts to reduce weight like following a diet or exercise schedule designed for weight loss. Information was also taken with regards to their personal medical history to assess their eligibility to participate in our study. Employees who confirmed having conditions or medical disorders as mentioned in exclusion list mentioned in Figure 4.1 were not eligible to take part in our study and hence were excluded. This information was taken on a health and lifestyle questionnaire from all employees to determine their eligibility. Also employees who were eligible to get enrolled in our study were encouraged to maintain their regular lifestyle, and not to consciously try to gain or lose weight throughout the study.

4.2.3: Study protocol and experimental design

Six hundred and fifty (650) bank employees who were screened in Phase I were further segregated and classified under the various categories of BMI after their data was analyzed.

Two BMI categories of our study interest namely Normal BMI and Obese Grade –I were purposively selected in view of our outlined objectives for phase –II. Out of 650 screened, there were Normal BMI: N=209 and Obese Grade-I: N=222 bank employees. Out of these 150 bank employees from Normal BMI and 150 bank employees from Obese Grade-I, without any medical or specified conditions as mentioned in inclusion and exclusion criteria were selected purposively for comparative phase II.

Comparative baseline data with information regarding socio-economic profile, general habits, dietary intake, physical activity pattern, persona medical history and family history was collected using pretested semi structured questionnaire (Appendix II). Subjects were screened for various parameters in terms of anthropometric, dietary, biophysical, biochemical and microbial parameters. Physical activity pattern was measured using WHO-Global physical activity questionnaire-2. Dietary analysis was done using 24hr dietary recall and food frequency questionnaire.

Prolegomenon assessment of six Gut satietogenic hormones Glucagon-like peptide (GLP-1), Glucose dependent-Insulinotropic Peptide (GIP), Peptide YY (PYY), Leptin, Ghrelin and Insulin] and Gut microbial profile in terms of *Lactobacilli, Bifidobacteria, Clostridium* and *Bacteriodes* were assessed for bank employees.

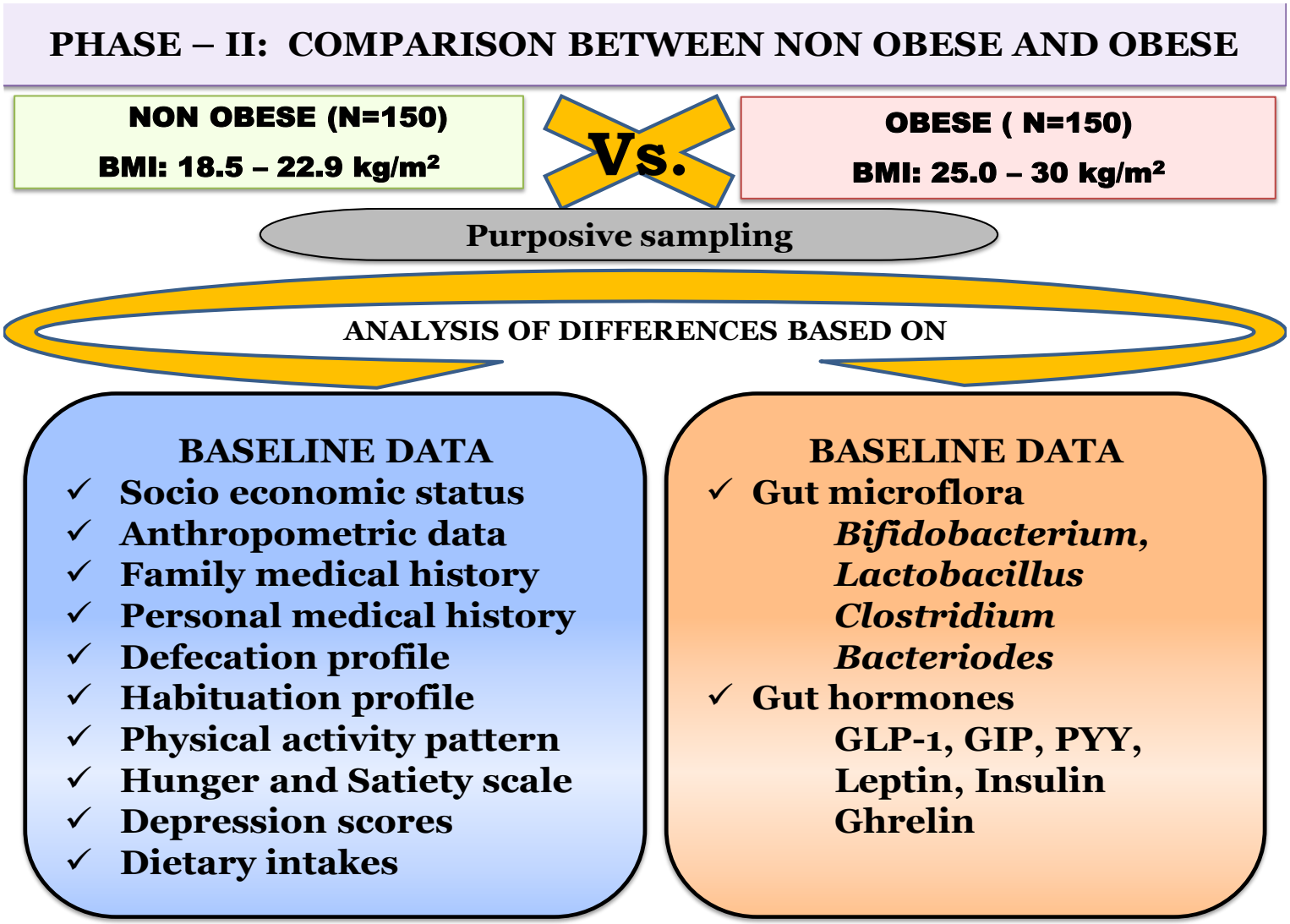


Figure 4.2: Experimental design for comparison between non-obese and obese bank employees

4.2.4: Interview based questionnaires

The socioeconomic status (SES)

The key to understand affordability in community-based studies focuses on socio-economic stratification with regards to use health services, amenities, and purchasing ability. To analyze the value system as expected for a particular level of education and occupation, total composite score and summation of data in SES of education, occupation and income is considered. It yields a score range of 3 – 29 and categorizes study participants in high, middle and low categories of SES. Also, it is a set norm that standard of living is directly proportional to income earned. Kuppuswamy's SES tool, proposed in 1976, is well established tool to determine socio-economic status and is also valuable for use in hospital and community-based research in India.

General information with regards to age sex, religion, educational level, marital status, income etc was collected from the subjects using the Kuppuswamy's socioeconomic status scale, 2010 (Bairwa, Rajput & Sachdeva, 2012; Singh, Sharma & Nagesh, 2017). (Appendix III).

Table 4.1: Score Cutoffs for Socio Economic Status

Scores	Codes	Socioeconomic class	Tick(√)
26-29	**A10CI**	**Upper (I)**	
16-25	**A10CII**	**Upper Middle (II)**	
11-15	**A10CIII**	**Lower middle (III)**	
5-10	**A10CIV**	**Upper lower (IV)**	
<5	**A10CV**	**Lower (V)**	

Defecation profile

Defecation is one of the regular human bodily functions that is rarely studied and rarely understood. Stephan (2011) has given five major criteria with specific signs and symptoms that might form the basis for a positive and more objective definition of gut health. The first and foremost criteria is effective digestion and absorption of food and the specific signs are a) normal nutritional status and effective absorption of food, water and minerals; b) regular bowel movement, normal transit time and no abdominal pain; c) normal stool consistency and rare nausea, vomiting, diarrhoea, constipation and bloating. Defecation pattern of the subjects was studied using a score card developed by Tokunaga et al. (1993). Before getting the score card filled the subjects were asked questions like: (1) 'Do you have constipation?' (2) 'How many times do you defecate in a day and /or week?' (3) Do you look at the stool before flushing it away?' The list of seven stool types (Table 4.2) that is, the Bristol stool form scale (Figure 4.3) was shown to subjects to identify the stool type.

To reduce embarrassment the researcher first explained the subjects by saying that "The stools that are passed vary quite a lot on daily basis and there are seven types which are commonly passed; I want you to look at this list and see if it includes the type of stool you usually pass'. Then after a suitable pause, researcher said 'Does this list include the type of stool you usually pass?' The researcher then asked 'Are your stools generally of the same type or so they vary?' followed by 'What is the type number of your usual stool?'

After this interview subjects were asked to rate the score card (Table 4.3) regarding their defecation profile. Information was collected using Bristol stool chart and defecation scores regarding the various aspects like frequency, quantity, odour, colour, hardness, and feeling after defecation (Oku & Nakamura, 2017, Stephan, 2011, Tokunaga et al., 1993).

Table 4.2: List of seven types of stool which was shown to each subject (Bristol stool form scale)

Type 1	**Separate hard lumps, like nuts**
Type 2	**Sausage shaped but lumpy**
Type 3	**Like a sausage or snake but with cracks on its surface**
Type 4	**Like a sausage or snake, smooth and soft**
Type 5	**Soft blobs with clear cut edges**
Type 6	**Fluffy pieces with ragged edges, a mushy stool**
Type 7	**Watery stools, no solid pieces**

Table 4.3: Score card for rating defecation pattern

DEFECATION PROFILE	CONDITIONS SCORE					
Constipation	Yes (1)	No (0)				
Frequency (times/day)	1		2		3	>3
Quantity of Stool	Small (1)		Middle (2)			Large (3)
Hardness of stool	Very hard (1)	Hard (2)	Medium (3)	Soft (4)	Muddy (5)	Watery (6)
Color of Stool	Blackish (1)		Middle (2)			Yellowish (3)
Odor of Stool	Strong (1)		Medium (2)			Weak (3)
Feeling after defecation	Bad (1)		Fine (2)			Very fine (3)
Regular use of Laxatives	Yes (1)	No (0)				

Table 4.4 : Score Cut –offs for Type of Defecation profile (Lewis & Heaton, 1997)	
Constipated	**≤ 07**
Normal Defecation	**08 – 13**
Watery Stools	**≥ 14**

Table 4.5 : Score Cut –offs for Defining Degree Of Constipation (Lewis & Heaton, 1997)	
Severe Constipation	**1 – 2**
Moderate Constipation	**3 – 5**
Mild Constipation	**6 – 7**

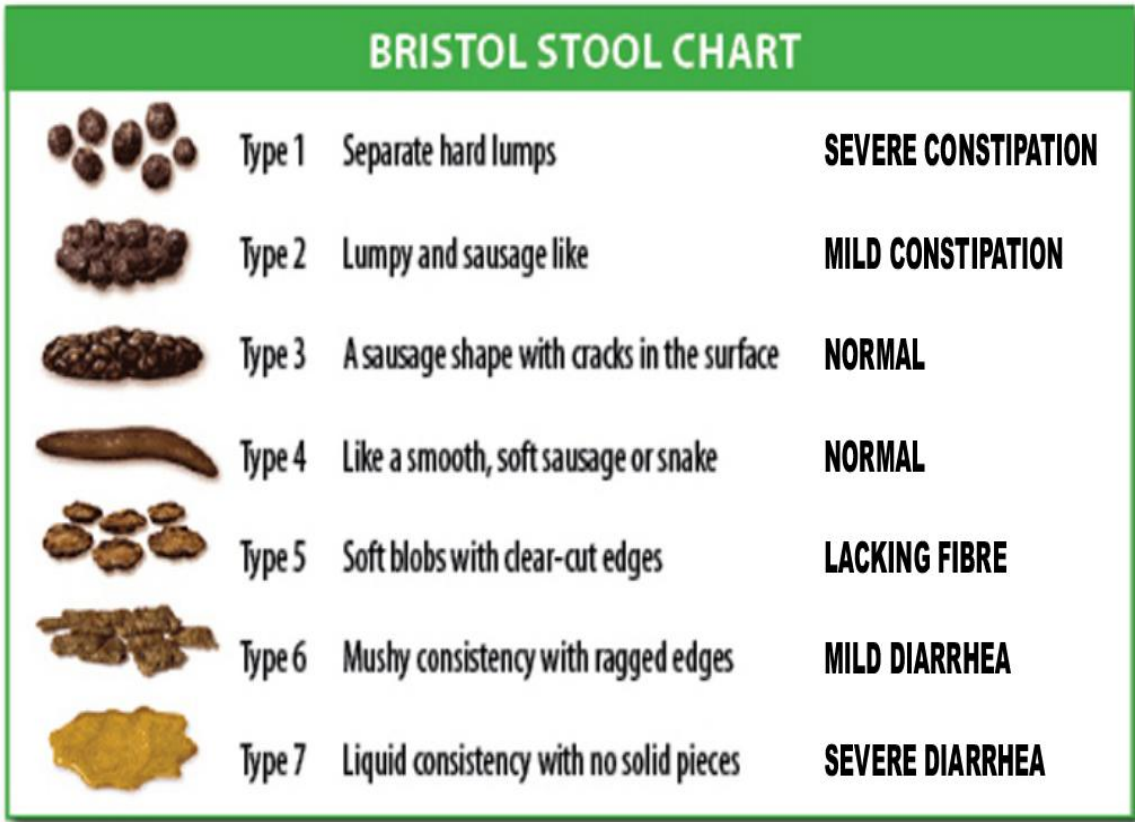


Figure 4.3a: Bristol Stool chart
Source and Developed by: Lewis & Heaton, 1997

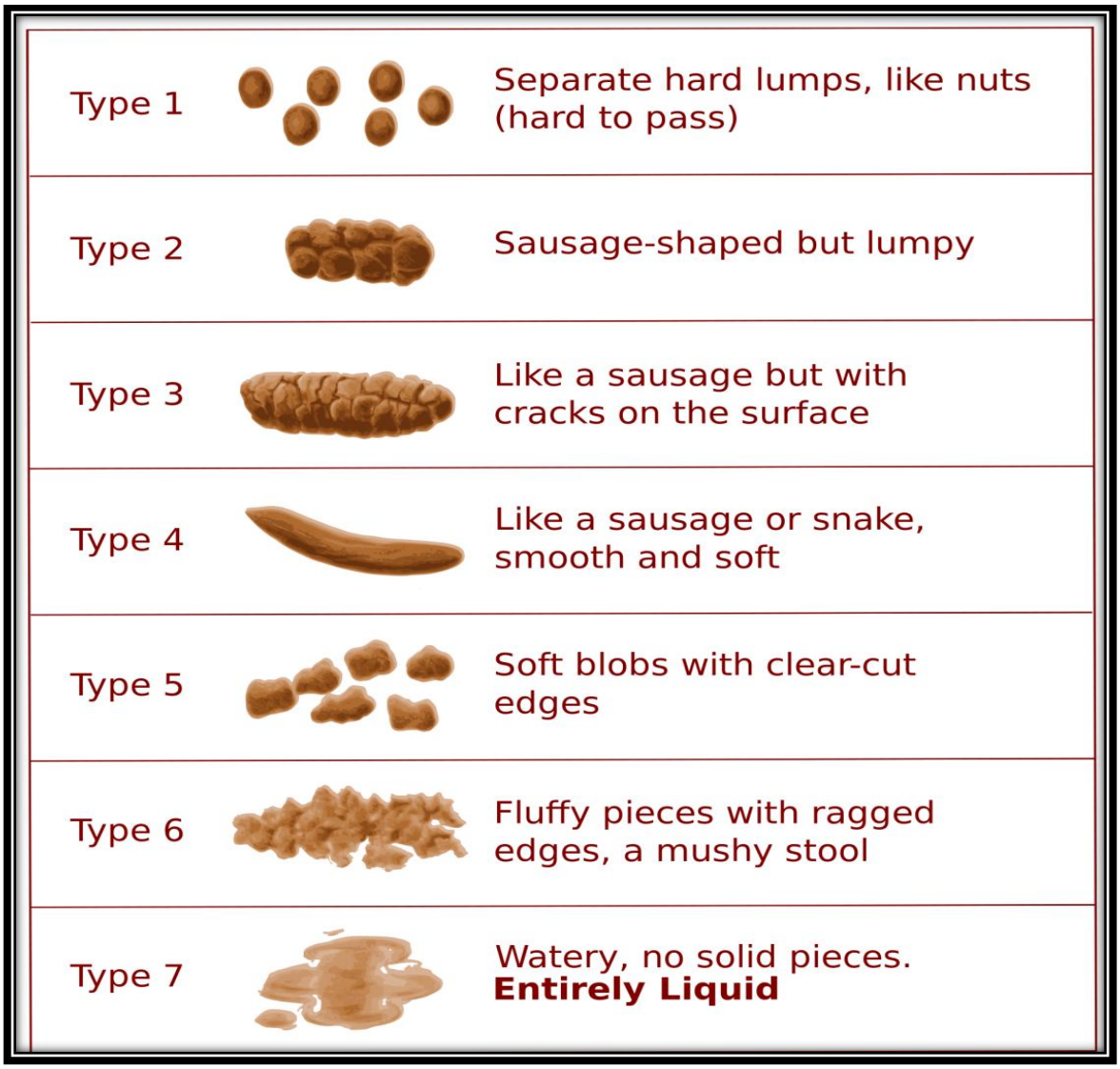


Figure 4.3b: B ristol Stool chart
Source and Developed by: Lewis & Heaton, 1997

General Physical Activity Questionnaire (GPAQ 2 – WHO 2007)

A checklist containing various types of activities along with the time spent to perform each activity was used to assess the activity pattern of the subjects. Responses to specific questions numbers from P1 – P15 given in GPAQ were recorded. Based on the responses given by subjects those responses were put in the formula given at the end of GPAQ questionnaire and the level of physical activity was calculated to be Low, Moderate or High. Physical activity level (PAL) was used as a composite index of physical activity

patterns and was calculated using formula as mentioned in Figure 4.4. Subjects who scored < 600 met min & < 3 days, were sedentary active. 600–1500 met min & 3–5 days was considered as moderately active and >1500 met min & 5–7 days as heavily active (Appendix – IV).

Total physical activity MET-minutes/week (= the sum of the total MET minutes of activity computed for each setting)

Equation: Total Physical Activity = [(P2 * P3 * 8) + (P5 * P6 * 4) + (P8 * P9 * 4) + (P11 * P12 * 8) + (P14 * P15* 4)]

Level of total physical activity	Physical activity cutoff value
High	• IF:(P2 + P11) >= 3 days AND Total physical activity MET minutes per week is >= 1500 **OR** • IF: (P2 + P5 + P8 + P11 + P14) >= 7 days AND total physical activity MET minutes per week is >= 3000
Moderate	• IF: (P2 + P11) >= 3 days AND ((P2 * P3) + (P11 * P12)) >= 60 minutes **OR** • IF: (P5 + P8 + P14) >= 5 days AND ((P5 * P6) + (P8 * P9) + (P14 * P15)>= 150 minutes **OR** • IF: (P2 + P5 + P8 + P11 + P14)>= 5 days AND Total physical activity MET minutes per week >= 600
Low	F: the value does not reach the criteria for either high or moderate levels of physical activity

Figure 4.4: Formula to Calculate Level of Total Physical Activity

Table 4.6: Criteria for - Days and Total Physical Activity Met/Min/Week

Physical Activity Level	Scores
Low	**< 600 met min & < 3 days**
Moderate	**600 –1500 met min & 3–5 days**
High	**>1500 met min & 5–7 days**

Hunger and Satiety Scale

A score card was used to rate the degree of hunger and satiety, before and after meals developed by Lisa Burgoon MS, RD, LD, sports nutritionist, sports-well centre, McKinley health centre, university of Illinois at Urbana – champain (1998). Scores are

from 1 – 10, where 1 stands for severe starvation and 10 stands for extreme fullness to level of bursting and pain.

Table 4.7: Hunger and Satiety Scale by Lisa Burgoon (1998)

Scale	Score
Famished, starving	1
Headache, weak, cranky, low energy	2
Want to eat now, stomach growls and feels empty	3
Hungry - but could wait to eat, starting to feel empty but not there yet	4
Not hungry, not full	5
Feeling satisfied, stomach feels full and comfortable	6
Feeling full, definitely don't need more food	7
Uncomfortably full	8
Stuffed, very uncomfortable	9
Bursting, painfully full	10

Table 4.8: Hunger and Satiety Score Card for Bank Employees

Sr. No	Meal time	Hunger	Satiety
1	**Breakfast**		
2	**Lunch**		
3	**Evening**		
4	**Dinner**		
5	**Total score**		
6	**Mean score**		

Depression Status

To understand depression we need to look into two primary components that is cognitive component (mood) and somatic component (sleep and appetite) (Engstrom, 2018).

How to measure Depression ?

One of the most respected and commonly used instruments for measuring depression is Becks Depression Inventory (BDI). BDI consists of 8 cognitive and 13 somatic sub scales for assessment of depression (Engstrom, 2018).

Cognitive sub-scale consists of: (Engstrom, 2018).

- Pessimism
- Past failures
- Guilty feelings
- Punishment feelings
- Self-dislike
- Suicidal thoughts or wishes
- Self-criticalness
- Worthlessness

Somatic sub-scale consists of: (Engstrom, 2018).

- Sadness
- Loss of pleasure
- Crying
- Agitation
- Loss of interest
- Indecisiveness
- Loss of energy
- Change in sleep patterns
- Irritability
- Change in appetite
- Concentration difficulties
- Tiredness and/or fatigue
- Loss of interest in sex.

Data on psychological background of bank employees was obtained using Becks Depression Inventory (BDI) which was included in the questionnaire. The subjects were classified as mild, moderate and severely depressed based on the BDI scores (Engstrom, 2018; Beck et al., 1961) (Appendix V).

Interpreting the beck depression inventory

To evaluate BDI scores we need to add up the all score's marked by employee for each of the twenty-one questions marked on the right-side of each question. The range of score would be from zero to highest total of sixty-three for the whole test. This would mean that employee either circled zero for all question or rated highest score of three for all twenty-one question. Score cut-offs for evaluating depression is mentioned in the table 4.9 below.

Scores	Levels of depression
01-10	These ups and downs are considered normal
11-16	Mild mood disturbance
17-20	**Borderline clinical depression**
21-30	Moderate depression
31-40	Severe depression over
40	Extreme depression

Note: A persistent score of 17 or above indicates medical treatment.

Dietary Recall Nutrient Calculations

A 24-h recall gathered from a group of individuals can be used to characterize the 'usual' (foods that the respondent consumer on a typical day) diet pattern of the population from which they are sampled, since intra-individual variation in diet is unimportant when examining group level dietary patterns. A three day 24-dietary recall was collected before and after the intervention.

This questionnaire (Appendix-VI) was interviewer administered, since it is seen that this method produce better results as the interviewer can probe for forgotten items or common diet patterns. The 24-h dietary recall is used to estimate the food intake of an individual over the period of 24 h, referring to previous day/night. The 24 h dietary recall was done for all the subjects to collect information on the intakes of calories, proteins fat, carbohydrates and fibers. Subjects were asked to provide details of all the major meals consumed throughout the previous day, along-with additional beverages, snacks, sweets, pickles, etc. along with added sugar and salt.

The subject was probed to remember any forgotten item, and it was made sure that no expression of opinion, feelings or suggestions was made could lead to affect respondent's answer. Subjects were shown the set of standardized set of utensils and encouraged to respond to the quantity, number and size of the food item consumed on the basis of the same. An account of raw ingredients used in each of the reported preparations was obtained and weighed on Braun Scale with 5 g sensitivity.

Nutrient content of diet was calculated using ICMR Food composition tables given in the nutritive value of Indian foods (IFCT, 2017; Gopalan et al., 2004) using Diet Soft Software (Kaur, 2007).

4.2.5: Anthropometric Measurements

Anthropometry is the measurement of body dimensions to characterize skeletal and tissue development, and effect relationship between nutrient and level of well-being of the body is assessed. In the present study, all anthropometric measurements were made using the guidelines adopted at the NIH sponsored Arlie Conference (Lohman et al., 1988).

Weight

A digital platform weighing scale (Omron) to the nearest 100gm was used to measure weight. The subject was weighed in standard office clothing, bare feet and without leaning against or holding anything. Scale was zeroed before taking any weight, and was calibrated using standard weights after every third subject. Calibration values were maintained (Robinson et al., 1988).

Height

It is a linear measurement made up of the sum of four components i.e. legs, pelvis, spine and skull. Stedometer was used to measure the height of the subjects. Stedometer was used to measure the height of the subjects. A convenient flat wall was identified at the bank site for the measurement of height. The subject was made to stand barefoot with the arms hanging freely by the side. Heels of the feet were placed together with the medial (inner) border of the feet at an angle of 60 degrees. The scapula and the buttock were ensured to be in contact with the measuring wall. The head was held in the Frankfort plane (with the tragus of the ear and the lateral angle of the eye in a horizontal line). Height was recorded to the nearest 0.1 cm after the subject inhaled fully and maintained the erect position without altering the load on the heels. In this position, a mark was made on the wall and height was recorded with a measuring tape. Two consecutive reading were taken. Height was recorded to the nearest 0.1 cm after the subject inhaled fully and maintained the erect position without altering the load on the heels. In this position, a mark was made on the wall and height was recorded with a measuring tape. Two consecutive reading were taken (Jellife, 1966).

Waist Circumference

Circumference of the waist is an important indicator of the risk of CVD when calculated with hip circumference to give waist-hip ratio (WHR) the subject was made to stand erect with the abdomen relaxed and the arms at the sides. The circumference was recorded using the Flexi tape at the narrowest part of the abdomen between the ribs and iliac crest. This was done with measurer facing the subject and identifying the natural waist (i.e. the point of narrowing). The measurement was taken to the nearest 0.1 cm at the end of a normal expiration, without the tape compressing the skin (Walker et al., 1996).

Hip Circumference

Hip circumference was measured at the point yielding the maximum circumference over the buttocks. The Flexi tape was placed around the buttocks in a horizontal plane at this level without compression the skin. The measurement was noted to the nearest 0.1 cm.

4.2.6: Computed Anthropometric Indices

Body Mass Index (BMI)

The convenient and valid measure for assessing the risk of cardiovascular disease is calculating BMI, that indicates adiposity and is found to be positively correlated with blood pressure and plasma lipid concentrations and therefore with the risk of cardiovascular disease (Brown et al., 2000). A relatively new classification of BMI has been recommended by WHO (2004) for the Asians. Under this, a BMI of more than 25 kg/m^2 is considered obese for Asian Indians in contrast to 30 for other population (WHO, 2004; Misra et al., 2009).

It is computed as-

$$\text{BMI } (kg/m2) = \frac{\text{Weight (kg)}}{\text{Height (m)}^2}$$

Table 4.10: Classification of Weight Status According to BMI in Asian Adults

BMI (kg/m^2)	Classification	Risk of Co-morbidities
< 18.5	Underweight	Low [but risk of other clinical complications]
18.5 – 22.9	Normal	Acceptable Risk
> 23	Overweight	
23.0 – 24.9	At Risk	Increased
25.0 – 29.9	Obesity Grade I	Moderate
≥ 30.0	Obesity Grade II	Severe

(Source: Misra et al., 2009; WHO/IASO/IOTF, 2000)

Waist- Hip Ratio (WHR)

This is one of the measures to assess central adiposity. High WHR often indicates an atherogenic lipid profile that tremendously enhances the cardiovascular risk (Suk et al., 2003). Males with WHR of ≥ 0.9 and Females with WHR of ≥ 0.85 were taken as cut-offs for central obesity (Misra et al., 2009; WHO, 2008).

Table 4.11: Cutoffs for WC, WHR and WSR for CVD risk

	MEN	WOMEN
Waist Circumference (WC)	**90 cm (35 inches)**	**80 cm (32 inches)**
Waist Hip Ratio (WHR)	**>0.9**	**>0.85**
Waist Stature Ratio (WSR / WHt.R)	**>0.55**	**>0.53**

Source: Waist Circumference and Waist–Hip Ratio: Report of a WHO Expert Consultation Geneva, 8–11 December 2008

Waist- Stature Ratio (WSR)

Waist stature ratio was calculated by diving waist circumference by height. WSR = Waist circumference (cm) / Height (cm). The cut off for abdominal obesity was taken as WSR ≥0.55 in males and WSR ≥0.53 females (Ashwell, Gunn, & Gibson, 2012; Schneider et al., 2010; Browning et al., 2010).

4.2.7: Biophysical Investigation

Blood Pressure

Measurement of blood pressure were taken after an employee achieved relaxed state and heartbeats settled down as he was made to sit down quietly for at least 5 minutes. The bare arm of the subject was supported and position at heart level. In position blood pressure was measured using digital blood pressure monitor UA-767PC (Saitama, Japan) on the right arm.

On each occasion two or more readings were averaged. Classification given by Joint National Commission VIII and American Heart Association, 2011 in publication named "Understanding blood pressure readings" was used. Desired Systolic BP was 90 -119 mmHg and Diastolic BP was 60 – 79 mmHg.

Table 4.12: JNC VIII - Classification of Blood Pressure in Adults (Age ≥ 18 years)

Classification	Systolic Blood Pressure (mmHg)		Diastolic Blood Pressure (mmHg)
Normal	**<120**	**AND**	**< 80**
Pre-hypertension	**120 – 139**	**OR**	**80 – 89**
Stage I HTN	**140 – 159**	**OR**	**90 – 99**
Stage II HTN	**≥ 160**	**OR**	**≥ 100**

Body composition analysis

Digital body fat monitor (Omron healthcare co. ltd Japan; model no. HBF – 306 –C1; SN: 2010100047 IUF) was used to measure body fat percentage, basal metabolic rate and body mass index. It also displayed graphical interpretation of body types like lean, lean normal, muscular, latent obesity and obese.

Method :

Both hands were placed around electrode hand grips. Thumbs were correctly positioned on top of grip (thumbs up) and palms were adhered firmly to both upper and lower electrodes. The start button was switched on and held till the results were displayed

Table 4.13: Gender-Wise Classification of Body Fat Percentage (Omron Manual)

Description	Body Fat% (Male)	Body Fat % (Female)
Essential fat	2-5 %	10–13%
Athletes	6–13%	14–20%
Fitness	14-17%	21-24%
Acceptable	18-24%	25-31%
Obese	**≥25%**	**≥32%**

4.2.8: Biochemical Evaluation and Assay Methods

Estimation of Random blood sugar

This test was performed using "Digital Aqua check" glucometer. The finger was first wiped with the cotton swab dipped in alcohol. The first drop of blood was discarded and the second drop of blood was placed on the strip attached to glucometer. The results were automatically displayed on the screen and recorded.

Estimation of Gut Satietogenic Hormones

Gut Incretins' and hormones were analyzed using Milliplex map human gut hormone panel kit (#HGT68K) of Millipore Merck Company. Gut Satietogenic Hormones were analyzed by using plasma sample. The method for preparation of plasma was followed as mentioned below.

Principle: ***(Source: Luminex kit manual #HGT68K)***

MILLIPLEX™ MAP was based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded beads known as microspheres.

- Luminex® uses proprietary techniques to internally colour-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly coloured bead sets can be created, each of which was coated with a specific capture antibody.

- ✪ After an analyte from a test sample was captured by the bead, a biotinylated detection antibody was introduced.
- ✪ The reaction mixture was then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- ✪ The microspheres were allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule.
- ✪ Finally, a high-speed digital-signal processor identifies each individual microsphere and quantifies the result of its bioassay based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

Sample collection and storage (Serum or Plasma)

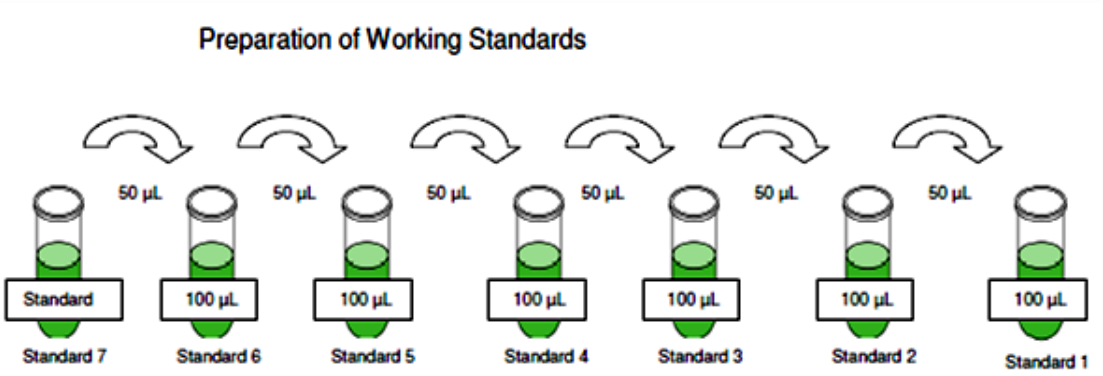
A. Method for Preparation of Plasma Samples (We used Plasma Samples for our study):

- ✪ Plasma collection using EDTA as an anticoagulant was used as recommended in the kit manual. After collecting blood, immediately DPP-IV inhibitor cocktail was added for GLP-1 measurement. Tube was inverted several times to mix properly.
- ✪ Blood sample was centrifuged for 10 minutes at 1000xg within 30 minutes of blood collection. Plasma was removed and assay was not used immediately. Aliquots were made in duplicate and samples were stored at £ -20°C.
- ✪ It is recommended to avoid multiple (>2) freeze/thaw cycles.
- ✪ As we used frozen samples, they were allowed to thaw completely. They were then mixed well by vortexing and centrifuged prior to use in the assay to remove particulates.

- For normal samples, no dilution was required. If dilution was necessary, we used serum matrix to dilute plasma samples prior to assay as recommend by the kit manual.

B. Method for Preparation Serum Samples:

- After collecting blood samples, invert tube several times to mix, immediately add DPPIV inhibitor (for GLP-1 measurement), Protease Inhibitor cocktail (for amylin measurement), and Serine protease inhibitor (for active ghrelin measurement). Use Millipore's DPPIV inhibitor, Sigma's Protease inhibitor cocktail, and Roche's Pefabloc SC (AEBSF).
- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at £ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- For normal samples, no dilution was required. If dilution was necessary, Serum Matrix was used to dilute serum samples prior to assay.



The serial dilutions result in the following concentrations of standards.

Standard Tube	GIP (pg/mL)	GLP-1, PYY, PP, Ghrelin (pg/mL)	Amylin (pg/mL)	Leptin, Insulin (pg/mL)
Standard 1	2.7	13.7	27.4	137
Standard 2	8.2	41.2	82.3	412
Standard 3	24.7	123.5	246.9	1235
Standard 4	74.1	370.4	740.7	3,704
Standard 5	222.2	1,111	2,222.2	11,111
Standard 6	666.7	3,333	6,666.7	33,333
Standard 7	2000	10,000	20,000	100,000

Figure 4.5: Preparation of working standard, serial dilution and concentrations of standards

Blood sampling

Blood samples were drawn from subjects practicing overnight fasting state. A cannula was inserted into the antecubital vein for drawing blood in a cooled EDTA-treated tube containing DPP-IV inhibitor (10 µg/ml blood; Millipore Merck). Tube was inverted several times to mix. Within 30 min, the blood was centrifuged at 1000 rpm for 10 min at 4ºC. Plasma was removed and 2 aliquots of samples were stored at -80ºC. All samples were stored in polypropylene tubes.

Blood samples were taken at the work place of the employees. Lab technician was hired to draw the blood and separate plasma using cooled collection tubes and portable centrifuge. The plasma samples were stored immediately in the dry-ice container and stored under -20^{o} C in deep-freezer in department.

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 0 (Background)	Standard 4	QC-1 Control	Etc.								
B	Standard 0 (Background)	Standard 4	QC-1 Control									
C	Standard 1	Standard 5	QC-2 Control									
D	Standard 1	Standard 5	QC-2 Control									
E	Standard 2	Standard 6	Sample 1									
F	Standard 2	Standard 6	Sample 1									
G	Standard 3	Standard 7	Sample 2									
H	Standard 3	Standard 7	Sample 2									

Figure 4.6: Preparation of working standard, serial dilution and concentrations of standards

Immunoassay procedure

- The Immunoassay procedure was conducted at the Millipore Merck Lab in Bangalore under the supervision of the Lab scientist of the Millipore Merck Company.
- Prior to beginning of the assay, it was imperative to read the kit protocol completely and to thoroughly understand the technical guidelines.
- Reagents were allowed to warm at room temperature (20-25°C) before use in the assay.
- Diagram was used for the placement of standards as shown in Figure 4.6 (Background, Standard 1, 2, 3, 4, 5, 6 & 7)
- We marked the control 1, control 2, and sample codes on well map worksheet in a vertical configuration using the print out of Figure 4.6 (Note: Most instruments will only read the 96-well plate vertically by default.) It was recommended to run the assay in duplicate however; we did single assay and not in duplicate due to large sample size.

✪ Filter plate was set on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate would not touch any surface.

✪ **INCUBATION STEPS:**

1. Filter plates were pre-wet by pipetting 200 µl of assay buffer into each well of the microtiter filter plate. Filter plate was then sealed and mixed on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Assay buffer was removed by using vacuum suction. **(NOTE: DO NOT INVERT PLATE.)** Excess assay buffer was blot from the bottom of the plate with an absorbent pad or paper towels.
3. In appropriate wells, 25 µl of each standard and control was added. For 0 pg/ml standard well (Background well), plain assay buffer was used.
4. In next step, 25 µl of assay buffer was added to the sample wells followed by 25 µl of sample into the appropriate wells.
5. For background, standards, and control wells, 25 µl of appropriate matrix solution was added. We used serum matrix provided in the kit for our plasma samples (same can be used for serum samples as well) as recommended in the kit manual. **Note:** When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Mixing Bottle was vortexed and 25 µl of the mixed beads were added to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
7. Plates were sealed with a plate sealer and were covered with the lid. A rubber band was wrapped around the plate holder, plate and lid and incubated with agitation on a plate shaker overnight (16-18 hours) at 4°C.
8. Fluid was gently removed by vacuum suction. **(NOTE: DO NOT INVERT PLATE.)**
9. Plates were washed 3 times with 200 µl/well of wash buffer. Wash buffer was removed by vacuum filtration between each wash. Excess wash buffer was blot from the bottom of the plate with an absorbent pad or paper towels.
10. Detection antibodies of 50 µl volume were added into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to the addition).

11. Plates were again sealed, covered with lid, and incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**
12. Streptavidin-Phycoerythrin of 50 µl volume was added to each well containing the 50 µl of detection antibodies.
13. Plates were again sealed, covered with lid, and incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
14. All contents were gently removed by vacuum suction. (NOTE: DO NOT INVERT PLATE.)
15. Plates were washed 3 times with 200 µl/well using wash buffer. Wash buffer was removed by vacuum filtration between each wash. Excess buffer was wiped on the bottom of the plate with a tissue.
16. Sheath Fluid of 100 µl volume was added to all wells. Beads were resuspended on a plate shaker for 5 minutes.
17. Plates were run on Luminex 200™. Luminex 100™ IS, or HTS can also be used if plates are compatible.
18. Median Fluorescent Intensity (MFI) data was saved and analyzed using a weighted 5-parameter logistic or spline curve-fitting method for calculating hormone concentrations in samples.

Assay characteristics

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated by the StatLIA®Immunoassay Analysis Software from Brendan Technologies. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Ghrelin - 1.8	Leptin - 157.2	GIP - 0.2
GLP-1 - 5.2	PYY - 8.4	Insulin - 44.5

Precision: Intra-assay precision is generated from the mean of the %CV's from 6 reportable results across two different concentrations of hormones in one experiment.

Inter-assay precision is generated from the mean of the %CV's from 6 reportable results each for two different concentrations of hormone across 6 different experiments.

Intra-assay variation (%CV) <11%

Inter-assay variation (% CV) <19%

Accuracy: Spike Recovery: The data represent mean percent recovery of 3 levels of spiked standards (low, medium and high) in serum matrices.

Ghrelin 85%	Leptin 102%	GIP 89%
GLP-1 83%	PYY 107%	Insulin 85%

Cross-Reactivity

According to the manufacturer, the antibody pairs in the panel are specific only to the desired analyte and exhibit no or negligible cross-reactivity with other analytes in the panel.

Plate 4.1: Luminex Instrument

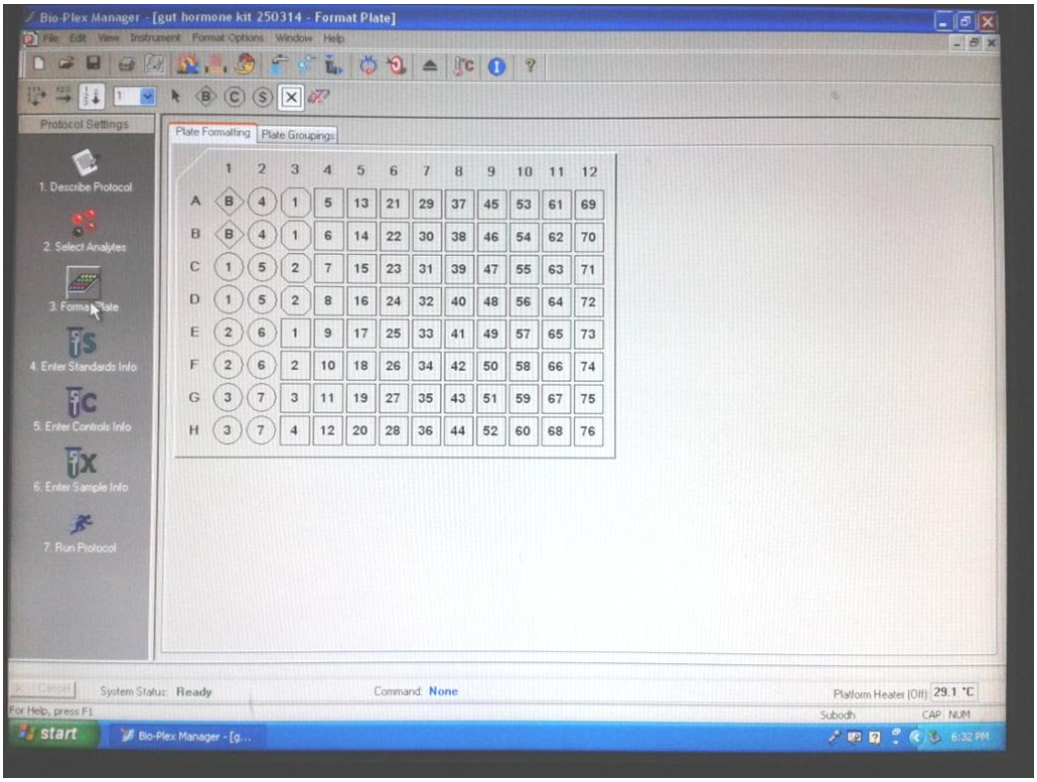
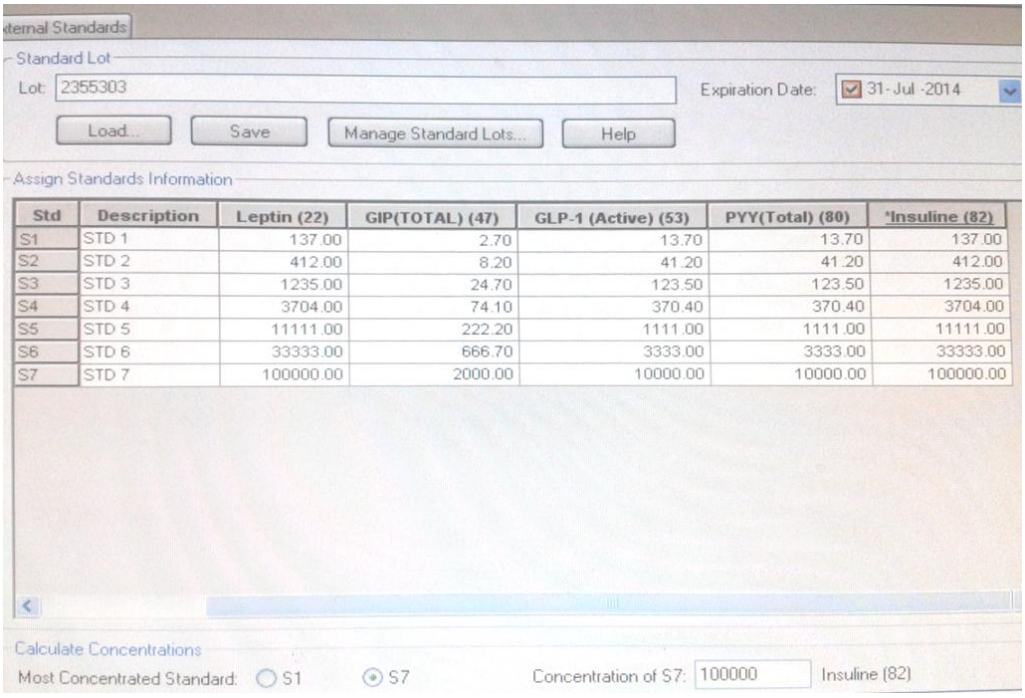
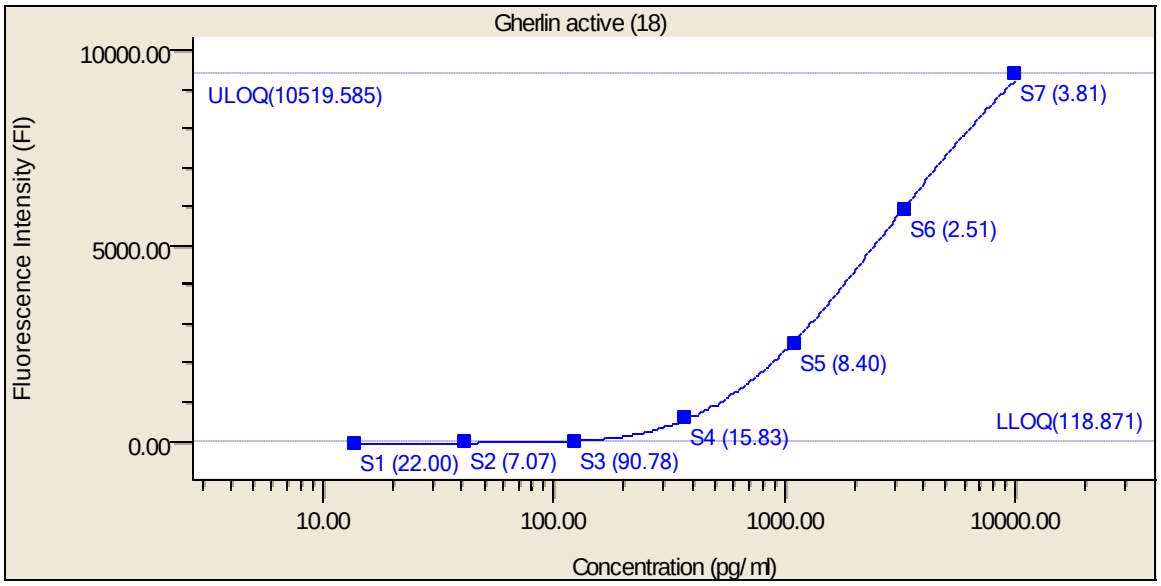


Plate 4.2: Well Map data entered into computer



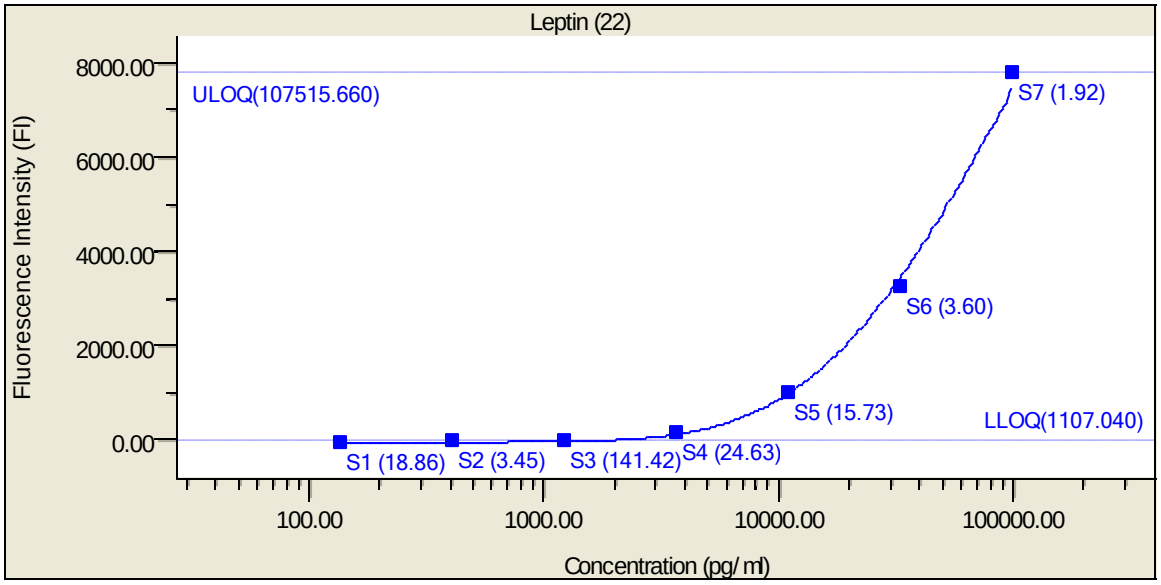
Std	Description	Leptin (22)	GIP(TOTAL) (47)	GLP-1 (Active) (53)	PYY(Total) (80)	*Insuline (82)
S1	STD 1	137.00	2.70	13.70	13.70	137.00
S2	STD 2	412.00	8.20	41.20	41.20	412.00
S3	STD 3	1235.00	24.70	123.50	123.50	1235.00
S4	STD 4	3704.00	74.10	370.40	370.40	3704.00
S5	STD 5	11111.00	222.20	1111.00	1111.00	11111.00
S6	STD 6	33333.00	666.70	3333.00	3333.00	33333.00
S7	STD 7	100000.00	2000.00	10000.00	10000.00	100000.00

Plate 4.3: Concentration of standards



Regression Type: Logistic - 5PL

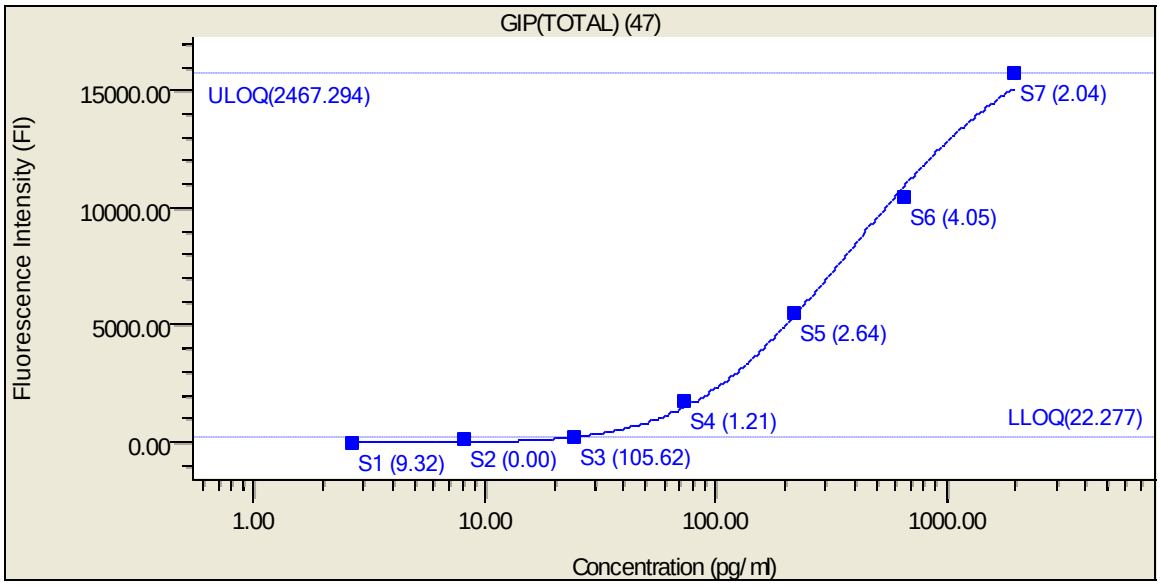
Std. Curve: FI = -0.262716 + (14032.6 + 0.262716) / ((1 + (Conc / 129.882)^-0.676113))^7.95944
FitProb. = 0.0000, ResVar. = 10.9055



Regression Type: Logistic - 5PL

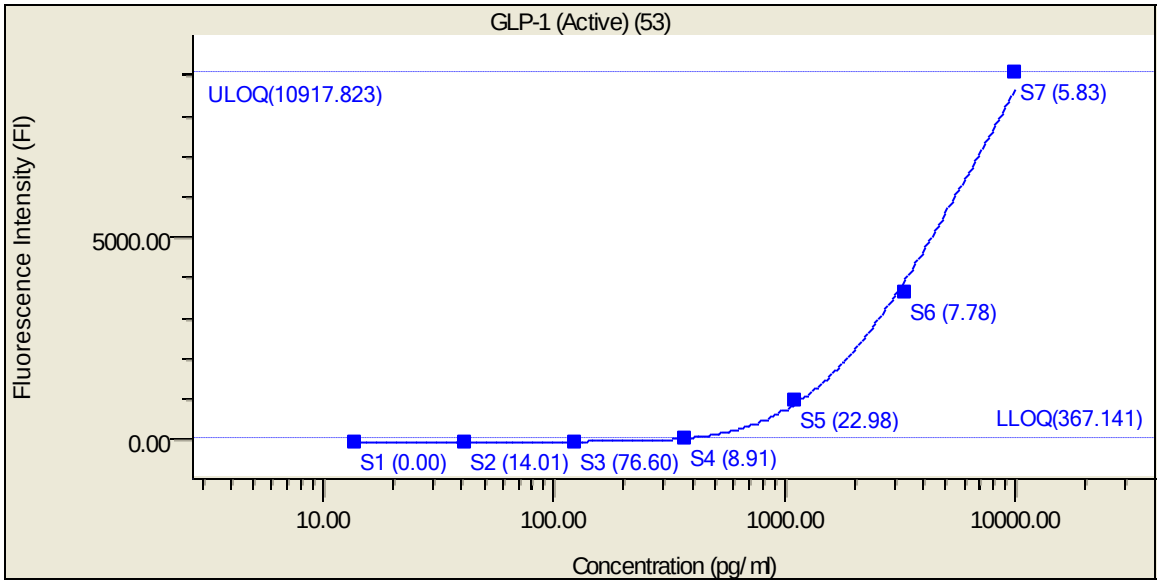
Std. Curve: FI = -4.32962 + (22203.2 + 4.32962) / ((1 + (Conc / 1542.21)^-0.519743))^10
FitProb. = 0.0003, ResVar. = 8.0413

Figure 4.7 A: Standard Curves for Six Gut Satietogenic Hormones



■ **Standard** ☐ **Partial Outlier** ☒ **Outlier**

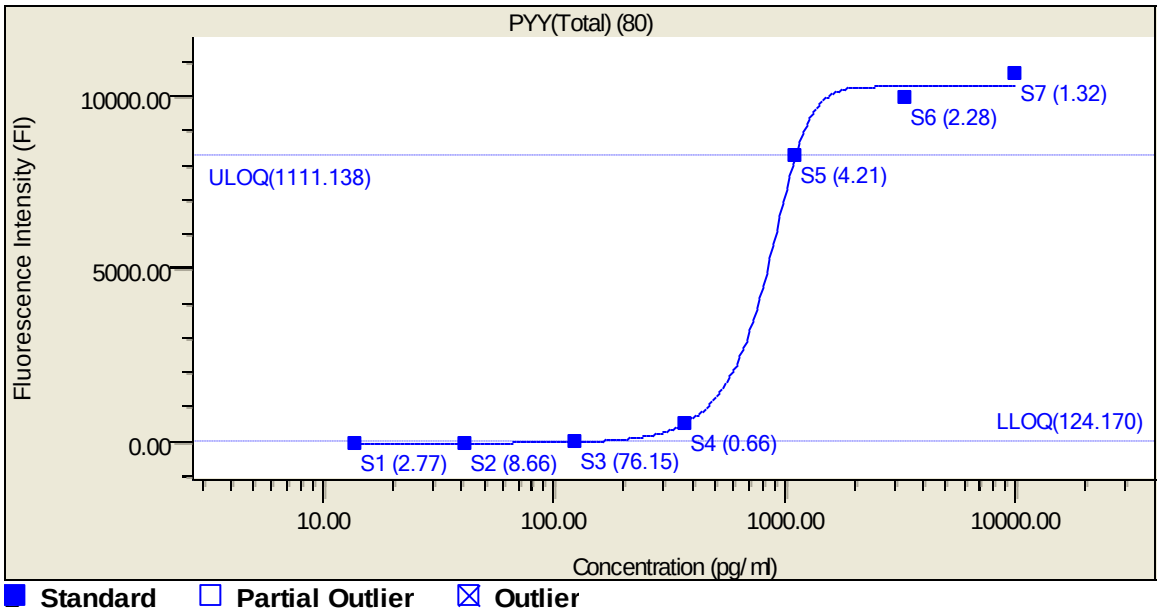
Regression Type: Logistic - 5PL
Std. Curve: FI = 43.0565 + (18762.1 - 43.0565) / ((1 + (Conc / 133.087)^-0.901233))^2.52132
FitProb. = 0.0000, ResVar. = 20.3480



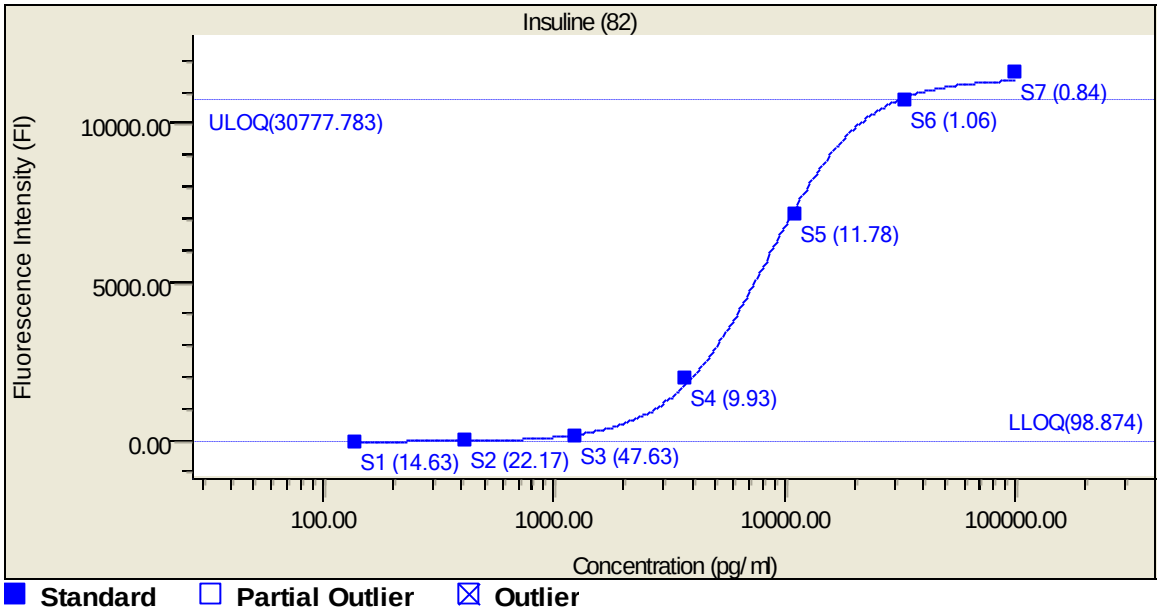
■ **Standard** ☐ **Partial Outlier** ☒ **Outlier**

Regression Type: Logistic - 5PL
Std. Curve: FI = -12.6716 + (18884.9 + 12.6716) / ((1 + (Conc / 234.744)^-0.67242))^10
FitProb. = 0.0031, ResVar. = 5.7650

Figure 4.7 B: Standard Curves for Six Gut Satietogenic Hormones



Regression Type: Logistic - 5PL
Std. Curve: FI = -1.17783 + (10334 + 1.17783) / ((1 + (Conc / 1061.98)^-7.03255))^0.392398
FitProb. = 0.4039, ResVar. = 0.9067



Regression Type: Logistic - 5PL
Std. Curve: FI = 4.87577 + (11475 - 4.87577) / ((1 + (Conc / 8332.89)^-2.08921))^0.996384
FitProb. = 0.0000, ResVar. = 11.5207

Figure 4.7 C: Standard Curves for Six Gut Satietogenic Hormones

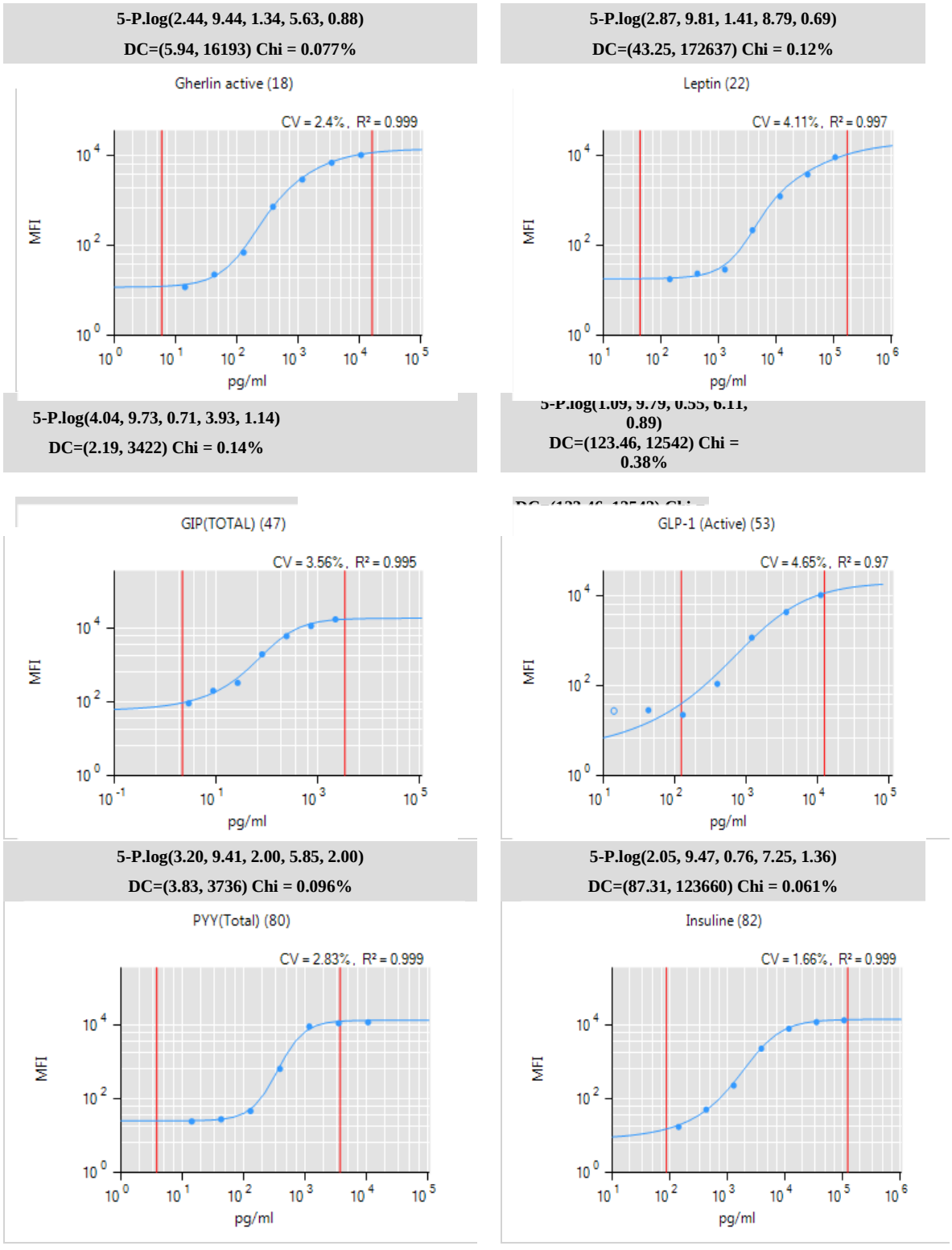


Figure 4.8: MILLIPLEX Analyst Detail Report

Table 4.14: MILLIPLEX Analyst Detail Report

Standard Curve					
Analyte	**Chi**	**R^2**	**CV**	**MinDC**	**MaxDC**
Gherlin active (18)	**0.077%**	**0.999**	**2.40%**	**5.94**	**16193**
Leptin (22)	**0.12%**	**0.997**	**4.11%**	**43.25**	**172637**
GIP (TOTAL) (47)	**0.14%**	**0.995**	**3.56%**	**2.19**	**3422**
GLP-1 (Active) (53)	**0.38%**	**0.97**	**4.65%**	**3.46**	**12542**
PYY(Total) (80)	**0.096%**	**0.999**	**2.83%**	**3.83**	**3736**
Insulin (82)	**0.061%**	**0.999**	**1.66%**	**87.31**	**123660**

4.2.9: Determination of gut microbiota

Gut microflora was determined with respect to four species namely *Bifidobacteria, Lactobacillus, Bacteriodes,* and *Clostridium* from the stool samples of bank employees.

a) Collection of Sample: Stool samples were collected in sterile container directly placed in a "Genbag Anaer®" device (Biomerieux, Marcy 1Etolle; France) and stored in dry ice. This helps the sample to remain in an anaerobic environment until it is processed at the laboratory. All samples were collected fresh and thereafter carried to the department's microbiology laboratory. The samples were processed immediately within 2 hours of collection.

b) Sterilization of Glass wares: All the petri dishes and the other glass wares such as beakers and conical flask were sterilized before use. The petri dishes were kept in the petri dish box and the other glassware were wrapped in a paper and kept in a hot air oven at 180^0 C for 2 hours for sterilization. The micro-tips were sterilized by autoclaving at 121^0C for 15 minutes at 15 lbs pressure. The other instruments which were used like the weighing balance and spatula were all sterilized by alcohol flaming using 70 % alcohol.

c) Preparation of the sample: One gram of fecal sample was weighed in a vacuum weighing balance and was used for the estimation.

d) Homogenization and Dilution: For the preparation of dilution blanks 1 g of peptone was dissolved in 1000 ml of distilled water (0.1% w/v peptone and 0.005% w/v of NaCl). This solution was dispensed in portion of 100 ml in 10 dilution bottles. These were autoclaved at 121^0C for 15 mins. The bottles were cooled at room temperature before putting them to use. One gram of stool sample was added in stomacher bag. To this 99ml of peptone water was added. Homogenization was done using a stomacher blender (Bag Mixer 400 VW) at 200 rpm for 1 minute.

e) Serial Dilution : Ten-fold Serial dilution was performed from 10^{-2} to 10^{-12} (WHO/FAO 1979)

f) Preparation and sterilization of media:

Lactobacillus **-** Hi Media MRS Agar M 641 (67.15 g in 1000 ml distilled water).

Bifidobacterium – Hi Media Bifidobacterium Agar M 1396 (49.3 g in 1000 ml distilled water).

Clostridium – Hi Media Anaerobic Agar M 228 (58 g in 1000 ml distilled water).

Bacteriodes – Hi Media Anaerobic Basal Agar M 1635 (45.9 g in 1000 ml distilled water).

The prepared media was autoclaved at 121^0C for 15 minutes and then poured into sterile petri plates and was allowed to set.

g) Plating: One gram of fresh fecal sample was accurately weighed and homogenized in 99 ml of 0.1% peptone water to provide 1% (w/v) fecal slurry. One ml of slurry was diluted serially in peptone water as shown in Figure 4.3.9.1. Then 0.1 ml of dilution was pipetted from each of the dilutions to the petri plates containing respective media. The above procedure was carried out inside laminar flow (as shown in plate 4.3.9.2, 4.3.9.4) that ensures a sterile environment thereby preventing contamination from outside.

h) Incubation: Anaerobic Jars were used for the petriplates of obligate anaerobes like *Bifidobacterium, Clostridium, and Bacteriode.* Gas pack was opened up and kept in the anaerobic jars. The Plates of *Lactobacillus were* kept in the dessicator. All plates were kept for Incubation at 37^0 C for 48 – 72 hours.

i) **Counting and Recording of data:** After 48 hrs. of incubation colonies were counted using a digital colony counter and data was recorded. Colonies that appeared in the range of 30–300 were converted in to log counts after multiplying with their dilution factors (Reynolds, J. 2015)

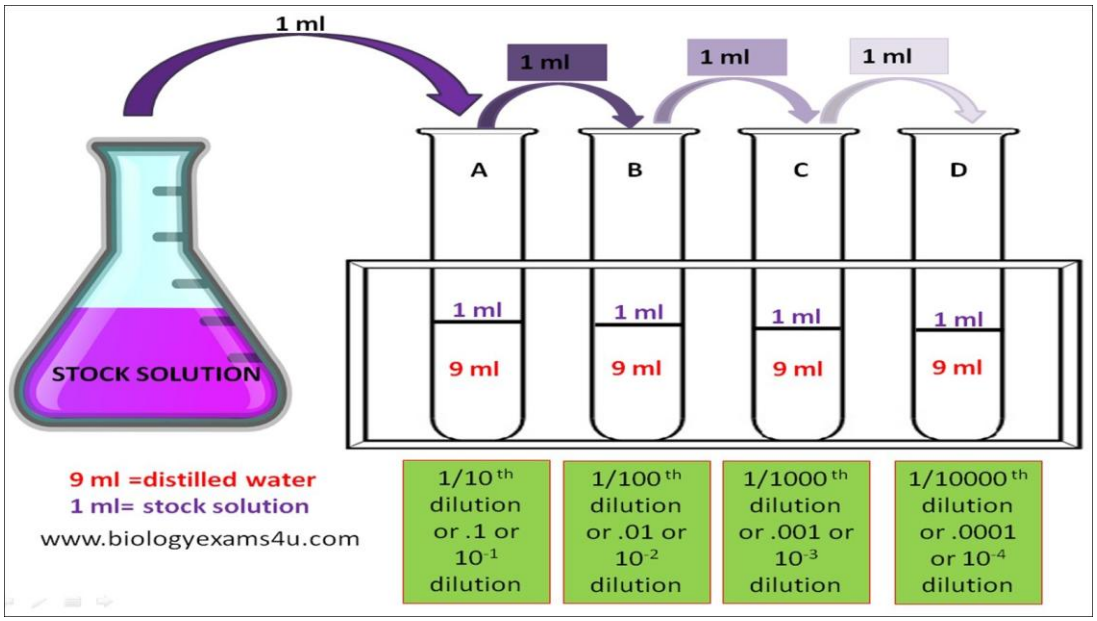


Figure 4.9: Serial Dilution technique

4.2.10: Statistical Analysis

The data was entered into Microsoft excel spread sheets and then subjected to appropriate statistical analysis. Frequency and percentages were calculated for background information. Microsoft Excel data analysis package was used to express results as mean and standard deviation. Paired t-test was used to calculate the difference between the means. ANOVA was performed to determine the significant differences in the mean scores. Percent increase and decrease was also determined by calculations to compare. Student "t" test of unequal variance was used to observe the difference that exists between the values. The significance levels were set at 5% by two sided tests. Student "t" test was performed for the comparison between two groups. Epi-info 7, version 7.2.0.1 was used to calculate chi-square values, odds ratio, relative risk and absolute risk reduction. Pearson's correlation was used to determine the relevant association between

the parameters. Statistical package for social sciences (SPSS 21.0v) software was used for obtaining results for regression analysis.

PHASE III – TO STUDY IMPACT OF FOS INTERVENTION FOR 90 DAYS IN OBESE SUBJECTS: A RANDOMIZED CONTROL TRIAL

4.3.1 Sample size calculation for Clinical trial (Schoenfeld, 2018; MGH Biostatistics Center, Harvard University)

The sample size was calculated using the software developed by Dr. David Schoenfeld with support from the MGH Mallinckrodt General Clinical Research Center, Harvard. The sample size was based on power calculations that used weight loss as the primary outcome. With an estimated weight loss of 1.0 kg and an SD of 1.0 kg based on 0.9 power to detect a significant difference (P=0.01, 2 sided), a minimum of 64 subjects in total were needed. We made an effort to collect data for more than 64 subjects. Total of 150 subjects were enrolled who were willing to participate in study (75 in each group – placebo and experimental).

Sample Size Calculation for Gut hormones

Gut hormones, with an estimated mean difference of 0.25 pg/ml and an SD of 0.25 pg.ml based on 0.9 power to detect a significant difference (P=0.05, 2 sided), a minimum of total 46 subjects (23 in each arm) were needed in both arms and data was collected for total 80 subjects (40 subjects in each arm).

4.3.2 Study Protocol

Current section of the study was designed to evaluate the impact of FOS intervention on obesity outcomes. Total of 150 obese employees who participated in phase-II continued further in our study and were enrolled for intervention RCT trial. Baseline data for these 150 obese bank employees was already collected in phase –II.

These 150 obese employees were randomly divided in two arms i.e. experimental and placebo arm. Experimental arm received FOS (20g) and placebo arm received maltodextrin (10 g) for period of 90 days. It was taken care that both the interventional products were provided equal calories (40 kcal / day).

Effect of FOS supplementation was studied on all parameters studied during baseline data collection like anthropometry parameters, biophysical parameters, defecation profile, hunger and satiety scores, depression scores, dietary intakes, fasting plasma levels of gut satietogenic hormones (GLP-1, GIP, PYY, Leptin, Ghrelin and Insulin), and gut microbiota (*Lactobacillus, Bifidobacteria, Clostridium and Bacteroides*).

During the study period there were total 8 dropouts. Five were from placebo group and three from experimental group. Post intervention the final sample size for statistical analysis was 72 in experimental group and 70 in placebo group. The Consort for RCT intervention phase –III of the study is depicted in Figure 4.13.

4.3.3 Study Food

FOS is "Generally Recognized as Safe". FOS, ingested at up to 20g/day in adults, appears to be safe and well tolerated (WHO, 2000).

Intervention and Comparator agent

Intervention agent: Fructooligosaccharide - Dose: 20 gm for 3 months (40 kcal / day).

Comparator agent: Maltodextrin - Dose: 10 gm for 3 months (40 kcal / day).

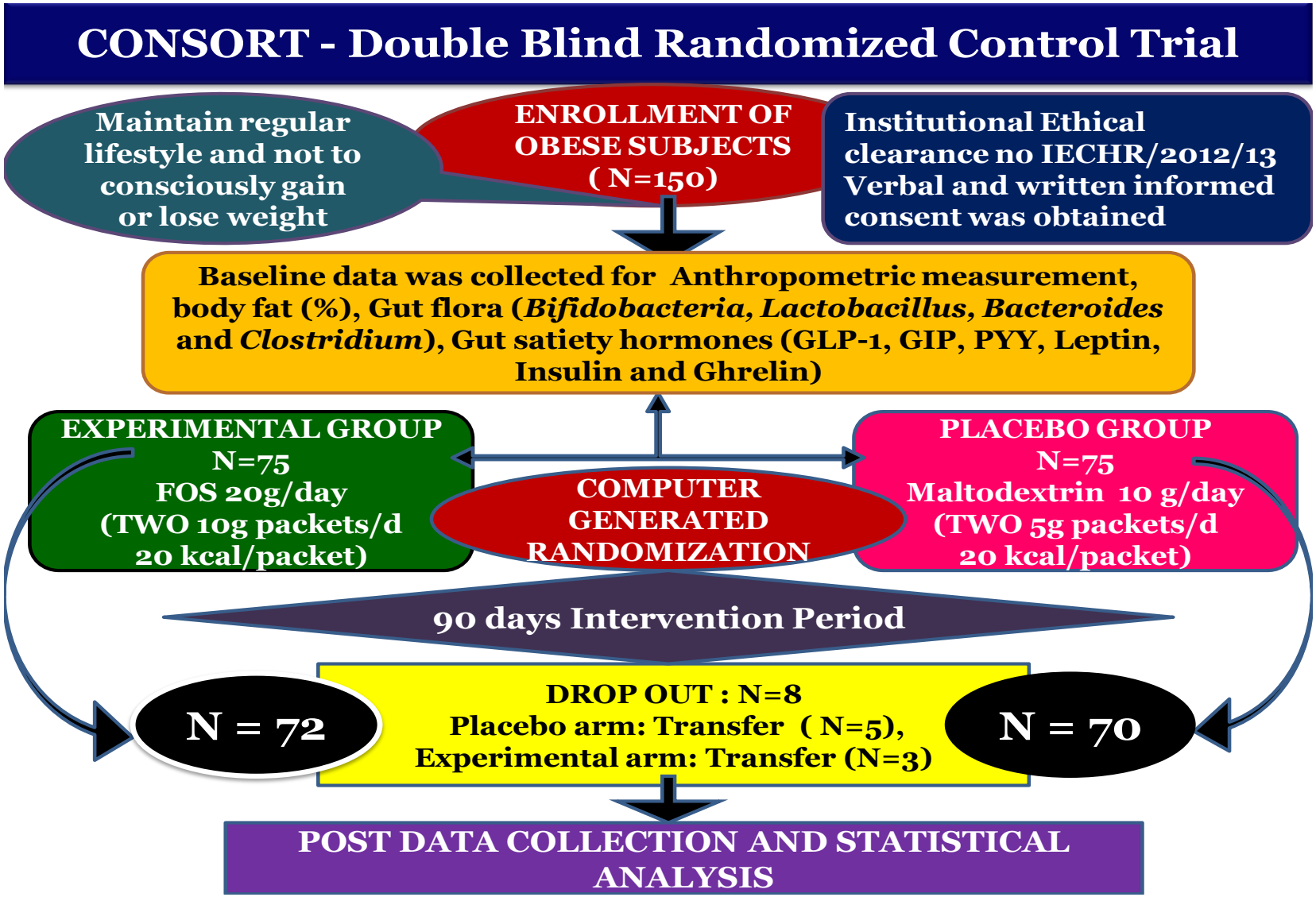


Figure 4.10: Intervention Phase – III CONSORT - Double Blind Placebo Control Trial

4.3.4: Method of Randomization (Clinical Trial Registry of India, 2018)

Study Design: Double-blind, Placebo- Controlled Randomized Trial

1) Method of generating randomization sequence:	**Computer generated randomization**
2) Method of allocation concealment:	**Centralized**
3) Who generated the allocation sequence:	**Staff from department who were not involved in the study**
4) Who enrolled participants:	**Investigator enrolled participants**
5) Who assigned participants to their groups:	**Random allocation using computer -generated randomization numbers**
6) Blinding/Masking:	**Participant, Investigator and Outcome assessor Blinded**

Method for Randomization

Obese subjects (n=150) were randomly assigned to groups that received either intervention of 20g FOS/d (FOS-P powder; provided by Meiji Co., Ltd, Tokyo, Japan) or an equicaloric amount of 10g comparator agent Maltodextrin for 90 days.

The main purpose of randomization is to eliminate selection bias and balance known or unknown confounding factors in order to create a control group that is as similar as possible to the treatment group. Methods for randomly assigning participants to groups, which limits bias, include the use of a table of random numbers and a computer program that generates random numbers. Methods of assignment that are prone to bias include alternating assignment or assignment by date of birth or hospital admission number. The study participants were enrolled by the investigators and they were randomly allocated using computer generated sequence.

Concealment of the randomization sequence is critical to prevent selection bias. Adequate allocation concealment is a pre-requisite for adequate blinding.

In our study the method of allocation concealment was centralized and the allocation sequence was done by the employee of department, not involved in the study. In this study the participant, investigator and outcome assessor were blinded.

4.3.5 Primary and Secondary Outcomes

Outcome	Timepoints
Weight Reduction in kg.	Zero day - Pre, 1st month, 2nd month and 3rd month - Post

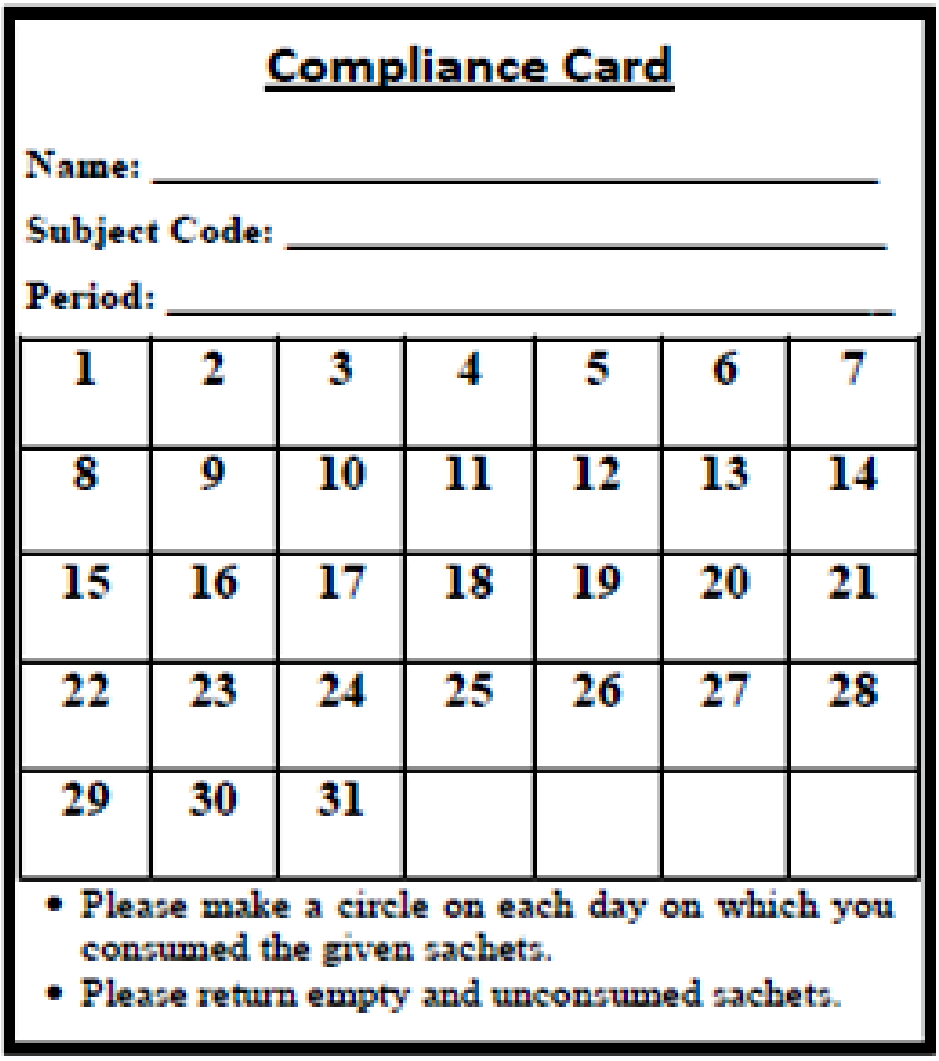
Outcome	Timepoints
Gut Hormones : GLP-1, GIP, PYY, Ghrelin, Leptin and Insulin	Zero day and 3rd Month [Pre and Post]
Gut Microflora: Bifidobacteria,Lactobacillus,Clostridium and Bacteroides	Zero day and 3rd Month [Pre and Post]
Defecation Profile	Zero day[Pre], 1st month, 2nd month and 3rd month [Post]
Hunger scores between 1 - 5	Zero day [Pre], 1st month, 2nd month and 3rd month [Post]
Satiety scores between 6 - 10	Zero day [Pre], 1st month, 2nd month and 3rd month [Post]
24 hour Dietary Recall for 3 consecutive days for Macro-nutrient and Fiber Intake	Zero day and 3rd month [Pre and Post]
Becks Depression Inventory Scores greater than 10	Zero day and 3rd month [Pre and Post]

Figure 4.11: Primary and Secondary Outcomes with Time-point of data collection and follow-up plan

4.3.6 Trial Monitoring Plan and compliance check

The FOS group received 20g divided into two packets of 10 g each providing 20 kcal/packet, and placebo group received 10 g maltodextrin divided into two packets of 5 g each providing 20 kcal/packet that were to be taken before meals. Both the FOS and placebo were provided to the subjects in identical opaque packages. Sachets were given

on weekly basis and total 14 sachets were provided to one subject in one week. Subjects were instructed to return all packets to assess compliance. A compliance card was also provided to all subjects and they were asked to tick (√) in the card on daily basis after consuming 2 sachets and the investigator visited once a week for checking the compliance and collection of empty sachets. At the end of 3 months all the ticks (√) were counted and compliance was calculated.



Compliance Card

Name: _____

Subject Code: _____

Period: _____

1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

- Please make a circle on each day on which you consumed the given sachets.
- Please return empty and unconsumed sachets.

Figure 4.12: Format of Compliance Card given to Obese Bank Employees

PHASE IV– ACCEPTABILITY TRIALS OF FOS ADDED POPULAR INDIAN RECIPES

From various strategies proposed for obesity management like physical, psychological, pharmaceutical; diet therapy plays a vital role in the management of obesity and is without any adverse health effect. In recent years the concept of addition of bioactive compound that exerts a positive effect on host health and its well-being beyond the nutritive value along with bringing a positive drift in the composition of gut-microflora focused largely on probiotics.

Recently, the use of bioactive compound in the matrix of functional food is the global consumer trend. Bioactive compounds are described as "naturally occurring chemical compounds that are contained in or derived from a plant, animal or marine source, that exert the desired health and wellness benefit" (Agriculture and Agri-Food Canada, 2009). As acknowledged by Health Canada 1998 and 2010 essential nutrients (e.g. vitamins and minerals) and non-nutrients (e.g. plant sterols), both are considered as bioactive compounds. Amongst various types of bioactive compounds, some of them are antioxidants, dietary fibre, omega-3 fatty acids, plant sterols, probiotic and prebiotic (Ye et al., 2010).

Currently, use of prebiotics as bioactive compounds is the new area emerging in the management of obesity. Fortification of foods and beverages with novel functional ingredients like Prebiotic Fructooligosaccharide is a recent development in this direction. Also, various health benefits of prebiotic FOS are evident from several studies, if consumed on regular basis (Roberfroid et al., 2010; Macfarlane & Cummings, 2006).

Prebiotic Fructooligosaccharide being a carbohydrate and having a sweet taste similar to that of sucrose contributes minimum calories as compared to sucrose (Yun, 1996). Apart from being classified as a sweetener, it is also classified as fibre and unlike other fibers; it does not have off-flavor and can be used to increase the fibre without increasing the viscosity. Fructooligosaccharide are also considered as bulking agents and fat substitutes, they have high solubility, and they do not have any after or artificial taste (Guggisberg et al., 2011; Crittenden & Playne, 1996).

4.1.1: Development of FOS incorporated food products

The study aimed to conduct acceptability trials of FOS addition at varying levels of 10g, 15g and 20g compared to standard recipe of steamed *Dudhi muthiya*, shallow fried *Vegetable Cheela*, baked *Handwa* and deepfried *Vegetable Mini Samosa* in terms of physical and organoleptic attributes. Experimental design for this phase is given in Figure 4.16

4.1.2: Selection of food products

Four products were selected on the basis of their cooking technique like steaming, shallow frying, baking and deep frying. Recipes were further selected based on popularity and frequency of consumption like *Dudhi muthiya*, *Vegetable Cheela*, *Handwa* and *Vegetable Mini Samosa*. In *Vegetable Mini Samosa* level of FOS addition was reduced to 5g, 10, and 15g instead of 10g, 15g, and 20g. All the products were studied for their physical and organoleptic attributes. All recipes were pre-standardized recipes and given by Professor Mini Sheth.

4.1.3: Procurement of Fructooligosaccharide (FOS)

Fructooligosaccharide was procured in powder form from Mitushi pharma, Ahmedabad. The FOS was food grade approved and was safe for human consumption. It was manufactured by Meiji Japan; 20 kg bag; Lot no.MMS 182-270. The specifications of the FOS are given in Table 4.15.

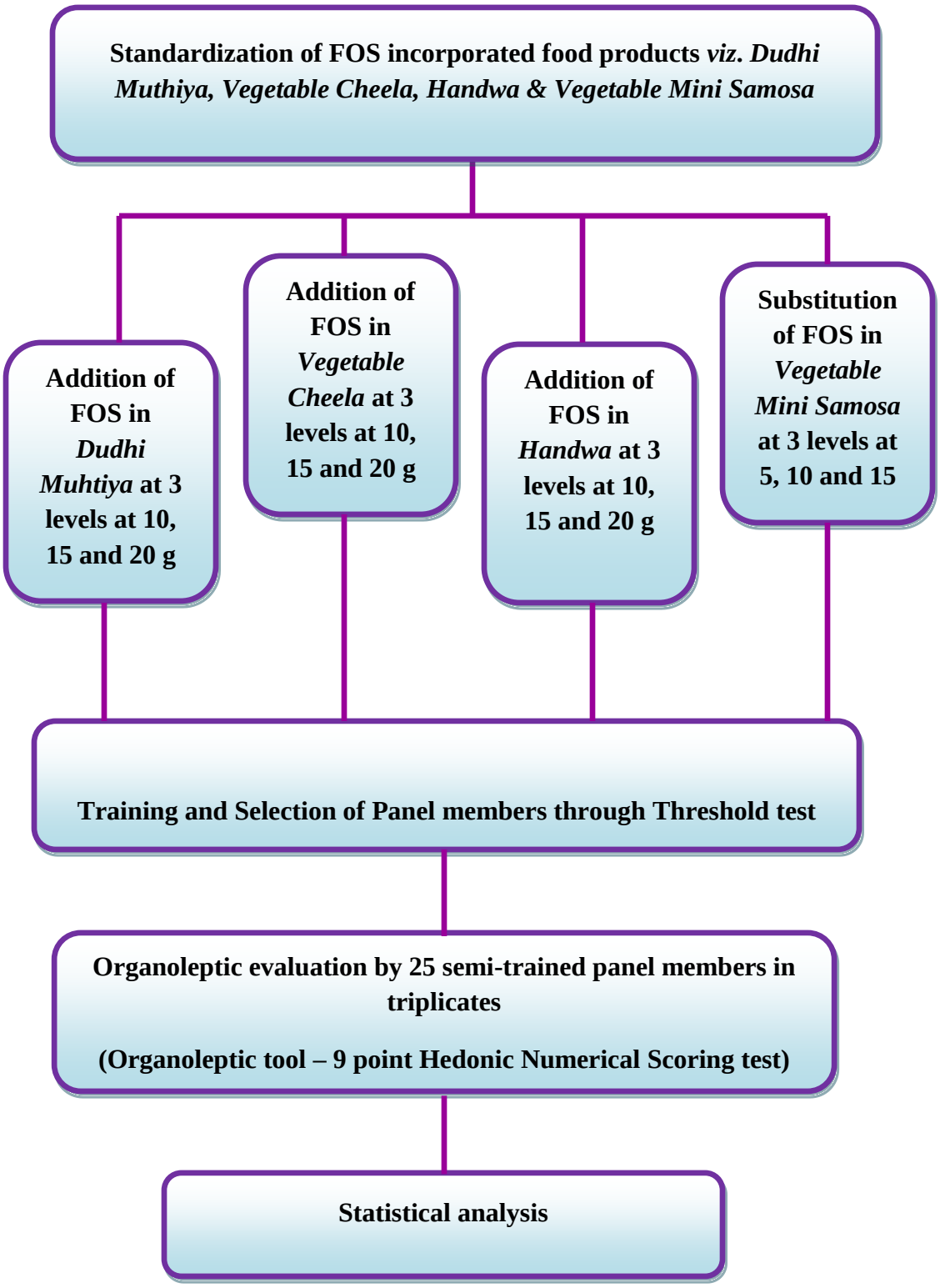


Figure 4.13: Experimental Design of Phase IV of the Study

Table 4.15: Specification and Technical Aspects of FOS Used for Product Development

Items	Results
1. Moisture (w/w %)	2.3
2. Carbohydrate composition (% dry basis)	
Glucose+Fructose+Sucrose	4.1%
Fructooligoscharrides	95.9%
3. Appreance	White powder
4. Granular size	40 mesh pass
5. Ash (w/w %)	Below 0.1
6. Total heavy metal (as pb) ppm	Below 1
7. Arsenic (As As_2O_3) ppm	Not detected (detection limit 1 ppm)
8. Foreign taste and odor	Free
9. Foreign substances	Free
10. Microbiological specification	
Mesophilic bacteria	0
Molds and yeast	0
Coliforms	Negative

Source : Meiji Co. Ltd., 2011

4.1.4: Procurement of raw ingredients

The list of raw ingredients and their sources according to the type of recipe is listed in the Table 4.16

Table 4.16: List of Raw Ingredients Along With Their Sources Used For Product Preparation

Product	Ingredients	Source/ Brand name
Dudhi Muthiya	Bottle gourd	Local vegetable market, Vadodara
	Fenugreek leaves	Local vegetable market, Vadodara
	Fresh coriander leaves	Local vegetable market, Vadodara
	Wheat	Wheat variety GW496
	Maize	D-mart brand
	Split chickpea	D-mart brand
	Ground Oil	Fortune brand
	Curd	Amul brand
	Turmeric powder	Everest brand
	Red chili powder	Everest brand
	Asafoetida	Ramdev brand
	Sesame seeds	D-mart brand
	Sugar	D-mart brand
	Salt	Tata brand
Vegetable Cheela	Yellow split moong dal	D-mart brand
	White split udad dal	D-mart brand
	Fresh Spinach	Local vegetable market, Vadodara
	Onion	Local vegetable market, Vadodara
	Green chillies	Local vegetable market, Vadodara
	Fresh corriander	Local vegetable market, Vadodara
	Cumin seeds	D-mart brand
	Turmeric	D-mart brand
	Salt	Tata brand
	Groundnut Oil	Fortune brand
Handwa	Parboiled rice	D-mart brand
	Split Redgram dal	D-mart brand
	Split chickpea dal	D-mart brand
	Fresh garlic, ginger, chillies	Local vegetable market, Vadodara
	Curd	Amul brand
	Mustard seeds	D-mart brand
	Sesame seeds	D-mart brand
	Turmeric powder	D-mart brand
	Salt	Tata brand
	Ground nut Oil	Fortune brand
Vegetable Mini Samosa	Refined wheat flour [Maida]	Uttam brand, Baroda
	Groundnut oil	Fortune brand
	Salt	Tata brand
Stuffing	Potato	Local vegetable market, Vadodara
	French Beans	Local vegetable market, Vadodara
	Carrot	Local vegetable market, Vadodara
	Spring onion	Local vegetable market, Vadodara
	Green chillies	Local vegetable market, Vadodara
	Green coriander leaves	Local vegetable market, Vadodara
	Red chilli powder	D-mart brand
	Garam masala [Hot spices]	D-mart brand
	Turmeric powder	D-mart brand
	Salt	Tata brand
	Groundnut oil	Fortune brand

4.1.5: Standardization and addition of fructooligosaccharide into selected products

A) Dudhi Muthiya:

To prepare standard product of *Dudhi Muthiya*, flour-mix was prepared using whole wheat, maize and split chickpea in ratio of 3:1:1 in natraj flour grinding machine set on mode-2 and using mesh number 4 to get a coarse flour. To 50g of flour-mix , 1.25g of ginger-garlic-chilli paste (GGC), 5g of fresh fenugreek leaves, 5g of fresh coriander leaves, 5g of sugar, 2.5g of salt, 50g of curd, 1 pinch of turmeric, 1 pinch of red chilli powder was added. After adding all ingredients it was mixed properly and kneaded in form of dough by adding 30 ml of water. Dough was further shaped into cylindrical rolls and was steamed for 15 minutes. As they cooled down, rolls were sliced into 1icnh x 1inch dimension. For garnshing, 10 ml of oil was heated in a small pan and 2 pinches of sesame seeds and 1 pinch of asafetida was added. As they started cracking, the entire content along with oil was poured on steamed *Dudhi Muthiya* as a garnish. Around 144g of yield was obtained for steamed *Dudhi Muthiya*. (Recipe by Professor. Mini Sheth, Guide) (Plate: 4.4). FOS was added at the stage of adding ingredients before kneading into dough at level of 10g, 15g and 20g for acceptability trials

Plate 4.4: Steamed *Dudhi Muthiya*

B) Vegetable Cheela

To prepare standard product of *Vegetable Cheela*, flour-mix was prepared using Yellow split moong dal and white split udad dal in natraj flour grinding machine set on mode-1 and using mesh number 4 to get desired coarse flour. For one serving of *Vegetable Cheela*, 40g of flour mix, 10g of chopped spinach, 5g of chopped onion, 2.5g of fresh coriander leaves, 2.5g of GGC paste, 0.5g of cumin seeds, 0.5g of turmeric and 2g of salt was added. To make a spreadable batter 80 ml of water was added slowly to flour-mix to avoid lumps. This batter was poured in three parts on hot plate and was shallow fried using 5 ml of oil on medium flame for 2 minutes (1 minute for each side) to get a golden crispy layer. Total yield of 3 medium sizes *Vegetable Cheela* was obtained with dimension of 13x13 x3 and total cooked weight of 116 gm (Recipe by Professor Mini Sheth, Guide) (Plate 4.5).

FOS was added at the stage of adding ingredients before stirring into a batter at level of 10g, 15g and 20g for acceptability trials.

Plate 4.5: Coded Shallow Fried *Vegetable Cheela* for organoleptic evaluation

C) Handwa

To prepare standard product of *Handwa*, flour-mix was prepared using parboiled rice, split redgram dal and split chickpea dal in natraj flour grinding machine set on mode-2 and using mesh number 4 to get desired coarse flour. For one serving of *Handwa*, 100g of

flour mix, 10g of chopped spinach, 5g of chopped onion, 2.5g of fresh coriander leaves, 5g of GGC paste, 15g of curd, 5g of sugar, 5g of salt, 1.5g of turmeric and 30ml of oil was added. No water was added to form a thick batter. Batter was allowed to sit for 2 hours for batter to ferment. After 2 hous, it was poured into baking tray and baked at 180 ºC for 20 min. Total yield of approximated 250g was obtained for *Handwa* (Recipe by Professor Mini Sheth, Guide) (Plate: 4.6).

FOS was added at the stage of adding ingredients before stirring into a batter at level of 10g, 15g and 20g for acceptability trials.

Plate 4.6: Coded Baked *Handwa* for organoleptic evaluation

D) Vegetable Mini Samosa

To prepare one serving of standard product of *Vegetable Mini Samosa*, recipe was followed in three steps. First step was to prepare dough for making outer covering of *Vegetable Mini Samosa*, second step was to prepare stuffing and third step was to assemble both, shape them and deep fry *Vegetable Mini Samosa*.

Dough preparation: 75g of refined wheat flour, 2.5 ml oil, 2g salt and 15ml of water was used for kneading it in dough. 3 dough balls were formed from 75g of flour. 4 strips were rolled out from one dough ball (3 dough balls X 4 strips = 12 strips in one serving).

Vegetable stuffing preparation: Ingredients used were 80g potato, 8g green chili, 33g French beans, 33g carrots, 8g spring onion, 15ml oil, 2g salt, 8g fresh coriander leaves, 1 pinch of garam masala (Indian hot spice mix), 1 pinch of red chili powder, 1 pinch of

turmeric powder. Vegetables were finely chopped and cooked in hot oil to get desired flavor and texture.

Assembling product: Dough was rolled thin and flat as much as possible into shape of large circle. It was then cut into strips of 2 inch width across length. Then each strip was initially folded from one corner to form a pocket of triangle shape. 12g of cooked stuffing was added to this pocket. Further it was continuously folded by the rest of the strip till the stuffing is completely covered from all corners and till the strip ends. Same procedure was repeated for rest all strips. 300 ml of oil was poured into deep pan for frying. Oil was heated on high flame till reached its smoking point. Flame was then turned on to medium flame and uncooked *Vegetable Mini Samosas* were deep fried into this oil for 2-3 minutes resulting in golden crispy *Vegetable Mini Samosas*. Total yield of 12 *Vegetable Mini Samosas* was obtained in one serving (Recipe by Professor Mini Sheth, Guide) (Plate 4.7 & 4.8). FOS was added into stuffing at level of 5g, 10g and 15g for acceptability trials.

Plate 4.7: Uncooked *Vegetable Mini Samosa*

Plate 4.8: Deep Fried *Vegetable Mini Samosa*

4.4.6: A. Sensory evaluation of FOS incorporated food products

Sensory evaluation is a scientific way to analyze consumer food products using human senses like sight, smell, taste, touch and hearing and applies principles of experimental design and statistical analysis. Prerequisite of this discipline is panel of human assessors on whom products are tested and responses are recorded by them. Large companies have dedicated sensory analysis department for consumer goods.

Similarly, sensory evaluation of our FOS incorporated products at varying levels *viz Dudhi Muthiya, Vegetable Cheela, Handwa and Vegetable Mini Samosa* was performed. Overall acceptability of the products was also assessed.

4.4.6: B. Evaluation of Physical Characteristics of FOS incorporated food products

Weight of raw and cooked product were recorded using digital weighing balance. Length (cm), width (cm) and Thickness (cm) was measured using a scale. Bulk density (g/cc) and Water absorption power (WAP %) was calculated. Time taken in minutes for shallow frying, Baking, Steaming was also recorded.

Bulk density -It is calculated by dividing the weight of the sample (cooked weight) by the volume (batter / dough).

Water absorption power- This property mainly refers to the amount of water that is required to be added to the flour to make the dough / batter of desired consistency.

Calculation:

$$WAP\ (ml) = W1 - W2$$

Where, W1 = amount of water measured initially; W2 = amount of water left

Yield of cooked product – is calculated by multiplying cooked weight of product with 100 and then dividing by the weight of the standard product.

4.4.7: Selection and training of judges for organoleptic evaluation

Screening of Panelists

Selection of panelist for sensory evaluation of test products was carried out at Department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda. Students pursuing post-graduation and above qualification were invited and interested candidates were subjected to threshold testing to qualify as a panel member.

Threshold test (Ranganna, 1995)

Threshold is defined as a stimulus scale at which a transition in a series or judgment occurs. For conducting this test, score card for the same was formulated and pre tested (Appendix VII (A), (B)). Each perspective panel member was given three sets of the solution i.e. Set 1, Set 2 and Set 3 having six solutions of different concentrations of salt, sugar and citric acid respectively and was arranged in random order (Plate 4.9). The participants were asked to identify and rank the samples in increasing order of concentration of taste from the test solutions offered. Three successive trials were conducted for screening of the panelists. Subjects who succeed to pass the threshold test were included further in evaluating the organoleptic characteristics of the food products.

Plate 4.9 A: Threshold Test Solutions

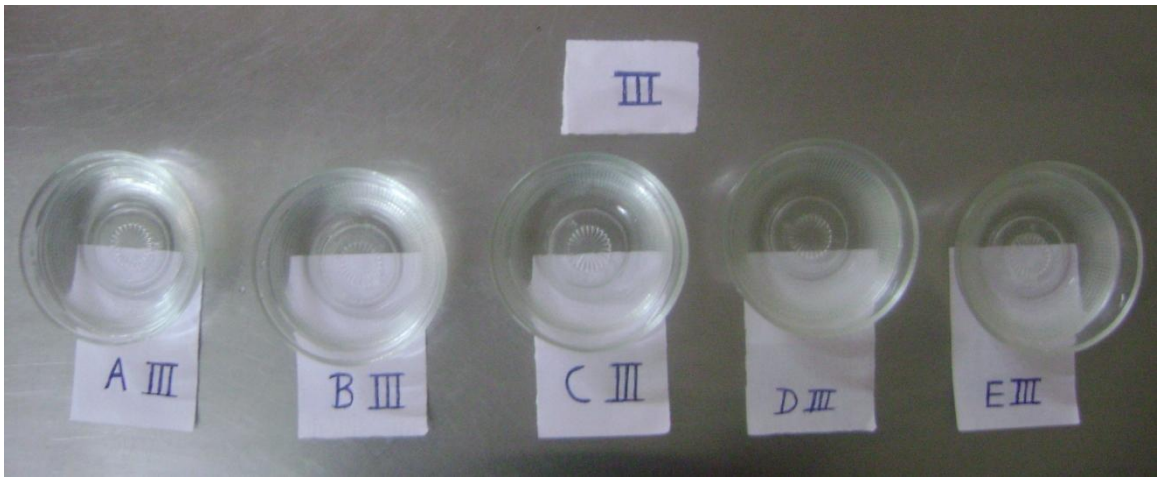


Plate 4.9 B: Threshold Test

Training of the selected panel members

A training tool was developed for imparting the basic knowledge and understanding of visual and organoleptic characteristics of the products. Training was given using the same.

4.4.8: Nine-point Hedonic Scale for Organoleptic evaluation

One of the most reliable and is widely used scale for measuring food acceptability is the 9-point hedonic scale. It was developed at the Quartermaster Food and Container Institute of the U.S. Armed Forces, by David Peryam and colleagues with a primary purpose of measuring the food preferences of soldiers. Immediately this scale gained popularity and was quickly adopted by the food industry.

Presently, this scale is used in wide range of products like personal care products, household products, and cosmetics and not just for measuring the acceptability of foods and beverages. To help justify the practice of analyzing the responses, the property of equal-interval and successive integer values (1,2,3… up to 9) helps in testing differences in average acceptability using parametric statistics.

Hedonic scale has been timely updated and has several extensions. When used with kids the verbal anchors range from "super good" to "super bad" making it "child friendly" and providing better rating performance in 5 – 10 year olds. In some cases "smiley" faces have also been used.

Application of this test was done for visual and organoleptic evaluation. Panelists were asked to evaluate products from "Liked Extremely to Disliked Extremely" and mention score accordingly for essential quality sensory attributes like texture, color, taste, mouth-feel etc (Appendix VIII).

4.4.9: Statistical analysis

Statistical analysis was performed using Microsoft Excel 2007. Results were expressed as mean values ± standard deviations of all the four products. ANOVA and student's t test was performed to determine the significant differences in various levels of FOS added and substituted food products. Percent increase and decrease was also determined to compare the FOS added and substituted products from standard products.

Plate 4.10: Setting Up for Organoleptic Evaluation

Plate 4.11: Panel Conducting Organoleptic Evaluation