



## HEp-2 / DFS70 Knock-out IFA

### PRODUCT INSERT

IVD	For in vitro diagnostic use		
REF	1108	HEp-2/DFS70 Knock-out Substrate Kit	60 Determinations
REF	1108-120	HEp-2/DFS70 Knock-out Substrate Kit	120 Determinations
REF	1108-240	HEp-2/DFS70 Knock-out Substrate Kit	240 Determinations

### INTENDED USE

Indirect immunofluorescence (IF) antibody test for the detection and quantitation of anti-nuclear antibodies (ANA) in human serum.

### SUMMARY AND EXPLANATION

**Antinuclear antibodies (ANA)**, detected by indirect immunofluorescence, aid in the diagnosis of connective tissue disorders including systemic lupus erythematosus (SLE), mixed connective tissue disease, Sjögren's syndrome and scleroderma<sup>1-5</sup>. ANA occur in about 95% of SLE patients as well as patients with other connective tissue diseases. ANA may also occur in other disorders such as chronic active hepatitis and primary biliary cirrhosis<sup>6-8</sup>.

**Anti-mitochondrial antibodies (AMA)** occur in over 90% of primary biliary cirrhosis cases, 3-11% of chronic active hepatitis patients and are absent in patients with extra-hepatic biliary obstruction and in other liver diseases. The universal presence of anti-mitochondrial antibodies in primary biliary cirrhosis and their virtual absence in extra-hepatic jaundice makes their detection of considerable value in the differential diagnosis<sup>6-12</sup>.

**Anti-smooth muscle antibodies (ASMA)** in high titer (>160) occur in the majority of cases of chronic active hepatitis and in intermediate titers (40-80) in acute viral hepatitis. Occasionally they may occur in cases of primary biliary cirrhosis where they are also found in intermediate titers. The significance of titers of 20-40 is doubtful since these titers may occur in normal individuals<sup>13,14</sup>.

**Anti-DFS70 Antibodies** produce a nuclear dense fine speckled immunofluorescence pattern (DFS70) on HEp-2 cells. These autoantibodies target a 70 kDa antigen also known as LEDGF (Lens Epithelium Derived Growth Factor) or psp1 gene product. These have been initially reported to occur in sera from patients with positive ANA tests but no clinical evidence of systemic autoimmune rheumatic disease (SARD). Their presence was initially documented in patients with certain inflammatory conditions and 'apparently' healthy individuals<sup>15-17</sup>. DFS70 antibodies produce a pattern that can be confused as homogeneous (associated with DNA, histones and nucleosomes) or fine speckled pattern associated with SARDs. On the slides provided with this kit, wild type (WT) HEp-2 are mixed with HEp-2 with the psp1 gene knocked out (KO) in 1:9 ratio. WT cells are able to detect all autoantibody specificities identical to conventional, non-engineered HEp-2. KO cells are able to detect all autoantibody specificities except DFS70 associated with psp1/LEDGF. The use of this product thereby provides additional functionality to aid in accurate discrimination of homogeneous, speckled and dense fine speckled patterns. This may

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provide the laboratory with more information to select appropriate confirmatory assays.

### PRINCIPLES OF PROCEDURE

In the indirect IF method used in this kit, patient sera are incubated on HEp-2 cell substrates to allow binding of antibodies. Any antibodies not bound are removed by rinsing. Bound antibodies of the IgG class are detected by incubation of the substrate with fluorescein-labeled, anti-human IgG conjugate. Reactions are observed under a fluorescence microscope equipped with appropriate filters. The presence of ANA, ASMA, and AMA is demonstrated by an apple green fluorescence of specific structures in the cells. A majority of the cells present have the psp1 gene knocked out which prevents formation of LEDGF binding sites in these cells. This allows differentiation of ANA homogeneous and speckled reaction patterns from DSF70 patterns<sup>18</sup>. The titers (the reciprocal of the highest dilution giving a positive reaction) are then determined by testing serial dilutions<sup>19</sup>.

### PRODUCT INFORMATION

#### Storage and preparation

Store all reagents at 2-8°C. Reagents are ready for use after equilibration to room temperature.

#### Materials provided

##### HEp-2/DFS70 Knock-out Substrate Kit

[REF] 1108	60 Determinations	
[REF] 1108-120	120 Determinations	
[REF] 1108-240	240 Determinations	
HEp 2 substrate	[SORB SLD 12]	Barcoded 12 well HEp-2/DFS70 knock out substrate slide (5x [REF] 1108, 10x [REF] 1108-120, 20x [REF] 1108-240)
1 x 0.5 ml	[CONTROL + ANA-HOMO]*	ANA positive control. Contains human serum.
1 x 0.5 ml	[CONTROL + DFS70]*	DFS70 antibody positive control. Contains human serum.
1 x 0.5 ml	[CONTROL -]*	Negative control. Contains human serum.
5 ml	[IgG-CONJ FITC EB]*	Anti-human IgG FITC conjugate containing Evan's blue counterstain. Protect from light. (1x [REF] 1108, 2x [REF] 1108-120, 3x [REF] 1108-240)
60 ml	[BUF]*	Buffered diluents. (1x [REF] 1108, 1108-120, 2x [REF] 1108-240)
vials	[BUF WASH]	Phosphate buffered saline (PBS). Dissolve each vial into 1L distilled or deionized water. (2x [REF] 1108, 1108-120, 3x [REF] 1108-240)
5 ml	[MOUNTING MEDIUM]*	Mounting medium. Do not freeze. (1x [REF] 1108, 1108-120, 2x [REF] 1108-240)
12 per box	[COVER SLD]	Coverslips. (1x [REF] 1108, 2x [REF] 1108-120, 4x [REF] 1108-240)


#### Optional Components


5 ml	[IgG-CONJ FITC EB]*	Anti-human IgG FITC conjugate. Protect from light. [REF] 2100
1 ml	[EVANS]	Evan's blue counterstain. [REF] 2510.

\* contains <0.1% NaN<sub>3</sub>

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### Symbols used on labels:


 Lot number


 Catalog number


 Use by



 Storage temperature

 Read instructions for use

 In vitro diagnostic use

 Manufacturer

 Number of Tests

 Warning. May cause cancer. Obtain special instructions before use. Do not handle until all safety precautions have been read and understood. Wear protective gloves, clothing, eye and face protection. IF exposed or concerned, get medical advice/attention. Store locked up. Dispose of contents/container to an approved waste disposal plant. Applies to  2510.

### Material required but not provided

- Fluorescence microscope
- Micropipette or Pasteur pipette
- Serological pipettes
- Staining dish (e.g. Coplin jar)
- Small test tubes (e.g. 13 x 75 mm) and test tube rack
- Distilled or deionized water
- 1 liter container
- Paper towels
- Incubation chamber

### WARNINGS AND PRECAUTIONS

For *in vitro* Diagnostic Use. All human derived components used have been tested for HbsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. All human serum specimens and human derived products should be treated as potentially hazardous, regard-less of their origin. Follow good laboratory practices in storing, dispensing and disposing of these materials<sup>20</sup>.

WARNING – Sodium azide (NaN<sub>3</sub>) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this insert to ensure valid results. Do not interchange kit components with those from other sources other than the same catalog number from Immco Diagnostics Inc. Do not use beyond expiration date.

### SPECIMEN COLLECTION AND PREPARATION

Only serum specimens should be used for this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of this test and should not be used. Store specimens at 2-8°C for no longer than one week. For longer storage, serum should be frozen at -20°C. Avoid repeated freezing and thawing of samples.

## EN PROCEDURE

### Test Method

#### A. Screening

Patient sera to be reacted on slides with HEp-2 substrate should be diluted using 1:40 screening dilution. On HEp-2 a specific reaction at 1:40 or greater titer is considered positive.

1. Dilute each patient serum 1:40 (10 µl serum + 390 µl diluent). Do not dilute Positive or Negative Controls. Save the undiluted sera to determine antibody titers if screening tests are found to be positive.
2. Allow pouches containing substrate slides to equilibrate to room temperature for 10-15 minutes. Carefully remove the slides without touching the substrate.
3. Label the slides and place them in an incubation chamber lined with paper towels moistened with water to prevent drying.
4. Invert dropper vial and gently squeeze to apply 1 drop (approximately 50 µl) of the Negative Control to well #1. Similarly apply 1 drop of ANA Positive Control to well #2. Avoid overfilling the wells.
5. Using a micropipette or Pasteur Pipette, apply 1 drop of patient's diluted serum (approximately 50 µl) to the other wells. Avoid overfilling the wells.
6. Place the lid on the incubation chamber and incubate slides 30 minutes at room temperature.
7. Remove a slide from the incubation chamber. Hold slide at tab end and rinse gently with approximately 10 ml PBS using a pipette, or rinse slide in beaker filled with PBS. Do not use wash bottle. Transfer slide immediately into Coplin jar and wash 10 minutes. Repeat process with all remaining slides.
8. Remove slide(s) from Coplin jar. Blot the edge of the slide on a paper towel to remove excess PBS. Place the slide in the incubation chamber. Immediately invert the Conjugate dropper vial and gently squeeze to apply 1 drop (approximately 50 µl) to each well.
9. Repeat steps **7 and 8** for each slide.
10. Replace the lid on the incubation chamber. Incubate 30 minutes at room temperature.
11. Remove a slide from incubator. Hold the slide at the tab end and dip the slide in a beaker containing PBS to remove excess conjugate. Place slide(s) in a staining dish filled with PBS for 10 minutes. If optional conjugate without counterstain is used (see optional components in Materials Provided Section), 2-3 drops of Evan's Blue counterstain may be added to the final wash. Repeat for the remaining slides. NOTE: Improper washing may lead to increased background fluorescence.
12. Remove a slide from the staining dish. Blot the edge of the slide on a paper towel to remove excess PBS. **To prevent slide from drying, proceed immediately with next step while slide is still wet.**
13. Mount the coverslip by applying **3 drops** of Mounting Medium evenly on the coverslip and place coverslip over slide. Avoid applying undue pressure and prevent lateral movement of the coverslip.
14. Repeat steps 12 and 13 for each slide.
15. Examine for specific fluorescence under a fluorescence microscope at a magnification of 200x or greater.

Slides may be read as soon as prepared. However, because of the presence of antifading agent in the mounting medium, no significant loss of staining intensity occurs if reading is delayed for up to 48 hours. Slides should be stored in the dark at 2-8°C.

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The clinician should consider the results of all positive indirect immunofluorescence tests along with the results of other laboratory tests and the clinical condition of the patient when making a diagnosis.

### EXPECTED VALUES

Tests for nuclear antibodies are used to screen for SLE and certain other immunologic disturbances. AMA occur in over 90% of cases of primary biliary cirrhosis and 3-11% of cases of chronic hepatitis. ASMA occur in the majority of cases of chronic active hepatitis.

**Table 2: Incidence of Antinuclear Antibodies (ANA) Detected by Indirect Immunofluorescence on HEp-2 Cells**

Clinical Condition	No. of Sera	% Positive
SLE	12	100
Subacute Cutaneous LE (SCLE)	7	86
Scleroderma	6	100
Rheumatoid Arthritis	10	50
Normal Controls	15	0

### PERFORMANCE CHARACTERISTICS

The ImmuGlo™ Autoantibody Test System was compared with another commercially available fluorescent antibody test using HEp-2 cells as a substrate. The comparison included 15 serum samples from normal subjects as well as sera from patients with the diagnosis of SLE, subacute cutaneous lupus erythematosus, scleroderma or rheumatoid arthritis. Sera were tested according to the procedure and screening dilution recommended by the manufacturer. These yielded comparable results as summarized below:

#### **Comparison of Kits Using HEp-2 Cell Substrate for the Detection of Antinuclear Antibodies**

Clinical Condition	No. of Sera	% Positive	
		Immco	Other
SLE	12	100	100
Subacute Cutaneous LE (SCLE)	7	85	85
Scleroderma	6	100	100
Rheumatoid Arthritis	10	50	30
Normal Controls	15	0	0

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### B. Endpoint Determination (titration)

A serum positive in the screening test may be further tested following steps 5 through 13 to determine the appropriate screening dilution for the substrate. Each test run should include the Positive and Negative Controls. Make serial two-fold dilutions starting at 1:40. The reciprocal of the highest dilution producing a positive reaction is the titer.

#### **Preparation of Serial Dilutions Starting at 1:40**

To create serial dilutions of a patient sample from 1:40 to 1:2560, begin by numbering six tubes from 1 through 6. More tubes may be used if a higher final dilution is desired. Add 3.9 ml of Sample Diluent to tube 1 and 0.2 ml to tubes 2 through 6. Pipette 0.1 ml of undiluted serum to tube 1 and mix thoroughly. Transfer 0.2 ml from tube 1 to tube 2 and mix thoroughly. Continue transferring 0.2 ml from one tube to the next after mixing to yield the dilutions depicted in the following table:

Tubes	1	2	3	4	5	6
Serum	0.1 ml					
	+					
Buffered Diluent	3.9 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
		↻	↻	↻	↻	↻
Transfer		0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Final dilution	1:40	1:80	1:160	1:320	1:640	1:1280 etc.

### QUALITY CONTROL

Both a Positive and Negative Control should be included with each test run. The Negative Control should show no specific fluorescence of the nuclei. The ANA Positive Control should have 2+ or greater staining intensity of the nuclei with a predominantly homogeneous pattern.

If expected results are not obtained, the run should be repeated. If inadequate results continue to occur with the controls, these may be due to:

- Turbidity. Discard and use another control
- Problems with the optical system of the fluorescence microscope. These may include: improper alignment, bulb beyond useful life expectancy, etc.
- Allowing the slide to dry during the procedure.

### INTERPRETATION OF RESULTS

Results should be reported as negative, positive, or, if end-point titer has been determined, positive with titer. It is recommended that patient samples demonstrating specific fluorescence reactions at a dilution of 1:40 be reported as positive. This should serve as a guide in the interpretation of results. Each laboratory must determine its own normal values to account for differences in microscopy systems, personnel and training.

Read only fields which contain specific staining of the HEp-2 cells and the patterns observed for ANA, AMA and ASMA. All other reactions should be reported as negative.

ANA can be quantified on the HEp-2 cells. The nuclear staining patterns observable include homogeneous, peripheral (rim), speckled, nucleolar and centromere. These nuclear staining patterns are described below. They may be one or a combination of several staining patterns. The latter are due to reactions to several different nuclear antigens.

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**Homogeneous:** The entire nucleus fluoresces evenly with a diffuse staining pattern.

**Nuclear Membranous:** The nuclear membrane stains most intensely as fine linear pattern with decreasing staining intensity of the nucleoplasm towards the center of the nucleus.

**Speckled:** Discrete coarse to fine round speckles fluoresce throughout the nucleus.

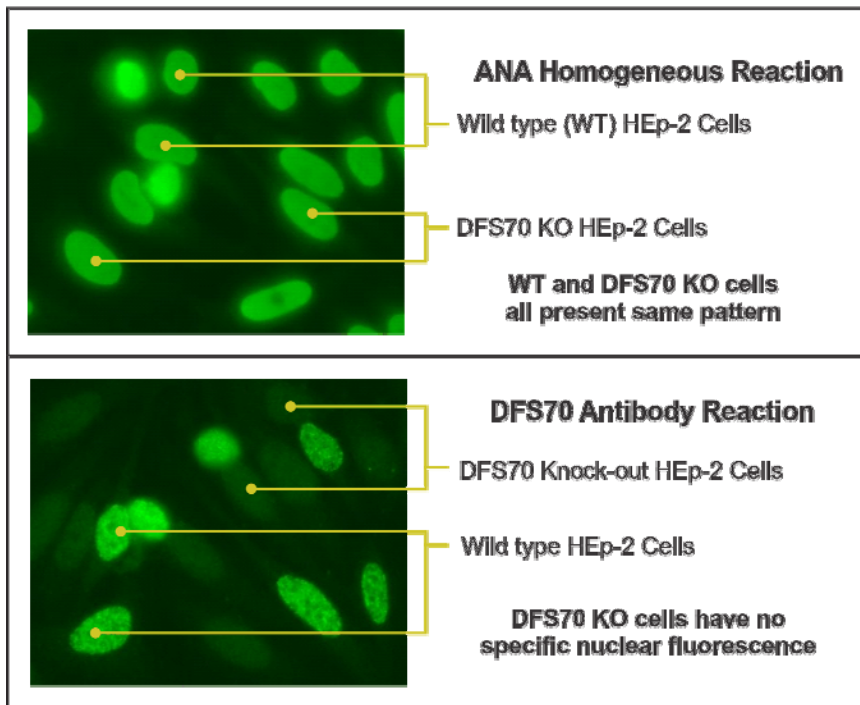
**Nucleolar:** The nucleoli stain as multiple solid bodies within the nucleus.

**Centromere:** Large speckles of finite number. Reactive antigen segregates with condensed chromosomes in cells undergoing mitosis<sup>5</sup>.

**DFS70:** Wild Type HEp-2: In ~10% of the cells in each well, a dense fine speckled pattern will be observed on the interphase nucleus. Chromatin associated staining is seen on mitotic nuclei. The remaining ~90% of HEp-2 cells have the psp1 gene encoding the LEDGF antigen knocked out and therefore will not producing a similar pattern.

If 10% of the HEp-2 cells have brighter fluorescence corresponding to DFS70 pattern and rest of the cells present additional patterns or fine speckled signal above cut-off, it indicates the presence of mixed patterns. Closer analysis of such patterns is essential to confirm what other antibody specificity is present in addition to the DFS70 pattern associated with LEDGF/psp1.

**Figure 1. Reference Reactions**



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The specificity of some of the antibodies giving the above staining patterns may be further identified by tests for antibodies to nDNA and to various extractable nuclear antigens. These may be of diagnostic significance as listed in Table 1.

**Table 1. Diagnostic Significance of Antinuclear Antibodies**

IF Staining Pattern	Nature of Antigen	Associated Disease
Homogeneous	dsDNA/Histones	SLE
Nuclear membranous	Laminins	SLE, vasculitis or chronic hepatitis
Speckled	RNP	SLE or MCTD*
	Sm	SLE
	SS-A/SS-B	SLE or Sjögren's Syndrome
	Scl-70	Scleroderma
Nucleolar	RNAP-I	Scleroderma
	Pm-Scl	
	RNA/probably U3 RNA	
Centromere/Kinetochores	inner and outer plates of kinetochores	CREST syndrome
DFS70 <sup>15-18</sup>	Dense Fine Speckled Pattern (positive in ~10% of cells or relatively higher fluorescence signal in ~10% of cells)	Negative association with systemic autoimmune diseases; reported in atopic dermatitis, alopecia areata, Vogt-Koyanagi-Harada (VKH) disease, sympathetic ophthalmia, Behcet's disease and atypical retinal degeneration.

\* Mixed Connective Tissue Disease

Detectable cytoplasmic antibodies include anti-mitochondrial antibodies (AMA) and anti-smooth muscle antibodies (ASMA). In an AMA pattern, the cytoplasm appears granular, whereas the ASMA pattern is a fibrillar network of staining throughout the cytoplasm. Both patterns should be reported as negative for ANA.

### LIMITATION OF THE PROCEDURE

In some cases, sera positive for ANA may either be very weak or negative at the initial screening dilution (prozone phenomenon). In such doubtful cases the sera should be screened at higher dilutions and, if positive, antibody titers determined.

In some cases the presence of two or more antibodies in a serum which are reactive with the same substrate may cause an interference in their detection by immunofluorescence. This interference may cause either failure to detect ANA or suppression of its titer if the interfering antibody has a higher titer than ANA. All ANA reactions should be reported.

The goat anti-human IgG FITC Conjugate supplied in this kit is primarily heavy chain specific but has some light chain activity. It reacts primarily with IgG class autoantibodies, but may, to a lesser degree, react with light chains of other classes such as IgM.

A positive ANA should not be considered diagnostic of SLE by itself. They also occur in patients with other connective tissue diseases and certain drugs such as procainamide and hydralazine may induce a positive ANA<sup>1</sup>. Moreover, sera of patients with malignancies and infectious diseases may also have positive ANA<sup>21</sup>.