

ENGLISH



AESKUSLIDES®
THE IFA PRODUCT LINE



INSTRUCTION MANUAL

AESKUSLIDES® ANCA Ethanol **AESKUSLIDES® ANCA Formalin**

Standard Ref.	Description	Tests
54.100.US	ANCA Ethanol (12 wells)	120
54.101.US	ANCA Formalin (12 wells)	120
54.050.US	ANCA Ethanol (6 wells)	60
54.051.US	ANCA Formalin (6 wells)	60
54.100.US.Demo	ANCA Ethanol (12 wells)	24
54.100.US.Bulk5	ANCA Ethanol (12 wells)	600
54.100.US.Bulk10	ANCA Ethanol (12 wells)	1200
54.101.US.Demo	ANCA Formalin (12 wells)	24
54.101.US.Bulk5	ANCA Formalin (12 wells)	600
54.101.US.Bulk10	ANCA Formalin (12 wells)	1200



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AESKUSLIDES® ANCA Ethanol **AESKUSLIDES® ANCA Formalin**

1. INTENDED USE

AESKUSLIDES ANCA is an indirect fluorescent antibody assay utilizing human neutrophil granulocyte coated slides, fixed with Ethanol or Formalin, as a substrate for the qualitative and semi-quantitative determination of anti-neutrophil cytoplasmic autoantibodies (ANCA) in human serum **by manual microscopy or with the HELIOS® AUTOMATED IFA SYSTEM.**

This in vitro diagnostic assay is used as an aid for the diagnosis of ANCA-associated vasculitides (AAV) in conjunction with other clinical and laboratory findings.

All suggested results obtained with the HELIOS AUTOMATED IFA SYSTEM must be confirmed by trained personnel.

2. CLINICAL APPLICATION

Anti-neutrophil cytoplasmic antibodies (ANCAs) are an important tool in the diagnosis of ANCA-associated vasculitides (AAV), comprising granulomatosis with polyangiitis (GPA, formerly Wegener's Granulomatosis), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome). Since those vasculitides lead to severe complications if untreated, early diagnosis is important for disease outcome. [1]

ANCAs are directed against different proteins in the cytoplasm of human neutrophil granulocytes. Indirect immunofluorescence (IIF) testing reveals two main patterns on ethanol-fixed human neutrophil granulocytes: the cytoplasmic ANCA (C-ANCA), and the perinuclear ANCA (P-ANCA). In patients with AAV the target antigens of these two main types of ANCAs have been identified. C-ANCAs are mainly directed against proteinase 3 (PR3), while P-ANCAs are mainly directed against myeloperoxidase (MPO). [2, 3] Moreover, only PR3-specific C-ANCAs and MPO-specific P-ANCAs are relevant for the diagnosis of GPA, MPA and EGPA. [4, 5]

However, ANCA IIF patterns can also be found in other diseases like inflammatory bowel diseases (e.g. Ulcerative Colitis), hepatobiliary disorders (e.g. Autoimmune Hepatitis), Rheumatoid Arthritis, and Systemic Lupus Erythematosus. [6, 7, 8, 9] Furthermore, the antigenic nature of ANCAs is not restricted to PR3 and MPO. Possible other antigens include lactoferrin, cathepsin G, elastase, and bactericidal permeability-increasing protein. Those ANCAs are described as atypical ANCA (A-ANCA) or very perinuclear ANCA and their IIF patterns resemble the P-ANCA pattern. However, they are not specifically associated with AAVs and may play a role in the diagnosis to other non-vasculitis diseases. [10, 11, 12]

In addition, the detection of antinuclear antibodies (ANA) in patient serum can result in the occurrence of ANCA patterns in IIF.

To discriminate C- and P-ANCAs from atypical ANCAs and ANAs, samples showing an ANCA pattern on ethanol-fixed cells should be retested on formalin-fixed cells. There, ANCA patterns caused by atypical ANCA and ANA usually become negative, while C-ANCA patterns keep their C-ANCA pattern and P-ANCA patterns are converted to a C-ANCA pattern. [13]

Furthermore, C- and P-ANCA patterns are not exclusively associated with PR3- and MPO-antigenicity, meaning that autoantibodies with other antigen specificities can also generate C- and P-ANCA patterns. However, only PR3-specific C-ANCAs and MPO-specific P-ANCAs are indicative of GPA and MPA/EGPA, respectively. [14, 15] Thus, current guidelines dictate that positive IIF results are to be confirmed by PR3 and MPO ELISA. [16, 17]

Principle of detection: The detection of antibodies is based on the principle of indirect immunofluorescence assay (IIFA). Glass microscope slides are coated with human neutrophil granulocytes fixed either with ethanol or formalin. If the patient's serum contains specific antibodies they will bind during the first incubation. After removing unbound material by washing steps, bound antibodies are detected by Fluorescein-conjugated anti-human immunoglobulins during the second incubation. A specific green fluorescent staining of antigen-antibody-complex can be visualized with the aid of a fluorescent microscope. [18]



3. MATERIALS

3.1 Materials provided

Catalog/ Ref.	Reagent	Quantity Ethanol fixation					Volume	Description	Ready to use
		54.100. US	54.100. US .Demo	54.100.US .Bulk5	54.100.US .Bulk10	54.050. US			
s54.100	Slides	10	2	50	100	10	-	ANCA Ethanol	YES
	Wells	12				6	-	Coated with neutrophil Cells fixed with Ethanol	
C54.100	Conjugate	1x		5x	10x	-	4 ml	Containing: BSA, Fluorescein (FITC) labelled goat anti-human IgG (Gamma Chain) Capped blue: slightly blue colored solution.	YES
C54.050	Conjugate	-		-	-	1x	2 ml	Containing: BSA, Fluorescein (FITC) labelled goat anti-human IgG (Gamma Chain) Capped blue: slightly blue colored solution.	YES
PC54.100	Positive ANCA c-ANCA pattern control	1x					0.5 ml	ANCA pattern control C-ANCA Capped red: colorless solution. Containing: Human serum (diluted), sodium azide <0.1% (preservative)	YES
PC54.101	Positive ANCA p-ANCA pattern control	1x					0.5 ml	ANCA pattern control P-ANCA Capped red: colorless solution. Containing: Human serum (diluted), sodium azide <0.1% (preservative)	YES
NCANCA	Negative Control	1x					0.5 ml	Capped green: colorless solution. Containing: Human serum (diluted), sodium azide <0.1% (preservative)	YES
EBIFA*	Evans Blue 0.2%	1x					3 ml	Capped white: Blue colored solution Containing: PBS, Evans Blue. Dilute the Evans Blue 0.2% 1:3000 in 1x WBIFA	NO
MMIFA	Mounting Medium	1x		3x	6x	1x	7 ml	Validated for use with the HELIOS® Capped white: colorless solution Containing: PBS, Glycerin.	YES
WBIFA	Wash buffer (10x)	1x		3x	6x	1x	100 ml	Capped white: colorless solution Dilute the concentrated buffer 1:10 in distilled water (e.g.: 100 ml + 900 ml). Containing: PBS, sodium azide (preservative).	NO
SBIFA	Sample buffer (1x)	1x		3x	6x	1x	70 ml	Capped white: colorless solution for the dilution of patient sera. Containing: BSA, PBS, sodium azide (preservative).	YES

*must be ordered separately



Catalog/ Ref.	Reagent	Quantity Formalin fixation					Volume	Description	Ready to use
		54.101. US	54.101. US .Demo	54.101.US .Bulk5	54.101.US .Bulk10	54.051. US			
s54.101	Slides	10	2	50	100	10	-	ANCA Formalin	YES
	Wells	12				6	-	Coated with neutrophil Cells fixed with Formalin	
C54.101	Conjugate	1x		5x	10x	-	4 ml	Containing: BSA, Fluorescein (FITC) labelled goat anti- human IgG (Gamma Chain) Capped blue: slightly blue colored solution.	YES
C54.051	Conjugate	-		- n	-	1x	2 ml	Containing: BSA, Fluorescein (FITC) labelled goat anti- human IgG (Gamma Chain) Capped blue: slightly blue colored solution.	YES
PC54. 100	Positive ANCA c-ANCA pattern control	1x					0.5 ml	ANCA pattern control C- ANCA Capped red: colorless solution. Containing: Human serum (diluted), sodium azide <0.1% (preservative)	YES
PC54. 101	Positive ANCA P-ANCA pattern control	1x					0.5 ml	ANCA pattern control P- ANCA Capped red: colorless solution. Containing: Human serum (diluted), sodium azide <0.1% (preservative)	YES
NCANCA	Negative Control	1x					0.5 ml	Capped green: colorless solution. Containing: Human serum (diluted), sodium azide <0.1% (preservative)	YES
EBIFA*	Evans Blue 0.2%	1x					3 ml	Capped white: Blue colored solution Containing: PBS, Evans Blue. Dilute the Evans Blue 0.2% 1:3000 in 1x WBIFA	NO
MMIFA	Mounting Medium	1x		3x	6x	1x	7 ml	Validated for use with the HELIOS® Capped white: colorless solution Containing: PBS, Glycerin.	YES
WBIFA	Wash buffer (10x)	1x		3x	6x	1x	100 ml	Capped white: colorless solution Dilute the concentrated buffer 1:10 in distilled water (e.g.: 100 ml + 900 ml). Containing: PBS, sodium azide (preservative).	NO
SBIFA	Sample buffer (1x)	1x		3x	6x	1x	70 ml	Capped white: colorless solution for the dilution of patient sera Containing: BSA, PBS, sodium azide (preservative).	YES

*must be ordered separately



3.2 Materials not provided

Catalog/Reference	Name	Description
IOS-1000	HELIOS® AUTOMATED IFA SYSTEM	The HELIOS AUTOMATED IFA SYSTEM is an automated system for immunofluorescence processing with image capturing with an integrated fluorescence microscope and software. The HELIOS is a further development of the HELMED IFA processor. The HELIOS will automatically perform: sample dilution, transfer of controls and diluted sample to wells, incubations, washing steps, conjugate addition, mounting medium addition, image acquisition, positive/negative pre-classification, estimate the End Point Titer, and assign pattern interpretation. For routine laboratory use by professional users under controlled environmental conditions. For more information, refer to the HELIOS AUTOMATED IFA SYSTEM Instruction Manual Catalogue #20140055. All suggested results obtained with the HELIOS AUTOMATED IFA SYSTEM must be confirmed by trained personnel.

The HELIOS AUTOMATED IFA SYSTEM can also be referred to as the HELIOS. The HELIOS is designed for routine laboratory use by professional users under controlled environmental conditions and under compliance with installation and maintenance requirements. The system performs immunofluorescence assays from patient samples, for use as aid in the diagnosis of various autoimmune diseases in conjunction with other clinical and laboratory findings.

4. STORAGE AND SHELF LIFE

Store all reagents at 2°C-8°C/35-46°F, protected from intense light. The expiration date of each component is indicated on the respective label. Do not use reagents beyond the expiration date.

Once prepared, reconstituted solutions are stable for 1 week at 2-8°C/35-46°F.

Ready to use reagents (sample diluent, conjugate, controls and mounting medium) should be used within the expiration date. Once opened, these reagents are stable for up to 7 months.

5. PRECAUTIONS OF USE / HEALTH HAZARD INFORMATION

THIS PRODUCT IS FOR IN VITRO DIAGNOSTIC USE ONLY. Thus, only staff trained and specially advised in methods of in vitro diagnostics may perform the kit. Although this product is not considered particularly toxic or dangerous in conditions of intended use, refer to the following for maximum safety:

1. Patient samples, as well as all materials coming into contact with them, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories", 1984 Edition.
2. This kit contains potentially hazardous components. Though kit reagents are not classified as being irritants to eyes and skin we recommend avoiding contact with eyes and skin and wearing disposable gloves.



3. All human source material used for some reagents of this kit (e.g. controls) have been tested by approved methods and found negative for HBsAg, Hepatitis C and HIV. However, no test can guarantee the absence of viral agents in such material completely. Thus handle kit controls and patient samples as if capable of transmitting infectious diseases and according to national requirements.
4. The kit contains material of animal origin (BSA, Immunoglobulin) as stated in the table of kit components, handle according to national requirements.
5. Do not pipette by mouth. Do not smoke, eat or drink when manipulating the kit.
6. Do not mix or substitute reagents from different lot numbers. This may lead to variations in the results.
7. Keep all bottles capped/closed after use to avoid bacterial contamination.
8. Always pipette all solutions with new sterile pipetting tips.
9. Never expose components to temperatures higher than 37°C / 98.6°F.
10. Never let the slide wells dry out during the entire procedure.
11. Never freeze the slides.
12. Each laboratory should establish its own in house controls upon its own techniques, controls, equipment and patient population according to its established procedures.
13. A clinical diagnosis must not be based on the results of the performed test only, but should be made by the physician after all clinical and laboratory findings have been evaluated.
14. Positive test results may not be valid in persons who have received blood transfusions or various blood products within the past several months [19].
15. Test results on specimens from immunosuppressed patients and pregnant women may be difficult to interpret [20, 21, 22].



6. KIT PROCEDURE

6.1 Sample Collection and Handling

Use freshly collected serum samples. Blood withdrawal must follow national requirements. Collect blood samples aseptically.

Lipemic, icteric, hemolysed or microbially contaminated specimens may cause interference.

Sera with particles should be cleared by low speed centrifugation (<1000 x g). Blood samples should be collected in clean, dry and empty tubes. After separation, the serum samples should be used during the first 8h and then stored tightly closed at 2-8°C/35-46°F up to 48h, or frozen at -20°C/-4°F for longer periods. Avoid repeated freezing and thawing. [23, 24]

6.2 Screening and Titrations

6.2.1 Suggested Screening dilution

Suggested screening dilution ratio:

1:20 (e.g. 20µL Serum + 380µL 1x Sample buffer)

It is recommended that each laboratory establishes its own screening dilution based on its own patient population and instrumentation used.

6.2.2 Titrations / Serial dilutions (Manual)

After screening the samples for the presence of ANCAs, a titration of ANCA-positive samples is recommended. The reciprocal of the highest dilution in which staining intensity just appears positive is expressed as endpoint titer.

Example for a twofold serial dilution:

- Prepare a 1:20 dilution of each patient's serum by adding 20µl serum to 380µl of 1x Sample Buffer (tube#1). Mix well.
- Add 200µl 1x Sample Buffer to tubes #2, #3, #4 and #5.
- Using a 200µl pipette, transfer 200µl from tube #1 to tube #2. Mix well. Using a new tip for each dilution, transfer 200µl from tube #2 to tube #3. Mix well. Repeat this for the remaining tubes. Thus, the tubes contain the following dilutions:

Tube #1 = 1:20

Tube #2 = 1:40

Tube #3 = 1:80

Tube #4 = 1:160

Tube #5 = 1:320

NOTE: The Positive and Negative Controls are intended to be used undiluted. However, if performing a semi-quantitative test, the Positive Control should be diluted as suggested above; assuming the contents of the bottle is equivalent to a dilution of 1:20.



6.2.3 Titrations / Serial dilutions (HELIOS)

Additional titrations can also be performed on the HELIOS by creating a worklist and selecting the desired titrations. See HELIOS AUTOMATED IFA SYSTEM Instruction Manual for more detailed instructions.

6.3 Reagent Preparation

Allow all components to reach room temperature (20 - 26°C / 64 – 79°F) before use and mix well (do not shake or introduce air bubbles).

6.3.1 Manual Assay Reagent Preparation

1. Dilute the concentrated wash buffer 1:10 with distilled water.
2. Dilute patient sera (1:20 dilution, according to the screening dilution chosen) with sample buffer.
3. Controls are ready to use.

6.3.2 Reagent Preparation for use with the HELIOS

1. Dilute the concentrated wash buffer 1:10 with distilled water.
2. Controls are ready to use.
3. The samples will be automatically diluted by the HELIOS.

6.4 Workflow

6.4.1 Manual Workflow

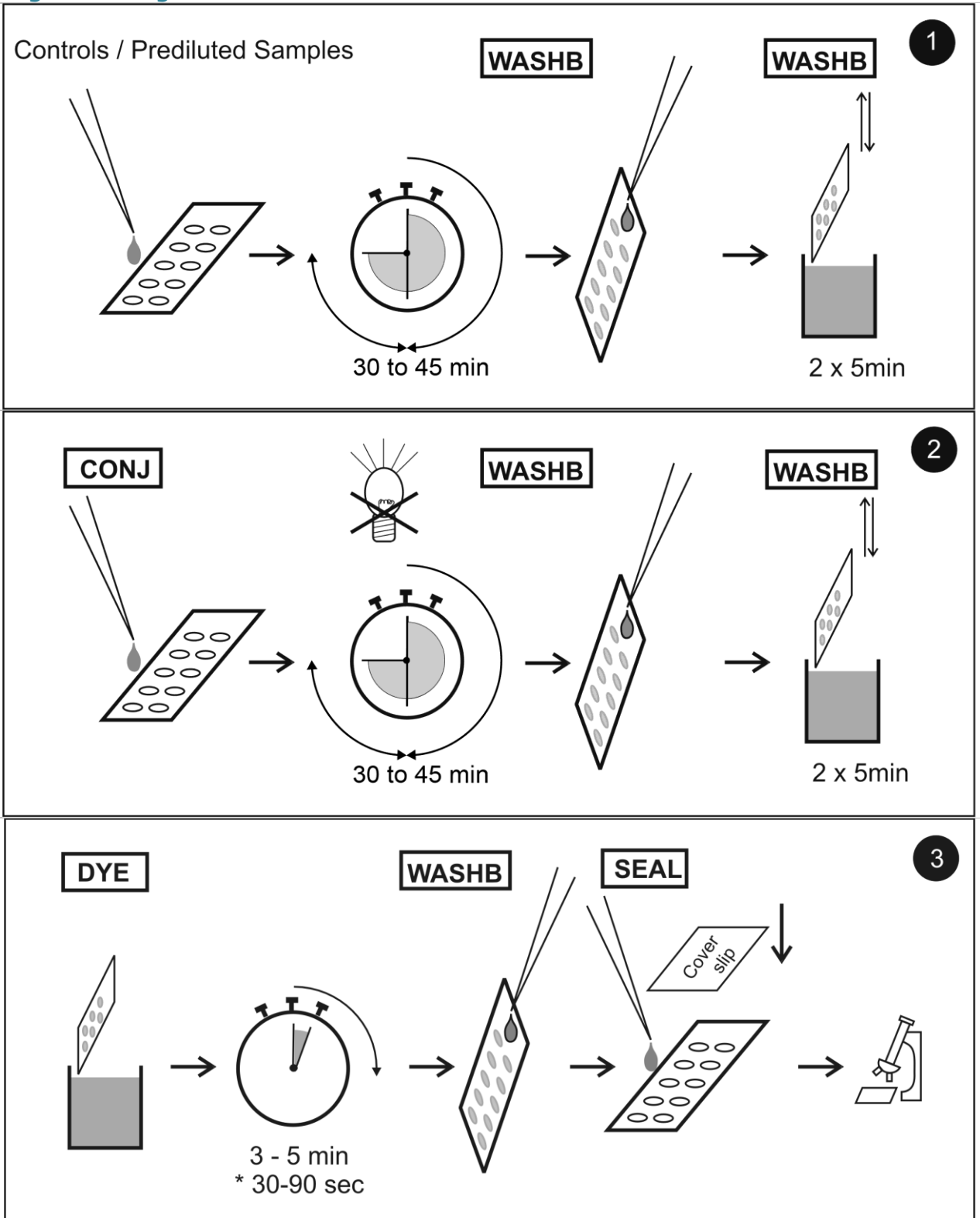
No.	Step description
1.	Remove required slide(s) from pouch(es) and mark them. Do not touch the wells. Do not allow the slides to dry out.
2.	Preparation of incubator tray: Place a small volume of deionized or distilled water in an incubator tray and place slide(s) on supports in the incubator tray. First incubation: Pipette an adequate volume of each diluted serum and controls (ready to use) into the appropriate wells, avoid direct contact of pipette with slide surface. Make sure that each well is completely covered with serum. It is important to use as much test material as is necessary to cover the well completely. However, avoid serum overlapping between wells as this may cause incorrect results. Incubate slide(s) 30 minutes (not exceeding 45 minutes) at room temperature in the moist incubator tray. Use consistent incubation times for the samples.
3.	Washing: After incubation, remove slides from incubator tray and rinse briefly with wash buffer using a squeeze wash bottle. Do not squirt buffer directly on the wells. NOTE: To prevent cross contamination, tilt the slide first towards one row and carefully run a stream of wash buffer along the midline of the slide, allowing the wash buffer to run off the lower edge of the slide. Then tilt the slide towards the other row, and repeat this procedure, allowing the wash buffer to run off what is now the lower edge of the slide. Wash slide(s) 10 minutes with wash buffer in a slide staining dish. Avoid direct contact of solid items with the substrate. For optimal results, change the buffer solution once after 5 minutes. Lift slide(s) out of staining dish and carefully remove excess washing buffer.



	NOTE: It is important that slide wells do not dry out during the procedure as this may lead to damage to the substrate. Do not blot or dry the slide in any manner or allow slide to sit without fluorescent antibody reagent for longer than a few seconds.
4.	Second incubation: After the washing procedure, return the slide immediately to incubator tray and cover each well with an adequate volume of conjugate and make sure that the well is covered completely. Incubate slide(s) for 30 minutes (not exceeding 45 minutes) at room temperature in the dark.
5.	Washing: After incubation, remove slides from the incubator tray and rinse briefly with wash buffer using a squeeze wash bottle. Do not squirt buffer directly on the wells. Wash slide(s) 10 minutes with wash buffer in a slide staining dish. For optimal results, change the buffer solution once after 5 minutes.
6.	*Optional counterstain: Dilute counterstain (Evans Blue) 1:3000 in Wash buffer and mix well. Tilt counterstain into the staining dish and incubate the slides in it. Evans Blue covers unspecific background fluorescence. Counter staining time: 30 to 90 seconds Remove slide(s) after the incubation time and rinse briefly with washing buffer. Remove excess washing buffer. Please do not blot or dry the slide in any manner.
7.	Mounting Medium: Add an adequate volume of mounting medium along midline of each slide. Carefully place coverslip in position, avoiding air bubbles.
8.	Reading: Read slide(s) immediately at 400 - 800 x total magnification with a fluorescent microscope (490 nm excitation filter, 510 nm barrier filter). If not read immediately, cover (protect from light) and store the slide(s) at 2-8°C/35-46°F.



Figure 1. Diagram of Manual Workflow



6.4.1 HELIOS Workflow

For more detailed instructions, refer to HELIOS® Automated IFA System Instruction Manual (Catalogue #20140055).

No.	Step description
1.	Activate the HELIOS software by double-clicking 
2.	Select Instrument. Select 
3.	Load samples into the sample racks.
4.	Remove required slide(s) from pouch(es) and label them. Do not touch the wells.
5.	Load reagents and slide(s) onto the HELIOS.
6.	Create a new worklist or open an existing worklist.
7.	Select test: AESKU_HELIOS_ANCAE 12W or 6W or/and AESKU_HELIOS_ANCAF 12W or 6W
8.	Select Screening mode and choose "Finish".
9.	If the "Scan Sample Barcodes When Creating New Worklist" and "Scan Rack Layout When Creating New Worklist" has been pre-selected, the HELIOS will scan the racks and read the sample barcodes. Otherwise, manually enter the Sample ID's in the worklist.
10.	Select Start. If "Start Loading Wizard Before Run" has been selected, the Reagent Wizard will provide prompts to double-check the Reagent placement. If it has not been pre-selected, the run will start right away.
11.	The HELIOS automatically processes the slides. The HELIOS image capturing starts automatically after the mounting medium has been added.
12.	HELIOS Result confirmation: Perform classification as described in the HELIOS Instruction Manual. All suggested results obtained with the HELIOS must be confirmed by trained personnel.



7 QUALITY CONTROL

Both a positive and negative serum control must be included in each run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Positive Control (2 Types):

Type 1: PC54.100 ANCA pattern control C-ANCA Capped red

- a. Viewed with a manual microscope: Using the positive C-ANCA pattern control serum as provided with the Aeskuslides ANCA (Ethanol/Formalin) test system, the neutrophils should exhibit a cytoplasmic staining pattern with a fluorescent intensity of 3+.
- b. Viewed with the HELIOS: Using the positive C-ANCA pattern control serum as provided with the Aeskuslides ANCA (Ethanol/Formalin) test system, the neutrophils should exhibit a cytoplasmic staining pattern with a strong fluorescent intensity when compared to the negative control. The HELIOS Image Capturing module should pre-classify the Positive Control as positive.

Type 2: PC54.101 ANCA pattern control P-ANCA Capped red

- a. Viewed with a manual microscope: Using the positive P-ANCA pattern control serum as provided with the Aeskuslides ANCA (Ethanol/Formalin) test system, the neutrophils should exhibit a perinuclear staining pattern with a fluorescent intensity of 3+.
- b. Viewed with the HELIOS: Using the positive P-ANCA pattern control serum as provided with the Aeskuslides ANCA (Ethanol/Formalin) test system, the neutrophils should exhibit a perinuclear staining pattern with a strong fluorescent intensity when compared to the negative control. The HELIOS Image Capturing module should pre-classify the Positive Control as positive.

Negative Control:

- a. Viewed with a manual microscope: Using the Aeskuslides negative control serum as provided with the Aeskuslides ANCA (Ethanol/Formalin) test system, the neutrophils should exhibit less than 1+ fluorescence and appear green.
- b. Viewed with the HELIOS: Using the Aeskuslides negative control serum as provided with the Aeskuslides ANCA (Ethanol/Formalin) test system, the neutrophils should appear green but not fluorescent when compared with the positive control. The HELIOS Image Capturing module should pre-classify the Negative Control as negative.

Each control must demonstrate the expected reaction in order to validate the test. If the controls fail to appear as described above, the test results should not be reported and the test should be repeated. If upon repeated testing the controls still fail to show the proper reaction, do not report test results.

Optional Titer Control

A certificate of analysis (CoA) included in each Aeskuslides ANCA (Ethanol/Formalin) kit for the Positive Control (PC) provides with it a calculated endpoint titer. As an additional (optional) control, the positive control can be used to test substrate sensitivity, check technique, conjugate quality and the microscope optical system by evaluating a set of PC dilutions.

To perform this optional sensitivity test, dilute the PC fourfold (+/-) with sample diluent. Include in the run one fourfold dilution above and one fourfold dilution below the PC endpoint titer.



Acceptance criterion: The final endpoint titer must be within +/- one dilution range around the CoA endpoint titer. If the diluted controls fail to show the proper reaction upon repeated testing, do not report the test results.

Example dilution scheme:

Values indicated on the CoA:

- PC pre-diluted at 1/20
- Calculated Endpoint Titer: 1/320

Dilution	1/40	1/80	1/160	1 /320	1/640
Sample diluent volume	50 µl	50 µl	50 µl	50 µl	50 µl
PC volume	50 µl	-	-	-	-

If the results are positive at one of the expected value dilutions (1/160, 1/320, or 1/640), the run passes the Optional Titer Control test. If the results are negative at all of the expected value dilutions, then Optional Titer Control test fails. Do not report test results.

Adapt the example schema to the endpoint titer value provided in the CoA (EPT values may differ between lots).

8 RESULTS

To receive utmost information about the antibodies present in the serum, samples should be tested on both AESKUSLIDES ANCA Ethanol and AESKUSLIDES ANCA Formalin. Based upon those results further antigen-specific solid phase assays should be run to confirm antigen specificity.

8.1 Positive/Negative Confirmation (Manual Microscope)

The **fluorescence intensity** level is the intensity of the specific fluorescence expressed as a numeric value. These values, if present, are reported as a number between "0" (no specific fluorescence) and "4+" (very strong visible reaction).

Intensity	Interpretation	
4+	high positive	maximal fluorescence, very strong visible reaction; brilliant yellow-green
3+	positive	strong visible reaction; less brilliant as 4+; yellow-green fluorescence
2+	positive	moderate visible reaction; definite but dull yellow-green fluorescence
1+	positive	weak visible reaction, very dim subdued fluorescence
0	negative	no specific fluorescence



The degree of fluorescent intensity is not clinically relevant and has only limited value as an indicator of titer. Differences in fluorescence microscope optics, filters and light sources may result in differences of 1+ or more fluorescent intensity when observing the same slide using different microscopes.

Positive Results

A sample is considered positive for ANCA antibodies if the cells exhibit $\geq 1+$ fluorescence and a discernible pattern at a sample dilution of 1:20 or greater.

Positive IIF results should be confirmed by antigen (MPO and PR3)-specific solid phase assays.

Negative Results

A serum dilution is considered negative for ANCA if the cells exhibit less than 1+ fluorescence and lacks a clearly discernible pattern.

Negative samples may exhibit fluorescent staining slightly greater than the Negative Control, but less than 1+.

A sample is interpreted as "borderline" by the user if the cells are not clearly positive or negative. "Unclassified" samples are samples which cannot be classified as positive, negative or borderline by the user. If that is the case, for example if there are no evaluable images, the user can manually select "unclassified". Borderline and unclassified samples should be measured again.

For **semi-quantitative** or **end point titer determination** a sample tested positive can be diluted serially as described in Chapter 6.2.

The result should be reported as the reciprocal of the last dilution in which 1+ apple-green fluorescent intensity with a clearly discernible staining pattern is detected.

8.2 Interpretation of Patterns

The two main **patterns** seen on an ethanol-fixed substrate are perinuclear (P-ANCA) and cytoplasmic (C-ANCA):

- C-ANCA presents as coarse speckled cytoplasmic fluorescence, often with accentuated staining between the nuclear lobes. This pattern is characteristic for antibodies reacting with PR3.
- P-ANCA presents as perinuclear staining with or without nuclear extension. This pattern is usually characteristic for antibodies reacting with MPO.

On formalin-fixed substrate, both MPO/P-ANCA and PR3/C-ANCA antibodies appear as coarse cytoplasmic granular staining with interlobular accentuation (C-ANCA).

Note that anti-nuclear antibody (ANA) positive samples may react with the nuclei of ethanol-fixed neutrophils, causing nuclear staining, that may mask or mimic the ANCA patterns. Furthermore, atypical ANCAs (A-ANCA) can occur and they can present as a perinuclear or very perinuclear staining on ethanol-fixed neutrophil substrate. ANAs and atypical ANCAs usually become negative on formalin-fixed neutrophils [25, 26, 27, 28].



8.3 User confirmation of HELIOS results

The HELIOS Result Confirmation module facilitates software-based documentation of positive/negative identification. For user classification purposes, the images are available for each well along with the pattern suggestion. **All suggested results obtained with the HELIOS must be confirmed by trained personnel.**

For further details on how to complete results confirmation, refer to the HELIOS Instruction Manual.

Determination of endpoint titer on HELIOS

After the first screening run, a titration of positive samples is strongly recommended. The HELIOS EPT Plus offers the possibility to estimate an endpoint titer for wells that were pre-classified as positive in a previous screening mode run. Supported by this endpoint titer estimation, the next dilution steps can easily be selected during the user classification process. Keep in mind that this is an estimated support value that can differ from the real endpoint titer of the sample and should only be used by trained personnel.

Furthermore, we recommend for the end point titer results obtained by titration of samples on HELIOS to be confirmed by the manual method, considering that the automated method (with reader confirmation) results in only an estimate of the end point titer.

9 LIMITATIONS OF PROCEDURE

1. All results must be confirmed by a trained operator.
2. Formalin ANCA fixation is generally used in conjunction with Ethanol ANCA fixation, and formalin fixation is not intended as a stand-alone test.
3. Decisions about treatment should not be based solely on ANCA IFA results. ANCA serological test results should be used in conjunction with information available from the clinical evaluation and other diagnostic information. Positive ANCA IFA results should be confirmed by MPO and PR3 ELISA. ANA positive samples may react with fixed neutrophils, generating fluorescence patterns resembling ANCA patterns.
4. Endpoint titers may vary between laboratories due to differences in type or condition of fluorescence microscope employed or assay procedure used. We recommend for the end point titer results obtained by titration of samples on HELIOS to be confirmed by the manual method, considering that the automated method (with reader confirmation) results in only an estimate of the end point titer.
5. If both the positive and negative control substrate cells are not visible when viewed using the fluorescence microscope, it may be necessary to replace or realign the light source and check the specific filters.
6. Cell culture substrate slides may exhibit nonspecific fluorescence due to contamination of antibodies or PBS rinse-wash solutions with bacteria or fungi. It is very important that personnel reading the staining results have experience in fluorescence microscopy.
7. In general titers of 1:20 and 1:40 are considered low titers, 1:80 and 1:160 are considered medium titers, and 1:320 and greater are considered high titers. It is recommended that each laboratory establish its own reference ranges based on its own population and instrumentation used.



Special Controls

1. For Prescription Use Only.
2. Only for use with reagents that are indicated for use with the assay.
3. The assay is for use by a trained operator in a clinical laboratory setting.
4. All software-aided results must be confirmed by the trained operator.
5. For use only by manual microscopy or with HELIOS AUTOMATED IFA SYSTEM.

10 EXPECTED VALUES

Each laboratory should establish their own normal control ranges dependent on their own techniques, controls, equipment, patient population and according to their own established procedures.

A definite clinical diagnosis should not be based on the results of the performed test only, but should be made by the physician after all clinical and laboratory findings have been evaluated.

To determine the prevalence of ANCA, a panel of 150 sera from healthy donors was tested with AESKUSLIDES ANCA. Each serum was tested at a dilution of 1:20. Slides were processed manually according to the IFU and subsequently analyzed at the microscope by two independent readers.

Normal Range Study	ANCA Ethanol			ANCA Formalin		
	Pos (%)*	Neg (%)	Total	Pos (%)	Neg (%)	Total
Reader 1	6 (4)	144 (96)	150	6 (4)	144 (96)	150
Reader 2	3 (2)	147 (98)	150	4 (2.7)	146 (97.3)	150

* [6, 29, 30, 31, 32]

11 SPECIFIC PERFORMANCE CHARACTERISTICS

Intra-Assay Precision at 1:20 Dilution AESKUSLIDES ANCA Ethanol

Average % Agreement (95% CI)	HELIOS	HELIOS w/ User Confirmation	Manual AESKUSLIDES
Positive Agreement	99.2 (97 - 99.8)	100 (99.2 - 100)	97.5 (95.7 - 98.6)
Positive Agreement w/o borderline samples	98.9 (96 - 99.7)	100 (98.9 - 100)	100 (98.9 - 100)
Negative Agreement	100 (94 - 100)	100 (96.9 - 100)	100 (96.9 - 100)
Overall Agreement	99.3 (97.6 - 99.8)	100 (99.4 - 100)	98 (96.5 - 98.9)
Overall Agreement w/o borderline samples	99.2 (97 - 99.8)	100 (99.2 - 100)	98.8 (96.4 - 99.6)
Pattern Agreement	74.2 (68.3 - 79.3)	94.8 (92.4 - 96.4)	96.9 (94.9 - 98.1)
Pattern Agreement w/o borderline samples	89.4 (84.1 - 93.1)	93.1 (89.9 - 95.3)	99.2 (97.6 - 99.7)
FI Agreement	na	na	98.5 (97.3 - 99.1)
FI Agreement w/o borderline samples	na	na	97.7 (95.9 - 98.7)

Inter-Assay Precision at 1:20 Dilution AESKUSLIDES ANCA Ethanol

% Agreement (95% CI)	HELIOS	HELIOS w/ User Confirmation	Manual AESKUSLIDES
Positive Agreement	90.1 (87.7 - 92.1)	93.7 (92.3 - 94.8)	94.6 (93.3 - 95.6)
Positive Agreement w/o borderline samples	96.3 (94.3 - 97.6)	99.3 (98.5 - 99.6)	100 (99.6 - 100)
Negative Agreement	90 (84.7 - 93.6)	98.3 (96.4 - 99.2)	100 (98.9 - 100)
Overall Agreement	90.1 (88 - 91.9)	94.6 (93.5 - 95.6)	95.7 (94.6 - 96.5)
Overall Agreement w/o borderline samples	94.7 (92.8 - 96.1)	99 (98.4 - 99.4)	100 (99.7 - 100)
Pattern Agreement	66.1 (62.6 - 69.5)	89.7 (88 - 91.2)	94.4 (93.1 - 95.5)
Pattern Agreement w/o borderline samples	81.3 (77.8 - 84.4)	94 (92.4 - 95.3)	99.7 (99.2 - 99.9)
FI Agreement	na	na	98.9 (98.4 - 99.3)
FI Agreement w/o borderline samples	na	na	98.9 (98.4 - 99.3)

Intra-Assay Precision at 1:20 Dilution AESKUSLIDES ANCA Formalin

%Agreement (95% CI)	HELIOS	HELIOS w/ User Confirmation	Manual AESKUSLIDES ANCA Formalin
Positive Agreement	100 (98.4 - 100)	100 (99.2 - 100)	100 (99.2 - 100)
Negative Agreement	93.3 (84.1 - 97.4)	95 (89.5 - 97.7)	94.2 (88.4 - 97.1)
Overall Agreement	98.7 (96.6 - 99.5)	99 (97.8 - 99.5)	98.8 (97.6 - 99.4)
Pattern Agreement	95.4 (92 - 97.4)	100 (99.2 - 100)	100 (99.2 - 100)
FI Agreement	na	na	100 (98.7 - 100)

Inter-Assay Precision at 1:20 Dilution AESKUSLIDES ANCA Formalin

% Agreement (95% CI)	HELIOS	HELIOS w/ User Confirmation	Manual AESKUSLIDES ANCA Formalin
Positive Agreement	96.7 (95.1 - 97.7)	97.5 (96.6 - 98.2)	99 (98.3 - 99.4)
Negative Agreement	77.2 (70.6 - 82.7)	94.4 (91.6 - 96.4)	98.1 (96 - 99.1)
Overall Agreement	92.8 (90.9 - 94.3)	96.9 (96 - 97.6)	98.8 (98.2 - 99.2)
Pattern Agreement	87.9 (85.3 - 90.1)	95.7 (94.5 - 96.6)	99 (98.3 - 99.4)
FI Agreement	na	na	97.7 (96.8 - 98.4)



Sensitivity/Specificity at 1:20 Dilution

Diagnostic sensitivity and specificity for AESKUSLIDES ANCA Ethanol:

AAV	Non-healthy Controls
% Sensitivity (95% CI)	% Specificity (95% CI)
Manual	
30.1 (27.1 - 33.4)	74.8 (73 - 76.6)
HELIOS with User Confirmation	
30.4 (27.3 - 33.6)	76.9 (75.1 - 78.6)
HELIOS	
26.4 (22.4 - 30.9)	81.7 (79.3 - 83.8)

Diagnostic sensitivity and specificity for AESKUSLIDES ANCA Formalin:

AAV	Non-healthy Controls
% Sensitivity (95% CI)	% Specificity (95% CI)
Manual	
41.1 (37 - 45.3)	92 (90.5 - 93.3)
HELIOS with User Confirmation	
44.4 (40.3 - 48.7)	91.8 (90.3 - 93.1)
HELIOS	
43.7 (37.9 - 49.7)	94.8 (93 - 96.2)

12 TROUBLESHOOTING

12.1 Troubleshooting - Manual

ERROR	POSSIBLE CAUSES	SOLUTION
Low cell density	-Cell lysis following prolonged contact with deionized water -Buffer squirted directly on the substrate in the well	Follow the recommended wash procedure
	Proteolytic enzymes have attacked the substrate	Inactivate serum
Uneven fluorescence	Serum dried in the well, fluorescence stronger at the edge	Always incubate in a humid environment
	Serum does not cover the test well	Apply an adequate volume of test material
	Cross-reaction between the wells	Avoid running between the wells in the first incubation
	Marking the slide with a wax pencil produces a film on the slide	Use a standard (non-wax) pencil
	Microscope incorrectly adjusted	Check the adjustment of the UV-lamp
Diffuse picture	Slide stored in refrigerator without cover	Seal slide with nail polish or paraffin wax
	I.F. Microscope is dirty. Possible scratches on the lens	Clean the microscope according to its instructions
Little or no fluorescence	Conjugate and slides thawed and refrozen	Conjugate and slides stored at 2°C-8°C/35-46°F.
	Controls diluted	Check instructions, use ready to use kit controls
	-Bacterial contamination of the sera or conjugate -Microscope not adjusted -pH-value of washing buffer too low (pH value 7.4 ± 0.2)	Check conditions
	FITC conjugate exposed to light	Store conjugate protected from the light
Background fluorescence	- Incorrectly washed - Slide dried out - Lipemic, hemolytic sera - Microscope error	- Check the washing instructions - Do not allow the slide to dry out - Use only fresh sera - Check correct filter / objective

12.2 Troubleshooting - HELIOS

ERROR	POSSIBLE CAUSES	SOLUTION
Low cell density	Cell lysis following prolonged contact with deionized water	-Refer to HELIOS User Manual -Perform weekly maintenance
	Proteolytic enzymes have attacked the substrate	Inactivate serum
Uneven fluorescence	Serum dried in the well, fluorescence stronger at the edge	-Refer to HELIOS User Manual -Perform weekly maintenance
	Serum does not cover the test well	
	Cross-reaction between the wells	-Refer to HELIOS User Manual
	Microscope incorrectly adjusted	
Diffuse picture	I.F. Microscope is dirty. Possible scratches on the lens	- Clean the microscope according to its instructions -Refer to HELIOS User Manual
Little or no fluorescence	Conjugate and slides thawed and refrozen	Conjugate and slides stored at 2°C-8°C/35-46°F.
	Controls diluted	Check instructions, use ready to use kit controls
	-Bacterial contamination of the sera or conjugate -Microscope not adjusted -pH-value of Washing buffer too low (pH value 7.4 ± 0.2)	Check conditions
	FITC conjugate exposed to light	Store conjugate protected from the light
Background fluorescence	- Incorrectly washed - Slide dried out - Lipemic, hemolytic sera - Microscope error	- Refer to HELIOS User Manual - Perform weekly maintenance - Use only fresh sera - Check correct filter / objective



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IVD	- Diagnosi in vitro	in vitro diagnostic use
	- Pour diagnostic in vitro	- Para uso diagnóstico in vitro
	- In Vitro Diagnostikum	- In Vitro Διαγνωστικό μέσο
	- Para uso Diagnóstico in vitro	
REF	“ Numero d'ordine	“ Catalogue number
	“ Référence Catalogue	“ Numéro de catálogo
	“ Bestellnummer	“ Αριθμός παραγγελίας
	“ Número de catálogo	
LOT	“ Descrizione lotto	“ Lot
	“ Lot	“ Lote
	“ Chargen Bezeichnung	“ Χαρακτηρισμός παρτίδας
	“ Lote	
CE	“ Conformità europea	“ EC Declaration of Conformity
	“ Déclaration CE de Conformité	“ Declaración CE de Conformidad
	“ Europäische Konformität	“ Ευρωπαϊκή συμφωνία
	“ Declaração CE de Conformidade	
	“ Rispettare le istruzioni per l'uso	“ See instructions for use
	“ Voir les instructions d'utilisation	“ Ver las instrucciones de uso
	“ Gebrauchsanweisung beachten	“ Λάβετε υπόψη τις οδηγίες χρήσης
	“ Ver as instruções de uso	
	“ Da utilizzarsi entro	“ Use by
	“ Utilise avant le	“ Utilizar antes de
	“ Verwendbar bis	“ Χρήση μέχρι
	“ Utilizar antes de	
	“ Conservare a 2-8°C	“ Store at 2-8°C (35-46°F)
	“ Conserver à 2-8°C	“ Conservar a 2-8°C
	“ Lagerung bei 2-8°C	“ Φυλάσσεται στους 2-8°C
	“ Conservar entre 2-8°C	
	“ Prodotto da	“ Manufactured by
	“ Fabriqué par	“ Fabricado por
	“ Hergestellt von	“ Κατασκευάζεται από
	“ Fabricado por	
DYE	“ Colorante Blue-Evans	“ Evans-Blue Dye
	“ coloration au Bleu Evans	“ Colorante Azul de Evans
	“ Evans-Blue Färbelösung	“ Evans Blue
	“ Evans Blue	
CONTROL +	“ Controllo positivo	“ Positive Control
	“ Contrôle Positif	“ Control Positivo
	“ Positiv Kontrolle	“ Θετικός ορός ελέγχου
	“ Controllo positivo	
CONTROL -	“ Controllo negativo	“ Negative Control
	“ Contrôle Négatif	“ Control Negativo
	“ Negativ Kontrolle	“ Αρνητικός ορός ελέγχου
	“ Controllo negativo	
SEAL	“ Mezzi di montaggio	“ Mounting media
	“ milieu de montage	“ Medio de montaje
	“ Mounting Medium	“ Μέσο μονιμοποίησης
	“ Meio de montagem	
CONJ	“ Coniugato	“ Conjugate
	“ Conjugué	“ Conjugado
	“ Konjugat	“ Σύζευγμα
	“ Conjugado	
	“ Vetrino per microscopio	“ Microscope slide
	“ lame de microscope	“ Portaobjetos
	“ Objektträger	“ Αντικειμενοφόρο πλακίδιο
	“ Lâmina	
WASHB 10x	“ Tampone di lavaggio	“ Wash Buffer
	“ Tampon de Lavage	“ Solução de lavagem
	“ Waschpuffer	“ Ρυθμιστικό διάλυμα πλύσης
	“ Solución de lavado	
SB 1x	“ Tampone di campione	“ Sample Buffer
	“ Tampon de Echantillons	“ Solução de Muestras
	“ Probenpuffer	“ Ρυθμιστικό διάλυμα δειγμάτων
	“ Solución de Muestras	
	“ XX determinazioni	“ XX tests
	“ XX tests	“ XX pruebas
	“ XX Bestimmungen	“ XX προσδιορισμοί
	“ XX Testes	



AESKU.DIAGNOSTICS
THE DIAGNOSTIC TOOL THAT WORKS

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Rx only

For Prescription Use Only