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# Does adding anti-F actin antibody assay to the testing protocol help in diagnosis and monitoring of patients with Autoimmune Hepatitis?

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77311392

A thesis submitted in partial fulfilment of the requirements of Leeds Beckett University for the degree of Master of Research.

July 2024

# Abstract

## Background

Autoimmune hepatitis (AIH) is a disease that affects around 0.025% of the population. Without treatment the autoimmune response can cause progressive liver damage and deterioration of liver function leading to cirrhosis which can ultimately prove fatal.

Diagnosis can be challenging due to the heterogeneity of presentation. Four diagnostic indicators are used raised immunoglobulins, absence of viral hepatitis, liver histology compatible with AIH and presence of autoantibodies (ANA, SMA or LKM).

## Objective

Smooth muscle antibodies are the predominant autoantibody found in AIH, with those directed against F-actin proposed to be the most clinically significant. This study assessed three F-actin specific assays to ascertain whether they could replace or enhance the current immunofluorescence testing method.

## Method

Sera of 133 patients who had liver autoantibody test requests were collected and tested using the current methodology and the three new assays. Current practice utilizes rodent liver kidney stomach tissue indirect immunofluorescence (IFA) microscopy to distinguish tubular staining pattern (F-actin specific) from that of a vascular staining pattern. This study assessed three F-actin specific assays (Euroimmun VSM47 IFA, INOVA NOVALite IgG F-actin IFA and the INOVA QUANTALite IgG F-actin ELISA).

All the results generated were designated positive, negative or equivocal to allow comparison between methods. Their performance was assessed against the current method and the clinical outcome (whether the patient had AIH). Qualitative results from the ELISA were analysed to determine optimal cutoff value using Youden's J.

## Results

All three assays showed strong correlation with a diagnosis of AIH ( $p < 0.05$ ). Cohens Kappa showed good correlation with the current method.

The specificity (Sp) and sensitivity (Se) of each as a stand-alone assay was similar or improved when compared to the current LKS method (Sp=81.31%, Se= 86.67%), VSM 47 (81.31%, 92.86%), NOVALite (85.98%, 85.71%), QUANTALite cutoff <20 (80.37%, 92.86%), QUANTALite cutoff <30 (93.46%, 85.71%), QUANTALite using optimal cutoff <28.18 (90.74%, 100%) with the QUANTALite using optimal cutoff <28.18AU showing the best performance. In all circumstances the specificity improved when a confirmatory second line test was added. The QUANTALite using optimal cutoff <28.18AU following a positive SMA (all patterns), identified 100% of AIH positive patients and showed to be 92% specific for the disease. The specificity was 98% if the initial pattern observed was Tubular type.

## Conclusions

All three assays would be suitable as first line screening for AIH-1, although the realities of liver autoantibody screening mean this would be impractical currently. All three assays would provide clinical benefit as a second line test, especially the ELISA with the altered cutoff of 28AU.

## Student's Declaration

Leeds Beckett University

School of Health

Research Project

Declaration of authenticity:

By signing below, I hereby confirm that the following thesis:

“Does adding anti-F actin antibody assay to the testing protocol help in diagnosis and monitoring of patients with Autoimmune Hepatitis?”

Signed: E Payne

Date: 17/07/2024

## Acknowledgments

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## List of Abbreviations

<b>Abbreviation</b>	<b>Expansion</b>
AIH	Autoimmune Hepatitis
AIH-1	Autoimmune Hepatitis Type 1
AIH-2	Autoimmune Hepatitis Type 2
ALD	Alcoholic Liver Disease
ALP	Alkaline Phosphatase
ALT	Alanine transaminase
AMA	Anti Mitochondrial antibody
ANA	Anti Nuclear Antibody
APS	Anti Phospholipid Syndrome
ASGPR	Asialoglycoprotein receptors
AST	Aspartate aminotransferase
AU	Arbitrary units
CIE	Counter immunoelectrophoresis
CLL	Chronic Lymphocytic Leukaemia
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
EBV	Epstein Barr Virus
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbant Assay
ENA	Extractable Nuclear Antigen
F-actin	Filamentous actin
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HEp2	Human epithelial type 2 cells
HLA	Human Leukocyte Antigen
IAIHG	International Autoimmune Hepatitis Group
IASL	International Association for the Study of the Liver
IBD	Inflammatory Bowel Disease
IgG	Immunoglobulin G
IHEP	Immunology Hepatology Clinic
IIF	Indirect Immunofluorescence
IMF	Intermediate filaments

LC-1	Liver Cytosol – 1 Antibody
LFT	Liver Function Test
LKM-1	Liver Kidney Microsomal antibody type 1
LKS	Liver Kidney Stomach
MF	Microfilaments
MT	Microtubules
NAFLD	None alcoholic fatty liver disease
pANCA	Perinuclear Anti Neutrophil Cytoplasmic Antibody
PBC	Primary Biliary Cholangitis
PBS	Phosphate Buffered Saline
PID	Primary Immunodeficiency
PSC	Primary Sclerosing Cholangitis
SLA/LP	Soluble Liver antigen / Liver pancreas antibody
SLE	Systemic Lupus Erythematosus
SMA	Smooth muscle antibody(ies)
T-SMA	Tubular Smooth Muscle Antibody
T1DM	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
VG-SMA	Vascular Glomerular Smooth Muscle Antibody
V-SMA	Vascular Smooth Muscle Antibody



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## Chapter 1 Introduction

The aim of the project is to determine whether the current method for the detection of smooth muscle antibodies using indirect immunofluorescence (IIF), could be replaced with either an actin antibody specific cell line substrate or Enzyme-linked Immunosorbant assay (ELISA), or whether adding one of these methods as a second line confirmatory test would aid diagnosis of Autoimmune Hepatitis.

### 1.1 Background of Autoimmune Hepatitis

Autoimmune Hepatitis (AIH) is a rare chronic condition found across all ethnic groups, that predominantly affects females, with a bimodal distribution between the ages of 10 and 30, and then 40-60 (Gatselis et al., 2015; Mack et al., 2020). The incidence in the Caucasian populations of western Europe and North America has been estimated to be 0.2-1.2 cases per 100,000, or 3 per 100,000 (Whalley et al., 2007) and as high as 10 in 100,000 according to the British society of Gastroenterology (Gleeson & Heneghan, 2011). Stable populations (native Alaskans and New Zealand) have shown to have even higher incidence, initial studies showing 42.9 and 24.5 per 100,000 respectively (Gatselis et al., 2015). Serial studies conducted in Japan and Sweden showed an increase in cases over a 6 – 12-year time frame (Tanaka, 2020). This suggests that the disease is either on the increase, detection and diagnosis is improving, or a combination of the two, and that there may be a strong genetic component to aetiology. Although as the name suggests this is an immune-mediated disease of the liver, initial clinical presentation can vary; some patients will be asymptomatic and only be discovered due to abnormal blood results obtained as part of a routine health check, whilst others will be in acute liver failure (Muratori et al., 2016). The majority of adults manifest with non-specific mild symptoms including an increasing feeling of fatigue, weight loss, cessation of menstruation (amenorrhea) and joint stiffness (arthralgia). In more severe cases, jaundice, nausea, excessive fatigue and abdominal pain may be seen (Terziroli Beretta-Piccoli et al., 2022a). The disease is characterized by elevated levels of Immunoglobulin G, raised alanine and aspartate aminotransferases, and the presence of circulating autoantibodies (directed against smooth muscle, nuclear antigens, or hepatic antigens) and with a histological finding of Interface Hepatitis (Liberal et al., 2013). Interface hepatitis is inflammation at the junction (the interface) between a portal tract (a triad of a bile duct, a branch of the portal vein and an arteriole) (Cardona, 2011) and the hepatocytes within a liver lobule. The portal tract is surrounded by fibrous tissue referred to as the limiting plate. It is the breaching of this which is termed Interface Hepatitis and is the hallmark of AIH (Covelli et al., 2021a). If left untreated AIH can lead to cirrhosis and liver failure requiring liver transplantation or in extreme cases death. Studies before the onset of routine corticosteroid treatment in 1970s put the 10-year mortality at >90% (MISTILIS et al., 1968). However, if detected early AIH can be successfully managed with immunosuppressant medication, treatment deemed successful once symptoms subside and Immunoglobulin G and transaminase levels return to within normal ranges (Komori, 2021; Terziroli Beretta-Piccoli et al., 2017). Despite this the 10-year mortality rate for those with autoimmune hepatitis is suggested to be 32% - 49% (Grønbaek et al., 2020).

The diagnosis of AIH can be challenging due to the heterogeneity of the presentation, with several differential diagnoses being possible (Harada et al., 2017; Mack et al., 2020; Manns et al., 2015). In 1992 a panel of experts from the International Association for the Study of the Liver (IASL) (Johnson & McFarlane, 1993) met to discuss the diagnostic criteria for AIH and devise a scoring system. The aim at this time was purely for research purposes, allowing the selection of similar patients to be grouped together for comparative studies between multiple centres. By 1999 the International Autoimmune Hepatitis Group (IAIHG) had formed and developed a new scoring system based on the clinical findings mentioned above and summarised in *Table 1.1* below. A diagnosis of AIH would require a score of 15 before steroid treatment and 17 if steroid treatment had already commenced. (Alvarez et al., 1999a).

*Table 1.1 1999 IAIHG scoring criteria for diagnosis of Autoimmune Hepatitis (Alvarez et al., 1999a) ALT = Alanine transaminase, AST = Aspartate transaminase, ALP = Alkaline phosphatase, IgG = Immunoglobulin Gamma, SMA = Smooth muscle antibodies, ANA = Anti-Nuclear Antibody, LKM = Liver Kidney Microsomal Antibody, HLA- Human Leukocyte Antigen, Anti SLA/LP = Anti Soluble Liver Antigen / Liver Pancreas, ASGPR = Asialoglycoprotein receptor 1, pANCA = Anti Neutrophil Cytoplasmic Antibody with perinuclear staining*

Parameter	Feature	Score
Sex	Female	+2
ALP:AST (or ALT) ratio	>3	-2
	1.5-3	0
	<1.5	+2
Serum globulins or IgG (times above normal)	>2	+3
	1.5-2	+2
	1-1.5	+1
	<1	0
ANA, SMA or anti-LKM titres	>1:80	+3
	1:80	+2
	1:40	+1
	<1:40	0
Anti-Mitochondrial Antibody	Positive	-4
Viral markers of infection	Positive	-3
	Negative	+3
Hepatotoxic drug history	Yes	-4

	No	+2
Average alcohol	<25g/day	+2
	>60g/day	-2
Histological Features	Interface Hepatitis	+3
	Plasma cells	+1
	Rosettes	+1
	None of the above	-5
	Biliary changes	-3
	Atypical changes (suggesting a different aetiology)	-3
Immune diseases	Thyroiditis, colitis, other	+2
HLA	DR3 or DR4	+1
Seropositivity for other autoantibodies	Anti SLA/LP, actin, ASGPR, pANCA	+2
Response to therapy	Remission	+2
	Relapse	+3


The 1999 scoring system was based on the expert opinion of the time but was still primarily used as a tool for research (Alvarez et al., 1999b). In 2008 the IAIHG met once more, this time to simplify the criteria, removing features deemed to be of questionable significance or unhelpful at the initial presentation stage (e.g. patient response to treatment) with the aim of introducing a diagnostic criterion for use in the clinical setting (Hennes et al., 2008a). The current criteria were defined using a retrospective cohort study involving patients from 11 centres in 10 countries across Europe, Asia and North and South America. The current criteria are outlined in *Table 1.2* below.

Table 1.2 IAIHG Revised scoring criteria for the diagnosis of AIH (Hennes et al., 2008b) AIH = Autoimmune Hepatitis type 1, SMA = smooth muscle antibodies, SLA/LP = Soluble Liver Antigen / Liver Pancreas antibody

Parameter	Feature	Score
ANA or SMA	$\geq 1:40$	1
ANA or SMA	$\geq 1:80$	
Or LKM	$\geq 1:40$	2
Or SLA/LP	Positive	
IgG	>Normal limit	1
	1.1 times the upper normal limit	2
Liver Histology	Compatible with AIH	1
	Typical of AIH	2
Absence of viral hepatitis	Yes	2
Total score		$\geq 6$ probable AIH
		$\geq 7$ definite AIH

Not all patients with AIH initially present with detectable autoantibodies and having viral hepatitis does not preclude an individual from developing AIH. Indeed, in China the incidence of Hepatitis B surface antigen is between 6 and 10% depending on the region (Wang et al., 2019) rendering the criteria less helpful in these population groups. In clinical settings the criteria can be therefore used as a guide without the stringent use of the scoring system and alongside other factors such as sex, alcohol consumption, and presence of comorbidities, which often include other immune mediated inflammatory disorders such as coeliac disease, ulcerative colitis, type 1 diabetes, and most commonly autoimmune thyroid disease (Lucey & Vierling, 2014).

## 1.2 The autoantibodies

In the 1950s the first cases of  hepatitis affecting females presenting with high gamma globulins, amenorrhea, and who showed symptom improvement upon administration of adrenocorticotrophic hormone, were described (Last, 1957; Mackay, 2008). These were referred to as cases of chronic active hepatitis (CAH). At the time most clinicians assumed the cause to be viral, even if there was no evidence of past or present infection. In 1956 Ian Mackay had identified, in the sera and ascites of patients with this presentation, the presence of Anti-Nuclear Antibody (ANA) a group of antibodies directed against proteins in the cell nucleus (Mackay, 2008). ANA had first been detected serendipitously in the form of L.E (Lupoid Erythematosus) cells in the bone marrow of patients suffering from “collagen diseases of the Lupus Erythematosus type” by Hargraves in the 1940’s (Hargraves et al., 1948). The L.E

cell is a cell monocyte or neutrophil in which the remnants of the nucleus (the L.E body) of a phagocytosed cell can be observed, making them distinct from other red and white blood cells on a blood film (Sathiavageesan & Rathnam, 2021). The phagocytosis of the denatured nuclei of damaged cells was later discovered to be mediated by the presence of ANA (Terziroli Beretta-Piccoli et al., 2018a), although the exact mechanism remains unknown (Pisetsky, 2012). The L.E cell was found to be present in other connective tissue diseases and not specific for SLE so became used as a marker for defining an underlying autoimmune basis for disease. The patients with CAH were shown to have L.E cell positivity (Mackay, 2008) meaning an autoimmune factor could be determined and there was now a method available to distinguish CAH from viral hepatitis. The term Lupoid Hepatitis was used to describe this condition, due to the presence of these cells and it sharing some similarities with systemic lupus erythematosus (SLE) including rash and arthralgia.

Systemic Lupus Erythematosus is a complex, immune mediated inflammatory disease that can affect all organs within the body and like AIH it can be varied in its clinical manifestations (Lahita, 2011). The introduction of immunofluorescence assays using rodent tissues substrate in 1950's (Iruere-Ventura & López-Hoyos, 2022) allowed better detection of ANA as these methods were more sensitive, and also allowed the visualisation of different patterns, which later showed to have some correlation with particular connective tissue diseases (Van Hoovels et al., 2020). The use of rodent tissue for ANA detection has been replaced in most laboratories with a human cell line derived from human laryngeal carcinoma, the HEp2 cell (Hernández-Flórez & Valor, 2018). The large cell nucleus and greater expression of some of the proteins targeted by ANAs allowed for better visualisation of ANA patterns (Hansson et al., 1996). The advent of immunofluorescent techniques lead to the discovery of smooth muscle antibodies in the mid-1960s (Johanet & Ballot, 2012; Toh, 1979). These antibodies were present in the sera of patients with Lupoid Hepatitis but not in the sera of patients with SLE, allowing Lupoid Hepatitis to be identified as a disease distinct from SLE whereupon it was renamed Autoimmune Hepatitis (although not officially until the 1990s). Throughout the 20<sup>th</sup> century as autoantibody testing and detection improved and became more routine, several more autoantibodies were identified. One such antibody is Liver Kidney Microsomal (LKM-1) which was discovered to have a strong link to AIH but in a different cohort of patients to the SMA/ANA positive group. LKM-1 positive patients tended to be younger and have a more even prevalence between the sexes (Lapierre & Alvarez, 2022; Smith et al., 1974). This has meant that AIH can be divided into 2 subgroups.

Type 1 AIH is the most common, accounting for around 80% of cases (Gossard & Lindor, 2012). This type affects both adults and children. The autoantibody profile is positive ANA and/or Smooth Muscle Antibodies. Type 2 AIH affects predominantly children, has an autoantibody profile consisting of LKM-1 and/or Liver Cytosol-1 (LC-1) antibodies and a more aggressive course (Invernizzi et al., 2012). The Anti -Soluble Liver Antigen (SLA) is found in 20-30% of cases of both Type 1 and Type 2 AIH and is the only AIH specific autoantibody (Terziroli Beretta-Piccoli et al., 2021).

The reference method for detection of liver autoantibodies is Indirect Immunofluorescence (IIF) (Vergani et al., 2004) using triple organ substrate, namely Liver Kidney and Stomach (LKS)



from a rodent, mounted onto wells on glass slides. The advantage of this substrate is that it allows the detection of several autoantibodies at once, including those found in other autoimmune liver diseases such as Primary Biliary Cirrhosis and Primary Sclerosing Cholangitis. The patient sera are diluted in Phosphate Buffered Saline (PBS) and applied to a well containing the LKS substrate. Any antibody directed against antigen found in the LKS tissue will bind. Wells are subsequently washed to remove any unbound patient antibody. A fluorochrome labelled anti human IgG conjugate is added and incubated further to allow the conjugate to bind to any human immunoglobulin present, slides are then washed and mounted with cover slips (Vergani et al., 2004). The tissue sections can then be studied by a suitably trained operator using a UV microscope to identify different autoantibody patterns. The reference methodology is followed at Leeds General Infirmary.

A summary of the antibodies found in AIH is detailed in the *Table 1.3*.

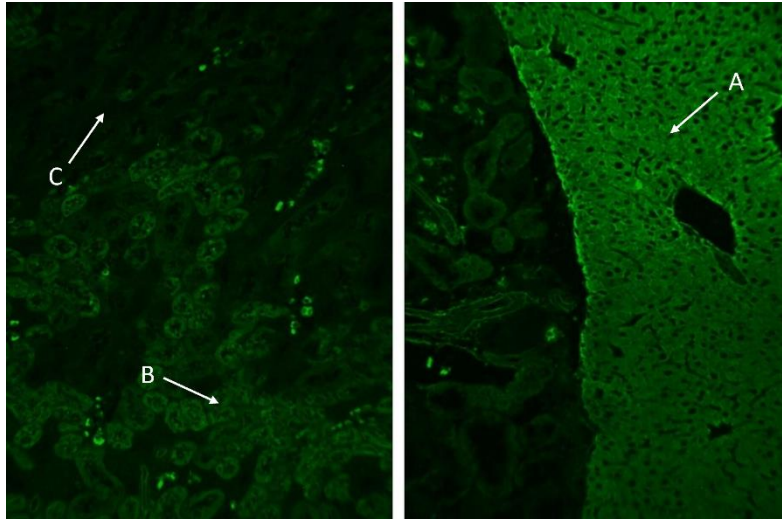
*Table 1.3 Clinical relevance of autoantibodies in autoimmune hepatitis (Terziroli Beretta-Piccoli et al., 2018a) AIH-1 = Autoimmune Hepatitis type 1, AIH-2 = Autoimmune Hepatitis type 2, SMA = smooth muscle antibodies, V-SMA = Vascular smooth muscle antibody pattern, VG-SMA = Vascular-Glomerular smooth muscle antibody pattern, VGT-SMA = Vascular Glomerular Tubular smooth muscle antibody pattern, HCV = Hepatitis C virus, SLE = Systemic Lupus Erythematosus, Anti SLA/LP = Anti Soluble Liver Antigen / Liver Pancreas, Anti-LKM = Liver Kidney Microsomal antibody, Anti LC-1 = Liver Cytosol antibody, p-ANCA = Anti Neutrophil Cytoplasmic Antibody with perinuclear staining*

Autoantibody	Target Antigen	Frequency in AIH		Clinical significance
		AIH1	AIH2	
ANA	Chromatin, Histones, centromere, double and single stranded DNA, cyclin-A and ribonucleoproteins. In approximately 30% of patients the target antigen remains unidentified.	75%	Rare	Found in 50% of patients with AIH-1, co-exists with SMA.  Commonly found in a range connective tissue disease (SLE, Sjögrens, Systemic Sclerosis)
SMA	Filamentous actin Desmin Vimentin Unknown in ~20% of cases	85-95%	Rare	VG-SMA and T-SMA patterns are specific for AIH-1 Often found in very high titres in patients with AIH-1  Low titre and V-SMA pattern found in drug induced hepatitis, viral hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis.

Anti-actin	Filamentous actin	75%	Unknown	Usually coexists with SMA in AIH-1 Typical of AIH-1
Anti LKM	Cytochrome P4502D6	Absent	Up to 90%	Diagnostic of AIH-2 (in absence of HCV infection) Very rare in Autoimmune sclerosing cholangitis
Anti -LC-1	Forminino-transferase cyclodeaminase	Very rare	Up to 60%	Diagnostic of AIH-2 (in absence of HCV infection)
Anti -SLA/LP	O-phosphoseryl-tRNA: selenocysteine-tRNA synthase (SEPSECS)	20-30%	20-30%	Highly specific (98.9%) Very rarely in HCV Prognostic of severe disease
pANCA	Unknown	50-96%	Absent	Could be the only antibody present in AIH1

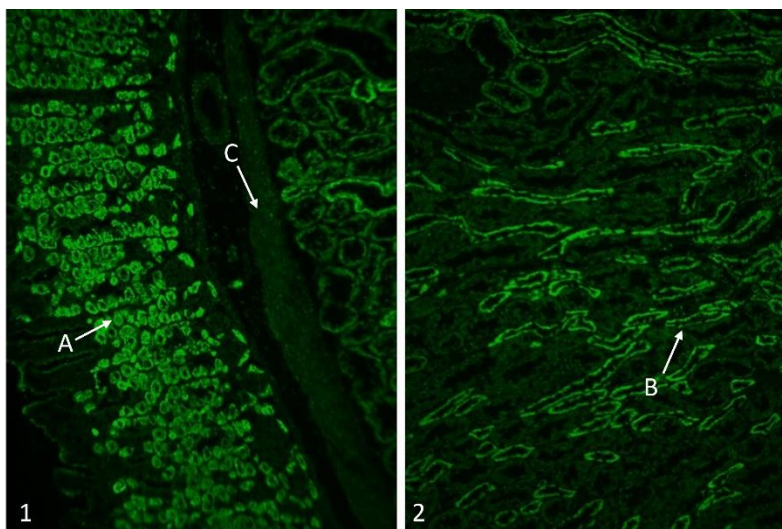
The reading or interpretation of IIF requires significant skill and several years of practice to become proficient and confident, (Sebode et al., 2018) and as with any practice involving human interpretation, the results can be subjective (Rigon et al., 2007; Terziroli Beretta-Piccoli et al., 2018).

LKM (Figure1.1), an antibody associated with Type 2 AIH is characterized by bright homogeneous staining of the hepatocyte cytoplasm and staining of the proximal renal tubules. The gastric parietal cells and stomach remain negative. The target antigen has been identified as the cytochrome P4502D6, (Gueguen et al., 1989) which has allowed molecular based assays to be developed aiding in the detection of this autoantibody. Molecular based assays have the benefit of being able to be automated and do not require an experienced operator to interpret the results. This assay can be in the form of an ELISA or Western Blot allowing an LKM antibody pattern observed on LKS tissue to be confirmed by a second method (Terziroli Beretta-Piccoli et al., 2018).



*Figure 1.1 Fluorescent pattern observed in LKM positive (A)= Homogenous staining of the Liver Hepatocytes (B) Staining of the proximal renal tubules of mouse kidney (C) Distal renal tubules remain unstained. Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x.*

Mitochondrial antibody (Figure 1.2) can be detected on LKS and is similar to LKM in that the kidney tubules fluoresce, however both proximal and distal tubules show staining, gastric parietal cells are stained, and the liver shows granular rather than homogenous staining. An LKM coexisting with Gastric parietal cell (GPC) antibody could be mistaken for mitochondrial antibody (AMA). AMA is an antibody associated with Primary Biliary Cirrhosis rather than AIH whilst GPC antibodies are found in Pernicious anaemia, underlining the importance of having highly trained personnel interpreting IIF.



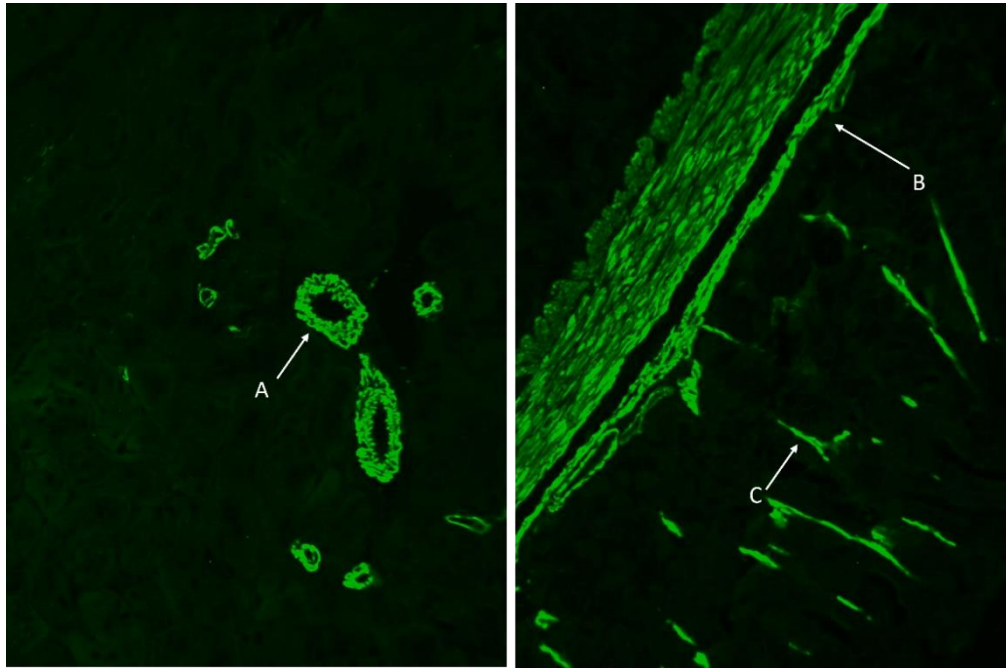
*Figure 1.2 Mitochondrial antibody on Mouse LKS tissue. Fluorescent staining of gastric parietal cells within the gastric mucosa of mouse stomach tissue (A). Fluorescent staining of both proximal and distal tubules of mouse kidney (B). No staining with the Muscularis Mucosa band (C). Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x.*

Liver cytosol type 1 antibody (LC-1) shows staining only on the liver tissue with the intensity reducing significantly towards the hepatic vein. This antibody is often found in conjunction with LKM which can mask its presence. Again, the identification of the target antigen (formiminotransferase cyclodeaminase) has allowed the development of an alternative molecular method of detection which can be used as a second-line confirmatory test or in some cases as the primary method.

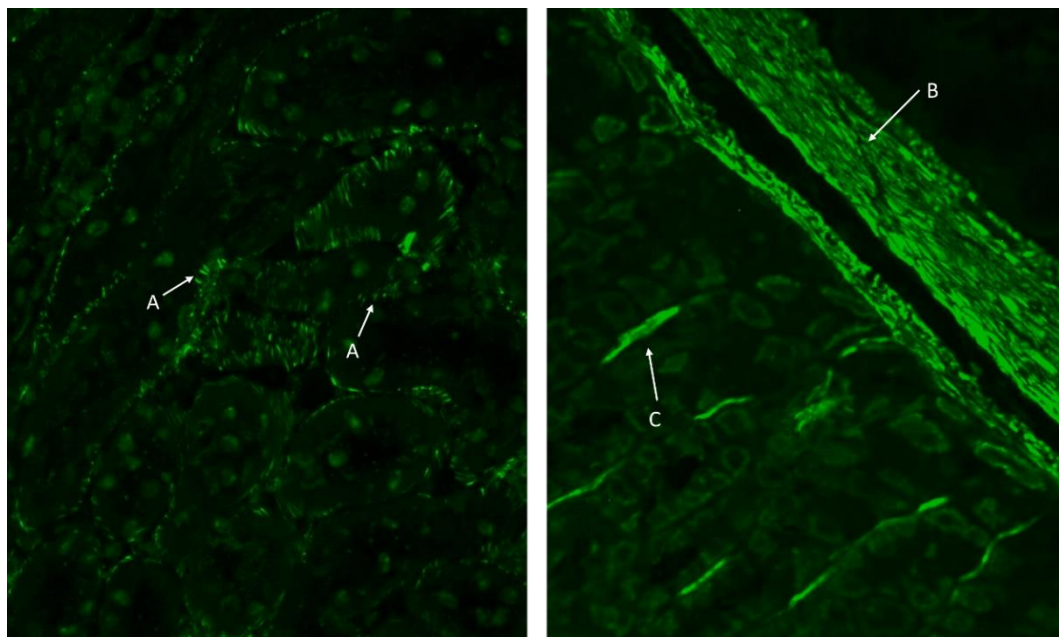
It is worth mentioning that the only AIH-specific antibody, SLA, cannot be detected on LKS substrate and relies on detection using the Western blot method.

ANA is detected alongside SMA in 50% of AIH-1 cases and as the only serological marker in some 10–15% of cases (Bogdanos et al., 2009). The IAIHG suggest screening for the presence of ANA using IIF LKS substrate, with the specificity being later defined using HEp2 cells, a human epithelial cell line characterized by a large nucleus allowing detection of a range of antinuclear antigens and their patterns. Three quarters of AIH patients show a homogenous nuclear pattern with nucleolar and speckled patterns being seen in the other 25% of cases (Terziroli Beretta-Piccoli et al., 2021). The IAIHG caution against using HEp2 as a first line method for detection of ANA in AIH, as studies have shown that a significant proportion of the healthy population have a positive ANA by HEp2, especially at lower dilutions (Esteves Hilário et al., 2004; Tan et al., 1997). If HEp2 cells are used it has been suggested a higher cut-off dilution of 1:160 should be used to mitigate this, Tan et al (1997) showed that positivity in healthy individuals dropped from 31.7% to just 5% when titre was increased from 1:40 to 1:160. However, given the volume of requests for ANA received in clinical laboratories, many have moved away from IIF and towards molecular methods such as Enzyme Immunoassay (EIA) and multiplex assay to detect known ANA specificities. Although often faster and requiring less skill, the use of molecular methods can result in missed ANA positives as the antigenic targets of ANAs in AIH is only partially understood (Terziroli Beretta-Piccoli et al., 2018).

Smooth muscle antibodies, first described on rodent stomach by Johnson et al., 1966, are found in the majority of cases of type 1 AIH. The use of rodent kidney tissue allowed them to be separated into subgroups by Bottazzo et al., 1976 (Christen et al., 2015). Three subgroups of smooth muscle antibody were defined based on observations made on rat kidney tissue: vascular (V-SMA) (*figure 1.3*), vascular-glomerular (VG-SMA), vascular-glomerular-tubular (T-SMA) (*figure 1.4*). As their names suggest, the vascular pattern shows fluorescent staining of the blood vessel walls, vascular-glomerular shows staining of vessel walls and the mesangium of the glomeruli, and finally tubular shows staining of the vessels, glomeruli and of the basement membrane of the renal tubules. Smooth muscle antibodies are detected using the triple rodent “liver, kidney, stomach” tissue substrate, and the smooth muscle antibody can also be detected in the stomach layer. The staining gives a “tree bark” or “alligator skin” appearance in the transverse and longitudinal muscle bands of the muscularis mucosa and appears as fine fibres in the smooth muscle of the gastric layer between the gastric parietal cells.



*Figure 1.3 Vascular Smooth Muscle Antibody. The blood vessels within the mouse kidney are fluorescing (A). The "alligator skin" appearance of the longitudinal and transverse muscle bands of the muscularis mucosa (B). Fine fibres of the gastric layer between the gastric parietal cells (C). Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x.*



*Figure 1.4 Tubular Smooth Muscle Antibody. Fine "matchstick like" actin fibres staining around the kidney tubules fluorescing (A). The "alligator skin" appearance of the longitudinal and transverse muscle bands of the muscularis mucosa (B). Fine fibres of the gastric layer between the gastric parietal cells (C). Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x.*

The pattern observed is of great significance as the VG-SMA and T-SMA patterns have been shown to be highly (though not entirely) specific for AIH (G. F. Bottazzo et al., 1976; Muratori et al., 2002; Galaski et al., 2021), whilst the V-SMA pattern can be found in a wide range of

conditions including viral hepatitis, fatty liver disease, drug induced liver injury, primary sclerosing cholangitis (Terziroli Beretta-Piccoli et al., 2021).

Smooth muscle antibody reacts with a range of smooth muscle cytoskeleton cellular antigens, which can be defined as microfilaments (MF), intermediate filaments (IMF) or microtubules (MT). The specific target in the MF was identified as actin in 1973 (Gabbiani, 1973) and then confirmed using absorption studies that showed complete absorption of sera with the VG-SMA and VGT-SMA patterns, but crucially not the V-SMA patterns (G. Bottazzo et al., 1976). Several other studies have shown a strong correlation between AIH-1 and actin antibodies (Granito et al., 2006; Muratori et al., 2002). The IMF targets are vimentin, desmin and cytokeratin, whilst the MT target is Tubulin (Villalta et al., 2009). Methods for the detection of MF, IMF and MT include IIF using cultured fibroblasts, IIF on vinblastine treated peripheral blood mononuclear cells (PBMCs), cryostat sections of liver from rats chronically injected with phalloidin and counter immunoelectrophoresis (CIE) to detect precipitating antibody to XR1 antigen (Czaja et al., 1996; Cassani et al., 1987). Cell culture requires specialised equipment such as HEPA filters, incubators, aseptic work area and liquid nitrogen for storage, in order to maintain a favourable artificial environment and ensure good reproducibility of the cloned cells (Thermofisher Scientific, 2020) whilst CIE is performed on a gel and so has a low sample throughput. Vinblastine treated PBMCs and sections of liver from rats injected with phalloidin would need to be produced in house as they are not commercially available and would require the laboratory to house and care for animals in accordance with the Animals (Scientific Procedures Act) (ASPA) (*The Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012*, 2012). As a consequence, these methods are only suitable for research settings and are not able to be translated into a clinical laboratory with large sample volume, as an example Leeds General Infirmary currently processes approximately 150 liver autoantibody screens per day.

Despite the target antigen most specific for AIH-1 being identified as actin, a successful molecular method for detection has yet to be developed. The ELISA developed thus far has proved to be unsatisfactory with the poor specificity being attributed to presence of denatured actin, or differences in the expression of actin epitopes in ELISA compared to tissues (Kurki et al., 1980; Villalta et al., 2009). As the actin is extracted, there could be a conformational change from filamentous actin (polymer form) to the monomeric G actin form. Once a conformational change has occurred, the antibodies would have reduced binding ability (Granito et al., 2006). Alteration of cut-offs allowing the assay to provide a similar level of specificity to the IIF method, has resulted in sensitivity reducing significantly, making this suitable as a confirmatory test only (Villalta et al., 2009).

Despite the criteria set out by the IAIHG there is very little standardisation of smooth muscle antibody testing. The criteria award points for positive SMA, regardless of pattern, at a dilution of 1:40. The original guidelines and research from several decades ago was based on the use of fresh rodent tissue (Bottazzo et al., 1976); in a clinical laboratory today, commercially available kits are used which utilise rodent tissue fixed using diverse fixing agents and different conjugates (Terziroli Beretta-Piccoli et al., 2022b). This tissue itself could be rat or mouse, with differences observed between the two, notably heterophile antibodies on rat tissue (Hawkins et al., 1977). These factors and local automated procedures can affect

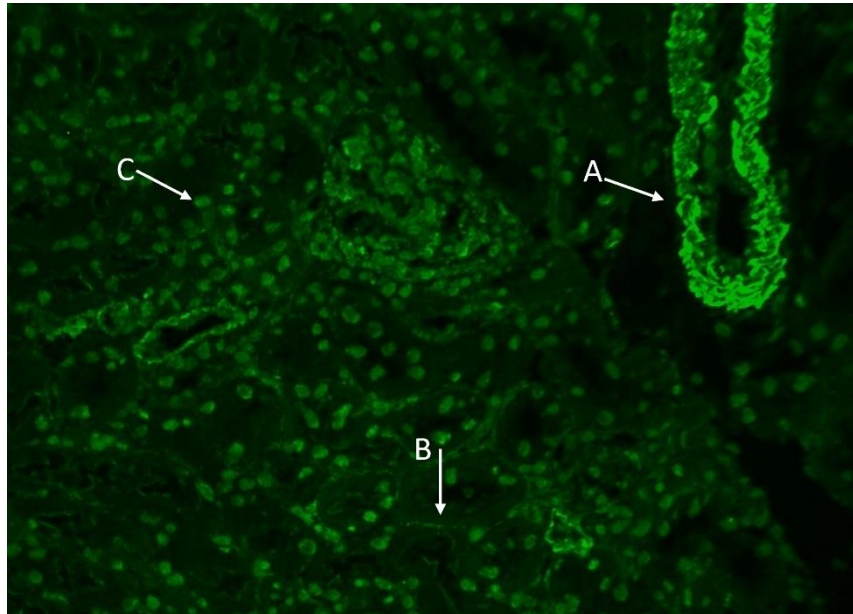


the choice of screening dilution and may require optimization in house, all attributing to the lack of standardization (Vergani et al., 2004).

### 1.3 Current Practice

The Clinical Immunology laboratory at Leeds General Infirmary (LGI) uses INOVA NOVALite mouse kidney, liver stomach slides (manufactured by INOVA for Werfen). The serum dilution and application of that and the conjugate is performed using the Quantalyser 2 slide processor (Werfen). Slides are read by suitably trained operators who are able to identify the many autoantibody patterns that can be demonstrated on this tissue. As LKS tissue can be used to detect several autoantibodies the patient sera are initially diluted 1:40 which is the dilution at which GPC, LKM and Mitochondrial antibodies are clinically relevant (Chazouilleres et al., 2022; Jin et al., 2022; Sebode, Weiler-Normann, et al., 2018). Any samples that display SMA staining are titred to 1:80 with only those still visible at 1:80 reported as positive, and their pattern classed as V-SMA or T-SMA. At LGI it is routine to use the Euroimmun Euroline Liver Profile 2 immunoblot to confirm new mitochondrial and LKM antibodies, and to identify others such as the LC-1 antibody and SLA antibody which can be difficult to detect on the LKS substrate. There is currently no second-line test to confirm T-SMA in use at LGI.

A strong positive T-SMA can be easily identified, but where the staining is weak or multiple patterns are observed at once it can be difficult to distinguish the crucial matchstick-like filamentous staining seen in the kidney tubules (*figure 1.5*). It could also be possible that due to natural variations in the expression of antigens within cell structures and the representation of said structures in the tissue sections, that sometimes the antigens of interest are not sufficiently available. However, it must be said that manufacturers must follow stringent quality control checks before kits are released to the market, so this may not be a routine issue. In the UK suppliers must comply with In Vitro Diagnostic Medical Devices (IVD) Directive (98/79/EC) (In Vitro Diagnostic Medical Devices (IVD) Directive (98/79/EC), 1998). It is also possible that for less experienced readers other staining within the kidney could be misinterpreted as tubular staining or could be missed entirely. A case in India identified a patient with acute liver failure who was positive for ANA, positive for actin antibodies using the VSM-47 substrate but who had a negative antibody result using the liver kidney stomach substrate (Mahto et al., 2023).



*Figure 1.5 Tubular Smooth Muscle antibody alongside ANA staining observed on mouse kidney tissue. Staining of the blood vessel within the kidney tubules (A). Fine matchstick like staining of actin filaments surrounding kidney tubules (B). Anti-Nuclear Antibody (ANA) staining observed in the cell nuclei. The presence of multiple antibodies can make distinguishing antibody patterns of interest challenging. Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x.*

With this in mind, a second line test could be beneficial to confirm F-actin positivity. Two methods currently available are the INOVA NOVALite IgG F-Actin IIF kit (Werfen) and the Euroimmun VSM47 (Euroimmun) which both use indirect immunofluorescence, yet despite both assays having been around since late 2000's neither has been used routinely in the UK. The INOVA NOVALite IgG F-Actin kit (Werfen) uses a cell line derived from rat intestine epithelial cells, whilst the VSM47 uses a cell line derived from the thoracic aorta of rat embryo. Samples containing antibodies against F-actin show a microfilamentous fluorescent pattern that can be easily distinguished. However, it cannot be ignored that in the current climate of shortages of suitably skilled operators and a move to larger more automated diagnostic laboratories, adding another interpretative and subjective method to the test repertoire could be problematic. ELISA offers a middle ground between the operator dependant IIF and surely the future goal of random-access automated testing. The ELISA would offer a solution to avoid the requirement for operators trained in the field of reading IIF and remove the subjectivity associated with an interpretive assay but would still require samples to be batched up for processing.

As previously stated, the T-SMA pattern is highly specific for AIH-1, but the number of T-SMA reported compared to V-SMA is relatively small. In the period 1<sup>st</sup> January 2023 until 31<sup>st</sup> December 2022, the SMA positivity at Leeds General Infirmary was approximately 10% (of which only 7.5% were T-SMA pattern, the large majority being the less specific V-SMA). The range of disorders in which V-SMA are found is broad, however it will be true that some of these patients with V-SMA antibody do have AIH-1. In the context of seropositivity with relation to V-SMA antibodies, it can be said that their presence is assessed within the entire



clinical picture. For example, V-SMA will carry more clinical significance in a young female patient with elevated ALT and another pre-existing autoimmune condition than in a middle-aged male patient with metabolic risk factors and excess alcohol intake who happens to have a mildly elevated ALT (Ngo et al., 2014; Tunio et al., 2021) Webb et al., 2021). If a patient has had liver autoantibodies requested in response to a raised ALT discovered as part of a routine health screening it could be that their symptoms are non-existent or very mild. The risk is that patients presenting with mild symptoms initially and who have borderline results not confirming AIH-1, may be sent back to GP and not present again until they have much more pronounced disease further down the line and when extensive damage has occurred. An untreated asymptomatic patient with mild disease has been shown to have a lower 10-year survival than a treated patient with severe symptomatic disease (Czaja, 2016). Accurate detection and interpretation of liver autoantibodies in the right clinical context aids swift, assured diagnosis of auto-immune liver disease and helps to reduce unnecessary additional investigations (at present at LGI, any autoantibody positivity results in reflex testing of Immunoglobulins) and un-warranted anxiety to patients. Unreliable liver auto antibody results can result in missed diagnoses, and patients subsequently presenting when their disease has progressed further and more damage has occurred (Bhumi & Wu, 2022).

#### 1.4 Study Objectives

This project will determine the clinical utility of three F-actin antibody specific assays and assess the viability of using these to replace the current method for the detection of SMA. It will also assess whether adding one of these methods as a second line confirmatory test could aid the diagnosis of Autoimmune Hepatitis.

The new assays include two actin antibody specific cell line substrates (VSM47 and NOVA Lite IgG F-actin), and QUANTA Lite IgG F-actin Enzyme-linked Immunosorbent assay (ELISA).

This will be performed in the following way.

- Sera from patients with T-SMA, will be tested using the Euroimmun VSM47, the INOVA NOVALite IgG F-actin cell line and the INOVA QUANTALite IgG ELISA to ensure that those patients who are labelled T-SMA, are in fact true actin antibody positive and that the current method is correctly identifying such patterns.
- Sera from patients with V-SMA, will be tested using the Euroimmun VSM47, the INOVA NOVALite IgG F-actin cell line and the INOVA QUANTALite IgG ELISA to ensure that those patients we are calling V-SMA do not have the more clinically significant actin antibody.

## Chapter 2 Sample selection

### 2.1 Sample collection

Sera of patients who had been routinely sent for liver autoantibody testing and subsequently tested positive for any of the antibodies detected using LKS substrate (GPC, Mitochondrial, LKM, V-SMA and T-SMA). Once routine testing was complete samples were stored at -20°C over a period of nine months.

As the aim of the study was to assess whether a second-line confirmatory test could aid the diagnosis of AIH, samples selected for the project were predominantly made up of T-SMA and V-SMA positives with some positive for GPC, Mitochondrial antibodies and LKM. A selection of samples from patients that had tested negative for all antibodies detected using LKS substrate were also set aside for the study. For a more complete medical history to be available, only samples from patients of Leeds Teaching Hospitals or the Leeds Primary care region were used, those received from other hospitals in the West Yorkshire region were excluded from the study. All samples that had a T-SMA or diagnosis of AIH were selected for testing, due to the low incidence of the disease (3 per 100,000) (Whalley et al., 2007) and antibody pattern observed at Leeds (1%). There were 3 paediatric male patients for whom 2 samples were available for testing (a pre and post treatment sample). In total 133 samples from 130 patients were selected for the study.

Of the 133 samples tested, 68 had previously tested positive for V-SMA (three with mitochondrial antibodies and two with GPC antibodies), 39 for T-SMA (one with mitochondrial antibodies and one with GPC antibodies), nine for mitochondrial antibodies (three with V-SMA, one with T-SMA) and two LKM positive patients. The number of confirmed AIH diagnosis was 26 (23 patients). Of these, 14 samples were taken pre-diagnosis and pre-treatment. A further 19 patients had tested negative for antibodies on LKS substrate. Both sexes were equally represented in the samples collected, albeit females having a higher proportion in the AIH+ group, samples were collected from patients from early childhood up to geriatric age groups (*Table 2.1*). Three paediatric patients had two samples each in the study, one pre-treatment and one post-treatment which is reflected in the sample demographics (*Table 2.1*) versus patient demographics (*Table 2.2*)

*Table 2.1 Demographics of the serum samples used in the study. The low median age reflects the fact that 3 paediatric patients have 2 samples each.*

	Number of samples	Median Age (years)	Sex
AIH-1	26	14 (7-78)	54% female
Not AIH	107	52 (4-88)	49% female
All patients	133	47	50% female

*Table 2.2 Demographics of the patients whose samples were used for testing in the study. The data is amended from Table 2.1 above to remove "double counting" of the patient' on whom two samples were received.*

	Number of patients	Median Age (years)	Sex
AIH-1	23	27 (7-78)	61% female
Not AIH	107	52 (4-88)	49% female
All patients	130	47	51% female

## 2.2 Considerations

It should be recognised that Leeds Teaching Hospitals is one of three specialist Adult Liver centres in Yorkshire and Humber region which covers a population of approximately 5.5 million people (Office for Health Improvement and Disparities, 2021), one of six Adult Liver and Transplant centres in England, and one of three specialist Paediatric liver centres in UK. This affected the median age of the AIH positive patients, but also allowed the collection of a greater number of AIH+ positive patient samples. Patients are often referred to Leeds after an initial diagnosis elsewhere and require a level of specialised care not able to be provided by their local hospital.

The retrospective nature of the study and sample collection method meant that it was not possible to acquire sero-negative samples from patients with AIH, nor was it possible to recruit "healthy controls" given the ethical considerations. Samples were collected based on positivity LKS substrate, the result of this being that samples were a mixture of initial pre-diagnosis, pre-treatment samples and post-diagnosis, post treatment monitoring samples. A true reflection of the population would contain many more LKS negative samples.

## Chapter 3 Testing protocol

### 3.1 Indirect immunofluorescence using Inova Diagnostics NOVA Lite ANA KSL mouse.

All samples were initially screened using the LKS substrate in the following manner. Patient sera was tested using Inova Diagnostics NOVA Lite ANA KSL mouse kit (508380.3). *Appendix A.*

The kits were allowed to reach room temperature (approximately 30 minutes). One bottle of PBS concentrate (25mL) was diluted in 975mL of distilled water and placed on a rotating mixer for 20 minutes to ensure thorough mixing. The Quantalyser 2 slide processor was used to dilute patient sera with PBS at a dilution of 1:40, and to apply 25 µL the diluted sera and the neat controls individual fields on the ready-to-use slides with 8 wells, each containing mouse liver, kidney and stomach (LKS) tissue section. All tests are verified as passed by using the kit Negative quality control (human derived ready for use negative control for IgG autoantibodies against F-actin, mitochondrial, LKM-1 and GPC, prediluted and mixed with 0.09% sodium

azide) and four internal quality controls, which were previous patient serum samples that had tested positive for the GPC, Mitochondrial, Tubular Smooth Muscle and LKM-1 antibodies which were prediluted and now ready for use.

The LKS slides were then incubated at room temperature for 30 minutes. The pre-programmed Quantalyser 2 then carried out an automated wash procedure using the PBS, before any remaining PBS was removed and 25 µL of fluorescein-labelled anti-human IgG goat conjugate containing Evans Blue and 0.09% sodium azide, was applied to each field. A further 30-minute room temperature incubation took place, before the wash step was repeated. Slides were then mounted onto coverslips using 3 drops of mounting medium ensuring there were no bubbles.

IIF was viewed and interpreted by 2 different operators using the Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x. All results were scored as VSMA, TSMA or Negative and were only accepted subject to IQC performing as described.

Any samples that tested positive at 1:40 for V-SMA or T-SMA we retested using the sample protocol as above but with a dilution of 1:80. Only those remaining positive at this dilution were reported to clinician as positive. Mitochondrial and LKM positive samples were further diluted using the above protocol but to dilutions 1:100, 1:500, 1:1000 and 1:2000.

### 3.2 Indirect immunofluorescence using Euroimmun VSM47 cells.

Patient sera was tested using the Euroimmun Anti-F Actin IIF kit (FA1651-1010). *Appendix B.*

The kits were allowed to reach room temperature (approximately 30 minutes). One sachet of PBS salt (pH 7.2) was dissolved in 1 litre of distilled water, mixed with 2mL of Tween 20 and placed on a rotating mixer for 20 minutes to ensure thorough mixing. Patient sera was then diluted 1:100 with the PBS-Tween (10.1 µL sera in 1000 µL PBS-Tween) and vortexed for 5 seconds to thoroughly mix.

The sera and controls (human derived ready for use positive control for autoantibodies against F-actin and human derived ready for use negative control for autoantibodies against F-actin) were then applied to contained ready to use BIOCHIP slides with 10 wells, each coated with VSM-47 cells (these cells are derived from rat embryonic thoracic aorta and strongly express F-actin) using the Euroimmun TITERPLANE technique which aims to standardize the incubation across all analysis fields. 30 µL of diluted sera was applied to each field on a glass reagent tray and template. Once all samples were applied, the individual slides were placed into recesses on the reagent tray and incubated for 30 minutes at room temperature.

Once initial incubation was completed slides were flushed with PBS-Tween, then immersed in a Coplin jar filled with PBS-Tween and placed on a gently rotating shaker (50rpm) for 5 minutes to remove unbound conjugate.

The reagent tray was used once again for conjugate application. 25 µL of FITC-labelled anti-human IgG goat conjugate (ready for use) was applied to each field on the reagent tray. Slides were removed from the Coplin jar one at a time, and the back and sides dried using a paper

towel, before being fitted into the recesses on the reagent tray and incubated out of direct sunlight at room temperature for 30 minutes.

Slides were flushed with PBS-Tween, then immersed in a Coplin jar filled with PBS-Tween and placed on a gently rotating shaker (50rpm) for 5 minutes to remove unbound conjugate. Slides were then removed from the washing cuvette and mounted onto glass cover slips with 3 drops of mounting medium, ensuring no bubbles formed.

IIF was reviewed and interpreted by 2 different operators using the Leica microscope fitted with Leica DMRB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x. All results were scored as Positive, Negative or Equivocal, and were only accepted subject to IQC performing as described. An equivocal result was recorded when the staining appeared weak or suffered interference from non-specific staining.

### 3.3 Indirect immunofluorescence using Inova Diagnostics NOVA Lite IgG F-Actin

Patient sera was tested using Inova Diagnostics NOVA Lite IgG F-Actin kit (708255). *Appendix C*.

The kits were allowed to reach room temperature (approximately 30 minutes). One bottle of PBS concentrate (25mL) was diluted in 975mL of distilled water and placed on a rotating mixer for 20 minutes to ensure thorough mixing. The Quantalyser 2 slide processor was then used to dilute patient sera with PBS at a dilution of 1:40, and then apply 25 µL the diluted sera and the neat controls (ready for use positive control for IgG autoantibodies against F-actin prediluted and mixed with 0.09% sodium azide, human derived ready for use negative control for IgG autoantibodies against F-actin prediluted and mixed with 0.09% sodium azide) to individual fields on the ready to use 6 well slides, each individual well coated with rat intestine epithelial cells. The slides were then incubated at room temperature for 30 minutes. The pre-programmed Quantalyser 2 then carried out an automated wash procedure using the PBS, before PBS was removed and 25 µL of FITC-labelled anti-human IgG goat conjugate was applied to each field. A further 30-minute room temperature incubation took place before the wash step was repeated. Slides were then mounted onto coverslips using 3 drops of mounting medium ensuring there were no bubbles.

IIF was reviewed and interpreted by 2 different operators using the Leica DM RB microscope fitted with a CoolLED pE-100 bulb at a magnification of 200x. All results were scored as Positive, Negative or Equivocal, and were only accepted subject to IQC performing as described. An equivocal result was recorded when the staining appeared weak or suffered interference from non-specific staining.

### 3.4 ELISA using Inova Diagnostics Quanta Lite IgG F-Actin

Patient sera was tested using the Inova Diagnostics Quanta Lite IgG F-Actin ELISA (708785).  
*Appendix D.*

The ELISA uses a cutoff sample of a predetermined concentration, in order to calculate the level of antibody in patient sera, rather than a standard curve.

All reagents were brought to room temperature (30 minutes) and mixed. Wash concentrate was diluted 1:40 by adding the 25mL contents of the HRP wash concentrate (40x concentrate) containing Tris-buffered saline and Tween-20, bottle to 975mL of distilled water and mixed thoroughly using a rotating mixer. Patient samples were prepared by adding 5 µL sera to 500 µL of HRP sample diluent containing Tris-buffered saline Tween-20, protein stabilizers and preservative (1:101 dilution) and vortexed to mix.

To ensure that any variation within the assay could be identified, 100 µL of each neat control (a prediluted ready to use Negative control made with preservative and human sera without antibodies to Actin, a prediluted ready to use Low Positive control made with preservative and human sera with antibodies to Actin, a prediluted ready to use High Positive control made with preservative and human sera with antibodies to Actin) and diluted patient sample was applied to the polystyrene microwell ELISA plate coated with a purified Actin antigen (12 x 8 wells) in duplicate, as shown in figure 3.1, and incubated at room temperature for 30 minutes.

HPC	P2	P6	P10	P14	P18	P22	P26	P30	P34	P38	P42
HPC	P2	P6	P10	P14	P18	P22	P26	P30	P34	P38	P42
NC	P3	P7	P11	P15	P19	P23	P27	P31	P35	P39	P43
NC	P3	P7	P11	P15	P19	P23	P27	P31	P35	P39	P43
LPC	P4	P8	P12	P16	P20	P24	P28	P32	P36	P40	P44
LPC	P4	P8	P12	P16	P20	P24	P28	P32	P36	P40	P44
P1	P5	P9	P13	P17	P21	P25	P29	P33	P37	P41	P45
P1	P5	P9	P13	P17	P21	P25	P29	P33	P37	P41	P45

*Figure 3.1 Graphic to show the order in which control and patient samples were applied to the 96 well plate. HPC = High Positive Control, LPC = Low Positive Control, NC = Negative control, P1= Patient 1, P2= Patient 2 etc. All samples were applied in duplicate.*

All contents of the wells was thoroughly aspirated, 300 µL of wash was added using a multichannel pipette before the contents of wells was once again aspirated. This wash procedure was performed a further two times (total 3 times) ensuring the wells were completely emptied after each wash step.

Using a multichannel pipette, 100 µL of HRP IgG conjugate (anti-human IgG, goat) containing buffer, protein stabilizers and preservative was added to each well and incubated for 30 minutes, after which time all contents of the wells was thoroughly aspirated, 300 µL of wash added using a multichannel pipette and the contents of wells was once again aspirated. This

wash procedure was performed a further two times (total 3 times) ensuring the wells were completely emptied after each wash step.

Using a multichannel pipette, 100 µL of TMB Chromagen was added to each well and incubated for 30 minutes in the dark.

Finally, 100 µL of HRP Stop Solution (0.344M Sulphuric Acid) was added to each well using the multichannel pipette. The plate was then lightly tapped to ensure mixing. A colour change from blue to yellow could be observed.

The plate absorbance of each well was read at 450nm using a protocol set up for this assay on the DS2. This ELISA does not use a standard curve to determine the actin antibody value, instead the result was then calculated by dividing the average optical density (OD) of the sample by the average OD of the Actin IgG ELISA Low Positive and multiplying by the number of units assigned to the Actin IgG ELISA Low Positive, in this kit the assigned unit value was 25AU.

The manufacturer states the following are required in order for the assay to pass quality control. The absorbance of the High Positive must have an absorbance greater than 1 and greater than the absorbance of the low positive. The negative control absorbance must be lower than that of the low positive and below 0.20. The low positive absorbance must be more than twice the absorbance of the negative control or above 0.25.

$$\text{Sample Value (units)} = \frac{\text{Sample OD}}{\text{Actin IgG ELISA Low Positive OD}} \times \text{Actin IgG ELISA Low Positive (units)}$$

*Figure 3.2 Equation to determine the AU values for F-actin antibody using the QUANTALite IgG Actin ELISA*

The manufacturer guidelines then classify the result according to the value produced:

Negative	<20AU
Weak Positive	20 – 30AU
Moderate to Strong Positive	>30AU

The manufacturer stated sensitivity and specificity are detailed in *Table 3.1*.

*Table 3.1 Manufacturer-stated sensitivity and specificity taken from the IFU (instructions for use) received with each kit. Information available regarding how these were calculated has been included. NOVALite IFU 628255 July 2020 Revision 3. QUANTALite IFU 62878 November 2018 Revision 6. Euroimmun VSM47 IFU FA\_1300-1\_A\_UK\_C23 Version 26/04/2022*

<b>Method</b>	<b>Sensitivity stated by manufacturer</b>	<b>Specificity stated by manufacturer</b>	<b>Information on how specificity and sensitivity were determined as stated in IFU.</b>
INOVA NOVALite IgG F-Actin	Not stated	99.2%	Specificity determined as only four of the 493 normal samples tested positive by IIF.  Ability to detect F-actin antibodies was determined by comparing to the QUANTALite F-actin ELISA result.
INOVA QUANTALite IgG F-Actin	72.4	88%	Many of these patients were undergoing immunosuppressive therapy prior to sample being drawn. Many patients had multiple draws. Specificity was calculated from the data provided in the IFU and not explicitly defined in the IFU.
Euroimmun VSM47	51.5%	100%	Specificity determined after testing 64 samples of which eight were AIH+, 30 had HCV, 16 coeliac disease, and 10 with steatohepatitis.  Sensitivity determined by testing 73 samples, 33 had AIH and for had PBC (10% of which had overlap with PBC). 51.5% of AIH+ patients tested positive.

All results were entered onto a master spreadsheet that contained the patient demographics, clinical information including whether a AIH diagnosis had been made and a date of diagnosis which meant it was possible to ascertain whether the sample was taken pre or post diagnosis.



### 3.5 Statistical Analysis

Positive predictive value (PPV), Negative predictive value (NPV), diagnostic sensitivity and specificity were calculated for each of the three new assays, and also for the existing method for both “all SMA” and the more clinically significant T-SMA pattern.

True positives (TP) were patients with a confirmed diagnosis of AIH, true negatives were those with negative antibody results and no disease. False positives were patient testing positive for antibody but with no overall AIH diagnosis (this included some patients for whom the clinicians strongly suspected AIH but were unable to confirm). The patients with an AIH diagnosis but a negative test result were false negatives (FN).

Positive predictive value (PPV), Negative predictive value (NPV), diagnostic sensitivity and specificity were calculated using the following equations with the total number of each classification being used e.g. if 24 FP patients and 54 TP the first equation would be  $54/54+24$ .

$$\text{PPV} = \text{TP} / (\text{TP} + \text{FP})$$

$$\text{NPV} = \text{TN} / (\text{FN} + \text{TN})$$

$$\text{Sensitivity} = \text{TP} / (\text{TP} + \text{FN})$$

$$\text{Specificity} = \text{TN} / (\text{TN} + \text{FP})$$

The data was then further trimmed to exclude any samples from patients with a known AIH diagnosis at time of sample being taken (as they would be receiving treatment), before being reanalysed. Autoantibody detection is primarily used to aid diagnosis, rather than monitor response to treatment. The PPV, NPV, sensitivity and specificity was then recalculated.

The agreement between the methods was compared using Cohen’s Kappa analysis. The Cohens Kappa is designed to compare two nominal variables where results fall into defined categories. This statistical test is used when results are subjective and corrects for agreement occurring by chance.

A ROC plot analysis was performed to determine the area under the curve (AUC). The ROC curve can be used to calculate the Youden’s J statistic. This test allows the optimal cutoff for a biomarker to be determined using the calculation  $(\text{Sensitivity} + \text{Specificity}) - 1$  for each ELISA result point on the ROC. This calculation will produce a number (the J statistic between 0 and 1 with 1 being a perfect test. The point with the highest J statistic is determined to be the optimal cutoff (Ruopp et al., 2008).

The PPV, NPV, sensitivity and specificity was then recalculated using the calculated optimal cutoff.

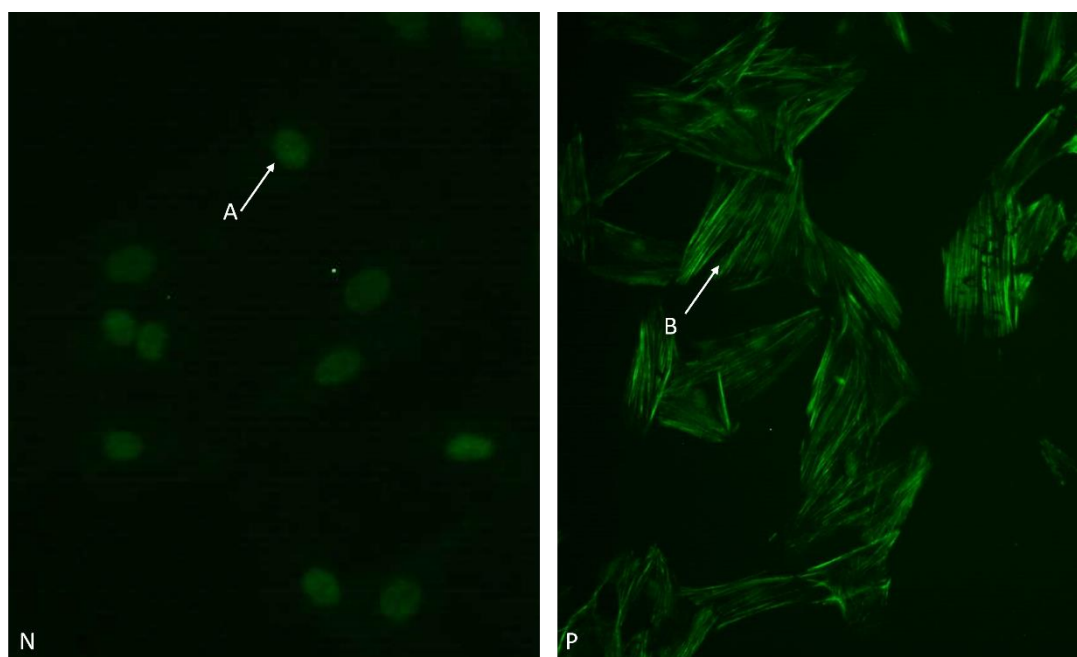
Chi square analysis was used to determine the significance of the results when using to diagnose for AIH. The significance level (p value) was set at 0.05 with anything falling below 0.05 being classed as statistically significant and not the result of chance.

Microsoft Excel and Analyse It were used for the statistical analysis.

## Chapter 4 Results

Three new assays were assessed in this study, two utilised indirect immunofluorescence (INOVA NOVALite and Euroimmun VSM47) and one used ELISA (INOVA QUANTALite), consequently the results generated were a mixture of quantitative and qualitative. The results yielded were allocated an overall interpretation of positive or negative, but in the methods using indirect immunofluorescence an equivocal result was given in certain cases where interpretation was more complicated. Using the same interpretation nomenclature allowed comparison between methods, with the ELISA the results were compared using two different cut-offs as per manufacturer instructions. In the case of the ELISA where a numerical result was obtained, further data analysis was completed. All results were collated alongside patient demographics and medical history (*Appendix E*).

Immunofluorescence patterns observed using the Euroimmun VSM47 and INOVA NOVALite kits can be shown in *figure 4.1* and *figure 4.2*, respectively. In this study the VSM47 pattern had a lower incidence of non-specific staining than the NOVALite, which facilitated clearer interpretation. This is reflected in the samples that were deemed equivocal, the more difficult-to-interpret NOVALite had more equivocal classifications than the VSM47 (22 versus 3 respectively).



*Figure 4.1 Immunofluorescence pattern detected on Euroimmun VSM 47 slides. N = Pattern observed when the result is negative. (A) points to a cell nucleus that is visible but not showing the actin antibody pattern. P is the pattern observed when a sample is positive for F-actin antibodies. The cell nuclei are surrounded by fluorescent micro-filamentous staining. Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a COOLLED pE-100 bulb at a magnification of 200x.*

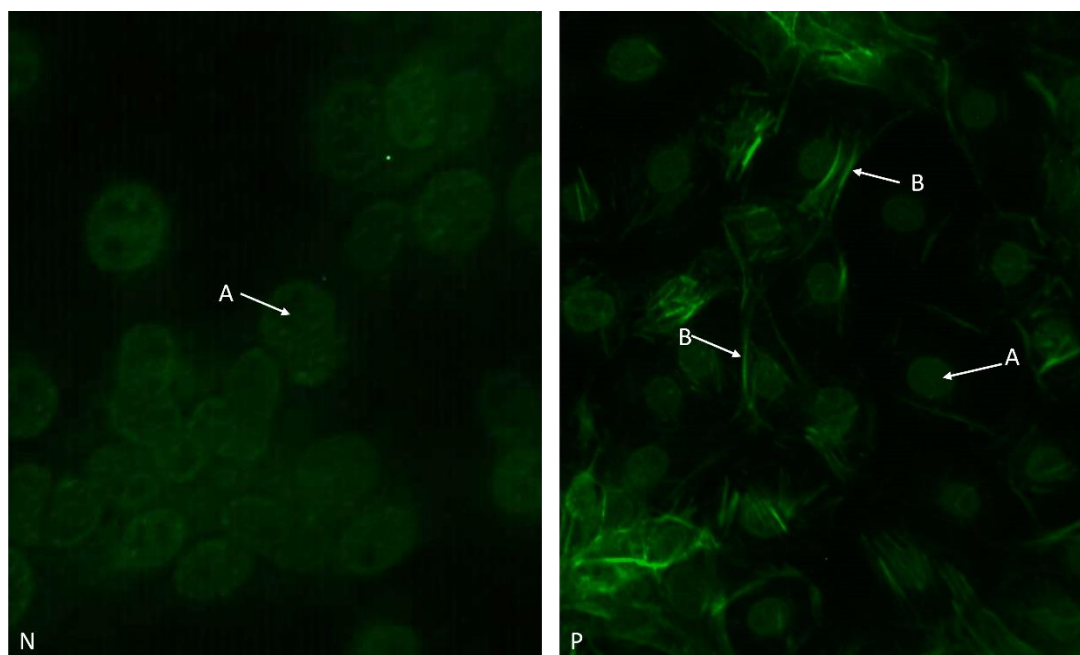


Figure 4.2 Immunofluorescence pattern detected on NOVALite F-actin slides. N is a negative sample; the nucleus of the cell can be seen (A) but displays very little fluorescence. P shows a positive sample. The filamentous staining (B) can be seen around the cell nucleus (A). Images captured by the author using a Kappa Zelos camera fitted to a Leica DMRB microscope fitted with a Cooled pE-100 bulb at a magnification of 200x.

The ELISA produced absorbance results that were converted into arbitrary unit values (as no international standard is available for F -actin antibody) using the equation in figure 3.2. The manufacturer suggests a cutoff of 20 AU for weak positive and 30 AU for a strong positive. As such the results displayed in the following Table 4.1 accounts for both cutoffs.

Table 4.1 Number of samples (n) and percentage of samples (%) that were positive for each method and AIH diagnosis status. In this Table any samples that were judged as weak/equivocal using the IIF methods are excluded from the overall positive count. T-SMA = Tubular Smooth Muscle Antibody, SMA = Smooth Muscle Antibody both vascular and tubular patterns.

Diagnosis	Testing Method											
	SMA+ (V&T)		T-SMA+		NOVALite F		VSM47+		QUANTALite		QUANTALite	
	n	%	n	%	n	%	n	%	n	%	n	%
AIH + (n=26)	24	92.3	19	73	14	53.8	16	61.5	17	65.3	14	53.8
AIH – (n=107)	83	77.6	20	18.7	15	14	20	18.7	21	19.6	7	6.5

Table 4.1 shows the number of samples that had a positive result for each method in both the AIH positive groups and in the group of patients with no diagnosis of AIH. In Table 4.1, results that were judged to be equivocal for the two new IIF methods were included in the negative group. Table 4.2 shows the difference if they were included in the positive group. The method of obtaining samples (positive selection of those with a previous SMA positive result) reflects the high proportion with a SMA.

Table 4.2 Number of samples (n) and percentage of samples (%) that tested positive for each method and AIH diagnosis status. In this Table any samples that were interpreted as weak/equivocal using the IIF methods are included in the overall positive count.

	Testing Method			
	NOVALite F		VSM47 + Actin +	
Diagnosis	n	%	N	%
AIH + (n=26)	18	69.2	17	65.4
AIH – (n=107)	33	30.8	25	23.4

#### 4.1 Statistical Analysis of entire data set

Chi square analysis (Table 4.3) showed, as expected, that all four F-Actin specific assays (T-SMA, VSM47, NOVALite and QUANTALite) demonstrated a strong correlation existed between a diagnosis of AIH and a positive result (p value <0.05). The detection of a none F-Actin specific SMA showed there to be no strong association between a positive SMA and AIH.

Table 4.3  $\chi^2$  analysis of all assays. Those with a p value < or equal to 0.05 show a strong correlation between a positive result and a diagnosis of AIH.

	NOVALite	VSM47	QUANTALite	T-SMA	SMA
$\chi^2$	19.45999	19.45238	21.4607117	29.85245	2.888718
p-value	0.00001	0.00001	<0.00001	<0.0001	0.089202

Table 4.4 Cohens  $\kappa$  statistical test value for agreement between all methods used for anti-actin detection, where IIF interpreted as equivocal is classified as negative. Values  $\leq 0$  as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement (McHugh, 2012) SMA= smooth muscle antibody, T-SMA = tubular smooth muscle antibody

		% agreement	Cohen's $\kappa$	Agreement
NOVALite	SMA	42	0.13	Slight
	T-SMA	89	0.71	Substantial
	QUANTALite >20	84	0.59	Moderate
	QUANTALite >30	85	0.52	Moderate
	VSM47	90	0.73	Substantial
QUANTALite >20	SMA	46	0.15	Slight
	T-SMA	85	0.63	Substantial
	VSM47	84	0.61	Substantial
QUANTALite >30	SMA	36	0.09	Slight
	T-SMA	84	0.54	Moderate
	VSM47	85	0.57	Moderate
VSM47	SMA	47	0.17	Slight
	T-SMA	93	0.81	Almost perfect

Table 4.5 Cohens  $\kappa$  statistical test value for agreement between all methods used for anti-actin detection, where IIF interpreted as equivocal is classified as positive. Values  $\leq 0$  as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement (McHugh, 2012). SMA= smooth muscle antibody, T-SMA = tubular smooth muscle antibody

		% agreement	Cohen's $\kappa$	Agreement
NOVALite	SMA	57	0.26	Fair
	T-SMA	84	0.64	Substantial
	QUANTALite >20	73	0.39	Fair
	QUANTALite >30	72	0.32	Fair
	VSM47	85	0.67	Substantial
VSM47	QUANTALite >20	83	0.59	Moderate
	QUANTALite >30	80	0.48	Moderate
	SMA	52	0.21	Fair
	T-SMA	89	0.74	Substantial

Cohen's kappa analysis (*Table 4.4 and Table 4.5*) showed a varied agreement between methods. According to the Cohen scale (McHugh, 2012) the agreement between the broader SMA pattern and the new methods was only slight, whereas the Tubular smooth muscle antibody pattern (which is F-Actin specific) had mostly substantial agreement and almost perfect agreement with the VSM47. The three new methods have been designed to be F-Actin specific and as anticipated correlation is higher. The correlation between the three new methods themselves ranges from moderate to substantial when equivocal results were classed as negative (*Table 4.4*) but only fair to moderate if the equivocal results were classed as positive (*table 4.5*).

Correlation between current and new methodology is important but the application of the method is of much greater significance. The positive predictive value (PPV), negative predictive value (NPV), specificity and sensitivity of each assay is shown in *Table 4.6*, the equivocal results are counted as negative in this instance. Using LKS substrate and reporting SMA as positive but without distinguishing the pattern is the most sensitive (92.31%), but the specificity is extremely low (22.43%). If only the T-SMA pattern is reported as a positive result, the sensitivity is lower (73.08%) but specificity greater (81.31%). The ELISA with a cutoff of 30 AU showed the greatest specificity (93.46) but the sensitivity is only 53.85%. The PPV value was higher for ELISA with a cutoff of 30AU (66.67) than the other methods by at least 20 %.

*Table 4.6 PPV, NPV, Specificity and Sensitivity of the four assays (and associated patterns where applicable) when weak/equivocal results are interpreted as negative. SMA= smooth muscle antibody, T-SMA = tubular smooth muscle antibody, PPV = positive predictive value, NPV = Negative predictive value*

	PPV (%)	NPV (%)	Specificity (%)	Sensitivity (%)
SMA	22.43	92.31	22.43	92.31
T-SMA	48.72	92.25	81.31	73.08
NOVALite F Actin IIF	48.28	88.46	85.98	53.85
VSM47 IIF	44.44	89.69	81.31	61.54
QUANTALite F Actin ELISA (>30AU)	66.67	89.29	93.46	53.85
QUANTALite F Actin ELISA (>20AU)	44.74	90.53	80.37	65.38

*Table 4.7* shows the positive predictive value, negative predictive value, specificity, and sensitivity of each assay if the IIF that was interpreted as positive or equivocal by the readers was classed as positive. The sensitivity has improved for both assays (increased from 53.85% to 69.23% for NOVALite and from 61.54% to 65.38% for VSM47) but with a reduction in specificity (85.98% to 69.16% for NOVALite and 81.31% to 76.64% for VSM47). This is to be expected with equivocal result as the IFA was ambiguous and likely had some non-specific staining interference.

Table 4.7 PPV, NPV, Specificity and Sensitivity of the four assays (and associated patterns where applicable) when weak/equivocal results are interpreted as positive. SMA= smooth muscle antibody, T-SMA = tubular smooth muscle antibody, PPV = positive predictive value, NPV = Negative predictive value

	PPV (%)	NPV (%)	Specificity (%)	Sensitivity (%)
NOVALite F Actin IIF	35.29	90.24	69.16	69.23
VSM47 IIF	40.48	90.11	76.64	65.38

## 4.2 Statistical analysis of Autoimmune Hepatitis Negative and Treatment Naïve Autoimmune Hepatitis patients

Data up until this point has included all the samples received and analysed, whether they be from treatment naïve pre-AIH diagnosis, post- AIH diagnosis or patients without AIH. As the presence of the antibody is more clinically significant in aiding diagnosis, than as a tool for monitoring, the data was trimmed to exclude samples from patients known to have AIH (and therefore be receiving treatment) at time of sample collection. The PPV, NPV, sensitivity and specificity data for this trimmed cohort are in *Table 4.8*.

The sensitivity for all assays has now improved, with the most interesting finding being that the VSM47 and ELISA (with a cut-off of 20 AU) method has surpassed the current method (T-SMA detection) in sensitivity level (92.86% in each case). The ELISA with a cutoff of 30AU and the NOVALite have sensitivity similar to that of T-SMA (85.71% for QUANTALite >30 and NOVALite versus 86.67% for T-SMA). Again, this data in *Table 4.8* has incorporated any IIF deemed to be equivocal into the negative group, *Table 4.9* shows that when this data is included in the positive group the specificity is reduced (to 69.16% for NOVALite and 75.7% for VSM47) and sensitivity remains the same.

Table 4.8 Pre AIH-diagnosis patients and non AIH patients. PPV, NPV, Specificity and Sensitivity of the four assays (and associated patterns where applicable) when weak/equivocal results are interpreted as negative. SMA= smooth muscle antibody, T-SMA = tubular smooth muscle antibody, PPV = positive predictive value, NPV = Negative predictive value

	PPV %	NPV %	Specificity %	Sensitivity %
SMA	14.43	100	22.43	100
T-SMA	39.39	97.75	81.31	86.67
NOVALite F Actin IIF	44.44	97.87	85.98	85.71
VSM47 IIF	39.39	98.86	81.31	92.86
QUANTALite F Actin ELISA (>30AU)	63.16	98.04	93.46	85.71
QUANTALite F Actin ELISA (>20AU)	38.24	98.85	80.37	92.86

Table 4.9 Pre AIH-diagnosis patients and non AIH patients. PPV, NPV, Specificity and Sensitivity of the four assays (and associated patterns where applicable) when weak/equivocal results are interpreted as positive.

	PPV (%)	NPV (%)	Specificity (%)	Sensitivity (%)
NOVALite F Actin IIF	26.67	97.37	69.16	85.71
VSM47 IIF	33.33	98.78	75.7	92.86

The quantitative data produced by the QUANTALite ELISA offered the opportunity to assess whether measuring the level of antibody could offer any indication of disease presence. ROC analysis (figure 4.3) showed that the F-actin ELISA has an AUC of 0.957 indicating that a positive result for this assay is a strong indicator of an AIH-1 diagnosis. Youden index indicated that the optimal cutoff in this data set was 28.18 AU. At this cutoff the specificity was 90.74% and the sensitivity was 100%.

Values for the ELISA F-Actin antibodies are significantly higher ( $p < 0.05$ ) in the treatment naïve pre-AIH diagnosis samples than in both the post-AIH diagnosis and none AIH groups, although as shown in figure 4.4, the values obtained in the none AIH group did on occasion reach similar levels to those found in the AIH group.

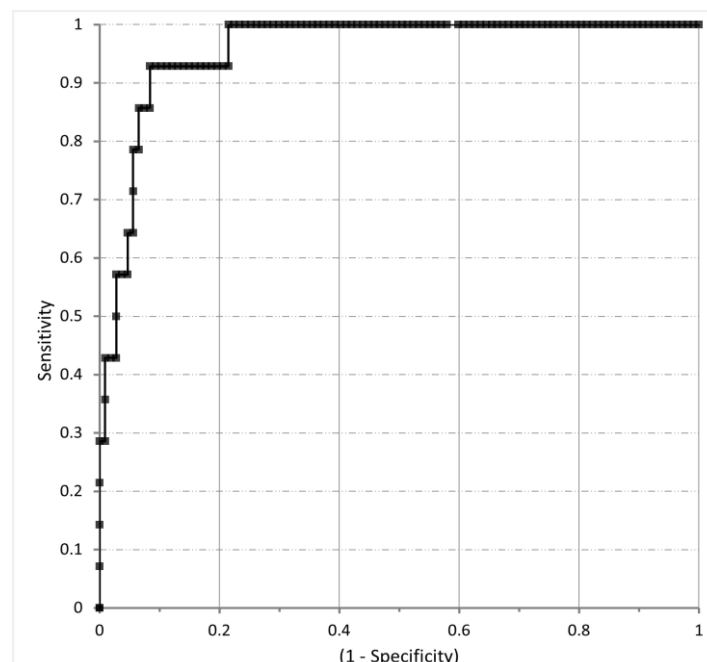


Figure 4.3 Receiver-operating curve showing diagnostic value of the F-Actin ELISA for patients with AIH. Data used include AIH - and treatment naïve AIH+ Area Under the Curve (AUC) = 0.957



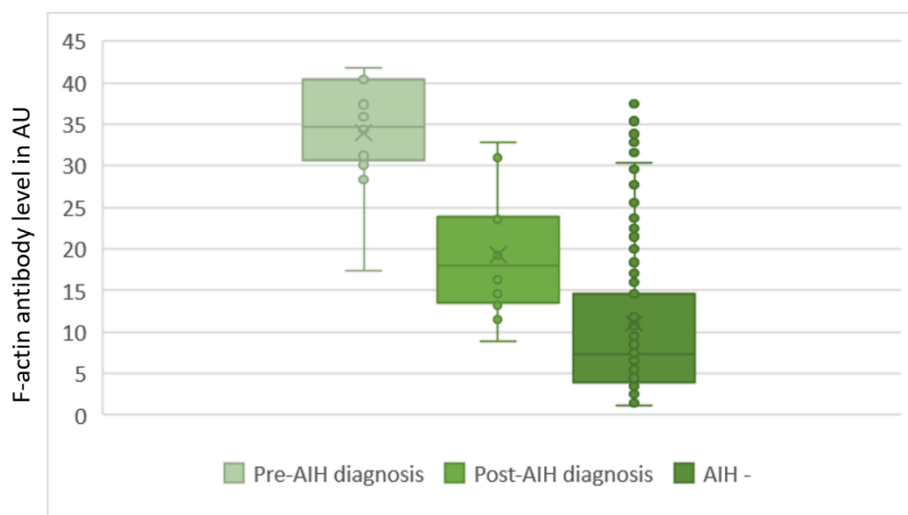


Figure 4.4 Whisker and Box plot demonstrating the median and range of the results obtained across three different patient groups; pre-AIH diagnosis, pos-AIH diagnosis and no diagnosis of AIH.

### 4.3 Addition of a new assay as a second-line test

The aim of the study has been to determine whether the addition of a second line assay would benefit the users and patients, with the concern that tubular staining could be missed by less experienced operators. Data in *Table 4.10* indicates that the addition of second assay would reduce the sensitivity for detecting AIH+ patients by between 7 and 14% depending on the assay combination used but that the specificity would increase. A positive SMA on LKS, followed by a positive result on one of the new methods increased specificity from 22% to between 84 to 95%. If the result on LKS was T-SMA the specificity rose to 97-99%. The greatest specificity (99%) was provided by a positive T-SMA on LKS, followed by a QUANTALite result of >30AU. The optimal cutoff gave a marginally reduced specificity of 98% but raised the sensitivity by over 10% from to 85.7%

Including or excluding the equivocal results did not affect the sensitivity of the assay but when the NOVALite and VSM47 equivocal results are included as positive in the “All SMA” group, the specificity decreases from 89% to 60% for NOVALite and 86% to 68% for VSM47. However, in the T-SMA only group, inclusion of the equivocal results the specificity remains the same 97% or slightly increases (97% to 99%). This reflects the widely held view that T-SMA pattern is more specific for AIH and again highlights the subjective nature of immunofluorescence.

Table 4.10 Sensitivity (Se) and Specificity (Sp) of using two methods as a consecutive test. Combined Se = SeT1xSeT2. Combined Sp = SpT1 + SpT2- (SpT1 \* Sp T2). Specificities in brackets when equivocal results are included in the positive count.

	Current method only	Combined serial testing of current method and new method				
	All SMA	NOVALite	VSM47	QUANTALite >20	QUANTALite >30	QUANTALite >28.18 (optimal cutoff)
Sensitivity (%)	100	85.7	92.8	92.9	85.7	100
Specificity (%)	22	89.1 (60)	85.5 (68)	84.8	94.9	92.8

	T-SMA	NOVALite	VSM47	QUANTALite >20	QUANTALite >30	QUANTALite >28.18 (optimal cutoff)
Sensitivity (%)	87	73.5 (74.3)	79.6 (80.5)	79.6	73.5	85.7
Specificity (%)	81	97.3 (94.2)	96.5 (95.5)	96.3	98.9	98.2

The utility of a second line confirmatory test is displayed in *Table 4.11* and *Table 4.12*. Of the 32 T-SMA positive samples, only twelve will go on to be diagnosed as having AIH-1, and of the 65 V-SMA samples this number will be only two. The initial concern was that tubular staining could be missed when interpreting SMA on LKS substrate. The 2<sup>nd</sup> line testing identified an AIH+ patient (patient 34) as having F-actin antibodies despite being only V-SMA on LKS substrate. Interestingly the patient had a normal IgG of 13g/L but an antibody level of 37.4 AU on the ELISA. Given the normal IgG and presence of the less specific V-SMA pattern it would be thought unlikely that this patient had AIH using the IAIHG scoring system, but the second line test may prompt further investigation. Conversely patient 10 had a V-SMA, was negative for all 2<sup>nd</sup> line assays but had a raised IgG of 21.3 and went on to be diagnosed at AIH+.

In the T-SMA group there were two patients subsequently diagnosed with AIH who were negative/weakly positive by a second line test. Patient 81 has a T-SMA but was negative by NOVALite, (although positive on VSM47 and had the second highest F-actin level by ELISA of 40.4 AU) and a much-raised IgG (over two times greater than upper limit of normal). Patient 71 had a mildly elevated IgG, positive result for VSM47 and NOVALite but the ELISA was only weak positive (range 20-30AU). Although at 28.3 AU this result was at the higher end of the weak positive range.

Patient 108 was negative for SMA but had a weak positive result (29.5 AU) using QUANTALite. This patient was being worked up for liver transplant, having been initially diagnosed as having PBC at another hospital.

Some patients did have a positive result for F-actin antibodies despite having no diagnosis of AIH. Five of these patients were positive (or weak positive) for all methods and had AU levels >27. Patient 91 had liver cancer, patients' 101 and 102 had normal IgG but continue to be monitored by the Immune liver disease clinic. Patient 99 has a strong indication of AIH but declined biopsy so diagnosis could not be confirmed, however their F-actin level was 28.18 AU which coincidentally is the optimal cutoff determined by Youden's J. Finally patient 106 had a rapid deterioration leading to death but had extensive liver damage.

*Table 4.11 Results of second line testing of patient samples after both initial V-SMA and T-SMA positive result on Liver Kidney Stomach (LKS) substrate. The number of patients with confirmed AIH diagnosis falling into each category is indicated. Pos =Positive, Equ = Equivocal and Neg = Negative*

Result from LKS substrate	NOVALite			VSM47			QUANTALite >20AU		QUANTALite >30AU	
	Pos	Equ	Neg	Pos	Equ	Neg	Pos	Neg	Pos	Neg
V-SMA n =65	4	12	49	5	5	55	9	56	3	64
	AIH+			AIH+			AIH+		AIH+	
	1	0	1	1	0	1	1	1	1	1
T-SMA n = 32	23	6	3	28	1	3	24	8	16	16
	AIH+			AIH+			AIH+		AIH+	
	11	0	1	12	0	0	13	0	11	1
NEG n = 24	0	0	24	0	0	24	1	23	0	24
	AIH+			AIH+			AIH+		AIH+	
	0	0	0	0	0	0	0	0	0	0

Table 4.12 Samples negative for F-actin antibodies using second line test. Column one is the number of patients who would be deemed to be low risk of AIH following a negative result for the second line assay. Column two is the number of patients with AIH who would be incorrectly identified as low likelihood of having AIH following a negative result for the second line assay.

Method of detection	True negative patients. AIH patients correctly suggested as not having AIH by second line test	False negative patients. AIH patients not identified by second line test
NOVALite	50 (48 V-SMA and 2 T-SMA)	2 (1 V-SMA and 1 T-SMA)
VSM47	57 (54 V-SMA)	1 (V-SMA)
QUANTALite >20AU	63 (55 V-SMA and 8 T-SMA)	1 (V-SMA)
QUANTALite >30AU	78 (63 V-SMA and 15 T-SMA)	2 (1 V-SMA and 1 T-SMA)

#### 4.4 Interference from other antibodies found in autoimmune liver disease and detected using LKS substrate.

Table 4.13 examines whether the presence of the other liver autoantibodies detected on LKS substrate (LKM and Mitochondrial) could affect the results of the second line test. The NOVALite and VSM47 did not show any positivity in the presence of these other autoantibodies. However, the QUANTALite did give three weak positive results in three patients with mitochondrial antibodies. Patient 100 was known to have AIH-1 with PBC overlap syndrome, patient 108 was Hepatitis B positive and patient 58 had fatty liver disease. LKM did not seem to have any effect on the F-actin antibody result, however only two samples were included in the study due to their rarity.

Table 4.13 Patient samples with a positive liver related antibody result other than SMA. Mito = mitochondrial antibody. The + indicates the titre at which the sample is positive. + = positive at 1:50, ++ = 1:100, +++ = 1:500, ++++ = >1:1000 -/+ = equivocal result

Patient	SMU	Mito	LKM	NOVALite	VSM47	QUANTALite	Diagnosis	Treatment naïve?
44	V-SMA	++	-	-/+	-	- (14.6)	Sclerosing Cholangitis	No
47	V-SMA	++++	-	-	-	- (9.4)	PBC	No
58	V-SMA	++	-	-	-	Weak+ (25.612)	Fatty liver	No
100	T-SMA	++	-	-	-	Weak (23.5)	+ AIH-1 with potential PBC overlap	No
108	-	++++	-	-	-	Weak (29.6)	+ Hep B	No
109	-	-	+	-	-	- (19.8)	AIH-2	No
111	-	++++	-	-	-	- (14.6)	AIH-1	No

112	-	++++	-	-	-	-(11.8)	No diagnosis available	Yes
114	-	++	-	-	-	-(6.6)	PBC	Yes
117	-	-	+++	-	-	-(4.8)	No diagnosis available	Yes
128	-	+++	-	-	-	-(2.2)	No diagnosis available but monitored by Hepatology	Yes

#### 4.5 Effect of treatment on antibody level

A pre and post treatment sample was analysed in three of the patients studied (*Table 4.14*). All 3 patients had IgG level return to the normal range after treatment but remained T-SMA positive. Patient A (samples 83 and 84) became equivocal for both VSM47 and NOVALite, Patient B (samples 76 and 77) remained positive for VSM47 and NOVALite, Patient C (samples 72 and 73) remained positive for NOVALite but became negative for VSM47. All three had a reduction in the F-actin level by ELISA, patient A and B had levels fall into the negative range whilst patient C remained strongly positive.

*Table 4.14 Autoantibody results for three patients before and after treatment. Patient A had samples 83&83, Patient B had samples 76&77 and Patient C had samples 72&73. QLite = QUANTALite, Pre= Pre-treatment, Post = Post=treatment, T-SMA= Tubular smooth muscle antibody, + = Positive, +/- = Equivocal, - = Negative*

Patient	SMA		IgG		QLite (AU)		VSM47		NOVALite	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A	T-SMA	T-SMA	28.7	11.3	35.9	19.3	+	+/-	+	+/-
B	T-SMA	T-SMA	18.7	12.9	31.8	16.9	+	+	+	+
C	T-SMA	T-SMA	57.4	14.6	40.4	32.8	+	-	+	+

## Chapter 5 Discussion

The purpose of this study was to assess whether adding a second line confirmatory test could aid in the diagnosis of AIH. In all cases performing the second line assay on samples with a positive SMA resulted in a decrease in sensitivity and an increase in specificity as some patients with AIH did not test positive for F-actin antibody. The greatest specificity observed was screen using the Liver Kidney Stomach (LKS) substrate to detect tubular smooth muscle (T-SMA) pattern followed by QUANTALite ELISA with a cutoff of 30AU. In this scenario the specificity was 99%. Although the overall sensitivity was reduced to 73.5% from 87% (TSMA) when the assays were used in combination, compared to current methodology, the reality would be that the clinician would still be in possession of the initial immunofluorescence result (as is currently the case) but would now have the added information provided by the second line assay. Using the optimal cutoff of 28.18AU increased the combined sensitivity to 85.7%, which is line with that TSMA and raises the combined specificity from 81.31% to 98.2%. This shows that a second line assay could be a valuable addition and may be of particular use for patients who are unwilling or unable to undergo liver biopsy, such as patient 99 seen in this study. Their initial liver autoantibody screen had a positive TSMA which according to this study is 81.31% specific for AIH, however with the addition of the QUANTALite assay with a cutoff of 28.18, this specificity rises to 98.2%. This could provide the clinician with more certainty that their diagnosis suspicions are correct.

The use of confirmatory second line assay is a proven and routine feature within autoimmune testing. Small vessel vasculitides such as Eosinophilic Granulomatosis with Polyangiitis (EGPA) (formally Churg-Strauss) and Granulomatosis with polyangiitis (GPA) (formally Wegener's Granulomatous) were first associated with anti neutrophil cytoplasmic antibodies (ANCA) in the 1980's (Jennette et al., 1989). The antibodies are identified by performing indirect immunofluorescence on ethanol fixed neutrophils with patient serum. Two main patterns are observed; perinuclear (p-ANCA) and cytoplasmic (c-ANCA) alongside other atypical patterns. Positive ANCA are found in several diseases other than the vasculitides including Crohn's and Ulcerative Colitis (Savige et al., 1999). In the late 1980's the specific antigens associated with ANCA associated vasculitides were identified, namely Myeloperoxidase (MPO), commonly seen alongside perinuclear ANCA IFA staining and associated with EGPA and other vasculitides, and Proteinase 3 (PR3) seen alongside cytoplasmic ANCA IFA staining and associated with GPA disease (Drooger et al., 2009). In 1999 an international consensus statement was released to confirm that antigen specific testing be performed routinely alongside IFA due to the high sensitivity and specificity of MPO and PR3 for small vessel vasculitis (Savige et al., 1999). In 2017 this was updated to suggested the antigen specific testing be performed as a first line screen (Bossuyt, Cohen Tervaert, et al., 2017). Higher antibody levels were shown to be a greater predictor of ANCA associated vasculitis (Bossuyt, Rasmussen, et al., 2017).

The agreement between the Tubular smooth muscle pattern and the F-actin specific assays was substantial in the majority of cases, indicating the predominant target antigen in T-SMA is F-actin which is in consensus with literature (Bottazzo et al., 1976; Granito et al., 2006; Toh, 1979; Toh et al., 2010). The consensus between T-SMA and QUANTALite dropped to “moderate” only when the cutoff was increased to 30AU, which could be explained by T-SMA having no designation between weak and strong. All IIF methods maintained substantial agreement with each other, perhaps as the actin target in these assays is in a native form. The agreement was slightly reduced between QUANTALite and the VSM47 substrate although remained moderate to substantial. An unexpected finding was that despite being produced by the same manufacturer, agreement between NOVALite and QUANTALite methods was only fair to moderate at best. An explanation for each assay obtaining slightly different results may be that the F-Actin antigens are derived from various sources. NOVALite uses rat intestine epithelial cells, QUANTALite uses highly purified F-Actin antigen, VSM47 uses rat embryo thoracic aorta, and the current method uses sections of mouse liver, kidney, and stomach tissue which could add to variability in detection, as there may be some mild conformational differences between each substrate.

The results of all three methods support the common view that V-SMA is not actin specific and therefore not indicative of AIH (Dalekos & Gatselis, 2023). However, the presence of only V-SMA does not preclude an individual from having AIH hepatitis, and this is evidenced in this study as two patients with only V-SMA do have a diagnosis of AIH.

The initial specificities and sensitivities determined from the data produced in this study are similar to the specificities and sensitivities provided by the manufacturers in the accompanying IFUs. The initial sensitivity was calculated before the dataset was trimmed to exclude the known AIH patients. The treatment status of the patient samples used in the VSM47 data provided by Euroimmun is not available, and the QUANTALite states that patients already undergoing treatment are included (the exact numbers of which are not elaborated upon). The NOVALite sensitivity is not provided by the manufacturer and the specificity was determined by a negative result being obtained for “normal samples” which suggests that these could be healthy controls rather than samples from patients with other underlying liver or autoimmune conditions. Once the data in this study was trimmed to exclude known AIH patients the sensitivity increased across all assays.

## 5.1 The Rationale

Autoimmune Hepatitis diagnosis can be challenging due to heterogeneity of the presentation. The diagnostic scoring system developed and then further refined in 2008 by the IAIHG (Hennes et al., 2008b), highlights the importance of accurate and timely autoantibody detection to aid identification of AIH. A swift diagnosis and access to treatment is essential to halt or delay disease progression by minimising liver damage, leading to a more favourable prognosis for the patient (Dalekos & Gatselis, 2023). The current criteria in the 2008 guidelines includes circulating autoantibodies, elevated IgG, absence of viral hepatitis and distinguishing

liver biopsy findings (Hennes et al., 2008b). It is clear that the presence of F-actin antibodies is associated with, and not diagnostic of AIH, and therefore results from these assays should be viewed within the wider context when determining an AIH diagnosis. Liver biopsy is still required for confirmation of disease, to ascertain if the presentation is acute or chronic and to inform treatment plan (Covelli et al., 2021b).

The antibodies that are described in type 1 AIH are ANA, Smooth muscle antibodies and SLA. With the exclusion of SLA (which is highly specific, less frequently observed and only detectable using solid phase assay), there are potentially several antigenic targets, many of which are yet to be identified at a molecular level (Terziroli Beretta-Piccoli et al., 2022b). Aside from F-actin, smooth muscle antibodies themselves can target Vimentin and Desmin and other unknown targets in 20% cases, and for the ANA positive samples the target antigen is unknown in approximately 30% cases (Terziroli Beretta-Piccoli et al., 2018a). This contrasts with LKM-1 antibodies found in type 2 AIH; the antigenic target was identified as Cytochrome P4502D6 allowing solid phase assays such as immunoblot and ELISA to be developed, these can be used as a confirmatory test or in case of the United States of America frequently as the first line screen (Ludwig et al., 2018). ANA are found more commonly in connective tissue disease and in these instances the antigenic targets and more specific disease associations have been ascertained. Smooth muscle antibodies are detected by indirect immunofluorescence using rodent liver, kidney and stomach tissue sections, a method that can be used to simultaneously detect several other autoantibodies found in liver disease (e.g. mitochondrial antibodies in PBC) and other disorders (gastric parietal cell antibodies in Pernicious anaemia and B12 deficiency) (Guo et al., 2020). This method utilizes microscopy which relies on highly trained laboratory personnel to identify the individual autoantibody patterns observed, is subjective and is poorly standardised. This can lead to inconsistencies in reporting, potentially between and within laboratories. Double reading of all slides can help to prevent this within a laboratory. Two operators independently read the slides and results are compared, any discrepancies are then discussed, and a third operator can help reach a consensus if required. Inter laboratory variations would be much harder to eliminate without regular consensus meetings. Smooth muscle antibodies are interpreted as being negative, vascular (V-SMA), vascular glomerular (VG-SMA) or vascular glomerular tubular (T-SMA) depending on the which structures within the tissue are displaying fluorescent reactivity, with the tubular pattern being strongly associated with AIH-1. Not all laboratories report all patterns and may use different screening dilutions. This lack of standardization makes comparing the results of the LKS substrate at Leeds to findings in other laboratories challenging. Meta-analysis by Zhang et al., 2014 indicated that the specificity of smooth muscle antibody was 92% and sensitivity was 59% which is similar to the findings at Leeds for tubular smooth muscle antibodies (81.31% and 73.08%) but not when the vascular smooth muscles antibodies are also included (22.43% and 92.31%). This meta analysis took into account IIF and other methodologies, and did not specify the SMA pattern. Data from (Villalta et al., 2008) showed TSMA to have a specificity of 96.1% and a sensitivity 57.6%, whilst a study



by Aubert et al., 2008 showed SMA specificity and sensitivity to both be around 74%. The discrepancies in the literature highlight the requirement for standardization of reporting.

At Leeds General Infirmary smooth muscle antibodies are reported as vascular or tubular, but other laboratories may choose not to report vascular smooth muscle, instead only reporting as smooth muscle positive if tubular or glomerular staining is observed. Vascular smooth muscle antibodies are associated with a myriad of conditions including alcoholic liver disease, viral infection and drug induced liver injury (Terziroli Beretta-Piccoli et al., 2022b). In some cases, the presence of multiple antibodies (whether clinically relevant or non-specific binding) can make antibodies of interest difficult to discern.

## 5.2 Ease of Use

The three new methods are directed specifically against F-actin which is the antigenic target said to be the most clinically significant in AIH-1 (Dalekos et al., 2022). As with the current LKS method, two use Indirect Immunofluorescence (IIF), however unlike the LKS substrate these have been designed to specifically identify F-actin autoantibodies. The VSM 47 kit from Euroimmun uses a cell line derived from rat embryo thoracic aorta. Both operators found this pattern simple to distinguish, with very little non-specific staining causing interference. Despite this there were still seven patient samples classified as equivocal, although only one was a true AIH patient and they were already receiving treatment.

The second IIF assay was the INOVA NOVALite which uses a cell line derived from rat intestine epithelial cells. In this case both operators found the IIF interpretation less straightforward. Immunofluorescence was observed that did not match the defined pattern of a positive F-actin sample, making it difficult to discern whether the F-actin pattern coexisted. This led to the number of “equivocal” classifications rising to 20.

In our experience the sensitivity increased and specificity was reduced when the equivocal results were included in the positive count, and this is something that still applied when the samples were broken down into different cohorts; initially all samples were included in statistical analysis but in subsequent analysis patients who were known to have AIH and were on treatment were removed from the data set to reflect the use of this test as a diagnostic tool rather than for monitoring. Specificity remained notably lower when equivocal results were included compared to the positive alone.

As alluded to previously, IIF is a subjective method, and it takes a certain amount of practice to become proficient. In this study the operators were both new to interpreting F-actin autoantibody using this substrate, and had only the positive control as a guide as to what pattern they should be aiming to identify. Some of those samples designated as equivocal did come from patients with AIH-1 and after more experience interpreting these patterns, discerning positive F-actin staining from non-specific staining will become less challenging.

The third assay in the study was the QUANTALite ELISA which uses purified actin antigen. ELISA works by measuring the optical density of the final mixture and comparing this to the optical density of a mixture with a known quantity of the analyte of interest, thus allowing quantitation of said analyte, in this instance F-actin antibody. One benefit of a quantitative assay is that it allows for specified negative, weak positive and positive ranges to be determined, acknowledging that autoimmunity is not always straightforward and does not always conform to a set of rules. Smooth muscle antibody presence is primarily used as a diagnostic indicator of AIH, although it may be used as a tool for monitoring disease progression or response to treatment (Bogdanos et al., 2008).

Once the patient is receiving treatment the goal is to see a normalization of liver enzymes (ALT and AST) and IgG levels (Harrington et al., 2022) (with this one may assume therefore a reduction in antibody titre). In laboratory studies diluting SMA, the pattern changed from T-SMA (vascular-glomerular-tubular staining) to VG-SMA (vascular-glomerular staining) to V-SMA with increasing dilutions (Galaski et al., 2021; Bottazzo et al., 1976). It can therefore be postulated that as the patient SMA antibody reduces the pattern visualised on IIF changes from T-SMA to V-SMA. The data obtained from the QUANTALite ELISA showed that the pre-treatment and post-treatment patients fell into 2 distinct groups regarding F-actin antibody level. The production of a quantitative result was of particular interest for the six samples that came from three different patients A, B and C. In each pair of samples, one was pre treatment and one post. The data suggests that antibody titre does decrease alongside the fall in IgG level, but that even a return to normal IgG level doesn't result in the disappearance of antibodies entirely. The treatment regime for AIH can be gruelling for patients. Long term use of immunosuppressive medications such as Azathioprine, Mycophenolate Mofetil, Corticosteroids and the more heavy weight Tacrolimus and Cyclosporine come with a range of side effects that can have a negative impact on quality of life. Side effects can range from acne, hirsutism, increased hypersensitivity, and nausea through to tremors, gastrointestinal disorders and malignancy (Ruiz & Kirk, 2015). As a result many patients, especially younger and adolescent patients, (Mieli-Vergani et al., 2018a) wish to see a secession immunosuppressant medication, however this can only be implemented once remission has been achieved. Currently response to treatment and remission in adults is indicated by a return to and maintenance of normal IgG and liver enzymes levels (Mack et al., 2020). In paediatric patients there must also be a sustained negative or extremely low antibody titre for three years. At this point a liver biopsy can be performed and if histological findings indicate resolution of hepatic inflammation, the immunosuppressive treatment can be withdrawn (Mieli-Vergani et al., 2018b). Couto et al., (2013) found that a persistent antibody titre of  $>1/80$  indicated inflammatory activity in the liver, despite there being signs of biochemical remission, which could indicate worse prognosis or necessitate more frequent follow up appointments, this ELISA assay could have the potential to be used for monitoring patients in this way. However, this contrasts to the findings of Czaja, 1999) who found no correlation to antibody titre and prognosis.

The initial data analysis that incorporated all samples including those patients already on treatment for AIH showed the three assays to have a reduced sensitivity (53.9 - 65.4%) but similar or increased specificity (at least 80%) compared to the current method (obtaining a T-SMA pattern on LKS) when only the samples that were unequivocally positive by IIF were included. In light of this, and in the context of using the assay to decide whether it is appropriate to biopsy a patient with a view to withdrawing immunosuppression, it is clear further study would need to be carried out to determine whether the assays are appropriate for this use. The sensitivity is calculated on the diagnosis of AIH and in the absence of data regarding histological findings and follow up it is not possible to draw conclusions at this time.

### 5.3 Reliance of skilled operators

One of the reasons for assessing the utility of a second line test was that the current method relies on the availability of highly skilled operators to interpret IIF. In last two decades pathology services within the UK have undergone transformation. Although a greater number of support worker grades of staff have been incorporated into the pathology laboratory, cost saving and financial strains have led to a decrease in the number of higher level posts available, inevitably leading to the loss of specialised skills and knowledge (Institute of Biomedical Scientists, 2023) and the workforce numbers have failed to keep up with the workload demand (Hayden Kath, 2023) . A new model described as “Hub and Spoke” has seen the centralisation of specialised services such as Immunology into one regional centre (Beastall, 2008). In West Yorkshire, the vast majority of Immunology workload and indeed all the IIF and other specialised testing moved to Leeds in 2016, with the idea that economies of scale would lead to financial savings and focus specialised staff in one location. Technological advancements such as faster automated slide processors and interfaced result reporting has gone some way towards alleviating the pressure of the additional work, however the interpretation of the IIF is still performed by individuals which is a time consuming process and ~~is and will remain,~~ remains a rate limiting step (Meroni et al., 2014). -Adding an extra IIF assay would increase the testing load on the slide processors, necessitate more staff time for reading slides, as well as require training and expertise in reading another tissue substrate, leading to an increase in the turnaround time for patient results, for both smooth muscle antibodies testing and other assays within the laboratory test repertoire.-

A relatively new advancement in the immunofluorescence technology sector that may one day relieve this potential bottleneck, is automated slide readers. These systems, of which there are now at least 6 models on the market, use automated image capture, analysis and mathematical pattern-recognition software algorithms to determine antibody positivity (Kim et al., 2019a) . Several studies have so far been carried out comparing manual and automated ANA detection on HEp2 substrate and have shown favourable results, particularly in distinguishing between negative and positive samples, and the identification of common patterns (Meroni et al., 2014). However, misidentification of mixed patterns or less common patterns indicates that this is some way from perfection (Bossuyt et al., 2013; Kivity et al.,

2012;). The goal is for automated slide reading to speed up IIF reading interpretation, perhaps by screening out negatives, so that only the positive results need to be validated by a trained operator and to improve intra and inter laboratory standardization with regards autoantibody detection (Kim et al., 2019b). Although as the studies so far have shown, the automated system performance is not currently adequate enough to replace manual interpretation, especially when it comes to rarer patterns, and not all tissue substrates are currently available on these systems. HEp2 uses a human derived cell line, whereas the SMA are detected on triple rodent tissue substrate which will make standardisation more challenging due to physiological differences between animals and the requirement to capture images relating to all three tissues, which suggest there is some way to go before automated slide reading becomes the norm. Routine image capture should begin as soon as reasonably practicable as it will allow for long term digital storage of an image of the patient results, as opposed to just the digital storage of its interpretation. This will allow full traceability and the ability to reassess at a later date if required.

Over the last five years Digital Pathology for Histology has been implemented across several sites in UK. This is part of a strategy by the Royal College of Pathologists (Royal College of Pathologists, 2019) to improve workflow in the laboratory, allow sharing of work across sites and potentially mitigate shortages of pathologist in some geographical regions. Digital images allow second opinions to be easily sought, without the need for the second pathologist to be in the same country. This suggest there is a possibility that a similar approach could be used in the autoimmunity scientific community.

In this study all ELISA was performed manually but in a routine medical laboratory setting ELISA are often completed using an automated ELIS A processor which allows testing to be performed by an unregistered support worker, with a registered member of staff only required to check the assay performance was acceptable before results being electronically released. Once the ELISA results are generated they can be saved as a hard copy and kept indefinitely, this is in contrast to current IIF processes in which the generated result i.e. the slide will rapidly deteriorate. This removes time pressures on operators and ensures that work produced can be revisited at a later date. Numerical results determined by a machine rather than a human do not require interpretation, e.g. if the result is 52 then it will remain 52 regardless of which operator views it, thus removing any ambiguity and subjectivity from the analysis. In the absence of routine image capture, it is almost impossible to reanalyse IIF at a later date, the sample will have been discarded once testing completed and the original slide no longer available for viewing. Metrological traceability (the traceability of all reagents and calibrations) is a requirement of ISO standard 15189 (Thelen et al., 2019), a standard to which all UKAS accredited laboratories should work. This requirement is so that patients and clinicians can be assured that the results being provided are of the highest standard, or in a situation where an original result needs to be revisited to confirm initial result all variables can be assessed fully (Schneider et al., 2017). In this circumstance, having the original data (or image) to refer to is essential although currently as with IIF not always possible.

#### 5.4 Utility as stand alone assays.

In the cohort of treatment naïve AIH+ and non AIH patients, the QUANTALite (cutoff of 20AU) and the VSM47 actually had greater sensitivity than the current methodology using the T-SMA pattern (both 92.86% compared to 86.6%7) which was similar to the findings of Galaski et al., 2021. In this study reporting all SMA (T-SMA and V-SMA) had a sensitivity of 100% but as expected the specificity was poor at only 22.4%, as it includes the V-SMA pattern which is nonspecific and can be observed in a range of clinical conditions including alcoholic liver, disease, fatty liver, and infection (Terziroli Beretta-Piccoli et al., 2018a). In contrast T-SMA specificity was 81.3%, which is why it is essential that the two patterns are distinguished. In the cases of the three new assays under review, the specificity was similar to that of T-SMA; QUANTALite (>20AU) 80.3%, VSM47 81.3%, NOVALite 85.98% but the specificity rose considerably (to 92.9%) when the cutoff was increased from 20AU to 30AU. The calculated optimal cutoff of 28.18 AU was 90.74%. Other studies (Chretien-Leprince et al., 2005; Galaski et al., 2021; Granito et al., 2006; Villalta et al., 2009) examining the effectiveness of QUANTALite F-actin ELISA had similar results for specificity however sensitivity showed more variation (63% to 74% at cutoff of 30AU). This could be due to the sample population, in this study the sensitivity increased from 60% to 92% when the post treatment AIH patients were removed from the sample, in the study by Galaski et al., 2021 studied it is stated that “large majority were treatment naïve”. Another potential reason for this difference could be that changes were made to the kit; the three first studies are from 2006 and 2007, whereas the later study is from 2021, it is plausible that changes were made to the formulation to improve sensitivity in this time period.

Despite statistical data suggesting all three of the new methods could be used as an alternative, it is unlikely that a decision would be made to move away from the Gold standard IIF using rodent tissue anytime soon. Initial presentation of autoimmune liver disease is varied, it can be non-specific such as joint pain, amenorrhea, weight loss, it can be more indicative of liver involvement with jaundice, oesophageal varices or in some cases further testing will have been indicated by an incidental finding at a routine checkup (Muratori et al., 2016). The LKS substrate allows simultaneous detection of multiple liver autoantibodies; Smooth muscle, Mitochondrial, LKM and LC-1 as well as the GPC antibodies associated with Pernicious Anaemia. It is unlikely that a single test used to screen for these antibodies linked to several conditions would be replaced with five separate investigations, more sample volume would be required, more time, there would be five assays to quality assure and it would likely cost more. However, in the future new assays may be developed that will be able to circumnavigate this issue. Multiplex technology is a well established method of analysing multiple antibodies in a single test. Panels currently exist for a range of autoimmune conditions including Coeliac disease, Anti-Phospholipid syndrome, Vasculitis and Connective tissue diseases (Tozzoli et al., 2013). Prior to the advent of multiplexing, the analysis of the Extractable Nuclear Antigens (ENAs) and double stranded DNA antibody (dsDNA) found in in connective tissue disease would have required an initial ANA screen, followed by an individual ELISA/EIA for each antibody of interest performed over several days (Eissfeller et al., 2005).

There are two groups of multiplex autoantibody testing commercially available; planar (such as microarray and immunoblot/line immunoassay) and non-planar (micro-particle flow based assays) (Tozzoli, 2007). Immunoblots and their counterparts utilise membranes coated with thin lines of purified specific antigens (Fritzler, 2006) with several different antigen types fixed to one strip. This one strip can then be incubated with patient serum before addition of a conjugate and finally substrate is added to allow visualisation of any antibody. The strips can then be scanned and a semi quantitative result generated. Despite being automated the processing time is similar to IIF but with a smaller capacity (an example that the Euroimmun Blot One can run forty three samples plus one quality control in a single three hour long run),(Euroimmun, 2022a, 2022b) making it unsuitable for high throughput for routine screening.

Microparticle based multiplex technology includes random access platforms such as the BioRad Bioplex 2200 or Werfen Aptiva. Microparticles are coated with a specific antigen of interest and individually labelled with fluorochrome to allow identification of each microparticle type (Hanly et al., 2010). A specific cocktail of antigen coated microparticles are included in each panel. The disease appropriate assay and therefore panel is selected and the patient serum is mixed with the microparticles before a wash step. Any remaining patient serum bound to the microparticle is labelled with a fluorescent conjugate. The particles are then channelled through a flow cytometer which uses a red laser to identify the bead type and a green laser to measure the reactivity of each bead (via the amount of patient serum remaining bound). Particle based assays are able to perform fast processing and results generation of a high volume of patient samples (Tozzoli & Villalta, 2014). The original workflow model at LGI of an initial ANA screen followed by an individual ELISA or EIA for each ENA would have taken at least two days but can now be completed in 45 minutes.

Based on this cohort of just 133 patient samples, it would not be appropriate to replace the current method of reporting the V-SMA and T-SMA antibody patterns, as one patient was observed who did have vascular smooth muscle antibodies and no actin antibodies at presentation. Maintaining current reporting procedure would ensure that SMA against antigens other than F-actin are still recorded albeit via the non-specific V-SMA result and would capture 96% off all AIH-1 patients.

## 5.5 Strengths and Limitations

This study suggests that the addition of a second line assay would be beneficial in the diagnosis of autoimmune hepatitis. All assays proved to be highly specific for F-actin antibodies which would make them a useful tool as a confirmatory test, particularly if the presence of another antibody on the LKS substrate made establishing tubular staining existence challenging. The specificity when adding a second line assay to positive T-SMA rose from 81% for the single LKS, to between 96 and 99% (depending on which assay was selected). The existence of T-SMA and an F-actin level of >30AU on ELISA had the greatest specificity at 99%. Frequently patients are tested for liver autoantibodies in primary care and upon receipt

of a positive result (whether V-SMA or T-SMA) the General Practitioner will seek the advice of Hepatology regarding the significance on a case by case basis depending on other patient findings. Of the 32 T-SMA samples, 16 of these tested negative or weak positive for F-actin antibodies and did not go on to have AIH. The one sample that was weak positive but did have AIH, had a F-actin level of 28.3AU which was at the upper end of the weak positive range. Having the second line assay could assist Hepatology with triaging patients and identifying those who need a faster follow up. In this study the addition of a second line assay only identified one AIH patient as having an F-actin antibodies despite only having the V-SMA pattern on LKS. This patient also had a normal IgG level which taken together with the V-SMA would not strongly suggest AIH, this second line assay positivity could have an impact on treatment and care for patients in this scenario. Although only observed in one patient in the study, it is fair to say that the proportions of V-SMA and T-SMA samples studied are not representative of their frequencies in everyday practice. At Leeds General Infirmary between 1<sup>st</sup> January 2023 and 31<sup>st</sup> December 2023, 10% of samples tested on LKS substrate were smooth muscle positive (2491 samples out of a total of 24562). Of these V-SMA accounted for 92.5% of SMA positive samples (2303 samples), and T-SMA for the remaining 7.5% (188 samples); in this study only 67% of samples were V-SMA. Further work would therefore be required to ascertain if adding a second line test routinely to all SMA, could help to identify V-SMA with underlying AIH.

Selection of only T-SMA positive samples for confirmation using ELISA as a second line assay, would be impractical. On average only three T-SMA are detected each week, the ELISA is designed to run 43 samples at a time and although the kits can be run using only a small proportion of the plate, the reagents that require making up such as wash buffer are only stable for 7 days. A solution could be to batch up positive T-SMA samples in order to run bigger volumes in one go but this would lead to an increase in turnaround time and potentially impede the clinical utility of the assay, which make it an unsuitable alternative. To meet clinical turnaround times the assay would be hugely wasteful of reagents and resources, and therefore expensive; and so despite the assay having a clinical benefit, from a practical standpoint, it is unlikely that the QUANTALite ELISA would fit in to the workflow of the laboratory.

Both the VSM47 and the NOVALite IIF methods would be more suitable than an ELISA in the context of very small sample numbers (e.g. only T-SMA having second line testing) but only subject to the availability of suitable quality control material. The VSM47 kit only provides sufficient positive and negative QC volume for two assay runs but the kit can perform up to 98 patient samples, so amendments would be required to make it fit for purpose in this setting. These methods also require the specialised interpretative skills that are in short supply across many medical laboratories.

The data collected and analysed in this study suggests that the second line assay only has clinical benefit if used to confirm that tubular staining observed on the LKS substrate is the result of actin antibody. It did not show that adding this assay to V-SMA positive samples would provide any further information (except for in one patient) to assist with diagnosis. The results of the IIF method were not always conclusive and require specialised skill. The

QUANTALite ELISA showed potential benefit with its ability to quantify actin antibody but would prove to be impractical in meeting the service needs. Therefore the second line assay needs to be free from subjectivity, able to be performed on smaller sample numbers and ideally provided quantitation. The F-actin antigen has recently been added to an extended autoimmune liver disease immunoblot panel from Euroimmun and had this been available earlier it would have been part of this study. This semi quantitative method allows simultaneous detection of several autoantibodies, it can be performed using a fully automated instrument which can carry out the assay and interpret the results using scanning software to measure the intensity of reaction bands (Euroimmun, 2022). This removes inter individual subjectivity and a requirement for specialised operators. As such this new assay is certainly one that holds interest.

## Chapter 6 Conclusion

The performance of all three assays but particularly the ELISA were positive. Unfortunately due to the limitations of the assays in this study, be they of a practical nature or a need for additional skills, it is unlikely that these assays will be incorporated into the laboratory in their current form. However, this study indicates that solid phase actin antibody assays do show promise for the future, proving to have similar or greater sensitivity and specificity than the current method of detecting T-SMA on LKS. If the success of these assays can be converted into high throughput random access technology, there is a strong possibility that immunofluorescence using LKS substrate could follow in the footsteps of ANA IIF screening, becoming if not a thing of the past, certainly a much less frequently performed assay.



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## NOVA Lite<sup>®</sup> ANA KSL Kit

For *In Vitro* Diagnostic Use  
CLIA Complexity: High



REF 708380, 708390  
508385.10, 508380.30,  
508380, 508385

Rx Only

### Intended Use

NOVA Lite ANA KSL is an indirect immunofluorescent assay for the screening and semi-quantitative determination of anti-nuclear antibodies (ANA), anti-mitochondrial (AMA), anti-smooth muscle (ASMA) and gastric parietal antibodies (GPA) in human serum. The presence of anti-nuclear antibodies can be used in conjunction with other serological tests and clinical findings to aid in the diagnosis of systemic lupus erythematosus (SLE) or other connective tissue or rheumatic diseases.

### Summary and Explanation of the test

The term "anti-nuclear antibodies" describes a variety of autoantibodies that react with constituents of cell nuclei including DNA, RNA and several proteins and ribonucleoproteins.<sup>1</sup> These autoantibodies occur with high frequency in patients with connective tissue or rheumatic diseases, especially systemic lupus erythematosus. Virtually all SLE patients are ANA positive. This diagnostic sensitivity has led to the incorporation of ANA testing into the 1982 Revised Criteria for the Classification of Systemic Lupus Erythematosus by an American College of Rheumatology subcommittee.<sup>2</sup> While ANA testing is an excellent screening test for SLE (a negative result virtually rules out active SLE<sup>3</sup>) it is by no means a specific test. Patients with other connective tissue diseases such as rheumatoid arthritis, scleroderma and dermatomyositis are frequently positive, and low ANA titers may be observed in other disease states and in the normal population.<sup>4</sup> Positive ANA results can occur following severe burns or viral infection and have been reported in some normal, healthy people, especially in older populations. Because of this lack of specificity, it is recommended that all ANA positive samples be titrated to endpoint and that more specific testing for autoantibodies to double stranded DNA (dsDNA) and extractable nuclear antigen (ENA) autoantibodies be performed.<sup>1-3</sup>

High levels of AMA are often detected in association with primary biliary cirrhosis. Low titers of AMA may be detected in other liver disorders such as chronic active hepatitis and cryptogenic cirrhosis.<sup>5,6</sup> ASMA is found in high titers in the serum of 70% of patients with chronic active hepatitis. In addition, 50% of these patients are positive for ANA, while 25% demonstrate low AMA titers. Low ASMA titers may be present in viral infections, malignancies, and normal individuals.<sup>5,7</sup>

GPA occurs in the serum of 90% of patients with pernicious anemia. With other clinical and laboratory data, a positive GPA result helps to distinguish autoimmune pernicious anemia from other megaloblastic anemias. Although detected in less than 2% of the normal population under 20 years of age, the incidence of GPA increases in women over the age of 40 and may be present in up to 16% of the normal population over 60 years of age.<sup>8,9</sup>

Indirect immunofluorescence is the reference method for ANA, AMA, ASMA and GPA testing. Common substrates are either thin sections of rodent organs or some type of cell line. The substrate chosen for NOVA Lite ANA KSL is optimally-fixed mouse kidney, stomach and liver sections. The conjugate is affinity purified anti-human IgG.

### Principles of the Procedure

In the indirect immunofluorescence technique, samples are incubated with antigen substrate and unreacted antibodies are washed off. The substrate is incubated with specific fluorescein labeled conjugate and then unbound reagent is washed off. When viewed through a fluorescence microscope, autoantibody positive samples will exhibit an apple green fluorescence corresponding to areas of the cell or nuclei where autoantibody has bound.

### Reagents

1. ANA KSL Slides (Mouse kidney/stomach/liver) substrate on 4 or 8 well slides, with dessicant

#### Kits only:

1. Anti-Human IgG Conjugate (Goat), fluorescein labeled in buffer containing Evans Blue and 0.09% sodium azide
2. ANA Homogeneous Pattern, 1 vial of buffer containing 0.09% sodium azide and human serum antibodies to the cell nucleus, prediluted
3. IFA System Negative Control, 1 vial of buffer containing 0.09% sodium azide and no human serum antibodies to ANA KSL, prediluted
4. PBS Concentrate (40x)
5. Mounting Medium, 0.09% sodium azide
6. Coverslips

## Warnings

1. All human source material used in the preparation of controls for this product has been tested and found negative for antibody to HIV, HBsAg, and HCV by FDA cleared methods. No test method, however, can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the ANA Homogeneous Pattern and IFA Systems Negative Control should be handled in the same manner as potentially infectious material.<sup>10</sup>
2. Sodium Azide is used as a preservative. Sodium Azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. Flush sinks, if used for reagent disposal, with large volumes of water to prevent azide build-up.
3. Use appropriate personal protective equipment while working with the reagents provided.
4. Spilled reagents should be cleaned up immediately. Observe all federal, state and local environmental regulations when disposing of wastes.

## Precautions

1. This product is for *In Vitro* Diagnostic Use.
2. Substitution of components other than those provided in this system may lead to inconsistent results.
3. Incomplete or inefficient washing of IFA wells may cause high background.
4. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
5. A variety of factors influence the assay performance. These include the starting temperature of the reagents, the strength of the microscope bulb used, the accuracy and reproducibility of the pipetting technique, the thoroughness of washing and the length of the incubation times during the assay. Careful attention to consistency is required to obtain accurate and reproducible results.
6. Over time, the Anti-Human IgG Conjugate may change in color due to exposure to light. However, the color change does not affect the assay performance.
7. Strict adherence to the protocol is recommended.

## Storage Conditions

1. Store all the kit reagents at 2 - 8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
2. Diluted PBS buffer is stable for 4 weeks at 2 - 8°C.

## Specimen Collection

This procedure should be performed with a serum specimen. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, or specimens containing visible particulates should not be used. Grossly hemolyzed or lipemic serum specimens should be avoided.

Following collection, the serum should be separated from the clot. CSLI (formerly NCCLS) Document H18-A2 recommends the following storage conditions for samples: 1) Store samples at room temperature no longer than 8 hours. 2) If the assay will not be completed within 8 hours, refrigerate the sample at 2 - 8°C. 3) If the assay will not be completed within 48 hrs, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing.

## Procedure

### Materials provided (kits)

#### 708380

- |    |   |
|----|---|
| 25 | ANA KSL Slide (Mouse Kidney/Stomach/Liver) (8 well) |
| 1  | 15mL FITC IgG Conjugate                             |
| 1  | 1.0mL ANA Homogeneous Pattern                       |
| 1  | 1.0mL IFA System Negative Control                   |
| 2  | 25mL PBS Concentrate (40x)                          |
| 1  | 10mL Mounting Medium                                |
| 1  | 25 Coverslips                                       |

#### 708390

- |    |   |
|----|---|
| 10 | ANA KSL Slide (Mouse Kidney/Stomach/Liver) (4 well) |
| 1  | 7mL FITC IgG Conjugate                              |
| 1  | 0.5 mL ANA Homogeneous Pattern                      |
| 1  | 0.5mL IFA System Negative Control                   |
| 1  | 25mL PBS Concentrate (40x)                          |
| 1  | 7mL Mounting Medium                                 |
| 1  | 10 Coverslips                                       |

### Materials provided (slides)

508385.10	10 x ANA KSL Slide (Mouse Kidney/Stomach/Liver) (4 well)
508380.30	30 x ANA KSL Slide (Mouse Kidney/Stomach/Liver) (8 well)
508385	1 x ANA KSL Slide (Mouse Kidney/Stomach/Liver) (4 well)
508380	1 x ANA KSL Slide (Mouse Kidney/Stomach/Liver) (8 well)

### Additional Materials Required But Not Provided

Micropipets to deliver 15-1000µL volume  
Distilled or deionized water  
Squeeze bottles or Pasteur pipets  
Moist chamber  
1L container (for diluting PBS)  
Coplin jar  
Fluorescence microscope with 495nm exciter and 515nm barrier filter

### Method

#### Before you start

1. Bring all reagents and samples to room temperature (20 – 26°C) and mix well.
2. **Dilute PBS Concentrate: IMPORTANT:** Dilute the PBS Concentrate 1:40 by adding the contents of the PBS Concentrate bottle to 975mL of distilled or deionized water and mix thoroughly. The PBS buffer is used for diluting patient samples and as a wash buffer. The diluted buffer can be stored for up to 4 weeks at 2 - 8°C.
3. **Dilute Patient Samples:**
  - a. **Initial Screening:** Dilute patient samples 1:20 with diluted PBS buffer (i.e., add 50µL of serum to 950µL of PBS buffer).
  - b. **Titration:** Make serial 2-fold dilutions from the initial screening dilution for all positive samples with diluted PBS buffer (i.e. 1:40, 1:80,... 1:5120).

#### Assay procedure

1. **Prepare Substrate Slides:** Allow the substrate slide to reach room temperature prior to removal from its pouch. Label it with pencil and place it in a suitable moist chamber. Add 1 drop (70 – 90µL) of the undiluted positive and the negative control to wells 1 and 2 respectively. Add 1 drop (70 – 90µL) of diluted patient sample to the remaining wells.
2. **Slide Incubation:** Incubate the slide for 30 ± 5 minutes in a moist chamber (a dampened paper towel placed flat on the bottom of a closed plastic or glass container) will maintain the proper humidity conditions. **Do not allow the substrate to dry out during the assay procedure.**
3. **Wash Slides:** After incubation, use a plastic squeeze bottle or pipet to gently wash off the serum with diluted PBS buffer. Orient the slide and stream of PBS buffer so as to minimize wash-over of samples between wells. **Avoid directing the stream directly onto the wells to prevent substrate damage.** If desired, place the slides in a Coplin jar of diluted PBS buffer for up to 5 minutes.
4. **Addition of Fluorescent Conjugate:** Shake off the excess PBS buffer. Place the slide back in the moist chamber and immediately cover each well with a drop of fluorescent conjugate. Incubate the slides for an additional 30 ± 5 minutes.
5. **Wash Slides:** Repeat Step 3.
6. **Coverslip:** Coverslip procedures vary from lab to lab; however, the following procedure is recommended:
  - a. Place a coverslip on a paper towel.
  - b. Apply mounting medium in a continuous line to the bottom edge of the coverslip.
  - c. Shake off the excess PBS buffer and touch the lower edge of the slide to the edge of the coverslip. Gently lower the slide onto the coverslip in such a way that the mounting medium flows to the top edge of the slide without air bubble formation or entrapment.

### Quality Control

The ANA Homogeneous Pattern and IFA System Negative Control should be run on every slide to insure that all reagents and procedures perform properly. Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at ≤ -70°C. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test results should be considered invalid and the assay repeated.

1. The ANA Homogeneous Pattern must be ≥ 3+.
2. The IFA System Negative Control must be negative.

### Interpretation of Results

**Negative Reaction.** A sample is considered negative if specific staining is equal to or less than the IFA System Negative Control. Samples can exhibit various degrees of background staining due to heterophile antibodies or low-level autoantibodies to cytoplasmic constituents such as contractile proteins.

**Positive Reaction.** Specific staining of cell constituents. A variety of staining patterns can be observed depending on antibody specificity.

Determine the fluorescence grade or intensity using these criteria:

- 4+ Brilliant apple green fluorescence
- 3+ Bright apple green fluorescence
- 2+ Clearly distinguishable positive fluorescence
- 1+ Lowest specific fluorescence that enables the nuclear or cytoplasmic staining to be clearly differentiated from the background fluorescence

**Pattern Interpretation.** A variety of patterns of nuclear and cytoplasmic staining can be exhibited depending on the types and relative amounts of autoantibodies present in the sample. The following types of staining patterns can be observed:

**Homogeneous:** A solid staining of the nucleus with or without apparent masking of the nucleoli.

**Nuclear antigens present:** dsDNA, ssDNA, histones

**Disease association:** High titers are suggestive of SLE; lower titers are suggestive of SLE or other connective tissue diseases.

**Peripheral:** A solid staining, primarily around the outer region of the nucleus, with weaker staining toward the center of the nucleus.

**Nuclear antigens present:** dsDNA, ssDNA, DNP, histone.

**Disease association:** High titers are suggestive of SLE; lower titers are suggestive of SLE or other connective tissue diseases.

**Speckled:** A fine or grainy appearing staining of the nucleus, generally without fluorescent staining of the nucleoli.

**Nuclear antigens present:** Sm, RNP, Scl-70, SS-A, SS-B, and other antigen/ antibody systems not yet characterized.

**Disease association:** High titers suggestive of SLE (Sm antibody), mixed connective tissue disease (RNP antibody), scleroderma (Scl-70 antibody), or Sjogren's syndrome-sicca complex (SS-B antibody); lower titers may be suggestive of other connective tissue diseases.

**Nucleolar:** Large coarse speckled staining within the nucleus, generally less than 6 in number per cell, with or without occasional fine speckles.

**Nuclear antigens present:** 4-6S RNA and other unknown nuclear antigens.

**Disease association:** High titers are prevalent in scleroderma and Sjogren's syndrome.

**Mitochondrial (AMA):** A discrete granular staining of the distal and/or proximal tubules of the kidney. The metabolically active parietal cells of the stomach contain high concentrations of mitochondria and can also stain positive.

**Antigen present:** Various types of mitochondrial antigens.

**Disease association:** High titers indicate primary biliary cirrhosis.

**Smooth muscle (ASMA):** A discrete staining of the inner muscular layers of blood vessels of the kidney and/or the muscularis layers of the stomach is considered specific for ASMA.

**Antigen present:** Actin.

**Disease association:** High titers are indicative of chronic active hepatitis, autoimmune hepatitis.

**Gastric parietal cell (GPA):** Staining of the parietal cell cytoplasm. These cells are located in the gastric mucosa of the stomach. Since AMA also stain parietal cells, the kidney tubules should also be checked before reporting GPA. If the sample is GPA positive, the kidney tubules will be negative.

**Antigen present:** Proton pump (h/K ATPase).

**Disease association:** These antibodies are found in pernicious anemia and atrophic gastritis.

It is important to caution the user about relying on patterns to determine autoantibody specificity, except for the nucleolar and centromere patterns in which each of the antigens is very well defined and their patterns are characteristic. Since many autoantibodies or combinations thereof can induce a homogenous or speckled pattern, it is recommended that specific, follow-up autoantibody testing (such as for dsDNA and ENA) be performed on all speckled or homogeneous samples.

## Limitations of the Procedure

1. High-titered ANA is suggestive of connective tissue disease but should not be considered diagnostic. The ANA result should be considered in combination with other serological results as well as the overall clinical history of the patient.
2. ANA patterns often change as the sample is titrated out to endpoint. This phenomenon is due to lower titer antibodies dropping below the sensitivity of the system as more dilute sample is tested.
3. A variety of external factors influence the test sensitivity including the type of fluorescence microscope used, the bulb strength and age, the magnification used, the filter system and the observer.
4. If a band pass filter is used instead of a 515 barrier filter, increased artifactual staining may be observed.
5. Only pencil should be used to label the slides. Use of any other writing material may cause artifactual staining.
6. All coplin jars used for slide washing should be free from all dye residues. Use of coplin jars containing dye residue may cause artifactual staining.
7. Results of this assay should be used in conjunction with clinical findings and other serological tests.
8. The assay performance characteristics have not been established for matrices other than serum.

Slides sold separately are classified as "Analyte specific reagents".

Except as a component of NOVA Lite ANA KSL Kit, analytical and performance characteristics are not established.

## Expected Values

Using the NOVA Lite ANA KSL test, a variety of connective tissue disease patients, patients with autoimmune liver diseases, patients with pernicious anemia as well as 200 random blood donors were tested. The results appear below:












Patient Group	Number	Number Positive* NOVA Lite ANA KSL			GPA
		ANA	AMA	ASMA	
SLE	105	101	5	9	1
Drug Induced Lupus	24	24	0	0	0
Rheumatoid Arthritis	40	28	1	1	0
Chronic Active Hepatitis	25	10	4	21	0
Primary Biliary Cirrhosis	30	5	27	3	0
Pernicious Anemia	15	0	0	0	14
Normals	200	3	2	6	1

\*Total number positive may exceed number tested due to multiple patterns in a single well.

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## Symbols Used

	In Vitro diagnostic medical device Producto sanitario para diagnóstico in vitro
	European Conformity Conformidad europea
	European Authorized Representative Representante europeo autorizado
	Prescription Only per US FDA Solo con receta, conforme a la FDA de EE. UU.
	Temperature Limitation Límites de temperatura
	Batch Code Código de lote
	Catalogue or part number Número de catálogo o componente
	Manufacturer Fabricante
	Use by Caducidad
	Contains Sufficient for <n> Tests Contiene suficiente para <n> Pruebas
	Consult instructions for use Consulte las instrucciones de uso

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August 2019  
Revision 21





**EUROIMMUN**

Medizinische  
Labordiagnostika  
AG



### IIFT: Liver Mosaics Instructions for use

ORDER NO.	ANTIBODIES AGAINST	SUBSTRATE	SPECIES	FORMAT SLIDES x FIELDS
FA 1300-1 to FA 1300-21 FA 1302-50 FA 1651 and FA 1710-1 (see page 20)	cell nuclei (ANA) liver antigens, cell nuclei (ANA) LKM + cell nuclei (ANA) LKM + mitochondria (AMA) smooth muscles (ASMA) F-actin soluble liver antigen/ liver-pancreas antigen (SLA/LP)	HEp-2-cells liver liver kidney stomach VSM47 cells transfected cells	human monkey rat rat rat rat EU 90	10 x 05 (050) 10 x 10 (100) 20 x 05 (100)

**Indication:** This test kit provides qualitative or semiquantitative in vitro determination of human antibodies of immunoglobulin class IgG in patient samples to support the diagnosis of autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and rheumatic diseases. The fluorescence is either evaluated using the fluorescence microscope (specifications see chapter "Incubation", section "Evaluation") or, following automated image recording by the EUROPattern microscope at the computer screen, optionally supported by the EUROPattern Classifier software. The product is designed for use as **IVD**.

**Test principle:** Test fields are incubated with diluted patient sample. If the reaction is positive, specific antibodies of classes IgA, IgG and IgM attach to the antigens. In a second step, the attached antibodies are stained with FITC-labelled anti-human antibodies and made visible with the fluorescence microscope.

**Contents of a test kit for 50 determinations (e.g. FA 1300-1005-2):**

Description	Format	Symbol
1. Slides, with a mosaic of BIOCHIPS (specifications see page 16)	10 slides	SLIDE
2. FITC-labelled anti-human IgG (goat), ready for use	1 x 1.5 ml	CONJUGATE
3. Positive control: autoantibodies against mitochondria (AMA-M2), human, ready for use	1 x 0.25ml	POS CONTROL
4. Positive control: autoantibodies against liver-kidney microsomes (LKM), human, ready for use	1 x 0.1 ml	POS CONTROL
5. Negative control: autoantibodies negative, human, ready for use	1 x 0.1 ml	NEG CONTROL
6. Salt for PBS pH 7.2	2 packs	PBS
7. Tween 20	2 x 2.0 ml	TWEEN 20
8. Mounting medium, ready for use	1 x 3.0 ml	GLYCEROL
9. Cover glasses (62 mm x 23 mm)	12 pieces	COVERGLASS
10. Instructions for use	1 booklet	---
Lot description		Storage temperature
In vitro diagnostic medical device		Unopened usable until

Single slides (e.g. EUROIMMUN order no. FB 1300-1005-2) are provided together with cover glasses. Additional positive control (e.g. EUROIMMUN order no. CA 1622-0502 and EUROIMMUN order no. CA 1320-0502) and negative control (e.g. EUROIMMUN order no. CA 1000-0502) can be ordered. Performance of the test requires reagent trays **TRAY**, which are not provided in the test kits. They are available from EUROIMMUN under the following order no.:

- ZZ 9999-0105-R Reagent trays for slides containing up to 5 fields,
- ZZ 9999-0110-R Reagent trays for slides containing up to 10 fields.

Updates with respect to the previous version are marked in grey.

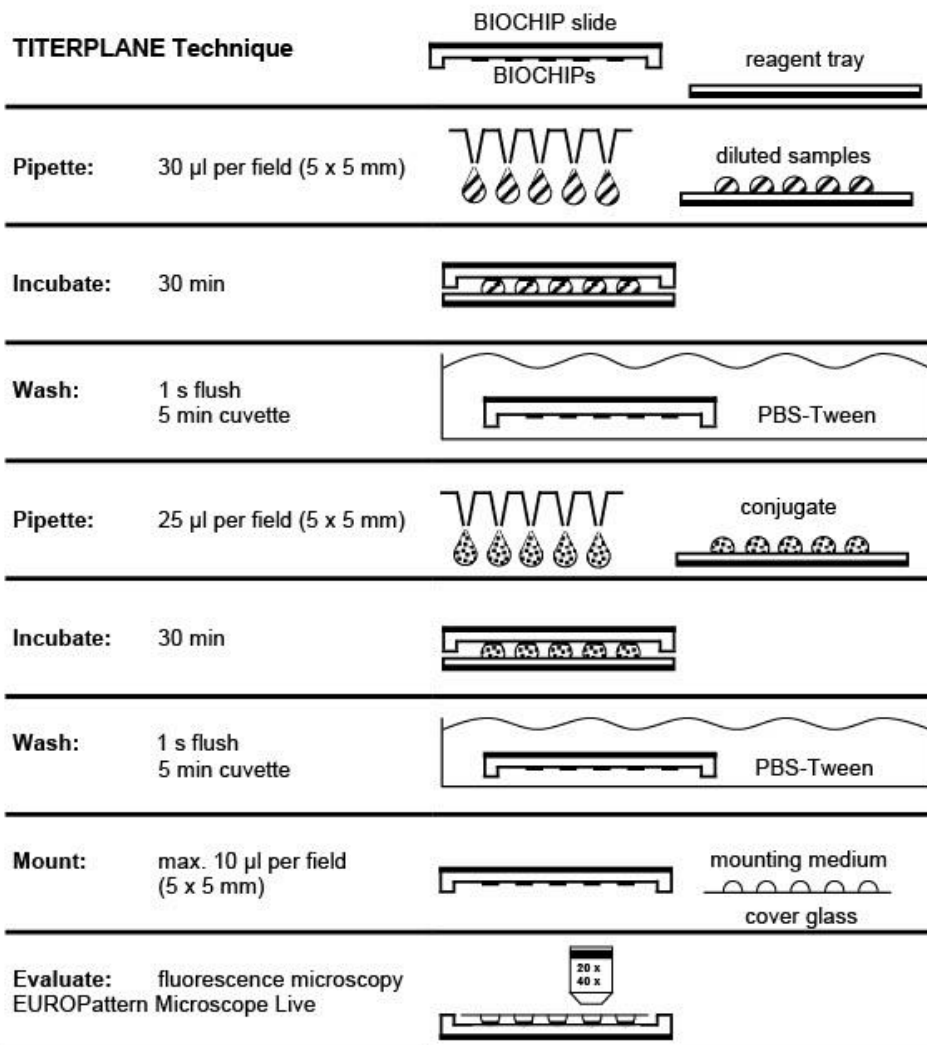
FA\_1300-1\_A\_UK\_C26.doc  
Version: 22/03/2024



### Performing the test (reaction fields 5 x 5 mm)

The TITERPLANE Technique was developed by EUROIMMUN in order to standardise immunological analyses: Samples or labelled antibodies are applied to the reaction fields of a reagent tray. The BIOCHIP slides are then placed into the recesses of the reagent tray, where all BIOCHIPs of the slide come into contact with the liquids, and the individual reactions commence simultaneously. Position and height of the droplets are exactly defined by the geometry of the system. As the fluids are confined to a closed space, there is no need to use a conventional "humidity chamber". It is possible to incubate any number of samples next to each other and simultaneously under identical conditions.

- Prepare:** The preparation of the reagents and of the serum and plasma samples is described on page 4 of these instructions for use.
- Pipette:** Apply a defined volume (see scheme next page) of **diluted sample** to each reaction field of the reagent tray, avoiding air bubbles. Transfer all samples to be tested before starting the incubation (up to 200 droplets). Use a polystyrene pipetting template.
- Incubate:** Start reactions by fitting the BIOCHIP slides into the corresponding recesses of the reagent tray. Ensure that each sample makes contact with its BIOCHIP and that the individual samples do not come into contact with each other.  
Incubate for **30 minutes** at room temperature (+18 °C to +25 °C).
- Wash:** Rinse the BIOCHIP slides with a flush of PBS-Tween using a beaker and immerse them immediately afterwards in a cuvette containing PBS-Tween for at least **5 minutes**. Shake with a rotary shaker if available. Wash max. 16 slides, then replace PBS-Tween with new buffer.
- Pipette:** Apply a defined volume (see scheme next page) of **conjugate** to each reaction field of a clean reagent tray. Add all droplets before continuing incubation. Use a stepper pipette. The conjugate should be mixed thoroughly before use. To save time, conjugate can be pipetted onto separate reagent trays during the incubation with the diluted sample.
- Incubate:** Remove one BIOCHIP slide from the cuvette. Within five seconds blot only the back and the long sides with a paper towel and immediately put the BIOCHIP slide into the recesses of the reagent tray. Do not dry the areas between the reaction fields. Check for correct contact between the BIOCHIPs and liquids. Then continue with the next BIOCHIP slide. From now on, protect the slides from direct sunlight.  
Incubate for **30 minutes** at room temperature (+18 °C to +25 °C).
- Wash:** Fill cuvette with new PBS-Tween. Rinse the BIOCHIP slides with a flush of PBS-Tween using a beaker and put them into the cuvette filled with the new PBS-Tween for at least **5 minutes**. Shake with a rotary shaker if available. Wash max. 16 slides, then replace PBS-Tween with new buffer.
- Mount:** Place mounting medium onto a cover glass – volume per reaction field see scheme next page. Use a polystyrene mounting tray. Remove one BIOCHIP slide from PBS-Tween and dry the back and all four sides with a paper towel. Put the BIOCHIP slide, with the BIOCHIPs facing downwards, onto the prepared cover glass. Check immediately that the cover glass is properly fitted into the recesses of the slide. Correct the position if necessary.
- Evaluate:** Evaluate the fluorescence on the microscope or on the automatically recorded images (optionally following the result suggestion by the software).  
The evaluation can be performed using a manual fluorescence microscope or the EUROPattern Microscope Live. Every result issued by the software must be verified by trained laboratory personnel.
- Manual microscopy:  
General recommendation: objective 20x (tissue sections, infected and transfected cells), 40x (cell substrates).  
Excitation filter: 450 – 490 nm, colour separator: 510 nm, blocking filter: 515 nm  
Light source: Mercury vapour lamp, 100 W, EUROIMMUN LED, EUROStar Bluelight  
EUROPattern Microscope Live:  
The image recording settings are defined by the microscope and the software.



**Automated incubation:** The test kit can be incubated by using automated devices, e.g. IF Sprinter, Sprinter XL, EUROLabLiquidHandler or others. The incubation and washing conditions programmed should be the same as described in the manual procedure. The test settings for EUROIMMUN devices are validated in combination with the kit. Any other combination has to be validated by the user. For details please refer to the device manual.





### Preparation and stability of reagents

**Note:** After initial opening, the reagents are stable until the expiry date when stored between +2 °C and +8 °C and protected from contamination, unless stated otherwise below.

- **Slides:** Ready for use. Remove the protective cover only when the slides have reached room temperature (+18 °C up to +25 °C; condensed water can damage the substrate). Do not touch the BIOCHIPS. If the protective cover is damaged, the slide must not be used for diagnostics. The slide must be disposed of after single use, even if not all incubation fields have been used.
- **FITC-labelled secondary antibody:** Ready for use. Before using for the first time, mix thoroughly. The conjugate is sensitive to light. Protect from sunlight.
- **Positive and negative controls:** Ready for use. Before using for the first time, mix thoroughly.
- **PBS-Tween:** 1 pack of "Salt for PBS" should be dissolved in 1 liter of distilled water (optimal: aqua pro infusione, aqua ad injectabilia) and mixed with 2 ml of Tween 20 (stir for 20 minutes until homogeneous). The prepared PBS-Tween can be stored at +2 °C to +8 °C, generally for 1 week. PBS-Tween should not be used if the solution becomes cloudy or contamination appears.
- **Mounting medium:** Ready for use.
- **Reagent trays:** The reaction fields of the reagent tray must be hydrophilic and the surrounding area hydrophobic. If necessary, leave in 2% Deconex 11 universal (EUROIMMUN order number: ZZ 9912-0101) for 12 hours. Afterwards rinse generously with water and dry. Cleaning: Rub reagent trays with 5% Extran MA 01 (EUROIMMUN order number: ZZ 9911-0130) and rinse with plenty of water. To disinfect: Spray reagent trays generously with Mikrozid AF (EUROIMMUN order number: ZZ 9921-0125), turn over and leave for 5 minutes. Afterwards, rinse generously with water and dry.

**Storage and stability:** The slides and the reagents should be stored at a temperature between +2 °C and +8 °C. Unopened, all test kit components are stable until the indicated expiry date.

**Waste disposal:** Patient samples, controls and slides are to be handled as potentially infectious materials. All reagents are to be disposed of in accordance with official disposal regulations.

**Warning:** The BIOCHIPS coated with antigen substrates have been treated with a disinfecting fixing agent. Neither HBsAg nor antibodies against HIV-1, HIV-2, and HCV could be detected in the control sera using appropriate ELISA or indirect immunofluorescence tests. **Nevertheless, all test system components should be handled as potentially infectious materials.** Some of the reagents also contain sodium azide in a non-declarable concentration. Avoid skin contact.

### Preparation and stability of samples

**Samples:** Human sera or EDTA, heparin or citrate plasma.

**Stability:** The patient samples to be investigated can generally be stored up to 14 days at a temperature between +2 °C and +8 °C. Diluted samples must be incubated within one working day.

**Recommended sample dilution for qualitative evaluation:** The sample to be investigated is diluted 1:100 in PBS-Tween. For example, dilute 10.1 µl sample in 1000 µl PBS-Tween and mix thoroughly, e.g. vortex for 4 seconds.



**Recommended sample dilution for semiquantitative evaluation:** The dilution of samples to be investigated is performed using PBS-Tween. Add 100 µl of PBS-Tween to each tube and mix with 11.1 µl of the next highest concentration, e.g. vortex for 2 seconds. EUROIMMUN recommends incubating samples from a dilution of 1:100.

Dilution	Dilution scheme	
1:10	100 µl PBS-Tween + 11.1 µl undiluted sample	
1:100	100 µl PBS-Tween + 11.1 µl 1:10 diluted sample	
1:1000	100 µl PBS-Tween + 11.1 µl 1:100 diluted sample	
⋮	⋮	

## Evaluation

**Magnification VSM47:** Eyepiece 10x, objective 20x (200x). This magnification deviates from our general recommendation given in the incubation protocol on page 2.

**Fluorescence pattern (positive reaction):** The fluorescence is either evaluated through the ocular or, following automated image recording by the EUROPattern microscope at the computer screen, optionally supported by the EUROPattern Classifier software (see table "EUROPattern"). Every result issued by the software must be verified by trained laboratory personnel.

**Anti-nuclear antibodies (ANA)** can be detected on numerous substrates. For targeted detection and differentiation of anti-nuclear antibodies, human epithelial cells (HEp-2 cells) and primate liver are used as a combined substrate. The cell nuclei show a strong fluorescence which is characterised by specific patterns. In the case of negative samples, the nuclei do not show any specific staining. In every field to be evaluated, interphase nuclei and different stages of division (mitotic stages) of the HEp-2 cells, as well as liver cells are to be investigated, if possible, in several areas.

The primate liver reacts with many different antibodies. Important are antibodies against **nuclear antigens (ANA)**, **ribosomal P-proteins**, **liver-specific protein (LSP)**, **liver cell membrane (LMA)**, **actin**, **bile ducts** and **endothelial cells**.

Antibodies against **liver-kidney microsomes (LKM)** react very well with rat liver and generate a smooth staining in the cytoplasm of the hepatocytes. In rat kidney, particularly in the cortex area, a finely granular fluorescence of the proximal tubules – recognisable by the luminal brush border – is visible. The distal tubules are negative. The fluorescence intensity of the liver cells is normally stronger than that of the proximal renal tubules.

Antibodies against **mitochondria (AMA)** can be determined using various histological substrates and HEp-2 cells. For the targeted identification of these antibodies, frozen sections of rat kidney are used as standard substrate. The cytoplasm of the proximal and distal tubule cells shows a granular, basally emphasised fluorescence. The glomeruli are only weakly stained by AMA. Any fluorescence of the luminal tubule sections (brush border) of the rat kidney is not taken into account.

With frozen sections of rat stomach antibodies against **smooth muscles (ASMA)** show a distinct cytoplasmic fluorescence of the tunica muscularis as well as the lamina muscularis mucosa and



the interglandular contractile fibrils of the tunica mucosa. In the case of negative samples, the contractile elements show no staining. Any fluorescence in other structures is not evaluated.

If specific antibodies against F-actin are present in the serum, the cytoskeleton shows a filamentous fluorescence. Essentially the same pattern must be obtained as for the positive control serum.

Antibodies against soluble liver antigen/liver-pancreas antigen (SLA/LP) do not react with tissue sections of monkey or rat liver. SLA/LP-transfected cells show a fine granular fluorescence of the entire cytoplasm that also overlays the nucleus. The nucleoli can be positive.

If the positive control shows no specific fluorescence pattern or the negative control shows a clear specific fluorescence, the results are not to be used and the test is to be repeated.

A large range of fluorescence images can be found on the EUROIMMUN website ([www.euroimmun.com](http://www.euroimmun.com)).

#### Recommended qualitative evaluation:

ANA reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against cell nuclei detectable in the patient sample.
Positive reaction at 1:100	Trace. For IF types: pattern homogeneous, centromeres, nuclear dots, Jo-1, typical patterns of SS-A/SS-B, Sm/RNP possible indication of various rheumatic and other diseases.
Positive reaction at 1:320	Positive. Indication of various rheumatic and other diseases.

Anti-LKM reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against cell nuclei and LKM detectable in the patient sample.
Positive reaction at 1:100	Positive. Indication of various rheumatic diseases and autoimmune hepatitis.

Anti-LSP reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against liver specific antigens detected in the patient sample.
Positive reaction at 1:100	Positive. Antibodies against liver specific antigens detected in the patient sample.

AMA reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against mitochondria detected in the patient sample.
Positive reaction at 1:100	Positive. Indication of various diseases, e.g. primary biliary liver cholangitis (PBC), autoimmune chronic-active hepatitis, several forms of systemic lupus erythematosus, lues and others (see clinical significance).





ASMA reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against smooth muscles detected in the patient sample.
Positive reaction at 1:100	Positive. Indication of various diseases, e.g. chronic-active hepatitis, viral hepatitis, infectious mononucleosis and others.

Anti-F-actin reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against F-actin detected in the patient sample.
Positive reaction at 1:100	Positive. With corresponding clinical symptoms indicative of autoimmune hepatitis type I.

Anti-SLA/LP reactivity (IgG)	Evaluation
No reaction at 1:100	Negative. No antibodies against SLA/LP detected in the patient sample.
Positive reaction at 1:100	Positive. With corresponding clinical symptoms indicative of autoimmune hepatitis type I.

**Recommended semiquantitative evaluation:** The titer is defined as the sample dilution factor for which specific fluorescence is just identifiable. This should be compared with the reaction obtained using an equivalently diluted negative serum.

Antibody titers can be determined according to the following table from the fluorescence of the different sample dilutions.

Fluorescence at				Antibody titer
1:10	1:100	1:1000	1:10,000	
weak	negative	negative	negative	1:10
moderate	negative	negative	negative	1:32
strong	weak	negative	negative	1:100
strong	moderate	negative	negative	1:320
strong	strong	weak	negative	1:1000
strong	strong	moderate	negative	1:3200
strong	strong	strong	weak	1:10,000
⋮	⋮	⋮	⋮	⋮

### Limitations of the procedure

1. A diagnosis should not be made based on a single test result. The clinical symptoms of the patient should always be taken into account along with the serological results by the physician.
2. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
3. Mishandling of slides during the staining procedure, especially allowing slides to dry between steps, may result in a "washed out" pattern appearance and/or a high level of background staining.
4. Coplin jars used for slide washing should be free from all residues. Use of Coplin jars containing residues may cause staining artefacts.
5. The light source, filters and optical unit of the fluorescence microscope can influence the sensitivity of the assay. Using traditional mercury vapour lamp systems, the performance of the microscope depends on correct maintenance, especially alignment of the lamp and replacement of the lamp after the recommended period of time. The EUROIMMUN fluorescence microscopes with LED BlueLight as the light source offer many advantages. For further information, please contact EUROIMMUN



### Test characteristics

**Antigen:** For the determination of antinuclear antibodies (ANA) by means of indirect immunofluorescence, human epithelial cells (HEp-2) are preferred nowadays. HEp-2 cells show a wide spectrum of human nuclear antigens, and assessment of the fluorescence pattern makes the pre-differentiation of a large number of antibodies possible. By using **primate liver** as an additional antigen substrate (frozen section), the spectrum of antibodies that can be differentiated is extended even further.

For the detection of autoantibodies against mitochondria (AMA) by indirect immunofluorescence, **rat kidney** is used as a standard substrate (frozen section). These antibodies mainly react with biochemically defined antigens of the mitochondrial membranes.

For the determination of autoantibodies against smooth muscles (ASMA) by indirect immunofluorescence, **rat stomach** is used as a standard substrate (frozen section). Some of the antibodies against smooth muscles are targeted towards actin and are tested using the substrate combination with HEp-2 cells and primate liver.

For the detection of autoantibodies against F-actin by indirect immunofluorescence, **VSM47 cells** (vascular smooth muscle) are used.

For monospecific detection of antibodies against **soluble liver antigen/liver-pancreas antigen (SLA/LP)** by indirect immunofluorescence **transfected cells** are used as the standard substrate.

**Measurement range:** The dilution starting point for this measurement system is 1:100. Samples can be further diluted by a factor of 10 so that the dilution series is 1:1000, 1:10,000 etc. There is no upper limit to the measurement range.

**Reproducibility:** Inter-lot, intra-assay and inter-assay reproducibility are ensured.

#### Cross-reactivity:

Cross-reactivities were examined for the following parameters:

Substrate	Ig class	Sample specificity (antibodies against)	n	Prevalence	
				Positive	%
SLA/LP	IgG	LKM-1	11	0	0
Stomach (rat)	IgG	CDC panel	11	0	0
HEp-2 cells (human)	IgG			0	0
Liver (monkey)	IgG			0	0
Kidney (rat)	IgG			0	0
Liver (rat)	IgG			0	0
Cross-reactions are unlikely.					

**VSM47 cells (rat):** there is no data known to EUROIMMUN in which cross-reactivities are described.

**Interference:** Haemolytic, lipaemic and icteric samples showed no influences on analysis results.





**Reference range:** Titer 1:<100

The following antibody prevalences were determined using a panel of samples from healthy blood donors (origin: Germany):

Substrate	Antibodies against	Conjugate	Prevalence	Cut-off	Number of samples
Stomach (rat):	ASMA	IgG	4.0%	1:100	300
Liver (monkey):	ANA AMA nuclear membrane ribosomes	IgG	4.5% 1.5% 0.5% 0.5%	1:100	200
Liver (rat):	ANA LKM	IgG	2% 0%	1:100	200
Kidney (rat):	AMA	IgG	1.0%	1:100	300
HEp-2 cells (human):	ANA	IgG	12.5%	1:100	200
VSM47 (rat):	F-actin	IgG	0.5%	1:100	207
Anti-SLA/LP Transfected cells:	SLA/LP	IgG	0%	1:100	205

**Method comparison:**

**IIFT: HEp-2**

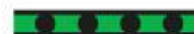
Overview of the tested samples / reference test systems:	n (IgG)
Sera from patients with mixed connective tissue disease	24
Sera from patients with Sjögren syndrome	24
Sera from patients with systemic sclerosis	25
Sera from patients with polymyositis/dermatomyositis	5
Sera from healthy blood donors	50
<b>Total number of samples</b>	<b>128</b>

**Evaluation:**

		Reference ELISA + Blot	
		positive	negative
<b>EUROIMMUN IIFT: HEp-2 (IgG)</b>	positive	78	0
	negative	0	50
<b>Specificity (IgG)</b>		100%	
<b>Sensitivity (IgG)</b>		100%	

**Liver (rat)**

Overview of the tested samples / reference test system:	n
Characterised samples from the national reference centre INSTAND (sample origin: Germany)	30
<b>Number of samples</b>	<b>30</b>

**Evaluation:**

n = 30		Reference	
		positive	negative
EUROIMMUN Liver (rat) LKM	positive	7	0
	negative	0	23
Positive agreement (Sensitivity) LKM		100%	
Negative agreement (Specificity) LKM		100%	

**Liver (rat) ANA**

Overview of the tested samples / reference test system:	n
Samples characterised as negative by EUROIMMUN IIFT: HEp-2 (origin: Germany) *	191
Samples characterised as positive by EUROIMMUN IIFT: HEp-2 (origin: Germany) *	37
<b>Number of samples</b>	<b>228</b>

\* The tests were performed exclusively with IgG. For IgG + PI, the results can also be guaranteed and need not be performed separately, as the data in Section 14 (Functional comparison IgG vs. IgG + PI) ensure functionality of the conjugate IgG + PI on the substrate.

**Evaluation:**

n = 228		Reference	
		positive	negative
EUROIMMUN Liver (rat) ANA	positive	22	0
	negative	15	191
Specificity (ANA)		100%	
Sensitivity (ANA)		59%	

**Stomach (rat) ASMA:**

Overview of the tested samples:	n
Characterised samples from the national reference centre INSTAND (origin: Germany), precharacterised with different analysis methods by the quality assessment institute	31

n = 31		INSTAND	
		positive	negative
EUROIMMUN IIFT: Stomach (rat) (IgG)	positive	6	0
	negative	0	25

Positive agreement (Sensitivity)		100%	
Negative agreement (Specificity)		100%	

Overview of the tested samples:	n
Characterised samples from the Clinical Immunological Laboratory Prof. Dr. med. Winfried Stöcker (origin: Germany), tested in reference to EUROIMMUN VSM47 IIFT	73

n = 73		VSM47 IIFT (IgG) EUROIMMUN AG	
		positive	negative
EUROIMMUN IIFT: Stomach (rat) (IgG)	positive	64	0
	negative	1	8

Positive agreement (Sensitivity)		98.5%	
Negative agreement (Specificity)		100%	

**Kidney (rat) AMA:**

Overview of the tested samples:	n
Characterised samples from the national reference centre INSTAND (origin: Germany), tested in reference to EUROIMMUN M2-3E ELISA	31

n = 31		EUROIMMUN M2-3E ELISA	
		positive	negative
EUROIMMUN	positive	12	0
IIFT: Kidney (rat) (IgG)	negative	0	19

Negative agreement (Specificity)	100%
Positive agreement (Sensitivity)	100%

Overview of the tested samples:	n
Characterised samples from the national reference centre INSTAND (sample origin: Germany), precharacterised with different analysis methods by the quality assessment institute	33

n = 33		INSTAND AMA	
		positive	negative
EUROIMMUN	positive	9	0
IIFT: Kidney (rat) (IgG)	negative	0	24

Negative agreement (Specificity)	100%
Positive agreement (Sensitivity)	100%

**Kidney (rat) LKM:**

Overview of the tested samples:	n
Characterised samples from the national reference centre INSTAND (origin: Germany), tested in reference to EUROIMMUN LKM ELISA	31

n = 31		EUROIMMUN LKM ELISA	
		positive	negative
EUROIMMUN	positive	2	0
IIFT: Kidney (rat) (IgG)	negative	0	29

Negative agreement (Specificity)	100%
Positive agreement (Sensitivity)	100%

Overview of the tested samples:	n
Characterised samples from the national reference centre INSTAND (sample origin: Germany), precharacterised with different analysis methods by the quality assessment institute	32

n = 32		INSTAND LKM	
		positive	negative
EUROIMMUN	positive	7	0
IIFT: Kidney (rat) (IgG)	negative	0	25

Negative agreement (Specificity)	100%
Positive agreement (Sensitivity)	100%



**Liver (monkey)**

Overview of the tested samples / reference test system:	n (IgG)
1. Characterised panel (actin antibody), Germany	37
2. Serum from healthy blood donors, Germany	41
<b>Total number of samples</b>	<b>78</b>

**Evaluation:**

n = 78		IIFT: HEp-2 EUROIMMUN	
		positive	negative
EUROIMMUN	positive	33	1
IIFT: Liver (monkey)	negative	0	44
<b>Specificity</b>		<b>97.8%</b>	
<b>Sensitivity</b>		<b>100%</b>	

**Clinical specificity and sensitivity:**

Antibodies Substrate	Ig class	Characterisation of samples Clinical panel of patient samples (origin of samples)	n	Prevalence	
				Positive	%
SLA/LP Transfected cells	IgG	Samples from patients: positive serologically precharacterised with Anti- SLA/LP ELISA (EUROIMMUN AG) or liver profile EUROLINE (EUROIMMUN AG); (origin: Germany, USA, Greece, Poland)	50	50	100%
		Samples from patients with autoimmune hepatitis type I (origin: Italy)	61	10	16.4%
		Samples from patients: negative serologically precharacterised with Anti- SLA/LP ELISA (EUROIMMUN AG) or liver profile EUROLINE (EUROIMMUN AG); (origin: Germany)	45	0	0%
		Samples from patients with autoimmune or infectious liver diseases (origin: Italy), Healthy blood donors (origin: Germany)	235	1	0.4%

**Clinical specificity Anti-F-Actin IIFT:**

Antibodies Substrate	Characterisation of samples Clinical panel of patient samples (reference: Autoimmunity; 41 (1) 105-110 (February 2008))	n	Positive	Prevalence
Anti-F- Actin IIFT	Autoimmune hepatitis type II	8	0	0%
	Chronic hepatitis C	30	0	0%
	Coeliac disease associated with increased transaminases	16	0	0%
	Steatohepatitis	10	0	0%
	<b>total</b>	<b>64</b>	<b>0</b>	<b>0%</b>

The clinical specificity amounted to 100%



**Clinical sensitivity Anti-F-Actin IIFT:**

Antibodies Substrate	Characterisation of samples Clinical panel of patient samples (reference: Autoimmunity; 41 (1) 105-110 (February 2008))	n	Positive	Prevalence
Anti-F- Actin IIFT	Autoimmune hepatitis type I	33	17	51.5%
	PBC*	40	5	12.5%

\* Approx. 10% of PCB patients show an overlap with AIH. The prevalence observed in this study (12,5%) agrees with the values given in the literature (Chazouillères O: **Overlap Syndromes**. Dig Dis, 33 Suppl 2: 181-7 (2015)).

The clinical sensitivity amounted to 51.5%.

**EUROPattern Classifier (software evaluation)**

Following automated image acquisition using a EUROPattern-compatible microscope, the evaluation of the fluorescence for the following test systems can be performed visually by the user on the computer screen or supported by means of the EUROPattern Classifier software. The performance data of the classifier can be found in the EUROPattern software.

Order no	Product	Automated image recording	EUROPattern Classifier (from software version)
FA 1300-####-1	IIFT: Liver Mosaic 1	EPM Live	Liver and kidney (rat): 2.3
FA 1300-####-8	IIFT: Liver Mosaic 8	EPM Live	Hep-2, Kidney (rat), Liver (rat), Stomach (rat), VSM47: 2.4 Liver (monkey): -
FA 1300-####-9	IIFT: Liver Mosaic 9	EPM Live	2.4
FA 1300-####-21	IIFT: Leber-Screen 1	EPM Live	-
FA 1302-####-50	Anti-Soluble Liver Antigen/Liver Pancreas Antigen (SLA/LP) IIFT	EPM Live	-
FA 1651-####	Anti-F-Actin IIFT	EPM Live	2.4
FA 1710-####-1	IIFT Mosaic: Stomach (Rat)/ VSM47 cells (anti-F-Actin)	EPM Live	2.4

**Clinical significance****Autoimmune liver diseases (AiLD)**

The determination of numerous autoantibodies (AAb) against liver-specific and systemic antigens is indispensable for the diagnosis of AiLD. A number of test procedures have become established for this application, for example IIFT, blot and ELISA. In particular, multiparameter profiles such as IIFT Mosaics and line blots (EUROLINE) are recommended.

To obtain a secure diagnosis of AiLD, it is absolutely essential to exclude viral hepatides (A-E), hepatitis accompanying other infections, toxic hepatides, for example caused by drugs or alcohol, metabolic liver diseases and hereditary diseases.

AiLD encompass the following diseases:

- Autoimmune hepatitis (AIH)
- Primary biliary cholangitis (PBC)
- Primary sclerosing cholangitis (PSC)

In addition, so-called overlap syndromes can occur, i.e. the simultaneous presence of two different AiLD or one AiLD in combination with another autoimmune disease, for example systemic sclerosis (SSc).

**Autoimmune hepatitis (AIH) with subtypes AIH type 1 and AIH type 2**

Like most autoimmune diseases AIH is characterised by a female predominance (>75%). The prevalence is given as 10 to 20 cases per 100,000 persons. In a study in Norway the incidence was determined as 19 cases per 1,000,000 inhabitants per year. A connection with other autoimmune syndromes is observed in around 50% of cases (overlap syndrome AIH with e.g. autoimmune thyroiditis or ulcerative colitis).

The disease often progresses to liver cirrhosis. Hepatocellular carcinoma can also develop. Despite suitable medication therapy, which is successful in up to 90% of patients, for around 10% of patients the last therapeutic option is a liver transplant. In 20% to 40% of affected individuals the AIH recurs even after transplantation (de novo AIH). The 5-year survival rate after transplantation is around 80% to 90%.

Up to 90% of AIH patients show pathological titers of at least one AAb. Due to the low prevalence of the highly specific AAb against SLA/LP (soluble liver antigen/liver pancreas antigen), which amounts to 15% to 30% in Europe and North America and around 7% in Japan, the investigation of other AAb is indispensable in suspected cases of AIH. These include in particular:

- AAb against cell nuclei (ANA), especially those that produce a homogeneous pattern
- AAb against smooth muscle (ASMA, important target antigen F-actin)
- AAb against dsDNA
- AAb against liver-kidney microsomes (LKM-1; target antigen cytochrome P450 IID6)
- AAb against cytosolic liver antigen type 1 (LC-1; target antigen formiminotransferase cyclodeaminase, FTCD)
- AAb against granulocytes (P-ANCA, perinuclear anti-neutrophil cytoplasmic antibody)

AAb against F-actin show the highest sensitivity (30 to 40%) of all AIH-associated AAb. They can only be determined with high specificity for AIH by IIFT. The specificity for AIH of SLA/LP AAb is 100%, for LC1 AAb it is near to 100%. AAb against LKM-1 can occur in viral hepatitis as well as in AIH. For patients who are positive for anti-SLA/LP and anti-Ro-52, a higher AIH activity with a more severe disease course is to be expected.

**Primary biliary cholangitis (PBC)**

PBC is a chronic non-suppurative destructive cholangitis with progressive inflammatory destruction of the small biliary ducts and liver cirrhosis in the final stage. 80% to 90% of patients are female, mainly between 20 and 60 years of age. In rare cases the disease also affects children. In Germany the prevalence is around 3 to 4 cases per 100,000 inhabitants. Ethnic differences (Caucasians, Africans, etc.) are minimal.

PBC can be subdivided into various stages using liver biopsy:

- Stage I: Inflammatory reaction of the so-called portal fields
- Stage II: Additional necroses
- Stage III: Formation of connective tissue-like septa
- Stage IV: Cirrhotic liver tissue changes  
(in around 6% of cases increased risk of hepatocellular carcinoma)

In the final stage of PBC (decompensated cirrhosis) only liver transplantation will save the patient's life. In around 75% of cases the transplant patients recover fully from PBC. Some patients, however, suffer a PBC relapse after transplantation, but only with a very slow disease course.

Nowadays three criteria are recommended, of which two must be present to diagnose PBC reliably: biochemical markers of cholestasis (increased level of AP and gamma-glutamyltransferase (γGT) in serum), histological features characteristic of PBC in liver biopsy, and the presence of PBC-specific AAb, in particular AAb against mitochondria (AMA) directed against the component M2 (family of oxo-acid dehydrogenases).

Furthermore, the additional determination of ANA is recommended, in particular AAb against nuclear dots (Sp100 und PML) and nuclear membrane (gp210), which are also of pathognomonic relevance. AAb against centromere proteins are found regularly in a proportion of patients with overlap syndrome with systemic sclerosis.



### Primary sclerosing cholangitis (PSC)

PSC is a chronic fibro-obliterative inflammation of the intra- and extra-hepatic bile ducts. It is a localised condition, which leads to the simultaneous occurrence of inflammatory scarred constrictions together with widening of ducts. Typical complications are repeated bacterial bile duct infections, which cause narrowing of the bile ducts and chronic biliary stasis, which in turn results in increasingly scarred tissue (fibrosis) with an end stage of liver cirrhosis.

This autoimmune disease occurs particularly frequently (85% of patients) together with chronic inflammatory bowel diseases, especially ulcerative colitis, whereby the intestinal disease generally precedes the PSC. There is also an association with extrahepatic diseases, especially with sicca syndrome, autoimmune thyroiditis, rheumatic diseases or retroperitoneal and mediastinal fibrosis.

Young men (around 65%) are predominantly affected, whereby the age at diagnosis is 25 to 50 years old. The occurrence in Germany amounts to 10:100,000 to 20:100,000 inhabitants.

In immunological serodiagnostics for PSC, the detection of AAb is conclusive, namely the detection of P-ANCA (perinuclear anti-neutrophil cytoplasmic antibodies). These AAb are predominantly directed against DNA-bound lactoferrin and occur in 50% of patients. Clinical chemical laboratory diagnostics in PSC patients generally show an increase in AP, GGT and bilirubin.

Table: Prevalences of AAb/test markers

Auto-immunologic test marker	Prevalence AIH	Prevalence PBC	Prevalence PSC	Prevalence overlap syndrome
ANA	40% - 60% (AIH) 100% (AIH - type 1)	5% - 50%	6% - 35%	5% - 100%
dsDNA	20% - 30%			
ASMA	40% - 50% (AIH) 60% - 90% (AIH - type 1)	25% - 50%	<10%	
SLA/LP	15% - 30% (Europe, North America) 7% (Japan)			<10%
Ro-52	5% - 19%	<5%		<10%
LC-1	10% (AIH) 35% (AIH - type 2)			
LKM-1	3% - 5% (AIH) 70% (AIH - type 2)			
AMA		85% - 95%	<5%	30% - 96%
M2-3E (BPO)	4%	90% - 95%		
Sp100		15% - 31%		
PML	4%	13%		
gp210	4%	26%		
CENP		10% - 25%		
P-ANCA	10%	6% - 28%	40% - 50%	





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## NOVA Lite<sup>®</sup> IgG F-Actin

For *In Vitro* Diagnostic Use

FOR EXPORT USE ONLY. NOT FOR SALE IN THE U.S.A



REF 708255

### Intended Use

The NOVA Lite IgG F-Actin kit is an indirect immunofluorescent assay (IFA) on rat intestine epithelial substrate for the screening and semi-quantitative determination of IgG anti-F-Actin antibodies in human serum. The presence of IgG anti-F-Actin antibodies can be used in conjunction with clinical findings and other laboratory tests to aid in the diagnosis of autoimmune liver diseases such as autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC).

### Summary and Explanation of the test

IgG anti-F-Actin autoantibodies are the main component of what have been called anti-smooth muscle antibodies (ASMA).<sup>1-6</sup> These antibodies are found in up to 80% of autoimmune hepatitis (AIH) patients and up to 30% of patients with primary biliary cirrhosis<sup>2,5</sup>, but may also be found, usually at low titers, in individuals without AIH. ASMA are heterogeneous and include antibodies to filamentous (F) and globular (G) forms of actin as well as non-actin components such as tubulin and intermediate filaments.<sup>3,4,7</sup> Several autoantibodies are associated with AIH. However, IgG antibodies to F-Actin are the most specific autoantibody for AIH.<sup>2,5</sup>

While IgG F-Actin ELISA tests, such as the QUANTA Lite Actin IgG ELISA, allow detection of IgG anti-F-Actin antibodies in an objective and quantifiable manner, some laboratories prefer IFA methodology to screen for IgG anti-F-Actin antibodies or to confirm their presence when detected by the conventional rodent kidney/stomach/liver (KSL) tissue sections. Differentiating anti-F-Actin from other antibodies to components of smooth muscle is often difficult using conventional IFA substrates such as KSL tissue sections.<sup>2,5</sup> The identification of anti-F-actin is an important aid in the diagnosis of AIH. At the moment, a "gold standard" method for the discrimination of anti-F-actin from other reactivities is not available.<sup>1,2</sup> Slides made with rat intestine epithelial cells prepared using proprietary growth and fixation methods offer a new substrate for detection of IgG anti-F-Actin antibodies by IFA. This substrate overcomes some of the limitations of other IFA procedures since the IgG anti-F-Actin pattern is distinct and other antibodies that cause an interfering pattern on KSL slides do not interfere on the rat epithelial cell substrate.

### Principles of the Procedure

In the indirect immunofluorescent technique, samples are incubated with the antigen substrate, and unbound antibodies are washed off. The substrate is then incubated with specific fluorescein labeled conjugate followed by a wash to remove the unbound reagent. When viewed through a fluorescence microscope, samples will exhibit an apple green fluorescence corresponding to areas where autoantibody has bound. A sample is considered positive if specific staining is observed in the F-Actin fibers surrounding most cells in a hexagonal pattern, and sometimes observed in the fibers crossing over cells.

### Reagents

1. F-Actin Slide; 6 wells/slide, with desiccant
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4. IFA System Negative Control, 1 vial of buffer containing 0.09% sodium azide and no human serum antibodies to F-Actin, prediluted
5. PBS Concentrate (40x), 2 vials
6. Mounting Medium, 0.09% sodium azide, 1 vial
7. Coverslips

### Warnings

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2. Sodium Azide is used as a preservative. Sodium Azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. Flush sinks, if used for reagent disposal, with large volumes of water to prevent azide build-up.
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## Precautions

1. This product is for *in vitro* diagnostic use.
2. Substitution of components other than those provided in this kit may lead to inconsistent results.
3. Incomplete or inefficient washing of IFA wells may cause high background.
4. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
5. A variety of factors influence the assay performance. These include the starting temperature of the reagents, the strength of the microscope bulb used, the accuracy and reproducibility of the pipetting technique, the thoroughness of washing and the length of the incubation times during the assay. Careful attention to consistency is required to obtain accurate and reproducible results.
6. Strict adherence to the protocol is recommended.

## Storage Conditions

1. Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
2. Diluted PBS buffer is stable for 4 weeks at 2-8°C.

## Specimen Collection

This procedure should be performed with a serum specimen. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, grossly hemolyzed, or lipemic specimens containing visible particulates should not be used.

Following collection, the serum should be separated from the clot. CLSI (NCCLS) Document H18-A3 recommends the following storage conditions for samples: 1) Store samples at room temperature no longer than 8 hours. 2) If the assay will not be completed within 8 hours, refrigerate the sample at 2-8°C. 3) If the assay will not be completed within 48 hours, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing.

## Procedure

### Materials provided

- 5 F-Actin Slide (6 well)
- 1 7mL FITC IgG Conjugate
- 1 0.8mL IgG F-Actin Positive Control
- 1 0.5mL IFA System Negative Control
- 2 25mL PBS Concentrate (40x)
- 1 7mL Mounting Medium
- 1 Package of 10 Coverslips

### Additional materials required but not provided

Micropipets to deliver 15-1000µL volume  
 Distilled or deionized water  
 Squeeze bottles or Pasteur pipets  
 Moist chamber  
 1L container (for diluting PBS)  
 Coplin jar  
 Fluorescent microscope with 495nm exciter and 515nm barrier filter

## Method

### Before you start

1. Bring all reagents and samples to room temperature (20-26°C).
2. **Dilute PBS Concentrate: IMPORTANT:** Dilute the PBS Concentrate 1:40 by adding the contents of the PBS Concentrate bottle to 975mL of distilled or deionized water and mix thoroughly. The PBS buffer is used for diluting patient samples and as a wash buffer. The diluted buffer can be stored for up to 4 weeks at 2-8°C.
3. **Dilute Patient Samples:**
  - a. **Initial Screening:** Dilute patient samples 1:40 with diluted PBS buffer (i.e., add 50µL of serum to 1.95mL of PBS buffer).
  - b. **Titration:** Make serial 2-fold dilutions from the initial screening dilution for all positive samples with diluted PBS buffer (i.e. 1:80, 1:160, 1:320... to endpoint).

### Assay procedure

1. **Prepare Substrate Slides:** Allow the substrate slide to reach room temperature prior to removal from its pouch. Label it with pencil and place it in a suitable moist chamber. Add 1 drop (20-25µL) of the undiluted positive and the negative control to wells 1 and 2 respectively. Add 1 drop (20-25µL) of diluted patient sample to the remaining wells.
2. **Slide Incubation:** Incubate the slide for 30 ± 5 minutes in a moist chamber (a dampened paper towel placed flat on the bottom of a closed plastic or glass container will maintain the proper humidity conditions). **Do not allow the substrate to dry out during the assay procedure.**
3. **Wash Slides:** After incubation, use a plastic squeeze bottle or pipet to gently wash off the serum with diluted PBS buffer. Orient the slide and stream of PBS buffer so as to minimize wash-over of samples between wells. **Avoid directing the stream directly onto the wells to prevent substrate damage.** If desired, place the slides in a Coplin jar of diluted PBS buffer for up to 5 minutes.
4. **Addition of Fluorescent Conjugate:** Shake off the excess PBS buffer. Place the slide back in the moist chamber and **immediately** cover each well with a drop of fluorescent conjugate. Incubate the slides for an additional 30 ± 5 minutes.
5. **Wash Slides:** Repeat Step 3.
6. **Coverslip:** Coverslip procedures vary from lab to lab; however, the following procedure is recommended:
  - a. Place a coverslip on a paper towel.
  - b. Apply mounting medium in a continuous line to the bottom edge of the coverslip.
  - c. Shake off the excess PBS buffer and touch the lower edge of the slide to the edge of the coverslip. Gently lower the slide onto the coverslip in such a way that the mounting medium flows to the top edge of the slide without air bubble formation or entrapment.

### Quality Control

The IgG F-Actin Positive Control and IFA System Negative Control should be run on every slide to ensure that all reagents and procedures perform properly. Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at ≤ -70°C. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test results should be considered invalid and the assay repeated.

1. The undiluted IgG F-Actin Antibody Positive Control must be ≥ 3+.
2. The IFA System Negative Control must be negative.

### Interpretation of Results

**Negative Reaction:** A sample is considered negative if specific staining as described below in the section "Positive Reaction" is less than or equal to the IFA System Negative Control. Samples can exhibit various degrees of specific or background staining to other cellular components, but be negative for IgG anti- F-Actin antibodies.

**Positive Reaction:** A sample is considered positive if specific staining at a greater intensity than the IFA System Negative Control is observed in the F-Actin fibers surrounding most cells in a hexagonal pattern, and sometimes observed in the fibers crossing over cells.

Determine the fluorescence grade or intensity using these criteria:

- 4+ Brilliant apple green fluorescence
- 3+ Bright apple green fluorescence
- 2+ Clearly distinguishable positive fluorescence
- 1+ Lowest specific fluorescence that enables the F-Actin staining to be clearly differentiated from the background fluorescence.

**Pattern Interpretation:** A variety of patterns of nuclear and/or cytoplasmic staining can be exhibited depending on the types and relative amounts of autoantibodies present in the sample. Only the pattern described above in the heading "positive reaction" should be considered positive for IgG anti-F-Actin antibodies. All other patterns should be considered negative.

### Limitations of the Procedure

1. High-titered IgG anti-F-Actin pattern is suggestive of AIH but should not be considered diagnostic. The IgG anti-F-Actin result should be considered in combination with other laboratory results as well as the overall clinical history of the patient.
2. A variety of external factors influence the test sensitivity including the type of fluorescence microscope used, the bulb strength and age, the magnification used, the filter system and the observer.
3. If a band pass filter is used instead of a 515nm barrier filter, increased artifactual staining may be observed.
4. Only pencil should be used to label the slides. Use of any other writing material may cause artifactual staining.
5. All coplin jars used for slide washing should be free from all dye residues. Use of coplin jars containing dye residue may cause artifactual staining.
6. The assay performance characteristics have been established for serum, but not for plasma or other specimen types.

### Expected Values

The ability of the NOVA Lite IgG F-Actin Kit to detect IgG F-Actin antibodies was evaluated by comparison to commercially available QUANTA Lite Actin IgG ELISA (Inova Diagnostics, Inc). ELISA results were determined to be positive if the patient sample was 20 units or greater and negative if less than 20 units.

### Normal Range

Four hundred ninety three samples from normal blood donors were run using the NOVA Lite IgG F-Actin kit. All but 4 of the 493 normal samples (99.2% specificity) were negative on NOVA Lite IgG F-Actin.

### Comparison between NOVA Lite IgG F-Actin IFA and QUANTA Lite Actin IgG ELISA

To determine the positive and negative percent agreement of the assays, 992 samples containing antibodies to a wide variety of antigens were tested with the NOVA Lite IgG F-Actin IFA kit and the QUANTA Lite Actin IgG ELISA. These samples included 493 normal blood donors, 60 patients with clinically defined diseases (rheumatoid arthritis, SLE, and scleroderma), 40 samples with defined antibodies (infectious diseases and autoantibodies) and 399 samples from patients suspected of having liver disease.

N=992	QUANTA Lite Actin IgG ELISA Positive	QUANTA Lite Actin IgG ELISA Negative	Total
NOVA Lite IgG F-Actin Positive	269	21**	290
NOVA Lite IgG F-Actin Negative	69*	633	702
Total	338	654	992



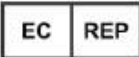







Positive % agreement 269/338 (80%)  
 Negative % agreement 633/654 (97%)  
 Overall % agreement 902/992 (91%)

\*39 of the 69 are weak positive ELISA

\*\* 14 of the 21 were 1+ IFA (low reactivity)

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## Symbols Used

	In Vitro diagnostic medical device Producto sanitario para diagnóstico in vitro
	European Conformity Conformidad europea
	European Authorized Representative Representante europeo autorizado
	Temperature Limitation Límites de temperatura
	Batch Code Código de lote
	Catalogue or part number Número de catálogo o componente
	Manufacturer Fabricante
	Use by Caducidad
	Contains Sufficient for <n> Tests Contiene suficiente para <n> Pruebas
	Consult instructions for use Consulte las instrucciones de uso

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8. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control/National Institute of Health, 2009, Fifth Edition.



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July 2020  
Revision 3



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## Storage Conditions

1. Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
2. Diluted PBS buffer is stable for 4 weeks at 2-8°C.

## Specimen Collection

This procedure should be performed with a serum specimen. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, grossly hemolyzed, or lipemic specimens containing visible particulates should not be used.

Following collection, the serum should be separated from the clot. CLSI (NCCLS) Document H18-A3 recommends the following storage conditions for samples: 1) Store samples at room temperature no longer than 8 hours. 2) If the assay will not be completed within 8 hours, refrigerate the sample at 2-8°C. 3) If the assay will not be completed within 48 hours, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing.

## Procedure

### Materials provided

- 5 F-Actin Slide (6 well)
- 1 7mL FITC IgG Conjugate
- 1 0.8mL IgG F-Actin Positive Control
- 1 0.5mL IFA System Negative Control
- 2 25mL PBS Concentrate (40x)
- 1 7mL Mounting Medium
- 1 Package of 10 Coverslips

### Additional materials required but not provided

Micropipets to deliver 15-1000µL volume  
 Distilled or deionized water  
 Squeeze bottles or Pasteur pipets  
 Moist chamber  
 1L container (for diluting PBS)  
 Coplin jar  
 Fluorescent microscope with 495nm exciter and 515nm barrier filter

## Method

### Before you start

1. Bring all reagents and samples to room temperature (20-26°C).
2. **Dilute PBS Concentrate: IMPORTANT:** Dilute the PBS Concentrate 1:40 by adding the contents of the PBS Concentrate bottle to 975mL of distilled or deionized water and mix thoroughly. The PBS buffer is used for diluting patient samples and as a wash buffer. The diluted buffer can be stored for up to 4 weeks at 2-8°C.
3. **Dilute Patient Samples:**
  - a. Initial Screening: Dilute patient samples 1:40 with diluted PBS buffer (i.e., add 50µL of serum to 1.95mL of PBS buffer).
  - b. Titration: Make serial 2-fold dilutions from the initial screening dilution for all positive samples with diluted PBS buffer (i.e. 1:80, 1:160, 1:320... to endpoint).

### Assay procedure

1. **Prepare Substrate Slides:** Allow the substrate slide to reach room temperature prior to removal from its pouch. Label it with pencil and place it in a suitable moist chamber. Add 1 drop (20-25µL) of the undiluted positive and the negative control to wells 1 and 2 respectively. Add 1 drop (20-25µL) of diluted patient sample to the remaining wells.
2. **Slide Incubation:** Incubate the slide for 30 ± 5 minutes in a moist chamber (a dampened paper towel placed flat on the bottom of a closed plastic or glass container will maintain the proper humidity conditions). **Do not allow the substrate to dry out during the assay procedure.**
3. **Wash Slides:** After incubation, use a plastic squeeze bottle or pipet to gently wash off the serum with diluted PBS buffer. Orient the slide and stream of PBS buffer so as to minimize wash-over of samples between wells. **Avoid directing the stream directly onto the wells to prevent substrate damage.** If desired, place the slides in a Coplin jar of diluted PBS buffer for up to 5 minutes.
4. **Addition of Fluorescent Conjugate:** Shake off the excess PBS buffer. Place the slide back in the moist chamber and immediately cover each well with a drop of fluorescent conjugate. Incubate the slides for an additional 30 ± 5 minutes.
5. **Wash Slides:** Repeat Step 3.
6. **Coverslip:** Coverslip procedures vary from lab to lab; however, the following procedure is recommended:
  - a. Place a coverslip on a paper towel.
  - b. Apply mounting medium in a continuous line to the bottom edge of the coverslip.
  - c. Shake off the excess PBS buffer and touch the lower edge of the slide to the edge of the coverslip. Gently lower the slide onto the coverslip in such a way that the mounting medium flows to the top edge of the slide without air bubble formation or entrapment.

### Quality Control

The IgG F-Actin Positive Control and IFA System Negative Control should be run on every slide to ensure that all reagents and procedures perform properly. Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at ≤ -70°C. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test results should be considered invalid and the assay repeated.

1. The undiluted IgG F-Actin Antibody Positive Control must be ≥ 3+.
2. The IFA System Negative Control must be negative.

### Interpretation of Results

**Negative Reaction:** A sample is considered negative if specific staining as described below in the section "Positive Reaction" is less than or equal to the IFA System Negative Control. Samples can exhibit various degrees of specific or background staining to other cellular components, but be negative for IgG anti- F-Actin antibodies.

**Positive Reaction:** A sample is considered positive if specific staining at a greater intensity than the IFA System Negative Control is observed in the F-Actin fibers surrounding most cells in a hexagonal pattern, and sometimes observed in the fibers crossing over cells.

Determine the fluorescence grade or intensity using these criteria:

- 4+ Brilliant apple green fluorescence
- 3+ Bright apple green fluorescence
- 2+ Clearly distinguishable positive fluorescence
- 1+ Lowest specific fluorescence that enables the F-Actin staining to be clearly differentiated from the background fluorescence.

**Pattern Interpretation:** A variety of patterns of nuclear and/or cytoplasmic staining can be exhibited depending on the types and relative amounts of autoantibodies present in the sample. Only the pattern described above in the heading "positive reaction" should be considered positive for IgG anti-F-Actin antibodies. All other patterns should be considered negative.

### Limitations of the Procedure

1. High-titered IgG anti-F-Actin pattern is suggestive of AIH but should not be considered diagnostic. The IgG anti-F-Actin result should be considered in combination with other laboratory results as well as the overall clinical history of the patient.
2. A variety of external factors influence the test sensitivity including the type of fluorescence microscope used, the bulb strength and age, the magnification used, the filter system and the observer.
3. If a band pass filter is used instead of a 515nm barrier filter, increased artifactual staining may be observed.
4. Only pencil should be used to label the slides. Use of any other writing material may cause artifactual staining.
5. All coplin jars used for slide washing should be free from all dye residues. Use of coplin jars containing dye residue may cause artifactual staining.
6. The assay performance characteristics have been established for serum, but not for plasma or other specimen types.

### Expected Values

The ability of the NOVA Lite IgG F-Actin Kit to detect IgG F-Actin antibodies was evaluated by comparison to commercially available QUANTA Lite Actin IgG ELISA (Inova Diagnostics, Inc). ELISA results were determined to be positive if the patient sample was 20 units or greater and negative if less than 20 units.

### Normal Range

Four hundred ninety three samples from normal blood donors were run using the NOVA Lite IgG F-Actin kit. All but 4 of the 493 normal samples (99.2% specificity) were negative on NOVA Lite IgG F-Actin.

### Comparison between NOVA Lite IgG F-Actin IFA and QUANTA Lite Actin IgG ELISA

To determine the positive and negative percent agreement of the assays, 992 samples containing antibodies to a wide variety of antigens were tested with the NOVA Lite IgG F-Actin IFA kit and the QUANTA Lite Actin IgG ELISA. These samples included 493 normal blood donors, 60 patients with clinically defined diseases (rheumatoid arthritis, SLE, and scleroderma), 40 samples with defined antibodies (infectious diseases and autoantibodies) and 399 samples from patients suspected of having liver disease.

N=992	QUANTA Lite Actin IgG ELISA Positive	QUANTA Lite Actin IgG ELISA Negative	Total
NOVA Lite IgG F-Actin Positive	269	21**	290
NOVA Lite IgG F-Actin Negative	69*	633	702
Total	338	654	992











Positive % agreement	269/338 (80%)
Negative % agreement	633/654 (97%)
Overall % agreement	902/992 (91%)

\*39 of the 69 are weak positive ELISA

\*\* 14 of the 21 were 1+ IFA (low reactivity)

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## Symbols Used

	In Vitro diagnostic medical device Producto sanitario para diagnóstico in vitro
	European Conformity Conformidad europea
	European Authorized Representative Representante europeo autorizado
	Temperature Limitation Límites de temperatura
	Batch Code Código de lote
	Catalogue or part number Número de catálogo o componente
	Manufacturer Fabricante
	Use by Caducidad
	Contains Sufficient for <n> Tests Contiene suficiente para <n> Pruebas
	Consult instructions for use Consulte las instrucciones de uso

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Appendix E Table of results for all sample tested, allocated ID number, AIH diagnosis, IgG level and clinical details.

Patient	SMA	Other antibodies	Age	SEX	NOVALite IFA	VSM 47 IFA	QUANTALite value	QUANTALite Result	AIH-1	Treatment Naïve	IgG (g/L)	Clinical details
1	VSMA		24	M	-	-	5.0	-	-		9.7	Paracetamol Overdose
2	VSMA		24	M	-	-	11.0	-	-		15.3	No follow up
3	VSMA		24	M	-	-	9.5	-	-		12.9	Fatty liver
4	VSMA		44	M	EQU	-	6.7	-	-		19	NAFLD
5	VSMA		40	M	EQU	-	3.7	-	-		14.2	HIV, Fatty liver
6	VSMA		49	F	-	-	12.2	-	-		10.8	Thrombocytopenia
7	VSMA		58	M	-	-	3.4	-	-		8.6	Fatty liver
8	VSMA		18	F	-	-	5.6	-	-		12.1	Gallstones
9	VSMA		13	M	-	EQU	29.7	WEAK	-		23.3	PBC
10	VSMA		39	M	-	-	17.3	-	Y	Y	21.3	AIH-1
11	VSMA		44	F	-	-	5.1	-	-		15.1	Breast nodule
12	VSMA	ANA	16	F	EQU	-	4.5	-	-		8.7	Under rheumatology
13	VSMA		21	M	-	-	24.2	WEAK	-		14	Raised LFT
14	VSMA		22	F	-	-	17.2	-	-		15.5	EBV Hepatitis
15	VSMA		21	M	-	-	4.7	-	-		9.6	Gastric ulcer, non cancerous condition of unknown origin
16	VSMA		23	F	-	-	26.2	WEAK	-		16.6	Acute CMV
17	VSMA		22	F	EQU	-	7.5	-	-		13.6	Fatty liver
18	VSMA	MPO	22	F	-	-	7.7	-	-		16.1	Peripheral neuropathy
19	VSMA		23	F	-	-	3.6	-	-		12.5	PID
20	VSMA		23	F	-	-	12.1	-	-		25.7	Borderline T2D
21	VSMA		41	F	-	-	5.7	-	-		14.4	NAFLD
22	VSMA		47	M	-	-	4.5	-	-		14.6	Fatty liver, Gallstones
23	VSMA		48	M	-	-	23.7	WEAK	-		21.7	Cirrhosis, ALD, post transplant
24	VSMA		46	F	-	-	3.8	-	-		11.7	T2DM, Fatty liver
25	VSMA		45	F	EQU	-	13.2	-	Y	N	15.1	
26	VSMA		34	M	-	-	7.5	-	-		10.2	No information
27	VSMA		70	M	EQU	EQU	14.6	-	-		20.9	ALD

28	VSMA		62	M	-	-	3.4	-	-	9.4	Fatty liver
29	VSMA		63	F	POS	POS	2.3	-	-	9.9	Obese
30	VSMA		62	F	-	-	7.6	-	-	9.1	Coeliac disease
31	VSMA		57	F	EQU	-	1.5	-	-	8	No information
32	VSMA		62	M	-	-	3.9	-	-	10.8	Fatty liver
33	VSMA		58	M	-	-	6.9	-	-	14.7	Alcohol related cirrhosis, T2DM, Gastro-oesophageal reflux disease
34	VSMA		68	F	POS	POS	37.4	POS	Y	Y	13
35	VSMA		66	M	-	EQU	11.7	-	-	13	Obese, cirrhosis, Fatty liver
36	VSMA		66	F	-	-	10.0	-	-	12.9	No further hepatology review raised ALT
37	VSMA		65	M	-	-	9.4	-	-	19.2	Fatty liver
38	VSMA		66	F	-	EQU	10.8	-	-	17.1	Juvenile arthritis, lupus nephritis T1DM
39	VSMA		67	M	-	-	5.6	-	-	9	Unable to follow up
40	VSMA		68	F	POS	EQU	16.0	-	-	13.9	Fatty liver
41	VSMA		69	M	EQU	-	9.3	-	-	13.6	COPD
42	VSMA		71	F	-	-	3.1	-	-	14.3	Stroke
43	VSMA		67	M	-	-	4.6	-	-	11.4	Fatty liver
44	VSMA	AMA ++	73	M	EQU	-	14.6	-	-	26.7	PSC
45	VSMA		71	F	-	-	33.9	POS	-	20.6	alcohol related liver disease
46	VSMA		71	M	EQU	POS	18.4	-	-	9.1	Unable to follow up
47	VSMA	AMA ++++	78	F	-	-	9.4	-	-	10	Likely PBC
48	VSMA		77	M	-	-	5.6	-	-	8.3	Fatty liver
49	VSMA		88	F	-	-	12.2	-	-	16.2	Unable to follow up
50	VSMA		75	F	EQU	-	4.4	-	-	10.2	Fatty liver
51	VSMA		75	M	-	-	8.8	-	Y	N	19.6
52	VSMA		27	M	EQU	-	16.3	-	Y	N	25.5
53	VSMA		56	M	-	-	6.5	-	-	14.2	Fatty liver, alcohol related
54	VSMA		54	M	EQU	POS	32.9	POS	-	10.3	Fatty liver, cardiac disease
55	VSMA		37	M	-	-	7.1	-	-	9.8	Fatty liver
56	VSMA		41	M	-	-	9.1	-	-	18.5	No information
57	VSMA		42	F	-	-	5.4	-	-	16.4	No information
58	VSMA	AMA ++	42	F	-	POS	25.6	WEAK	-	16.8	Fatty liver

59	VSMA	52	M	-	-	9.5	-	-	9.3	Lung cancer	
60	VSMA	36	M	-	-	3.0	-	-	11.2	IBD	
61	VSMA	53	F	POS	-	12.5	-	-	13.5	Fatty liver, T2DM	
62	VSMA	52	F	-	-	3.7	-	-	12.1	Abdominal pain	
63	VSMA	40	F	-	-	3.4	-	-	13.4	HCV, Hypothyroidism	
64	VSMA	36	F	-	-	11.8	-	-	13	No information	
65	VSMA	36	M	EQU	-	9.3	-	-	14.4	Fatty liver	
66	VSMA	47	M	-	-	26.0	WEAK	-	32	HBV	
67	VSMA	47	F	-	-	8.7	-	-	17	No information	
68	VSMA	41	M	-	-	3.0	-	-	17.5	Sarcoma	
69	TSMA	7	F	POS	POS	34.4	POS	Y	Y	24.2	
70	TSMA	13	F	-	POS	24.0	WEAK	Y	N	11.5	Coeliac disease
71	TSMA	12	F	POS	POS	28.3	WEAK	Y	Y	16.2	
72	TSMA	10	M	POS	POS	40.4	POS	Y	Y	57.4	
73	TSMA	11	M	POS	-	32.8	POS	Y	N	14.6	
74	TSMA	47	F	POS	POS	35.0	POS	Y	Y	22.7	Presented with acute liver failure
75	TSMA	14	F	POS	POS	41.7	POS	Y	Y	25.4	
76	TSMA	8	M	POS	POS	31.8	POS	Y	Y	18.7	
77	TSMA	9	M	POS	POS	16.9	-	Y	N	12.9	AIH-1 with overlap (bile duct involvement)
78	TSMA	46	F	EQU	POS	12.4	-	-	11.9	CLL	
79	TSMA	58	F	EQU	POS	7.4	-	-	15.3	Breast carcinoma	
80	TSMA	13	M	POS	POS	30.9	POS	Y	Y	25.2	
81	TSMA	13	F	-	POS	40.4	POS	Y	Y	36.7	IBD
82	TSMA	14	F	-	-	11.4	-	Y	N	11.9	Biliary features suggestive of overlap syndrome (PSC)
83	TSMA	13	M	EQU	EQU	19.3	-	Y	N	11.3	IBD
84	TSMA	13	M	POS	POS	35.9	POS	Y	Y	28.7	
85	TSMA	19	M	EQU	-	5.6	-	-	16.6	Fatigue post covid	
86	TSMA	48	M	POS	POS	40.9	POS	Y	Y	26.7	
87	TSMA	54	F	POS	POS	21.9	WEAK	-	15	Discharged no further information	
88	TSMA	59	M	EQU	EQU	24.4	WEAK	-	17.5	Monitored by IHEP, no biopsy until LFT become abnormal	
89	TSMA	59	F	POS	POS	1.2	-	-	8.4	T2DM, NAFLD	

90	TSMA		61	M	POS	POS	6.3	-	-		9.2	Fatty liver
91	TSMA		70	M	POS	POS	30.4	POS	-		23	Liver cancer
92	TSMA		71	F	POS	-	20.0	WEAK	-		18.3	Biopsy no features of AIH
93	TSMA		73	F	-	POS	35.4	POS	-		12.2	Adrenal nodule, neoplasm of breast
94	TSMA		73	M	EQU	POS	17.0	-	-		17.7	Stroke, deceased
95	TSMA		71	F	POS	POS	21.5	WEAK	-		14.4	Skin carcinoma, Fatty liver, Diverticulitis
96	TSMA		81	F	-	-	31.6	POS	-		14.8	Pancreatic cyst, Previous breast cancer
97	TSMA		76	M	EQU	POS	9.2	-	-		8.7	No information
98	TSMA		81	M	POS	POS	22.5	WEAK	-		17.8	High alcohol intake
99	TSMA		78	M	POS	POS	28.2	WEAK	-		20.2	Declined biopsy so unable to confirm strong indication of AIH, Haemochromatosis,
100	TSMA	AMA ++	78	M	-	-	23.5	WEAK	Y	N	17.3	Possibly also PBC but histology Unclear
101	TSMA		49	F	POS	POS	27.7	WEAK	-		12.5	Monitored by IHEP, bile calculi
102	TSMA		57	F	POS	POS	35.5	POS	-		15.3	Monitored by IHEP
103	TSMA		35	F	POS	POS	30.1	POS	Y	Y	20.9	PBC overlap
104	TSMA		43	F	POS	POS	31.2	POS	Y	Y	60.8	
105	TSMA		55	F	EQU	POS	31.0	POS	Y	N	16	
106	TSMA		52	F	POS	POS	37.5	POS	-		30.2	Rapid deterioration leading to death, extensive liver damage, SLE
107	TSMA		29	F	POS	POS	4.5	-	-		11.1	Potential live liver donor
108	-	AMA ++++	54	F	-	-	29.6	WEAK	-			HBV
109	-	LKM +	12	F	-	-	19.8	-	Y	N		
110	-		4	M	-	-	18.5	-	-		30.3	"Super urgent" liver Transplant following acute liver failure signs of immune mediated liver injury
111	-	AMA ++++	66	F	-	-	14.6	-	Y	N	27	PBC/AIH overlap, liver Transplant 2002
112	-	AMA ++++	41	F	-	-	11.8	-	-			No information
113	-		27	F	-	-	8.5	-	-			T1DM
114	-	AMA ++	35	F	-	-	6.6	-	-		NA	PBC, APS
115	-		54	M	-	-	6.6	-	Y	N		PBC/AIH overlap, liver Transplant 2002
116	-		55	F	-	-	5.5	-	-			PBC

117	-	LKM +++	57	F	-	-	4.8	-	-	No information		
118	-		41	M	-	-	4.5	-	-	12.5	Overweight, liver scarring	
119	-		53	F	-	-	4.2	-	-	11	HCV	
120	-		41	M	-	-	3.9	-	-	7.5	Fatty liver	
121	-		61	M	-	-	3.8	-	-	Fatty liver		
122	-		41	M	-	-	3.7	-	-	No information		
123	-		25	M	-	-	3.6	-	-	HBV		
124	-		73	F	-	-	3.6	-	-	11.1	Fatty liver	
125	-		17	F	-	-	3.5	-	-	HCV		
126	-		23	M	-	-	3.5	-	-	NA	Fatty liver, alcohol related	
127	-		55	F	-	-	3.0	-	-	11.4	Fatty liver	
128	-	AMA +++	40	M	-	-	2.9	-	-	N	NA	Monitored by Hepatology but LFT normalised
129	-		53	M	-	-	2.6	-	-	14	ALD	
130	-		33	F	-	-	2.5	-	-	Connective tissue disease		
131	-		17	M	-	-	2.0	-	-	NA	Alcohol related liver damage	
132	-		37	M	-	-	2.0	-	-	Adenoma		
133	-		31	M	-	-	1.4	-	-	N	No information	