11-9-2016

Reproducibility and Reliability of Chromatin Immunoprecipitation in Clinical Research

Kelli McDonough

Reproducibility and Reliability of Chromatin Immunoprecipitation in Clinical Research

by

Kelli McDonough

Thesis

Submitted to the School of Health Sciences

Eastern Michigan University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Clinical Research Administration

Thesis Committee:

Irwin Martin, Ph.D., Eastern Michigan University

Timothy Cornell, M.D., University of Michigan

Thomas Shanley, M.D., Northwestern University

November 9, 2016

Ypsilanti, Michigan

Abstract

Association between histones and DNA is crucial for many cellular functions such as gene transcription and epigenetic silencing. Changes to chromatin structure influence gene expression via histone modifications. Chromatin immunoprecipitation (ChIP) is an experimental technique used to investigate the interactions between histones and DNA. ChIP determines the specific location in the genome that histone modifications are associated with, indicating the target of the histone modifiers. Despite the appeal of ChIP as an in vitro technique, there are limitations to its use. There is variability from one preparation to the next, given the many steps and reagents used throughout the technique. No one has been able to demonstrate consistent results in a clinical population using white blood cells. It was established, for the first time, the normal variability of ChIP results in THP-1 cells, Jurkat cells, TF-1a, and human male circulating leukocytes over time for histone modifications H3k4me3 and H3k9Ac.

CHROMATIN IMMUNOPRECIPITATON

Table of Contents

CHROMATIN IMMUNOPRECIPITATON

List of Tables

List of Figures

Chapter 1: Introduction and Background

Introduction

The term epigenesis first appears in 1651, used by English physician William Harvey (1578–1657) in his *Exercitationes*, and again 1653, in the *English Anatomical Exercitations*. He defined epigenesis to mean the "additament of parts budding out of another" in his argument that all living beings gradually developed from eggs into complex beings based on Aristotle's theory of embryonic development (Zumbo, 2013). From this, C.H. Waddington coined the term epigenetics in 1942, as pertaining to the differentiation of cells from their pluripotent state during embryonic development (Waddington, 1942). Waddington coined this term before the discovery of the physical nature of genes and their role in heredity. Instead, he was theorizing on how genes and their environment interact to produce a phenotype. The word "epigenetics" has been defined and redefined based on the knowledge of biology and genetics at the time. The term has further evolved as contemporary biologists refer to epigenetics simply as how genetic material can be activated or deactivated based on their environment. However, this definition doesn't include processes that may be transient and not heritable. In 2008, a consensus definition was created to define the epigenetic trait as "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al., 2009). Currently, the National Institutes of Health (NIH) Roadmap Epigenomics Project maintains "…epigenetics refers to both heritable changes in gene activity and expression and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable" (NIH, 2010). The NIH definition will be used for the purposes of this study.

Epigenetic Processes

Epigenetics is a method of genetic control by means other than an individual's DNA sequence. Epigenetic changes alter gene expression and determine which genes are transcribed for protein translation. Control of gene expression is important when considering that all of an organism's cells have the same DNA, but cells differentiate into numerous cell types each with unique characteristics and functions based on the patterns of gene expression. Both properties of the DNA and components of the cell's environment influence these expression patterns as is readily apparent in embryonic development. Cells in an embryo have the option to differentiate based on their genetics, but the influences from their environment play an important role in determining their development. Multiple layers of epigenetic influence are required to ensure proper development in the embryo. Epigenetic states have to be first established and then maintained throughout development to ensure maintenance and carry-over of the developmental identity of each cell as the embryo develops (Das, 2016). Cells, tissues, and organs differ because epigenetic control determines which genes are turned off, or repressed, and which genes are turned on, or expressed (Simmons, 2008). Specifically, epigenetic regulation is important for X-chromosome inactivation in female mammals ensuring females do not have twice the number of X-chromosome gene products as males (Egger et al., 2004).

Epigenetic processes are natural and essential in regulating many cellular functions and therefore can contribute to pathology if disrupted, leading to major adverse health and behavioral effects. A growing body of evidence shows that inappropriate epigenetic processes are key contributors to autoimmune diseases and cancer (Wen et al., 2008). Epigenetic mechanisms help establish cellular identities, and failure of the proper preservation of epigenetic marks results in inappropriate activation or inhibition of various cellular signaling pathways leading to pathology

of cancer and other disorders (You & Jones, 2012). Epigenetic processes can be divided into three systems that interact with each other to regulate gene expression patterns related to healthy and diseased states: 1) DNA methylation, 2) RNA-associated silencing, and 3) histone modifications (Egger et al., 2004). This study focused on the epigenetic histone modifications.

Histone Modification

Chromatin is the state in which DNA is packaged within the cell. The nucleosome is the foundational unit of chromatin and is composed of an octamer of four core histones—H3, H4, H2A, H2B—that act as a spool for DNA to wrap around (Simmons, 2008). The core histones are globular except for their N-terminal tails, which are unstructured and can be modified. When histones are modified, they influence how chromatin is arranged in three-dimensional space. This arrangement determines whether the promoter region of a gene that is associated with the DNA will be transcribed, i.e., whether the gene is turned on. When chromatin is condensed, creating the complex called heterochromatin, the promoter region of the DNA is not available for transcription, and the gene is repressed, or turned off. When chromatin is not condensed and the DNA is loosely spooled around the histones exposing promoter regions of genes, DNA transcription can occur, allowing for gene expression (Simmons, 2008).

Several post-translational covalent modifications of the N-terminal tails of each histone have been identified and include acetylation, methylation, ubiquination, sumoylation, and phosphorylation. The two main histone modifications are acetylation and methylation, resulting in adding either an acetyl or methyl group, respectively, to the amino acid lysine within the histone tail. Lysine acetylation is usually associated with active chromatin, while deacetylation

is generally associated with condensed chromatin. On the other hand, histone methylation can be a marker for both active and inactive regions of chromatin (Simmons, 2008). For example, methylation of a particular lysine (K9) on a specific histone (H3) that marks silent DNA is widely distributed throughout heterochromatin. This type of epigenetic change is responsible for the inactivated X chromosome of females. In contrast, methylation of a different lysine (K4) on the same histone (H3) is a marker for active genes (Egger et al., 2004). There are over 60 different residues on histones where modifications have been detected (Kouzarides, 2007). This number is misleading, however, because multiple modifications can take place on a single histone, influencing the 3-D structure of the histone. In addition to multiple modifications, methylation at lysines within the histones may take one of three forms: mono-, di-, or trimethylation (Kouzarides, 2007). The timing of modifications will depend on signaling conditions in the cell based on factors such as an organism's age, diet, and exposure to chemicals.

Histone modifications have been linked to a number of chromatin-dependent processes, including replication, DNA repair, and gene transcription (Karlic et al., 2010). These modifications may be dynamic as the transcriptional state of a gene changes, particularly during cell differentiation. However, the ability of these chromatin modifications to persist through cell division and thus allow a cell to "remember" its transcriptional profile and, by extension, its cellular identity, defines epigenetic regulation (Zediak et al., 2011).

The concept that a gene phenotype can be maintained over a long period of time and is heritable through histone modification is thought to be due to the fact that the DNA is not completely stripped of its nucleosome after replication. This means the remaining modified histones can act as templates to initiate the same modification of new histones during replication

(Turner, 2002). Thus, any modification to the histones can be transferred to daughter cells during replication allowing for the transfer of the "histone code."

Figure 1 demonstrates how DNA is packaged into nucleosomes and how modifications to the histone tail dictate gene activity. Chromatin structure is dynamic in that it constantly changes in response to external stimuli. Substantial evidence has been accumulated to suggest that the cellular state is closely related to the chromatin state, particularly modifications of histones. In addition to defining and controlling gene expression patterns, chromatin modifications also determine a cells' response to environmental or developmental cues regarding its transcriptional output (Jayani et al., 2010).
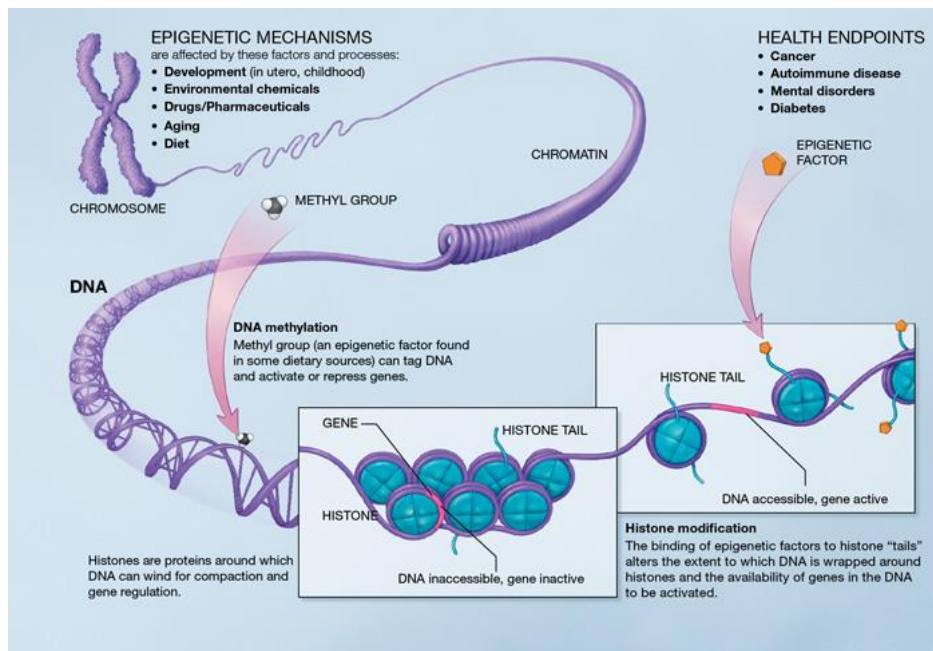
Figure 1. Histone modification occurs when the binding of epigenetic factors to histone "tails" alters the extent to which DNA is wrapped around histones and the availability of genes in the DNA to be activated (NIH, 2013).

Histones

This study focused on two specific histone modifications: methylation of lysine 4 on histone 3 (H3k4me3) and acetylation of lysine 9 on histone 3 (H3k9Ac). H3k4 was first discovered in trout testes in 1975, ten years after lysine methylation of histones was first described (Honda et al., 1975; Murray, 1964). Subsequent studies linked H3k4 to transcriptional activation in a variety of eukaryotic species (Ruthenburg, 2006). It is thought that high levels of H3k4me3 are associated with the promoter regions of most active genes and that there is a strong positive relationship between this modification, transcription rates, and histone acetylation (Eissenberg & Shilatifard, 2009). Methylation may affect DNA accessibility in two ways: by affecting a conformation change of the histone directly or by creating a binding site for proteins that will then alter the properties of the chromatin for transcription (Eissenberg & Shilatifard, 2009).

One of the most studied modifications of histones is acetylation of specific lysine (K) residues, which generally correlates with gene activation. The level of histone acetylation is regulated by the activity of both histone acetyl transferases (HATs) and histone deacetylases (HDACs), which acetylate and deacetylate lysine residues of the N-terminal histone tails, respectively (Karmodiya, 2012). Histone acetylation plays essential roles in transcriptional initiation and elongation and is known to open the chromatin structure at the respective promoter sites to activate transcription (Hansen, 2006). H3K9Ac, which is a histone modification associated with open chromatin, is enriched at gene promoters with various genomic features and highly correlates with gene expression (Qiao, 2014). H3K9Ac is present at different sets of gene promoters associated with cell identities and enables active transcription with cell type-specific patterns (2014).

Several methods have been developed to address questions concerning the interactions involved in chromosome structure and nuclear organization. Histones are part of maintaining the genomic landscape within the cell (Jayani et al., 2010). The spatial and temporal changes involved in histone modifications dictate the active, inactive, or poised status of a particular gene. Earlier methods developed to study the DNA-protein interactions involve *in vitro* methods; however, these have limited use because they do not operate within the context of the cell (Das et al., 2004). Chromatin immunoprecipitation (ChIP) has become a widely used technique in determining the location of various transcription factors, histones, and other proteins.

Chromatin Immunoprecipitation

A key determinant in deciding the merit of a technique used to study histone modifications and gene activity is the ability to provide direct evidence that proteins are associated in time and space with specific genome regions (Orlando, 2000). Chromatin immunoprecipitation (ChIP) has become the technique of choice to investigate protein-DNA interactions inside the cell (Collas, 2010). The principle of the ChIP assay is demonstrated in Figure 2. DNA and proteins are cross-linked with formaldehyde to covalently attach proteins to DNA sequences. The formaldehyde cross-links the proteins and DNA very closely to each other, thus making the assay suitable for looking at proteins, such as histones, that bind directly to DNA. Because the proteins are captured at the sites of their binding with the DNA, ChIP helps to detect DNA-protein interactions within living cells. The chromatin is then fragmented using sonication, into segments of 200–1000 base pair (bp). Antibodies specific to the protein of interest are used to precipitate protein-DNA complexes. The cross-link is reversed, the protein is

digested, and the DNA is released from the complex, purified, and analyzed using various

follow-up techniques (Collas, 2010).
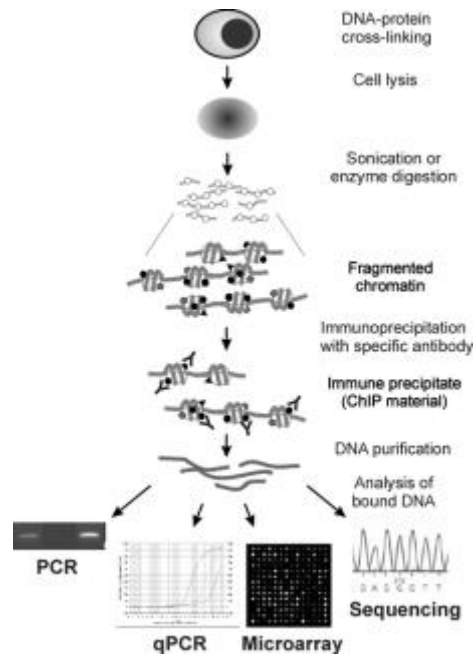
Figure 2. Chromatin Immunoprecipitation (ChIP) assay and analysis techniques (Collas, 2010). Copyright by Humana Press. Reprinted with Permission.

For a long time, a major disadvantage of ChIP has been the requirement for large cell

numbers, typically 20 to 30 million cells. This large cell number is necessary to compensate for

the loss of cells after cross-linking, as well as the overall inefficiency of ChIP. The need for large

cell numbers has limited the application of ChIP in sample from humans, potentially preventing

its use in non-tissue clinical samples. Alterations to conventional ChIP protocols are aimed at

making the technique applicable to smaller cell numbers (Collas, 2010).

Purpose of the Study

While ChIP is a versatile technique, it requires the optimization of several processing

conditions for successful DNA isolation (Das, 2004) and subsequent accurate results. For almost

two decades, ChIP remained a cumbersome protocol, requiring several days and large numbers

of cells—multimillion cells per immunoprecipitation. ChIP assays typically involve extensive sample handling leading to a loss of DNA and allowing for technical errors and decreased consistency between replicates (Collas, 2010). This limits our understanding of ChIP results reported in scientific literature, typically reported as fold changes or a percentage of DNA input. It is important to determine if the results of ChIP assays are biological or technical in nature. The vast majority of studies done using ChIP in the clinical setting involve tissue biopsies, with ideal yields of 10 million cells (O'Neill, 2006). This may mask any technical issues and inconsistencies between samples. With the development of less invasive biopsy techniques and clinical diagnostics requiring less biological material, it becomes imperative that ChIP assays adapt to fewer cells. One aim of this study was to demonstrate the biological and technical variability present when using a small cell number in ChIP assays.

Because epigenetics has become an expanding field of study in immunology, studies indicate that epigenetic mechanisms can govern immune responses, productive and deleterious. In particular, histone methylation has been implicated in the development of acquired immune responses, such as the long-term suppression of the host's immune system after severe sepsis (Wen, 2008). However, most of this evidence has been acquired using *in vitro* studies associated with T cell activity. Recent studies conducted on white blood cell (WBC) populations, particularly peripheral blood mononuclear cells (PBMCs) from human subjects still use large cell numbers (>10 million), requiring 10 milliliters of blood (Zhang, 2009; Wen 2008). In a healthy adult, there is a range 0.84–2.64 x 10^6 PBMCs per mL of whole blood (Mayo Clinic, 2016). That value increases in the pediatric population, from 1.5 x 10^6 to as many as 1.1 x 10^7 cells/mL (Mayo Clinic, 2016). That number drops precipitously with an acute immune response as the cells leave the peripheral blood to respond to the source of inflammation. Based on extensive

literature searches using PubMed and sources cited within scientific papers, there are no studies demonstrating consistent ChIP results in a clinical population using WBCs in small numbers. Those few studies done on WBCs give no indication of what researchers can expect for a control or "normal" individual. The second aim of this study was to demonstrate that variability in small cell number ChIP assays on WBCs is due to individual changes over time while defining normal ranges for subjects serving as controls.

Significance of the Study

Establishing a normal range from ChIP assays performed on control organisms becomes important when considering the fact that many therapeutics, particularly in cancer, involve disrupting histone modifications. Histone deacetylase inhibitors (HDACi), in particular, have been intensely scrutinized for the past decade. As previously described, acetylation has been linked to a chromatin state that is poised for transcription or that corresponds to actively transcribed genomic regions (Minucci & Pelicci, 2004). The HDACi are a small group of molecules that target histone deacetylases (HDACs), preventing those genomic regions associated with acetylated histones from being repressed. Many HDACi have entered Phase I through III clinical trials and two, vorinostat and romidepsin, have the approval of the Food and Drug Administration (FDA) in the treatment of lymphomas (Slingerland, 2014). Epidrugs are drugs that inhibit or activate disease-associated epigenetic proteins for ameliorating, curing, or preventing the disease (Ivanov et al., 2014). Table 1 shows HDACi epidrugs currently in clinical use and under investigation. HDACi are also currently under investigation for the treatment of schizophrenia (Hasan et al., 2013). In the field of immunology, HDACi are under investigation

for the treatment of rheumatoid arthritis, systemic mastocytosis, acute myeloid leukemia, and

lupus (Grabiec et al., 2012; Abdulkadir et al., 2015; Lubbert, 2015; Guo et al., 2014).

Table 1. *Epidrugs Currently Approved or in Clinical Trials* (Ivanov et al, 2014).

HDAC Inhibitors	Clinical Application
Valproic Acid	Promising nontoxic and effective therapy for MDS in combination with hydralazine (Phase II trial)
	Under evaluation in metastatic cervical cancer in combination with hydralazine (Phase III trial)
	Combined with ada-dC for non-small-cell lung cancer (Phase I trial)
Suberoylanilide hydroxamic acid (vorinostat, Zolinza)	Approved by the FDA in 2006 for treatment of advanced cutaneous T-cell lymphoma
	Reversed hormone resistance in patients with ER metastatic breast cancer, when co-administered with tamoxifen (Phase II trial)
Romidepsin (depsipeptide Istodax)	Approved by the FDA in 2009 for treatment of advanced cutaneous T-cell lymphoma
Sodium butyrate	Induces antimicrobial peptide LL-37 in the rectum of shigellosis patients (Phase II trial)
Panobinostat	Promising results in monotherapy of heavily pretreated Hodgkin's lymphoma patients (Phase II trial)
	Recaptures responses in bortezomib-resistant multiple myeloma patients (Phase II trial)
Entinostat	Improved survival in women with ER advanced breast cancer when added to exemestane (Phase II trial)
Mocetinostat	Promising effect in monotherapy of relapsed Hodgkin's lymphoma (Phase II trial)
Selisistat	Under evaluation for treatment of Huntington's disease (Phase II trial)

Histone methyltransferases are being investigated in relation to gliomas, autoimmune

disorders, breast cancer, and pulmonary viral infections (Erfani et al., 2015; Xia et al., 2013; Salz

et al., 2015; de Almeida Nagata et al., 2015). The primary method in determining the activity of

these histone modifiers is ChIP. Understanding the impact any of these molecules may have on

epigenetic processes may be crucial. For example, HDACi are not without risks; HDACs are an

integral part in transcriptional silencing of HIV and inhibiting them results in a disruption of HIV

latency (Shirakawa, 2013). Understanding the potential risks, as well as the benefits, is essential to the success of any of these molecules as clinical treatments.

Blood volume was another area of potential significance for this study. In previous studies, 10 mL of blood was taken from individuals in order to get enough cells to satisfy the demands of the ChIP assay. However, if an investigator wanted to use the ChIP assay to look at histone modifications in the presence or absence of a drug or other physiological stimuli in the pediatric population, that would pose a significant problem. Most, if not all institutional review boards (IRB) impose limits on how much blood may be removed from an individual over a three-month period based on body weight. If blood is needed for multiple diagnostics, it becomes essential that ChIP assays can be done in smaller cell numbers.

Limitations of the Study

The ChIP assay is useful for studying DNA-protein interaction, but it is more of a qualitative study than a quantitative approach. It is possible to determine if a specific DNA sequence is associated with a specific protein, but it's difficult to establish that the association is true for every cell in the cell lysate. The quantification of DNA may be misleading because the crosslinking with formaldehyde is never complete. If that were the case, it would be impossible to isolate and purify DNA fragments from the cross-linked nuclear DNA. This means that there will be variability in the crosslinking from one sample preparation to the next. Another factor is the immunoprecipitation step. From one experiment to the next, the antibody may not bind efficiently to the entire antigen in the sample. Finally, there may be variability in the washing step. Washing the beads can result in the removal of the antibody or the antigen from the beads (Das et al., 2004).

In addition to limitations in the ChIP assay, there are limitations to this study. The use of cells in culture may provide a different picture than that of primary cells isolated from an organism. Cell lines commercially available for culturing are immortalized or transformed due to a genetic alteration that allows them to divide indefinitely. The sources of these cell lines are often cancers and after a period of growth in culture, cell characteristics can change. This can result in inconsistent ChIP data, as there is already a shift in the genetic and epigenetic machinery in these cell lines. Using primary cells from human subjects presents its own challenges. A study to determine the normal ranges of histone modifications in healthy subjects may require a number larger than used in this investigation.

Chapter 2: Review of Related Literature

Chromatin immunoprecipitation (ChIP) is a powerful tool in the application of antibody techniques to epigenetic research providing researchers a detailed analysis of histone modifications. However, conventional ChIP requires large numbers of cells, limiting its application to cell culture models that provide large numbers of homogenous cells (O'Neill et al., 2005). This study focused on primary cells isolated from whole blood, specifically peripheral blood mononuclear cells (PBMCs).

A small number of studies conducted in the last ten years used PBMCs in their investigations into epigenetic modifications using ChIP-qPCR. Bosch and her associates (2002) conducted one of the earliest studies in PBMCs by collected 120 mL of blood from healthy human volunteers to study interleukin-8 (IL-8) transcription regulation in response to *in vitro* stimulation to dengue fever. They showed increased IL-8 transcription and production using western blot to demonstrate an increase in histone acetylation. In 2007, Miao and associates isolated 50 mL of blood from adults characterized as healthy, with type I diabetes, or type II diabetes. They reported their ChIP data in arbitrary units, comparing healthy adults to those with either form of diabetes, demonstrating that H3k4me2 distribution patterns are comparable among individuals (Miao, 2007). Sullivan (2007) used monocytes isolated from an undisclosed volume of blood drawn from adult patients diagnosed with lupus and healthy volunteers. She expressed her data as normalized units, doing a side-by-side comparison of patient versus control in their expression of tumor necrosis factor alpha (TNFα) to show that H4 acetylation and H3k4me3 increased early in transcription. In 2008, Miao again used 50 mL of blood from healthy volunteers to study histone modification at core genes, using ratios to analyze ChIP data. Zhang (2009) collected 10 mL of blood from 15 adults suffering from minimal change nephrotic

14

syndrome, an immunologically mediated disease. This trend of using large volumes of blood continues presently.

Only two studies have used smaller volumes of blood. The first was in 2008 by Wen, who isolated T cells from mouse spleen to investigate the relationship between histone modifications and interleukin-12 expression. Wen and his colleagues cross-linked 1 million cells for their ChIP assays, similar to the aims of this study. They also expressed their results as a percent of total DNA. However, their numbers raise questions. In many of their assays, the percentages calculated range from 0.1 to 0.8% to show histone status at the promoter regions of their genes of interest. In the process of doing a literature search, it was clear that most reports of percent input in ChIP assays range from 10% to 80%, as in Gurvich's study from 2004 and in Gemelli's study in 2014. This raises questions on just how Wen chose to calculate his data. Also, Wen used a 2% input DNA isolation for use in their qPCR rather than the standard 1%. Using more input DNA drives the percent input value down.

In the second study, Cornell et al. (2012) isolated PBMCs from whole blood using a protocol similar to the one used in this study. They collected 4 mL of blood from pediatric patients undergoing cardiopulmonary bypass (CPB) into cell preparation tubes to separate PBMCs from red blood cells. However, in analyzing the ChIP data, fold changed, rather than percent input, was used to compare post-CPB to pre-CPB. This method of analysis was appropriate for the purposes of that study. However, it presents no insight as to what "normal" values look like in healthy individuals. Nor does it, or any study, show a temporal profile for histone modification changes over time in healthy individuals.

Chapter 3: Research Design and Methodology

Reproducibility

Due to the inherent variability involved in any *in vivo* study, it is important to demonstrate the reproducibility of chromatin immunoprecipitation (ChIP) using a cell culture model. This provides investigators with a baseline for determining if variability seen in human participant samples is due to technique or biological variability. Using a ChIP assay suitable for low cell numbers from Dahl and Collas (2007) as shown in Figure 3, changes in two histone modifications on the promoters of three genes regulated by the immune environment were monitored. ChIP was used on three cell culture lines, Jurkat (Clone E6-1), THP-1, and TF-1a cells (American Type Culture Collection, Manassas, VA, www.atcc.org), to demonstrate the reproducible nature of the technique in controlled cell populations.

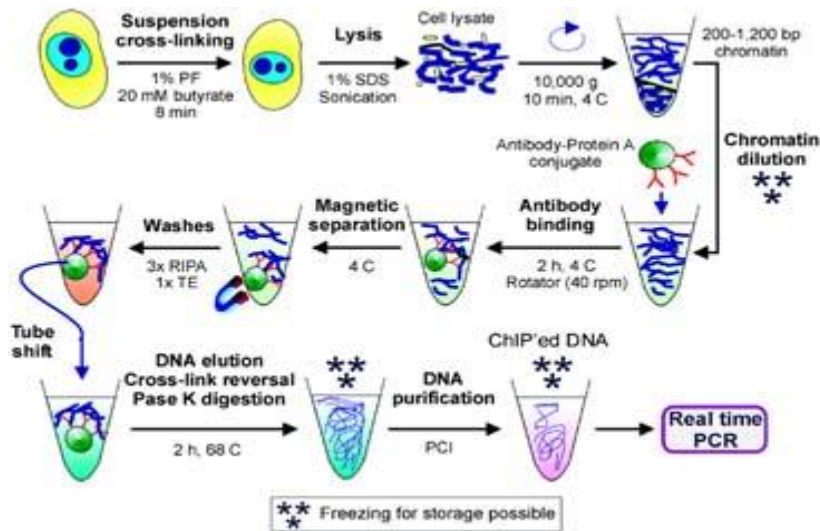*Figure 3.* Summary of the ChIP procedure (Dahl and Collas, 2007). Copyright 2007 by John Wiley and Sons. Reprinted with permission.

Jurkat cells, T cells established from acute T cell leukemia, were cultured in RPMI-1640 supplemented with 10% fetal calf serum. Cells were passaged every 3 days such that cell density did not exceed 3×10^6 cells/ml. THP-1 cells, monocytes derived from acute monocytic leukemia,

were cultured in RPMI-1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol to a final concentration of 0.05 mM. Cells were passaged every 3 days so as to maintain a cell density between 4×10^5 and 8×10^5 cells/mL. TF-1a cells, lymphoblasts established from erythroleukemia, were cultured in RPMI-1640 supplemented with 10% fetal calf serum. Cells were passaged every 3 days to maintain a cell density between 3×10^5 and 3×10^6 cells/mL. These cell lines were chosen to mimic the cell types found when isolating peripheral blood mononuclear cells (PBMCs) from human participants.

DNA-Protein Crosslinking. Cells were harvested and processed as follows. Each of the cell lines was cultured in 150 cm^3 tissue culture flasks, in a total volume of 75 mL of media. Within 12 hours of passage, two aliquots of $1\text{-}2 \times 10^6$ cells were isolated from the flasks and the volume of cells removed was replaced with fresh media. Subsequent aliquots were removed from the flasks the following two days, for a total of 6 aliquots from 3 consecutive days from the same passage. Cells were centrifuged and washed with phosphate-buffered saline (PBS), then resuspended to $1\text{-}2 \times 10^6$ cells per 500 microliters of PBS. Formaldehyde was added to 1% (vol/vol) and allowed to fix the cells for 10 minutes at room temperature. The reaction was stopped with 125 mM glycine for 5 minutes at room temperature. Cells were placed on ice or at 4°C and processed as follows. Cells were washed by centrifugation and suspension in PBS. Cells were lysed for 5 minutes in 150 uL of lysis buffer (50 mM Tris-HCL, pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitor cocktail, 1 mM PMSF) containing 20 mM sodium butyrate. The cells were then flash frozen in liquid nitrogen and stored at -80°C.

Antibody-Bead Complexes. Antibodies against H3k9Ac (catalog number C15410004) and H3k4me3 (catalog number C15410003-50) were acquired from Diagenode (Denville, NJ, www.diagenode.com). Paramagnetic beads (Dynabeads Protein A; ThermoFisher Scientific,

CHROMATIN IMMUNOPRECIPITATON

Waltham, MA, www.thermofisher.com) were washed twice in Pierce RIPA buffer

(ThermoFisher Scientific) and resuspended in 1 volume of RIPA buffer. Beads (10 μL) were

added to 90 μL of RIPA buffer and 2 μg of primary antibody in a 0.2 mL tube and incubated on a

rotator (HulaMixer; ThermoFisher Scientific) for 2 hours at 4°C. The tubes were in 8-tube strips

and handled in parallel in an aluminum magnetic rack (DiaMag 0.2 mL, Diagenode) and chilled

on ice.

Sonication. One aliquot from each day of cell isolation was sonicated for 15 x 30 seconds

in ice-cold water (Bioruptor; Diagenode) with 30 second pauses to produce chromatin fragments

of ~200 base pairs. The lysate was centrifuged at 10,000 x g for 10 minutes, and the supernatant

was collected into fresh tubes. Chromatin was diluted to a concentration of $1-2 \times 10^6$ cells/mL.

Immunoprecipitation. The tubes containing the antibody-bead complexes were placed in

magnets to hold the complexes to the tube wall. The supernatant was removed and replaced with

100 μL of sheared chromatin containing material from 10^5 cell equivalents. The beads were

released into the chromatin suspension and rotated at 40 rpm overnight at 4°C.

Washes. Immune complexes were washed three times by capturing the beads with the

magnet and releasing them in fresh 100 μL of RIPA buffer and once in TE buffer (10 mM Tris-

HCl, pH 8.0, 10 mM EDTA). Each wash lasted 4 minutes at 4°C.

DNA Elution, Crosslink Reversal, Proteinase K Digestion. Chromatin suspended in TE

was transferred to a new 1.5 mL tube to reduce background in the end product. The beads were

captured by magnet (DiaMag 1.5 mL, Diagenode), the TE was removed, and 100 μL of elution

buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM sodium butyrate, 50mM NaCl)

containing 1% SDS and 50 μg/mL proteinase K (Sigma-Aldrich, St. Louis, MO,

www.sigmaaldrich.com) were added. Samples were incubated for 2 hours at 68°C on a

Thermomixer (Eppendorf North America, Hauppauge, NY, www.eppendorf.com) at 1300 rpm. After capturing the beads, the supernatant was recovered for DNA isolation. ChIP is assumed to precipitate less than 1% of nucleosomes, so a 1/100 dilution of the diluted sheared chromatin was treated to DNA elution, crosslink reversal, and proteinase K digestion in parallel with the immunoprecipitated samples.

 DNA Isolation. The DNA was extracted by adding 180 μL Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, www.beckmancoulter.com) to the chromatin to incubate for 2 minutes. The beads were washed twice by capture and release into 70% ethanol. After the last wash, the beads were allowed to dry to ensure the removal of the ethanol. The beads were then resuspended in UltraPure water (ThermoFisher Scientific). The beads were captured again and the supernatant, containing DNA, was recovered.

 Polymerase Chain Reaction. Input DNA and immunoprecipitated DNA were analyzed in triplicate by real-time PCR on a StepOnePlus Real-Time PCR System (ThermoFisher Scientific) using *Power* SYBR Green PCR Master Mix (ThermoFisher Scientific). PCR Conditions were 95°C for 3 minutes and 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The ChIP assay was repeated 24 hours later on the second aliquot from each day of cell isolation as a means of examining technical reproducibility.

 The DNA primers chosen for real-time PCR, shown in Table 2, are the promoter regions for cytokines that regulate inflammation. Tumor necrosis factor alpha (TNFα) has a major role in early inflammatory responses as a pro-inflammatory cytokine (Sullivan, 2007). Interleukin-10 (IL10) is also an important cytokine in the regulation of immune responses. It serves mainly as an anti-inflammatory cytokine, functioning to inhibit the production of pro-inflammatory cytokines. IL10 is also noted to have pro-inflammatory capabilities, promoting B cell

proliferation and antibody production (Larsson et al., 2012). Interleukin-12 (IL12) is another important cytokine that is important in the pro-inflammatory immune process. It is composed of two subunits, p35 and p40, and its protein is functional as a heterodimer of p35 and p40 as well as a homodimer of p40 (Kalinski et al., 1997). Primer pairs were used to amplify the promoters of TNFα, IL12 p35, and IL12 p40; two primer pairs were used to amplify the promoter of IL10, IL10-233, and IL10-531.

Table 2. *Real-time ChIP Primers Used in This Study*.

Gene	Forward primer 5' – 3' Reverse primer 5' – 3'
TNFα kB3	**TACCGCTTCCTCCAGATGAG** **TGCTGGCTGGGTGTGCCAA**
IL-10 233	GCTGTAATGCAGAAGTTCATGTTC AGGGAGGCCTCTTCATTCA
IL-10 531	GGGACAGCTGAAGAGGTGGA CCTCAAAGTTCCCAAGCAGC
IL-12 p35	**CCTGGCATCTAGTGAGCCAT** **GTGTCTAAGCTACCCCGCC**
IL-12 p40	**TATTCCCCACCCAAAAGTCA** **CTGCTGTTGCTGGTACTGGA**

Reliability

Once reproducibility was demonstrated in cell culture, investigation continued in a group of five healthy participants, all Caucasian males aged 25–50. Stored samples were obtained from a study, HUM00074806, approved by the IRB at the University of Michigan (Appendix A). Eastern Michigan University IRB determined that this study did not require review because samples were previously obtained (Appendix B). Blood was drawn from participants on three consecutive days and then once a week for three weeks. Four milliliters was drawn via venipuncture in a laboratory setting on each day and placed into cell preparation tubes (CPT; Becton Dickinson, Franklin Lakes, NJ, www.bd.com) with sodium citrate. Peripheral blood

mononuclear cells (PBMCs) were isolated from these blood samples for use in ChIP by

centrifuging tubes at 3000 rpm for 30 minutes. The plasma layer was discarded and cells in the

remaining buffy coat were removed to 1.5 mL tubes. The cells were counted and divided into

aliquots of 2 x 10^6 cells per milliliter. The samples were then immunoprecipitated using the ChIP

assay detailed above. One aliquot from each individual's six time points were

immunoprecipitated in parallel to minimize any technical variability.

Statistical Analysis

      % of Input. Once the ChIP assay was complete, real-time PCR (qPCR) was performed on

each of the cell lines using all the above DNA primers. The qPCR technique did not quantify the

amount of PCR product at the end of the PCR reaction. Rather, the initial amount of template

DNA was calculated from the kinetics of the PCR reaction. The accumulation of PCR product

was measured every cycle using DNA-dye based detection chemistry (Haring et al., 2007). In

this study, *Power* SYBR green was used as it fluoresces when bound to double-stranded DNA

and the amount of fluorescence was proportional to the amount of double-stranded DNA. The

number of cycles needed to reach a certain amount of PCR product, the cycle threshold or CT

value, was used in calculations. The efficacy of chromatin immunoprecipitation of a particular

genomic locus was calculated as a percentage of starting material:

      % (ChIP/ Total input) = 2^[(Ct(x%input) – log(x%)/log2) – Ct(ChIP)] x 100%

Ct(ChIP) and Ct(x%input) were CT values obtained from qPCR for the immunoprecipitated

sample and input sample. Log(x%)/log2 accounts for the dilution of the input, in this study the

dilution is 1:100, so the value was 6.6. The study used percent input for all of the statistical

analysis.

In understanding the qPCR data from the cell culture portion of the study, two means of analysis were performed. First, all percent input values were aggregated to study how genes behaved at either histone modification site. The mean, standard deviation, and coefficient of variation were calculated. Second, a paired t test were applied to the two replicates from each day of cell isolation to determine whether the mean of the differences between the two match samples varies significantly.

In analyzing the qPCR data from the human samples, the data points for each individual were paired, in that several observations were linked to a specific individual. This was directly related to the fact that there was a genetic component to the response. This automatically eliminated any independent sample tests. There were more than two observations linked to an individual, which ruled out two sample testing. Because it was assumed that data meet assumptions of normality and homoscedasticity, two-way ANOVA was the preferred method of statistical analysis for understanding the influence of individual epigenomics and time on histone modification at each gene promoter location. In addition, the mean, standard deviation, and coefficient of variation were calculated for each participant in order to understand the reliability of the ChIP assay in normal human participants.

Chapter 4: Presentation and Analysis of Data

The first step in analyzing the chromatin immunoprecipitation (ChIP) assay is to

determine the efficacy of the cell shearing. After the shearing step, a 10 μl aliquot of chromatin

was removed from each tube and digested with proteinase K at 55°C for an hour. The chromatin

was then loaded on a 2% agarose gel, along with a 100 bp ladder. Using electrophoresis,

fragments of DNA were separated by size. As shown in Figure 4, the bands across cell types

were consistently under 500 bp in length, indicating successful chromatin shearing.

500 bp ➔

Jurkat   **TF-1a**   **THP-1**
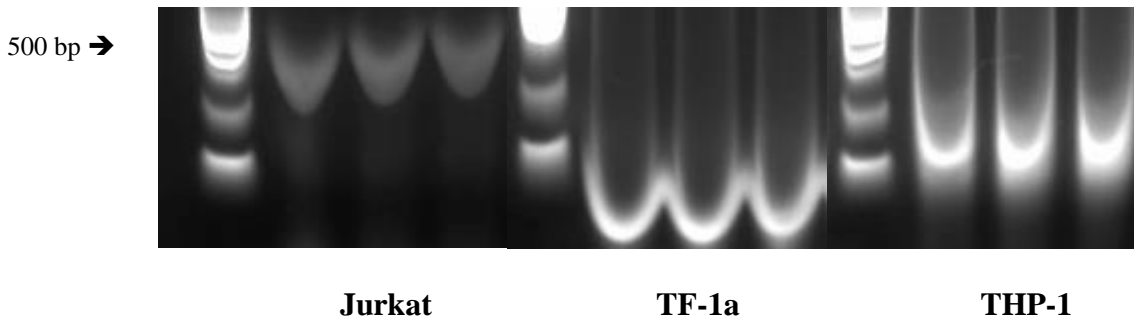
Figure 4. Image of sheared DNA from the three cell lines.

The percent input values for all three cell isolations and their two aliquots were combined

to calculate the overall mean for each cell line, at each gene's promoter region(s) at each histone

modification site. Figure 5 illustrates the mean and standard deviation for each target of interest

in THP-1 cells. One pattern that presents itself is the higher percentage of input at each promoter

for H3k4me3 compared to that of H3k9Ac. This may be due the fact that histone acetylation is

thought to strongly correlate with gene activation while histone methylation is associated with

both gene activation and repression. Because the cells are believed to be in a homeostatic

environment with no immune system signals in which to respond, gene activity is minimal.
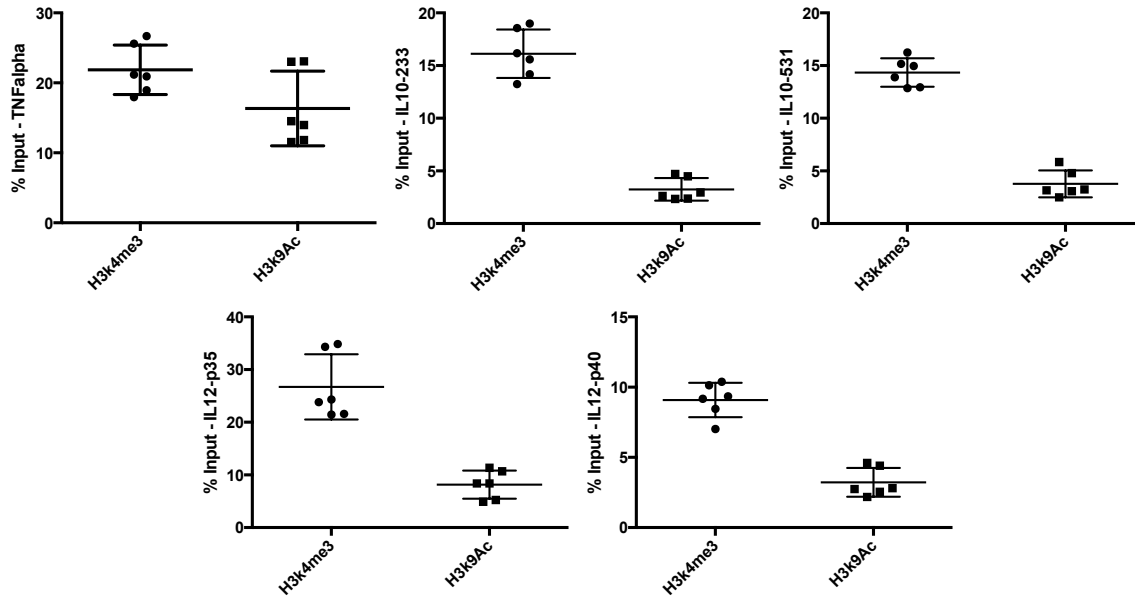
Figure 5. Mean with standard deviation of all ChIP assays on THP-1 cells.

Looking at the numerical analysis in Table 3, the standard deviation ranges from 1.3 to 6.2% for H3k4me3 and 1.1 to 5.3% for H3k9Ac. The coefficient of variation has a relatively wide range at H3k4me3, 9.4 to 23.2%. This suggests that there is some precision in the assay based on the relatively low percentages, and it is reproducible with good success. The coefficient of variation for H3k9Ac suggests that there is less precision, with values in the 30s, but the assay is highly reproducible given that the range in numbers is relatively tight.

Table 3. *Statistical Analysis of THP-1 Cells at Each Promoter and Histone Modification.*

H3k4me3	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	21.88	16.13	14.34	26.73	9.084
Standard Deviation	3.533	2.306	1.346	6.201	1.227
Coeff. Of Variation	16.15%	14.30%	9.38%	23.20%	13.50%
H3k9Ac	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	16.32	3.25	3.768	8.161	3.213
Standard Deviation	5.334	1.071	1.278	2.669	1.024
Coeff. Of Variation	32.68%	32.97%	33.91%	32.70%	31.88%
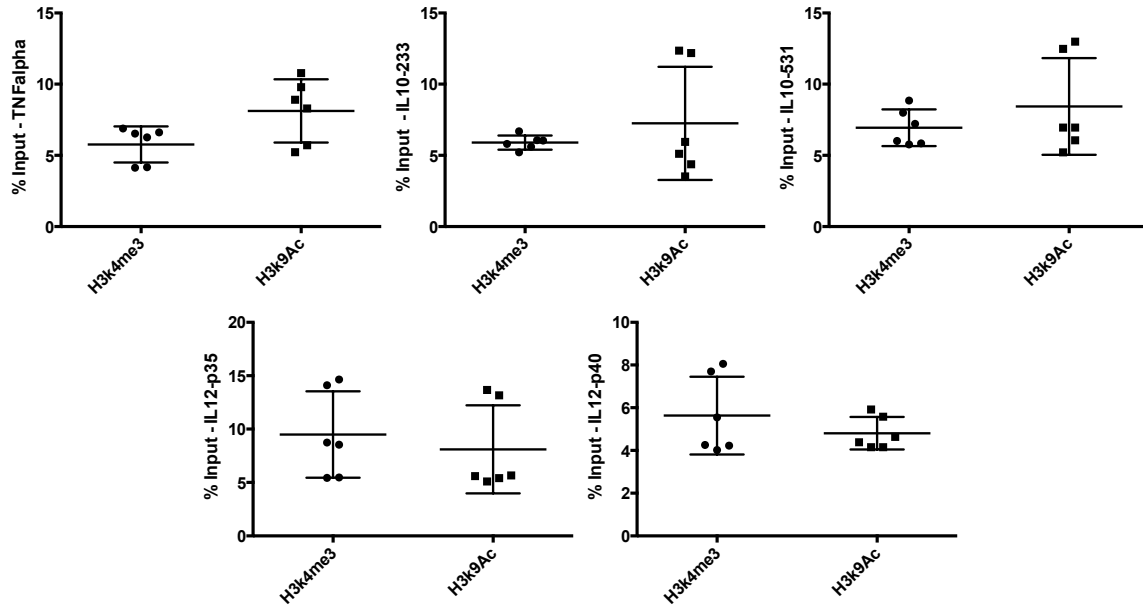
Figure 6. Mean with standard deviation of all ChIP assays on Jurkat cells.

Analysis of the Jurkat cells, in Figure 6 above, again shows minimal gene activity. However, there is a wider spread between the values suggesting less success in repeating the assay than in THP-1 cells. The numerical data, in Table 4, supports this belief. The wide ranges in the coefficient of variation for both H3k4me3 and H3k9Ac demonstrate a lack of precision and reproducibility in this cell line. Because of the many variables inherent in the ChIP assay, it's difficult to pinpoint a reason as to the high variation in some gene targets and not others.

Table 4. *Statistical Analysis of Jurkat Cells at Each Promoter and Histone Modification.*

H3k4me3	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	5.764	5.9	6.944	9.491	5.633
Standard Deviation	1.267	0.4964	1.287	4.046	1.823
Coeff. Of Variation	21.98%	8.41%	18.54%	42.64%	32.35%
H3k9Ac	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	8.117	7.249	8.438	8.098	4.803
Standard Deviation	2.222	3.972	3.391	4.125	0.7613
Coeff. Of Variation	27.38%	54.78%	40.19%	50.94%	15.85%

In looking at the TF-1a ChIP assays, there is still minimal gene activity, as shown by the percent input in Figure 7. There is less variability at each gene than in Jurkat cells, but more than in THP-1 cells. While the values in Table 5 are not tight for all genes at H3k4me3, there is good reproducibility and there is good precision at both promoter regions of IL10 and at promoter of IL12-p35. At H3k9Ac, the numbers suggest reproducibility and precision of the assays is not very good, especially at both IL12 promoters.
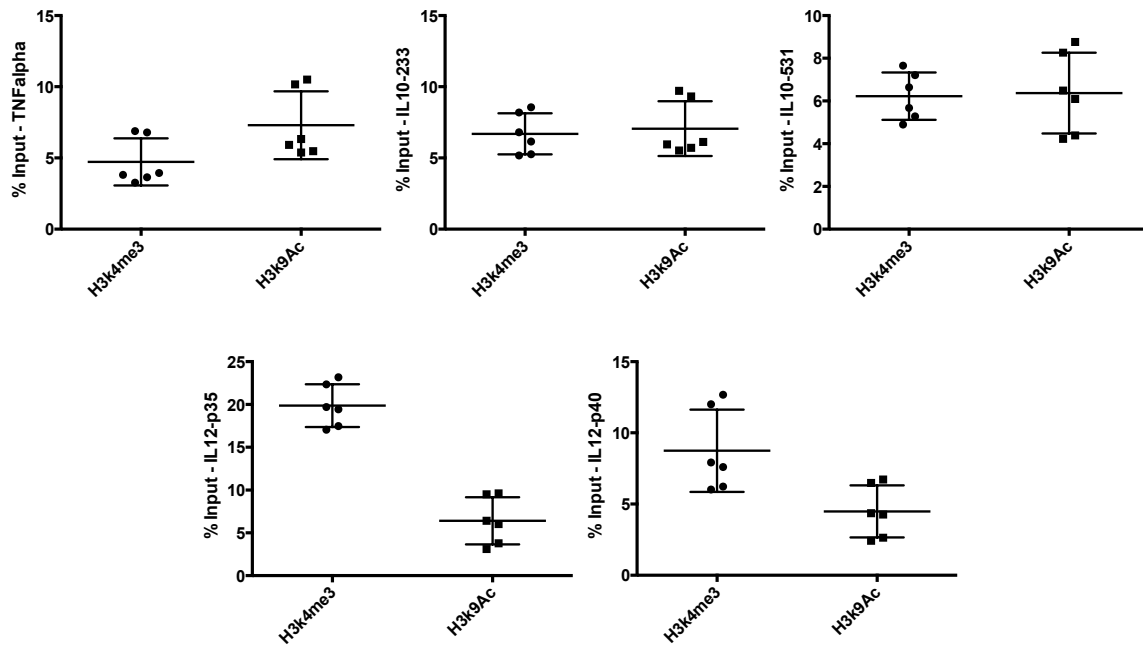
Figure 7. Mean with standard deviation of all ChIP assays on TF-1a cells.

Table 5. *Statistical Analysis of TF-1a Cells at Each Promoter and Histone Modification.*

H3k4me3	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	4.722	6.689	6.224	19.86	8.741
Standard Deviation	1.654	1.441	1.109	2.492	2.891
Coeff. Of Variation	35.03%	21.54%	17.83%	12.55%	33.07%
H3k9Ac	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	7.294	7.057	6.37	6.408	4.481
Standard Deviation	2.381	1.921	1.893	2.747	1.83
Coeff. Of Variation	32.64%	27.23%	29.72%	42.87%	40.85%

Another means of looking at the reproducibility of the ChIP assay was to perform assays on the same cell isolations at two different times. Analyzing that data using paired t-tests shows that the assay is, with a few exceptions, highly reproducible. A correlation coefficient was also calculated to determine how effective the pairing was between the two aliquots. None of the t-tests suggested any significant differences between the paired samples. Several correlation coefficients were significant at $p = 0.05$, as indicated by an *, and $p = 0.01$, indicated by **, in Figures 8, 9, and 10. In the THP-1 cells, it is interesting to note that none of the r values went below 0.9. This observation, paired with the values discussed above in Table 3, suggests that in THP-1 cells the ChIP assay is highly reproducible if not necessarily highly precise. One could argue that the differences between cell isolation days are the product of biology rather than technique. However, there is no pattern of increased or decreased percent input values based on the day of cell isolation. In the THP-1 cells in Figure 8, half of the graphs show an increase in percent input in Day 2 of cell isolation and then a decrease in Day 3. If the differences between days were to be attributable to biology, one would expect a stronger, more consistent pattern.

Similarly in Jurkat cells, the variability seen in the above data is mirrored in Figure 9. There is more variability in the r values, with ranges from 0.7460 to 1.000. Only half of the graphs show consistency in the change in percent input between cell isolation days as in THP-1 cells. This pattern is again repeated in TF-1a cells, as shown in Figure 10. In these two cell lines, the little consistency there is shows a decrease in percent input from Day 1 to Day 2, followed by an increase from Day 2 to Day 3, with the percent input on Day 3 being larger than Day 1. This all suggests that the ChIP assay is highly reproducible and there is the possibility that the changes seen in percent input may be due to biology, specifically the potentially altered epigenetic machinery in immortalized cell lines.

Figure 8. Comparison between aliquots of same THP-1 cells assayed on different days. The lines represent the three different days the cells were isolated and crosslinked.

Figure 9. Comparison between aliquots of same Jurkat cells assayed on different days. The lines represent the three different days the cells were isolated and crosslinked.
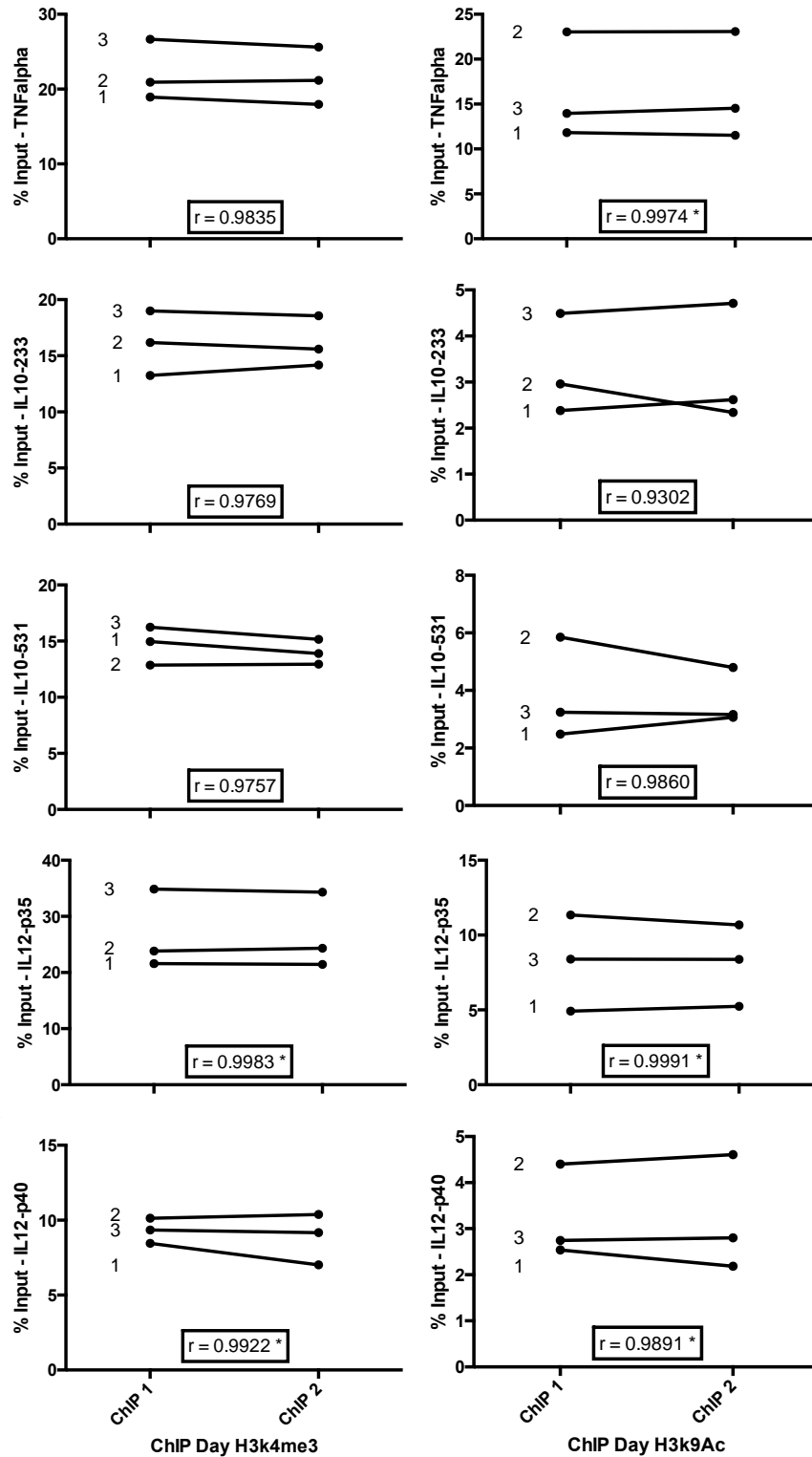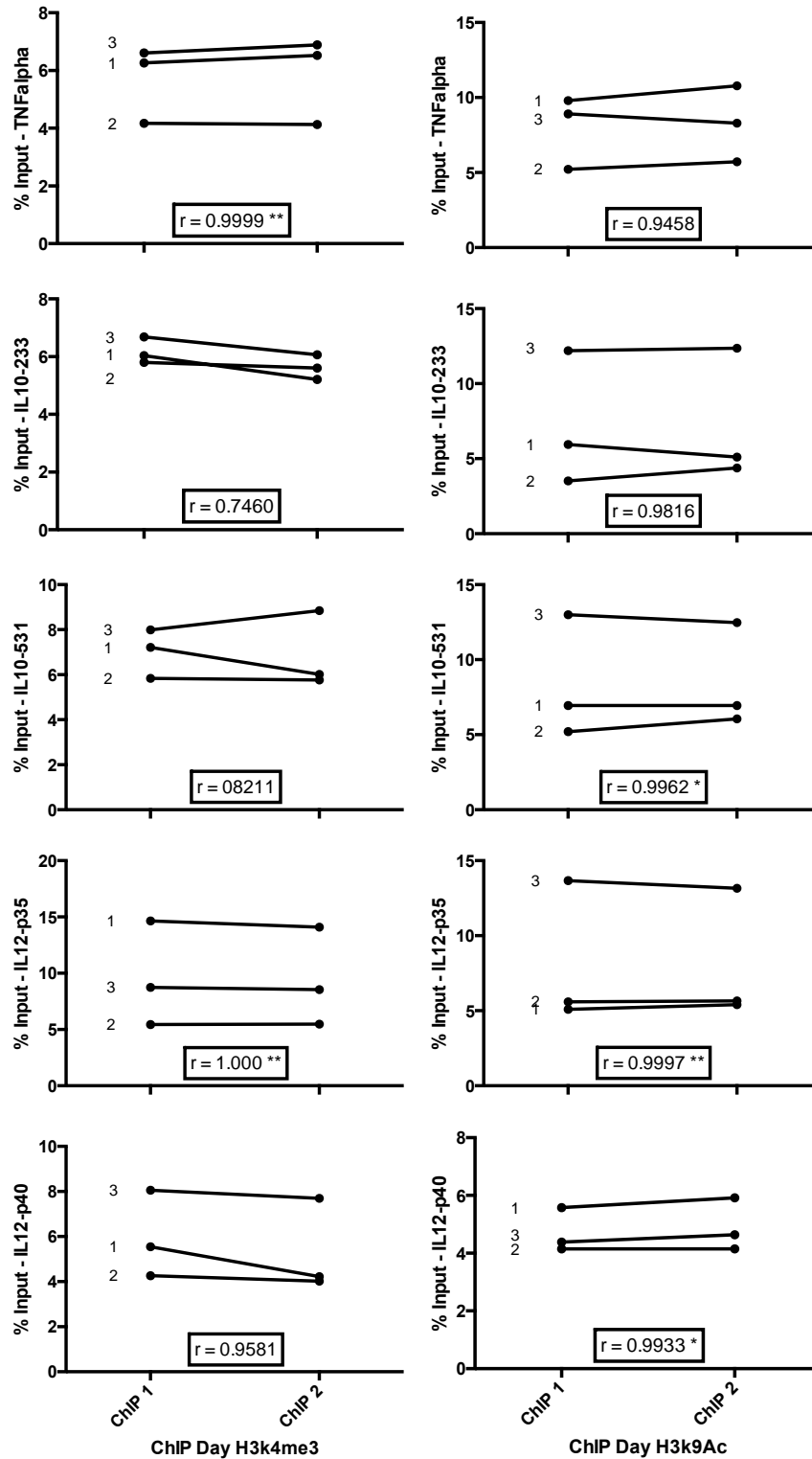
Figure 10. Comparison between aliquots of same TF-1a cells assayed on different days. The lines represent the three different days the cells were isolated and crosslinked.
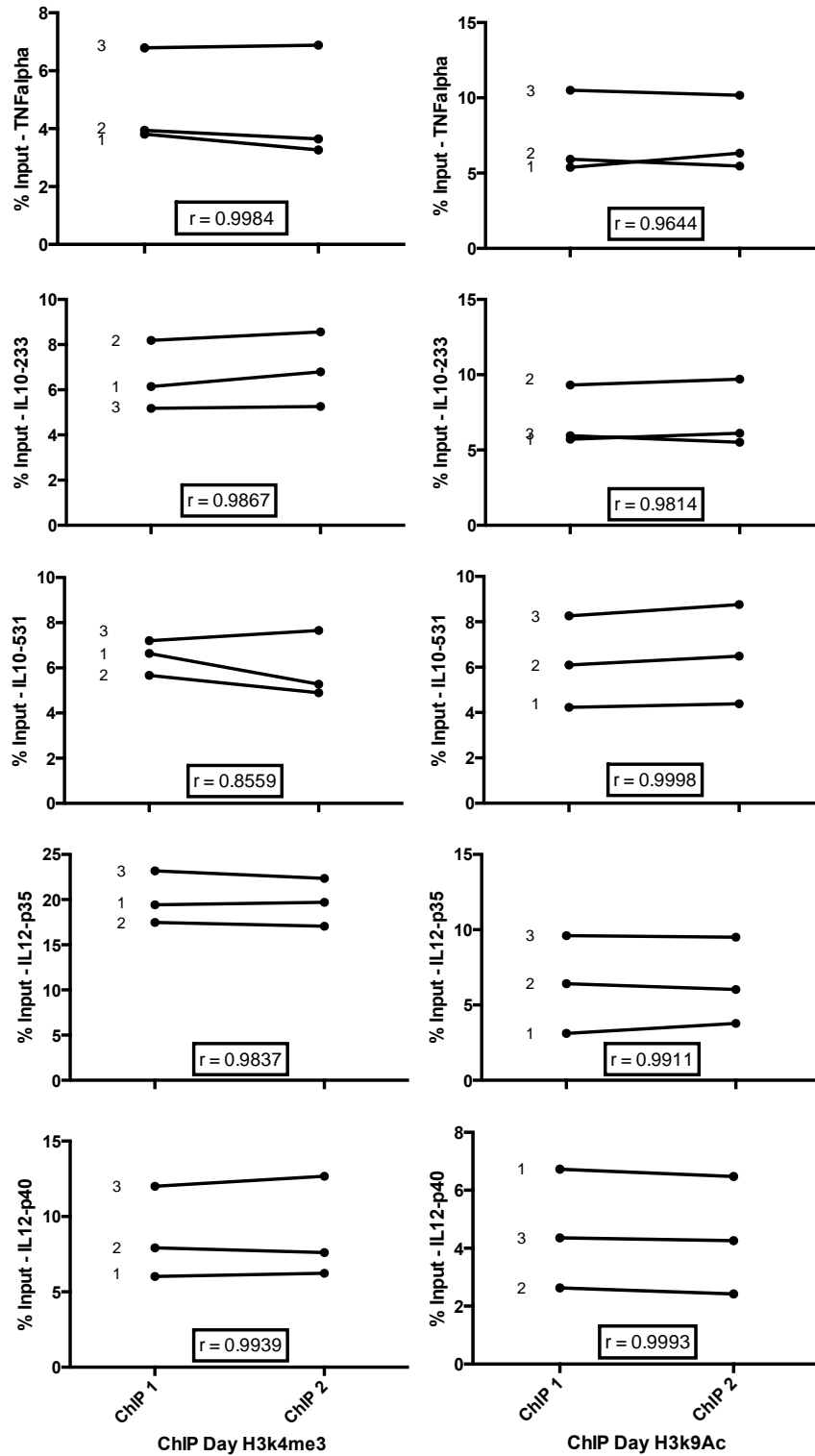
CHROMATIN IMMUNOPRECIPITATON

Initial analysis of histone modification in normal individuals involves establishing whether the data fulfills the requirements for normal distribution. Normal distribution is important, statistically, because most biological variables have a normal distribution. In addition, many statistical methods assume sample distribution is normal. Figure 11 illustrates the variation in all samples collected for each histone modification at each gene promoter site. Table 6 shows that variability ranges from a tight 13.90% to a wider 37.87%. Using the D'Agastino & Pearson omnibus normality test indicates that the data are normally distributed.
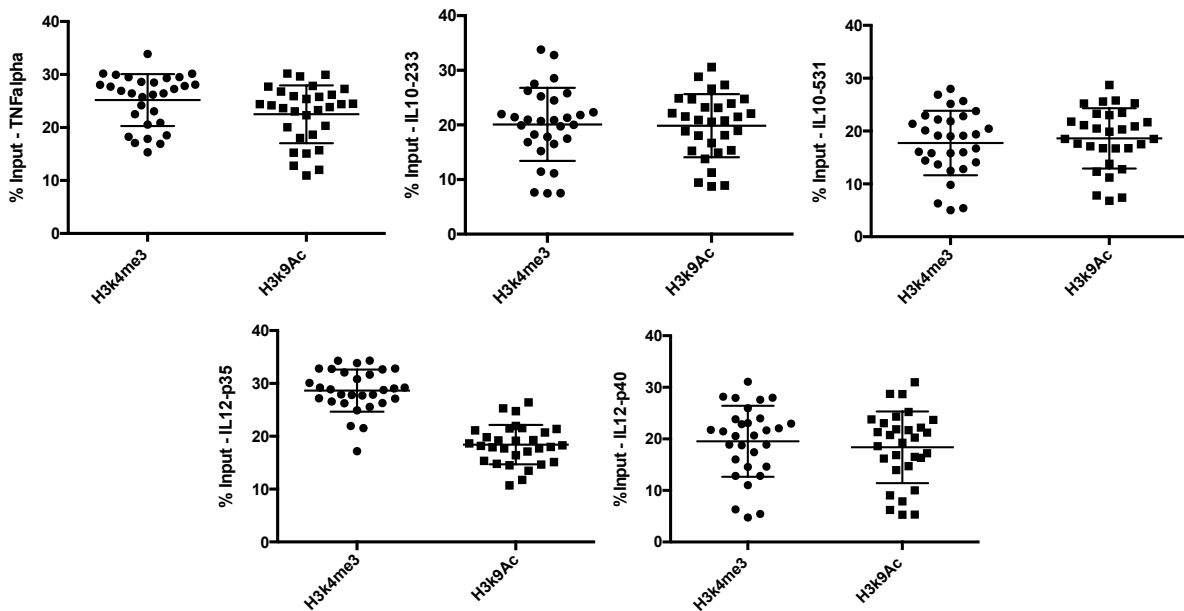
Figure 11. Mean with standard deviation of all ChIP assays on healthy individuals.

Table 6 *Statistical Analysis of PBMCs at Each Promoter and Histone Modification.*

H3k4me3	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	25.18	20.09	15.47	28.64	19.52
Standard Deviation	4.902	6.686	6.099	3.981	6.884
Coeff. Of Variation	19.47%	33.27%	34.37%	13.90%	35.26%
Normality	Yes	Yes	Yes	Yes	Yes
H3k9Ac	TNFalpha	IL10-233	IL10-531	IL12-p35	IL12-p40
Mean	20.47	19.86	18.61	18.41	18.37
Standard Deviation	5.454	5.791	5.695	3.741	6.957
Coeff. Of Variation	24.23%	29.15%	30.61%	20.32%	37.87%
Normality	Yes	Yes	Yes	Yes	Yes

CHROMATIN IMMUNOPRECIPITATON

Two-way analysis of variance (ANOVA) was performed on the data in Figure 12 to examine the influence of individuals and time on epigenetic changes for each histone and gene loci. While there is no statistical significance of time on the percent input calculated for each individual, there is statistical significance regarding the differences between individuals at all promoter regions except at H3k4me3 at the TNFα promoter. This demonstrates the inherent biological variability present in ChIP analysis. Regarding technical variability, it is important to investigate epigenetic changes within each individual.
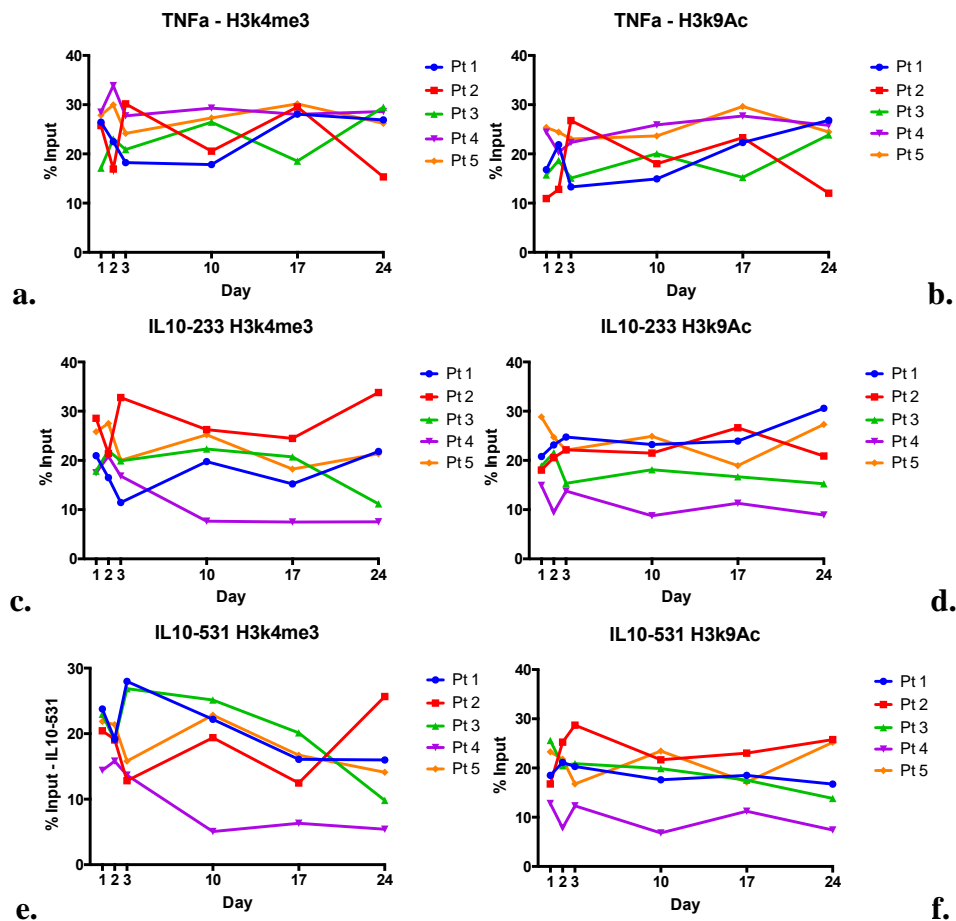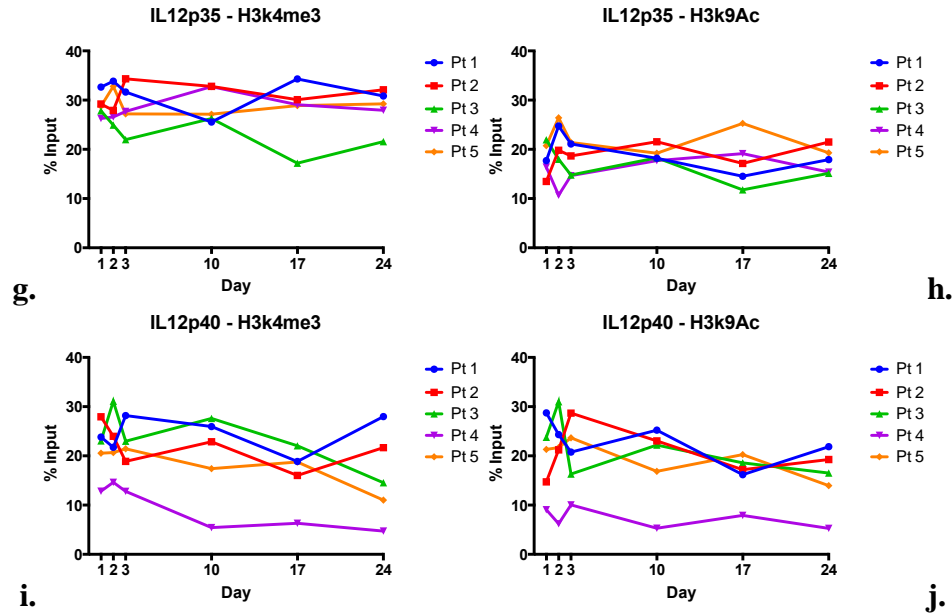
Figure 12. Histone modification at gene loci over time between participants.

Overall, the amount of variability is relatively low for each individual, under 30%, shown in Table 7. There are a few exceptions, particularly Subject 4 at IL10-233 and IL12-p40, both at H3k4me3. It is difficult to determine if the high variability at these two locations for this one individual is attributable to biological changes or technical problems; a problem inherent in using ChIP. In looking across values for each histone modification and promoter combination, it is interesting to note that there is minimal variation at H3k4me3 at the IL12-p35 promoter site. However, in general, there is slightly more variability associated with the modification of H3k4 than there is at H3k9 across all five patients.

Table 7. *Statistical Analysis of Human Participants at Each Promoter.*

TNFalpha					
H3k4me3	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	23.34	23.04	22.58	29.35	27.61
Standard Deviation	4.515	6.372	4.737	2.281	2.283
Coeff. Of Variation	19.35%	27.66%	20.98%	7.77%	8.27%
H3k9Ac	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	19.34	17.31	18.09	24.41	25.11
Standard Deviation	5.173	6.556	3.467	2.687	2.357
Coeff. Of Variation	26.74%	37.88%	19.16%	11.00%	9.39%
IL10-233					
H3k4me3	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	17.61	27.88	18.99	12.98	23.02
Standard Deviation	3.96	4.806	4.155	6.096	3.677
Coeff. Of Variation	22.48%	17.24%	21.89%	46.97%	15.97%
H3k9Ac	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	24.4	21.63	17.63	11.19	24.46
Standard Deviation	3.305	2.822	2.405	2.636	3.568
Coeff. Of Variation	13.54%	13.05%	13.64%	23.56%	14.58%
IL10-531					
H3k4me3	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	20.86	18.32	20.66	10.12	18.79
Standard Deviation	4.699	4.993	6.072	5.007	3.676
Coeff. Of Variation	22.53%	27.26%	29.40%	49.49%	19.57%
H3k9Ac	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	18.8	23.53	19.7	9.73	21.27
Standard Deviation	1.65	4.113	3.902	2.677	3.526
Coeff. Of Variation	8.77%	17.48%	19.81%	27.51%	16.58%
IL12-p35					
H3k4me3	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	31.47	31.07	23.29	28.38	29
Standard Deviation	3.172	2.414	3.851	2.359	2.065
Coeff. Of Variation	10.08%	7.77%	16.54%	8.31%	7.12%
H3k9Ac	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	19.03	18.69	16.64	15.66	22.05
Standard Deviation	3.491	3.062	3.524	2.919	3.071
Coeff. Of Variation	18.35%	16.39%	21.17%	18.64%	13.93%
IL12-p40					
H3k4me3	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	24.43	21.89	23.55	9.452	18.3
Standard Deviation	3.672	4.133	5.605	4.417	3.858
Coeff. Of Variation	15.03%	18.88%	23.80%	46.72%	21.08%
H3k9Ac	**Subject 1**	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**
Mean	22.85	20.69	21.39	7.298	19.62
Standard Deviation	4.292	4.883	5.584	2.01	3.57
Coeff. Of Variation	18.78%	23.60%	26.10%	27.55%	18.19%

Chapter 5: Summary, Conclusions, and Recommendations for Further Research

The human genome contains 23,000 genes that must be expressed in specific cells at precise times. Cells manage gene expression by wrapping DNA around clusters of globular histone proteins to form nucleosomes (Rodenhiser, 2006). In general, DNA is wrapped around nucleosomes, which are arranged as regularly spaced beads (146 bp DNA/nucleosome) along the DNA. Typically, nucleosomes consist of a histone octamer that includes histones H2A/B, H3, and H4, with N-terminal histone tails protruding from the nucleosomes (Rodenhiser, 2006). There is a large body of evidence showing that modifications of the histone tails provide signals that are recognized by specific binding proteins that in turn influence gene expression and other chromatin functions. Specific sets of histone modifications and/or variants are associated with genes that are actively transcribed or are repressed, a phenomenon defined as the "histone code" (Vanden Berghe, 2006). This histone code results in a conformation change in the chromatin: genes are inactivated (switched off) when the chromatin is condensed (silent), and they are expressed (switched on) when chromatin is open (active); (Roundtree, 2001).

Histone-modifying enzymes are recruited to ensure that a receptive DNA region is either accessible for transcription or that DNA is targeted for silencing. Lysine methylation displays the highest degree of complexity among known covalent modifications, and each site of methylation can influence gene activity independently (Hirst & Marra, 2009). Thus, an epigenetic "tag" is placed on targeted DNA, marking it with a special status that specifically activates or silences genes. These reversible modifications ensure that specific genes can be expressed or silenced depending on specific developmental or biochemical cues, such as changes in hormone levels,

inflammation, dietary components, or drug exposures (Rodenhiser, 2006). Epigenetic

mechanisms regulate DNA accessibility throughout a person's lifetime.

Research has shown that epigenetic mechanisms provide an "extra" layer of

transcriptional control that regulates how genes are expressed. Many of these transcriptional

changes occur during development and are subsequently retained through cell proliferation.

Epigenetic changes can also arise in adults either by random change or under the effects of the

environment, aging, chronic inflammation, stress, viral/bacterial infections, diet, hormones, and

toxins (Vande Berghe, 2006). Epigenetic abnormalities have been found to be causative factors

in cancer, genetic disorders, and pediatric syndromes as well as contributing factors in

autoimmune diseases and aging (Egger et al, 2004). The activation of the immune response

involves stepwise epigenetic changes, which allow individual cells to mount a specific immune

response that can be maintained over multiple cell generations. For example, shifts in both

acetylation and methylation of lysines are required to coordinate DNA accessibility and permit

recombination, thereby allowing cells to mount an immune response against a specific antigen

(Rodenhiser, 2006).

One of the most intensely studied lysine modifications is histone H3 lysine 4

trimethylation (H3K4me3). H3K4me3 has been shown to recruit downstream effectors, rather

than directly affect transcription (Hirst and Marra, 2009). Downstream areas of interest in the

study of inflammation are the promoter sites for TNFα, IL-10, IL-12. Sullivan (2007) was able

to demonstrate that TNFα expression increased when Histone 3 was acetylated and that

inhibition of histone methylation reduced TNFα expression in cells capable of producing TNFα.

Larrson's study (2012) showed increased IL10 expression when Histone 3 was acetylated and

also when Histone 3 lysine 4 was trimethlyated. Jin (2016) recently demonstrated that IL12

expression increased with trimethylation of H3k4, and Lu et al. (2005) showed that Histone 3 acetylation stimulated expression of the IL12 p40 promoter.

Chromatin Immunoprecipitation (ChIP) is a type of immunoprecipitation experimental technique used to investigate the interaction between proteins and DNA in the cell. Association between proteins and DNA is crucial for many vital cellular functions such as gene transcription, DNA replication and recombination, repair, segregation, chromosomal stability, cell cycle progression, and epigenetic silencing. It is important to know the genomic targets of DNA-binding proteins and the mechanisms by which they control and guide gene regulation pathways and cellular proliferation (Das et al., 2004). ChIP aims to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone modifiers. Because of the sensitive nature of the technique, it is difficult to adapt to a clinical setting or for high-throughput screening. This is especially difficult in the pediatric population due to limitations in tissue sample sizes. The goal of this study was to demonstrate reproducibility with small cell volumes (~1 million cells) and reliability in small cell numbers isolated whole blood drawn from healthy adult volunteers.

This study presents data that establishes, for the first time, the normal variability of ChIP results in THP-1 cells, Jurkat cells, TF-1a cells, and human male circulating leukocytes over time for H3k4me3 and H3k9Ac. The results from this study allow us to begin establishing a normal range for ChIP assays, which is important in interpreting future ChIP results from patient samples. Several therapeutics involve disruption histone modifications, so an understanding of what occurs in a healthy individual becomes important in determining the safety and efficacy of clinical intervention using these therapeutics. This study provides a baseline for understanding

the role of histone modification and gene expression. The study needs to be expanded beyond

more than five male individuals to include healthy females, the elderly, and children.

References

Abdulkadir, H., Grootens, J., Kjellander, M., Lindberg E. H., Nilsson, G., Ungerstedt, J. (2015). Histone deacetylase inhibitor SAHA mediates epigenetic silencing of KIT D816V mutated systemic mastocytosis primary mast cells and selective apoptosis of mutated cells. *Blood*, 126(23), 2834.

Berger, S. L., Kouzarides, T., Shiekhattar, R., Shilatifard, A. (2009). An operational definition of epigenetics. *Genes and Development*, 23(7), 781-783.

Bosch, I., Xhaja, K., Estevez, L., Raines, G., Melichar, H., Warke, R.V., … Rothman, A.L. (2002). Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. *Journal of Virology*, 76(11), 5588-5597.

Collas, Philippe. (2010). The current state of chromatin immunoprecipitation. *Moleculara Biotechnology*, 45, 87-100.

Dahl, John and Collas, Philippe. (2007). Q^{2}ChIP, a quick and quantitative chromatin immunoprecipitation assay, unravels epigenetic dynamics of developmentally regulated genes in human carcinoma cells. *Stem Cells*, 25, 1037-1046.

Das, P.M., Ramachandran, K., Singal, R. (2004). Chromatin immunoprecipitation assay. *BioTechniques*, 37, 961-969.

Das, Ankita. (2016). Epigenetics and development. *Epigenetics, the Environment, and Children's Health Across Lifespans*. David Hollar, ed. Springer International Publishing: Switzerland.

de Almeida Nagata, D. E., Ting, H. A., Cavassani, K. A., Schaller, M. A., Mukherjee, S, Ptaschinski, C., … Lukacs, N.W. (2015). Epigenetic control of Foxp3 by SMYD3 H3k4 histone methyltransferase controls iTreg development and regulates pathogenic T-cell responses during pulmonary viral infection. *Mucosal Immunology*, 8, 1131-1143.

Eissenbeg, Joel C. and Shilatifard, Ali. (2009). Histone H3 lysine 4 (H3K4) methylation in development and differentiation. Developmental Biology, 339, 240-249.

Egger, G., Gangning, L., Aparicio, A., Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature, 429*, 457-463.

Erfani, P., Tome Garcia, J., Canoll, P., Tsankova, N. (2015). Ash2L and p300 mediate histone H3 modifications at EGFR during its development silencing and re-expression in gliomas. *Cancer Research*, 76(5), 3831-3840.

Gemelli, C., Zanocco Marani, T., Bicciato, S., Mazza, E. M. C., Boraschi, D., Salsi, V., … Grande, A. (2014). MafB is a downstream target of the IL-10/STAT3 signaling pathway, involved in the regulation of macrophage deactivation. *Biochimica et Biophysica Acta*, 1843, 955-964.

Grabiec, A. M, Krausz, S., de Jager, W., Burakowski, T., Groot, D., … Sanders, M.E. (2010). Histone deacetylase inhibitors suppress inflammatory activation of rheumatoid arthritis patient synovial macrophages and tissue. *Journal of Immunology*, 184, 2718-2728.

Guo, Y. ,Sawalha, A.H., Lu, Q. (2014). Epigenetics in the treatment of systemic lupus erythematosus: Potential clinical application. *Clinical Immunology*, 155(1), 79-90.

Gurvich, N., Tsygankova, O. M., Meinkoth, J. L., Klein, P. S. (2004). Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Research*, 64, 1079-1086.

Hansen, Jeffrey C. (2006). Linking genome structure and function through specific histone

    acetylation. *ACS Chemical Biology*, 1(2), 69-72.

Hasan, A., Mitchell, A., Schneider, A., Halene, T., Akbarian, S. (2013). Epigenetic dysregulation

    in schizophrenia: Molecular and clinical aspects of histone deacetylase inhibitors.

    European Archives of Psychiatry and Clinical Neuroscience, 263(4), 273-284.

Hirst, Martin and Marra, Marco. (2009). Epigenetics and human disease. *International Journal

    of Biochemistry and Cell Biology,* 41, 136-146.

Honda, B. M., Candido, P. M., Dixon, G. H. (1975). Histone methylation: Its occurrence in

    different cell types and relation to histone H4 metabolism in developing trout testis.

    Journal of Biological Chemistry, 250, 8686-8689.

Ivanov, M., Barragan, I., Ingelman-Sunderberg, M. (2014). Epigenetic mechanisms of

    importance for drug treatment. *Trends in Pharmacological Sciences*, 35(8), 384-396.

Jayani, R. S., Ramanujam, P. L., Galande, S. (2010). Studying histone modifications and their

    genomic functions by employing chromatin immunoprecipitation and immunoblotting.

    Methods in Cell Biology, 98, 35-56.

Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G., Kapsenberg, M. L. (1997). IL-12

    deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2

    cytokine production in maturing human naïve T helper cells. *Journal of Immunology*,

    159, 28-35.

Karlic, R., Chung, H. R., Lassere, J., Vlahovicek, K., Vingron, M. (2010). Histone modification

    levels are predictive for gene expression. *Proceedings of the National Academy of

    Sciences*, 107(7), 2926-2931.

Karmodiya, K., Krebs, A. R., Oulad-Abdelghani, M., Kimura, H., Tora, L. (2012). H3k9 and

    H3k14 acetylation co-occur at many gene regulatory elements, while H3k14ac marks a

    subset of inactive inducible promoters in mouse embryonic stem cells. *BMC Genomics*,

    13, 424.

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell*, 128, 693-705.

Larrson, L., Thorbert-Mros, S., Rymo, L., Berglundh, T. (2012). Influence of epigenetic

    modifications of the interleukin-10 promoter on IL10 gene expression. *European Journal*

    of Oral Sciences, 120, 14-20.

Lubbert, Michael and Kuendgen, Andrea. (2015). Combining DNA methlytransferase and

    histone deacetylase inhibition to treat acute myeloid leukemia/myelodysplastic syndrome:

    Achievements and challenges. *Cancer*, 121(4), 498-501.

Mayo Clinic. (2016). CBC with differential, blood. http://www.mayomedicallaboratories.com/test

    info/pediatric/refvalues/reference.php?unit_code=9109. Accessed February 10, 2016.

Miao, F., Wu, X., Zhang, L., Yuan, Y. C., Riggs, A. D., Natarajan, R. (2007). Genome-wide

    analysis of histone lysine methylation variations cause by diabetic conditions in human

    monocytes. *Journal of Biological Chemistry*,282(18), 13854-13863.

Miao, F., Wu, X., Zhang, L., Riggs, A. D., Natarajan, R. (2008). Histone methylation patterns

    are cell-type specific in human monocytes and lymphocytes and well maintained at core

    genes. *Journal of Immunology*, 180(4), 2264-2269.

Minucci, Saverio and Pelicci, Pier Guiseppe. (2006). Histone deacetylase inhibitors and the

    promise of epigenetic (and more) treatments for cancer. *Nature Reviews Cancer*, 6; 38

    51.

Murray, K. (1964). The occurrence of iε-N-methyl lysine in histones. *Biochemistry*, 3(1), 10-15.

National Institutes of Health. (2010). NIH roadmap epigenomics mapping consortium.

http://www.roadmapepigenomics.org/. Accessed February 2, 1026.

National Institutes of Health. (2013). A scientific illustration of how epigenetic mechanisms can

affect health. http://commonfund.nih.gov/epigenomics/figure. Accessed February 5,

2016.

O'Neill, L. P., VerMilyea, M. D., Turner, B. M. (2006). Epigenetic characterization of the early

embryo with a chromatin immunoprecipitation protocol applicable to small cell

populations. Nature Genetics, 38(7), 835-841.

Orlando, Valerio. (2000). Mapping chromosomal proteins in vivo by formaldehyde-crosslinked

chromatin immunoprecipitation. *Trends in Biochemical Sciences*, 25, 99-104.

Qiao, Y., Wang, R., Yang, X., Tang, K., Jing, N. (2014). Dual roles of histone H3 lysine 9

acetylation in human embryonic stem cell pluripotency and neural differentiation.

Journal of Biological Chemistry, 290, 2508-2520.

Rodenhiser, David and Mann, Melissa. (2006). Epigenetics and human disease: Translating basic

biology into clinical applications. *Canadian Medical Association Journal, 175(3)*, 341

348.

Roundtree, M.R., Bachman, K. E., Herman, J. G., Baylin, S. B. (2001). DNA methylation,

chromatin inheritance, and cancer. *Oncogene,* 20, 3156-3165.

Ruthenburg, A. J., Allis, C. D., Wysocka, J. (2006). Methylation of lysine 4 on histone H3:

Intricacy of writing and reading a single epigenetic mark. *Molecular Cell*, 25(1), 15-30.

Salz, T. Deng, C., Pampo, C., Siemann, D., Qiu, Y., Brown, K., Huang, S.. (2015). Histone methyltransferase hSETD1A is a novel regulator of metastasis in breast cancer. *Molecular Cancer Research*, 13, 461-469.

Shirakawa, K., Chavez, L., Shweta, H., Calvanese, V., Verdin, E. (2013). Reactivation of latent HIV by histone deacetylase inhibitors. *Trends in Microbiology*, 21(6), 277-285.

Simmons, Danielle. (2008). Epigenetic influences and disease. *Nature Education,* 1(1), 6.

Slingerland, M., Guchelaar, H. J., Gelderblom, H. (2015). Histone deacetylase inhibitors: An overview of the clinical studies in solid tumors. *Anti-Cancer Drugs*, 25(2), 140-149.

Sullivan, K. E., Reddy, A. B. M., Dietzmann, K., Suriano, A. R., Kocieda, V. P., Stewart, M., Bhatia, M. (2007). Epigenetic regulation of Tumor Necrosis Factor alpha. *Molecular and Cellular Biology*, 27(14), 5147-5160.

Turner, Bryan M. (2002). Cellular memory and the histone code. *Cell*, 111(3), 285-291.

Vanden Berghe, W., Ndlovu, M. N., Hoya-Arias, R., Dijsselbloem, N., Gerlo, S., Haegeman, G. (2006). Keeping up NF-kB appearances: Epigenetic control of immunity or inflammation-triggered epigenetics. *Biochemical Pharmacology, 72*, 1114-1131.

Waddington, C.H. (1942). The epigenotype. *Endeavor* 1, 18-20.

Wen, H., Dou, Y., Hogaboam, C., Kunkel, S. L. (2008). Epigenetic regulation of dendritic cell derived interleukin-12 facilitates immunosuppression after a severe innate immune response. *Blood*, 111(4), 1797-1804.

Xia, M., Liu, J., Wu, X., Liu, S., Li, G., Han, C., … Cao, X. (2013). Histone methlytransferase Ash1l suppresses interleukin-6 production and inflammatory autoimmune diseases by inducing the ubiquitin-editing enzyme A20. *Immunity*, 39(3), 470-481.

You, Jueng Soo and Jones, Peter A. (2012). Cancer genetics and epigenetics: Two sides of the same coin? *Cancer Cell*, 22, 9-20.

Zediak, V. P., Wherry, E. J., Berger, S. L. (2011). The contribution of epigenetic memory to immunologic memory. *Current Opinion in Genetics and Development*. 21, 154-159.

Zhang, L. Dai, Y., Lu, J., Zhang, Y., Wang, L. (2009). Genome-wide analysis of histone H3 lysine 4 trimethylation in peripheral blood mononuclear cells of minimal change nephrotic syndrome patients. *American Journal of Nephrology*, 30, 505-513.

Zumbo, Paul. (2013). Preformationism vs. epigenesis. http://physiology.med.cornell.edu/faculty/mason/lab/zumbo/files/ZUMBO_preformationsm_epigenesis_DRAFT.pdf. Accessed February 2, 2016.

CHROMATIN IMMUNOPRECIPITATON

APPENDIX

CHROMATIN IMMUNOPRECIPITATON

Appendix A: University of Michigan IRB Approval Letter

To: Dr. Timothy Cornell

From:

Michael	Geisser
Alan	Sugar

Cc:

Kelli	McDonough
Walker	McHugh
Nicholas	Harris
Timothy	Cornell
Michael	Heung
Tak For	Yu
Neal	Blatt
Moni	Weber

 Subject: Scheduled Continuing Review [CR00047161] Approved for [HUM00074806]

SUBMISSION INFORMATION:
Study Title: Effects of Blood Product Transfusion on Immune Function
Full Study Title (if applicable):
Study eResearch ID: HUM00074806
SCR eResearch ID: CR00047161
SCR Title: HUM00074806_Continuing Review - Tue Mar 24 12:04:46 EDT 2015
Date of this Notification from IRB:4/10/2015
Review: Expedited
Date Approval for this SCR: 4/9/2015
Current IRB Approval Period: 4/9/2015 - 4/8/2016
Expiration Date: Approval for this expires **at 11:59 p.m. on 4/8/2016**
**UM Federalwide Assurance:FWA00004969 (For the current FWA expiration date, please
visit the UM HRPP Webpage)**
OHRP IRB Registration Number(s): IRB00001995

CHROMATIN IMMUNOPRECIPITATON

Approved Risk Level(s) as of this Continuing Report:

Name   Risk Level
HUM00074806 No more than minimal risk

NOTICE OF IRB APPROVAL AND CONDITIONS:
The IRBMED has reviewed and approved the scheduled continuing review (SCR) submitted for the study referenced above. The IRB determined that the proposed research continues to conform with applicable guidelines, State and federal regulations, and the University of Michigan's Federalwide Assurance (FWA) with the Department of Health and Human Services (HHS). You must conduct this study in accordance with the description and information provided in the approved application and associated documents.

APPROVAL PERIOD AND EXPIRATION DATE:
The updated approval period for this study is listed above. Please note the expiration date. If the approval lapses, you may not conduct work on this study until appropriate approval has been re-established, except as necessary to eliminate apparent immediate hazards to research subjects or others. Should the latter occur, you must notify the IRB Office as soon as possible.

IMPORTANT REMINDERS AND ADDITIONAL INFORMATION FOR INVESTIGATORS

APPROVED STUDY DOCUMENTS:
You must use any date-stamped versions of recruitment materials and informed consent documents available in the eResearch workspace (referenced above). Date-stamped materials are available in the "Currently Approved Documents" section on the "Documents" tab.

In accordance with 45 CFR 46.111 and IRB practice, consent document(s) and process are considered as part of Continuing Review to ensure accuracy and completeness. The dates on the consent documents, if applicable, have been updated to reflect the date of Continuing Review approval.

RENEWAL/TERMINATION:
At least two months prior to the expiration date, you should submit a continuing review application either to renew or terminate the study. Failure to allow sufficient time for IRB review may result in a lapse of approval that may also affect any funding associated with the study.

CHROMATIN IMMUNOPRECIPITATON

AMENDMENTS:
All proposed changes to the study (e.g., personnel, procedures, or documents), must be approved in advance by the IRB through the amendment process, except as necessary to eliminate apparent immediate hazards to research subjects or others. Should the latter occur, you must notify the IRB Office as soon as possible.

AEs/ORIOs:
You must continue to inform the IRB of all unanticipated events, adverse events (AEs), and other reportable information and occurrences (ORIOs). These include but are not limited to events and/or information that may have physical, psychological, social, legal, or economic impact on the research subjects or others.
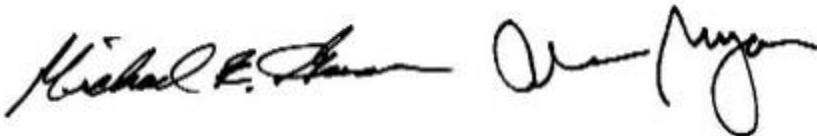
Investigators and research staff are responsible for reporting information concerning the approved research to the IRB in a timely fashion, understanding and adhering to the reporting guidance (http://medicine.umich.edu/medschool/research/office-research/institutional-review-boards/guidance/adverse-events-aes-other-reportable-information-and-occurrences-orios-and-other-required-reporting), and not implementing any changes to the research without IRB approval of the change via an amendment submission. When changes are necessary to eliminate apparent immediate hazards to the subject, implement the change and report via an ORIO and/or amendment submission within 7 days after the action is taken. This includes all information with the potential to impact the risk or benefit assessments of the research.

SUBMITTING VIA eRESEARCH:
You can access the online forms for continuing review, amendments, and AE/ORIO reporting in the eResearch workspace for this approved study, referenced above.

MORE INFORMATION:
You can find additional information about UM's Human Research Protection Program (HRPP) in the Operations Manual and other documents available at: http://hrpp.umich.edu.

Michael Geisser
Co-chair, IRBMED

Alan Sugar
Co-chair, IRBMED

Appendix B: Eastern Michigan University IRB Not Research Letter

RESEARCH @ EMU

UHSRC Determination: NO REVIEW REQUIRED

DATE: June 29, 2016

TO: Kelli McDonough, Eastern Michigan university

Re: UHSRC: # 904014-1

Category: Not Human Subject Research

Title: Reproducibility and reliability of chromatin immunoprecipitation in clinical research

Your project, entitled **Reproducibility and reliability of chromatin immunoprecipitation in clinical research**, does not require UHSRC review in accordance with federal regulation 45 CFR 46.102 because you are using already-obtained blood samples. UHSRC policy states that you, as the Principal Investigator, are responsible for protecting the rights and welfare of your research subjects and conducting your study as described in your protocol.

Modifications: You may make changes to your study without submitting for review. However, if you plan to publish your data or collect human subject data that are generalizable, you must submit a **Human Subjects Approval Request Form** and obtain approval prior to implementation. The form is available through IRBNet on the UHSRC website.

Good luck in your research. If we can be of further assistance, please contact us at 734-487-3090 or via e-mail at human.subjects@emich.edu. Thank you for your cooperation.

Sincerely,

Sonia Chawla, PhD

Reaserch Compliance Officer

CHROMATIN IMMUNOPRECIPITATON