Preparation of HEp-2 cells for Antinuclear Antibody Detection using Immunofluorescent Technique

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ชื่อวิทยานิพนธ์ การเตรียมเซลล์ชนิด HEp-2 สำหรับตรวจหาแอนตินิวเคลียร์แอนติบอดีโดยวิธีอิมมูโนฟลูออเรสเซนส์

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บทคัดย่อ

การตรวจคัดกรอง antinuclear antibody (ANA) โดยวิธี Immunofluorescent (IF) โดยใช้เซลล์เพาะเลี้ยงชนิด HEp-2 เป็นแหล่งของแอนติเจนเป็นวิธีที่นิยมใช้โดยทั่วไป ซึ่งส่วนใหญ่เป็นน้ำยาสำเร็จรูป ส่วนการเตรียมเซลล์ขึ้นใช้เองรวมทั้งชนิดของน้ำยารักษาสภาพที่ใช้ในขั้นตอนการเตรียมเซลล์ยังมีการศึกษาค่อนข้างน้อย งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาหาสภาวะเหมาะสมในการเตรียมเซลล์เพาะเลี้ยงชนิด HEp-2 สำหรับตรวจหา ANA ขึ้นใช้เองในห้องปฏิบัติการ และเปรียบเทียบวิธีรักษาสภาพของเซลล์สี่แบบคือ 1) methanol-acetone 10 นาที 2) 1% paraformaldehyde 5 นาที ตามด้วย methanol-acetone 10 นาที 3) 1% paraformaldehyde 30 นาที ตามด้วย methanol-acetone 10 นาที และ 4) 1% paraformaldehyde 5 นาที ตามด้วย 0.5%Triton X-100 เป็นเวลา 5 นาที โดยเพาะเลี้ยงเซลล์บนสไลด์แก้วชนิด 12 หลุม และเลี้ยงเซลล์ในเวลาที่แตกต่างกัน คือ 6, 12, 24, 36, 42 และ 48 ชั่วโมง เลือกเซลล์ในระยะที่มีการกระจายตัวดี นำมารักษาสภาพเซลล์ด้วยวิธีรักษาสภาพทั้งสี่แบบและย้อมด้วยวิธี IF ดูผลการติดสีเรืองแสงของ ANA ในรูปแบบที่พบได้บ่อย 5 รูปแบบ จากนั้นทำการทดสอบกับตัวอย่างผู้ป่วยที่ทราบรูปแบบแล้วรูปแบบละ5 ราย และตัวอย่างที่ให้ผลลบ 5 ราย เปรียบเทียบกับน้ำยาสำเร็จรูป ผลการศึกษาพบว่าเซลล์ที่เลี้ยงเป็นเวลา 42 ชั่วโมง ให้ผลการย้อมที่ดีที่สุด โดยเซลล์เกาะติดแน่นบนสไลด์และกระจายตัวดีประมาณ 70-80% confluence และหลังจากย้อมเซลล์และสังเกตด้วยกล้องจุลทรรศน์ที่กำลังขยาย 400X พบว่ามีเซลล์ในระยะแบ่งตัว (mitotic cells) ประมาณ 2-5 เซลล์ และจากการศึกษาการติดสีเรืองแสงของเซลล์รูปแบบต่างๆ พบว่าเซลล์ที่รักษาสภาพด้วยวิธีที่ 1 คือ methanol-acetone และวิธีที่ 2 คือ 1% paraformaldehyde 5 นาที ตามด้วย methanol-acetone 10 นาที ให้ผลรูปแบบ homogeneous, speckle, nucleolar และ centromere ได้ถูกต้องตรงกันกับชุดตรวจสำเร็จรูป และวิธีที่ 1 ให้ผลรูปแบบ speckle และ nucleolar ที่มีความคมชัดกว่าวิธีอื่นๆ พบว่าเซลล์ที่รักษาสภาพด้วย 1% paraformaldehyde นั้น มี background สูงกว่า และวิธีรักษาสภาพทั้งสองแบบ

ไม่สามารถให้ผล nuclear membrane ที่ถูกต้องในผู้ป่วยจำนวน 1 ราย เนื่องจากมีการติดสีในส่วนของไซโตพลาสซึมมาบดบังรูปแบบการเรืองแสง แต่เมื่อนำตัวอย่างตรวจรายนี้ไปทดสอบกับเซลล์ที่รักษาสภาพด้วยวิธีที่สี่คือ 1% paraformaldehyde 5 นาทีและใช้ Triton-X100 เป็น permeabilizer พบว่าให้ผลได้ถูกต้อง ส่วนวิธีรักษาสภาพแบบที่สามคือ 1% paraformaldehyde 30 นาทีตามด้วย methanol-acetone 10 นาทีนั้นพบว่า มี background สูงกว่าวิธีอื่นๆ ไม่เหมาะสำหรับใช้ในการทดสอบ โดยสรุป การศึกษานี้ทำให้ทราบสภาวะที่เหมาะสมในการเตรียมเซลล์และพบว่า การรักษาสภาพสไลด์ด้วย methanol-acetone ให้ผลที่ดี และคาดว่าจะสามารถนำไปใช้ในการพัฒนาชุดตรวจ ANA ขึ้นใช้เองในห้องปฏิบัติการต่อไป

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ABSTRACT

The antinuclear antibody (ANA) screening method that is widely used is the immunofluorescent (IF) assay using human epithelial cell lines (HEp-2) cells as substrate antigen. However, most of the IFA-ANA tests are commercial products. The practical data for HEp-2 cells preparation and fixative reagents for in-house preparation were rarely described in the literatures. The proposes of this study were to determine the optimal condition in the preparation of HEp-2 cells culture for detecting antinuclear antibody by IFA and to compare four fixative protocols including 1) methanol-acetone for 10 minutes 2) 1% paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes 3) 1% paraformaldehyde for 30 minutes followed by methanol-acetone for 10 minutes and 4)1% paraformaldehyde for 5 minute followed by 0.5% Triton X-100 for 5 minutes. HEp-2 cells were cultured on 12-wells glass slide and harvested at different time points (6, 12, 24, 36, 42 and 48 hours). The cultured cells which showed well confluence were selected for fixing with four fixatives protocols. Next, the fixed cells were stained by IF method with known clinical samples of the five common ANA patterns and compared with commercial kit. The results showed that the most optimum harvesting condition for fixation and IF staining was at 42 hours. At this time, the spreading of cells on slide was 70-80% confluences with 2-5 mitotic cells observing under microscope at 400x magnification. For ANA pattern results, the fixation protocol 1 and 2 demonstrated the correct ANA positive patterns for homogeneous, speckle, nucleolar and centromere pattern. Protocol 1 showed brighter and sharper intensity fluorescence staining for speckle and nucleolar pattern than the other protocols. 1% paraformaldehyde fixed cells demonstrated higher background than the methanol-acetone fixed

cells. In one case of nuclear membrane pattern, in both protocol 1 and 2, the staining pattern could not be interpreted due to masking of cytoplasm staining. This problem was solved with fixation protocol 4: 1% paraformaldehyde and permebilyzed with Triton X-100. The present study suggested that methanol-acetone was the effective fixative for ANA screening using IF method. This protocol would be useful for in-house ANA test development.

CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (Continued)

LIST OF ABBREVIATION AND SYMBOLS

∀C	=	Degree celsius
σl	=	Microliters
ANA	=	Antinuclear antibodies
ATCC	=	American type culture collection
BSA	=	Bovine serum albumin
CTD	=	Connective tissue disease
dsDNA	=	Double stranded deoxyribose nucleic acid
ELISA	=	Ensyme linked immunosorbent assay
ENA	=	Extractable nuclear antigen
et al.	=	et ali (Latin) and others
FBS	=	Fetal bovine serum
FITC	=	Fluorescein isothiocyanate
HEp-2	=	Human epithelial cell line: type 2
IF	=	Immunofluorescence
IgG	=	Immunoglobulin G
MCTD	=	Mixed connective tissue disorders
ml	=	Milliliter
NOR-90	=	Nucleolus organizing region protein 90kDa
PBS	=	Phosphase buffer saline
PCNA	=	Proliferating cell nuclear antigen
PM-Scl	=	Polymyositis-Scleroderma
Scl-70	=	Scleroderma antigen-70kDa
SLE	=	Systemic lupus erythematosus
Sm	=	Smith ENA
SS-A	=	Sjogren's Syndrom-antigen A
SS-B	=	Sjogren's Syndrom-antigen B

CHAPTER 1

INTRODUCTION

Background and Rationale

Antinuclear antibodies (ANA) are a spectrum of autoantibodies that react against a variety of nuclear components of cells, such as nuclear membrane, nucleoplasm, nucleoli, and nuclear organelles (Desplat-Jego et al., 2007). These antibodies are found in patients who have systemic or organ specific autoimmune diseases and in a variety of infections. The positive ANA test at low titer were also found in some other diseases and in healthy persons (Adams & Mutasim, 2000). The ANA test is used for screening, diagnosing, and monitoring of autoimmune diseases, such as systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome (SS), scleroderma, polymyositis, ect. (Adams & Mutasim, 2000; Desplat-Jego et al., 2007)

The most common methods to determine ANA are immunofluorescence (IF) assay and enzyme-linked immunosorbent assay (ELISA) (Egner, 2000). IFA is performed by incubating patient sera with substrate cells such as rodent tissue or human epithelial cell line. Bound antibodies are detected by incubation with a dye-conjugated anti-human immunoglobulin and visualized by fluorescent microscopy. Intensive trainings are required for this method to identify various patterns of fluorescent staining, which are correlated with different disease states. In contrast to the other method, ELISA has become to be the most widely used for routine ANA screening. It is easier, less labor intensive, cheaper than the IF assay and could be performed by automatic machine. Briefly, specific antigens (or mixtures of antigens) are bound to ELISA microtiter plates and incubated with patient sera. Then, the complexes are bound with an enzyme-conjugated anti-human antibody that results in a color change when react with the substrate. This method has the advantage of greater sensitivity and less subjective. However, in interpretation the results from ELISA method (negative or positive) have to be confirmed with IF assay to obtain specific ANA patterns which are important for clinical assessment. These certain patterns are

associated with the presence of autoantibodies to nuclear antigens which are associated with specific diseases (Adams & Mutasim, 2000).

As several reasons mentioned above, the IF assay test is considered to be gold standard. Initially, animal tissue such as rodent tissue was used as substrate antigens. Later, human cell lines, in particular human epithelial cell lines (HEp-2) cells were used instead of animal tissue with many superior advantages. Compared to rodent tissue, HEp-2 cells have higher sensitivity and provide clearer patterns of reactivity. In addition, HEp-2 cells substrate allows recognition of autoantibodies directed against antigens in all phases of mitosis which is not possible if using rodent tissue (Bradwell et al., 1999). At present, many IF assay commercial kits are available and therefore are more commonly used than in-house preparation (Ghosh et al., 2007). This may due to laborious process for maintenance of cultured cells. In addition, in-house preparation was rarely described in the literatures.

The preparation processes of HEp-2 cells coated slides consist of cultivation of HEp-2 cells on microscopic slides or on the other adhesive surfaces and the fixation steps to preserve cells structure and nuclear antigens. There are two major methods of fixation: fixation by precipitation and fixation by cross-linking. For fixation by precipitation, alcohol or acetone is used as precipitating agents. The activity of these chemical agents is to dehydrate and destroy the hydrophobic interaction of proteins resulting in loss of protein structures. Another method, fixations by cross linking, formaldehyde, paraformaldehyde or glutaraldehyde is used. These agents create a chemical covalent bond between proteins and make up the network. With cross linking fixation, the cells morphology is better preserved. However, an additional process to create the permeability of cells using permeabilized reagents (such as Triton X-100) may be needed. The selection of a specific fixation protocol generally depends on several factors, such as cell types of antigens, types of fluorescent antibody used in test and the staining procedure (Spector et al., 2005).

There were three previous studies reported about the fixatives. The first study reported that acetone was more efficient than alcohol/acetone solution in screening for anti-SS-A (Ro) antibodies with a sensitivity of 97.5% and 81.3% respectively (Monce et al., 1994). The second study demonstrated the performance of varieties of fixatives. The study revealed that aldehyde fixations which caused linking of protein chains provide normal cell size, the normal

staining of cells and nuclei. However when using the combination of methanol and acetone or acetone alone, a marked change in the size of nuclei and nucleoli were noted (Hahm & Anderer, 2006). The third study by Tsiakalou and co-workers evaluated alternative protocol using formaldehyde-fixed HeLa and Hep2 cells. The study presented a better resolution of nuclear membrane pattern than the commercial available HEp-2 cells (Tsiakalou et al., 2006).

Based on all above evidences, formaldehyde seems to be an efficient fixative. However, the fixed cells must be stained right after the fixation or kept in a buffer solution at 4∀C (Hahm & Anderer, 2006). This disadvantage may be overcome by using different fixatives which can be kept for a longer time in a stock of cell preparations. These fixative include alcohol–acetone, acetic acid–ethanol or combination with formaldehyde and alcohol (Hahm & Anderer, 2006)

The objective of this study for determination the optimal conditions in the preparation of HEp-2 cells culture on glass slides as substrates for ANA detection by IF technique. In addition, two fixatives, methanol-acetone and paraformaldehyde for our 'in house' HEp-2 slides were performed to determine their efficacy in preserving nuclear antigens which are associated to five basic ANA patterns including homogeneous, speckled, nucleolar, nuclear membrane, and centromere pattern. This study will provide important data for developing in-house ANA screening by IF technique for routine testing.

Review of Literatures

1. Historical aspects of ANA

In 1941, Klemperer and co-workers first described systemic lupus erythematosus (SLE) as one of the connective tissue diseases. In 1948, Hargrave and co-workers had noted the presence of previously unknown cells in the bone marrow of a patient with SLE. They called these "LE cells" and described them as mature polymorphonuclear leukocytes which had phagocytosed the liberated nuclear material of another leukocyte (Kavanaugh et al., 2000). This extremely important discovery laid the foundation of research on ANA. Since then, ANA has

been divided into specific subtypes based on the nuclear or cytoplasmic components that being attacked i.e. anti-DNA, anti-histones, etc (Kumar et al., 2009).

2. Biology of Antinuclear Antibodies

ANA are the spectrum of autoantibodies that react with various nuclear molecules, including DNA, ribonucleic acid (RNA), histones, acidic nuclear proteins or complexes of these molecular elements (Desplat-Jego, 2007). The presence of antinuclear antibodies in patient serum appears to be associated with various factors including genetic predisposition (such as the histocompatibility locus DR3), environmental agents (ultraviolet light, viruses, certain drugs and chemicals and intravenous drug use), estrogen-androgen balance, chronic infections, neoplasms and advancing age. Aging cannot be over emphasized as a factor associated with the presence of antinuclear antibodies (White & Robbins, 1987). Clinicians generally correlate the presence of antinuclear antibodies with systemic connective tissue diseases particularly systemic lupus erythematosus and rheumatic disease. These antibodies can also be detected in persons with other immunologically mediated diseases such as Hashimoto's thyroiditis and immunopathic chronic active hepatitis (White & Robbins, 1987; Satoh et al., 2009).

3. Type of ANA

ANA consists of many types of antibodies. They can be classified by their target antigens. At present, ANA had been categorized into 2 main groups (Kumar et al., 2009).

3.1 Autoantibodies to DNA and histones group

These include antibodies against single and double stranded DNA (dsDNA). They were discovered since 1957. The significant levels of anti-dsDNA antibodies are considered to be confirmatory in the diagnosis of SLE. Anti-histones antibodies were reported in 1971 which are indicative of drug-induced SLE (Fishbein et al., 1979).

3.2 Autoantibodies to extractable nuclear antigens (ENA) group

Besides DNA and histones, autoantibodies may also target to other nuclear antigens. These nuclear antigens were named ENA as they were originally extracted from the nuclei with saline (Tan & Kunke, 1966; Fishbein et al., 1979). The first type of ENA autoantibody found in 1966 was autoantibody to Smith antigen (Sm) which was considered to be specific for SLE (Tan & Kunke, 1966). Thereafter, other subtypes of ENA i.e. ribonucleoproteins (RNP), SSA/Ro, or SSB/La, Scl-70, Jo-1 and PM1 were more clearly identified (Kumar et al., 2009, Conrad et al., 2002). Although most of this anti-ENA is disease specific, their sensitivities and specificities are varied depending on the type of underlying connective tissue diseases (CTD).

4. Techniques for ANA detection

Identification of ANA is an important part of clinical medicine and clinical immunology. The techniques that are widely used for ANA screening are indirect immunofluorescent (IF) assay and enzyme linked immunosorbent assay (ELISA). Other new methods such as flow cytometry and nanotechnology involving antigen arrays are still in experimental stages (Kumar et al., 2009; Hanly et al., 2010).

4.1 IF assay

IF technique is simple, reliable and highly sensitive for screening purposes in the detection of ANA .This technique is currently a preferred method for detecting ANA. Technically in brief, patient's sera are used in different dilutions and then incubated with the substrate cells. The bound antibodies, the fluorescein-conjugated anti-human immunoglobulin are tagged to the ANA and visualized through a fluorescent microscope. Various staining patterns are apparent depending on the types of antibodies in the samples (Fishbein et al., 1979). Ideally, the source of substrate cells should be cells from human cell lines such as the human epithelial cell line and HEp-2. However, due to the financial cost of continual maintenance of the cell lines, rodent tissue

cells e.g. rat liver have been used with some limitations (Adams & Mutasim, 2000; Kumar et al., 2009).

The patterns of ANA by IF staining are divided into three groups (Kumar et al., 2009).

1. Nuclear patterns are homogeneous, speckled (fine and coarse), peripheral/rim, nucleolar, centromeric, PCNA (proliferating cell nuclear antigen), nuclear dots, nuclear membrane, diffuse grainy.

2. Cytoplasmic patterns are speckled, mitochondrial-like, ribosomal-like, Golgi apparatus, lysosomal-like, cytoskeletal filaments (actin, vimentin, cytokeratin)

3. Mitotic patterns are mitotic spindle, centrosomes, NuMA (nuclear mitotic apparatus), midbody, CENP-F (centromere protein)

Among these patterns, the homogenous, speckled, peripheral and nucleolar patterns are more commonly observed and have a clinical importance (White & Robbins, 1987).

4.2 ELISA

ELISA is the choice of method for ANA detection. Over the past few years, a number of investigators and commercial organizations have attempted to develop solid phase immunoassays for the detection of ANA and the specific nuclear antibodies. The ELISA methods are more convenient to handling, easier and cheaper to perform. They have been standardized with IF assay using fixed HEp-2 cells as a substrate (Jaskowski et al., 1996).There are two types of ELISA methods that are currently used including generic assay and antigen specific assay (Kumar et al., 2009). Generic assay is used for screening purpose and antigen specific assay is used to detect specific ANA that reacts with a single autoantigen i.e. dsDNA, SS-A/Ro, SS-B/La, Scl-70, Sm, Sm/RNP etc (Kumar et al., 2009).

In antigen specific assay, multiple antigens are coated on to microtiter plates, usually a combination of SSA/Ro, SSB/La, Sm, and U1-RNP, with many also including Jo-1 and Scl70. This new test is both highly specific and sensitive and substantially decreases the time involved when screening large numbers of patient samples. The test is simple, can be applied to automation and does not require highly trained operators in contrast to those who recognize

microscopic patterns in IF assay. The ELISA is therefore, becoming the most widely used method not only for routine screening but also for detection of specific ANA. The test kits that presently available in the market either utilize extracts of tissue containing various nuclear components or the molecules synthesized by recombinant technology. The synthesized DNA molecule may include individual recombinant molecules such as SS-A/Ro, or combinations of molecules which increase the sensitivity of the test. In a recent study, the performance of ELISA test had been compared with the "gold standard" IF test. The agreement that a serum is ANA positive was 87% to 95% when comparing the ELISA with IF test (Jaskowski et al., 1996). The sensitivity of the various ELISAs was 69% to 98% and the specificity ranged between 81% and 98%. These figures were reached by using the sera that had a positive at the dilution of 1:160 by the IF test. The above comparison figures were much lower with IF titer of 1:40. However, evaluation of the technique is still needed because there are variety of commercial test kits which have varied sensitivities and specificities (Adams & Mutasim, 2000; Solomon et al., 2002). In addition, the results of ELISA screening method are only presumptive (negative or positive for ANA). IF test have to be performed to examine staining pattern which provide additional information for clinical assessment (Kumar et al., 2009).

5. HEp-2 cell

5.1 The advantage of HEp-2 cells

The use of HEp-2 cells (human laryngeal epithelium cancer cell line) has provided an important alternative substrate to rodent tissue for ANA testing. The HEP-2 cells substrate allow recognition of autoantibodies direct against in all phase of mitosis, which is not possible using rodent tissue section. For example, centromere positive sera generally reported as ‘speckled’ pattern on rodent tissues (Bradwell et al., 1999; Robert et al., 2003).

HEp-2 cells have many advantages over rodent tissue:

1. They are more sensitive substrates that allow identification of many patterns.
2. Human origin ensures better specificity than animal tissues.

3. The nuclei are much larger so that complex nuclear details can be seen.
4. The cell monolayer ensures that all nuclei are visible.
5. Cell division rates are higher so that antigens produced only in cell division are easily located eg. , centromere and mitotic spindle patterns.
6. There is no obscuring intercellular matrix.
7. Antigen distribution is uniform.

HEp-2 cells allow recognition of over 30 different nuclear and cytoplasmic patterns which represent up to 100 different autoantibodies. Some patterns are antigen specific but some patterns are represented for several different autoantibodies. (Bradwell et al., 1999).

5.2 Structure of HEp-2 cells

Immunofluorescent patterns seen on individual HEp-2 cells depend on whereabouts the cells are in their reproductive cycle. For instance, patterns that unique to cells in mitosis will be restricted to that small proportion of the cycle when the cell is dividing. Since most autoantibodies are react against cell in interphase. The cultured HEp-2 cells should be large at this stage. Synchronised cultures might not contain cells at all stages in the cycle and should generally be avoided. The HEp-2 cell cycle at optimal growth is approximately 36 hours. There are two phases, interphase and mitosis phase (Bradwell et al., 1999).

5.2.1 The cell during interphase

In the interphase nuclei, the chromosomes from a fibrillar network of chromatin, more or less uniformly distributed throughout the nucleoplasm and delimited by the nuclear membrane. Only the nucleoli are well differentiated. Cytoplasmic organelles and fibrous structures are most visible at this stage and tend to largely disappear or change their appearance during mitosis. The outlines of various components in eukaryotic cells are shown in Figure 1 (Bradwell et al., 1999).

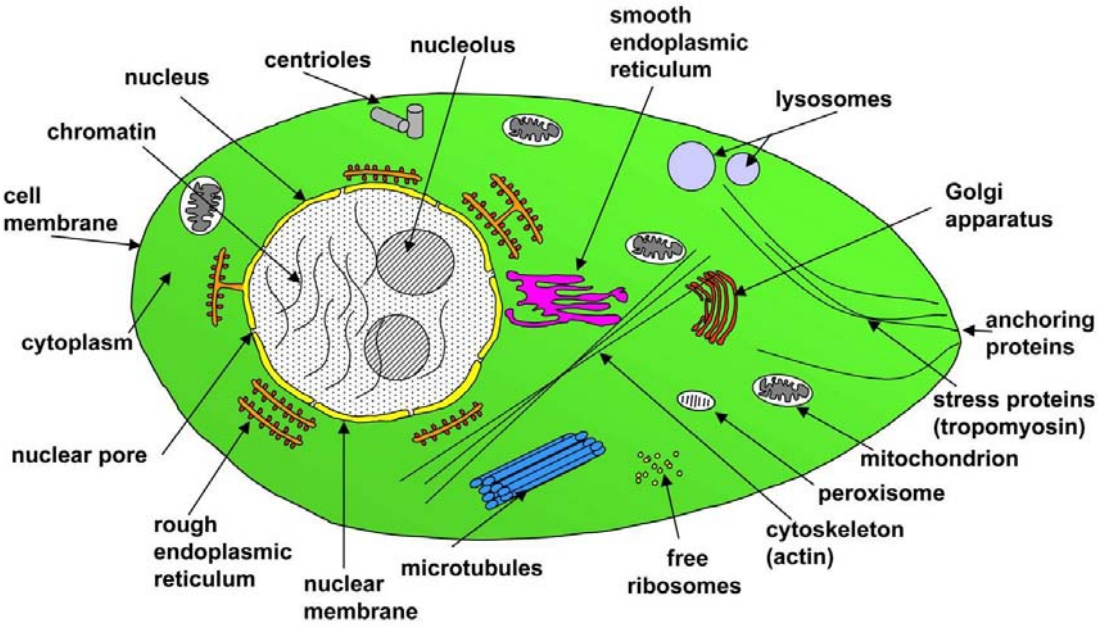


Figure 1. The cell during interphase (Bradwell et al., 1999)

5.2.2 The cell during division

Mitosis occurs during 10-15% of the cell cycle and is divided into four successive phase: prophase, metaphase, anaphase and telophase. During prophase, DNA condensation gives rise to the appearance of individual chromatids whist the nucleolar contents are distributed throughout the nucleoplasm. Meanwhile, the mitotic spindles form around the nucleus and terminate at the polar centrioles (or centromeres). Metaphase is characteristised by condense chromosomes at the equator of the spindle apparatus through the action of microtubules. In anaphase, each paired of chromatids splits at the centromere and chromatids migrate separately to each pole of the spindle. In telophase the nuclear membrane reforms around each of the two daughter nuclei. The nucleoli reappear and the chromosomes become decondensed in the newly reformed nuclei (Bradwell et al., 1999). The cell during cell cycle is shown in figure 2.

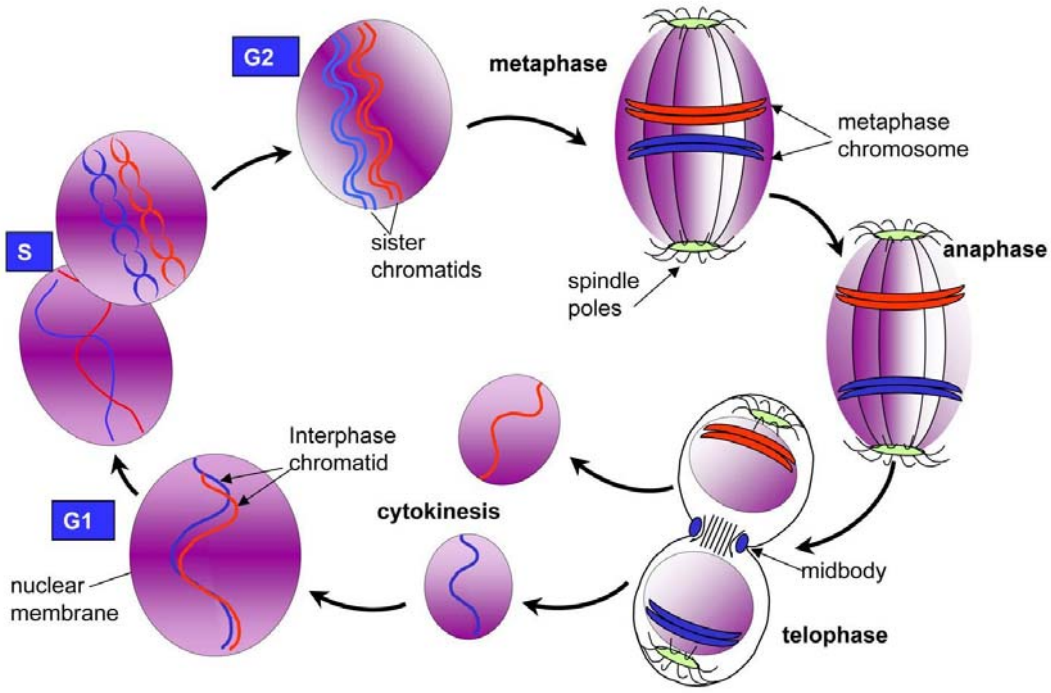


Figure 2. The cell during the cell cycle (Bradwell et al., 1999)

5.3 Diseases and autoantibodies associated with HEp-2 patterns

Antinuclear and anti-cytoplasmic antibodies are found in many diseases of which a few are listed below in Table 1. Some of these are discussed in more detail under the relevant specific HEp-2 patterns. Interpretation of positive results in patients should be considered with several factors. Elderly and pregnant people frequently have ANA, albeit in low titer, as many patients with tumors, chronic infections and may other illnesses. Autoantibodies may also appear months or years before overt manifestations of an autoimmune disorder. While ANA tests are diagnostically important, results must be interpreted in context of the clinical information. The diseases associations were shown in Table 2 (Bradwell et al., 1999).

Table 1. Principal associations of HEp-2 positive sera (Bradwell et al., 1999)

Diseases	% Incidence
Systemic kupus erythematosus	95-100
Primary bulirary cirrhosis	95-100
Lupoid hepatitis	95-100
Felty's syndrome	95-100
Rheumatoid arthritis	> 95
Sjogren' syndrome	>95
Systemic sclerosis (scleroderma)	>90
Neonatal lupus syndrome	< 90
Drug associated SLE like syndrome	< 50
Dermatomyositis, polymyositis	30-40
Chronic infection	10-50
Chronic discoid lupus	5-50
Juvenile arthritis	15-30
Neoplastic disease	10-30
Normal old age	< 30
Polyarteritis nodosa	15-25
Healthy relatives of SLE patients	25
Pregnancy	5-10
Rheumatic fever	< 5
Healthy population	< 5

Table 2. The various fluorescence patterns of antinuclear antibodies on HEp-2 cells with auto-antigens and associated diseases (Bradwell et al., 1999)

Fluorescence on HEp-2 cells		Antigens	Clinical associations
Nuclear	Homogeneous	dsDNA, histone	Lupus erythematosus
	Homogeneous and nuclear membrane	dsDNA,	Lupus erythematosus
	Linear nuclear membrane		Chronic autoimmune diseases, Lupus erythematosus
	Punctate nuclear membrane	Nuclear pore glycoproteins	Primary biliary cirrhosis Polymyositis
	Thick grain speckled, matrix	Nuclear matrix proteins hnRNP	Sharp's syndrome (MCTD) Lupus erythematosus Chronic rheumatological disease
	Speckled, large grains	U1-snRNP (or Sm)	Sharp's syndrome (MCTD) Lupus erythematosus
	Speckled, fine grains	SSA/SSB RNA polymerases II and III	Lupus erythematosus Sjögren's syndrome
	Speckled, fine nuclear and nucleolar grains	ADP-ribose polymerase	Sjögren's syndrome Lung cancer Liver disease and polyneuropathy
	Speckled, very fine grains	Scl-70	Systemic scleroderma
	Nuclear dots, 2-6	P80-coilin+snRNP	Primary biliary cirrhosis, hepatitis
	Multiple nuclear dots	Protein Sp100	Primary biliary cirrhosis, Sjögren's syndrome, rarely lupus erythematosus

Table 2. The various fluorescence patterns of antinuclear antibodies on HEp-2 cells with auto-antigens and associated diseases (continued)

Fluorescence on HEp-2 cells		Antigens	Clinical associations
Nucleolar	Homogeneous (+ nucleus)	PM-Scl	Polymyositis-Scleroderma
	Homogeneous	Ku, ADP-ribose polymerase	Polymyositis-Scleroderma Sjögren's syndrome, other
	Granular	U3-snRNP, Fibrillarin	5% systemic scleroderma
	Speckled	RNA pol I	30% systemic scleroderma
	Speckled with mitotic dots	NOR-90	Scleroderma with Raynaud's phenomenon
Cell cycle-linked	Nuclear and mitotic dots	Centromeres	CREST syndrome
	Pleiomorphic nucleus	PCNA 1-3%	lupus erythematosus
	2 dots	Centrioles Centrosomes	Chronic rheumatological disease Viral infections

5.4 Characteristics of common patterns

A variety of patterns may be found in nuclear and cytoplasmatic images depending on the types and relative amounts of sample autoantibodies. The most common patterns are homogeneous, speckle, nucleolar, nuclear membrane and centromere. Characteristic of these patterns are described in Table 3 (Bradwell et al., 1999).

Table 3. Characteristics of five commons ANA HEp-2 patterns by IF assay (Bradwell et al., 1999)

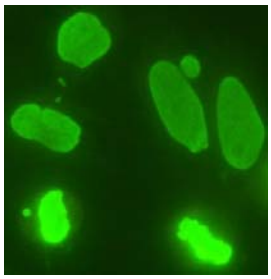
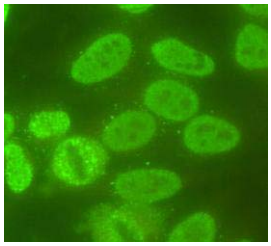
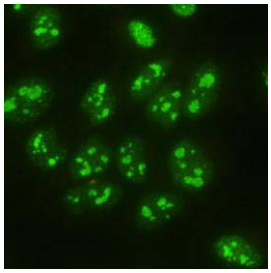
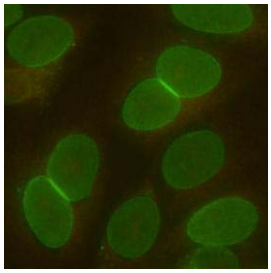
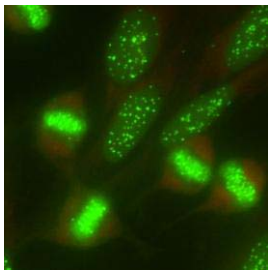
Pattern	Characteristics of pattern
Homogeneous 	Diffuse staining of the entire nucleus, with or without apparent masking of the nucleoli. Especially as the antibody reaches its endpoint the pattern may appear granular. The chromosome region of mitotic cells exhibits a bright positive staining pattern
Speckled 	Fluorescent aggregates throughout the nucleus which can be very fine to very coarse depending on the type of antibody present. More than one type of speckle may be seen in one specimen. The chromosome region of mitotic cells is usually negative.
Nucleolar 	Fluorescent staining of the nucleoli within the nucleus, sharply separated from the unstained nucleoplasm. The nucleolar fluorescence may be homogeneous, speckled or clumpy. Frequently accompanied by a speckled pattern.

Table 3. Characteristics of five commons ANA HEp-2 patterns by IF assay (continued)

Pattern	Characteristics of pattern
Nuclear membrane 	Fine, linear fluorescence of the inner and outer nuclear membranes with less homogeneous staining. In mitosis the fluorescence is diffusely localized in the cytoplasm while the cytoplasm while the chromatin is negative.
Centromere 	Discrete uniform speckles throughout the nucleus, the number corresponds to a multiple of the normal chromosome number. The staining pattern of the mitotic cells will follow that of the chromosomes, with pairs of dots arranging themselves in an equatorial plane during metaphase and then moving towards their respective centrosomes during anaphase.

6. Fixatives

6.1 Type of chemical fixatives

The purpose of fixation is to preserve tissues or cells permanently in as life-like a state as possible and to preserve important antigens. Varieties of fixatives are available for use. There are two types of fixatives which common used for HEp-2 cell fixation (Hahm & Anderer, 2006).

6.1.1 Crosslinking fixatives

Cross linking fixatives act by creating covalent chemical bonds between proteins in tissue. This anchors soluble proteins to the cytoskeleton and lends additional rigidity to the tissue. This cross-linkage does not harm the structure of proteins greatly, so that antigenicity is not lost. The standard solution is 10% neutral buffered formalin that is approximate 3.7% formaldehyde in phosphate buffered saline. This fixative could be made from paraformaldehyde. It is a polymerized form of formaldehyde, usually obtained as a fine white powder, which is depolymerized back to formalin when heated (Spector et al., 2005; O'Leary et al., 2009).

6.1.2 Precipitating fixatives

Precipitating (or denaturing) fixatives act by reducing the solubility of protein molecules and by disrupting the hydrophobic interactions that give many proteins their tertiary structure. The most common precipitating fixatives are ethanol, methanol and acetone. They are commonly used to fix frozen sections and smears (Spector et al., 2005)

6.2 Fixatives of HEp-2 cells preparation for ANA detection by IF assay

Up to now, a detailed protocol about fixatives that is used for HEp-2 preparation for ANA detection is not available in the literatures. Only few reports are disclosed. There were

three previous studies reported about the fixatives. The first study reported that acetone was more efficient than alcohol/acetone solution in screening for anti-SS-A (Ro) antibodies (speckle pattern) with a sensitivity of 97.5% and 81.3% respectively (Monce et al., 1994). The second study demonstrated the performance of varieties of fixatives. The study revealed that aldehyde fixations which caused linking of protein chains provide normal cell size and normal staining of cells nuclei. In contrast when using the combination of methanol and acetone or acetone alone, a marked change in the size of nuclei and nucleoli were noted (Hahm & Anderer, 2006). However, this study used Haematoxylin–Eosin Staining and DAPI-Staining. Further experiments are necessary to find out which fixation procedure best preserves the important antigens and is effective for ANA detection. The third study by Tsiakalou and co-workers in 2006 focused on rim-like/peripheral pattern (nuclear membrane pattern) which correlated with autoantibodies against nuclear envelope proteins. The nuclear membrane pattern was reported to provide additional information for diagnosing chronic fatigue syndrome, primary biliary cirrhosis and lupus or lupus-like syndrome. In some cases, this nuclear membrane pattern may be masked by the presence of autoantibodies against other cytoplasmic or nucleoplasmic elements This study demonstrated a better resolution of nuclear membrane pattern when using formaldehyde-fixed HeLa and Hep2 cells compared to the commercial available (Tsiakalou et al., 2006).

Objectives

1. To determine the optimal conditions in the preparation of HEp-2 cells culture on microscopic slides as substrates for ANA detection by IF technique.
2. To determine the efficacy of fixatives in preserving nuclear antigens which are associated to five basic ANA patterns by comparison four fixative protocols including 1) methanol-acetone for 10 minutes 2) 1%paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes 3) 1%paraformaldehyde for 30 minutes followed by methanol-acetone for 10 minutes and 4)1%paraformaldehyde for 5 minute followed by 0.5% Triton X-100.

CHAPTER 2

RESEARCH METHODOLOGY

Materials

1. Samples

1.1 Known negative clinical sera for ANA five samples.

1.2 Known positive clinical sera for ANA cases for five common patterns including: homogeneous, speckle, nucleolar, nuclear membrane and centromere (five samples for each pattern). All samples were obtained from routine laboratory of Division of Immunology and Virology Department of Pathology, Faculty of Medicine.

2. Positive controls

Positive controls of five ANA patterns including homogeneous, speckle, nucleolar, nuclear membrane and centromere pattern (EUROIMMUN Medizinische Labordiagnostika AG)

3. Cell culture

HEp-2 cell (human laryngeal epithelium cancer cell line, CCL-23) was purchased from American Type Culture Collection (ATCC, USA). The cells were grown in MEM supplemented with 10% fetal bovine serum, 100 U/ ml penicillin and 100 mg/ ml streptomycin. HEp-2 cells were maintained in humidified 37 ∀C incubator with 5% CO_2.

4. Commercial kit for ANA screening test

Mosaic HEp-20-10/ Liver (Monkey) (EUROIMMUN Medizinische Labordiagnostika AG)

5. Cell culture media and supplements

5.1 Culture medium : Modified Eagle Medium (MEM, GIBCO BRL)

5.2 Fetal bovine serum (GIBCO BRL)

5.3 Antibiotics (Sigma)

- Penicillin
- Streptomycin

5.4 0.25% Trypsin (GIBCO BRL)

6. Reagents

6.1 Fixatives

- Absolute Methanol (RCI LABSCAN)
- Acetone (RCI LABSCAN)
- paraformaldehyde (MERCK)

6.2 Antibody (Polyclonal Rabbit Anti-Human IgG/FITC, Dako)

6.3 Permeabilizer : Triton X-100 (Sigma)

6.4 Bovine serum albumin (BSA); Fraction V (Sigma)

6.5 Tween20 (Sigma)

6.6 Phosphate Buffer Saline (PBS) pH 7.4 (NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 1.76 mmol/L)

6.7 Mounting media (90% glycerol in PBS buffer pH 7.4, 0.01% anti-fading agent (DABCO)

7. Equipments

7.1 Microscopic glass slides (Thermo SCIENTIFIC)

7.2 Culture flask (NUNC)

Methods

1. Preparation of cells on microscopic slides

HEp-2 cells were grown in MEM complete medium in T-25 culture flask. A monolayer of cells were trypsinized by using 0.25% trypsin-EDTA and prepared the cells to obtain the final concentration of 3.0×10^5 cells / ml cells suspension.

Ten microliters of HEp-2 cells suspension was dropped into the well area of the sterile 12-well microscopic glass slide and spread to cover the specific area of the well, given an approximately 3000 cells/ well.

The slides contained cells suspension in each well areas were incubated in the humidified 37 ∀C incubator with 5% CO_2 for 1 hour 30 minutes for allowing the cells to attachment. Next an approximately 25 σl of MEM growth medium were added into each well.

The cells culture slides were continued to incubate in humidified 37 ∀C incubator with 5% CO_2. A few drops of MEM growth medium were added into each well for every 12 hours. The culture slides were harvested for further step testing at difference time points (6, 12, 24, 36, 42 and 48 hours).

To determine the appropriated time to harvest cultured cells. The cultured cells were observed by phase contrast microscope. The criterion are 1) The cultured cells showed well distribution and cell confluents were more than 70% 2) Numbers of mitoses are at least 2-3 cells per visual observing at 400x magnification.

2. Optimization of cells harvesting for fixation and staining

To determine the appropriate time of cultured cells harvesting for fixation and staining, HEp-2 cells cultured at different times of incubation were fixed with four fixation protocols as described below. Then, the fixed slides were stained by IF method using positive control sera of homogeneous pattern. The staining cells were observed by fluorescence microscope. Percentage of cell confluences and number of mitotic cells were evaluated.

2.1 Cells fixation

The cultured slide was rinsed 3 times with the PBS buffer. The further washing step was continued by dipped the slides in 50 ml PBS buffer for 10 times. Then, the slide was immersed in 1X PBS buffer (pH 7.4) until the next fixation step being performed. Cells were fixed using one of protocol 1, 2, 3 or 4 as described below:

Protocol 1: Methanol-Acetone fixation

The pre-washed slide was immersed into 50 ml of cold Methanol-Acetone (1:1, pre-cool at -20∀C) for 10 minutes at -20 ∀C. Then washed the cell-culture slide 3 times with 50 ml of PBS buffer and keep the slide in the PBS buffer until the next step being processed.

Protocol 2: 1% Paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes fixation

The slide was immersed in 50 ml 1% paraformaldehyde-PBS buffer solution (PBS buffer contains 1% paraformaldehyde) at room temperature for 5 minutes and washed with 50 ml of PBS buffer for 5 minutes, 5 times. For, permeabilization step, slide was immersed in cold Methanol-Acetone solution (1:1; pre-cool the solution at -20∀C) for 10 minutes at -20 ∀C and then washed 3 time with 50 ml of PBS buffer. Slide was kept in PBS buffer until the next step being performed.

Protocol 3: 1% Paraformaldehyde for 30 minutes followed by methanol-acetone for 10 minutes fixation

The slide was immersed in 50 ml of 1% paraformaldehyde-PBS buffer solution at room temperature for 30 minutes and washed with 50 ml of PBS buffer for 5 minutes, 5 times. After permeabilization and washing as after as described in protocol 2, slide was kept in PBS buffer until the next step being performed.

Protocol 4: 1% Paraformaldehyde for 5 minutes followed by 0.5% Triton X-100 fixation for 5 minutes fixation

The slide was immersed in 50 ml of 1% paraformaldehyde-PBS buffer solution at room temperature for 5 minutes. and washed with 50 ml of PBS buffer for 5 minutes, 5 times. For, permeabilization step, slide was immersed in the in 0.5 % Triton X-100 solution at room temperature for 5 minutes followed by 3 brief rinse with PBS buffer. Slide was kept in PBS buffer until the next step being performed.

2.2 IF staining

The patient sera or control sera were diluted to 1: 80 with diluents buffer (containing 1X PBS buffer pH 7.4, 0.5% Triton X-100 and 1% bovine serum albumin). The slide was removed from PBS buffer. The trace residue PBS buffer around the wells was removed. The slide was kept in wet condition throughout the whole processes.

Ten microliters of diluted sample 10 σl was applied to the each well of cell-culture slide. The slide was incubated with diluted serum at room temperature for 30 minutes. Then slide was washed 3 times, 5 minutes each with washing buffer (containing 1X PBS buffer pH 7.4 contain 0.2% tween20). The washing buffer was removed from slide. The trace residue PBS buffer around the wells was removed

Ten microliters of diluted conjugate, dilution 1:300 (Polyclonal Rabbit Anti-Human IgG/ FITC, Dako) was added to each well of the slides. Slide was incubated in dark, at room temperature for 30 minutes. The slides were washed 3 times, 5 minutes each with washing buffer. The washing buffer was drain from the slide. Next, the slide was mounted with mounting media and the test area was covered with cover glass. The slide was examined with the fluorescent microscope.

CHAPTER 3

RESULTS

1. Cells confluence and number of mitotic cells on microscopic slide

To determine the appropriate time of cultured cells harvesting, percentage of cell confluences and number of mitotic cells were observed by phase contrast microscope. The cultured slide was observed at different time points, 6, 12, 24, 36, 42 and 48 hours after incubation. The well prepared slides should have more than 70% confluence and at least 2-3 mitotic cells per visual field at 400x magnification. The results showed that the cultured cells at 12, 24, 36 and 42 hours were suitable times for harvesting. At these times, the cultured slides showed 70-100% cells confluence and 2-7 mitotic cells. While the cultured cells at 48 hours showed high confluence and adequate mitotic cells but there were some dead cells (Table 4 and Figure 3).

Table 4. Percentage of cell confluence and number of mitotic cells on microscopic slide

Incubation time (hour)	Confluent (%)	Mitotic cell (Number/400X magnification)
6	50-60	0
12	70-80	2-3
24	80-90	2-3
36	80-90	3-5
42	90-100	3-7
48	> 100	3-7

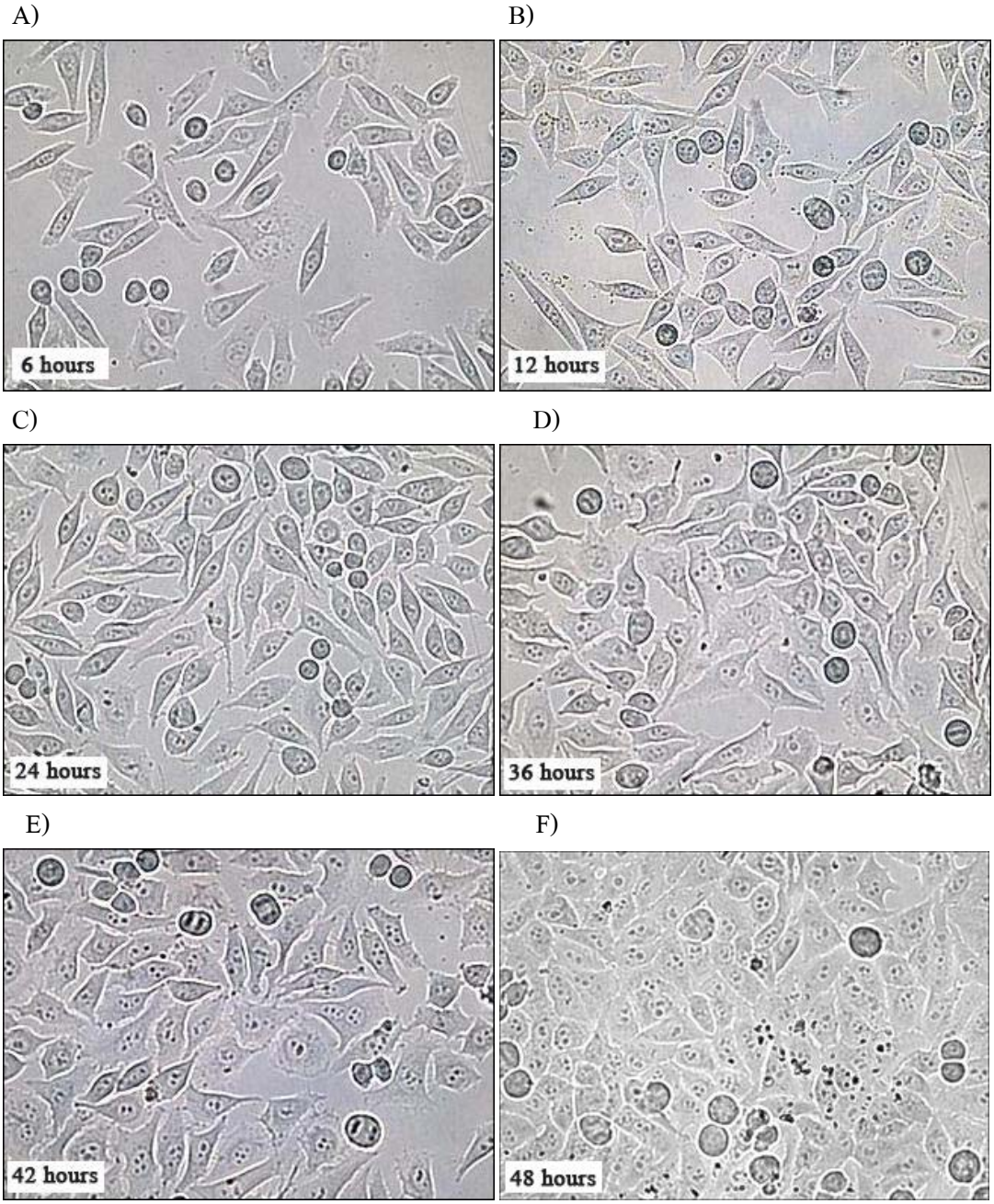


Figure 3. HEp-2 cells cultured on glass slide after different times of incubation.

At (A) 6 hours, (B) 12 hours, (C) 24 hours, (D) 36 hours, (E) 42 hours and (F) 48 hours of incubation.

2. Cells confluence and number of mitotic cells on microscopic slide after fixation and staining.

To determine the appropriate time of harvesting cultured cells for fixation and ANA-IFA testing, four different times of incubation (12, 24, 36, 42 hours) were chosen for fixing with four fixation protocols. Protocol 1: methanol-acetone fixation for 10 minutes, Protocol 2: 1% paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes, Protocol 3: 1% paraformaldehyde for 30 minutes followed by methanol-acetone for 10 minutes, Protocol 4 : 1% paraformaldehyde for 5 minutes followed by 0.5% Triton X-100 for 5 minutes. Then, the fixed slides were stained by IF method using positive control sera of homogeneous pattern. The staining cells were observed by fluorescence microscope. Percentage of cell confluences and number of mitotic cells were evaluated. The criteria for good preparation slides were those described in the previous section (> 70% confluence and at least 2-3 mitotic cells/400x magnification).

The results showed that, after fixation and staining, cell density and amount of mitotic cells on microscopic slides were less than the original slide. These results were found in all of incubation times and four fixation protocols. The slides of 12, 24 and 36 hours demonstrated 0-2 mitotic cells/400x magnification which less than the criteria. The slide of 42 hours incubation revealed cell confluence of 70-80% and 2-5 mitotic cells/400x magnification. Therefore, the most effective time of cell harvesting for fixation and IF staining is at 42 hours (Table 5 and Figure 4-7).

Table 5. Percentage of cells confluence and number of mitotic cells on microscopic slide after fixation and IF staining

Fixation Protocol	Incubation time (hours)	Confluent (%)	Mitotic cell (Number/ 400x magnification)
1 (methanol-acetone for 10 minutes)	12	50-60	0-1
	24	50-60	0-1
	36	70-80	1-2
	42	70-80	2-5
2 (1% paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes)	12	50-60	0-1
	24	50-60	0-1
	36	70-80	1-2
	42	70-80	2-5
3 (1% paraformaldehyde for 30 minutes followed by methanol-acetone for 10 minutes)	12	50-60	0-1
	24	50-60	0-1
	36	70-80	1-2
	42	70-80	2-5
4 (1% paraformaldehyde for 30 minutes followed by 0.5%Triton X-100 for 5 minutes)	12	50-60	0-1
	24	50-60	0-1
	36	70-80	1-2
	42	70-80	2-5

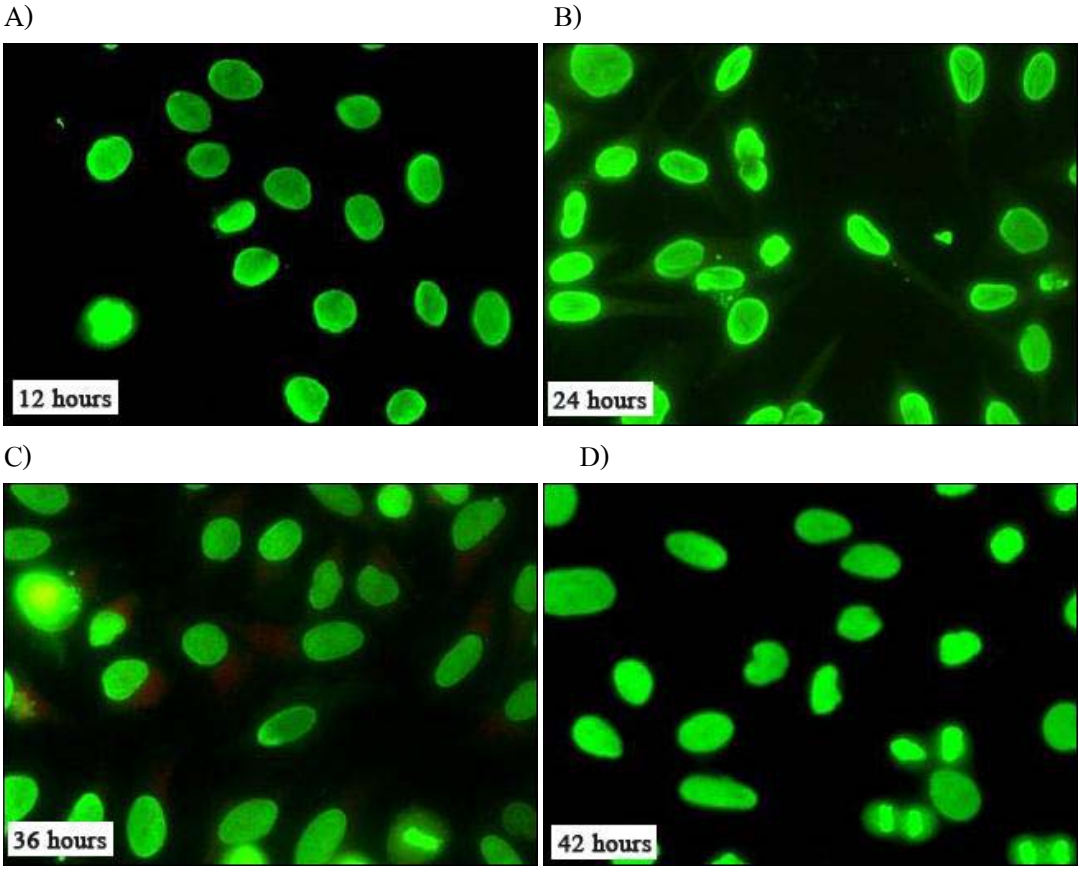


Figure 4. HEp-2 cells on glass slide at different times of incubation after fixation using protocol 1 (methanol-acetone 10 minutes) and staining by IF method. Cells were observed under 400x magnification of fluorescence microscope at (A) 12 hours, (B) 24 hours, (C) 36 hours and (D) 42 hours of incubation.

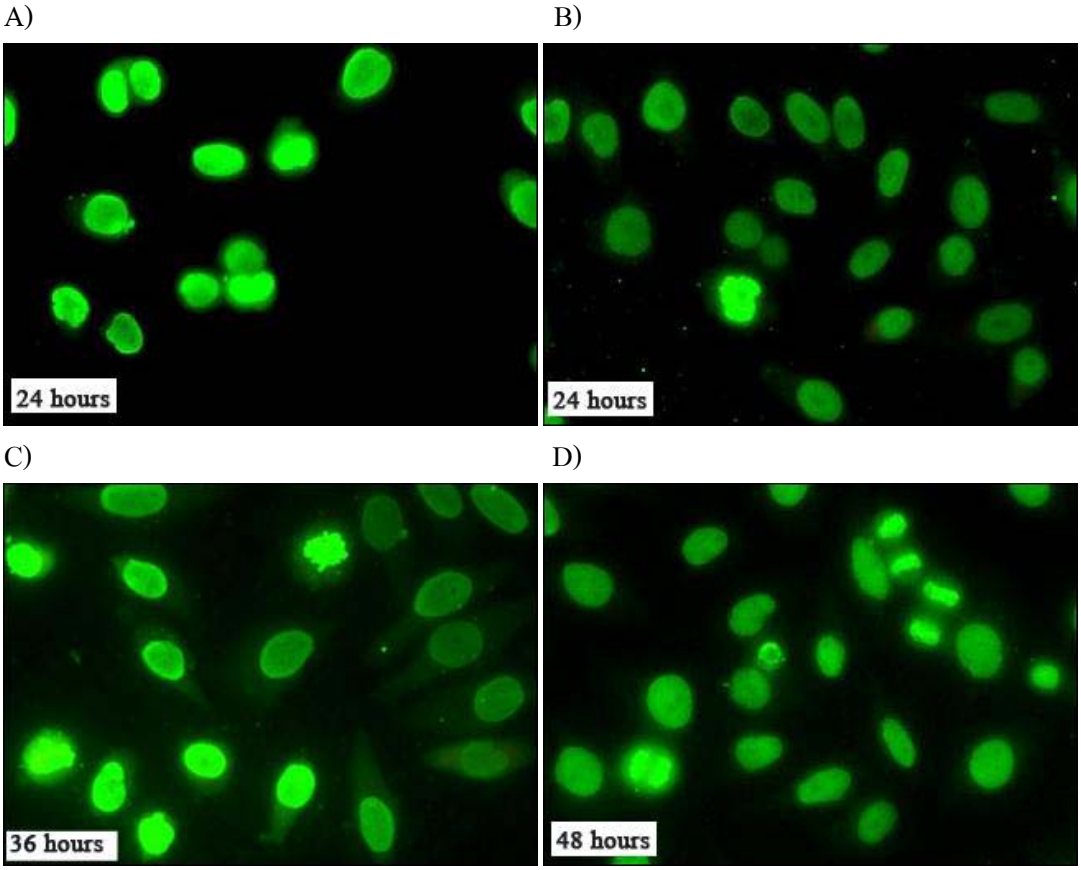


Figure 5. HEp-2 cells on glass slide at different times of incubation after fixation using protocol 2 (1% paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes fixation) and staining by IF method. Cells were observed under 400x magnification of fluorescence microscope at (A) 12 hours, (B) 24 hours, (C) 36 hours and (D) 42 hours of incubation.

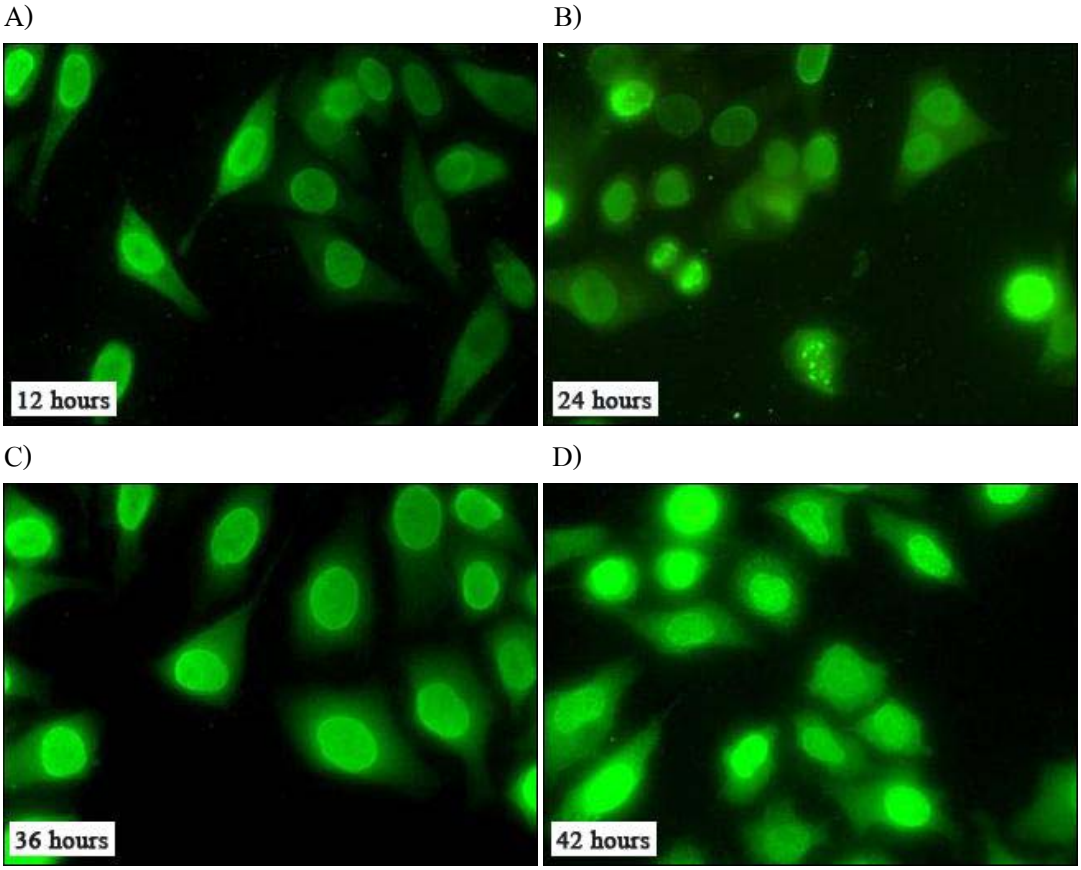


Figure 6. HEp-2 cells on glass slide at different times of incubation after fixation using protocol 3 (1% paraformaldehyde for 30 minutes followed by methanol-acetone for 10 minutes fixation) and staining by IF method. Cells were observed at 400x magnification of fluorescence microscope at (A) 12 hours, (B) 24 hours, (C) 36 hours and (D) 42 hours of incubation.

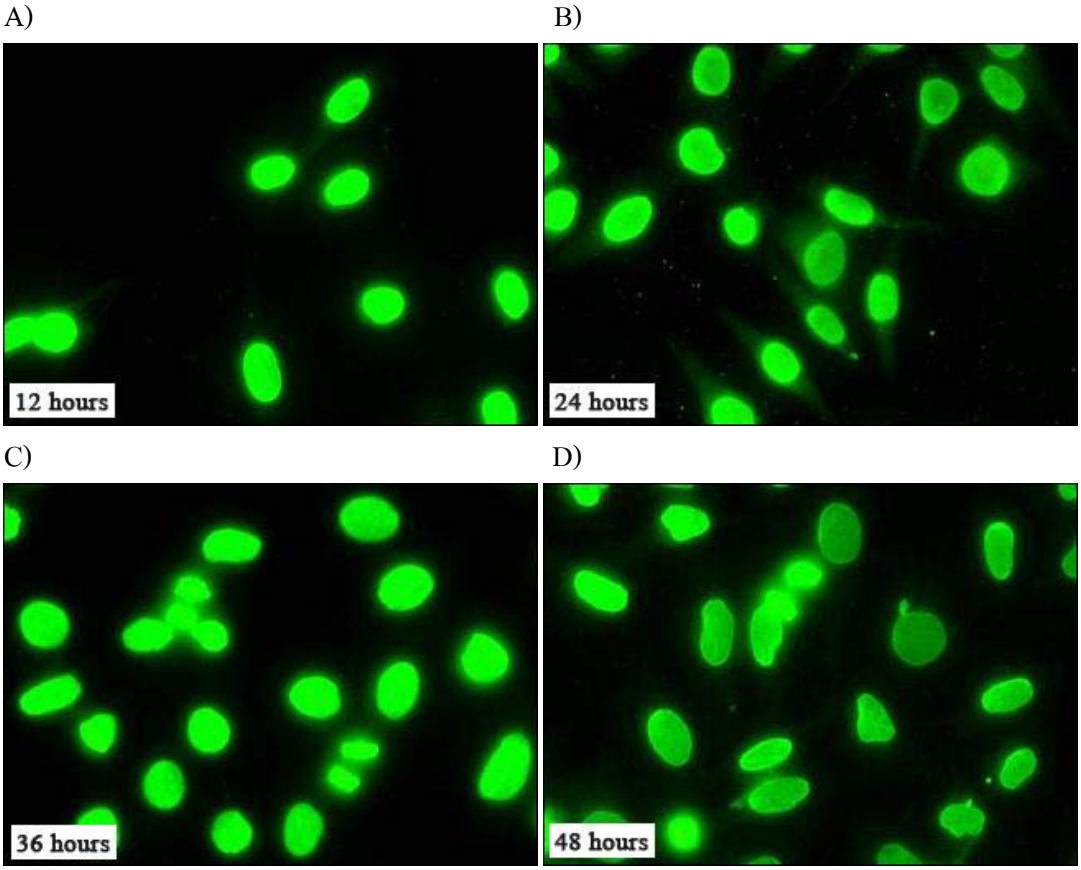


Figure 7. HEp-2 cells on glass slide at different times of incubation after fixation using protocol 4 (1% paraformaldehyde for 5 minutes followed by 0.5% Triton X-100 for 5 minutes fixation) and staining by IF method. Cells were observed under 400x magnification of fluorescence microscope at (A) 12 hours, (B) 24 hours, (C) 36 hours and (D) 42 hours of incubation.

3. Five ANA patterns comparing among four fixation protocols and commercial kit.

To determine the appropriate fixation protocol for ANA detection by IF staining, cultured HEp-2 cells at 42 hours of incubation were chosen for fixing with four fixation protocols. Then the fixed slide was stained by IF method using positive controls for five ANA patterns: homogeneous, speckled, nucleoli, nuclear membrane and centromere pattern. The stained cells were observed by fluorescence microscope at 400x magnification. The results were compared with HEp-2 cells commercial kit. The criteria for determination the appropriated fixation protocol for next experiment were that the stained cells should demonstrated distinct characteristic of each ANA pattern as shown in Table 3. Fluorescence background, brightness and sharpness of the fluorescence intensity were evaluated.

The results showed that all fixation protocols demonstrated distinct characteristic of five ANA patterns (Figure 8-12). However, protocol 1 and protocol 2 showed sharper and brighter picture of speckle pattern than protocol 3 and protocol 4 (Figure 9). For nucleolar, nuclear membrane and centromere pattern, protocol 1, protocol 2 and protocol 4 showed clear fluorescents background, while high intensity background was found in protocol 3 (Figure 10-12). Therefore, fixation protocol 1 and protocol 2 represented better staining than protocol 3 and protocol 4 for all five patterns.

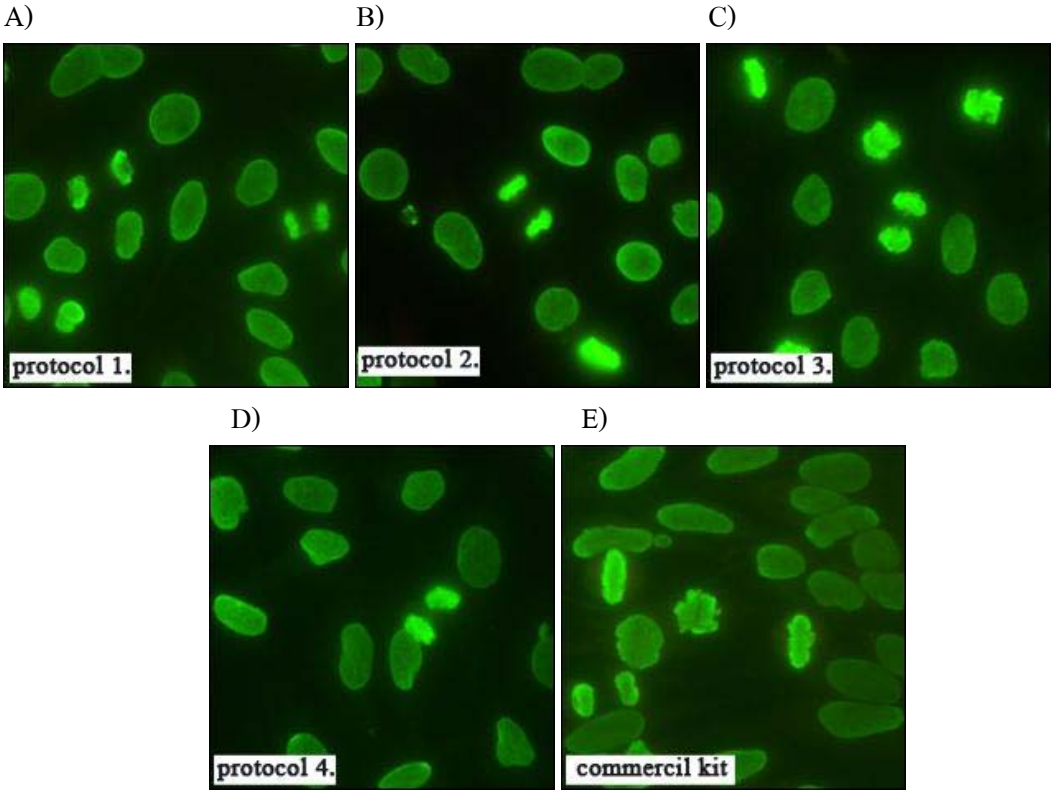


Figure 8. Fixed HEp-2 cells and staining by IF method with positive control of homogeneous pattern. Fixation protocol 1-4 (A-D) and commercial kit (E).The characteristics of diffuse nuclear staining and bright chromosome staining of mitotic cells were found in all four fixation protocols and commercial slides.

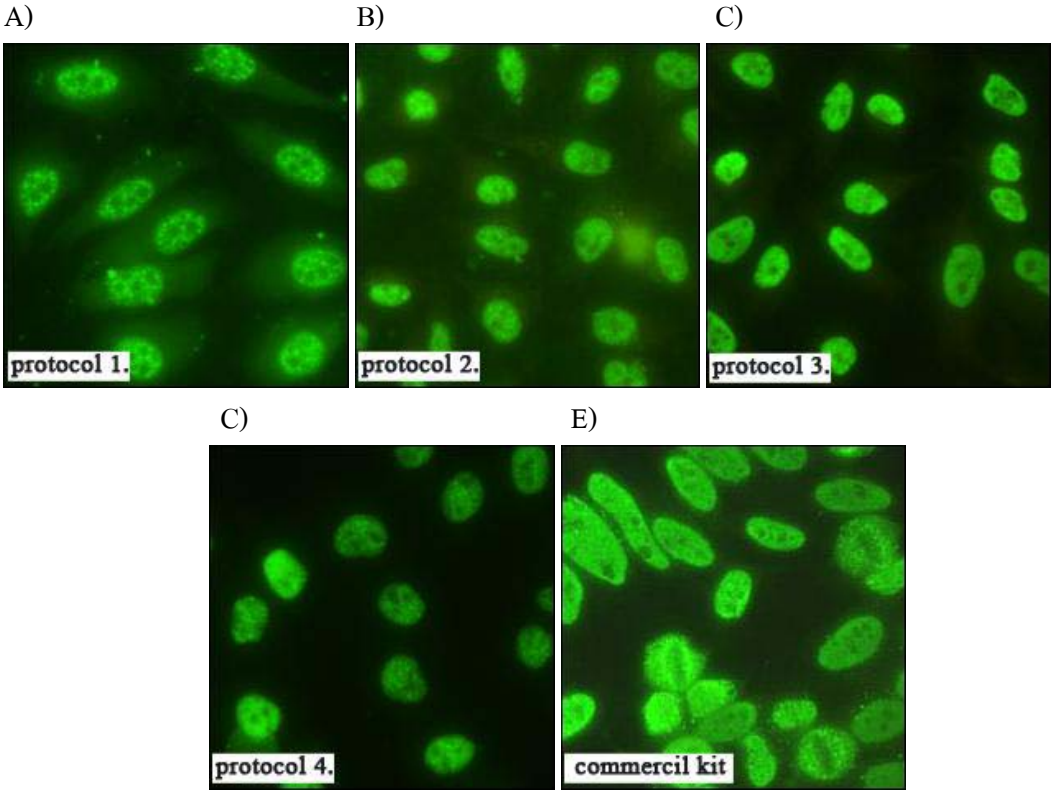


Figure 9. Fixed HEp-2 cells and staining by IF method with positive control of speckle pattern. Fixation protocol 1-4 (A-D) and commercial kit (E). The characteristics of granular aggregate staining throughout the nucleus and negative chromosome staining in mitotic cells were found in all four fixation protocols staining cells. Brighter and sharper fluorescents staining were observed in fixation protocol 1 and commercial slides.

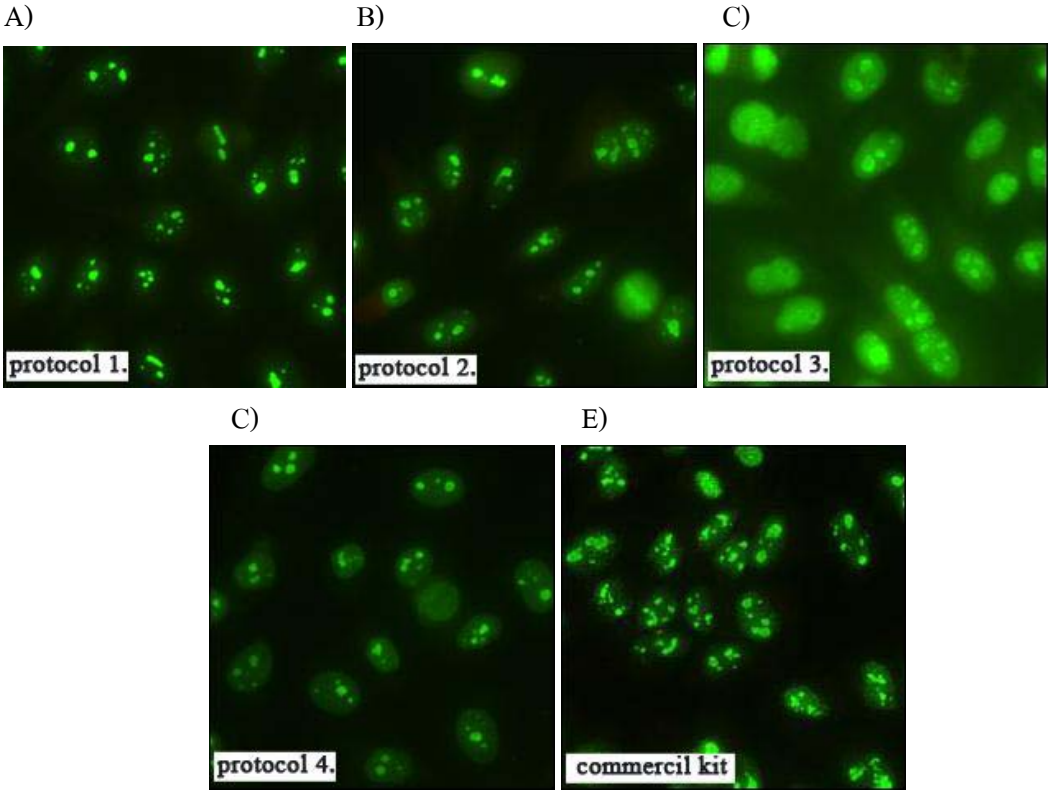


Figure 10. Fixed HEp-2 cells and staining by IF method with positive control of nucleolar pattern. Fixation protocol 1-4 (A-D) and commercial kit (E). The characteristics of nuclolar pattern, fluorescent staining of the nucleoli within the nucleus and distinct contrast with unstained nucleoplasm were found in all four fixation protocols. Brighter and sharper fluorescents staining were found in fixation protocol 1, protocol 2, protocol 4 and commercial slides, while protocol 3 showed high fluorescent background.

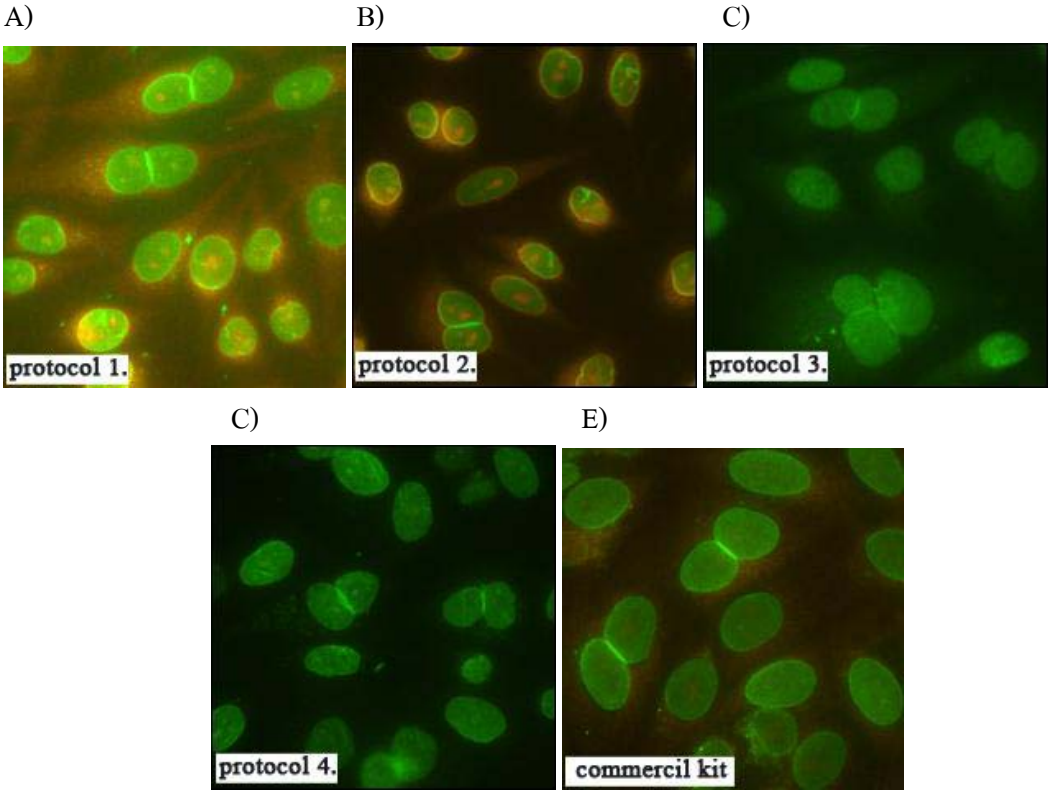


Figure 11. Fixed HEp-2 cells and staining by IF method with positive control of nuclear membrane pattern. Fixation protocol 1-4 (A-D) and commercial kit (E). The characteristics of distinct linear nuclear membrane staining and negative chromosome staining were observed in all four fixation protocols. Protocol 1, protocol 2 and protocol 4 represented brighter and sharper fluorescent staining than protocol 3. High fluorescent background was seen in protocol 3 fixed slides.

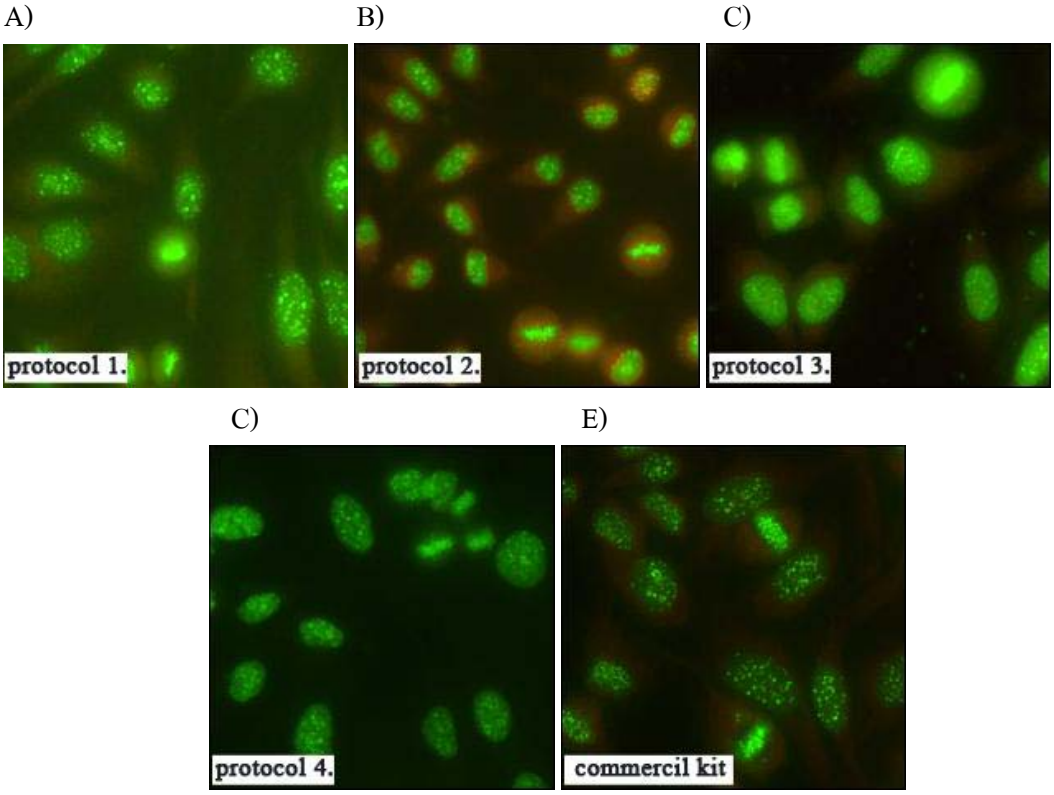


Figure 12. Fixed HEp-2 cells and staining by IF method with positive control of centromere pattern. Fixation protocol 1-4 (A-D) and commercial kit (E). The characteristics of discrete speckles staining throughout the nucleus and positive chromosomes staining with pairs of dots arranged in an equatorial plane of metaphase were observed in all four fixation protocols. Protocol 1, Protocol 2 and commercial kit demonstrated brighter and sharper fluorescent staining than protocol 3. High fluorescent background was seen in protocol 3.

4. ANA-IF staining with clinical samples comparing among two fixation protocols and commercial kit.

The fixation protocols 1 and 2 were chosen for staining with known negative and positive clinical samples of five ANA patterns (five samples for each pattern). The staining cells were observed by fluorescence microscope at 400x magnification. The results were compared with HEp-2 cells commercial kit. The distinction of each ANA pattern, background fluorescence, brightness and sharpness of the fluorescence intensity were included for evaluation.

4.1 Negative samples: All five samples demonstrated negative IF staining for both of the two fixation protocols and commercial kit (Figure 13).

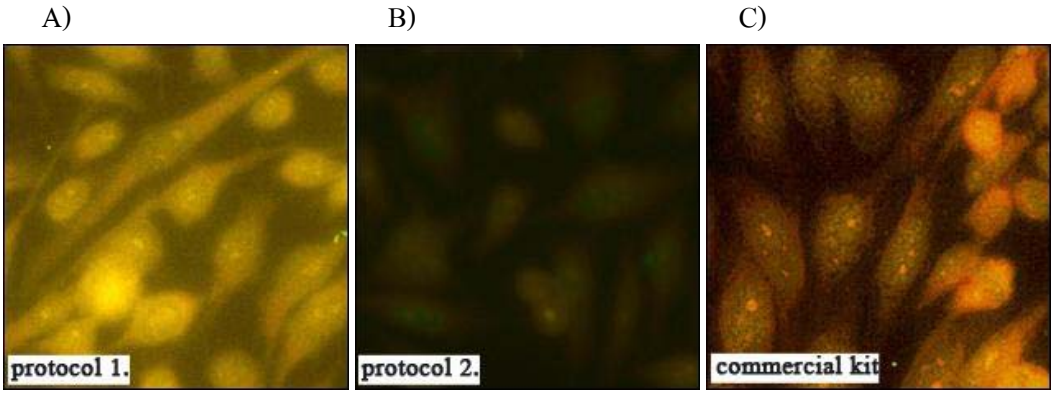


Figure 13. Fixed HEp-2 cells and staining by IF method with negative samples.
Fixation protocol 1-2 (A-B) and commercial kit (C). All slides showed negative fluorescent staining.

4.2 Positive samples of homogeneous pattern: All five samples were shown positive homogeneous pattern for both of the two fixation protocols and commercial kit (Figure 14).

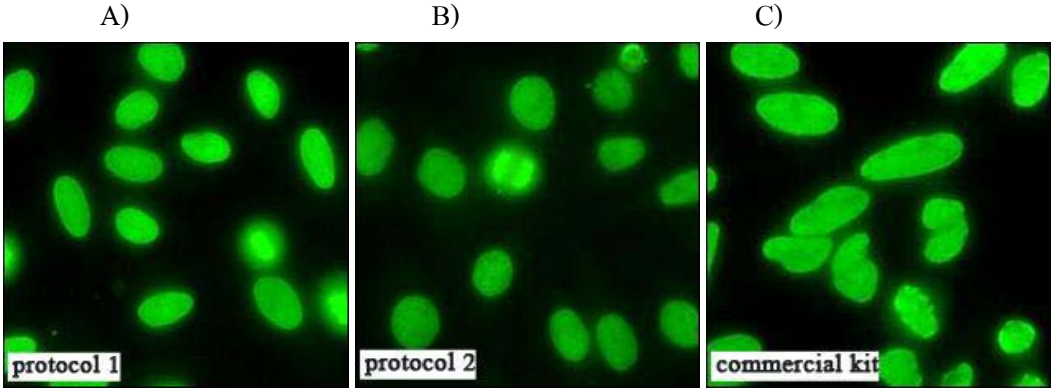


Figure 14. Fixed HEp-2 cells and staining by IF method with positive clinical samples of homogeneous pattern. Fixation protocol 1-2 (A-B) and commercial kit (C). All slides showed distinct characteristic homogeneous nuclear fluorescent staining.

4.3 Positive samples of speckle patterns: All five samples showed positive speckle pattern for both of the two fixation protocols and commercial kit. However, slightly brighter and sharper fluorescents staining were found in protocol 1 and commercial kit (Figure 15).

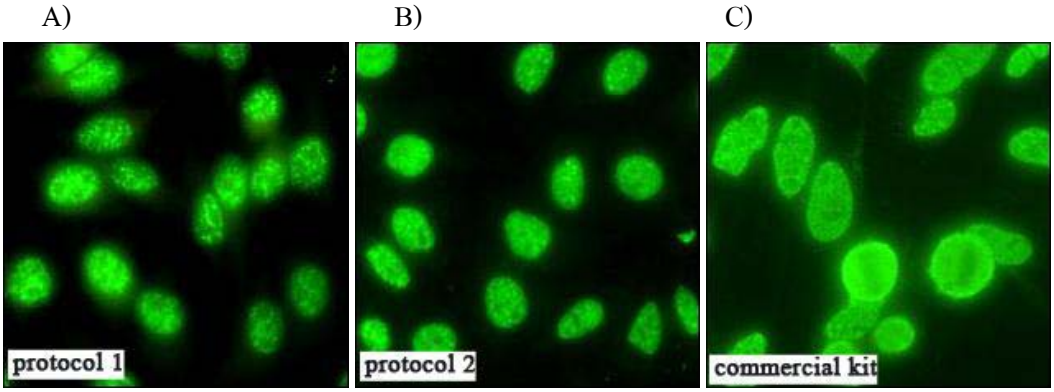


Figure 15. Fixed HEp-2 cells and staining by IF method with positive clinical samples of speckle pattern. Fixation protocol 1-2 (A-B) and commercial kit (C). Protocol 1 and commercial kit demonstrated slightly brighter and sharper fluorescent staining than protocol 2.

4.4 Positive samples of nucleolar patterns: All samples were shown positive nucleolar pattern for both of the two fixation protocols and commercial kit. Brighter and sharper fluorescents staining were found in commercial kit. Minimal cytoplasmic staining was found in fixation protocol 1 and protocol 2 (Figure 16).

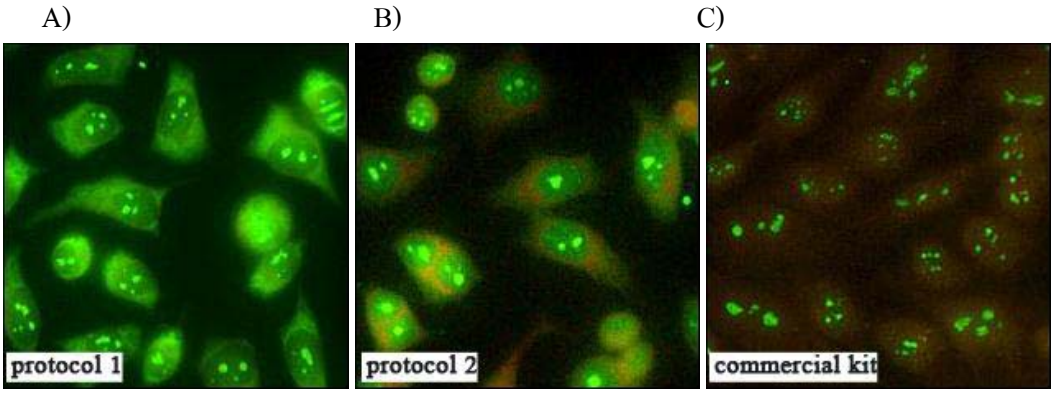


Figure 16. Fixed HEp-2 cells and staining by IF method with positive clinical samples of nucleolar pattern. Fixation protocol 1-2 (A-B) and commercial kit (C). Commercial kit demonstrated brighter and sharper intensity IF staining than protocol 1 and protocol 2. Protocol 1 and protocol 2 kits showed slight cytoplasmic staining.

4.5 Positive samples of nuclear membrane pattern: Four samples showed positive nuclear membrane pattern for both two fixation protocols and commercial kit (Figure 17). There was one sample demonstrated undetermined results with cytoplasmic staining in both fixation protocol 1 and protocol 2, while commercial kit showed positive result with slightly cytoplasmic staining (Figure 19). This sample was retested using fixation protocol 4 (1% paraformaldehyde for 5 minutes and 0.5%Triton X-100 for 5 minutes).

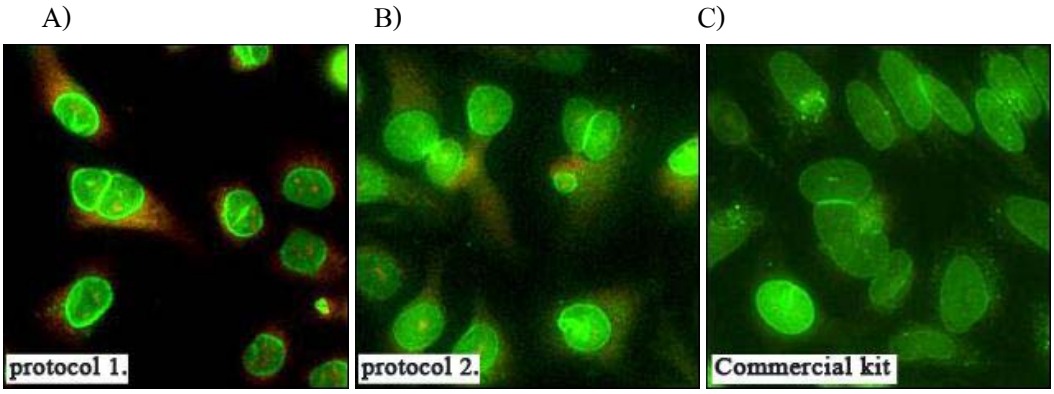


Figure 17. Fixed HEp-2 cells and staining by IF method with positive samples of nuclear membrane pattern. Fixation protocol 1-2 (A-B) and commercial kit (C). All of HEp-2 cells showed characteristic of nuclear membrane IF staining.

4.6 Positive samples of centromere pattern: All five samples demonstrated positive centromere pattern for both two fixation protocols and commercial kit. Brighter and sharper fluorescents staining were found in commercial kit (Figure 18).

A) B) C)

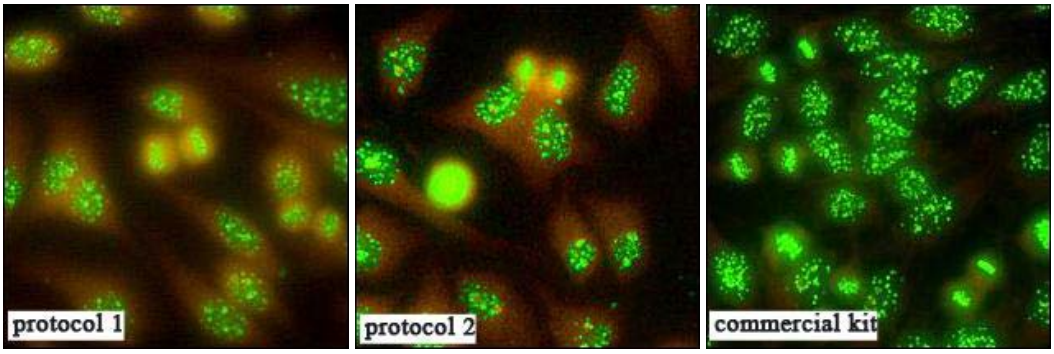


Figure 18. Fixed HEp-2 cells and staining by IF method with positive samples of centromere pattern. Fixation protocol 1-2 (A-B) and commercial kit (C). Commercial kit demonstrated brighter and sharper fluorescent staining than protocol 1 and protocol 2.

5. ANA-IF staining with known positive clinical sample for nuclear membrane pattern with cytoplasmic staining.

One positive sample for nuclear membrane pattern which also showed cytoplasmic staining was retested using fixation protocol 4 (1% paraformaldehyde for 5 minutes and 0.5%Triton X-100 for 5 minutes). The results showed that protocol 1 and protocol 2 demonstrated cytoplasmic staining. The ANA pattern could not be interpreted (Figure 19A-B). While commercial slide showed positive nuclear membrane pattern (with slight cytoplasmic staining) (Figure 19D). The cytoplasmic staining was removed and distinct nuclear membrane pattern was demonstrated when retesting the sample with Protocol 4 (Figure 19C).

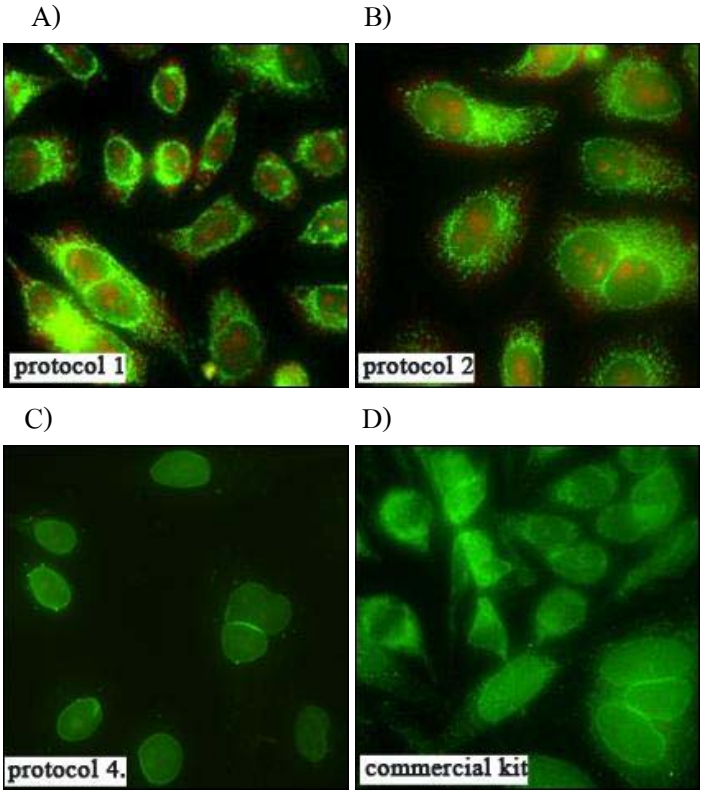


Figure 19. Fixed HEp-2 cells and staining by IF method with positive samples of nuclear membrane pattern with cytoplasmic staining. Fixation protocol 1-2 (A-B), protocol 4 (C) and commercial kit (D). Protocol 1 and protocol 2 represented unclear nuclear membrane pattern with cytoplasmic staining while commercial kit showed slight cytoplasmic staining. Protocol 4 showed distinct nuclear membrane staining without cytoplasmic staining.

CHAPTER 4

DISCUSSION

Antinuclear antibodies (ANA) are useful diagnostic tools for several autoimmune diseases (Tozzoli et al., 2002). The method that is widely used for ANA screening is the immunofluorescent (IF) assay using HEp-2 cells as substrate antigen. Methods used for cell preparation on microscopic slide are important for ANA pattern analysis. The processes of cell preparation consist of two important steps, cultivation of HEp-2 cells on microscopic slides and fixation of cultured cells to preserve cells structure and nuclear antigens. However the practical data for HEp-2 cells preparations were rarely described in the literatures and fixative reagents for in-house preparation remain unclear. The objectives of this study were to determine the optimal conditions in the preparation of HEp-2 cells on microscopic slides as substrates for ANA detection by IF technique. In addition, two fixatives, methanol-acetone and paraformaldehyde as four different fixation protocols were evaluated for their efficacies in preserving nuclear antigens. Fixations were evaluated against five basic ANA patterns including homogeneous, speckled, nucleolar, nuclear membrane, and centromere pattern. The criteria mentioned by Sack and co-worker (Sack et al., 2009) were used as quality control for HEp-2 cells preparation including 1) cell density and distribution on the microscope slide 2) number of mitoses, at least 2 to 3 mitoses per visual field at 400× magnification 3) maintenance of morphology, 4) low background fluorescence and 5) expression of target antigens for relevant autoantibodies.

HEp-2 cells preparation on glass slide

The processing of HEp-2 cell preparation was reported by Hahm and co-worker (Hahm & Anderer, 2006). The report revealed that Hep-2 cell could be cultured on microscopic slide. However, the techniques of cell preparation were not described in detail. In this study, HEp-2 cells preparations on microscopic slide were demonstrated, 3,000 HEp-2 cells/well were seeded on 12 wells microscopic slide. The harvesting condition for fixation and IF staining was at 42 hours of incubation. At this time, after fixation and IF staining, there were 70-80 % confluence and 2-5 mitotic cells/ 400x magnification. After 42 hours of incubation, the cell confluence was too high and there were some dead cells. The results were consistent with the previous report (Hahm & Anderer, 2006) which used the condition at 2 days of incubation for harvesting.

The numbers of mitotic cells on the in-house HEp-2 slides were less than the commercial slides which there were 5-7 cells. However the numbers of mitoses are still in the criteria of quality control described above. This may be because of using different cell preparation method. Normally, mitosis occurs during 10-15% of the cell cycle. Actually, the number of mitoses can be accelerated by a method of cell synchronization (Davis et al., 2001). However, other suggested that, synchronized cultures might not contain cells at all stages in the cycle and should generally be avoided (Bradwell et al., 1999).

Fixation of slides

Fixatives are important to keep cells on microscopic slide and to preserve cell structure and nuclear antigens. There are two major types of fixative, precipitation (alcohol or acetone) and cross-linking fixatives (formaldehyde, paraformaldehyde or glutaraldehyde). The selection of a specific fixation protocol generally depends on several factors, such as type of cell, types of antigens, types of fluorescent antibody used in test and the staining procedure.

The activities of precipitating agents are dehydration and destroying the hydrophobic interaction of proteins resulting in loss of protein structures. The advantages of these fixatives are one step fixing and shorter time fixation. In addition, the fixed cells could be kept at -20 C for longer time. The previous study reported that acetone was more efficient than alcohol/acetone solution in screening for speckle pattern (anti-SS-A (Ro) (Monce et al., 1994). Another report revealed that cell nucleolar was not demonstrated when using acetone fixative (Haematoxilin-Eosin staining). While using methanol combination with acetone (1:1) as a fixative presented better result, cell nucleolar were distinct demonstrated with larger size. The present study showed that methanol-acetone fixation demonstrated distinct characteristic of ANA pattern including homogeneous, speckle, nucleolar and centromere pattern with brighter and sharper fluorescent staining than paraformaldehyde fixation especially for speckle pattern which consistent with the previous study.

Paraformaldehyde, cross-linking fixative, create a chemical covalent bond between proteins and makes up the network. With cross linking fixation, the cells morphology is better preserved. However, an additional process to create the permeability of cells using permeabilized reagents (such as Triton X-100) may be needed. Another disadvantage is high background fluorescent may be occur in case of residual aldehyde groups. In present study, 1% paraformaldehyde was used followed by the premeabilizer, methanol-acetone. The reason for designing this protocol is that 1% paraformadehyde is a mild fixative which could not fix mitochondrial antigen, result in reduced cytoplasmic staining. Furthermore, the fixed slide which used methanol-acetone as permeabilizer could be kept for longer time. For fixing incubation time, 5 and 30 minutes were tested in this study. There was a report revealed that, the intensity of the staining of the nucleus after 10 minutes fixation was different from cell to cell, whereas after 20 to 30 minutes, a more homogeneous staining pattern appeared (Hahm & Anderer, 2006). The present results of paraformaldehyde fixation showed characteristic of ANA patterns including homogeneous, speckle, nuclolar and centromere with higher fluorescent background than

methanol-acetone fixation especially for nucleolar pattern. Moreover, highest fluorescent backgrounds were found with 1% paraformaldehyde for 30 minutes fixation, this may be because of residual aldehyde groups on microscopic slide which was difficult to remove as described above. This result was different from the study of Hahm and Anderer in 2006 which demonstrated that fixation by paraformaldehyde for 30 minutes showed the better intensity staining than 10 minutes. However the staining of the two studies was different. The previous study used Haemotaxilin-Eosin staining while the present study used ANA-IF staining which is more specific than the first one.

For nuclear membrane pattern, the detection of this pattern is based on the characteristic nuclear membrane (rim-like) fluorescence. This pattern may be masked by the presence of autoantibodies directed against other cytoplasmic or nucleoplasmic elements. The cytoplasmic staining will be reduced by using mild fixative such as 1% paraformadehyde as describe above. In this study, one case of nuclear membrane pattern, the staining pattern could not be interpreted due to masking of cytoplasm staining. This result occurred with both methanol-acetone fixation and 1% paraformaldehyde followed with methanol-acetone fixation. Although the mild fixative, 1% paraformaldehyde was used (in the second protocol), but the cytoplasm staining was still observed. This may be because of the permeabilizer, methanol-acetone that acted as premeabilizer and fixative at the same time. The problem was solved by using alternative permebilizer, Triton-X-100 which was mentioned by Tsiakalou and co-worker (Tsiakalou et al., 2006). The result showed distinct nuclear membrane pattern with unstained cytoplasm.

Comparison with commercial HEp-2 slide

In-house HEp-2 slide for ANA detection was compared with commercial HEp-2 slide using the criteria as described above (Sack et al., 2009). Adequacy of cell density and well distribution were found in both type of slides. The number of mitosis of the in-house HEp-2 slide

was less than the commercial HEp-2 slide, however adequate amount were found. Cell morphology of the in-house HEp-2 cells for any preparation lots was not significantly different. The in-house HEp-2 slide demonstrated higher background fluorescence than commercial HEp-2 slide, while commercial HEp-2 slide showed sharper and brighter fluorescent intensity. This may be because of using different kind of microscopic slides. Special plastic slides are used in commercial kit that non-specific binding might be better removed than glass slide. For expression of target antigens of relevant autoantibodies, ANA by IF method were performed using known negative and positive samples of five common ANA patterns including homogeneous, speckle, nucleolar, nuclear membrane and centromere pattern. The in-house HEp-2 slide with protocol 1 and protocol 2 demonstrated the characteristic of each pattern as seen in the commercial HEp-2 slide, except case of the nuclear membrane with cytoplasmic staining. Nevertheless, only five samples of five patterns were tested in this study. Evaluation in larger sample set is required to obtain sensitivity and specificity of the test.

CHAPTER 5

CONCLUSIONS

In the present study, HEp-2 cell slide preparation for ANA detection by IF technique and four fixative protocols were studied. The experiments were concluded in below;

1. The total amount of HEp-2 cell concentration/ well of 12 wells microscopic slide were 3,000 cells in the volume of 10 σl.
2. The optimal time to harvest cultured cells for fixation and IF staining was 42 hours of incubation. After staining, the spreading of cells on slide was 70-80% confluences and amount of mitotic cells were 2-5 cells observing under microscope at 400x magnification.
3. About the fixation protocols :
   3.1 The methanol-acetone for 10 minutes and the 1% paraformaldehyde for 5 minutes followed by methanol-acetone for 10 minutes protocols demonstrated the characteristics of five ANA patterns tested including homogeneous, speckle, nucleolar, centromere and nuclear membrane pattern (exception in cases of cytoplasm was stained).
   3.2 In case of there were both of nuclear and cytoplasmic autoantibodies, the 1% paraformaldehyde for 5 minute followed by 0.5% Triton X-100 for 5 minutes demonstrated the distinct nuclear membrane pattern without cytoplasmic staining,
   3.3 The methanol-acetone for 10 minutes fixation showed brighter and sharper fluorescence staining of speckle and nucleolar pattern than the paraformaldehyde fixation.

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APPENDIX

1. Chemical stock solution and buffer

10 % BSA stock solution

BSA	10	g
1X PBS PH 7.4	90	g

Dissolve the ingredient, adjust volume to 100 ml. Filter and store the solution in the refrigerator (2-8 °C).

4% Paraformaldehyde

Paraformaldehyde	4	g
Distilled water	80	ml

Dissolve paraformaldehyde in the distilled water by heating to 60°C in the hood. Keep the beaker covered with foil while heating. Add 1N NaOH (approximately 1 ml) to completely depolymerize and clear solution. Let cool to room temperature, and adjust final volume to 100 ml. Filter (to remove flocculant) and label with formaldehyde caution labels. Store the solution in the refrigerator (2-8 °C).

Phosphate buffer saline (PBS): 1X pH 7.4

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g

Dissolve the ingredients in 800 ml of distilled water. Adjust the Ph TO 7.4 with HCl. Add distilled water to 1,000 ml.

2. Solution for fixation

1% Paraformaldehyde

Dilute 4 % paraformaldehyde stock solution 1:4 with PBS pH 7.4

Methanol-Acetone 1:1

Absolute Methanol	50	ml
Acetone	50	ml

Mix and store the solution at -20 °C until use.

0.5 % Triton X-100

Triton X-100	0.5	ml
1 X PBS pH 7.4	100	ml

Mix by vigorous shaking and warm to 37 deg briefly. Store the solution in the dark to prevent photo-oxidation.

3. Solution for IF staining

Diluent buffer of IF staining

0.5 % Triton X-100	99	ml
10% BSA	1	ml

Mix by vigorous shaking.

Washing buffer of IF staining

Tween20	2	ml
1X PBS pH 7.4	1,000	ml

Mix by vigorous shaking.

4. Commercial kit for ANA screening test

Mosaic HEp-20-10/ Liver (Monkey)

(EUROIMMUN Medizinische Labordiagnostika AG)

Contents:

1. Slides, each containing 5x2 BIOCHIPs coated with HEp-20-10 cells and primate liver
2. Fluorescein-labelled anti-human IgG (goat), ready for use
3. Positive control: autoantibodies against cell nuclei (ANA), control serum with titer information, human, ready for use
4. Negative control: autoantibody-negative, human, ready for use
5. Salt for PBS pH 7.2
6. Tween 20
7. Embedding medium, ready for use
8. Cover glasses (62 mm x 23 mm)
9. Instruction booklet

VITAE

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