Anti-Phospholipase A_2 receptor (PLA_2R) IIFT
Instructions for the indirect immunofluorescence test

ORDER NO.	ANTIBODIES AGAINST	SUBSTRATE	SPECIES	FORMAT SLIDES x FIELDS
FA 1254-1003-50 FA 1254-1005-50 FA 1254-1010-50	phospholipase A_2 receptor (PLA_2R)	transfected cells control transfection	EU 90	10 x 03 (030) 10 x 05 (050) 10 x 10 (100)

Indication: This test kit provides qualitative or semiquantitative in vitro determination of human antibodies of immunoglobulin class IgG against phospholipase A_2 receptor (PLA_2R) in patient samples to support the diagnosis of primary membranous nephropathy (pMN).

Application: Autoantibodies of class IgG against PLA_2R are highly specific for the diagnosis of pMN. They can be detected in 70 to 75% of patients in the serum or plasma. A titer increase, decrease or disappearance precedes a change in the clinical status. Thus, the quantitative determination has a high predictive value with respect to clinical remission or relapse and risk estimation after kidney transplantation. The success of therapy can be assessed by means of the anti-PLA_2R antibody titer course.

Test principle: PLA_2R-expressing cells are incubated with diluted patient samples. 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 a fluorescence microscope.

Contents of a test kit for 50 determinations (e.g. FA 1254-1005-50):

Description	Format	Symbol
1. Slides, each containing 5 x 2 BIOCHIPs coated with PLA_2R-transfected and control-transfected cells	10 slides	SLIDE
2. FITC-labelled anti-human IgG (goat), ready for use	1 x 1.5 ml	CONJUGATE
3. Positive control: anti-PLA_2R, ready for use	1 x 0.1 ml	POS CONTROL
4. Negative control: autoantibody negative, 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 diagnostic medical device	CE	Unopened usable until

Single slides (e.g. EUROIMMUN order no. FB 1254-1005-50) are provided together with cover glasses. Additional positive control (e.g. EUROIMMUN order no. CA 1254-0101) and negative control (e.g. EUROIMMUN 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 (5 x 5 mm)

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.

Modifications to the former version are marked in grey.

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 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 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 **25 µl 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.

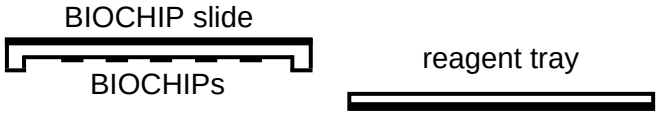
Incubate: Remove one BIOCHIP slide from the cuvette. Within five seconds blot only the back and the long sides with a paper towel and immediately put the BIOCHIP slide into the recesses of the reagent tray. Do not dry the areas between the reaction fields. Check for correct contact between the BIOCHIPs 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.

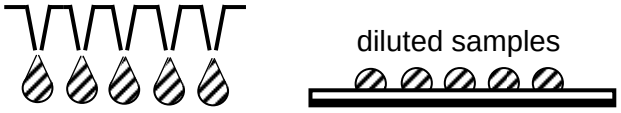
Mount: Place mounting medium onto a cover glass – drops of **max. 10 µl per reaction field**. Use a polystyrene mounting tray. Remove one BIOCHIP slide from PBS-Tween and dry the back and all four sides with a paper towel. Put the BIOCHIP slide, with the BIOCHIPs facing downwards, onto the prepared cover glass. Check immediately that the cover glass is properly fitted into the recesses of the slide. Correct the position if necessary.

Evaluate: Read the fluorescence with the microscope.
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.

TITERPLANE Technique

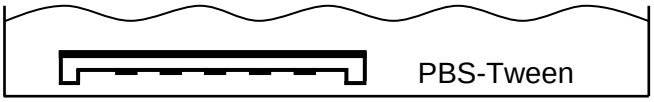


Pipette:	30 µl per field



Incubate:	30 min

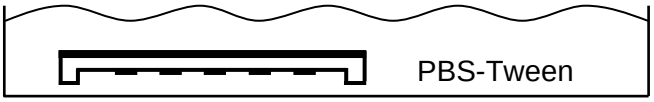
Wash:	1 s flush 5 min cuvette



Pipette:	25 µl per field

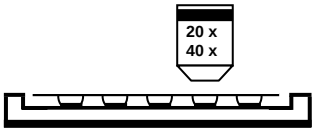
Incubate:	30 min

Wash:	1 s flush 5 min cuvette



Mount:	max. 10 µl per field

Evaluate:	fluorescence microscopy



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.

- **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.

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 not declarable concentration. Avoid skin contact.

Preparation and stability of samples

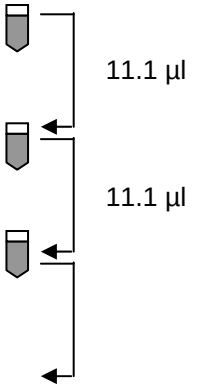
Samples: Human sera or EDTA, heparin or citrate plasma.

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 1:10 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 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 1:10.

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
⋮	⋮



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

Evaluation

Fluorescence pattern (positive reaction): Antibodies against **phospholipase A_2 receptor (PLA_2R)** react with the transfected cells of the substrate. They produce a flat, smooth to fine granular fluorescence of the cell with an accent of the cell membrane. The area of the cell nucleus is only slightly stained.

If all cells are stained, i.e. also control-transfected cells, antibodies against 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:

Anti-PLA_2R reactivity (IgG)	**Evaluation**
No reaction at 1:10	Negative. No antibodies against PLA_2R detected in the patient sample.
Positive reaction at 1:10	Positive. Indication of primary membranous nephropathy.

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	1:1,000	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	1:1,000
strong	strong	moderate	negative	1:3,200
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. Contact EUROIMMUN for details.

Test characteristics

Antigens: For the detection of autoantibodies against **phospholipase A_2 receptor (PLA_2R)** by indirect immunofluorescence, specifically transfected cells of EU 90 are used as standard substrate.

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, 1:1,000, 1:10,000 etc. There is no upper limit to the measurement range.

Reproducibility:

Reproducibility	Inter-lot	Intra-assay	Inter-assay
Minimum requirement	3 lots x 3 samples x 1 run x single determination: max. ± 1 titer step	1 lot x 3 samples x 1 run x tenfold determination: max. ± 1 titer step	1 lot x 3 samples x 2 runs x double determination: max. ± 1 titer step
PLA_2R (transfected cells)	Maximum deviation ± 1 titer step	Maximum deviation ± 1 titer step	Maximum deviation ± 1 titer step

Cross-reactivity: No cross-reacitivities were found.

Substrate	Ig class	Specifity of samples	n	Prevalence	
				Positive	%
PLA_2R (transfected cells)	IgG	HBsAg-positive	10	0	0
		anti-GBM-positive	10	0	0
		pANCA-positive	10	0	0
		cANCA-positive	10	0	0

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

Reference range: Titer 1:< 10

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
PLA_2R (transfected cells)	PLA_2R	IgG	0%	1:10	302

Clinical sensitivity and specificity:

Substrate	Ig class	Sample characterisation/ Clinical patient collectives (origin of samples)	n	Positive	Prevalence
PLA_2R	IgG	Patients with primary membranous glomerulonephritis (pMGN) (origin: Netherlands, Germany)	302	259	85.8%
		Patients with clinically confirmed idiopathic membranous nephropathy (IMN)*	100	52	52%
		Patients with a secondary form of membranous glomerulonephritis (sMGN) (origin: Germany)	47	0	0%
		Patients with a secondary form of membranous glomerulonephritis (sMGN) (n = 17), patients with biopsy-proven non-membranous glomerular renal diseases (n = 90), healthy blood donors (n = 153)*	260	0	0%
		Patients with other diseases (rheumatoid arthritis, thyroiditis, systemic sclerosis, systemic lupus erythematosus, psoriatic arthritis), healthy blood donors (n = 100) (origin: Germany, UK)	254	0	0%

***** *Reference: Hoxha E, Harendza S, Zahner G, Panzer U, Steinmetz O, Fechner K, Helmchen U and Stahl R.* ***An immunofluorescence test for phospholipase-A_2-receptor antibodies and its clinical usefulness in patients with membranous glomerulonephritis. Nephrol Dial Transplant. 2011 Aug;26(8):2526-32.***

FDA Study:

Sensitivity and specificity: Clinical studies were performed in cooperation with different sites. A total of 560 clinically characterised samples (275 from pMGN patients, 285 from control groups) were investigated for anti-PLA_2R antibodies (IgG). pMGN diagnosis was based on renal biopsy and was considered to be idiopathic/primary when no secondary cause of MN was suspected on the basis of clinical and laboratory criteria. The samples were withdrawn within 8 weeks after biopsy, before treatment; excluding patients who had been or were currently being treated with immunosuppressive drugs. Patients with history of drug usage and neoplasias were also excluded from the study. With the EUROIMMUN Anti-PLA_2R IFA using the cut-off dilution of 1:10, a sensitivity of 77.1% (95% C.I.: 71.7 to 81.9%) was found in pMGN, which is within the expected range of approximately 70% of anti-PLA_2R reported in the scientific literature. Specificity was 100.0% (95% C.I.: 98.7 to 100.0%). The results are shown in the tables below.

n = 560		Clinical diagnosis	
		positive	negative
EUROIMMUN Anti-PLA_2R IIFT	positive	212	0
	negative	63	285

Panel	Anti-PLA_2R IIFT	
	n	positive (%)
Primary membranous glomerulonephritis	275	212 (77.1%)
Secondary membranous glomerulonephritis	68	0 (0%)
Non-membranous glomerulonephritides	63	0 (0%)
Systemic lupus erythematosus	30	0 (0%)
Systemic sclerosis	30	0 (0%)
Psoriasis arthritis	30	0 (0%)
Rheumatoid arthritis	14	0 (0%)
Thyroiditis	50	0 (0%)

Clinical significance

The detection of autoantibodies against phospholipase A_2 receptors (PLA_2R) is useful in the diagnosis of primary membranous glomerulonephritis (MGN, pMGN), which has formerly been known as idiopathic membranous nephropathy (IMN). MGN is a chronic inflammatory disease of the renal corpuscle (glomeruli), which is accompanied by a progressive reduction in kidney function.

The autoimmune mechanism of MGN, which was first discovered and described in 2009, is the result of autoantibodies reacting with PLA_2R (transmembrane glycoproteins), which are expressed in human glomeruli on the surface of podocytes. They are involved in regulatory processes in the cell following phospholipase binding. Up until now two main groups of PLA_2R have been described (types M and N), with the M type of the PLA_2 receptor being identified as the major target antigen of autoantibodies. Autoantibodies against PLA_2R are suspected to be pathogenetically relevant, although the exact pathogenesis is still unknown. Circulating autoantibodies expressed by MGN patients bind to PLA_2R on podocytes. Immune complexes are produced in situ in the area of the glomerular basement membrane. There, they might trigger complement activation, which causes damage to podocytes and the blood-urine barrier. This leads to protein entering the primary urine, and subsequently proteinuria. Alternatively, the autoantibodies can also act as receptor agonists or antagonists and damage the podocyte architecture and, hence, the barrier function.

MGN is the most frequent kidney disorder with nephrotic syndrome. With increasing proteinuria, the long-term risk of kidney failure with major morbidity and mortality becomes higher, particularly in connection with thromboembolic and cardiovascular complications.

MGN is prevalent in all ethnic groups and genders, with men over 40 years of age and of white skin colour being more frequently affected. In young women with suspected MGN, lupus nephritis should be considered. MGN is rare in children (only 2 to 3% of kidney disorders in children).

Symptoms in MGN:
- Around 80% of MGN patients suffer from nephrotic syndrome with sometimes severe oedema in the legs and eye lids, weight gain and reduced urination.
- Around 20% of patients have proteinuria without any additional symptoms.
- Around 50% of patients have microscopic haematuria, albuminuria and glucosuria.
- Around 70% of patients show normal blood pressure and kidney function at the onset of the disease.

MGN can proceed in many different ways. In around one third of cases the disease heals spontaneously. In a further third it is stagnant and in the final third it progresses to chronic kidney failure. Acute kidney failure is rare. In chronic cases, complete kidney failure (terminal kidney insufficiency) occurs after five years in approx. 15% of untreated patients, after ten years in approx. 35% and after 15 years in approx. 40%.

Primary membranous glomerulonephritis (pMGN) should be discriminated from secondary membranous glomerulonephritis (sMGN), which is a secondary (accompanying) disease in infections, in drug therapy or abuse or intake of toxins, in collagenosis and other autoimmune diseases and in tumours. sMGN improves with treatment of the underlying disease. The treatment of MGN as an independent disease improves prognosis, particularly with respect to nephrotic syndrome and hypertonicity.

Diagnosis of MGN is made by kidney puncture, histological examination and electron microscopy of the kidney tissue. The deposition of immune complexes on the outside of the glomerular basement membrane is characteristic for the disease.

Serological diagnosis of MGN, however, is less time-consuming and less stressful for the patient. The identification and characterisation of PLA_2R (type M) as the target antigen of circulating antibodies in MGN has proven to be of major importance for non-invasive diagnostics. Autoantibodies of class IgG against PLA_2R are highly specific for the diagnosis of primary MGN. They can be detected in the serum of up to 70% to 75% patients. They are not exhibited by healthy blood donors or patients with lupus or IgA nephritis.

RC-IFT and ELISA are available for the determination of autoantibodies against PLA_2R.The Anti-PLA_2R RC-IFT uses transfected cells as standard substrate. The Anti-PLA_2R ELISA is based on purified human recombinant receptor from transfected cells. RC-IFT and ELISA are suited for qualitative and quantitative detection of human autoantibodies of class IgG against PLA_2R. The success of therapeutic measures can be assessed by means of the anti-PLA_2R titer. A titer increase, decrease or disappearance generally precedes a change in the clinical status. Thus, the determination of the autoantibody titer has a high predictive value with respect to clinical remission or relapse and estimation of the risk of relapse after kidney transplantation.

Literature references

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