Oesophagus (Rat)
Instructions for the indirect immunofluorescence test

ORDER NO.	ANTIBODIES AGAINST	SUBSTRATE	SPECIES	FORMAT SLIDES x FIELDS
FA 1503–1003 FA 1503–1005 FA 1503–1010 FA 1503–2005 FA 1503-2010	keratin („RA keratin", filaggrin)	oesophagus	rat	10 x 03 (030) 10 x 05 (050) 10 x 10 (100) 20 x 05 (100) 20 x 10 (200)

Indication: Rheumatoid arthritis (RA).

Test principle: This test kit is designed exclusively for the in vitro determination of human antibodies in serum or plasma. The determination can be performed qualitatively or quantitatively.

Frozen sections of rat oesophagus are incubated with diluted patient sample. If the reaction is positive, specific antibodies of classes IgA, IgG and IgM attach to oesophagus antigens. In a second step, the attached antibodies are stained with fluorescein-labelled anti-human antibodies and made visible with the fluorescence microscope.

Contents of a test system for 50 determinations: FA 1503-1005 (IgG)

Description	Format	Symbol
1. Slides, each containing 5 BIOCHIPs coated with frozen sections of rat oesophagus	10 slides	SLIDE
2. Fluorescein-labelled anti-human IgG (goat), ready for use	1 x 1.5 ml	CONJUGATE
3. Positive control: autoantibodies against keratin, human, ready for use	1 x 0.1 ml	POS CONTROL
4. Negative control: autoantibody negative, human, ready for use	1 x 0.1 ml	NEG CONTROL
5. Salt for PBS pH 7.2	2 packs	PBS
6. Tween 20	2 x 2.0 ml	TWEEN 20
7. Mounting medium, ready for use	1 x 3.0 ml	GLYCEROL
8. Cover glasses (62 mm x 23 mm)	12 pieces	COVERGLASS
9. Instruction booklet	1 booklet	---

LOT	Lot description		Storage temperature
IVD	In vitro diagnostics	CE	Unopened usable until

Single slides (e.g., EUROIMMUN order no. FB 1503-1005) are provided together with cover glasses. Additional positive control (e.g., order no. CA 1508-0101) and negative control (e.g., order no. CA 1000-0101) 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 9999-0110 Reagent trays for slides containing up to 10 fields

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.

Performing the test (reaction fields 5 x 5 mm)

The **TITERPLANE Technique** was developed by EUROIMMUN in order to standardize 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 fluids, 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 this test instruction.

Pipette: Apply **30 µl 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 min** 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 min**. Shake with a rotary shaker if available. Wash max. 16 slides then replace PBS-Tween with new buffer.

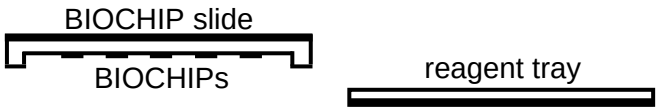
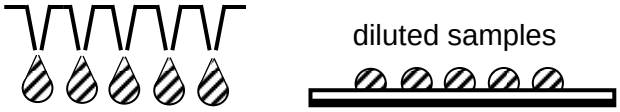
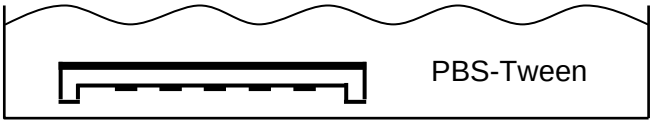
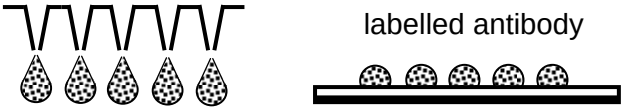
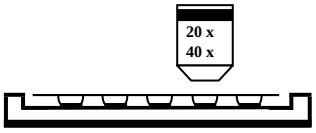
Pipette: Apply **25 µl of fluorescein labelled anti-human globulin** to each reaction field of a clean reagent tray. Add all droplets before continuing incubation. Use a stepper pipette. The labelled anti-human serum should be mixed before use. To save time, conjugate can be pipetted onto separate reagent trays during the incubation with the diluted sample.

Incubate: Remove one BIOCHIP Slide from 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 min** 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 min**. Shake with a rotary shaker if available. 10 drops of Evans Blue for each 150 ml phosphate buffer can be added for counterstaining. Wash max. 16 slides then replace PBS-Tween with new buffer.

Mount: Place mounting medium onto a cover glass - drops of **max. 10 µl per reaction field**. Use a polystyrene mounting tray. Remove one BIOCHIP Slide from PBS Tween and dry the back, all four sides, as well as the surface around, but not between the reaction fields 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: Read the fluorescence with the microscope.
General recommendation: Objective 20x (tissue sections, infected and transfected cells), 40x (cell substrates).
Excitation filter: 488 nm, color separator: 510 nm, blocking filter: 520 nm.
Light source: mercury vapor lamp, 100 W, EUROIMMUN LED, EUROStar Bluelight.

TITERPLANE Technique		
Pipette:	30 µl per field	
Incubate:	30 min	
Wash:	1 s flush 5 min cuvette	
Pipette:	25 µl per field	
Incubate:	30 min	
Wash:	1 s flush 5 min cuvette	
Mount:	max. 10 µl per field	
Evaluate:	fluorescence microscopy	

Preparation and stability of reagents

Note: The individual reagents of one lot are matched with one another and should not generally be swapped with reagents of another lot. 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 (condensed water can damage the substrate). Mark with a felt-tip pen. Do not touch the BIOCHIPs. After the protective cover has been opened, the slide should be incubated within 15 minutes. If the protective cover is damaged, the slide must not be used for diagnostics.

- **Fluorescein-labelled secondary antibody (FITC):** 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 min 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**: Reaction fields of the reagent tray must be hydrophilic and surrounding area hydrophobic. If necessary, wipe with Extran MA 01 (Merck) and rinse generously with water. To disinfect: Immerse in Sekusept Extra (Henkel) (3% in water) for 1 hour. After disinfection rinse generously with water and dry with absorbent paper.

Warning: 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 the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of serum and plasma samples

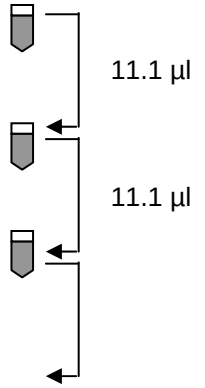
Samples: Human sera or EDTA, heparin or citrate plasma.

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 1:10 in PBS-Tween. For example, dilute 11.1 µl sample in 100 µl PBS-Tween and mix thoroughly, e.g., vortex for 4 seconds.

Recommended Serum dilution for semiquantitative evaluation: The dilution of samples to be investigated is performed using PBS-Tween. For each add 100 µl of PBS-Tween to the 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 1:10.

Dilution	Dilution sheme
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
⋮	⋮



After every two dilution steps, a new pipette tip should be used to prevent carryover.

Evaluation

Fluorescence pattern (positive reaction): Antibodies against „RA keratin" (filaggrin) react with frozen sections of rat oesophagus. A fine, linear fluorescence around the cells of the stratum corneum becomes visible. The pattern shown is essentially the same as that obtained for the positive control serum. Any reactions with other structures are not assessed.

If the cell nuclei or the cytoplasm of all cells are stained, antinuclear antibodies or antibodies against mitochondria and other cell antigens are present.

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)

Recommended qualitative evaluation:

IgG reactivity	**Evaluation**
No reaction at 1:10	Negative. No antibodies against „RA keratin" detected in the patient samples.
Positive reaction at 1:10	Positive. Indication of rheumatoid arthritis.

Recommended semiquantitative evaluation: The titer is defined as the sample dilution factor for which specific fluorescence is just identifiable. This should be compared to the reaction obtained with 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	1:10000	
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	1:10000
⋮	⋮	⋮	⋮	⋮

For diagnosis the clinical symptoms of the patient should always be taken into account along with the serological results.

Limitations of the procedure

1. A diagnosis should not be made 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 residue 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. Contact EUROIMMUN for details.

Test characteristics

Antigen: For the determination of autoantibodies against „RA keratin" (filaggrin) by indirect immunofluorescence, frozen sections of rat oesophagus can be used. These antibodies are specific for rheumatoid arthritis. The specificity of the test is 95 to 99%. The determination of these autoantibodies provides supplementary diagnostic findings in cases where rheumatoid arthritis is suspected, particularly when the result of the standard test for antibodies against rheumatoid factors is negative.

Measurement range: The dilution starting point for this measurement system is 1:10. Samples can be further diluted by a factor of 10 so that the dilution series is 1:100, 1:1000, 1:10000 etc. There is no upper limit to the measurement range.

Intra-assay reproducibility: Inter-lot reproducibility was tested with more than 10 different lots. In quantitative evaluation of results, the deviation amounted to no more than ± 1 fluorescence intensity level for all samples. Intra-assay reproducibility can therefore also be guaranteed. The intensity of the specific fluorescence as a numeric value is called fluorescence intensity level by EUROIMMUN. These values can reach from “0“ (no specific fluorescence) to “5“ (extremely strong specific fluorescence).

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Cross reactivity: There is no data known to EUROIMMUN in which cross reactivities are described.

Interference: Hemolytic, lipemic and icteric samples had no influence on analysis results.

Reference range: Titer 1: < 10

Specifity: The specifity of this test system is 97%. Reference: Anti-CCP-ELISA, n = 82 samples (origin: Germany).

Sensitivity: The sensitivity of this test system is 92%. Reference: Anti-CCP-ELISA, n = 82 samples (origin: Germany).

Clinical significance

In cases of rheumatoid arthritis (RA), various circulating antibodies can be detected. The serological investigation usually consists only of the determination of antibodies against rheumatoid factors (RF). An association of so-called anti-keratin antibodies with this disease has been known for some time. Antibodies against profilaggrin have also been found in RA patients.

Biochemical and molecular biology investigations have shown that these "RA Keratin" specific antibodies are directed against the human target antigen filaggrin of the skin. in the stratum granulosum, the polyprotein profilaggrin is synthesized as a phosphorylated precursor from at least 10 tandem filaggrin units in the keratinocytes and stored in the keratohyaline granula. In the further course of differentiation from granular to keratinised cells, profilaggrin is converted into filaggrin molecules. The filaggrin proteins have a functional participation in the aggregation of cytokeratin filaments to form macrofibrils.

Antibodies against filaggrin can be detected in about 50% of all patients with rheumatoid arthritis (sensitivity 36 - 69%). These antibodies can already be detected in the early stage of the disease. Approximately 30% of RF-negative patients are positive for anti-filaggrin antibodies. Of relevance for assessment are antibodies of the immunoglobulin class IgG, whereby the subclasses mainly represented are IgG1 and IgG4.

Various studies have shown that the determination of antibodies against filaggrin in the early phase of RA is an indicator for the progressive development of the clinical course of the disease. The antibody titer correlates with the activity of the disease, high titers seem to be pathognomonic for rheumatoid arthritis. Investigation into anti-filaggrin antibodies increases the sensitivity of serological testing of patients with suspected RA and takes on a prognostic significance in the early phase of the disease.

In the case of RA patients, it is possible to find antinuclear antibodies (histones, U1-nRNP, ssDNA) and antibodies against smooth muscle in addition. Sporadically, antibodies against filaggrin can also be detected in other rheumatoid diseases, such as systemic lupus erythematosus, systemic sclerosis and ankylosing spondylitis.

Reference list

1. Kühne, S.A.M., Seidel, W., Häntzschel, H.: **Diagnostische Relevanz von Antikeratin-Antikörpern.** Zeitschrift für Rheumatologie, 57 (Suppl I): 42 (1998).

2. Salvador, G., Gomez, A., Vinas, O., Ercilla, G., Canete, J.D., Munoz-Gomez, J., Sanmarti, R.: **Prevalence and clinical significance of anti-cyclic citrullinated peptide and antikeratin antibodies in palindromic rheumatism. An abortive form of rheumatoid arthritis?** Rheumatology 42: 972-975 (2003).

3. Serre, G., Vincent, C.: **Filaggrin (Keratin) Autoantibodies.** In: Peter, J.B., Shoenfeld, Y. Autoantibodies. Elsevier, Amsterdam: 271-276 (1996).

4. Youinou, P., Le Goff, P., Maran, R.: **Perinuclear Factor (Profilaggrin) Autoantibodies.** In: Peter, J.B., Shoenfeld, Y. Autoantibodies. Elsevier, Amsterdam: 618-623 (1996).